SELECTIVE EXPANSION OF GP91^{phox} GENE-MODIFIED MURINE HAEMATOPOEITIC STEM CELLS

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Selektive Expansion von GP91^{phox} Gen-modifizieten Murinen Hämatopoetischen Stammzellen

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" Scientists are painfully aware that they do not know everything but they think they can often recognize nonsense when they come across it."

- Francis Crick -

For my parents Jun Chen and Mingqing He

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Abbreviations

ΔLNGFR	truncated low-affinity nerve growth factor receptor
µg	micro gram
µl	micro litre
aa	amino acid
ab	antibody
amp	ampicillin
bp	base pair
cd	cluster of differentiation
CFU-GM	Granulocyte-Macrophage Colony Forming Units
cip	calf intestine phosphatase
CMV	cytomegalovirus
CRU	competitive repopulating unit
D	donor
dH_2O	desterilised water
DMEM	Dulbecco's Modified Eagles Medium
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic Acid
dNTP	desoxynucleotidtriphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylendiamin tetra acet acid
eGFP	enhanced green fluorescence protein
EGFR	epidermal growth factor receptor
EMSA	electrophoretic mobility shift assay
env	envelope
ES	embryonic stem cell
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FKBP	FK506 binding protein

g	gram
G418	geneticin
gag	group specific antigen
GM-CFC	granulocyte-macrophage colony forming cells
GvHD	graft versus host disease
h/hrs	hour(s)
HA	hemagglutinin
HBS	HEPES buffered saline
HBSS	Hank's buffered saline solution
HEPES	N-2-hydroxy ethyl piperazin solution
HIV	human immunodeficiency virus
hmpl	human thrombopoietin receptor
hu	human
Ig	immunglobulin
JAK	janus protein tyrosine kinase
LB-medium	Luria-Bertani-Medium
LM-PCR	ligation-mediated PCR
LT-HSC	long term repopulating-HSC
M	molar
MAPK	mitogen activated protein kinase
mBM	murine bone marrow
mg	milli gram
ml	milli liter
mIL-3	murine interleukin 3
mIL-6	murine interleukin 6
MNC	mononuclear cells
mpl	thrombopoietin receptor
mSCF	murine stem cell factor
MW	molecular weight
neo	neomycin phosphotransferase
ng	nano gram

NK-cell	natural killer cell
p75NTR	p75 neurotrophin receptor
PB	peripheral blood
PCR	polymerase chain reaction
PLCγ	phospholipase Cγ
pol	reverse transcriptase
poly A	polyadenylation signal
PRL	prolactin
PrlR	prolactin receptor
rh	recombinant human
RNA	ribonuclein acid
rpm	rounds per minute
RPMI	Roswell Park Memorial Institute
RT	room temperature
RT-PCR	reverse-transcriptase polymerase chain reaction
SCID	severe combined immune deficiency
SDS	sodium dodecyl sulfat
sec	second
SFFV	spleen focus forming virus
STAT	signal transducers and activator of transcription
ST-HSC	short term repopulating-HSC
TAE	tris-acetat-EDTA
TE	tris-EDTA
TEMED	tetra-methyl-ethylen-diamin
Tpl	transplantation
Tris	2-amino-2-hydroxymethylpropan-1,3-diol
TU	transducing units
U	units
V	volts
vol	volume
WB	western blotting

WPRE	post-transcriptional regulatory element of woodchuck hepatitis virus
x-CGD	x-linked chronic granulomatous disease

1. Introduction

1.1 Haematopoiesis

The haematopoietic system is responsible for the production and maintenance of all types of blood and immune cells. It contains a supply of a range of different lineages: lymphoid cells, including B-lymphocytes, T-lymphocytes and nature killer cells; myeloid cells, including granulocytes, monocytes and macrophages; red blood cells (erythrocytes) and platelets (thrombocytes) (Figure 1.1). These mature blood cells have only limited life spans ranging from less than a day for granulocytes, to longer than 4 months for erythrocytes and up to several years for thrombocytes or memory T-cells (Abkowitz et al., 1996; Gunsilus et al., 2001). Hence, a source of cells is needed which is preserved throughout an individual's entire life. This source of cells is the multipotent haematopoietic stem cells (HSCs), and the process of continuous formation of blood cells is known as haematopoiesis. In adults, the dynamic process of haematopoiesis takes place in the bone marrow (BM), providing the haematopoietic cells with necessary supplies for survival, proliferation and differentiation (Kondo et al., 2003). The amount of active BM is about 2600 g, with roughly 1.3×10^{12} marrow cells. Every day, the HSCs are responsible for the formation of an estimated 1 trillion (10^{12}) mature blood cells of different lineages (Figure 1.1). These terminally differentiated blood cells are required for a variety of important functions, such as the transportat of oxygen and CO_2 by the red blood cells, fighting infections (killing of pathological microbes e.g. by granulocytes and macrophages), immune surveillance (B- and T-cells) and stopping bleeding by the platelets. In addition to the great productivity, haematopoiesis is remarkably stable, i.e. even in periods of extraordinary demand, blood cell production normally never becomes exhausted throughout life.

1.1.1 Haematopoietic Stem Cells

Haematopoiesis is generally regarded as an irreversible hierarchy with just a few HSCs at the top of the tree. These HSCs can give rise to early progenitors committed to the myeloid and the lymphoid lineages that proliferate and progressively differentiate into fully mature blood cells (Weissman et al., 2000) (Figure 1.1). The existence of HSCs was established in the early 1950s (Cole et al., 1952; Lorenz et al., 1951) and the first candidate HSC, the colony-forming unit-spleen (CFU-S), was described by Till and McCulloch in 1961.

With this historic experiment, the early haematopoietic progenitors were identified and their clonogenic nature was detected, in that they are capable of forming colonies (CFU-S) post-transplantation in the spleen of ablated mice, as well as repopulating the ablated recipients (Till et al., 1961). Becker and colleagues first reported the clonal origin of mouse BM HSCs in 1960 (Becker et al., 1963). However, in the late 1970s, data from different groups indicated that the CFU-S cells do not represent the most primitive HSCs (Hodgson et al., 1979; Jones et al., 1990; Jones et al., 1996; Ploemacher et al., 1989). Later, evidence of the presence of HSCs was obtained by *in vivo* tracking of progeny from foetal liver cells marked with retroviruses in transplanted mice (Jordan et al., 1990). A turning point in the search for HSCs came in 1988, when the Weissman group used a combination of monoclonal antibodies against cell surface markers to successfully characterise and isolate murine HSCs by fluorescence activated cell sorting (FACS) (Spangrude et al., 1988). This led to rapid developments in the identification of HSCs. However, the exact identity of stem cells is still not known. At present, the definition of a HSC depends on three unique and important functional requirements, as described by Kondo and Weissman (Kondo et al., 2003). First, HSCs have the ability to self-renew, i.e. they can divide into two daughter cells, of which at least one remains identical to the parental cell. Second, HSCs can differentiate into all haematopoietic blood cell lineages, therefore, they are multipotent. Third, HSCs can reconstitute long-term and thereby rescue an ablated recipient.

In the murine haematopoietic system, HSCs can be divided into the more primitive long-term repopulating (LT-HSC) and the less primitive short-term repopulating (ST-HSC) stem cells based on their function in transplantation (Morrison et al., 1994; Kondo et al., 2003) (Figure 1.1). Both types of HSCs are derived from multipotent stem cells, which can be isolated from various tissues in foetal and adult animals. Multipotent stem cells are tissue specific and include, among others, HSCs, neuronal stem cells and hepatic stem cells (Kondo et al., 2003). During the progressive development of haematopoiesis from LT-HSC to mature blood cells, the self-renewal ability of the cells decreases, while their differentiation capability increases.

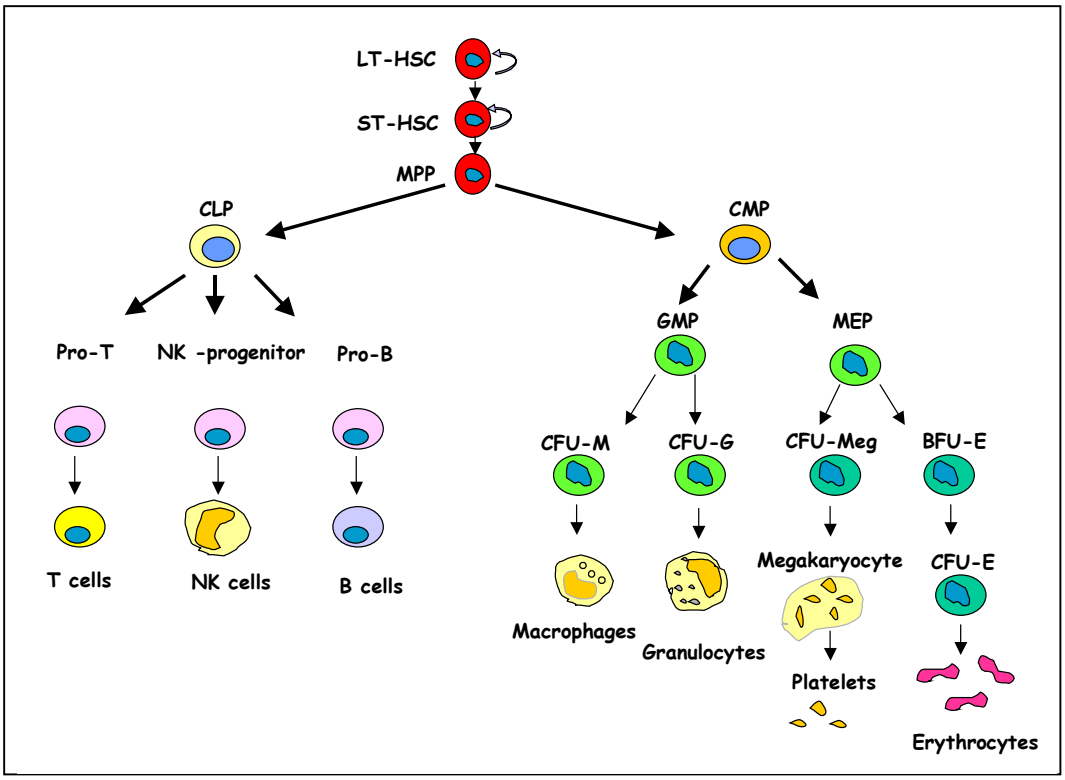


Figure 1.1: A schematic demonstration of the haematopoiesis of an adult mouse. The pluripotent HSC gives rise to multipotent progenitors that in turn give rise to committed progenitors with the final output being the mature, functional blood cells. Not all of the linear relationships in this figure have been proved. In the human haematopoiesis tree, the CMPs can directly give rise to CFU-Eo and CFU-Basso which yield the mature eosinophilic and basophilic cells (schematic presentation modified from Kondo et al., 2003). Abbreviations: CLP, common lymphoid progenitors; CMP, common myeloid progenitors; CFU, colony forming unit; GMP, granulocyte-macrophage progenitors; BFU, burst-forming unit erythroid; MEP, megakaryocyte-erythrocyte progenitor; MPP, multipotent progenitors.

1.1.2 Identification of HSCs and Progenitor Cells

HSCs and their progenitor cells have been extensively identified and enriched through size fractionation with density-gradient centrifugation, the use of cell-cycle active drugs, such as 5-FU (5-fluorouracil), and FACS. Presently, FACS analysis based on cell surface markers is the most commonly used method for identifying HSCs. The most important cell surface markers of murine haematopoietic stem and progenitor cells are shown in Table 1.1 (Kondo et al., 2003). All HSCs lack the expression of lineage-specific cell surface markers found on differentiated blood cells (lineage negative; Lin^-) but express the stem cell antigen-1 (Sca1) and show low or negative expression of the differentiation marker Thy-1 (Uchida et al., 1992). These $Lin^-/Sca1^+/Thy\text{-}1^{lo/-}$ (LST) stem cells fulfil all stem cell criteria and allow long-term repopulation in lethally irradiated mice (Spangrude et al., 1988; Spangrude et al., 1990; Uchida et al., 1992). In addition, numerous other cell surface antigens have also been used in

an effort to isolate a more primitive HSC. For example, the co-selection of c-kit further enriches for HSCs. c-kit$^+$ murine BM progenitors were found to possess high haematopoietic potential. Hence, many researchers have utilised Lin$^-$/Sca1$^+$/c-kit$^+$ (LSK) cells as a source of murine HSCs (Ikuta et al., 1992; Orlic et al., 1993). Single Lin$^-$/Sca1$^+$/Thy-1$^{lo/-}$/c-kit$^+$ HSC cells give rise to complete long-term multilineage reconstitution and self-renewal in irradiated mice (Smith et al., 1992; Wagers et al., 2003). However, recent studies in mice and humans have provided evidence indicating that very early pluripotent HSCs display only low expression of c-kit (= c-kit^{lo}, phenotypically c-kit$^-$, with c-kit still detectable by RT-PCR) (Ikehara et al., 2000; Gunji et al., 1993; Katayama et al., 1993). The expression pattern of surface markers like Thy1.1 and Flk2 allow different cell types to be distinguished e.g. mature HSCs from a population with extensive self-renewal potential (Thy1.1^{lo}/Flk2$^-$) or a multipotent progenitor population with limited self-renewal ability (Thy1.1$^-$/Flk2$^+$) (Christensen et al., 2001).

The human HSC has not been dissected as scrupulously as the mouse HSC due to the lack of an optimal stem cell assay. Human LT-HSCs express CD34 but not the surface marker CD38, and therefore exist within the CD34$^+$/CD38$^-$ cell population (Morrison et al., 1995). In contrast, mouse LT-HSCs express CD34 at negative to low levels (Osawa et al., 1996). However, the stem cell pool is most likely a heterogeneous population and recent data demonstrate that candidate human HSCs can also be found within the CD34$^-$ population (Zanjani et al., 1998; Bhatia et al., 1998). Furthermore, the murine HSC has a reversible CD34 expression depending on its activation status (Ogawa et al., 2002).

The downstream progeny of murine HSCs have also been characterised based on the expression profile of cell surface markers. Thus lineage-restricted oligopotent progenitor cells for lymphoid (common lymphoid progenitor, CLP) and myeloid (common myeloid progenitor cells, CMP), and granulocyte-monocyte progenitor (GMP), and megakaryocyte-erythrocyte progenitor (MEP) lineages have been identified and are listed in Table 1.1 (Kondo et al., 1997; Na Nakorn et al., 2002).

Purification and isolation of murine and human HSCs is not an easy task because of the limited frequencies of these cells. For example, the frequency of long- and short-term repopulating cells in BM is roughly 1 in 10,000 and 1 in 2,000, respectively (Reya, 2003). Most isolation strategies rely on the expression of specific cell surface antigens, as described above. However, other properties of HSCs can also be utilised to isolate these primitive cells. The majority of HSCs are believed to be in a quiescent, non-cycling state (Ogawa et al., 1993). Based on this theory, sorting for HSCs residing in the G0/G1 cell cycle phase with low

levels of RNA, measured with different stains like the DNA stain Hoechst 33342, can be used to purify these cells (Goodell et al., 1996).

Cell type		Cell surface marker
Multipotent	LT-HSC	Lin^-, $Sca1^+$, $c\text{-}kit^+$, $Thy\text{-}1^{lo}$, $IL\text{-}7Ra^-$, $FLK2^-$, $CD34^{-/lo}$
	ST-HSC	Lin^-, $Mac1^{lo}$, $Sca1^+$, $c\text{-}kit^+$, $Thy\text{-}1^{lo}$, $IL\text{-}7Ra^-$, $FLK2^+$, $CD34^+$
	MPP	Lin^-, $Mac1^{lo}$, $Sca1^+$, $c\text{-}kit^+$, $Thy\text{-}1^-$, $IL\text{-}7Ra^-$, $FLK2^+$, $CD34^+$
Oligopotent	CLP	Lin^-, $Sca1^{lo}$, $c\text{-}kit^{lo}$, $Thy\text{-}1^-$, $IL\text{-}7Ra^+$, $FLK2^+$, $CD24^{lo}$, $CD27^+$, $CD34^+$, $CD43^+$, TdT^+
	CMP	Lin^-, $Sca1^-$, $c\text{-}kit^+$, $Thy\text{-}1^-$, $IL\text{-}7Ra^-$, $CD34^+$, $F_{c\gamma}RII/III^{lo}$
Single lineage	Pro T	Lin^-, $CD4^{lo/-}$, $CD25^-$, $CD44^+$, $c\text{-}kit^+$, $Thy\text{-}1^-$, $IL\text{-}7Ra^+$, $FLK2^+$, $CD34^+$
	NK progenitor	$NK1.1^-$, $IL\text{-}2R\beta^+$
	Pro B	$B220^+$, $CD19^-$, $CD24^-$, $CD43^+$, $AA4.1^+$, $IL\text{-}7Ra^-$
	GMP	Lin^-, $Sca1^-$, $c\text{-}kit^+$, $Thy\text{-}1^-$, $IL\text{-}7Ra^-$, $CD34^+$, $F_{c\gamma}RII/III^+$
	MEP	Lin^-, $Sca1^-$, $c\text{-}kit^+$, $Thy\text{-}1^-$, $IL\text{-}7Ra^-$, $CD34^-$, $F_{c\gamma}RII/III^{lo}$
	Meg progenitor	Lin^-, $Sca1^-$, $c\text{-}kit^+$, $Thy\text{-}1^-$, $IL\text{-}7Ra^-$, $CD34^{lo}$, $F_{c\gamma}RII/III^{lo}$, $CD9^+$

Table 1.1: Cell surface markers of murine HSCs and progenitor cells. The different cell surface markers that have been identified from undifferentiated stem cell to differentiated lineage cells. Most abbreviations are explained in the main text (modified from Kondo et al., 2003).

So far, the primary source of murine or human HSCs for therapeutic applications has been the adult bone marrow (BM). In human stem cell transplantation studies, the preferred cell source is BM cells from the peripheral blood (PB) after mobilisation of progenitor and stem cells from the BM to the PB through treatment with cytokines such as granulocyte-colony stimulating factor (G-CSF). These mobilised cells can then be harvested by blood aphaeresis. Additionally, there is an increased number of short-term-repopulating-HSCs (ST-HSCs) in this population, and these cells are reported to contribute to the transplantation efficiency (Korbling et al., 2001). Another source for human HSCs is umbilical cord blood (CB). These cells are potentially the best source for transplantation since they have been demonstrated to have a competitive advantage over BM cells (Holyoake et al., 1999), but the collected cell numbers are limited.

1.1.3 Functional Assays for Haematopoietic Stem and Progenitor Cells

A continuum of cells with altering phenotype and properties is produced in the haematopoietic system during the progression from stem to differentiated cells. A variety of *in vitro* and *in vivo* assays have been developed for murine and human cells in order to measure quantitative and qualitative properties of primitive haematopoietic stem and progenitor cells (Figure 1.2). These assays are based on the high proliferative and clonogenic characteristics of primitive haematopoietic cells separating them from the more mature cells that possess a narrower haematopoietic potential.

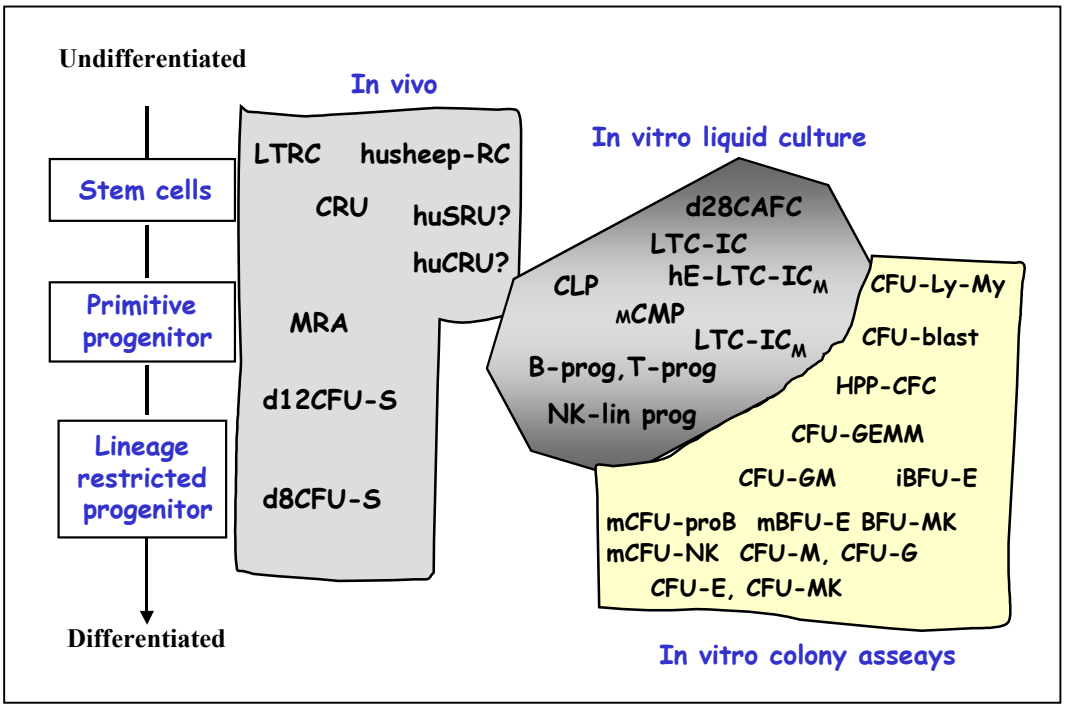


Figure 1.2: Categorisation of distinct classes of haematopoietic stem and progenitor cells in *in vitro* and *in vivo* assays. According to the type of assay, 3 methodological families can be distinguished: *in vivo* transplantation assay (left), *in vitro* long-term culture assay (middle) and *in vitro* short-term colony assay (right). The cells are aligned on the vertical axis based on their maturation stage, from undifferentiated stem cells to differentiated lineage-restricted progenitors. “hu” designates cells identified solely in human tissues and “m” indicates murine assays (adapted from Coulombel, 2004).

1.1.3.1 *In Vitro* Assays

So far, no perfect *in vitro* assay is available which allows analysis of all the haematopoietic characteristics of HSCs. Nevertheless, for the routine analysis of early haematopoiesis, a few assays for primitive progenitors “correlating” to the numbers of the

HSCs have been developed. These comprise short-term *in vitro* colony assays based on semi-solid media such as methylcellulose and long-term *in vitro* assays including LTC-IC (long-term culture-initiating cells) and CAFC (cobblestone area-forming cells). These are performed in the presence of feeder cells which are necessary for the long-term culture of HSCs.

In human HSC colonies, several types of progenitors, e.g. erythroid, granulocytic, macrophagic and megakaryocytic can be distinguished on the basis of their sensitivity to cytokines, the time required to produce differentiated cells and their size, colour and composition (see web page www.stemcell.com). For murine cells, however, it is more difficult to discriminate the colour and appearance of the different colonies. Several classes of multipotent progenitors, e.g. colony-forming unit granulocyte-erythroid-macrophage-megakaryocyte (CFU-GEMM), blast progenitors and lymphomyeloid progenitors, have been identified (Ash et al., 1981; Leary and Ogawa, 1987; Lacaud et al., 1998) because of the presence of more than one lineage of differentiated cells and/or progenitors in a single colony. Nevertheless, the lifespan of the viscous medium is too short (no more than 3 weeks) to allow the detection of more immature progenitors with this method. For long-term *in vitro* assays, both human and mouse, two different cell types have been identified: LTC-IC (or human ELTC-IC) and CAFC. LTC-IC are obtained by culturing the cells for a long time of about 5–8 weeks (about 12 weeks for ELTC-IC) on an irradiated stroma layer, during which mature cells die and a selection for the most primitive cells occurs. Further committed cells present in the input populations will not survive culture. The haematopoietic ability of surviving primitive cells can then be measured by their colony forming ability in methylcellulose cultures. The CAFC assay is another measurement for primitive haematopoietic progenitors based on a similar principle to the LTC-IC assay. The CAFCs integrate in an adherent layer of feeder cells and form a cobblestone area, which is defined as a group of flattened, optically dense cells tightly associated with the adherent feeder cells (Breems et al., 1994; Ploemacher et al., 1989). Murine CAFCs are counted on day 28–35 after plating.

1.1.3.2 *In Vivo* Assays

The *in vitro* assays described above are important for measuring certain functions of haematopoietic progenitor cells. However, *in vitro* assays characterise an artificial system that can not take into account the effects from stimuli and other environmental surroundings found *in vivo*. Hence, the final assessment of HSC capacity is the ability to repopulate a recipient.

This method can be most easily performed when using the murine system. Here three transplantation assays have been established (Coulombel, 2004):

- Short-lived cell assay, such as day 12 CFU-S (colony forming unit-spleen). The CFU-S assay was the first to be described for assessing murine HSC activity *in vivo*. Macroscopic colonies visible on the spleen 8–12 days post-transplantation were shown to contain cells which contributed to the rescue of lethally irradiated mice (Till et al., 1961). However, experimental data demonstrated that long-term reconstituting HSCs (LTR-HSCs) could not be found in CFU-S colony forming units (Na Nakorn et al., 2002). Currently, the CFU-S assay is used mainly for measurements of primitive multipotent myeloid progenitors but not as an assay for true HSCs.
- Pre-CFU-S and marrow-repopulating activity (MRA) assay. Two weeks post-transplantation, BM cells of recipients are re-transplanted into secondary recipients. The ability to produce CFU-S in secondary recipients is analysed (Hodgson et al., 1979; Lord et al., 1993).
- Identification and quantification of long-term reconstituting cells (LTRCs) based on the competitive repopulating unit (CRU) assay. This assessment is the most stringent assay for murine LTR-HSCs and can be performed in a competitive transplantation with a limiting dilution approach, thereby measuring the repopulating capability of a single stem cell (Harrison, 1980; Harrison et al., 1993; Szilvassy et al., 1990).

The murine transplantation assay allows systematic analysis of short-term (<6 weeks) and long-term (3–4 months) engraftment with the option of serial transplants into secondary or tertiary recipients as the ultimate test for LTR-HSC activity. Importantly, transplantation experiments also determine the homing ability of HSCs to the BM, a feature not accessible in *in vitro* assays (Szilvassy et al., 2001). For human HSCs, similar transplantation studies have been established using xenograft models in immune-deficient mice, such as the non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mouse model (Vormoor et al., 1994).

1.1.4 HSCs as Targets for Gene Therapy

Based on their capability to self-renew and to give rise to all haematopoietic lineages, HSCs are an attractive and well-studied target cell population for gene therapy of haematopoietic disorders like chronic granulomatous disease (see chapter 1.2), severe

combined immunodeficiency (X-SCID) and others. These cells are easily isolated from haematopoietic tissues. They can be concentrated, enriched and readily delivered back to the patients by autologous transplantation methods which have been routinely performed for over 40 years. As mentioned earlier, HSCs are long-lived, have repopulation potential and are pluripotent. They could contribute to the correction of defects in all haematopoietic lineages and probably non-haematopoietic cell lineage via transdifferentiation (Vollweiler et al., 2003).

A primary focus of studies in the field of gene transfer to HSCs is targeting primitive stem cells without losing their unique stem cell properties during the *ex vivo* transduction period. Various cytokine combinations have been considered preclinically for their ability to improve gene transfer efficiency and to expand primitive HSCs while maintaining their repopulating capability in model systems (Laneuville et al., 1988; Dick et al., 1991; Nolta et al., 1996; Bhatia et al., 1997). Different cytokines, such as stem cell factor (SCF), Flt-3 ligand (Flt-3), thrombopoietin (TPO), megakaryocyte growth and development factor (MGDF), interleukin-3, -6 and -11 (IL-3, IL-6, IL-11) have been used in many gene transfer protocols for murine and human HSCs (Table 1.2). SCF has been demonstrated to have a critical role in haematopoiesis and its maintenance, both in human and murine system (Broudy, 1997), and Flt-3 can induce the expansion of haematopoietic cells based on stimulation of tyrosine kinase activity (Gilliland and Griffin, 2002). The utilisation of IL-3 in the *ex vivo*-expansion of HSCs has been considered as an attractive possibility (Ivanovic, 2004), although it has been reported that IL-3 could be associated with a diminished repopulating ability in mice (Yonemura et al., 1996; Wognum et al., 2000). It has been suggested that a combination of cytokines including IL-3, IL-6 and SCF may be more beneficial for improving gene transfer efficiency, and may preserve the characteristic properties of target HSCs (Kiem et al., 1997; Van Beusechem et al., 1997; Ivanovic 2004). However, many combinations of cytokines with activity on early haematopoietic stem/progenitor cells have been utilised for *ex vivo* stem cell transduction and expansion. So far, no consensus on the optimal combination has been reached (Horn et al., 2004). The optimal combinations of cytokines are dependent on the vector type, cell type and experimental goal. It is commonly agreed that SCF, TPO Flt-3 and IL-3 are the optimal cytokine combination for clinical trials for gene therapy of diseases such as X-CGD (see below), X-SCID and adenosine deaminase deficiency (ADA).

Origin of HSCs	Cytokine combination	Reference
Human	SCF, Flt-3, TPO	Schilz et al., 2000 Schiedlmeier et al.,2000
	SCF, Flt-3, IL-3, TPO	Cavazzana-Calvo et al., 2000 Bruno et al., 2001
	SCF, Flt-3, IL-6, TPO	Hossle et al., 2002 Piacibello et al., 2002
	SCF, Flt-3, IL-3, IL-6	Demaison et al., 2002 Schilz et al., 2000
	SCF, G-CSF, TPO	Hanenberg et al., 1997 Leurs et al., 2003
	SCF, Flt-3, IL-3, IL-6, G-CSF	Hennemann et al., 1999 Conneally et al., 1998
Baboon	SCF, Flt-3, G-CSF, MGDF	Kiem et al., 2002
	SCF, G-CSF, MGDF	Horn et al., 2002
	Il-3, IL-6, SCF	Kurre et al., 2001
Murine	SCF, Flt-3, IL-11	Mackarehtschian et al., 1995 Miller et al., 1990
	SCF, IL-3, IL-6	Mikkola et al.,2000
	SCF, IL-3, IL-6, Flt-3, TPO	Moreau-Gaudry et al., 2001
	SCF, IL-6	Dinauer et al., 1999 Hara et al., 2004
	SCF, Flt-3, IL-3, IL-11	Li et al., 2003
	SCF, TPO, IL-6, Flt-3	Kurre et al., 2004
	SCF, Flt-3, IL-6	Schwickerath et al., 2004

Table 1.2: Summary of cytokine combinations established in various studies to maintain the properties of HSCs.

1.2 Chronic Granulomatous Disease

Chronic granulomatous disease (CGD) is a rare inherited disorder of phagocyte function, based on a genetic defect in 1 of four genes encoding the subunits of the nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase, which is required for superoxide (O_2^-) production. The disease is characterised by an increased susceptibility to bacterial and fungal infections. The incidence of CGD is about 1 in 200,000 all over the world (Winkelstein et al., 2000). However, it is likely that this disease continues to be under-diagnosed, which would explain discrepancies in incidence. About 70% of CGD patients

inherit the disease as a characteristic sex-linked defect in the recessive gene found on the X-chromosome from the mother (Rae et al., 1998).

1.2.1 Clinical Features and Diagnosis of X-linked CGD

The symptoms of this disorder do not appear until 3 months of age. In the UK, the median age of patients at the time of diagnosis is 3 years (Jones, 2002). Patients with X-linked CGD (X-CGD) suffer from recurrent infections with certain bacteria, such as *Staphylococcus*, *Salmonella* and *Pseudomonas* or fungi, such as *Aspergillus*. *Staphylococcus aureus* is most frequently isolated from CGD patients, but it is also reported that the most common causes of death are pneumonia and/or sepsis due to infection with *Aspergillus* or *Bacillus cepacia* (Winkelstein et al., 2000). Some bacteria and fungi generate their own hydrogen peroxide, which phagocytes can use to kill the microbes themselves. Other invaders quickly destroy any hydrogen peroxide they produce so that the scavenger cells that lack NADPH oxidase can not do their job. Although these bacteria and fungi can lead to infections anywhere in the body, they most often target the lung, lymph nodes, skin, liver, gastrointestinal tract, nostrils, mouth or bones in the arms and legs (Winkelstein et al., 2000). Recurrent chronic inflammations lead to the development of characteristic tumour-like masses called granulomas, which can cause serious problems by preventing the passage of food through the oesophagus, stomach and intestine, as well as by blocking urine flow from the kidneys and bladder.

CGD is diagnosed by the demonstration of absent or markedly deficient respiratory burst activity in phagocytes. The oldest and most widely used assay is the nitroblue tetrazolium dye reduction (NBT) test (Ochs et al., 1973). This assay assesses the ability of activated neutrophils to produce superoxide, which reduces the water-soluble, yellow NBT dye to insoluble, blue formazan. The test is useful for the identification of female carriers having a mixed population of NBT-positive and -negative cells. Usually, the presence of as few as 2–5 % of normal neutrophils is sufficient to maintain a mild clinical phenotype, an aspect of great importance for any gene therapeutic approach (see chapter 1.3.3). The dihydrorhodamine (DHR) assay (Vowells et al., 1995) is a quick and convenient assay based on flow cytometric analysis. This test assesses the ability of stimulated phagocytes to produce superoxide that can oxidize dihydrorhodamine to rhodamine. Rhodamine releases a fluorescent signal that can be detected by a flow cytometer. A lack of fluorescence confirms the clinical diagnosis of CGD. The most widely used quantitative measurement of superoxide production is the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome c (Babior et al., 1973). In addition, a chemiluminescent-based assay has been developed which

involves the SOD-inhibitable reduction of lucigenin or catalysed peroxidation of luminol (Porter et al., 1992). This assay is less quantitative but has greater specificity for determining NADPH oxidase activity. Conformation of the molecular defect can be obtained by sequencing the relevant gene (Roos et al., 1996).

1.2.2 Molecular Background of CGD

The NADPH oxidase is a phagosomal and plasma membrane-associated enzyme complex. In resting neutrophils, it is dormant and its components are localised in different parts of the cell. The active enzyme complex is rapidly assembled following neutrophil activation by a variety of inflammatory stimuli, e.g. by the binding of microorganisms to cell surface receptors. It has been demonstrated that oxidase activation needs at least five different protein factors (De Leo et al., 1996). During neutrophil stimulation, the cytosolic factors $p47^{phox}$, $p67^{phox}$/$p40^{phox}$ and GTPase Rac1/2 translocate to the plasma membrane and associate with cytochrome b558, which consists of $p22^{phox}$ and heavily glycosylated $gp91^{phox}$ subunits. The $gp91^{phox}$ subunit is the redox centre of the oxidase and contains two heme prosthetic groups in the hydrophobic N-terminus of the protein embedded within the membrane. The C-terminus contains a flavoprotein domain with binding sites for flavin and NADPH (Babior et al., 1999). A schematic representation of the inactive and active NADPH oxidase complex is displayed in Figure 1.3.

CGD patients lack NADPH oxidase activity as a result of the absence of one of its components. If one of the components is defective, the activity of NADPH oxidase is significantly decreased, which also results in the CGD phenotype (Jurkowska et al., 2004). X-CGD cases result from defects in the CYBB gene that encodes the $gp91^{phox}$ subunit. The remaining cases are autosomal recessive and caused by defects in the CYBA, NCF-1 or NCF-2 genes (Table 1.3).

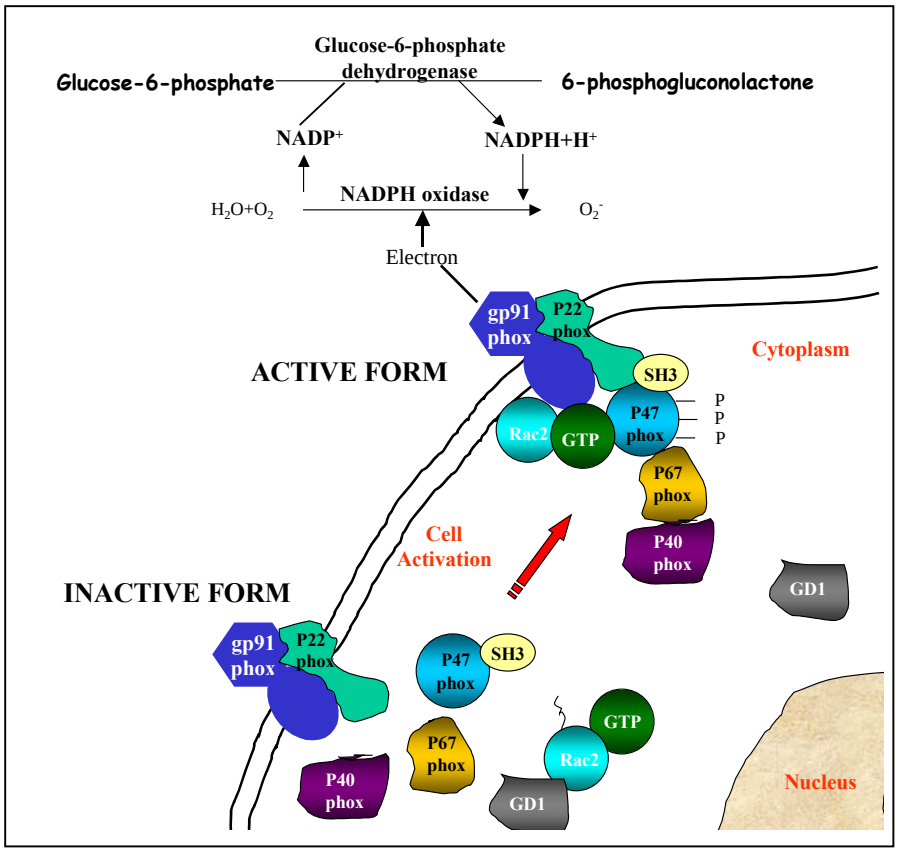


Figure 1.3: Schematic representation of the inactive and active NADPH oxidase complex. The membrane assembly of activated NADPH oxidase occurs upon stimulation. The known functional components involved in activation are shown: the integral membrane cytochrome b558 consisting of the gp91^{phox} and p22^{phox} subunits; the cytosolic components p47^{phox} and p67^{phox}/p40^{phox}, and the Rac2 GTPase in complex with the regulatory protein GDP dissociation inhibitor (GDI). Multiple phosphorylations of p47^{phox} are depicted by attached p groups. The wavy line on Rac2 indicates the C-terminal geranylgeranyl isoprenyl group and associated polybasic domain (modified from Lekstrom-Himes and Gallin, 2000).

Gene	Protein	Cellular localisation	Assembling domains	Aberrant in CGD (%)
CYBB	gp91^{phox}	membrane	-	~ 65
NCF1	p47^{phox}	cytosol	two proline-rich, two SH3 motifs	~ 25
NCF2	p67^{phox}	cytosol	one proline-rich, two SH3 motifs	~ 5
CYBA	p22^{phox}	membrane	one proline-rich motif	~ 5
NCF4	p40^{phox}	cytosol	one SH3 motif	-

Table 1.3: Characteristics of NADPH oxidase components of CGD patients. The genes most commonly mutated in CGD patients are given. An internationally maintained database, the CYBB base (see web page http://www.uta.fi/imt/bioinfo/CYBBbase/), lists 304 different mutations within this gene (adapted from Jurkowska et al., 2004).

1.2.3 Treatment and Prognosis of CGD

The most important principles for treatment of CGD patients are prevention and aggressive treatment of infections, usually through antimicrobial chemoprophylaxis, such as with cotrimoxazole, and antifungal prophylaxis, such as with itraconazole (Petropoulou et al., 1994; Gallin et al., 2003). It has also been reported that interferon-gamma (IFN-γ) can restore NADPH oxidase activity in a small number of patients with X-CGD (Ezekowitz et al., 1988); however, this is not a routine treatment. Topical and systemic steroids are major components of therapy for the inflammatory complications of CGD, but unfortunately, a significant number of patients become dependant on these drugs. In cases where patients are resistant to conventional treatments, neutrophils from leukophoresed donors can be administered, with similar drawbacks as those described for BM cell transplants below.

For over 40 years, allogeneic haematopoietic stem cell transplantation (HSCT) has been used to treat a wide range of primary immunodeficiencies, haematological disorders and metabolic conditions. It can also be performed for the treatment of CGD patients (Seger et al., 2002). Nevertheless, high rates of morbidity, mortality and graft failure limit the broad usage of this method. Morbidity and mortality result on the one hand from the high degree of immunosuppression required to achieve engraftment, and on the other hand from graft versus host disease (GvHD). Despite modern tissue typing techniques and prophylaxis, GvHD remains one of the major complications of allogeneic HSCT, with a rate of about 25%. Additionally, the limited availability of matched donors has restricted the application. Attempts are being made to reduce toxicity related to conditioning and to use stem cells derived from CB as an alternative source with a significantly lower rate of GvHD (Han et al., 1995; Risdon et al., 1995). The better immunological tolerance of this source of haematopoietic progenitor cells (HPC) has permitted an expansion of the donor pool, and wider representation for ethnic minorities, who have a much smaller pool of possible donors for BM transplants. CB offers additional advantages over BM in unrelated transplantations, such as a low transmission rate of infectious diseases and the immediate availability of the product. However, the major current barrier to a wider use of CB transplantations is the limited cell dose of CB units, especially for adults (Benito et al., 2004).

Altogether, there is currently no cure for CGD with drug treatment and HSCT is limited due to severe drawbacks, another alternative method like gene therapy could be an option for the treatment of X-CGD patients (see chapter 1.3.3).

1.3 Gene therapy

Principally, gene therapy can be defined as the transfer of genetic material into cells in order to treat or prevent a disease, or to slow its progression (Mountain, 2000; Somia and Verma, 2000; Pfeifer and Verma, 2001). This novel therapeutic strategy has been regarded as a potential revolution in medicine. The reason for this is that gene therapy aims to treat or correct the source of a genetic disease. A mutated gene could be replaced by its normal functional counterpart, rather than treating the effects of the defective gene product, as many drugs do (Friedmann, 1989; Mountain, 2000). Gene therapy was originally conceived as a possible treatment option for hereditary single gene defects. Today, it has been demonstrated that many complex diseases, such as cancer (Cristiano, 1998), cardiovascular diseases (Isner, 2002), neurodegenerative disorders (Baekelandt et al., 2000) and infectious diseases like AIDS (Bunnell and Morgan, 1998), could also be targeted via gene transfer. In this technique, a defective gene is replaced or supplemented by a functional, normal gene using recombinant DNA technology. The functional gene copy provides the corrected enzyme or protein and the cause of the disease is consequently eliminated. Different strategies for gene therapy have been developed (Driskell et al., 2003):

- Gene replacement: A normal gene is inserted into a non-specific location of the genome. This strategy is currently the standard approach in gene therapy, due to its relative simplicity.
- Gene repair: Mutated sequences in the genomic DNA are directly repaired or replaced by a normal gene. This method is considered as the ultimate goal of gene therapy, but even today the efficiency of gene repair techniques is so low that clinical applications are not realistic.
- Gene reprogramming: A functional correction of the defect is reached by modification of endogenously encoded mutated RNA transcripts. Unfortunately, this is also equally inefficient at this time.

To date, gene replacement is the most common type used in clinically approved gene therapy trials, and it is used in the current study. In all of these trials, the genes of interest were transferred into somatic cells. In principle, there are three techniques that can be used, based on the mode of delivery of the gene to the target tissue (Anderson, 1998):

- Indirectly, *ex vivo*: Cells are modified in culture and then transplanted back into the patient.

- Directly, *in situ*: Gene delivery vectors are injected or infused into the target tissue.
- *In vivo*: Gene transfer vectors are directly injected into the bloodstream.

The *ex vivo* method has been proposed for gene therapy of haematopoietic diseases such as CGD and SCID.

A number of factors may influence a successful gene therapy approach, such as the identification of a suitable therapeutic gene, targeting the tissue associated with the disease and a delivery system that can transfer the gene efficiently into the host genome in a locus that does not disturb other gene functions or, most importantly, lead to insertional oncogenesis.

1.3.1 Retroviral Gene Transfer Vector Systems

Two types of vector system, i.e. non-viral and viral, are used for gene therapy applications. Non-viral methods involve either the direct introduction of naked DNA into target cells, or the use of liposomes or other molecules that can transport DNA into cells. However, most of these approaches suffer from inefficient delivery and transient expression of the gene. Transiently expressed cDNA plasmids are usually lost or degraded by the targeted cell itself. Viral vectors are more effective, delivering genes into the target cells with high efficiency, due to their natural infectious properties. Promoting integration into the host cell genome also provides the opportunity for long-term expression (Walter et al., 2000; Gardlik et al., 2005).

There are also drawbacks in the use of viral gene therapy vectors, such as immunogenicity, the problem of specific targeting, and the duration and level of gene expression in the cells. Nevertheless, the progress of viral vectors into clinical trials has been eased through current advances in vector design, pseudotyping of viruses with envelopes specific for certain cell types, the use of target cell-specific promoters, the development of viral packaging cell lines and the incorporation of reagents to improve transduction efficiencies. In gene therapy studies, different vectors are chosen based on their properties and the experimental goal. A summary of various viral vectors and their properties is given in Table 1.4. Gammaretroviral vectors based on the murine leukaemia virus (MLV) and lentiviral vectors based on the human immunodeficiency virus (HIV) are attractive tools for gene therapy due to their large packaging capacity, high transduction efficiency, long-term expression of the transgene and to the possibility of pseudotyping the vector with a range of envelopes.

Table 1.4: Summary of properties of viral vectors used for gene transfer

Features	Gammaretroviral	Lentiviral	HSV-1	Adenoviral	AAV
Genetic material	RNA	RNA	dsDNA	dsDNA	ssDNA
Maximum insert size	8 kb	8 kb	40 kb	~ 30 kb	<5 kb
Tropism	Dividing cells only	Broad	Strong for neurons	Broad	Broad with possible exception of HSC
Vector genome form	Integrated	Integrated	Episomal	Episomal	Episomal (>90%) Integrated (<10%)
Duration of expression *in vivo*	Long	Long	Short	Short	Long
Immunological problems	Not immunogenic	Few	Extensive	Extensive	Few
Pre-existing host immunity	Unlikely	Unlikely except AIDS patients	Yes	Yes	Yes
Other advantages	Can be tissue specific Stable expression	Tissue-specific Stable expression High titre (10^{10} p.f.u./ml)	Transduce non-dividing cells	High titre High transduction efficiency	Stable expression Wild type integrates at specific site
Problems	Possible insertional mutagenesis Silencing of LTR promoter	Possible insertional mutagenesis Ethical considerations	Lytic replication Transient expression Low titre	Possible hepatotoxicity Transient expression Lack of tissue specificity Vector immunogenicity	Loss of specific integration Difficult to purify vector Low titre Possible insertional mutagenesis

(Adapted from Merma and Somia et al., 1997; Thomas, Ehrhardt and Kay, 2003; Tendeloo et al., 2000)

1.3.1.1 Gammaretroviral Vectors

One of the first classes of viral vectors to be developed were those based on gammaretroviruses, previously known as oncoretroviruses. These viral vectors have been the most widely used in clinical trials so far, particularly for *ex vivo* transduction of HSCs. Gammaretroviral vectors are derived from different simple type C murine retroviruses, such as MLV, spleen necrosis virus and Rous sarcoma virus. MLV-based vectors are most frequently utilised (Kootstra and Verma, 2003). These vectors have been modified for safety and to generate space for carrying the therapeutic gene. Important features of gammaretroviral vectors include:

- First, for reasons of safety, the endogenous viral genes necessary for viral replication and budding from the cell are removed.
- Second, viral genes can be replaced by a therapeutic gene and expressed under the control of the long terminal repeat (LTR) promoter.
- Third, the natural viral envelope can be replaced e.g. by those from the gibbon ape leukaemia virus (GALV Env) and the vesicular stomatitis virus (VSV-G) (pseudotyping).

Retroviral vectors have also been modified to incorporate an internal promoter to increase specificity of transgene expression and to improve vector safety. The minimal cytomegalovirus (CMV) promoter, a very strong promoter sequence, was one of the first promoters used in haematopoietic cells. However, it was observed that this promoter could not provide high transduction efficiency and long-term expression of the transgene because of gene silencing (Sutton et al., 1998; Uschida et al., 1998; Case et al., 1999). An alternative promoter element is derived from the polycythemic strain of the spleen focus forming virus (SFFV). This contains a potent murine retroviral enhancer sequence important for gene expression in myeloerythroid haematopoietic cells (Baum et al., 1995). Numerous studies have demonstrated high levels of transgene expression and low levels of gene silencing in haematopoietic cells transduced with gammaretroviral vectors incorporating the SFFV promoter (Grez et al., 1990; Danno et al., 1999; Hildinger et al., 1999; Flasshove et al., 2000). In addition, the development of self-inactivating (SIN) vectors further reduced the possibility of vector mobilisation and recombination with defective retroviruses in the target cells (Yu et al., 1986; Kraunus et al., 2004). Figure 1.4 shows a comparison of the wild type MLV genome and a retroviral vector developed for gene transfer into mammalian cells.

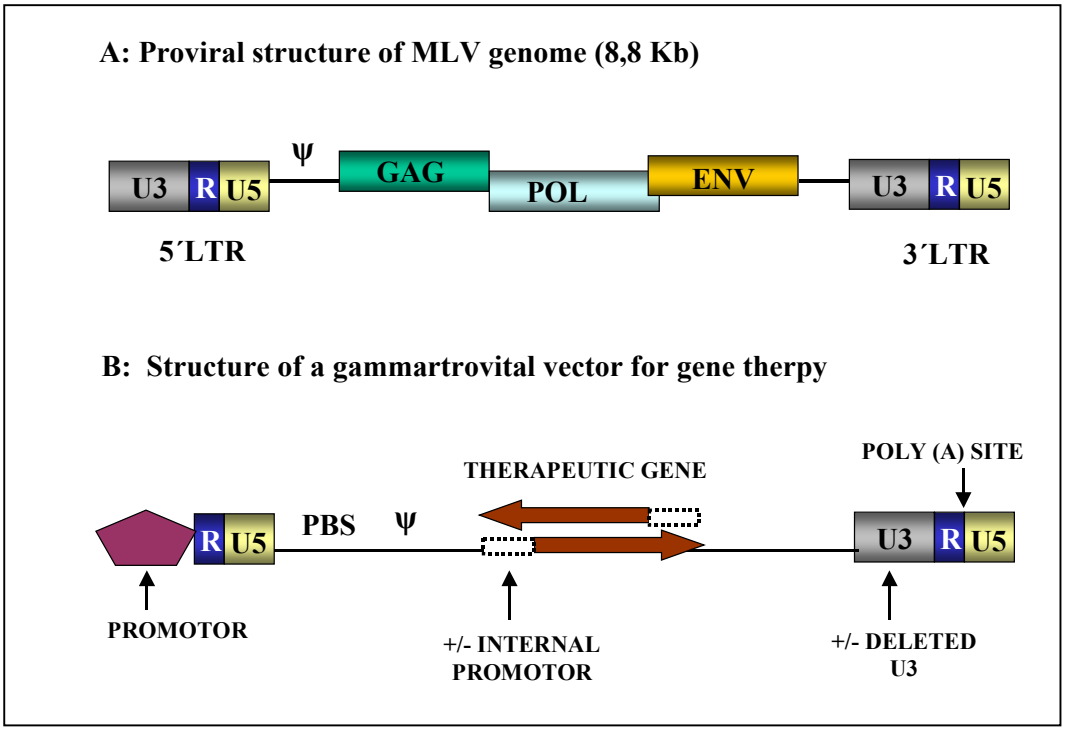


Figure 1.4: Schematic presentation of the proviral structure of the MLV genome (A) and a modified gammaretroviral vector (B). In the MLV provirus, *gag*, *pol* and *env* coding regions are flanked by LTRs. The LTR contains three regions, U3, R and U5. These regions are necessary for reverse transcription, proviral integration and transcriptional activation. ψ indicates the packaging signal. In the recombinant vector (B), the genomic RNA is transcribed using the 5'U3 or an internal heterologous promoter. The primer binding site (PBS) is indicated in the figure (schematic diagram modified from Hu and Pathak, 2000; Pages et al., 2004; Kootstra and Verma, 2003).

Retroviral vectors are packaged into infectious particles from so called packaging cell lines. Such a cell line contains the necessary structural genes *gag*, *pol* and *env* (Tendeloo et al., 2001). The *gag* (group specific antigen) gene encodes the viral core proteins and the *pol* (RNA-dependent DNA polymerase) gene encodes the viral replication enzymes. These two genes are expressed as a Gag/Pol fusion glycoprotein, which is cleaved by a viral protease. The *env* (envelope) gene encodes the viral envelope glycoprotein, which is subsequently cleaved by cellular proteases into the external envelope glycoprotein and transmembrane protein. The constructs encoding these genes lack the packaging signal (ψ) which normally allows the viral RNA to be distinguished from other RNA in the cell. The recombinant vector containing the gene of interest lacks the structural genes *gag*, *pol* and *env*, but has retained the LTR sequences, which are required for viral integration and transcription, and the packaging signal. The packaging signal ψ is located downstream of the 5'-LTR in the construct incorporating the therapeutic gene. This ensures that only the viral genomic RNA encoding the therapeutic gene is

packaged (but not the *gag*, *pol* and *env* genes) into virions to be transferred to the target cells (see Figure 1.5). Once it has been established, the packaging cell line can provide a continuous source of high titre vector (Verma and Somia, 1997; Hu and Pathak, 2000; Buchschacher and Wong-Staal, 2000; Vollweiler et al., 2003; Tendeloo et al., 2001).

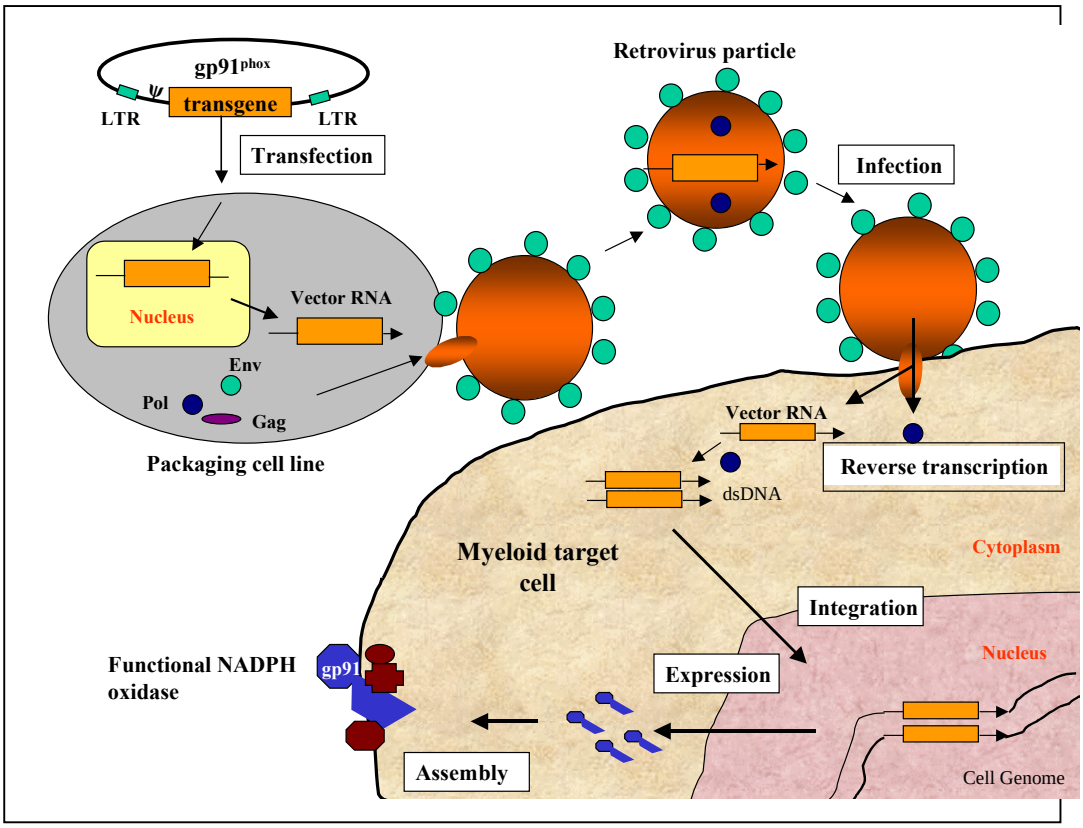


Figure 1.5: Principles of the function of the retroviral packaging cell line and transduction of target cells. The eukaryotic expression plasmid encoding the therapeutic gene (e.g., gp91^{phox} for X-CGD), lacking the essential genes for replication (*gag*, *pol* and *env*) but containing a packaging signal ψ, LTR promoter sequences and enhancer regions, is introduced into the packaging cell line by calcium phosphate transfection. The packaging cell line is constructed such that the viral core proteins (*gag*), envelope (*env*), and polymerase and reverse transcriptase (*pol*) are constitutively expressed and therefore provided *in trans*. These *trans*-acting factors are needed for the packaging of expressed vector RNA into retroviral particles. After transduction of target cells, the transferred vector RNA is reverse-transcribed into double-stranded DNA for random integration into the cell's genome and subsequent expression of the transgene (adapted from Hossle, Seger and Steinhoff, 2002).

For the pre-integration complex to gain access to the chromatin and for efficient transduction, gammaretroviral vectors are strictly dependent on the disruption of the nuclear membrane that occurs during mitosis (Miller et al., 1990). This prevents their application for the transduction of quiescent cells, such as HSCs, muscle, brain, lung and liver cells. For this reason,

there has been considerable interest in the development of vectors such as those based on HIV type 1 (HIV-1), which are able to infect non-dividing cells.

1.3.1.2 Lentiviral Gene Transfer

In contrast to gammaretroviruses, lentiviruses have a more complex genome and, consequently, a more complex replication cycle. Besides the structural proteins Gag, Pol and Env, HIV-1 encodes two regulatory proteins, Tat and Rev, essential for efficient viral expression, and four accessory proteins termed Vpr, Vpu, Nef and Vif (Figure 1.5). The Rev protein interacts with a region of viral RNA known as the Rev-responsive element (RRE). This element controls the amount of spliced RNA and promotes the transport of viral RNA from the cell nucleus to the cytoplasm (Kondo et al., 1995). Modification of the HIV genome with the aim of developing lentiviral vectors started over 15 years ago (Page et al, 1990). Great efforts have been directed towards improving the biological safety of the vectors to minimise the possibility of replication-competent lentivirus production. Similar in design to gammaretroviral vectors, lentiviral vectors are produced by separating the sequences required in *cis* for packaging (packaging signal, *psi*), reverse transcription (primer binding site) and transcriptional control (LTR) from those encoding viral structural and enzymatic proteins (Miller et al., 1993). In addition, the RRE is included in the transfer vector. Three generations of lentiviral vector system have been developed with the view of improving biosafety while retaining properties essential for efficient gene transfer. The first and second generation of packaging constructs still contained *tat* and *rev* genes, and all or some of the accessory elements, although the U3 regions of the 5'- and 3'-LTRs were replaced with a minimal CMV promoter and a polyadenylation signal from the insulin gene, respectively, and part of the packaging sequence was replaced with 5' splice donor and acceptor sites. At this stage, the HIV accessory proteins were demonstrated to be critical fir HIV pathogenesis (Cullen et al., 1998). In order to improve the safety of lentiviral vectors, a so-called third generation of lentiviral packaging systems were constructed. These are devoid of all accessory genes not essential for transduction (except *gag*, *pol* and *env*) in order to prevent viral recombination. In the absence of a functional HIV-LTR and with expression of the transgene being driven by an internal promoter, the presence of *tat* was no longer necessary (Dull et al., 1998; Zufferey et al., 1997 and 1998). Furthermore, the *rev* gene was also deleted from the packaging plasmid and incorporated in a separate construct, generating a four-plasmid expression system (Dull et al., 1998). At the same time, SIN lentiviral vectors were developed to further increase biosafety (Miyoshi et al., 1998, Zufferey et al., 1998). In these vector constructs, the 3'-LTR contains a

133 bp deletion which is transferred to the 5'-LTR during reverse transcription and integration, resulting in elimination of expression from the HIV-1-LTR and allowing expression from an internal promoter. This inactivation design significantly improves the safety of HIV-derived vectors, as it reduces the risk of the formation of replication-competent lentiviruses and hinders recombination with wild type HIV in infected host cells. More recently, another *cis*-acting sequence, the central polypurine tract (cPPT) from the *pol* open reading frame (ORF), was added back into the vector. This sequence has been demonstrated to improve nuclear import of the proviral DNA and subsequently accelerates transduction (Follenzi et al., 2000; Zennou et al., 2000; Demaison et al., 2002).

Like gammaretroviral vectors, the lentiviral vectors have also been modified to incorporate an internal promoter, such as the SFFV promoter for efficient expression in haematopoietic cells. Improvement of transgene expression has also been achieved by the addition of elements that act post-transcriptionally. The incorporation of the post-transcriptional regulatory element (PRE) of the woodchuck hepatitis virus (WPRE) has substantially increased the levels of expression from HIV-1 derived vectors (Huang and Liang et al., 1993; Donello et al., 1996; Zufferey et al., 1999; Demaison et al., 2002). Schematic representations of the HIV-1 wild type genome and a typical SIN-lentiviral vector are shown in Figure 1.6.

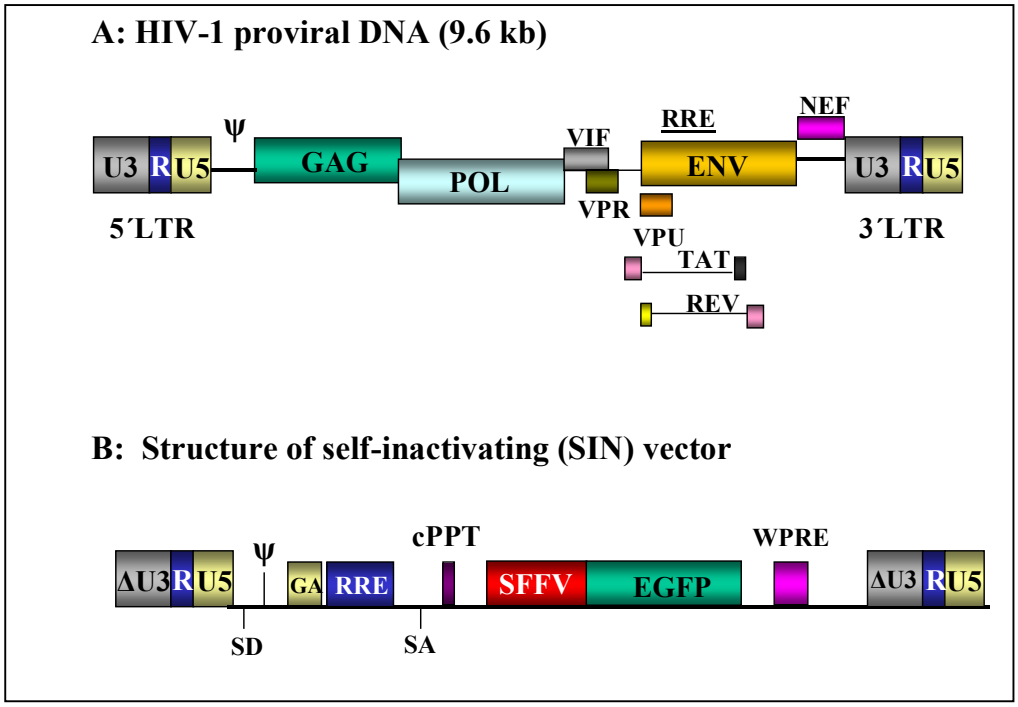


Figure 1.6: Schematic representation of the HIV-1 provirus (A) and a modified SIN-lentiviral vector used in this study (B). Besides the *gag*, *pol* and *env* genes, the HIV-1 proviral DNA contains the six additional genes *vif*, *vpr*, *vpu*, *tat*, *rev* and *nef*, and the RRE element. In the HIV-based SIN-lentiviral vector, the viral *gag*, *pol* and *env* have been replaced by promoter and transgene sequence and are flanked by the viral

LTRs. Packaging of viral RNA genome is ensured by the presence of a packaging signal from the 5' untranslated region of the *gag* ORF. In addition, two *cis*-acting sequences RRE and cPPT are present in the vector. The 3' LTR contains a large deletion in the U3 region (depicted as ΔU3) to prevent transcription from the LTR. A WPRE element downstream from the transgene is used to improve transcription efficiency. The SFFV promoter is used in this example as an internal promoter to drive the expression of enhanced GFP (eGFP) (modified from Kootstra and Verma, 2003; Gaspar et al., 2004).

For the production of lentiviral vector particles, the natural HIV-1 Env glycoprotein has been replaced by the vesicular stomatitis virus glycoprotein (VSV-G), which allows high transduction of haematopoietic cells (Kootstra and Verma, 2003). The VSV-G protein has a broad host range and is expressed from a individual construct during vector production (Naldini et al., 1997; Zufferey et al., 1997). Figure 1.7 summarises the lentiviral vector production with a SIN-vector.

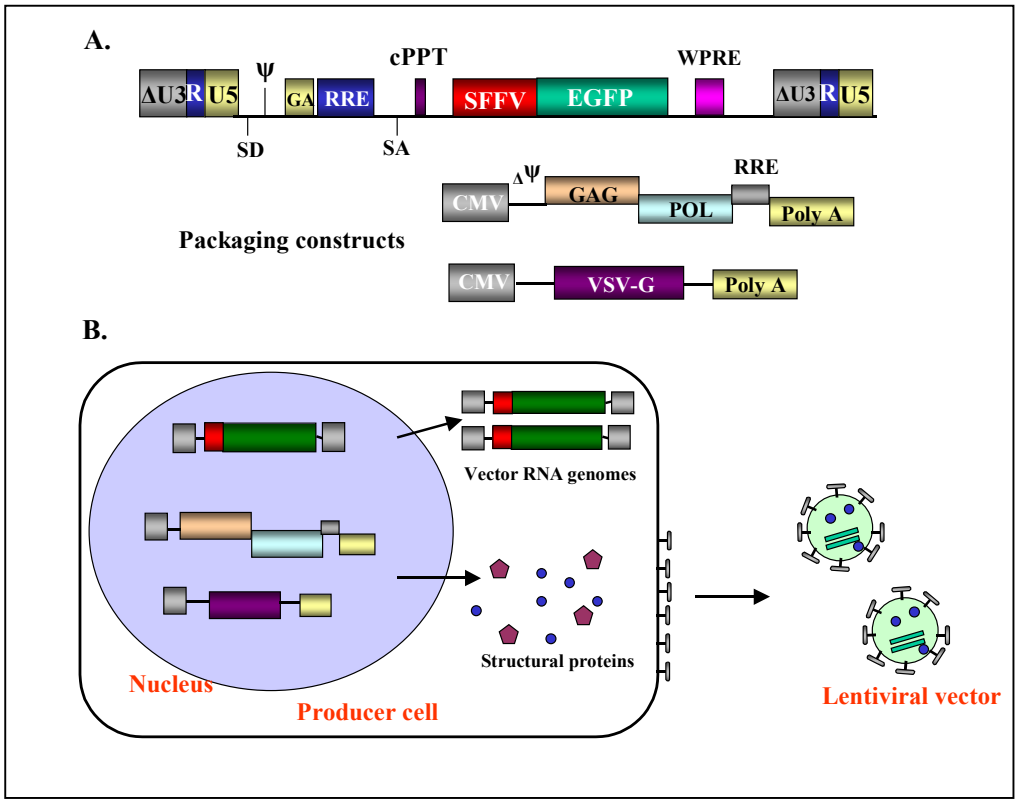


Figure 1.7: Representation of lentiviral vector production: (A) A HIV-based SIN-lentiviral vector (see legend for Figure 1.6). The lentiviral packaging system consists of two constructs encoding Gag/Pol and VSV-G. (B) Lentiviral vectors are produced by transient transfection of the vector constructs together with the packaging constructs into producer cells. Vector RNA is packaged by Gag/Pol precursor proteins at the cellular membrane. Subsequently, the vector particles bud through the cellular membrane, obtaining the viral envelope that contains the VSV-G glycoprotein (modified from Kootstra and Verma, 2003).

1.3.2 CGD as a Gene Therapy Model

An alternative treatment for CGD is the genetic transfer of a functional copy of the defective gene to haematopoietic progenitor cells. Some of the features that make X-CGD an attractive candidate for a gene therapy strategy are (Roos et al., 1992; Woodman et al., 1995, Bu-Ghanim et al., 1995):

- X-CGD is a monogenic disease that is well characterised at the molecular level.
- Constitutive expression of $gp91^{phox}$ is likely to be effective for reconstitution of the NADPH oxidase.
- Transduction and engraftment of haematopoietic stem/progenitor cells should provide a long-term source of functional monocytic cells.
- The level of transduction required for a therapeutic benefit is low, both in terms of protein expression within the cell and the percentage of functional cells. A small population (about 5%) of corrected neutrophils with normal NADPH oxidase activity should be sufficient to correct or at least abrogate the clinical phenotype.
- There are well-established laboratory tests and mouse models available for testing the efficiency of gene transfer vectors *in vivo*.

1.3.2.1 *In Vitro* and *In Vivo* CGD Gene Therapy Experiments

In the past 20 years, *in vitro* systems allowing reconstitution of the respiratory burst oxidase activity in human CGD leukocytes using gene transfer technology have been well established. Various gene transfer vectors and different cell lines have been utilised. Epstein-Barr virus (EBV)-transformed B-cell lines from CGD patients that express small amounts of the NADPH oxidase protein have been used as a model by a number of groups. Respiratory burst activity has been corrected in all 4 genetic subgroups using retroviral and/or plasmid-based vectors containing appropriate cDNA (Maly et al., 1993; Porter et al., 1993; Chanock et al., 1996; Iwata et al., 1998). More recently, a human myeloid X-CGD cell line, generated by gene targeting, has been used in several studies. For example, $gp91^{phox}$ expression and superoxide formation has been quantitatively evaluated in retrovirus-reconstituted cells (Becker et al., 1998; Kume et al., 1994). Similarly successful results were also achieved with retrovirally transduced myeloid progenitors from PB or BM obtained from CGD patients. Functional expression of proviral transcripts was documented *in vitro* using diverse methods to monitor reconstitution of NADPH oxidase activity (Porter et al., 1996; Weil et al., 1997). A recent study demonstrated that

phagocyte superoxide levels was corrected to levels (68.9%) similar to those of normal cells after transduction of human $CD34^+$ marrow cells with a murine stem cell virus-based retroviral vector expressing $gp91^{phox}$ (Grez et al., 2000). In addition, the development of HIV-based vectors has significantly increased the transduction efficiency *in vitro*. One study reported transduction of up to 63% of cells from a human X-CGD cell line using a HIV-1-based lentiviral vector containing $gp91^{phox}$ (Saulnier et al., 2000). Recently, the use of third-generation SIN $gp91^{phox}$ lentiviral vectors allowed correction of superoxide production in transduced $CD34^+$ PB cells from X-CGD patients to 53% of normal levels, which was lower than that obtained with retroviral vectors. However, the lentivirus-transduced stem cells produced higher numbers of gene corrected neutrophils than the retroviral vector-transduced cells when transplanted into immunodeficient mice (Roesler et al., 2002).

The feasibility of a gene therapy approach for X-CGD was revealed in the $gp91^{phox-/-}$ (X-CGD) knock out mouse model. The $gp91^{phox}$-deficient mice were generated by the disruption of the X-linked murine CYBB locus. The mice have a similar phenotype to human CGD in that they lack phagocyte superoxide production and are susceptible to infections with *Staphylococcus aureus* and *Aspergillus fumigatus* (Morgenstern et al., 1997). BM cells from the X-CGD mice were transduced with retrovirus vectors containing the $gp91^{phox}$ cDNA and transplanted into lethally irradiated syngeneic X-CGD recipients. NADPH oxidase activity was detected in 50–80% of circulating neutrophils by the NBT test 12–14 weeks post-transplantation and persisted for at least 18 months; gene-corrected neutrophils were also maintained in secondary recipients (Ding et al., 1996; Dinauer et al., 1999, 2001). A study by Sadat et al. (2003) demonstrated that high levels of NADPH oxidase activity could be achieved in transplanted X-CGD mice using a bicistronic vector containing $gp91^{phox}$ and the ΔLNGFR marker. Additionally, partial reconstitution of NADPH oxidase activity after retroviral gene transfer was shown to improve host protection against bacterial and fungal infections in X-CGD mice, when a sufficient number of cells was corrected (Bjorgvinsdottir et al., 1997; Dinauer et al., 2001).

1.3.2.2 Phase I Clinical Trials of CGD Gene Therapy

A limited number of human clinical trials of gene therapy in CGD patients have been performed in the USA and Germany. Malech et al. (1997, 2000) conducted two clinical trials of *ex vivo* gene therapy to treat five patients with autosomal recessive $p47^{phox}$-deficient and five patients with X-linked $gp91^{phox}$-deficient CGD. Autologous $CD34^+$ stem cells from cytokine-mobilised PB were used as the targets for retroviral-mediated gene transfer of the normal $p47^{phox}$

and gp91^{phox} cDNAs. The mobilised PB CD34^{+} cells were collected by aphaeresis and transduced over a 3-day period *ex vivo*. The transduction rates were 20% for p47^{phox}-transduced cells and 70% for gp91^{phox}-transduced cells. Afterwards, the transduced cells were transfused back into the patients. In eight of ten patients, peak levels of 0.004 to 0.13% oxidase-corrected PB neutrophils and monocytes were observed at 3–6 weeks, with the effect lasting several months per cycle of gene therapy. However, the number of circulating gene-corrected neutrophils declined over time (Malech et al., 1997, 2000). More recently, research groups from Frankfurt am Main, Germany have conducted clinical phase I treatment of 2 adult X-CGD patients by gene transfer. G-CSF mobilised CD34^{+} PB cells were collected and transduced with gammaretroviral vectors containing normal gp91^{phox} cDNA. Five days later, the transduced cells were re-infused into the patients, which had received a non-myeloablative dose of busulfan prior to re-infusion. To date, over 16 months after gene therapy, the treatment has been well tolerated and no adverse effects have been observed in either patient. A significant reconstitution of NADPH oxidase activity was observed in granulocytes from both patients with the NBT and DHR tests. In addition, the corrected cells were able to eliminate bacterial and fungal infections *in vitro* and *in vivo* (Manuel Grez, personal communication).

1.3.3 Drawbacks of Gene Therapy for Inherited Diseases of the Haematopoietic System

The idea of HSC gene therapy is as exciting as that of stem cell transplantation itself. The development of techniques for transferring and expressing genes in HSCs has indicated that new genes can be expressed in repopulating stem cells of multiple species. Optimisation of the vector design, i.e. utilisation of pseudotyped vectors and of lentiviral vectors, has opened up whole new areas of possible clinical applications. In addition, the use of diverse cytokine combinations and fibronectin fragments has greatly improved the transduction efficiency of HSCs (Vollweiler et al., 2003). However, gene therapy has not fulfilled the expectations promised by the press in the early 1980s. There are still some limitations that inhibit the ability to reach and to maintain therapeutically relevant levels of genetically modified cells and their progeny after gene delivery. The major challenges at present are (Hossle et al., 2002):

- Low frequency and quiescent nature of target cells. As described in chapter 1.1, there are only limited numbers of true HSCs (about 1 in 10^6 BM cells), which are in the G_0 phase of the cell cycle with densely packed DNA and an intact nuclear membrane.

- Poor engraftment of transduced cells after cell activation with cytokines. Activated cells have been shown to possess lower engraftment capacity than unmanipulated cells by competitive assay (Qin et al., 1999).
- Lack of selective growth advantage post-engraftment and unpredictable expression of the transgene in transduced cells (see chapter 1.4).
- Unexpected insertional mutagenesis and multiple random integrations.

1.4 Selection of Genetically Modified HSCs

In principle selective expansion of stem cells could be reached by inhibition of cell differentiation, activation of proliferation signals and anti-apoptotic signals, or maintenance of stem cell properties. As mentioned earlier, the lack of a selective growth advantage of genetically modified cells is a major limitation of gene therapy of HSCs. A natural selective advantage of gene-transduced cells occurs in only a very few diseases, for example in the case of X-linked SCID. However, in most other haematopoietic genetic diseases there is an unfortunate competition between transduced and non-transduced cells which leads to low engraftment of therapeutic gene modified cells (Choi et al., 2000). Therefore, introduction of an artificial selection advantage will be needed to increase the number of genetically modified cells *ex vivo* and/or *in vivo*.

Various strategies have been developed to selectively expand gene-modified cells *in vitro* and *in vivo*. A number of selective markers have been used for *in vitro* selection, such as the neomycin resistance gene (neo), murine heat shock antigen CD24, low affinity nerve growth factor receptor (ΔLNGFR) and fluorescent proteins (e.g. green fluorescence protein). Transduced cells can be positively selected through the use of drugs such as G 418, a neomycin analogue, or on the basis of FACS sorting (Dick et al., 1985; Migita et al., 1995; Berger et al., 2003; Kalberer et al., 2000). However, these approaches are seriously limited. Normally the frequency of transduced cells is very low and elimination of all nontransduced cells would probably result in a graft unable to support a normal haematopoiesis. So far, several strategies have been reported for *in vivo* selective expansion of gene-modified HSCs, and these are outlined in Table 1.5. As indicated in Table 1.5, the strategies can be classified into two categories. One involves the transfer of a drug-resistance gene and elimination of non-transduced cells with a relevant cytotoxic compound, normally an anti-tumour agent. The other confers a direct growth advantage on the target HSCs. The latter approach can be divided into two subclasses based on

whether the growth advantage is derived from an overexpressed intrinsic protein e. g HOXB4 gene or a chimeric cytokine receptor controlled by an artificial ligand (Kume et al., 2002).

Category	Strategy	Example
Passive selection	Drug resistance	ABC transporters (e.g. MDR-1)
		DNA repair (e.g. MGMT)
		Folate metabolism (e.g. DHFR variants)
Direct growth advantage	Fusion protein plus drug	Steroid-based (e.g. Oestrogen receptor)
		FKBP-based (e.g. AP20187)
	Intrinsic factor	HOXB4

Table 1.5: Strategies for *in vivo* expansion of genetically modified cells. MDR, multidrug resistance gene; DHFR, dihydrofolate reductase gene; MGMT, O^6-methylguanine-DNA methyl-transferase gene; FKBP, FK506-binding protein 12; HOXB4, HoxB4 transcription factor; ABC, ATP-binding cassette (modified from Kume, 2002; Milsom and Fairbairn, 2004)

1.4.1 The Homeobox B4 Transcription Factor as a Candidate for Selective Expansion of Gene-Modified HSCs

Various complex combinatory effects of external and internal signals regulate the proliferation and differentiation of haematopoietic cells. The regulatory machinery includes haematopoietic growth factors (cytokines), cell cycle regulators and transcription factors. The function of transcription factors is to bind to specific DNA sequence motifs upstream of the target gene and regulate its transcription, resulting in RNA transcripts. The homeobox (HOX) transcription factors are recognised as important players in murine and human haematopoiesis.

There are two main groups of HOX genes in mammals, class I and class II. There are 39 class I HOX genes known, organised in four clusters, A–D, each cluster containing 9–11 genes on four different chromosomes (Scott, 1992; Boncinelli et al., 1989; Grier et al., 2005). Figure 1.8 shows the organisation of the HOX clusters. All haematopoietic progenitors express HOX genes in a pattern characteristic for the lineage and stage of differentiation of the cell. For example, at least 22 of the 39 HOX genes are expressed in human $CD34^+$ cells (Sauvageau et al., 1994; Lawrence and Largman, 1992). The function of HOX genes in haematopoiesis has been extensively investigated using overexpression strategies. It has been reported that retroviral overexpression of HOXA5, HOXA9 and HOXA10 leads to perturbation of myeloid and/or lymphoid haematopoiesis, both in murine and human haematopoietic cells (Crooks et al., 1999; Lawrence et al., 1997; Thorsteinsdottir et al., 2002; Schiedlmeier et al., 2003).

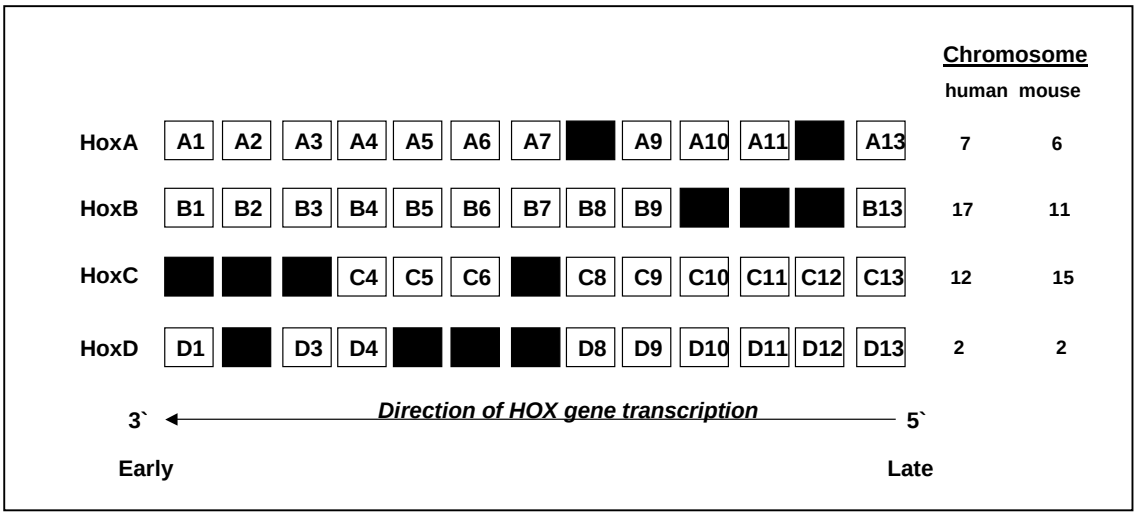


Figure 1.8: Schematic representation of the chromosomal organisation of mammalian HOX genes. Groups 1–13 are called paralogue groups and share high sequence homology in the homeodomain. Black boxes indicate the lack of a gene (modified from Grier et al., 2005).

The two HOX genes from the HOXB group, i.e. HOXB3 and HOXB4, are strongly expressed in BM cells enriched for pluripotent progenitors (Giampaolo et al., 1994). Even though expression patterns for these two genes during haematopoiesis are similar, results from retroviral-engineered overexpression are clearly contrasting. In murine BM cells, overexpression of HOXB3 causes a myeloproliferative disorder that can give rise to myeloid leukaemia and disruption of lymphoid differentiation, increased granulopoiesis and a reduced number of pro-B clonogenic progenitors (Sauvageau et al., 1997). However, most reports demonstrate that overexpression of HOXB4 does not result in a malignant phenotype but instead strongly enhances the regenerative capability of the stem cell pool. When overexpressed by retroviral gene transfer, HOXB4 showed an enhanced ability to regenerate the most primitive stem cell compartment in serial transplantation studies. This resulted in a 50–80 fold increase in the number of transplantable pluripotent HSCs in both primary and secondary recipients (Sauvageau et al., 1995; Thorsteinsdottir et al., 1999; Antonchuk et al., 2001). Additionally, HOXB4-mediated *ex vivo* expansion of adult murine HSCs resulted in 1000-fold higher levels relative to controls and a 40-fold net increase in HSCs. This demonstrated that the unique effects of HOXB4 on HSC regeneration are greater than any other known regulatory factor or cytokine combination. A recent study has also shown that HOXB4 can induce rapid *ex vivo* expansion of transduced HSCs (Antonchuk et al., 2002) and the use of a soluble recombinant HOXB4 protein led to strong *ex vivo* expansion of HSCs (Krosl et al., 2003). A study of mice deficient in the HOXB4 gene demonstrated a clear connection between this gene and the stem cell compartment since HOXB4 deficiency led to reduced regeneration of LTR-HSCs (Björnsson et al., 2001).

These observed actions have potential important clinical implications and could be utilised to correct the low number of HSCs after BM cell transplantation. However, Schiedlmeier et al. (2003) reported that, in addition to expansion of the CB stem cell pool, strong ectopic expression of HOXB4 hampered myeloerythroid differentiation and caused a reduction in B-lineage output in transplanted NOD/SCID mice. In this study, modified FMEV-based retroviral gene transfer vector was used for transduction of human CB cells, resulting in 7-fold higher HOXB4 levels than originally described by Sauvageau et al. (1995). However, a study by another group using a MSCV-based retroviral vector showed that enforced HOXB4 expression increased the number of primitive human repopulating cells by 3–4 fold without perturbing the lymphomyeloid reconstitution *in vivo* (Buske et al., 2002). Therefore, the suitability of HOXB4 for enhancing the stem cell pool in therapeutic applications appears to depend upon the level of expression.

The molecular mechanisms governing HOXB4 expression are unknown, but essential DNA binding sites have been identified in the HOXB4 promoter region. The upstream stimulating factors (USF)-1 and -2 bind to this sequence and induce proliferation in response to cytokines that stimulate self-renewal rather than differentiation (Giannola et al., 2000). A more recent study using the FDCP-mix cell line demonstrated that ectopic overexpression of HOXB4 can elicit an *in vitro* differentiation delay in murine BM cells, a process which might be essential for HSC expansion, but may not be easily detectable *in vivo* (Milsom et al., 2005).

1.4.2 Drug Resistance Genes as Selective Cassettes for Gene-Modified HSCs

The idea for this strategy comes from marrow protection in cancer patients undergoing treatment with anti-cancer drugs. The principle of the method is shown in Figure 1.9. Cells expressing a drug resistance gene would tolerate the selective pressure of the relevant cytotoxic drug, whereas non-transduced cells of sensitive tissues would be eliminated. The relative number of transduced cells would thereby increase. A number of vectors have been developed to transfer various drug-resistance factors into HSCs with a view to protecting the BM compartments against collateral toxicity (Rafferty et al., 1996; Milsom and Fairbairn, 2004). The most extensively studied drug resistance gene is the multidrug resistance gene 1 (MDR-1), which confers resistance to a variety of chemotherapeutic drugs, such as anthracyclines. Clinical trials of MDR-1 gene therapy have been carried out and are characterised by low transduction frequencies in repopulating cells and short-term engraftment of MDR-1-positive cells. However, protection of the gene-transduced cells from chemotherapy-induced myeloablation could be achieved in some patients (Hanania et al., 1996; Hesdorffer et al., 1998; Cowan et al., 1999;

Moscow et al; 1999). Other drug resistance genes, including mutants of dihydrofolate reductase (DHFR) and O^6-methylguanine-DNA methyltransferase (MGMT), are also utilised in preclinical trials for selection of gene-modified HSCs (Allay et al., 1998; Sawai et al., 2001).

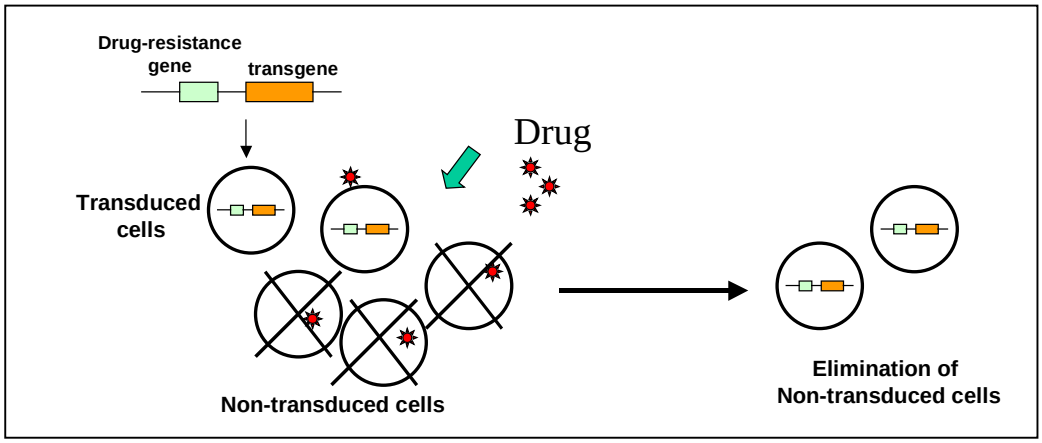


Figure 1.9: Principle of passive expansion. Non-transduced cells are eliminated by cytotoxic drugs, whereas cells carrying a drug-resistance gene survive and repopulate.

Although attractive in theory, there are significant drawbacks to this approach. No true expansion of the desired transduced cells is achieved, only elimination of non-transduced cells. An elimination of >95% of non-transduced cells would result in aplastic anaemia. Additionally, the host would be exposed to recurrent cycles of chemotherapy with the accompanying side effects on non-haematopoietic cells.

1.4.3 Direct Growth Advantage with Chimeric Receptors from Cytokine or Growth Factor Receptors

A less toxic approach to expand genetically modified cells is to confer a direct growth advantage to transduced cells (Figure 1.10). For this purpose, conditional systems that regulate the fate of genetically modified cells based on protein dimerization have been developed. Protein dimerization is an important physiological process in mammalian cells and controls many behaviours, such as proliferation, differentiation and apoptosis (Scott, 2000). The conditional system consists of two main members: a fusion protein and a drug (or hormone). The fusion protein contains a growth factor receptor signalling domain which is linked to one or more drug-binding sites (or hormone receptor). By administering a small synthetic molecule (chemical inducer of dimerization, CID), therapeutically relevant expansion of the target cells can be attained. The principle and mechanism of this positive selection is outlined in Figure 1.10. The

treatment with the dimerizer could be performed repeatedly over time with an optimal titred dose and its effect must be reversible (Ito et al., 1997; Matsuda et al., 1999; Milsom 2004).

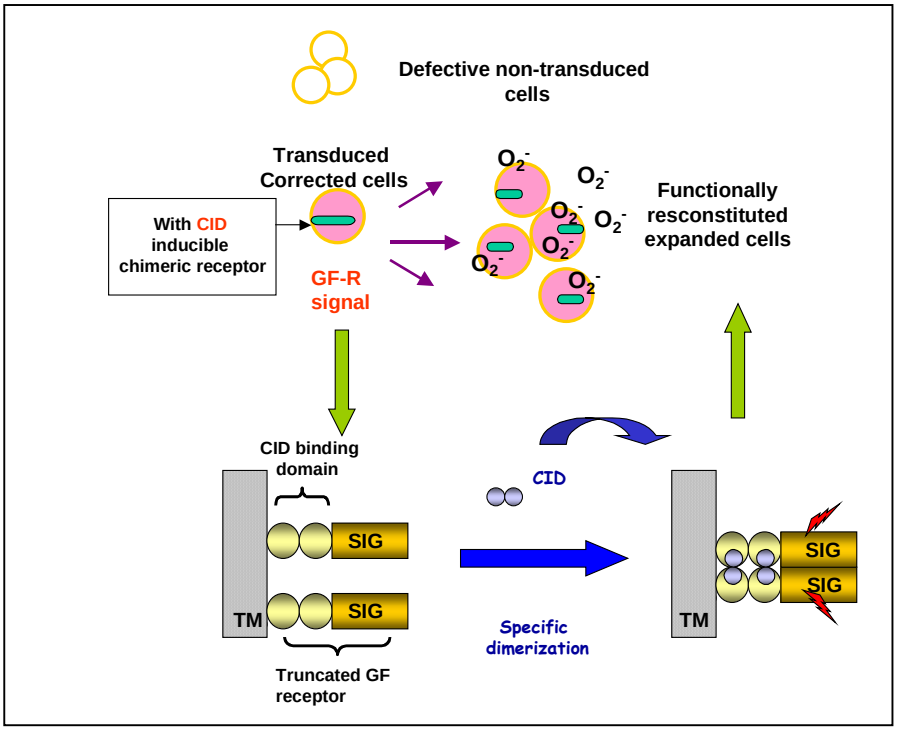


Figure 1.10: Schematic representation of dimerizer-induced selective expansion of transduced cells. Corrected cells, transduced with the therapeutic gene, are co-transduced with a fusion protein containing the signal transduction domain (SIG) from a truncated growth factor receptor (GF-R) and a CID binding domain. The specific binding of the CID to its domain causes dimerization of the SIG, giving rise to transduced cell proliferation. This can be detected by e.g. expression of the $gp91^{phox}$ protein and its product O_2^- (modified from Hossele et al., 2002).

The most intensively researched fusion protein used in haematopoietic cell lines and murine BM cells is derived from the truncated G-CSF and oestrogen binding sites. Binding of oestrogen (or tamoxifen in the case of the tamoxifen-responsive mutant of the oestrogen receptor) leads to activation of the G-CSF receptor (Xu et al., 1999; Matsuda et al., 1999). Oestrogen-dependent growth has been observed in transduced IL-3-dependent BaF/3 cells and primary murine BM progenitors (Ito et al., 1997). Selective expansion has also been demonstrated in primary and secondary BM recipients in mouse transplantation studies (Kume et al., 2003). Additionally, following *in vivo* administration of oestrogen in non-human primates, selection for gene-modified cells was achieved in one of two animals (Hanazono et al., 2002). Recently, tamoxifen-inducible, *in vitro* expansion of haematopoietic cells following transduction with a chimeric version of the human thrombopoietin receptor (hmpl) has been reported (Nagashima et al., 2003).

Another extensively characterised system is based on the immunolipophilin FKBP12, which binds the immunosuppressive agent FK506. The fusion protein is cross-linked by a CID, which leads to activation of the signalling transduction domain. Early work, using an FK506-binding domain (FKBP) fused to the signalling domain of the erythropoietin receptor, showed a significant growth-factor dependent selective effect in haematopoietic cell lines (Blau et al., 1997). Furthermore, a mutant version of FKBP (F36V) was developed which binds derivatives of FK506 (e.g. AP20187) that can not bind to endogenous FKBP12 (see web page www.ariad.com). Use of a protein containing F36V fused with the signalling domain of hmpl led to a marked selective expansion of gene-modified haematopoietic progenitor cells *in vitro*, and the effect was superior to that of a fusion protein containing the G-CSF receptor and flat-3 (Jin et al., 1998; Zheng et al., 2001). Clear evidence of selection could also be found in *in vivo* murine studies using the hmpl dimerizer system (Jin et al., 2000). In this study, the murine haematopoietic cells were expanded by introduction of a fusion protein consisting of FKBP and the hmpl cytoplasmic domain. In serial samples of BM taken before and after drug treatment of animals, an increased number of gene-modified GM-CFCs was observed post-selection; however, selection was not based on true stem cells. Experiments in large animals using this system have also shown clear evidence for selection of gene-modified (GFP^+) cells, although expression was transient and mostly restricted to the erythroid and platelet compartments where hmpl signalling plays an important role (Neff et al., 2002).

1.5 Epidermal Growth Factor Receptor and Prolactin Receptor

As described above, data from *in vitro* and *in vivo* experiments have so far not indicated a true expansion of HSCs through the use of dimerizer systems with a signalling domain from the G-CSF, thrombopoietin (mpl) or other receptors. Therefore, we looked for other signalling domains which may induce selective expansion of early HSCs with the dimerizer system. Two growth factor receptors, the epidermal growth factor receptor (EGFR) and the prolactin receptor (PrlR) were used in our studies,. These growth factor receptors belong to the family of cell surface receptors, which integrate a multitude of extracellular signals such as environmental stresses, growth factors, neuropeptides or hormones, and thereby regulate a large diversity of signalling pathways and cell responses. The structure, signalling pathway, and physiological, pathophysiological functions of both receptors are explained in following text.

1.5.1 Epidermal Growth Factor Receptor

The EGFR is a well-known signal transducer that has been well conserved during evolution. It induces a wide range of cellular processes, including cell fate determination, proliferation, cell migration and apoptosis (Carpenter et al., 2000). The activity of the EGFR is subject to modulation by multiple positive and negative regulators and is transduced into the nucleus by several conserved signalling cassettes.

1.5.1.1 Structure and Signalling Pathway of EGFR

The EGFR belongs to a subgroup of cell surface receptors, the receptor tyrosine kinases (RTKs), which possess an intrinsic tyrosine kinase activity. The RTK subgroup consists of four members: EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3 and HER4/ErbB4. The EGFR was the first cell surface signalling protein and proto-oncogene to be characterised (Ullrich et al., 1984). All members of the EGFR family are composed of an extracellular ligand-binding domain, a single membrane-spanning domain and a cytoplasmic domain containing a conserved protein tyrosine kinase core, flanked by regulatory sequences (Schlessinger, 2002) (Figure 1.10). The extracellular domain has two cysteine-rich domains, CRI and CRII, which are responsible for ligand binding. Three conserved sequence domains have been recognised within the cytoplasmic region. The juxtamembrane region is required for feedback reduction by the protein kinase. It is followed by the tyrosine kinase domain, a Src homology domain 1 (SH1). This is the most conserved region among the EGFR proteins and it mediates auto-phosphorylation of the carboxy-terminal tail on six tyrosine residues. EGFR can be activated by a large group of EGF-related growth factors, such as EGF and transforming growth factor α (TGF-α). Binding of EGF to EGFR leads to the dimerization of the receptor to form a complex containing two ligand molecules and two receptor molecules. This activates several conserved signalling cassettes that are required to transmit the signal from the receptor to the nucleus or to the cytoplasm. The first important pathway to be activated is the Ras-mitogen-activated protein kinase or MAP kinase pathway. When this pathway is activated, the SOS guanine nucleotide exchange factor is recruited to the plasma membrane via adaptors such as Grb2 and Drk. SOS stimulates the exchange of GTP to GDP on the small G-protein Ras. Subsequently, activated Ras stimulates the MAP kinase pathway to promote cell proliferation. Phospholipase Cγ (PLCγ) and phosphatidylinositol 3-kinase (PI3-kinase) can also transduce the EGFR activity responsible for cell motility (Bogdan and Klämbt, 2001). Figure 1.11 presents the structure and the major signalling pathways of EGFR.

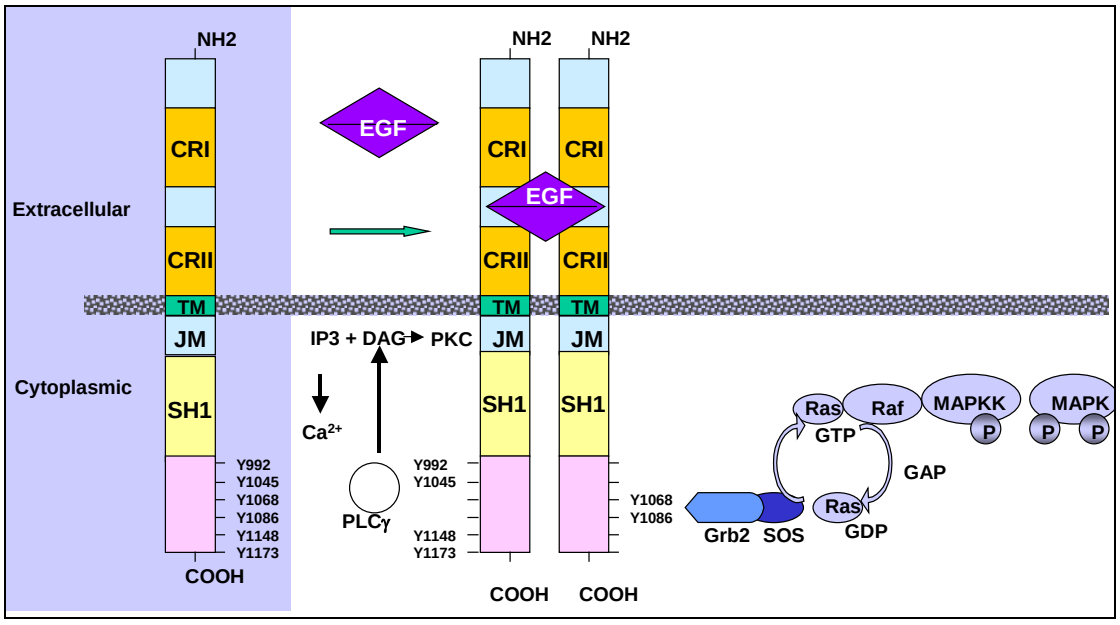


Figure 1.11: Schematic representation of EGFR structure and signalling transduction pathways. At the plasma membrane, the non-stimulated EGFR is present as a monomer of 1186 amino acids (left). Upon binding of extracellular ligand, EGFR undergoes dimerization resulting in *trans*-autophosphorylation of its cytoplasmic domain. EGFR activity is transduced to the cell via cascades such as the Ras-Raf-MAP pathway, PLCγ and PI3-kinase (see text). The positions of 6 tyrosine residues are shown. CR, cysteine-rich domain; TM, transmembrane region; JM, juxtamembrane region (modified from Bogdan and Klämbt, 2001).

1.5.1.2 Physiological and Pathophysiological Roles of EGFR

The basic functions of the pleiotropic signal transducer EGFR execute multiple roles during cell fate determination, differentiation, proliferation and apoptosis. This is evident from the analysis of genetically modified mice. Mice lacking EGFR usually die during the first postnatal weeks as a result of respiratory problems. They also show gastrointestinal phenotypes, thin skin and hair-follicle defects (Miettingen et al., 1995; Sibilia and Wagner, 1995).

However, various studies have made clear that EGFR overexpression or mutations contribute to the development of many human cancers. The ErbB1 protein is a prototype of the EGFR family and its identification provided one of the first links between an activated oncogene and the development of cancer. The expression status of ErbB1 is also changed in a large number of tumours and it has been used as a prognostic marker for many years (Olayioye et al., 2000). As many as 77% of colorectal cancer cases demonstrate a significant overexpression of EGFR (Vallbohmer and Lenz., 2005). In human breast cancer, gene amplification of another member of the EGFR family (HER2) is correlated with a shorter overall survival and relapse-free survival (Slamon et al., 1987). Many studies have demonstrated a role of EGFR mutants in tumour

development. Several deletions in the extra- and intracellular domains have been found in glioblastomas (Ekstrand et al., 1992), non-small-cell lung carcinomas (Garcia de Palazzo et al., 1993), breast cancer (Wikstrand et al., 1995) and ovarian carcinomas (Moscatello et al., 1995).

In general, the EGFR is thought to be either not expressed or expressed at low levels in haematopoietic cells. A recent study has shown that it is expressed at a low level in the murine cytokine-dependent haematopoietic cell line FDC-P1 but not in the murine IL-3 dependent cell line FL5. EGF induces a mild effect on DNA synthesis and ERK activation in EGFR-positive FDC-P1 cells but not in EGFR-negative FL5.12 cells (Shelton et al., 2005). A direct correlation between EGFR and haematopoietic cells cancers has not been observed.

1.5.2 Prolactin and its Receptor

The natural ligand of PrlR, prolactin (Prl), is a paradoxical hormone. It is secreted by the anterior pituitary gland and was originally identified by its ability to stimulate the development of the mammary gland and lactation (Freeman et al., 2000). Prl has a similar amino acid sequence to the two other peptide hormones, growth hormone (GH) and placental lactogen (PL) (Goffin et al., 1996). More recently, based on both molecular and functional evidence from studies of the immune system, the Prl/GH/PL hormones have been linked to a further extended family of proteins, referred to as haematopoietic cytokines (Horseman and Yu-Lee, 1994). The functions of PRL are effected through binding to its receptor. The PrlR is a single membrane-bound protein belonging to the class I cytokine receptor subfamily (Bazan et al., 1990). Just like their respective ligands, the PrlR and growth hormone receptor share several structural and functional features despite their low (30%) sequence homology (Goffin et al., 1998). The PrlR structure is explained below.

1.5.2.1 Structure and Signalling Pathway of PrlR

PrlR is a membrane-bound protein and contains an extracellular domain, a transmembrane domain and an intracellular domain. Because PrlR can be transcribed from different alternative promoters and from diverse splicing of non-coding and coding exons, various isoforms have been described from different tissues (Ali et al., 1991; Davis and Linzer, 1989). Although the length and composition of the cytoplasmic domains of PrlR are different, the extracellular domains are identical (Lesueur et al., 1991) (Figure 1.11). Three major PrlR isoforms, termed short (291 aa), intermediate (393 aa) and long (591 aa), have been detected in rats. For mice, one long and three short forms have been reported (Clarke and Linzer, 1993;

Bole-Feysot et al., 1998). The extracellular domain of PrlR can be divided into an NH_2-terminal D1 containing the conserved feature of two pairs of disulphide bonds (Cys-) and a membrane-proximal D2 subdomain with a "WS motif". Each PRL molecule has two binding sites and activation of PrlR involves ligand-induced sequential receptor dimerization (Bole-Feysot et al., 1998). The role of the 24 amino acid transmembrane domain in activation is not clear. The intracellular domain is a key player in the signal transduction pathway and differs in length and composition among the various isoforms. However, there are two relatively conserved regions, the proline-rich motif region Box 1 and the less conserved Box 2.

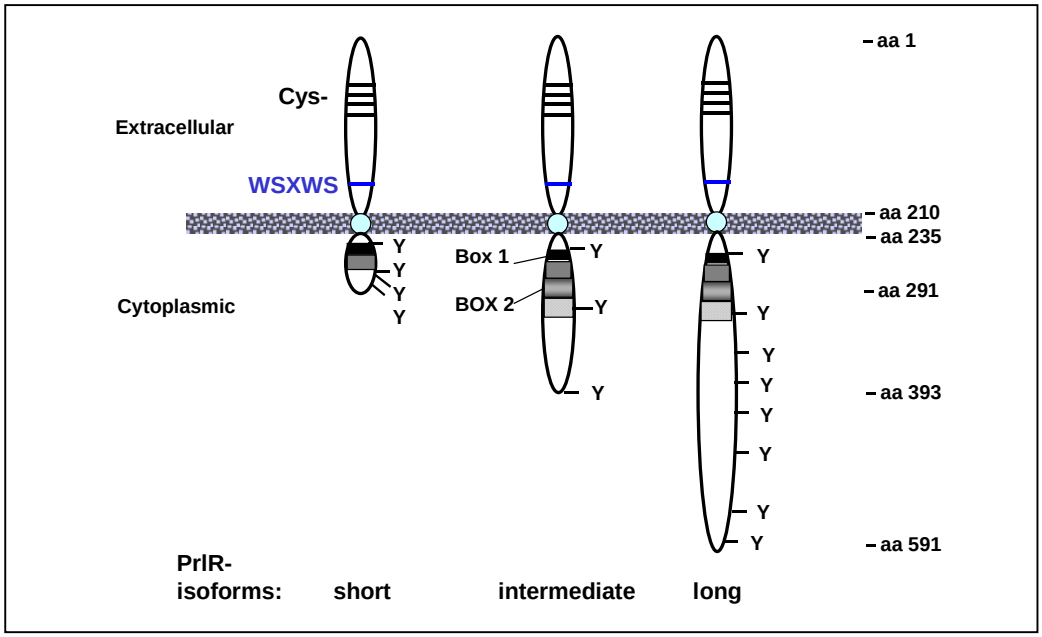


Figure 1.12: Structure of the rat PrlR and isoforms. The short and long isoforms result from alternative splicing, while the intermediate form represents a deletion mutant of the long isoform. Tyrosine residues are indicated by –Y, cysteine residues by Cys- and the tryptophan-serine-X-tryptophan-serine motif by "WSXWS". aa, amino acids (modified from Clevenger et al., 1998; Bole-Feysot et al., 1998).

The function of the Prl/PrlR complex is mediated by the receptor-associated signalling proteins. In contrast to the EGFR, the PrlR *per se* lacks intrinsic enzymatic activity. Ligand-induced dimerization of the PrlR complex serves to activate the associated signalling cascades, which include (Freeman et al., 2000; Clevenger et al., 1998; Bole-Feysot et al., 1998):

- JAK/STAT pathway: STAT1, STAT3, STAT5a and STAT5b, members of the signal transducer and activator of transcription family, are the central transducer molecules of the signal transduction pathways initiated by PrlR activation (Goffin et al., 1998). Binding of PRL to its receptor activates phosphorylation of the STAT proteins by the receptor-associated JAK kinase, triggering their dimerization. Finally, the STAT dimers translocate into the nucleus and activate a STAT DNA-binding motif in the promoter of a target gene.

- Mitogen-activated protein kinase (MAPK) cascade: Phosphorylation of JAK2 kinase enables the association of the signalling adaptor protein SHC with PrlR, which in turn activates the Shc/Grb2/Vav/Sos/Ras/Rac/Raf/MEK/MAPK signalling cascades. This triggers activation of a wide range of transcription factors.
- Src kinase: PRL also induces the activation of members of the Src kinase family, such as Fyn, which is involved in the tyrosine phosphorylation of PI3-kinase.

A schematic representation of major signalling transduction pathways induced by the Prl/PrlR complex is shown in Figure 1.13.

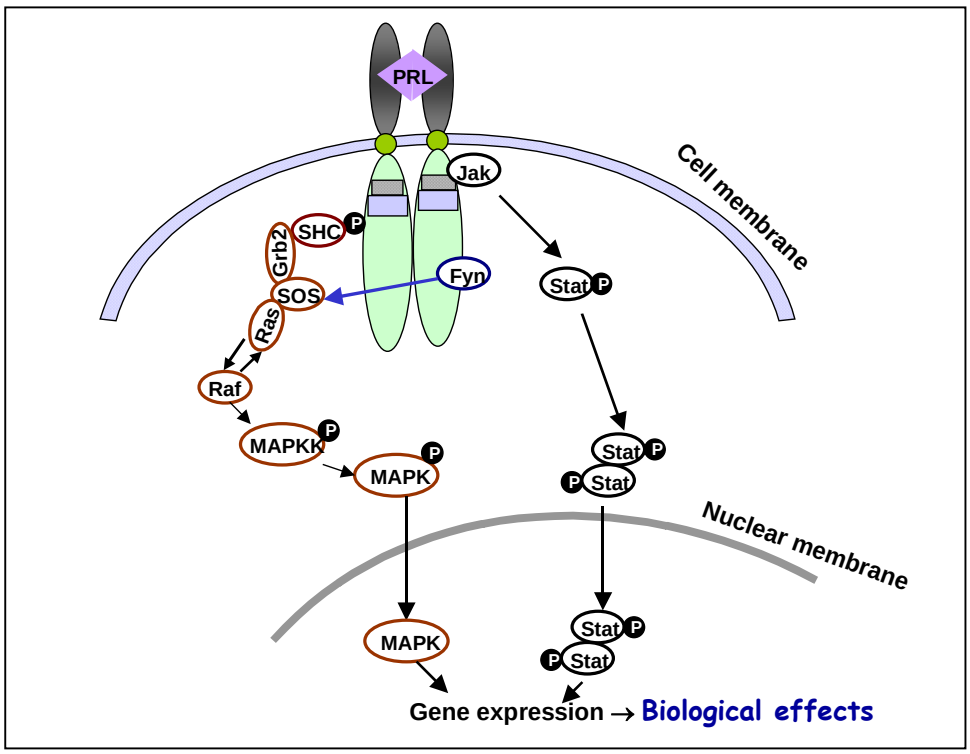


Figure 1.13: Signal transduction pathways initiated by PrlR activation. Ligand-induced activation of the PrlR complex has been found to activate the JAK/STAT (black), Shc-MAPK (red) and Fyn (blue) pathways.

1.5.2.2 Functions of Prl/PrlR in Haematopoiesis

Prl, historically known as the pituitary hormone of lactation, has now been found in more than 300 disconnected actions, which could be correlated to the quasi-ubiquitous distribution of its receptor. It has been demonstrated that this hormone has numerous and various functions attributed to six broad categories: (i) reproduction and lactation, (ii) growth and development, (iii) endocrinology and metabolism, (iv) brain and behaviour, (v) immunomodulation and (vi) electrolyte balance (Bole-Feysot et al., 1998).

The effect of PRL on the immune system is similar to that of cytokines and was first described in avian and murine species. In the presence of mitogen, PRL can act as a co-mitogen

for T- and B-cells of human or murine origin, and also for NK cells and macrophages (Nagy and Berczi., 1991; Skwarlo-Sonta, 1990; Matera et al., 1992). In human studies, a transient, but significant reduction in the overall response of isolated PB lymphocytes to mitogens was observed following a decrease in PRL levels (Devins et al., 1992; Bailey and Burchett, 1997). However, data from Prl/PrlR double knock out mice revealed no obvious defect in the haematopoietic system, and the haematopoietic organs, such as PB, spleen, thymus and lymph nodes, revealed normal T- and B-cell numbers. This evidence indicates that PRL alone is not required for the development of a normal number of immune system cells (Horseman et al., 1997; Ormandy et al., 1997).

Despite the fact that it does not play a key role in the haematopoietic system, evidence indicates that Prl and its receptors exist in many types of BM-derived cells, such as T-cells, B-cells, NK cells, monocytes and granulocytes (Sabharwal et al., 1992). *In vivo* experiments have indicated that the physiological level of PRL can correct the fatal anaemia in hypophysectomised rats induced by administration of PRL antibodies, and restore normal myeloid and erythroid parameters (Nagy and Berczi, 1991). In normal mice, administration of recombinant human PRL (r-hPrl) can increase splenic haematopoiesis with a small, but significant increase in BM progenitors (Woody et al., 1999) and promote T-cell function (Murphy et al., 1993). Syngeneic BM transplantation in mice has shown that Prl can accelerate myeloid, erythroid and platelet recovery in the BM, spleen and PB, as well as thymic recovery. PRL can also enhance myeloid and erythroid recovery following myelosuppression induced by reverse transcriptase inhibitors, such as AZT (Woody et al., 1999). *In vitro* data from BM stromal cells, which have been shown to both produce PRL and express PrlR, has demonstrated the promoting function of PRL in haematopoiesis (McAveney et al., 1996).

Activation of the PrlR in the haematopoietic system also leads to biological effects, such as cell proliferation, survival and inhibition of apoptosis via the signal transduction pathways described above (see chapter 1.5.2.1). Evidence for PrlR expression and a direct action of Prl on haematopoietic stem and/or progenitor cells is less clear. One group has demonstrated that the $CD34^+$ population expresses PrlRs and addition of PRL to these cells in colony assays enhanced the growth of granulocytic and erythrocytic progenitors (Bellone et al., 1995). In contrast, another group using r-hPrl in mouse BM cells did not find any evidence of Prl effects on progenitor numbers (Woody et al., 1999). Further experiments are necessary to investigate the contribution of Prl/PrlR to haematopoietic stem/progenitor cell function.

2. Goals of The Present Study

Low transduction efficiency *ex vivo* and the disappearance of gene-modified transplanted HSCs *in vivo* are still critical issues that need to be addressed before gene therapy approaches can be used to correct genetic defects. The aim of this project was to develop a positive selection system to expand gp91^{phox} gene-modified murine HSCs *in vitro*, *ex vivo* and *in vivo*. This selection should be inducible to allow controllable expansion of the cells.

At first, a positive selection system based on the homeobox transcription factor HOXB4, an essential factor for stem cell expansion, was created. A bicistronic gammaretroviral vector, coding for HOXB4 and the therapeutic gene gp91^{phox}, was designed and its influence on cell proliferation and selective expansion of transduced HSCs was analysed. The ability of these cells to contribute to normal haematopoiesis *in vivo* was then tested. Most importantly, the amount of gp91^{phox}-transgene expression in the peripheral blood of transplanted mice, indicating successful gene correction, was investigated

The second strategy aimed to produce inducible expansion of gp91^{phox}-transduced HSCs. For this purpose, a dimerizer system based on fusion proteins containing two FBKP12 drug-binding domains and the intracellular signalling domain of the EGF or PrlR was designed. The following tasks were performed:

a) Construction of a lentiviral vector containing the FKBP12 and signalling domains of the EGF or PRL receptor and examination of the resulting inducible/reversible cell proliferation and transgene expression in transduced BaF/3 cells.
b) Generation of a bicistronic gammaretroviral vector containing the tested dimerizer system and therapeutic gp91^{phox} gene, and assessment of the effect on cell proliferation and gp91^{phox} transgene expression in transduced BaF/3 cells.
c) Analysis of the influence on selection capacity of Sca1^{+} murine haematopoietic stem/progenitor cells *ex vivo*. Furthermore, transgene expression, normal multilineage differentiation and polycolonality of the *ex vivo*-expanded HSCs were determined after selection.
d) Assessment of cell engraftment and gene transfer efficiency in peripheral blood, spleen and bone marrow cells in mouse transplantation models 8 weeks post transplantation.

3. Materials and Methods

3.1 Materials for Molecular Biological Experiments

3.1.1 Equipments and Necessary Materials

ACL^{TM}-analysis system	Instrumentation Laboratories, Lexington, MA, USA
analysis balance	Sartorius, Göttingen, Germany
autoclave	Integra Biosciences, Baar, Switzerland
autoradiographie-film (Hyperfilm^{TM} MP)	Amersham Pharmacia, Little Chalfont, UK
incubator	Heraeus, Hanau, Germany
electrophoresis chamber	
- for agarose gel (Horizon 11-14)	Life Technologies, Karlsruhe, Germany
- for SDS-PAGE (Mini Protean 3)	Bio-Rad, Munich, Germany
- for EMSA	Bio-Rad, Munich, Germany
FACS-Scan	Becton Dickinson, Heidelberg, Germany
FACS-Calibur	Becton Dickinson, Heidelberg, Germany
fluorescence microscope	Nikon, Düsseldorf, Germany
tissue culture flask	Greiner, Frickenhausen, Germany
hot plate	Techne, Princeton, NJ, USA
magnetic particle concentrator	Dynal, Hamburg, Germany
needle	Becton Dickinson, Heidelberg, Germany
cell strainer (100 µm, 40 µm)	Becton Dickinson, Heidelberg, Germany
cryocontainer	Nalgene, Rochester, NY, USA
cryotube	Nalgene, Rochester, NY, USA
light microscope	Zeiss, Göttingen; Leica, Wetzlar, Germany
PCR-block (GeneAmp 2400)	Perkin-Elmer, Weiterstadt, Germany
pH-meter	Knick, Berlin, Germany
pipettes	Becton Dickinson, Heidelberg, Germany
pipette helper	Hirschmann Laborgeräte, Eberstadt, Germany
reaction tube	Eppendorf, Hamburg; Sarstedt, Nümbrecht, Germany
injection	Becton Dickinson, Heidelberg, Germany

stericup-filter	Millipore, Eschborn, Germany
sterilbank	Heraeus, Hanau, Germany
sterilfilter (0,22 µm; 0,45 µm)	Millipore, Eschborn, Germany
table centrifuge	Eppendorf, Hamburg, Germany
vortexer	Janke & Kunkel, Staufen, Germany
water bath	Gesellschaft für Labortechnik, Burgwedel, Germany
whatman-Paper	Schleicher & Schuell, Dassel, Germany
cell culture flasks	Greiner, Frickenhausen; Becton Dickinson, Heidelberg, Germany
cell culture dishes	Greiner, Frickenhausen; Becton Dickinson, Heidelberg, Germany
cell scraper	Corning, Wiesbaden, Germany
irradiation instrument	Department of Radiology, University Frankfurt, Germany

3.1.2 Chemicals and Reagents for Cell Culture

ammonium chloride	StemCell Technologies, Vancouver, Canada
AP20187	ARIAD Pharmaceuticals, Inc. Cambridge, UK
calcium chloride	Sigma, Taufkirchen, Germany
chloroquine	Sigma, Taufkirchen, Germany
dinatriumhydrogenphosphate	Merck, Darmstadt, Germany
DMEM	Dulbecco's Modified Eagle Medium (High Glucose); Life Technologies (#21969-035), Karlsruhe, Germany
DMSO	Dimethylsulfoxid; Sigma, Taufkirchen, Germany
DNase I (RNase-free)	Roche (#776785), Mannheim, Germany
dNTPs	New England Biolabs, Frankfurt, Germany
EasySep-system	Stemcell Technologies, Vancouver, Canada
EDTA	Sigma, Taufkirchen, Germany
enfluran	2-Chloro-1.1.2-trifluoro-

	ethyldifluoromethylether (Ethrane); Abbott, Wiesbaden, Germany
FBS	Fetal Bovine Serum, Seromed (#S0115), Berlin, Germany & StemCell Technologies (#06400), Vancouver, Canada
formaldehyde	Roth, Karlsruhe, Germany
gelatine (powder)	Merck, Darmstadt, Germany
glutamine	L-Glutamine; BioWhittaker, Verviers, Belgium
HBSS	Hanks' Balanced Salt Solution; Sigma (#H6648), Taufkirchen, Germany
HEPES	Sigma, Taufkirchen, Germany
HSA	Human Serum Albumin (20% solution); DRK-Blutspendedienst Niedersachsen, Springe, Germany
IMDM/2% FBS	Iscove's Modified Dulbecco's Medium with 2% FBS; StemCell Technologies, Vancouver, Canada
MethoCult GF M3434	StemCell Technologies, Vancouver, Canada
sodium acid	Roth, Karlsruhe, Germany
sodium chloride	Roth, Karlsruhe, Germany
PBS	Dulbecco's Phosphate Buffered Saline (ohne Ca^{2+} und Mg^{2+}); BioWhittaker, Verviers, Belgium
penicillin-Streptomycin	BioWhittaker, Verviers, Belgium
polybrene	Sigma, Taufkirchen, Germany
poly-L-lysine solution	Sigma, Diagnaostics INC., St. Lous, USA
protaminsulfate	Sigma (#2162), Taufkirchen, Germany
retronectin	Takara, Shiga, Japan
RPMI 1640	RPMI 1640 (without Glutamine); Life Technologies (#31870-025), Karlsruhe, Germany
StemSep-Systeme	StemCell Technologies, Vancouver, Canada
tris	Roth, Karlsruhe, Germany

trypan blue	0,4% solution; Sigma, Taufkirchen, Germany
trypsine -EDTA	Trypsine-EDTA in HBSS; Life Technologies (#25300-054), Karlsruhe, Germany
sterile water for tissue culture	Sigma (#W3500), Taufkirchen, Germany

3.1.3 Enzymes

alkaline phosphatase (CIP)	New England Biolabs, Frankfurt, Germany
ampli-Taq-DNA-polymerase	Biosystems, Weiterstadt, Germany
Klenow-Fragment DNA-polymerase I	New England Biolabs, Frankfurt, Germany
Pfu Turbo DNA-polymerase	Stategene, Amsterdam, Netherlands
RNase A	Sigma, Taufkirchen, Germany
restriction endonucleases	New England Biolabs, Frankfurt, Germany
T4 DNA-ligase	New England Biolabs, Frankfurt, Germany

3.1.4 Cytokines

rmIL-3 (recombinant murine interleukin 3)	PeproTech, Frankfurt, Germany
rmIL-6 (recombinant murine interleukin 6)	PeproTech, Frankfurt, Germany
rmSCF (recombinant murine stem cell factor)	PeproTech, Frankfurt, Germany
WEHI-medium (conditioned RPMI-medium with mIL-3)	GSH, Laboratory Dr.Grez, Frankfurt, Germany

3.1.5 Antibodies

3.1.5.1 Antibodies for Western Blot and Intracellular Staining

anti-FKBP	ABR Affinity Bioreagents Inc., CO, USA
monoclonal anti-α-Tubulin	Sigma, Taufkirchen, Germany
488 goat anti-rabbit IgG1	Molecular Probes, Eugene, Oregon, USA
546 goat anti-mouse IgG1	Molecular Probes, Eugene,Oregon, USA
anti-Plcγ	Sigma, Taufkirchen, Germany
anti-phospho-STAT5A/B(Y694)	Cell Signalling Technology, MA, USA
anti-STAT5	BD Transduction Laboratories, Lexington, KY, USA

anti-PI3-Kinase	BD Transduction Laboratories, Lexington, KY, USA
polyclonal Rabbit anti-Stat5a	Zymed Laboratories Inc, San Francisco, CA, USA
anti-maus IgG-peroxidase	BD Transduction Laboratories, Lexington, KY, USA
anti-rabbit IgG-peroxidase	BD Transduction Laboratories, Lexington, KY, USA

3.1.5.2 Antibodies for FACS-analyse

7D5 antibody (anti-gp91^{phox})	GSH, Loboratory Grez, Frankfurt, Germany
rat anti-mouse IgG1(RAM IgG1)-PE	Becton Dickinson Immunocytometry Systems, San Jose, CA, USA
anti-mouse CD3-Biotin	BD Pharmingen, Heidelberg, Germany
anti-mouse B220-Biotin	BD Pharmingen, Heidelberg, Germany
anti-mouse Gr1-Biotin	BD Pharmingen, Heidelberg, Germany
streptavidin-APC	BD Pharmingen, Heidelberg, Germany
anti-LNGFR(C40-1457)-PE	BD Pharmingen, Heidelberg, Germany
rat anti-mouse IgG1-APC	BD Pharmingen, Heidelberg, Germany
anti-CD45.1-PE	BD Pharmingen, Heidelberg, Germany
anti-mouse Sca1-PE	BD Pharmingen, Heidelberg, Germany
anti-mouse c-kit-PE	BD Pharmingen, Heidelberg, Germany
anti-mouse IgG1-PE (Isotype)	BD Pharmingen, Heidelberg, Germany
anti-mouse IgG1(pure)	BD Pharmingen, Heidelberg, Germany
anti-mouse IgG2a-biotin	BD Pharmingen, Heidelberg, Germany

3.1.6 Reagents and Enzymes for Molecular Biological Experiments

acryl amide for SDS-PAGE	Rotipherese® Gel 30 (30% Acrylamid, 0,8% Bisacrylamid); Roth, Karlsruhe, Germany
agarose	Roth (#T846.3), Karlsruhe, Germany
alkaline phosphatase	New England Biolabs, Frankfurt, Germany
ampicillin	Roche, Mannheim, Germany

APS	Ammonium peroxydisulfate; Merck, Darmstadt, Germany
bromphenol blue	Sigma, Taufkirchen, Germany
bradford-reagent	Bio-Rad Laboratories, Inc., CA, USA
chloroform	Roth, Karlsruhe, Germany
DNA isolation kit	DNeasy™ Tissue Kit; Qiagen, Hilden, Germany
dried milk powder	Roth, Karlsruhe, Germany
ECL-solution	SuperSignal ® West Pico Chemiluminescent Substrate; Pierce, Rockford, IL, USA
acetic acid	Roth, Karlsruhe, Germany
alcohol (ethanol, 100%)	Roth, Karlsruhe, Germany
ethidium bromide	Roth, Karlsruhe, Germany
glycerol	Roth, Karlsruhe, Germany
glycine	Roth, Karlsruhe, Germany
glycogen	Roche (#901393), Mannheim, Germany
IPTG	Isopropyl-β-D-1-thiogalactopyranoside; Sigma, Taufkirchen, Germany
isopropanol	Roth, Karlsruhe, Germany
potassium acetate	Sigma, Taufkirchen, Germany
LB Agar	Life Technologies (#22700-025), Karlsruhe, Germany
LB Broth Base	Life Technologies (#12780-029), Karlsruhe
manganese chloride	Sigma, Taufkirchen, Germany
marker	
- DNA	Smart Ladder; Eurogentec, Seraing, Belgium
- Protein	BenchMark™ Prestained Protein Ladder; Invitrogen Life Technologies, Karlsruhe, Germany Bio-Rad Laboratories, Inc., CA, USA
methanol	Roth, Karlsruhe, Germany
sodium acetate	Sigma, Taufkirchen, Germany
sodium hydroxide	Merck, Darmstadt, Germany

PCI	Phenol/Chloroform/Isoamylalcohol (25:24:1); Roth, Karlsruhe, Germany
PCR-Reagents	
- AmpliTaq	Perkin Elmer, Weiterstadt, Germany
- AmpliTaq Gold	Perkin Elmer, Weiterstadt, Germany
- GeneAmp® PCR-buffer	Perkin Elmer, Weiterstadt, Germany
- Magnesium chloride	Perkin Elmer, Weiterstadt, Germany
- PfuTurbo DNA polymerase	Stratagene, Amsterdam, The Netherlands
plasmid-maxipreparation kits	JetStar, Hannover, Germany
protease inhibitor cocktail tablets, Complete (mini)	Boehringer Mannheim, Germany
PVDF-membrane Roti®-PVDF	Roth, Karlsruhe, Germany
QIAquick- gel extraktion kits	Qiagen, Hilden, Germany
RotiLoad1 loading buffer	Roth, Karlsruhe, Germany
rubidium chloride	Sigma, Taufkirchen, Germany
SDS, sodium didecyl sulfate	Roth, Karlsruhe, Germany
To-Pro-3 iodide	Eugene, Oregon, USA
TEMED	N,N,N',N'-Tetramethylethylendiamin; Roth, Karlsruhe, Germany
triton X-100	Sigma, Taufkirchen, Germany

3.1.7 Oligonucleotides

3.1.7.1 Primer for PCR

gp91(human):

5'-Primer (gpfor01): TTGTACGTGGGCAGACCGCAGAGA

3'-Primer (gprev02): CCAAAGGGCCCATCAACCGCTATC

Length of PCR fragments: 510 bp

β-Actin (mouse):

5'-Primer (ACT-DFI5): CTA AGG CCA ACC GTG AAA AG

3'-Primer (ACT-DFI3): TGA TAG ATG GGC ACA GTG TG

Length of PCR fragments: 166 bp

F2PrlR:

5'-Primer: TCG AGA CTA GAG GAG TGC AGG TGG

3'-Primer: CTT GCC CTT CTC TAG CAG ATG AG
Length of PCR fragments: 428 bp

3.1.7.2 Oligonucleotides for Cloning

Amplification of intracellular PrlR:
PrlRforSpe: 5'- GGA CTA GTA AGG GTT ATA GCA TGA CC-3'
PrlRrevKpn : 5' – GGG GTA CCT CAG TGA AAG GAG TGC – 3'
Length of PCR fragment: 1036 bp

Amplification of intracellular EGFR:
EGFRSpe-for: 5'- GAA ACT AGT CGA AGG CGC CAC ATC G – 3'
EGFRKpn-rev: 5' – GGG GTA CCG CTC ATG CTC CAA TAA ATT CAC – 3'
Length of PCR fragment: 1700 bp

Amplification of HOXB4 gene:
HOXBamHIrev: 5' – CGG GAT CCC TAG AGC GCG CGG GGG CCT CCA – 3'
HOXHindIIIfor: 5' – CCC AAG CTT GGC CAG AAA TTA ATG GC – 3'
Length of PCR fragment: 789bp

3.1.7.3 Oligonucleotides for Sequencing

Sequencing of the F2PrlR construct:
SFFV for1: 5' – GCT TCT GCT TCC CGA GCT C – 3'
F2PrlRfor: 5'- GCT CTA GAG CCA CCA TGG GCG TCC AAG – 3'

Sequencing of the F2 construct:
SFFV for1: 5' – GCT TCT GCT TCC CGA GCT C – 3'
gsF2for: 5' – CCC AAG CTT GCC ACC ATG GGC GTC CAA GTC – 3'
gsF2rev: 5' – ACT CCT CTA GTC TCG AGC TTC – 3'

Sequencing of the NF2PrlR construct:
PrlRrev1: 5' – GCC CTT CTC TAG CAG ATG – 3'
PrlRfor1: 5' – GCT GGA TTA CCT GGA TCC – 3'

Sequencing of the NF2EGFR construct:

EGFRfor1: 5’ – GGC ATC TTT AAG GGC TCC – 3’

EGFRrev1: 5’ – CCA CTG GGT GTA AGA GGC – 3’

Sequencing of the gsHOXB4 construct:

HOXfor1: 5’- GTT CCT CCC CAC TCC CCG ACC-3’

HOXrev1: 5’-TGC CCC CCG CAC GCA CGC GGG -3’

3.1.7.4 Primers for Integration Test (LM-PCR)

Blunt-end Adapter:

SL_linker_for: 5’-GTAATACGACTCACTATAGGGCACTATAGGGCACGCGTGGT-3’

SL_linker_rev: 5’-ACCACGCGTGCCCTATAGT-3’ (with 5’ phosphate)

Primer against Adapter:

SL-AP1: 5’-GTAATACGACTCACTATAGGGC-3’

SL-AP2: 5’-ACTATAGGGCACGCGTGGT-3’

Primer against SFFV-LTR:

SL-R1-bio: 5’-TGGCCCAACGTTAGCTATTTTCATGTA-3’ (with 5’ biotin)

SL-R1: 5’-TGGCCCAACGTTAGCTATTTTCATGTA-3’

SL-R2-: 5’-CCTTGATCTGAACTTCTCTATTCTTGGTTTG-3’

Sequencing primers for inserts of TOPO-Vectors:

M13_for (-40): 5’-GTTTTCCCAGTCACGAC-3’

M13_for (-20): 5’-GTAAAACGACGGCCAG-3’

M13_rev: 5’-CAGGAAACAGCTATGAC -3’

3.1.8 Vectors

pBluescript KS	Stratagene, CA, USA
pCR□2.1-TOPO	Invitrogen™, Life Technologies, Karlsruhe, Germany
$pCMV_4F_v2E$	Ariad, UK
pSF91-GFP2AHOXB4	Christopher Baum, MHH, Hannover, Germany

pSF71gp	GSH, Laboratory Grez, Frankfurt, Germany
pSxgs-NF2hmpl	Stefan Stein, GSH; Frankfurt
pSxgs-NF2d3YF	Stefan Stein, GSH; Frankfurt
pS-NF2hmpl	Stefan Stein, GSH; Frankfurt
pSxgs-LΔS	Stefan Stein, GSH; Frankfurt

3.1.9 Bacterial *E.Coli* Strains

one Shot™ (TOP 10F'): Invitrogen, Karlsruhe, Germany

Genotype:F'{*lac*/^{q} TN 10(Tet^{R})}*mcr*A □(*mrr-hsd*/RMS-*mcr*BC) □80*lacZ*□M15 □*lac*X74 *deo*R *rec*A1 *ara*D139 □(*ara-leu*) 7697 ga/U ga/K rpsL endA1 nupG

Stbl2™: Life Technologies, Karlsruhe, Germany

Genotype: F *mcr*A Δ(*mcr*BC-hsd/RMS-mrr) recA1 endA1 gyrA96 thi supE44 relA1 λ` Δ(lac-proAB)

DH5a: Stratagene, CA, USA

Genotype. F ¢80dlacZΔM15 Δ(lacZYA-argF) U169 deoR recA1 endA1 hsdR17(r_k^-, m_k^+) PhoA supE44 λ$^-$ thi-1 gyrA96 relA1

3.1.10 Medium for Bacterial Culture

3.1.10.1 5 x LB-liquid medium

To produce 5x LB-liquid medium, 100 g LB Broth Base per litre dH_2O was weighted and autoclaved. Before usage 400 ml dH_2O was mixed with 100 ml 5x LB medium together with equivalent antibiotics of choice.

3.1.10.2 LB-Agar

For the production of LB-Agar, 32 g LB-agar per litre dH_2O was weighted and autoclaved. After cooling of the agar in a 50°C water bath, the corresponding antibiotic was added to the agar. The mixture was applied into 10 cm petri-dishes (about 15 ml/dish). After gelation of the agar, the 1 x agar-dishes can be stored at 4° C for further 4 weeks.

3.1.11 Cell lines

293 T	human embryonic kidney, DSMZ No. ACC 305
Phoenix-E	modified 293T , Phoenix^{TM} Retroviral system, Orbigen, Canada
SC1	mouse embryonic fibroblast, ATCC No. CRL-1404
BaF/3	mouse pro B cells, DSMZ No. ACC 300

3.2 Materials for animal experiments

3.2.1 Mice

The C57BL/6NCr1 (Ly5.2) mice (age between 6-8 weeks, all female) were purchased from Charles River Laboratories GmbH in Munich, Germany and served as the recipient mice for all the animal experiments.

The donor mice C57BL/6 (Ly5.1) were obtained from the Jackson Laboratory, USA, and were further bred under IVC condition in the animal house of Georg-Speyer-Haus, Frankfurt.

3.2.2 Materials for Preparation of Mice

enfluran	2-Chloro-1.1.2-trifluoro-ethyldifluoromethylether; Abbott, Wiesbaden
heparin	Ratiopharm, Germany
EDTA	Roth, Germany
preparation set	Roth, Germany
water bath (45°C)	Lauda, Germany
micro isolator cages	EBECO, Germany
injection syringe with needle (1 ml and 0.5 ml)	BD, Germany
animal food	SNIFF, Specialisation GmbH, Soest, Germany

3.3 Molecular Biological Methods

3.3.1 Working with *E.coli* and Preparation of Plasmid DNA and Genomic DNA

3.3.1.1 Transformation of Competent Bacterial Strains

Transfomation is the method to infiltrate DNA into bacteria. $CaCl_2$-competent *E. coli* (50 µl) were thawed on ice, 1-25 ng plasmid DNA in 0.5-2 µl volume were added and the suspension was mixed gently with pipeting up and down and incubated on ice for 30 minutes. Cells were heat-shocked in a water bath at 42° C for 45-60 seconds, then immediately cooled on ice for 2 minutes. Finally 250 µl SOC medium (supplied from Invitrogen) was added. For expressing the resistance gene and subsequent selective outgrowth, bacteria were incubated for 1 hour at 37° C in the shaker. Then 100 µl of the preparation were plated on a LB-agar plate, which contains the antibiotic necessary for selection of transformed cells. Those plates were incubated overnight at 37° C in the incubator. Normally the plasmids, which were used in this study, have a coded sequence for the ampicillin resistance gene. Thereby positive selection of bacteria that inoculated the plasmid DNA is possible. After 24 hours single colony inoculums were cultured overnight in 3 ml of LB-Ampicillin (100mg/ml) on a shaking incubator (175 rpm) at 37° C.

3.3.1.2 Preparation of plasmid DNA (Mini prep)

This method is suitable for the preparation of small amounts of plasmid DNA (5µg-10µg). 2 ml overnight bacterial cultures were pelleted by centrifugation at 13.000 rpm for 5 minutes and resuspended in 300 µl buffer 1 (10 mM HCL, pH 8; 10 mM EDTA) containing of 100mg/ml RNAase. After incubation for 5 minutes in RT, 300 µl buffer 2 (200 mM NaOH; 1% [w/v] SDS) were added. The cells were lysed throughout careful invention of the cell suspension. 300 µl buffer 3 (3 M KAc, pH 4.8) were added into the tube, in order to neutralize the samples. The samples were incubated 5 minutes on ice. Further the mixture was centrifuged at 13.000 rpm, 4°C for 15 minutes, in order to remove the precipitated proteins, membrane components and the chromosomal DNA. The supernatant containing the low molecular plasmid DNA was transferred into a new Eppendorf tube and precipitated by addition of 0.6 ml isopropanol. The samples were centrifuged at 13.000 rpm, 4°C for 20 minutes. The obtained plasmid DNA pellets were washed 2 times with 70% ethanol and dried at RT. At last the DNA was resuspended in 30 µl TE (10 mM Tris, pH8.0; 10 mM EDTA) or dH_2O.

3.3.1.3 Plasmid Maxi-Preparation via JETstar Genomed-columns (Combination of an Anion Exchange Resin)

This method is suitable for DNA plasmid preparation yields of 50 µg - 500µg. 3 ml overnight bacterial culture was added to 300 ml LB-Ampicillin and incubated overnight by shaking at 175 rpm, 37°C. Bacteria were pelleted by centrifugation at 5000 rpm, 4°C for 10 minutes (JA20-rotor, Beckman centrifuge) and resuspended by adding of 10 ml buffer 1(50 mM Tris; 10 mM EDTA, pH 8.0; 0.1 mg/ml RNase), further lysed by a 5 minute incubation at RT in 10 ml buffer 2 (200 mM NaOH; 1 % (w/v) SDS). Consequently adding 10 ml buffer 3 (3.1 M potassium acetate, pH 5.0) and incubating 10 minutes on ice neutralized the sample. The precipitated proteins, membrane components and genomic DNA were removed via centrifugation at 15.000 rpm, 4°C for 10 minutes (JA10, Beckman centrifuge). The supernatant was applied to an already with buffer 4 (600 mM NaCl ; 100 mM sodium acetate, pH 5.0; 0.15 % Triton X-100)with buffer 4) equilibrated column and run by gravity flow. The column was washed with buffer 5 (800 mM NaCl 100 mM; sodium acetate, pH 5.0) to remove the non-DNA fractions and the plasmid DNA was eluated using a high salt solution (1250 mM NaCl, 100 mM Tris-HCl, pH 8.5) and then de-salted by precipitation with isopropanol. Then plasmid DNA pellets were washed in 70% ethanol and then resuspended in dH_2O or TE. The concentration of plasmid DNA was measured by a spectrometer with the wavelength of 260 nm and stored in –20° C.

3.3.1.4 Preparation of Genomic DNA from Cultured Eukaryotic Cells with DNeasy Kit (Qiagen)

Genomic DNA from various cell types was prepared by using the blood and cell culture DNeasy kit from Qiagen. The isolation of total DNA is based on the silicagel membrane technology. All the buffers are designed for optimal binding of DNA to the membrane after cell lysis. Finally the DNA is eluted after repeated washing steps from the membrane via change of the pH-value.

Maximal $5x10^6$ cells were centrifuged at 1500 rpm, 4 °C for 5 minutes and resuspended in 200 µl 1x PBS after washing one time with ice cold 1x PBS. Thereafter 20 µl RNase (20 mg/ml) was added to the cell suspension. The suspension was incubated for 2 minutes at RT. Then 20 µl proteinase K and 200 µl Buffer AL were added. The mixture was vortexed and incubated at 70°C for 10 minutes. After that 200 µl 100 % ethanol was added to the sample, which was mixed by vortexing and transferred to the column, which was placed in a 2 ml collection tube. The sample was centrifuged at 9000 rpm for 1 minute, further the membrane bound DNA was washed with 500 µl Buffer AW1 at 9000 rpm for 1 minute and

AW2 at 13.000 rpm for 3 minutes separately. Then the flow-through was discarded and the DNA was then eluted from the membrane with 200 µl elution solution via an additional centrifugation step (9000 rpm; 1 minute). The yield of DNA could be increased when the elution step had been repeated. The concentration of the DNA was determined with a spectrometer in the wavelength of 260 nm. The DNA was stored at 4°C.

3.3.2 Enzymatic Modification of Nucleotide Acids

3.3.2.1 Restriction of Plasmid DNA

The restriction endonucleases used for restriction of plasmid DNA belong to the type II endonucleases, which can recognize short DNA sequences and cleave double stranded DNA at specific sites. Thereby either blunt ends or sticky ends can be obtained.

Restriction endonuclease cleavage is accomplished simply by incubating the enzymes with DNA in the appropriate reaction conditions. 0.5 µg – 1 µg plasmid-DNA was used for one restriction reaction. The DNA was incubated together with 5-fold excess of enzyme (5 U enzyme/µg DNA), with the appropriate restriction buffer for 1-2 hours at 37° C. For purification of DNA fragments from a restriction reaction the DNA was isolated by phenol-chloroform extraction. Running on a 1% agarose gel separated the digested DNA fragments.

3.3.2.2 Dephosphorylation of Linear Plasmid-DNA by Alkaline Phosphatase CIP (calf intestinal phosphatase)

This method can be used to decrease the self-ligation of the cut plamid DNA. The CIP-treated fragments lack the 5’ phosphoryl-termini, which are required by ligases for proper ligation. The reaction was done directly after enzymatic restriction of the plasmid DNA by addition of the related CIP-buffer and 1 µl (0.5 U/µg vector) CIP and incubation at 37° C for 1 hour. The following phenol-chloroform extraction or gel extraction was performed to purify the DNA fragments.

3.3.2.3 Fill-in of 5’-Overhangs to Form Blunt Ends by Klenow-Reaction

The 5’overhang of DNA ends can be filled up by Klenow-fragment, a large subunit of the DNA polymerase I of *E. coli*. The enzyme is active in all the NEB buffers and was incubated with the restricted DNA (1 U/µg DNA) and dNTPs for 15 minutes at 25° C. The

enzyme activity was stopped by adding EDTA to a final concentration of 10 mM and heating at 75° C for 20 minutes.

3.3.2.4 Phenol-Chloroform Extraction for Purification of DNA Fragment

To purify the DNA fragments without using the gel extraction method the phenol-chloroform extraction was used. The DNA sample was filled up to a volume of 200 µl with dH_2O (or 1x TE) and the same volume of a mixture of phenol/chloroform/isoamylalkohol (25/24/1) was added. The reaction was centrifuged (3 minutes; 13.000 rpm; RT) to separate the water phase. The upper water phase containing the DNA was transferred into a new tube loaded previously with 200µl chloroform. After well mixture, the reaction was centrifuged again (3 minutes; 13.000 rpm; RT). The upper water phase was transferred to a new tube. Then 1 µl glycogen, 1/10 volume 3 M NaAc (pH=7) and 2 volumes of 100% ethanol were added, in order to precipitate the DNA. The sample was stored at –80° C for 30 minutes. After further centrifugation of the sample for 30 minutes at 13.000 rpm, 4° C, the DNA was precipitated. The DNA pellet was washed 2 times with 70% ethanol, dried at RT and resuspended in an appreciate volume of 1x TE.

3.3.2.5 Ligation of DNA Fragments

Cut DNA and vector fragments can be combined with T4 DNA ligase. T4 DNA ligase catalyses the formation of a phosphor-diester bonds between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA. 15 µl reaction was done with 25-50 ng vector, 3-6 fold molar excess of insert, 1.5 µl 10x ligase buffer and 1 U T4 DNA ligase overnight at 16°C and 7,5 µl of the reaction was used for the transformation with chemically competent E.Coli carried out ligation. To control for the self-ligation an additionally vector-only ligation reaction was included.

3.3.2.6 Transformation of TOPO-Ligation Product in TOP 10F' *E.coli*

1-2 µl TOPO-ligation product was added into 50 µl on the ice melted "One shot Top 10F' Cells with carefully mixing. The mixture was incubated for 30 minutes on ice followed by a 30 seconds heat shock in a 42°C water bath. After two minutes incubation on ice, 250 µl SOC medium were added to the sample, which was then further incubated for 45 minutes at

37°C and rotation. 10-50 µl of the sample was plated on the LB-Ampcillin plates, which were spread with 40 µl IPTG-Gal (40 mg/ml) solution prior to use. The plates were incubated in a 37°C incubator overnight. The white colonies containing the ligated DNA-fragment were picked for the colony-PCR analysis.

3.4 Polymerase Chain Reaction (PCR)

3.4.1 PCR of Plasmid DNA for Amplification of DNA Sequences

The purpose of a PCR is to make a huge number of copies of a special gene, e.g. in this study to amplify the sequences of the intracellular domain of the PrlR, EGFR and HOXB4 for cloning of the various constructs. The reagents and standard procedure of the PCR reaction are described in the following. Dependent on the various primers, the annealing temperature is changed between 57° C - 62° C. Also the length of the DNA, which should be amplified, plays an important role. Normally, the large fragments need longer fractions of time for each single reaction step.

The PCR reaction is divided into 3 steps. The second part, the amplification, is repeated for 25 - 40 times (cycles). This is carried out on an automated cycler machine (Perkin Elmer), which can heat up and cool down the tubes with the reaction mixtures in a very short time. In the first step, the denaturation, the DNA double strand melt and open to single strand DNA at 94°C. Thereby the bonds of the strand primer to the single strand DNA is possible, this step is called annealing. Furthermore the polymerase can attach and start copying the template.

After the PCR reaction, the products were applied on an agarose gel and the length of the amplified DNA sequences were controlled. The correct products were purified either with the DNA isolation kit (Qiagen) or with the phenol-chloroform extraction (3.3.2.4). Further the PCR product was cut out with the corresponding restriction enzymes prior to cloning.

Required reagents:

DNA polymerase	2,5 U/µl	*Pfu Turbo- polymerase* with the supplied reaction buffer (10x)
Primers	0.4 µM	sense-/antisense primers (10 µM)
dNTP	0.2 mM	dATP, dCTP, dGTP, dTTP (5mM each)

Standard PCR protocol:

PCR reaction	PCR program	
10-100 ng plasmid-DNA	5 minutes at 94° C	
5 µl buffer (10x buffer with 20 mM $MgSO_2$)		
2 µl sense primer (10 µM)	1 minute at 94° C	25-35 cycles
2 µl antisense primer (10 µM)	1 minute at 55° C	
2 µl dNTPs (5 mM)	2 minutes at 72° C	
1 µl Pfu Turbo-polymerase (2.5 U/µl)		
	15 minutes at 72° C	
Add H_2O to 50 µl		

3.4.2 PCR on Genomic DNA for Determination of Integration of Viral Vector

The principle is similar as explained in chapter 3.4.1. To determine $gp91^{phox}$ and F2PrlR fusion gene sequences the following protocol was used:

PCR reaction	PCR program	
100-200 ng genomicDNA	10 minutes at 95° C	
5 µl buffer (10x buffer with 20 mM MgSO2)		
2 µl sense primer (10 µM)	1 minutes at 95° C	30-35 cycles
2 µl antisense primer (10 µM)	1 minute at 55° C	
2 µl dNTPs (5 mM)	1 minute at 72° C	
1 µl Tag DNA polymerase (2.5 U/µl)		
	15 minutes at 72° C	
Add H_2O to 50 µl		

3.4.3 Ligation Mediated PCR (LM-PCR)

Ligation mediated PCR is a modified PCR method to proof of the provirus integration in host-genomic DNA. It is also suitable for the test of clonality of tranduced cells.

Required primers: See chapter 3.1.7.4

Required buffers and reagents:

10x restriction buffer 1	NEB, Cambridge, UK
10x native Pfu buffer	Stratagene, CA, USA
Dynalbeads M-280 Streptavidin (10 µg/µl)	Dynal, Hamburg, Germany
2x BW buffer	10 mM Tris pH7.5; 1 mM EDTA; 2 M NaCl
5x annealing linker	0.5 M Tris pH 7.4; 0.35 M $MgCl_2$
Extensor Hi-fidelity PCR master mix	AB gene, Hamburg, Germany

Preparation of ligation mix with polylinker: 40 µl H_2O, 20 µl primer linker 1 (200 pmol/µl) and 20 µl primer linker 2 (200 pmol/µl) were incubated for 5 minutes at 70°C. Then 20 µl 5x annealing buffer were added. After that, the samples were incubated again for 5 minutes at 70°C and further incubation overnight at RT. The aliquots were stored at -20°C.

250 ng genomic DNA prepared from *ex vivo* cultured mBM cells under cytokine or selective condition was mixed together with 3 µl 10x restriction buffer, 0.5 µl RNase (Roche), 0.5 µl Tsp5091 restrictase and H_2O to an end volume of 30 µl. Samples were incubated for 2 hours at 65°C in water bath. After that, the mixture was cleaned with MiniElute Cleanup Reaction Kit (Qiagen) and acquired to 10 µl elution. 10 µl elute inclusive different DNA fragments was mixed with 2 µl 10x native Pfu buffer, 0.8 µl 5 mM dNTPs, 1 µl primer A1RV-bio (0.25 pmol/µl), 1 µl native pfu DNA polymerase and 5.2 µl H_2O to an end volume of 20 µl. The biotin-labeled primer was incorporated into the fragment throughout 1 circle PCR program as listed in Table 3.4.1

Table 3.4.1: 1 step PCR program for binding of biotin-labelled primer

95°C	5 minutes
65°C	30 minutes
72°C	15 minutes
4°C	∞

Samples were cleaned again with the MinElute Cleanup Reaction Kit and acquired to a volume of 40 µl. Twenty µl Dynalbeads were washed 2 times with 100 µl dH_2O and resuspended in 40 µl 2x BW buffer, then together mixed with 40 µl elutes. After 1 hour incubation at RT and rotation (800 rpm), the mixture was washed 2 times with 100 µl water and resuspended in 10 µl *Ligation Mix,* then incubated at 800 rpm, 16°C for a process of 2 minutes on, 5 minutes off at rotation overnight. Half of the ligation mix (5 µl) was mixed with 2 µl NEB buffer 2, 0,2 µl restriction enzyme HindIII and 12,8 µl H_2O, then incubated for 1 hour at 37°C. Thereafter, the samples were washed 2 times with 100 µl H_2O in the magnetic

particle concentrator (Dynal) and resuspended in 5 µl H_2O. The first integration PCR (Int 1-PCR) was performed in an end volume of 25 µl according to Table 3.4.2.

Table 3.4.2: Reaction materials and program of Int 1-PCR

PCR reaction	PCR program	
1µl DNA from HindIII cut ligation mix	2 minutes at 94° C	
12.5 µl extensor Hi-fidelity PCR master mix	15 seconds at 94° C	
1 µl SL AP1 primer (25 pmol/µl)	30 seconds at 60° C	30 cycles
1 µl A2RV primer (25 pmol/µl)	2 minutes at 68° C	
9.5 µl H_2O	10 minutes at 68° C	
	4°C ∞	

The products from the Int 1-PCR were diluted 1:50 and 1 µl was used as the template DNA for the second nested PCR (Int 2-PCR) as shown in Table 3.4.3.

Table 3.4.3: Reaction materials and program of Int 2-PCR (nested PCR)

PCR reaction	PCR program	
1µl DNA (1:50 diluted from Int1-PCR)	2 minutes at 94° C	
12,5 µl extensor Hi-fidelity PCR master mix	15 seconds at 94° C	
1 µl A3 RV primer (25 pmol/µl)	30 seconds at 60° C	30 cycles
1 µl SL AP2 primer (25 pmol/µl)	2 minutes at 68° C	
9.5 µl H_2O	10 minutes at 68° C	
	4°C ∞	

The PCR products were analysed on 2.5% agarose gels. The DNA fragments, obtained from the selective expanded cells, were ligated into TOPO-pCR-2.1 vector (3.3.2.5) and transformed into TOP 10F' *E Coli.* (3.3.2.6). The white colonies were picked for the "colony-PCR" according to Table 3.4.4.

Table 3.4.4: Colony-PCR: reaction material and PCR program

PCR reaction	PCR program	
1 white colony	10 minutes at 94° C	
5 µl buffer (10x buffer with 20 mM $MgSO_2$)		
1 µl M13 rev (10 µM)	1 minute at 94° C	
1 µl M 13 for (-40) (10 µM)	1 minute at 53° C	30-35 cycles
2 µl dNTPs (5 mM)	2 minutes at 72° C	
0.3 µl Taq DNA polymerase (5 U/µl)		
	5 minutes at 72° C	
Add 50 µl H_2O		

The PCR-products were analysed on 2% agarose gels. The products resulting in a single band were purified with NucleoSpin®Extract II Kit (Machery-Nagel) before sequencing using M13 (-20) forward and M13 reverse primer.

3.5 Working with Proteins

3.5.1 Preparation of Whole Cell Lysates

The used protein extraction buffer is modified from a protocol of Dinauer et al.(Yu et al., 1999). Briefly, cells were harvested, centrifuged at 1500 rpm, 4°C and 5 minutes, then washed 3 times in ice cold 1x PBS, resuspended for10-20 x 10^6 cells/ml either in triton buffer (50 mM Tris-HCl, pH 7.4; 1% Triton; 150 mM NaCl; 2 mM EDTA) for determination by Western blotting with anti-FKBP or in Ripa buffer (120 mM NaCl; 50 mM Tris, pH 8.0; 1% Triton; 0.5% Deoxycholat; 0.1% SDS) for EMSA. The mixture was transferred into a previously cooled 1,5 ml tube. The lysates were kept on ice for 15 minutes before centrifugation at 13000 - 15000 rpm, 4°C for 5 minutes. The protease inhibitors (BOE protease inhibitor, Rothe) were added into the buffer before usage.

The supernatants were aliquoted into 1.5 ml centrifuge tubes and stored at –80°C until usage. For determination of protein phosphorylation, the phophotase inhibitors were added into the lysis buffer before preparation of the protein extracts.

3.5.2 Bradford Protein Assay

Duplicates of each protein sample were measured in 96-well micro titre plates. Per well 149 µl H_2O, 30 µl working reagent (Bradford solution, supplied from BioRad) and 1 µl sample or protein standard (BSA, 6 different protein concentrations from 0.125 mg/ml to 8 mg/ml) were combined, incubated for 5 minutes at RT and then absorbance was measured at 595 nm wave length on a plate reader (SoftMaxpro). The protein concentration of each sample was calculated using the parameters obtained from linear regression of the protein standard values.

3.5.3 Western Blotting

Western blotting is based on the one-dimensional gel electrophoresis of proteins and can be provide information about the molecular size of a certain protein and the purity of a protein extraction, as well as the number and molecular size of subunits. The whole procedure of western blotting comprises gel electrophoresis, transfer of the proteins to a polyvinylidene

difluoride (PVDF) membrane, incubation of the membrane with specific antibodies and finally the development and detection of specific signals.

3.5.3.1 Discontinuous SDS-PAGE

The gel concentration of the SDS-PAGE gel should conform to the molecular size of the protein of interest as shown below

Molecular weight	Gel concentration
< 30 KD	15%
30-50 KD	12%
50-100 KD	10%
> 100 KD	8%

Required reagents for preparation of SDS gels:

Table 3.1: Solutions for preparing resolving gels (for 5 ml mini-gels):

	acryl amide mix 30%	1.5 M tris (pH 8,8)	10% SDS	Ammonium persulfate 10%	TEMED	H_2O
8% gel	1.3 ml	1.3 ml	0.05 ml	0.05 ml	0.003 ml	2.3 ml
10% gel	1.7 ml	1.3 ml	0.05 ml	0.05 ml	0.002 ml	1.9 ml
12% gel	2.0 ml	1.3 ml	0.05 ml	0.05 ml	0.002 ml	1.6 ml

Table 3.2: Solutions for preparing 5% stacking gels (for 2 ml):

Acryl amide mix, 30%	1,0 M tris (pH 6,8)	10% SDS	Ammonium persulfate 10%	TEMED	H_2O
0.33ml	0.25 ml	0.02 ml	0.02 ml	0.002 ml	1.4 ml

The separating gel was cast at first according to Table 3.1 and overlaid with water-saturated isobutanol until polymerisation. Then isobutanol was exchanged by stacking gel according to Table 3.2 on top of the separating gel. After complete polymerisation, the gel was put into the electrophoresis tank and mounted with running buffer (25 mM Tris-Base; 192 mM Glycine; 0.1% [w/v] SDS; pH 8.3). Meanwhile, the protein samples were mixed with an equal volume 4x sample buffer (laemli buffer), boiled for 5 minutes at 95° C and loaded into the lanes. Gels were run at first about 30 minutes at 80 volts throughout the stacking gel and furthermore at 120 volts for 3-4 hours.

3.5.3.2 Western Blotting

After separation by SDS-PAGE (3.5.3.1), proteins were blotted via electrophoresis onto a PVDF membrane (Roti-PVDF transfer membrane, Roth) using a semi-dry technique (Towbin *et al.*, 1979). The membrane was cut to gel size and soaked with methanol, then placed on top of 3 sheets of 1 sponge and 2 filter paper soaked with 1x blotting buffer (25 mM Tris-Base; 192 mM Glycin; 20 [v/v] Methanol) (bottom, on the anode). The SDS-PAGE gel was removed from the glass plates and placed on top of the membrane. Another 2 sheets of filter paper and 1 sheet sponge soaked with blotting buffer were put on top of the gel, followed by the cathode. Protein transfer was conducted for 2 hours at 120 volts.

3.5.3.3 Immunostaining of Blotted Proteins

Blotted membranes were blocked at RT for 45 minutes either with 1x TBST (1% Tween-20 in TBS) plus 5% low fat dried milk (for STAT5 phosphorylation) or 1x PBST (1% Tween-20 in PBS) plus 5% milk powder (for FKBP protein). Blots were then washed two times for 5 minutes in TBST (or PBST) and incubated with the primary antibody overnight at 4°C. FKBP was stained with anti-FKBP diluted 1:1000 in 2% milk powder, STAT5 were detected with anti-STAT5 diluted 1:5000 in 4% milk powder. Phospho-STAT5 was stained with anti-phospho-STAT5 diluted 1:1000 in 2% milk powder. After washing the membrane three times for 10 minutes with TBST or PBST, respectively, the blots were incubated with the corresponding secondary antibody diluted 1:10000 at RT for 1 hour (either HRP-conjugated goat anti-rabbit antibody for FKBP, anti-Phospho-STAT5 or HRP-conjugated goat anti-mouse for anti-STAT5). At last the blots were washed again three times for 10 minutes with 1x TBST or 1x PBST and bound antibodies were visualized using the ECL-solution (Pierce). To control for equal protein loading of the different lanes, the blots were stripped with 0.1 M glycine (pH 2.9) by RT for 30 minutes and stained furthermore with anti-PLC-gamma or anti-PI3 kinase according to manufacturer's instructions.

3.5.4 Electrophoretic Mobility Shift Assay (EMSA)

In the nucleus, gene expression is controlled by DNA-binding transcription factors which bind to characteristic DNA motifs to initiate or repress transcription. The electrophoretic mobility shift assay (EMSA) is a powerful tool for evaluating DNA-protein interactions. It is based on the principle that when subjected to electrophoresis in native poly

acrylamide gels, free DNA has a different electrophoretic mobility than a DNA-protein complex. The process of the experiment contains steps below:

- Annealing of oligonucleotides: Equal amount of complementary single-stranded synthetic oligonucleotides (2 x 10^{-10} moles of each) was prepared to a final volume of 10 µl by adding TE (pH 8.0). The mixture was heated for 5 minutes at 70°C and then allowed to cool to room temperature (overnight at RT). The annealed oligos with final concentration of 2 x 10^{-11} moles/µl were stored at 4°C for a short time or at –20°C for a long time.
- Labelling the probe (30 µl): 100-200 ng of annealed oligos, 3 µl 10x T4 polynucleotid kinase buffer, 7.5 µl □^{32}P-ATP (50 µCi (3000µCi/mmol), Amersham, Pharmacia, Freiburg, Germany) and 4 µl T4 polynucleotid kinase were mixed on ice by adding sterile H_2O to a end volume of 30 µl. The mixture was incubated for 1 hour at 37°C. After that, 1 µl 0.5 M EDTA (pH 8.0) was added to stop the reaction. Thereafter, 20 µl H_2O was added to an end volume of 50 µl.
- Preparation of binding reaction mixture without antibody: 100000 cpm/µl radiolabelled probe was mixed with fresh prepared incubation buffer (10 mM HEPES pH 7.9; 50 mM KCl; 1 mM DTT; 1 mM EDTA pH 8.0; 10% glycerol; 0.1 µg/µl poly (dl-C); 0.5 µg/µl BSA) to an end volume of 10 µl. The probe sample was then mixed well with 10 µl from 8 µg whole cell extract, 1µl 10x incubation buffer and sterile H_2O.
- Preparation of binding reaction mixture with antibody: 10 µl probe mixture was mixed with 10 µl reaction from 8 µg whole cell extract, 1 µl specific antibody (STAT5a and STAT5b respectively) and 1µl 10x incubation buffer and sterile H_2O.
- Separation of the samples with 5% polyacrylamide gel: The samples were incubated for 30 minutes at 30°C, then 5 µl 6x DNA loading buffer was added. After centrifugation, the samples were loaded into gel and further running in electrophoresis buffer (250 mM Tris; 192mM glycine; 50 mM EDTA pH 8.0) out for 2 hrs at 100 volts.
- After running, gel was rinsed at first with 120 ml fixing solution, then washed with 500 ml sterile H_2O. After that, the gel was attached to a piece of Whatman paper and dried on a vacuum gel dryer with heat for 2 hours
- The dry gel with paper was exposed to films.

3.6 Cell Culture

3.6.1 Cell Culture Conditions and Passaging of Cells

Retroviral producer cell line Phoenix E is based on 293T cell line, a human embryonic kidney cell line transformed with adenovirus E1a which carries a temperature sensitive T antigen co-selected with neomycin. This cell line is highly transfectable using the calcium phosphate mediated transfection method. The producer line was created by placing constructs capable of producing *gag-pol* and *env* proteins for ecotopic virus particle production in 293T cells. SC1 cell line for titre determination is a cell line derived from mouse embryonic fibroblast cells.

BaF/3 cell line, used for cell proliferation assays and functional assays for the constructs, is a mouse pro-B cell line, established from peripheral blood derived from BALb/c mice. These cells grow under stimulation with murine IL-3 (10 ng/ml).

If not noted otherwise, cells like SC1 or Phoenix E were cultured in DMEM supplemented with L-glutamine (4 mM), antibiotics (50 U/ml penicillin and 50 mg/ml streptomycin) and 10% FCS. BaF/3 cells were cultured in RPMI 1640 with the same amount of L-glutamine, antibiotics and FCS and further with 10 ng/ml murine IL-3 or with 10% conditioned medium (WEHI medium) containing mIL-3. Cells were cultured at 37°C, 5% CO_2 and 95% relative humidity in an incubator. Before usage FCS was heat-inactivated for 30 minutes at 56°C.

3.6.2 Assessing Cell Viability by Trypan Blue Exclusion

The number of viable and dead cells was determined by trypan blue exclusion. The cell suspension was diluted with trypan blue solution (0.2 % (w/v) trypan blue in 0.9% NaCl solution) and cells were counted in a Neubauer haematocytometer. The concentration of viable cells was then calculated using the equation:

Number of viable cells/ml: $N \times F \times 10^4$

N = average of unstained cells per corner square
(1 mm^2 containing 16 sub-squares)
F = dilution factor

3.6.3 Freezing and Thawing Cells

Cells were harvested and suspended in freezing medium (90% FCS; 10% DMSO) in 1-10 x 10^6 cells/ml. Such suspension was preserved in cryo-vials. To allow gradual freezing at

a rate of 1°C per hour, the vials were placed in an isopropanol-filled cryocontainer and frozen at –80° C for 24 hours. For long-term storage, samples were then transferred to the liquid nitrogen (-196° C). To recover the frozen cells, the cell suspension was thawed quickly in a water bath at 37° C. To avoid toxic effects of DMSO to viability, the cell suspension was transferred into a falcon tube containing 15-25 ml warm 1x PBS with 20% FCS as soon as thawed and consequently the cells were spun down (6-8 minutes at 1200 rpm and 4° C) and resuspended in fresh medium. For the thawing of mouse bone marrow cells, 40 U/ml DNase were added into the thawing medium.

3.6.4 Primary Mouse Bone Marrow Cells

Fresh prepared murine bone marrow (mBM) cells (see 3.7.2) were cultured in RPMI 1640 (complete components described in capter 3.6.1) with 10 ng/ml mIL-3, 50 ng/ml mIL-6 and 50 ng/ml mSCF. The cell density after isolation or enrichment was kept minimum at 1x 10^6 cells/ml in a 24-well plate.

3.6.5 Retroviral Virus Particle Production via Calcium Phosphate Mediated Transfection

The calcium phosphate mediated transfection method is based on the ability of the producer cells to take up the DNA calcium phosphate precipitate via endocytosis. Phoenix E cells carry the MLV *gag, pol* and *env* gene, which are necessary for the packaging of the retroviral vectors. Another packaging construct M57 (M. v. Laer, Frankfurt/GSH), which also contains the MLV *gag-pol* sequences, was co-transfected into the producer cells, in order to increase the transfection efficiency (Figure 3.1).

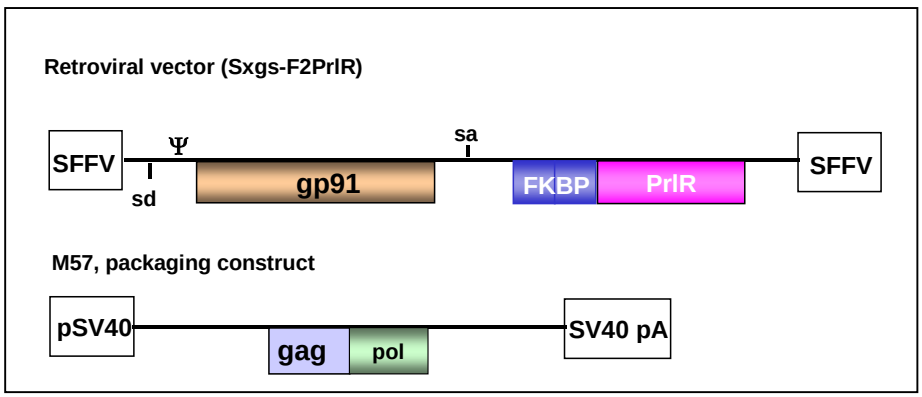


Figure. 3.1: Retroviral construct for virus production: As an example, construct of Sxgs-F2PrlR and helper plasmid with *gag-pol* elements are shown**.** Except of the retroviral cassettes, all the retroviral plasmids have a bacterial repopulation origin and an ampicillin resistance gene.

Phoenix E producer cells were seeded 1 day before transfection at a density of 3.5-4 x 10^6 in 10 cm diameter dishes and allowed to grow to 60% confluence (sub confluent). Two hours before transfection, the cells were provided with fresh medium. 10 µg expression construct DNA, 3 µg M57 helper plasmid, 50 µl $CaCl_2$ (2,5 M) were pipetted together with 0.1x TE H_2O to the end volume of 500 µl. Then 500 µl 2x HBS (50 mM HEPES pH 7.05; 280 mM NaCl; 1.5 mM Na_2HPO_4; pH 7,12) were added drop wise into the solution under continuously blubbering. The precipitate was put aside for 5 minutes before drop wise giving it onto the Phoenix E cells. In order to increase the transfection efficiency, 2 minutes before transfection, 1 µl chloroquine (100 mM) was added into the DMEM medium. 6-8 hours after transfection, the medium was exchanged by 5 ml fresh RPMI medium, in order to concentrate viral particles. 24 h after medium exchange, viral supernatant was harvested by filtration through a 0.22 µm filter, which was soaked with sterile water before usage. The supernatant, containing viral particles, was aliquoted and stored at – 80° C. In the case of a good viability of the producer cells, the supernatant was harvested a second time under the same conditions.

The titre of retroviral particles used in this study was determined by transduction of SC1 cells. Briefly, one day before titre determination, 5x 10^4 SC1 cells were seeded in 24-well plate, on the day of titre determination, 1 ml fresh DMEM was changed and virus supernatants were added into the well duplicates with the dilutions 1:10 (100 µl virus supernatant) and 1:100 (10 µl virus supernatant). To increase the infection efficiency, 10 µl polybrene (from stock solution 400 mg/ml) was added into the wells. 3 days after infection of SC1 cells, FACS staining with equivalent antibodies was performed. The percentage of positive cells was between 5-15 %. The following equation was used for calculation of titre:

Titre (TU/ml) $= N \times A \times F$

N: cell number at day of infection

A: percent of positive cells

F: dilution factor

3.6.6 Lentiviral Virus Particle Production via Calcium Phosphate Mediated Transfection of 293t Cells

The lentiviral virus particles were produced by 293T cells cotransfection with plasmids carry out the *gag*, *p*ol (pCMVΔR8.91) and *env* (pMD.G) gene (Romain Zufferey, Genf; Zufferey et al., 1997) as shown in Figure 3.2.

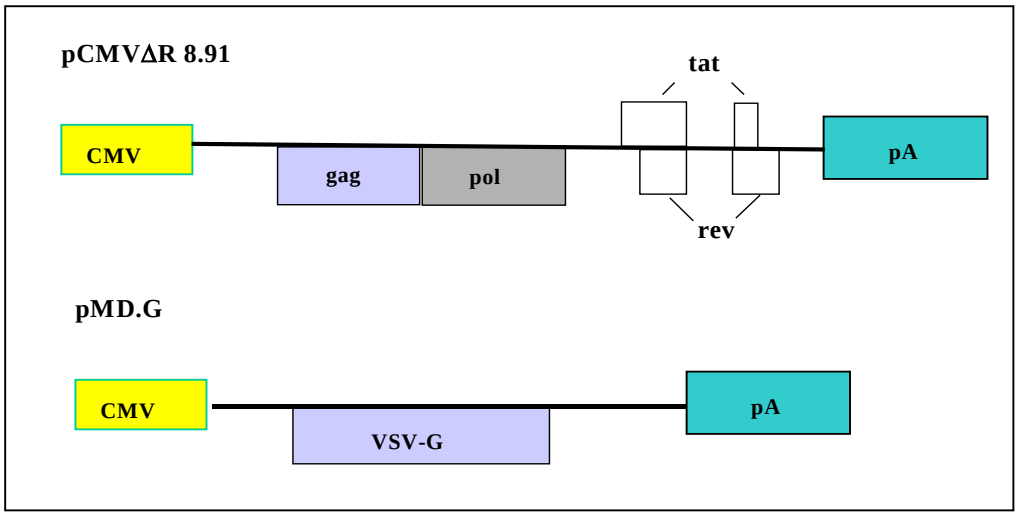


Figure. 3.2: Structure of lentiviral helper plamids: Except the lentiviral cassettes, both helper plasmids have a bacterial repopulation origin, a SV40-repopulation origin and an ampicillin resistance gene.

293T cells were seeded as described in chapter 3.6.5. 10 µg expression construct DNA, 6.3 µg pCMV8.91 and 3,7 µg pMD.G helper plasmid were cotransfected in sub-confluent 293T cells in the same way as explained above. The obtained lentiviral supernatants were aliquoted and stored at – 80° C.

3.6.7 Transduction of BaF/3 Cells and Primary mBM cells with Retroviral Particles

For the transduction of BaF/3 and primary mBM cells, the definitely MOI (multiplicity of infection) was used to control the number of virus particles which transduced the cells like below.

$$\textit{MOI} = \frac{\textit{Virus titre (TU/ml) x volume of the used virus supernatent (ml)}}{\textit{Number of the cells}}$$

BaF/3 cells were transduced according to the standard spin protocol. 1x 10^5 cells were seeded in 24-well plates and a definitely amount of virus supernatant with MOI = 5 was added into RPMI medium supplemented with mIL-3 (10 ng/ml) and 4µg/ml of protaminsulfate. Then the cells were centrifuged at 32° C and 2500 rpm for 90 minutes and incubated in the incubator for 6-8 hours. At last fresh complete RPMI medium containing all necessary cytokines was added. 3 days after transduction, the transduction efficiency was measured by FACS-staining analysis (see chapter 3.8).

The mBM cells ($Sca1^+$ or total mBM cells) were transduced as described by Christopher Baum (protocol kindly provided by Prof. Dr. Christopher Baum). Fresh isolated and enriched Sca1 positive mBM cells were pre-stimulated with cytokines for 48 hours before the first transduction cycle. One 24-well plate (non-tissue coated) was coated with 400 µl retronectin per well (from a 50 µg/ml stock solution, Takara, Japan) and further incubated for 2 hours at RT. Then retronectin solution was discarded and 1 ml H_2O containing 2% HSA was added in order to block the plate for 30 minutes at RT. Thereafter, HSA solution was discarded and the wells were washed with 1 ml HBSS/2.5% (v/v), 1M Hepes and 1 ml 1x PBS. The 1x PBS remained in the wells until the viral supernatant was added. The viral supernatant was thawed quickly in a 37°C water bath and added into the previous retronectin coated wells with an MOI of 5 (the total volume of the viral supernatant never exceeded 1,5 ml). The virus-loaded plate was centrifuged at 2000 rpm for 30 minutes at 4° C. After that, the supernatant was carefully discarded and the new virus supernatant was added. This centrifugation process was repeated 2 times. The pre-stimulated mouse $Sca1^+$ BM cells were diluted to a concentration of 4 x 10^5 cells/ml and 500 µl of the cell suspension were then added into the prepared wells after removing of the viral supernatant. During the following overnight incubation at 37°C in the incubator, the virus infected the cells. Within the next two days infection was repeated twice like described below: one time infected cells were resuspended well and transferred into a new virus-coated well. The used well was washed 2 times with 1x PBS to collect remaining cells. Those were added in 300 µl fresh cytokine medium to the already transferred cell suspension. 24 - 36 hours after transduction, the cells were collected for FACS-analysis of transduction efficiency, CFU-assay (see chapter 3.6.7) and potentially transplantation (see chapter 3.7.4).

3.6.8 Methylcellulose Based CFU-assay (*StemCell Technologies Inc*)

This method was used to determine the potential of haematopoietic progenitor cells in total bone marrow or peripheral blood to form colonies in semi-solid medium. Methylcellulose based medium allows cells, derived from the same progenitor cell, to build colonies. The process of CFU-assay is showed below in Figure 3.3.

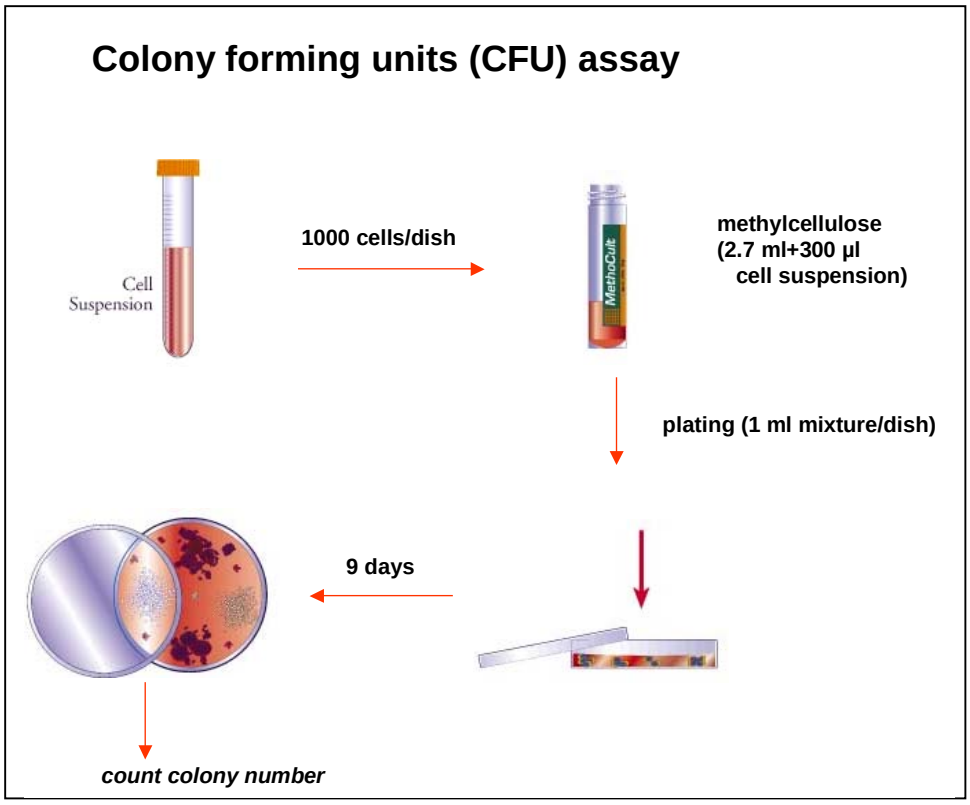


Figure 3.3: Methylcellulose based CFU-assay. 1000 cells/dish Sca1$^+$ mBM cells were diluted with IMDM medium in the volume of 300 µl, and then added into previously aliquoted 2.7 ml methylcellulose in a poly tube. After well vortexing the samples were allowed to dissipate 5-10 minutes until the air bubbles disappeared. Then 1.0 ml mixtures were dispensed into each of two 35 mm culture dishes using a 3 ml syringe and a 16 gauge blunt-end needle. The dishes were gently rotated to spread the methylcellulose evenly. Then the dishes were placed in a 100 mm petri dish with a third 35 mm dish containing 3 to 4 ml of sterile water. This plate was incubated for 9-10 days at 37° C and 5% CO_2 in the incubator. When counting the colonies, the cell populations over 50 cells per colony were calculated as one colony.

3.6.9 Selective Expansion of Transduced BaF/3 Cells and mBM Cells with Small Molecular Dimerizer AP20187

To achieve selective expansion of transduced cells, we employed in this study the FKBP/AP20187 system from the company *Ariad (Cambrige, Uk)*. This homodimerization kit is based on the modified human protein FKBP12 (FKBP, for FK506 binding protein) and its small binding molecule AP20187. This molecule has the benefit to bind with subnanomolar affinity to FKBP and is suitable for *in vitro* and *in vivo* experiments. The structure and the mechanism of AP20187 are shown in Figure 3.4.

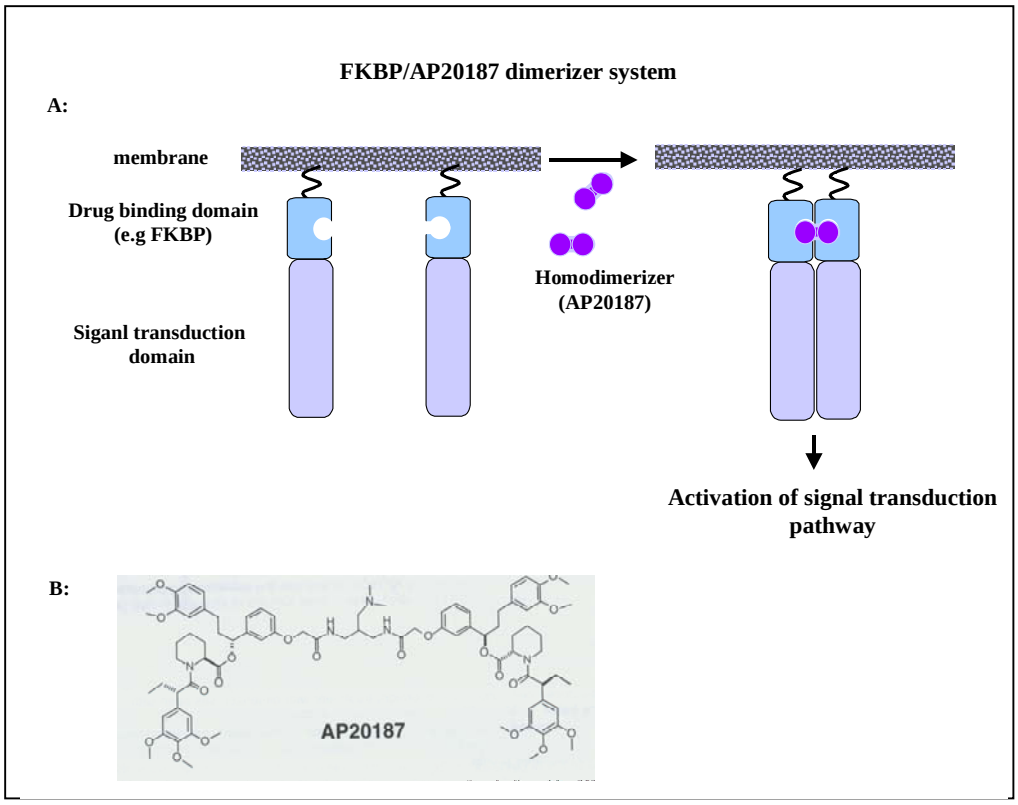


Figure 3.4: Structure and mechanism of the FKBP/AP20187 system. A: The signal transduction domain can be activated after drug binding domain (FKBP) binding with dimerizer AP20187. B: Chemical structure of small dimerizer AP20187.

3 days after transduction with retroviral or lentiviral vectors, AP20187 induced proliferation was investigated. 6 x 10^5 transduced BaF/3 cells were washed three times with 1 x PBS, then divided in 3 wells in a 24-well plate in a total end volume of 1 ml under three different conditions: RPMI; RPMI + mIL-3 (10 ng/ml); RPMI + AP20187 (10 nM). The cell number was counted (see chapter 3.6.2) in the interval of 2-3 days and the cumulative cell number was calculated. The transduced mBM cells were washed three times with 1x PBS and then cultured under following conditions: RPMI + cytokines (see chapter 3.6.4), RPMI + mSCF (50 ng/ml) + AP20187 (10 nM). The cell number was determined weekly. To proof for reversibility of the effect of AP20187 on the transduced cells, 3 weeks after selection, the AP20187 was removed from the culture by extensive washing with 1xPBS. The cells were further cultured with RPMI (for BaF/3 cells) or RPMI + mSCF (50 ng/ml for mBM cells) for 3 weeks and the cumulative cell number was calculated as described in chapter 3.6.2.

3.7 Animal Experiments

3.7.1 Breeding of Animals

All mice used in the experiments were bred in the animal centre of the Georg-Speyer-Haus (Frankfurt, Germany). The health status of the animal centre was monitored based on the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA). Minimal of 3 days before transplantation, the C57BL/6 inbred female mice were purchased from Charles River Laboratories. They were bred in standard sterile cages (maximal 5 mice /cage) and feed with standard rodent diets and water. The donor mice Ly 5.1 were purchased from Jackson Laboratory/USA, and further maintained in the animal centre. For the bone marrow cell preparation, mice of an age between 6-8 weeks were chosen. All experiments were performed out according to the German Animal Protection Law (Tierschutz: Genehmigungs-aktenzeichen: 211-2531-31/2002).

3.7.2 Preparation of Murine Bone Marrow Cells

The mice chosen for the bone marrow cell preparation were killed after narcosis with enfluran. The bone marrow from femurs and tibias of each mouse was isolated by flushing with 25 ml preparation buffer (1x PBS + 5 % FCS+1 % pen-strep) using a 23-gauge needle (3 mice in a 50 ml tube). Then the bone marrow cells were suspended well and centrifuged at 1200 rpm and 4° C for 5 minutes. The cell pellet was resuspended in 1 ml preparation buffer. To lyse the erythrocytes, 9 volumes of lysis buffer (0.8% NH_4Cl; 10 µM EDTA) were added to the cell suspension and mixed well by vortexing. After that mixture was incubated for 10 minutes at RT. 30 ml preparation buffer was added to the tube after lysis and centrifuged for 6 minutes at 1200 rpm and 4° C. This washing step was repeated 1 time and cell pellet was resuspended in 5 ml preparation buffer. Thereafter the cell suspension was filtered through a nylon mesh (BD, 100 µm filter) into a fresh 50 ml tube, in order to remove the big pieces of bone or fat tissues. The cell number was determined and the bone marrow cells were ready to go for the further purpose or for freezing.

3.7.3 Enrichment of Sca1 Positive Cells from Total mBM Cells with *EasySep™* kit (*StemCell Technology*)

$Sca1^+$ mouse haematopoietic progenitor cells can be enriched from total mBM cells via immunomagnetic cell selection procedure, which requires a PE-conjugated monoclonal

$Sca1^+$ antibody. Fresh prepared mBM cells were counted with Tuekish' solution and suspended at a concentration of 1 x 10^8 cells/ml in buffer containing 1x PBS, 1 mM EDTA and 2% FCS. Murine FcR blocker (applied from *EasySep* kit) was added at a concentration of 10 µl/ml to the cells and the sample was well mixed. Then Sca1- PE labelling reagent was added at a concentration of 15 µl/ml. After well mixing the cells were incubated at 4° C for 15 minutes. The sample was washed once with 10-fold excess buffer and resuspended in half of the original volume, further transferred in a 12 x 75 mm polystyrene tube to properly fit into the EasySep™ Magnet. Thereafter 100 µl/ml PE-selection cocktail was added to the sample and the sample was incubated at 4° C for 15 minutes. At last, EasySep™ Magnet nanoparticles were added at 50 µl/ml to the sample and the mixture was further incubated at room temperature for 10 minutes. The total sample volume was brought up to 2.5 ml by adding buffer. Finally the tube was placed into the magnet and set aside for 5 minutes. Then the supernatant fraction was poured off and the magnetically labelled cells remained inside the tube. The tube containing the negative sample was removed from the magnet and 2,5 ml buffer was added. The sample was mixed by gently pipetting up and down 2-3 times and placed into the magnet again. This process was repeated 3-6 times and at the end the selected cells were suspended in the suitable culture medium for further experiments.

3.7.4 Bone Marrow Transplantation of Genetically Modified Cells

At the day of transplantation, the recipient mice (C57BL/6, Ly5.2) were sublethally irradiated with 8.0 Gy from a ^{137}Cs γ-ray source at the University Hospital Frankfurt/Main, Germany. The cells for transplantation with the appropriate cell number were washed 3 times with 1x PBS, and then kept on ice before transplantation. 2-4 hours after irradiation, transplantation was performed through the injection of the cells into the tail vein of the recipient mice. The analysis of the mice was carried out 8-10 weeks after transplantation.

3.8 Flow Cytometry

To characterize the phenotype of the different cell types, the cell surface expression of a number of membrane proteins was assessed by flow cytometry. For determination of surface expression, 1-2 x 10^5 cells per staining reaction were washed twice for 5 minutes with 3 ml of ice-cold FACS buffer (1x PBS; 1% FBS; 0.1% Sodium acid) and immunostained for 20 minutes at 4° C with a selection of biotin, fluorescein-isothiocyanate (FITC)- and

phycoerythrin (PE)-conjugated antibodies or non-conjugated antibodies as listed in Table 3.3. Cells stained with non-conjugated primary antibodies had to be incubated with a secondary antibody like rat anti mouse IgG1-PE, IgG1-APC or streptavindin-APC (SA-APC) for 15 minutes in 4° C. Between two incubation steps the cells were washed once with 1x PBS. At least, stained cells were fixed with 200 µl fixing buffer (1x PBS; 1% Formaldehyde) after washing process. For the samples from mouse primary bone marrow cells or peripheral blood, the Fc-block was added into each sample (2 µl from 1:10 diluted solution) before staining in order to block the non-specific binding positions.

Table 3.3: Antibodies for FACS analysis.

Cell surface expressed protein	Primary antibody	Secondary antibody
gp91	human 7D5	RAM IgG1-PE; RAM IgG1-APC
CD 45.1	mouse α-CD45.1-PE	-
LNGFR	human α-LNGFR-PE	-
CD 3	mouse α-CD3-biotin	Streptavidin-APC
Gr-1	mouse α-Gr1-biotin	Streptavidin-APC
B220	mouse α-B220-biotin	Streptavidin-APC
Sca1	mouse α-Sca1-PE	-
c-kit	mouse α-ckit-PE	-
Fc-block	mouse α-CD16/CD32	-

3.9 Intracellular Immunostaining

The localization of proteins in cells can be proved by indirect immunofluorescence with proper antibodies. This method relies on the following steps: fixation of cells; exposition of the cells to the primary antibody of interest; removing of the unbound antibody; incubation of the cells with a fluorescence-tagged secondary antibody.

The sterilized coverslips (24 mm) used for the staining of SC1 cells were coated with 1 % polylysine for 5 minutes at room temperature. After 2 times washing with water, the coverslips were dried properly at least for 2 hours at room temperature. 2 x 10^5 SC1 cells were plated on a coverslip placed in a 6-well. After 24 hours the cells were transfected with pure plasmid DNA and incubated for 2 days. At the day of staining, the cells were washed 2 times with 1 x PBS for 5 minutes and fixed in fixing buffer (1x PBS + 3% formaldehyde) for 15 minutes. After two further washing steps (each step 5 minutes), the cells were permeabilized with permeability buffer (1x PBS + 0,1% triton) for 4 minutes and then

furthermore washed 2 times with 1x PBS for 5 minutes. After 5 minutes blocking of the cells in blocking buffer (the cells were incubated for 40 minutes at room temperature with the first antibody (e.g. anti-FKBP) diluted 1:1000 in blocking buffer. After 2 washing steps with blocking buffer (1x PBS + 3% BSA) 5 minutes each step. Cells were incubated with the secondary antibody (1:5000 - 1:10000 diluted) in blocking buffer for 40 minutes at room temperature. Finally, the cells were washed again 2 times with blocking buffer for 5 minutes. The coverslips with cells were transferred on a glass-slide and mounted in mounting medium (Vectashield, Vector Lab., CA, USA) with DAPI. The slide was now ready for viewing on the fluorescence microscope. As a negative control cells stained only with a secondary antibody were used.

4. Results

Gene transfer in haematopoietic stem cells (HSCs) holds the promise of providing a long-standing cure for many inherited and acquired blood cell disorders. However, the low efficiency of gene transfer into human HSCs with retroviral vectors is one of the major obstacles associated with HSC gene therapy. Therefore, methods aimed at achieving selective expansion of genetically modified HSCs are required (see chapter 1.3). Additionally, the development of gene and cell therapies would benefit substantially from methods allowing specific control over the growth of genetically modified cells after transduction and transplantation. In this study, 2 separate positive selection systems were developed for the expansion of genetically modified murine HSCs to attain therapeutic levels of cells expressing the transgene. One system relies on the constitutive overexpression of HOXB4. The other strategy makes use of an inducible selection system. Chemically induced dimerization of a cytokine receptor activates function of the selection marker genes epidermal growth factor (EGF) receptor and prolactin-receptor. Cell proliferation, transgene expression and differentiation potential were measured *in vitro* and / or *in vivo* for both strategies.

4.1 HOXB4 Induces Selective Expansion of gp91^{phox} Gene-Modified Murine HSCs

The homeodomain-containing transcription factors of the HOX gene family, regulators of pattern formation and tissue identity during embryogenesis, have also been identified as regulators of haematopoietic cell proliferation and differentiation (Pekins et al., 1993; Sauvageau et al., 1995; Thorsteinsdottir et al., 1997). The most interesting member of the homeobox gene family in the haematopoietic system is HOXB4. Ectopic expression of this gene with retroviral vectors has led to a 1000-fold expansion of HSCs in transplantation studies *in vivo* (Sauvageau et al., 1995; Antochuck et al., 2001) and, importantly, no haematological abnormalities were found in a large group of mice transplanted with HOXB4-transduced HSCs. In contrast, overexpression of HOXB3, for example, can induce myeloid leukaemia and perturbed lymphopoiesis (Thorsteinsdottir et al., 1999). This prompted us to investigate whether expression of HOXB4 can also increase the proportion of HSCs expressing the therapeutic gene gp91^{phox}, when both genes are co-expressed in HSCs.

4.1.1 Cloning and Titre of Bicistronic Construct Sxgs-HOXB4

The bicistronic retroviral construct Sxgs-HOXB4 was cloned as indicated in Figure 4.1.1. The initial construct containing the full-length HOXB4 sequence was provided by Christopher Baum (Hannover Medical School). In this construct, the enhanced green fluorescence protein (eGFP) sequence was fused with HOXB4 via 2A sequences and served as the marker gene for expression. The retroviral vector Sxgs-dLN from Dr. Stefan Stein in our laboratory was used as the cloning vector.

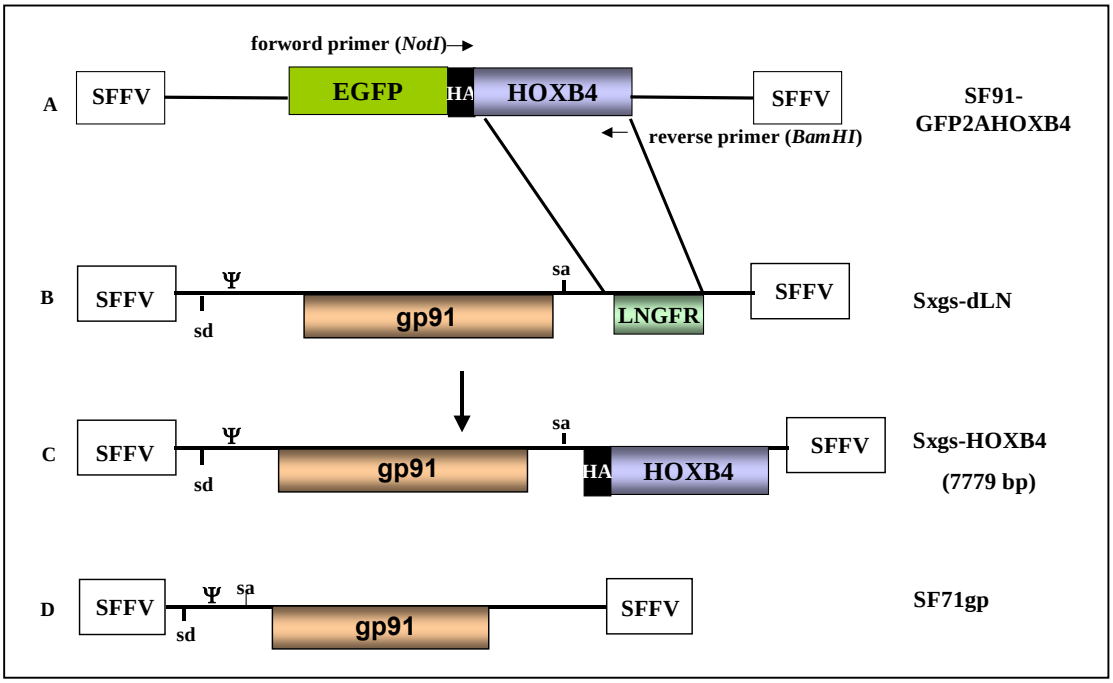


Figure 4.1.1: Cloning of the bicistronic construct Sxgs-HOXB4. All constructs were derived from a MLV (murine leukaemia virus)-based retroviral vector under the control of the SFFV promoter. A: Amplification of the full-length *HOXB4* gene sequence with PCR primers designed with *Not*I and *Bam*HI restriction sites. B: Backbone of the retroviral cloning vector Sxgs-LΔS. The therapeutic gene gp91^{phox} is located between the viral splice sites and the gene for ΔLNGFR (truncated low affinity receptor for human nerve growth factor). This vector was cut with *Not*I and *Bam*HI and ligated with the PCR product from step A. C: Final construct Sxgs-HOXB4. The full-length HOXB4 sequence and HA tag from A was cloned into Sxgs-LΔS, forming the new construct containing gp91^{phox} and *HOXB4*. D: Control construct SF71gp with the human gp91^{phox} sequence. SFFV: spleen focus forming virus LTR; EGFR: epidermal growth factor receptor; HA: influenza haemagglutinin tag; sd: splice donor; sa: splice acceptor; ψ: packaging signal.

SF71gp, which only contains the gp91^{phox} gene (Figure 4.1.1 D), served as a control construct for the following *in vitro* and *in vivo* experiments. Ecotropic retroviral particles

were generated in Phoenix-E producer cells as described in chapter 3.6.4, and titres of the viral supernatants were determined in the murine fibroblast cell line SC1. Figure 4.1.2 shows gp91^{phox} gene expression in SC1 cells transduced with supernatants diluted 1:100. The titres ranged from 4×10^5 to 16×10^5 for different vector preparations (Table 4.1.1).

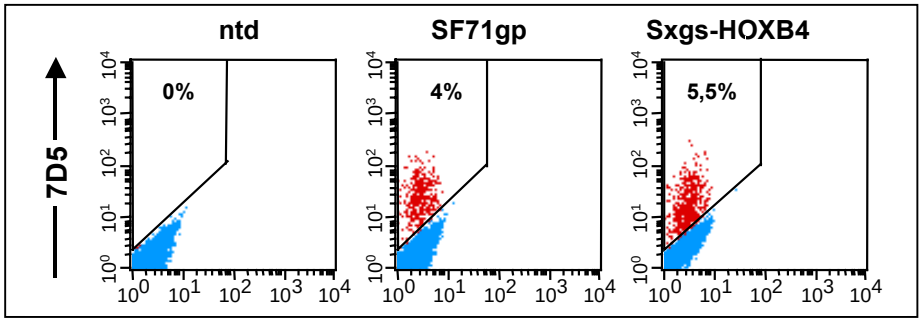


Figure 4.1.2: Titre determination of SF71gp and Sxgs-HOXB4 retroviral particles. 1 day before transduction, 5×10^4 SC1 cells in 1ml DMEM were plated per well of a 24-well plate. Twenty to 24 hours after plating, the viral particles were added to the SC1 cells at dilutions of 1:10 (100 µl) and 1:100 (10 µl). Three days after transduction, the gp91^{phox}-positive cells were detected by FACS analysis with the anti-gp91^{phox} antibody 7D5. The percentage of 7D5-positive cells, which ranged between 2 and 20%, was used to calculate of the titres in transduction units (TU)/ml. SF71gp-transduced SC1 cells were 4% positive (middle) and Sxgs-HOXB4-transduced cells were 5% positive (right). The viral particles used here were diluted 1:100. ntd: non-transduced cells.

Table 4.1.1: Range of titres of SF71gp and Sxgs-HOXB4 retroviral vectors

Constructs	SF71gp	Sxgs-HOXB4
Titre on SC1 cells TU/ml	4–13 × 10^5	5–16 × 10^5

4.1.2 *In Vitro* Functional Analysis of Sxgs-HOXB4 Transduced Primary BM Cells

To test the ability of HOXB4 to exert expansion of HSCs, functional analyses were carried out with Sxgs-HOXB4-transduced cells. Total murine bone marrow (mBM) cells were freshly prepared from C57BL/6 mice. After 2–3 days prestimulation with the cytokine combination mIL-3 (10 ng/ml), mIL-6 (50 ng/ml) and murine stem cell factor (50 ng/ml) (SCF/IL-3/IL-6), the mBM cells were transduced for 3 days with either Sxgs-HOXB4 or SF71gp. The transduction efficiency is shown in Figure 4.1.4. Following transduction, cells were cultured in a cytokine combination of SCF/IL-3/IL-6 for 3 weeks. The proliferation potential and transgene expression are illustrated in Figures 4.1.3 and 4.1.4.

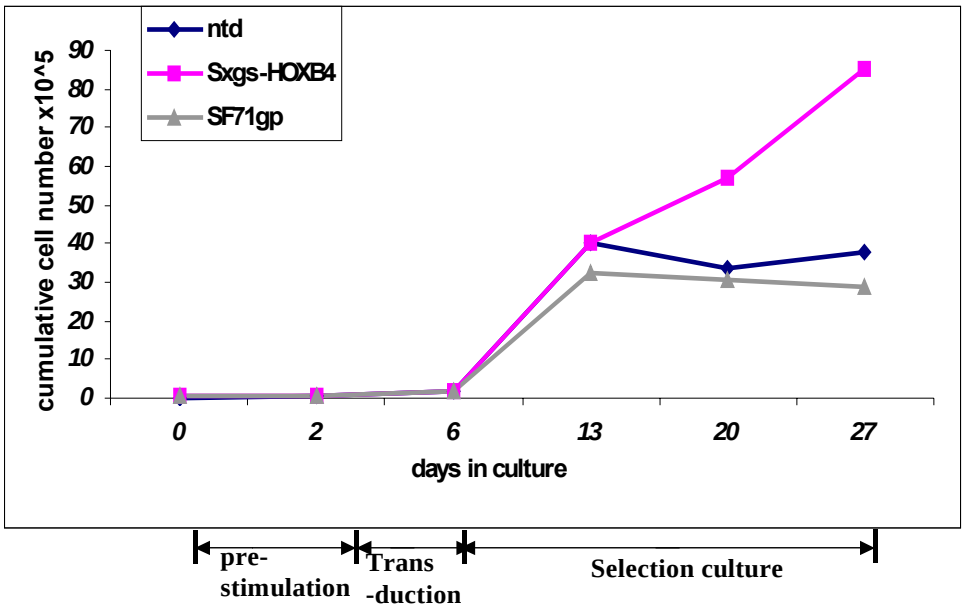


Figure 4.1.3: Cell proliferation of Sxgs-HOXB4-transduced primary mBM cells. Total mBM cells were transduced as explained in the text. After transduction, the cells were cultured for an additional 3 weeks in a standard cytokine combination. Pink line: proliferation curve of cells transduced with Sxgs-HOXB4. Blue line: proliferation curve of non-transduced cells. Grey line: proliferation curve of SF71gp-transduced cells.

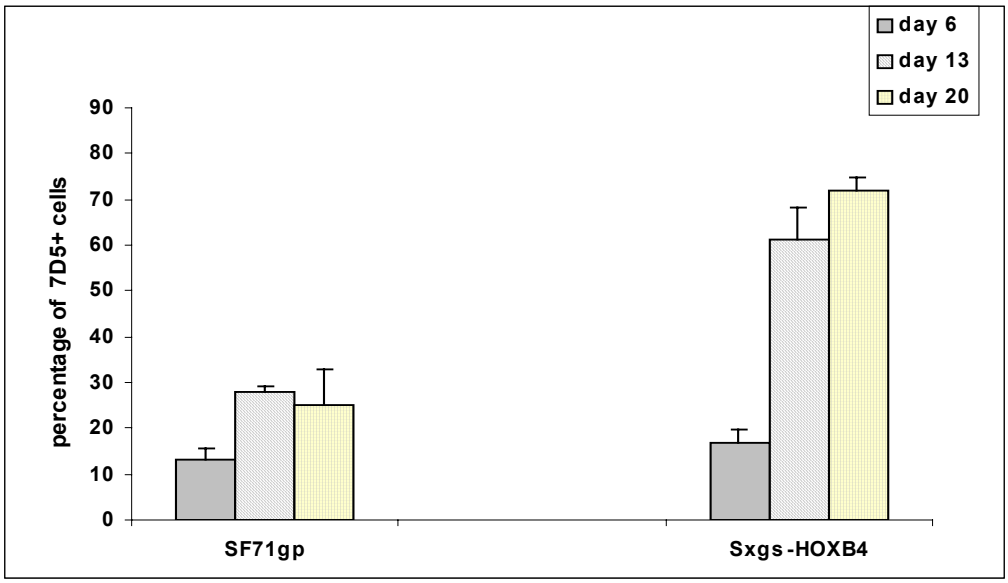


Figure 4.1.4: Transgene expression of Sxgs-HOXB4- and SF71gp-transduced primary BM cells. The percentage of 7D5-positive cells was calculated from FACS analysis with the 7D5 anti-gp91^{phox} antibody on days 6, 13 and 20.

As shown above, 3 weeks after transduction, the number of Sxgs-HOXB4-transduced cells had increased by about 8-fold compared to day 6. In contrast, the number of non-transduced or SF71gp-transduced cells increased by about 3.5 fold during the first culture

week, after which the cells stopped proliferating. The significant proliferation of Sxgs-HOXB4-transduced cells was mirrored by the gp91^{phox} transgene expression (Figure 4.1.4), which was 7-fold higher after 2 weeks expansion. Cells transduced with the control construct showed only a minor increase (1 fold) in 7D5-positive cells (Figure 4.1.4).

The standard *in vitro* method for identifying murine haematopoietic progenitor cells is the methylcellulose based CFU-assay, which allows the formation of colonies from one single progenitor cell in semi-solid medium. Primary mBM cells transduced with the Sxgs-HOXB4 and control constructs were plated in methylcellulose containing the appropriate cytokines 1 day and 1 week after transduction. After 10 days incubation, clusters of 50 or more cells were counted as 1 colony. In addition, the morphology of the colonies was investigated under the microscope (Figure 4.1.5).

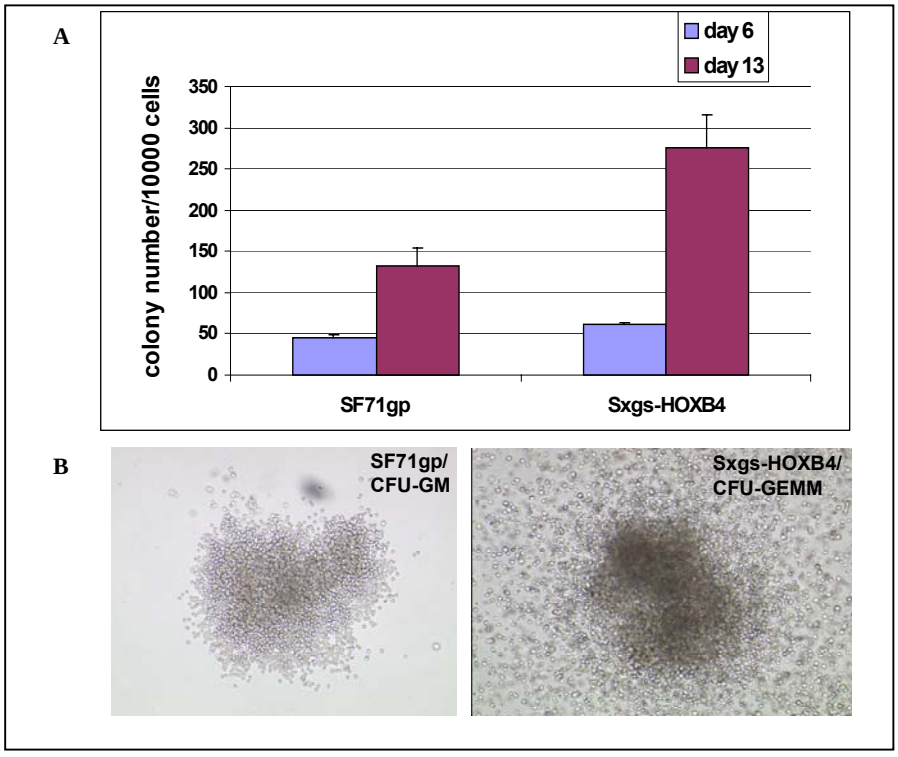


Figure 4.1.5: Colony forming unit (CFU) assay in methylcellulose. A: Colony number. Transduced cells were plated in methylcellulose (*StemCell*) at a concentration of 5000/35cm dish 1 and 6 days after transduction. After 10 days incubation at 37°C, the total colony number was determined. B: Colony morphology was observed under the microscope. Right: colony from Sxgs-HOXB4-transduced cells. Left: colony from cells transduced with the control construct.

As illustrated in Figure 4.1.5, the total colony number of Sxgs-HOXB4-transduced cells had increased to 62 colonies/1000 cells 1 week after transduction, almost 2-fold higher than the colony number counted on day 6. In contrast the colony number of mock-transduced

cells remained at a low level. Colonies from expanded Sxgs-HOXB4-transduced cells included early progenitor cell colonies, such as CFU-GEMM, BFU and CFU-GM, whereas those from cells transduced with the control construct were dominated by CFU-GM colonies.

4.1.3 *In Vivo* Experiments

As demonstrated above, expression of the Sxgs-HOXB4 construct in murine primary BM cells led to the selection of modified cells, especially of haematopoietic progenitor cells. Previously, it was reported that expression of HOXB4 caused 50–1000 fold expansions of murine haematopoietic cells *in vivo*, whereas no strong proliferation advantage could be observed *ex vivo* (Antochuck et al., 2002). To investigate whether our construct has a similar effect *in vivo*, we performed transplantation experiments in mice. In this setting, the constructs SF71gp and SF91-GFP2AHOXB4 served as the negative and positive controls.

Fresh total mBM cells from the C57BL/6 Ly5.2 mice (see chapter 3) were prepared and enriched with an anti-Sca1 antibody as described in the *EasySep* kit (StemCell Technology; see chapter 3.7.3). After enrichment, the percentage of $Sca1^+$ cells was about 87%. These cells were cultured with a cytokine combination of SCF/IL-3/IL-6 for BM cells for 3 days. Pre-stimulated cells were then incubated with retroviral particles containing Sxgs-HOXB4, SF71gp and SF91-GFP2AHOXB4 vectors. The titres of the viral particles, multiplicity of infection (MOI) and transduction efficiencies are listed in Table 4.1.2.

Table 4.1.2: Titres of viral particles, MOI and transduction efficiencies for *in vivo* experiments

	Titre (TU/ml)	MOI (total)	7D5+/GFP+ cells
Sxgs-HOXB4	8×10^5	18	11%
SF71gp	12×10^5	18	11%
SF91-GFP2AHOXB4	5×10^5	18	36%

One day after transduction, the transduced cells from all groups were injected i.v. into 8.0 Gy-irradiated C57BL/6 recipient mice. All the mice survived until analysis 8–10 weeks after transplantation, when the percentages of transgene-expressing cells (7D5-positive or GFP-positive cells) in the BM cells were determined. The results from all the recipients and average values from the 3 groups are shown in Table 4.1.3 and Figure 4.1.6. In addition, 2 irradiated but non-transplanted mice were used as a control for the irradiation dose. These mice died 12 and 14 days after irradiation.

Table 4.1.3: Engraftment of the transplanted cells in recipients

Mouse number	Construct	% 7D5+ or GFP$^+$ cells in total BM cells	**Mouse number**	Construct	% 7D5+ cells in tatal BM cells
No.1	ntd	0	**No.11**	Sxgs-HOXB4	0.2
No.2	ntd	0	**No.12**	Sxgs-HOXB4	0.3
No.3	SF71gp	4,5	**No.13**	Sxgs-HOXB4	2.4
No.4	SF71gp	5,6	**No.14**	Sxgs-HOXB4	0.3
No.5	SF71gp	3,3	**No.15**	Sxgs-HOXB4	2.6
No.6	SF71gp	13,3	**No.16**	Sxgs-HOXB4	1.8
No.7	SF91-GFP2AHOXB4	78	**No.17**	Sxgs-HOXB4	0.5
No.8	SF91-GFP2AHOXB4	79	**No.18**	Sxgs-HOXB4	0.9
No.9	SF91-GFP2AHOXB4	79	**No.19**	ntpl	-
No.10	SF91-GFP2AHOXB4	83	**No.20**	ntpl	-

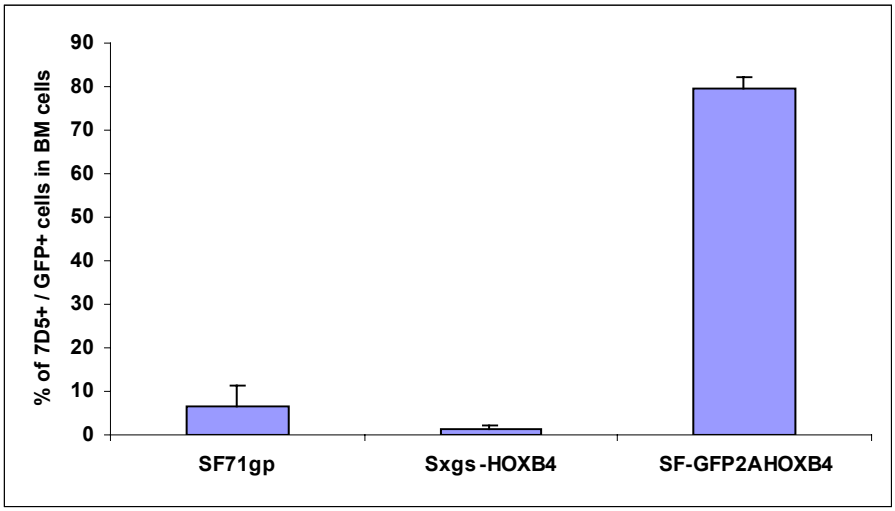


Figure 4.1.6: Average engraftment for each experimental group of recipient mice. Average values and standard deviations of the percentage of 7D5-positive or GFP-positive cells were calculated from the data in Table 4.1.3.

As shown above, the transgene expression levels of the transplanted mice differ significantly. Cells transduced with the positive control construct SF91-GFP2AHOXB4 repopulated recipient mice with about 80% GFP transgene expression. However, the expression of the gp91^{phox} transgene in recipient mice, which were transplanted with cells transduced with our construct Sxgs-HOXB4, was very low, only about 1%. This value is even lower than the number of gp91^{phox}-positive cells in recipients of cells transduced with the negative construct lacking HOXB4 sequences (about 5%). Therefore, high transgene

expressing cells cannot be observed in recipient mice transplanted with Sxgs-HOXB4-transduced Sca1+ cells.

In summary, overexpression of the retroviral vector Sxgs-HOXB4 in primary BM cells *in vitro* can lead to a 3–4-fold higher cell proliferation with high numbers of $gp91^{phox}$ expressing cells compared to cells transduced with the control construct. The expanded cell population contained a high number of progenitor cells, as determined by CFU-assay. Nonetheless, high transgene expressing cells were not determined in the BM cells of recipient mice transplanted with Sxgs-HOXB4-transduced cells, although 11% of the transduced Sca1+ BM cells were $gp91^{phox}$ positive.

It has been reported that high ectopic expression of HOXB4 impairs lymphoid and myeloid differentiation in transplanted mice (Schiedlmeier et al., 2003). The function of HOXB4 is dependent on the expression level, which we cannot quantitatively control. My aim, therefore, became the development of a retroviral vector that is suitable for the controllable selective expansion of gene-modified cells.

4. 2 Testing of Truncated Forms of Epidermal Growth Factor Receptor (EGFR) and Prolactin Receptor (PrlR) as Selection Molecules

The receptors for EGF and prolactin are membrane-bound proteins. Following ligand-induced dimerization, the intracellular region serves as the signal transduction domain, which is important for cell proliferation, survival and other physiologic functions (see Introduction, chapters 1.1 and 1.4). If dimerization can be controlled with chemical inducers of dimerization (CID), then the proliferation advantage of EGFR and PrlR can also be used for the selective expansion of gene-modified HSCs. In the following approach, activation of receptor signalling was coupled with inducible dimerization of the fusion constructs S-NF2/PrlR and S-NF2/EGFR, allowing it to be controlled by the addition of activators, such as the drug AP20187 (Ariad, www.ariad.com).

4.2.1 Lentiviral Vectors Containing Selectable domains Derived from the EGF and Prolactin Receptors

The human EGFR cDNA and the mouse PrlR cDNA are shown in Figures 4.2.1 and 4.2.2. The intracellular domains of both receptors were amplified using PCR with appropriate primer pairs, as described in chapter 3.1.7, and cut with the restriction enzymes *Spe*I and

*Kpn*I. The lentiviral cloning vector contained the marker gene LNGFR in frame with 2 copies of FKBP (see description in chapter 3.6.8) and the intracellular domain of human thrombopoietin receptor (hmpl). The cloning strategy is shown in Figure 4.2.1. The resulting constructs, S-NF2EGFR and S-NF2PrlR, were used for further functional analysis.

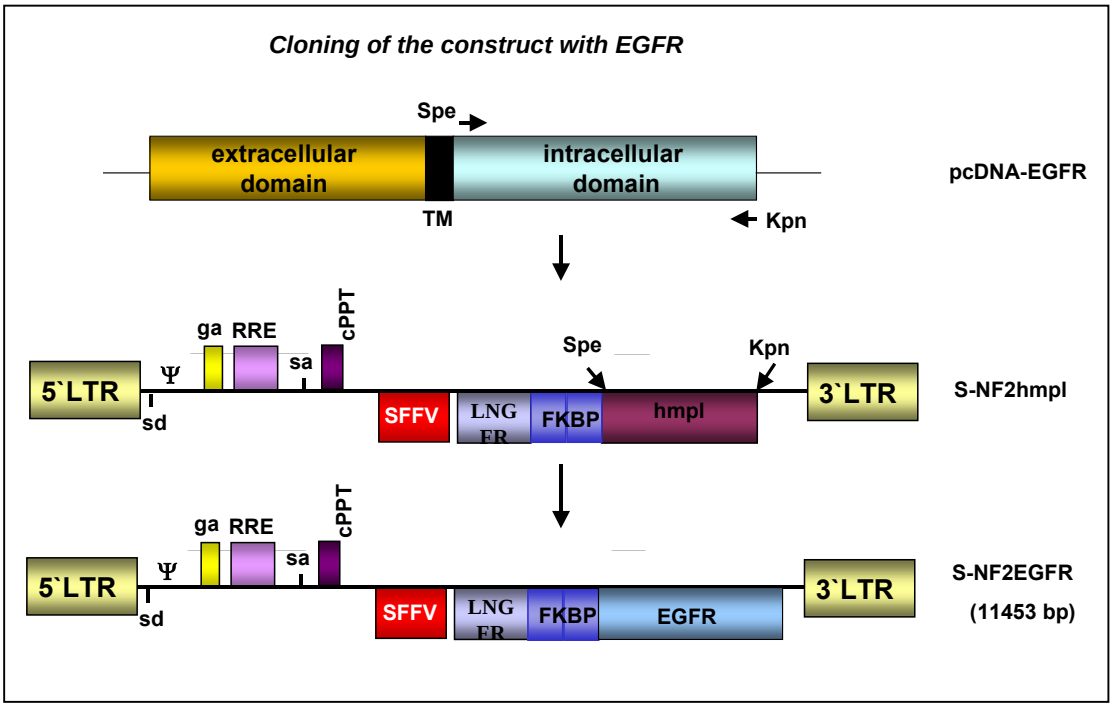


Figure 4.2.1: Cloning of S-NF2EGFR. The sequence encompassing the intracellular domain of the human EGFR was amplified by PCR to introduce *Spe*I and *Kpn*I restriction sites. This product was cloned into *Spe*I- and *Kpn*I-digested vector DNA. Positive clones were detected by restriction digest and appropriate cloning was proven by DNA sequencing.

TM = transmembrane domain; sd = splice donor; ga = gag; RRE = rev response element; sa = splice acceptor; cPPT = central polypurine tract; SFFV = promoter/enhancer from spleen focus forming virus; Ψ: packaging signal.

The lentiviral vector SEW, which contains the eGFP sequence as a marker gene in place of the selection cassette, served as a control construct for these experiments.

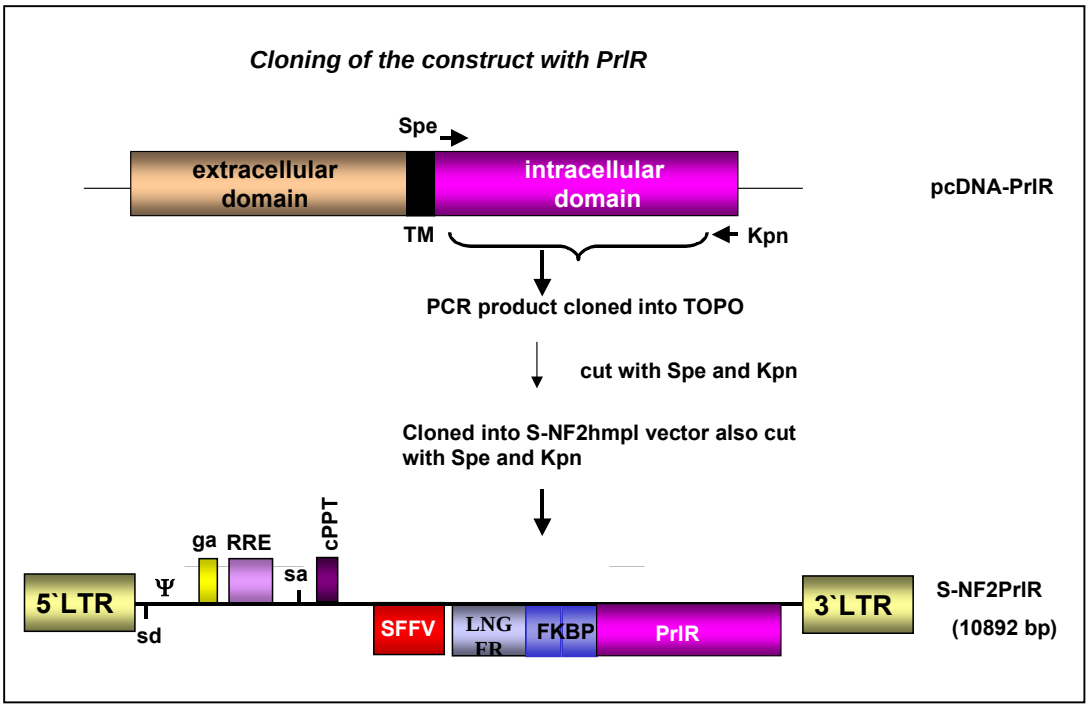


Figure 4.2.2: Cloning of S-NF2PrlR. The intracellular domain of murine PrlR was amplified by PCR and subcloned into the TOPO vector pCR2.1 (Invitrogen). This was then cleaved with *Spe*I and *Kpn*I and the DNA-fragment containing the PrlR was cloned into *Spe*I- and *Kpn*I-digested S-NF2hmpl vector DNA resulting in the new construct S-NF2PrlR. Abbreviations are as in Figure 4.2.1.

4.2.2 Lentiviral Vector Production and Titre Determination on 293T Cells

The lentiviral particles were produced as described in chapter 3.6.6 and their titre was determined on 293T cells (Figure 4.2.3). LNGFR-positive cells were measured using FACS analysis with an appropriate antibody (Figure 4.2.3 A) or direct determination of green fluorescence-positive cells (Figure 4.2.3 B). Table 4.2.1 gives a summary of all titres determined for S-NF2EGFR, S-NF2PrlR and SEW from different experiments.

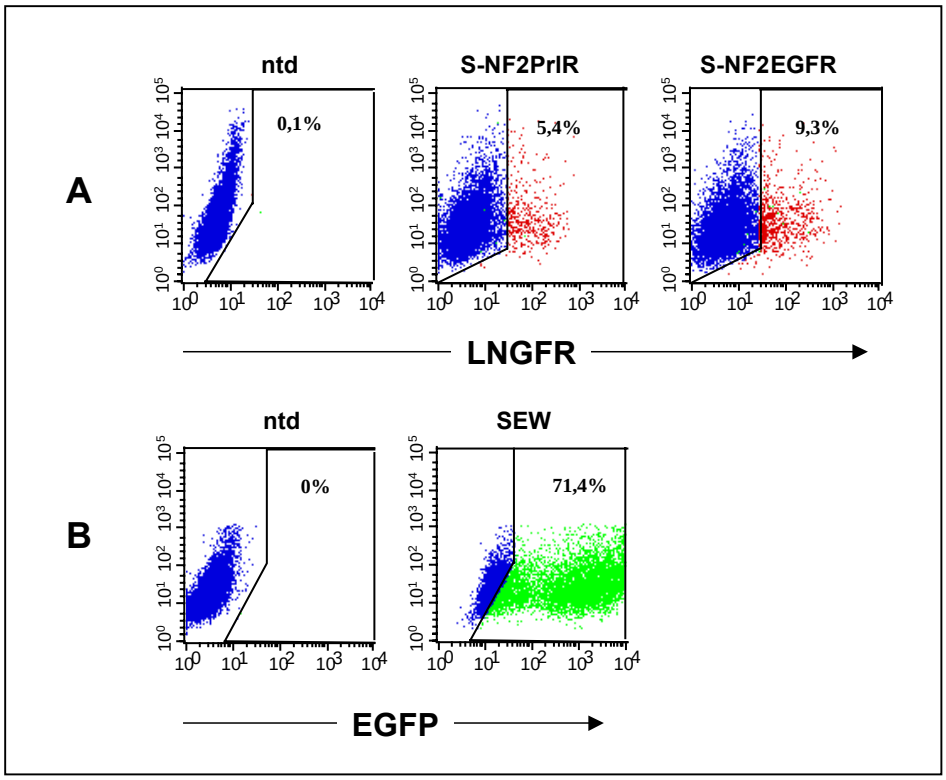


Figure 4.2.3:Titre determination on 293T cells. The virus particles were diluted 1:10 and 1:100, and added to plated 293T cells. Three days after transduction, the cells transduced with the S-NF2PrlR and S-NF2EGFR constructs were prepared for FACS analysis with anti-LNGFR-PE antibody. For the SEW-transduced cells, eGFP-positive cells were measured without staining. A: In this experiment (dilution 1:100), the titres of S-NF2PrlR and S-NF2EGFR were 5.4×10^5 and 9.3×10^5 TU/ml, respectively. B: The titre from control SEW-transduced cells (dilution again 1:100) was over 7.1×10^6 TU/ml.

Table 4.2.1: Range of virus titres from lentiviral constructs

Construct	Titre (TU/ml)
S-NF2EGFR	$2–7 \times 10^5$
S-NF2PrlR	$5–12 \times 10^5$
SEW	$7–13 \times 10^6$

4.2.3 Selective Proliferation Advantage Supplied by S-NF2EGFR and S-NF2PrlR in Transduced BaF/3 Cells

To test whether the fusion proteins allow control of proliferation via dimerization, we used lentiviral particles containing the fusion constructs to transduce BaF/3 cells. BaF/3 is a mouse pro-B cell line, which continuously requires mouse interleukin 3 (mIL-3) for growth. In this study, 5% conditioned WEHI medium was used as a source for mIL-3. AP20187 was used at a concentration of 10 nM. The MOI applied and transduction efficiency is indicated in Table 4.2.2.

Table 4.2.2: The MOI and percentage of 7D5-positive cells in lentiviral vector-transduced BaF/3 cells.

Construct	MOI	Transduction efficiency
S-NF2EGFR	5	21%
S-NF2PrlR	5	19%
SEW	20	21%

The proliferation rates of transduced cell were compared during 2 weeks of culture with and without AP20187. As shown in Figure 4.2.4, cells transduced with S-NF2EGFR and stimulated with AP20187 had a high proliferation rate over the 17 days, similar to that of control cultures in conditioned WEHI medium. The S-NF2PrlR-transduced cells stimulated with AP20187 did not expand significantly in the first 10 days, but started to expand on day 10. The control SEW-transduced cells underwent cell death 1 week after the WEHI conditioned medium had been replaced with AP20187. A high percentage of both S-NF2EGFR- and S-NF2PrlR-selected cells expressed the LNGFR marker gene (Figure 4.2.5). In contrast, non-selected cells (cultured with WEHI medium, without AP20187) did not show an increase in LNGFR-positive cells. Similarly, SEW-transduced cells maintained only low levels of transgene expression (eGFP+ cells) after 2 weeks in culture.

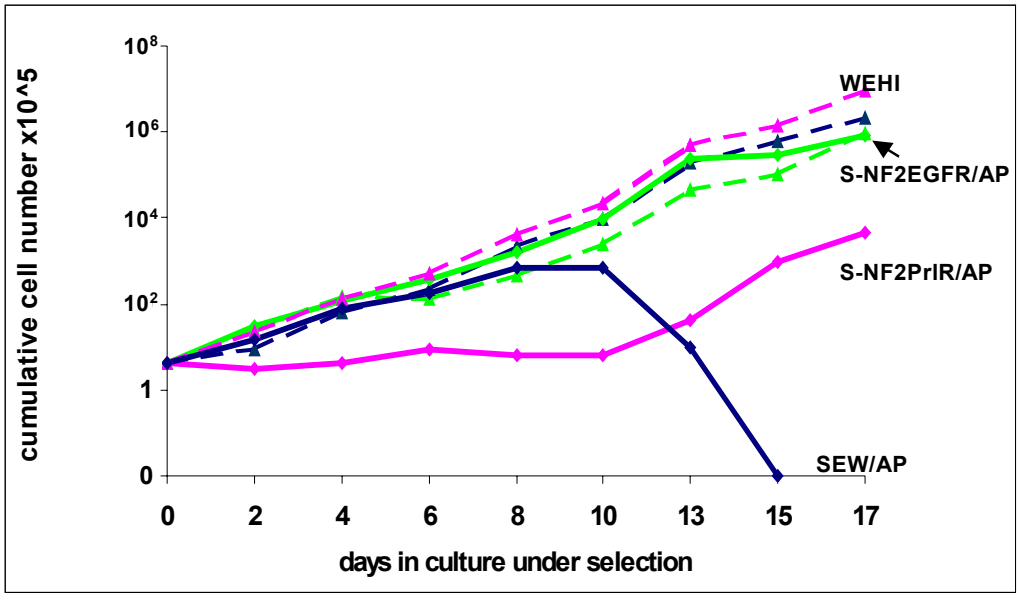


Fig 4.2.4: Transduced BaF/3 cell proliferation under stimulation with AP20187. BaF/3 cells were transduced with SEW (blue), S-NF2PrlR (pink) and S-NF2EGFR (green). Three days after transduction, transduced cells were washed extensively with PBS and cultured further with either conditioned WEHI medium (containing mIL-3) (dotted lines) or 10 nM AP20187 (without mIL-3) (solid lines). The cell number was counted every 2 days by trypan blue dye exclusion.

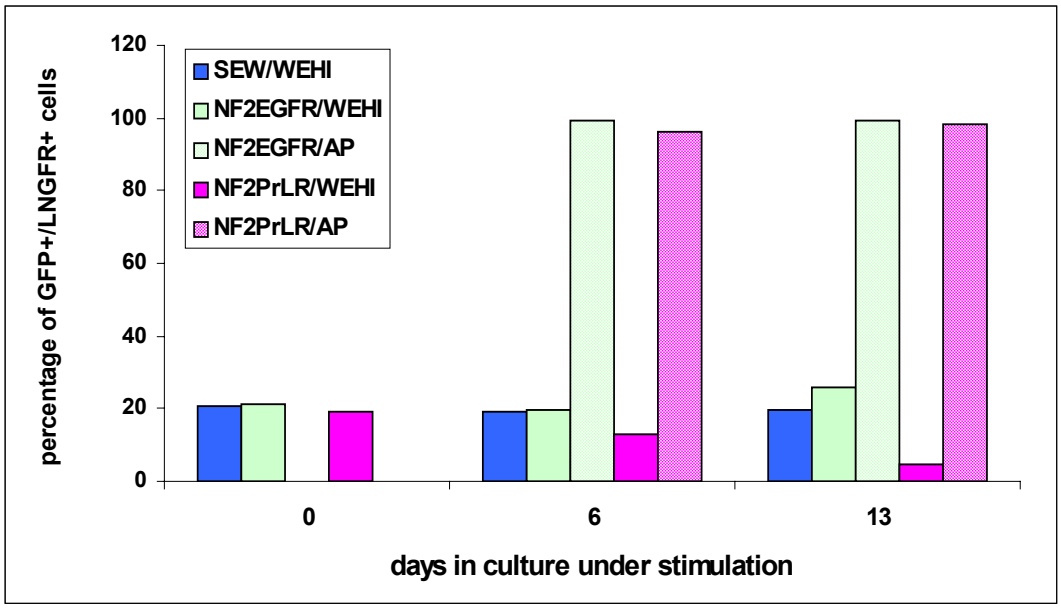


Fig 4.2.5: Transgene expression in selected and non-selected transduced BaF/3 cells. The percentage of transgene-expressing cells (LNGFR+ cells or eGFP+ cells) was determined by FACS analysis with antibodies against human LNGFR for S-NF2PrlR- and S-NF2EGFR-transduced cells or direct fluorescence for SEW-transduced cells at the indicated time points.

As demonstrated in Figure 4.2.5, 2 weeks after selection, the S-NF2EGFR- and S-NF2PrlR-transduced cells showed about 99% (dotted green columns) and 96% (dotted pink columns) LNGFR-positive cells, respectively. In contrast, the S-NF2EGFR-transduced cells cultured in WEHI medium (green columns) did not show any major increase in LNGFR transgene expression. S-NF2PrlR-transduced cells showed a slight decrease in LNGFR-positive cells in conditioned WEHI medium (pink columns).

4.2.4 Reversibility of Dimerizer-Induced Selective Expansion of BaF/3 Cells Expressing EGFR and PrlR Fusion Genes

EGFR and PrlR have putative roles in tumour development, and are often found to be overexpressed in tumour samples, for example PrLR in breast cancer cells (Clevenger et al., 2003). Therefore, reversibility of AP20187-dependent proliferation cells is an important feature of this strategy. To test the reversibility of S-NF2EGFR- and S-NF2PrlR-mediated proliferation, we removed the chemical dimerizer AP20187 from the culture medium of the selected and expanded BaF/3 cells. Proliferation rates of these cells are demonstrated in Figure 4.2.6.

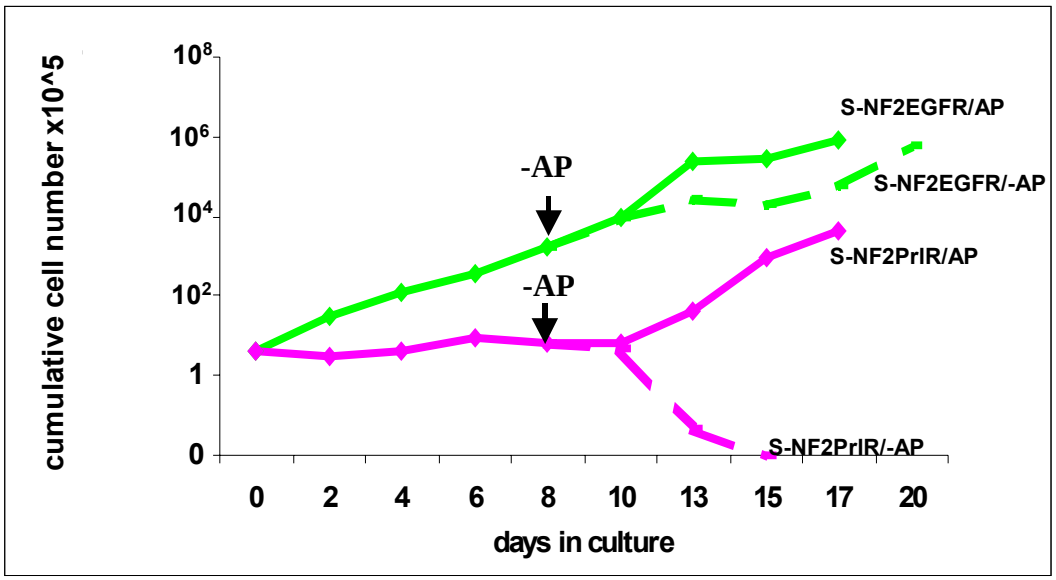


Figure 4.2.6: Removal of AP20187 from the selected BaF/3 cell cultures. This experiment was carried out using the proliferation test as described in Figure 4.2.4. On day 8 after the start of selection (indicated by the arrow in the figure), the cells were washed 3 times with PBS. One sample of the cells was cultured further with AP20187 (continuous line), while the other was cultured without (dotted line). The *y*-axis shows the calculated cumulative cell number.

As demonstrated in Figure 4.2.6, selected S-NF2EGFR-transduced BaF/3 cells continued to proliferate independently even after removal of the dimerizer AP20187 from the medium, whereas proliferation of S-NF2PrLR-transduced cells was entirely dependent on the presence of AP20187.

4.2.5 Protein Detection of Fusion Constructs by Western Blotting

Expression of the fusion proteins was proved by western blotting with an anti-human FKBP antibody (see description in chapter 3.5.3). The expected sizes of the NF2EGFR and NF2PrlR fusion proteins are about 110 kDa and 92 kDa, respectively.

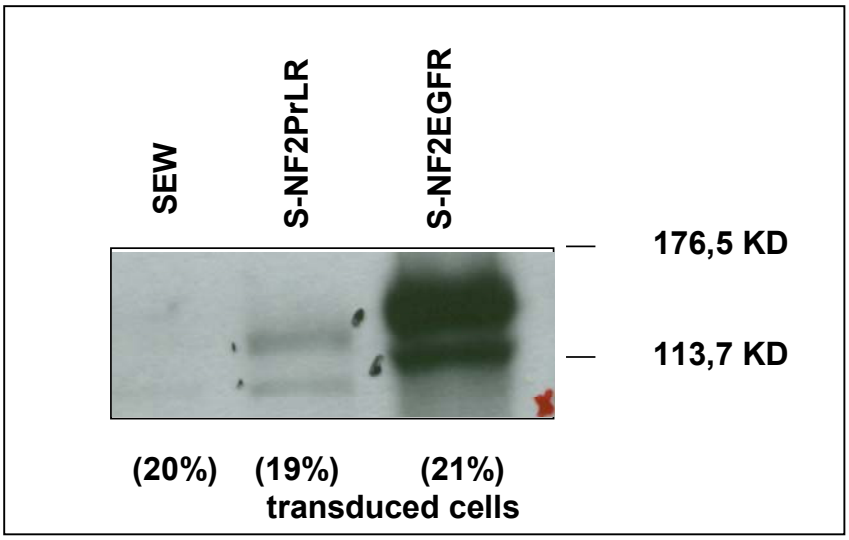


Figure. 4.2.7: Protein detection in cell extracts from transduced BaF/3 cells by western blotting. Whole cell extracts were prepared from SEW-, S-NF2PrlR- and S-NF2EGFR-transduced BaF/3 cells with Triton-X lysis buffer. The proteins were separated by SDS-PAGE, blotted onto a PVDF membrane and incubated with a polyclonal antibody against human FKBP (anti-FKBP12, ABR Affinity Bioreagents). In SEW transduced cells, no FKBP protein was observed (lane 1). Fusion protein expression was detected in S-NF2PrlR- (lane 2) and S-NF2EGFR-transduced cells (lane 3) as a double band. Arrows indicate the expected bands.

As illustrated in Figure 4.2.7, the fusion proteins NF2PrlR and NF2EGFR were detected at the expected sizes of 92 kDa and 110 kDa, respectively. In addition, a higher band signal was observed with both constructs, probably due to glycosylation of the LNGFR part of the fusion protein (Junker et al., 2003).

4.3 Development of a Bicistronic Retroviral Vector Consisting of the Therapeutic Gene gp91^{phox} and a PrlR-Based Selection Cassette

As shown in chapter 4.2, overexpression of the S-NF2EGFR construct led to a proliferation of BaF/3 cells that could no longer be controlled. These results indicate that, despite the advantages of the desired strong proliferation and anti-apoptosis activity, the intracellular domain of EGFR is not suitable for the selective expansion of HSCs, because activation of the selection construct became independent of the dimerizer. In contrast, overexpression of the fusion gene S-NF2PrlR, caused a selective expansion of modified BaF/3 cells that could be switched off by removing the small dimerizer AP20187. Therefore, all vectors used in the following experiments contain a selection cassette derived from the intracellular domain of the PrlR. This cassette was cloned together with the cDNA for the

therapeutic gene gp91^{phox}. As described in chapter 1.3, lentiviral vectors have the striking advantage of also being able to target non-dividing cells. However, the safety of these vectors for human gene therapy approaches is not yet fully established. We therefore used gammaretroviral vectors based on MLV (murine leukaemia virus), which have become a safe and powerful tool for efficient *in vitro*, *ex vivo* and *in vivo* gene transfer into proliferating cells.

4.3.1 Cloning of Retroviral Constructs Sxgs-NF2 and Sxgs-NF2PrlR

The control vector Sxgs-NF2 was obtained by inserting a PCR-amplified 1×FKBP element into the large *Xho*I- and *Bam*HI-restricted fragment of the retroviral vector Sxgs-NF2hmpl, as shown in Figure 4.3.1. The functional vector Sxgs-NF2PrlR was derived from the lentiviral vector S-NF2PrlR and the retroviral vector Sxgs-NF2d3YF containing the sequences for 2xFKBP, the truncated LNGFR, and, downstream of the coding sequence for the therapeutic gene gp91^{phox}. The cloning steps are shown in Figure 4.3.2.

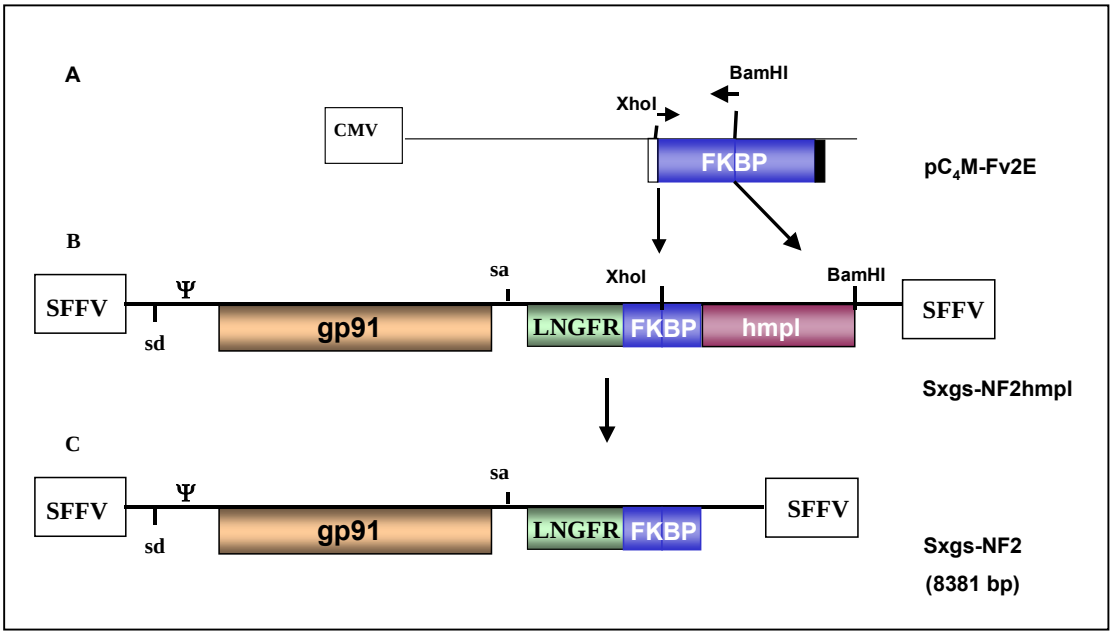


Figure 4.3.1: Cloning of the control vector Sxgs-NF2. One copy FKBP sequence was amplified by PCR using pC$_4$M-Fv2E as a template (Ariad). The PCR primers were equipped with *Xho*I and *Bam*HI restriction sites. The amplified fragment was cloned into the retroviral vector Sxgs-NF2d3YF, which was also cut with *Xho*I and *Bam*HI. A: Structure of template plasmid pC$_4$M-Fv2E. B: Structure of bicistronic cloning vector Sxgs-NF2hmpl containing gp91^{phox} (upstream) and the fusion construct with LNGFR, two copies of FKBP and the human thrombopoietin receptor. Expression of the proteins is controlled by the SFFV promoter and the viral splice donor, splice acceptor system (sd/sa). C: Control vector Sxgs-NF2 with the therapeutic gene

gp91^{phox} and the fusion construct lacking a receptor domain. Numbers indicate length of the entire plasmid in base pairs.

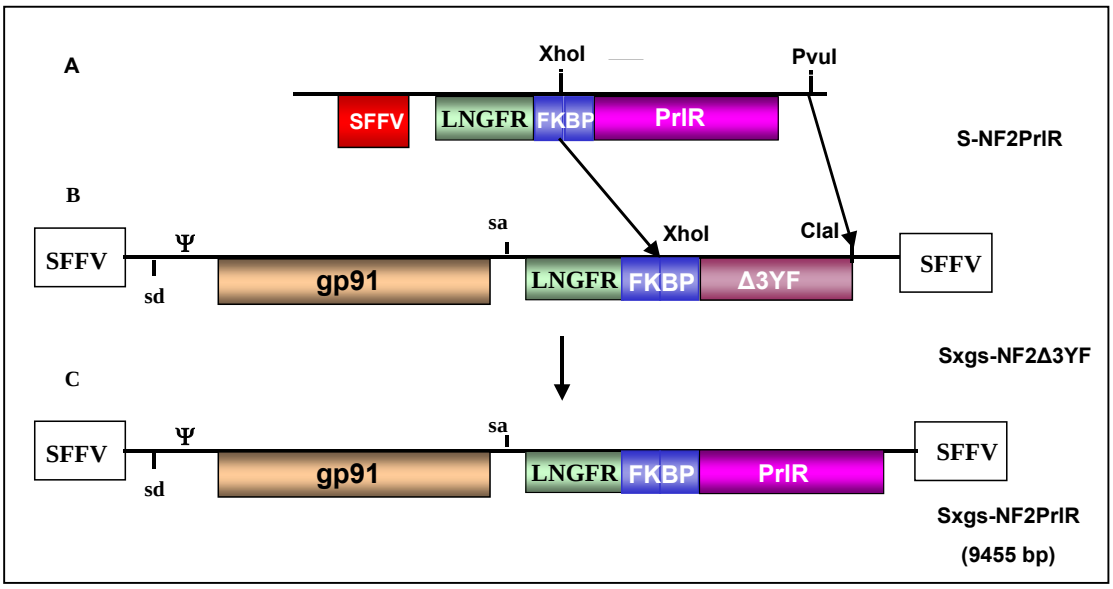


Figure 4.3.2: Cloning of the functional vector Sxgs-NF2PrlR. The insert consisting of one copy FKBP and the intracellular domain of PrlR was cut from the lentiviral vector S-NF2PrlR with *Pvu*I. The fragment was then blunt-ended using the Klenow reaction. Thereafter, the fragment was restricted with *Xho*I and cloned into the vector Sxgs-NF2d3YF between the *Xho*I and blunted *Cla*I restriction sites. A: Scheme of S-NF2PrlR (see also Figure 4.2.2). B: Cloning vector Sxgs-NF2d3YF. C: Functional vector Sxgs-NF2PrlR with the therapeutic gene gp91^{phox} and the fusion construct NF2PrlR.

4.3.2 Production and Titre Determination of Sxgs-NF2 and Sxgs-NF2PrlR Retroviral Supernatants

Retroviral particles were produced in the ecotropic cell line Phoenix E (see chapter 3.6.6). The titre of the virus particles was determined by detecting gp91^{phox} expression in transduced SC1 cells. Cells were stained with the 7D5 antibody and analysed by FACS. An example is shown in Figure 4.3.3. Similar results were also obtained by the detection of LNGFR-positive cells.

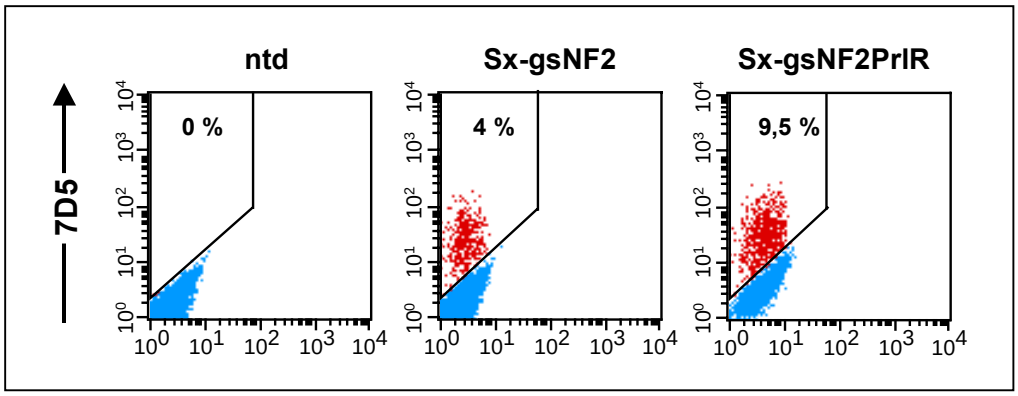


Figure 4.3.3: Titre determination of virus particles carrying the constructs Sxgs-NF2 and Sxgs-NF2PrlR. One day before transduction, 5×10^4 SC1 cells per well were plated in 24-well plates. The virus particles were diluted 1:10 and 1:100 (10 and 100 µl), and added into the wells with 1 ml fresh medium. Three days after transduction, 1×10^5 transduced cells were used for FACS analysis after staining with 40 µl 7D5 antibody and 4 µl anti-RAM IgG-PE as the secondary antibody. The percentage of 7D5-positive cells was used to calculate the virus titre. Left: non-transduced SC1 cells (ntd) were used as the negative control. Middle: Percentage of 7D5-positive cells in Sxgs-NF2-transduced cells. Right: Percentage of 7D5-positive cells in Sxgs-NF2PrlR-transduced cells. The formula from chapter 3 was used to calculate titres in TU/ml. In the example shown, the titres were 4×10^5 TU/ml for the Sxgs-NF2 construct and 9.5×10^5 TU/ml for the Sxgs-NF2PrlR construct.

4.3.3 Functional Analysis of the Sxgs-NF2 and Sxgs-NF2PrlR Constructs in Transduced Murine BaF/3 Cells

To test the Sxgs-NF2PrlR construct, we first transduced it into the mouse pro-B cell line BaF/3 using retroviral particles. Cell proliferation was measured by cumulative cell numbers during 15 days stimulation with AP20187 (Figure 4.3.4). In addition, transgene expression ($gp91^{phox}$-positive cells) was also analysed by FACS with 7D5 antibody (Figure 4.3.5).

As illustrated in Figure 4.3.4, the number of Sxgs-NF2PrlR-transduced BaF/3 cells increased by about 120-fold during culture with AP20187. The selected cell population contained a high percentage of 7D5-positive cells (about 84% after 5 days stimulation and 94% after 14 days) (Figure 4.3.5). In contrast, the Sxgs-NF2-transduced cells underwent cell death within 4 days of stimulation with AP20187. Cells transduced with both constructs and cultured with mIL-3 expanded very strongly. However, the percentage of 7D5-positive cells in these expanded populations decreased during cultivation.

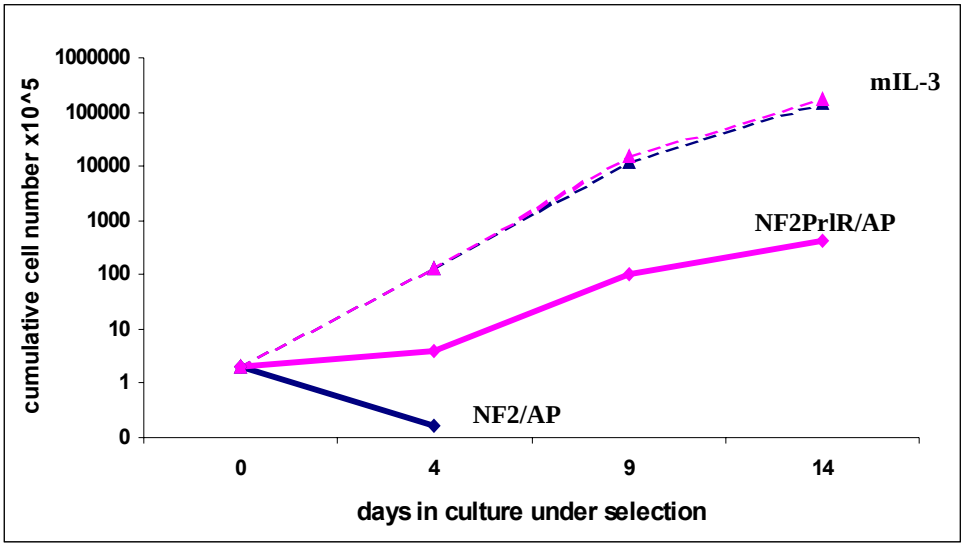


Figure 4.3.4: AP20187-induced cell proliferation of Sxgs-NF2PrlR-transduced BaF/3 cells. 2×10^5 BaF/3 cells were transduced with Sxgs-NF2PrlR and Sxgs-NF2 viral particles at a MOI of 5. Three days after transduction, the cells were washed 3 times with PBS and then cultured under 2 different conditions: either with mIL-3 (10 ng/ml) or with AP20187 (10 nM). Cell concentrations were counted at an interval of 5 days and are plotted as cumulative cell numbers. Dotted line: Cell proliferation under stimulation with mIL-3. Pink line: Sxgs-NF2PrlR-transduced cells cultured with AP20187. Blue line: Sxgs-NF2-transduced cells cultured with AP20187.

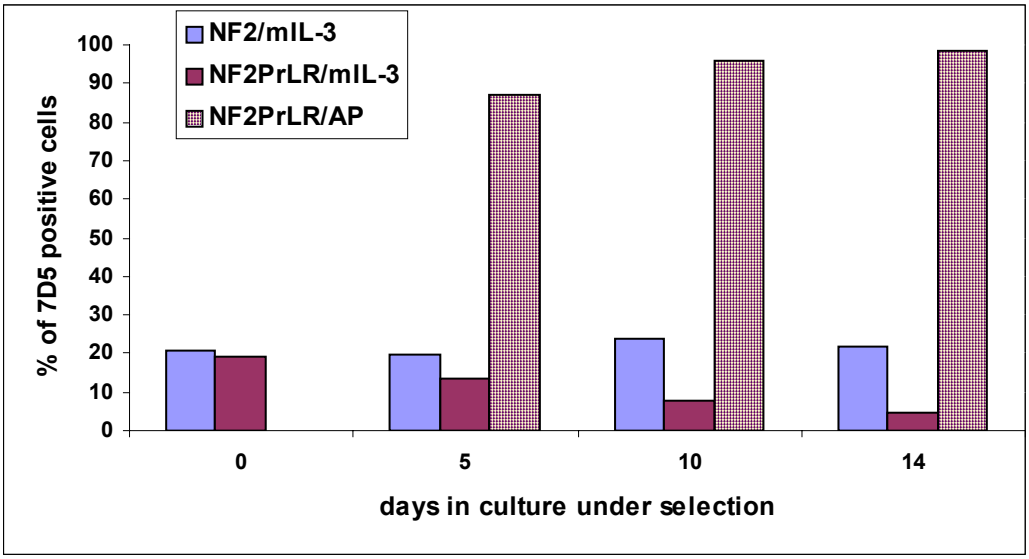


Figure 4.3.5: AP20187-induced selection of Sxgs-NF2PrlR-transduced BaF/3 cells. The transduced cells were treated as described in the legend for Figure 4.3.4. At various time points, cells were stained with 7D5 antibody and analysed by FACS. Blue column: Sxgs-NF2-transduced cells cultured with mIL-3. Pink column: Cells transduced with Sxgs-NF2PrlR and cultured with mIL-3. Pink hatched column: Cells transduced with Sxgs-NF2PrlR and stimulated with AP20187.

4.3.4 Functional Analysis of the Sxgs-NF2 and Sxgs-NF2PrlR Constructs in Transduced Murine Primary BM Cells

Experiments were carried out in mBM cells, in order to confirm the effect of Sxgs-NF2PrlR in primary cells. Total mBM cells were prepared as described in chapter 3.7.2. The cells were pre-stimulated with a cytokine combination consisting of 10 ng/ml mIL-3, 50 ng/ml mIL-6 and 50 ng/ml mSCF for 2 days. The cells were then transduced with Sxgs-NF2 and Sxgs-NF2PrlR retroviral particles. Three days after transduction, 7D5-positive cells were quantified by FACS analysis. Thereafter, the cells were washed and further cultured in the presence either AP20187 (10 nM) + mSCF (50 ng/ml) or the cytokine combination mIL-3, mIL-6 and mSCF. Figures 4.3.6 and 4.3.7 show the resulting cell proliferation curves and transgene expression profiles respectively.

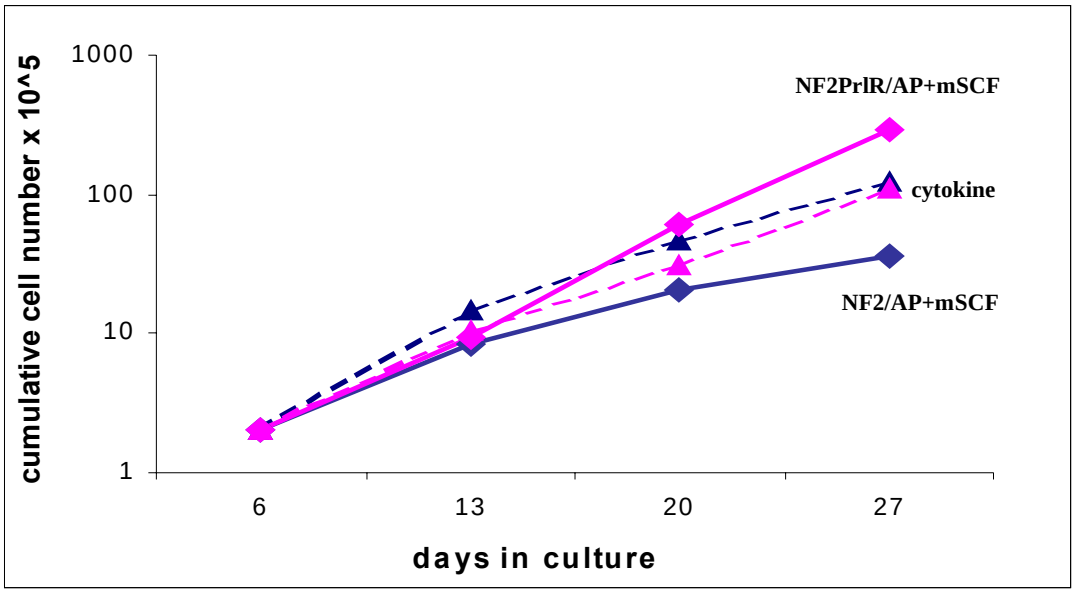


Figure 4.3.6: AP20187-induced cell proliferation of Sxgs-NF2PrlR- and Sxgs-NF2-transduced mBM cells. 2×10^5 total mBM cells were transduced with Sxgs-NF2PrlR and Sxgs-NF2 at a MOI of 10. Three days after transduction (on day 6), the cells were washed 3 times with PBS and then cultured with either AP20187 (10 nM) + mSCF (50 ng/ml) (solid lines) or the cytokine combination mIL-3 (10 ng/ml), mIL-6 (50 ng/ml) and mSCF (50 ng/ml) (dotted lines). Cell numbers were cumulative calculated every week. Dotted line: Cell proliferating under stimulation with the cytokine combination. Pink line: Sxgs-NF2PrlR-transduced cells. Blue line: Sxgs-NF2-transduced cells.

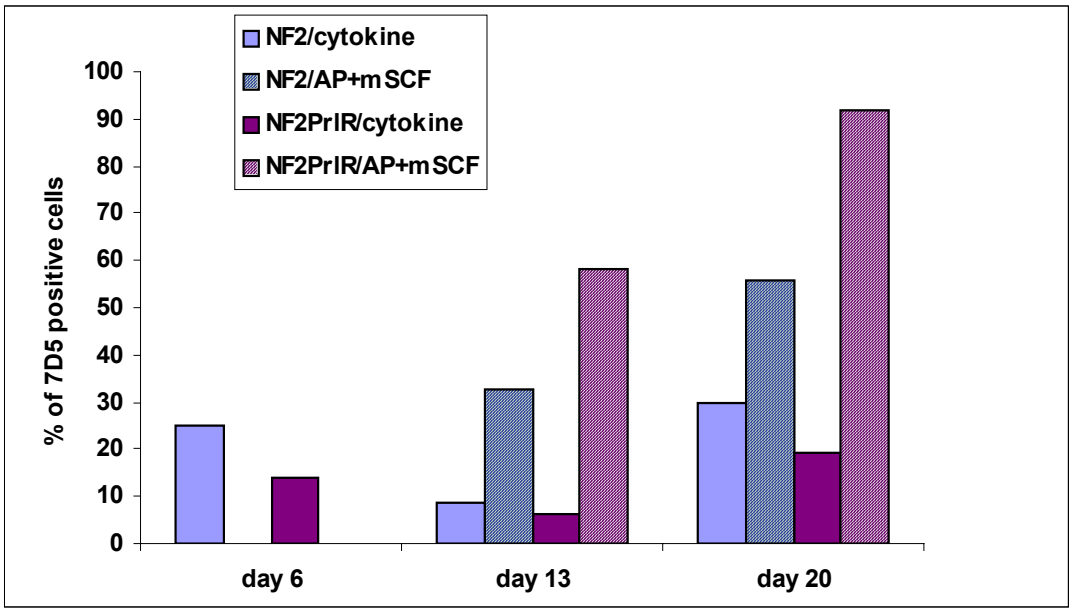


Figure 4.3.7: AP20187-induced selection of Sxgs-NF2PrlR- and Sxgs-NF2-transduced mBM cells. The transduced cells were treated as described in the legend for Figure 4.3.6. At different time points, cells were stained with 7D5 antibody and analysed by FACS. Blue columns: Sxgs-NF2-transduced cells cultured with the cytokine combination (solid) or with AP20187 + mSCF (hatched). Red columns: Cells transduced with Sxgs-NF2PrlR and cultured with the cytokine combination (solid) or with AP20187 + mSCF (hatched).

As demonstrated in Figures 4.3.6 and 4.3.7, Sxgs-NF2PrlR-transduced cells proliferated more strongly under stimulation with AP20187 than with the cytokine combination. Additionally, the percentage of 7D5-positive cells increased from an initial 13% to 92% after 2 weeks stimulation with AP20187. However, the control Sxgs-NF2-transduced mBM cells also expanded under stimulation with AP20187, albeit not as strongly as those growing in the presence of the cytokine combination. This was also accompanied by an increase in 7D5-positive cells from 25 to 55% after 2 weeks stimulation with AP20187 + mSCF. In contrast to the cells under dimerizer-induced selection, cells transduced with either construct did not show any increased number of 7D5-positive cells when cultured with the cytokine combination.

The characteristics of the expanded cell populations were determined in methylcellulose-based CFU progenitor assays (Figure 4.3.8).

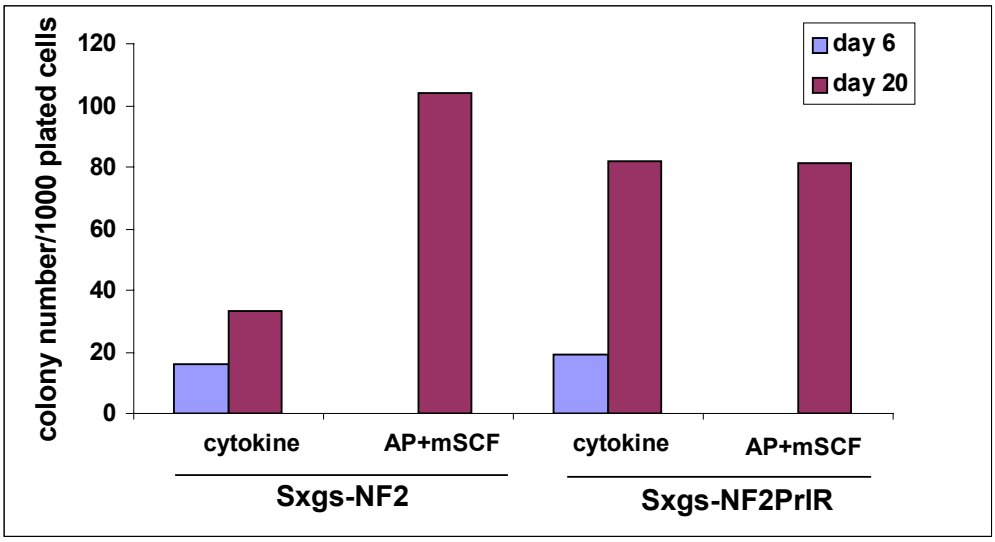


Figure 4.3.8: CFU assay of transduced mBM cells on days 6 and 20. On day 6, 3 days after transduction (also the start of selection), 1000 transduced cells for each construct were plated in 10 cm dishes with 1 ml methylcellulose medium. After 10 days incubation, the total colony numbers were counted. On day 20 (after 2 weeks selection), 3000 transduced cells for each construct and culture condition were plated.

As illustrated in Figure 4.3.8, after 2 weeks selection with AP20187, cells transduced with the control construct Sxgs-NF2 showed 6-fold higher total colony numbers, while the cytokine cultured cells had an 2-fold increase of CFUs. The Sxgs-NF2PrlR-transduced cells have about 4-fold increase in colony numbers either after 2 weeks selection with AP20187 or cultured with the cytokine combination.

4.4 Development of Bicistronic Retroviral Vectors Sxgs-F2 and Sxgs-F2PrlR Containing an Intracellular Signalling Molecule

For unknown reasons, the negative control construct Sxgs-NF2 also increased the proliferation of transduced primary BM cells under AP20187 stimulation. Since this effect was possibly due to the presence of dLNGFR, this sequence was removed from the Sxgs-NF2 and Sxgs-NF2PrlR constructs, and the resulting novel bicistronic constructs were analysed. The upstream gene $gp91^{phox}$ was used as the marker gene in all the following experiments.

4.4.1 Cloning of Constructs Sxgs-F2PrlR and Sxgs-F2

The novel vector was cloned by replacing the LNGFR and one copy FKBP sequence of Sxgs-NF2PrlR with a PCR product coding for one copy FKBP and containing *Xba*I and *Xho*I restriction sites, as shown in Figure 4.4.1. Sxgs-F2 served as the control vector and was cloned as described in Figure 4.4.2.

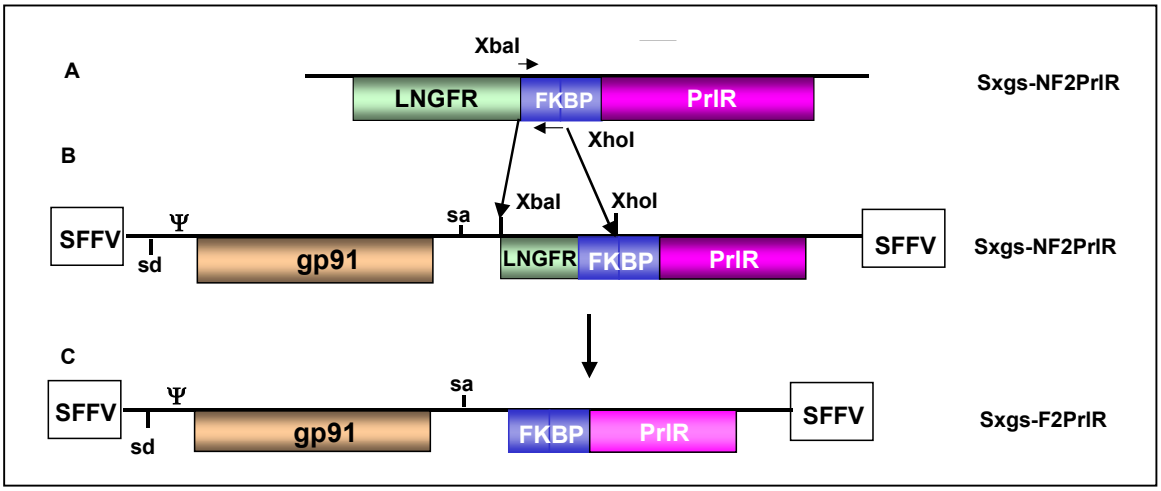


Figure 4.4.1: Cloning strategy for the novel construct Sxgs-F2PrlR. The 1×FKBP fragment was amplified with 2 PCR primers designed with *Xba*I and *Xho*I restriction sites at the ends (see small arrows in A) using Sxgs-NF2PrlR as a template. This PCR product was subcloned into the pCR2.1-TOPO vector, cut with the enzymes *Xba*I and *Xho*I, and ligated to the large *Xba*I– *Xho*I DNA fragment from Sxgs-NF2PrlR (B) resulting in the new vector Sxgs-F2PrlR that lacks the LNGFR marker gene (C).

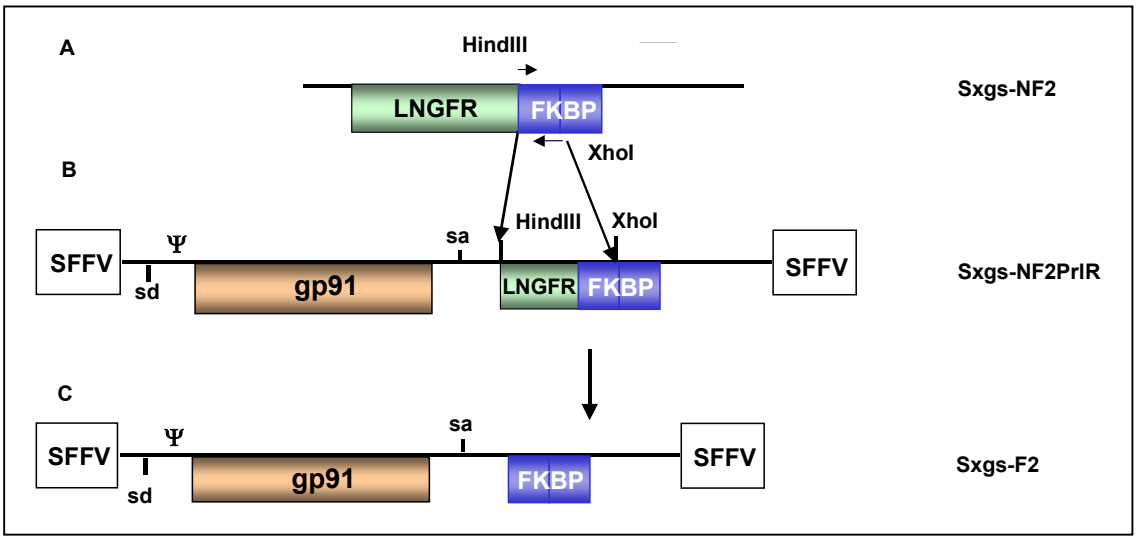


Figure 4.4.2: Cloning of the control construct Sxgs-F2. The 1×FKBP fragment was amplified with 2 PCR primers designed with *Hind*III and *Xho*I restriction sites at the end (see arrows in A) using Sxgs-NF2 as a template. The PCR product was subcloned into the pCR2.1-TOPO vector, cut with the enzymes

*Hind*III and *Xho*I, and ligated to the large *Hind*III–*Xho*I DNA fragment from Sxgs-NF2 (B) resulting in the new vector Sxgs-F2 which lacks the LNGFR marker gene (C).

4.4.2 Virus Particle Production in Phoenix-E Cells and Titre Determination on SC1 Cells

Retroviral virus particles were produced in Phoenix-E cells as described in chapter 3.6.6. Titre determination was carried out on SC1 cells with viral supernatant diluted 1:10 and 1:100. Figure 4.4.3 shows a FACS analysis of the titre determination for Sxgs-F2PrlR- and control construct Sxgs-F2-transduced cells.

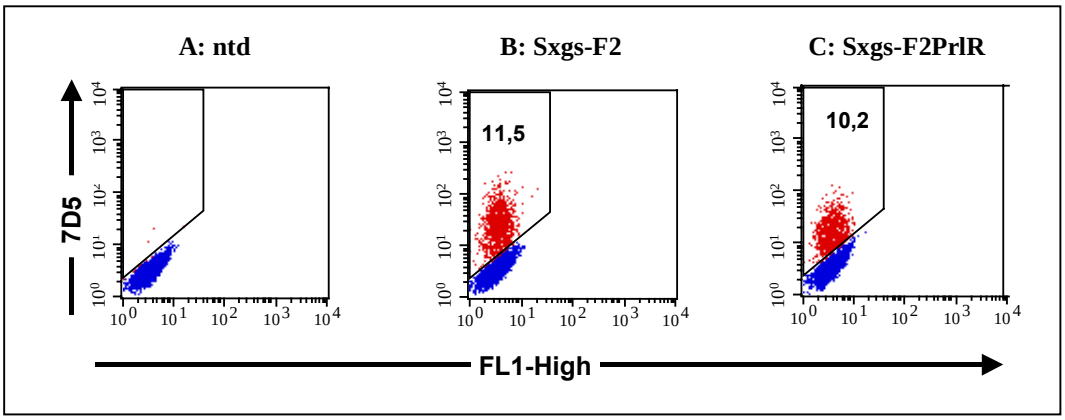


Figure 4.4.3: FACS analysis for the titre determination of Sxgs-F2PrlR and Sxgs-F2 retroviral vectors on SC1 cells. One day before transduction, 5×10^4 SC1 cells per well were plated in 24-well plates. The virus particles were given to the wells at a dilution of 1:100 in 1 ml fresh medium. Three days after transduction, 1×10^5 transduced cells were used for FACS staining with 40 µl 7D5 antibody and 4 µl RAM IgG1-PE as the secondary antibody. The percentage of 7D5-positive cells was used to calculate the titre (see chapter 3.6.6). A: ntd SC1 as the negative control. B: Percentage of 7D5-positive cells in Sxgs-F2-transduced cells. C: Percentage of 7D5-positive cells in Sxgs-F2PrlR-transduced cells.

With this method, retroviral virus particles were produced at different time points in different fractions. Titre determination was carried out for all fractions separately and the range of values for both retroviral vectors is shown in Table 4.4.1.

Table 4.4.1: Titre determination on SC1 cells for Sxgs-F2PrlR and Sxgs-F2 from different experiments. Viral supernatants were stored at –80°C before use.

Constructs	Titre on SC1 cells (TU/ml)
Sxgs-F2PrlR	$1.0–2.3 \times 10^6$
Sxgs-F2	$1.2–2.5 \times 10^6$

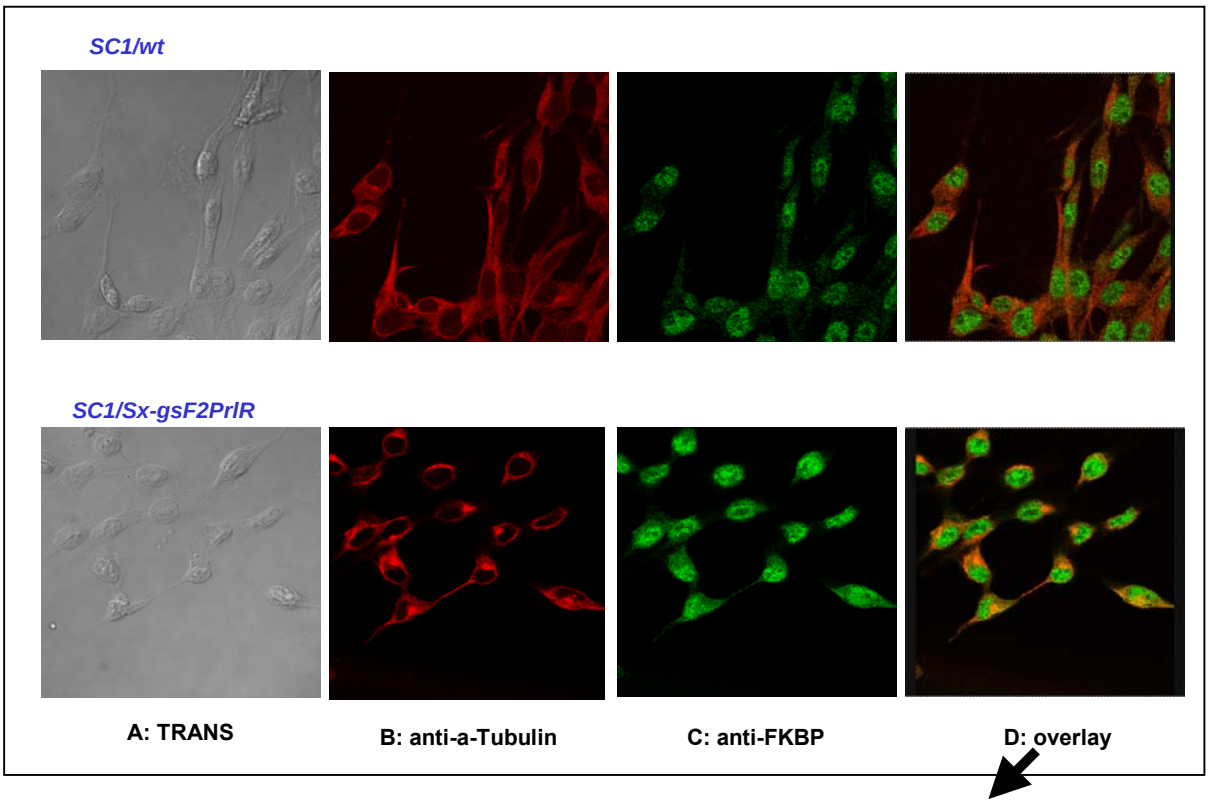
4.4.3 Cytoplasmic Localisation of Novel Fusion Protein F2PrlR

The truncated form of the prolactin receptor used as the selection molecule in the Sxgs-F2PrlR construct lacks a membrane anchor signal and should therefore be located in the cytoplasm of transduced cells. To verify cytoplasmic localisation, murine SC1 cells were transduced with Sxgs-F2PrlR retroviral vectors and localisation of the truncated receptor was examined by laser confocal microscopy through detection with a specific anti-human FKBP antibody. In addition, the cytoplasmic indicator protein α-tubulin was stained to visualise the cytoplasm. As shown in Figure 4.4.4, the endogenous FKBP protein localises mainly to the nucleus, while an additional strong fluorescence signal was also detectable in the cytoplasm of the Sxgs-F2PrlR-transduced cells. Furthermore, overlay of both fluorescence signals indicated colocalisation in the cytoplasm of Sxgs-F2PrlR-transduced cells, but not in wild type cells. We conclude from this experiment that the truncated prolactin receptor localises to the cytoplasm of transduced cells.

4.4.4 Selective Expansion of Transduced BaF/3 Cells Caused by the Sxgs-F2PrlR Construct Containing $gp91^{phox}$

Retroviral particles containing the novel construct Sxgs-F2PrlR were used to transduce BaF/3 cells to investigate the function of the FKBP-prolactin receptor fusion protein which lacks the LNGFR sequence. BaF/3 cells were transduced as described in chapter 3.6.7 with an MOI of 5 (1×10^6 TU per 2×10^5 cells). Three days after transduction, the number of $gp91^{phox}$-positive cells, was determined by FACS analysis. Transduced cells were then washed 3 times with PBS and cultured for 14 days with either mIL-3 (10 ng/ml) or AP20187 (10 nM). Growth kinetics and transgene expression (7D5+ cells) from 2 independent experiments (A and B) are shown in Figure 4.4.5 and 4.4.6 respectively. In total, 4 expansion experiments were performed with BaF/3 cells, displaying similar outcomes.

I



II

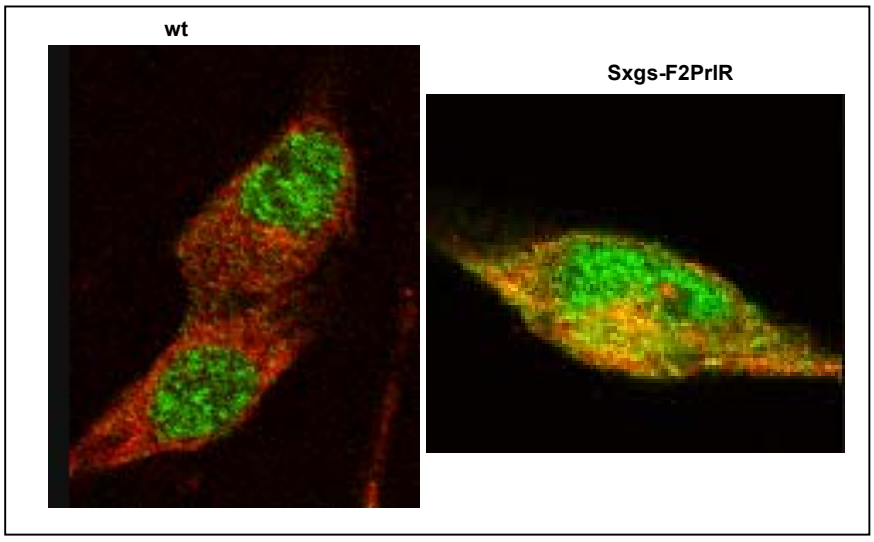
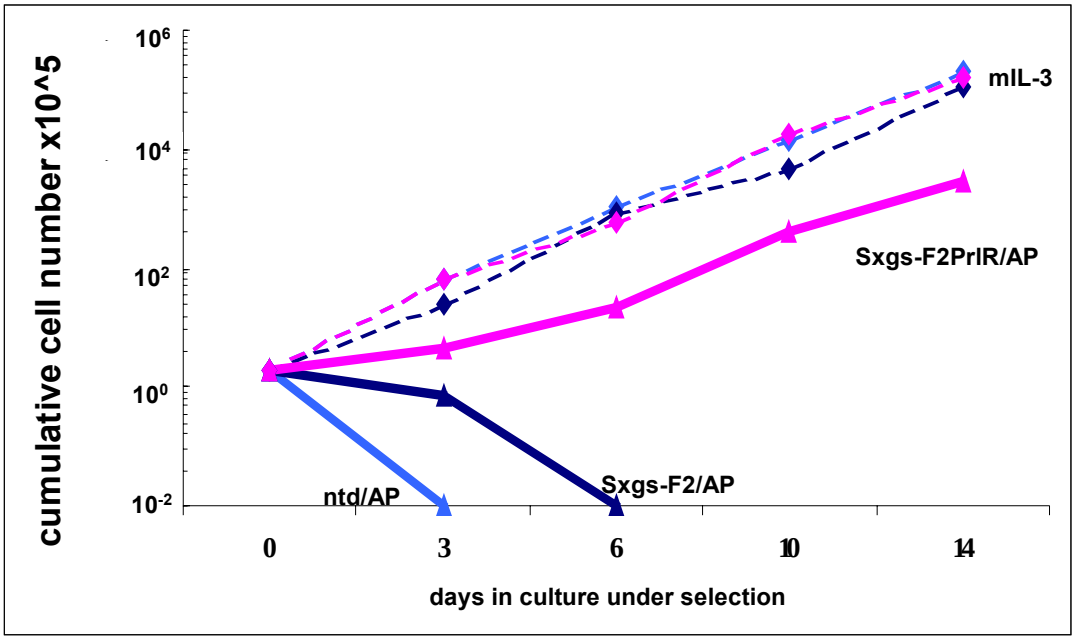


Figure 4.4.4: The F2PrlR fusion protein resides in the cytoplasm of Sxgs-F2PrlR-transduced murine SC1 cells. I: Murine SC1 cells were transduced with Sxgs-F2PrlR retroviral particles diluted 1:10. Three days after transduction, transduced and wild type SC1 cells were stained with a polyclonal anti-human FKBP12 antibody. A 488-goat anti-rabbit IgG1 antibody (green fluorescence) was used as secondary antibody. The cytoplasmic protein α-tubulin was specifically stained with a monoclonal antibody and visualised with a Alixa 546-goat anti-mouse IgG secondary antibody (red fluorescence; Molecular Probes). A: Transmission pictures without UV light. B: Images of the cytoplasmic protein α-tubulin. C: Images of

the FKBP protein. D: Overlay of α-tubulin and FKBP. **II:** Enlargement of wild type and Sxgs-F2PrlR-transduced SC1 cells from image I (number D). Colocalisation of green and red fluorescence is depicted by the yellow staining in the cytoplasm of Sxgs-F2PrlR-transduced cells.

A



B

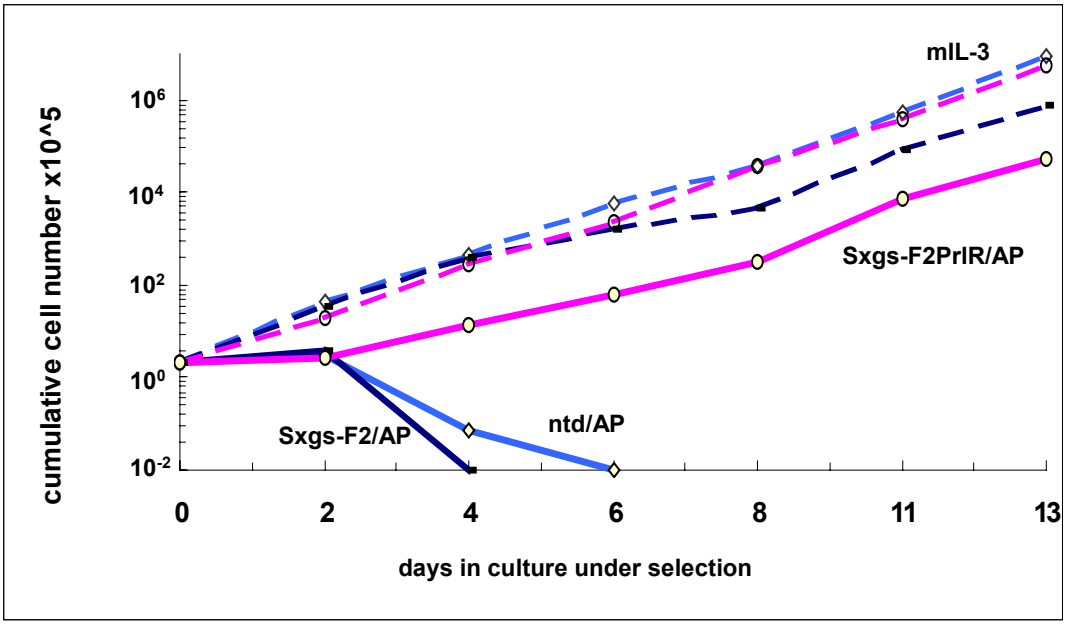
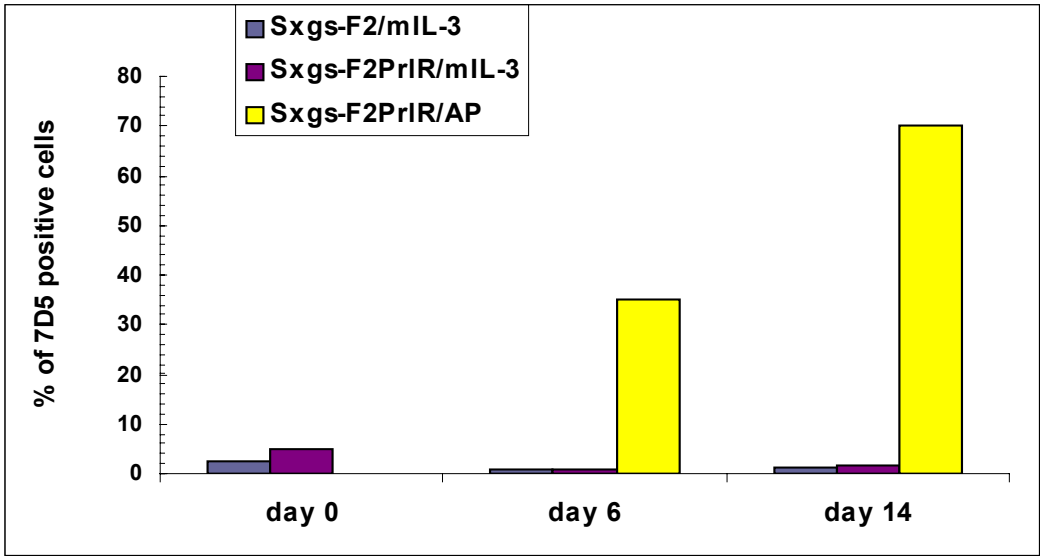


Figure 4.4.5: Proliferation advantage of Sxgs-F2PrlR-transduced BaF/3 cells stimulated with AP20187. Three days after transduction, the non-transduced (ntd) Sxgs-F2-transduced and Sxgs-F2PrlR-transduced cells were washed and cultured with either mIL-3 (10 ng/ml) (broken lines) or AP20187 (10 nM)(solid lines). The cumulative cell numbers were calculated over 2 weeks at an interval of 2–3 days by trypan blue staining. Pink line: Sxgs-F2PrlR-transduced cells. Light and dark blue line: Ntd and Sxgs-F2-transduced cells, respectively. A and B indicate two separate experiments.

In the experiments shown, the total number of Sxgs-F2PrlR-transduced cells increased 1500-fold (experiment A) and 27000-fold (experiment B) after 14–15 days stimulation with AP20187. The rate of proliferation was similar to that of cells cultured with the growth factor mIL-3, albeit with a short lag phase (roughly 2 days in both experiments) treated with AP20187. Non-transduced and control Sxgs-F2-transduced cells underwent cell death within 1 week of culture with AP20187.

Transgene expression, i.e. the percentage of 7D5-positive cells in the transduced cell population, was analysed by flow cytometry. As shown in Figure 4.4.6, after 2 weeks of selection, the percentage of Sxgs-F2PrlR-transduced cells expressing the marker gene $gp91^{phox}$ increased from 3 to 70% (experiment A) and 38 to 82% (experiment B) (column F2PrlR/AP). No increase occurred in Sxgs-F2-transduced cells (column F2/AP). In the mIL3 cultures (columns F2/mIL-3 and F2PrlR/mIL-3), the number of $gp91^{phox}$-positive cells decreased after 2 weeks.

A:



B:

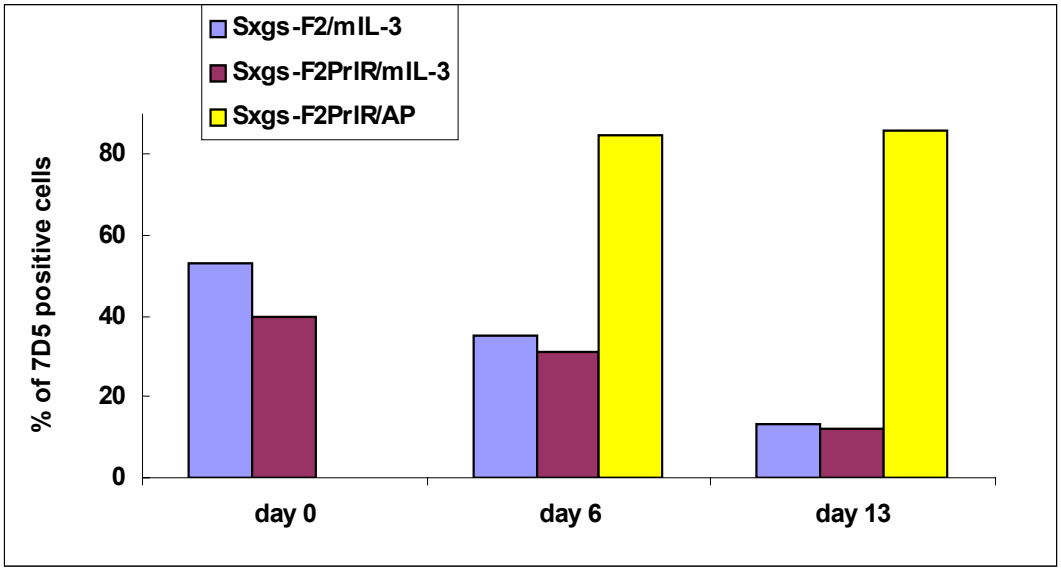


Figure 4.4.6: AP20187-induced selection of F2PrlR- and gp91^{phox}-expressing BaF/3 cells. FACS analysis was carried out on day 0 (3 days after transduction), day 6 (1 week of stimulation with AP20187) and day 14 (2 weeks of stimulation with AP20187) with the anti-7D5 antibody. The different columns represent the different constructs and different culture conditions. Blue: Sxgs-F2-transduced cells stimulated with mIL-3. Red: Sxgs-F2PrlR-transduced cells stimulated with mIL-3. Yellow: Sxgs-F2PrlR-transduced cells stimulated with AP20187.

4.4.5 The Proliferation Effect Is Reversible After Removal of the Dimerizer

As described previously in chapter 4.2, the reversibility of AP20187-induced proliferation of the transduced cells is a crucial prerequisite for the potential clinical use of the dimerizer construct Sxgs-F2PrlR. Thus, after 14 days of stimulation, AP20187 was removed from the BaF/3 culture and the cells were cultured for another 2 weeks in RPMI with 10% FCS only. As shown in figure 4.4.7, the proliferation of Sxgs-F2PrlR-transduced BaF/3 cells ceased within 3 weeks after removal of the stimulator, although they proliferated during the first two weeks. This is probably dependent on the half-life of the small molecule AP20187 under cell culture conditions. The non-transduced control cells rapidly underwent cell death after removal of mIL-3 from the culture medium. In contrast, the Sxgs-F2PrlR-transduced cells that were further cultured with mIL-3 expanded extensively.

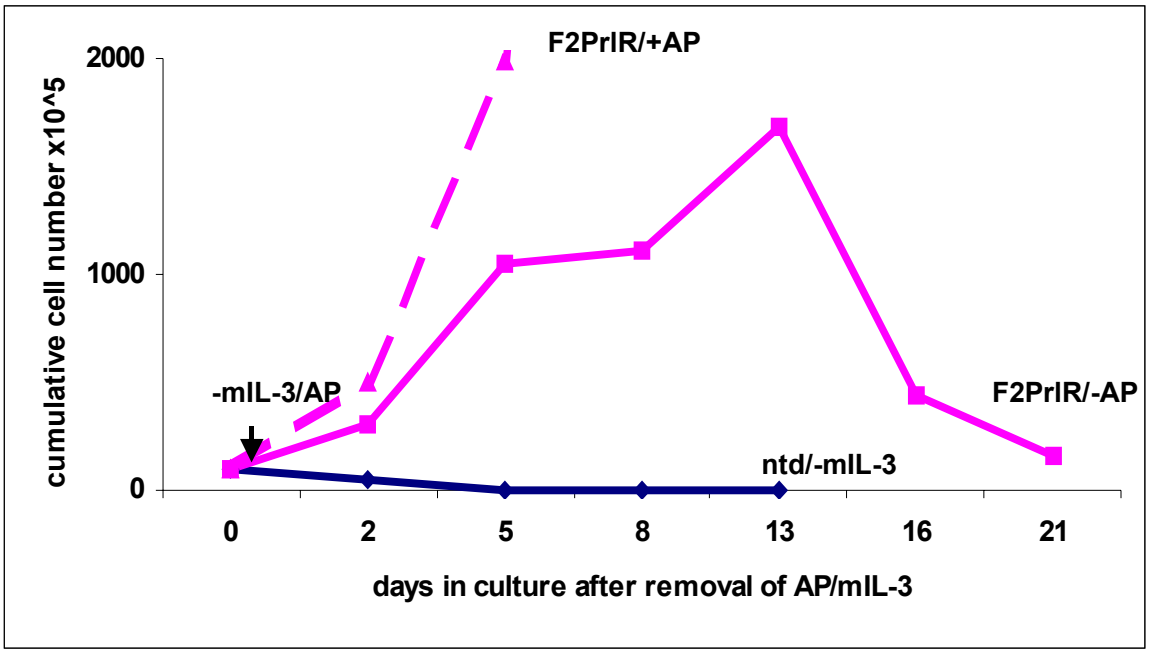


Figure 4.4.7: The effect of AP20187 on Sxgs-F2PrlR-transduced BAF/3 cells is reversible. After being stimulated with AP20187 for 2 weeks, the Sxgs-F2PrlR-transduced BaF/3 were extensively washed and further cultured either with (pink dotted line) or without AP20187 (pink line) for a further 3 weeks. Cell number was determined by trypan blue dye exclusion. Blue line: ntd control cells after removal of mIL-3.

4.4.6 Identification of Fusion Protein Expression via Western Blotting

Expression of the fusion protein F2PrlR in selected and non-selected transduced BaF/3 cells was determined using western blotting with a specific anti-FKBP antibody. As shown before, while proliferation of BaF/3 cells is dependent on mIL-3, Sxgs-F2PrlR-transduced BaF/3 cells can be selected with AP20187. As illustrated in Figure 4.4.8, a weak and a strong band corresponding to the expected molecular weight of the fusion protein (~ 69 kDa) were observed in samples from the non-selected and selected cells, respectively. The significant difference in signal intensity can be explained by the high number of transgene-expressing cells in selected (80%) versus non-selected cells (20%), indicating a selection advantage for Sxgs-F2PrlR-transduced BaF/3 cells.

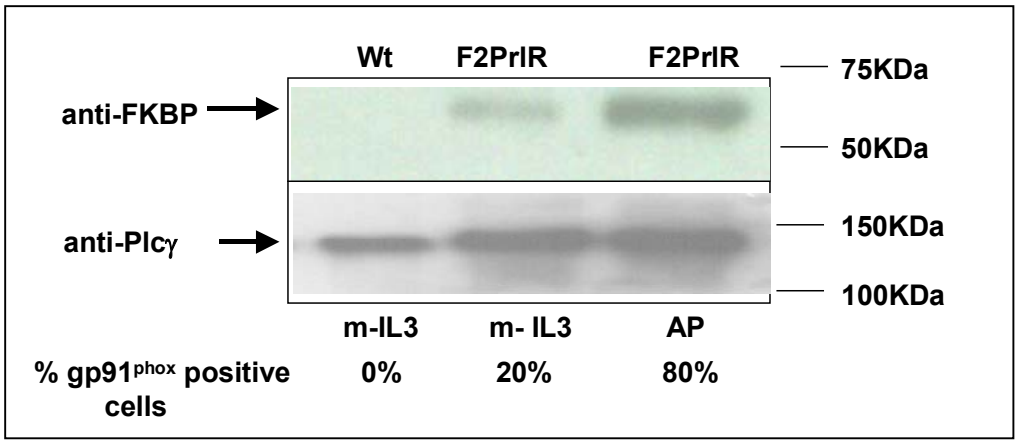


Figure 4.4.8: Fusion protein expression in transduced and selected BaF/3 cells. Cell extracts were prepared from BaF/3 wild type cells (lane 1), Sxgs-F2PrlR-transduced cells without stimulation (20% transduced cells, lane 2) and Sxgs-F2PrlR-transduced cells stimulated with AP20187 for 14 days (80% transduced cells, lane 3). The proteins were separated in a 10% SDS-PAGE gel and blotted onto a PVSF membrane. An antibody against human FKBP was used to detect the fusion protein (~ 69 kDa). Lower blot: After stripping the membrane, similar amounts of PLC-γ protein (~ 148 kDa) were detected with a monoclonal anti-PLC-γ antibody, indicating equal loading.

4.4.7 Examination of Downstream Signal Transduction Pathways Activated by the Sxgs-F2PrlR Construct

The wild type prolactin receptor was identified as a specific high-affinity membrane-bound protein (Posner et al., 1974). The unusual localisation of the prolactin receptor fusion construct in this study prompted us to ask how the signal transduction cascade induced by Sxgs-F2PrlR compares to that triggered by the wild type prolactin receptor. A well-known substrate activated by the prolactin receptor is the signal transducer and activator of transcription 5, STAT5 (Goffin et al., 1998). The 2 isoforms of this protein, STAT5a and STAT5b, are activated by tyrosine phosphorylation in response to a wide variety of cytokines after ligand-induced receptor dimerization. We therefore investigated whether AP20187-induced cell proliferation is caused by activation of STAT5, similarly to that caused by prolactin receptor overexpression and stimulation with prolactin, for example in BaF/3 cells (Pallard et al., 1995). Cell extracts from restimulated Sxgs-F2- and Sxgs-F2PrlR-transduced BaF/3 cells treated with different stimulation conditions were examined for STAT5 activation by western blotting with antibodies against STAT5 and phosphorylated STAT5 (phospho-STAT5) (Figure 4.4.9).

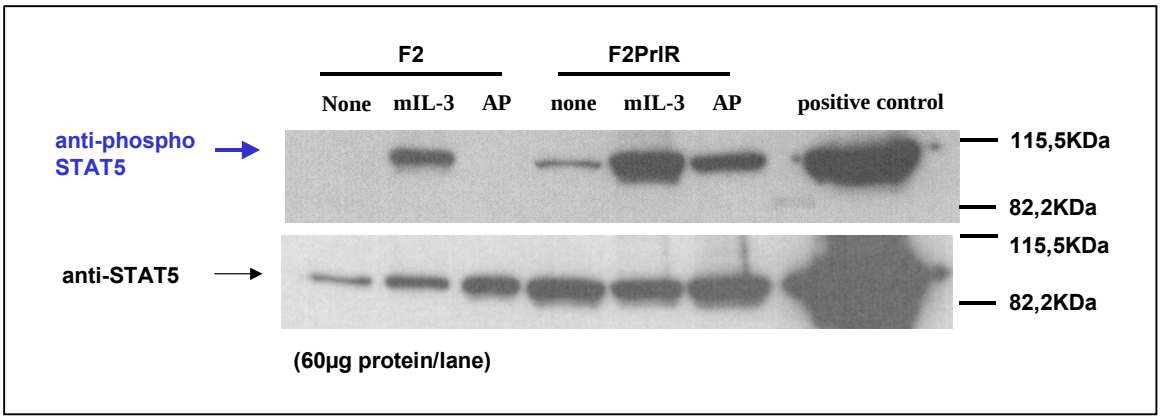


Figure 4.4.9: Induction of STAT5 phosphorylation by addition of AP20187 to BaF/3 cells expressing Sxgs-F2PrlR. Sxgs-F2-transduced BaF/3 cells (cultured with mIL-3, 25% transgene expression, left 3 lanes) and Sxgs-F2PrlR-transduced cells (cultured for 2 weeks with AP20187, 85% transgene expression, middle 3 lanes) were deprived of mIL-3 or AP20187 for 24 hours and starved of FCS for a further 4 hours. The cells were then restimulated with either mIL-3 or AP20187 for 10 min. Extracts were prepared and equal protein amounts (60 µg per sample) were separated in an 8% SDS-PAGE gel. Western blotting was performed with an antibody against phospho-STAT5. After stripping the membrane, an antibody against STAT5 was used to determine the amount of the STAT5 protein. The right lane (+) shows a positive control obtained from cell extracts of wild type prolactin receptor-transfected cells stimulated with prolactin used to confirm the quality of antibodies.

After restimulation with AP20187, only the cell extract from Sxgs-F2PrlR-transduced cells showed a strong signal corresponding to the expected molecular weight (~ 110 kDa) when analysed with the anti-phospho-STAT5 antibody. When restimulated with conditioned medium containing mIL-3, both cell populations showed strong STAT5 phosphorylation. Sxgs-F2PrlR-transduced cells that were not restimulated also displayed a weak signal with the anti-phospho-STAT5 antibody. This indicates that STAT5 activation induced by AP20187 in Sxgs-F2PrlR-transduced BaF/3 cells was not completely switched off after 28 hours of starvation following a previous 14 days stimulation.

Phosphorylated STAT5 migrates to the nucleus of cells, where it binds to the promoter region of several target genes and activates transcription (Rane et al., 2002). One of the targets of prolactin receptor-induced signalling is the ß-casein gene. Therefore, the binding activity of STAT5 to the ß-casein promoter after dimerization of the truncated cytosolic form of the prolactin receptor was investigated using the electrophoretic mobility shift assay (EMSA) (Figure 4.4.10). A radioactively labelled oligonucleotide derived from the STAT5 binding site in the ß-casein promoter was incubated with cell extracts obtained from transduced BaF/3 and control cells.

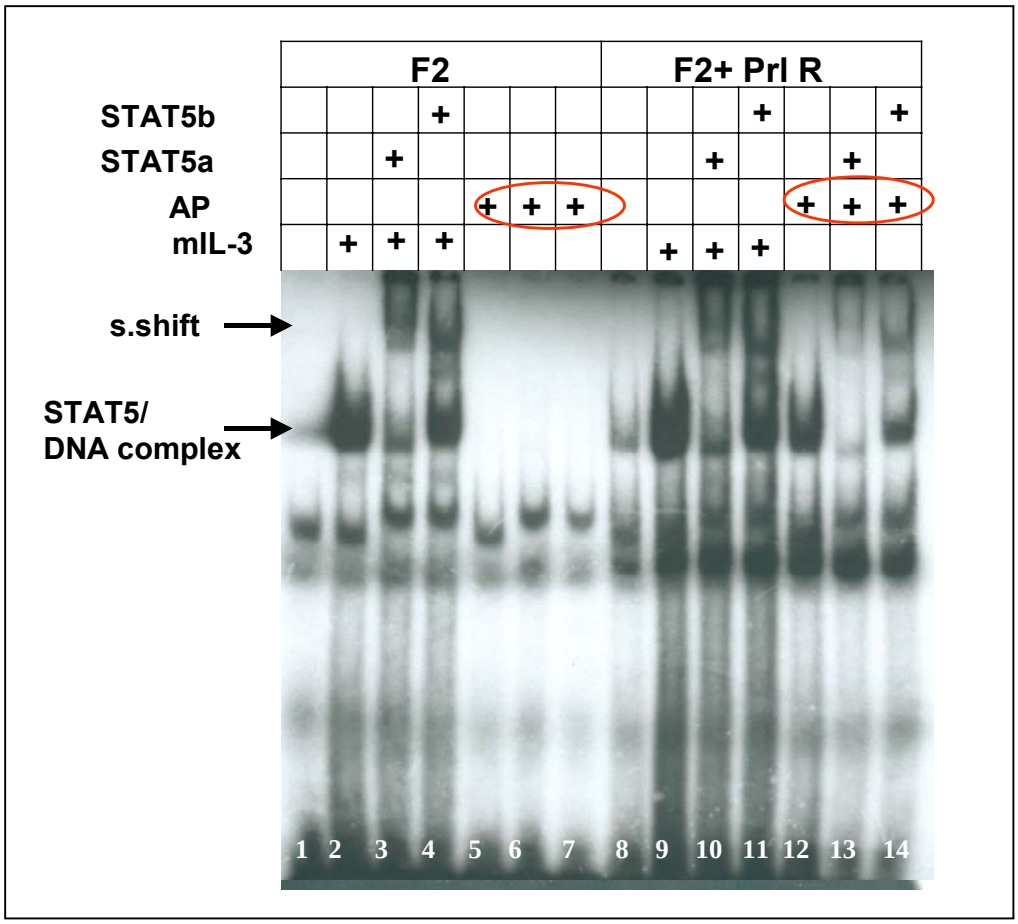


Figure 4.4.10: Signalling from the fusion receptor induces binding of STAT5 to its target gene promoter sequence. Whole cell extracts (WCE) were prepared by resuspending cells in lysis buffer after treating as described in Figure 4.4.7. WCE (8 µg per condition) were incubated for 20 minutes at 30°C with radiolabelled (T4 polynucleotide and γ^{32}P-ATP) ß-casein oligonucleotide (5' – agatttctaggaattcaaatc). The protein-DNA complex was separated in a 5 % polyacrylamide gel in 0.25× TBE buffer and the radioactive signal was visualised by film exposure. Lanes 1–7: Control Sxgs-F2-transduced cells stimulated with mIL-3 or AP20187. Lanes 8–14: Sxgs-F2PrlR-transduced cells stimulated with mIL-3 or AP20187. Binding of the antibodies against STAT5a and STAT5b to the STAT5/DNA complex is revealed by the respective supershifts of the complex (lanes 6, 7 and 13, 14).

If the DNA (from the ß-casein) binds the STAT5a or STAT5b antibody, these protein/DNA complexes migrated more slowly than free DNA molecular when subjected to non-denaturing polyacrylamide gel electropjoresis. Therefore, a supershift could be observed. As shown in Figure 4.4.10, binding of STAT5 to the DNA probe (from ß-casein) is made evident by the retarded migration of the radiolabelled probe in the gel. Moreover, supershifted bands were observed in WCE from AP20187-stimulated, F2PrlR-transduced cells, similar to those from the positive control cells which were stimulated with mIL-3. In contrast, control non-stimulated, Sxgs-F2-transduced cells showed no binding activity. The weak signal observed in SXgs-F2PrlR-transduced cells that had not been restimulated with AP20187 (lane

8) was presumably due to the fact that the effect of AP20187 was not completely switched off within 28 hours after removal of the dimerizer.

In summary, overexpression of the Sxgs-F2PrlR construct in BaF/3 cells led to a significant selective expansion of the transduced cells after stimulation with the dimerizer AP20187. The AP20187-induced cell proliferation might be a consequence of the activation of the STAT5 signalling pathway, similar to STAT5-derived proliferation of prolactin-stimulated BaF/3 cells transduced with the full-length prolactin receptor construct (Pallard et al., 1995). In addition, the effect of AP20187 was reversible after withdrawal of the dimerizer.

4.4.8 Selective Proliferation Advantage of Sxgs-F2PrlR-Transduced Primary Sca1+ mBM Cells

The Sxgs-F2PrlR construct demonstrates a significant selection effect in transduced BaF/3 cells under stimulation with AP20187. Moreover, this effect is reversible after withdrawal of the drug. Subsequently, we therefore went on to investigate the constructs in the context of primary mBM cells and *in vivo* mouse models. As already mentioned in the Introduction (chapter 1.1), adult mBM contains different cells types, ranging from early pluripotent HSCs to committed differentiated cells. The HSCs have the capacity to self-renew, maintain their progeny and to differentiate into all haematopoietic cell lineages. A variety of different phenotypic markers can be used to separate HSCs from other cell types or at least to enrich cell populations for these HSCs. Sca1 and c-kit are commonly accepted as markers for mouse HSCs and progenitor cells (see Introduction chapter 1.1). In the following studies, Sca1-positive cells were employed for the *ex vivo* and *in vivo* experiments. Sca1+ cells were isolated from the BM cells of C57BL/6 Ly5.1 mice using commercially available kits (*StemCell* Technology) and showed high purity (96% Sca1+ cells after enrichment) (Figure 4.4.11). To allow effective retroviral transduction, the isolated BM cells were pre-stimulated for 48–60 hours in the presence of a cytokine combination consisting of 50 ng/ml mSCF, 50 ng/ml mIL-6 and 10 ng/ml mIL-3 (described as cytokines in the following text).

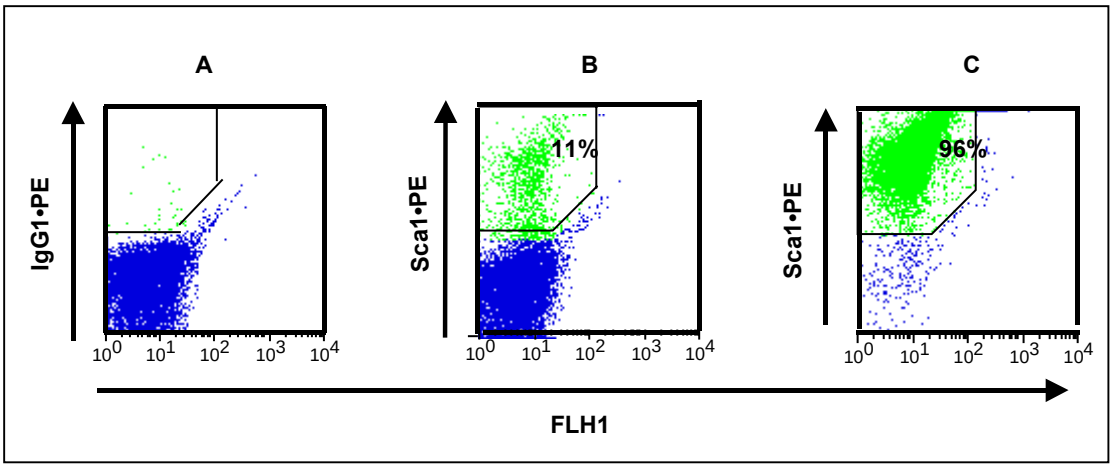
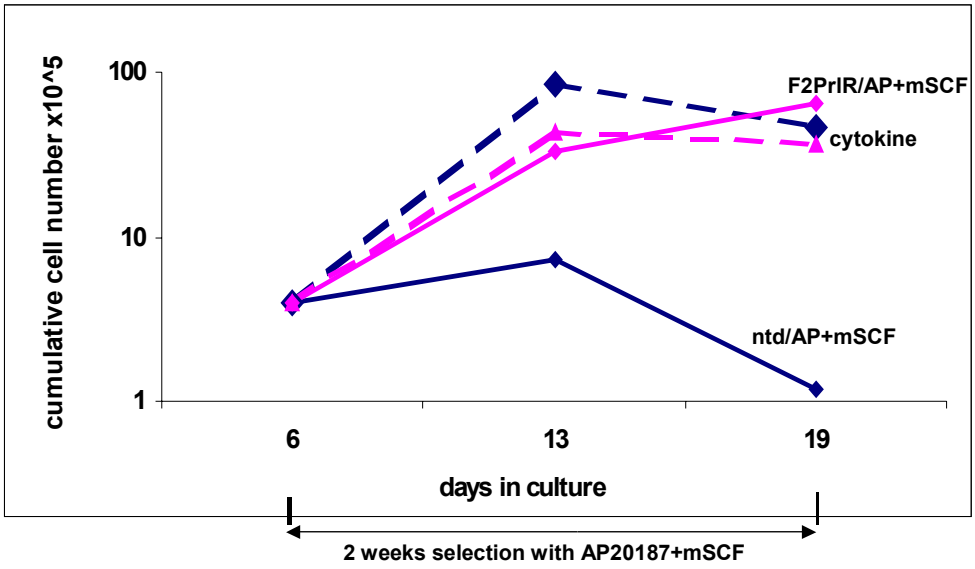


Figure 4.4.11: Enrichment of Sca1+ cells from primary BM cells (C57BL/6 Ly5.1). Sca1+ BM cells were separated from whole BM as described in chapter 3.7.3. Cells were stained with PE-conjugated anti-mouse Sca1 antibody and analysed by FACS. A: Isotype control. B: Percentage of Sca1+ cells before enrichment (11%). C: Percentage of Sca1+ cells after enrichment (96%).

The prestimulated Sca1+ cells were then transduced with retroviral particles carrying Sxgs-F2PrlR or the control construct Sxgs-F2 as described in chapter 3.6.7. One day after transduction, the transduction efficiency was determined with anti-7D5 antibody by FACS analysis. The results are shown in Figure 4.4.13. A sample of the Sxgs-F2PrlR-transduced cells was transplanted into recipient C57BL/6 Ly5.2 mice (see Figure 4.4.18). Remaining transduced cells were washed 3 times with PBS and cultured for a further 2 weeks in the presence of cytokines (cytokine in Figure 4.4.12) or 10 nM AP20187 + 50 ng/ml mSCF (F2PrlR/AP+mSCF and ntd/AP+mSCF in Figure 4.4.12). At the end of the 2-week culture period, the characteristics of non-selected and selected Sxgs-F2PrlR-transduced cells were determined via FACS analysis and methylcellulose-based CFU assay (see Figures 4.4.12, 4.4.13, 4.4.14). The expanded cells were used for a second transplantation series of C57BL/6 Ly5.2 mice (Figure 4.4.18).

A:



B:

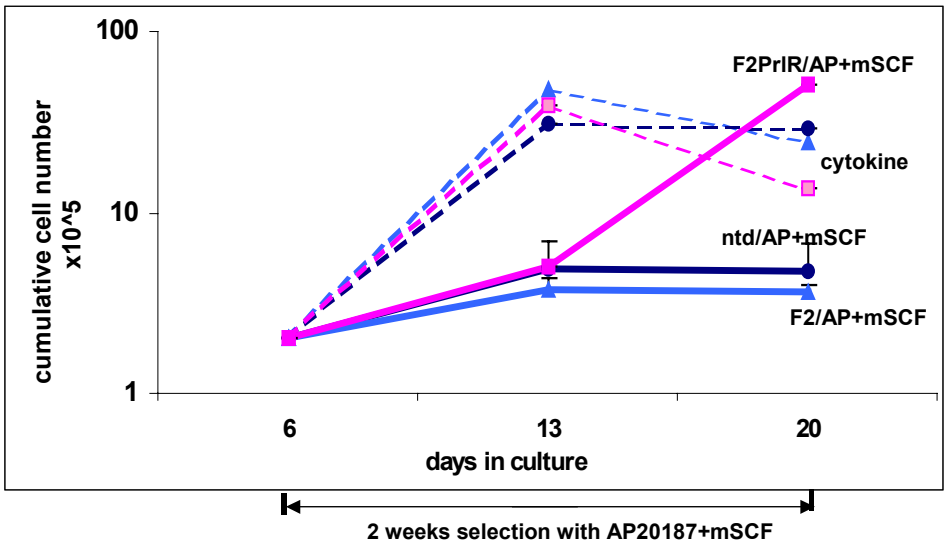
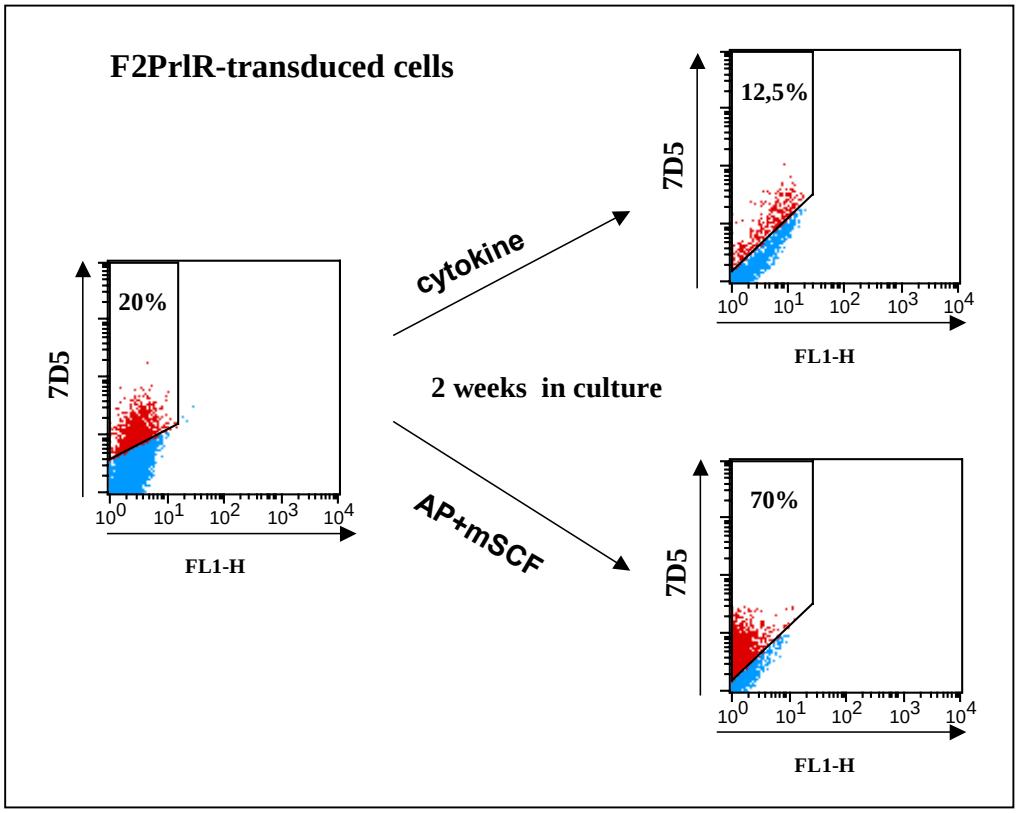


Figure 4.4.12: Selective proliferation advantage of Sxgs-F2PrlR-transduced Sca1+ primary mBM cells stimulated with AP20187. Sca1+ cells were isolated from C57BL/6 Ly5.1 mice, pre-stimulated for 2 days and transduced with the Sxgs-F2PrlR and Sxgs-F2 retroviral constructs. On day 6 (1 day after transduction), the cells were placed under 2 different conditions: with standard cytokine combination (dotted lines) or under selection with 10 nM AP + 50 ng/ml mSCF (solid lines), and further cultured for 13–14 days. Cumulative cell numbers were counted every week by trypan blue. Pink line: Proliferation of Sxgs-F2PrlR-transduced cells. Dark and light blue lines: Non-transduced and control Sxgs-F2-transduced cells, respectively. The results in B were obtained from 2 experiments.

As illustrated in Figure 4.4.12, the number of Sxgs-F2PrlR-transduced cells increased under stimulation with AP20187 to about 50–60-fold above starting cell numbers. An enhanced proliferation occurred in the second week of selection. In contrast, non-transduced

and Sxgs-F2-transduced cells survived for the 2 weeks stimulation with AP20187 and mSCF; however, they showed no overall proliferation. In principle, all cells stimulated with the cytokine combination (Il-3, IL-6, mSCF) expanded in the first week, while during the second week they stopped proliferating completely.To determine whether a selection of the cells had occurred, the percentage of gp91^{phox}-positive cells in transduced *ex vivo* cells was analysed by FACS. The results from one experiment are given in Figure 4.4.13.

As demonstrated in Figure 4.4.13, during the 2 weeks of AP29187-induced expansion, the percentage of 7D5-positive cells in the Sxgs-F2PrlR-transduced cell population increased from 20 to70%, while that of cells cultured in the presence of the cytokine combination decreased slightly during same time period. A similar decrease in 7D5-positive cells was observed in the control Sxgs-F2-transduced cells in the presence of both cytokines and the AP20187 + mSCF selection mix.



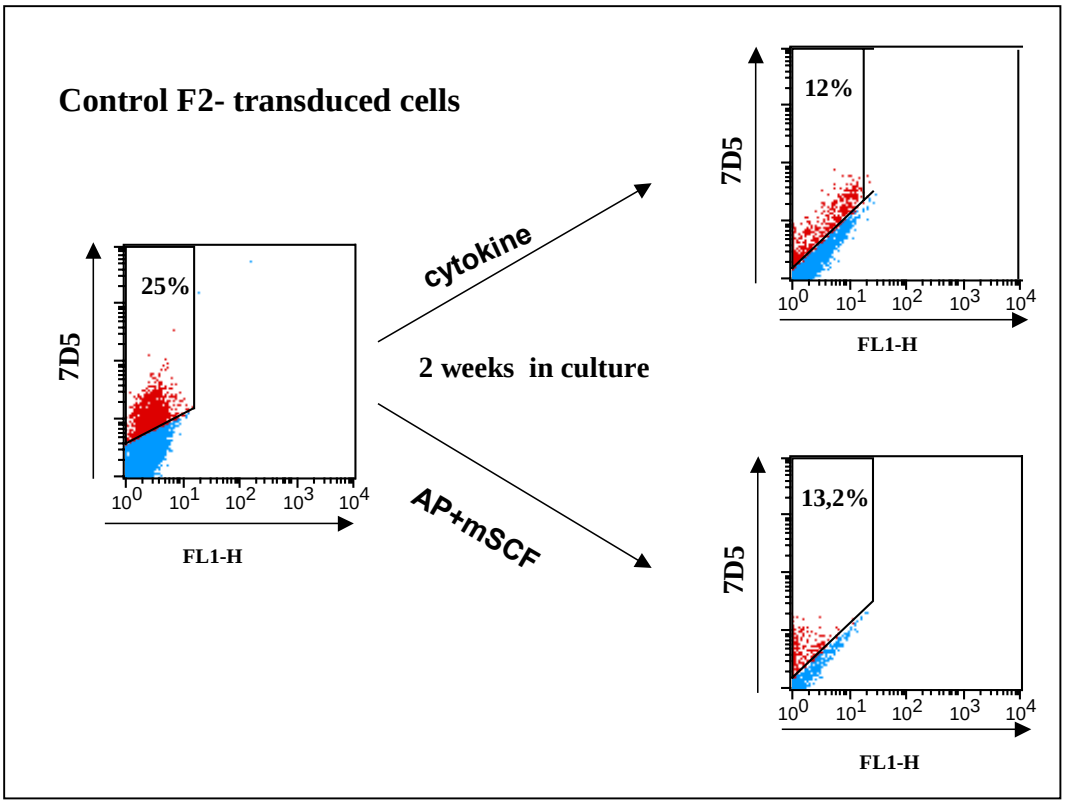


Figure 4.4.13: 7D5 transgene expression (7D5+ cells) of transduced Sca1+ primary mBM cells measured 1 day after transduction and after an additional 2 weeks in the presence of cytokines or AP20187 + mSCF. The transduced cells were treated as described in the legend for Figure 4.4.12. One day after transduction and after 2 weeks selection, 1×10^5 Sxgs-F2PrlR- (top) or Sxgs-F2-transduced cells (bottom) for each culture condition were used for FACS analysis.

4.4.9 The Selected Sxgs-F2PrlR-Transduced Cells Maintain Multilineage Differentiation Ability

The ability of the selected cells to keep multilineage differentiation is very important to reduce the risk of leukaemia. Flow cytometric analysis of the myeloid cell surface marker Gr1, the B-cell surface marker B220 and the T-cell surface marker CD3 in combination with staining of the transgene expression marker 7D5 was performed and the results are shown in Figure 4.4.14.

As illustrated in Fig. 4 4.14, the non-selected cells stimulated with the cytokine combination showed a lower proportion of $gp91^{phox}$-positive cells than the selected cells, as in Figure 4.4.13. 7D5-positive cells were found in all lineages under stimulation with cytokines or with AP20187 + mSCF. This suggests that the AP20187-stimulated cells retained the ability to differentiate into multiple cell lineages. However, the percentages of Gr1, B220 and CD3 cells in the 7D5-positive cells selected with AP20187 were 2.7, 24.4 and 1.2%. These

values are significantly lower than those of the cells stimulated with the cytokine combination, which were 35.9, 70.5 and 17.8%, respectively.

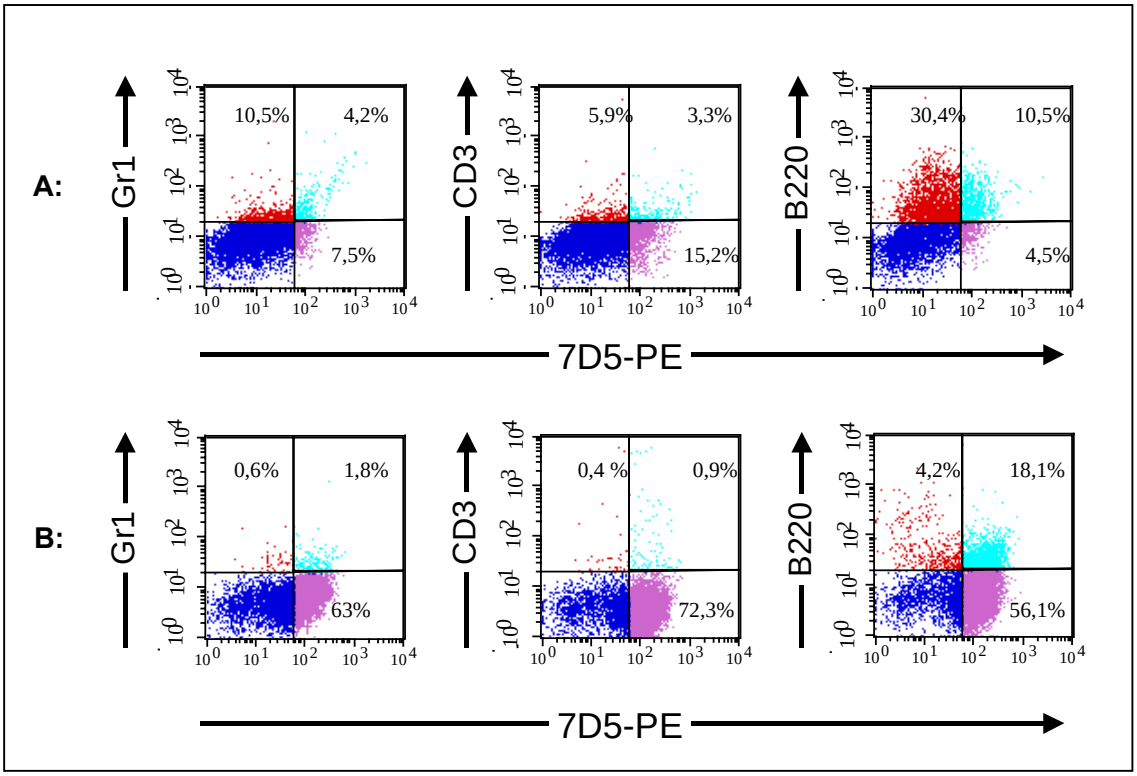
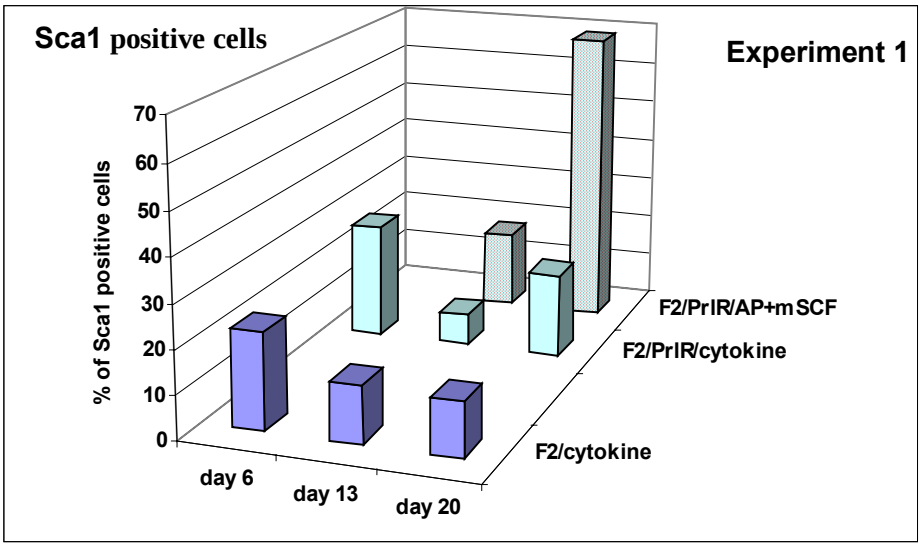
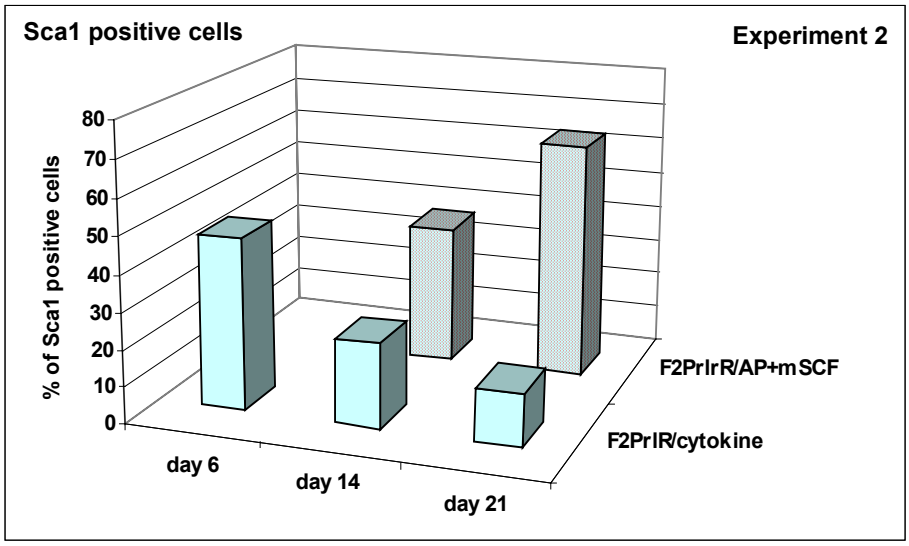


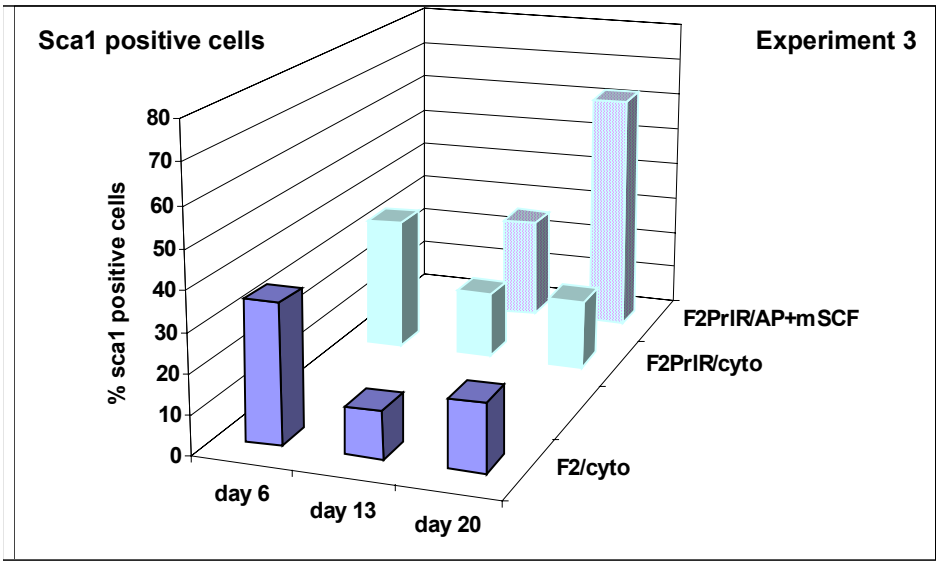
Figure 4.4.14: Flow cytometric analysis of cell surface lineage markers in non-selected and selected Sxgs-F2PrlR-transduced, Sca1+ mBM cells. The transduced cells were cultured for 2 weeks in the presence of cytokines (A) or selectively expanded for 2 weeks under stimulation with AP20187and mSCF (B). The non-selected and selected cells were then separately incubated with biotin-conjugated lineage marker antibodies and APC-conjugated streptavidin as the secondary reagent. 7D5-PE was co-incubated with each sample to determine transgene expression.

4.4.10 Maintenance of Early Haematopoietic Marker Gene Expression in *ex vivo*-Cultured Cells

As outlined above, the Sxgs-F2PrlR-transduced Sca1+ primary BM cells had a selective proliferation advantage during 2 weeks stimulation with AP20187 and mSCF. To characterise the features of these expanded cells, the expression profiles of early haematopoietic markers (Sca1, c-kit) during *in vitro* culture was analysed by flow cytometry. Figure 4.4.15 shows the data from 3 separate experiments.


Sca1 positive cells
Experiment 1
% of Sca1 positive cells
70
60
50
40
30
20
10
0
day 6
day 13
day 20
F2/PrIR/AP+mSCF
F2/PrIR/cytokine
F2/cytokine


Sca1 positive cells
Experiment 2
% of Sca1 positive cells
80
70
60
50
40
30
20
10
0
day 6
day 14
day 21
F2PrIrR/AP+mSCF
F2PrIR/cytokine


Sca1 positive cells
Experiment 3
% sca1 positive cells
80
70
60
50
40
30
20
10
0
day 6
day 13
day 20
F2PrIR/AP+mSCF
F2PrIR/cyto
F2/cyto

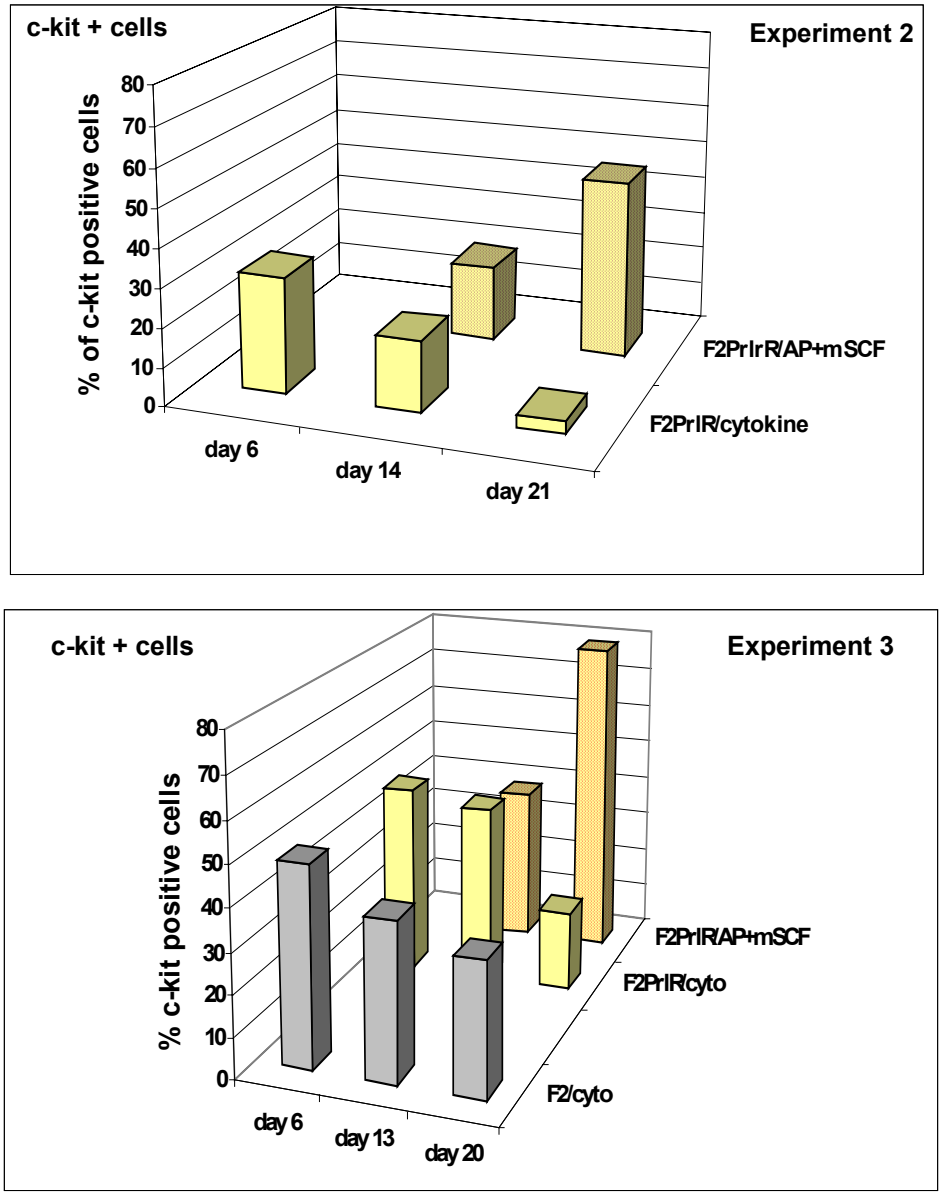


Figure 4.4.15: Analysis of progenitor cell phenotype in transduced Sca1+ primary mBM cells 1 day after transduction and 2 weeks after stimulation with AP20187. Three separate experiments (1-3) were performed. One day after transduction (day 6 in culture), 1 week (day 13 in experiments 1 and 3, day 14 in experiment 2) and 2 weeks (day 20 in experiments 1 and 3, day 21 in experiment 2) after selection, 1×10^5 cells from each culture condition were used for FACS staining with anti-Sca1-PE or with anti-c-kit antibody (experiments 2 and 3).

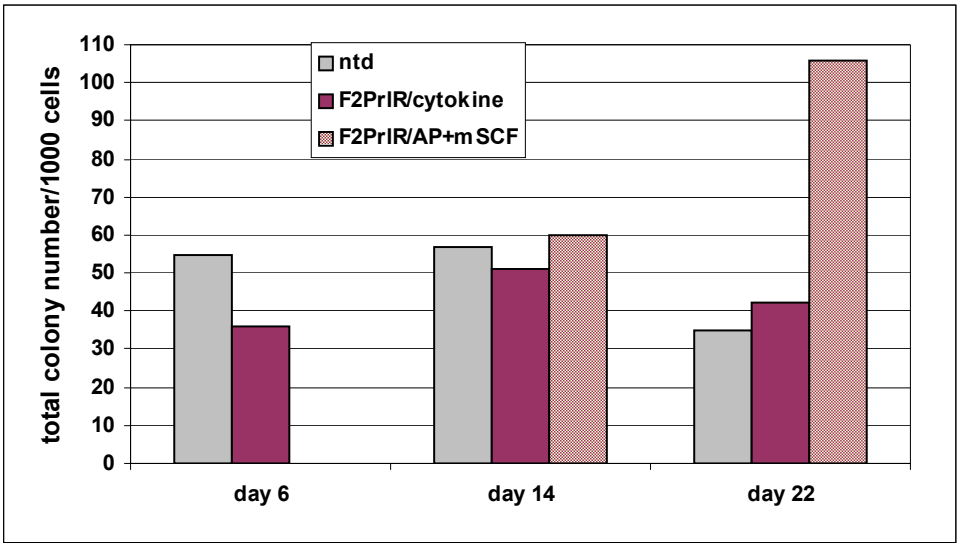
As indicated in Figure 4.4.15, the percentage of Sca1+ cells had dropped from the initial over 95% in the freshly isolated and purified BM cells to 28–45% at beginning of selection (3 independent experiments). As expected, the number of Sca1+ cells went down to 10–30% during the following 2 weeks of culture in the presence of the cytokine mix. Nevertheless, the Sxgs-F2PrlR-transduced and AP20187-expanded mBM cells showed a

significant increase in the proportion of Sca1+ cells, up to 55–65% during the same culture period. A similar kinetic was also observed for the c-kit marker in experiments B and C.

4.4.11 CFU Assay of *ex vivo* Selectively Expanded BM Cells

As mentioned previously in 4.3.2, the method usually utilised to calculate the number of progenitor cells in haematopoietic systems *in vitro* is the methylcellulose-based CFU assay. CFU assays were, therefore, performed to calculate the number of haematopoietic progenitors in the expansion cultures. On day 6 (1 day after transduction), day 14 (after 1 week stimulation) and day 22 (or day 20, after 2 weeks stimulation), an aliquot of the cells was plated on methylcellulose semisolid medium and incubated for 10 days. The total colony number per 1000 plated cells from 2 independent experiments is shown in Figure 4.4.16.

A:



B:

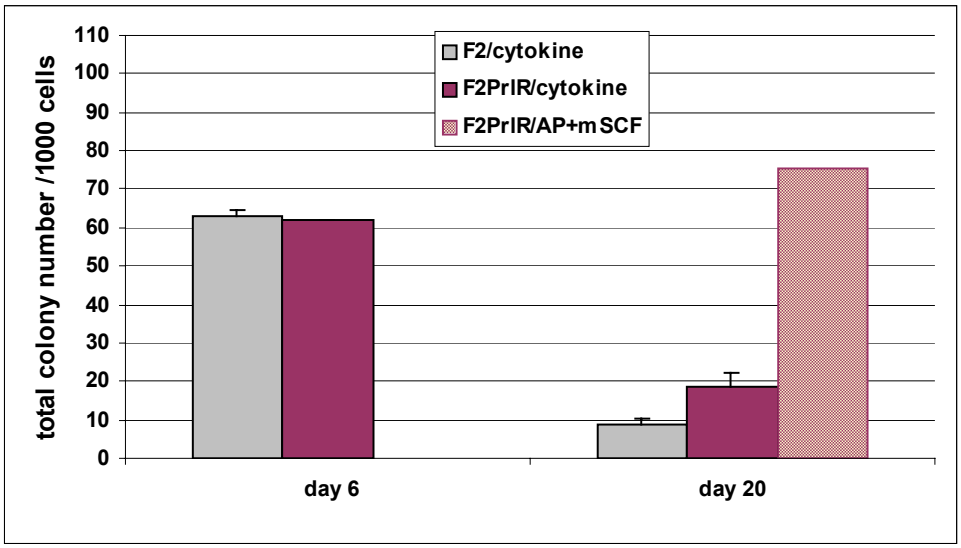


Figure 4.4.16: Increased colony numbers in selected Sxgs-F2PrlR-transduced cells obtained by primary CFU assay in methylcellulose The transduced cells were plated in methylcellulose at a concentration of 5000 cell/ml methylcellulose on day 6 (1 day after transduction), day 14 (after 1 week selection) and day 22 (or day 20, after 2 weeks selection). After 10 days incubation, the total colony numbers were counted and calculated as the colony number/1000 plated cells. Grey column: control Sxgs-F2-transduced or ntd cells. Red column: Sxgs-F2PrlR-transduced cells cultured in the presence of cytokines. Red dotted column: Sxgs-F2PrlR-transduced cells stimulated with AP20187.

As demonstrated in Figure 4.4.16, the colony number from Sxgs-F2PrlR-transduced cells stimulated with AP20187 + mSCF increased from day 6 to day 22 (experiment A) and day 20 (experiment B), whereas those from non-selected and non-transduced cells decreased in this period. The total colony number from the cells cultured with AP20187 + mSCF for 2 weeks was about 3.5-fold (experiment A) and 7-fold (experiment B) higher than that from the cells cultured for 2 weeks with the cytokine mix. No increase in colony number was observed for control Sxgs-F2-transduced or ntd cells under the same culture conditions. These results suggest that AP20187-stimulated, transduced Sca1+ cells contain more progenitor cells than the non-selected and ntd cells.

In summary, as illustrated in Figures 4.4.15 and 4.4.16, the selective expansion of Sxgs-F2PrlR-transduced murine Sca1+ cells stimulated with AP20187 was significant, based on the proliferation of haematopoietic progenitor cells.

4.4.12 Polyclonal Provirus Integration of Genomic DNA in AP20187-Induced Selective Expansion of Murine HSCs Transduced with Sxgs-F2PrlR Retroviral Particles

Ligation mediated (LM)-PCR is the technique available to map provirus integration sites in the genomic DNA of transduced cells. The polyclonal proliferation of transduced cells is an important pre-requisite for the use of retroviral vectors for gene therapy (see Introduction, chapter 1). To characterise the clonality and integration sites in the expanded transduced cells, the LM-PCR technique was adapted to the vector system used in this study. Special primers were designed to amplify a region of the 5'LTR-sequences in the retroviral vector. Genomic DNA was prepared from *ex vivo*-selected and non-selected mBM cells, and LM-PCR was performed with 250 ng genomic DNA, as described in chapter 3.4.4. The schematic representation of LM-PCR used in this study is shown in Figure 4.4.17.

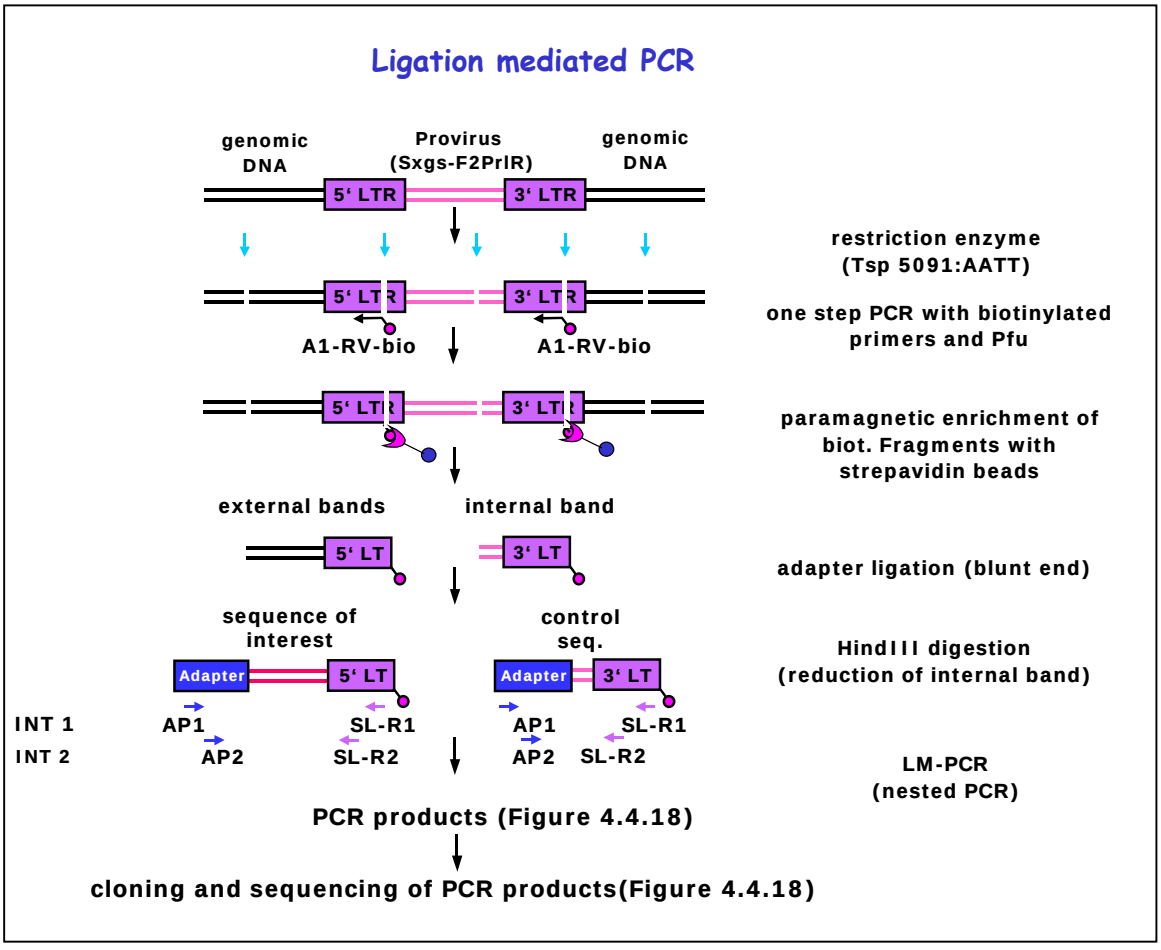
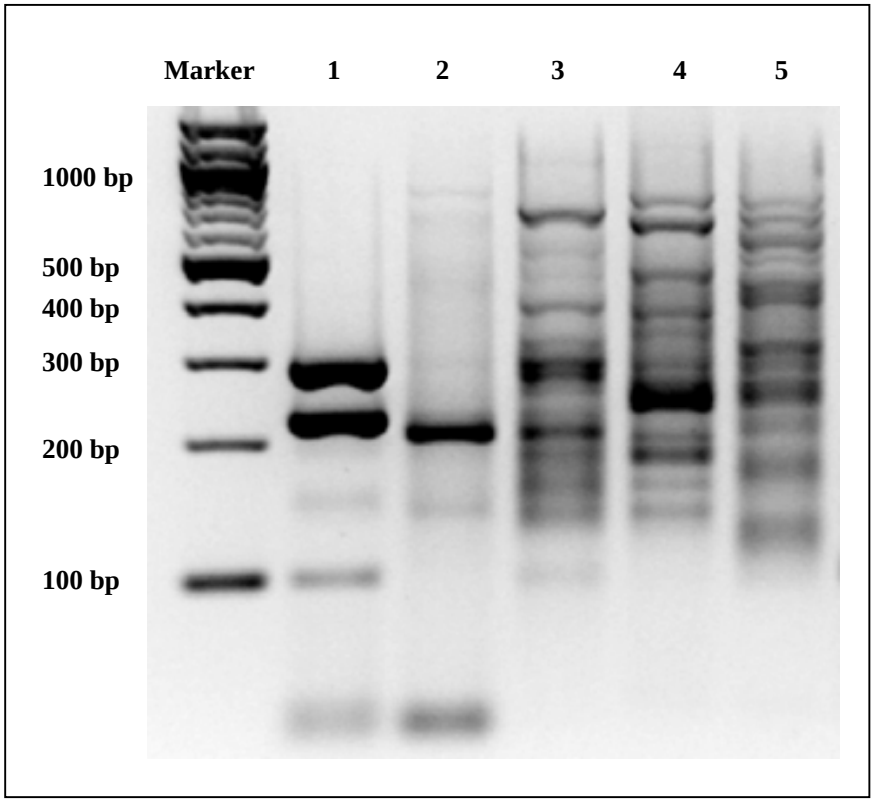


Figure 4.4.17: Schematic representation of LM-PCR: The genomic DNA was digested with four-cutter enzyme Tsp5091 and further combined with biotinylated special primer. The biotinylated fragments were enriched with strepavidin beads. A special adaptor was added into the sequence of interest and the expected bands were amplified with nested PCR. The details of this representation were described in chapter 3.4.3.

Genomic DNA from the samples was digested with the restriction enzyme *Tsp*5091, a four-cutter which should generate DNA fragments of an average length of ~300 bp. Digestion with the unique cutter Hind*III* reduced background by cutting the internal 3'LTR band, allowing specific amplification of the 5'LTR sequences. Polyclonal proliferation of expanded cells and identities of integration sites are shown in Figure 4.4.18 and Table 4.4.2. As shown in Figure 4.4.18, multiple bands representing different integration sites of Sxgs-F2PrlR provirus were observed by LM-PCR analysis of the AP20187-expanded mBM cells clearly. Most of the amplified DNA fragments ranged from 1000 to 100 bp. In contrast, only 4 background bands were obtained from genomic DNA of wild type mBM cells. Furthermore, the genomic DNA from non-selected cells showed only 1 strong signal at ~ 230 bp. The DNA fragments from selected cells (Figure 4.4.18 A, lane 5) were transformed into the TOPO cloning vector, in order to sequence integration sites. As shown in Figure4.4.17 B, out of 13 selected colonies, 12 showed a single band signal of variable size after colony PCR. The DNA fragments amplified from each single colony were purified and sequenced (Table 4.4.2). Ten of the 12 sequenced integrations could be found in the mouse genome database (see Table 4.4.2). The location of genes within 100 kb and their distance to the integration site are listed. Genes containing integration sites are shown in bold type.

A



B

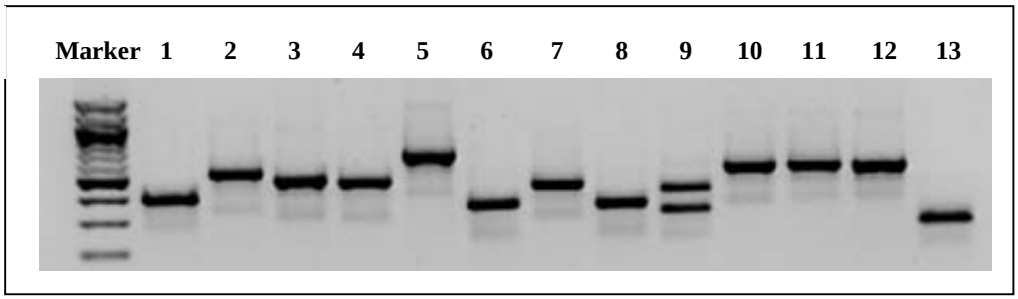


Figure 4.4.18: Polyclonality of *ex vivo* selectively expanded murine HSCs shown by LM-PCR. A: Gel electrophoresis of 5'LTR integration site analysis of genomic DNA from wild type mBM cells (lane 1); Sxgs-F2PrlR-transduced, Sca1-positive mBM cells, cultured *ex vivo* for 2 weeks in the presence of cytokines (lane 2), Sxgs-F2PrlR-transduced, Sca1-positive mBM cells, stimulated for 4 weeks with AP20187 (lane 4), and Sxgs-F2PrlR-transduced, Sca1-positive mBM cells, stimulated for 2 weeks with AP20187 (lanes 3 and 5) Genomic DNA was digested with the restriction enzyme *Tsp*5091 and amplified with the LTR sequencing primer and the adapter primer at the end of the DNA fragments. The PCR products were cut with the restriction enzyme *Hind*III to digest the 3'LTR fragments. The 5'LTR DNA fragments were then amplified by nested PCR. B: Amplified PCR products from single picked colonies. The PCR product from lane 5 (A) was cloned into the TOPO cloning vector and positive colonies were picked directly for carrying out a colony-PCR with TOPO-primers (M13 rev, M13 for). Numbers correspond to the clone numbers in Table 4.4.2.

Table 4.4.2: Insertion site analysis of selected clones from Sxgs-F2PrlR-transduced murine HSCs after 2 weeks stimulation with AP20187

Clone number	Genomic length(bp)	Mouse chr.	Gene	Protein family	Distance & location
1	88	4(qD3)	2610510H1Rik	nd	7.7 kb, 5'
			Padi4	Peptidylarginine deiminase, type IV	70 kb, 5'
			Padi6	Peptidylarginine deiminase, type VI	89 kb, 5'
2	232	15(qA1)	**PrlR**	**Prolactin receptor**	**intron**
			A1987712	hypothetical protein	68 kb, 5'
3	188	non-random	Tmlhe	Trimethyl hydroxylase epsilon	38 kb,5'
4	164	9(qA5.2)	D630044F24Rik	Hypothetical protein LOC 38273	5.1 kb, 3'
			RP525	Ribosomal protein S25	71 kb, 3'
			Trappc4	Trafficking protein particle complex 4	98 kb, 5'
5	365	18(qE3)	*no gene within 100 kb*		
6,8	49	2(qA3)	Abca2	ATP-binding cassette sub-family A	1.1 kb, 5'
			Fut7	Fucosyltransferase 7	22 kb, 3'
7	148	17(qE4)	*no gene within 100 kb*		
9	nd	nd	nd	nd	nd
10,11,12	247	6(qF2)	**Grcc2f**	**Gene rich cluster c2f gene**	**intron**
			Bcap37	B-cell receptor associated protein 37	11 kb, 5'
			Grcc3f	Gene rich cluster c3f gene	5.7 kb, 3'
			Hcph	Hemopoietic cell phosphatase	2.2 kb, 5'

All insertion site sequences detected in the different clones were aligned to the mouse genome using mouse genome Blast (http://genome.ucsc.edu). Length denotes the size of the genomic DNA part of the LM-PCR amplification without linker and LTR sequence; nd.: not determined; chr, chromosome.

As indicated in Table 4.4.2, from the 12 selected and sequenced colonies, clones 10, 11 and 12 have the same size and are located all on chromosome 6. Clones 6 and 8 also have the same size and are located on chromosome 2. All other clones are located on different chromosomes. Only clone 2 and clones 10–12 are located within genes, namely PrlR and Grcc2, respectively. The other indicated genes are located within 100 kb up- or downstream of the integration site. This result suggests that the *ex vivo* selectively expanded mBM cells transduced with Sxgs-F2PrlR have proliferated polyclonaly and show multiple, different integration sites.

4.4.13 Expanded Cells Retain the Capacity to Repopulate the Bone Marrow of Lethally Irradiated Mice

As shown earlier, the Sxgs-F2PrlR-transduced cells presented a clear selective proliferation after 2 weeks stimulation with AP20187 + mSCF *ex vivo*. This expansion seemed to be primarily due to proliferation of progenitor cells, as judged by standardised short-term colony assay and staining of the progenitor cells for Sca1 and c-kit expression. However, the identification of true HSCs, which have the ability both to durably repopulate myeloid and lymphoid lineages and to self-renew, has to be done using *in vivo* assays, i.e. mouse transplantation experiments (Benveniste et al., 2003; Matsuzaki et al., 2004). In order to clarify and extend the *in vitro* data, cells expanded *ex vivo* under different culture conditions were transplanted into sublethally irradiated C57BL/6 Ly 5.2 mice. As described in 4.4.4, the Sca1-positive mBM cells from C57BL/6 Ly 5.1 mice were transduced with Sxgs-F2PrlR retroviral particles. One day after transduction (on day 6 in culture), a first transplantation was carried out with transduced and non-transduced cells, as indicated in Figure 4.4.19. In addition, transduced cells were cultured under selective and non-selective conditions. After 2 weeks of selection, a second transplantation series was carried out with selected, non-selected and non-transduced cells (Figure 4.4.19). Eight to 10 weeks after transplantation, cells from the bone marrow (BM), peripheral blood (PB) and spleen of individual recipient mice were analysed. Figure 4.4.19 and Table 4.4.3 give an overview of the experimental outline and the results obtained from the transplantation study in terms of

engraftment level (% of CD45.1-positive cells) and transgene expression (percentage of 7D5-positive cells).

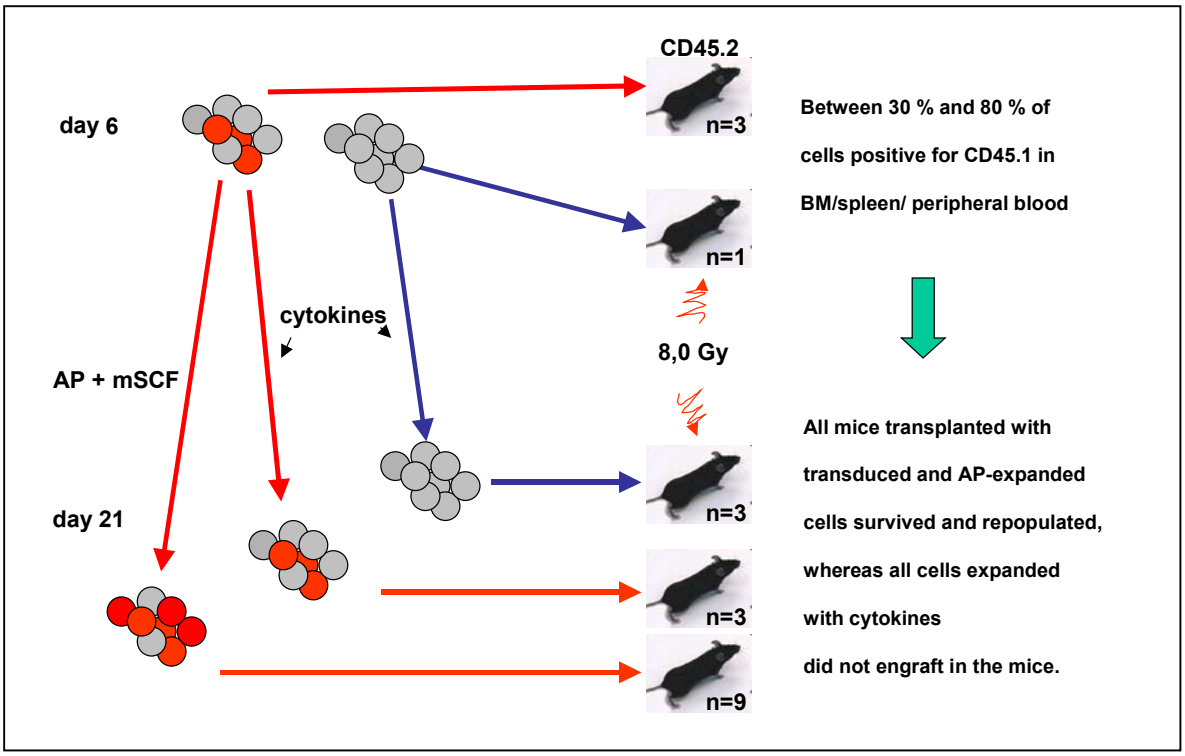


Figure 4.4.19: Study design of the transplantation with selected and non-selected cells. Day 6: One day after transduction, 1×10^6 transduced or non-transduced cells were administrated i.v. into preconditioned (8.0 Gy-irradiated) recipient mice. Day 21: After 2 weeks *ex vivo* expansion of transduced cells under different conditions, 1×10^6 selected or non-selected transduced cells, or non-transduced cells were transplanted into recipient mice.
n: number of individual mice treated per condition.

As shown in Figure 4.4.19, all mice transplanted with transduced cells (day 6) survived (8–10 weeks post-transplantation) until analysis. However, all mice transplanted with cytokine-stimulated, Sxgs-F2PrlR-transduced cells (day 21) died 12–16 days after transplantation. Two mice transplanted with non-transduced cells which had also been cultured for 2 weeks in cytokine medium died 14 days after transplantation. Unexpectedly, 1 mouse in this group survived until analysis. In contrast, all mice transplanted with AP20187-stimulated, Sxgs-F2PrlR-transduced cells survived until analysis 8–10 weeks post-transplantation. This result suggests that the population of AP20187-stimulated cells contained high frequencies of progenitor cells which could repopulate irradiated recipient mice. Two lethally irradiated but non-transplanted mice were included in both transplantation experiments as a control for the efficiency of the irradiation dose. All non-transplanted mice

died 12–14 days after irradiation. The surviving recipient mice were used for multiple analyses 8–10 weeks after transplantation. The results are summarised in Table 4.4.3.

Table 4.4.3: Repopulation efficiency and transgene expression in transplanted mice Cells from different tissues (BM, PB and spleen) were analysed by FACS with anti-CD45.1-PE antibody for detection of the Ly5.1 mouse cells (donor cells) and anti-7D5-PE antibody for detection of $gp91^{phox}$-positive cells. PB samples (10-20 µl) were stained after erythrocytes lysis using Pharmingen Lysis buffer (BD Pharmingen). For the BM and spleen, 1×10^5 cells were used for each staining after preparation of NH_4Cl-lysed cell populations. T1No.1: mouse number 1 from transplantation 1. No1: mouse number 1 from transplantation 2.

A: Percentage of CD45.1-positive and 7D5-positive cells in PB, BM and spleen. First transplantation on day 6

Mouse no:	Vector	Culture condition	PB cells		BM cells		Spleen cells	
			CD45.1	7D5	CD45.1	7D5	CD45.1	7D5
T1No.1	F2PrlR	cytokines	54.8	3.2	11.1	0	41.1	3.2
T1No.2	F2PrlR	cytokines	90.6	2.7	78.1	1.8	76.1	2.1
T1No.3	F2PrlR	cytokines	79.4	3.0	41.5	3	69.5	3.0
T1No.4	ntd.	cytokines	95.7	-	92.5	-	85.5	-

B: Percentage of CD45.1 positive cells and 7D5 positive cells in PB, BM and spleen. Transplantation on day 21

Mouse no:	Vector	Culture condition	PB cells		BM cells		Spleen cells	
			CD45.1	7D5	CD45.1	7D5	CD45.1	7D5
No.1	F2PrlR	AP+mSCF	5.2	0	1	0	5.5	0
No.2	F2PrlR	AP+mSCF	80.5	1.7	51.7	1.3	75.5	1.3
No.3	F2PrlR	AP+mSCF	60.5	1.1	17.9	1.0	53.2	0.8
No.4	F2PrlR	AP+mSCF	33.4	1.3	2.1	0	26.5	2.1
No.5	F2PrlR	AP+mSCF	17.2	0	1.8	0.2	12.7	1.2
No.6	F2PrlR	AP+mSCF	56.5	5.5	49.1	3.8	58.5	3
No.7	F2PrlR	AP+mSCF	62.3	3.4	2.8	0.2	13.1	0.8
No.8	F2PrlR	AP+mSCF	21.5	0	22.5	1.2	50.7	2
No.9	F2PrlR	AP+mSCF	74.1	1.5	31.5	1.2	79.4	4.4
No.10	ntd.	cytokines	0	0	0	0	0.7	0

As illustrated in Table 4.4.3 A, all the surviving mice from the first transplantation had a clear engraftment ranging from 54 to 95.7% in PB cells, 11 to 92.5% in BM cells and 41 to 85% in spleen. However, the percentage of transgene-expressing cells, that is 7D5-positive cells, was very low (<4%). Interestingly, mouse that was transplanted with non-transduced cells had a higher engraftment level in all organs than those transplanted with transduced cells. In contrast to these results, the transduced cells that had been cultured for a further 2 weeks with the cytokine combination could not repopulate recipient mice (Figure 4.4.19 and Table 4.4.3 B). Nevertheless, the transduced cells stimulated with AP20187 for 2

weeks were able to establish a new haematopoiesis for the irradiated recipient mice. All mice in the group transplanted with dimerizer-expanded BM cells had a repopulation efficiency ranging from 1–80% in the 3 organs analysed. In addition, engraftment was usually much lower in BM cells than in PB and spleen cells. As in the first transplantation, gp91^{phox} transgene expression in this group of transplanted mice was very low (< 5% of the cells).

4.4.14 Transgene Analysis of Genomic DNA from Recipient Mice Post-Transplantation

After 2 weeks stimulation with AP20187, the transduced cells had a large transgene-expressing population with 70% 7D5-positive cells. After transplantation, however, although the repopulation efficiency was over 50% in some organs, the number of 7D5+ cells was very low. These observations prompted us to use a more sensitive analysis method i.e. PCR, to determine transgene expression in transplant recipient mice. The human gp91^{phox} sequence was amplified from genomic DNA in different tissues and the results are shown in Figure 4.4.20.

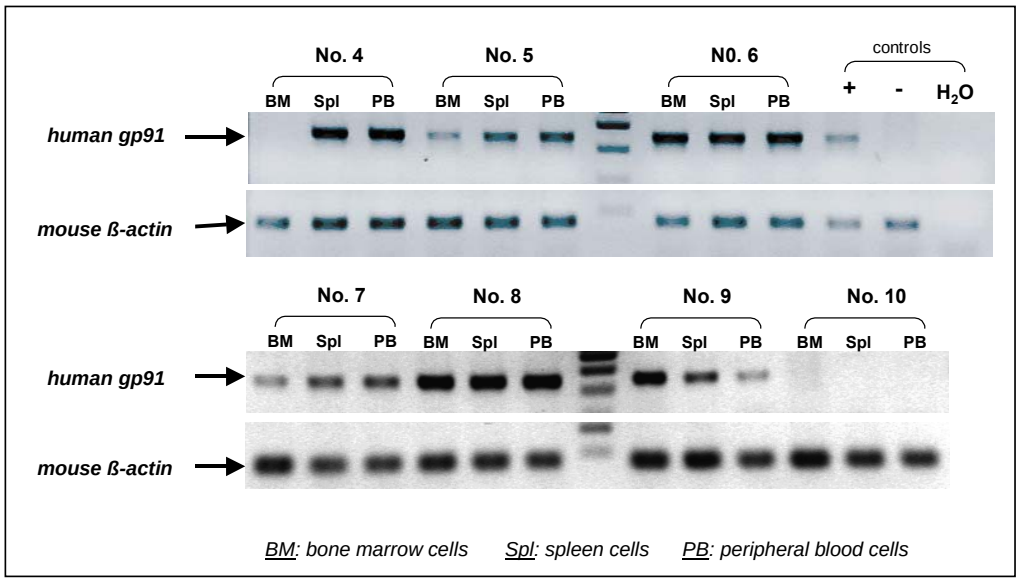


Figure 4.4.20: PCR analysis of the human gp91^{phox} sequence in different organs of transplanted mice. Genomic DNA was prepared from BM, PB and spleen cells of transplanted mice (No.4–No.10) 10 weeks post transplantation. PCRs were carried out with 100 ng genomic DNA. Human gp91^{phox} gene-specific primers were used to produce a 510 bp amplificate (see Figure 4.4.22). Mouse ß-actin sequences were amplified under the same PCR conditions as a control for the amount of DNA loaded. Genomic DNA for the positive control was prepared from SF71gp retroviral vector-transduced X-CGD cells containing 80% 7D5-positive cells. The negative control was genomic DNA from BM cells of a wild type C57BL/6 mouse.

As shown in Figure 4.4.20, human gp91^{phox} sequences were observed in all samples (except BM from mouse No.4) from mice (No.4–No.9) that had been transplanted with Sxgs-F2PrlR-transduced cells previously selected for 2 weeks with AP20187 and mSCF. No signal for human gp91^{phox} could be detected in all analysed organs of mouse No.10. This mouse had not received gp91^{phox}-expressing cells and served as a negative control. These results indicate that gp91^{phox}-containing cells may contribute to the reconstitution of haematopoiesis in transplanted mice.

4.4.15 *Ex vivo* Restimulation of BM Cells from Recipient Mice Post-Transplantation

For unknown reasons, the percentage of 7D5-positive cells in the BM cells of transplanted mice was found to be very low. We, therefore, investigated whether these cells can be reselected under stimulation with AP20187. To answer this question, BM cells from mice No.5–No.9 were cultured *ex vivo* for 2 weeks either with the cytokine combination or under stimulation with AP20187 + mSCF. The results are shown in Figure 4.4.21.

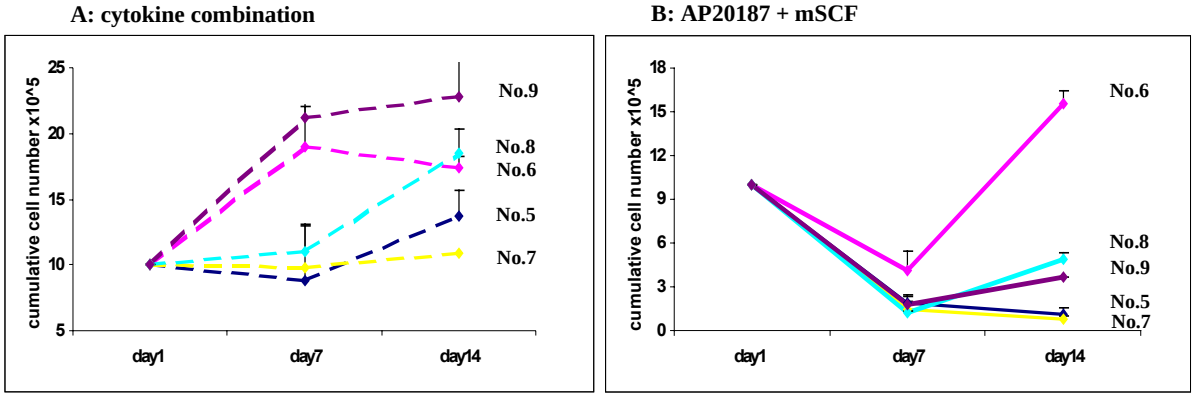


Figure 4.4.21: E*x vivo* proliferation rate of mBM cells from transplanted mice under cytokine- and AP20187-stimulation conditions. BM cells were prepared from transplanted mice No.5–No.9 and 1×10^6 cells from each mouse were cultured for 2 weeks with either the cytokine combination (A) or AP20187 + mSCF (B). The cell number was counted each week. The colours of the lines represent samples from different mice, as indicated in the figure.

As indicated in Figure 4.4.21, the cytokine-stimulated cells from different mice proliferated within 2 weeks. In contrast, the number of cells cultured with AP20187 and mSCF strongly decreased during the first week. However, in the second week, the cells from mice No.6, No.8 and No.9 began to proliferate, in particular those from mouse No.6. The cells from mice No.5 and No.7 continued to decrease in number during the second week of stimulation with AP20187. These results suggest that a proportion of the BM cells from mice

No.6, No.8 and No.9 reacted upon stimulation with AP20187. Expression of the fusion protein F2PrlR most likely contributed to the proliferation of these cells. To verify this hypothesis, BM cells stimulated for 2 weeks either with cytokines or with AP20187 from mice No. 6, No.8 and No.9 were stained with anti-7D5 antibody for FACS analysis. In addition, PCR reactions for human gp91^{phox} and for the fusion protein F2PrlR were performed with genomic DNA. The results are shown in Figures 4.4.22 and 4.4.23.

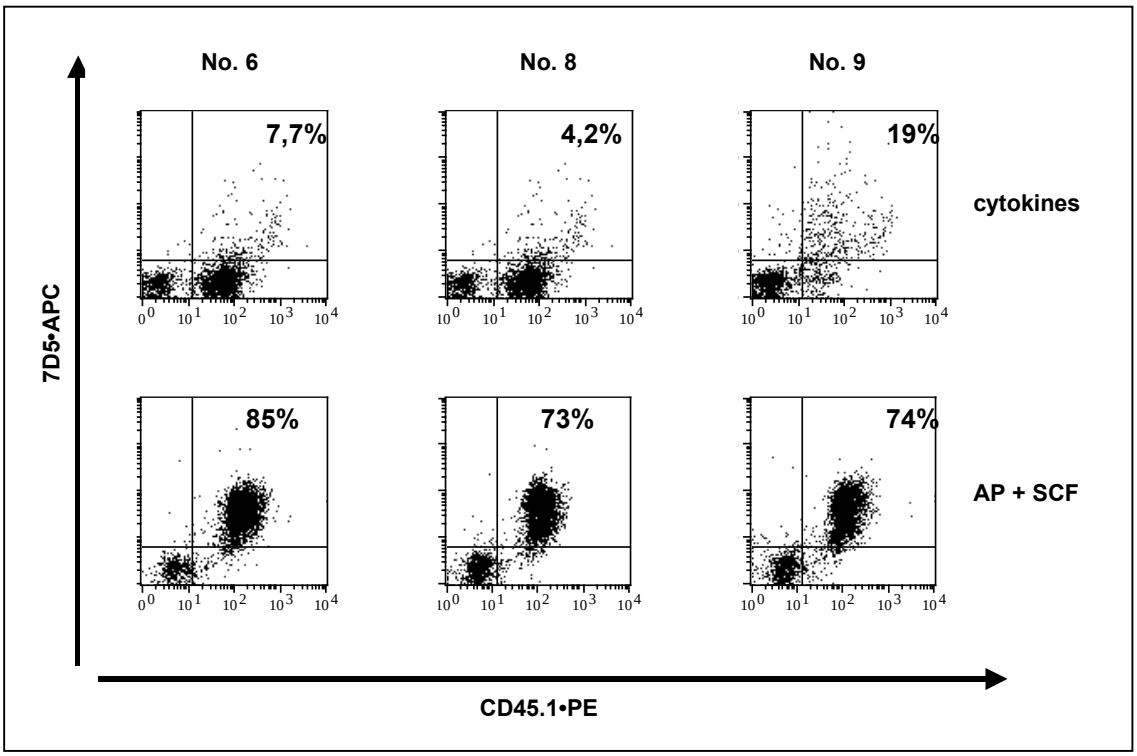


Figure 4.4.22: FACS analysis of *ex vivo*-selected mBM cells from transplanted mice. 1×10^5 cells per sample were used for each staining. For double staining, cells were incubated with anti-7D5-APC and anti-CD45.1-PE antibodies. After staining, cells were analysed in 3-colour mode with a FACS Calibur. The mouse number is indicated at the top of the figure. The upper plots show cells cultured with the cytokine combination and the lower plots show cells selected with AP20187. The percentage of 7D5/CD45.1 double-positive cells is shown in each

As demonstrated in Figure 4.4.22, the cells stimulated with AP20187 + mSCF show high levels of 7D5-positive cells (over 70%) in donor cell populations (CD45.1-positive cells) from all analysed mice. In contrast, the cells from mice No.6, No.8 and No.9 that were stimulated with cytokines showed low levels of 7D5-positive cells (7.7, 4.2 and 19%). In the PCR analysis shown in Figure 4.4.23, significantly stronger signals for gp91^{phox} and F2PrlR were observed in AP20187-stimulated cells from mice No.8 and No.9 than in cells cultured

with cytokines. However, genomic DNA from the 2 populations of cells from mouse No.6 gave rise to equal signal intensities for both gp91^{phox} and F2PrlR.

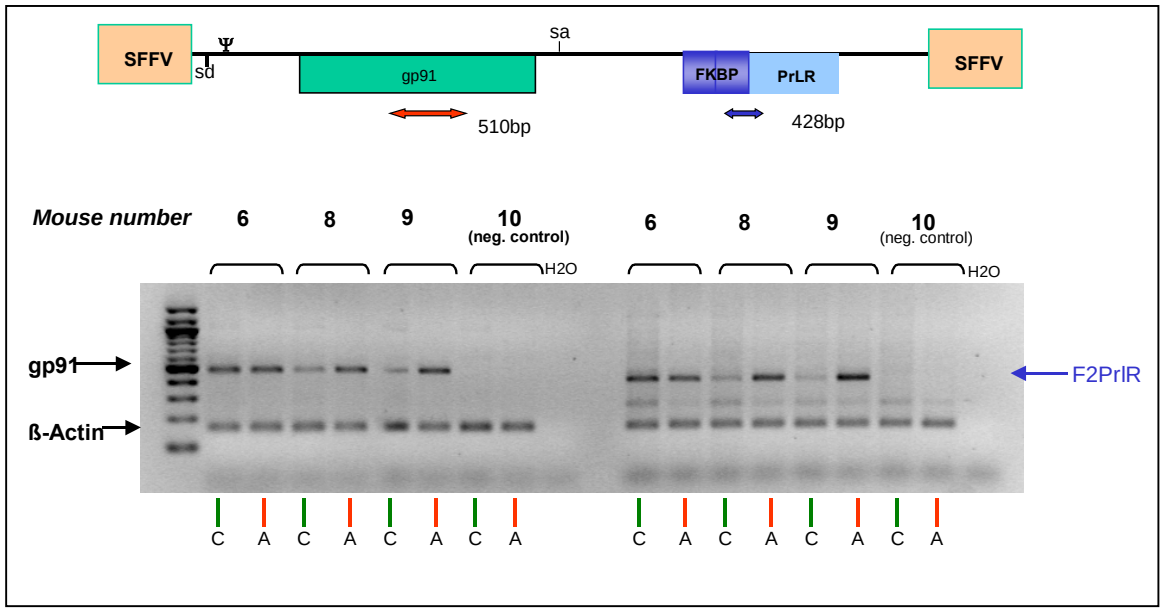


Figure 4.4.23: PCR analysis of gp91^{phox} and fusion protein F2PrlR sequences in *ex vivo*-cultured BM cells from mice No.6, No.8 and No.9. Genomic DNA was prepared from BM cells from mice No.6, No.8 and No.9 cultured under different conditions, and 100 ng was used for each PCR reaction. The PCR reaction was carried out as described in chapter 3.4.2. The sample from mouse No.10 served as a negative control. The sizes and positions of the 2 PCR products are shown as red and blue arrows in the upper part of the figure. At the bottom of the figure, the PCR products for gp91^{phox} (left) and F2PrlR (right) are shown. C: Cells cultured with cytokines. A: Cells cultured with AP20187 + mSCF. PCR-reactions for ß-actin sequences were performed as a control for equal DNA loading.

4.4.16 Determination of Progenitor Cells in *ex vivo*-Cultured BM Cells from Recipient Mice using the CFU Assay

As shown above, mBM cells from 3 transplanted mice could be selected for Sxgs-F2PrlR-transduced cells for a second time *ex vivo*. As already shown, *ex vivo*-expanded cells cultured for 2 weeks under stimulation with AP20187 before transplantation contained high numbers of progenitor cells compared to cells stimulated with the cytokine combination. Therefore, we asked whether BM cells from the transplanted mice would show equally high numbers of progenitor cells after 2 weeks of *ex vivo* culture in selection medium. For this purpose, CFU-assays in methylcellulose were performed with *ex vivo*-cultured cells from 3 transplanted mice. The results from the second plating are given in Figure 4.4.24.

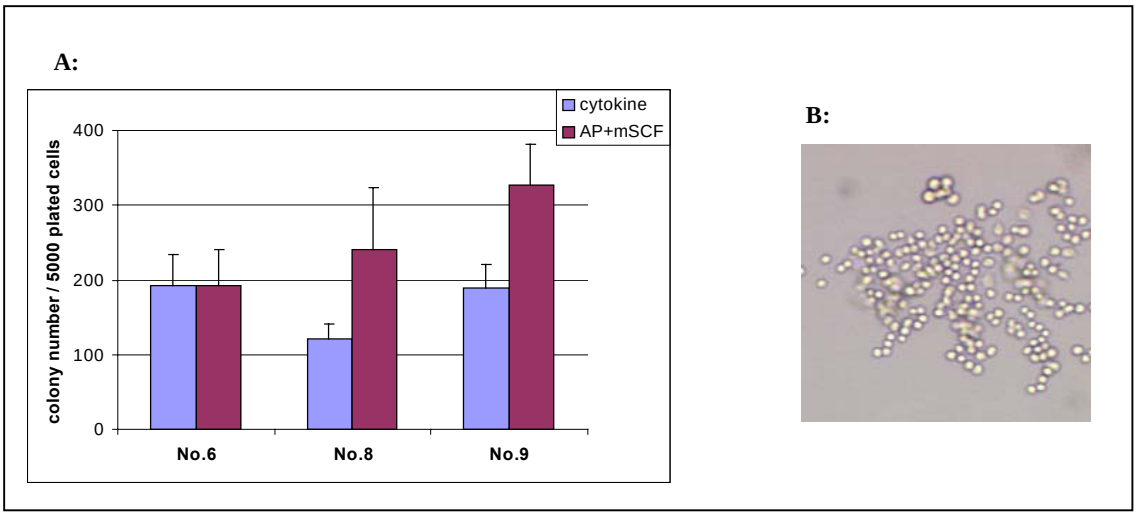


Figure 4.4.24: CFU assay in methylcellulose: Second plating of *ex vivo*-cultured BM cells from mice No.6, No.8 and No.9. After 2 weeks of *ex vivo* culture of the BM cells under different conditions, 10000 cells per sample were plated in methylcellulose as described in chapter 3.6.8. The total colony number of the first plating was very high. Thus, second plating was carried out with 5000 cells per sample. Ten days after plating, the total colony number in each dish was counted. A: Colony number from the second methylcellulose plating for all analysed mice. B: Typical morphology of one colony under the microscope.

As observed in the second CFU assay, the total colony number from cells from mice No.8 and No.9, selected for 2 weeks with AP20187, was about 2-fold higher than that from cells stimulated with the cytokine combination. Despite the significance of the results obtained from mice No.8 and No.9, the colony numbers from BM cells from mouse No.6 did not show any difference between the 2 culture conditions. To determine whether the progenitor cells that generated the colonies, contained the transgene, we picked single colonies and performed PCR reactions with gp91^{phox}-specific primer pairs. As shown in Table 4.4.5 and Figure 4.4.25 for the 3 mice No.6, No.8 and No.9, 100, 80 and 100% of colonies from the cells stimulated with AP20187 were gp91^{phox}-positive. However, only 60, 30 and 10% positive colonies were found in cytokine-stimulated cells. The PCR analysis of colonies from mouse No.9 is shown in Figure 4.4.24.

Table 4.4.4: Percentage of gp91^{phox}-positive colonies in picked colonies from mice No.6, No.8 and No.9.

Mouse number	AP + mSCF	Cytokine
No.6	100%	40%
No.8	80%	30%
No.9	100%	10%

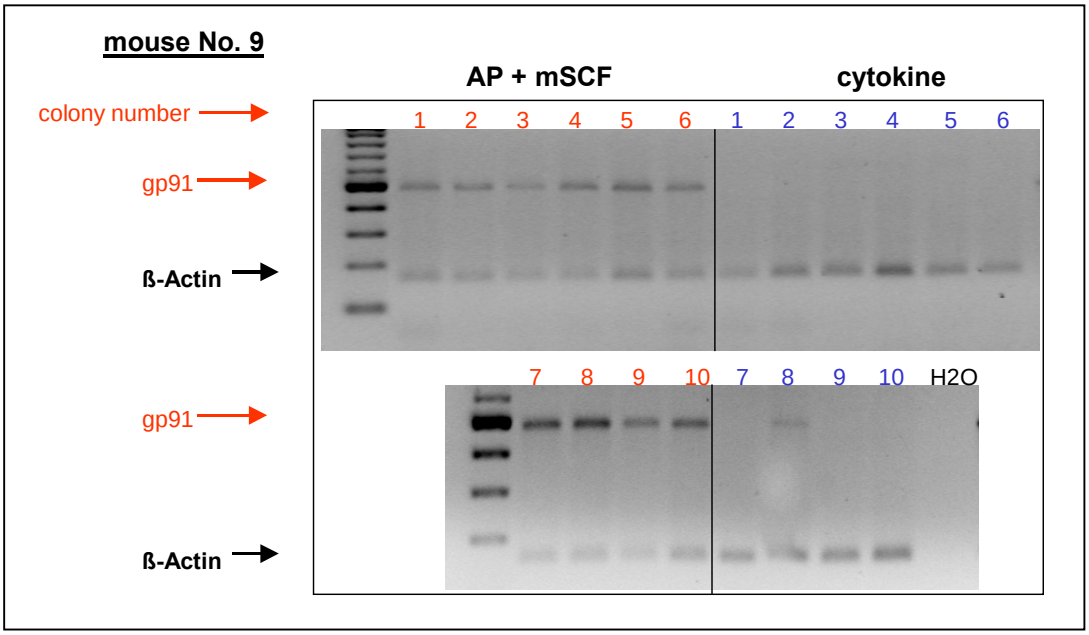


Figure 4.4.25: PCR analysis of gp91^{phox} sequences in picked colonies from mouse No. 9. Ten colonies from each sample were picked for analysis and the PCR reaction was performed as described in chapter 3.4.2. The figure shows the results obtained from mouse No.9 as an example. The PCR reaction for mouse ß-actin was carried out with ß-actin primer pairs in the same reaction to ensure equal DNA levels.

In summary of chapter 4.4, overexpression of Sxgs-F2PrlR retroviral construct led to a significant reversible selective expansion of transduced BAF/3 and primary mBM cells. In mouse transplantation experiments, *ex vivo*-expanded cells repopulated the bone marrow of lethally irradiated mice suggesting that the *ex vivo* expansion took place at the level of haematopoietic stem and/or progenitor cells. Genomic gp91^{phox} sequences were detected in the bone marrow, spleen and peripheral blood cells of transplanted animals, indicating that gp91^{phox}-containing cells most likely contributed to the reconstitution of haematopoiesis in these mice.

5. Discussion

One of the main limitations of gene transfer into HSCs for the treatment of inherited disorders like X-CGD is the inefficiency of gene transfer into early haematopoietic progenitor and stem cells (Kume et al., 2003). Over many years, methods have been developed to improve the efficiency of gene transfer and expression in HSCs *ex vivo* and *in vivo*. The aim of this study was to develop a novel strategy to expand transduced murine HSCs. Strategies using different selection molecules, which play important roles in the maintenance of HSC self-renewal and proliferation, were investigated.

The first strategy is based on the intrinsic transcription factor HOXB4, which can enhance self-renewal of human and murine HSCs, as observed in numerous *in vitro* and *in vivo* studies (Antonchuk et al., 2001, 2002; Sauvageau et al., 1995; Thorsteinsdottir et al., 1999; Björnssen et al., 2002). A bicistronic vector derived from the backbone of the gammaretroviral vector SF71, coding for HOXB4 and the therapeutic gene $gp91^{phox}$, was designed. The second strategy used in the present study is based on an inducible dimerizer system consisting of an artificial drug-binding component and a signalling domain from either the EGFR or the PrlR. Addition or removal of the small molecular drug AP20187 controls activation of both constructs. To compare the efficiency and capability of each selection strategy, the following questions were examined throughout:

- Does the appropriate selection system work in HSCs *ex vivo*?
- Are *ex vivo*-selected HSCs able to repopulate lethally irradiated recipient mice?
- Which of the strategies is most suitable for selective expansion of genetically modified murine HSCs?

5.1 HOXB4 induced *ex vivo* murine HSC proliferation but did not confer a growth advantage *in vivo*

The first system used in the present study is based on the HOXB4 transcription factor. A large number of reports indicate that HOX genes act as key regulators for the balance between self-renewal and committed differentiation of haematopoietic stem/progenitor cells. In 1994, Sauvageau et al. reported that HOXB4 is expressed at high levels in primitive haematopoietic cells and becomes down-regulated in more mature cells. One year later, the same group published data showing that HOXB4 overexpression in murine BM cells resulted

in increased numbers of CFU-C (*in vitro* clonogenic progenitors), CFU-S_{12} (day 12 colony-forming units in spleen) and long-term repopulating cells in primary and secondary recipients (Sauvageau et al., 1995). In competitive repopulation experiments, HOXB4-transduced cells outperformed control cells (Antonchuk et al., 2001). Recently, it was reported that transient expression of HOXB4 in mouse embryonic stem and yolk sac cells, which give rise to haematopoiesis, led to definitive haematopoietic potential and allowed stable and long-term haematopoietic engraftment of adult mice (Kyba et al., 2002). In the present study, overexpression of HOXB4 and $gp91^{phox}$ from bicistronic retroviral vectors in primary BM cells led to a 3–4-fold increase in cell number compared to mock-transduced cells under the same culture conditions. The cell number increased from 2×10^5 to 86×10^5 within 2 weeks after transduction in culture under stimulation with the cytokine combination mIL-3, mIL-6 and mSCF. Supporting these results, the proportion of transgene-expressing cells (7D5-positive cells) clearly increased from 16%, one day after transduction, to 70%, 2 weeks later. These results are comparable to data from Humphries and colleagues (Antonchuk et al., 2002). In their study, overexpression of a HOXB4-IRES-eGFP fusion construct led to a 2–3-fold *ex vivo* proliferation advantage in 5-FU-treated mBM cells compared to mock (GFP)-transduced cells. The percentage of GFP-positive cells increased 2.7-fold, from 30 to 80%, while that of mock-transduced cells increased only 1.3-fold, from 45 to 60%. In particular, the CFU from the HOXB4-transduced cells increased 2–3-fold after 14 days cultivation in both studies. However, Antonchuk et al. (2002) reported a significant 41-fold enhancement in CRU *in vivo*, compared to a 29-fold and 58-fold decrease for non-transduced and mock-transduced cells, respectively. In our case, after 7 days of *ex vivo* cultivation, HOXB4-transduced haematopoietic stem/progenitor cells did not have any selection advantage in irradiated recipient mice 8–10 weeks post-transplantation. Only 1% $gp91^{phox}$-positive cells could be detected in the PB via FACS analysis. Several circumstances could have contributed to our findings: First, the transduction efficiency of HSCs may be too low to achieve a functional selective effect *in vivo*. Only 11% of the transduced cell population were $gp91^{phox}$-positive. Secondly, the expression level of HOXB4 in the target cells is extremely important for its function. Too high an expression, such as in adenovirus-transduced cells, could lead to perturbation of the cells, whereas too low an expression might have no effect *in vivo*. In the vector used in this study, expression of the upstream therapeutic gene and downstream selective element HOXB4 are controlled by splice donor and acceptor sites. It has been reported that the transgenes are expressed at different levels with this vector system. When the expression levels of the therapeutic gene are high, the downstream gene for the selection

cassette is expressed at a much lower intensity (Hildinger et al., 1999). Similarly, our FACS analysis revealed that expression levels of the downstream protein (ΔLNGFR) reached only 40% after *ex vivo* selection, while therapeutic gene ($gp91^{phox}$) expression levels were 70% (data not shown). To clarify these observations western blot and genomic DNA PCR analysis should be carried out. Milsom and Fairbairn (2004) suggested that for certain selectable markers, such as drug-resistance-genes, HOXB4 and perhaps chemically inducible receptors, the level of expression required to achieve effective selection is likely to be high. Thirdly, an *ex vivo* cultivation and pre-selection (e.g. to about 20–30%) of the transduced cells may be required for a further sufficient selection *in vivo.*

In the light of the intriguing report by Schiedlmeier et al. (2003), the use of HOXB4 as a selection molecule has to be re-discussed carefully. Schiedlmeier et al. provided data showing that high-level expression of HOXB4 in mice impairs myeloerythroid differentiation and further reduces the B-cell output of transduced human CD34-positive CB cells transplanted into NOD/SCID mice. In contrast, Buske et al. (2002) reported that overexpression of HOXB4 in human cells led to an increase in the number of progenitor cells, as shown by increased secondary plating capacity of colony-forming cells and an increased number of NOD/SCID repopulating cells. No evidence of any disruption in the differentiation potential of the engrafting human HSCs was observed, although an increased production of B-cells was seen *in vitro*. These contradictory results and the poor expression levels in our transplanted mice prompted us to change the selection molecule. Furthermore, the effect of HOXB4 may be strongly influenced by the expression level of the protein. Definitive control of HOXB4 expression from the bicistronic Sxgs-HOXB4 retroviral vector cannot be controlled and we, therefore, focused our attention on approaches allowing an inducible action of another selection molecule.

5.2 A Fusion Protein Containing the Intracellular Signalling Domain of the EGFR Constitutively Induced Proliferation of BaF/3 Cells

As described in the introduction (chapter 1.5.1), constitutively active mutants or overexpression of EGF or its receptor play a crucial role in the development of numerous human tumours. EGF receptors are often constitutively stimulated in cancer cells in the presence of EGF ligands secreted by the cells themselves (Salomon et al., 1995). However, less evidence is available that overexpression of EGFR alone has oncogenic potential in

haematopoietic cells. In the present study, overexpression of the lentiviral dimerization construct S-NF2EGFR led to a huge proliferation advantage in transduced BaF/3 cells after stimulation by the dimerizer molecule AP20187. The cell number increased about 5×10^5-fold, from 2×10^5 to 1×10^{11}, after 18 days of stimulation. This was the highest rate observed with any of the constructs used in this study. In the same experiment, the percentage of 7D5-positive cells significantly increased from 20 to 99% after AP20187 stimulation. In opposition to the excellent selection capacity, the proliferation promoting effect could not be switched off after removal of AP20187 and it facilitated an uncontrolled proliferation of transduced BaF/3 cells for more than 12 days. Hence, cell proliferation induced by activation of the EGFR construct by AP20187 may be potentially oncogenic. The reasons for this are still not clear, although the following suppositions may have contributed. It has been reported that EGFR deletion mutants, which lack the extracellular domain, resemble the v-erb-B oncoprotein and have been detected in xenograft models derived from malignant human gliomas (Sugawa et al., 1990; Moscatello et al., 1995), breast carcinomas (Wilkstrand et al., 1995), non-small-cell lung carcinomas (Garcia de Pallazzo et al., 1993) and ovarian tumours (Moscatello et al., 1995). These deletions represent receptor mutants that maintain constitutive active kinase function, thereby stimulating cellular proliferation in the absence of any ligands (Ekstrand et al., 1994). In our fusion construct, the extracellular domain sequence of the EGFR is completely replaced by a truncated LNGFR sequence. After AP20187-induced dimerization, this structural alteration may lead to constitutive autophosphorylation of tyrosine residues in the intracellular kinase signalling domain, causing a further uncontrollable cell proliferation of transduced cells. Analysing the phosphorylation status of the EGFR after removal of AP20187 by western blotting experiments could prove this hypothesis. Furthermore, it is conceivable that the large number of EGFR proteins produced by overexpression leads to assembly and dimerization without the dimerizer AP20187, at least to some extent, which may be sufficient to propel cell proliferation. One could verify this mechanistic assumption by appropriate co-immunoprecipitation experiments in the presence and absence of AP20187. As a result of the observed oncogenic potential, I did not continue with the EGFR signalling domain construct.

5.3 Dimerizer-Induced PrlR Signalling Promotes Expansion of Murine HSCs

In contrast to overexpression of the lentiviral vector S-NF2EGFR, overexpression of S-NF2PrlR, containing the intracellular domain of the PrlR as the selection cassette, led to inducible, and most importantly, reversible selective expansion of transduced BaF/3 cells. This result prompted us to investigate this system further using the backbone of a gammaretroviral vector that is currently utilised in clinical trials due to its safety and efficient transduction.

The truncated LNGFR sequence is not an appropriate marker gene when fused to the 2×FKBP dimerizer system

As mentioned earlier, the bicistronic retroviral vectors used in this study contain splice donor and acceptor elements to control the co-expression of the therapeutic gene $gp91^{phox}$ and a selection cassette. At first, vectors were designed with a truncated LNGFR (ΔLNGFR) as a marker gene to be co-expressed with the selection cassette in order to allow detection of transduced cells by FACS analysis. Surprisingly, expression of the Sxgs-NF2 construct, which lacks any proliferation-conferring signalling domain, in primary BM cells led to significant expansion of transduced cells under stimulation with AP20187+mSCF. Under this treatment, cell numbers increased from 2×10^5 to 37×10^5 and the percentage of transgene-expressing cells (7D5-positive) rose from 25 to 56%. The total Sxgs-F2PrlR-transduced cell number increased about 150-fold under selective conditions, from 2×10^5 to 296×10^5. Simultaneously, the percentage of 7D5-positive cells rose from 14 to 92%. Although the selective expansion of mock-transduced BM cells was not as strong as that of Sxgs-NF2PrlR-transduced cells, the mock construct-driven proliferation was obviously too high. In addition, methylcellulose-based CFU assays indicated increased progenitor numbers in Sxgs-NF2-transduced mBM cells after 2 weeks *ex vivo* stimulation with AP20187+mSCF. The colony number rose from 16 to 104 per 1000 plated cells. These numbers were even higher than those of Sxgs-NF2PrlR-transduced mBM cells. Here the colony numbers increased from 19 to 82 per 1000 plated cells. Three independent experiments showed similar results. For unknown reasons, the ΔLNGFR element seems to contribute to the proliferation advantage of transduced mBM cells under stimulation with AP20187 and mSCF. Interestingly, this effect was never observed in Sxgs-NF2 transuced BaF/3 cells. ΔLNGFR was derived from the p75

neurotrophin receptor (p75NTR), which lacks the intracellular domain and juxtamembrane region of the LNGFR (Mavilio et al., 1994). The ΔLNGFR *per se* is broadly used as a marker gene for various gene therapy studies in haematopoietic cells (Junker et al., 2003; Sadat et al., 2003) and other tissues (Ohkubo et al., 2001). To date, there is no evidence from those studies that overexpression of ΔLNGFR plays any role in proliferation. However data obtained in our laboratory from dimerizer constructs containing different deletions of the human thrombopoietin receptor (hmpl) showed similar auto-selective proliferation effects in transduced primary BM cells in serum-containing medium over about 99 days (Stefan Stein, personal communication). This may be due dimerization of ΔLNGFR/FKBP12 monomers induced by one of the numerous serum factors utilised for primary BM cell cultivation. It has also been reported that in cells expressing tyrosine kinase receptors for different neurotrophin ligands (NTs), association of p75NTR creates a heterodimeric receptor complex with increased ligand affinity that is not dependent on the presence of the cytoplasmic residues (Lee et al., 2001). More recently in one report, it was shown that retroviral overexpression of modified LNGFR may increase the risk of leukaemia development in mice (Li et al., 2002). However, no solid evidence for an increased incidence of leukaemia was observed in a large population of animals, or in patients transplanted with ΔLNGFR-modified cells (Bonini et al., 2003). Apparently, the leukaemia observed by Li et al. may have been in part due to retroviral insertional up-regulation of the Evi-1 gene in haematopoietic cells, which has been implicated in the pathogenesis of human myelodysplastic syndrome and acute myeloid leukaemia (Baum et al., 2003). To minimise factors influencing our positive selection effect, derived from activation of the PrlR fusion construct, a new construct lacking the ΔLNGFR sequence was cloned and investigated. This was possible because 7D5 antibody was established to monitor $gp91^{phox}$ expression, thus allowing the expression of the therapeutic gene also to be used as marker gene.

Inducible and reversible selective proliferation effect achieved with the F2PrlR fusion protein

The inducible dimerizer system used in the present study is based on the immunophilin FKBP12 and the CID AP20187. Certain properties of this system make it suitable for selective expansion of genetically modified haematopoietic cells. First, FKBP12 is a small gene (180 bp cDNA), easily packaged into a retroviral vector together with the fusion signal domain and the therapeutic gene. Second, FKBP12 contains a cavity, engineered

by introducing a single phenylalanine-to-valine substitution at amino acid position 36 (F36V), thereby providing lock-and-key specificity for AP20187. Third, chemical modification of the CID AP20187 allows it to bind specifically to the adapted FKBP12. Together these modifications circumvent toxicity that might arise from binding to endogenous FKBP12. In addition, a fusion protein with 2 modified FKBP domains can enhance the binding activity of AP20187, thereby increasing the selective expansion effect of this system (Ariad **www.ariad.com**). For these reasons, the system was very attractive for the inducible selection system I was interested in.

Deletion of the extracellular ΔLNGFR sequence in the novel Sxgs-F2PrlR construct led to loss of the membrane anchor amino acids. The assumption that the fusion protein would be located in the cytoplasm was verified by intracellular staining with specific antibody against FKBP. Results of confocal laser scanning studies indicate that the fusion protein F2PrlR located mainly in the cytoplasm. A. Blau and colleagues developed similar constructs with a mono- or oligo-FKBP12 fused to the intracellular domain of mpl (Jin et al., 1998, 2000; Richard et al., 2003). This group showed that membrane localization is not required for the Mpl fusion protein (Otto et al., 2001). Our results demonstrate that the F2PrlR fusion protein localises mainly in the cytoplasm, in contrast to the wild type PrlR anchored in the plasma membrane.

Examination of this truncated PrlR dimerizer system was performed in transduced BaF/3 cells, the growth of which is dependent on mIL-3 stimulation. The data show that PrlR-construct-transduced BaF/3 cells proliferated very strongly in the presence of AP20187 without mIL-3. The cumulative cell number increased about 100–4000-fold during 2 weeks AP20187 stimulation. In contrast, mock-transduced BaF/3 cells died within 4–6 days. Also, the number of $gp91^{phox}$-positive, Sxgs-F2PrlR-transduced cells, measured by FACS analysis, increased about 2.5–14-fold under AP20187 treatment, whereas the percentage of $gp91^{phox}$-positive, mock-transduced cells decreased under the same conditions. Similar results have also been achieved with dimerizer systems containing signal domains derived from hmpl, G-CSFR and Flt-3 (Jin et al., 1998; Zeng et al., 2001). Importantly, the proliferation potential of expanded BaF/3 cells in the present study could be switched off after removal of AP20187. This is a necessary condition for the utilisation of the CID-mediated dimerizer system. The fusion protein should not undergo constitutive dimerization and must be strictly dependent on the presence of CID for spatial convenience and therefore activation (Neff and Blau, 2001).

The ex vivo-expanded murine HSCs maintained stem/progenitor cell characteristics with polyclonal proliferation potential

The CID-mediated expansion of primary haematopoietic cells has been well investigated using the mpl construct. Jin et al. (1998) reported that total mBM cells transduced with a vector encoding an FKBP12-mpl fusion construct failed to grow in the absence of dimerizer or growth factors, and died within –14 days. In contrast, the CID (FK1012) dramatically stimulated cell proliferation for up to nearly a year in culture. An initial brief phase of multilineage differentiation was followed by a longer phase of preferential megakaryocytic differentiation *ex vivo*. Instead of total BM cells, Sca1-positive mBM cells were employed in the present study for all *ex vivo* and *in vivo* experiments, because of the well established preparation method and high enrichment of murine early stem/progenitors. Our data showed that, after transduction and subsequent selection for 2 weeks, Sxgs-F2PrlR-transduced cells cultured in the presence of AP20187 and mSCF expanded 50–60-fold, and showed 5–10-fold higher numbers than cells cultured with the cytokine combination. Additionally, 70% of the AP20187+mSCF-stimulated cells expressed the transgene, compared with only 12.5% of cytokine-stimulated cell populations. Furthermore, stem/progenitor capacities of the Sxgs-F2PrlR-transduced, AP20187+mSCF-stimulated cells were analysed *ex vivo* by FACS using the stem cells markers Sca1 and c-kit. AP20187+mSCF-stimulated cells maintained high numbers of Sca1-positive cells (about 60–78%), whereas the numbers in cytokine-treated, Sxgs-F2PrlR- or mock-transduced cell populations decreased (<10%) after 2 weeks culture. The same results were observed using c-kit. Similar data were not provided in the study of Jin et al. described above. Further methylcellulose-based CFU assays showed significantly increased colony numbers from genetically modified cells after 2 weeks *ex vivo* CID stimulation, both in the study of Jin et al. and in the present approach. Thus, our data clearly demonstrate that Sxgs-F2PrlR-transduced cells maintained stem/progenitor cell capabilities after 2 weeks *ex vivo* selection with AP20187+mSCF. In addition, multilineage differentiation of AP20187+mSCF-stimulated cells was verified by FACS analysis of the lineage markers Gr-1 for myeloid cells, B220 for B-cells and CD3 for T-cells. The percentage of Gr-1$^+$, B220$^+$ and CD3$^+$ cells in gp91^{phox}-positive cell populations were 2.7, 24 and 1.2%, respectively. The percentages in cells cultivated with cytokines were significantly higher, 30, 70 and 17.8%, respectively. These results provided further evidence for a high proportion of lineage-negative stem and/or progenitor cells in AP20187+mSCF-stimulated BM cells. In this aspect, multilineage

differentiation in AP20187-stimulated cells is comparable to data from Jin et al. (2001). In their study, the CID stimulated the expansion of multiple cell lineages, including granulocytes and macrophages, during the first 2 weeks of culture. After 6 weeks of selection, the megakaryocytes emerged as the dominant phenotype. Long-term *ex vivo* culture of AP20187+mSCF stimulation was not investigated in the present study. The cells cultured *ex vivo* for 2 weeks were transplanted into mice.

Elevated expression levels of Prl and its receptor have been observed in lymphohaematopoietic cells, granulocytes, macrophages and stroma cells, which support haematopoiesis (Welniak et al., 2001). Clevenger et al. (1998) reported that the PrlR plays an important role in the immune system by stimulating both cell proliferation and survival. This was verified by determining the activity of PrlR-induced signal transduction pathways. Among them, STAT5, Src family kinase and c-myc were found to be activated in T-, B-, mono- and polymorphonuclear cells (Dominguez-Caceres et al., 2004; Dogusan et al., 2001, 2003). However, no clear evidence was found that the PrlR plays a direct role in stem cell proliferation and maintenance. The present study demonstrates for the first time that activation of a PrlR construct is able to stimulate murine haematopoietic stem and/or progenitor cell proliferation *ex vivo*. A similar effect was observed for the mpl dimerizer system (Jin et al., 1998; Richard et al., 2003) and the steroid-based selective proliferation system (Kume et al., 2003).

For the matter of safety, polyclonality is an important condition for the administration of selected HSCs to patients. LM-PCR represents a well-established method to determine the position of provirus integration after gene delivery. In the present study, integration-site analysis by LM-PCR indicated that the AP20187 response was followed by proliferation and selection of various provirus-containing transduced mBM cell clones. Subsequently, through the analysis of the DNA fragments using Mouse Genome BLAST, the integration positions of the 13 colonies investigated were determined. Proviral sequences of the retroviral vector were found to be integrated into 8 different chromosomes at diverse positions in various genes. This clearly ruled out a monoclonal, eventually oncogenic, expansion of PrlR-construct-transduced cells. Recently, Du et al. (2005) reported that defective oncogenic retroviruses induce leukaemia in part through insertional mutagenesis of cooperating cancer genes. A few genes, relevant to the development of mouse leukaemia, have been identified (Du et al., 2005). None of the eight genes identified in the present study have been found to play a role in leukaemia development compared with the identified genes in Du's study. To answer the

question of whether integration of the retroviral vectors activated genes with oncogenic potential, a higher number of samples should be examined.

AP20187 induced selective expansion of haematopoietic cells by activation of the truncated PrlR construct and subsequently STAT5 phosphorylation

Normally, growth factor receptor-induced cell signal transduction depends on membrane anchored receptor proteins. Ligand binding to the extracellular domain of the receptor activates intracellular signal cascades. Prolactin activates three important pathways termed the JAK/STAT, the Ras/Raf/MAPK and the Fyn/Sos/Vav pathways (Clevenger et al., 1998). The present study demonstrated that the PrlR construct is mainly localised to the cytoplasm. In order to test whether AP20187-induced activation of the cytoplasmic PrlR construct leads to activation of the same signal transduction pathways as wild type membrane anchored PrlR, I investigated the JAK/STAT pathway, particularly the activation status of STAT5. This was because the JAK/STAT pathway, especially STAT5, has fundamental roles in apoptosis, differentiation and proliferation of haematopoietic cells. The results showed that enhanced phosphorylation of STAT5 can be detected in the cell lysates of PrlR-construct transduced BaF/3 cells under AP20187 stimulation. In addition, enhanced binding activity of STAT5 to the promoter of its target gene ß-casein could be clearly verified by EMSA assay in AP20187-stimulated cell populations. Most likely, AP20187-induced PrlR-construct-signalling activates the same signal transduction pathways as the natural ligand Prl in haematopoietic cells. However, a weak signal for STAT5 phosphorylation and STAT5-DNA binding activity were obtained from starved transduced cells without AP20187 stimulation. It is possible that the effect of AP20187 could not be completely turned off due to the short starving period of 28 hours after a long stimulation period of 2 weeks. For various reasons, such as the half-life of AP20187, ending PrlR-construct activity takes some time, but the cells finally stopped proliferating about 2 weeks after removal of AP20187. Over the past few years, the STAT5 transcription factor has been recognised as a key transcription factor for proliferation and survival of haematopoietic cells. Most recently, Kato et al. (2005) reported that activated forms of STAT5 led to a dramatic expansion of multipotential progenitors and promoted HSC self-renewal *ex vivo*; however, they induced a foetal myeloproliferative disease *in vivo*. Proleukaemia effects of activated STAT5 have also been reported by other groups (Mulloy et al., 2005; Morrigl et al., 2005). In our system, the advantageous effect of STAT5 activation in expanding HSCs can be switched on by AP20187 treatment, while long-

term, leukaemia-promoting effects can avoid by removing AP20187, resulting in inactivation of PrlR-construct-driven STAT5 activity. The other signal transduction pathways were not investigated in the present study. It will be helpful to assess the phosphorylation status of MAPK, MAPKK and other transcription factors (such as c-myc, SOS and Ras/Raf), which may also influence PrlR-construct-induced cell activation. Activation of the PrlR construct may exert its effect through activation of STAT5. Experiments in STAT5-deficient murine HSCs could clarify a key role of STAT5 in PrlR-construct-induced proliferation.

PrlR-construct-expanded HSCs can repopulate myeloablated recipient mice

Sxgs-F2PrlR-transduced mBM cells maintained stem and/or progenitor cell characteristics during 2 weeks stimulation with AP20187+mSCF *ex vivo*. To answer the question of whether these expanded cells are still able to contribute to repopulation of lethally irradiated recipient mice, AP20187-selected and unselected cells were injected directly into such mice. The donor and recipient strains are phenotypically distinguishable on the basis of allelic differences at the Ly5 locus. Donor mice are Ly5.1 homozygous, whereas recipient mice are Ly5.2 homozygous. All the recipients that were transplanted with cells one day after transduction (i.e. 6 days in culture) survived until analysis. In contrast, all recipient mice that were injected with cells cultured for a further 2 weeks with cytokines died 12–14 days post-transplantation. On the other hand, all mice transplanted with mBM cells stimulated for 2 weeks with AP20187 survived until analysis. Clearly, there were enough progenitor cells in selected cell populations for rescue of myeloablated mice, whereas non-selected cells lost their stem/progenitor cell ability after 2 weeks cultivation with cytokines. The repopulation efficiency (Ly5.1-positive cells) of 9 analysed transplanted mice ranged between 5.2 and 80.5% in PB cells, 1 and 51.5% in BM cells, and 5.5 and 79.4% in spleen cells. Nevertheless, transgene-expressing cells were detected at very low level (<5%) in all organs. These results were quite unexpected, since 30% of the AP20187-stimulated mBM cells were 7D5 - and Sca1-double positive before transplantation. Therefore, theoretically at 50% engraftment there should be 15% 7D5-positive cells in the peripheral blood of recipient mice. The reasons for these results are not clear. One explanation could be that the number of early stem cells in AP20187-selected cells was much lower than 30%. FACS analysis of transgene expression from the inducible hmpl-dimerizer system showed over 15% GFP^+ cells in mice 3 months post-transplantation (Jin et al. 1998, 2001; Richard et al 2003). However, in this system, the cell growth switch was induced by a fusion protein containing the marker gene GFP. In the

present study, the marker gene and selection cassette were expressed from a bicistronic vector, with unequal expression levels. Constructing a gp91^{phox} fusion protein including a selection cassette did not solve this problem. The gp91^{phox} selection marker fusion protein was not expressed properly. In contrast to our approach, in the study of Jin et al., AP20187 treatment was performed *in vivo*. Another possibility could be due to limitations of the analysis method. Human gp91^{phox} expression in our recipient mice was measured with the specific 7D5 antibody. This antibody allows good detection of human gp91^{phox} by FACS analysis in a few murine cell lines, such as the mouse fibroblast cell line SC1, but not in others, such as NIH3T3 cells. The reasons for this difference are not clear. gp91^{phox} expression can also be determined in transduced primary BM cells *ex vivo.* The sensitivity of this method for samples collected from PB, BM and spleen is not known. Practically, handling of the samples e.g. lysing the red cells with NH_4Cl, may also have an influence on the sensitivity of the 7D5 antibody. Determination of gp91^{phox} protein expression in cell lysates from different organs by western blotting with anti-gp91^{phox} antibody could be useful. Another reason for low numbers of gp91^{phox}-expressing cells may be gene silencing *in vivo*. Gene silencing is a limitation for retroviral vectors, arising as a consequence of random integration into the genome. Transgene expression is influenced by chromosomal sequences adjacent to the integration site and is inversely correlated with CpG methylation. Vector integration into heterochromatin may induce inhibition of transcription (position-dependent silencing) or may result in varying expression in progeny cells (position-effect variegation) (Karpen, 1994). It is commonly agreed that gene silencing tends to occur in undifferentiated cells like HSCs that go through a long differentiation process (Baum et al., 2003). In order to clarify whether the gene-modified HSCs contributed to the repopulation of recipients, PCR analysis with primers pairs specific for gp91^{phox} was carried out with genomic DNA from BM, spleen and PB cells. The results showed a strong gp91^{phox} signal in all analysed tissues, with one exception in BM cells from mouse No.4. Therefore, gene-modified HSCs did contribute to the repopulation of irradiated recipient mice. Furthermore, BM cells from 3 recipient mice could be re-stimulated with AP20187+mSCF *ex vivo*. In two analysed mice, stimulated cells showed stronger signals with gp91^{phox} or F2PrlR fusion protein primers than the non-stimulated cells in PCR analysis, and also higher colony numbers in the CFU assay. In contrast, samples from stimulated and non-stimulated cells from one mouse showed equal signals in PCR analysis and the same colony numbers in the CFU assay. Gene silencing may possibly have occurred in this mouse. It would be very interesting to determine whether this

increase in gp91^{phox} expressing cells is due to a reselection of few gp91^{phox} expressing cells or to a possible inactivation of the silencing process by the restimulation with the dimerizer.

Transplantation of *ex vivo*-stimulated mBM cells into recipient mice demonstrated that the selectively expanded BM cells contained sufficient stem/progenitor cells to repopulate irradiated recipients. However, no clear increase in early HSC numbers after AP20187 stimulation was seen. Most importantly, the number of early HSCs should be estimated with CRU assays. As described in the introduction, the CRU assay is considered to be the best available method for analysing early murine HSCs. To compare HSC numbers in Sxgs-F2PrlR-transduced cells before and after selection, CRU assays should be carried out one day after transduction and 2 weeks after selection with AP20187+mSCF. We expect an increased number of CRUs in AP20187-stimulated cells. The CFU-S assay could then be used to determine the number of myeloid progenitor cells.

5.4 Perspectives

In the development of gene transfer approaches targeting HSCs, methods allowing selective expansion of genetically modified cells could improve their performance in clinical trials. As seen in this and various other studies, the HOXB4 transcription factor acts as an important positive regulator of HSC self-renewal. However, high-level of HOXB4 overexpression may have a negative effect on the output of mature blood cells by preventing or perturbing differentiation. Recently, new findings suggest that HOXB4 *per se*, when delivered directly as an oligopeptide, has the ability to expand murine and human HSCs, and to retain their *in vivo* potential for differentiation and long-term repopulation (Krosl et al., 2003; Amsellem et al., 2003). These findings boost the potential clinical application of HOXB4 oligopeptides for expansion of HSCs. gp91^{phox}-transduced cells could be isolated by FACS cell sorting and stem cells could then be expanded *ex vivo* by treatment with HOXB4 oligopeptides before re-infusion into X-CGD patients. This strategy would allow removal of the HOXB4 selection molecule from the retroviral transduction vector, thus limiting toxicity issues and perturbation of differentiation.

Recently, the Poly comb group (PcG) gene Bmi-1 has been implicated in the maintenance of haematopoietic and leukemic stem cells (Ohta et al., 2002; Park et al., 2003, Iwama et al., 2004). A progressive postnatal decrease in the number of early HSCs has been observed in Bmi-1 knockout mice (Park et al., 2003, Iwama et al., 2004). Retroviral overexpression of Bmi-1 in murine HSCs clearly mediated cell proliferation, which was largely restricted to the primitive haematopoietic cells. A net 56- to 80-fold CFU-GMEM

expansion and 15- to 35-fold higher repopulation activity were obtained in the Bmi-1 transduced cultures (Iwana et al., 2004). Therefore, Bmi-1 could be used as a novel molecular target for the selective expansion of gp91^{phox} gene transduced HSCs.

In the presented experiments, the signalling domain of the PrlR was employed for the first time as a cell growth switch contributing to the selective expansion of genetically modified HSCs. The findings in this study demonstrate the feasibility of conditionally enlarging the gp91^{phox} gene-modified progenitor cell population *ex vivo*, and, as such, provide a new strategy for gene and cell therapy of stem cells. This approach may circumvent some of the major difficulties of stem cell gene therapy, among which is the extremely low efficiency of gene transfer into low cycling early stem cells. Whether the *ex vivo* selection induced by the signalling domain from the PrlR led to high numbers of early stem cells remains to be determined. AP20187 administration for *in vivo* selection appears to be the next step for investigation. *In vivo* administration of AP20187 has been established using selection vectors based on the hmpl cell-growth switch, for both mice and large animals (Jin et al., 2000; Richard et al., 2002), and a JAK2 cell-growth switch in mice (Zhao et al., 2004). The hmpl construct demonstrated a clear selective proliferation advantage in dimerizer construct-transduced cells throughout a 7-day course of AP20187 treatment (Jin et al., 2000), albeit an apparent preference for differentiation along the megakaryocytic lineage was observed. Since gene therapy of x-CGD patients would require a proliferation advantage in the macrophage lineage, a therapeutic approach using the selective cassette with the hmpl construct is not suitable. The biological responses to AP20187 treatment *in vi*vo could be kept within a desired range by titrating the dose. Trangene-expressing cells in different organs could be easily detected by FACS analysis. Further experiments should investigate the possibility of *in vivo* positive selection of gene-modified stem cells to the desired range. AP20187 lacks apparent toxicity and, therefore, could potentially be used repeatedly over time. *In vivo* administration could avoid some of the unexpected effects of *ex vivo* culturing, such as culture conditions, quality of the cytokines and microorganism contamination.

6. Summary

Selective Expansion of Gp91^{phox} Gene-Modified Murine Haematopoietic Stem Cells

Haematopoietic stem cells (HSCs) are regarded as the prime target for gene therapy of inherited and acquired disorders of the blood system, e.g. X-linked chronic granulomatous disease (X-CGD). The major reason for this is that HSCs posses the ability to self renew as well as the potential to differentiate into all lineage-specific cell types. However, the need to reach and to maintain sufficient therapeutic levels of genetically modified stem cells and their progeny after gene delivery still presents major challenges for current HSC gene therapy approaches. In particular, one of the main limitations for most genetic defects is the lack of a selective growth advantage of gene-modified cells after engraftment. *In vitro* and *in vivo* methods have been developed that focus on either positive or negative selection of HSCs. An artificial selection advantage can be conferred to transduced HSCs by incorporating a selection marker in addition to the therapeutic transgene. In the present study, two novel strategies for positive selection of murine gp91^{phox} gene-modified haematopoietic stem cells were developed and tested, bearing in mind that with selective growth advantage, the possibility of uncontrolled proliferation arises.

The first strategy to be investigated was based on the homeobox transcription factor HOXB4, which plays an important role in the control of haematopoietic stem cell proliferation and differentiation. Overexpression of a retroviral bicistronic construct containing the therapeutic gene gp91^{phox} and HOXB4 in murine primary bone marrow cells led to a significant 3–4-fold expansion of transduced cells *ex vivo*. The numbers of transgene-expressing cells increased 2–3-fold after 2 weeks cultivation under cytokine stimulation. Furthermore, the clonogenic progenitor cell assay (CFU assay) demonstrated that the number of colony-forming cells had increased to levels 2-fold higher than those of mock-transduced cells after 1 week of culture, thereby augmenting the presence of a significant number of stem/progenitor cells in the selected cell population. However, in our experiments, HOXB4-overexpressing murine HSCs did not show any repopulating advantage in transplanted recipient mice over control construct-transduced HSCs. These results indicate that selective expansion of gp91^{phox} gene-modified HSCs can be induced by the HOXB4 transcription factor *ex vivo* but not *in vivo*. This is possibly dependent on HOXB4 expression levels, which are too low *in vivo* to achieve selection.

The second strategy made use of a chemically inducible dimerizer system consisting of the therapeutic gene $gp91^{phox}$ and a fusion protein, containing sequences from a growth factor receptor signalling domain (epidermal growth factor receptor, EGFR, or prolactin receptor, PrlR) and the drug binding protein FKBP12, as the selection cassette. This strategy aimed to allow inducible selection that could be easily switched off. The activity of these fusion proteins is controlled through the small molecular dimerizer AP20187. Transduction of BaF/3 cells with lentiviral vectors expressing the EGFR construct induced proliferation and led to complete selection within 18 days (99%). However, removing AP20187 could not turn off proliferation. This construct is, therefore, not suitable as a selection cassette for the expansion of gene-modified HSCs due to its oncogenic potential. Transduction of the construct containing the intracellular domain of PrlR caused significant selective expansion of AP20187-treated BaF/3 cells. Following expression in cells, the fusion protein, which lacks membrane-anchoring sequences, mainly localized to the cytoplasm. Evidence was found to indicate that activated STAT5 might be responsible for this effect. Upon expression of the prolactin construct, phosphorylation of STAT5 and its DNA-binding activity to a ß-casein promoter sequence was strongly increased. Importantly, the induced proliferation was reversible after removal of AP20187. Transduced $Sca1^{+}$ bone marrow cells obtained from C57BL/6-CD45.1 mice could be expanded about 20–100-fold *ex vivo* in the presence of AP20187 and mSCF without losing progenitor cell features and the capability to contribute to all lineages of the haematopoietic system. To exclude oncogenic outgrowth of one single clone, the polyclonality of selected cells was proven by ligation-mediated PCR (LM-PCR) analysis. In mouse transplantation experiments, *ex vivo*-expanded cells repopulated the bone marrow of lethally irradiated mice suggesting that the *ex vivo* expansion took place at the level of haematopoietic stem and/or progenitor cells. Genomic $gp91^{phox}$ sequences were detected in the bone marrow, spleen and peripheral blood cells of transplanted animals, indicating that $gp91^{phox}$-containing cells most likely contributed to the reconstitution of haematopoiesis in these mice.

7. Selektive Expansion von gp91^{phox} Gen-modifizierten murinen hämatopoetischen Stammzellen

Stammzellen stellen die entscheidenden Zielzellen bei Gentherapie-Strategien zur Behandlung von erworbenen und angeborenen Krankheiten des Blutsystems, wie zum Beispiel der X-chromosomal vererbten chronischen Granulomatose, dar. Diese Stammzellen können später in alle verschiedenen Blutzellreihen differenzieren und gleichzeitig den Stammzellpool erhalten (Selbsterneuerung). Allerdings bereitet es immer noch große Probleme, eine genügend große Anzahl dieser Stammzellen genetisch zu modifizieren. Dies stellt eines der Hauptprobleme aktueller Gentherapie Studien dar. Des weiteren bedeutet der Gentransfer vieler therapeutischer Gene für die Zelle kein Vorteil in Bezug auf das Zellwachstum, nachdem sich die gen-modifizierten und transplantierten Zellen im Körper angesiedelt haben. Ein zusätzliches Selektions-Molekül könnte der Zelle einen Proliferationsvorteil verschaffen und so zur Expansion der wenigen gen-modifizierten Zellen führen. In der vorliegenden Arbeit wurden für eine selektive Expansion von genetisch modifizierten murinen hämatopoetischen Stammzellen zwei neue Selektionssysteme entwickelt.

In der ersten Strategie wurde die Funktion des intrinsischen Transkriptionsfaktors HOXB4 genutzt, der eine zentrale Rolle in Zellvermehrung und Differenzierung von hämatopoetischen Stammzellen spielt. Zunächst wurde ein bicistronisches gammaretrovirales Vektorkonstrukt, bestehend aus dem therapeutischen Gen gp91^{phox} und HOXB4, kloniert. Wachstumskurven haben gezeigt, dass die Überexpression dieses retroviralen Vektors in murinen hämatopoetischen Stammzellen *ex vivo* in einem signifikanten 3 bis 4-fachen Zellzahl-Anstieg resultierte. Die FACS-Analyse dieser Zellen hat ergeben, dass, nach zwei Wochen unter Stimulation mit den Stammzellzytokinen IL-6, IL-3 und SCF, die Anzahl der gp91^{phox} Transgen exprimierenden Zellen ebenfalls 2 bis 3-fach anstieg. Des weiteren zeigt der auf Methylcellulose basierende *Colony forming units* (CFU) - Assay, dass die Anzahl der koloniebildenden Zellen nach einer Woche auf das zweifache anstieg, verglichen mit Kontroll-Vektor transduzierten Zellen. Dies belegt eine signifikante Anzahl an Stamm- und/oder Vorläuferzellen in der selektierten Zellpopulation. Allerdings haben diese selektionierten Zellen nicht die Fähigkeit, in bestrahlte, myeloablatierte Empfängermäuse transplantiert das hämatopoetische System wieder aufzubauen. Der prozentuale Anteil der

gp91^{phox} exprimierenden Zellen im Knochenmark dieser Mäuse betrug 8 bis 10 Wochen nach Transplantation deutlich weniger als 5%. Diese Ergebnisse belegen, dass gp91^{phox} Gen-modifizierte hämatopoetische Stammzellen zwar *ex vivo* mittels Überexpression von HOXB4 selektioniert und vermehrt werden können, *in vivo* aber keinen Vorteil gegenüber nicht modifizierten Zellen besitzen. Möglicherweise beruht diese Diskrepanz auf einem zu niedrigen Expressionslevel von HOXB4 in dem murinen *in vivo* - Modell.

Die zweite Strategie beruht auf einem induzierbaren Dimerisierungs-System. Dazu wurden zunächst lentivirale Fusionskonstrukte, bestehend aus FKBP12 und einer Signaldomäne entweder des EGF-Rezeptors oder des Prolaktin-Rezeptors, kloniert. FKBP12, eine modifizierte Form des für das Immunsystem wichtigen FK506-binding Proteins, fungiert hier als induzierbare, durch Zugabe des kleinen Dimerisierungsmoleküls AP20187, anschaltbare Dimerisierungsdomäne. Dimerisierung von zwei Fusionprotein-Monomeren sollte die jeweilige Signaltransduktionskaskade anschalten. Die chemische Substanz AP20187 kann sowohl in Zellkultur als auch in *in vivo* Mausmodell-Experimenten ohne toxische Nebenwirkungen verwendet werden. Durch dieses System sollte die selektionierende Wirkung der Signaldomäne kontrollierbar an- und abschaltbar gemacht werden. Die Aktivität des Fusionsproteins ist also durch Zugabe und Wegnahme des chemischen Moleküls AP20187 steuerbar. Lentivirale Vektoren, die das EGFR-Konstrukt exprimierten, induzierten in BaF/3 Zellen deutlich gesteigertes Zellwachstum und führten zu einer kompletten Selektion gen-modifizierter Zellen innerhalb von 18 Tagen von 20% transduzierter auf 99% gp91^{phox} tragender Zellen. Diese induzierte Proliferation ließ sich jedoch nach Wegnahme des Moleküls AP20187 nicht abschalten. Auch nach 2 Wochen AP20187 Entzug teilen sich die Zellen mit fast ungeminderter Schnelligkeit weiter. Aufgrund dieser unkontrollierbaren und möglicherweise onkogenen Eigenschaften schien uns dieses Selektionsmodell als nicht anwendbar. Das weitere Konstrukt, welches anstelle der EGF-Rezeptor-Signaldomäne die komplette intrazelluläre Phosphotyrosinkinase-Domäne des Prolaktin-Rezeptors enthält, verursachte nach AP20187 Stimulation ebenfalls eine deutliche Proliferationssteigerung und eine Selektion von 19% zu 96% gp91^{phox} positiver BaF/3 Zellen. Dieser Proliferationsvorteil konnte durch Entnahme des AP20187 wieder ausgeschaltet werden. Bereits 2 Tage nach Entzug der AP20187 Stimulation nimmt die Zellzahl deutlich ab. Nach diesem Test in BaF/3 Zellen wurde ein neuer bicistronischer retroviraler Vektor kloniert, bestehend aus dem therapeutische Gen gp91^{phox} und dem selektionsvermittelndem Prolaktinrezeptor-Fusionskonstrukt (PrlR-Konstrukt). Nach Überexpression des Konstruktes in transduzierten

murinen SC1 Zellen haben konfokale Lasermikroskopiestudien gezeigt, dass dieses PrlR-Fusionsprotein, welches keine membranverankernden Sequenzen des Prolaktinrezeptors mehr besitzt, hauptsächlich im Zytoplasma der Zelle lokalisiert ist. Von den wesentlichen Prolaktin-aktivierten Signalwegen, dem Jak/Stat-Signalweg, dem Ras/Raf/MAP-Kinase-Signalweg und dem Fyn/Sos/Vav-Signalweg, wurde der Jak/Stat-Signalweg untersucht, da er eine zentrale Rolle in der Hämatopoese spielt. Möglicherweise verursacht das aktivierte PrlR-Konstrukt gesteigerte Proliferation und Stammzellselektion durch Aktivierung des STAT5-Signalweges. Es wurde bereits in STAT5 Knockout Maus-Modellen gezeigt, dass STAT5 eine wichtige Rolle für den Erhalt der hämatopoetische Stammzellen spielt. Die vorgelegten Ergebnisse zeigen, dass nach Expression des PrlR-Konstruktes und Stimulation mit AP20187 ein gesteigerter STAT5-Phosphorylierungsstatus und eine vermehrte Bindung von STAT5 an Promotorsequenzen des STAT5 Zielgens ß-Casein in BaF/3 Zellen resultieren. AP20187 induzierte Aktivität des PrlR-Konstruktes vermag auch gänzlich ohne IL-3 Stimulation STAT5 in vergleichbarer Stärke zu phophorylieren. In Kontroll-Vektor transduzierten Zellen waren hingegen kein Signal detektierbar. In dieser Zelllinie vermag die Expression des PrlR-Konstruktes nach zwei Wochen AP20187 Stimulation die Proliferation um den Faktor 1.500 bis 27.000 zu steigern. Simultan wurde in FACS- Analysen eine Zunahme der $gp91^{phox}$ Expression von 3% auf 70% beobachtet. Dieser proliferationssteigernde Effekt ist, wie auch zuvor für das lentivirale Konstrukt beobachtet, nach Wegnahme des AP20187 reversibel, was eine wichtige Eigenschaft im Hinblick auf weitere Anwendungen, möglicherweise in humanen Studien, darstellt. Auf Proteinebene kann in den transduzierten BaF/3 Zellen nach Selektion mit AP20187 ein deutlich stärkeres Signal für das PrlR-Konstruktes nachgewiesen werden. Auch gen-modifizierte murine Stammzellen können unter AP20187 Stimulation *ex vivo* um den Faktor 20 bis 100 expandiert werden, ohne ihre Stammzelleigenschaften und die Fähigkeit zu allen Blutzellen der verschiedenen Reihen beizutragen, zu verlieren. In der FACS-Analyse wurde deutlich beobachtet, dass PrlR-Konstrukt modifizierte Stammzellen unter AP20187 Stimulation wichtige Stammzell-Oberflächenmarker wie Sca1 und c-kit über einen Zeitraum von 2 Wochen in hohen Levels exprimieren. Während in der Abwesenheit von AP20187 die Stammzellen in die Differenzierung eintreten, behalten AP20187 stimulierte Zellen den Stammzellphänotyp bei. Hier nimmt die Zahl der Sca1- und c-kit-positiven Zellen von 20% auf 70% und von 12% auf 40% zu. In Methylcellulose basierten Assays (CFU-Assays) können diese Stammzellen, ähnlich wie im Knochenmark, Kolonien von Vorläuferzellen aller Blutzellreihen bilden. Die selektionierten murinen Zellen wurden auf die Fähigkeit, Kolonien zu bilden, hin untersucht. Nach 14-tägiger Inkubation bildeten

AP20187 stimulierte PrlR-Konstrukt gen-modifizierte primäre Mause-Knochenmarkzellen deutlich mehr Kolonien aus. Während Kontrollzellen ohne AP20187 Stimulation lediglich 15 Kolonien pro 1.000 ausplattierten Zellen bilden, liegt die Zahl unter AP20187 Stimulation bei 75 Kolonien. In weiterführenden FACS-Analysen dieser Zellen wird ersichtlich, dass PrlR-Konstrukt transduzierte Zellen unter AP20187 Stimulation dennoch zu einem kleinen Ausmaß in die verschiedenen Blutzell-Richtungen differenzieren können. Sie sind zu einem geringen Teil positiv für die Oberflächenmarker Gr-1 (myeloische Zellreihe) mit 2,7%, B220 (B-Zellen) mit 24% und CD3 (T-Zellen) mit 1,2%. Allerdings ist die Oberflächenmarker-Expression deutlich schwächer als in PrlR-Konstrukt-transduzierten Zellen unter alleiniger Zytokin-Stimulation ohne AP20187. Der prozentuale Anteil der Gr-1, B220 und CD3 positiven Zellen beträgt hier 30%, 70% und 17,8%. Vermutlich veranlassen die Expression des PrlR-Konstruktes und die gleichzeitige Aktivierung durch AP20187 die Stammzelle zur ständigen Selbsterneuerung. Simultan wird dabei die Differenzierung hin zu den verschiedenen Vorläuferzellen unterdrückt. Dies würde erklären, warum es zu einer Anreicherung der Stammzellpopulation kommt. Um ferner eine mögliche onkogene Eigenschaft der PrlR-Konstrukt-Aktivierung auszuschließen, wurden die zweiwöchig selektionierten Mause-Knochenmark-Zellen auf ihre Klonalität mit der Ligation-vermittelnde PCR (LM-PCR)-Methode hin untersucht. Es kann klar gezeigt werden, dass es innerhalb der selektionierten Zellen zahlreiche hervorgegangene Klone gibt und die retrovirale Vektorsequenz auf unterschiedlichen Chromosomen in verschiedenen Gen-Abschnitten integriert wurde. Diese polyklonale Situation spricht gegen eine mögliche onkogene Potenz des PrlR-Fusionsproteins.

In anschließend durchgeführten Maus-Transplantationsexperimenten können *ex vivo* expandierte Knochenmarkzellen das Knochenmark von tödlich bestrahlten Mäusen wieder herstellen. Während die Kontroll-Mäuse, die mit unselektionierte Zellen transplantiert wurden, 12-14 Tage nach der Transplantation gestorben sind, haben Mäuse, die mit AP20187 stimulierten Zellen transplantiert wurden, bis zum Abschluss der Versuche (8-10 Wochen nach Transplantation) überlebt. Dieses Ergebnis zeigt, dass es eine ausreichende Anzahl an frühen Stammzellen in der AP20187 stimulierten Zellenpopulation gab, um das Blutsystem der tödlich bestrahlten, myeloablatierten Empfängermäuse wiederherzustellen. Das Engraftment der Spenderzellen lag, gemessen in peripheren Blutzellen bei 5% bis 80%, bei 1% bis 50% in Knochenmarkzellen und bei 5,5% bis 80% in Milz-Zellen. Allerdings war die transgene Experessionsrate, gemessen als $gp91^{phox}$ Expression in der FACS-Analyse, im Bereich von 5%, in allen Mäusen sehr niedrig, sogar in Mäusen mit hohem Entgraftment.

Möglicherweise stellt ein großer Anteil der *ex vivo* selektionierten Zellen zwar Sca1 positive Vorläuferzellen, aber keine frühen, pluripotenten Stammzellen, dar und geht durch Differenzierung verloren. Die Tatsachen, dass diese Mäuse überlebt haben und Gen-modifizierte Zellen in Knochenmark, Milz wie auch peripherem Blut gefunden wurden, legt nahe, dass die *ex vivo* Selektion auch auf früher Stammzellebene stattgefunden haben muss. In genomischer DNA aus Zellen von Knochenmark, Milz und peripheren Blut der transplantierten Mäuse konnten mittels PCR Sequenzen von $gp91^{phox}$ nachgewiesen werden. Somit tragen die gen-modifizierten murinen Stammzellen zur Rekonstitution der Hämatopoese bei. Weiterhin wurden von 5 Mäusen, transplantiert mit PrlR-Konstrukt transduzierten und *ex vivo* AP20187 selektioneierten Stammzellen, 10 Wochen nach Transplantation Knochenmarkzellen entnommen und wieder *ex vivo* unter AP20187 Stimulation kultiviert. Knochenmarkzellen von 3 der 5 Mäuse konnten *ex vivo* wieder unter AP20187 Stimulation deutlich vermehrt und selektioniert werden. Der prozentuale Anteil der $gp91^{phox}$ exprimierenden Zellen stieg von anfangs weniger 5% auf über 70%. In Gegensatz dazu haben die nur mit Zytokinen kultivierten Knochenmarkzellen nach zwei Wochen Kultivierung lediglich einen Anteil von 10% $gp91^{phox}$ exprimierender Zellen. Diese *ex vivo* unter AP20187 wieder re-stimulierten versus unstimulierten Knochenmark-Zellen wurden ebenfalls in Methylzellulose-Assays auf Koloniebildung hin untersucht. AP20187 stimulierte Zellen von 2 Mäusen zeigten signifikant höhere Kolonie-Anzahlen als die der unselektionierten Zellen. Die PCR-Analysen mit Primer-Paaren sowohl für das $gp91^{phox}$ Transgen als auch für das PrlR-Selektionsmolekühl zeigten, dass 100% der Kolonien aus AP20187 stimulierten Zellen für beide Gene starke Signale aufweisen. Dagegen ist dies bei lediglich 10% der Kolonien aus unselektionierten Zellen nachgewiesen worden.

Zusammengefasst konnte gezeigt werden, dass selektive Expansion von murinen hämatopoetischen Stammzellen mit Hilfe von verschiedenen aktivierten Signalmolekülen *ex vivo* erreicht werden kann. Als besonders vielversprechend hat sich das in seiner Aktivität regulierbare PrlR-Konstrukt erwiesen. Zum einen kann es nach erfolgter positiver Selektion abgeschalten werden, zum anderen können die Gen-modifizierten Stammzellen zur Rekonstitution der kompletten Hämatopoese beitragen. Weitere Versuche und Verbesserungen können die Sicherheit und Effizienz dieses System steigern. Insgesamt bekräftigen die gezeigten Ergebnisse die Strategie, Gen-modifizierte Zellen zusätzlich zum therapeutischen Gen mit einem Selektionsmolekül für eine *ex vivo* und/oder *in vivo* Selektion auszustatten.

8. References

Abkowitz JL, Catlin SN, Guttorp P. Evidence that hematopoiesis may be a stochastic process in vivo. Nat Med. 1996;2:190-197

Ali S, Pellegrini I, Kelly PA. A prolactin-dependent immune cell line (Nb2) expresses a mutant form of prolactin receptor. J Biol Chem. 1991;266:20110-20117

Allay JA, Persons DA, Galipeau J, Riberdy JM, Ashmun RA, Blakley RL, Sorrentino BP. In vivo selection of retrovirally transduced hematopoietic stem cells. Nat Med. 1998; 4:1136-1143

Amsellem S, Pflumio F, Bardinet D, Izac B, Charneau P, Romeo PH, Dubart-Kupperschmitt A, Fichelson S. Ex vivo expansion of human hematopoietic stem cells by direct delivery of the HOXB4 homeoprotein. Nat Med. 2003;9:1423-1427

Anderson WF. Human gene therapy. Nature. 1998;392:25-30

Antonchuk J, Sauvageau G, Humphries RK. HOXB4 overexpression mediates very rapid stem cell regeneration and competitive hematopoietic repopulation. Exp Hematol. 2001;29:1125-1134

Antonchuk J, Sauvageau G, Humphries RK. HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. Cell. 2002;109:39-45

Ash RC, Detrick DA, Zanjani ED. Studies of human pluripotential hemopoietic stem cells (CFU-GEMM) in vitro. Blood. 1981;58:309-316

Babior BM. NADPH oxidase: an update. Blood. 1999;93:1464-1476

Babior BM, Kipnes RS, Curnutte JT. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. J Clin Invest. 1973;52:741-744

Bailey AR, Burchett KR. Effect of low-dose dopamine on serum concentrations of prolactin in critically ill patients. Br J Anaesth. 1997;78:97-99

Baum C, Dullmann J, Li Z, Fehse B, Meyer J, Williams DA, von Kalle C. Side effects of retroviral gene transfer into hematopoietic stem cells. Blood. 2003;101:2099-2114

Baum C, Hegewisch-Becker S, Eckert HG, Stocking C, Ostertag W. Novel retroviral vectors for efficient expression of the multidrug resistance (mdr-1) gene in early hematopoietic cells. J Virol. 1995;69:7541-7547

Bazan JF. Structural design and molecular evolution of a cytokine receptor superfamily. Proc Natl Acad Sci U S A. 1990;87:6934-6938

Becker AJ, Mc CE, Till JE. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature. 1963;197:452-454

Becker S, Wasser S, Hauses M, Hossle JP, Ott MG, Dinauer MC, Ganser A, Hoelzer D, Seger R, Grez M. Correction of respiratory burst activity in X-linked chronic granulomatous cells to therapeutically relevant levels after gene transfer into bone marrow CD34+ cells. Hum Gene Ther. 1998;9:1561-1570

Bellone G, Geuna M, Carbone A, Silvestri S, Foa R, Emanuelli G, Matera L. Regulatory action of prolactin on the in vitro growth of CD34+ve human hemopoietic progenitor cells. J Cell Physiol. 1995;163:221-231

Benito AI, Diaz MA, Gonzalez-Vicent M, Sevilla J, Madero L. Hematopoietic stem cell transplantation using umbilical cord blood progenitors: review of current clinical results. Bone Marrow Transplant. 2004;33:675-690

Bhatia M, Bonnet D, Murdoch B, Gan OI, Dick JE. A newly discovered class of human hematopoietic cells with SCID-repopulating activity. Nat Med. 1998;4:1038-1045

Bhatia M, Wang JC, Kapp U, Bonnet D, Dick JE. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proc Natl Acad Sci U S A. 1997;94:5320-5325

Bjorgvinsdottir H, Ding C, Pech N, Gifford MA, Li LL, Dinauer MC. Retroviral-mediated gene transfer of gp91phox into bone marrow cells rescues defect in host defense against Aspergillus fumigatus in murine X-linked chronic granulomatous disease. Blood. 1997;89:41-48

Bjornsson JM, Andersson E, Lundstrom P, Larsson N, Xu X, Repetowska E, Humphries RK, Karlsson S. Proliferation of primitive myeloid progenitors can be reversibly induced by HOXA10. Blood. 2001;98:3301-3308

Blau CA, Peterson KR, Drachman JG, Spencer DM. A proliferation switch for genetically modified cells. Proc Natl Acad Sci U S A. 1997;94:3076-3081

Bogdan S, Klambt C. Epidermal growth factor receptor signaling. Curr Biol. 2001;11:R292-295

Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA. Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocr Rev. 1998;19:225-268

Boncinelli E, Acampora D, Pannese M, D'Esposito M, Somma R, Gaudino G, Stornaiuolo A, Cafiero M, Faiella A, Simeone A. Organization of human class I homeobox genes. Genome. 1989;31:745-756

Bonetta L. Leukemia case triggers tighter gene-therapy controls. Nat Med. 2002;8:1189

Bonini C, Grez M, Traversari C, Ciceri F, Marktel S, Ferrari G, Dinauer M, Sadat M, Aiuti A, Deola S, Radrizzani M, Hagenbeek A, Apperley J, Ebeling S, Martens A, Kolb HJ, Weber M, Lotti F, Grande A, Weissinger E, Bueren JA, Lamana M, Falkenburg JH, Heemskerk MH, Austin T, Kornblau S, Marini F, Benati C, Magnani Z, Cazzaniga S, Toma S, Gallo-Stampino C, Introna M, Slavin S, Greenberg PD, Bregni M, Mavilio F, Bordignon C. Safety of retroviral gene marking with a truncated NGF receptor. Nat Med. 2003;9:367-369

Breems DA, Blokland EA, Neben S, Ploemacher RE. Frequency analysis of human primitive haematopoietic stem cell subsets using a cobblestone area forming cell assay. Leukemia. 1994;8:1095-1104

Broudy VC. Stem cell factor and hematopoiesis. Blood. 1997;90:1345-1364

Bruno S, Gammaitoni L, Gunetti M, Sanavio F, Fagioli F, Aglietta M, Piacibello W. Different growth factor requirements for the ex vivo amplification of transplantable human cord blood cells in a NOD/SCID mouse model. J Biol Regul Homeost Agents. 2001;15:38-48

Bu-Ghanim HN, Segal AW, Keep NH, Casimir CM. Molecular analysis in three cases of X91- variant chronic granulomatous disease. Blood. 1995;86:3575-3582

Buske C, Feuring-Buske M, Antonchuk J, Rosten P, Hogge DE, Eaves CJ, Humphries RK. Overexpression of HOXA10 perturbs human lymphomyelopoiesis in vitro and in vivo. Blood. 2001;97:2286-2292

Carpenter G. The EGF receptor: a nexus for trafficking and signaling. Bioessays. 2000;22:697-707

Case SS, Price MA, Jordan CT, Yu XJ, Wang L, Bauer G, Haas DL, Xu D, Stripecke R, Naldini L, Kohn DB, Crooks GM. Stable transduction of quiescent CD34(+)CD38(-) human hematopoietic cells by HIV-1-based lentiviral vectors. Proc Natl Acad Sci U S A. 1999;96:2988-2993

Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, Gross F, Yvon E, Nusbaum P, Selz F, Hue C, Certain S, Casanova JL, Bousso P, Deist FL, Fischer A. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science. 2000;288:669-672

Chanock SJ, Faust LR, Barrett D, Christensen B, Newburger PE, Babior BM. Partial reconstitution of the respiratory burst oxidase in lymphoblastoid B cell lines lacking p67-phox after transfection with an expression vector containing wild-type and mutant p67 -phox cDNAs: Deletions of the carboxy and amino terminal residues of p67-phox are not required for activity. Exp Hematol. 1996;24:531-536

Choi. Inverse PCR analysis of unique inserts found in granulomatous disease (Abstract). Blood. 2000;96

Christensen JL, Weissman IL. Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. Proc Natl Acad Sci U S A. 2001;98:14541-14546

Clarke DL, Linzer DI. Changes in prolactin receptor expression during pregnancy in the mouse ovary. Endocrinology. 1993;133:224-232

Conneally E, Eaves CJ, Humphries RK. Efficient retroviral-mediated gene transfer to human cord blood stem cells with in vivo repopulating potential. Blood. 1998;91:3487-3493

Cristiano RJ. Viral and non-viral vectors for cancer gene therapy. Anticancer Res. 1998;18:3241-3245

Crooks GM, Fuller J, Petersen D, Izadi P, Malik P, Pattengale PK, Kohn DB, Gasson JC. Constitutive HOXA5 expression inhibits erythropoiesis and increases myelopoiesis from human hematopoietic progenitors. Blood. 1999;94:519-528

Cullen BR. Retroviruses as model systems for the study of nuclear RNA export pathways. Virology. 1998;249:203-210

Danno S, Itoh K, Baum C, Ostertag W, Ohnishi N, Kido T, Tomiwa K, Matsuda T, Fujita J. Efficient gene transfer by hybrid retroviral vectors to murine spermatogenic cells. Hum Gene Ther. 1999;10:1819-1831

Dao MA, Nolta JA. Use of the bnx/hu xenograft model of human hematopoiesis to optimize methods for retroviral-mediated stem cell transduction (Review). Int J Mol Med. 1998;1:257-264

Davis JA, Linzer DI. Expression of multiple forms of the prolactin receptor in mouse liver. Mol Endocrinol. 1989;3:674-680

De Leo FR, Ulman KV, Davis AR, Jutila KL, Quinn MT. Assembly of the human neutrophil NADPH oxidase involves binding of p67phox and flavocytochrome b to a common functional domain in p47phox. J Biol Chem. 1996;271:17013-17020

Demaison C, Parsley K, Brouns G, Scherr M, Battmer K, Kinnon C, Grez M, Thrasher AJ. High-level transduction and gene expression in hematopoietic repopulating cells using a human immunodeficiency [correction of imunodeficiency] virus type 1-based lentiviral vector containing an internal spleen focus forming virus promoter. Hum Gene Ther. 2002;13:803-813

Devins SS, Miller A, Herndon BL, O'Toole L, Reisz G. Effects of dopamine on T-lymphocyte proliferative responses and serum prolactin concentrations in critically ill patients. Crit Care Med. 1992;20:1644-1649

Dick JE, Kamel-Reid S, Murdoch B, Doedens M. Gene transfer into normal human hematopoietic cells using in vitro and in vivo assays. Blood. 1991;78:624-634

Dillo W, Brassel F, Becker H. [Possibilities and limitations of CT angiography in comparison to DSA in intracranial aneurysm]. Rofo. 1996;165:227-231

Dinauer MC, Gifford MA, Pech N, Li LL, Emshwiller P. Variable correction of host defense following gene transfer and bone marrow transplantation in murine X-linked chronic granulomatous disease. Blood. 2001;97:3738-3745

Dinauer MC, Li LL, Bjorgvinsdottir H, Ding C, Pech N. Long-term correction of phagocyte NADPH oxidase activity by retroviral-mediated gene transfer in murine X-linked chronic granulomatous disease. Blood. 1999;94:914-922

Ding C, Kume A, Bjorgvinsdottir H, Hawley RG, Pech N, Dinauer MC. High-level reconstitution of respiratory burst activity in a human X-linked chronic granulomatous disease (X-CGD) cell line and correction of murine X-CGD bone marrow cells by retroviral-mediated gene transfer of human gp91phox. Blood. 1996;88:1834-1840

Dogusan Z, Hooghe R, Verdood P, Hooghe-Peters EL. Cytokine-like effects of prolactin in human mononuclear and polymorphonuclear leukocytes. J Neuroimmunol. 2001;120:58-66

Dogusan Z, Martens N, Stinissen P, Hellings N, Demotte N, Hooghe R, Hooghe-Peters E. Effects of prolactin on cloned human T-lymphocytes. Endocrine. 2003;20:171-176

Dominguez-Caceres MA, Garcia-Martinez JM, Calcabrini A, Gonzalez L, Porque PG, Leon J, Martin-Perez J. Prolactin induces c-Myc expression and cell survival through activation of Src/Akt pathway in lymphoid cells. Oncogene. 2004;23:7378-7390

Donello JE, Beeche AA, Smith GJ, 3rd, Lucero GR, Hope TJ. The hepatitis B virus posttranscriptional regulatory element is composed of two subelements. J Virol. 1996;70:4345-4351

Driskell RA, Engelhardt JF. Current status of gene therapy for inherited lung diseases. Annu Rev Physiol. 2003;65:585-612

Du Y, Spence SE, Jenkins NA, Copeland NG. Cooperating cancer gene identification via oncogenic retrovirus-induced insertional mutagenesis. Blood. 2005

Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, Naldini L. A third-generation lentivirus vector with a conditional packaging system. J Virol. 1998;72:8463-8471

Ekstrand AJ, Longo N, Hamid ML, Olson JJ, Liu L, Collins VP, James CD. Functional characterization of an EGF receptor with a truncated extracellular domain expressed in glioblastomas with EGFR gene amplification. Oncogene. 1994;9:2313-2320

Ekstrand AJ, Sugawa N, James CD, Collins VP. Amplified and rearranged epidermal growth factor receptor genes in human glioblastomas reveal deletions of sequences encoding portions of the N- and/or C-terminal tails. Proc Natl Acad Sci U S A. 1992;89:4309-4313

Ezekowitz RA, Newburger PE. New perspectives in chronic granulomatous disease. J Clin Immunol. 1988;8:419-425

Flasshove M, Bardenheuer W, Schneider A, Hirsch G, Bach P, Bury C, Moritz T, Seeber S, Opalka B. Type and position of promoter elements in retroviral vectors have substantial effects on the expression level of an enhanced green fluorescent protein reporter gene. J Cancer Res Clin Oncol. 2000;126:391-399

Follenzi A, Ailles LE, Bakovic S, Geuna M, Naldini L. Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. Nat Genet. 2000;25:217-222

Freeman ME, Kanyicska B, Lerant A, Nagy G. Prolactin: structure, function, and regulation of secretion. Physiol Rev. 2000;80:1523-1631

Gallin JI, Alling DW, Malech HL, Wesley R, Koziol D, Marciano B, Eisenstein EM, Turner ML, DeCarlo ES, Starling JM, Holland SM. Itraconazole to prevent fungal infections in chronic granulomatous disease. N Engl J Med. 2003;348:2416-2422

Garcia de Palazzo IE, Adams GP, Sundareshan P, Wong AJ, Testa JR, Bigner DD, Weiner LM. Expression of mutated epidermal growth factor receptor by non-small cell lung carcinomas. Cancer Res. 1993;53:3217-3220

Gardlik R, Palffy R, Hodosy J, Lukacs J, Turna J, Celec P. Vectors and delivery systems in gene therapy. Med Sci Monit. 2005;11:RA110-121

Gaspar HB, Parsley KL, Howe S, King D, Gilmour KC, Sinclair J, Brouns G, Schmidt M, Von Kalle C, Barington T, Jakobsen MA, Christensen HO, Al Ghonaium A, White HN, Smith JL, Levinsky RJ, Ali RR, Kinnon C, Thrasher AJ. Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. Lancet. 2004;364:2181-2187

Giampaolo A, Sterpetti P, Bulgarini D, Samoggia P, Pelosi E, Valtieri M, Peschle C. Key functional role and lineage-specific expression of selected HOXB genes in purified hematopoietic progenitor differentiation. Blood. 1994;84:3637-3647

Giannola DM, Shlomchik WD, Jegathesan M, Liebowitz D, Abrams CS, Kadesch T, Dancis A, Emerson SG. Hematopoietic expression of HOXB4 is regulated in normal and leukemic stem cells through transcriptional activation of the HOXB4 promoter by upstream stimulating factor (USF)-1 and USF-2. J Exp Med. 2000;192:1479-1490

Gilliland DG, Griffin JD. The roles of FLT3 in hematopoiesis and leukemia. Blood. 2002;100:1532-1542

Goffin V, Bouchard B, Ormandy CJ, Weimann E, Ferrag F, Touraine P, Bole-Feysot C, Maaskant RA, Clement-Lacroix P, Edery M, Binart N, Kelly PA. Prolactin: a hormone at the crossroads of neuroimmunoendocrinology. Ann N Y Acad Sci. 1998;840:498-509

Goffin V, Kelly PA. Prolactin and growth hormone receptors. Clin Endocrinol (Oxf). 1996;45:247-255

Goffin V BB, Ormandy CJ, Weimann E, Ferrag F, Touraine P, Bole-Feysot C, Maaskant RA,Clement-Lacroix P,Edery M, Binnart N, and Kelly PA. Prolactin: a hormone at the crossroads of neuroimmunoendocrinology. Ann NY Acad Sci. 1998:498-509

Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med. 1996;183:1797-1806

Grez M, Akgun E, Hilberg F, Ostertag W. Embryonic stem cell virus, a recombinant murine retrovirus with expression in embryonic stem cells. Proc Natl Acad Sci U S A. 1990;87:9202-9206

Grez M, Becker S, Saulnier S, Knoss H, Ott MG, Maurer A, Dinauer MC, Hoelzer D, Seger R, Hossle JP. Gene therapy of chronic granulomatous disease. Bone Marrow Transplant. 2000;25 Suppl 2:S99-104

Grier DG, Thompson A, Kwasniewska A, McGonigle GJ, Halliday HL, Lappin TR. The pathophysiology of HOX genes and their role in cancer. J Pathol. 2005;205:154-171

Gunji Y, Nakamura M, Osawa H, Nagayoshi K, Nakauchi H, Miura Y, Yanagisawa M, Suda T. Human primitive hematopoietic progenitor cells are more enriched in KITlow cells than in KIThigh cells. Blood. 1993;82:3283-3289

Gunsilius E, Gastl G, Petzer AL. Hematopoietic stem cells. Biomed Pharmacother. 2001;55:186-194

Han P, Hodge G, Story C, Xu X. Phenotypic analysis of functional T-lymphocyte subtypes and natural killer cells in human cord blood: relevance to umbilical cord blood transplantation. Br J Haematol. 1995;89:733-740

Hanazono Y, Nagashima T, Takatoku M, Shibata H, Ageyama N, Asano T, Ueda Y, Dunbar CE, Kume A, Terao K, Hasegawa M, Ozawa K. In vivo selective expansion of gene-modified hematopoietic cells in a nonhuman primate model. Gene Ther. 2002;9:1055-1064

Hanenberg H, Hashino K, Konishi H, Hock RA, Kato I, Williams DA. Optimization of fibronectin-assisted retroviral gene transfer into human CD34+ hematopoietic cells. Hum Gene Ther. 1997;8:2193-2206

Hara T, Kume A, Hanazono Y, Mizukami H, Okada T, Tsurumi H, Moriwaki H, Ueda Y, Hasegawa M, Ozawa K. Expansion of genetically corrected neutrophils in chronic granulomatous disease mice by cotransferring a therapeutic gene and a selective amplifier gene. Gene Ther. 2004;11:1370-1377

Harrison DE. Competitive repopulation: a new assay for long-term stem cell functional capacity. Blood. 1980;55:77-81

Harrison DE. Competitive repopulation in unirradiated normal recipients. Blood. 1993;81:2473-2474

Hennemann B, Conneally E, Pawliuk R, Leboulch P, Rose-John S, Reid D, Chuo JY, Humphries RK, Eaves CJ. Optimization of retroviral-mediated gene transfer to human NOD/SCID mouse repopulating cord blood cells through a systematic analysis of protocol variables. Exp Hematol. 1999;27:817-825

Hildinger M, Abel KL, Ostertag W, Baum C. Design of 5' untranslated sequences in retroviral vectors developed for medical use. J Virol. 1999;73:4083-4089

Hodgson GS, Bradley TR. Properties of haematopoietic stem cells surviving 5-fluorouracil treatment: evidence for a pre-CFU-S cell? Nature. 1979;281:381-382

Holyoake TL, Nicolini FE, Eaves CJ. Functional differences between transplantable human hematopoietic stem cells from fetal liver, cord blood, and adult marrow. Exp Hematol. 1999;27:1418-1427

Horn PA, Morris JC, Neff T, Kiem HP. Stem cell gene transfer-efficacy and safety in large animal studies. Mol Ther. 2004;10:417-431

Horn PA, Topp MS, Morris JC, Riddell SR, Kiem HP. Highly efficient gene transfer into baboon marrow repopulating cells using GALV-pseudotype oncoretroviral vectors produced by human packaging cells. Blood. 2002;100:3960-3967

Horseman ND. Famine to feast--growth hormone and prolactin signal transducers. Endocrinology. 1994;135:1289-1291

Horseman ND, Zhao W, Montecino-Rodriguez E, Tanaka M, Nakashima K, Engle SJ, Smith F, Markoff E, Dorshkind K. Defective mammopoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene. Embo J. 1997;16:6926-6935

Hossle JP, Seger RA, Steinhoff D. Gene therapy of hematopoietic stem cells: strategies for improvement. News Physiol Sci. 2002;17:87-92

Huang J, Liang TJ. A novel hepatitis B virus (HBV) genetic element with Rev response element-like properties that is essential for expression of HBV gene products. Mol Cell Biol. 1993;13:7476-7486

Ikehara S. Pluripotent hemopoietic stem cells in mice and humans. Proc Soc Exp Biol Med. 2000;223:149-155

Ikuta K, Weissman IL. Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. Proc Natl Acad Sci U S A. 1992;89:1502-1506

Ito K, Ueda Y, Kokubun M, Urabe M, Inaba T, Mano H, Hamada H, Kitamura T, Mizoguchi H, Sakata T, Hasegawa M, Ozawa K. Development of a novel selective amplifier gene for controllable expansion of transduced hematopoietic cells. Blood. 1997;90:3884-3892

Ivanovic Z. Interleukin-3 and ex vivo maintenance of hematopoietic stem cells: facts and controversies. Eur Cytokine Netw. 2004;15:6-13

Iwama A, Oguro H, Nigishi M, Kato Y, Morita Y, Tsukui H, Ema H, Kamijo T, Katohfukui Y, Koseki H, Van Lohuizen M, Nakauchi H. Enhanced self-renewal of hematopoietic stem cells mediated by the polycomb gene product Bmi-1. Immunity. 2004; 21: 843-851

Iwata M, Nunoi H, Matsuda I, Kanegasaki S, Tsuruo T, Sugimoto Y. Drug-selected complete restoration of superoxide generation in Epstein-Barr virus-transformed B cells from p47phox-deficient chronic granulomatous disease patients by using a bicistronic retrovirus vector encoding a human multi-drug resistance gene (MDR1) and the p47phox gene. Hum Genet. 1998;103:419-423

Jin L, Siritanaratkul N, Emery DW, Richard RE, Kaushansky K, Papayannopoulou T, Blau CA. Targeted expansion of genetically modified bone marrow cells. Proc Natl Acad Sci U S A. 1998;95:8093-8097

Jin L, Zeng H, Chien S, Otto KG, Richard RE, Emery DW, Blau CA. In vivo selection using a cell-growth switch. Nat Genet. 2000;26:64-66

Jones RJ, Collector MI, Barber JP, Vala MS, Fackler MJ, May WS, Griffin CA, Hawkins AL, Zehnbauer BA, Hilton J, Colvin OM, Sharkis SJ. Characterization of mouse lymphohematopoietic stem cells lacking spleen colony-forming activity. Blood. 1996;88:487-491

Jones RJ, Wagner JE, Celano P, Zicha MS, Sharkis SJ. Separation of pluripotent haematopoietic stem cells from spleen colony-forming cells. Nature. 1990;347:188-189

Junker K, Koehl U, Zimmerman S, Stein S, Schwabe D, Klingebiel T, Grez M. Kinetics of cell death in T lymphocytes genetically modified with two novel suicide fusion genes. Gene Ther. 2003;10:1189-1197

Karpen GH. Position-effect variegation and the new biology of heterochromatin. Curr Opin Genet Dev. 1994;4:281-291

Katayama N, Shih JP, Nishikawa S, Kina T, Clark SC, Ogawa M. Stage-specific expression of c-kit protein by murine hematopoietic progenitors. Blood. 1993;82:2353-2360

Kato Y, Iwama A, Tadokoro Y, Shimoda K, Minoguchi M, Akira S, Tanaka M, Miyajima A, Kitamura T, Nakauchi H. Selective activation of STAT5 unveils its role in stem cell self-renewal in normal and leukemic hematopoiesis. J Exp Med. 2005;202:169-179

Kiem HP, Heyward S, Winkler A, Potter J, Allen JM, Miller AD, Andrews RG. Gene transfer into marrow repopulating cells: comparison between amphotropic and gibbon ape leukemia virus pseudotyped retroviral vectors in a competitive repopulation assay in baboons. Blood. 1997;90:4638-4645

Kiem HP, Rasko JE, Morris J, Peterson L, Kurre P, Andrews RG. Ex vivo selection for oncoretrovirally transduced green fluorescent protein-expressing CD34-enriched cells increases short-term engraftment of transduced cells in baboons. Hum Gene Ther. 2002;13:891-899

Kondo E, Mammano F, Cohen EA, Gottlinger HG. The p6gag domain of human immunodeficiency virus type 1 is sufficient for the incorporation of Vpr into heterologous viral particles. J Virol. 1995;69:2759-2764

Kondo M, Wagers AJ, Manz MG, Prohaska SS, Scherer DC, Beilhack GF, Shizuru JA, Weissman IL. Biology of hematopoietic stem cells and progenitors: implications for clinical application. Annu Rev Immunol. 2003;21:759-806

Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell. 1997;91:661-672

Kootstra NA, Verma IM. Gene therapy with viral vectors. Annu Rev Pharmacol Toxicol. 2003;43:413-439

Korbling M, Anderlini P. Peripheral blood stem cell versus bone marrow allotransplantation: does the source of hematopoietic stem cells matter? Blood. 2001;98:2900-2908

Kraunus J, Schaumann DH, Meyer J, Modlich U, Fehse B, Brandenburg G, von Laer D, Klump H, Schambach A, Bohne J, Baum C. Self-inactivating retroviral vectors with improved RNA processing. Gene Ther. 2004;11:1568-1578

Krosl J, Austin P, Beslu N, Kroon E, Humphries RK, Sauvageau G. In vitro expansion of hematopoietic stem cells by recombinant TAT-HOXB4 protein. Nat Med. 2003;9:1428-1432

Kume A, Dinauer MC. Retrovirus-mediated reconstitution of respiratory burst activity in X-linked chronic granulomatous disease cells. Blood. 1994;84:3311-3316

Kume A, Hanazono Y, Mizukami H, Okada T, Ozawa K. Selective expansion of transduced cells for hematopoietic stem cell gene therapy. Int J Hematol. 2002;76:299-304

Kume A, Koremoto M, Xu R, Okada T, Mizukami H, Hanazono Y, Hasegawa M, Ozawa K. In vivo expansion of transduced murine hematopoietic cells with a selective amplifier gene. J Gene Med. 2003;5:175-181

Kurre P, Anandakumar P, Harkey MA, Thomasson B, Kiem HP. Efficient marking of murine long-term repopulating stem cells targeting unseparated marrow cells at low lentiviral vector particle concentration. Mol Ther. 2004;9:914-922

Kurre P, Morris J, Horn PA, Harkey MA, Andrews RG, Kiem HP. Gene transfer into baboon repopulating cells: A comparison of Flt-3 Ligand and megakaryocyte growth and development factor versus IL-3 during ex vivo transduction. Mol Ther. 2001;3:920-927

Kyba M, Perlingeiro RC, Daley GQ. HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. Cell. 2002;109:29-37

Lacaud G, Carlsson L, Keller G. Identification of a fetal hematopoietic precursor with B cell, T cell, and macrophage potential. Immunity. 1998;9:827-838

Laneuville P, Chang W, Kamel-Reid S, Fauser AA, Dick JE. High-efficiency gene transfer and expression in normal human hematopoietic cells with retrovirus vectors. Blood. 1988;71:811-814

Lawrence HJ, Helgason CD, Sauvageau G, Fong S, Izon DJ, Humphries RK, Largman C. Mice bearing a targeted interruption of the homeobox gene HOXA9 have defects in myeloid, erythroid, and lymphoid hematopoiesis. Blood. 1997;89:1922-1930

Lawrence HJ, Largman C. Homeobox genes in normal hematopoiesis and leukemia. Blood. 1992;80:2445-2453

Leary AG, Ogawa M. Blast cell colony assay for umbilical cord blood and adult bone marrow progenitors. Blood. 1987;69:953-956

Lee FS, Kim AH, Khursigara G, Chao MV. The uniqueness of being a neurotrophin receptor. Curr Opin Neurobiol. 2001;11:281-286

Lesueur L, Edery M, Ali S, Paly J, Kelly PA, Djiane J. Comparison of long and short forms of the prolactin receptor on prolactin-induced milk protein gene transcription. Proc Natl Acad Sci U S A. 1991;88:824-828

Leurs J, Wissing BM, Nerme V, Schatteman K, Bjorquist P, Hendriks D. Different mechanisms contribute to the biphasic pattern of carboxypeptidase U (TAFIa) generation during in vitro clot lysis in human plasma. Thromb Haemost. 2003;89:264-271

Li Z, Dullmann J, Schiedlmeier B, Schmidt M, von Kalle C, Meyer J, Forster M, Stocking C, Wahlers A, Frank O, Ostertag W, Kuhlcke K, Eckert HG, Fehse B, Baum C. Murine leukemia induced by retroviral gene marking. Science. 2002;296:497

Li Z, Schwieger M, Lange C, Kraunus J, Sun H, van den Akker E, Modlich U, Serinsoz E, Will E, von Laer D, Stocking C, Fehse B, Schiedlmeier B, Baum C. Predictable and efficient retroviral gene

transfer into murine bone marrow repopulating cells using a defined vector dose. Exp Hematol. 2003;31:1206-1214

Lord BI, Woolford LB. Proliferation of spleen colony forming units (CFU-S8, CFU-S13) and cells with marrow repopulating ability. Stem Cells. 1993;11:212-217

Mackarehtschian K, Hardin JD, Moore KA, Boast S, Goff SP, Lemischka IR. Targeted disruption of the flk2/flt3 gene leads to deficiencies in primitive hematopoietic progenitors. Immunity. 1995;3:147-161

Malech HL. Use of serum-free medium with fibronectin fragment enhanced transduction in a system of gas permeable plastic containers to achieve high levels of retrovirus transduction at clinical scale. Stem Cells. 2000;18:155-156

Malech HL, Maples PB, Whiting-Theobald N, Linton GF, Sekhsaria S, Vowells SJ, Li F, Miller JA, DeCarlo E, Holland SM, Leitman SF, Carter CS, Butz RE, Read EJ, Fleisher TA, Schneiderman RD, Van Epps DE, Spratt SK, Maack CA, Rokovich JA, Cohen LK, Gallin JI. Prolonged production of NADPH oxidase-corrected granulocytes after gene therapy of chronic granulomatous disease. Proc Natl Acad Sci U S A. 1997;94:12133-12138

Maly FE, Schuerer-Maly CC, Quilliam L, Cochrane CG, Newburger PE, Curnutte JT, Gifford M, Dinauer MC. Restitution of superoxide generation in autosomal cytochrome-negative chronic granulomatous disease (A22(0) CGD)-derived B lymphocyte cell lines by transfection with p22phax cDNA. J Exp Med. 1993;178:2047-2053

Matera L, Cesano A, Bellone G, Oberholtzer E. Modulatory effect of prolactin on the resting and mitogen-induced activity of T, B, and NK lymphocytes. Brain Behav Immun. 1992;6:409-417

Matsuda KM, Kume A, Ueda Y, Urabe M, Hasegawa M, Ozawa K. Development of a modified selective amplifier gene for hematopoietic stem cell gene therapy. Gene Ther. 1999;6:1038-1044

Mavilio F, Ferrari G, Rossini S, Nobili N, Bonini C, Casorati G, Traversari C, Bordignon C. Peripheral blood lymphocytes as target cells of retroviral vector-mediated gene transfer. Blood. 1994;83:1988-1997

McAveney KM, Gimble JM, Yu-Lee L. Prolactin receptor expression during adipocyte differentiation of bone marrow stroma. Endocrinology. 1996;137:5723-5726

Miettinen PJ, Berger JE, Meneses J, Phung Y, Pedersen RA, Werb Z, Derynck R. Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. Nature. 1995;376:337-341

Mikkola H, Woods NB, Sjogren M, Helgadottir H, Hamaguchi I, Jacobsen SE, Trono D, Karlsson S. Lentivirus gene transfer in murine hematopoietic progenitor cells is compromised by a delay in proviral integration and results in transduction mosaicism and heterogeneous gene expression in progeny cells. J Virol. 2000;74:11911-11918

Miller AD, Miller DG, Garcia JV, Lynch CM. Use of retroviral vectors for gene transfer and expression. Methods Enzymol. 1993;217:581-599

Miller DG, Adam MA, Miller AD. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. Mol Cell Biol. 1990;10:4239-4242

Milsom MD, Duxbury R, Gagen D, Humphries RK, Schmidt M, von-Kalle C, Fairbairn LJ. Overexpression of HOXB4 confers a myelo-erythroid differentiation delay in vitro. Leukemia. 2005;19:148-153

Milsom MD, Fairbairn LJ. Protection and selection for gene therapy in the hematopoietic system. J Gene Med. 2004;6:133-146

Miyoshi H, Blomer U, Takahashi M, Gage FH, Verma IM. Development of a self-inactivating lentivirus vector. J Virol. 1998;72:8150-8157

Moreau-Gaudry F, Xia P, Jiang G, Perelman NP, Bauer G, Ellis J, Surinya KH, Mavilio F, Shen CK, Malik P. High-level erythroid-specific gene expression in primary human and murine hematopoietic cells with self-inactivating lentiviral vectors. Blood. 2001;98:2664-2672

Morgenstern DE, Gifford MA, Li LL, Doerschuk CM, Dinauer MC. Absence of respiratory burst in X-linked chronic granulomatous disease mice leads to abnormalities in both host defense and inflammatory response to Aspergillus fumigatus. J Exp Med. 1997;185:207-218

Morrison SJ, Uchida N, Weissman IL. The biology of hematopoietic stem cells. Annu Rev Cell Dev Biol. 1995;11:35-71

Morrison SJ, Weissman IL. The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. Immunity. 1994;1:661-673

Moscatello DK, Holgado-Madruga M, Godwin AK, Ramirez G, Gunn G, Zoltick PW, Biegel JA, Hayes RL, Wong AJ. Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. Cancer Res. 1995;55:5536-5539

Mountain A. Gene therapy: the first decade. Trends Biotechnol. 2000;18:119-128

Mulloy JC, Jankovic V, Wunderlich M, Delwel R, Cammenga J, Krejci O, Zhao H, Valk PJ, Lowenberg B, Nimer SD. AML1-ETO fusion protein up-regulates TRKA mRNA expression in human CD34+ cells, allowing nerve growth factor-induced expansion. Proc Natl Acad Sci U S A. 2005;102:4016-4021

Murphy WJ, Durum SK, Longo DL. Differential effects of growth hormone and prolactin on murine T cell development and function. J Exp Med. 1993;178:231-236

Na Nakorn T, Traver D, Weissman IL, Akashi K. Myeloerythroid-restricted progenitors are sufficient to confer radioprotection and provide the majority of day 8 CFU-S. J Clin Invest. 2002;109:1579-1585

Nagy E, Berczi I. Hypophysectomized rats depend on residual prolactin for survival. Endocrinology. 1991;128:2776-2784

Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science. 1996;272:263-267

Neff T, Horn PA, Valli VE, Gown AM, Wardwell S, Wood BL, von Kalle C, Schmidt M, Peterson LJ, Morris JC, Richard RE, Clackson T, Kiem HP, Blau CA. Pharmacologically regulated in vivo selection in a large animal. Blood. 2002;100:2026-2031

Nolta JA, Dao MA, Wells S, Smogorzewska EM, Kohn DB. Transduction of pluripotent human hematopoietic stem cells demonstrated by clonal analysis after engraftment in immune-deficient mice. Proc Natl Acad Sci U S A. 1996;93:2414-2419

Ochs HD, Igo RP. The NBT slide test: a simple screening method for detecting chronic granulomatous disease and female carriers. J Pediatr. 1973;83:77-82

Ogawa M. Differentiation and proliferation of hematopoietic stem cells. Blood. 1993;81:2844-2853

Ogawa M. Changing phenotypes of hematopoietic stem cells. Exp Hematol. 2002;30:3-6

Ohkubo T, Barcena A, Smith CA, Harrison MR, Muench MO. High-efficiency retroviral transduction of fetal liver CD38-CD34++ cells: implications for in utero and ex utero gene therapy. Fetal Diagn Ther. 2001;16:299-307

Ohta H, Sawada A, Kim JY, Tokimasa S, Nishiguchi S, Humphries RK, Hara J, Takihara Y. J. Exp. Med. 2002; 195: 759-770

Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. Embo J. 2000;19:3159-3167

Orlic D, Fischer R, Nishikawa S, Nienhuis AW, Bodine DM. Purification and characterization of heterogeneous pluripotent hematopoietic stem cell populations expressing high levels of c-kit receptor. Blood. 1993;82:762-770

Ormandy CJ, Camus A, Barra J, Damotte D, Lucas B, Buteau H, Edery M, Brousse N, Babinet C, Binart N, Kelly PA. Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. Genes Dev. 1997;11:167-178

Osawa M, Hanada K, Hamada H, Nakauchi H. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science 1996; 273:242-245

Otto KG, Broudy VC, Lin NL, Parganas E, Luthi JN, Drachman JG, Ihle JN, Blau CA: Membrane localization is not required for Mpl function in normal haematopoietic cells. Blood 2001; 98: 2077-2083

Park IK, Qian D, Kiel M, Becker MW, Pihalija M, Weissman IL, Morrison SJ, Clarke MF: Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. Nature 2003; 423(6937): 231-3

Page KA, Landau NR, Littman DR. Construction and use of a human immunodeficiency virus vector for analysis of virus infectivity. J Virol. 1990; 64:5270-5276

Pallard C, Gouilleux F, Charon M, Groner B, Gisselbrecht S, Dusanter-Fourt I. Interleukin-3, erythropoietin, and prolactin activate a STAT5-like factor in lymphoid cells. J Biol Chem. 1995; 270:15942-15945

Pekins AC CS. Conditional immortalization of mouse myelomonocytic, megakaryocytic and mast cell progenitors by Hox-2.4 homeobox gene. Embo J. 1993;12:3835-3846

Petropoulou T, Liese J, Tintelnot K, Gahr M, Belohradsky BH. [Long-term treatment of patients with itraconazole for the prevention of Aspergillus infections in patients with chronic granulomatous disease (CGD)]. Mycoses. 1994;37 Suppl 2:64-69

Pfeifer A, Verma IM. Gene therapy: promises and problems. Annu Rev Genomics Hum Genet. 2001;2:177-211

Piacibello W, Bruno S, Sanavio F, Droetto S, Gunetti M, Ailles L, Santoni de Sio F, Viale A, Gammaitoni L, Lombardo A, Naldini L, Aglietta M. Lentiviral gene transfer and ex vivo expansion of human primitive stem cells capable of primary, secondary, and tertiary multilineage repopulation in NOD/SCID mice. Nonobese diabetic/severe combined immunodeficient. Blood. 2002;100:4391-4400

Ploemacher RE, Brons RH. Separation of CFU-S from primitive cells responsible for reconstitution of the bone marrow hemopoietic stem cell compartment following irradiation: evidence for a pre-CFU-S cell. Exp Hematol. 1989;17:263-266

Porter CD, Parkar MH, Collins MK, Levinsky RJ, Kinnon C. Superoxide production by normal and chronic granulomatous disease (CGD) patient-derived EBV-transformed B cell lines measured by chemiluminescence-based assays. J Immunol Methods. 1992;155:151-157

Porter CD, Parkar MH, Collins MK, Levinsky RJ, Kinnon C. Efficient retroviral transduction of human bone marrow progenitor and long-term culture-initiating cells: partial reconstitution of cells from patients with X-linked chronic granulomatous disease by gp91-phox expression. Blood. 1996;87:3722-3730

Porter CD, Parkar MH, Levinsky RJ, Collins MK, Kinnon C. X-linked chronic granulomatous disease: correction of NADPH oxidase defect by retrovirus-mediated expression of gp91-phox. Blood. 1993;82:2196-2202

Posner BI KP, Shiu RP, Friesen HG. Studies of insulin, growth hormone and prolactin binding: tissue distribution, species variation and characterization. Endocrinology. 1974:521-531

Qin S, Ward M, Raftopoulos H, Tang H, Bradley B, Hesdorffer C, Bank A. Competitive repopulation of retrovirally transduced haemopoietic stem cells. Br J Haematol. 1999;107:162-168

Rae J, Newburger PE, Dinauer MC, Noack D, Hopkins PJ, Kuruto R, Curnutte JT. X-Linked chronic granulomatous disease: mutations in the CYBB gene encoding the gp91-phox component of respiratory-burst oxidase. Am J Hum Genet. 1998;62:1320-1331

Rafferty JA, Hickson I, Chinnasamy N, Lashford LS, Margison GP, Dexter TM, Fairbairn LJ. Chemoprotection of normal tissues by transfer of drug resistance genes. Cancer Metastasis Rev. 1996;15:365-383

Rane SG RE. JAKs,STATs and Src kinase in hematopoiesis. Oncogene. 2002:3334-3358

Reya T. Regulation of hematopoietic stem cell self-renewal. Recent Prog Horm Res. 2003;58:283-295

Richard RE, Blau CA. Small-molecule-directed mpl signaling can complement growth factors to selectively expand genetically modified cord blood cells. Stem Cells. 2003;21:71-78

Risdon G, Gaddy J, Horie M, Broxmeyer HE. Alloantigen priming induces a state of unresponsiveness in human umbilical cord blood T cells. Proc Natl Acad Sci U S A. 1995;92:2413-2417

Roesler J, Brenner S, Bukovsky AA, Whiting-Theobald N, Dull T, Kelly M, Civin CI, Malech HL. Third-generation, self-inactivating gp91(phox) lentivector corrects the oxidase defect in NOD/SCID mouse-repopulating peripheral blood-mobilized CD34+ cells from patients with X-linked chronic granulomatous disease. Blood. 2002;100:4381-4390

Roos D, de Boer M, Borregard N, Bjerrum OW, Valerius NH, Seger RA, Muhlebach T, Belohradsky BH, Weening RS. Chronic granulomatous disease with partial deficiency of cytochrome b558 and incomplete respiratory burst: variants of the X-linked, cytochrome b558-negative form of the disease. J Leukoc Biol. 1992;51:164-171

Roos D, de Boer M, Kuribayashi F, Meischl C, Weening RS, Segal AW, Ahlin A, Nemet K, Hossle JP, Bernatowska-Matuszkiewicz E, Middleton-Price H. Mutations in the X-linked and autosomal recessive forms of chronic granulomatous disease. Blood. 1996;87:1663-1681

Sabharwal P, Glaser R, Lafuse W, Varma S, Liu Q, Arkins S, Kooijman R, Kutz L, Kelley KW, Malarkey WB. Prolactin synthesized and secreted by human peripheral blood mononuclear cells: an autocrine growth factor for lymphoproliferation. Proc Natl Acad Sci U S A. 1992;89:7713-7716

Sadat MA, Pech N, Saulnier S, Leroy BA, Hossle JP, Grez M, Dinauer MC. Long-term high-level reconstitution of NADPH oxidase activity in murine X-linked chronic granulomatous disease using a bicistronic vector expressing gp91phox and a Delta LNGFR cell surface marker. Hum Gene Ther. 2003;14:651-666

Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol. 1995;19:183-232

Saulnier SO, Steinhoff D, Dinauer MC, Zufferey R, Trono D, Seger RA, Hossle JP. Lentivirus-mediated gene transfer of gp91phox corrects chronic granulomatous disease (CGD) phenotype in human X-CGD cells. J Gene Med. 2000;2:317-325

Sauvageau G, Lansdorp PM, Eaves CJ, Hogge DE, Dragowska WH, Reid DS, Largman C, Lawrence HJ, Humphries RK. Differential expression of homeobox genes in functionally distinct CD34+ subpopulations of human bone marrow cells. Proc Natl Acad Sci U S A. 1994;91:12223-12227

Sauvageau G, Thorsteinsdottir U, Eaves CJ, Lawrence HJ, Largman C, Lansdorp PM, Humphries RK. Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo. Genes Dev. 1995;9:1753-1765

Sauvageau G, Thorsteinsdottir U, Hough MR, Hugo P, Lawrence HJ, Largman C, Humphries RK. Overexpression of HOXB3 in hematopoietic cells causes defective lymphoid development and progressive myeloproliferation. Immunity. 1997;6:13-22

Sawai N, Zhou S, Vanin EF, Houghton P, Brent TP, Sorrentino BP. Protection and in vivo selection of hematopoietic stem cells using temozolomide, O6-benzylguanine, and an alkyltransferase-expressing retroviral vector. Mol Ther. 2001;3:78-87

Schiedlmeier B, Klump H, Will E, Arman-Kalcek G, Li Z, Wang Z, Rimek A, Friel J, Baum C, Ostertag W. High-level ectopic HOXB4 expression confers a profound in vivo competitive growth advantage on human cord blood CD34+ cells, but impairs lymphomyeloid differentiation. Blood. 2003;101:1759-1768

Schiedlmeier B, Wermann K, Kuhlcke K, Eckert HG, Baum C, Fruehauf S, Zeller WJ. Human multidrug resistance-1 gene transfer to long-term repopulating human mobilized peripheral blood progenitor cells. Bone Marrow Transplant. 2000;25 Suppl 2:S118-124

Schilz AJ, Schiedlmeier B, Kuhlcke K, Fruehauf S, Lindemann C, Zeller WJ, Grez M, Fauser AA, Baum C, Eckert HG. MDR1 gene expression in NOD/SCID repopulating cells after retroviral gene transfer under clinically relevant conditions. Mol Ther. 2000;2:609-618

Schlessinger J. Ligand-induced, receptor-mediated dimerization and activation of EGF receptor. Cell. 2002;110:669-672

Schmidt M, Hoffmann G, Wissler M, Lemke N, Mussig A, Glimm H, Williams DA, Ragg S, Hesemann CU, von Kalle C. Detection and direct genomic sequencing of multiple rare unknown flanking DNA in highly complex samples. Hum Gene Ther. 2001;12:743-749

Schwickerath O, Brouns G, Thrasher A, Kinnon C, Roes J, Casimir C. Enhancer-deleted retroviral vectors restore high levels of superoxide generation in a mouse model of CGD. J Gene Med. 2004;6:603-615

Scott MP. Vertebrate homeobox gene nomenclature. Cell. 1992;71:551-553

Seger RA, Gungor T, Belohradsky BH, Blanche S, Bordigoni P, Di Bartolomeo P, Flood T, Landais P, Muller S, Ozsahin H, Passwell JH, Porta F, Slavin S, Wulffraat N, Zintl F, Nagler A, Cant A, Fischer A. Treatment of chronic granulomatous disease with myeloablative conditioning and an unmodified hemopoietic allograft: a survey of the European experience, 1985-2000. Blood. 2002;100:4344-4350

Sibilia M, Wagner EF. Strain-dependent epithelial defects in mice lacking the EGF receptor. Science. 1995;269:234-238

Skwarlo-Sonta K. Mitogenic effect of prolactin on chicken lymphocytes in vitro. Immunol Lett. 1990;24:171-177

Smith LG, Weissman IL, Heimfeld S. Clonal analysis of hematopoietic stem-cell differentiation in vivo. Proc Natl Acad Sci U S A. 1991;88:2788-2792

Somia N, Verma IM. Gene therapy: trials and tribulations. Nat Rev Genet. 2000;1:91-99

Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. Science. 1988;241:58-62

Spangrude GJ, Smith L, Uchida N, Ikuta K, Heimfeld S, Friedman J, Weissman IL. Mouse hematopoietic stem cells. Blood. 1991;78:1395-1402

Sugawa N, Ekstrand AJ, James CD, Collins VP. Identical splicing of aberrant epidermal growth factor receptor transcripts from amplified rearranged genes in human glioblastomas. Proc Natl Acad Sci U S A. 1990;87:8602-8606

Sutton RE, Wu HT, Rigg R, Bohnlein E, Brown PO. Human immunodeficiency virus type 1 vectors efficiently transduce human hematopoietic stem cells. J Virol. 1998;72:5781-5788

Szilvassy SJ, Humphries RK, Lansdorp PM, Eaves AC, Eaves CJ. Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulation strategy. Proc Natl Acad Sci U S A. 1990;87:8736-8740

Szilvassy SJ, Meyerrose TE, Ragland PL, Grimes B. Differential homing and engraftment properties of hematopoietic progenitor cells from murine bone marrow, mobilized peripheral blood, and fetal liver. Blood. 2001;98:2108-2115

Thomas CE, Ehrhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. Nat Rev Genet. 2003;4:346-358

Thorsteinsdottir U, Mamo A, Kroon E, Jerome L, Bijl J, Lawrence HJ, Humphries K, Sauvageau G. Overexpression of the myeloid leukemia-associated Hoxa9 gene in bone marrow cells induces stem cell expansion. Blood. 2002;99:121-129

Thorsteinsdottir U, Sauvageau G, Humphries RK. Hox homeobox genes as regulators of normal and leukemic hematopoiesis. Hematol Oncol Clin North Am. 1997;11:1221-1237

Thorsteinsdottir U, Sauvageau G, Humphries RK. Enhanced in vivo regenerative potential of HOXB4-transduced hematopoietic stem cells with regulation of their pool size. Blood. 1999;94:2605-2612

Till JE, Mc CE. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res. 1961;14:213-222

Uchida N, Sutton RE, Friera AM, He D, Reitsma MJ, Chang WC, Veres G, Scollay R, Weissman IL. HIV, but not murine leukemia virus, vectors mediate high efficiency gene transfer into freshly isolated G0/G1 human hematopoietic stem cells. Proc Natl Acad Sci U S A. 1998;95:11939-11944

Uchida N, Weissman IL. Searching for hematopoietic stem cells: evidence that Thy-1.1lo Lin- Sca-1+ cells are the only stem cells in C57BL/Ka-Thy-1.1 bone marrow. J Exp Med. 1992;175:175-184

Ullrich A, Coussens L, Hayflick JS, Dull TJ, Gray A, Tam AW, Lee J, Yarden Y, Libermann TA, Schlessinger J, et al. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature. 1984;309:418-425

Vallbohmer D, Lenz HJ. Epidermal growth factor receptor as a target for chemotherapy. Clin Colorectal Cancer. 2005;5 Suppl 1:S19-27

van Beusechem VW, Bart-Baumeister JA, Hoogerbrugge PM, Valerio D. Influence of interleukin-3, interleukin-6, and stem cell factor on retroviral transduction of rhesus monkey CD34+ hematopoietic progenitor cells measured in vitro and in vivo. Gene Ther. 1995;2:245-255

Van Tendeloo VF, Van Broeckhoven C, Berneman ZN. Gene therapy: principles and applications to hematopoietic cells. Leukemia. 2001;15:523-544

Verma IM, Somia N. Gene therapy -- promises, problems and prospects. Nature. 1997;389:239-242

Vollweiler JL, Zielske SP, Reese JS, Gerson SL. Hematopoietic stem cell gene therapy: progress toward therapeutic targets. Bone Marrow Transplant. 2003;32:1-7

Vormoor J, Lapidot T, Pflumio F, Risdon G, Patterson B, Broxmeyer HE, Dick JE. Immature human cord blood progenitors engraft and proliferate to high levels in severe combined immunodeficient mice. Blood. 1994;83:2489-2497

Vowells SJ, Sekhsaria S, Malech HL, Shalit M, Fleisher TA. Flow cytometric analysis of the granulocyte respiratory burst: a comparison study of fluorescent probes. J Immunol Methods. 1995;178:89-97

Wagers AJ, Sherwood RI, Christensen JL, Weissman IL. Little evidence for developmental plasticity of adult hematopoietic stem cells. Science. 2002;297:2256-2259

Walther W, Stein U. Viral vectors for gene transfer: a review of their use in the treatment of human diseases. Drugs. 2000;60:249-271

Weil WM, Linton GF, Whiting-Theobald N, Vowells SJ, Rafferty SP, Li F, Malech HL. Genetic correction of p67phox deficient chronic granulomatous disease using peripheral blood progenitor cells as a target for retrovirus mediated gene transfer. Blood. 1997;89:1754-1761

Weissman IL. Translating stem and progenitor cell biology to the clinic: barriers and opportunities. Science. 2000;287:1442-1446

Wikstrand CJ, Hale LP, Batra SK, Hill ML, Humphrey PA, Kurpad SN, McLendon RE, Moscatello D, Pegram CN, Reist CJ, et al. Monoclonal antibodies against EGFRvIII are tumor specific and react with breast and lung carcinomas and malignant gliomas. Cancer Res. 1995;55:3140-3148

Winkelstein JA, Marino MC, Johnston RB, Jr., Boyle J, Curnutte J, Gallin JI, Malech HL, Holland SM, Ochs H, Quie P, Buckley RH, Foster CB, Chanock SJ, Dickler H. Chronic granulomatous disease. Report on a national registry of 368 patients. Medicine (Baltimore). 2000;79:155-169

Wognum AW, Visser TP, Peters K, Bierhuizen MF, Wagemaker G. Stimulation of mouse bone marrow cells with kit ligand, FLT3 ligand, and thrombopoietin leads to efficient retrovirus-mediated gene transfer to stem cells, whereas interleukin 3 and interleukin 11 reduce transduction of short- and long-term repopulating cells. Hum Gene Ther. 2000;11:2129-2141

Woodman RC, Newburger PE, Anklesaria P, Erickson RW, Rae J, Cohen MS, Curnutte JT. A new X-linked variant of chronic granulomatous disease characterized by the existence of a normal clone of respiratory burst-competent phagocytic cells. Blood. 1995;85:231-241

Woody MA, Welniak LA, Sun R, Tian ZG, Henry M, Richards S, Raziuddin A, Longo DL, Murphy WJ. Prolactin exerts hematopoietic growth-promoting effects in vivo and partially counteracts myelosuppression by azidothymidine. Exp Hematol. 1999;27:811-816

Xu R, Kume A, Matsuda KM, Ueda Y, Kodaira H, Ogasawara Y, Urabe M, Kato I, Hasegawa M, Ozawa K. A selective amplifier gene for tamoxifen-inducible expansion of hematopoietic cells. J Gene Med. 1999;1:236-244

Yonemura Y, Ku H, Hirayama F, Souza LM, Ogawa M. Interleukin 3 or interleukin 1 abrogates the reconstituting ability of hematopoietic stem cells. Proc Natl Acad Sci U S A. 1996;93:4040-4044

Yu L, Cross AR, Zhen L, Dinauer MC. Functional analysis of NADPH oxidase in granulocytic cells expressing a delta488-497 gp91(phox) deletion mutant. Blood. 1999;94:2497-2504

Yu SF, von Ruden T, Kantoff PW, Garber C, Seiberg M, Ruther U, Anderson WF, Wagner EF, Gilboa E. Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells. Proc Natl Acad Sci U S A. 1986;83:3194-3198

Zanjani ED, Almeida-Porada G, Livingston AG, Flake AW, Ogawa M. Human bone marrow CD34- cells engraft in vivo and undergo multilineage expression that includes giving rise to CD34+ cells. Exp Hematol. 1998;26:353-360

Zeng H, Masuko M, Jin L, Neff T, Otto KG, Blau CA. Receptor specificity in the self-renewal and differentiation of primary multipotential hemopoietic cells. Blood. 2001;98:328-334

Zennou V, Petit C, Guetard D, Nerhbass U, Montagnier L, Charneau P. HIV-1 genome nuclear import is mediated by a central DNA flap. Cell. 2000;101:173-185

Zufferey R, Donello JE, Trono D, Hope TJ. Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. J Virol. 1999;73:2886-2892

Zufferey R, Dull T, Mandel RJ, Bukovsky A, Quiroz D, Naldini L, Trono D. Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J Virol. 1998;72:9873-9880

Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat Biotechnol. 1997;15:871-875

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1. Selective Expansion of gene modified murine haematopoietic stem cells *ex vivo* and *in vivo*

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Poster on XII Annual Congress of ESGT, 4-7 November 2004, Tampere, Finnland

2. Selective expansion of gp91^{phox}-gene modified murine haematopoietic stem cells by dimerizer induced activation of chimerical prolactin receptor

Linping Chen, Stefan Stein, Daniela Baus, Robert Pick, Christian Wichmann and Manuel Grez
(In preparation)

3. **Targeting the oligomerization domain of ETO interferes with AML1/ETO oncogenic activity in t(8;21)-positive leukemic cells**

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4. Application of a human multidrug transporter (ABCG2) as selectable marker in gene therapy

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Selektive Expansion von gp91^{phox} Gen-modifizierten murinen hämatopoetischen Stammzellen

Hämatopoetische Stammzellen stellen die entscheidenden Zielzellen für Gentherapie-Strategien zur Behandlung von erworbenen und angeborenen Krankheiten des Blutsystems, wie zum Beispiel der X-chromosomal vererbten chronischen Granulomatose, dar. Diese Stammzellen können später in alle verschiedenen Blutzellreihen differenzieren und gleichzeitig den Stammzellpool erhalten (Selbsterneuerung). Allerdings bereitet es immer noch große Probleme, eine genügend große Anzahl dieser Stammzellen genetisch zu modifizieren. Des weiteren bedeutet der Gentransfer vieler therapeutischer Gene, nachdem die gen-modifizierten und transplantierten Zellen sich im Körper angesiedelt haben, für die Zelle kein Vorteil in Bezug auf das Zellwachstum. Ein zusätzliches Selektions-Molekül könnte der Zelle einen Proliferationsvorteil verschaffen und so zur Expansion der wenigen gen-modifizierten Zellen führen. In der vorliegenden Arbeit wurden für eine selektive Expansion von genetisch modifizierten murinen hämatopoetischen Stammzellen zwei neue Selektionssysteme entwickelt.

In der ersten Strategie wurde die Funktion des intrinsischen Transkriptionsfaktors HOXB4 genutzt, der eine zentrale Rolle in Zellvermehrung und Differenzierung von hämatopoetischen Stammzellen spielt. Die Überexpression eines bicistronischen gammaretroviralen Vektors, kodierend für das therapeutische Gen gp91^{phox} und HOXB4, in murinen hämatopoetischen Stammzellen *ex vivo* resultierte in einem signifikanten 3 bis 4-fachen Zellzahl-Anstieg. Zugleich stieg die Anzahl der gp91^{phox} Transgen exprimierenden Zellen 2 bis 3-fach an. CFU-Assays zeigten eine signifikante Anzahl an Stamm- und/oder Vorläuferzellen in der HOXB4-selektierten Zellpopulation. Allerdings haben diese selektionierten Zellen nicht die Fähigkeit, in bestrahlte, myeloablatierte Empfängermäuse transplantiert, das hämatopoetische System wieder aufzubauen. Diese Ergebnisse belegen, dass gp91^{phox} gen-modifizierte hämatopoetische Stammzellen zwar *ex vivo* mittels Überexpression von HOXB4 selektioniert und vermehrt werden können, *in vivo* aber keinen Vorteil gegenüber nicht modifizierten Zellen besitzen. Möglicherweise beruht diese Diskrepanz auf einem zu niedrigen Expressionslevel von HOXB4 in dem murinen *in vivo* - Modell.

Die Zweite Strategie beruht auf einem induzierbaren Dimerisierungs-System. Dieses umfasst das therapeutische Gen gp91^{phox} und ein Fusionkonstrukt bestehend aus einer „Drug-binding“-Domäne (FKBP12) und einer Signaldomäne entweder des EGF-Rezeptors oder des

Prolaktin-Rezeptors. Durch dieses System sollte die selektionierende Wirkung der Signaldomäne kontrollierbar an- und abschaltbar durch Zugabe bzw. Wegnahme des chemischen Moleküls AP20187 gemacht werden. Es hat sich gezeigt, dass die Überexpression von EGFR-lentiviraler Vektoren zu unkontrollierbaren Vermehrung von transduzierten murinen BaF/3 Zellen führte. Deshalb wurde dieses Selektionsmodell als nicht anwendbar erachtet. Im Gegensatz dazu führte die Transduktion des PrlR-lentiviralen Konstruktes zu einer deutlichen Proliferationssteigerung der AP20187-behandlten BaF/3 Zellen. Darüber hinaus konnte dieser Proliferationsvorteil durch Entnahme des AP20187 wieder abgeschaltet werden. Weiterhin wurde ein neuer bizistronischer retroviraler Vektor, bestehend aus dem therapeutische Gen $gp91^{phox}$ und dem selektionsvermittelndem PrlR-Konstrukt, kloniert. Es wurde nachgewiesen, dass dieses PrlR-Fusionsprotein, welches keine membranverankernden Sequenzen des Prolaktinrezeptors mehr besitzt, hauptsächlich im Zytoplasma der Zelle lokalisiert ist. Möglicherweise verursacht das aktivierte PrlR-Konstrukt gesteigerte Proliferation und Stammzellselektion durch Aktivierung des STAT5-Signalweges. Die vorgelegten Ergebnisse zeigen, dass nach Expression des PrlR-Konstruktes und Stimulation mit AP20187 ein gesteigerter STAT5-Phosphorylierungsstatus und eine vermehrte Bindung von STAT5 an Promotorsequenzen des STAT5 Zielgens ß-Casein in BaF/3 Zellen resultieren. Auch Gen-modifizierte murine Stammzellen können unter AP20187 Stimulation *ex vivo* um den Faktor 20 bis 100 expandiert werden, ohne ihre Stammzelleigenschaften und die Fähigkeit, zu allen Blutzellen der verschiedenen Reihen beizutragen, zu verlieren. Um ferner eine mögliche onkogene Eigenschaft der PrlR-Konstrukt-Aktivierung auszuschließen, wurde Polyklonalität der selektionierten Zellen durch LM-PCR-Methode nachgewiesen. In anschließend durchgeführten Maus-Transplantations-experimenten konnten *ex vivo* expandierte Knochenmarkzellen das Knochenmark von tödlich bestrahlten Mäusen wiederherstellen. Diese Ergebnisse zeigen, dass eine ausreichende Anzahl an frühen Stammzellen in der AP20187 stimulierten Zellenpopulation vorhanden war, um das Blutsystem der tödlich bestrahlten, myeloablatierten Empfängermäuse wiederherzustellen. Die Tatsache, dass diese Mäuse überlebt haben und Gen-modifizierte Zellen in Knochenmark, Milz wie auch peripherem Blut gefunden wurden, legt nahe, dass die *ex vivo* Selektion auch auf früher Stammzellebene stattgefunden haben muss. Außerdem konnten Knochenmarkzellen von 3 der 5 Mäuse *ex vivo* wieder unter AP20187 Stimulation deutlich vermehrt und re-selektioniert werden. Somit tragen die Gen-modifizierten murinen Stammzellen zur Rekonstitution der Hämatopoese bei.