Targeted cell entry of lentiviral vectors

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1 Introduction

1.1 Gene transfer into eukaryotic cells by lentiviral vectors

Lentiviral vectors allow stable long-term transgene expression in non-dividing cells and tissues. This property has made them ideal gene delivery vehicles for research and therapeutic applications (Cockrell and Kafri, 2007), including clinical trials (Levine et al., 2006). However, further efforts in vector design are required to improve safety and efficacy of lentiviral mediated gene transfer. Special attention has to be given to measures that restrict gene transfer to the cell type relevant for a particular therapeutic application. Ideally, gene transfer into non-target populations is already restricted at the step of cell entry. However, an effective and universally applicable system for cell entry targeting of lentiviral vectors is still not available. Therefore, this thesis deals with the development of an efficient cell entry targeting system for lentiviral vectors.

1.1.1 Lentiviral vectors

Lentiviral vectors are derived from lentiviruses which belong to the family *Retroviridae*. Lentiviruses, with their most famous member the human immunodeficiency virus-1 (HIV-1), are enveloped viruses of about 100 nm in diameter with a diploid, positive sense, single stranded RNA genome of 7 to 13 kb per monomer. They replicate through a DNA intermediate that becomes integrated into the host cell genome (Goff, 2007). The envelope, consisting of host cell membrane and viral envelope proteins (Env), surrounds the capsid, a protein core, harbouring the viral replication enzymes and the RNA genome (Figure 1). The Env protein interacts specifically with the viral receptor(s) on the host cell surface, which triggers conformational changes in Env resulting in pH-independent fusion of the viral and the cellular membrane. Therefore, the host range (tropism) of a given lentivirus is determined by the Env protein.

The lentiviral genome consists mainly of the genes *gag/pol* and *env*. All structural proteins like the membrane associated matrix protein, the core forming capsid protein and the nucleocapsid protein, which binds to the viral RNA, are encoded in the *gag*

region. The *pol* gene provides all viral enzymes, namely the protease, the reverse transcriptase and the integrase, whereas the *env* gene encodes the Env.

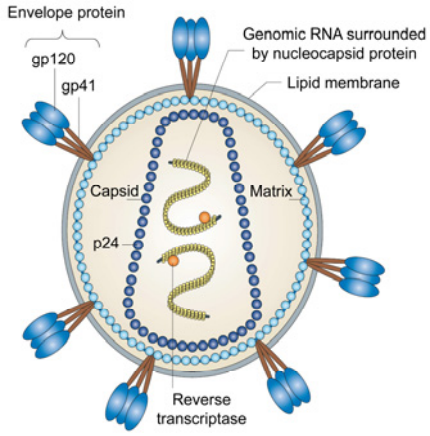

Figure 1: Schematic representation of HIV-1 as an example for a typical lentivirus. The following features are indicated: the membrane associated matrix, the capsid composed of the capsid protein p24 harbouring the viral RNA genome, associated with nucleocapsid proteins as well as the reverse transcriptase and the surrounding cell-derived lipid membrane with inserted viral envelope proteins, consisting of the surface unit (gp120) and the non-covalently bound transmembrane unit (gp41). (modified after Karlsson Hedestam et al. 2008, Nature Reviews Microbiology (Karlsson Hedestam et al., 2008)).

The viral genome is flanked by two identical long terminal repeats (LTRs) which carry the promoter, the transcription termination, the poly-adenylation signals and at the ends the attachment sites necessary for viral integration. The encapsidation signal psi (Ψ) mediates the packaging of the genomic RNA into viral particles. This genome structure is common to all of the seven genera of the *Retroviridae* family, to which also the simple γ-retroviruses with their prototype murine leukaemia virus (MLV) belong. But in contrast to γ-retroviruses, lentiviruses like HIV-1 have additional accessory genes, namely *tat*, *rev*, *nef*, *vif*, *vpu* and *vpr* (Freed and Martin, 2007) (Figure 2).

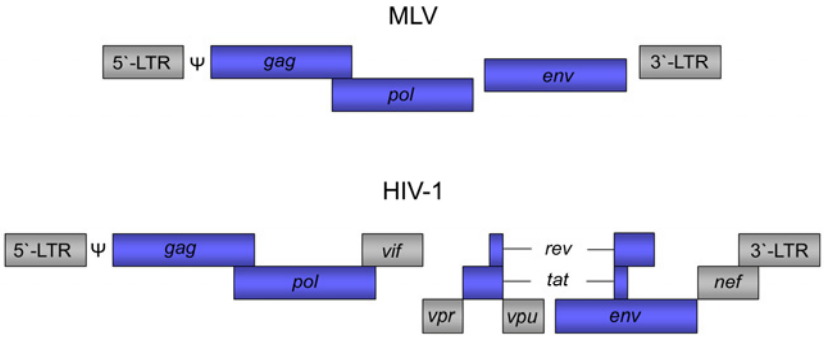

Figure 2: Schematic representation of the MLV and HIV-1 genome. LTR: long terminal repeat, Ψ: encapsidation signal psi (modified after Breckpot et al. 2007, Gene Therapy (Breckpot et al., 2007)).

An important feature of lentiviruses, which further discriminates them from γ-retroviruses, is their ability to infect not only dividing but also non-dividing and terminally differentiated cells (Lewis et al., 1992). They do not need the mitotic dissolution of the nuclear membrane, instead they most likely rely on the cellular nuclear import proteins to pass through the nuclear pore (Bukrinsky, 2004). An exception are e.g. quiescent primary human lymphocytes that require a minimal stimulation with cytokines or other factors to become transduced by lentiviral vectors (Korin and Zack, 1998; Serafini et al., 2004; Unutmaz et al., 1999).

The main advantages in using retroviral vectors in gene therapy, which means gene delivery into cells with the purpose of a therapeutic benefit, are efficient gene transfer and their ability to integrate the foreign gene into the host`s chromosome thereby achieving transmission of the transgene to the progenies of the modified cells. Due to their relative simple genome structure (Figure 2) first retroviral vectors were derived from MLV (Miller, 1997). Accordingly, MLV derived vectors were also the first to be used in human gene therapy trials for the efficacious treatment of so far incurable inherited diseases, as e.g. severe combined immunodeficiency (Cavazzana-Calvo et al., 2000; Cavazzana-Calvo and Fischer, 2007). However, because of their ability to mediate gene transfer into mitotically inactive cells, like neurons, lentiviral vectors are becoming more and more popular (Cockrell and Kafri, 2007). In contrast to lentiviruses, lentiviral vectors have packaged therapeutic or marker genes instead of the viral genome. Consequently, they are replication deficient, which means that they do not form progeny viruses, but are able to transfer any type of genetic information into mammalian cells and to integrate it into the cellular genome. This process is called transduction, in contrast to infection mediated by replication competent viruses (Figure 3) (Buchholz et al., 2008).

To generate lentiviral vectors basically three components must be provided in so called packaging cells: a transfer vector consisting of the gene of interest, the Ψ-site and the two LTRs and additionally the core and the envelope proteins to form particles that have packaged the transfer vector (Figure 3b). To avoid the occurrence of replication competent lentiviruses through recombination events (Hu and Temin, 1990), the genes encoding the core (*gag*/*pol*) and the envelope proteins (*env*) are usually split onto two separate plasmids that both lack a functional Ψ-site, for which reason they are not packaged into the vector particles. Since for the generation of lentiviral vectors, from all accessory proteins, only Tat and Rev are essential

(Zufferey et al., 1997), all other accessory proteins are deleted from the packaging plasmid, just leaving *gag*/*pol*, *tat* and *rev* (Figure 3b). Tat activates the LTRs leading to more efficient viral RNA production and Rev interacts with the Rev responsive element of the viral RNA in order to deliver unspliced viral RNA from the cell nucleus to the cytoplasm (Nekhai and Jeang, 2006). Different designs are possible for the transfer vector, e.g. the transcription of the transfer gene can be directly under the control of the viral LTRs or under the control of any additional heterologous promoter of interest. Thereby, insertions of up to a total size of about 7.5 kb are possible (Verma and Somia, 1997).

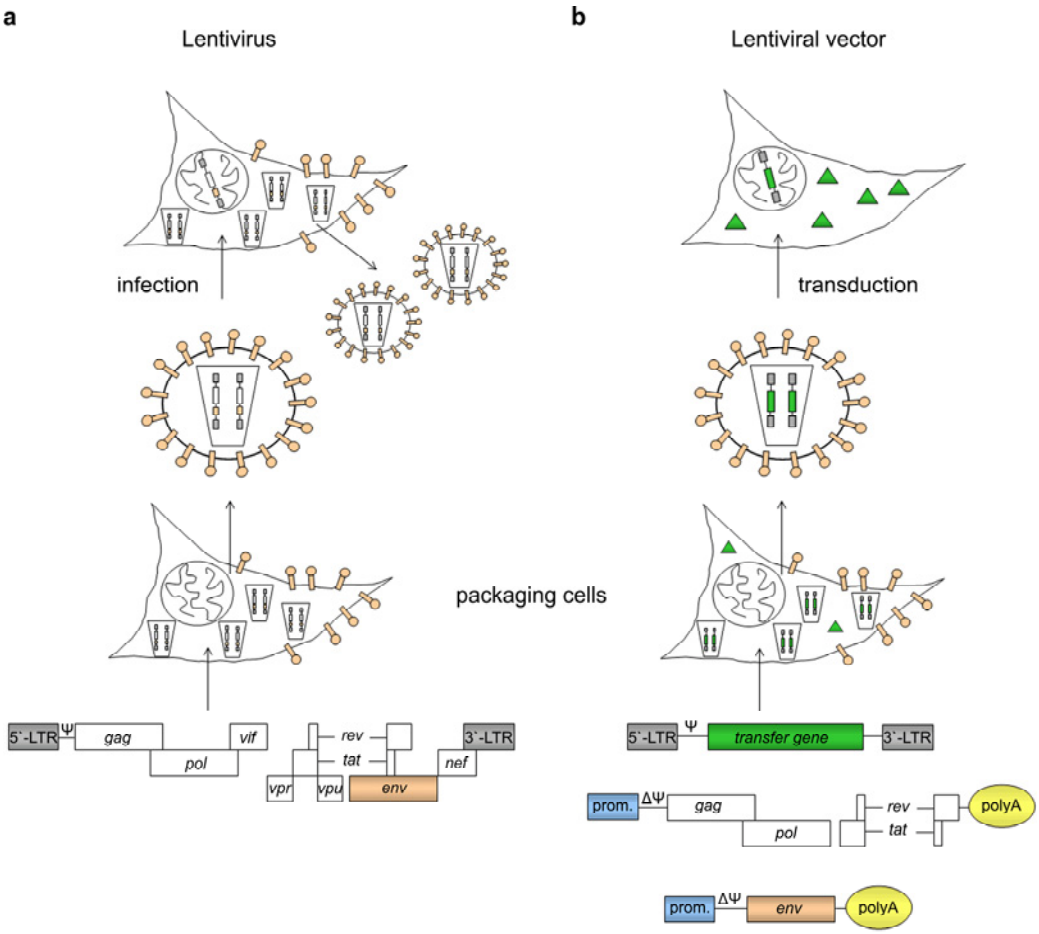

Figure 3: Lentivirus versus lentiviral vector. (a) After transfection of the whole virus genome into so called packaging cells, lentiviruses are released into the supernatant and can be used for infection of target cells. Due to the chromosomal integration of their genome into the target cell population, they enable the cells to produce viral progeny. **(b)** The DNA constructs required for lentiviral vector production are the transfer vector plasmid, encoding the transfer gene (top), the packaging plasmid harbouring the *gag*/*pol*, *tat* and *rev* genes under the control of a heterologous promoter (prom.) (middle) and the Env expression plasmid (bottom). The presence (Ψ) or absence (ΔΨ) of the packaging signal is indicated. Packaging cells transfected with these three plasmids release lentiviral vectors that have two RNA copies of the transfer vector packaged which can be transferred and after reverse transcription integrated into the target cell (transduction). The expressed protein encoded by the transfer gene is shown as green triangles. As none of the viral proteins is expressed in the target cells, no vector progeny is released. LTR: long terminal repeat; polyA: polyadenylation signal.

For particle formation, the two plasmids encoding the structural components and the transfer vector plasmid are co-expressed after transient or stable transfection in packaging cells. Then, the vector particles, which have packaged the transfer vector RNA but not the genetic information of the core and envelope proteins, bud from the cell membrane. Hence, their envelope consists of the cellular lipid-bilayer and viral Env, which is expressed on the cell surface (Figure 3b). After release into the cell culture supernatant, the vector particles can be used directly or upon concentration for transduction of target cells.

The choice of envelope glycoprotein determines which cell type will be preferentially entered. Lentiviral vectors offer the possibility to incorporate not only homologous glycoproteins but also heterologous ones derived from other enveloped viruses, an approach termed pseudotyping. Moreover, engineering of the glycoproteins can be performed to restrict the tropism to a target cell population of interest.

1.1.2 Pseudotyping of lentiviral vectors

The host range of lentiviral vectors can be easily altered by incorporation of heterologous glycoproteins into the lentiviral envelope, a process called pseudotyping. Such particles possess the tropism of the virus from which the envelope glycoprotein is derived (Cronin et al., 2005). Two mechanisms have been suggested by which the glycoproteins become incorporated into the lentiviral particles. In the passive model of incorporation no direct interactions between the glycoproteins and the viral core proteins are necessary, but sufficient amounts of glycoproteins must be provided at the site of budding (Pickl et al., 2001) and their cytoplasmic tail may not sterically interfere with viral assembly or virion morphology (Swanstrom and Wills, 1997). Accordingly, glycoproteins with short cytoplasmic tails, like the one of the vesicular stomatitis virus (VSV), are more likely passively incorporated than glycoproteins with a long cytoplasmic tail. In contrast, in the active model of incorporation the glycoprotein cytoplasmic tail interacts directly with the viral core proteins or indirect via a cellular factor, which leads to successful pseudotyping. Although the exact mechanism of glycoprotein incorporation is not fully understood, there is abundant evidence in literature supporting the importance of the cytoplasmic tail in lentiviral assembly (Cosson, 1996; Freed, 1998; Murakami and Freed, 2000). Not only Env proteins of the *Retroviridae* family can heterologously pseudotype

lentiviral vectors (Liu et al., 2004; Reiser et al., 1996; Stitz et al., 2000). Up to date, glycoproteins from almost any family of mammalian enveloped viruses have been functionally incorporated into lentiviral particles. Examples include, but are not limited to orthomyxoviruses, filoviruses, alphaviruses and baculoviruses (Cronin et al., 2005; McKay et al., 2006). There is also one example in which envelope proteins derived from a paramyxovirus were incorporated into lentiviral particles (Kobayashi et al., 2003). Among the first and still most widely used glycoprotein for pseudotyping of lentiviral vectors is the glycoprotein of vesicular stomatitis virus (VSV-G) (Cronin et al., 2005). Due to their high stability and broad tropism that covers amongst others all human cell types, such vectors have become effectively the standard for evaluating the efficiency of other pseudotypes.

In some cases, pseudotyping can only be achieved upon modifying the cytoplasmic tail of the protein of interest. For example, the relatively short 30-40 amino acids long cytoplasmic tails of mammalian γ- and δ-retroviral Env proteins harbour a 15-20 amino acid long carboxy-terminal peptide, named R peptide, that renders the Env protein fusion inactive unless being cleaved off by the viral protease during particle budding (Bobkova et al., 2002). While the R-peptide of the MLV Env becomes cleaved by the HIV-1 protease thus allowing straight forward pseudotyping of HIV-1 particles, failure of R-peptide cleavage was determined as main reason preventing pseudotyping of HIV-1 particles with the Env proteins of the gibbon ape leukaemia virus (GALV) or the RD114 virus (Merten et al., 2005; Christodoulopoulos and Cannon, 2001; Sandrin et al., 2004; Stitz et al., 2000). In an other example, the two glycoproteins of the paramyxoviral Sendai virus, namely fusion (F) and hemagglutinin-neuraminidase (HN) protein, became incorporated into a simian immunodeficiency virus (SIV) derived lentiviral vector after truncation of the F protein cytoplasmic tail and by addition of the cytoplasmic tail of SIV Env to the cytoplasmic tail of the HN protein, respectively (Kobayashi et al., 2003).

In most cases, the lentiviral tropism is broadened through pseudotyping and sometimes the heterologous glycoproteins allow a preferential gene transfer into specific tissues. For example envelope proteins of viruses that infect via the airway epithelia, like Ebola virus or influenza virus, are useful for the transduction of this kind of cells (Kobinger et al., 2001; Medina et al., 2003; McKay et al., 2006). But there are only few glycoproteins that allow selective transduction of a special cell population, like HIV-1 Env, which is specialised for CD4-positive T cells. Therefore, attempts to

engineer viral glycoproteins in terms of receptor usage were initiated over 15 years ago. However, this protein engineering task proved to be difficult, especially in respect of glycoproteins that combine the receptor attachment and membrane fusion function, such as the lentiviral Env or VSV-G (Buchholz et al., 2008; Sandrin et al., 2003; Verhoeyen and Cosset, 2004).

1.1.3 Envelope engineering of retroviral vectors

Since lentiviral vectors allow stable integration of a transgene and its propagation into daughter cells as well as the transduction of non-dividing cells, they are the most promising tools for gene therapy. But due to their genome integration, also serious adverse effects, like insertional mutagenesis, can occur (Loewen and Poeschla, 2005). Furthermore, is it often not desirable to express the therapeutic protein in healthy cells, especially when a suicide gene like the one of the herpes simplex virus thymidine kinase is transferred.

Therefore, altering receptor usage by envelope engineering has become an important research field. In principle, envelope engineering in contrast to envelope substitution is not restricted by the availability of viral glycoproteins evolved by nature but should allow the universal generation of retargeted vectors. Initial approaches to alter receptor usage consisted of the insertion of various ligand types, like growth factors, hormones, peptides or single-chain antibodies (scAb) in several locations on the retroviral Env such that instead of, or in addition to the natural receptor a chosen cell surface molecule mediates cell entry (Buchholz et al., 2008; Sandrin et al., 2003; Verhoeyen and Cosset, 2004). In particular scAbs are favorable polypeptides for display on glycoproteins. They are composed of the variable domains of the heavy and the light chain of an antibody molecule connected via a long glycine-serine linker, thus forming a single antigen binding site (Figure 4). By choosing a scAb with a target antigen expressed exclusively on the relevant cell type, in principle, redirection of gene transfer to any desired cell fraction should be possible.

Many of the above mentioned chimeric Env proteins folded correctly, were stably incorporated into the retroviral vectors and allowed efficient binding of the vectors to the respective cell surface molecule. However, cell entry into the desired cell population via the retargeted receptor was either absent or very inefficient, which is

most likely due to a loss of coupling between retargeted binding and fusion activity (Sandrin et al., 2003).

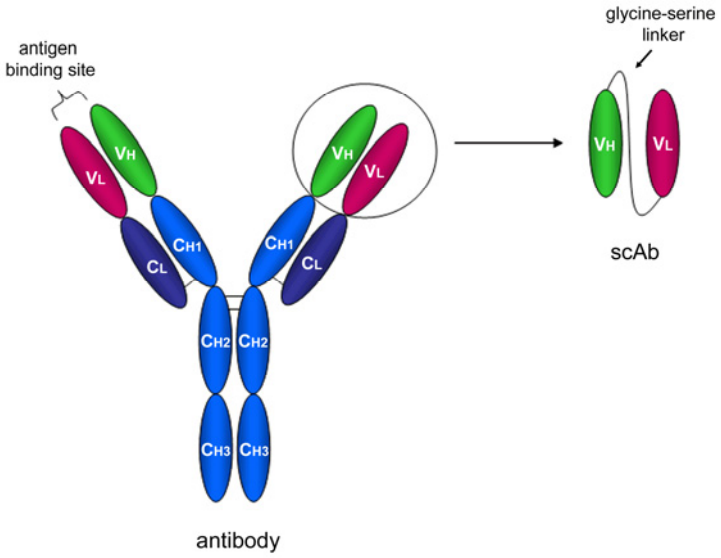

Figure 4: Schematic representation of an antibody and the derived single-chain antibody (scAb). An antibody of the IgG class consists of two identical heavy (H) and two identical light (L) chain polypeptides which are linked by disulfide bonds. The variable domains of the heavy and light chains (V_H and V_L) form the two identical antigen binding sites, whereas the constant regions (C_{H1-3} and C_L) mediate the immune effector functions of the antibody. The scAb consists only of one of the V_H and V_L domains linked by a long glycine-serine-linker.

Alternative targeting strategies have been developed based on specific requirements such as the surface expression or release of a special protease by the target cell (Hartl et al., 2005; Szecsi et al., 2006). Thereby, the displayed ligand is used as blocking domain that prevents cell entry via the native receptor. The linker between ligand and glycoprotein contains a protease cleavage site, so that cell entry proceeds upon cleavage and release of the blocking domain from the glycoprotein. Targeting is determined by the proteases expressed, e.g. matrix metalloproteases, which are over expressed in certain tumor cells. The disadvantage of this system is the limited availability of proteases restricted to distinct cell populations.

Recently, lentiviral vectors have been pseudotyped with engineered Sindbis virus glycoproteins unable to recognise their natural receptor and modified to either non-covalently bind a monoclonal antibody directed against a surface antigen, or to become co-incorporated into vector particles together with a complete antibody molecule (Morizono et al., 2005; Yang et al., 2006). In these approaches, the membrane fusion is mediated by the low pH in the endosomes after endocytosing of

the lentiviral vector upon antigen binding. Although such vector particles showed indeed promising targeting capabilities, these strategies suffer from the non-covalent linkage to the antibody, or the requirement for the targeted receptor to become endocytosed upon vector binding to activate the membrane fusion function of the Sindbis virus glycoprotein by low pH. In conclusion, until now no universal cell entry targeting strategy for lentiviral vectors that allows the redirection of gene transfer to any desired cell population is available.

1.2 Measles virus cell entry

Measles virus (MV), a member of the genus *Morbillivirus* in the family *Paramyxoviridae*, is the causative agent for measles, a disease characterised by fever, cough, conjunctivitis and a generalised maculopapular rash. The infection also causes a transient immunosuppression which begins just after the rash and lasts until the virus is cleared by the immune system after about two weeks. Apart from acute measles, in about one in 100,000 cases, a late (several years after acute infection) and severe disease, triggered by MV after asymptomatic persistence in the central nervous system, occurs and is known as subacute sclerosing panencephalitis (SSPE) (Yanagi et al., 2006). MV is an enveloped virus with a non-segmented, negative-strand RNA genome of about 16 kb which is tightly bound to nucleocapsid proteins and the viral polymerase complex. It also possesses the envelope associated matrix protein and the two envelope glycoproteins, namely the fusion (F) and the hemagglutinin (H) protein (Figure 5a) (Schneider-Schaulies and ter Meulen, 2002), which are of utmost importance for this thesis and will be described in detail later on.

MV was first isolated in 1954 using a primary culture of human kidney cells (Enders and Peebles, 1954). This first isolate, the attenuated Edmonston strain, is the progenitor of the currently used live vaccine strains. In 1993, CD46 (membrane cofactor protein; MCP), a type I transmembrane protein and complement regulatory molecule that is ubiquitously expressed on all human cells except erythrocytes, was shown to act as a cellular receptor for the Edmonston strain of MV. However, wildtype MV clinical isolates are unable to enter cells via CD46. Their receptor is the signaling lymphocyte activation molecule (SLAM or CD150) (Yanagi et al., 2006). SLAM, which can also be used by the vaccine MV strains as receptor, is a type I

transmembrane protein of the immunoglobulin superfamily. It is expressed on various cells of the immune system, namely immature thymocytes, memory T cells, B cells, macrophages and mature dendritic cells. Stimulation with antigens or mitogens upregulates SLAM expression on T and B cells (Veillette, 2006). SLAM signalling, together with T cell receptor engagement, regulates the production of T_H2 cytokines such as interleukin (IL)-4 and IL-13 by CD4-positive T cells (Yanagi et al., 2006).

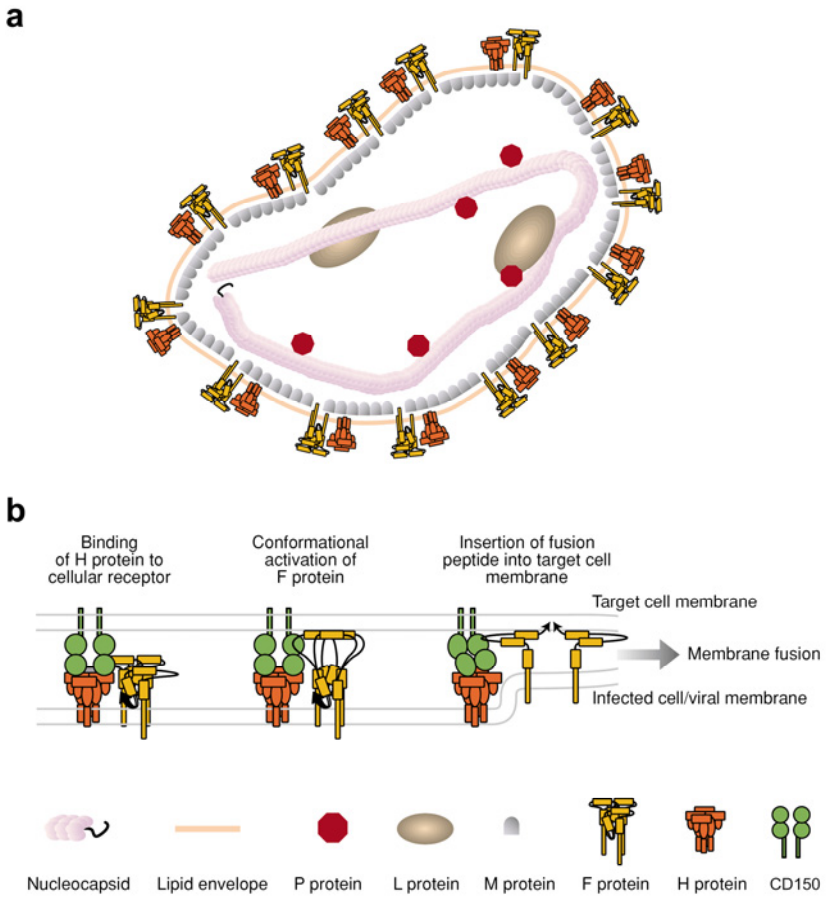

Figure 5: Schematic representation of the MV particle and the mechanism of membrane fusion. **(a)** MV is a pleomorphic particle of about 200 nm in diameter consisting of the RNA genome tightly bound to nucleocapsid proteins (N) and the viral polymerase complex, namely the large protein (L) and the phosphoprotein (P). The matrix protein (M) covers the inner side of the lipid envelope from which the two viral glycoproteins, the fusion (F) and the hemagglutinin (H) protein, project. The M protein is also thought to interact with the cytoplasmic tails of the viral glycoproteins. **(b)** Model of MV-induced membrane fusion and conformational changes within the F protein. The H protein mediates attachment to the cellular surface receptor and also provides a fusion helper function. Interaction of the H protein with its cellular receptor (CD150 is shown here) triggers conformational changes within the F protein that lead to insertion of the hydrophobic fusion domain (N-terminus of F; represented by arrowheads) into the cell membrane. Then intramolecular rearrangements leading to the formation of a coiled-coil structure within this subunit occur, caused by the interaction of two α-helical domains. In this way, the membranes to be fused are brought into close proximity, which is a prerequisite for the mixing of the outer leaflets (hemifusion) and subsequent fusion (modified after Schneider-Schaulies and ter Meulen 2002, Expert. Rev. Mol. Med. (Schneider-Schaulies and ter Meulen, 2002)).

The MV envelope glycoproteins are responsible for MV receptor interaction and adjacent membrane fusion: The F protein, a type I transmembrane protein, is synthesised as a precursor protein (F_0), and is proteolytically cleaved and activated by the furin protease in the *trans*-Golgi compartment into the disulphide-bonded F_1-F_2 heterodimer (Bolt and Pedersen, 1998). The F protein is responsible for fusion between the virus and the host cell membrane. In contrast, the H protein, a type II transmembrane protein, has a dual role: it mediates the attachment of the virus to the cell surface through interaction with the MV receptors and it provides a helper function for membrane fusion, for which residues in the membrane proximal part of its cytoplasmic tail are essential (Moll et al., 2002). The membrane fusion is pH-independent, takes place directly at the cell surface and requires a complex of an H protein tetramer and an F protein trimer (Plemper et al., 2001). Thus, in contrast to the lentiviral Env, which provides both, the fusion and the receptor binding function, these two mechanisms are separated on two proteins in MV.

After receptor binding of the H protein, a conformational change in H causes a subsequent conformational change within the F protein (Yanagi et al., 2006) which leads to the insertion of its hydrophobic fusion domain into the cell membrane and to pairing of two amphipatic α-helical domains, thereby bringing the membranes to be fused into close proximity (Figure 5b). Consequently, the cell and viral membrane fuse (Schneider-Schaulies and ter Meulen, 2002). The viral particle context is not required for membrane fusion as infected cells or cells only expressing the MV H and F glycoproteins on their cell surface can mediate cell-cell fusion, when at least one MV receptor is present. Thereby a multinucleate cellular mass, called syncytium, is produced (Herschke et al., 2007; Moll et al., 2002). In conclusion, MV, like lentiviruses, belongs to the virus class that mediates pH-independent fusion directly at the cell membrane which discriminates it from the viruses that depend on receptor-mediated endocytosis for productive entry into their host cell, such as Sindbis virus and VSV (Roche et al., 2008; Yang et al., 2006).

1.3 Engineering measles virus cell entry

In contrast to the lentiviral vector system, a very efficient cell entry targeting system was recently established for MV (Nakamura et al., 2005). With no loss in cell entry capacity this method seems to allow retargeting of MV to any desired cell type.

As described above, cells infected with MV mediate cell-cell fusion resulting in strong syncytia formation leading to cell death. Hence, attenuated vaccine MV is emerging as a promising oncolytic platform. To avoid unwanted damage to non-cancer tissues that would also become infected via CD46 and SLAM, the H protein was engineered to restrict and retarget MV cell entry (Nakamura et al., 2005). In a first step, SLAM and CD46 contact residues were mutated to generate an H protein that is blind for these receptors. The respective substitutions are R533A to restrict entry via SLAM and Y481A, S548L and F549S to ablate CD46 interaction. Then, different scAbs were fused to the H ectodomain (C-terminus). These modified H proteins were cloned into the MV genome to generate fully retargeted oncolytic MVs. Such retargeted MVs infect MV receptor-negative cell lines when these cells express the cognate cell surface antigen whereas they are unable to infect MV receptor-positive but antigen-negative cells (Nakamura et al., 2005). Thereby, the efficiency of retargeted cell entry is comparable to that of unmodified MV (Figure 6). Furthermore, retargeted MVs selectively spread through antigen-positive tumor cells *in vivo* (Hammond et al., 2001; Nakamura et al., 2005; Paraskevakou et al., 2007; Ungerechts et al., 2007).

As attenuated MV has shown therapeutic potential as a replicating oncolytic virus in models of non-Hodgkin`s lymphoma (Grote et al., 2001) also a scAb directed against CD20, which is overexpressed on most B cell lymphomas, was displayed on the MV H protein (Bucheit et al., 2003; Ungerechts et al., 2007). Although Bucheit et al. used an H protein as display platform that could still recognise the MV receptors, they demonstrated with MV receptor-negative but CD20-positive cell lines for the first time that MV entry can be mediated through interaction with a receptor with four membrane-spanning domains, like CD20 (Bucheit et al., 2003). The native MV receptors are single transmembrane domain proteins. MVs with targeted entry to CD20, which is exclusively expressed on B cells (Cragg et al., 2005), might represent a useful tool in therapy of lymphomas. Besides scAbs also ligands can be displayed on the MV H protein. For example, H proteins displaying on their ectodomain epidermal growth factor (EGF) mediated MV cell entry through the EGF receptor (EGFR), which is overexpressed on many cancer tissues (Schneider et al., 2000).

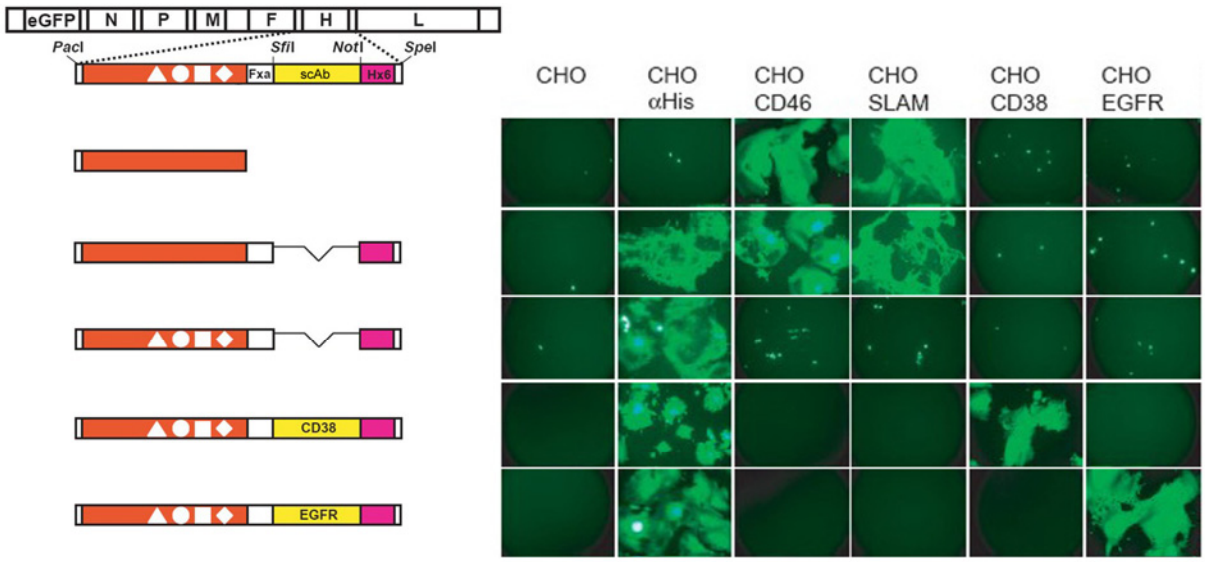

Figure 6: Retargeted MVs infect cell lines via the targeted receptor *in vitro*. (left) Schematic representation of the recombinant MV genome showing the modified H protein. The scAb, directed against CD38 or the epidermal growth factor receptor (EGFR), is flanked by *Sfi*I/*Not*I restriction sites and is displayed as a C-terminal extension of the mutated H glycoprotein. White triangle, circle, square and diamond represent Y481A, R533A, S548L and F549S mutations in the H protein that ablate entry via SLAM and CD46, respectively. Also indicated is a factor Xa (Fxa) cleavage site in front of the scAb and a six-histidine peptide (Hx6) at its C-terminus. Furthermore, the MV genome carries an additional transcription unit coding for the enhanced green fluorescent protein (eGFP) marker gene. (right) CHO cells that express no MV receptors and no targeted receptor and CHO transfectands positive for an anti-His antibody (αHis), CD46, SLAM, CD38 or EGFR were infected with the retargeted or control MVs and were photographed 2 d later. Obviously, the retargeted MVs infect MV receptor-negative cell lines when these cells express the respective cell surface antigen whereas they are unable to infect MV receptor-positive but antigen-negative cells (modified after Nakamura et al. 2005, Nature Biotechnology (Nakamura et al., 2005)).

1.4 Objective

The aim of this thesis was the development of an efficient and compliant cell entry targeting system for lentiviral vectors. It was hypothesised that the retargeting system of measles virus (MV) can be transferred to lentiviral vectors, by pseudotyping HIV-1 vector particles with the MV glycoproteins. To test this hypothesis, plasmids encoding the MV glycoproteins F and H, respectively, or a native receptor blind H protein with a displayed single-chain antibody (scAb) directed against the B cell surface marker CD20 were available.

For the establishment of MV-HIV pseudotypes, cytoplasmic tail truncation mutants of the H and F protein had to be screened, to identify variants that allow efficient pseudotyping of lentiviral vectors. Then, these MV pseudotyped HIV-1 particles should be characterised in terms of glycoprotein incorporation, mediation of stable

gene transfer and expression and receptor usage. Furthermore, the titers of these pseudotypes should be optimised on MV receptor-positive cell lines.

In a next step, retargeted lentiviral vector particles should be generated. Therefore, SLAM and CD46 blind H proteins with an optimally truncated cytoplasmic tail that allows incorporation into HIV-1 vector particles, displaying at their ectodomain either the epidermal growth factor (EGF) ligand or a scAb directed against CD20 had to be generated. Together with the optimally cytoplasmic tail truncated F protein HIV-1 vector particles retargeted to the EGF receptor or CD20 should be produced.

The targeting capability of these vectors should be determined on different target receptor-positive and -negative cell lines either in separate approaches or in mixed cell culture. Also the question if the CD20-retargeted vector can mediate selective cell killing should be addressed. As transduction of primary human cells is a crucial feature for a clinical application, finally, the ability of the CD20-retargeted HIV-1 vector particles to selectively transduce primary human B cells had to be investigated. Also unstimulated primary human B cells should be included to address if the new entry pathway via CD20 eventually enables transduction of quiescent B cells. This will be of special importance as until now, quiescent lymphocytes had been resistant to transduction by any type of retro- or lentiviral vector.

Such CD20-retargeted HIV-1 vectors will be of great use for a number of applications ranging from the genetic modification of B cells for investigating basic questions in immunology to therapeutic strategies such as the treatment of inherited B-cell disorders or lymphomas. Furthermore, as this novel targeting strategy for lentiviral vectors should be easily adaptable to many target molecules of interest by extending the H protein with appropriate ligands or scAbs, such retargeted vectors may improve safety and efficacy of lentiviral mediated gene transfer in gene therapy.

2 Material and Methods

2.1 Material

2.1.1 Chemicals and consumables

Unless otherwise noted, all used chemicals were obtained in p.a. quality from the companies Merck, Sigma-Aldrich, Roth, Fluka or Serva. The consumables for cell culture and molecular biology were obtained from the companies Greiner, Nunc, Eppendorf, BD™ or Sarstedt.

2.1.2 Enzymes, inhibitors, antibiotics and cytokines

enzymes	**source of supply**
restriction endonucleases	New England Biolabs
T4-DNA-ligase	New England Biolabs
Taq-DNA-polymerase	5 PRIME
PfuUltra™ High-Fidelity (HF)-DNA-polymerase	Stratagene
deoxyribonuclease I (DNase I)	Invitrogen

inhibitors	**source of supply**
azidothymidine (AZT)	Sigma-Aldrich
fusion-inhibiting peptide (FIP) Z-D-Phe-Phe-Gly-OH	Bachem
protease inhibitor cocktail complete	Roche

antibiotics	**source of supply**
ampicillin	Roche
geneticin (G418)	Gibco
puromycin	Sigma-Aldrich
penicillin	Biochrom AG
streptomycin	Biochrom AG

The used cytokines IL-2, IL-4 and IL-10 as well as CD40 ligand were obtained from R&D Systems.

2.1.3 Kits

kits	source of supply
QIAprep® Spin Miniprep Kit	Qiagen
EndoFree® Plasmid Maxi Kit	Qiagen
Jetstar 2.0 Mega Kit	Genomed
Geneclean® Turbo Kit	Q-Biogene
DNeasy® Blood and Tissue Kit	Qiagen
Dynal® B cell negative isolation Kit	Invitrogen

2.1.4 Antibodies and antisera

name	dilution application	source of supply
F431 rabbit anti-F polyclonal serum	1:1,000 western blot	R. Cattaneo, Mayo Clinic, USA
H606 rabbit anti-H polyclonal serum	1:2,000 western blot	R. Cattaneo, Mayo Clinic, USA
mouse anti-HIV-1 p24 mAb	1:1,000 western blot	ZeptoMetrix
HRP conjugated rabbit anti-mouse Immunoglobulins	1:2,000 western blot	DakoCytomation
HRP conjugated goat anti-rabbit Immunoglobulins	1:2,000 western blot	DakoCytomation
Y503 mouse anti-F mAb	1:100 FACS	R. Cattaneo, Mayo Clinic, USA
K83 mouse anti-H mAb supernatant of hybridoma K83	1:10 FACS	J. Schneider-Schaulies, University of Würzburg
R-Phycoerythrin (PE) conjugated anti-mouse IgG (whole molecule) $F(ab')_2$ fragment	1:50 FACS	Sigma-Aldrich
PE conjugated mouse anti-human CD19 mAb	1:10 FACS	DakoCytomation
PE-Cy5, FITC or PE conjugated mouse anti-human CD20 mAb	1:10 FACS	BD Pharmingen™
FITC conjugated mouse anti-human CD69 mAb	1:10 FACS	DakoCytomation
PE conjugated mouse anti-human CD69 mAb	1:10 FACS	BioLegend
PE conjugated mouse anti-human CD71 mAb	1:10 FACS	BD Pharmingen™
PE conjugated mouse anti-human CD86 mAb	1:10 FACS	BD Pharmingen™
PE conjugated mouse anti-human CD25 mAb	1:10 FACS	BD Pharmingen™
PE conjugated mouse anti-human CD34 mAb	1:10 FACS	EuroBioSciences

PE-Cy5, FITC or PE conjugated mouse $IgG_{2b,\kappa}$ isotype control	1:10 FACS	BD Pharmingen™
FITC or PE conjugated mouse $IgG_{1,\kappa}$ isotype control	1:10 FACS	BD Pharmingen™
PE conjugated mouse $IgG_{2a,\kappa}$ isotype control	1:10 FACS	BD Pharmingen™

2.1.5 Plasmids

name	characterisation	source of supply
peHcΔ14	shuttle vector with the coding region for MV H with a truncated cytoplasmic tail (ct) of 14 aa	R. Cattaneo, Mayo Clinic, USA (Cathomen et al., 1998)
p(+)MV-FcΔ24	full-length MV genomic plasmid with the coding region for F with a truncated ct of 24 aa	R. Cattaneo, Mayo Clinic, USA (Cathomen et al., 1998)
pE-Mo	full-length MoMLV genomic plasmid with the EGF ligand DNA fused to the N-terminal coding region of Env	C.J. Buchholz, Paul-Ehrlich-Institut (Buchholz et al., 1998)
glycoprotein expression plasmids		
pCG-H (10.2)	encodes MV H under control of the CMV promoter	R. Cattaneo, Mayo Clinic, USA (Cathomen et al., 1995)
pCG-F (10.1)	encodes MV F under control of the CMV promoter	R. Cattaneo, Mayo Clinic, USA (Cathomen et al., 1995)
pCG-HcΔ14	encodes MV H with a truncated ct of 14 aa under control of the CMV promoter	this thesis
pCG-HcΔ15 to pCG-HcΔ24	encodes MV H with a truncated ct of the indicated number of aa under control of the CMV promoter	A. Maisner, Philipps University of Marburg (Moll et al., 2002)
pCG-HcΔ21+A pCG-HcΔ24+4A pCG-HcΔ26+6A pCG-HcΔ30+10A	encodes MV H with a truncated ct of 21, 24, 26 or 30 aa and one, four, six or ten added alanine after the start methionine of the MV H protein	A. Maisner, Philipps University of Marburg (Moll et al., 2002)
pCG-FcΔ24	encodes MV F with a truncated ct of 24 aa under control of the CMV promoter	this thesis
pCG-FcΔ30	encodes MV F with a truncated ct of 30 aa under control of the CMV promoter	A. Maisner, Philipps University of Marburg (Moll et al., 2002)
pCG-Hmut-6His	encodes MV H with the four point mutations Y481A, R533A, S548L, F549S	R. Cattaneo, Mayo Clinic, USA
pCG-HmutΔ18	encodes MV H with the four point mutations Y481A, R533A, S548L,	this thesis

	F549S and a truncated ct of 18 aa	
pCG-HmutXαCD20-6His	encodes MV H with the four point mutations Y481A, R533A, S548L, F549S and the αCD20-scAb fused to its C-terminus	R. Cattaneo, Mayo Clinic, USA (Ungerechts et al., 2007)
pCG-H-αCD20 (10.7)	encodes MV H with the four point mutations Y481A, R533A, S548L, F549S, a truncated ct of 18 aa and the αCD20-scAb fused to its C-terminus	this thesis
pCG-H-αEGFR (10.8)	encodes MV H with the four point mutations Y481A, R533A, S548L, F549S, a truncated ct of 18 aa and the ligand EGF fused to its C-terminus	this thesis
pMD.G2 (10.5)	encodes VSV-G	D. Trono, Tronolab, Switzerland
pHIT123	encodes the Env of the Moloney MLV	(Soneoka et al., 1995)
	packaging plasmids	
pCMVΔR8.9 (10.3)	HIV-1 packaging plasmid	U. Blömer, University Hospital Kiel (Zufferey et al., 1997)
pHIT60	MLV packaging plasmid	A.J. Kingsman, University of Oxford, UK (Soneoka et al., 1995)
SIV10+	SIVmac packaging plasmid	F.-L. Cosset, University of Lyon, France (Negre et al., 2000)
	transfer vector plasmids	
pHR`-CMV-GFP (10.4)	HIV-1 packagable vector encoding GFP under control of the CMV promoter	U. Blömer, University Hospital Kiel (Miyoshi et al., 1997)
pSEW (10.6)	HIV-1 packagable vector encoding GFP under control of the SFFV promoter	M. Gretz, Georg-Speyer-Haus (Demaison et al., 2002)
pS-CD34TK39-W (10.9)	HIV-1 packagable vector encoding the CD34TK39 fusion protein under control of the SFFV promoter	this thesis
pM71tCD34tk39m	MPSV packagable vector encoding the CD34TK39 fusion protein under control of the viral LTRs	M. Gretz, Georg-Speyer-Haus (Junker et al., 2003)
pSFG-eGFP	MLV packagable vector encoding GFP under control of the viral LTRs	A. Schwantes, Paul-Ehrlich-Institut
GAE-sffv-gfp-wpre	SIVmac packagable vector encoding GFP under control of the SFFV promoter	D. von Laer, Georg-Speyer-Haus

2.1.6 Oligonucleotides

All oligonucleotides were synthesised from the company Eurofins MWG Operon.

name	5`→ 3`sequence
PR-CD34TK-for	AGGCGCGCCAGTCCTCCGATTGACTGAGTCGCCCGGGGG GGATCCACCGGTCGCCACCATGCCGCGGGGCTGGACC
PR-CD34TK-rev	GTGCCTGCAGGTCAGTTAGCCTCCCCCATC
ALUs	AAACCCACGCATGACACAACACTG
HIV-AluPCRas	CGGGCGCCACTGCTAGAGATTTT
SEW-LTR1s	ACTGGAAGGGCTAATTCACTCC
SEW-LTR1as	TGCTAGAGATTTTCCACACTGACT
β-Act-for	ATGATATCGCCGCGCTCGTCGTC
β-Act-rev	TTCTCGCGGTTGGCCTTGGGGTTCAG

2.1.7 Bacterial strains and culture media

name	genotyp	source of supply
Top10	F^-, *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), Φ80*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *ara*D139, Δ(*ara-leu*)7697, *gal*U, *gal*K, *rps*L (Str^R), *end*A1, *nup*G	Invitrogen

Luria-Bertani (LB) medium

Bacto-Trypton	1.0% (w/v)
yeast extract	0.5% (w/v)
NaCl	1.0% (w/v)
pH 7.0	

S.O.C. medium (Invitrogen)

Tryptone	2.0% (w/v)
yeast extract	0.5% (w/v)
NaCl	10 mM
KCl	2.5 mM
$MgCl_2$	10 mM
$MgSO_4$	10 mM
glucose	20 mM

2.1.8 Cell lines and culture media

name	characterisation	source of supply
HEK-293T	human embryonic kidney cell line genetically engineered to express the large T antigen	ICLC HTL04001
HT1080	human fibrosarcoma cell line	ATCC CCL-121
HT1080-CD20	human fibrosarcoma cell line genetically engineered to express CD20	R. Cattaneo, Mayo Clinic, USA
CHO-K1	chinese hamster ovary cell line	ATCC CCL-61
CHO-SLAM	chinese hamster ovary cell line genetically engineered to express	Y. Yanagi, Kyushu University, Japan

	human SLAM	
CHO-CD46 (previously termed CHO-BC1)	chinese hamster ovary cell line genetically engineered to express human CD46	C.J. Buchholz, Paul-Ehrlich-Institut
CHO-CD20	chinese hamster ovary cell line genetically engineered to express human CD20	R. Cattaneo, Mayo Clinic, USA
CHO-EGFR	chinese hamster ovary cell line genetically engineered to express human EGFR	R. Cattaneo, Mayo Clinic, USA
A-431	human epidermoid carcinoma cell line	ATCC CRL-1555
Daudi	human Burkitt`s lymphoma cell line	ECACC 85011437
Raji	human Burkitt`s lymphoma cell line	ATCC CCL-86
K-562	human chronic myelogenous leukaemia cell line	ATCC CCL-243
A3.01	continuous human T cell line	(Folks et al., 1985)

Culture medium for HEK-293T, HT1080, A-431 and CHO-K1 cells

Dulbecco`s modified Eagle medium (DMEM) obtained from Biochrom AG supplemented with 10% fetal calf serum (FCS; Biochrom AG) and 2 mM L-glutamine (Biochrom AG)

Culture medium for Daudi, Raji, K-562 and A3.01 cells

RPMI 1640 medium obtained from Biochrom AG supplemented with 10% FCS and 2 mM L-glutamine

Culture medium for CHO-SLAM cells

RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine and 0.5 mg/ml G418

Culture medium for CHO-CD46, CHO-EGFR, CHO-CD20 and HT1080-CD20 cells

DMEM supplemented with 10% FCS, 2 mM L-glutamine and 1.2 mg/ml G418 (CHO-CD46) or 1.0 mg/ml G418 (CHO-EGFR) or 3 µg/ml puromycin (CHO-CD20, HT1080-CD20)

2.2 Methods of molecular biology

2.2.1 Cultivation of bacteria

Liquid culture

Bacteria were grown in LB medium supplemented with 0.1 mg/ml ampicillin (LB_{Amp}) either at 37°C over night or at 25°C for 72 h and 180 rpm in a bacteria shaker (innova^{TM} 4200, New Brunswick scientific).

Culture plate

The cultivation on LB_{Amp} plates (1% (w/v) Bacto-Trypton, 0.5% (w/v) yeast extract, 1% NaCl, 50 µg/ml ampicillin, 1.5% (w/v) agar agar) was performed by applying bacteria onto the plates and subsequent incubation at 37°C or 25°C in a bacteria incubator (innova^{TM} 4200, New Brunswick scientific) until bacteria colonies were visible. The overgrown plates were stored for up to one month at 4°C.

2.2.2 Restriction and ligation of plasmid DNA

All DNA restrictions were performed using commercially available type II restriction endonucleases from New England Biolabs (NEB) according to the manufacturer's instructions.

Standard restriction reaction for preparative purposes

10 µg DNA
10 U (units) restriction enzyme (each, in case of a double digest)
5 µl 10x buffer (NEB buffer 1-4, corresponding to the used restriction enzyme/s)
5 µl 10x BSA (NEB, only if required by the applied enzyme/s)
ad 50 µl aqua bidest

The restriction sample was incubated for three hours or over night at the temperature optimum of the used restriction enzyme. In case of a double digest with restriction enzymes needing different NEB buffers, the optimal buffer for the double digest was chosen. In case of different optimal temperatures, the enzymes were added sequentially and incubation was performed at the respective temperature optimum for three hours each. Then, the restriction sample was applied to agarose gel

electrophoresis (2.2.5) and the desired DNA fragment was purified from the gel (2.2.6).

Standard restriction reaction for analytic purposes

1 µg DNA
5 U restriction enzyme (each, in case of a double digest)
2 µl 10x buffer (NEB buffer 1-4, corresponding to the used restriction enzyme/s)
2 µl 10x BSA (NEB; only if required by the applied enzyme/s)
ad 20 µl aqua bidest

Incubation was performed for 90 min or over night at the temperature optimum of the applied enzyme. Double digests were performed as described for preparative purposes. Then, the restriction sample was analysed by agarose gel electrophoresis (2.2.5).

Ligation of DNA fragments

Recombination of DNA is usually performed by ligation of two double stranded nucleic acid molecules exhibiting complementary overhangs or blunt ends, which is the case, when the DNA fragments of interest were digested with the same restriction endonucleases. For ligation, the T4-DNA-ligase, which catalyses the formation of phosphodiester bonds between the fragments under consumption of ATP was used. The following reaction mixture has been used as standard sample:

approximately 0.1 µg DNA (molar ratio of backbone to insert = 1:3)
1 µl 10x ligase buffer (NEB)
400 U T4-DNA-ligase
ad 10 µl aqua bidest

The reaction mix was incubated at 16°C over night. Then, the ligated DNA was directly used for the transformation of competent bacteria or was frozen at -20°C.

2.2.3 Generation and transformation of competent bacteria

Transformation of *E. coli* (K12-derived safety strains) is the method of choice to amplify plasmid DNA through cellular replication. For this purpose, bacteria have to

be pretreated in a special manner to become competent for introduction of foreign DNA.

For the generation of chemically competent Top10 bacteria 2.5 ml of an over night culture were used to inoculate 100 ml LB medium, which were subsequently incubated at 37°C and 180 rpm in a bacteria shaker (innova^{TM} 4200, New Brunswick scientific). Cells were allowed to grow to an OD_{550} of about 0.5 - 0.55 reaching the logarithmic growth phase. Then the culture was incubated on ice for 5 min, divided into two portions and pelleted at 4,000 rpm for 10 min at 4°C (Multifuge 3S-R, Heraeus). Next, the pellets were each resuspended in 20 ml TFB1 buffer (sterile filtrated solution of 30 mM KOAc, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerine, pH adjusted to 5.8 with HAc), incubated on ice for 5 min and once again pelleted as above. Subsequently the cells were resuspended each in 2 ml TFB2 buffer (sterile filtrated solution of 10 mM MOPS, 75 mM $CaCl_2$, 10 mM $RbCl_2$, 15% glycerine, pH adjusted to 6.5 using KOH-solution) and incubated on ice for 15 min. Afterwards the suspension was portioned á 100 µl into 1.5 ml reaction tubes and frozen at -80°C.

To transform the chemically competent *E. coli* bacteria, the cells were thawed on ice and approximately 50 ng DNA or the whole 10 µl ligation reaction (2.2.2) were added to one aliquot. After further incubation on ice for 30 min, a heat shock at 42°C for 45 sec was performed in a thermoblock (eppendorf). Then 500 µl of pre-warmed (37°C) S.O.C. medium were added and the sample before it was incubated at 600 rpm for 60 min at 37°C in a thermoblock (eppendorf). Then the bacteria suspension was applied to LB_{Amp} plates (2.2.1) and incubated at 37°C over night or at 25°C for 72 h.

2.2.4 Plasmid preparation

Preparation of plasmids from transformed bacteria was performed using the QIAprep® Spin Miniprep kit, the EndoFree® Plasmid Maxi kit or the Jetstar 2.0 Mega kit according to the manufacturer's instructions. The basic principle of these kits is binding of DNA to silica gel membranes in the presence of a high concentration of chaotropic salt or to anion exchange columns. Thus, all cellular compounds such as proteins can be washed away, whereas the DNA is retained within the columns from which it can be diluted with water or appropriate buffers.

For purification of low amounts of DNA (Miniprep), 5 ml LB_{Amp} medium were inoculated with one bacteria clone and incubated over night at 37°C (2.2.1). The next day, bacteria were harvested at 3,000 rpm for 10 min at RT (Multifuge 3S-R, Heraeus). The resulting pellet was used for the preparation of plasmid DNA according to the manufacturer's instructions of the QIAprep® Spin Miniprep kit.
For extraction of larger amounts of DNA (Maxiprep or Megaprep) 200 ml or 2 l LB_{Amp} medium were inoculated and cultivated over night at 37°C or for 72 h at 25°C (2.2.1). Bacteria were harvested at 4,000 rpm for 10 min at RT (Sorvall RC 26 plus). The resulting pellets were used for the preparation of plasmid DNA according to the manufacturer's instructions of the EndoFree® Plasmid Maxi kit or Jetstar 2.0 Mega kit. Finally, the concentration of the isolated plasmid DNA was determined photometrically (GeneQuant pro, Amersham Biosciences) at absorption A_{260}.

2.2.5 Agarose gel electrophoresis

Agarose gel electrophoresis allows the separation of DNA molecules by their size. Polymerised agarose acts like a molecular sieve, for which reason the negatively charged DNA migrates through agarose gels in a size dependent manner after applying an electric current.
For fragments >1 kb, 0.7% - 1% agarose gels were used, whereas 1.5% - 2% agarose gels were used for smaller fragments. The gels were produced by adding the corresponding amount of agarose to 100 ml TAE buffer (40 mM Tris-Acetat, 1 mM EDTA, pH 7.5) and heating of the emulsion in a microwave oven until the solid agarose became solved. Afterwards, 50 µg/ml ethidium bromide that intercalates into DNA strands and can be visualised under UV light, were added and the gel was casted into a tray, in which it polymerised within about 20 min.
DNA samples were mixed with 0.2 volumes 5x sample buffer (30% glycerine and 1% brome phenol blue and xylenxyanol in 5x TAE buffer) and applied to the gel. As marker, 1.0 µg 2-log ladder (NEB) was used. Electrophoresis was performed at 100 V for approximately 45 min in a Bio-Rad WIDE MINI-SUB® cell GT chamber. Afterwards the fragments were photographically documented under UV light. If desired, DNA fragments were isolated from the gel as described below.

2.2.6 Isolation of DNA fragments from agarose gels

Purification of DNA fragments from agarose gels was performed using the Geneclean® Turbo kit according to the manufacturer's instructions. This kit is based on binding of DNA to silica gel membranes in the presence of a high concentration of chaotropic salt. After electrophoretic separation, the DNA fragment of interest was cut out of the gel, transferred into a 1.5 ml reaction tube and purified according to the manual.

2.2.7 Nucleic acid sequencing

Nucleic acid sequencing was performed at the company Eurofins MWG Operon. For this purpose, DNA samples containing approximately 1 µg plasmid DNA were lyophilised in a Speedvac sc 100 (Savant) and sent via regular mail together with appropriate primers of 10 pmol/µl to the company.

2.2.8 DNase I digestion of vector particles

Vector particles used for the transduction of adherent eukaryotic cell lines or primary human B cells, from which afterwards genomic DNA was isolated, were incubated with DNase I in advance. This way plasmid DNA from the vector particle producing cells that might be attached to the vector particles was digested and could not lead to false positive results in PCR of the isolated genomic DNA. The following reaction mixture has been used as standard sample:

30 µl vector particles
4 µl 10x DNase I reaction buffer (Invitrogen)
1 U DNase I (Invitrogen)
ad 40 µl PBS (Biochrom AG)

The reaction sample was incubated for 1 h at RT and then directly used for transduction (2.3.8; 2.3.9).

2.2.9 Isolation of genomic DNA

Genomic DNA was isolated from adherent eukaryotic cell lines or primary human B cells using the DNeasy® Blood and Tissue kit according to the manufacturer's instructions. The kit is based on binding of DNA to silica gel membrane columns in the presence of a high concentration of chaotropic salt. Thus, DNA is retained, whereas all other cellular components are washed away.
Adherent cells were trypsinised (PBS (Biochrom AG), 100 mM EDTA, 0.25% Trypsin-Melnick) within the well of a 48 well plate and the resulting cell suspension was centrifuged at 3,000 rpm, RT, for 3 min (Heraeus Fresco 17). Then, the cells were resuspended in 1 ml PBS (Biochrom AG) and centrifuged as described above. Approximately $2x10^5$ - $5x10^5$ detached cells or primary human B cells (centrifuged as described above) were resuspended in 200 µl PBS and applied to the purification procedure described in the manual with the following modification: For elution of the DNA from the columns 100 µl of buffer AE were used instead of 200 µl to increase the DNA concentration. Finally, the concentration of the DNA was determined photometrically (GeneQuant pro, Amersham Biosciences) at absorption A_{260}.

2.2.10 Polymerase chain reaction (PCR)

PCR allows the amplification of specific DNA sequences from different origins, such as plasmid, genomic or complementary DNA (Mullis and Faloona, 1987; Saiki et al., 1985). The amplified fragment can be used for further molecular biological methods. One of the most used DNA-dependent DNA-polymerases is the thermally stable *Taq*-DNA-polymerase isolated from *Thermophilus aquaticus*. Using appropriate buffers, oligonucleotides (primers), deoxynucleotides and cycling conditions, the *Taq*-DNA-polymerase can amplify a DNA fragment (template) bordered by the forward and reverse primer in an exponential manner. A typical PCR cycle contains a denaturising step at 94°C or 95°C, leading to the dissociation of the double stranded template. The following hybridisation step allows primer annealing to the complementary sequences on the single stranded template. The hybridisation temperature T_H is adjusted according to length and G/C-A/T content of the primers. It can be calculated roughly corresponding to the Wallace rule (Suggs et al., 1981): $T_H = 4x(G+C) + 2x(A+T) - 5$. After hybridisation, DNA elongation is performed at the

temperature optimum of the used DNA-polymerase. The elongation time is chosen according to the length of the amplificate, for the DNA-polymerases used in this thesis approximately 1 min / 1 kb. By repeating this cycle sequence, the template is amplified in an exponential manner.

In this thesis, the *Taq*-DNA-polymerase and the *PfuUltra*™ HF-DNA-polymerase were used. In contrast to the *Taq*-DNA-polymerase, the *PfuUltra*™ HF-DNA-polymerase has an 3´-5´exonuclease activity that reduces the error rate.

For amplification of the *cd34tk39*-gene a standard PCR mix (100 µl) containing the following ingredients and a standard PCR protocol was used:

0.5 µg template (pM71tCD34tk39m)			
10 µl 10x *PfuUltra*™ HF reaction buffer (Stratagene)	94°C	2 min	
10 pmol PR-CD34TK-for (forward primer)	94°C	20 sec	↰
10 pmol PR-CD34TK-rev (reverse primer)	55°C	30 sec	15x
200 µM of each deoxynucleotid	72°C	125 sec	↲
5 U *PfuUltra*™ HF-DNA-polymerase (Stratagene)	72°C	7 min	
ad 100 µl aqua bidest	subsequent cool down to 4°C		

To verify chromosomal integration of vector sequences transferred by $MV_{\alpha CD20}$-HIV vectors or VSV-G-HIV vectors, a two-step PCR amplification assay (two-step *Alu*-PCR) was performed (Chun et al., 1997).

For integration analysis, isolated genomic DNA from transduced and untransduced cells was used as template. In the first step, the primers ALUs and HIV-AluPCRas that bind to cellular genomic *Alu* and proviral *gag* sequences (ψ-site coding region in the transfer vector), respectively, were used, for which reason only after integration of the transfer vector sequences, amplificates were obtained. As these amplificates were of different sizes, due to different integration sites in the genomic DNA, a second transfer vector specific PCR was performed. For this purpose, in the second step, 1/10 of the PCR product from the first step was used as template and the transfer vector-specific primers SEW-LTR1s and SEW-LTR1as binding in the LTR were applied. Integration of vector DNA was indicated, if a significant increase in the transfer vector-specific signal was obtained by two steps of amplification, compared to that attained without a preceding *Alu*-PCR. β-Actin sequences were amplified to demonstrate the integrity of isolated genomic DNA. The following PCR protocols were performed:

1. step: *Alu*-PCR

90 ng genomic DNA	
10 pmol ALUs (forward primer)	95°C 3 min
10 pmol HIV-AluPCRas (reverse primer)	95°C 30 sec ←
5 µl 10x *Taq* buffer advanced (5 PRIME)	60°C 30 sec | 35x
2.5 U *Taq*-DNA-polymerase (5 PRIME)	68°C 2 min ←
200 µM of each deoxynucleotid	68°C 7 min
300 µM $MgCl_2$	subsequent cool down to 4°C
ad 50 µl aqua bidest	

2. step: transfer vector-specific PCR

5 µl PCR product from step 1	
or 9 ng genomic DNA	95°C 3 min
10 pmol primer SEW-LTR1s (forward primer)	95°C 30 sec ←
10 pmol primer SEW-LTR1as (reverse primer)	60°C 30 sec | 30x
5 µl 10x *Taq* buffer advanced (5 PRIME)	68°C 40 sec ←
2.5 U *Taq*-DNA-polymerase (5 PRIME)	subsequent cool down to 4°C
200 µM of each deoxynucleotid	
ad 50 µl aqua bidest	

β-Actin PCR

90 ng genomic DNA	
12.5 pmol β-Act-for (forward primer)	94°C 3 min
12.5 pmol β-Act-rev (reverse primer)	94°C 1 min ←
5 µl 10x *Taq* buffer advanced (5 PRIME)	58.8°C 1 min | 35x
2.5 U *Taq*-DNA-polymerase (5 PRIME)	68°C 2 min ←
200 µM of each deoxynucleotid	68°C 7 min
ad 50 µl aqua bidest	subsequent cool down to 4°C

All PCR reactions were performed using an Eppendorf Mastercycler gradient (Eppendorf) or DNA Engine Peltier Thermal Cycler (Bio-Rad) and were subsequently analysed by agarose gel electrophoresis (2.2.5) or stored at -20°C.

2.3 Cell culture and virological methods

2.3.1 Cultivation of cell lines

Cell lines were cultivated in the appropriate medium (2.1.8) in an incubator (BBD 6220, Heraeus) at 37°C, 5% CO_2 and saturated water atmosphere and were passaged twice a week. For this purpose, adherent cells were trypsinised (PBS (Biochrom AG), 100 mM EDTA, 0.25% Trypsin-Melnick) before an appropriate fraction of the resulting suspension was seeded into a new culture flask with fresh medium.

2.3.2 Freezing and thawing of cultured cells

For storage, cells were kept in liquid nitrogen.

Freezing

Adherent cells were trypsinised (PBS (Biochrom AG), 100 mM EDTA, 0.25% Trypsin-Melnick) and resuspended in the appropriate medium before they were, like suspension cells, centrifuged (800 rpm for 5 min at 4°C in a Varifuge 3.0 RS) to pellet the cells. These were then resuspended in 4°C cold freezing medium (50% FCS, 10% DMSO and 40% DMEM or 40% RPMI, steril filtrated), aliquoted á approximately $1.5x10^6$ cells into cryotubes and frozen in a 5100 Cryo 1°C Freezing Container (Nalgene) at -80°C. After 24 h the cells were transferred into liquid nitrogen.

Thawing

Cryotubes were incubated in a water bath at 37°C until the ice thawed. Then the cell suspension was immediately transferred into a 15 ml falcon tube with 15 ml pre-warmed medium. To exclude the cytotoxic DMSO, cells were subsequently centrifuged (800 rpm for 4 min at RT, Varifuge 3.0 RS), resuspended in fresh medium (2.1.8) and seeded into appropriate cell culture flasks.

2.3.3 Isolation of human peripheral blood mononuclear cells (PBMC)

PBMCs were isolated from human blood (Buffy Coats obtained from the Blutspendedienst Hessen, Frankfurt a. M.) by density centrifugation using Histopaque®-1077 (Sigma-Aldrich). For this purpose, in a 50 ml falcon tube, 15 ml cold Histopaque (4°C) were overlaid with 25 ml of a 1:1 mixture of human blood and PBS (Biochrom AG). Through centrifugation at 1,800 rpm and RT for 30 min (without break; Varifuge 3.0 RS) the red blood cells and granulocytes were pelleted. Above the Histopaque solution the lymphocytes, monocytes and macrophages concentrated within the whitish "lymphocyte-ring" above which the plasma resided. The "lymphocyte-ring" was collected into 50 ml falcon tubes (5 ml per falcon) and resuspended in 45 ml PBS per tube. A centrifugation step at 1,500 rpm, 10 min, RT (Varifuge 3.0 RS) followed. The cells were once more washed with 50 ml PBS (centrifugation at 1,200 rpm, 10 min, RT, Varifuge 3.0 RS) and then collected into one single 50 ml falcon tube, in which they were washed again with 50 ml PBS (see above). Next, remained erythrocytes were lysed through incubation in 10 ml 0.86% ammonium chloride solution at 37°C for 20 min. Then, the cells were washed twice with PBS as described above. Finally, they were resuspended in 50 ml PBS and counted in a Neubauer counting chamber, before they were applied for B cell isolation.

2.3.4 Isolation and activation of primary human B cells

Primary human B cells were isolated from fresh human PBMCs with the Dynal® B-cell negative isolation kit following the manufacturer's instructions. With this kit, untouched B cells were isolated from PBMCs by depleting T cells, natural killer cells, monocytes, granulocytes and erythrocytes. For this purpose, a mixture of monoclonal antibodies against unwanted cells was added to the PBMCs, followed by magnetic Dynabeads® that bind to the antibodies. Then, the cells were applied to a magnetic field, which catches the Dynabeads® bound cells, only leaving the B cells in solution. These were pelleted at 1,500 rpm and RT for 10 min (Varifuge 3.0 RS). Then, isolated B cells were either activated for 48 h in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 50 mg/ml streptomycin, 100 U/ml penicillin, 25 mM HEPES as well as 300 ng/ml CD40 ligand, 50 ng/ml IL-2, 10 ng/ml IL-4 and 10 ng/ml IL-10 or

used directly for transduction. In the latter case, cells were transferred into RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 50 mg/ml streptomycin, 100 U/ml penicillin, 25 mM HEPES without any cytokines. The respective media were also used during transduction (2.3.9).

2.3.5 Isolation of mixed primary human B and T cells

A mixture of primary human CD20/CD19 double-positive B and CD3-positive T cells was obtained from U. Köhl (Goethe-University Hospital Frankfurt a. M.) by immunomagnetic purification of peripheral blood stem cells from healthy donors. Briefly, apheresis products were harvested after granulocyte-colony stimulating factor stimulation and performed using a COBE Spectra (Gambro, Lakewood, USA). After platelet reduction, cells were labeled with anti-CD3 and anti-CD19 antibodies ($CD3^+$ $CD19^+$ Microbeads, Miltenyi/Biotec) for 30 min. Then, T and B cells were depleted immunomagnetically on the CliniMacs system (Miltenyi/Biotec) under GMP conditions according to the manufacturer's instructions (positive selection of B and T cells). While the fraction including stem cells, monocytes, natural killer and dendritic cells was used for haploidentical stem cell transplantation, the fraction consisting of a mixture of highly purified CD19-positive B and CD3-positive T cells was provided for this thesis. The lymphocytes were activated as described above (2.3.4) and used for transduction experiments (2.3.9). Informed consent was given by the donors and use of the samples has been approved by the Goethe-University Hospital Ethics Committee.

2.3.6 Production and concentration of vector particles

Vector particles were generated by transient transfection of HEK-293T cells with the packaging plasmid, the transfer vector plasmid and the glycoprotein expression plasmid/s. The plasmid DNA was introduced into the cells by calcium phosphate transfection. The procedure is based on slow mixing of HEPES-buffered saline (HBS) containing sodium phosphate with a $CaCl_2$ solution containing the DNA. A DNA-calcium phosphate co-precipitate forms, which adheres to the cell surface and is taken up by the cell, presumably by endocytosis.

Twenty-four hours before transfection, $6.5x10^6$ HEK-293T cells were seeded into a T75 flask. One hour before transfection the medium was replaced by 7 ml fresh medium (DMEM supplemented with 10% FCS and 2 mM L-glutamine). In total, 8 µg of the two plasmids encoding either a F or a H protein variant of MV (unless otherwise noted, for the experiments described in chapters 3.1 to 3.1.5 4 µg of each plasmid were used, apart from that, for production of MV-HIV vectors 1 µg H protein variant encoding plasmid and 7 µg pCG-FcΔ30 were used and for the production of targeting vectors 2 µg H protein variant encoding plasmid and 6 µg pCG-FcΔ30 were used), 10.72 µg of the packaging plasmid and 11.27 µg of the transfer vector plasmid were mixed. The plasmid DNA was filled up with H_2O (Sigma-Aldrich W-3500; this special water was also used for the generation of the $CaCl_2$ solution and the 2x HBS buffer) to 450 µl. Then, 50 µl 2.5 M $CaCl_2$ solution were added. While vortexing the DNA-$CaCl_2$ solution, 500 µl 2x HBS Buffer (281 mM NaCl; 100 mM HEPES; 1.5 mM Na_2HPO_4, pH 7.12) were added dropwise. Afterwards the solution was vortexed for an additional minute. Then, the precipitate was added to the cells. After 17 h medium was replaced by 12 ml fresh medium. Twenty-four hours afterwards, the cell supernatant, containing the pseudotyped lentiviral vector particles, was filtered (0.45 µm filter) and 300 µl thereof were directly used for transduction. The remaining supernatant was concentrated by centrifugation at 3,600 rpm and 4°C for at least 24 h (Varifuge 3.0 RS). The pellet was resuspended in 120 µl FCS-free medium or PBS (Biochrom AG). Vector particle aliquots were stored at -80°C. Vector particles pseudotyped with the VSV-G protein or the Env protein of Moloney MLV were produced by co-transfection of 4.55 µg of the plasmid pMD.G2 encoding VSV-G or pHIT123 encoding MoMLV Env, 8.45 µg of packaging plasmid and 13.00 µg of transfer vector plasmid.

2.3.7 Transfection of cells in six well plates

Transfection of cells in six well plates was also performed by calcium phosphate transfection. Twenty-four hours before transfection, $8x10^5$ cells were seeded into a single well of a six well plate. One hour before transfection, the medium was replaced by 1 ml fresh medium per well. In total, 8 µg plasmid DNA was added with H_2O (Sigma-Aldrich W-3500) to 180 µl. If needed DNA content was filled up to 8 µg with empty pCG-1 vector. The transfection was performed as described above (2.3.6) with

20 µl $CaCl_2$ solution and 200 µl 2x HBS buffer. Twenty-four hours later, transfected cells were analysed.

2.3.8 Transduction of adherent cell lines and titration of vector particles

For transduction about $5.8x10^4$ cells were seeded into a single well of a 48 well plate. On the next day, vector particle stocks were serially diluted in 1:10 steps with medium and a total of 250 µl of the dilutions, including 8 µg/ml of the cationic polymer polybrene (hexadimethrine bromide; Sigma-Aldrich) that enhances transduction efficiency by neutralising the charge repulsion between the vector particles and cell surface (Davis et al., 2004), were added per well. In advance the old medium was removed from the cells. After incubation for 2.5 - 3 h, the transduction reaction mix was replaced by 1 ml of fresh medium (2.1.8). After 48 - 72 h titers were calculated by determining the number of GFP-fluorescent cells under the fluorescence microscope. The counted cells per well were multiplied by the dilution factor and the factor 4.0 (used 250 µl x 4.0 = 1 ml) to obtain transducing units (t.u.)/ml. Alternatively, titers were determined by FACS analysis based on the indicated percentage of green fluorescent cells. For both methods dilutions were chosen, in which about 10-20% of the cells were transduced.

2.3.9 Transduction of suspension cells and primary human lymphocytes

Twenty-four hours before transduction, a 48 well plate was coated with the extracellular matrix molecule fibronectin (BD™) to enable co-adhesion of vector particles and target cells to fibronectin resulting in a large increase in local virus titer presented to the cells (Moritz et al., 1996). For this purpose, 500 µl 5 µg/ml fibronectin containing medium were added into a single well of the 48 well plate and incubated for 24 h at 37°C in an incubator (BBD 6220, Heraeus). For transduction, the fibronectin was pre-coated with half of the vector particles used for transduction diluted in 120 µl medium by centrifugation of the plate at 2,000 rpm and 4°C for 20 min (Multifuge 3S-R, Heraeus). The other half of the vector particles was diluted in 180 µl medium supplemented with 4 µg/ml protamine sulfate (Sigma-Aldrich) that is a

polycation exhibiting the same function as polybrene (2.3.8) but is less cell toxic. If not otherwise noted, $1.0x10^5$ cells of a suspension cell line or $5.0x10^4$ primary human lymphocytes were added before the transduction mix was added to the respective wells pre-coated with the vector particles. A centrifugation step at 1,000 rpm and 32°C for 90 min (Multifuge 3S-R, Heraeus) followed. After 1 h incubation at 37°C in a cell culture incubator (BBD 6220, Heraeus), 700 µl medium per well were added. 48 h or 72 h after transduction the cells were analysed by FACS. The used media are depicted in chapters 2.1.8 and 2.3.4.

2.3.10 Fluorescence activated cell sorting (FACS)

FACS analysis allows to assay cell populations for surface expression of proteins or GFP-expression. The method makes use of scattered light and fluorescence of GFP or fluorofor labelled antibodies directed against cell surface proteins. The cells are excited with a laser beam and the fluorescence that is dependent from the used fluorofore or GFP is detected.

FACS analysis was performed on the Galaxy flow cytometry system (Dako). For this purpose, adherent cells were detached by incubation with PBS-Trypsin solution (PBS (Biochrom AG), 100 mM EDTA, 0.25% Trypsin-Melnick). Approximately $1x10^5$ - $5x10^5$ cells in suspension were pelleted by centrifugation at 3,500 rpm, 4°C, 3 min (Heraeus Fresco 17). Then they were washed in 1 ml FACS washing buffer (PBS, 1% FCS, 0.1% NaN_3; centrifugation as described above) and incubated with the appropriate antibody/antibodies (2.1.4) diluted in PBS (Biochrom AG) for 30 min at 4°C in the dark. If the antibody was not directly labelled with a fluorofor, after two washing steps with FACS washing buffer, the cells were incubated with the labelled secondary antibody for 30 min at 4°C in the dark. After antibody incubation, the cells were washed twice in 1 ml FACS washing buffer and were finally fixed in 200 µl PBS/1% paraformaldehyde. If only GFP-fluorescence was detected, the cells were directly fixed after the first washing step. The samples were either directly use for FACS analysis or stored up to one week at 4°C in the dark. Data were analysed with the FloMax program version 2.0 (partec). To determine the rate of unspecific staining by the antibodies, cells were also incubated with isotype controls conjugated with the same fluorofor as the applied antibody. By using differently labelled antibodies, multiple cell surface proteins were detected simultaneously on one cell.

2.4 Methods of protein biochemistry

2.4.1 SDS-polyacrylamide-gelelectrophoresis

The SDS-polyacrylamide-gelelectrophoresis (SDS-PAGE) (modified after (Laemmli, 1970)) allows the separation of protein mixtures according to the molecular weight of the proteins. The basic principle includes binding of multiple molecules of the anionic detergent sodium dodecyl sulfate (SDS) via hydrophobic interactions to denatured protein molecules. That way, irrespective of their native charges, the denatured proteins acquire an excess of negative charge on their surface and can thus be applied to electrophoresis. For this purpose, the samples were loaded on polyacrylamid gels, which act like molecular sieves, similar to agarose gels (2.2.5).
To denature the proteins and allow binding of SDS, the samples were mixed with the appropriate amount of 2x urea sample buffer (5% SDS, 8 M urea, 200 mM Tris-HCl, 0.1 mM EDTA, 0.03% bromphenol blue, 2.5% dithiothreitol, pH 8.0) and heated for 10 min at 95°C. Then, they were loaded on 10% SDS polyacrylamid gels within a Bio-Rad Mini Protean II chamber filled with SDS running buffer (36 g glycine, 7.75 g Tris, 1.25 ml 20% SDS, ad 250 ml aqua bidest). As protein standard 10 µl of the Precision Plus Protein™ Standard Kaleidoscope™ marker from Bio-Rad were used.

Ingredients of a 10% SDS polyacrylamid gel

ingredients	**resolving gel**	**stacking gel**
30% acrylamide Roti-gel (Roth)	5 ml	1.65 ml
1 M Tris pH 8.8	5.85 ml	-
1 M Tris pH 6.8	-	1.25 ml
50% glycerine	1.3 ml	-
10% SDS	0.17 ml	0.1 ml
aqua bidest	2.68 ml	7 ml
Temed	12.5 µl	12.5 µl
20% APS	40 µl	40 µl

SDS-PAGE was performed at 80 V until the samples entered the resolving gel using a Bio-Rad Power Pac 200. Then, 130 V were applied until the dye front had left the resolving gel.

2.4.2 Western blot analysis

The western blot technique is a method which enables the transfer of proteins onto protein-binding surfaces such as nitrocellulose membranes (Towbin et al., 1979). This transfer, also termed blot, enables the specific visualisation of proteins of interest by immunostaining. Usually, proteins which have been separated by SDS-PAGE are applied to western blot analysis.

The transfer of the proteins from the SDS polyacrylamid gels onto nitrocellulose membranes (Amersham Biosciences) was performed electrophoretically within a Bio-Rad Trans-Blot® SD Semi-Dry transfer cell according to the manufacturer's instructions. In advance, six 3 mm Whatman filter papers (Schleicher & Schuell), membrane/s and SDS polyacrylamid gel/s were shortly incubated in transfer buffer (48 mM Tris, 39 mM glycine, 20 % methanol, ad 1,000 ml aqua bidest). After blotting at 110 mA for 90 min (1 blot) or 200 mA for 90 min (2 blots), unspecific binding sites were blocked with 10% horse serum in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.1% Tween 20) for 1 h at RT. For specific protein staining, antibodies diluted in 5% horse serum in TBST have been used (2.1.4). Staining was performed over night at 4°C. Then, the membranes were washed three times for 10 min at RT with TBST before they were incubated for 1 h at RT with the appropriate horseradish peroxidase (HRP) conjugated secondary antibodies (2.1.4) diluted in 5% horse serum in TBST. After washing as described above, detection of H and F proteins was performed using the SuperSignal West Pico Luminol kit (Pierce) according to the manufacturer's instructions. For detection of p24, the Amersham ECL Plus Western Blotting detection reagent (GE Healthcare) was used according to the manufacturer's instructions. The reagents contain a HRP substrate that emits light during conversion into the product by the HRP conjugated secondary antibodies. Hence the signal can be visualised, using chemiluminescence films (Amersham Biosciences). The latter ones were exposed to the substrate-incubated membrane/s for 5 sec - 30 min depending on the signal intensities.

2.4.3 Preparation of cell lysates

Thick grown cells in T75 culture flasks were washed ones with 5 ml ice-cold PBS (Biochrom AG). Then, carefully 1.6 ml ice-cold lysis buffer (50 mM Tris pH 8.0, 62.5

mM EDTA, 1% NP-40, 0.4% sodium-deoxycholate, 40 µl/ml protease inhibitor cocktail complete (25x)) was added to the cells (nuclei should remain intact on the flask bottom). After incubation for 5 min at 4°C, the lysate was transferred into a 2 ml reaction tube and centrifuged at 13,000 rpm, 4°C for 2 min (Heraeus Fresco 17) to get rid of the cell debris. The supernatant was transferred into a new 2 ml reaction tube and either directly used for Bradford assay or frozen at -20°C.

2.4.4 Bradford assay

To determine the protein concentration in cell lysates the Quick Start™ Bradford Dye reagent from Bio-Rad was used according to the manufacturer's instructions for 1 ml cuvetts with the exception that sample and 1x dye was mixed in 1:1 ratio. The Bradford assay is a protein quantification method that involves the binding of Coomassie Brilliant Blue G-250 dye to proteins (Bradford et al., 1976). The dye exists in three forms: cationic (red), neutral (green), and anionic (blue) (Compton and Jones, 1985). Under acidic conditions, the dye is predominantly in the doubly protonated red cationic form (A_{max} = 470 nm). However, when the dye binds to proteins, it is converted to a stable unprotonated blue form (A_{max} = 595 nm) (Sedmak and Grossberg, 1977). This blue protein-dye form was detected at 595 nm using a spectrophotometer (GeneQuant pro, Amersham Biosciences). With the absorption at 595 nm and a standard curve the protein concentration was determined. Cell lysates were diluted 1:100 before they were applied to the assay.

3 Results

This thesis describes the development of an efficient cell entry targeting system for lentiviral vectors, which allows in principle retargeting to every cell surface molecule of interest. The very competent MV targeting system was transferred to lentiviral vectors by pseudotyping them with the MV H and F glycoproteins. To achieve pseudotyping, in a first step, the cytoplasmic tails of the H and F proteins were specifically truncated. Then, for proof of principle, lentiviral vectors that entered selectively epidermal growth factor (EGF) receptor-positive or CD20-positive cells were generated by using native receptor blind H proteins with an optimally truncated cytoplasmic tail and the EGF ligand or a CD20-specific scAb displayed at their ectodomain.

3.1 Pseudotyping of retroviral vector particles with the MV glycoproteins

The first step for the generation of retargeted retroviral vector particles was the pseudotyping with MV glycoproteins. For this purpose, the MV H and F genes derived from the NSe variant of the recombinant attenuated MV vaccine strain Edmonston B were used (Cathomen et al., 1995).

For the production of lentiviral vector particles, HEK-293T cells are transiently transfected with a packaging plasmid, a transfer vector plasmid and an envelope expression plasmid (1.1.1 Figure 3). To adapt this procedure for MV pseudotyped particles, the commonly used VSV-G expression plasmid was initially substituted by equal amounts of the F and H expression plasmids pCG-F (10.1) and pCG-H (10.2). Besides lentiviral HIV-1 vector particles also retroviral MLV vector particles were generated. For the production of HIV-1 vectors the following plasmids were co-transfected into HEK-293T cells: the HIV-1 packaging plasmid CMVΔR8.9 (10.3), the green fluorescent protein (GFP) transfer vector plasmid HR´-CMV-GFP (10.4), pCG-F and pCG-H. For the production of MLV vectors pHIT60, pSFG-eGFP, pCG-F and pCG-H were co-transfected. In parallel, as positive control, HIV-1 and MLV vector particles pseudotyped with VSV-G were generated by co-transfection of the VSV-G encoding pMD.G2 (10.5) along with the packaging and transfer plasmids. After 48 h, vector particle containing cell supernatants were harvested as described (2.3.6) and

used for the transduction of HT1080 cells, either directly or after concentration (2.3.8).

Twenty-four hours after transfection all HEK-293T packaging cells that were co-transfected with pCG-F and pCG-H showed strong syncytia formation (Figure 7 and data not shown), which is typical for cells expressing both MV glycoproteins and at least one MV receptor.

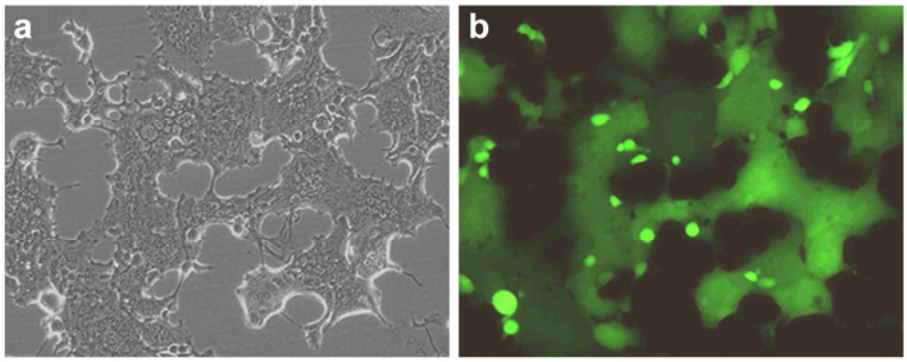

Figure 7: Syncytia formation of HEK-293T packaging cells. CD46-positive HEK-293T cells were co-transfected with the HIV-1 packaging plasmid CMVΔR8.9, the GFP transfer vector plasmid HR'-CMV-GFP and plasmids encoding the MV F and H glycoproteins, respectively. After 24 h, syncytia formation was detected by **(a)** reflected-light and **(b)** fluorescence microscopy (100x magnification).

HIV-1 as well as MLV vector particles that were released from these F/H transfected cells were unable to mediate any detectable *gfp*-gene transfer into the CD46-positive HT1080 cells, while particles pseudotyped with VSV-G reached titers of 10^9 transducing units (t.u.)/ml (Figure 8 and data not shown).

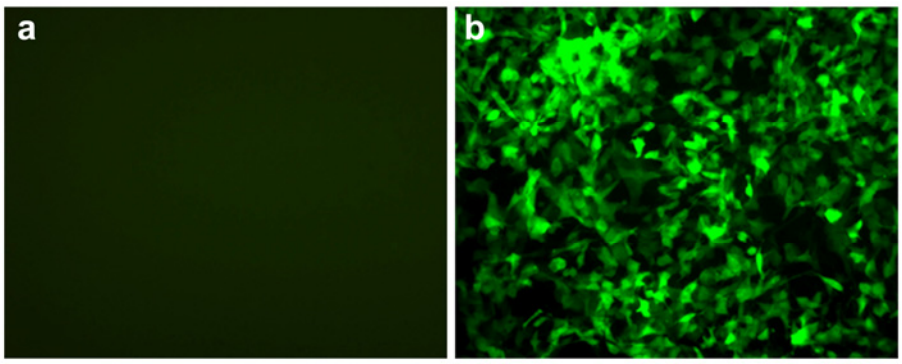

Figure 8: Transduction of HT1080 cells. Concentrated cell supernatants of HIV-1 vector particle producing HEK-293T cells were used for the transduction of HT1080 cells. After 48 h, cells incubated with HIV-1 particles produced in the presence of **(a)** the unmodified MV glycoproteins and **(b)** VSV-G, respectively, were analysed under the fluorescence microscope for GFP-expression (100x magnification).

Hence, although the MV H and F glycoproteins were able to form functional fusion complexes in HEK-293T packaging cells, they did not pseudotype HIV-1 or MLV vectors.

3.1.1 Truncation of the MV H and F protein cytoplasmic tails and screening for variants that pseudotype lentiviral vectors

As it was not possible to produce retroviral vectors pseudotyped with the unmodified MV glycoproteins, it was assumed that sequences in the cytoplasmic tail of the MV H and F proteins prevent pseudotyping. For this reason, MV glycoprotein cytoplasmic tail variants (Figure 9) that were characterised previously in terms of fusion function (Moll et al., 2002; Cathomen et al., 1998) were screened for their ability to pseudotype retroviral vectors. Thereby, all H protein variants with stepwise cytoplasmic tail truncations by less than 21 amino acids (aa) still own fusion helper function in a cell-cell fusion assay with CD46-positive Vero cells, whereas H proteins with truncations by more than 20 aa showed impaired cell-cell fusion when they were co-expressed with the F protein (Moll et al., 2002). In variants HcΔ21+A and HcΔ24+4A the fusion helper function was restored by substituting the critical aa with alanine (Moll et al., 2002) (Figure 9).

The plasmids pCG-HcΔ14 and pCG-FcΔ24, encoding MV H or F protein variants with cytoplasmic tails truncated by 14 and 24 aa, respectively, were generated by replacing the respective glycoprotein gene in pCG-H or pCG-F after *Pac*I/*Spe*I and *Nar*I/*Pac*I digestion, respectively, with the HcΔ14 or FcΔ24 coding region removed from peHcΔ14 and p(+)MV-FcΔ24 (Cathomen et al., 1998), respectively. The production of all other plasmids encoding cytoplasmic tail variants was described previously (Moll et al., 2002).

The 15 H protein variants carrying stepwise truncations and amino acid exchanges in their cytoplasmic tails and the two F protein variants (Figure 9) were screened in all combinations for their ability to pseudotype HIV-1 vector particles. Because it was speculated that the absence of syncytia formation in the packaging cells might be of advantage for pseudotype production, also the fusion helper function impaired H protein variants were included into the screen. Furthermore, the impairment of fusion helper function might be less pronounced for particle-cell fusion than for cell-cell fusion.

a

F	RGRCNKKGEQVGMSRPGLKPDLTGTSKSYVRSL*
FcΔ24	RGRCNKKGE*
FcΔ30	RGR*

b

		fusion helper function
H	MSPQRDRINAFYKDNPHPKGSRIVINREHLMIDR	+
HcΔ14	M.............PHPKGSRIVINREHLMIDR	+
HcΔ15	M.............HPKGSRIVINREHLMIDR	+
HcΔ16	M.............PKGSRIVINREHLMIDR	+
HcΔ17	M.............KGSRIVINREHLMIDR	+
HcΔ18	M.............GSRIVINREHLMIDR	+
HcΔ19	M.............SRIVINREHLMIDR	+
HcΔ20	M.............RIVINREHLMIDR	+
HcΔ21	M.............IVINREHLMIDR	-
HcΔ22	M.............VINREHLMIDR	-
HcΔ23	M.............INREHLMIDR	-
HcΔ24	M.............NREHLMIDR	-
HcΔ21+A	M.............AIVINREHLMIDR	+
HcΔ24+4A	M.............AAAANREHLMIDR	+
HcΔ26+6A	M.............AAAAAAEHLMIDR	-
HcΔ30+10A	M.............AAAAAAAAAAIDR	-

Figure 9: Overview on the MV F and H protein variants used for the pseudotyping screen. Amino acid sequences of the cytoplasmic tails of the **(a)** F protein and **(b)** H protein variants are shown. Note that H is a type II transmembrane protein. Presence (+) or absence (-) of the fusion helper function of each H variant as determined by Moll et al. (Moll et al., 2002) is indicated.

For the screen, pseudotyped HIV-1 particles were produced by co-transfection of HEK-293T cells with the HIV-1 packaging plasmid CMVΔR8.9, the GFP transfer vector plasmid HR`-CMV-GFP and the two plasmids encoding the modified H and F proteins, respectively. Thereby all combinations of MV H and F protein variants were assayed for pseudotype generation. Forty-eight hours after transfection, the vector particle containing cell supernatants were used directly or after concentration for the transduction of HT1080 cells and after further 48 h the respective titers were determined under the fluorescence microscope (2.3.8).

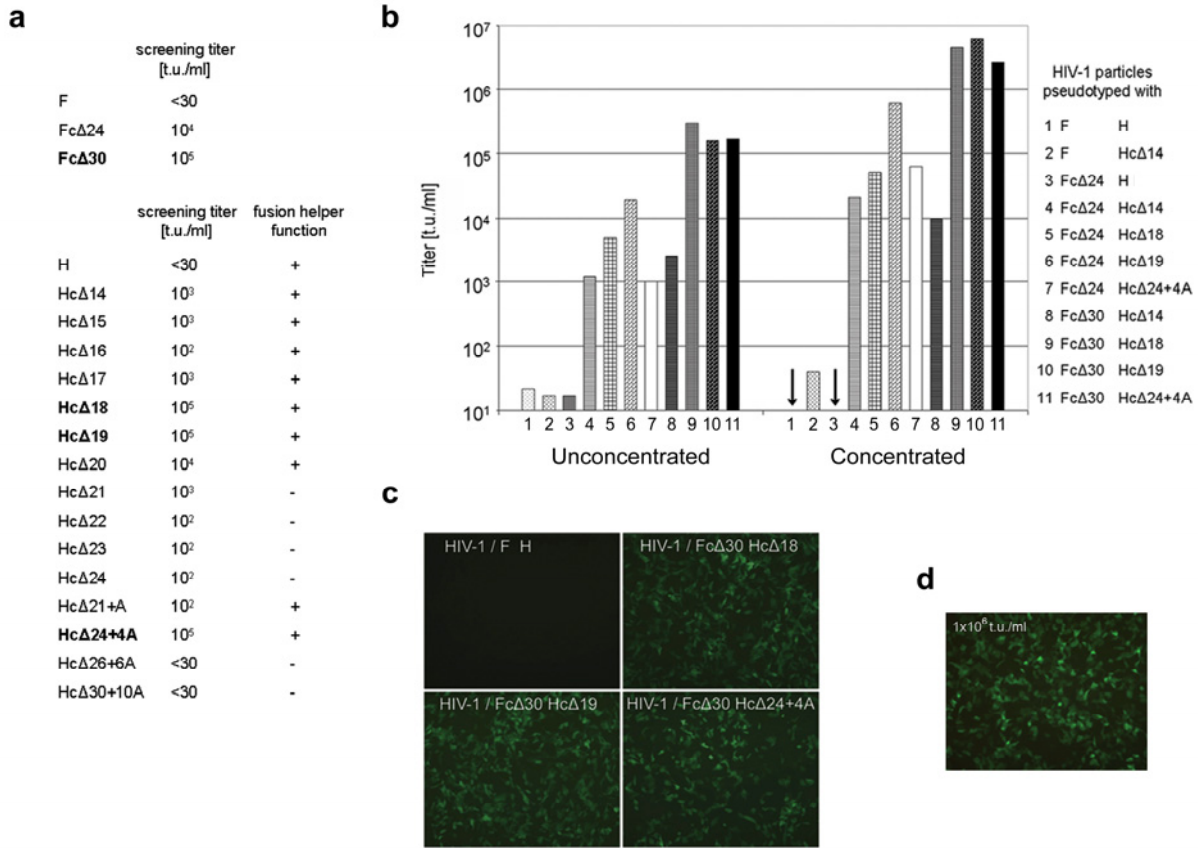

Figure 10: Screening for MV H and F protein variants that efficiently pseudotype lentiviral vector particles. HEK-293T cells were co-transfected with pCMVΔR8.9, pHR'-CMV-GFP and plasmids encoding the indicated F and H protein variant, respectively. After 48 h, cell supernatants were used for the transduction of HT1080 cells either directly or upon concentration and after further 48 h the respective titers were determined under the fluorescence microscope. **(a)** The best titer of unconcentrated pseudotyped HIV-1 vectors for each possible F/H combination that was tested is shown (screening titer [t.u./ml]). Presence (+) or absence (-) of the fusion helper function of each H variant as determined by Moll et al. (Moll et al., 2002) is indicated. Variants used for further studies are depicted in bold. **(b)** Screening titers of selected unconcentrated and concentrated pseudotype vectors are shown. The arrows indicate titers below 10 t.u./ml. **(c)** Representative pictures of HT1080 cells transduced by the indicated pseudotypes (concentrated particles) are shown (100x magnification) **(d)** HEK-293T cells were co-transfected with the SIVmac packaging plasmid SIV10+, the SIVmac transfer vector plasmid GAE-sffv-gfp-wpre and the two plasmids encoding the HcΔ18 and FcΔ30 protein variants, respectively. After 48 h, concentrated cell supernatants containing HcΔ18/FcΔ30 pseudotyped SIVmac vector particles were used for the transduction of HT1080 cells. A representative picture of *gfp*-transduced HT1080 cells is shown (100x magnification). Transduced cells were quantified by fluorescence microscopy and FACS analysis. The titer is indicated.

Twenty-four hours after transfection, all vector particle producing HEK-293T cells showed strong syncytia formation, except when an H protein variant with impaired fusion helper function (Figure 9) was used (data not shown). But only when the cytoplasmic tails of both glycoproteins were truncated, pseudotyped HIV-1 vector particles, mediating transfer of the *gfp*-gene into HT1080 cells, were produced (Figure 10a, b). Highest titers were obtained when the F protein cytoplasmic tail was truncated by 30 residues just leaving three aa, two of which being positively charged.

Among the H protein variants, there was a clear peak of optimal truncation when 18 or 19 residues were deleted (variants HcΔ18 and HcΔ19). Further truncation reduced titers, although replacing some of the deleted residues by alanine could restore optimal titers in case of variant HcΔ24+4A.

All three H protein variants that allowed most efficient pseudotype formation are also active in fusion helper function (Moll et al., 2002) (Figure 10a). Thus, the screening identified three combinations, namely HcΔ18/FcΔ30, HcΔ19/FcΔ30 and HcΔ24+4A/FcΔ30 that allowed most efficient pseudotyping of HIV-1 vector particles with titers of about 10^5 t.u./ml on HT1080 cells. After concentration, titers above 10^6 t.u./ml were obtained (Figure 10b, c). Also other lentiviral vectors, like simian immunodeficiency virus (SIVmac) derived ones, could be pseudotyped with the identified HcΔ18 and FcΔ30 protein variants with a titer of $1x10^6$ t.u./ml on HT1080 cells (Figure 10d), demonstrating the flexibility of the system.

Apparently, the cytoplasmic tail of the MV H and F protein contains sequences that prevent pseudotyping of lentiviral vectors. Only after optimal truncation of both cytoplasmic tails efficient pseudotyping of lentiviral vectors with the MV glycoproteins was possible. Highest titers with above 10^6 t.u./ml, using concentrated vector particles, were obtained with the combinations HcΔ18/FcΔ30, HcΔ19/FcΔ30 and HcΔ24+4A/FcΔ30. Obviously, the critical step was the identification of H protein cytoplasmic tail truncation mutants that allowed pseudotyping while retaining the fusion helper function. Below, HIV-1 vector particles pseudotyped with the MV envelope proteins will be referred to as MV-HIV vectors.

3.1.2 Screening for MV H and F protein variants able to efficiently pseudotype MLV vector particles

Next, the MV glycoprotein cytoplasmic tail truncation mutants were assayed to pseudotype MLV vectors.

The screen was performed as described above for HIV-1 vectors with the exception that the H protein variants with impaired fusion helper function were not included. For production of vector particles, HEK-293T cells were co-transfected with the MLV packaging plasmid HIT60, the GFP transfer vector pSFG-eGFP and the two plasmids encoding the H and F protein variants, respectively. Forty-eight hours after transfection, cell supernatants were collected and used for the transduction of

HT1080 cells. After further 48 h the respective titers were determined under the fluorescence microscope.

As described before for the HIV-1 vectors, 24 h after transfection, syncytia formation of the vector particle producing cells was observed (data not shown). However, in contrast to MV-HIV vector particles, which reached titers above 10^5 t.u./ml, MLV particles were not efficiently pseudotyped with any of the tested glycoprotein combinations. The best titer of only $3x10^3$ t.u./ml was observed for the combination HcΔ24+4A/FcΔ24 (Figure 11).

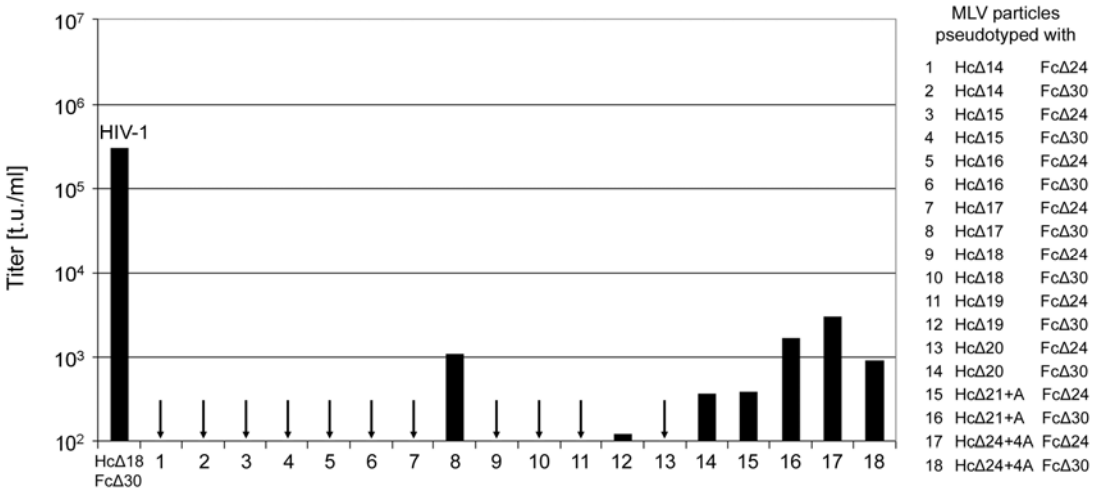

Figure 11: Screening for MV H and F protein variants that efficiently pseudotype MLV particles. HEK-293T cells were co-transfected with pHIT60, pSFG-eGFP and the plasmids encoding the indicated F and H protein variants, respectively. After 48 h, the cell supernatants were used for the transduction of HT1080 cells. Screening titers of unconcentrated pseudotype vectors are shown. The arrows indicate titers below 10^2 t.u./ml. For comparison, the titer of HcΔ18/FcΔ30 pseudotyped HIV-1 vectors, obtained in the screen depicted in Figure 10, is shown.

Thus, MV H and F cytoplasmic tail truncation mutants were identified that could pseudotype HIV-1 vector particles. However, the same variants as well as all other tested MV glycoprotein variants could not efficiently pseudotype MLV vector particles. Obviously, the restriction factors for the formation of lentiviral and γ-retroviral MV pseudotypes, respectively, are different. All further experiments were performed with HIV-1 vector particles.

3.1.3 Biochemical analysis confirmed the formation of HIV-1 pseudotypes

HIV-1 vector particles pseudotyped with the HcΔ18/FcΔ30 protein variants mediated efficient gene transfer into HT1080 cells, whereas HIV-1 particles produced in the presence of the unmodified MV glycoproteins did not. Thus, it was now investigated if the cytoplasmic tail truncations had enabled or enhanced the incorporation of the MV glycoproteins into the viral particles.

For this purpose, MV-HIV vector particles were generated in presence of the truncated HcΔ18 and FcΔ30 protein variants or the unmodified H and F proteins. They were concentrated and purified over a 20% sucrose cushion (2 ml) by centrifugation at 3,450xg and 4°C for at least 24 h. In parallel, the respective HEK-293T packaging cells were lysed and their protein content determined by Bradford assay (2.4.3; 2.4.4). The purified particles as well as the cell lysates were then applied to SDS-polyacrylamide-gelelectrophoresis and analysed by western blot (2.4.1; 2.4.2). For standardisation of the vector particles, p24, which builds the HIV-1 capsid, was analysed besides F and H. In parallel, cell surface expression of the MV glycoproteins, which is essential for their incorporation into vector particles, was determined by FACS analysis of HEK-293T cells, co-transfected in a six well plate with the HcΔ18 and FcΔ30 or the unmodified H and F proteins (2.3.7). As transfection control, 0.5 µg of a GFP-encoding plasmid was added to each well. To avoid syncytia formation, 0.2 mM fusion-inhibiting peptide (FIP)-containing medium was added to the cells 4 h after transfection. FIP interacts with the MV F protein, thereby inhibiting its fusion capability (Firsching et al., 1999; Richardson and Choppin, 1983). Twenty-four hours after transfection the cells were stained with a primary antibody directed against the H or F protein and anti-mouse PE-conjugated secondary antibody and were analysed by FACS (2.3.10).

In the cell lysates, the truncated proteins HcΔ18 and FcΔ30 as well as the unmodified MV H and F proteins were readily detectable and migrated according to their expected molecular weights (Figure 12a). Although especially for FcΔ30, the western blot analysis suggested increased protein expression levels upon cytoplasmic tail truncation (Figure 12a), there was no significant difference in cell surface expression levels between H and HcΔ18 or F and FcΔ30, respectively (Figure 13). Differences in transfection efficiencies were excluded, as similar GFP-

expression levels were obtained (data not shown). In HIV-1 vector particles, only low amounts of unmodified H protein and no unmodified F protein were detectable. The HcΔ18 and FcΔ30 proteins, in contrast, were present in significant amounts, demonstrating that cytoplasmic tail truncation had enhanced particle incorporation (Figure 12b).

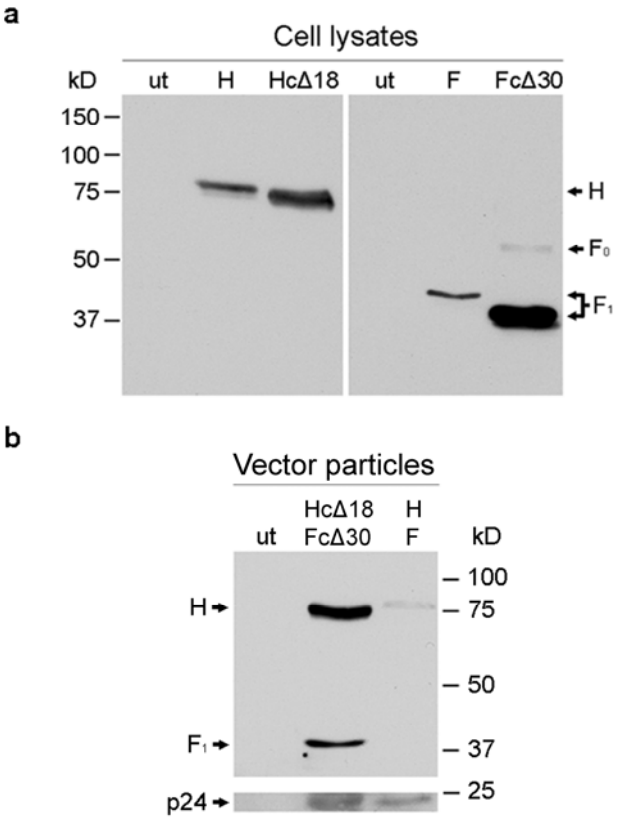

Figure 12: Incorporation of modified MV glycoproteins into HIV-1 vector particles. Western blot analysis of **(a)** cell lysates of HEK-293T packaging cells and **(b)** the respective produced HIV-1 vector particles pseudotyped with the indicated proteins. The blotted proteins were analysed utilizing anti-F, anti-H and anti-p24 antibodies, respectively. Either cell lysate or concentrated cell supernatant from untransfected HEK-293T cells were loaded as negative control (ut).

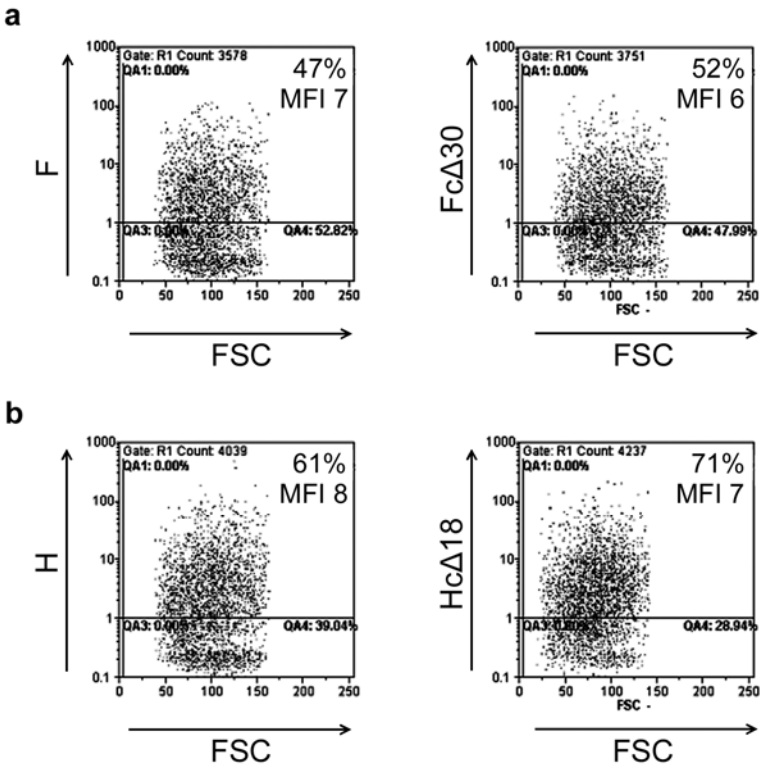

Figure 13: Cell surface expression of unmodified versus modified MV glycoproteins. In a six well plate, HEK-293T cells were co-transfected with 3 µg pCG-F and 3 µg pCG-H or 3 µg pCG-FcΔ30 and 3 µg pCG-HcΔ18. As transfection control also 0.5 µg of a GFP-encoding plasmid were added to each well. To avoid syncytia formation, 4 h after transfection 0.2 mM FIP-containing medium was added to the cells. Twenty hours later the cells were stained against **(a)** F and **(b)** H protein, respectively, and analysed by FACS. Transfection efficiency was similar for both approaches as determined by GFP-expression (not shown). MFI: mean fluorescence intensity.

Hence, sequences in the cytoplasmic tail of H and especially F seem to prevent incorporation of the unmodified MV glycoproteins into the vector particles. Their truncation enhanced particle incorporation. Thus, reduced or absent particle incorporation can be concluded as most likely reason for absence of pseudotyping with the unmodified H and F proteins.

3.1.4 MV-HIV vectors show the same tropism as MV

So far, it has been demonstrated that concentrated HIV-1 particles pseudotyped with selected modified MV glycoproteins can mediate efficient gene transfer into HT1080 cell with titers above 10^6 t.u./ml (3.1.1). Next, the question if MV pseudotyped HIV-1 vector particles have the same receptor usage as MV was addressed.

For this purpose, concentrated MV-HIV vector particles pseudotyped with the HcΔ18/FcΔ30, HcΔ19/FcΔ30 or HcΔ24+4A/FcΔ30 proteins, respectively, were used

for the transduction of cell lines being positive or negative for the MV receptors (Table 1).

The transduction of the different cell lines revealed that receptor usage of the pseudotyped particles is identical to that of MV (Figure 14; Table 1), e.g. CHO-K1 cells that express neither of the MV receptors (Nakamura et al., 2005) remained untransduced, whereas CHO cells stably transfected with SLAM or CD46 (Buchholz et al., 1996; Tatsuo et al., 2000) became GFP-positive (Figure 14).

Thus, by pseudotyping HIV-1 particles with the MV glycoproteins, MV receptor usage was transferred to these particles.

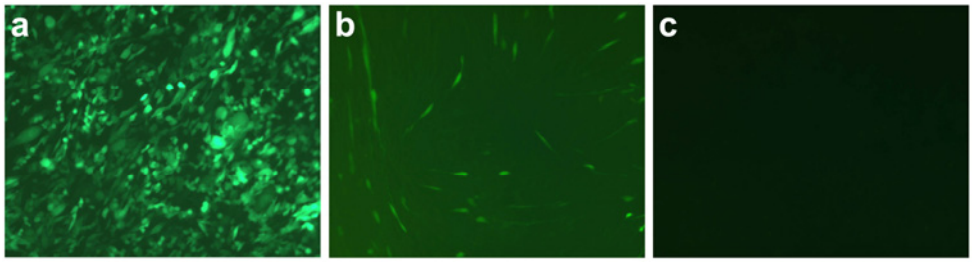

Figure 14: Receptor specificity of HIV-1 vector particles pseudotyped with the modified MV glycoproteins. CHO cells stably expressing **(a)** SLAM or **(b)** CD46 or **(c)** the parental CHO-K1 cells expressing neither of the two receptors were transduced with concentrated HIV-1 particles pseudotyped with the HcΔ18/FcΔ30 glycoproteins. Cells were analysed by fluorescence microscopy 48 h after transduction and representative pictures were taken at 100x magnification.

Table 1: Transduction of different cell lines with MV-HIV vector particles[a]

envelope proteins	HEK-293T[b] [t.u./ml]	A-431[b] [t.u./ml]	Daudi[c] [t.u./ml]	A3.01[c] [t.u./ml]	CHO-SLAM [t.u./ml]	CHO-CD46 [t.u./ml]	CHO-K1[d] [t.u./ml]
FcΔ30 HcΔ18	$1.2x10^6$	$8.5x10^{5f}$	nd	$6.0x10^4$	$7.3x10^6$	$5.4x10^5$	<30[e]
FcΔ30 HcΔ19	$2.0x10^5$	nd	$1.1x10^{6e}$	$2.0x10^5$	nd	nd	<30
FcΔ30 HcΔ24+4A	$1.6x10^6$	$4.1x10^5$	nd	nd	nd	nd	<30

[a] concentrated cell supernatants were used
[b] cell lines expressing CD46
[c] cell lines expressing CD46 and SLAM
[d] CHO-K1 cells do not express CD46 and SLAM
[e] average of two experiments
[f] average of four experiments
[nd] not determined

3.1.5 Stable gene transfer by MV-HIV vector particles

MV-HIV vectors showed high titers on different cell lines expressing the MV receptors CD46 and/or SLAM. To exclude that the observed titers were due to pseudotransduction, which means GFP-protein transfer from the producer to the target cells, instead of viral integration, the sensitivity of gene transfer towards a reverse transcriptase inhibitor (Liu et al., 1996) was determined. A contribution of pseudotransduction to the titers was not unlikely because of syncytia formation and thus enhanced vesicle production of the vector particle producing cells. During azidothymidine (AZT) incubation reverse transcription of the *gfp*-transfer vector in transduced cells**,** and thus GFP-expression, is prevented whereas GFP-protein transfer is unaffected.

HT1080 cells were transduced with concentrated MV-HIV vector particles pseudotyped with HcΔ18/FcΔ30, HcΔ19/FcΔ30 and HcΔ24+4A/FcΔ30, respectively, or as positive control VSV-G-HIV vectors either in the presence or absence of 10 µM AZT in the medium. The respective HT1080 cells were also pre-incubated for one hour in 10 µM AZT-containing medium. Forty-eight hours after transduction, titers with and without AZT incubation were compared and the relative titer reduction in presence of AZT was determined.

In presence of AZT, the titers of the MV pseudotyped HIV-1 particles decreased by 90-97%, which was in the same range as the 99% titer reduction observed for VSV-G pseudotyped vector particles (Figure 15a).

To further verify gene expression stability, the suspension B cell line Daudi was transduced by HIV-1 particles pseudotyped with the HcΔ18/FcΔ30 proteins (2.3.9) and adjacent, the number of GFP-positive cells was determined over a period of 15 days. For this experiment, the applied vector particles were not produced in presence of the lentiviral transfer vector plasmid HR`-CMV-GFP, but in presence of the transfer vector plasmid SEW (10.6), encoding GFP. This plasmid is optimised for expression in hematopoietic cells (Demaison et al., 2002).

A constant level of 12% - 20% GFP-positive Daudi cells was detectable over the whole observation period (Figure 15b).

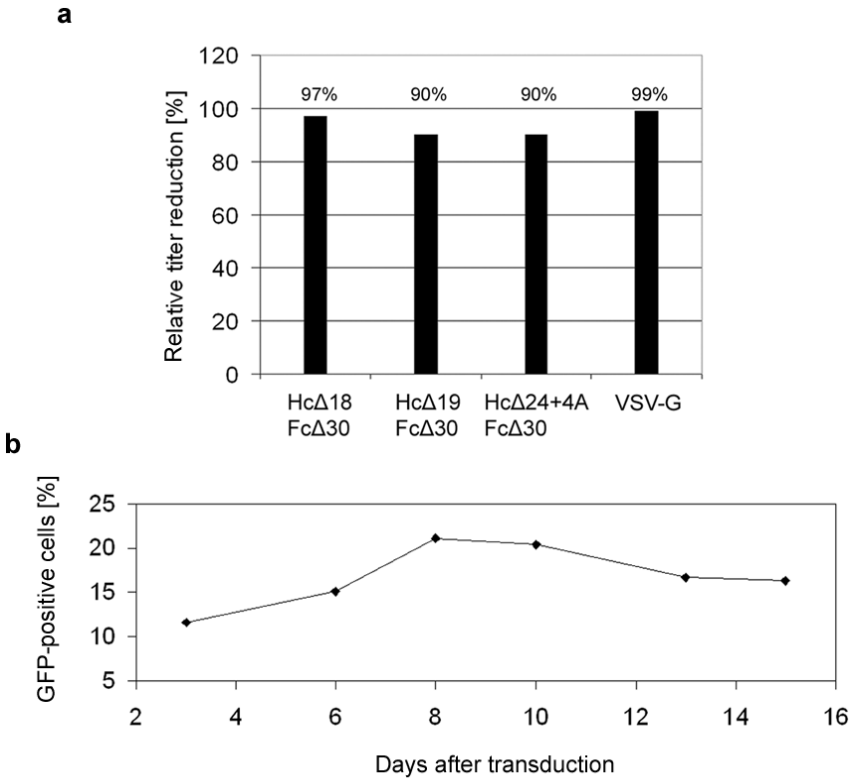

Figure 15: Stable gene transfer by MV-HIV vector particles. (a) HT1080 cells were transduced in the presence or absence of 10 µM AZT by concentrated HIV-1 vector particles pseudotyped with the indicated glycoproteins. The relative titer reduction in presence of AZT is shown. **(b)** To follow the stability of gene integration and expression, Daudi cells were cultivated for 15 days after transduction with HIV-1 vector particles pseudotyped with the HcΔ18 and FcΔ30 protein. The percentage of GFP-positive cells was determined by FACS analysis at the indicated time points.

Because a titer reduction of almost 100% was observed in presence of AZT, the data demonstrate that pseudotransduction contributed negligibly to the titers of the MV pseudotyped HIV-1 particles. Furthermore, the stability of gene integration and expression mediated by the pseudotypes was confirmed by the long-lasting GFP-expression in transduced Daudi cell over a period of 15 days.

3.1.6 Identification of an optimal H to F ratio

Concentrated MV-HIV vector particles reached titers above 10^6 t.u./ml on HT1080 cells (3.1.1). Next, it was tried to enhance titers to enable even more efficient gene transfer. During MV infection less H than F mRNA is produced (Cattaneo et al., 1987; Plumet et al., 2005), therefore it was expected that altering the ratio of the amounts of H and F plasmids in the packaging cells might lead to titer enhancement. All MV pseudotyped HIV-1 particles that were used for the experiments described above

were produced by co-transfection of the same amounts of H and F protein encoding plasmids, respectively.

To determine the optimal H to F ratio in the packaging cells, HEK-293T cells were co-transfected with pCMVΔR8.9, pHR`-CMV-GFP and different ratios of pCG-HcΔ19 and pCG-FcΔ30, respectively. Forty-eight hours later, the respective cell supernatants containing the HcΔ19/FcΔ30 pseudotyped HIV-1 particles were used for the transduction of HT1080 cells. After 48 h, the titer of the pseudotypes was determined under the fluorescence microscope and normalised to that obtained after transfection of a 1:1 ratio of pCG-HcΔ19 and pCG-FcΔ30.

Compared to vector particles generated upon transfection of equivalent amounts of both plasmids, increasing the relative level of pCG-HcΔ19 reduced titers. In contrast, higher levels of pCG-FcΔ30 considerably increased titers. The optimal ratio was determined to be seven-fold more F than H plasmid, which resulted in a more than ten-fold increase in titer (Figure 16).

Thus, it was possible to enhance the titer of HcΔ19/FcΔ30 pseudotyped HIV-1 vector particles on HT1080 cells just by varying the ratio of the HcΔ19 and FcΔ30 encoding plasmids during vector particle production. It is noteworthy that for optimal pseudotyping more F protein than H protein mRNA is needed, which reflects the natural situation in MV.

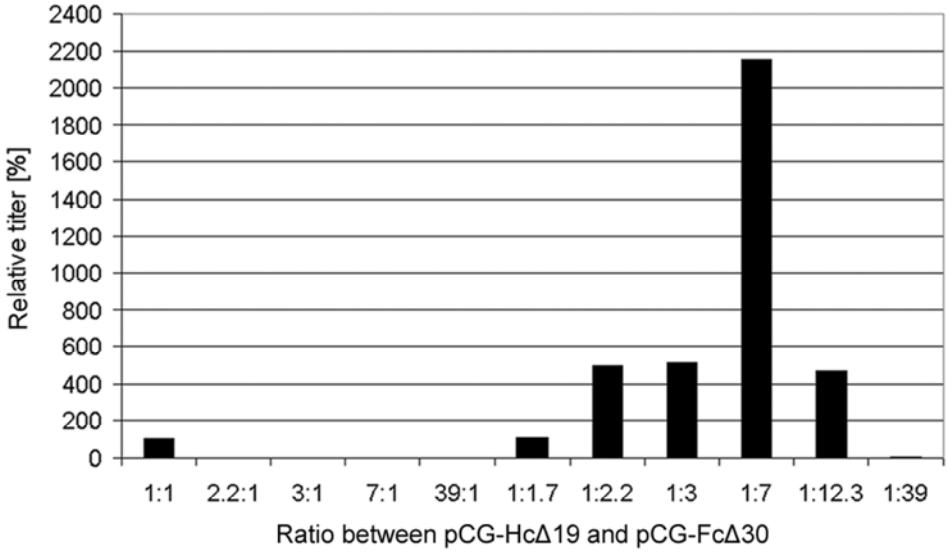

Figure 16: Titer optimisation of HcΔ19/FcΔ30 pseudotyped HIV-1 particles. The ratio of the plasmids encoding the HcΔ19 or FcΔ30 protein variant, used for vector particle production in HEK-293T cells, was varied as indicated. Each produced pseudotyped vector was titrated on HT1080 cells and its titer normalised to that obtained after transfection of a 1:1 ratio of pCG-HcΔ19 and pCG-FcΔ30 (100%).

In this first part of the thesis, MV H and F cytoplasmic tail truncation mutants, namely HcΔ18/FcΔ30, HcΔ19/FcΔ30, HcΔ24+4A/FcΔ30 were identified that efficiently pseudotyped HIV-1 vector particles. The incorporation of the glycoprotein variants HcΔ18/FcΔ30 was verified by western blot analysis and stable gene transfer, mediated by the MV-HIV vector particles, was confirmed by reverse transcriptase inhibition and long-term cultivation. As no detectable difference between the three H protein cytoplasmic tail truncation mutants in respect of pseudotyping efficiency was observed, all further experiments were performed with the HcΔ18 variant.

3.2 Retargeting of MV-HIV vector particles

In the first part of the results section it was demonstrated that HIV-1 vector particles can be pseudotyped with the MV glycoproteins after optimal truncation of their cytoplasmic tails. These particles mediated efficient and stable gene transfer into MV receptor-positive but not MV receptor-negative cell lines. Titers were enhanced, when an optimal ratio of 1:7 for plasmids encoding the H and F protein variants, respectively, was used during vector particle production. Now, based on the described pseudotyping with the MV glycoprotein variants, retargeted HIV-1 vector particles were generated. For proof of principle, the EGF receptor (EGFR) and the B cell surface marker CD20 (Cragg et al., 2005) were chosen as targets.

3.2.1 Generation of MV-HIV targeting vectors

For the production of targeting vectors specific for the EGFR or human CD20, the cytoplasmic tail of the H protein variant that was mutated in the CD46 (Y481A, S548L and F549S) and SLAM (R533A) interaction regions (Nakamura et al., 2005), thus unable to recognise its native receptors, was truncated by 18 amino acids (HmutΔ18). At its ectodomain either EGF or a scAb directed against human CD20 (αCD20-scAb) was displayed, resulting in the constructs H-αEGFR and H-αCD20, respectively (Figure 17).

The pCG-HmutΔ18 plasmid and the pCG-H-αCD20 plasmid (10.7) were constructed by subcloning the *Pac*I/*Nhe*I fragment of pCG-HcΔ18, coding for the truncated H cytoplasmic tail, into pCG-Hmut-6His and pCG-HmutXαCD20-6His (Ungerechts et al., 2007), respectively. To generate pCG-H-αEGFR (10.8), the αCD20-scAb coding

region in pCG-H-αCD20 was replaced upon *Sfi*I/*Not*I digestion by the EGF ligand coding region removed from pE-Mo (Buchholz et al., 1998).

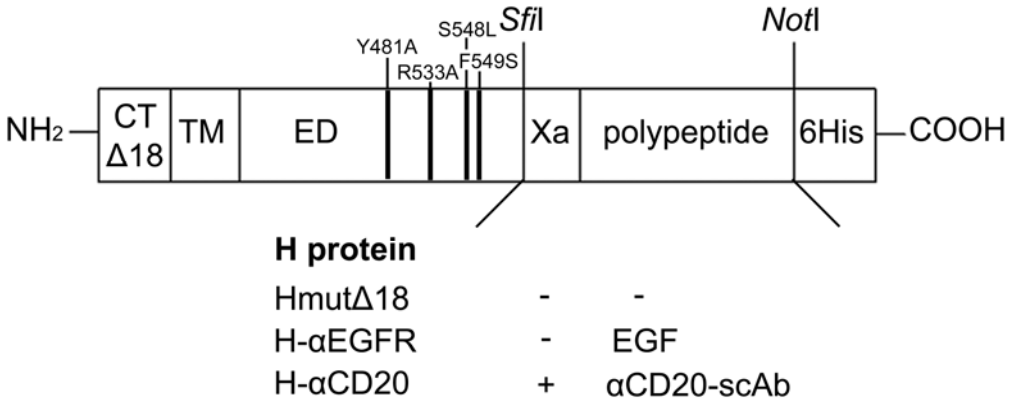

Figure 17: Schematic drawing of the modified H protein variants that were used for retargeting. The following modifications in the H protein are indicated: four point mutations in the ectodomain (ED) ablating CD46 and SLAM interaction, truncation of the cytoplasmic tail by 18 amino acids (CTΔ18), a factor Xa cleavage site (Xa) and the displayed polypeptide with a C-terminal His_6-Tag (6His). The position of the transmembrane domain (TM) is also indicated as well as the position of the restriction sites for *Sfi*I and *Not*I, respectively.

For the generation of the targeting vectors $MV_{\alpha EGFR}$-HIV and $MV_{\alpha CD20}$-HIV, the plasmids pCG-H-αEGFR and pCG-H-αCD20, respectively, were co-transfected with pCG-FcΔ30, pCMVΔR8.9 and pHR`-CMV-GFP into HEK-293T cells. After 48 h, the vector particle containing cell supernatants were concentrated and then used for the transduction of target receptor-positive and -negative cell lines (3.2.2 and 3.2.4).

3.2.2 Transduction of EGFR-positive and -negative cell lines

After generation of the plasmid encoding the H-αEGFR protein (Figure 17) that should specifically interact with the EGFR through its displayed EGF ligand, now, the targeting potential of HIV-1 vectors, pseudotyped with H-αEGFR and FcΔ30 was investigated.

For the production of $MV_{\alpha EGFR}$-HIV vector particles the same amounts of pCG-H-αEGFR and pCG-FcΔ30 were co-transfected with the HIV-1 packaging and transfer vector plasmid into HEK-293T cells. The untargeted MV-HIV vector was produced as control, using the optimised ratio of seven times more pCG-FcΔ30 than pCG-HcΔ18 for vector particle generation (3.1.6). To evaluate the targeting potential of the $MV_{\alpha EGFR}$-HIV vector, transduction of a panel of CHO cell lines stably expressing

EGFR, CD46 or SLAM as well as the human cell line A-431 naturally overexpressing EGFR (Blakely et al., 2000; Haigler et al., 1978) was performed. Therefore, in a first step, both vector types were titrated on A-431 cells (2.3.8), which are susceptible for both vector types. Then, serial dilutions containing equivalent amounts of A-431 transducing units of $MV_{\alpha EGFR}$-HIV or MV-HIV vectors were applied to the above mentioned cell lines. Titers were calculated under the fluorescence microscope, based on dilutions in which significantly less than every cell expressed GFP.

The background level of transduction ($5x10^2$ t.u./ml) was defined by applying the same amounts of HIV-1 vectors pseudotyped with the Env protein of the ecotropic Moloney MLV, which lacks a receptor on human and CHO cells (data not shown). Also HmutΔ18/FcΔ30 pseudotyped HIV-1 vectors did not show transduction above background on any of the cell lines tested (data not shown).

The $MV_{\alpha EGFR}$-HIV vector transduced CHO-EGFR cells, while transduction of CHO-CD46 and CHO-SLAM cells remained at background levels even when the highest possible amount of vector particles was applied (Figure 18). In contrast, MV-HIV vector particles efficiently transduced all cell lines expressing MV receptors, while CHO-EGFR cells were only transduced at background level (Figure 18).

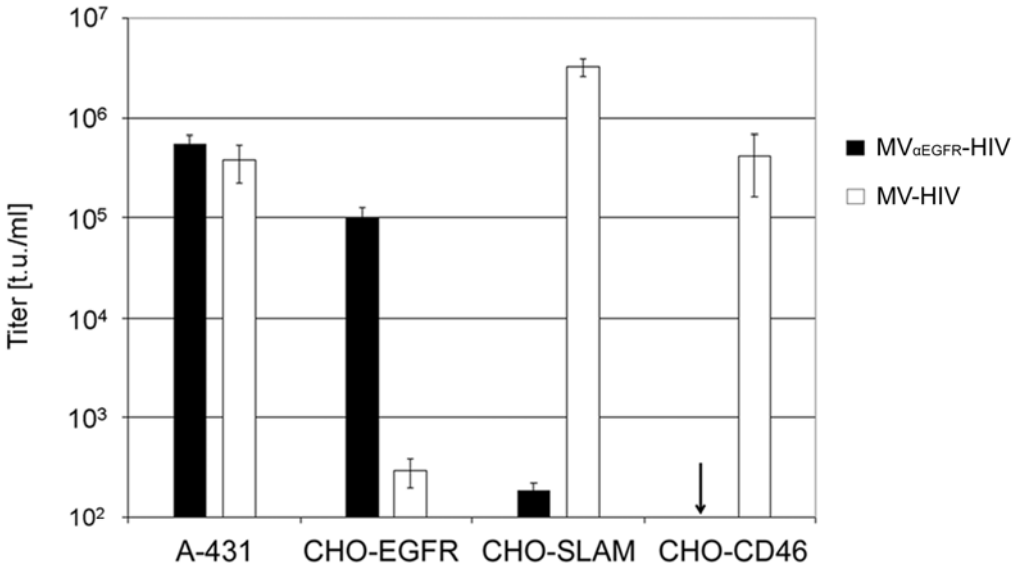

Figure 18: Targeting of EGFR-positive cell lines. Concentrated HIV-1 vector particles pseudotyped with H-αEGFR and FcΔ30 were used for the transduction of EGFR-positive and -negative cell lines. As control, vector particles pseudotyped with HcΔ18 and FcΔ30 were used. The titers are mean values from four independent experiments with standard deviation. The arrow indicates a titer below 10^2 t.u./ml.

These data clearly demonstrate that cell entry of the $MV_{\alpha EGFR}$-HIV vectors occurred via the retargeted receptor as EGFR-positive cell lines were transduced about 10^3-fold more efficiently by the targeting vectors than EGFR-negative cell lines. Even by the lowest applied dilution (highest vector dose) no transduction above background was detected for the $MV_{\alpha EGFR}$-HIV vector on CHO-SLAM and CHO-CD46 cells. Accordingly, the display of a ligand like EGF on a CD46 and SLAM blind H protein can mediate cell entry of the respective pseudotyped particles through the retargeted receptor.

3.2.3 Identification of an optimal H-αCD20 to FcΔ30 ratio

Having shown that $MV_{\alpha EGFR}$-HIV vector particles can mediate selective gene transfer into EGFR-positive cell lines, the targeting potential of $MV_{\alpha CD20}$-HIV vector particles was investigated next. As demonstrated for HcΔ19/FcΔ30 pseudotyped HIV-1 particles in chapter 3.1.6 a fine tuned balance of the relative amounts of F and H encoding plasmids has to be maintained in the packaging cells to allow most efficient formation of pseudotyped HIV-1 vectors. Therefore, the titer of $MV_{\alpha CD20}$-HIV particles on target receptor-positive cell lines was optimised by identification of an optimal pCG-H-αCD20 to pCG-FcΔ30 ratio during vector particle production in a first step. It was assumed that the optimal ratio differs from the one obtained for HcΔ19 and FcΔ30, as the H-αCD20 protein showed a reduced cell surface expression compared to the HcΔ18 protein (diploma thesis of Inna Kneiske).

To determine the optimal ratio, HEK-293T cells were co-transfected with pCMVΔR8.9, pHR`-CMV-GFP and different ratios of pCG-H-αCD20 and pCG-FcΔ30. The respective cell supernatants containing the CD20-retargeted HIV-1 particles were used for the transduction of HT1080 cells stably expressing CD20 (HT1080-CD20 cells). Measured titers were then normalised to the one obtained upon transfection of the same amounts of pCG-H-αCD20 and pCG-FcΔ30.

A clear peak in titer was observed, when a ratio of 1:3 of pCG-H-αCD20 to pCG-FcΔ30 was used for vector generation. At this optimised ratio, the titer of $MV_{\alpha CD20}$-HIV vector particles on HT1080-CD20 cells had increased by about five-fold (Figure 19). Interestingly, titers were drastically decreased when the optimal ratio for the production of MV-HIV vector particles was used (1:7 H/F ratio) (Figure 19).

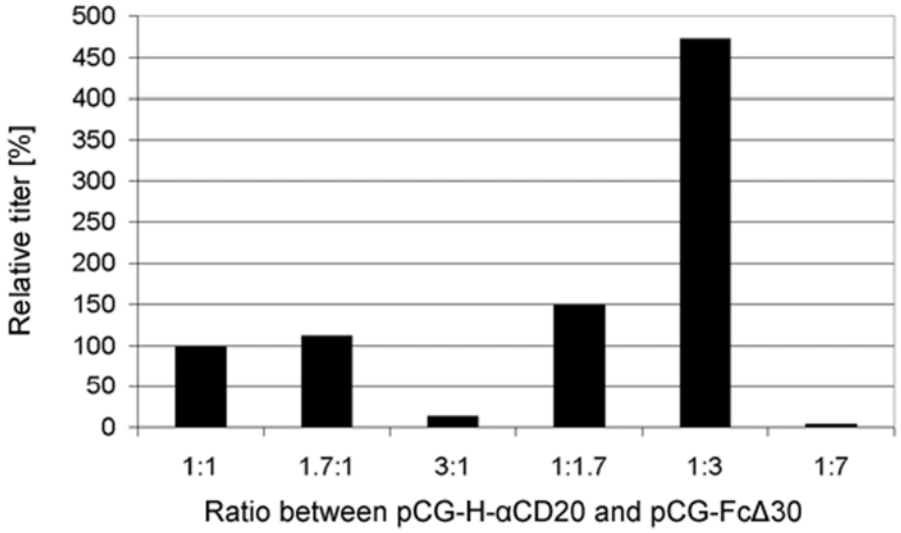

Figure 19: Titer enhancement of $MV_{\alpha CD20}$-HIV vector particles. To optimise titers, the ratio of the plasmids encoding H-αCD20 and FcΔ30, respectively, used for vector particle production in HEK-293T cells was varied as indicated. Each pseudotype vector produced was titrated on HT1080-CD20 cells. The titer of vector particles produced after transfection of a 1:1 ratio of pCG-H-αCD20 to pCG-FcΔ30 was set to 100%. The titers of all other vector types were normalised to this value.

Hence, the optimal H to F plasmid ratio for the production of $MV_{\alpha CD20}$-HIV particles is different from the one obtained for the generation of the untargeted MV-HIV vector particles. Obviously, relatively more H protein is needed when a scAb is fused to its ectodomain. This is most likely due to the reduced cell surface expression of such H proteins (diploma thesis of Inna Kneiske), which can be compensated by applying higher amounts of the corresponding expression plasmid.

3.2.4 Transduction of CD20-positive and -negative cell lines

After identification of optimal conditions for the production of $MV_{\alpha CD20}$-HIV vectors, the targeting capability of these vectors was investigated.

For this purpose, $MV_{\alpha CD20}$-HIV vectors and as control MV-HIV vectors were produced (2.3.6). In order to apply equivalent amounts of transducing units of $MV_{\alpha CD20}$-HIV and MV-HIV vectors, both vector stocks were titrated on HT1080-CD20 cells. Then, CHO cell lines expressing CD20, CD46 or SLAM as well as HT1080 and HT1080-CD20 cells were transduced with serial dilutions containing equivalent amounts of $MV_{\alpha CD20}$-HIV or MV-HIV vectors, before 48 h later titers were determined (2.3.8).

The titer of the $MV_{\alpha CD20}$-HIV vector on HT1080-CD20 cells was above 10^6 t.u./ml and thus almost three magnitudes higher than the titer on the parental CD20-negative

HT1080 cells (Figure 20). Similar observations were made on the CHO cell lines, which were only transduced when CD20 was expressed. The MV-HIV vector, in contrast, transduced the different MV receptor-positive cell types with similar efficiency but did not transduce CHO-CD20 cells (Figure 20).

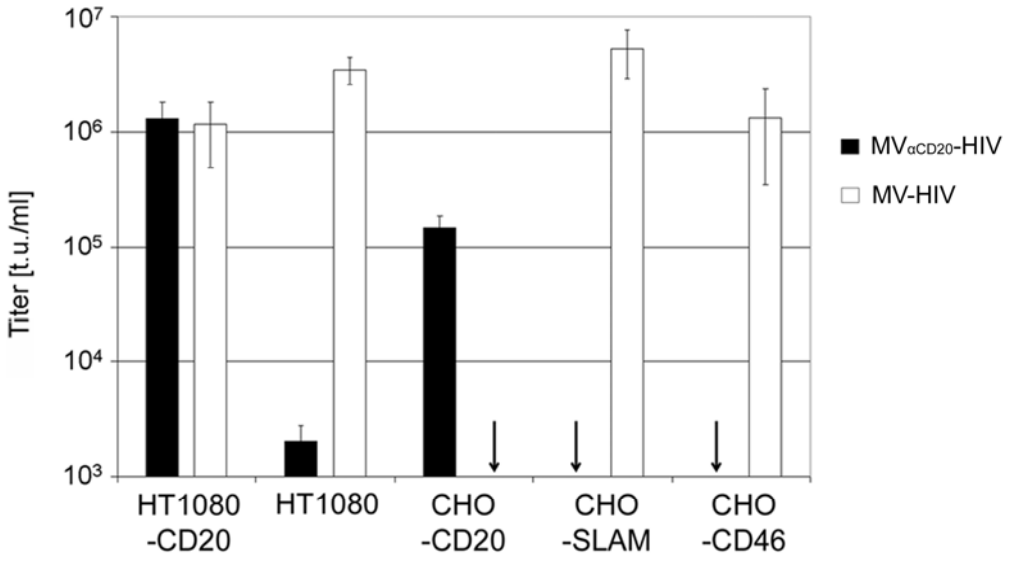

Figure 20: Targeting of CD20-positive cell lines. Concentrated HIV-1 particles pseudotyped with H-αCD20 and FcΔ30 were used for the transduction of the CD20-positive and -negative HT1080 and CHO cell lines. As control, MV-HIV particles were used. The titers are based on mean values of four independent experiments with standard deviation. The arrows indicate titers below 10^3 t.u./ml.

These data clearly demonstrate that cell entry of the $MV_{\alpha CD20}$-HIV vectors occurred via the retargeted receptor. CD20-positive cell lines were transduced at least 10^3-fold more efficiently by the targeting vectors than CD20-negative cell lines independent from the vector dose. Hence, also scAbs can mediate selective cell entry of MV pseudotyped HIV-1 particles, when they are displayed on a cytoplasmic tail truncated, CD46 and SLAM blind H protein. The maximal reached titers of the $MV_{\alpha CD20}$-HIV vector particles were even slightly higher than those of $MV_{\alpha EGFR}$-HIV vector particles (Figure 18, Figure 20).

3.2.5 Fusion via CD20 is pH-independent

It has been shown that $MV_{\alpha CD20}$-HIV particles enter specifically CD20-positive cells via interaction with the retargeted CD20 receptor. Upon recognition of its native receptors, MV mediates pH-independent membrane fusion directly at the cell

membrane. Now, the question was addressed, if also fusion via CD20 is pH-independent.

Therefore, HT1080-CD20 and HT1080 cells were transfected in a six well plate under neutral pH-conditions with the plasmids pCG-HcΔ18/pCG-FcΔ30 or pCG-H-αCD20/pCG-FcΔ30 and pSEW, encoding GFP, as transfection control. Twenty-four hours later syncytium formation was documented under the microscope.

Transfection efficiency was similar in all four samples (data not shown). Syncytia formation was observed in HT1080-CD20 cells but not in HT1080 cells after transfection with pCG-H-αCD20 and pCG-FcΔ30 (Figure 21). However, when pCG-HcΔ18 and pCG-FcΔ30 were co-transfected, both cell lines showed extensive syncytia formation (Figure 21).

Hence, fusion via CD20 was pH-independent as demonstrated by cell-cell fusion in CD20-positive cells at neutral pH. Furthermore, also in the cell-cell fusion model the membrane fusion is specific, as only the CD20-positive cell line showed syncytia formation after transfection with pCG-H-αCD20 and pCG-FcΔ30.

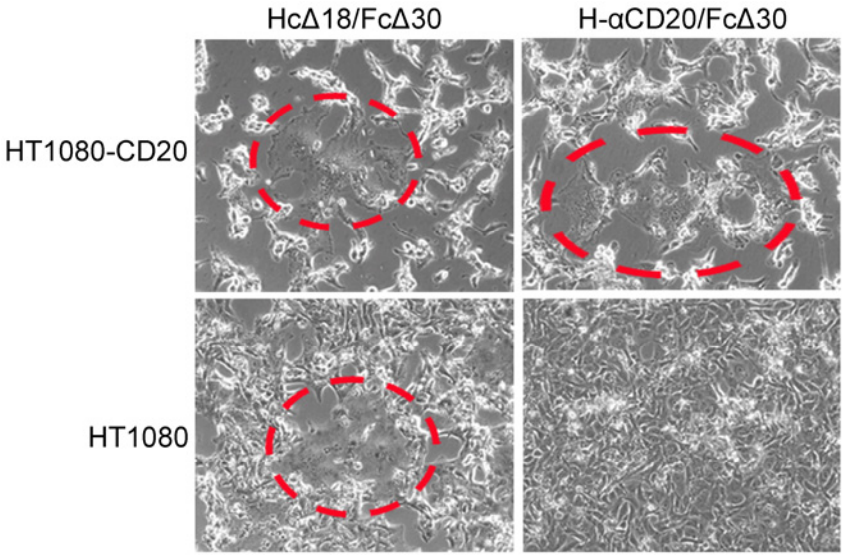

Figure 21: CD20-dependent membrane fusion at neutral pH. HT1080-CD20 (upper panel) and HT1080 cells (lower panel) were transfected in a six well plate with plasmids (1.5 µg each) pCG-HcΔ18/pCG-FcΔ30 or pCG-H-αCD20/pCG-FcΔ30 and 1 µg pSEW as transfection control. Twenty-four hours later syncytium formation (highlighted by red circles) was documented under the microscope at 100x magnification.

3.2.6 Exclusion of pseudotransduction by $MV_{\alpha EGFR}$-HIV and $MV_{\alpha CD20}$-HIV vectors

After confirmation of the targeting capability of the $MV_{\alpha EGFR}$-HIV and $MV_{\alpha CD20}$-HIV vector particles, it was now investigated, if pseudotransduction has contributed to the observed titers on the target receptor-positive cell lines. As already described in chapter 3.1.5, the reverse transcriptase inhibitor AZT was used to address this question.

A-431 and HT1080-CD20 cells, respectively, were transduced by $MV_{\alpha EGFR}$-HIV or $MV_{\alpha CD20}$-HIV vectors, either in presence or absence of 10 µM AZT. In the first case, both cell lines were also pre-incubated for one hour with 10 µM AZT-containing medium. In addition, also MV-HIV particles and HIV-1 particles pseudotyped with VSV-G (VSV-G-HIV) were incubated with both cell lines with and without AZT. Then, titers in presence and absence of AZT were compared and the relative titer reduction under AZT incubation determined.

In presence of AZT, the relative titers of $MV_{\alpha EGFR}$-HIV vector particles on A-431 cells (Figure 22a) and $MV_{\alpha CD20}$-HIV vector particles on HT1080-CD20 cells (Figure 22b) as well as the titers of MV-HIV and VSV-G-HIV vector particles on both cell lines decreased by 98%-100%, (Figure 22).

Consequently, pseudotransduction contributed, if at all, negligibly to the titers.

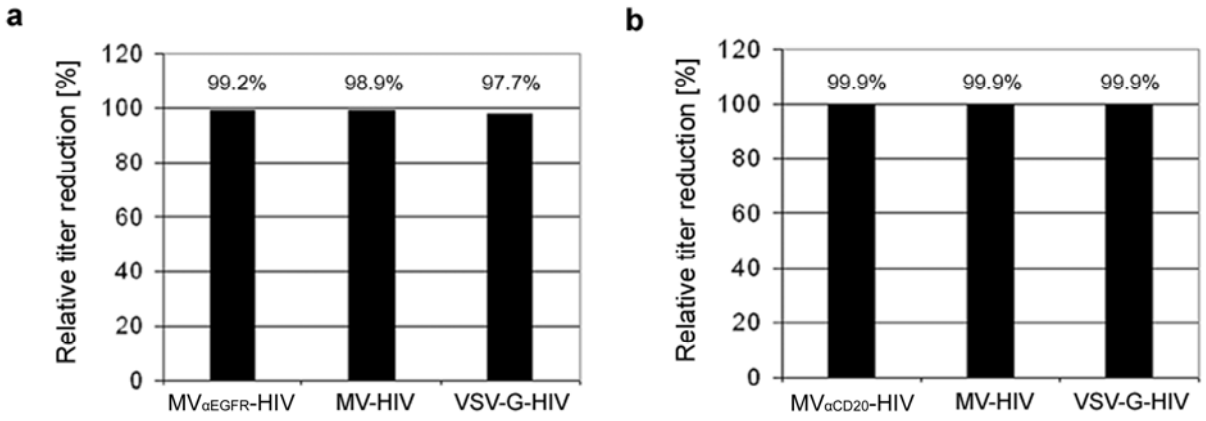

Figure 22: Exclusion of pseudotransduction by $MV_{\alpha EGFR}$-HIV and $MV_{\alpha CD20}$-HIV particles. In the presence or absence of 10 µM AZT in the medium **(a)** A-431 cells were transduced by concentrated $MV_{\alpha EGFR}$-HIV, MV-HIV or VSV-G-HIV vector particles and **(b)** HT1080-CD20 cells were transduced by $MV_{\alpha CD20}$-HIV, MV-HIV or VSV-G-HIV vector particles. The relative titer reduction in presence of AZT is shown.

3.2.7 Targeting of cell lines naturally expressing CD20

The results described in chapter 3.2.4 demonstrate that $MV_{\alpha CD20}$-HIV targeting vectors enter selectively into cell lines that had been genetically modified to express CD20. Now, it was analysed, if $MV_{\alpha CD20}$-HIV vectors also mediate selective and stable gene transfer into cell lines that do naturally express CD20. Furthermore, the ability of the targeting vector to selectively transduce CD20-positive cells in mixed cell culture with CD20-negative cells was investigated.

The following transductions were performed: The CD20-positive B cell line Daudi and the CD20-negative myeloid cell line K-562 were separately incubated with the $MV_{\alpha CD20}$-HIV vector particles at a multiplicity of infection (MOI) of 0.5 or as control VSV-G-HIV vector particles at an MOI of 5 (2.3.9). As transfer vector plasmid for vector particle production pSEW was used. Forty-eight hours after transduction the percentage of GFP-positive cells was analysed by FACS. Furthermore, to verify gene expression stability, the CD20-positive B cell line Raji was transduced with $MV_{\alpha CD20}$-HIV vector particles and the number of GFP-positive cells was determined over a period of 19 days by FACS analysis. In a next step, a 1:1 mixture of the CD20-positive Raji and the CD20-negative K-562 cell line was transduced with the retargeted vector particles or the VSV-G-HIV particles. After 48 h, the cells were stained against CD20 and the percentage of $CD20^+/GFP^+$ and $CD20^-/GFP^+$ cells was determined by FACS analysis, respectively (2.3.10).

As expected, VSV-G-HIV particles transduced both of the separately incubated cell lines efficiently (Figure 23a). In contrast, the $MV_{\alpha CD20}$-HIV vectors selectively transduced Daudi cells, whereas K-562 cells remained largely GFP-negative (Figure 23b). Remarkably, the transduction efficiency of Daudi cells was slightly higher with the $MV_{\alpha CD20}$-HIV particles than with the VSV-G-HIV particles. In addition, the Raji cells transduced by the $MV_{\alpha CD20}$-HIV vector showed a constant level of 17%-30% GFP-positive cells over the whole observation period (Figure 23c). Furthermore, also in the mixed cell culture the $MV_{\alpha CD20}$-HIV particles transduced nearly exclusively the CD20-positive Raji cells, whereas the CD20-negative K-562 cells remained mostly untransduced (Figure 24a). On the other side, VSV-G-HIV particles mediated *gfp*-gene transfer into both cell lines (Figure 24b).

Accordingly, also cells naturally expressing CD20 were efficiently transduced by the $MV_{\alpha CD20}$-HIV particles. Moreover, the targeting vector was able to discriminate between CD20-positive and -negative cells even in mixed cell cultures. The stability

of gene integration and expression mediated by the targeting vector was confirmed by stable GFP-expression in transduced Raji cells over a period of 19 days. Thus, it was demonstrated that CD20-retargeted HIV-1 vector particles had been generated that mediated efficient, selective and stable gene transfer into CD20-positive cells.

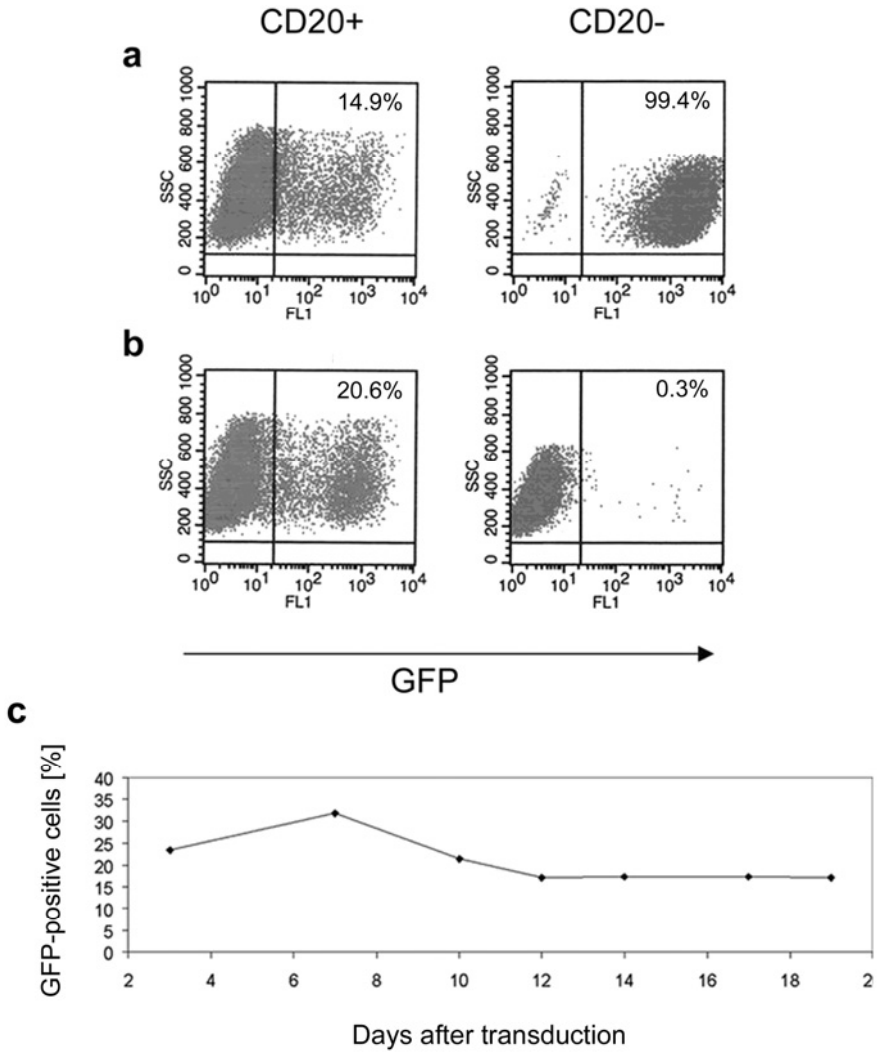

Figure 23: Selective and stable transduction of CD20-positive B cell lines. Concentrated **(a)** VSV-G-HIV (MOI 5) or **(b)** $MV_{\alpha CD20}$-HIV vector particles (MOI 0.5) were used for the transduction of CD20-positive Daudi cells and CD20-negative K-562 cells, respectively. The indicated percentages of GFP-positive cells were determined 72 h later by FACS analysis. **(c)** To follow the stability of gene integration and expression, Raji cells were cultivated for 19 days after transduction with $MV_{\alpha CD20}$-HIV vectors (MOI 0.1). The percentage of GFP-positive cells was determined by FACS analysis at the indicated time points.

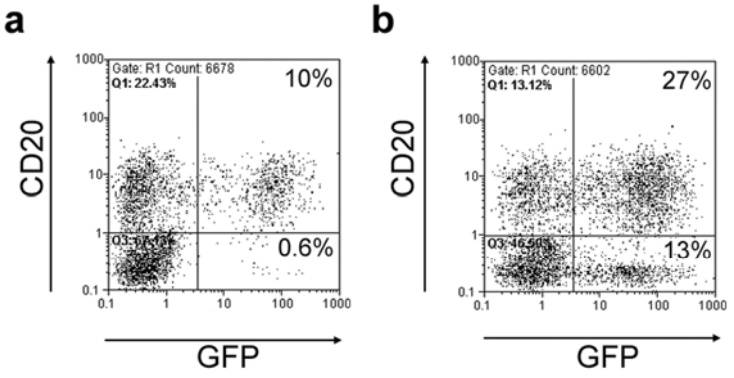

Figure 24: Selective transduction of CD20-positive cells in mixed cell culture. 1x10^5 CD20-positive Raji cells and 1x10^5 CD20-negative K-562 cells were mixed and incubated with **(a)** MV$_{\alpha CD20}$-HIV (MOI 0.5) or **(b)** VSV-G HIV (MOI 5) particles. After 48 h, the cells were stained against CD20 and the percentage of CD20$^+$/GFP$^+$ and CD20$^-$/GFP$^+$ cells was determined by FACS analysis, respectively.

3.3 Selective killing of CD20-positive cells in a mixed cell population

Having demonstrated that the MV$_{\alpha CD20}$-HIV vector efficiently targets CD20-positive cells in a mixed cell population, the question raised if the targeting capability of the vector can be applied to selectively kill CD20-positive cells in a mixture of CD20-positive and -negative cells.

3.3.1 Setting up the experiment

To allow cell killing, a suicide gene coding for a fusion protein (CD34TK39) composed of a hypersensitive mutant of the herpes simplex virus thymidine kinase (TK39) and a truncated version of the cell surface antigen CD34 was used (Junker et al., 2003). The thymidine kinase metabolises the inactive drug gancyclovir (GCV) into a chemical derivate, which is toxic for dividing cells and furthermore, transduced cells can be detected by CD34-expression. The beauty of the TK39/GCV system is that it can be used as a therapeutic agent for the treatment of e.g. cancer (Nasu et al., 2000; Miyake et al., 2007).

The HIV-1 transfer vector plasmid S-CD34TK39-W (10.9), encoding the fusion protein, was constructed by PCR amplification of the *cd34tk39* coding region of the plasmid M71tCD34tk39m (Junker et al., 2003) with the primers PR-CD34TK-for and PR-CD34TK-rev (2.2.10) and subsequent ligation of the PCR fragment into the

*Asc*I/*Sbf*I digested lentiviral transfer vector plasmid SEW (Demaison et al., 2002), thereby replacing the *gfp*-gene.

Next, the functionality of the fusion protein in context of the lentiviral transfer vector was verified and the killing capacity of $MV_{\alpha CD20}$-HIV vector particles with the packaged *cd34tk39*-gene ($MV_{\alpha CD20}$-$HIV_{cd34tk39}$) was analysed. In a first step, HEK-293T cells were transfected in a six well plate with pSEW or pS-CD34TK39-W followed by incubation in 10 µM GCV-containing medium 24 h after transfection. Two days and five days afterwards cell propagation was monitored under the microscope. Twenty-four hours after transfection, about 70% of the HEK-293T cells that were transfected with pSEW in a six well plate were GFP-positive (data not shown). While in presence of GCV cell propagation was obvious for these cells (Figure 25a), the cell density of *cd34tk39*-transfected HEK-293T cells was remarkably reduced, with nearly all cells killed after 5 days of GCV-incubation (Figure 25b).

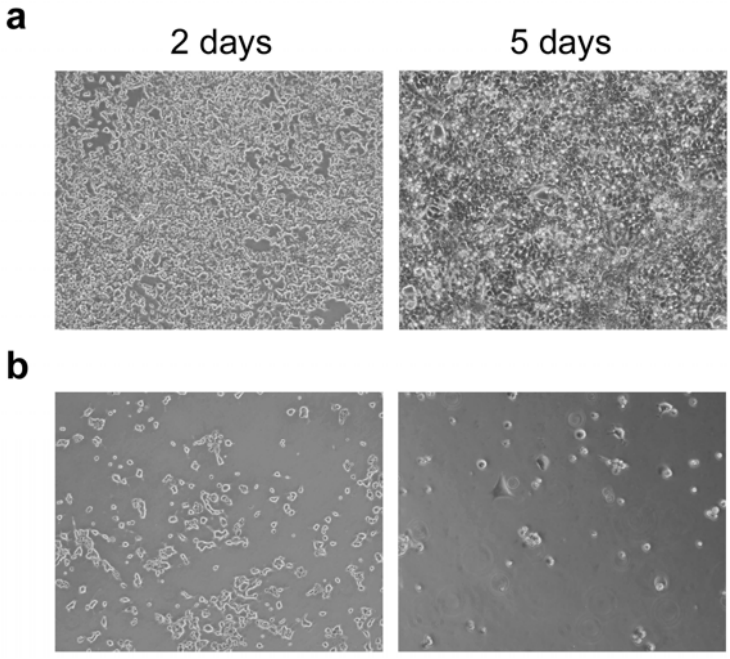

Figure 25: The *cd34tk39*-gene is functional in the backbone of pS-CD34TK39-W. HEK-293T cells were transfected in a six well plate with **(a)** 3 µg pSEW or **(b)** 3 µg pS-CD34TK39-W. Twenty-four hours later, medium containing 10 µM GCV was added and changed every 24 h against fresh GCV-containing medium. Two days and five days afterwards, the cells were analysed under the microscope at 100x magnification.

Then, $MV_{\alpha CD20}$-$HIV_{cd34tk39}$ particles were produced by co-transfection of HEK-293T cells with three-fold more pCG-FcΔ30 than pCG-H-αCD20 together with pCMVΔR8.9 and pS-CD34TK39-W. After 48 h, concentrated cell supernatant was used for the

transduction of Raji cells. Because transduced cells express CD34 on their cell surface, they can be distinguished from untransduced cells. Thus, the percentage of CD34-positive cells was determined ten days after transduction by FACS analysis. Afterwards, half of the cells were transferred into 10 µM GCV-containing medium with a medium exchange every 24 h while the other half was left as control in GCV-free medium. After 48 h, 72 h and 96 h the cells were stained against CD34 and analysed by FACS (2.3.10).

Ten days after transduction of Raji cells with $MV_{\alpha CD20}$-$HIV_{cd34tk39}$ vectors, about 27% CD34-positive cells were observed (Figure 26; time point 0 h). After 48 h of GCV-incubation, only about 16% CD34-positive cells were left and after four days (96 h) in GCV-containing medium nearly all CD34-positive cells were killed. In contrast, a constant level between 22% and 27% CD34-positive cells was present over the whole observation period in absence of GCV (Figure 26).

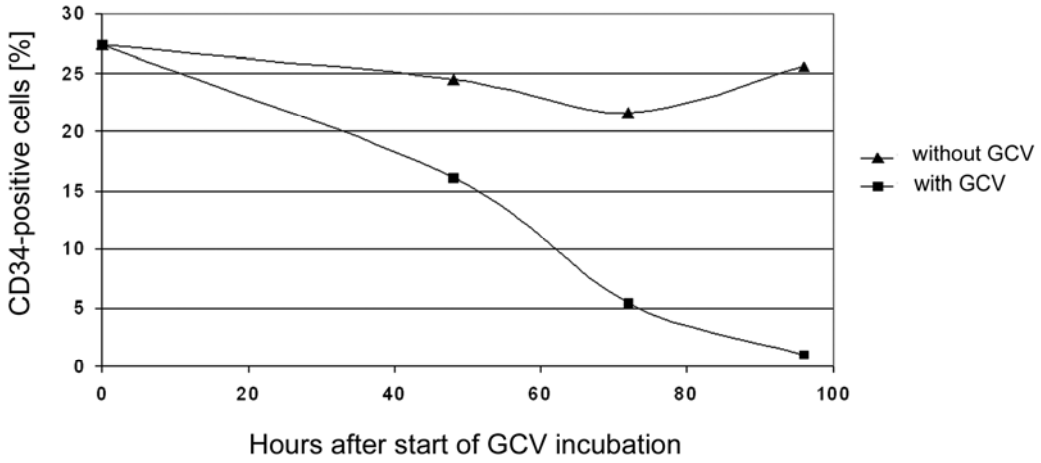

Figure 26: Killing of CD20-positive suspension cells by $MV_{\alpha CD20}$-$HIV_{cd34tk39}$ vector particles. Concentrated $MV_{\alpha CD20}$-$HIV_{cd34tk39}$ particles that were produced by co-transfection of pCMVΔR8.9, pS-CD34TK39-W, pCG-FcΔ30 and pCG-H-αCD20 were used for the transduction of $2x10^3$ Raji cells. After ten days the amount of CD34-positive cells was determined by FACS analysis (time point 0 h) and half of the cells were transferred into 10 µM GCV-containing medium while the other half was left as control in GCV-free medium. Every 24 h the GCV-containing medium was exchanged. At time points 48 h, 72 h and 96 h the percentages of CD34-positive cells were determined by FACS analysis.

These data demonstrate that the cd34*tk39*-gene is functional in the backbone of the lentiviral transfer vector, although the bystander effect of the TK39/GCV system (Zhang et al., 2008) might have supported the killing of the adherent HEK-293T cells. To avoid the influence of the bystander effect during the $MV_{\alpha CD20}$-$HIV_{cd34tk39}$ vector mediated killing, CD20-positive suspension cells were used for this experiment. Also

here, nearly all cells transduced with the *cd34tk39*-gene were killed after four days of GCV-incubation, demonstrating the ability of the $MV_{\alpha CD20}$-$HIV_{cd34tk39}$ vector to mediate efficient cell killing.

3.3.2 Selective killing of CD20-positive cells

After generation of the transfer vector encoding the fusion protein CD34TK39 and the demonstration of efficient cell killing by $MV_{\alpha CD20}$-$HIV_{cd34tk39}$ vectors, the selectivity of killing was investigated next.

Therefore, $MV_{\alpha CD20}$-$HIV_{cd34tk39}$ vector particles and as control also $MV_{\alpha CD20}$-HIV vector particles having packaged the pSEW transfer vector RNA were produced. The concentrated vector particles were separately applied to a 1:1 mixture of CD20-positive Raji and CD20-negative K-562 cells. Five days later, the percentage of GFP/CD20 and CD34/CD20 double-positive cells, respectively, was determined by FACS analysis (2.3.10). Then, half of the cells of both approaches were incubated in 10 µM GCV-containing medium while the other half was left as control in GCV-free medium. At different time points after start of GCV treatment the relative number of GFP/CD20 and CD34/CD20 double-positive cells was determined by FACS analysis, respectively.

Both CD20-retargeted vector particles selectively transduced the CD20-positive cell fraction. Five days after transduction, there were about 20% GFP/CD20 and 30% CD34/CD20 double-positive cells whereas only about 1-2.5% of the CD20-negative cells were transduced (Figure 27; time point 0 h). Normalised to the total cell number, this means that 32% and 55% of the CD20-positive cells, respectively, and 4% and 6% of the CD20-negative cells, respectively, had become GFP- or CD34-positive. Without GCV, the transduction patterns remained similar over time, demonstrating the stable integration and expression of the transferred genes and the viability of transduced cells. However, there was a slight increase in the number of reporter gene-positive CD20-negative cells in absence of cell killing (Figure 27), suggesting that transduction by the $MV_{\alpha CD20}$-HIV vectors may have resulted in loss of the CD20 surface marker in a small fraction of the cells. As expected, incubation with GCV had no influence on the fraction of GFP/CD20 double-positive cells (Figure 27a). In contrast, the CD20-positive cell fraction transduced with the *cd34tk39*-gene was

efficiently and selectively killed by GCV (Figure 27b): Starting with 32% double-positive cells, after six days almost all double positive cells had disappeared.

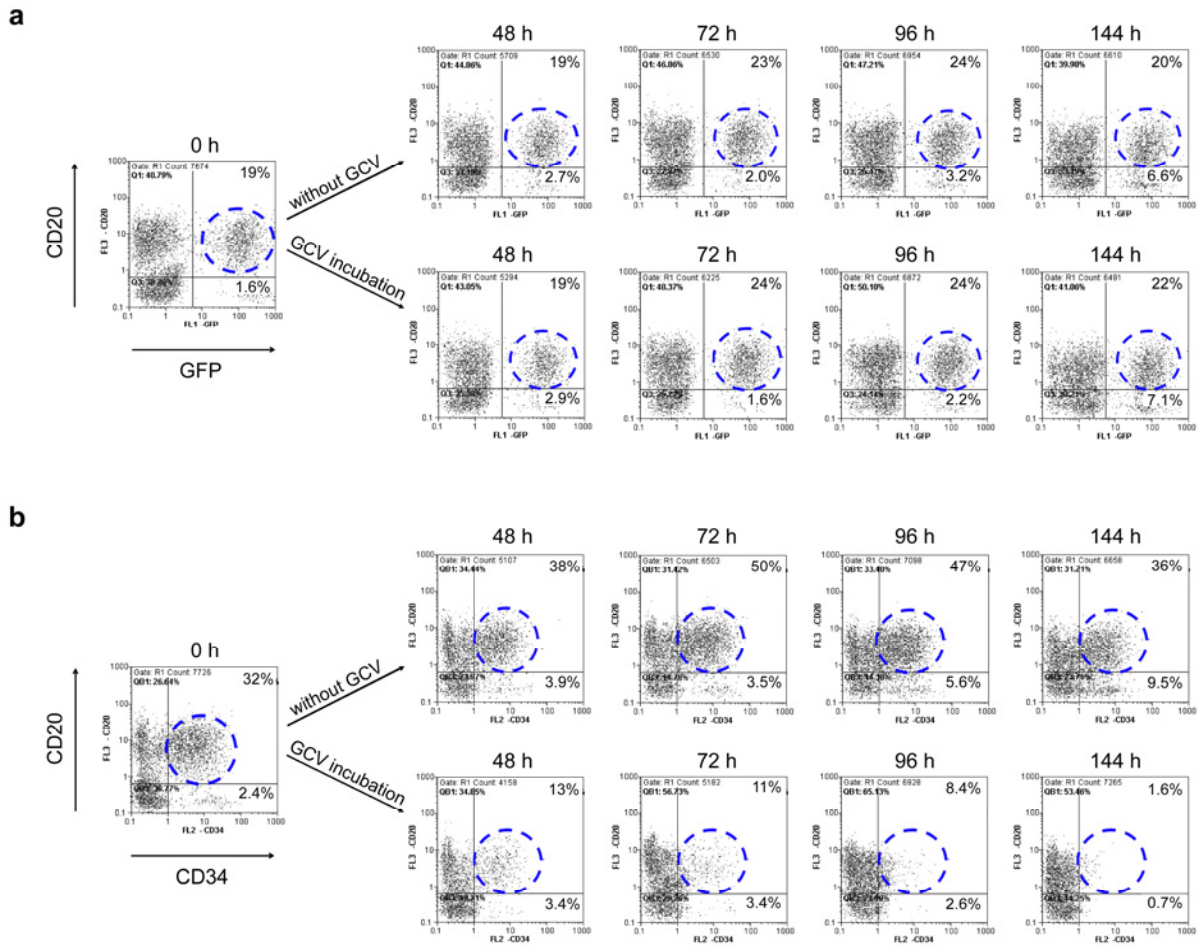

Figure 27: Selective killing of CD20-positive cells in a mixed cell population. $2x10^3$ CD20-positive Raji and $2x10^3$ CD20-negative K-562 cells were mixed and then transduced with the $MV_{\alpha CD20}$-HIV vector having packaged either **(a)** the *gfp*-gene or **(b)** the *cd34tk39*-gene. Five days after transduction (time point 0 h), half of the transduced cells were incubated in 10 µM GCV-containing medium while the other half was left as control in GCV-free medium. Every 24 h the GCV-containing medium was exchanged. At time point 0 h and at the indicated time points, the percentages of GFP/CD20 and CD34/CD20 double-positive cells (highlighted by blue circles) were determined by FACS analysis, respectively.

Accordingly, the CD20-targeting vector can mediate selective and efficient killing of CD20-positive cells in mixed cell populations**.** This demonstrated that the targeting capability of $MV_{\alpha CD20}$-HIV vectors can be applied for selective transfer of a therapeutic gene, like *cd34tk39*, into target cell populations, leading to a selective expression and effect of the therapeutic agent.

3.4 Transduction of primary human B cells

Having demonstrated the selective and efficient gene transfer of $MV_{\alpha CD20}$-HIV vectors into different CD20-positive cell lines, the potential of the targeting vector to transduce primary human cells was investigated next. The efficient transduction of primary human cells is a crucial feature for a future application of the vector in the clinic. Naturally, CD20 is exclusively expressed on B cells (Cragg et al., 2005), which were therefore used as target population.

3.4.1 Transduction of activated primary human B cells

As transduction of primary human B cells with any retro- or lentiviral vector has only been observed after activation of these cells (Serafini et al., 2004), in a first step, also in this study, activated B cells were used.

Therefore, primary human B cells were isolated by negative depletion from peripheral blood mononuclear cells (PBMCs) (2.3.4). Then, they were activated for 48 h with a cytokine cocktail consisting of 300 ng/ml CD40 ligand, 50 ng/ml IL-2, 10 ng/ml IL-4 and 10 ng/ml IL-10 and were analysed by FACS for the presence of the early activation marker CD69 (Bose et al., 2008; Damle et al., 2002). Next, the B cells were incubated at an MOI of 2 with concentrated $MV_{\alpha CD20}$-HIV particles produced by co-transfection of HEK-293T cells with three times more pCG-FcΔ30 than pCG-H-αCD20 together with pCMVΔR8.9 and pSEW. For comparison, also VSV-G-HIV particles with packaged *gfp*-gene derived from pSEW were used for the transduction of activated primary human B cells at an MOI of 2 and 150 (2.3.9). After 72 h, the cells were stained for the B cell markers CD20 and CD19 and the percentage of CD20/CD19/GFP triple-positive cells was analysed by FACS.

After incubation with the cytokine cocktail, 82% of the cells were CD69-positive (Figure 28) and thus activated. Furthermore, most of the isolated, activated cells were double-positive for the B cells markers CD20 and CD19 (Figure 29). Remarkably, more than 70% of these B cells were GFP-positive after transduction with the $MV_{\alpha CD20}$-HIV vector (Figure 29a), whereas only about 20% had become GFP-positive with the VSV-G-HIV vector (Figure 29b). This result, of $MV_{\alpha CD20}$-HIV vectors being much more efficient in mediating gene transfer into primary human B

cells than VSV-G-HIV particles, was confirmed with B cells of three further donors (Table 2).

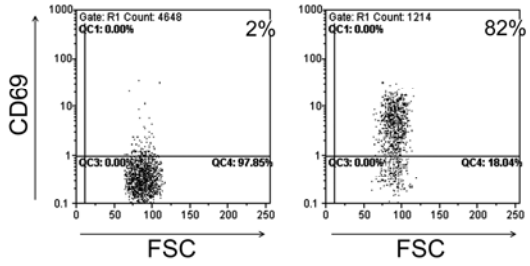

Figure 28: CD69-expression of activated primary human B cells. Isolated primary human B cells were activated for 48 h with 300 ng/ml CD40 ligand, 50 ng/ml IL-2, 10 ng/ml IL-4 and 10 ng/ml IL-10 and then analysed by FACS for the expression of the early activation marker CD69. left: B cells incubated with FITC conjugated IgG_1,κ isotype control; right: B cells incubated with FITC conjugated anti-CD69 antibody.

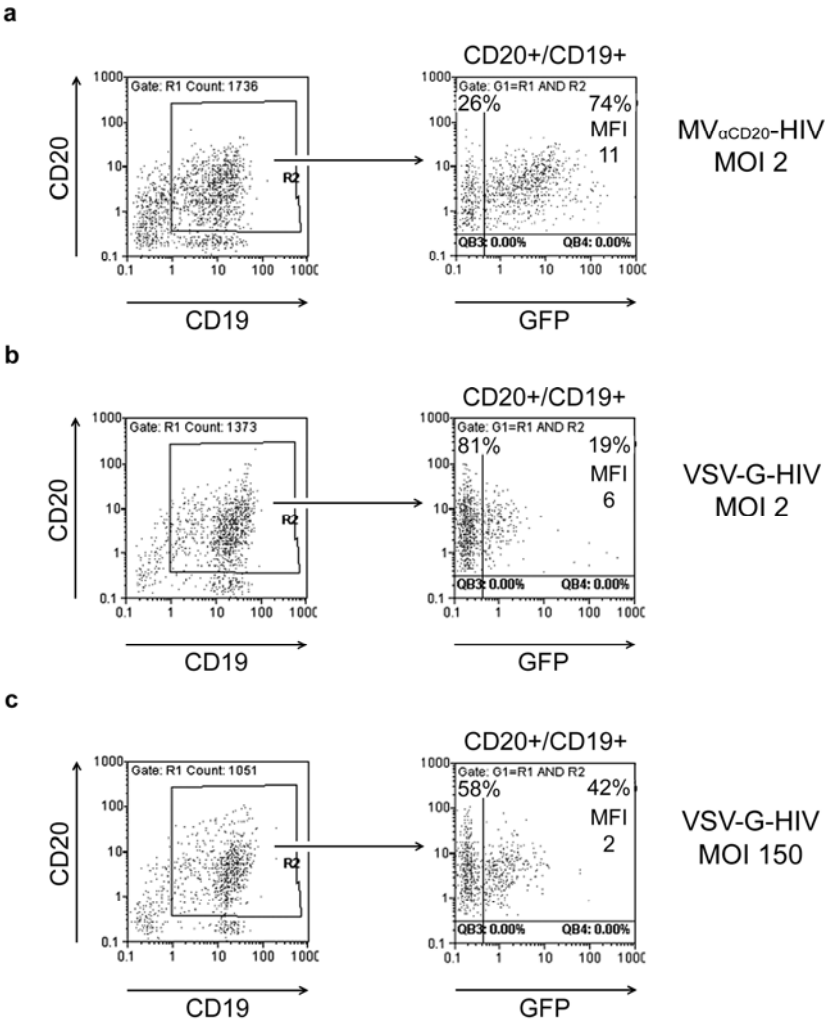

Figure 29: Transduction of activated primary human B lymphocytes. Primary human B cells were isolated from human PBMCs, activated for 48 h and then transduced with **(a)** the $MV_{\alpha CD20}$-HIV vector at an MOI of 2. In parallel, B cells were transduced with the VSV-G-HIV vector at an MOI of **(b)** 2 or **(c)** 150. Seventy-two hours after transduction, the percentage of CD20/CD19/GFP triple-positive cells and the respective GFP mean fluorescence intensity (MFI) was determined by FACS analysis.

Table 2: Transduction of activated primary human B cells from different donors

	transduced B cells [%]		
donor	$MV_{\alpha CD20}$-HIV MOI 2	VSV-G-HIV MOI 2	VSV-G-HIV MOI 150
1	74	19	42
2	32	nd	8 (MOI 510)
3	42	12	16
4	35	3	7

[nd] not determined

Increasing the MOI of the VSV-G-HIV vector to 150 raised the fraction of GFP-positive B cells about two-fold (Figure 29c). However, the GFP mean fluorescence intensity (MFI) of cells transduced by the VSV-G-HIV vector was considerably lower compared to the $MV_{\alpha CD20}$-HIV transduced cells (Figure 29).

In conclusion, it has been shown, that $MV_{\alpha CD20}$-HIV particles can transduce activated primary human B cells with a remarkably high efficiency, which is above any transduction rate described before for any type of retro- or lentiviral vector (Bovia et al., 2003; Janssens et al., 2003).

3.4.2 Chromosomal integration of vector sequences transferred by $MV_{\alpha CD20}$-HIV particles

Having observed this unexpected high transduction efficiency of $MV_{\alpha CD20}$-HIV vectors on primary human B cells, the question raised if the transferred vector sequences are indeed integrated into the B cell genome. For this reason a two-step PCR amplification assay (two-step *Alu*-PCR) was performed (Chun et al., 1997).

Therefore, in a first step, activated primary human B cells and as control HT1080-CD20 cells were transduced with $MV_{\alpha CD20}$-HIV particles at an MOI of 2 and 0.7, respectively (2.3.8; 2.3.9). In parallel, primary human B cells were also transduced with VSV-G-HIV particles at an MOI of 150. To avoid contamination with the transfer vector plasmid DNA (pSEW) from the packaging cells, the used vector particles were incubated with DNase I before transduction (2.2.8). Six days after transduction, the genomic DNA of the cells was isolated using the DNeasy® Blood and Tissue kit

(Qiagen) (2.2.9). For integration analysis, the isolated genomic DNA was then applied to a two-step *Alu*-PCR (2.2.10). Therefore, the primers ALUs and HIV-AluPCRas that bind to cellular genomic *Alu* and proviral *gag* sequences (ψ-site coding region in the transfer vector), respectively, in the first cycle and the transfer vector-specific primers SEW-LTR1s and SEW-LTR1as binding in the LTR in the second cycle were used. Integration of vector DNA is indicated, if a significant increase in the transfer vector-specific signal is obtained by two steps of amplification, compared to that attained without a preceding *Alu*-PCR.

Integration of vector sequences, transferred by $MV_{\alpha CD20}$-HIV particles, was detected in both, transduced primary human B cells and HT1080-CD20 cells, whereas no signal was detected in untransduced control cells or PCR controls (Figure 30). In contrast, primary human B cells transduced by the VSV-G-HIV vector were negative in the two-step *Alu*-PCR (data not shown).

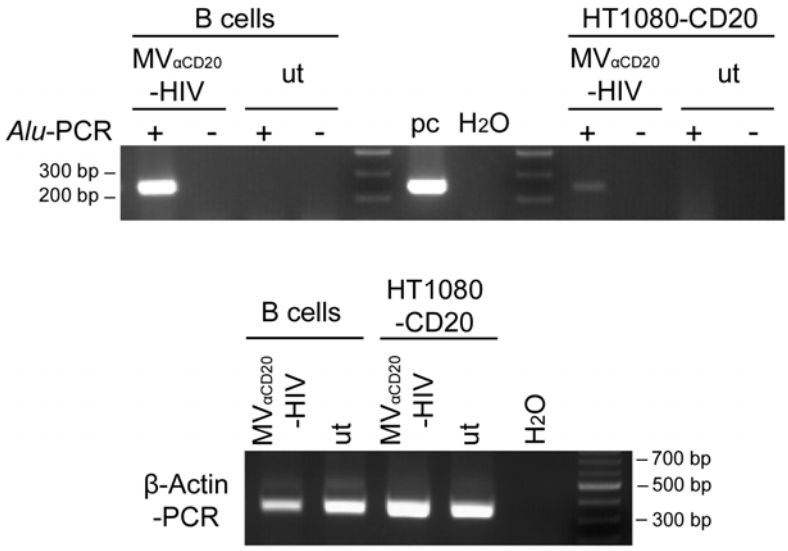

Figure 30: Chromosomal integration of transfer vector DNA in cells transduced by $MV_{\alpha CD20}$-HIV particles. Genomic DNA of primary human B cells and HT1080-CD20 cells that were transduced by $MV_{\alpha CD20}$-HIV particles at an MOI of 2 and 0.7, respectively, was isolated six days after transduction and analysed via a two-step *Alu*-PCR protocol for genomic integration of transfer vector sequences. To avoid contamination with pSEW from the packaging cells, the used vector particles were incubated with DNase I before transduction. β-Actin sequences were amplified to demonstrate the integrity of isolated genomic DNA. Amplification products were 236 bp for transfer vector-derived sequences and 390 bp for β-Actin sequences. ut, genomic DNA of untransduced cells; pc, positive control for the second amplification step (50 ng pSEW); +, two-step *Alu*-PCR protocol; -, transfer vector-specific PCR (second round) without previous *Alu*-PCR.

Accordingly, chromosomal integration of vector sequences transferred by the $MV_{\alpha CD20}$-HIV particles was demonstrated in primary human B cells as well as

HT1080-CD20 cells. The weaker signal obtained for the HT1080-CD20 cells can be explained by the lower MOI used for the transduction of these cells. Thus, it was verified, that the high amount of GFP-positive primary human B cells, gained after transduction with $MV_{\alpha CD20}$-HIV particles, was due to viral integration and not to pseudotransduction. In contrast, the absence of an amplificate after the two-step *Alu*-PCR for primary human B cells transduced by VSV-G-HIV particles indicates a significant contribution of pseudotransduction to the GFP-fluorescence obtained with this vector.

3.4.3 Selective transduction of B cells in primary human lymphocytes

Confirming the ability of the targeting vector to efficiently transduce primary human B cells, next, it was investigated if the $MV_{\alpha CD20}$-HIV vector discriminates between CD20-positive and -negative primary human cells in mixed lymphocytes.

Therefore, CD20/CD19 double-positive B cells and CD3-positive T cells were isolated from human blood via CD19 and CD3 antibody mediated immunomagnetic selection (2.3.5) and were activated with the same cytokine cocktail as described above. Then, the cell mixture containing about 70% T cells and 5% B cells (data not shown) was transduced with the $MV_{\alpha CD20}$-HIV vector and the VSV-G-HIV vector, respectively. Forty-eight hours after transduction, the lymphocyte mixture was stained against the B cell markers CD20 and CD19 and the percentages of $CD20^+/CD19^+/GFP^+$ and $CD20^-/CD19^-/GFP^+$ cells were determined by FACS analysis.

With the $MV_{\alpha CD20}$-HIV vector about 40% of the B cells had become GFP-positive, while the CD20/CD19 double-negative cell fraction, mainly consisting of T cells, remained GFP-negative (Figure 31a). In contrast, the VSV-G-HIV vector transduced both cell fractions roughly at equal efficiency (Figure 31b) and did not discriminate between the two cell populations. These data have been confirmed with a B and T cell mixture of a second donor (data not shown).

Thus, the $MV_{\alpha CD20}$-HIV vector effectively targets primary human CD20-positive cells, even when the target cell population is highly underrepresented.

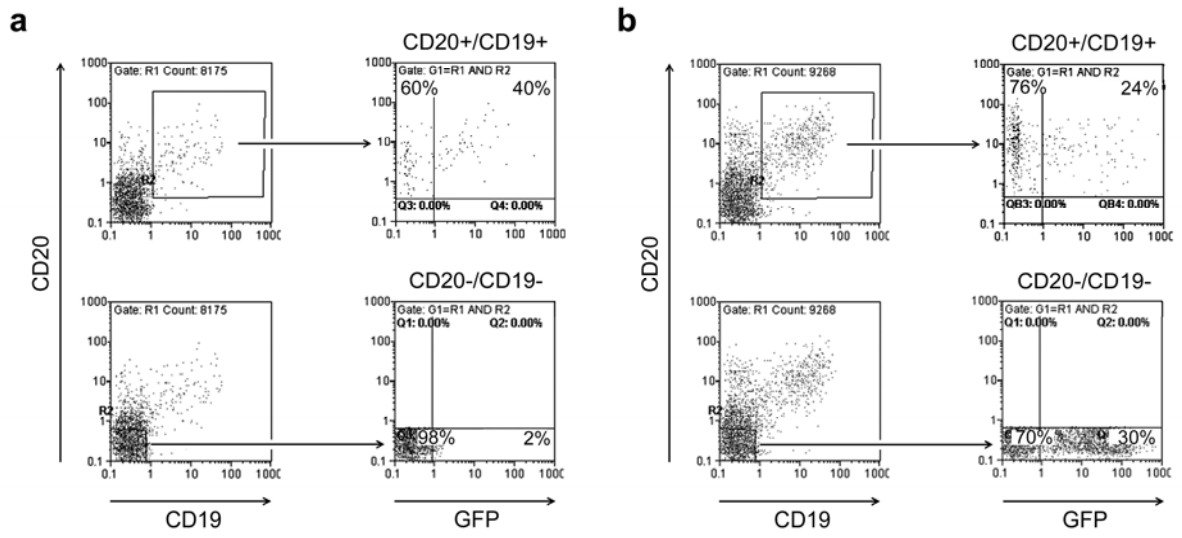

Figure 31: Selective transduction of primary human B cells in mixed lymphocytes. Primary human B and T cells were isolated from human blood by positive selection and incubated with cytokines activating B and T cells. Then, the cell mixture was transduced with **(a)** the $MV_{\alpha CD20}$-HIV vector or **(b)** the VSV-G-HIV vector at an MOI of 2. Forty-eight hours after transduction, the percentage of $CD20^+/CD19^+/GFP^+$ cells (upper panels) and $CD20^-/CD19^-/GFP^+$ cells (bottom panels) was determined by FACS analysis.

3.4.4 Transduction of unstimulated primary human B cells

The unexpected highly efficient transduction of activated primary human B cells by the $MV_{\alpha CD20}$-HIV vector prompted to test if the vector can mediate gene transfer into unstimulated primary human B cells.

For this purpose, primary human B cells were isolated from human PBMCs by negative depletion, were stained against the early activation marker CD69 (Bose et al., 2008; Damle et al., 2002) and analysed by FACS. Next, the B cells were transduced by $MV_{\alpha CD20}$-HIV and VSV-G-HIV particles, respectively, without ever coming in contact with any activating cytokines, neither before nor after transduction. After 48 h, the cells were stained against the B cell markers CD20 and CD19 and the percentage of CD20/CD19/GFP triple-positive cells was determined by FACS analysis.

The unstimulated state of the isolated B cells was confirmed by the absence of the early activation marker CD69 (Figure 32a). As expected, these cells were completely resistant against transduction with the VSV-G-HIV vector even at MOIs of 150 or higher (Figure 32b; Table 3). Most surprisingly, the $MV_{\alpha CD20}$-HIV vector transduced unstimulated B cells derived from five different donors at efficiencies of around 20% (Figure 32c; Table 3).

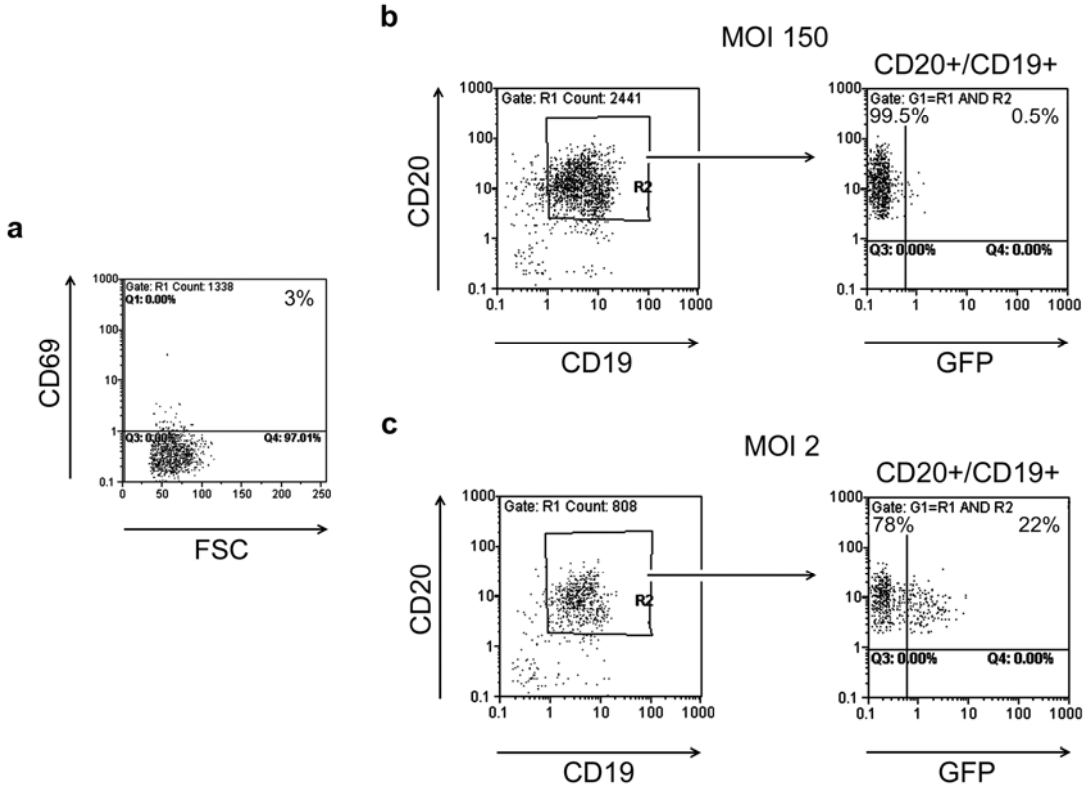

Figure 32: Transduction of unstimulated primary human B lymphocytes. Primary human B cells were isolated from human PBMCs, shown to be negative for CD69 **(a)**, and were then transduced with **(b)** the VSV-G-HIV vector at an MOI of 150 or **(c)** the $MV_{\alpha CD20}$-HIV vector at an MOI of 2. Forty-eight hours after transduction, the percentage of CD20/CD19/GFP triple-positive cells was determined by FACS analysis.

Table 3: Transduction of unstimulated primary human B cells from different donors

	transduced unstimulated B cells [%]		
donor	$MV_{\alpha CD20}$-HIV MOI 2	VSV-G-HIV MOI 2	VSV-G-HIV MOI 150
1	19	0	0.4
2	25	nd	0 (MOI 350)
3	22	0.2	0.5
4	23	0.6	nd
5	30	0	nd

[nd] not determined

In conclusion, it was demonstrated that the $MV_{\alpha CD20}$-HIV vector transduced activated primary human B cells with a surprisingly high efficiency. Even in a mixture of primary

lymphocytes, with highly underrepresented B cells, the $MV_{\alpha CD20}$-HIV vector mediated selective gene transfer into the B cell fraction. Even more remarkably was the observation that the $MV_{\alpha CD20}$-HIV particles transduced unstimulated primary human B cells with efficiencies of around 20% although until then unstimulated (quiescent) primary human B cells were resistant against transduction by any type of retro- or lentiviral vector (Serafini et al., 2004).

3.5 Induced activation of unstimulated primary human B cells by $MV_{\alpha CD20}$-HIV transduction

Having shown that $MV_{\alpha CD20}$-HIV vectors transduce activated primary human B cells much more efficiently than VSV-G-HIV vectors, which were believed to be the most promising vectors for the transduction of primary human B cells (Bovia et al., 2003; Janssens et al., 2003) and above all the observation that even unstimulated primary human B cells can be transduced by the CD20-targeting vector, let to the question of the difference between the $MV_{\alpha CD20}$-HIV and other pseudotype vectors, like VSV-G-HIV. One possible explanation for the ability of $MV_{\alpha CD20}$-HIV vectors to efficiently transduce unstimulated primary human B cells is a stimulatory signal induced by CD20-binding. Increasing evidence for a physiological role of CD20 as a regulator of cell growth and differentiation supports this view (Riley and Sliwkowski, 2000; Cragg et al., 2005).

3.5.1 Influence of $MV_{\alpha CD20}$-HIV particles on different activation markers

To answer the question if $MV_{\alpha CD20}$-HIV particles induce activating stimuli in primary human B cells, the expression patterns of the activation markers CD25, CD69, CD71 (Damle et al., 2002) and CD86 (Yokozeki et al., 1996) of untransduced B cells and B cells transduced by $MV_{\alpha CD20}$-HIV or VSV-G-HIV particles were analysed by FACS.
Therefore, primary human B cells were isolated from PBMCs and immediately transduced with $MV_{\alpha CD20}$-HIV or VSV-G-HIV particles without any pre-activation (2.3.9). Forty-eight hours later, the cells were stained against CD25, CD69, CD71 or CD86 and the expression of the different activation markers as well as the transduction efficiency, determined by GFP-expression, was analysed by FACS.

Again, VSV-G-HIV particles could not mediate gene transfer above background level, whereas the $MV_{\alpha CD20}$-HIV particles transduced 23% of the unstimulated primary human B cells (Table 3 donor 4). There was no detectable difference of CD25-expression between untransduced B cells and B cells transduced by $MV_{\alpha CD20}$-HIV or VSV-G-HIV vectors (data not shown). In contrast, a slight enhancement of CD69 and CD86 and a moderate enhancement of CD71 expression of B cells transduced by the $MV_{\alpha CD20}$-HIV particles compared to untransduced and VSV-G-HIV transduced B cells was observed (Figure 33). This rise in expression was confirmed for CD69 and CD71 with B cells from further donors (Table 4).

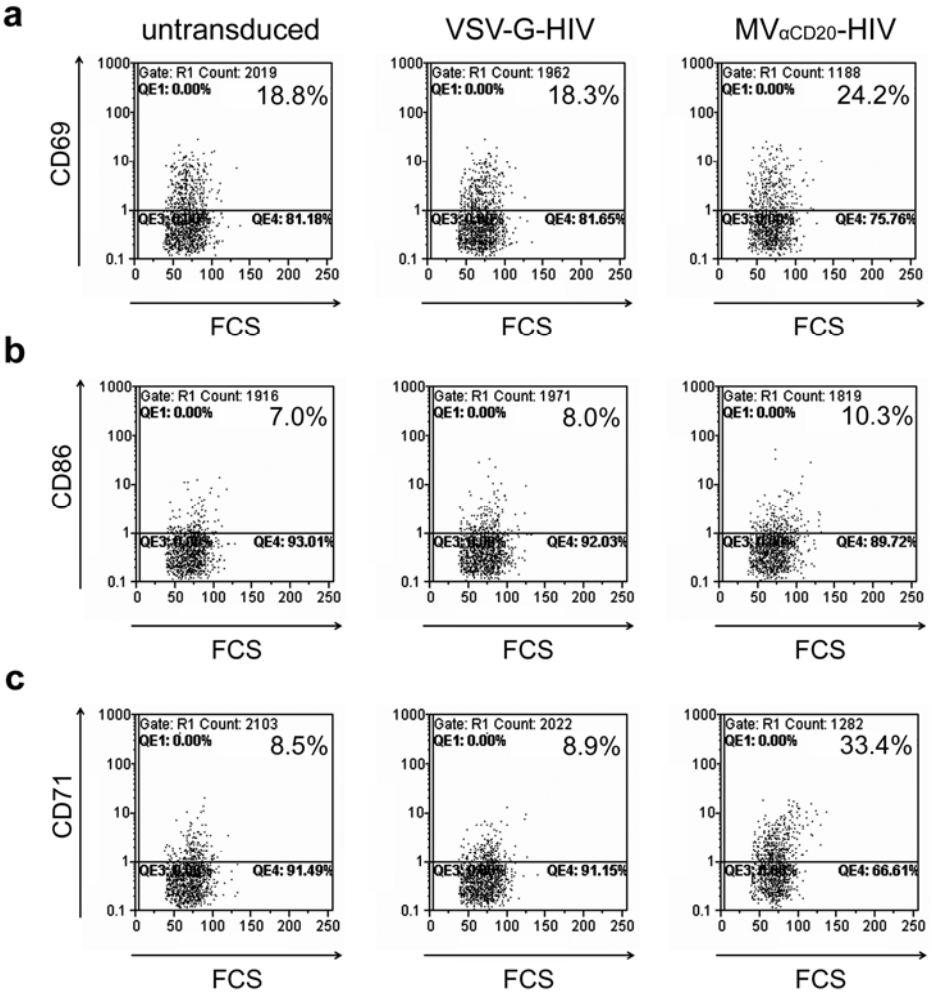

Figure 33: Expression of different activation markers in transduced primary human B cells. Unstimulated primary human B cells were incubated with VSV-G-HIV or $MV_{\alpha CD20}$-HIV particles at an MOI of 2. After 48 h, the expression of the activation markers **(a)** CD69, **(b)** CD86 and **(c)** CD71 was analysed by FACS for untransduced (left panel), VSV-G-HIV transduced (middle panel) and $MV_{\alpha CD20}$-HIV transduced (right panel) B cells.

Table 4: Expression of different activation markers in transduced primary human B cells from different donors.

transduction state	CD69[1] [%]	CD69[2] [%]	CD69[3] [%]	CD71[2] [%]	CD71[3] [%]	CD86[2] [%]	Transduction efficiency [%]
untransduced	9.0	18.8	5.7	8.5	0.0	7.0	-
VSV-G-HIV	8.7	18.3	5.4	8.9	0.0	8.0	0.0 - 0.6
$MV_{\alpha CD20}$-HIV	14.0	24.2	7.2	33.4	2.9	10.0	22.0 - 30.0

[1] primary human B cells from donor 1
[2] primary human B cells from donor 2
[3] primary human B cells from donor 3

In conclusion, three of the four tested activation markers showed an enhanced expression after transduction of unstimulated primary human B cells with the $MV_{\alpha CD20}$-HIV vectors, which indicates an influence of $MV_{\alpha CD20}$-HIV transduction on the activation status of primary human B cells.

3.5.2 Co-transduction of VSV-G-HIV and $MV_{\alpha CD20}$-$HIV_{cd34tk39}$ vectors

The results described in the previous chapter indicate an activating stimulus of $MV_{\alpha CD20}$-HIV transduction on unstimulated primary human B cells. Consequently, such cells might now be transducible by VSV-G-HIV vector particles. To test this hypothesis, unstimulated primary human B cells were incubated with a mixture of VSV-G-HIV and $MV_{\alpha CD20}$-$HIV_{cd34tk39}$ (3.3) vector particles.

For this purpose, VSV-G-HIV vectors were produced by co-transfection of pMD.G2, pCMVΔR8.9 and pSEW, whereas $MV_{\alpha CD20}$-$HIV_{cd34tk39}$ particles were generated by co-transfection of pCG-FcΔ30 and pCG-H-αCD20 together with pCMVΔR8.9 and pS-CD34TK39-W. In parallel, also $MV_{\alpha CD20}$-HIV particles using pSEW instead of pS-CD34TK39-W during transfection were produced. Thus, VSV-G-HIV and $MV_{\alpha CD20}$-HIV particles have packaged the *gfp*-gene, whereas $MV_{\alpha CD20}$-$HIV_{cd34tk39}$ particles transfer the *cd34tk39*-gene. Next, primary human B cells were isolated from PBMCs by negative depletion and were immediately transduced by a mixture of VSV-G-HIV and $MV_{\alpha CD20}$-$HIV_{cd34tk39}$ particles (both at an MOI of 2). Consequently, cells transduced by VSV-G-HIV vectors express GFP, whereas cells transduced by $MV_{\alpha CD20}$-$HIV_{cd34tk39}$ vectors do not. As controls, two further B cell aliquots were

separately transduced with VSV-G-HIV (MOI 2) or $MV_{\alpha CD20}$-HIV (MOI 2) vectors. After 48 h, the cells were stained for the B cell markers CD20 and CD19 and the percentage of CD20/CD19/GFP triple-positive cells was determined by FACS analysis.

While the $MV_{\alpha CD20}$-HIV particles mediated *gfp*-gene transfer into 50% of the unstimulated primary human B cells, only background transduction was observed after VSV-G-HIV incubation (Table 5 experiment 1). In contrast, after co-incubation with VSV-G-HIV and $MV_{\alpha CD20}$-$HIV_{cd34tk39}$ particles, 4% of the unstimulated B cells had become GFP-positive (Table 5 experiment 1). More or less the same amount of CD20/CD19/GFP triple-positive B cells after co-transduction with VSV-G-HIV and $MV_{\alpha CD20}$-$HIV_{cd34tk39}$ particles was observed with B cells from a second donor (Table 5 experiment 2).

Table 5: GFP-positive primary human B cells after co-transduction with VSV-G-HIV and $MV_{\alpha CD20}$-$HIV_{cd34tk39}$ particles.

	transduction efficiency [%]	
vector type	experiment 1	experiment 2
$MV_{\alpha CD20}$-HIV	50	not done
VSV-G-HIV	0.6	0
VSV-G-HIV $MV_{\alpha CD20}$-$HIV_{cd34tk39}$	4	7

Taken together, these results and the results from chapter 3.5.1 indicate that $MV_{\alpha CD20}$-HIV particles induce an activating stimulus in unstimulated primary human B cells, which allowed moderate gene transfer by VSV-G-HIV particles into these cells.

4 Discussion

Since lentiviral vectors allow stable integration of a transgene into the host cell genome and its propagation into daughter cells and furthermore the transduction of most non-dividing cells, they are the most promising tools for gene therapy. However, further efforts in vector design are required to improve safety and efficiency of lentiviral mediated gene transfer. One aspect is the restriction of gene transfer to the cell type relevant for a particular therapeutic application already at the step of cell entry. Furthermore, lentiviral vectors able to transduce quiescent lymphocytes are desirable. For this reason, the development of an efficient and compliant cell entry targeting system for lentiviral vectors is described in this thesis. The very competent MV targeting system was transferred to lentiviral vectors by pseudotyping them with the MV H and F glycoproteins. This novel approach allowed for the first time the retargeting of a lentiviral vector to a cell surface molecule of interest as well as the transduction of quiescent B lymphocytes.

4.1 Setting up the system

The first step for the generation of retargeted lentiviral vector particles was their pseudotyping with the MV glycoproteins. As the unmodified MV H and F proteins did not pseudotype HIV-1 vectors (3.1), it was assumed that sequences in their cytoplasmic tails prevent pseudotyping. In both postulated mechanisms of pseudotype formation the glycoprotein cytoplasmic tail plays an important role. In the passive incorporation model, in which no direct interactions between virus proteins and glycoproteins are needed, but sufficient amounts of glycoproteins must be provided at the site of budding, the glycoprotein cytoplasmic tail has no active function but should not sterically hinder vector particle incorporation and morphology (Swanstrom and Wills, 1997). In the active model, it directly interacts with the viral core proteins or indirectly via cellular factors, which leads to pseudotyping.

At the beginning of this thesis there was only a single description of lentiviral pseudotype formation with glycoproteins of a paramyxovirus, namely Sendai virus, available. Thereby, truncation of the F protein cytoplasmic tail and addition of the cytoplasmic tail of the lentiviral Env to the cytoplasmic tail of the HN protein were necessary to allow pseudotyping (Kobayashi et al., 2003). For the generation of HIV-

1 vectors pseudotyped with the MV glycoproteins, in a first attempt, this strategy was adapted for this thesis. However, no MV-HIV vectors could be generated with this approach (data not shown). For this reason also the cytoplasmic tail of the H protein was truncated.

Fifteen H protein variants carrying stepwise truncations and amino acid exchanges in their cytoplasmic tails and two F protein variants (3.1.1 Figure 9) were screened in all combinations for their ability to pseudotype HIV-1 vector particles. The screen identified three combinations that led to efficient pseudotype formation, namely HcΔ18/FcΔ30, HcΔ19/FcΔ30 and HcΔ24+4A/FcΔ30 (3.1.1). The F cytoplasmic tail has to be truncated by 30 aa, leaving just 3 aa, two of which are positively charged. In transmembrane proteins such membrane proximal, positively charged residues are often needed to stabilise their integration into the lipid-bilayer (Dalbey, 1990). The functionality of the FcΔ30 protein in respect of membrane fusion has been shown previously (Moll et al., 2002). Furthermore MV F proteins with a premature stop codon, resulting in a cytoplasmic tail truncated by 24 aa, are found in MV isolates from patients with SSPE (Cathomen et al., 1998).

Among the H protein variants, there was a clear peak of optimal truncation when 18 or 19 residues were deleted (variants HcΔ18 and HcΔ19). Further truncation reduced titers, although replacing some of the deleted residues by alanine could restore optimal titers in case of variant HcΔ24+4A. Obviously, the critical step was the identification of H protein cytoplasmic tail truncation mutants that allowed pseudotyping while retaining fusion helper function located in the membrane proximal region of the H protein cytoplasmic tail (Moll et al., 2002). All of the three H protein variants that allowed most efficient pseudotype formation are still active in fusion helper function (Moll et al., 2002). Apparently, the cytoplasmic tails of the unmodified MV H and F proteins contain sequences that prevent pseudotyping of lentiviral vectors. Western blot analysis of HcΔ18/FcΔ30 pseudotyped HIV-1 vector particles in comparison to HIV-1 particles produced in the presence of the unmodified H and F proteins demonstrated that these perturbing sequences in the cytoplasmic tails of H and especially F prevent incorporation of the unmodified MV glycoproteins into viral particles (3.1.3).

Although the overall expression levels of the truncated proteins in the packaging cells appeared increased compared to the unmodified proteins, cell surface expression levels, which are regarded more relevant for pseudotyping did not differ (3.1.3). Thus,

the interference of cytoplasmic tail sequences with particle incorporation is most likely the reason for absence of pseudotyping with the unmodified H and F proteins. Interestingly, these same variants as well as other tested MV glycoprotein variants could not efficiently pseudotype MLV particles (3.1.2). Obviously, the restriction factors for the formation of lentiviral and γ-retroviral MV pseudotypes, respectively, are different. There is indeed evidence in literature for a different behaviour of MLV and HIV-1 vector particles in respect of pseudotype formation. For example, the unmodified glycoproteins of GALV and RD114 virus readily form MLV pseudotypes, because the MLV protease can cleave their R peptide, located in their cytoplasmic tails, but are unable to pseudotype HIV-1 particles. Only after truncation or modification of the R peptide sequence, these envelope proteins form HIV-1 pseudotypes (Christodoulopoulos and Cannon, 2001; Merten et al., 2005; Sandrin et al., 2004; Stitz et al., 2000).

A few month after our publication of the MV-HIV pseudotype formation (Funke et al., 2008), also the group around François-Loïc Cosset published data demonstrating pseudotyping of HIV-1 vectors with MV glycoproteins at titers comparable to ours (Frecha et al., 2008). While they also used FcΔ30, they identified HcΔ24 as the best candidate for pseudotyping. Pseudotype formation with this H protein truncation mutant was very inefficient in the screen described in this thesis (3.1.1). Furthermore, HcΔ24 is strongly impaired in the fusion helper function (Moll et al., 2002), for which reason it is even more surprising that it allowed efficient pseudotype formation. Another discrepancy is the observation by Frecha et al. that HcΔ24/FcΔ30 also pseudotype MLV particles (Frecha et al., 2008). As mentioned above, in this thesis, for MV-MLV vector particle pseudotyping no gene transfer above background levels was observed, also including the HcΔ24/FcΔ30 combination (data not shown). Taken together, Frecha et al. confirmed the data in this thesis that MV-HIV pseudotypes are formed upon cytoplasmic tail truncations. In contrast to our data, a fusion helper function deficient H protein mutant was active and MV-MLV pseudotypes could be generated. Both issues are not in accordance with our results and can not be explained at the moment.

An interesting observation was the increase in MV-HIV titers, when the ratio of the plasmids encoding FcΔ30 and HcΔ19, respectively, was optimised for vector particle production. The optimal ratio was determined to be seven-fold more F than H plasmid, which resulted in a more than ten-fold increase in titer compared to particles

produced in presence of the same amounts of pCG-FcΔ30 and pCG-HcΔ19 (3.1.6). Although it was known before that more F than H mRNA is present in MV infected cells (Cattaneo et al., 1987; Plumet et al., 2005), the extent of titer reduction observed when suboptimal H:F ratios were applied, was unexpected. Obviously, a fine tuned balance of the relative amounts of F and H has to be maintained in the packaging cells to allow most efficient formation of pseudotyped HIV-1 vector particles.

In conclusion, the successful pseudotyping of HIV-1 vector particles with modified MV glycoproteins was demonstrated. These particles mediated efficient and stable gene transfer into MV receptor-positive but not MV receptor-negative cell lines. This paved the way for the generation of retargeted HIV-1 vector particles. For proof of principle, the EGF receptor (EGFR) and the B cell surface marker CD20 (Cragg et al., 2005) were chosen as targets.

4.2 Cell entry targeting of lentiviral vectors

The extensive range of target tissues/cell types transducible by lentiviral vectors *in vitro* and *in vivo* makes them an attractive tool for treating a number of genetic disorders. However, insertional mutagenesis, vector mobilization and germ-line transmission of vector sequences are all potential hazards that could arise as a consequence of the use of lentiviral vectors in gene therapy (Manilla et al., 2005). Accordingly, efforts in vector design are required to improve safety of lentiviral mediated gene transfer, for example, by restricting cell entry to the cell population important for a respective therapeutically application. For this reason, a lentiviral cell entry targeting system based on the described pseudotyping with the MV glycoproteins was developed in this thesis.

For the production of MV-HIV vectors specific for EGFR or human CD20, the cytoplasmic tail of an H protein variant that was mutated in the CD46 (Y481A, S548L and F549S) and SLAM (R533A) interaction regions (Nakamura et al., 2005), thus unable to recognise its native receptors, was truncated by 18 amino acids (HmutΔ18). At its ectodomain either EGF or a scAb directed against human CD20 (αCD20-scAb) was displayed, resulting in the constructs H-αEGFR and H-αCD20, respectively (3.2.1). For the generation of the targeting vectors $MV_{\alpha EGFR}$-HIV and $MV_{\alpha CD20}$-HIV, the plasmids pCG-H-αEGFR and pCG-H-αCD20, respectively, were

co-transfected with pCG-FcΔ30, the HIV-1 packaging construct pCMVΔR8.9 and the GFP transfer vector plasmid HR`-CMV-GFP or SEW into HEK-293T cells. The vector particle containing cell supernatants were concentrated and then used for the transduction of target receptor-positive and -negative cell lines (Figure 34).

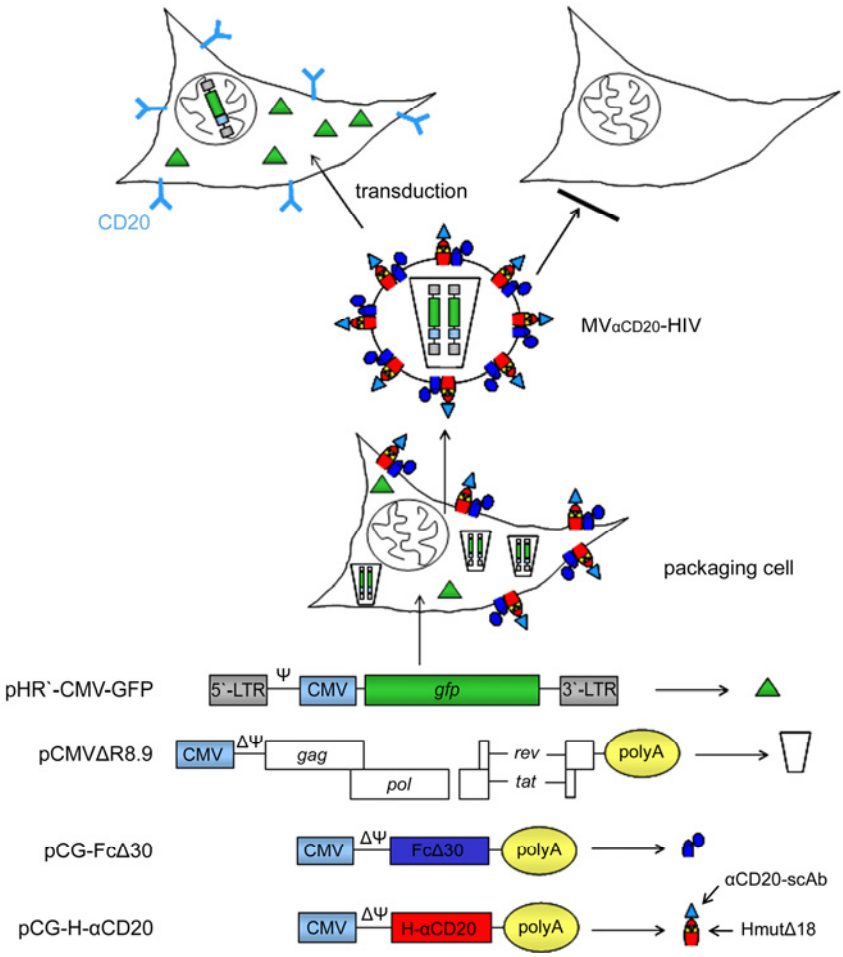

Figure 34: Production of retargeted $MV_{αCD20}$-HIV vectors. For the generation of $MV_{αCD20}$-HIV vector particles the glycoprotein expression plasmids pCG-H-αCD20 and pCG-FcΔ30, the HIV-1 packaging plasmid CMVΔR8.9 and the GFP transfer vector pHR`-CMV-GFP are transiently co-transfected into HEK-293T cells (packaging cells). Then, the packaging cells release CD20-retargeted lentiviral vector particles pseudotyped with FcΔ30 and H-αCD20. They have two RNA copies of the transfer vector packaged, which can be transferred and after reverse transcription integrated into CD20-positive cells (transduction). In contrast, CD20-negative cells are not transduced from these particles. The expressed GFP, encoded by the transfer gene, is shown as green triangles. Presence (Ψ) or absence (ΔΨ) of the packaging signal is indicated. LTR: long terminal repeat; CMV: cytomegalovirus promoter; polyA: polyadenylation signal.

Use of the targeted receptors EGFR and CD20, respectively, for cell entry was verified by the highly increased titers (about 1,000-fold) of $MV_{αEGFR}$-HIV and $MV_{αCD20}$-HIV vectors on CHO cells expressing the target receptors compared to the parental CHO cells or CHO cells positive for SLAM or CD46 (3.2.2; 3.2.4). Hence, the high

selectivity and flexibility of the novel retargeting system for lentiviral vectors was demonstrated by targeting two different types of cell surface molecules, namely the EGFR, a typical type I transmembrane protein which becomes rapidly endocytosed upon ligand binding, and CD20, a membrane tetraspan protein usually not internalised upon antibody binding (Blakely et al., 2000; Cosset et al., 1995; Cragg et al., 2005; Glennie et al., 2007). Furthermore, either a natural ligand (EGF) or a scAb can be displayed as C-terminal extension of the HmutΔ18 protein for retargeting.

Similarly as with MV-HIV vectors (3.1.6), also the titers of $MV_{\alpha CD20}$-HIV particles were enhanced after identification of an optimal pCG-H-αCD20 to pCG-FcΔ30 ratio, which is 1:3 (3.2.3). However, the optimal H to F plasmid ratio for the production of $MV_{\alpha CD20}$-HIV vector particles was different from the one determined for untargeted MV-HIV vector particles. Obviously, relatively more H protein is needed when a scAb is fused to its ectodomain. This is most likely due to the reduced cell surface expression of such H proteins (diploma thesis of Inna Kneiske), which can be compensated by applying higher amounts of the corresponding expression plasmid. Probably, the displayed scAb destabilises the H protein leading to a higher degradation rate and thus reduced cell surface expression. Data obtained in our laboratory indicate that each displayed scAb has a different effect on H protein surface expression, for which reason the optimal glycoprotein plasmid ratio might differ between diverse H protein display constructs (Buchholz, personal communication).

For $MV_{\alpha CD20}$-HIV vectors selective long-term *gfp*-gene transfer into cell lines naturally expressing CD20 was demonstrated in mixed cell culture of CD20-positive and negative cell lines (3.2.7). Virtual absence of background transduction of non-target cells with this system was demonstrated by the selective killing of CD20-positive human lymphocytes while co-cultured CD20-negative cells proliferated unaffectedly (3.3.2). This experiment also verified the ability of the $MV_{\alpha CD20}$-HIV vector particles to selectively transfer therapeutic genes, like a suicide gene (Nasu et al., 2000; Miyake et al., 2007), into the relevant cell population leading to a restricted effect of the expressed therapeutic agent.

Membrane fusion via CD20 was pH-independent as confirmed by the formation of syncytia under neutral pH conditions in HT1080-CD20, but not HT1080 cells upon co-transfection of plasmids encoding H-αCD20 and FcΔ30 (3.2.5). Hence, the pH-independent MV membrane fusion machinery is able to use totally different types of

cell surface proteins as receptor, and may thus be exploited for the targeting of lentiviral vectors to any desired cell surface molecule. Compared to another cell entry targeting system for lentiviral vectors (see below), MV-HIV vectors do not depend on receptor-mediated endocytosis of the vector upon antigen binding to mediate fusion via the low pH in the endosomes.

In parallel to this thesis a lentiviral cell entry targeting system based on the co-incorporation of a native receptor blind Sindbis virus glycoprotein, which mediates pH-dependent fusion, and a complete antibody molecule has been developed (Yang et al., 2006). Interestingly, also in this system CD20 was used as target molecule. Although numerous reports have demonstrated the lack of CD20 modulation after antibody binding, there is some evidence that it can be endocytosed with a slow kinetic under certain circumstances (Cragg et al., 2005; Press et al., 1989). Transduction of 293T-CD20 cells yielded similar titers with these vectors as with our MV glycoprotein-based targeting vectors on HT1080-CD20 cells. However, background transduction on control cells seems higher with the Sindbis vector pseudotypes (Yang et al., 2006). Transductions of mixed cultures of target and non-target cell lines were not performed and the selective transduction of primary human B lymphocytes lacked some important controls, like the demonstration that the CD20-negative T lymphocytes can be generally transduced with a lentiviral vector after stimulation with lipopolysaccharide only, which was the reagent used for activation of their primary lymphocytes. Lymphocytes require stimulation with cytokines or other factors to make them susceptible for transduction by lentiviral vectors (Serafini et al., 2004; Unutmaz et al., 1999).

In contrast to Yang et al. in this thesis a surprisingly efficient transduction of activated primary human B lymphocytes using the CD20-targeted vector was achieved, reaching more than 70% GFP-positive cells upon a single transduction cycle (3.4.1). Previously, difficulties in achieving efficient transduction of primary human B lymphocytes have been described. All studies agreed in VSV-G being the optimal envelope for B cell transduction but even under carefully optimised conditions never reached transduction levels higher than 20% (Bovia et al., 2003; Janssens et al., 2003). When the same culture and activation conditions were applied as in this thesis, only 2.3% transduced B cells were obtained (Bovia et al., 2003). Although in this thesis about 42% GFP-positive B cells were observed after applying VSV-G-HIV particles at an MOI of 150 (3.4.1), at such high MOIs pseudotransduction may have

significantly contributed to the GFP-fluorescence of VSV-G-HIV vector transduced cells (Gallardo et al., 1997). The considerably lower MFI of VSV-G-HIV vector transduced B cells, compared to B cells transduced by $MV_{\alpha CD20}$-HIV particles, supports this possibility.

Thus, the MV glycoproteins modified with an αCD20-scAb are much more efficient in mediating gene transfer into primary human B lymphocytes than the VSV-G protein. Moreover, gene transfer is very selective, demonstrated by the transduction of mixed lymphocytes with highly underrepresented B cells. With the $MV_{\alpha CD20}$-HIV vector only the B cells had become GFP-positive, while the T cell fraction remained GFP-negative. In contrast, the VSV-G-HIV vector transduced both cell fractions roughly at equal efficiency and did not discriminate between the two cell populations (3.4.3). Chromosomal integration of transfer vector sequences in primary human B cells transduced by the $MV_{\alpha CD20}$-HIV vector has been demonstrated by a two step *Alu*-PCR (3.4.2), which verifies that the high amount of GFP-positive primary human B cells was due to viral integration and not to pseudotransduction. In contrast, the absence of an amplificate after the two-step *Alu*-PCR for primary human B cells transduced by VSV-G-HIV particles (data not shown) supports a significant contribution of pseudotransduction to the GFP-fluorescence obtained with this vector.

Hence, with the novel targeting system based on the MV glycoproteins a highly selective gene transfer into specific target cell populations with an unprecedented degree of efficiency is possible. Furthermore, this system should be widely applicable, by displaying different scAbs or ligands on the modified H protein. In contrast to previously ineffective targeting strategies that used for example MLV Env as display platform, the reason for the success of this novel targeting system lays presumably in the separation of the receptor recognition and fusion functions onto two different proteins.

4.3 MV-HIV vectors and the transduction of quiescent lymphocytes

Quiescent cells reside in the G_0 phase of the cell-cycle. In comparison to cells in G_1 phase, this state is characterised by lower rates of transcription, translation and metabolism as well as reduced cell size (Yusuf and Fruman, 2003). In quiescent naïve lymphocytes, antigen recognition with appropriate co-stimulation triggers exit from G_0 phase, increased size and metabolism and progression through the cell

cycle. Presumably, quiescence in naïve lymphocytes acts to reduce the resources (energy and space) required to maintain a vast repertoire of T and B cells. Quiescence might also protect cells from accumulating metabolic damage as well as genetic changes that could result in malignancy (Yusuf and Fruman, 2003). Lentiviral vectors can transduce many types of non-proliferating cells, but primary human lymphocytes require stimulation with cytokines or other factors leading to exit from the G_0 status and transition to G_{1b} phase to become transduced by these vectors. In resting lymphocytes post-entry steps like completion of reverse transcription, nuclear import and chromosomal integration of the transgene do not occur (Korin and Zack, 1998; Serafini et al., 2004; Stevenson et al., 1992; Unutmaz et al., 1999).

It was therefore highly unexpected that $MV_{\alpha CD20}$-HIV vector particles transduced unstimulated, quiescent B cells from five different donors with efficiencies of about 20%. VSV-G-HIV vector particles were unable to transduce these cells even if applied at 100-fold higher MOIs than $MV_{\alpha CD20}$-HIV vectors (3.4.4). Hence, $MV_{\alpha CD20}$-HIV vectors do not only allow restricted transduction of CD20-positive cells but also transduction of unstimulated, resting primary human B cells. What is the molecular mechanism behind this unexpected result? Post-entry events in MV and HIV infected T lymphocytes as well as CD20-dependent signaling events in B cells, together with some initial experimental data might give an explanation:

T cells

In contrast to HIV-1 derived lentiviral vectors, HIV-1 itself can infect resting T cells *in vivo*, for which it needs beside CD4 the chemokine co-receptor CXCR4. Static cortical actin in quiescent T cells represents a post-entry barrier to HIV-1 infection, but upon binding, HIV-1 utilizes the CXCR4 signaling pathway to activate a cellular actin-depolymerising factor, namely cofilin, to overcome this restriction (Yoder et al., 2008). Cofilin is the primary molecule regulating cortical actin dynamics. In cycling cells, cofilin is constitutively active to facilitate constant remodeling of the actin cytoskeleton. In contrast, in resting CD4-positive T cells cofilin is largely inactive, implying a less dynamic cortical actin. Cofilin dephosphorylation and thus activation by CXCR4 signaling and adjacent actin dynamics are required post-entry processes for HIV-1 infection (Yoder et al., 2008). Probably, lentiviruses use the actin cytoskeleton for transfer of their viral genome from the peripheral regions of the cell to the microtubule network for reverse transcription and nuclear import.

Furthermore, Naghavi et al. showed that another cytoskeletal factor, namely moesin, a member of the ezrin/radixin/moesin proteins, also directly influences lentiviral infection of cells (Naghavi et al., 2007). Moesin, which partially resides in lipid rafts (Müller et al., 2006), acts as a cross-linker between plasma membrane and actin filaments, as well as a signal transducer in responses involving cytoskeletal remodeling. Moesin overexpression was found to downregulate the formation of stable microtubules, whereas knockdown of moesin increased stable microtubule formation (Naghavi et al., 2007). Naghavi et al. demonstrated that moesin overexpression blocks infection by HIV-1 before the initiation of reverse transcription while moesin knockdown enhances infection. They state that in the absence of stable microtubules, due to active moesin, functional reverse transcription complexes are not formed. Consistent with this, phosphorylation levels and activity of moesin are high in quiescent lymphocytes but decrease after stimulation with cytokines (Müller et al., 2006).

Importantly, Müller et al. investigated the influence of MV on cofilin and moesin (Müller et al., 2006): In T cells that had been in contact with the MV glycoproteins cofilin and moesin became dephosphorylated. This way cofilin became activated leading to actin rearrangement and moesin became inactivated leading to enhanced formation of stable microtubules. As mentioned above, both processes are crucial for the transport of HIV-1 core particles to the cell nucleus (Naghavi et al., 2007; Yoder et al., 2008).

Shortly after publication of some results of this thesis (Funke et al., 2008) also Frecha et al. (Frecha et al., 2008) published data about the pseudotyping of lentiviral vectors with MV glycoproteins (4.1). They showed that untargeted MV-HIV vectors are able to transduce quiescent primary human T cells. However, in contrast to B cells transduced by the $MV_{\alpha CD20}$-HIV vectors (3.5), Frecha et al. showed evidence that MV-HIV transduced T cells were not activated but remained in a quiescent state after transduction. Their data indicate that entry into primary human T cells happened primarily through SLAM. Even though SLAM and CD46 can act as co-stimulatory molecules for lymphocyte activation, their engagement has no mitogenic effect on freshly isolated T-cells (Cocks et al., 1995; Zaffran et al., 2001). Although Frecha et al. gave no mechanistic explanation for their observation, they confirmed our observation that MV glycoprotein pseudotyped HIV-1 vectors can transduce quiescent lymphocytes.

B cells

It is likely that also in B cells one of the blocks for quiescent B cell transduction is static cortical actin. Like T cells, also B cells must at least enter G_{1b} cell-cycle phase for transduction by all so far known lentiviral vectors (Serafini et al., 2004). During this state actin rearrangements take place (Brancolini and Schneider, 1994), which presumably dissolve the actin block. Although it is clear that $MV_{\alpha CD20}$-HIV vectors bind CD20 and enter cells via CD20, interactions with the natural receptors SLAM and CD46 cannot be completely ruled out. It is e.g. known that R533, which is mutated to prevent entry via SLAM, does not directly contact SLAM, but is involved in the H protein conformational changes that occur after SLAM binding (Navaratnarajah et al., 2008). It is therefore supposed that residual binding of the MV glycoproteins to SLAM and/or CD46 would lead to dephosphorylation of cofilin and moesin (Hao et al., 2008; Müller et al., 2006; Lanemo Myhrinder et al., 2008; Masumoto et al., 1998), resulting in actin rearrangement and microtubule formation (Naghavi et al., 2007; Yoder et al., 2008). $MV_{\alpha CD20}$-HIV vector particles can then enter unstimulated B cells via CD20 binding and due to the dissolved actin block and enhanced microtubule formation integrate their transgene into the host cell genome (Figure 35).

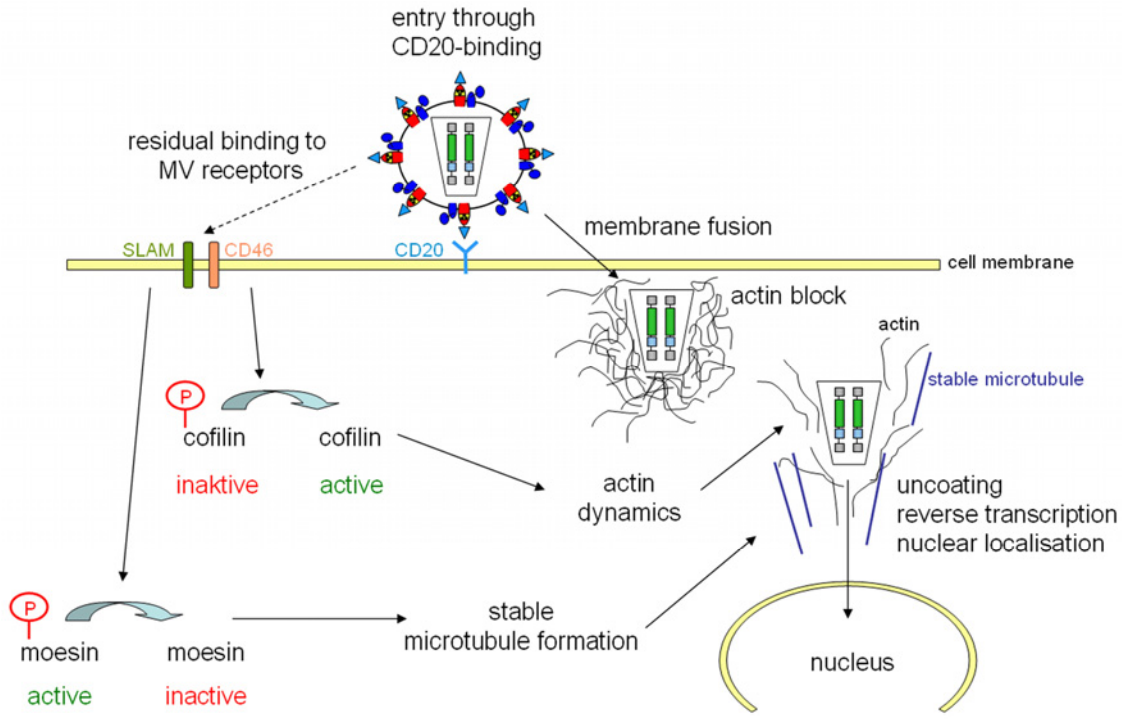

Figure 35: Hypothetical model for the mechanism of $MV_{\alpha CD20}$-HIV transduction of quiescent B cells. The MV glycoproteins of $MV_{\alpha CD20}$-HIV vectors exhibit residual interaction with CD46 and SLAM, which leads to dephosphorylation of cofilin and moesin, resulting in actin dynamics and enhanced stable microtubule formation. Entry of $MV_{\alpha CD20}$-HIV vector particles into resting B cells occurs through CD20. After membrane fusion, the post-entry block in resting B cells, consisting of static cortical actin,

is dissolved through the activation of cofilin. Moesin inactivation enhances formation of stable microtubules. Together, this enables uncoating, reverse transcription and nuclear localisation with transgene integration (transduction).

This mechanism relies on cytoskeleton rearrangement in absence of B cell activation. Alternatively, contact of vector particles with quiescent cells may result in mitogenic activation. It has been previously shown that lentiviral vectors engineered to display cytokines, like interleukin-7, thrombopoietin or stem cell factor on their surface can induce mitogenic stimuli in resting lymphocytes or haematopoietic stem cells and thus enable lentiviral vector transduction (Verhoeyen et al., 2003; Verhoeyen et al., 2005).

How about CD20 as signaling molecule? Although the anti-CD20 monoclonal antibody rituximab, which is routinely used for the treatment of e.g. non-Hodgkin`s lymphoma (Cheson and Leonard, 2008), is successfully used in immunotherapy, knowledge about the physiological function of CD20 is still limited. A natural ligand has not been identified and CD20 knockout mice are basically unaffected (Cragg et al., 2005). However, there is increasing evidence for a physiological role of CD20 as a regulator of cell growth and differentiation (Cragg et al., 2005; Riley and Sliwkowski, 2000). Recent data indicate that human CD20 acts as a capacitance or store-operated cation (SOC) channel involved in B cell antigen receptor (BCR) signal transduction, triggered to open when the intracellular stores are depleted (Li et al., 2003; Walshe et al., 2008; Janas et al., 2005). It is known that B cell activation requires sustained elevation of cytoplasmic free calcium, achieved through a combination of its release from intracellular stores and influx of extracellular calcium via membrane channels (Cragg et al., 2005).

Some CD20-specific mAbs, like 1F5, provoke G_0 phase to G_1 phase transition and induce elevated cytoplasmic free calcium levels (Cragg et al., 2005). The amino acid sequences of the variable chains of the αCD20-scAb used in this thesis (Bucheit et al., 2003) and that of the antibody 1F5 are ~88% identical. Walshe et al. (Walshe et al., 2008) suggest the following mechanism of anti-CD20 mAb mediated calcium flux: In the resting state, CD20 and BCR are separated. Following ligation of CD20 by anti-CD20 monoclonal antibodies and hyper-cross-linking, CD20 molecules cluster together and associate with BCR. This tight clustering of CD20/BCR complexes mediates BCR downstream signaling leading to release of calcium from intracellular stores. This release of intracellular calcium results in open CD20 channels causing

an influx of extracellular calcium, which leads to transition from the G_0 phase to the G_1 phase of the cell-cycle (Craxton et al., 2007). The multivalent CD20 binding as it occurs with lentiviral vector particles possibly further potentiates this effect that requires cross-linking of CD20 molecules (Janas et al., 2005).

Indeed, the activation markers CD69, CD86 and CD71 became upregulated when resting B cells where incubated with $MV_{\alpha CD20}$-HIV vectors but not with VSV-G-HIV vectors (3.5.1). Moreover, pre-incubation of unstimulated B cells with CD20-targeted MV-HIV vectors allowed a low but significant level of transduction by VSV-G-HIV vector particles (3.5.2). Thus, the CD20-targeted MV-HIV vector particles must have induced an activating stimulus in the resting B cells which made them susceptible for transduction by VSV-G-HIV particles.

Hence, an alternative mechanism for the transduction of unstimulated B cells by $MV_{\alpha CD20}$-HIV vector particles involves binding and hyper-cross-linking of CD20 molecules by the vector particles, which leads to CD20/BCR clustering. This results in influx of extracellular calcium and finally B cell activation. Such B cells are then susceptible for completion of reverse transcription, nuclear import and chromosomal transgene integration (transduction) by lentiviral vectors (Figure 36).

However, further experiments are needed to confirm these two hypothetical models (Figure 35; Figure 36) and to decide which molecular events are more relevant or if a combination of both takes place. For example, calcium influx into B cells after or during transduction with $MV_{\alpha CD20}$-HIV vector particles will have to be measured. Furthermore, different steps in the moesin and cofilin pathways can be blocked, to see if these pathways are indeed involved in the transduction of quiescent B cells.

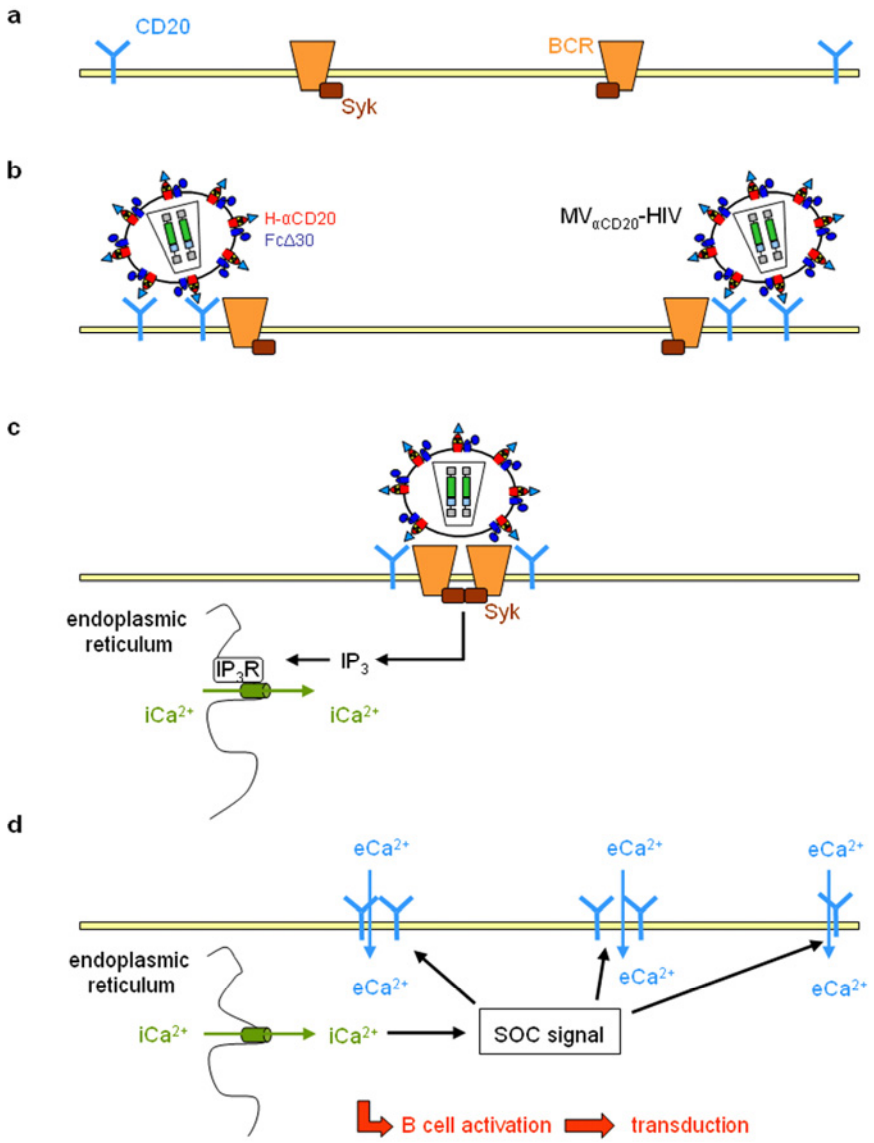

Figure 36: Hypothetical model for the stimulation of quiescent B cells by $MV_{\alpha CD20}$-HIV vectors. In the resting state, CD20 and BCR are separated **(a)** whereas after CD20-binding by $MV_{\alpha CD20}$-HIV particles the CD20 molecules cluster and associate with BCR **(b)**. The tight clustering of CD20/BCR complexes, which is potentiated through hyper-cross-linking of CD20 molecules by $MV_{\alpha CD20}$-HIV particles, results in clustering of the protein-tyrosine kinase Syk which activates the phospholipase C_γ that generates inositol trisphosphate (IP_3). Through IP_3 interaction with IP_3 receptors (IP_3R) calcium from intracellular stores (iCa^{2+}) is released **(c)**. This release of iCa^{2+} results in SOC channel opening (including CD20) causing an influx of extracellular calcium (eCa^{2+}), prolonging the calcium flux which functions as an activating stimulus making the B cells susceptible for transgene integration mediated by $MV_{\alpha CD20}$-HIV vector particles **(d)**. (modified after Walshe et al. 2008, JBC (Walshe et al., 2008))

4.4 Outlook

Whatsoever the molecular mechanism is that allows B cell transduction by $MV_{\alpha CD20}$-HIV particles, obviously, MV glycoproteins are especially suited for pseudotyping of lentiviral vectors as both viruses mediate pH-independent fusion and induce

cytoskeleton rearrangements in lymphocytes. Such vectors will enable a number of novel applications ranging from the genetic modification of B cells for investigating basic questions in immunology to therapeutic strategies such as the treatment of inherited B cell disorders or lymphomas. Until now, lymphocytes had to be stimulated for transduction with lentiviral vectors, which can lead to unwanted changes in lymphocyte subsets.

The MV-HIV vectors that were developed in this thesis are based on glycoproteins derived from a MV vaccine strain (3.1.1). By applying the same cytoplasmic tail truncations, we also generated HIV-1 vectors pseudotyped with the glycoproteins derived from the wildtype MV strain IC-B, recombinant clone IC323 (Takeda et al., 2000), that does not use CD46 as natural receptor. Such pseudotypes selectively enter SLAM-positive cell types (Funke et al., in revision). Presumably due to the absence of syncytia formation in the packaging cells, about ten-fold higher titers were obtained with these pseudotypes compared to the vaccine strain MV pseudotypes described in this thesis. Therefore, even more effective retargeted MV-HIV vectors may become available in future, using wildtype MV glycoproteins as display platform.

Moreover, as the MV fusion process is pH-independent, in principle, every cell surface molecule can be targeted by displaying the respective ligand or scAb on a native receptor blind H protein. Therefore, it should now be possible to tailor lentiviral vectors for highly selective gene transfer into any target cell population, including quiescent lymphocytes, with an unprecedented degree of efficiency (Baum, 2008).

5 Summary

Lentiviral vectors mediate gene transfer into dividing and most non-dividing cells. One exception are e.g. quiescent lymphocytes. Thereby, they stably integrate the transgene into the host cell genome. For this reason, lentiviral vectors are a promising tool for gene therapy. However, safety and efficiency of lentiviral mediated gene transfer still needs to be optimised. Ideally, cell entry should be restricted to the cell population relevant for a particular therapeutic application. Furthermore, lentiviral vectors able to transduce quiescent lymphocytes are desirable. Although many approaches were followed to engineer retroviral envelope proteins, an effective and universally applicable system for retargeting of lentiviral cell entry is still not available. Just before the experimental work of this thesis was started, retargeting of measles virus (MV) cell entry was achieved. This virus has two types of envelope glycoproteins, the hemagglutinin (H) protein responsible for receptor recognition and the fusion (F) protein mediating membrane fusion. For retargeting, the H protein was mutated in its interaction sites for the native MV receptors and a ligand or a single-chain antibody (scAb) was fused to its ectodomain.

It was hypothesised that the retargeting system of MV can be transferred to lentiviral vectors by pseudotyping human immunodeficiency virus-1 (HIV-1) derived vector particles with the MV glycoproteins. As the unmodified MV glycoproteins did not pseudotype HIV-1 vectors, two F and 15 H protein variants carrying stepwise truncations or amino acid (aa) exchanges in their cytoplasmic tails were screened for their ability to form functional MV-HIV pseudotypes. The combinations HcΔ18/FcΔ30, HcΔ19/FcΔ30 and HcΔ24+4A/FcΔ30 led to most efficient pseudotype formation with titers above 10^6 transducing units (t.u.)/ml on HT1080 cells, using concentrated vector particles. Thereby, the F cytoplasmic tail was truncated by 30 aa, just leaving 3 aa and the H protein cytoplasmic tail was truncated by 18, 19 or 24 residues with four added alanines after the start methionine in the latter case. Western blot analysis indicated that particle incorporation of the MV glycoproteins was enhanced upon truncation of their cytoplasmic tails. With the MV-HIV vectors high titers on different human cell lines expressing one or both MV receptors were obtained, whereas MV receptor-negative cells remained untransduced. Titers were enhanced using an optimal H to F plasmid ratio (1:7) during vector particle production. Stability of gene transfer was verified by reverse transcriptase inhibition and long-term cultivation.

Based on the described pseudotyping with the MV glycoprotein variants, HIV-1 vector particles retargeted to the epidermal growth factor receptor (EGFR) or the B cell surface marker CD20 were generated. For the production of the retargeted vectors $MV_{\alpha EGFR}$-HIV and $MV_{\alpha CD20}$-HIV, respectively, FcΔ30 together with a native receptor blind HcΔ18 protein, displaying at its ectodomain either the ligand EGF or a scAb directed against CD20 were

used. With these vectors, gene transfer into target receptor-positive cells was several orders of magnitude more efficient than into control cells. High target versus non-target cell discrimination with almost complete absence of background transduction of non-target cells was also demonstrated in mixed cell populations, where the CD20-targeting vector selectively eliminated CD20-positive cells upon suicide gene transfer. Remarkably, transduction of activated primary human CD20-positive B cells was much more efficient with the $MV_{\alpha CD20}$-HIV vector than with the standard pseudotype vector VSV-G-HIV. Even more surprisingly, $MV_{\alpha CD20}$-HIV vectors were able to transduce quiescent primary human B cells, which until then had been resistant towards lentiviral gene transfer.

The most critical step during the production of MV-HIV pseudotypes was the identification of H protein cytoplasmic tail mutants that allowed pseudotyping while retaining the fusion helper function. In contrast to previously inefficient targeting strategies, the reason for the success of this novel targeting system must be based on the separation of the receptor recognition and fusion functions onto two different proteins. Furthermore, with the CD20-targeting vector transduction of quiescent B cells was demonstrated for the first time. Own data and literature data suggest that CD20 binding and hyper-cross-linking by the vector particles results in calcium influx and thus activation of quiescent B cells. Alternatively this feature may be based on a residual binding activity of the MV glycoproteins to the native MV receptors that is insufficient for entry but induces cytoskeleton rearrangements dissolving the post-entry block of HIV vectors.

Hence, in this thesis efficient retargeting of lentiviral vectors and transduction of quiescent cells was combined. This novel targeting strategy should be easily adaptable to many other target molecules by extending the modified MV H protein with appropriate specific domains or scAbs. It should now be possible to tailor lentiviral vectors for highly selective gene transfer into any desired target cell population with an unprecedented degree of efficiency.

6 Summary (German, long)

Lentivirale Vektoren sind von Lentiviren, wie beispielsweise dem Humanen Immundefizienz Virus-1 (HIV-1) abgeleitet. Im Gegensatz zu diesen sind sie allerdings replikations-inkompetent, das heißt, sie können keine Nachkommen erzeugen. Sie können aber die genetische Information für ein therapeutisches Gen oder ein Markergen in Zielzellen transferieren, ein Prozess, der sich Transduktion nennt. Lentivirale Vektoren können sowohl mitotisch aktive wie auch die meisten mitotisch inaktiven Zellen transduzieren. Eine Ausnahme bilden z.B. ruhende Lymphozyten. Dabei integrieren sie das Transfergen stabil ins Genom der Wirtszelle. Aus diesem Grund sind lentivirale Vektoren sehr gut für die Gentherapie geeignet. Allerdings muss die Effizienz und die Sicherheit des lentiviral vermittelten Gentransfers noch optimiert werden, ehe eine breite klinische Anwendung möglich ist. Zum Beispiel sollte der Zelleintritt auf die Zielzellpopulation einer bestimmten therapeutischen Anwendung beschränkt werden können. Außerdem wären lentivirale Vektoren, die ruhende Lymphozyten transduzieren können, wünschenswert.

Das Wirtszellspektrum (Tropismus) lentiviraler Vektoren wird durch ihr Hüllprotein bestimmt. Durch Einbau von heterologen Hüllproteinen anderer umhüllter Viren, ein Prozess der sich Pseudotypisierung nennt, kann der lentivirale Tropismus verändert werden. Eines der ersten und bis jetzt am meisten genutzten Hüllproteine für die Pseudotypisierung ist das Glykoprotein (G) des vesikulären Stomatitisvirus (VSV). Auf Grund der Stabilität und des breiten Tropismus von VSV-G-HIV Vektoren werden diese als „Goldstandard“ bezeichnet, an dem andere lentivirale Pseudotypen gemessen werden. In den vergangenen 15 Jahren wurde intensiv auf dem Gebiet des gezielten Zelleintritts (*targeting*) retroviraler und lentiviraler Vektoren geforscht. Allerdings gibt es bis heute noch kein effizientes und universell anwendbares *targeting*-System. Es wurden z.B. verschiedene Polypeptide, wie Wachstumsfaktoren, Hormone oder einkettige Antikörperfragmente (*single-chain antibodies*; scAb) an die extrazelluläre Domäne des retroviralen Hüllproteins (*envelope protein*; Env) fusioniert. Durch die Präsentation der verschiedenen Moleküle auf Env, sollte anstatt des natürlichen Rezeptors ein ausgewähltes Zelloberflächenprotein den Zelleintritt vermitteln. Obwohl die meisten dieser chimären Envs an das jeweilige Zielmolekül binden konnten, konnte diese Bindung keinen effizienten Zelleintritt der Vektoren vermitteln.

Dagegen kann der Zelleintritt des Masernvirus (MV) sehr effizient umgeleitet werden. Dieses besitzt zwei glykosylierte Hüllproteine, das Hämagglutinin (H)-Protein, welches mit den MV-Rezeptoren CD46 und *signaling lymphocyte activation molecule* (SLAM) interagiert und das für die pH-unabhängige Membranfusion zuständige F-Protein. Um gezielten Zelleintritt vermitteln zu können, wurde das H-Protein in den Rezeptorerkennungsregionen mutiert und ein scAb oder ein Ligand auf seiner Ektodomäne präsentiert.

Die vorliegende Arbeit basiert auf der Hypothese, dass das effiziente *targeting*-System von MV auf lentivirale Vektoren übertragen werden kann, indem vom HIV-1 abgeleitete Vektoren mit den MV-Hüllproteinen pseudotypisiert werden.
Dazu mussten in einem ersten Schritt MV-Glykoproteine mit verkürzten zytoplasmatischen Domänen auf ihre Fähigkeit lentivirale Vektoren zu pseudotypisieren getestet werden. Basierend darauf, sollten MV-HIV Vektoren hergestellt werden, die gezielten Zelleintritt in Zellen vermitteln, die den epidermalen Wachstumsfaktor-Rezeptor (EGFR) oder das B-Zell-spezifische Oberflächenprotein CD20 exprimieren. Die Selektivität dieser Vektoren sollte mit Rezeptor-positiven und -negativen Zelllinien, in einzelnen Ansätzen sowie in Mischkulturen, verifiziert werden. Die CD20-spezifischen Vektoren sollten des Weitern auf ihre Fähigkeit getestet werden, Gentransfer in primäre humane CD20-positive B-Zellen zu vermitteln. Schließlich sollte überprüft werden ob durch den neuen Eintrittsweg über CD20 nun auch ruhende B-Zellen transduziert werden können, die bis dahin resistent gegen lentiviral vermittelten Gentransfer waren.

Da sich zunächst mit den unmodifizierten MV-Hüllproteinen keine MV-HIV Pseudotypen erzeugen ließen, wurde angenommen, dass Sequenzen in den zytoplasmatischen Domänen der beiden Glykoproteine die Pseudotypisierung von lentiviralen Vektoren verhindern. Es gibt mehrere Beispiele in der Literatur, die eine entscheidende Rolle der zytoplasmatischen Domäne im lentiviralen Hüllproteineinbau induzieren. Aus diesem Grund wurden zwei F-Protein und 15 H-Proteinvarianten mit stufenweise gekürzten zytoplasmatischen Domänen und Aminosäureaustauschen auf ihrer Fähigkeit getestet, lentivirale Vektoren effizient zu pseudotypisieren. Die Kombinationen HcΔ18/FcΔ30, HcΔ19/FcΔ30 und HcΔ24+4A/FcΔ30 erwiesen sich als am geeignetsten. Die entsprechenden ankonzentrierten Pseudotyp-Vektoren zeigten Titer von über 10^6 transduzierenden Einheiten pro Milliliter (*transducing units/ml*; t.u./ml) auf HT1080 Zellen. Dabei war eine Verkürzung der zytoplasmatischen Domäne des F-Proteins um 30 Aminosäuren und des H-Proteins um 18, 19 oder 24 Aminosäuren notwendig, wobei im letzten Fall vier Alanine nach dem Start-Methionin addiert wurden. Westernblot-Analysen zeigten, dass die Inkorporation der MV-Glykoproteine in die Vektorpartikel durch die Kürzung ihrer zytoplasmatischen Domänen verstärkt wurde.
Während andere lentivirale Vektoren, z.B. vom Simianen Immundefizienz Virus abgeleitet, mit der gleichen Effizienz wie HIV-1 Vektoren mit den identifizierten H- und F-Proteinmutanten pseudotypisiert werden konnten, wurden vom Murinen Leukämievirus (MLV) abgeleitete γ-retrovirale Vektoren nicht von diesen und auch von keiner der anderen getesteten MV-Glykoproteinvarianten effizient pseudotypisiert. Der beste Titer ($3x10^3$ t.u./ml) wurde mit der Kombination HcΔ24+4A/FcΔ24 erreicht. Alle weiteren Experimente wurden daher mit HIV-1 Vektoren durchgeführt.

Erwartungsgemäß vermittelten MV-HIV Vektoren effizienten Gentransfer in MV-Rezeptor-positive Zelllinien während MV-Rezeptor-negative Zellen untransduziert blieben. Interessanter Weise konnte ihr Titer durch Einstellen eines optimalen H- zu F-Plasmidverhältnisses in den vektorpartikelproduzierenden Zellen deutlich verbessert werden. Die Titration des Plasmidverhältnisses ergab, dass im Idealfall siebenmal mehr F-Plasmid als H-Plasmid eingesetzt werden muss. Dadurch wurde eine zehnfache Titererhöhung im Vergleich zu Vektorpartikeln, bei denen für die Produktion gleiche Mengen der beiden Plasmide eingesetzt wurden, erreicht. Hierbei ist erwähnenswert, dass auch in MV-infizierten Zellen mehr F- als H-Protein mRNA produziert wird. Durch Inhibition der Reversen Transkription konnte Pseudotransduktion, das heißt Proteintransfer von den vektorpartikel-produzierenden Zellen zu den Zielzellen, anstelle der Integration des Transfervektors, ausgeschlossen werden. Des Weiteren konnte durch Langzeitkultivierung die Stabilität des Gentransfers und der Genexpression bestätigt werden.

Nachdem die Pseudotypisierung der lentiviralen Vektoren mit MV-Hüllproteinen etabliert war, sollten basierend darauf in einem nächsten Schritt lentivirale *targeting*-Vektoren erzeugt werden. Zu diesem Zweck wurde als *targeting*-Domäne der epidermale Wachstumsfaktor (EGF) bzw. ein gegen das B-Zell-spezifische Oberflächenmolekül CD20 gerichteter scAb auf einem HcΔ18-Protein, das in den MV-Rezeptorerkennungsregionen mutiert ist und damit keinen Zelleintritt über die MV-Rezeptoren CD46 und SLAM vermitteln kann, präsentiert. Zusammen mit FcΔ30 konnten so die *targeting*-Vektoren $MV_{\alpha EGFR}$-HIV und $MV_{\alpha CD20}$-HIV hergestellt werden. Diese transduzierten effizient CHO Zelllinien, die genetisch so verändert worden waren, dass sie den EGFR oder CD20 exprimieren, während die parentalen CHO-K1 Zellen sowie CHO-CD46 und CHO-SLAM Zellen nicht transduziert wurden. Dadurch wurde die Nutzung des ausgewählten Zelloberflächenmoleküls für den Zelleintritt nachgewiesen. Auch Zelllinien, die natürlicherweise CD20 exprimieren wurden selektiv von den $MV_{\alpha CD20}$-HIV Vektoren mit Titern von über 10^6 t.u./ml transduziert, sowohl in einzelnen Ansätzen, wie auch in Mischkulturen von CD20-positiven und CD20-negativen Zelllinien. Ebenso wie der Titer der MV-HIV Vektoren, konnte auch der Titer der $MV_{\alpha CD20}$-HIV Vektoren erhöht werden, indem ein optimales Verhältnis der MV-Hüllprotein-kodierenden Plasmide während der Vektorpartikelproduktion eingesetzt wurde. Im Gegensatz zu den MV-HIV Vektoren lag hier das optimale H:F Plasmidverhältnis bei 1:3 und nicht bei 1:7. Somit wird in Relation mehr H-Protein benötigt, wenn ein scAb an seine Ektodomäne fusioniert ist, was vermutlich auf eine verringerte Oberflächenexpression solcher H-Proteine zurückzuführen ist.

Die Selektivität des von $MV_{\alpha CD20}$-HIV Vektoren vermittelten Gentransfers wurde in Mischkulturen aus CD20-positiven und -negativen Zellen untersucht. Dabei zeigte sich, dass $MV_{\alpha CD20}$-HIV Vektoren nach Transfer eines Suizidgens selektiv CD20-positive Zellen abtöten können, während co-kultivierte CD20-negative Zellen unbeeinflusst weiter wachsen. Somit

wurde nachgewiesen, dass ein therapeutisches Gen selektiv durch diese Vektoren übertragen werden kann und sich dessen Wirkung auf die Zielzellpopulation beschränkt.

Für eine klinische Anwendbarkeit solcher *targeting*-Vektoren ist die Transduktion primärer humaner Zellen bedeutend, was oftmals eine größere Herausforderung darstellt als der Gentransfer in Zelllinien. Weil CD20 exklusiv auf B-Zellen exprimiert ist, wurden diese als Zielzellpopulation verwendet. Auch wenn lentivirale Vektoren viele mitotisch inaktive Zellarten transduzieren können, brauchen ruhende Lymphozyten einen aktivierenden Stimulus, der sie aus der G_0-Phase des Zellzykluses mindestens in die G_{1b}-Phase transferiert. Aus diesem Grund wurden die B-Zellen nach ihrer Isolierung aus frischen humanen peripheren blutmononukleären Zellen zunächst mit einem Zytokincocktail aktiviert, bevor sie mit $MV_{\alpha CD20}$-HIV Vektoren und als Kontrolle VSV-G-HIV Vektoren inkubiert wurden. Überraschender Weise vermittelten die $MV_{\alpha CD20}$-HIV Vektoren einen sehr viel effizienteren Gentransfer in aktivierte primäre humane B-Zellen als VSV-G-HIV Vektoren. Nach einer einzelnen Transduktionsrunde und einer verwendeten Multiplizität der Infektion (MOI) von 2 wurden 70% der aktivierten primären humanen B-Zellen vom $MV_{\alpha CD20}$-HIV Vektor transduziert, während VSV-G-HIV Vektoren mit der gleichen MOI nur in 20% der Zellen ihr Transfergen übertrugen. Auch unter Verwendung einer MOI von 150 konnten VSV-G-HIV Vektoren nur 40% der B-Zellen transduzieren, wobei Pseudotransduktion bei einer solch hohen MOI nicht ausgeschlossen werden kann. Dies zeigt das herausragende Potential der $MV_{\alpha CD20}$-HIV Vektoren für die Transduktion dieses Zelltyps.

Des Weiteren war der Gentransfer sehr selektiv: In einer Mischkultur aus CD20-positiven primären humanen B-Zellen und CD20-negativen primären humanen T-Zellen wurden nahezu ausschließlich die B-Zellen vom $MV_{\alpha CD20}$-HIV Vektor transduziert, obwohl diese stark unterrepräsentiert waren. Dagegen diskriminierten VSV-G-HIV Vektoren nicht zwischen den beiden Zellpopulationen, die mit gleicher Effizienz transduziert wurden. Durch eine zwei Schritte *Alu*-PCR konnte die chromosomale Integration der vom $MV_{\alpha CD20}$-HIV Vektor übertragenen Transfervektorsequenzen nachgewiesen werden, wodurch Pseudotransduktion als Ursache für die hohe Transduktionsrate ausgeschlossen wurde.

Auf Grund der effizienten Transduktion der aktivierten primären humanen B-Zellen stellte sich die Frage, ob $MV_{\alpha CD20}$-HIV Vektoren auch unstimulierte, ruhende B-Zellen transduzieren können. Überraschender Weise konnten $MV_{\alpha CD20}$-HIV Vektoren unstimulierte B Zellen mit einer Effizienz von ~20% transduzieren, während den Erwartungen entsprechend, VSV-G-HIV Vektoren selbst bei einer 100-fach höheren MOI keinen Gentransfer in diese Zellen vermitteln konnten.

Ruhende B-Zellen, die von $MV_{\alpha CD20}$-HIV Vektoren transduziert worden sind, wiesen eine höhere Expression der Aktivierungsmarker CD69, CD86 und CD71 auf als untransduzierte oder mit VSV-G-HIV Vektoren inkubierte Zellen. VSV-G-HIV Vektoren konnten ruhende B-

Zellen mit einer geringen aber signifikanten Effizienz transduzieren, wenn diese mit $MV_{\alpha CD20}$-HIV Vektoren co-inkubiert wurden. Zusammengenommen spricht dies dafür, dass die B-Zellen durch Inkubation mit $MV_{\alpha CD20}$-HIV Vektoren einen aktivierenden Stimulus erhalten, der sie dann suszeptibel für lentiviral vermittelten Gentransfer macht.

In dieser Arbeit wurde basierend auf der Pseudotypisierung von lentiviralen Vektoren mit den MV-Hüllproteinen ein effizientes *targeting*-System entwickelt. Als kritischster Schritt für die Pseudotypisierung erwies sich die Identifizierung von H-Proteinmutanten, deren zytoplasmatische Domäne einerseits die Pseudotypisierung von HIV-1 Vektoren erlaubte, andererseits aber noch die Fusionshelfer-Funktion besaß. Im Gegensatz zu früheren ineffizienten Strategien basiert der Erfolg des hier beschriebenen Systems wahrscheinlich auf der Verteilung der Funktionen Rezeptorerkennung und Membranfusion auf zwei unterschiedliche Proteine.
Parallel zu dieser Arbeit wurde ein auf dem Sindbis-Virus Hüllprotein basierendes *targeting*-System in der Arbeitsgruppe von David Baltimore entwickelt. In diesem wurde ein komplettes Antikörpermolekül zusammen mit dem Sindbis-Virus Hüllprotein, welches blind für seine natürlichen Rezeptoren war, in lentivirale Vektoren eingebaut. Der Nachteil dieses Systems beruht vor allem auf der pH-abhängigen Fusion des Hüllproteins. Dieses ist darauf angewiesen, dass der Vektor nach Antigenbindung endozytiert wird, damit es die Membranfusion in Endosomen mit einem niedrigen pH durchführen kann.
Kurz nachdem Teile dieser Arbeit veröffentlicht wurden, publizierte auch die Arbeitsgruppe um François-Loïc Cosset Ergebnisse, welche die Pseudotypisierung von HIV-1 Vektoren mit MV-Glykoproteinen beschreiben. Auch wenn sie ein H-Protein mit einer stark beeinträchtigten Fusionshelfer-Funktion als besten Pseudotypisierungskandidaten identifizierten, bestätigen sie die hier gezeigten Ergebnisse, dass die Kürzung der zytoplasmatischen Domänen des H- und des F-Proteins Pseudotypisierung von lentiviralen Vektoren ermöglicht.
Das auf den MV-HIV Vektoren basierende *targeting*-System ermöglicht sogar erstmalig die Transduktion ruhender B-Zellen. In ruhenden Lymphozyten bildet kortikales Aktin einen Post-Eintrittsblock für HIV-1 Vektoren. Es wurde publiziert, dass die MV-Hüllproteine nach Kontakt mit T-Zellen Zytoskelettumordnungen auslösen, die auch das kortikale Aktin betreffen. Dementsprechend beruht ein hypothetisches Modell für die Transduktion ruhender B-Zellen auf residualer Bindung der MV-Glykoproteine an ihre nativen Rezeptoren. Dies bewirkt keinen Zelleintritt, aber könnte für den Gentransfer in ruhende Zellen notwendige Zytoskelettumordnungen verursachen. Des Weitern lösen $MV_{\alpha CD20}$-HIV Vektoren durch CD20-Bindung und -Verlinkung vermutlich einen Kalziumeinstrom und damit B-Zellaktivierung aus, was die B-Zellen suszeptibel für lentivirale Vektoren macht. Für das zweite Modell spricht unter anderem, dass B-Zellen die von dem $MV_{\alpha CD20}$-HIV Vektor

transduziert wurden, eine erhöhte Expression der Aktivierungsmarker CD69, CD86 und CD71 aufwiesen.

Das in dieser Arbeit entwickelte *targeting*-System erlaubt erstmalig einen effizienten, gezielten Zelleintritt lentiviraler Vektoren in Kombination mit Gentransfer in ruhende Lymphozyten. Darüber hinaus sollte es leicht auf jedes beliebige Zielmoleküle übertragbar sein, indem entsprechende Liganden oder scAbs an die Ektodomäne des modifizierten H-Proteins fusioniert werden. Schon in dieser Arbeit wurden zwei vollkommen unterschiedliche Zelloberflächenproteine, wie der EGFR und CD20, zum Zelleintritt verwendet. Entsprechend sollte jetzt die Generierung lentiviraler Vektoren für den selektiven Gentransfer in jede beliebige Zielzellpopulationen mit einem bis jetzt noch nie dagewesenen Grad an Effizienz möglich sein.

7 Summary (German, short)

Lentivirale Vektoren können sowohl mitotisch aktive wie auch die meisten mitotisch inaktiven Zellen transduzieren. Eine Ausnahme bilden z.B. ruhenden Lymphozyten. Dabei integrieren sie das Transfergen stabil ins Genom der Wirtszelle. Aus diesem Grund sind lentivirale Vektoren sehr gut für die Gentherapie geeignet. Allerdings müssen die Effizienz und Sicherheit des lentiviral vermittelten Gentransfers weiter optimiert werden. Zum Beispiel sollte der Zelleintritt auf die Zielzellpopulation einer bestimmten therapeutischen Anwendung beschränkt sein. Außerdem wären lentivirale Vektoren, die ruhende Lymphozyten transduzieren können, wünschenswert. Allerdings gibt es bis heute, trotz intensiver Forschung auf dem Gebiet des gezielten Zelleintritts (*targeting*), noch kein effizientes und universell anwendbares *targeting*-System für lentivirale Vektoren. Der Zelleintritt des Masernvirus (MV) kann dagegen sehr effizient umgeleitet werden. Dieses besitzt zwei Hüllproteine, das Hämagglutinin (H)-Protein, welches Bindung an die MV-Rezeptoren vermittelt, und das für die Membranfusion verantwortliche F-Protein. Um gezielten Zelleintritt vermitteln zu können, wird das H-Protein in den Rezeptorerkennungsregionen mutiert und ein einkettiges Antikörperfragment (scAb) oder ein Ligand an seine Ektodomäne fusioniert.

Die vorliegende Arbeit basiert auf der Hypothese, dass das effiziente *targeting*-System von MV auf lentivirale Vektoren übertragen werden kann, indem vom Humanen Immundefizienz Virus-1 (HIV-1) abgeleitete Vektoren mit den MV-Hüllproteinen pseudotypisiert werden. Da sich zunächst mit den unmodifizierten MV-Hüllproteinen keine MV-HIV Pseudotypen erzeugen ließen, wurden 15 H- und zwei F-Proteinvarianten mit stufenweise gekürzten zytoplasmatischen Domänen und Aminosäure(AS)austauschen auf ihre Fähigkeit getestet, HIV-1 Vektoren zu pseudotypisieren. Die Kombinationen HcΔ18/FcΔ30, HcΔ19/FcΔ30 und HcΔ24+4A/FcΔ30 führten zur effizientesten Pseudotypisierung mit Titern der ankonzentrierten Partikel von über 10^6 transduzierenden Einheiten (t.u.)/ml. Dabei war eine Verkürzung der zytoplasmatischen Domäne des F-Proteins um 30 AS und des H-Proteins um 18, 19 oder 24 AS notwendig, wobei im letzten Fall vier Alanine nach dem Start-Methionin addiert wurden. Westernblot-Analysen zeigten, dass die Inkorporation der MV-Hüllproteine in die Vektorpartikel durch die Kürzung der zytoplasmatischen Domänen verstärkt wurde. Erwartungsgemäß spiegelten MV-HIV Vektoren auf unterschiedlichen MV Rezeptor-positiven und -negativen Zelllinien den Tropismus des MV wider. Interessanterweise konnte ihr Titer durch Einstellen eines optimalen Plasmidverhältnisses von H zu F (1:7) in den vektorpartikelproduzierenden Zellen deutlich verbessert werden. Die stabile Integration des Transfergens wurde durch Inhibition der Reversen Transkription und durch Langzeitkultivierung verifiziert.

Als nächstes wurden HIV-1 Vektoren hergestellt, die zum Zelleintritt den epidermalen Wachstumsfaktor-Rezeptor (EGFR) bzw. das B-Zell-spezifische Oberflächenmolekül CD20 nutzten. Dafür wurde FcΔ30 zusammen mit einem HcΔ18-Protein, das für seine nativen Rezeptoren verblindet wurde und an seiner Ektodomäne den Liganden EGF oder ein scAb gegen CD20 präsentiert, zur Produktion der *targeting*-Vektoren $MV_{\alpha EGFR}$-HIV und $MV_{\alpha CD20}$-HIV eingesetzt. Die Verwendung des EGFR bzw. von CD20 für den Zelleintritt konnte mit Rezeptor-positiven bzw. -negativen Zelllinien bestätigt werden. Dass CD20-negative Zellen nicht transduziert wurden, wurde in Mischkulturen demonstriert, in denen der $MV_{\alpha CD20}$-HIV Vektor nach Suizidgentransfer selektiv CD20-positive Zellen eliminierte. Bemerkenswerter Weise wurden aktivierte primäre humane B-Zellen vom $MV_{\alpha CD20}$-HIV Vektor mit einer deutlich höheren Effizienz transduziert als vom Standard-Pseudotypvektor VSV-G-HIV. Noch überraschender war, dass $MV_{\alpha CD20}$-HIV Vektoren ruhende B-Zellen transduzierten, die sich bis dato als resistent gegen den lentiviralen Gentransfer erwiesen hatten.

Als kritischster Schritt der MV-HIV Produktion erwies sich die Identifizierung von H-Varianten, die einerseits Pseudotypisierung erlaubten, andererseits aber noch die Fusionshelfer-Funktion besaßen. Im Gegensatz zu früheren ineffizienten *targeting*-Strategien basiert der Erfolg des in dieser Arbeit entwickelten Systems wahrscheinlich auf der Verteilung der Funktionen Rezeptorerkennung und Membranfusion auf zwei unterschiedliche Proteine. Sogar die Transduktion ruhender B-Zellen ist nun möglich. Der zugrundeliegende Mechanismus könnte auf residualer Bindung der MV-Hüllproteine an die MV-Rezeptoren beruhen. Dies bewirkt keinen Zelleintritt, aber könnte für den Gentransfer in ruhende Zellen notwendige Zytoskelettumordnungen verursachen. Alternativ oder zusätzlich könnten $MV_{\alpha CD20}$-HIV Vektoren durch CD20-Bindung und -Verlinkung einen Kalziumeinstrom und damit B-Zellaktivierung auslösen.
Das in dieser Arbeit entwickelte *targeting*-System erlaubt erstmalig einen gezielten und effizienten Zelleintritt lentiviraler Vektoren in ruhende Lymphozyten. Dies kann als Basis dienen, dieses System auf beliebige andere Zielmoleküle zu übertragen, indem entsprechende Liganden oder scAbs an die Ektodomäne des modifizierten H-Proteins fusioniert werden.

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9 Abbreviations

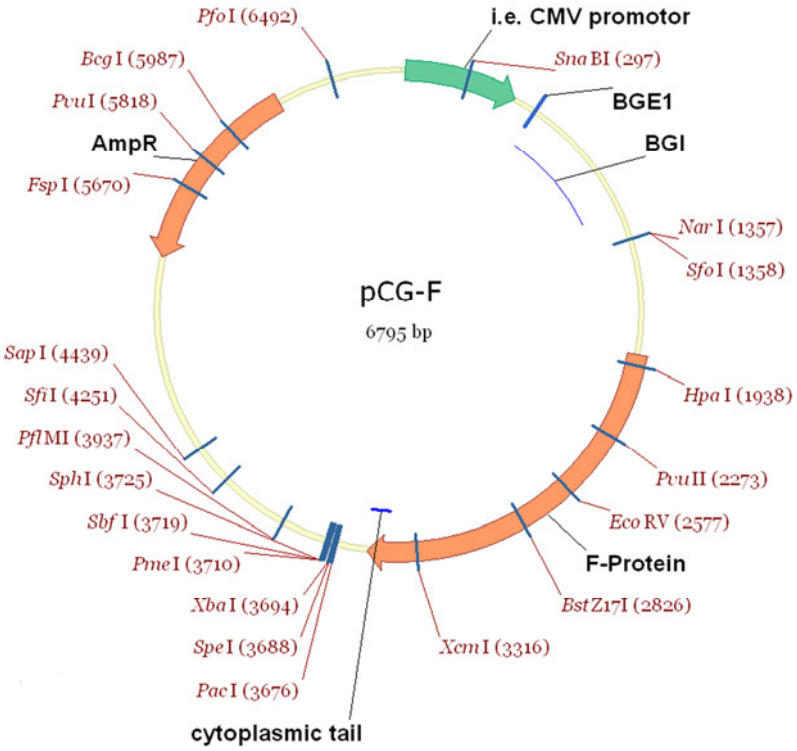
α	anti-
αCD20-scAb	single-chain antibody directed against human CD20
A	absorption or amper
aa	amino acids
Ac	acetate
Amp	ampicillin
APS	ammonium peroxydisulfate
ATCC	American Type Culture Collection
ATP	adenosine-5'-triphosphate
AZT	azidothymidine
BCR	B cell antigen receptor
°C	degree Celsius
ca.	circa
CD34TK39	fusion protein composed of TK39 and a truncated version of CD34
CMV	cytomegalovirus
ct	cytoplasmic tail
DMEM	Dulbecco`s modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
DTT	dithio-1,4-threithol
ECACC	European Collection of Cell Cultures
ECL	enhanced chemiluminescence
E. coli	Escherichia coli
EDTA	ethylene-diamine-tetra-acetate
e.g.	for example
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Env	envelope protein
et al.	and others
F	fusion protein
FACS	fluorescence activated cell sorting

FCS	fetal calf serum
FIP	fusion-inhibiting peptide
FITC	fluorescence isothiocyanate
g	gram or gravitational acceleration
gag	group specific antigen
GALV	gibbon ape leukaemia virus
GCV	gancyclovir
GFP	green fluorescent protein
h	hour
H	hemagglutinin protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV-1	human immunodeficiency virus-1
HRP	horseradish peroxidase
ICLC	Interlab Cell Line Collection
IL	interleukin
kD	kilodalton
l	liter
LB	Luria-Bertani
LTR	long terminal repeat
m	milli-
M	Molar
mAb	monoclonal antibody
MFI	mean fluorescence intensity
µ	micro-
min	minute
MLV	murine leukaemia virus
MOI	multiplicity of infection
MoMLV	Moloney murine leukaemia virus
MOPS	morpholinepropanesulfonate
MPSV	myeloproliferative sarcoma virus
MV	measles virus
n	nano-
NEB	New England Biolabs
OD	optical density

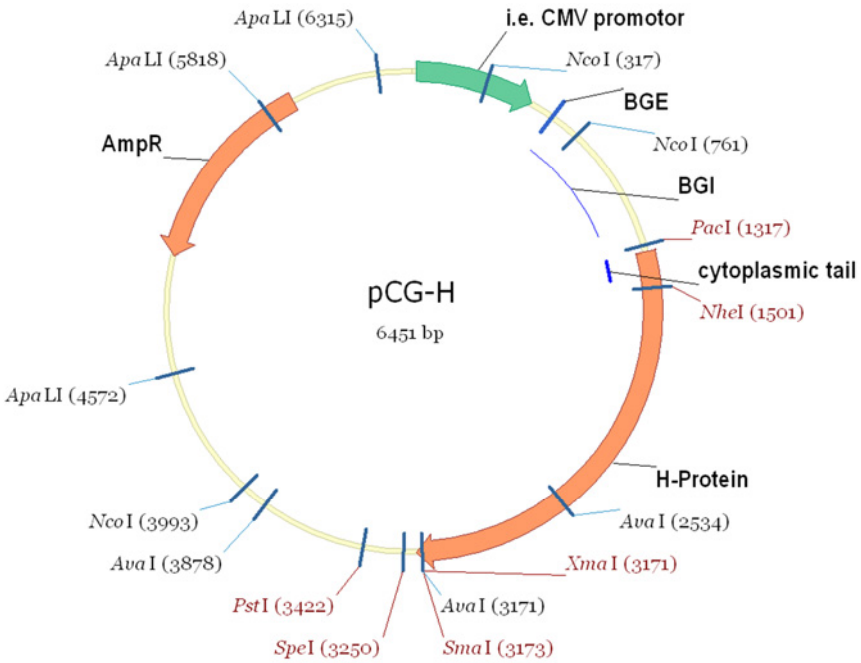
p.a.	pro analysis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	R-Phycoerythrin
pol	polymerase
Ψ	psi-packaging signal of retroviral genomic RNA
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	culture medium developed in the “Roswell Park Memorial Institute”
RT	room temperature
scAb	single-chain antibody
SDS	sodium dodecyl sulfate
sec	second
SFFV	spleen focus forming virus
SIVmac	simian immunodeficiency virus
SLAM	signaling lymphocyte activation molecule
SOC channel	store-operated cation channel
SSPE	subacute sclerosing panencephalitis
TEMED	tetramethylethylenediamine
TK39	hypersensitive mutant of the herpes simplex virus thymidine kinase
Tris	tris(hydroxymethyl)aminomethane
t.u.	transducing units
U	unit
UV	ultraviolet
V	volt
VSV-G	glycoprotein of vesicular stomatitis virus

10 Appendix

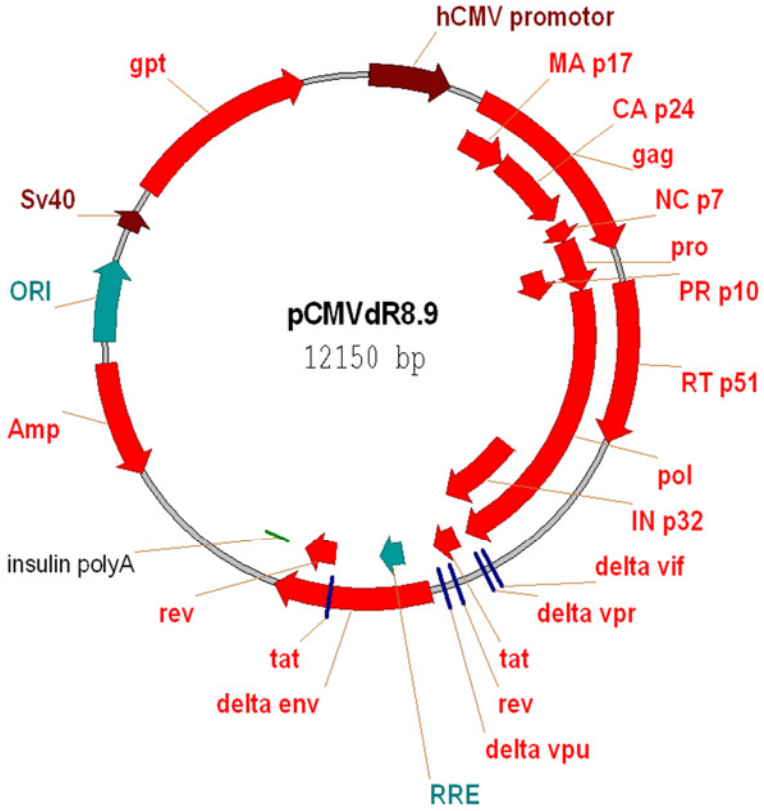
10.1 Plasmid map of pCG-F



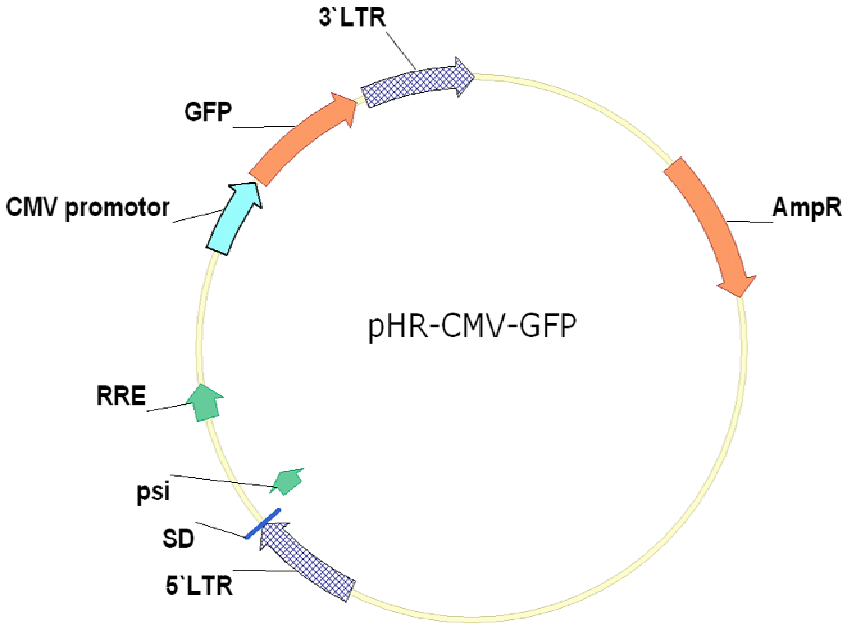
10.2 Plasmid map of pCG-H



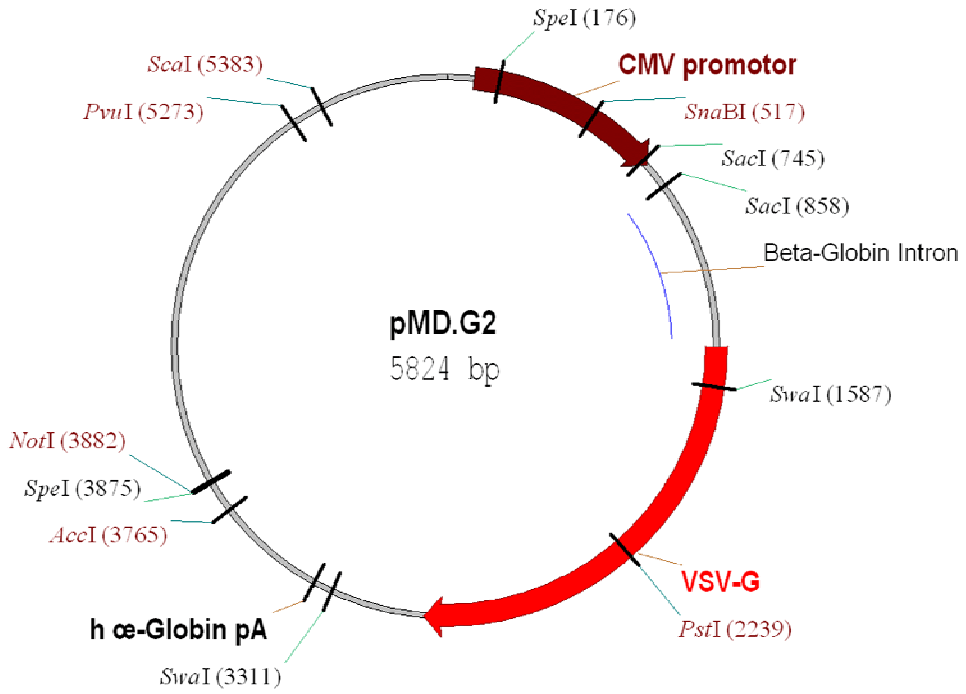
10.3 Plasmid map of pCMVΔR8.9



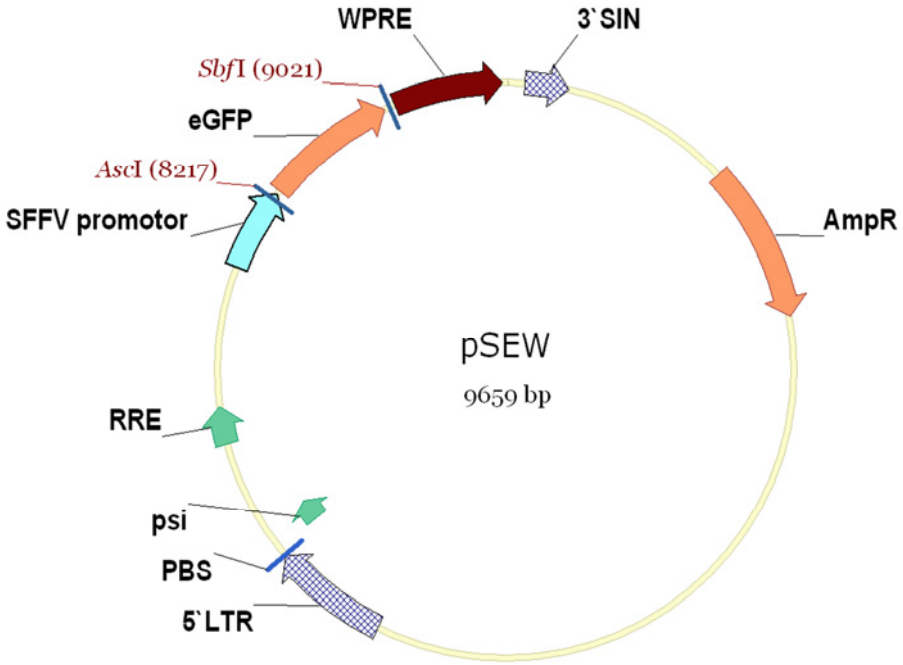
10.4 Plasmid map of pHR`-CMV-GFP



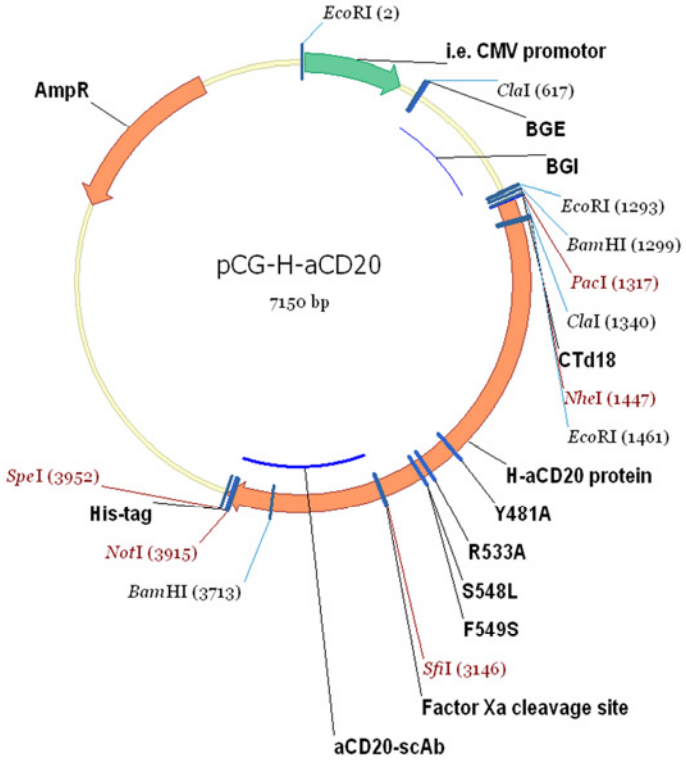
10.5 Plasmid map of pMD.G2



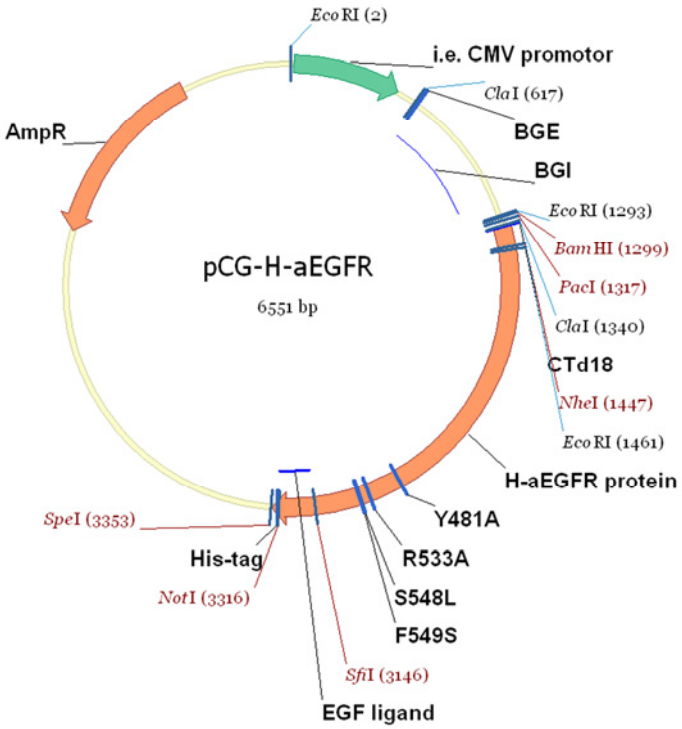
10.6 Plasmid map of pSEW



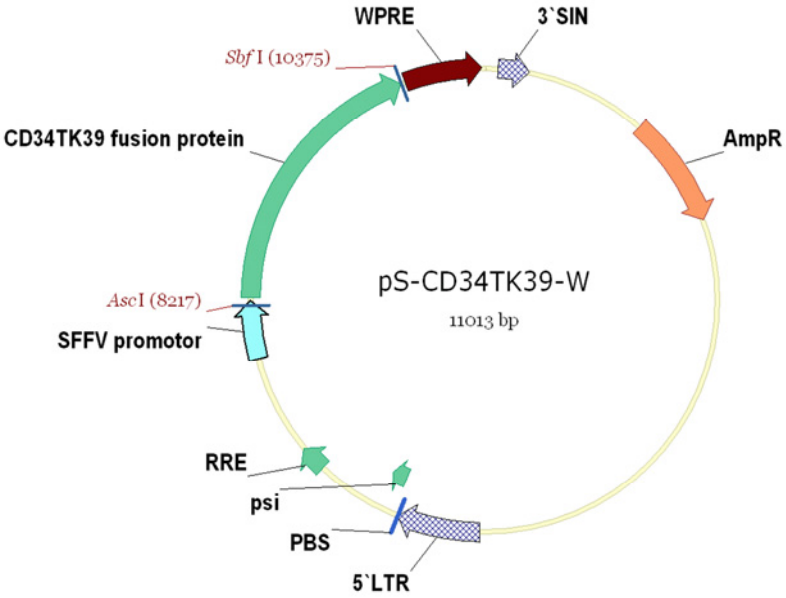
10.7 Plasmid map of pCG-H-αCD20



10.8 Plasmid map of pCG-H-αEGFR



10.9 Plasmid map of pS-CD34TK39-W



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12 Lebenslauf

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13 Publikationen

Originalartikel

Sabrina Funke, Andrea Maisner, Michael D. Mühlebach, Ulrike Koehl, Manuel Grez, Roberto Cattaneo, Klaus Cichutek, Christian J. Buchholz (2008) Targeted cell entry of lentiviral vectors. *Molecular Therapy*, Vol. 16 (8) p. 1427-1436.
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Impact Factor: 4,812

Übersichtsartikel

Christian J. Buchholz, Lydia J. Duerner, Sabrina Funke, Irene C. Schneider (2008) Retroviral Display and High Throughput Screening. *Combinatorial Chemistry & High Throughput Screening,* Vol. 11 (2) p. 99-110.

Patentanmeldungen

Europäische Patentanmeldung am 27.09.2006 (06020257.9; Veröffentlichungsnr. 1975239) und PCT-Anmeldung am 26.09.2007 (PCT/EP2007/008384; Veröffentlichungsnr. WO 2008/037458 A3) mit dem Titel „Pseudotyping of retroviral vectors, methods for production and use thereof for targeted gene transfer and high-throughput screening" Erfinder: Christian J. Buchholz, Sabrina Funke, Klaus Cichutek, Roberto Cattaneo

Vorträge auf internationalen Konferenzen

Sabrina Funke, Andrea Maisner, Michael D. Mühlebach, Ulrike Koehl, Manuel Grez, Roberto Cattaneo, Klaus Cichutek, Christian J. Buchholz

Vortragstitel: Targeted cell entry of lentiviral vectors

11. Jahrestagung der Amerikanischen Gesellschaft für Gentherapie in Boston, MA, USA, 28/05/2008 - 01/06/2008

Sabrina Funke, Andrea Maisner, Manuel Grez, Roberto Cattaneo, Christian J. Buchholz, Klaus Cichutek

Vortragstitel: Effective and flexible cell entry targeting of lentiviral vectors through pseudotyping with variant measles virus H and F proteins

18. Jahrestagung der Gesellschaft für Virologie e.V. in Heidelberg,
05/03/2008 - 08/03/2008

Sabrina Funke, Andrea Maisner, Manuel Grez, Roberto Cattaneo, Klaus Cichutek, Christian J. Buchholz

Vortragstitel: Cell entry targeting of lentiviral vectors through pseudotyping with the measles virus H and F proteins

Konferenz “In vivo barriers to gene delivery” in den Cold Spring Harbor Laboratories, NY, USA, 26/11/2007 - 29/11/2007

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Vortragstitel: Cell entry targeting of lentiviral vectors through pseudotyping with the measles virus H and F proteins

14. Jahrestagung der Deutschen Gesellschaft für Gentherapie e.V. in Heidelberg, 18/07/2007 - 20/07/2007

Posterpräsentationen

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Postertitel: Cell entry targeting of lentiviral vectors through pseudotyping with the measles virus H and F proteins

10. Jahrestagung der Amerikanischen Gesellschaft für Gentherapie in Seattle, WA, USA, 30/05/2007 - 03/06/2007

Eidesstattliche Versicherung

Ich erkläre hiermit an Eides Statt, dass ich die vorgelegte Dissertation mit dem Titel

„Targeted cell entry of lentiviral vectors“

selbständig angefertigt und mich anderer Hilfsmittel als der in ihr angegebenen nicht bedient habe, insbesondere, dass aus Schriften Entlehnungen, soweit sie in der Dissertation nicht ausdrücklich als solche mit Angabe der betreffenden Schrift bezeichnet sind, nicht stattgefunden haben.

Frankfurt am Main, den 15.06.2009

……..
Sabrina Funke