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MINNA KAIKKONEN

Engineering Baculo- and Lentiviral Vectors for Enhanced and Targeted Gene Delivery

Doctoral dissertation

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Department of Biotechnology and Molecular Medicine
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ABSTRACT

One of the major goals of gene therapy is the development of vectors able to precisely deliver a gene of interest to specific cells or organs *in vivo.* In this study we aimed at introducing more efficient and targetable baculo- and lentiviral vectors to the field of gene therapy. In addition, we studied the effects of baculovirus nuclear entry and viral gene transcription in human cells.

In the first article we show that a 21- amino acid EctoDomain in conjunction with transmembrane and cytoplasmic tail domains of VSV-G (VSV-GED), deprived of its tropism-mediating epitope, augments baculovirus-mediated gene-delivery to vertebrate cells *in vitro* and *in vivo*. We suggest that VSV-GED enhances baculovirus transduction by potentiating the membrane fusion activity of baculovirus envelope protein gp64. However, VSV-GED does not provide cell specificity and this is why other targeting strategies were sought.

An ideal targeting strategy would use a general system eliminating the need to engineer new vectors for each new ligand. The use of (strept)avidin and its extraordinary tight interaction with biotin (Kd ~ 10^{-13}-10^{-15}M) could offer an effective approach. In the second article we developed a targeting strategy based on metabolical biotinylation of baculovirus vectors. This was achieved by displaying a small biotin acceptor peptide, BAP, fused either to different sites in the baculovirus glycoprotein gp64 or to VSV-GED. Transduction efficiencies of different contructs showed significant differences highlighting the importance in choosing the peptide insertion site. Only vectors displaying BAP inserted at amino acid position 283 of the gp64 protein showed improved transduction when targeted to cancer cell lines with biotinylated ligands or antibodies. These vectors could also be concentrated by streptavidin conjugated paramagnetic particles to reach titers up to 10^{10} pfu/ml.

For applications requiring long-term transgene expression development of targeted lentiviral vectors is of great importance. In the third article we constructed lentiviral vectors displaying avidin and streptavidin fused to VSV-GED, codisplayed with gp64. We present data on targeting of these lentivirus vectors to transferrin, epidermal growth factor and CD46 receptors overexpressed on tumor cells *in vitro*. Further, we demonstrate the capability of avidin-display in non-invasive imaging *in vivo*.

The insect baculoviruses have the ability to transduce mammalian cell lines without replication. However, the baculovirus transduction can lead to the expression of some baculoviral immediate early genes in mammalian cells. In the last article we further studied the transcription and expression of viral immediate-early genes in human cells and examined the interactions between viral components and subnuclear structures.

In conclusion, this work presents a simple means to enhance baculoviral gene transfer by VSV-GED pseudotyping and gives the proof of principle of the utility of avidin-biotin display as a versatile tool for targeting baculo- and lentivirus transduction. This conjugate-based strategy is readily adaptable for different targets in order to increase the gene delivery for *ex vivo* and *in vivo* applications. Finally, we elucidated the intranuclear events followed by baculovirus transduction in human cells. Together these results provide new insights into the future design of safer and more specific gene therapy vectors.

National Library of Medicine Classification: QU 195, QU 350, QU 470, QU 475, QU 55.7, QW 162, QW 168.5.H6, QZ 52, WN 185, WN 203
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ABBREVIATIONS

*Ac*MNPV	*Autographa californica* multiple nuclear polyhedrovirus
Ad	adenovirus
ATCC	American type culture Collection
ATP	adenosine triphosphate
AVD	avidin
BAP	biotin acceptor peptide
BEVS	baculovirus expression vector system
BirA	bacterial biotin ligase
BIV	bovine immunodeficiency virus
BV	budded virus
CAR	coxsackie virus and adenovirus receptor
CB	cajal body
CEA	carsinoembryonic antigen
CMV	cytomegalovirus
CNS	central nervous system
CP	choroid plexux
CRAd	conditionally replicating adenovirus
CTD	cytoplasmic tail domain
CT	computed tomography
DC	dendritic cell
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
EIAV	equine infectious anemia virus
ER	endoplasmic reticulum
Fab	antigen-binding fragment of immunoglobulin
Fc	crystallizable fragment of immunoglobulin
FIV	feline immunodeficiency virus
FGFR1	fibroblast growth factor receptor
OV	occluded virus
GALV	gibbon ape leukemia virus
GFP	green fluorescent protein
GP64	baculovirus major envelope protein
GV	granulovirus
HBV	hepatitis B virus
hD2R	human dopamine receptor
HGFR	hepatocyte growh factor receptor
hHF	human heavy chain ferritin
HIV	human immunodeficiency Virus
hNIS	human sodium iodine symporter
HS	heparan sulfate
HSC	hematopoietic stem cells
HSPG	heparin sulfate proteoglycan
HSV	herpes simplex virus
HS	heparan sulfate
HSC	hematopoietic stem cells
HSPG	heparin sulfate proteoglycan
HSV	herpes simplex virus
ie	immediate early gene
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
IP	infectious virus particle
ITR	inverted terminal repeats
JSRV	Jaagsiekte sheep retrovirus
LamR	laminin receptor
lef	late expression factor gene
LCMV	lymphocytic choriomeningitis virus
LTR	long terminal repeat
MHC	major histocompatibility complex
MLV	murine leukemia virus
MOI	multiplicity of infection
MRI	magnetic resonance imaging
NB	nuclear body
NPV	nucleopolyhedrovirus
ODV	occlusion derived virus
ORF	open reading frame
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PEG	polyethylene glycol
PEI	polyethylenimine
PET	positron emission tomography
PFU	plaque forming unit
p.i.	post-infection
PIB	polyhedral inclusion body
PIC	pre-integration complex
PML	promyelotic
PMP	paramagnetic particle
PS	phosphatidylserine
PSTCD	*P. shermanii* transcarboxylase domain
p.t.	post-transduction
R1	relaxation rate (1/T1)
R2	relaxation rate (1/T2)
RD114	feline endogenous virus
RGD	arginine-glycine-aspartic acid
RNA	ribonucleic acid
RRE	Rev-responsive element
RRV	Ross River virus
SA	streptavidin
scFv	single chain variable fragment
SFV	Semliki Forest virus
SIN	self-inactivating lentivirus
SIV	simian immunodeficiency virus
SMC	smooth muscle cells
SPECT	single photon emission tomography
T1	time 1, longitudinal relaxation time
T2	time 2, transverse relaxation time
TfR	transferrin receptor
TM	transmembrane
TNF	tumor necrosis factor
TP	total virus particle
VCAM	vascular cell adhesion molecule
VSV-G	*vesicular stomatitis* virus G protein
VSV-GED	VSV-G EctoDomain
VV	vaccinia virus
WT	wild-type
X-SCID	X-linked severe combined Immunodeficiency
ZZ-domain	IgG binding domain of protein A

LIST OF ORIGINAL PUBLICATIONS

I Kaikkonen MU*, Räty JK*, Airenne KJ, Wirth T, Heikura T, Ylä-Herttuala S
Truncated vesicular stomatitis virus G protein improves baculovirus transduction efficiency *in vitro* and *in vivo*.
Gene Therapy 2006 Feb;13(4):304-12.

II Kaikkonen MU, Viholainen J, Närvänen A, Ylä-Herttuala S, Airenne KJ
Targeting and purification of metabolically biotinylated baculoviruses
Human Gene Therapy 2008 Jun;19(6): In Press

III Kaikkonen MU*, Lesch HP*, Pikkarainen J, Räty JK, Vuorio T, Huhtala T, Taavitsainen M, Laitinen T, Tuunanen P, Gröhn O, Närvänen A, Airenne KJ, Ylä-Herttuala S
Avidin-displaying lentiviruses as versatile tools for targeting and dual-imaging of gene delivery
Submitted for publication

IV Laakkonen JP, Kaikkonen MU, Ronkainen PH, Ihalainen TO, Niskanen EA, Häkkinen M, Salminen M, Kulomaa MS, Ylä-Herttuala S, Airenne KJ, Vihinen-Ranta M.
Baculovirus-mediated immediate-early gene expression and nuclear reorganization in human cells.
Cellular Microbiology 2008 10(3): 667–681

**equal contribution*

INTRODUCTION

Gene therapy is a process by which nucleic acids are delivered into the cells with the goal of treating or curing a disease. Gene therapy was initially developed to treat monogenic diseases by replacing the missing or defective gene with the functional one. However, over the last decade, more emphasis has been put on the possibilities of treating a broader spectrum of disease conditions, such as cardiovascular diseases and cancer.

Major limiting factor in gene therapy continues to be the poor performance of vectors and their inability to precisely deliver a gene of interest to specific cells or organs *in vivo*. Viral vectors are known to be the most efficient tools for gene transfer. Because different diseases require either transient or persistent expression of the therapeutic gene, a single vector system is unlikely to be sufficient for all gene therapy purposes. Due to this, the development of a more general targeting method, applicable to different vector types, would be of great value for future evolution of gene therapy.

In this work we developed novel viral vectors for enhanced and targeted gene delivery. We studied the utility of avidin and biotin display for vector targeting, purification and imaging of viral biodistribution and transgene expression. Baculoviral and lentiviral vectors were chosen as technology platforms to improve their potential use for therapeutic purposes.

2. REVIEW OF THE LITERATURE

2.1 Gene therapy

Gene therapy is a process by which DNA encoding specific proteins is delivered into the cells to treat or cure a disease. In comparison to classical medicines, gene therapy has the potential to mediate the highest possible level of therapeutic specificity. Over the last two decades gene therapy has moved from preclinical to clinical studies ranging from single gene disorders to more complex diseases such as cancer and cardiovascular disorders (Figure 1). Every year around 100 clinical trials are approved worldwide.

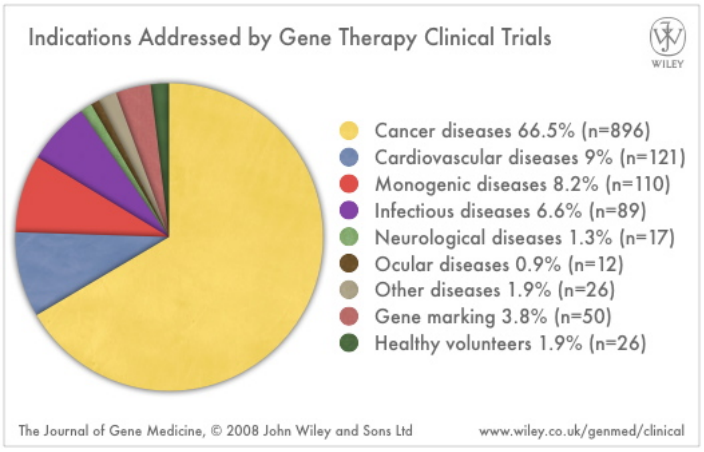

Figure 1. The indications addressed by gene therapy clinical trials.

2.2 Gene delivery vectors

In practice, we face the problem in realizing the concept of gene therapy: the gene delivery into target cells is very ineffective and presents a formidable challenge. Vectors that have been developed to overcome these obstacles include nonviral and viral vectors. Viral vectors have been reported as the most efficient tools for gene transfer *in vitro* and *in vivo*. Most of the clinical trials have focused on the use of vectors based on mammalian viruses, such as retroviruses, adenoviruses, adeno-associated viruses, vaccinia viruses and herpes simplex viruses (Figure 2). Their advantage is the natural adaptation to mammalian hosts. On the other hand, practical use of viral vectors is often limited by the emergence of replication competent viruses, cytotoxicity and immune responses, which presents a minimal problem for nonviral vectors. It is thus evident that the currently used classes of vectors have their own characteristics, advantages, drawbacks and applications. The next chapters will introduce some of the current vectors with a special focus on baculovirus and lentivirus.

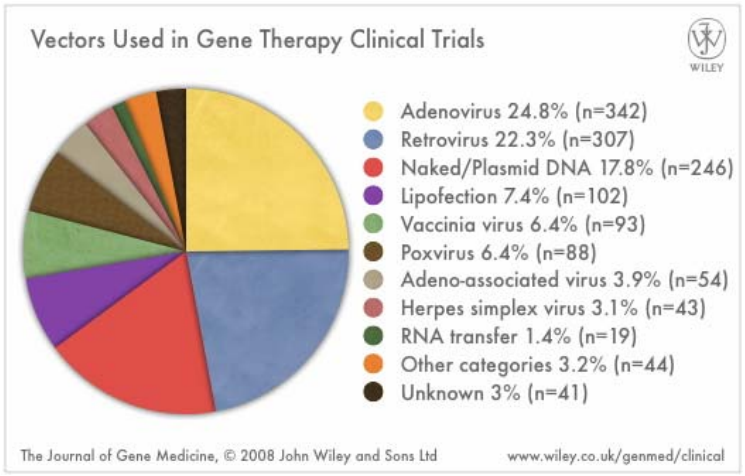

Figure 2. Vectors used in gene therapy clinical trials.

2.2.1 Baculoviruses

The virus family *Baculoviridae* has been known for hundreds of years. They comprise a diverse group of over 600 viruses, which infect only arthropod hosts. Studies since 1920's have acknowledged baculoviruses as effective natural insecticides against forestry and agriculture pests (Black et al., 1997). The research into the biology of these viruses and ways of improving them as a pest control method has lead to extensive studies of baculovirus genetics, ecology (Miller, 1997) and biosafety (Burges et al., 1980; Kost and Condreay, 2002).

Since the late-1980's the baculovirus expression vector system (BEVS) became a popular choice for the production of numerous recombinant proteins in insect culture and larvae (Kost et al., 2005). This technology has also led to the development of baculovirus surface display for the proper presentation of antigens, construction of eukaryotic libraries and for the enhancement of baculovirus-mediated transduction (Makela and Oker-Blom, 2006; Oker-Blom et al., 2003). As with other eukaryotic expression systems, baculovirus expression of heterologous genes permits folding, post-translational modification and oligomerization in manners that are often similar to those that occur in mammalian cells (Kost et al., 2005). Moreover, the flexibility of the capsid system allows insertion of very large genes into the *Ac*MNPV genome and the expression of heterologous proteins under the control of strong viral p10 or polyhedrin promoter enables high production levels (Fraser, 1986).

In the early 1980 it was discovered that baculoviruses can enter into non-host cells, including many mammalian cells, without infectious reproduction. A few years later it was discovered that baculoviruses containing mammalian expression cassettes can transduce mammalian cells (Carbonell et al., 1985). During the late 1990s several studies confirmed the initial findings and the list of suitable target cells has continued to expand (Hu, 2006). Since then baculoviruses have gained popularity as potential vectors for both *in vitro* and *in vivo* gene therapy.

2.2.1.1 Virion structure

Members of the *Baculoviridae* are divided into two genera, *Granulovirus* (GV) and *Nucleopolyhedrovirus* (NPV) (Miller, 1997). The NPVs can be further divided into two groups: single-nucleopolyhedroviruses (SNPV) containing a single nucleocapsid per virion, and multiple-nucleopolyhedroviruses (MNPV) containing multiple nucleocapsids. Both the SNPVs and MNPVs can contain numerous virions per polyhedral inclusion body (PIB).

The most extensively studied baculovirus, *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV), is a large enveloped virus with a double-stranded, circular genome of 134 kb. Its genome has been sequenced and predicted to contain 154 open reading frames (Ayres et al., 1994). They have a distinctive rod shaped nucleocapsid averaging 25-50nm in diameter and 250-300 nm in length (Harrap, 1972b; Williams and Faulkner, 1997). Baculoviruses exist in two distinct forms involved in different phases of its natural life cycle. The form responsible for the horizontal spread of viruses between insect hosts is the occlusion derived virions (ODV) whereas budded viruses (BVs) are necessary for the propagation within the insect (Williams and Faulkner, 1997).

BVs and ODVs differ in lipid and protein components of their envelopes but the capsid composition is similar (Figure 3); only ODV-EC27 is found exclusively on the ODV capsids (Funk et al., 1997). Vp39 (orf89), p80 (orf104) and p24 (orf129) represent the major capsid proteins and orf1629 (orf9) encloses the capsid structure (Funk et al, 1997; Braunagel et al, 1996a) whereas DNA binding protein p6.9 (orf100) participates in the condensation of the viral genome inside the nucleocapsids (Figure 3) (Wilson & Consigli, 1985). As the ODVs are not produced during the production of baculovirus vectors due to deletion of polyhedrin gene, the next chapter will concentrate on the composition of envelope of the BV with a special focus on the major envelope protein gp64.

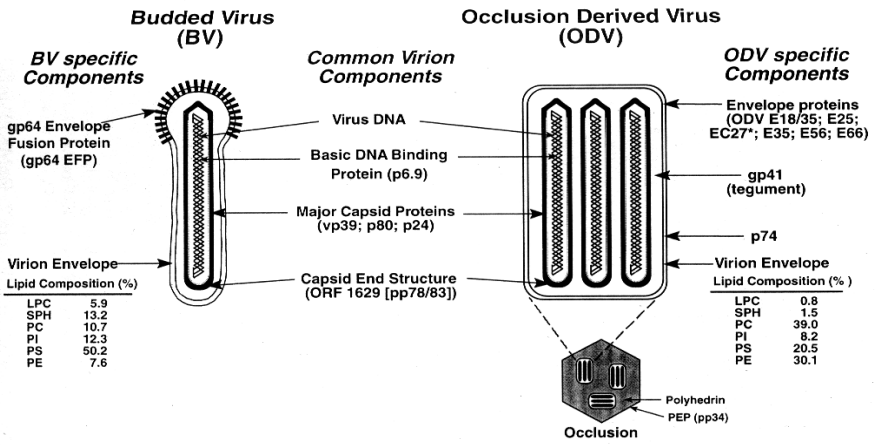

Figure 3. Baculovirus structural proteins on the budded and occlusion-derived virus (Funk et al., 1997)

2.2.1.2 Major envelope glycoprotein gp64

Budded virions contain one nucleocapsid surrounded by an envelope with gp64 major envelope protein found associated at one pole of the virus particles as peplomer structures (Figure 3). One virion is estimated to contain ~1000 gp64 peplomers (Wickham et al., 1990). The Gp64 exists as a disulfide-linked trimer with a molecular mass of 175 kDa (Oomens et al., 1995). The gp64 protein contains an N-terminal signal peptide and a C-terminal anchor domain. Gp64 accumulates at the plasma membrane during the early and late phases of infection, 8 and 24 hours p.i. (Blissard & Rohrmann, 1989; Monsma et al 1996; Monsma & Blissard, 1995; Volkman & Knudson, 1986). Nucleocapsids become surrounded by gp64-containing plasma membrane during budding from the cell surface in the late phase of infection. Furthermore, gp64 is required for efficient viral budding (Oomens and Blissard, 1999) and cell-to-cell transmission (Monsma et al., 1996). Gp64 mediates also virus binding to cell surface (Duisit et al., 1999; Ghosh et al., 2002; Hefferon et al., 1999; Hofmann et al., 1995) and low-pH-dependent membrane fusion (Blissard and Wenz, 1992). Successful membrane fusion requires the assembly of stable gp64 trimers into multiprotein aggregates in cell-cell contact regions (Markovic et al., 1998).

2.2.1.3 Baculovirus life cycle

The baculovirus infection is initiated by ODVs in the gut epithelium (Figure 4). Occluded virions in large PIBs are protected from the environmental factors by a crystalline polyhedrin matrix (Braunagel and Summers, 1994; Harrap, 1972a), but in the alkaline midgut of insect larva the matrix is solubilized and the occluded viruses are released (Harrap et al, 1974). Occluded viruses enter the midgut epithelial cells via direct membrane fusion (Granados, 1978; Summers, 1971). Transcription of viral genes begins immediately after the virus DNA is transported to the nucleus.

Baculovirus infection can be divided into three phases, early (0-6 h post-infection), late (6-24 h p.i.) and very late phase (18-24 to 72 h p.i.) (Williams and Faulkner, 1997). During the early phase of infection genes involved in the regulation of the replication cascade and those involved in preventing host responses are expressed. Early genes of the baculovirus are transcribed by the host RNA polymerase (Friesen, 1997). The late phase viral gene expression includes the replication of the viral DNA, the shutdown of host cell transcription and translation and the production of the budded form of the virus (Williams and Faulkner, 1997). The switch from early to late gene expression involves the appearance of a novel virus-induced RNA polymerase activity (Yang et al., 1991). In the very late phase the virus becomes occluded in the protein polyhedrin and the polyhedral envelope (calyx) is produced. Polyhedral inclusion bodies are released by cell lysis and the spreading of infection by adsorptive endocytosis leads eventually to the death of larva and the release of PIBs into the environment (Granados and Lawler, 1981). The cycle begins again when new insect ingests infected food.

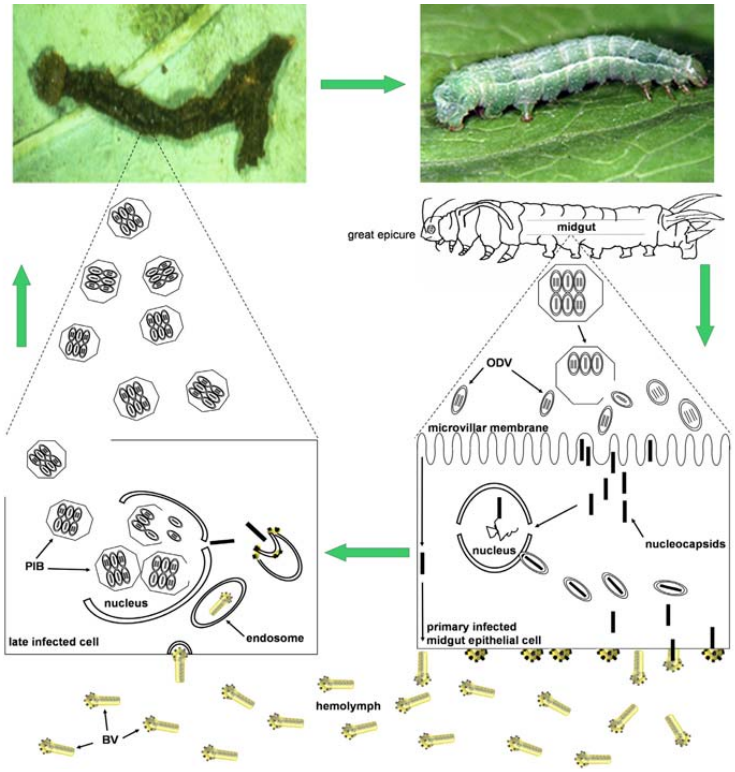

Figure 4. Baculovirus life cycle consisting of the primary infection (on right) and the secondary infection (on left) (Airenne et al., 2008).

2.2.1.3 Baculovirus entry and gene delivery

Budded viruses attach to and enter insect cells by absorptive endocytosis (Blissard and Wenz, 1992; Volkman and Goldsmith, 1985; Wang et al., 1997) followed by internalization into clathrin-coated vesicles. Recent observations in vertebrate cells also suggest involvement of macropinocytosis and caveolae (Long et al., 2006; Matilainen et al., 2005)

The sheer number of mammalian cell lines that can be transduced by baculovirus vectors suggests that uptake of baculovirus by mammalian cells is a general phenomenon. The nature of the cell surface molecule that interacts with baculovirus is unclear but the involvement of receptors (Hofmann et al., 1995), electrostatic interactions (Duisit et al., 1999) and phospholipids (Tani et al., 2001) has been proposed. One possible explanation for these contradictory results is that mechanisms of virus-cell interactions are different between cell types.

Following endosomal escape, nucleocapsids traverse the cytoplasm potentially with the help of actin filaments and enter the nucleus (van Loo et al., 2001) where the viral genome is released in response to the phosphorylation of basic core protein p6.9 (Funk and Consigli, 1993; Wilson and Consigli, 1985).

Baculoviruses are gaining popularity as potential vectors for gene transfer technology (Table 1). They are easily manipulated and produced in high titers (10^{10}-10^{12} pfu/ml). The inherent inability of baculoviruses to replicate in mammalian cells and low cytotoxicity and lack of pre-existing immunity makes them good candidates for gene therapy *in vivo* (Hu, 2006). The transient nature of baculovirus-mediated gene delivery makes it an attractive candidate for the treatment of cancer (Song and Boyce, 2001; Wang et al., 2006) and cardiovascular diseases (Airenne et al., 2000; Grassi et al., 2006). A number of studies have also implicated the potential use of baculoviruses for bone (Chuang et al., 2007) and cartilage tissue engineering (Sung et al., 2007) and for gene delivery into nervous system (Lehtolainen et al., 2002b; Sarkis et al., 2000; Tani et al., 2003; Wang et al., 2007).

Even though considerable progress has been made in elucidating the biology of baculovirus vectors, some limitations regarding the efficacy and specificity of these vectors have slowed their widespread applications. The major hurdle for baculovirus-mediated transduction lies in the stage of nuclear entrance since the viral DNA is unable to enter the nucleus of many vertebrate cells (Kukkonen et al., 2003; Volkman and Goldsmith, 1983). This might be due to the inability of the virus to escape from endosomes (Barsoum et al., 1997) or blockage of the transport or entry into the nucleus (Kukkonen et al., 2003; van Loo et al., 2001). It has been suggested that microtubules may constitute a barrier to nucleocapsid transport towards the nucleus in the cytoplasm (Salminen et al., 2005).

Attempts to enhance baculovirus-mediated gene delivery have mainly focused on the virion surface modifications (Makela and Oker-Blom, 2006), promoter choices (Spenger et al., 2004; Wang et al., 2006), insertion of transgene expression enhancing elements (Mahonen et al., 2007; Venkaiah et al., 2004) and optimization of the transduction protocol *in vitro* (Condreay et al., 1999; Hsu et al., 2004; Shen et al., 2007). Despite these advances, *in vivo* gene delivery is still unsatisfactory. One obstacle is the inactivation of baculovirus by serum complement (Hofmann and Strauss, 1998). Different strategies have been pursued to overcome the problem of complement: to inactivate the complement system for the period of infection, to generate complement-resistant vectors (Huser et al., 2001) and to deliver viruses into immunopriviledged areas (Haeseleer et al., 2001; Lehtolainen et al., 2002b; Sarkis et al., 2000) or to sites where the exposure to the complement can be avoided (Airenne et al., 2000; Sandig et al., 1996).

Baculovirus transduction leads to transient expression peaking at 3-5 days (Airenne et al., 2000; Lehtolainen et al., 2002b) and can last up to 200 days in the absence of complement (Pieroni et al., 2001). The gradual disappearance of the transgene expression is attributed to the degradation of baculoviral DNA (Ho et al., 2004). The transgene expression has been substantially prolonged by using baculovirus hybrid vectors, taking advantage of AAV ITRs necessary for replication and integration (Palombo et al., 1998; Wang and Wang, 2005; Zeng et al., 2007), or viruses capable of episomal replication (Shan et al., 2006).

Even though baculoviruses are non-pathogenic to humans, recent evidence suggests that baculovirus transduction can induce the expression of some baculoviral immediate early genes in mammalian cells, namely *ie-0*, *ie-1*, *pe-38*, *gp64* and *p35* (Fujita et al., 2006; Kitajima et al., 2006). All these genes belong to the essential (*p143*, *ie-1*, *lef-1*, *lef-2* and *lef-3*) or to the stimulatory (*dnapol*, *p35*, *ie-2*, *lef-7*, and *pe38*) genes involved in viral replication in the host cells (Kool et al., 1994; Lu

and Miller, 1995). This has shown to alter the expression profiles of mammalian genes although the physiology of the cells is not altered (Fujita et al., 2006; Kenoutis et al., 2006). Furthermore, administration of baculovirus induces expression of interferons and cytokines such as TNF-α, IL- 1α, IL-1β and IL-6 (Abe et al., 2003; Abe et al., 2005; Gronowski et al., 1999). These safety issues have to be taken into consideration when designing new vectors and therapies but also open new avenues for baculovirus-based vaccination and cancer immunotherapy (Kitajima and Takaku, 2008).

2.2.2 Retro- and lentiviruses

Retroviridae is a large family of enveloped RNA viruses found in all vertebrates. The most peculiar feature of retroviruses is their ability to integrate the viral genome into the host chromosomal DNA, which can lead to lifelong expression. Retroviruses are currently classified into seven genera based on nucleotide sequence relationship: alpharetroviruses, betaretrovirus, gammaretroviruses, deltaretroviruses, episilonretroviruses, spumaviruses and lentiviruses (Goff, 2001).

Gammaretroviruses, based on the murine leukaemia virus (MLV), are among the first viral delivery systems developed for gene therapy applications in 1990. Over the past decade, however, lentiviruses have gained a lot of attention due to their ability to transduce non-dividing cells. The advantages and disadvantages of these vectors are listed in Table 1.

2.2.2.1 Lentivirus structure and genome

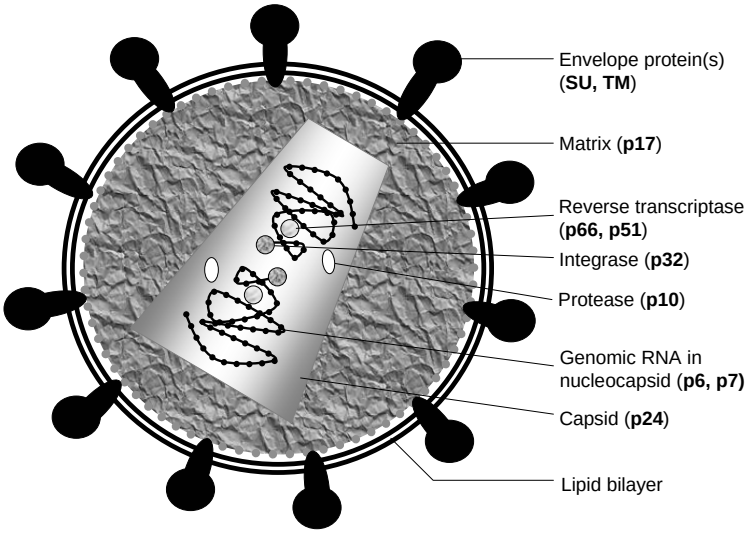

Figure 5. Structure of lentivirus with major viral proteins presented.

Lentivirus virions are roughly spherical particles with a diameter of 100-150 nm. Lentivirus genome is diploid and contains two plus-stranded RNA copies of its genome. Like other members of the retroviral family, the HIV genome contains the *gag, pol* and *env* genes (Wang et al., 2000). The *env* encodes for complex envelope protein, which consists of an outer protruding surface protein (SU) and a stem transmembrane protein (TM) (Figure 5). The *gag* gene products produce the protein core of viral particles consisting of p17 (matrix), p24 (capsid), p7 and p6 (nucleocapsid). In addition to nucleocapsid, the major elements contained within the viral core are two single strands of 9 kb RNA genome and three enzyme proteins, p66/p51 (reverse transcriptase), p11 (protease) and p32 (integrase), encoded by the *pol* gene (Figure 5). In addition, several other nonstructural proteins which serve regulatory functions including *tat*, *rev*, *nef*, *vif*, *vpu* and *vpr* are encoded by the HIV genome (Wang et al., 2000). Tat induces the transcriptional activation of the promoter situated at the long terminal repeat (LTR) whereas Rev plays a role in the nuclear export of viral mRNAs. The other accessory proteins Nef, Vif, Vpu and Vpr contribute to the replication and persistence of infection *in vivo* (Seelamgari et al., 2004). There are also a number of *cis*-acting elements required at different stages of the virus life cycle including the LTRs, packaging and dimerization signal (Ψ), Rev-responsive element (RRE), and the central polypurine tract (cPPT) (Wang et al., 2000).

Lentivirus vectors. The general strategy in designing lentiviral vectors for gene therapy is based on the deletion and alteration of the native viral sequences, in order to prevent the generation of replication competent retroviruses. The state-of-the-art 3rd generation lentivirus vector system consists of four plasmids (Figure 6) (Delenda, 2004). The minimal transgene expression cassette contains the LTRs, packaging signal, a heterologous promoter and the transgene. Three additional plasmids provide the factors required for virus production and packaging (*gag*, *pol*, *rev*, *env*). The envelope proteins are typically replaced by a heterologous viral glycoprotein, most commonly *vesicular stomatitis* virus G-protein (VSV-G) (Naldini et al., 1996b), to modify the host range of the vector. An important safety feature is also the deletion of the promoter-enhancer region form the 3' LTR preventing transcription from this region and subsequent viral replication (self-inactivating vector; SIN) (Miyoshi et al., 1998).

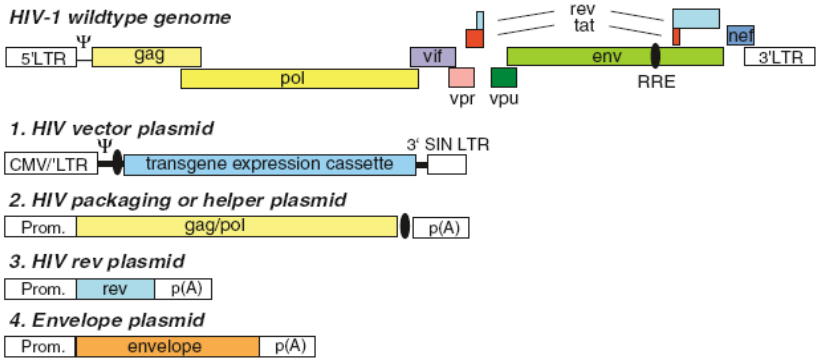

Figure 6. The four-plasmid transfection system for lentivirus production. The vector plasmid (1), the packaging plasmid (2), rev (3), and an envelope plasmid (4) are needed for HIV vector production. The packaging signal (Ψ), the rev-binding element (RRE) are indicated. (Sinn et al., 2005b)

2.2.2.2 Lentivirus life cycle

Lentivirus infection is initiated by binding of the virion surface protein (SU) to the cognate receptor (Figure 7). The SU protein is attached to the virus by a non-covalent binding to the transmembrane protein (TM) which anchors the complex in the lentiviral envelope. SU receptor binding triggers conformational changes in the TM protein leading to the fusion between the viral membrane and the host cell membrane (Freed and Martin, 2007). For HIV-1, however, the binding of SU (gp120) to CD4 receptor is followed by the exposure of a chemokine receptor (CXCR4 or CCR5) binding site on SU protein and only subsequent binding to this co-receptor is able to trigger TM(gp41)-mediated fusion (Nisole and Saib, 2004) .

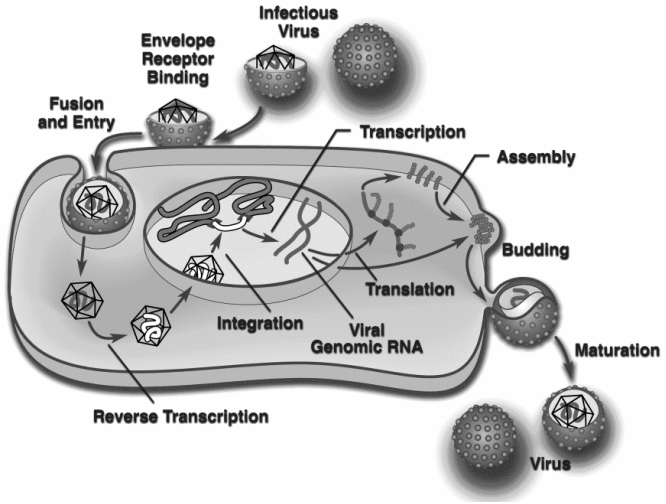

Figure 7. The lentivirus replication cycle (http://www.retrovirus.info).

The fusion of viral and cellular membranes delivers the viral core into the cytoplasm, where it undergoes a partial and progressive disassembly leading to the generation of pre-integration complexes (PICs). Subsequently the viral RNA is reverse transcribed into double-stranded cDNA in a process mediated by viral reverse transcriptase enzyme (Figure 7). The PIC associated cDNA enters the nucleus with the help of integrase, matrix protein and Vpr (Sherman and Greene, 2002). In the nucleus, the integrase protein catalyzes the integration of the viral cDNA into the host genome (Freed and Martin, 2007).

Transcriptional regulation of HIV-1 gene expression is controlled by several host cell transcription factors and the viral Tat protein (Rohr et al., 2003). In the early phase of viral gene expression the newly transcribed mRNA is spliced by the cellular splicing machinery into multiply spliced transcripts, which mainly produces Tat, Rev and Nef proteins. When Rev has accumulated to a critical level the mRNA production shifts from multiply spliced to the singly spliced and unspliced

transcripts (e.g. *gag*, *vif*, *env*), characteristic of the late phase of gene expression. The Rev binding to RRE leads to the nuclear export of the late-phase transcripts (Freed and Martin, 2007).

Following the production of viral structural proteins, the virus particle is assembled at the plasma membrane (Figure 7) (Bukrinskaya, 2004). In this process the Gag and Gag-Pol polyproteins interact with each other by protein-protein interaction, most probably via the capsid protein domain (Gelderblom, 1991). The viral genome is packaged in a process in which the packaging signal is recognized by the nucleocapsid protein domain of the Gag protein (Zhang et al., 1998). The mature HIV particles bud from the host membrane ready to infect another cell and to begin the replication process all over again.

2.2.2.3 Lentivirus as a gene therapy vector

Lentiviruses have gained much attention as a gene delivery tool over the past decade due to their ability to transduce non-dividing cells, giving rise to first clinical trials in 2001. Lentivirus development has mainly focused on human immunodeficiency virus type 1 (HIV-1) and improvements of the vector have enabled efficient *in vivo* and *ex vivo* gene delivery to many tissues. However, also non-human pathogens, such as feline (FIV), simian (SIV) and bovine (BIV) immunodeficiency virus and equine anemia infectious virus (EIAV) are currently being investigated for gene therapy due to their safety advantages (Romano, 2005).

Lentiviral vectors are rapidly becoming the vectors of choice for hematopoietic stem cell (HSC) gene therapy due to capacity to transduce also quiescent cells, in which state most of HSCs are (Naldini et al., 1996b). Lentiviral vectors can also deliver genes to HSCs with a superior efficiency to MLV vectors without affecting the repopulating capacity of these cells (Kay et al., 2001). Consequently the first clinical studies with HIV-based lentiviral vectors were concentrated on delivering anti-HIV-, antisense- or RNAi- genes to the HSCs of HIV infected patients (http://www.wiley.co.uk/genmed/clinical/). Lentiviral gene transfer to HSC has been also proposed to provide a potential cure for many inherited diseases such as sickle cell disease (Pawliuk et al., 2001) and chronic granulomatous disease (Roesler et al., 2002) and β-thalassaemia (Imren et al., 2002; May et al., 2002; Vacek et al., 2003) where progression towards the clinic can be seen (Bank et al., 2005).

Another promising target area for lentivirus-mediated gene delivery is the brain. The VSV-G pseudotyped vectors based on HIV (Kordower et al., 1999; Naldini et al., 1996a), FIV (Alisky et al., 2000) and EIAV (Mitrophanous et al., 1999) vectors were shown to efficiently transduce neurons in various areas of the brain while leading to long-lived transgene expression. Since then, several studies have demonstrated convincing therapeutic efficacy of lentivirus-mediated gene delivery in animal models of lysosomal enzyme deficiency disorders, Huntington's disease, Alzheimer's disease and Parkinson's disease (Wong et al., 2006).

Like MLV, HIV integrates randomly into the host genome. This poses a risk of insertional mutagenesis as was demonstrated by the appearance of several cases of leukaemia in the gene therapy trials for X-SCID (Gaspar et al., 2004; Hacein-Bey-Abina et al., 2003; Wilson, 2008). This malignant

transformation is likely related to gammaretroviruses' inherent disposition to integration near the 5'ends of transcription units leading to proto-oncogene activation (Wu et al., 2003). In contrast, lentiviruses strongly favor integration within active transcription units which might be a safer alternative (Mitchell et al., 2004; Schroder et al., 2002). In addition, the careful design of 3rd generation SIN vectors might further reduce the risk of insertional gene-inactivation or proto-oncogene deregulation in the case of lentiviruses (Miyoshi et al., 1998; Thornhill et al., 2008). Other strategies to overcome this problem have led to the development of non-integrating (Philpott and Thrasher, 2007) or site-specifically integrating vectors (Bushman, 1994; Tan et al., 2006).

2.2.3 Adenoviruses

Adenoviruses (Ad) belong to the family of *Adenoviridae* which to date includes 51 immunologically distinct human adenovirus serotypes (A-F) that can cause human infections ranging from respiratory disease, and conjunctivitis, to gastroenteritis. Replication defective viruses based on subgroup C adenovirus type 5 (Ad5) and type 2 (Ad2) are the most widely used for gene transfer in many applications (Shenk, 2001).

Adenoviruses are double-stranded DNA viruses with a genome of 36 kb (Chroboczek et al., 1992). The virions are nonenveloped and icosahedral in shape with a diameter of 70-90 nm. The viral capsid containins four principal protein components: the hexon, fiber, penton base and protein IX (Figure 8). Ads enter the host cells by receptor-mediated endocytosis. Initial interaction with the host cells is mediated by the fiber protein and the coxsackie virus and adenovirus receptor (CAR) (Bergelson et al., 1997) and subsequent internalization results from the activation of α_v integrin by penton base (Wickham et al., 1993).

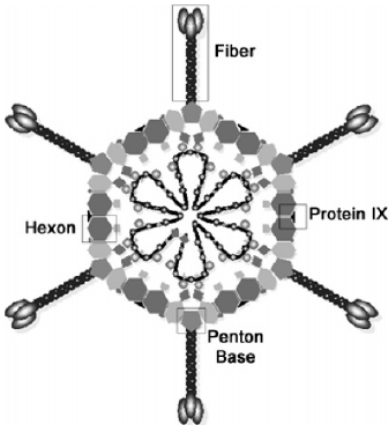

Figure 8. Structure of adenovirus as a simplified cross-section of the capsid showing the capsid proteins and adenovirus genome (Noureddini and Curiel, 2005).

Adenoviruses have passed retroviruses as the most commonly applied viral vectors with over 340 clinical trials finished or ongoing (Figure 2). The major advantages of adenovirus vectors is the the large DNA insertion capacity, the easy generation and purification of high titer stock (10^{10}-10^{12} pfu/ml) (Table 1). They have a broad host range and can efficiently transfer genes into both dividing and non-dividing cells. Adenoviral genomes do not integrate into the host genome, making them safe vectors for transient gene expression. However, both natural immunity against adenovirus (Chen et al., 2000) and acute inflammatory (Knowles et al., 1995; Yei et al., 1994) and immunological responses (Zoltick et al., 2001) have limited the current clinical applications to few areas such as localized cancer and cardiovascular gene therapy. The concomitant knowledge about adenovirus biology has led to the development of conditionally replicative adenoviruses (CRAds) which exhibit tumor specific amplification resulting in lysis of the cancer cells (Heise and Kirn, 2000). On the other side of the scope, the aspiration to enlargen the field of adenovirus application has led to development of methods to minimize the viral gene content (gutless Ads), to decrease the immunogenicity of the vectors and to retarget the vector tropism (Campos and Barry, 2007; Ghosh et al., 2006).

2.2.4 Adeno-associated viruses (AAVs)

AAV is one of the smallest viruses with a non-enveloped icosahedral capsid of approximately 20-25 nm in diameter belonging to the *parvoviridae* family (Xie et al., 2002). To date, at least 11 serologically distinct AAVs have been identified from humans or primates (Mori et al., 2004). The most extensively studied AAV is AAV type 2 (AAV-2), it also being the most common in active clinical trials (Coura and Nardi, 2007). The most peculiar feature of AAV is its dependence on helper viruses (e.g. adenovirus or herpes virus) for productive infection (Muzyczka and Berns, 2001). It infects humans and some other primate species but the virus has not been linked to any human diseases. Despite the nonpathogenic nature of virions, most humans are seropositive to AAV which may limit the gene delivery efficiency *in vivo*. The pros and cons of AAV vectors are listed in more detail in Table 1.

The AAV has a linear 5 kB single-stranded genome of either plus or minus polarity. The genome harbors two open reading frames (ORFs): one encoding for Rep proteins involved in regulation of replication and transcription and the other for virus capsid proteins VP1, VP2 and VP3 which form the virion in ratio (1:1:10) (Muzyczka and Berns, 2001). AAV-2 capsid proteins mediate the virion attachment to heparin sulfate proteoglycan (HSPG), fibroblast growth factor receptor-1 (FGFR1), integrin $\alpha_v\beta_5$ and hepatocyte growh factor receptor (HGFR) on host cell membrane and subsequent endocytosis through clathrin-coated pits (Kashiwakura et al., 2005; Qing et al., 1999; Summerford and Samulski, 1998). The ends of the genome form short inverted terminal repeats (ITRs), which serve as origins of viral replication. The ITRs are the only *cis* element required for replication and packaging of the virus and therefore all the other elements, provided in *trans*, have been deleted from the recombinant AAV-2 vectors (rAAV-2).

The wild type AAV-2 is able to integrate into the genome of the host with the help of Rep proteins with a site preference on human chromosome 19 (Samulski et al., 1991). However, even in the absence of *rep*-genes on AAV-2 vectors the rAAV genome has been shown to persist in episomal (Afione et al., 1996; Yang et al., 1999) or integrated (Nakai et al., 2001) form. The mechanism lying beneath integration has been thought to involve the host cell DNA break repair machinery, which inserts rAAV genome into existing chromosomal breaks (Nakai et al., 2003). For gene therapy applications, this feature of rAAV is a double-edged sword: rAAVs can maintain high levels of transgene expression but at the same time insertional mutagenesis becomes an issue. However, since rAAVs don't create but instead insert into existing chromosomal breaks, they could be considered safer than retroviruses.

rAAV has shown great potential for the gene delivery to muscle, brain, liver and eye. The current clinical trials are mainly concentrated for the treatment of monogenic diseases, especially cystic fibrosis, hemophilia B, retinal degeneration, and cancer (http://www.wiley.co.uk/genmed/clinical/). The results so far have shown that rAAVs are safe and efficient tools for gene delivery but the therapeutic benefit to human patients is still limited by the inadequate organ-specific transgene expression (Coura and Nardi, 2007).

2.2.5 Other viruses

Over 200 clinical trials have been conducted with less conventional viral vectors including poxviruses, herpes simplex virus, Semliki forest virus, Sendai virus, Simian virus, measles virus, poliovirus, flavivirus and Venezuelan equine encephalitis Newcastle disease virus. The former two represent the vast majority of the studies and will be discussed in more detail.

Poxviruses are enveloped viruses which can infect as a family both vertebrate and invertebrate animals (Moss, 2001). Vaccinia virus (VV) is the prototypical recombinant poxvirus. Vaccinia virus has been used clinically as a vaccine for smallpox since the late 18th century, and has thus a well known biology and extensive clinical experience (Niemialtowski et al., 1996). Recombinant VVs, as non-replicating viral vectors, have been demonstrated to have great potential as vaccines due to their safety, low cytotoxicity, high level of protein expression and ability to generate potent antibody and T-cell responses. A number of clinical trials using recombinant VV as vaccines have shown promising results for treating HIV and cancer (Moroziewicz and Kaufman, 2005). On the other hand, replicating VVa are promising candidates for oncolytic virotherapy (Thorne et al., 2005).

Herpes simplex virus (HSV) is a human infecting pathogen with a double stranded genome of 152 kB (Whitley, 2001). Among the herpes viruses, HSV-1 is an attractive vector for gene transfer to the nervous system because the natural infection leads to lifelong persistence of viral genomes in neurons in which the latent phase and lytic phase alternates. Two types of vectors have been developed for gene therapy applications: replication defective vectors, whose cytotoxicity has been abolished by deleting lytic gene products, and amplicon vectors, which are plasmids packaged into HSV particles with the aid of a helper virus (Whitley, 2001). Logically, these vectors have been primarily used for neuronal gene delivery for the treatment of neuropathies (Parkinson's disease, pain,

stroke) and lysosomal storage disorders (Berto et al., 2005). The majority of the clinical studies with HSV-1 are, however, concentrated on cancer therapy. For these applications, the HSV-1 vectors high infectivity and inherent cytotoxicity is harnessed to conditionally drive viral replication in tumor cells leading eventually to cell lysis (Shen and Nemunaitis, 2006). Similarly, other viruses have been studied as candidates for the oncolytic viral treatment of tumors, including Newcastle disease virus, reovirus, measles virus, Semliki forest virus, sindbisvirus, *vesicular stomatitis* virus, influenza virus and poliovirus (Kelly and Russell, 2007).

2.2.6 Nonviral vectors

Nonviral vectors represent an attractive alternative to viral vectors due to the ease of large-scale production, large insertion capacity, stability, flexibility and lack of immune response. Nonviral gene delivery can be divided into two broad categories: naked DNA delivery by a physical method and delivery by a complex of DNA with a cationic carrier. The latter group can be further divided into lipoplexes (cationic lipid/DNA complex) and polyplexes (cationic polymer/DNA complex) and more recently to the lipid-polymer hybrid systems (Gao et al., 2007).

The physical approaches consist direct delivery of DNA to the cytoplasm of target cells by microinjection, gene gun, electroporation, sonoporation or laser irradiation (Mehier-Humbert and Guy, 2005). For systemic administration, however, the plasmid DNA needs to be protected from the nucleases and mononuclear phagocyte system (Kawabata et al., 1995; Mahato et al., 1995). Therefore, plasmid DNA is often shielded from the degradation by cationic compounds.

Cationic lipoplexes (Felgner et al., 1987) and polyplexes (Wu and Wu, 1987) were introduced already in 1987 and are today the most studied strategy for nonviral gene delivery. These compounds condense and decrease the negative charge of DNA and thus facilitate its interaction with the cell membrane. Following binding, endocytosis or endocytosis-like mechanisms are proposed to be responsible for the entry of lipoplexes and polyplexes (Elouahabi and Ruysschaert, 2005). One of the major bottlenecks for effective transfection has been the subsequent release of DNA-complexes from the endosomes. This has been circumvented by the use fusogenic “helper” lipids such as dioleoylphosphatidylethanolamine (DOPE) (Farhood et al., 1995) or polymers with intrinsic endosomolytic activity, the most popular being polyethylenimine (PEI) (Boussif et al., 1995). Both vectors have shown excellent efficiency in cell culture but the *in vivo* gene delivery is still unsatisfactory. In addition, the *in vivo* administration can sometimes lead to aggregation, toxicity and acute immune responses (Gao et al., 2007). Various strategies have evolved to overcome these problems, the most promising being the shielding of the cationic compounds with polyethyleneglycol (PEG) (Ambegia et al., 2005; Kichler, 2004; Song et al., 2002).

Despite some drawbacks, efficient *in vivo* gene delivery has been achieved to the lungs, brain, kidney and tumors and some of the vectors have undergone clinical trials for the treatment of cancer and cystic fibrosis (Nishikawa and Hashida, 2002)(www.wiley.co.uk/genmed/clinical/).

In the future, the combination of the best features of viral and non-viral vector systems by creating chemically modified viral vectors or synthetic virus-like systems could provide significant therapeutic benefits over the traditional vector systems (Boeckle and Wagner, 2006).

Table 1. Properties of the most common gene delivery vectors (Gao et al., 2007; Kootstra and Verma, 2003; Moroziewicz and Kaufman, 2005; Waehler et al., 2007).

	Pros	Cons
Baculovirus	• High titers (10^{10}-10^{12} pfu/ml) • Large insertion capacity > 100 kB • Non-human pathogen, safety	• Inactivation by complement • Immunogenic • Large size
Retro-and lentiviruses	• Stable gene expression • Insert capacity 8-9 kB • No pre-existing immunity • Moderate titers 10^{6}-10^{10} TU/ml	• Risk of insertional mutagenesis • Risk of replication competent virus formation • Inactivation by complement
Adenovirus	• High titers (10^{10}-10^{12} pfu/ml) • Insert capacity 7-8 kB, for gutless vectors 36 kB • Broad tropism • High short-term gene expression • Oncolytic potential	• Pre-existing immunity: neutralizing antibodies • Acute inflammatory and immunological responses • Complicated vector genome
Adeno-associated virus (AAV)	• Stable gene expression possible • Nonpathogenic • Highly stable virions • Small size (22 nm) • No need for viral genes in vectors	• Small insert capacity, 4.6 kB • Slow onset of gene expression • Risk of insertional mutagenesis • Production requires helper viruses • Large-scale production difficult
Vaccinia	• Well established safety profile • Oncolytic potential	• Immunogenicity
Herpes simplex virus (HSV)	• Long-term expression in neuronal cells, neurotropism • High titers, 10^{8}-10^{11} pfu/ml • Transgene capacity 30 kB, for amplicons 152 kB • Oncolytic potential	• Host immune responses, inflammation and toxicity • Complicated vector genome
Nonviral vectors	• Low degree of toxicity, non-infectious • Easy and simple production • High efficiency *in vitro* • No insert size limit	• Low transfection effic. *in vivo* • Only transient expression • For some vectors acute immunity, toxicity, aggregation *in vivo*

2.3 Targeted gene delivery

Key issues for future development of gene therapy include improved gene delivery and targeting. In theory, targeted therapeutic gene delivery can be achieved by targeting entry of the vector (transductional targeting) or by targeting the gene expression (transcriptional targeting) to certain cell types or tissues. Transcriptional targeting has been shown to be highly feasible in the context of most viral vectors (Miller and Whelan, 1997). It provides a safety net by limiting the transgene expression

to specific target cells using tissue specific promoters. However, transcriptional targeting does not obviate the need for transductional targeting which is essential for allowing the administered therapeutic dose to be reduced, thereby lessening toxic side effects and costs for the treatment. Thereby the focus of this chapter will be on targeted transduction.

2.3.1 Targeting of membrane-enveloped viruses

Targeted gene delivery is currently the most attractive concept to achieve specificity and, in principle, this strategy is applicable for all current vectors (Waehler et al., 2007). The outer surface of virus, through its interaction with cellular receptors, plays a major role in determining the tropism of the virus. There are several strategies for modifying the binding characteristics of membrane-enveloped virus vectors and most of the studies have been conducted using retro- and lentiviruses because they are highly permissive for incorporation of heterologous attachment proteins. In theory, all of these targeting approaches can be extrapolated to other enveloped viruses on condition that the virus budding, fusion activity and infectivity is not compromised. The focus of this chapter will therefore be on these vectors with an extension to baculovirus.

Pseudotyping. The simplest form of transductional targeting consists of changing the virus surface protein itself for the envelope or capsid protein of another virus which is not of the same genus. This approach is called pseudotyping. One of the most commonly used pseudotyping tools is *Vesicular stomatitis virus* G protein, VSV-G (chapter 2.3.1.1). It is routinely used to broaden the target range and enhance the transduction efficiency of retroviruses (Emi et al., 1991) and HIV-1-, HIV-2-, SIV-, FIV-, EIAV- and BIV- based lentiviruses (Cronin et al., 2005; Naldini et al., 1996b; Reiser et al., 1996). Significant advantage of VSV-G pseudotyping is its ability to confer high vector particle stability allowing virus concentration by ultracentrifugation (Burns et al., 1993). There are also several reports of VSV-G pseudotyped baculoviruses which show improved transduction efficiency (Barsoum et al., 1997; Tani et al., 2001; Tani et al., 2003). Unfortunately, VSV-G is cytotoxic to producer cell lines (Burns et al., 1993; Ory et al., 1996; Schauber et al., 2004) and there have also been reports where the VSV-G included in the viral envelope increased the toxicity of the vector (Facciabene et al., 2004; Park et al., 2000; Watson et al., 2002). Together these features can limit the clinical use of VSV-G and alternative glycoproteins have been extensively studied. Some of the most prominent lentivirus pseudotypes and their target cells/organs are presented in Table 2. These include glycoproteins from the families rhabdoviridae, arenaviridae, togaviridae, filoviridae, paramyxoviridae, orthomyxoviridae, and hepadnaviridae (Cronin et al., 2005). In addition, lentiviral vectors pseudotyped with baculovirus envelope glycoprotein gp64 have been produced. Gp64-displaying HIV-1 vectors were produced at similar titers to VSV-G with no associated cytotoxicity and concetration by ultracentrifugation was well tolerated (Kumar et al., 2003). These vectors transduced efficiently various cell types, with a tropism restriction against hematopoietic cell types (Schauber et al., 2004). More recently, two reports have demonstrated the utility Gp64 pseudotyped FIV-vectors for hepatocyte and nasal epithelia targeting (Kang et al., 2005; Sinn et al., 2005; Sinn et al., 2007).

Table 2. Cell and organ preferences of lentivirus pseudotypes. Modified from (Cronin et al., 2005).

Glycoproteins (Genus)	Target cell/organ	Remarks	References
VSV-G (Rhabdoviridae)	Liver	Toxicity issues	(Park, 2003)
	CNS	Targets primary neurons	(Blomer et al., 1997)
	Retina	Photoreceptors and retinal pigm. epithelium	(Auricchio et al., 2001; Miyoshi et al., 1997)
Rabies (Rhabdoviridae)	CNS	Retro- and anterograde axonal transport	(Mazarakis et al., 2001; Wong et al., 2004)
	Cancer	Neuroblastoma	(Steffens et al., 2004)
Mokola (Rhabdoviridae)	CNS	Neurons	(Desmaris et al., 2001; Watson et al., 2002)
	Retina	Retinal pigm. epithelium	(Auricchio et al., 2001)
	Muscle	Cardiomyocytes	(MacKenzie et al., 2002)
	Cancer	Neuroblastoma	(Steffens et al., 2004)
LCMV (Arenaviridae)	Liver	Non-toxic	(Park, 2003)
	CNS	Neural progenitor cells	(Stein et al., 2005)
		Astrocytes	(Miletic et al., 2004)
	Pancreas	Islet cells	(Kobinger et al., 2004)
	Cancer	Malignant glioma	(Miletic et al., 2004; Steffens et al., 2004)
RRV (Togaviridae)	Liver	Nonhepatocytes, nontoxic	(Kang et al., 2002)
	CNS	Neuroglial cells	(Kang et al., 2002)
		Complement resistance	(Strang et al., 2005)
Ebola (Filoviridae)	Lung airway epithelia	Apical surface preference	(Kobinger et al., 2001)
	Muscle	Cardiomyocytes	(MacKenzie et al., 2002)
Marburg (Filoviridae)	Lung airway epithelia	Apical surface preference	(Sinn et al., 2003)
JSRV (Betaretrovirus)	Lung	Alveolar type II cells	(Sinn et al., 2005a)
MLV (Gammaretrovirus)	Cancer	Neuroblastoma	(Steffens et al., 2004)
GALV (Gammaretrovirus)	Hematopoietic system	Increased serum stability	(Sandrin et al., 2002)
	Cancer	Fusogenic glycoprotein	(Diaz et al., 2000)
RD114 (Gammaretrovirus)	Hematopoietic system	Less toxic and more efficient than VSV-G	(Sandrin et al., 2002)
Sendai (Paramyxoviridae)	Lung airway epithelia	Apical and basolateral surfaces	(Kobayashi et al., 2003)
Influenza A (Orthomyxoviridae)	Airway epithelia	Apical surface preference	(Sinn et al., 2005)
HBV (Hepadnaviridae)	Liver	Primary hepatocytes	(Chai et al., 2007)
Baculovirus (Baculoviridae)	Liver	Non-toxic	(Kang et al., 2005)
	Airway epithelia	Apical surface preference	(Sinn et al., 2005)

Despite enhancing the transduction efficiency, pseudotyping often provides a wide host range and lacks sufficient target cell specificity. Therefore other strategies have been sought, based the modification of envelope glycoproteins genetically or by using bispecific adaptor-molecules.

Genetic and adaptor-based targeting of retro- and lentiviruses. Retargeting based on genetic modification of the glycoproteins was first tested with retroviral vectors (Russell et al., 1993). Efforts to target retroviral vectors have concentrated largely on engineering the natural retroviral envelope proteins such as the ecotropic murine leukemia virus MLV protein. Several strategies have been taken to produce targeted envelope proteins. For direct targeting by host range extension, envelope glycoproteins are modified to incorporate heterologous proteins or ligands. This can be achieved by replacing the natural receptor-binding domain of SU protein with the targeting molecule. A wide range of receptors have been targeted this way but most of the derivatives were unable to trigger the subsequent fusion leading to low gene transfer efficiency (Benedict et al., 1999; Zhao et al., 1999a). Therefore, another approach was developed which consisted of leaving the native receptor binding domain intact while conferring the SU protein with an additional binding moiety, called "tethering". On this basis, the insertion of collagen-binding ligand into the SU of amphotropic MuLV was shown to enhance retrovirus binding and tranduction of human endothelial cells *in vitro* (Hall et al., 1997; Hall et al., 2000; Liu et al., 2000). Moreover, these vectors could localise gene delivery to sites of balloon-injured carotid arteries and in the angiogenic tumor vasculature in human cancer xenografts in nude mice (Gordon et al., 2001a; Gordon et al., 2001b).

Two strategies have been developed for targeting retroviral vectors by host-range restriction; inverse targeting and protease targeting. Inverse targeting involves the selective inhibition of infectivity on cells expressing the targeted receptor, whereas protease targeting selectively reactivates the inhibition imposed by inverse targeting. Several ligands displayed at the N-terminus of retroviral envelope glycoproteins have been shown to inhibit infectivity on cells expressing the targeted receptor. A well-studied example of this comes from amphotropic vectors displaying epidermal growth factor (EGF) which are sequestered on EGF receptor-positive (EGFR) cells through redirection to lysosomal degradation but remain fully infectious on EGFR-negative cells (Cosset et al., 1995). Similar receptor-mediated sequestration has been observed for vectors displaying stem cell factor (Fielding et al., 1998; Fielding et al., 2000), insulin-like growth factor (Chadwick et al., 1999; Fielding et al., 2000) and CD33 (Zhao et al., 1999a). Alternatively, the sequestration can be dismantled by separating the ligand and the virus envelope protein by the recognition site for a cell-surface specific protease. For example, the EGF-displaying retroviruses carrying a matrix-metalloproteinase (MMP) cleavage site could preferentially infect EGFR-positive MMP-rich target cells *in vitro* and *in vivo* (Peng et al., 1997; Peng et al., 1999). Similarly, protease targeting has been achieved by the display of single-chain variable fragment (scFv) directed against carcinoembryonic antigen and c-Met receptor frequently overexpressed on tumor cells (Chowdhury et al., 2004; Solly et al., 2005).

Since the retroviral Env requires interaction with their native receptor to activate fusion activity, binding to artificial target molecule does not activate the fusion step. Therefore most of the direct or indirect targeting strategies have suffered from low titer and/or specificity (Verhoeyen and Cosset, 2004). Ideally, binding and fusion functions can be broken into two separate molecules. The

key to the method is choosing a viral glycoprotein that mediates fusion in response to low pH and a cellular receptor that is efficiently endocytosed after antibody binding. In this regard, a binding-defective mutant of hemagglutinin of influenza A was coexpressed with a binding-competent but fusion-defective MLV Env containing Flt-3 targeting ligand resulting in enhanced entry into Flt-3-expressing cells (Lin et al., 2001). Similarly, the Sindbis virus envelope protein E2 responsible for cell binding was engineered to replace the receptor binding region with the Fc binding domain of protein A (ZZ domain) (Morizono et al., 2001). When E2 was coexpressed with the E1 fusion protein on the retroviral and lentiviral surface and conjugated to targeting antibody, successful retargeting to mouse metastatic melanoma and prostate cancer bone metastases was achieved after intravenous injection (Morizono and Chen, 2005; Morizono et al., 2005; Pariente et al., 2007). Futhermore, coexpression of E2 binding-mutant, E1 and a chimeric anti-CD20 antibody with the human membrane-bound IgG constant region demonstrated a strictly targeted transduction of CD20-positive cells *in vitro* and *in vivo* (Yang et al., 2006).

Adapter-based concept of virus targeting consists of the formation of a 'molecular bridge' between the vector and a cell surface receptor constitutes. One such approach has been described for the Env protein of avian leukosis virus (ALV) that combine the EGF targeting domain with the extracellular domain of the ALV receptor (Snitkovsky and Young, 1998; Snitkovsky et al., 2000). Thus this bifunctional bridge proteins binds virions to specific cell surface molecules and the receptor moiety triggers the normal fusion process. Other cell surface receptors targeted this way include the vascular endothelial growth factor and heregulin receptor (Snitkovsky and Young, 2002; Snitkovsky et al., 2001).

Genetic targeting of baculoviruses. Surface modification of baculovirus particles has been demonstrated by epitope insertions into the baculovirus glycoprotein gp64. Extensive mutagenesis revealed permissive insertion sites to be located between amino acid positions 274 and 283, whereas N-terminal fusions resulted in weaker epitope display. In this regard, successful peptide-display has been achieved with the biotin mimic streptagII, the ELDKWA peptide of the gp41 of HIV-1 and the RGD-motif (Ernst et al., 2000; Ernst et al., 2006; Spenger et al., 2002). A recent study also demonstrated the utility of the Gp64 peptide display for the targeting of lentiviral vectors, using a peptide derived from the hepatitis B virus PreS1 protein, with known affinity for hepatocytes (Markusic et al., 2007).

Most of the studies aiming at altering the baculovirus tropism have consisted of the fusion of heterologous proteins and ligand-binding moieties to an extra copy of the gp64 gene (Boublik et al., 1995). Using this strategy, Mottershead and colleagues constructed vectors displaying either functional scFv or a synthetic IgG binding domain (ZZ domain) of protein A (Mottershead et al., 2000). Specific binding to target cells was achieved although no enhancement of viral entry or gene transfer was observed (Ojala et al., 2001). In fact, only few studies based on the N-terminal fusions of gp64 have resulted in enhanced transduction efficiency including the display of RGD-peptide and avidin (Matilainen et al., 2006; Raty et al., 2004). This could be partly due to the fact that gp64-based fusion constructs must compete for space with the wild type gp64 leading to low level of incorporation of gp64-fusions on baculovirus surface (Boublik et al., 1995). On this basis, improvements in the expression of synthetic IgG binding domains on the baculovirus surface was

achieved by fusing the ZZ sequence to VSV-G membrane anchor which successfully incorporated into virions (Ojala et al., 2004). Similarly, tumor homing peptides were displayed on the baculovirus surface resulting in vectors with significantly improved binding and transgene delivery to human carcinoma cells (Makela et al., 2006).

2.3.1.1 Vesicular stomatitis virus G protein

All rhabdoviruses encode a membrane glycoprotein of about 500 amino acids. Approximately 1200 VSV glycoprotein molecules are organized into 400 trimeric spikes anchored in the viral envelope (Doms et al., 1987; Kreis and Lodish, 1986). The VSV-G protein is a type I membrane glycoprotein synthesized as a precursor of 511 amino acids (Indiana), which is cleaved from its 16 amino acid signal sequence after insertion into the endoplasmic reticulum (ER) (Lingappa et al., 1978). Most of the amino acids are exposed on the virion surface, while 20 hydrophobic amino acids span the membrane and 29-amino acid cytoplasmic domain extends into the virion (Figure 9). There are two N-linked glycosylation sites on the extracellular domain and a single molecule of palmitate on the cytoplasmic domain (Rose et al., 1984; Schmidt and Schlesinger, 1979). The absence of glycosylation sites leads to formation of large disulfide bonded aggregates (Doms et al., 1988; Machamer and Rose, 1988), whereas the function of palmitate is not known (Whitt and Rose, 1991).

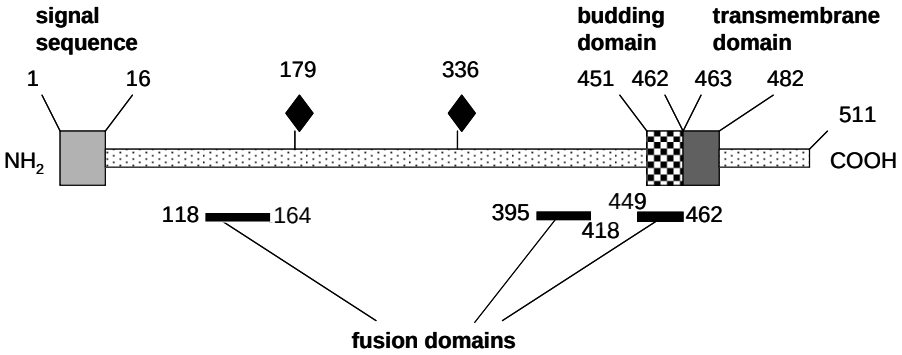

Figure 9. Schematic presentation of the vesicular stomatitis virus glycoprotein illustrating the functional domains [(Whitt and Rose, 1991) with modification].

Roles of all three domains of the VSV-G protein have been analyzed by mutagenesis (Figure 9). Mutations in the extracellular domain prevent correct folding, which leads to failed transport and aggregation in the ER before trimerization of monomers (Doms et al., 1988). Mutations in the transmembrane domain have similar effects, whereas mutations in the cytoplasmic domain do not seem to affect the folding of the extracellular domain (Doms et al., 1988). However, mutations in the cytoplasmic domain reduce the transport rate of VSV-G protein from the ER (Rose and Bergmann,

1983). This led to the discovery of transport signals (tyrosine based and di-acidic motifs) in the cytoplasmic tail responsible of promoting G protein export from the ER (Sevier et al., 2000).

The VSV host range extends from nearly all mammals to insects, suggesting that the receptor for this virus is a widely distributed molecule. Binding to phospholipids seems to be important for rhabdovirus infection since phospholipids from cellular membranes inhibit attachment and infection of rabies virus and VSV (Conti et al., 1988; Mastromarino et al., 1987; Schlegel et al., 1982). Further studies have led to the conclusion that the receptor for VSV-G is a ubiquitous membrane lipid, phosphatidylserine (PS) (Carneiro et al., 2002; Schlegel et al., 1983). The PS-binding site of the VSV-G protein has since been mapped to a p2-like peptide (residues 134 to 161) containing contiguous heptad repeats followed by a short segment containing positively charged amino acids (Carneiro et al., 2003; Coll, 1997). Positive charges in this segment might participate in electrostatic interactions with the negatively charged phospholipid PS during membrane recognition.

Unlike most viral glycoproteins, VSV-G protein has no obvious region in the amino acid sequence responsible for fusion activity (i.e. fusion peptide). Mutational analysis has indicated that amino acids between 118 and 139 could be the putative fusion domain (Li et al., 1993; Whitt et al., 1991; Zhang and Ghosh, 1994). Furthermore, recent studies have proposed that the PS-binding region of VSV-G (residues 134 to 161) is not only involved in membrane recognition but is also of crucial for membrane fusion probably through the protonation of its His residues (Carneiro et al., 2006; Carneiro et al., 2006). Another region of G protein, encompassing residues 395 to 418 has been identified as a segment that affects the fusogenic activity of the protein by influencing the low-pH-induced conformational changes (Li et al., 1993; Shokralla et al., 1998). In addition, it has also been shown that not only the ectodomain but also the membrane anchoring domain is required for VSV fusion activity (Cleverley and Lenard, 1998; Odell et al., 1997). Moreover, Jeetendra *et al* provided evidence that membrane-proximal stem region of VSV-G protein ectodomain (GS i.e. G stem), together with transmembrane and cytoplasmic domains can potentiate the membrane fusion activity when coexpressed with heterologous viral fusion proteins (Jeetendra et al., 2002). The G-stem was shown to be able to mediate binding of the VSV to target membranes in a manner similar to wild-type VSV. This could bring two membranes in close proximity to induce lipid mixing and initiate the fusion reaction. It was determined that only 14 amino acids of the GS were sufficient to induce hemifusion. A more recent study further elucidated the role of membrane proximal region of protein G showing that the membrane proximal region is not essential for G protein oligomerization, transport to the cell surface, or incorporation into virus particles but it is essential for acid-induced membrane fusion activity and virus infectivity (Jeetendra et al., 2003). Altogether these results suggested that in the case of VSV-G glycoprotein, the fusogenic activity may involve several spatially separated regions in the extracellular domain of the protein (Figure 9).

Early models of VSV assembly postulated the importance of interaction between VSV-G protein and ribonucleoprotein core (Metsikko and Simons, 1986). This conclusion was based on the evidence showing that G proteins with truncated cytoplasmic tails were not efficiently incorporated to the virions (Whitt et al., 1989) and that addition of cytoplasmic tails to heterologous viral glycoproteins was sufficient for their incorporation into virus particles (Owens and Rose, 1993). However, VSV-G protein is not needed for virus budding although it does enhance it (Knipe et al.,

1977; Mebatsion et al., 1996; Schnell et al., 1997; Takada et al., 1997). In fact addition of G protein cytoplasmic tail to heterologous proteins does not increase virus budding or glycoprotein incorporation (Kahn et al., 1999; Schnell et al., 1996) and G proteins with truncated or chimeric cytoplasmic tails produce infectious VSV (Schnell et al., 1998). These results support the idea that small cytoplasmic tail of VSV-G is important for VSV budding but no specific amino acid sequence is required. Identification of the budding domain in the membrane-proximal region of the G ectodomain supports this idea (Robison and Whitt, 2000). The report demonstrated a generation of series of recombinant VSVs, which expressed chimeric glycoproteins having truncated stem sequences. The recombinant viruses having 12 or more membrane-proximal residues, including transmembrane and cytoplasmic tail domains, produced near-wild-type levels of virus particles.

2.3.2 Targeted capsid viruses

The targeting of capsid viruses, adenoviruses and AAV, is generally more straightforward due to the lack of non-specific interaction mediated by the host-derived lipid membrane. This makes the rational design of targeted capsid vectors easier but requires detailed knowledge about the capsid structure and factors vital for its integrity and functionality. Two distinct approaches have been employed to transductionally target Ad and AAV vectors: adapter molecule-based targeting and targeting achieved via structural manipulation of the capsid by genetic means.

Adaptor-based targeting of adenoviruses. Adaptor proteins used to target capsid viruses include bi-specific antibodies, chemical conjugates between antibody fragments (Fab) and cell-selective ligands, Fab-cell specific antibody conjugates, Fab-peptide ligand conjugates and recombinant fusion proteins that incorporate Fabs and peptide ligands (Waehler et al., 2007). The first *in vitro* demonstration of Ad targeting via the adapter method used a bispecific conjugate of neutralizing Fab chemically linked to folate (Douglas et al., 1996). The results showed that Fab fragment alone blocked 99% of the wild type adenoviral infection but the folate restored the infectivity in folate receptor expressing cells. This technology was also applied to re-target adenoviral gene delivery to Kaposi's sarcoma cells via Fab conjugated to fibroblast growth factor (Goldman et al., 1997). Importantly, this targeting system also reduced hepatic toxicity and resulted in increased survival in a melanoma xenograft mouse model (Gu et al., 1999). Other Fab–ligand conjugates targeted against epidermal growth factor (EGF), epithelial cell adhesion molecule, tumor-associated glycoprotein-67, and CD40 have been employed in a similar manner with promising results (Haisma et al., 1999; Hakkarainen et al., 2003; Heideman et al., 2001; Kelly et al., 2000; Miller et al., 1998; Tillman et al., 1999). Further testing of lung targeting adapters such as anti-knob Fab chemically conjugated to a monoclonal antibody against angiotensin-converting enzyme or sCAR fused to a single-chain antibody directed against carcinoembryonic antigen provided evidence on the feasibility of this approach *in vivo* (Li et al., 2007; Reynolds et al., 2000). In both cases the reporter transgene expression was significantly increased compared to the untargeted vector while liver transduction was reduced.

Adaptor-based targeting of AAVs. The feasibility to target AAV-2 using adaptor molecules was shown by Bartlett *et al* (Bartlett et al., 1999). They generated a bispecific antibody molecule by a chemical crosslink of the Fab arms of the monoclonal antibodies against integrin and AAV-2 capsid. This targeting vector successfully transduced cells positive for the targeting receptor, whereas the transduction of receptor-negative cells was reduced by 90%.

Pseudotyping. Two basic strategies have been employed for genetic tropism modification of the capsid vectors: pseudotyping and ligand incorporation into the capsid proteins. Pseudotyping has mainly been achieved by substituting some or all coat proteins with homologous proteins of other serotypes, called serotype switching or serotype chimerism (Choi et al., 2005; Stone and Lieber, 2006). Such functional incorporation of the viral attachment protein into a protein capsid instead of a lipid bilayer poses a challenge. The tropisms of various Ad and AAV serotypes are presented in Tables 3 and 4.

Table 3. Comparison of the cellular receptors and tropism of the alternative adenovirus serotypes in comparison to Ad5 (* knob exchange only, **Ad2-based).

Ad serotype	Primary receptor	Secondary receptor	Tropism	References
Ad2	CAR	HS		
Ad3	CD80, CD86	CD46	ovarian* and squamous cancer*, glioma*, B cells*, vascular SMCs	(Kanerva et al., 2002a; Kawakami et al., 2003; Ulasov et al., 2007; Von Seggern et al., 2000)
Ad5	CAR	MHC-1 α2, VCAM-1, HS		
Ad7	CD80, CD86	CD46		
Ad8	sialic acid			
Ad11	CD46		HSCs and immature DCs, endothelial cells, prostatic cancer and laryngeal cancer	(Mei et al., 2004; Stecher et al., 2001; Stone and Lieber, 2006; Zhang et al., 2003)
Ad14	CD46			
Ad16	CD46		synovial tissues, vascular endothelial cells, and SMCs, chondrocytes, cancer stem cells	(Goossens et al., 2001; Havenga et al., 2001; Havenga et al., 2002; Skog et al., 2007)
Ad17			human airway epithelia** neurons**	(Chillon et al., 1999; Zabner et al., 1999)
Ad19	sialic acid			
Ad21	CD46			
Ad35	CD46		HSC (CD34+), DCs, melanocytes, follicle dermal papilla cells, cancer	(Havenga et al., 2002; Ni et al., 2006;Rea et al., 2001; Shayakhmetov et al., 2000; Yotnda et al., 2001)
Ad37	sialic acid		HSC (CD34+)	(Mei et al., 2004)
Ad50	CD46		HSC, myoblasts, cancer	(Havenga et al., 2002)

Altered vector tropism has been reported by substitution of the Ad5 fiber protein into that of Ad3, Ad7, Ad11, Ad16, Ad17, Ad35 and others (Chillon et al., 1999; Gall et al., 1996; Goossens et al., 2001; Havenga et al., 2001; Mizuguchi and Hayakawa, 2002; Rea et al., 2001; Shayakhmetov et al., 2000; Stecher et al., 2001; Stevenson et al., 1997; Zabner et al., 1999). Moreover, pseudotyping of Ad has been achieved by replacing the fiber knob domain from that of another serotype (serotype chimerism). This kind of Ad5/3 vector has proven useful for retargeting Ad5 to low-CAR primary ovarian carsinoma cells *in vitro* and *in vivo* (Kanerva et al., 2002a; Kanerva et al., 2002b). Furthermore, recent data from combination treatment with oncolytic Ad5/3 and chemotherapy has shown convincing preclinical efficacy in orthotopic model for advanced ovarian cancer(Raki et al., 2005; Raki et al., 2007). An important advantage of fiber-pseudotyped Ad vectors is the reduced the innate immune response following systemic delivery (Schoggins et al., 2005).

Similarly, studies using vectors from alternative AAV serotypes such as AAV-1, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAV-8 and AAV-9 have shown different potency and tropism (Davidson et al., 2000; Gao et al., 2006; Halbert et al., 2001; Handa et al., 2000; Seiler et al., 2006; Vandendriessche et al., 2007; Xiao et al., 1999; Zabner et al., 2000; Zhong et al., 2006). Also mosaic capsid AAVs composed of a mixture of viral capsids from different serotypes have been used to combine the advantages of parental serotypes (Rabinowitz et al., 2004; Xiao et al., 1999).

Table 4. Comparison of the cellular receptors and tropism of the alternative AAV serotypes in relation to AAV-2.

AAV serotype	Primary receptor	Secondary receptor	Tropism	References
AAV-1	N-linked sialic acid		skeletal muscle, HSC	(Xiao et al., 1999; Zhong et al., 2006)
AAV-2	HSPG	FGFR1,integrin αvβ5, HGFR	CNS	(Davidson et al., 2000)
AAV-3	HSPG	FGFR1	HSC, megacaryocytes	(Handa et al., 2000)
AAV-4	O-linked sialic acid	PDGF receptor	airway epithelia, CNS, retinal pigmented epithelium	(Davidson et al., 2000; Halbert et al., 2001; Rolling et al., 2006)
AAV-5	N-linked sialic acid		airway epithelia, CNS	(Davidson et al., 2000; Zabner et al., 2000)
AAV-6	N-linked sialic acid		airway epithelia	(Seiler et al., 2006; Xiao et al., 1999)
AAV-7			liver, skeletal muscle	(Gao et al., 2002; Gao et al., 2006)
AAV-8	LamR		liver, skeletal muscle	(Gao et al., 2002; Gao et al., 2006; Vandendriessche et al., 2007)
AAV-9			lung, liver, cardiac muscle	(Inagaki et al., 2006; Vandendriessche et al., 2007)

Genetic targeting of adenoviruses. To date, modification of the Ad fiber protein has been the most common approach used to genetically alter adenoviral tropism. Two approaches have been used for this purpose: addition of foreign peptides to the HI loop or C-terminus of the fiber knob (Figure 8) or design of knobless viruses. Expanded tropism has been reported for Ad vectors with C-terminal integrin-binding RGD motifs and polylysine ligands whereas other peptide ligands were ineffective in this context due to steric hindrance (Wickham et al., 1997). Consequently the HI-loop, tolerating insertions up to 100 amino acids with minimal negative effects on virion integrity, became the preferential site for ligand incorporation (Belousova et al., 2002).Dmitriev *et al.* introduced an integrin-binding RGD peptide into the HI-loop, resulting in vector with enhanced gene delivery to ovarian cancer cell lines and primary tumors (Dmitriev et al., 1998; Hemminki et al., 2001; Hemminki et al., 2002). Recent studies have demonstrated the high potential of this vector for the treatment of ovarian cancer in murine ovarian cancer models (Mahasreshti et al., 2006). Other targeting peptides inserted in the HI loop of the fiber included ones having high affinity for vascular endothelial cells, cancer cells, transferrin receptor and vascular smooth muscle cells (Nicklin et al., 2000; Nicklin et al., 2003; Work et al., 2004; Xia et al., 2000).

Another approach to achieve CAR-independent tropism consisted of ablating the fiber and/or knob domain (Figure 8), hereby the fiber is re-trimerized by an alternative trimerization motif such as T4 fibritin protein (Krasnykh et al., 2001). A de-knobbing strategy was employed by Magnussen *et al*. wherein an RGD motif was utilized to achieve selective infection of integrin-expressing cell lines *in vitro* (Magnusson et al., 2001). In another approach the entire fiber was replaced with T4 fibritin fused with a trimeric CD40 ligand (Belousova et al., 2003). Notably, this vector provided CD40-specific gene delivery in vivo following systemic delivery (Izumi et al., 2005)

In addition to fiber modifications, capsid protein hexon as well as minor capsid protein polypeptide IX have been used as platforms for incorporation of targeting peptides (Figure 8). They are both attractive locales for peptide ligand incorporation due to their surface exposure and abundance (240 hexon/80 pIX homotrimers per virion). Vigne *et al*. exploited hexon hypervariable region 5 as a site for incorporation of an integrin-binding RGD motif, demonstrating an enhanced, fiber independent transduction to low-CAR vascular smooth muscle cells (Vigne et al., 1999). The C-terminus of pIX has been used to insert poly-lysine, FLAG and RGD motifs, resulting in augmented, CAR-independent gene transfer (Dmitriev et al., 2002; Vellinga et al., 2004).

Genetic targeting of AAVs. The first attempts to alter the tropism of AAV-2 genetically was based on the insertion of scFv or receptor-specific ligands at the N-terminus of VP proteins. Although successful targeting was achieved using CD34 scFv, serpin receptor ligand, human luteinizing hormone peptide, it was of low efficiency and virus titers were affected (Shi et al., 2001; Wu et al., 2000; Yang et al., 1998). The more successful demonstration of genetic capsid modifications were achieved by inserting targeting peptides in different sites inside VP1, VP2 and VP3 sequences determined by sequence alignment between AAV-2 and other parvoviruses or systematic insertional mutagenesis. Peptides specific for integrin receptors, endothelial cells, atherosclerotic plaques and tumors have been employed for targeting of AAV-2 with promising results (Grifman et al., 2001; Nicklin et al., 2001; Shi and Bartlett, 2003; White et al., 2007).

Combination of genetic and adaptor-based targeting of Ads and AAVs. Also more general targeting systems have been developed for Ad- and AAV-vectors embodying elements of both genetic modification and adapter-based targeting. These studies incorporated the IgG domain of *Staphylococcus aureus* protein A on viral capsids enabling these vectors to form stable complexes with a wide variety of targeting molecules containing the Fc region of Ig. Genetic incorporation of this ZZ domain on VP3 on AAV-2 vectors led to specific transduction of distinct human hematopoietic cell lines using targeting antibodies against CD29, c-kit receptor, and CXCR4 (Ried et al., 2002). Similarly, other research groups incorporated the protein A domain into the fiber C-terminus or HI-loop of Ad vectors (Henning et al., 2005; Korokhov et al., 2003; Volpers et al., 2003). The results demonstrated a successful targeting and activation of dendritic cells via an Fc-single-chain antibody directed against CD40 (Korokhov et al., 2005). In addition, this system was used to target ovarian cancer cells via an antibody directed against mesothelin, as well as the pulmonary endothelium in a rat model *in vitro* (Balyasnikova et al., 2005; Breidenbach et al., 2005). Despite the flexibility of this system for the screening of numerous targeting antibodies, these systems still face some issues regarding the stability of the virus-adaptor complex *in vivo* and the fact that polyclonal Igs in the bloodstream might replace the targeting molecule.

2.3.3 Targeting non-viral vectors

The intrinsic properties of cationic lipoplexes and polyplexes complicate their systemic administration. For some applications the positive charge can be harnessed to target sites of increased vascular growth such as tumors, but for most applications this unspecific interaction leads to unwanted accumulation in the lung tissue associated with toxicity (Kircheis et al., 1999). Similarly, the size of the carrier can limit organ access and modulate biodistribution at the cellular level. Thus for successful targeting, these intrinsic properties of non-viral vectors need to be minimized. In this regard, the hydrophilic polymer polyethylene glycol (PEG) has been used to shield lipoplexes (Hofland et al., 2002; Monck et al., 2000) and polyplexes (Blessing et al., 2001; Ogris et al., 1999; Wolschek et al., 2002). PEG-shielding reduced gene transfer efficiency of complexes, but the efficiency was at least partly restored by incorporation of targeting ligands. Other approaches have used the serum protein transferrin to create negatively charged liposomes (Simoes et al., 1998) or to mask the positive charge of PEI-DNA complexes (Kircheis et al., 2001).

Specific targeting of the shielded non-viral vectors has most often taken advantage of natural ligand-receptor interactions. On this basis, systemic targeting of tumors was demonstrated using the folic acid receptor (Hofland et al., 2002; Jeong et al., 2005a), transferrin receptor (Bartlett et al., 2007; Hildebrandt et al., 2003; Kircheis et al., 1999; Xu et al., 1999) or EGFR (Wolschek et al., 2002) as a target. However, a potential drawback of endogenous targeting ligands is the presence of circulating ligands and binding to soluble receptors and receptors in nontarget tissue. Antibodies or antibody fragments might help to avoid some of these problems. As an example, antibodies have been used to target pancreas islet beta cells (Jeong et al., 2005b), human ovarian carcinoma cells (Merdan

et al., 2003), breast cancer cells (Germershaus et al., 2006) and glioma cells (Zhang et al., 2002), even though the *in vivo* data is still limited.

Despite these proof-of-principle studies, the transduction efficiencies of non-viral vectors remain low compared to viral vectors and particle shielding often adds to this problem. On this account, design of synthetic virus-like systems, combining the natural mechanisms exploited by viruses for membrane binding, endosomal escape and navigation through the nuclear pore, could improve the targeting potential of non-viral vector systems in the future (Wagner, 2004; Walker et al., 2005).

2.3.4 Avidin and streptavidin- biotin technology

Chicken avidin and bacterial streptavidin are widely used tools in the field of life sciences due to their strong specific affinity for biotin (Kd ~10^{13}-10^{15} M^{-1}) and ability to bind 4 biotins per (strept)avidin molecule. There is no interaction between the carboxy-containing side chain of biotin and avidin, which allows the modification of both counterparts (Bayer and Wilchek, 1994): Biotin can be modified chemically and attached to a wide variety of probes and binders, whereas avidin can be derivatised with other molecular probes or reporter groups of different types, without interfering with their interaction together. Together these features form the crux of (strept)avidin-biotin technology.

2.3.4.1 Avidin and streptavidin

Avidin is found in oviparous vertebrates including various birds, reptiles and amphibia, but no analogous protein has been detected in mammalian species (Elo, 1980). Chicken avidin, isolated from the hen egg white, and streptavidin, secreted by several species of *Streptomyces*, are functionally (Green, 1975; Green, 1990) and structurally (Livnah et al., 1993; Weber et al., 1989) analogous proteins. The main biological function of (strept)avidin is to bind biotin, vital enzymatic cofactor also known as vitamin H (Green, 1990). Both proteins form tetrameric complexes of approximately 60 kDa in which each subunit can bind one molecule of biotin with extremely high affinity (Kd ~10^{13}-10^{15} M^{-1}). This interaction is primarily thought to represent a natural defence mechanism against biotin requiring microbes (Board and Fuller, 1974; Elo et al., 1980) but also additional roles have been suggested for avidin (Elo and Korpela, 1984; Zerega et al., 2001). In addition to their exceptional ligand binding characteristics, avidin and streptavidin are exceptionally stable against high concentrations of denaturing agents, proteases, wide range of pH and temperature (Green, 1990).

Despite similarities, avidin and streptavidin differ in their primary amino acid sequence (41 % similarity), glycosylation, pI (Green, 1990), immunological reactivity and pharmacokinetics. Each avidin monomer has a single oligosaccharide moiety whereas streptavidin is devoid of sugars. Avidin is a basic protein with a high isoelectric point (pI) 10.5 whereas streptavidin has a mildly acidic isoelectric point (pI) of ~6. Owing to these dissimilarities, streptavidin has the advantage of lower

nonspecific binding to lectin-like- and negatively charged molecules than avidin. This has been further shown to affect the pharmacokinetics of these proteins with avidin eliciting a shorter plasma half-life compared to streptavidin (Marshall et al., 1995; Rosebrough and Hartley, 1996; Rosebrough, 1993; Schechter et al., 1990). The glycosylation is responsible for avidin's tendency to accumulate in the liver, whereas its accumulation in the kidneys is mainly due to high pI (Yao et al., 1999). Streptavidin shows accumulation to kidneys (Schechter et al., 1990). Both proteins are immunogenic (Knox et al., 2000; Subramanian and Adiga, 1997; Weiden and Breitz, 2001)

2.3.4.2 Modified avidins

Streptavidin was hoped to eliminate the non-specific backround binding to extraneous material accounted with avidin due to high pI and the presence of oligosaccharide moiety. However, streptavidin has been found to interact in biotin-independent manner with the integrins and related cell surface receptors (Alon et al., 1990). To overcome these problems, and thus to improve the immunological and pharmacokinetic properties of (strept)avidin, a plethora of studies have focused on developing novel chemically and genetically engineered forms of avidin and streptavidin (Figure 10).

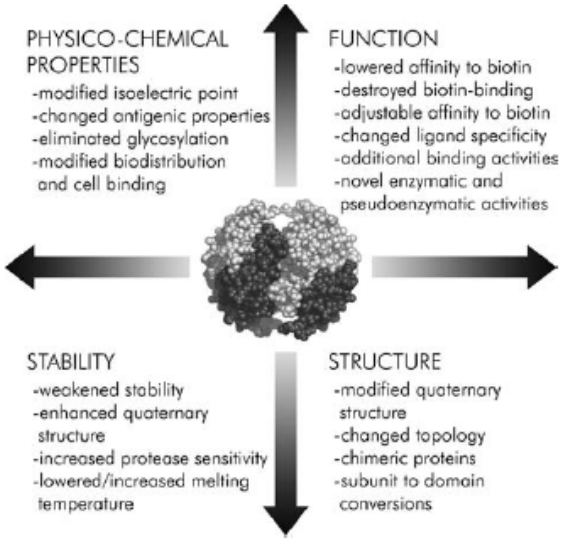

Figure 10. Engineering approaches used to modify the structural and functional properties of (strept)avidin (Laitinen et al., 2006).

Early attempts to reduce the charge of avidin consisted of formylation (Guesdon et al., 1979), acetylation (Kaplan et al., 1983) or succinylation (Finn et al., 1984) via covalent attachment to the available lysines of avidin. However, this blocked free amino groups which are often used in preparing avidin-conjugates.

The modification of avidin via arginines resolved the problem by leaving lysines available for subsequent interaction. Commercialized neutral avidin derivatives prepared this manner include ExtrAvidin (Sigma-Aldrich, St. Louis, MO, USA), NeutraLite Avidin (Belovo Chemicals, Bastogne, Belgium) and NeutrAvidin (Pierce, Rockford, IL, USA). Marttila et al, have further reported the construction of series of avidin charge mutants with pIs ranging from 4.7 to 9.4 by site-directed mutagenesis replacing arginines and lysines with neutral or acidic amino acids (Marttila et al., 1998). These mutants showed reduction of non-specific binding, while retaining their thermal stability and the ability to tightly bind biotin.

To further reduce the non-specific interaction with extraneous macromolecules, the oligosaccharide moiety of avidin can be removed chemically or enzymatically (Bayer and Wilchek, 1994). Enzymatic deglycosylation was used to develop NeutraLite Avidin, which consequently lacks the carbohydrates, exhibits neutral pI and bears free lysines for potential attachment of probes. In a more recent study Marttila *et al* produced an avidin mutant lacking the oligosaccharide moiety by genetic means. They combined this sugarless mutant with the avidin charge mutant (pI 4.7) to further improve the non-specific binding characteristics of avidin (Marttila et al., 2000).

Although most of the applications of (strept)avidin-biotin technology are based on the particularly high affinity between (strept)avidin and biotin, mutants with reduced affinity and reversible binding would be of great value for affinity-based separation of biotinylated molecules. To this end, two types of modifications have been extensively studied: binding-site mutants and interface mutants (Laitinen et al., 2006). To date, numerous binding-site mutants from streptavidin and avidin have been created with affinities for biotin ranging from near wild-type to 6 orders of magnitude weaker values (Chilkoti et al., 1995; Klumb et al., 1998; Marttila et al., 2003; Sano and Cantor, 1995).

Modification of the interfaces of (strept)avidin monomers has additionally provided solutions to aggregation problems due to the cross-linking by tetravalent (strept)avidin. Laitinen *et al* demonstrated the production of dimeric (Laitinen et al., 1999) and monomeric biotin-binding forms which are tetramerized upon biotin binding (Laitinen et al., 2001) or remain in the monomeric state (Laitinen et al., 2003). The affinity for biotin binding was decreased to Kd ~ 10^{-8} M in the case of dimeric avidin and to Kd ~10^{-7} M for monomeric avidin known to remain as a monomer upon biotin binding. Following these studies, monomeric forms of streptavidin have been developed (Qureshi et al., 2001; Wu and Wong, 2005)

Avidin has been further engineered for improved characteristics and this work continues to widen the field of applicability of avidin-biotin system. For example recent work by Kulomaa and co-workers has demonstrated the production of dual-and single-chain avidins or avidins with dual-affinity (Hytonen et al., 2005; Nordlund et al., 2004; Nordlund et al., 2005). In another case, Howarth *et al* contructed a streptavidin which binds biotin in monovalent fashion while retaining its high affinity (Howarth et al., 2006).

2.3.4.3 Biotin and biotinylation

Biotin is a small (244 Da) water-soluble vitamin H synthetized by bacteria, yeasts, molds, algae and some plants, but required by all forms of life (Mock, 1996). Biotin has been shown to play an essential role in regulating gene expression in *E.coli* and in mammalian cells (Cronan, 1989; Rodriguez-Melendez and Zempleni, 2003). Moreover, in mammalians more than 2000 biotin-dependent genes have been identified and biotinylation of histones plays an essential role in cell proliferation, gene silencing and cellular response to DNA damage (Zempleni, 2005).

Biotin is bound to cellular carboxylases and decarboxylases, which catalyze the transfer of CO_2 to and between metabolites in gluconeogenesis, lipogenesis, amino acid degradation and energy transduction (Knowles, 1989; Samols et al., 1988). The attachment of biotin to the ε-amino group of a specific lysine moiety in carboxylases is catalyzed by biotin protein ligase in an ATP-dependent reaction (Figure 11) (Otsuka and Abelson, 1978). The number of biotinated carboxylases varies from species to species: In *E.coli*, the bacterial biotin ligase BirA biotinylates only one protein called acetyl-CoA carboxylase whereas in mammalian cells acetylCoA- carboxylase, methyl crotonyt-CoA carboxylase, propionyl-CoA carboxylase and puryvate carboxylase are biotinylated (Cronan, 1990).

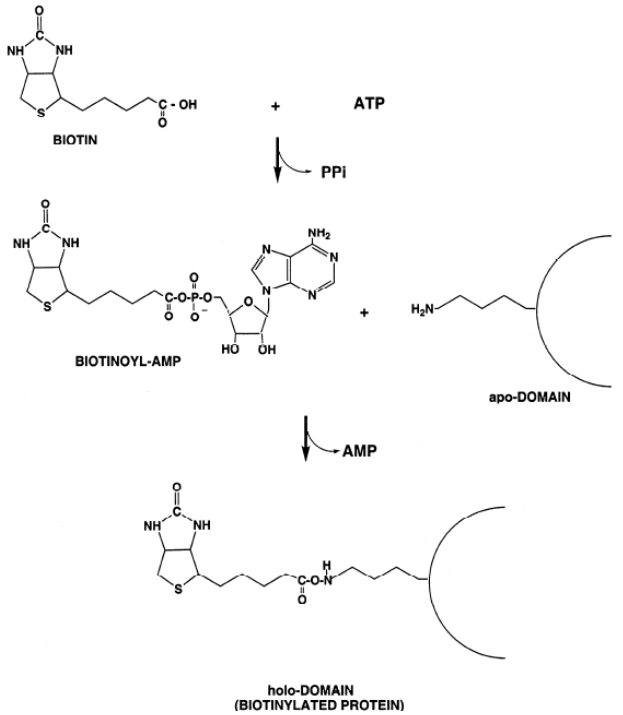

Figure 11. The biotin protein ligase reaction. In the first step, biotin protein ligase catalyses the attack of an oxygen atom of the biotin carboxylate on phosphate of ATP to form biotinoyl-AMP. Next, the nucleophilic ε-amino group of the lysine on apo-form of the biotin acceptor domain attacks the carbon atom on biotinoyl-AMP thus forming an amide bond between biotin and biotin accepting domain (Chapman-Smith and Cronan, 1999).

Biotinylation of proteins is an attractive alternative to epitope tagging due to the strong (strept)avidin-biotin interaction. While a wide range of chemical biotinylation techniques exist, they are limited by the fact that chemical biotinylation are not site-specific, require prior purification of the substrate and can lead to inactivation of the target protein (Stolz et al., 1998). Given the difficulties in chemical biotinylation, many efforts are now devoted to developing systems exploiting the natures own biotinylation machinery. Early studies using this approach took advantage of the 1.3S subunit of *Propioni bacterium shermanii* transcarboxylase (PSTCD) which is naturally biotinylated at lysine 89 (Cronan, 1990). The expression of PSTCD fusion protein led to its enzymatic biotinylation in *E.coli* and *Saccharomyces cerevisiae*. Later on, this approach has been expanded to mammalian cells and animals (Parrott and Barry, 2000; Parrott and Barry, 2001). Isolation of new shorter biotin acceptor peptide (BAP) substrates (ca. 13-20 residues) for BirA have further increased the appeal of this system for purification applications (Duffy et al., 1998; Schatz, 1993). Metabolically biotinylated gene therapy vectors will be discussed in the next chapter.

2.3.4.4 (Strept)avidin-biotin technology in gene therapy

The specific characteristics of avidin have been shown to be of great advantage in drug targeting: The high positive charge of avidin augments the efficiency of cellular uptake of biotin-coated particles (Pardridge and Boado, 1991), whereas incubation of bioconjugated avidin with biotinylated cell lines results in rapid surface attachment and endocytosis with efficiencies approaching 100% (Wojda et al., 1999). In addition, (strept)avidin has been demonstrated to accumulate into specific tissues (Rosebrough and Hartley, 1996; Rosebrough, 1993; Schechter et al., 1990), especially to tumors *in vivo* (Yao et al., 1998). Consequently, avidin alone can enable some targeting of gene therapy vectors to specific tissues, while tissue-specificity can be altered by biochemical modification of the protein.

The strong avidin-biotin interaction can be used to develop targeted therapies by the biotinylation of ligands or tissues *in vivo* (De La Fuente et al., 1997; Hoya et al., 2001; Singh et al., 2005). Several studies have shown promising results using monoclonal antibodies in targeting of biotinylated therapeutic or diagnostic compounds via avidin in experimental animals (Corti et al., 1998; Guttinger et al., 2000; Wu and Pardridge, 1999). One such approach, called pretargeted radioimmunotherapy, separates the administration of the monoclonal antibodies from that of a low-molecular-weight radionuclide ligand. This multistep (2 or 3 steps) approach has shown to improve tumor: normal tissue radiation dose ratios since the targeting molecule administered first is not radiolabeled. Subsequent administration of (strept)avidin removes the excess circulating antibodies or binds to tumor cells preparing them to receive radiolabelled biotin. This strategy has already been followed in clinical trials for the treatment of malignant glioma demontstrating impeding of cancer progression without significant toxicity (Grana et al., 2002; Paganelli et al., 1999; Paganelli et al., 2001; Paganelli et al., 2006). Other similar studies are being conducted for the treatment of colon cancer, B-cell lymphoma and gastrointestinal malignancies (Forero et al., 2004; Knox et al., 2000; Linden et al., 2005; Shen et al., 2005).

Another potential approach for targeting tumors or tissues is to introduce recombinant-avidin gene into specific tissues by local gene transfer which consequently can sequester and concentrate considerable amounts of therapeutic biotin conjugates *in vitro* and *in vivo* (Lehtolainen et al., 2002a; Lehtolainen et al., 2003; Walker et al., 1996)

In addition, (strept)avidin-biotin technology can be adapted to improve vector targeting (Waehler et al., 2007). It provides substantial advantage over other adaptor systems by assuring sufficient stability of the vector-adaptor complex even under physiological conditions (Pereboeva et al., 2007). Early studies with retroviruses used streptavidin-bound antibodies specific for both viral and cell membrane epitopes provided the proof-of-principle even though only low transduction efficiencies were attained (Etienne-Julan et al., 1992; Roux et al., 1989). Since that, the (strept)avidin-biotin-based targeting has followed two different approaches depicted in figure 12. Firstly, gene therapy vectors can be biotinylated either chemically or metabolically, while bringing the biotinylated targeting molecule in conjunction to avidin (Barry et al., 2003). First studies by Smith and collegues were performed with a chemically biotinylated adenovirus vector. They demonstrated successfull vector targeting to hematopoetic cells through an avidin bridge carrying biotinylated c-Kit receptor ligand, resulting up to 2400-fold increase in reporter gene expression (Smith et al., 1999). Later on, chemically biotinylated retrovirus, AAV and vaccinia virus have been created, all of which exhibited significantly increased transduction of target cells (Ponnazhagan et al., 2002; Purow and Staveley-O'Carroll, 2005; Zhong et al., 2001).

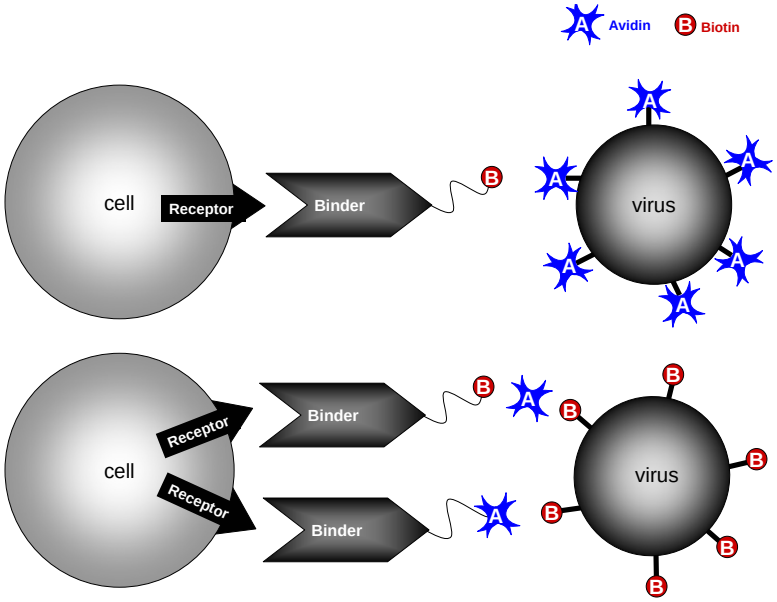

Figure 12. The principle of (strept)avidin-biotin technology in vector targeting. The vector can either carry avidin or biotin on its surface. This allows attachment of biotinylated or avidinylated targeting molecules (binders; e.g. cell surface ligands, antibodies).

However, chemical biotinylation often leads to non-specific labelling, inactivation of target proteins and require prior purification of the virus. Therefore, metabolic biotinylation, achieved directly in living cells, is considered a more promising approach (Cronan, 1990). A number of studies have shown that metabolic biotinylation provides an efficient means to target gene therapy vectors such as adenovirus (Campos and Barry, 2006; Campos et al., 2004; Maguire et al., 2006; Parrott et al., 2003) and AAV (Arnold et al., 2006; Stachler and Bartlett, 2006). A recent report by Pereboeva and coworkers also provided evidence on the applicability of metabolically biotinylated adenovirus vectors for *in vivo* retargeting (Pereboeva et al., 2007). Owing to the large availability of (strept)avidin-biotin-based purification methods, metabolical biotinylation of gene therapy vectors has also been applied for virus concentration (Arnold et al., 2006; Campos and Barry, 2006; Chan et al., 2005; Nesbeth et al., 2006; Stachler and Bartlett, 2006).

An opposite targeting approach consists of avidin displaying vectors (Figure 12). To this end, chemically avidinylated PEI-vectors (Wojda and Miller, 2000) and adenoviruses (Park et al., 2008) together with genetically engineered avidin displaying baculoviruses have been described (Raty et al., 2004). In the latter study, Räty and collegues expressed an avidin-gp64 fusion protein on the baculovirus surface thus providing binding sites to biotinylated targeting ligands (Raty et al., 2004). This 2-step system is easier to control than the 3-step process using the avidin-bridge between desired biotinylated molecules and offers better valence for covering since all four biotin-binding sites are available for biotinylated ligands. Avidin displaying baculovirus showed a 5-fold increase in transduction efficiency of rat glioma cells and a 26-fold increase in rabbit aortic smooth muscle cells compared to wild-type virus without major cytotoxicity. Enhanced transduction was also observed with biotinylated cells and biotinylated EGF enabled targeting to EGFR expressing cells. In addition, the use of biotinylated paramagnetic particles allowed magnetic targeting.

In addition to providing great potential for therapeutic purposes, the avidin-biotin technology has also been exploited for *in vivo* imaging purposes. The pretargeted radioimmunotherapy for cancer treatment described above, has been widely applied for simultaneous radioimmunoimaging (Cauchon et al., 2007; Hama et al., 2007; Li et al., 2005; Sharkey et al., 2005). Furthermore, conjugation of baculovirus vector to biotinylated iron oxide particles or radionuclides has enabled imaging of the viral particle biodistribution by magnetic resonance imaging and single photon emission computed tomography *in vivo* (Raty et al., 2006; Raty et al., 2007). Similarly, cells expressing metabolically biotinylated cell-surface receptors can be visualized with labeled streptavidin moieties (Tannous et al., 2006).

2.4 Detection of viral particles and gene expression

Molecular imaging techniques are defined as the visual characterization and quantification of biological processes at the cellular and subcellular levels within a living organism. The three main methodologies developed for noninvasive *in vivo* imaging include optical imaging, magnetic resonance imaging (MRI) and nuclear imaging [single photon emission tomography (SPECT), and

positron emission tomography (PET)]. Each of these technologies has its own advantages and drawbacks as listed in Table 5.

Current imaging methods in gene therapy can be divided into biodistribution and reporter gene imaging. Reporter gene imaging visualizes the transgene expression whereas biodistribution demonstrates the vector location throughout the body. It is often desirable to obtain images with both imaging modalities to obtain conclusive data on the safety and efficacy of gene therapy.

Table 5. Characteristics of different imaging modalities [Modified from (Massoud and Gambhir, 2003)].

Imaging technique	Electro-magnetic radiation	Spatial reso-lution	Temporal resolution	Sensiti-vity	Advantages	Disadvantages
Fluores-cence imaging	visible light or near-infrared	2-3 mm	seconds to minutes	10^{-9}-10^{-12} M	High sensitivity, low cost	relatively low resolution, imaging depth <1 cm
Biolumi-nescence imaging	visible light	3-5 mm	seconds to minutes	10^{-15}-10^{-17} M	High sensitivity, quick, low cost, high throughput	low resolution, 2D imaging only, imaging depth 1-2 mm
PET	high-energy γ-rays	1-2 mm	10 sec - minutes	10^{-11}-10^{-12} M	sensitive, isotopes can substitute natural atoms	PET cyclotron or generator needed, relatively low resolution, radiation
SPECT	lower-energy γ-rays	1-2 mm	minutes	10^{-10}-10^{-11} M	many probes available, simultaneous multiple probe imaging	relatively low resolution, radiation
MRI	radiowaves	25-100μm	minutes - hours	10^{-3}-10^{-5} M	high resolution, combines morphological and functional imaging	relatively low sensitivity, long scan and posprocessing time

2.4.1 Optical imaging

Optical imaging is a modality that is cost-effective, rapid, easy to use, and can be readily applied to studying transductional efficiency of gene therapy vectors *in vivo*. Optical methods can be divided into fluorescence- and bioluminescence-based reporter gene systems. A number of fluorescence reporter genes eg. different variants of the *Aqueorea Victoria* green fluorescent protein (GFP) (Contag et al., 2000; Ellenberg et al., 1999; Falk and Lauf, 2001; Hadjantonakis and Nagy, 2001) and red fluorescent proteins from *Discosoma* species (dsRed1 and dsRed2) (Campbell et al., 2002; Mathieu and El-Battari, 2003) have been described and applied in optical imaging of tumors and

metastases (Bharali et al., 2005; Bouvet et al., 2002; Yang et al., 2000). However, bioluminescence reporter imaging currently remains more sensitive for whole-body imaging: the most commonly used are the Firefly and Renilla luciferase genes (Choy et al., 2003; Gross and Piwnica-Worms, 2005; Wilson and Hastings, 1998; Yu et al., 2003). Expression of luciferase has been specifically used to image the growth kinetics of transformed tumor cells (Liang et al., 2004; Rehemtulla et al., 2002) and to monitor viral gene delivery (Berraondo et al., 2006) and replication (Yamamoto et al., 2006).

2.4.2 PET/SPECT

Nuclear imaging is based on the administration and detection of decaying radioisotopes. The decay of a radioisotope emits a positron or gamma ray which produces detectable two (positron emission tomography, PET) or single high-energy photons (single photon emission tomography, SPECT).

PET is based on the use of contrast agents (tracers) including radioisotopes ^{11}C, ^{13}N, ^{15}O, ^{18}F ^{64}Cu, ^{68}Ga, ^{76}Br and ^{94m}Tc (Massoud and Gambhir, 2003). Positrons emitted by the tracers are annihilated in tissue by collision with an electron. It results in an emission of two high-energy photons detected by PET detector array. The sensitivity of PET is relatively high, in the range of 10^{-11}-10^{-12} M (Mandl et al., 2002). PET provides a spatial resolution of 1-2 mm enabling small animal micro-PET applications.

SPECT is similar to PET, but acquires information on the concentration of gamma emitting radionucleotides like ^{111}In, ^{133}Xe, ^{99m}Tc ^{123}I, ^{125}I, and ^{131}I (Rosenthal et al., 1995). These heavy isotopes have longer decay times than PET contrast agents and are more readily available (Mandl et al., 2002). However, SPECT tracers can also be taken up by other areas than their target area and simultaneous imaging by computer tomography (CT) may be required (Labbe, 2003). SPECT is at least a log order less sensitive than PET even though the spatial resolution is near the same (Massoud and Gambhir, 2003).

Direct labeling of virus particles allows the systemic distribution of the virus to be detected by nuclear imaging. Labeling herpes simplex viruses with ^{111}In enabled to trace the organ distribution of the viruses after intravenous administration (Schellingerhout et al., 1998). The same group also performed mass distribution studies of HSV vectors in an experimental brain tumor model to evaluate and compare different delivery modalities (Schellingerhout et al., 2000). Imaging techniques for other viral vectors have also been developed based on iodination (Frost, 1977; Markwell and Fox, 1978; Montelaro and Rueckert, 1975; Moore et al., 1974) and the biotin-avidin system (Skulstad et al., 1995). In several of these studies, however, the viral labeling was reported to affect the infectivity of the vectors. Recently, a less detrimental approach, based on the display of avidin on the baculovirus surface, was introduced (Raty et al., 2007). The avidin-display enabled baculovirus labeling with ^{99m}Tc-polylys-ser-DTPA-biotin, and visualization of viral biodistribution and kinetics after various administration routes.

Reporter genes for nuclear imaging can encode for intracellular enzymes, extracellular or intracellular receptors or cell membrane transporters capable of irreversibly binding or transporting a

radiolabeled or paramagnetic probe. Wild-type HSV1 *thymidine kinase* (*tk*) (Tjuvajev et al., 1998) and its mutant version HSV-*sr39tk* (Gambhir et al., 2000), are the most commonly used enzyme-based marker genes in transductional imaging. Unlike human thymidine kinases, these enzymes have less substrate specificity and can selectively phosphorylate a wide range of compounds. Imaging of TK expression is based on the use of iodinated or fluorinated acycloguanosines (eg, ganciclovir ; penciclovir; ^{18}F -labeled 9-[4-fluoro-(hydroxymethyl)butyl]guanine (Gambhir et al., 1999; Jacobs et al., 2001; Namavari et al., 2000) as well as thymidine analogues (eg, 2'-fluoro-2'-deoxy-1-beta-D-arabinofuranosyl-5-iodouracil, 2'-^{18}F-fluoro-5-ethyl-1-beta-D-arabinofuranosyl-uracil) (Buursma et al., 2006; Yaghoubi et al., 2001). When HSV-TK is present in the cells, the substrates become phosphorylated and thus trapped. Cellular retention of radioactivity indicates the transfection and this has been widely used to monitor the gene therapy of cancer (Blasberg and Tjuvajev, 1999; Deng et al., 2006; Tseng et al., 2006; Yaghoubi et al., 2005). Alternative enzyme-based reporter systems are xanthine-phophoribotransferase (Doubrovin et al., 2003) and cytosine deaminase (Haberkorn et al., 1996).

Use of extracellular receptors or cell membrane transporters as reporter genes eliminates the need for the probe to penetrate into a cell. The human dopamine receptor is an example of such reporter gene (h*D2R*). h*D2R* expression is largely limited to the striatal-nigral system of the brain, which makes it a good candidate for transductional imaging. h*D2R* expressing cells and tissues can be imaged through the accumulation of an established probe, ^{18}F fluoroethyl spiperone (MacLaren et al., 1999). Somatostatin receptor subtype II is another receptor-based reporter gene, which has a naturally limited expression to carcinoid tumors (Rogers et al., 1999; Rogers et al., 2000). Both receptors can be imaged simultaneously with HSV-tk providing a platform for indirect imaging of therapeutic gene expression (Chen et al., 2004; Verwijnen et al., 2004).

Human sodium iodine symporter (h*NIS*), iodine-transporter in thyroid follicular cells, provides an opportunity to image with both PET (124I-iodine) and gamma camera (^{123}I-iodine or ^{99}mTc-pertechnetate) (Haberkorn et al., 2001). hNIS has been used for imaging adenovirus biodistribution and gene therapy (Dwyer et al., 2006; Groot-Wassink et al., 2002; Lee et al., 2004; Yang et al., 2004). More recently it has been suggested as a potential candidate for monitoring cardiac gene therapy (Miyagawa et al., 2005a; Miyagawa et al., 2005b). Another transporter-based reporter system, norepinephrine transporter, has been utilized to image transduced tumors (Altmann et al., 2003; Buursma et al., 2005).

2.4.3 MRI

Magnetic resonance imaging has the advantage of high spatial resolution (10-100µm) but the natural insensitivity for label detection requires robust amplification techniques. MRI is based on the detection of molecules that possess the property of nuclear spin, which align themselves when exposed to magnetic field. Following a radiofrequency pulse, the nuclear spins return to their baseline

orientation with a relaxation time corresponding to their physicochemical environment which can be measured (Massoud and Gambhir, 2003).

Contrast agents used in MRI can be divided into two categories which modify either T1 or T2 relaxation time constants, proton density or nuclear polarization to attain improved sensitivity. T1 contrast agents produce a positive signal enhancement on MRI images and are usually based on paramagnetic ions or stable free radical molecules (Potter, 2002). T2 contrast agents, on the other hand, decrease the signal in T2 weighted MRI seen as the darkening of image. T2 contrast agents are generally based on ferromagnetic of superparamagnetic nanoparticles. These particles can differ in their core size, coating thickness and coating material, all of which affect their biodistribution (Wang et al., 2001).

Most current transductional MRI strategies are based on coupling a reporter transgene with a specific probe. Initially, enzyme-based reporters were applied to monitor the changes in phosphor metabolism by arginine and creatine kinases (Koretsky et al., 1990; Walter et al., 2000) and fluorine metabolism by the enzyme activity of cytosine deaminase (Stegman et al., 1999) and β-galactosidase (Louie, 2006). Also approaches based on targeting the contrast agent to the cell surface using appropriate ligands have been developed. For example, after overexpression of inflammatory adhesion molecule or non-endogenous H2K(k) antigen, antibodies against these molecules conjugated to a superparamagnetic iron oxide particle, generated strong negative contrast (Bulte et al., 1998; So et al., 2005).

The most common reporter genes for MRI are based on proteins involved in iron metabolism, such as tyrosinase, transferrin receptor and ferritin. All cells use iron and they attain it from the circulating blood where it is bound to transferrin. Transferrin receptors throughout the body engulf and internalize both the protein and the iron attached to it. Once inside, the cell transfers the iron to ferritin, the internal iron storage molecule. Both these proteins can thus be used as reporter genes for MRI. Transferrin receptor overexpression can be probed with superparamagnetic agents (Moore et al., 2001; Weissleder et al., 2000) whereas ferritin is itself made superparamagnetic by the cell eliminating the need for an exogenous contrast agent (Genove et al., 2005). Ferritin is a ubiquitous intracellular iron storage protein that consists of 24 subunits of the heavy (H) and light (L) type (Arosio and Levi, 2002). This multi-subunit protein is capable of containing as many as 4,500 atoms of iron within a hydrous ferric oxide core. The H and L subunits have different functional specificity: the L-chain enhances the stability of the iron core while the H-chain has a metal-binding site which confers ferroxidase activity (Fe2+ oxidation to Fe3+) to the protein and accelerates iron incorporation (Levi et al., 1993; Levi et al., 1994). To date, ferritin expression has enabled the imaging of adenovirus transduction in mouse brain (Genove et al., 2005), and detection of transcriptional regulation of gene expression in glioma tumor models (Cohen et al., 2005) and transgenic animals (Cohen et al., 2007). The third approach for detecting transgenes by MRI relying on cellular accumulation of iron takes advantage of tyrosinase gene expression which leads to the production of metal-binding metabolite, melanin (Weissleder et al., 1997).

3. AIMS

The general objective of this study was to improve the efficiency, targetability and safety of baculo- and lentiviral vectors in order to extend the applicability of these viruses in the field gene therapy. A special emphasis was put on the development of generally applicable strategies for virus targeting, purification and non-invasive imaging based on avidin-biotin technology.

More specifically, the aims of this study were:

(I) To study if the VSV-GED could enhance the transduction efficiency of baculovirus vectors when coexpressed with gp64.

(II) To develop metabolically biotinylated baculovirus vectors by displaying a biotin acceptor peptide (BAP) fused either to different sites in the baculovirus glycoprotein gp64 or VSV-GED and to evaluate the utility of these vectors for virus targeting and purification applications.

(III) To create targetable lentivirus vectors displaying (strept)avidin-VSV-GED fusion proteins and to assess the utility of these vectors for *in vivo* imaging of virus particle biodistribution and transduction using SPECT/CT and MRI.

(IV) To analyze the transcription and expression of baculoviral immediate early genes in human cells and to examine the interactions between viral components and subnuclear structures after viral transduction.

4. MATERIALS AND METHODS

4.1 Methods

Table 6. Methods used in the studies.

Method	Desription	Study No.
DNA cloning	Vector design and construction	I- IV
Production of baculovirus vectors	Baculovirus production	I,II,IV
	Virus concentration: Ultracentrifugation	I,II,IV
	Magnetic capture	II
	Baculovirus titering: End-point dilution	I,II,IV
Production of lentivirus vectors	Lentivirus production	III
	Virus concentration: Ultracentrifugation	III
	Lentivirus titering: p24 ELISA	III
	Flow cytometry	III
Characterization of baculovirus vectors	SDS-PAGE and Immunoblotting	I, II
	ELISA	II
Characterization of lentivirus vectors	SDS-PAGE and Immunoblotting	III
	ELISA	III
***In vitro* experiments**	Transduction efficiency: β-galactosidase staining	I, II
	Luminescent β-galactosidase assay	I, II
	Flow cytometry	III
	Immunofluorescence labeling, confocal microscopy	IV
	Cell targeting with ligands and antibodies	II,III
	Magnetic targeting	II
	Cytotoxicity assay	I
	Endocytosis blocking	I
	Syncutium formation assay	I
	Magnetic resonance imaging	III
	RNA extraction, RT-PCR	III, IV
***In vivo* experiments**	Stereotactic injections to rat brain	I, III
	Intramuscular injections to rabbit hind limb	I
	Magnetic resonance imaging	III
	SPECT/CT imaging	III
Histochemical analyzes	β-galactosidase staining	I, III
	Antibody staining	III
	Iron staining	III
Statistical methods	Mean SD	I- IV
	Unpaired *t* test	I- IV
	ANOVA	II,III

4.2 Plasmids and DNA oligomers

The plasmids used to generate the recombinant baculo- and lentiviruses are summarized in Table 7. The DNA oligomers used in cloning and sequencing are listed in Table 8, whereas the individual cloning steps are described more thoroughly in the original publications.

Table 7. Plasmids used in studies I-IV.

Plasmid	Reference	Description	Study
pFastBac-1	Invitrogen, Carlsbad, CA, USA	Backbone for baculovirus constructs	I, II
pBacSurf-1	Novagen, Madison, WI, USA	Source of gp64	II
Baavi	(Raty et al., 2004)	Source of avidin and LacZ	III
PFD27	(Laitinen et al., 2003)	Source of monomeric avidin	III
pGEM-streptavidin	M. Kulomaa, University of Tampere, Finland	Source of streptavidin	III
pDONR201	Invitrogen, Carlsbad, CA, USA	Intermediate cloning of BirA	II
pCMV-VSVG	T. Friedmann, UCSD, La Jolla, CA, USA	Source of VSV-G, Lentivirus production	I II, III
pRSV-Rev	Tronolab, Lausanne, Switzerland	Lentivirus production	III
pMDLg/pRRE	Tronolab, Lausanne, Switzerland	Lentivirus production	III
pLV-GFP	(Makinen et al., 2006)	Lentivirus production, transfer vector	III
pENTR™221-Ferritin	RZPD German Resource Center for Genome Research, Berlin , Germany	Source of ferritin	III
pBOB-CAG	I. Verma, Salk Institute, La Jolla, CA, USA	Lentivirus production, transfer vector backbone	III
H2B-EYFP	J. Langowski, German Cancer Research Center, Heidelberg, Germany	Detection of human histone protein H2B expression	IV
pEGFP-sp100	G. Dellaire, The hospital for Sick Children, Toronto, Canada	Detection of promyelocytic nuclear bodies	IV
EGFP-PAB2	M. Carmo-Fonseca (Intituto de Medica Molecular, Lisbon, Portugal	Detection of nuclear speckles	IV
NPM-EGFP	M. Laiho, University of Helsinki, Finland	Detection of nucleophosmin	IV

Table 8. DNA oligomers used in the studies.

Sequence	Desription	Usage	Study
GGGGTGATACTGGGCTATCCAA	*VSV-GED* 5'forward	Cloning	I,II
AGATCTTTACTTTCCAAGTCGGTTCA	*VSV-GED* 3'reverse	Cloning	I,II
GGAAGTTCACCATAGTTTTTCCAC	*VSV-G* 5'forward	Cloning	I
GAAGGAGATAACATGAGATCT AAGGATAACACCGTGCCACTG	*BirA* 5'forward	Cloning	II
TTTAGTGATGGTGATGGTGA TGTTTTTCTGCACTACGCAGGG	*BirA* 3'reverse	Cloning	II
GATCCGTAAGCGCTATTGTTTTATATGTGCTTT TGGCGGCGGCGGCGCATTCTGCCTTTGCGA	*Gp64* signal sequence linker	Cloning	II
GATCTCGCAAAGGCAGAATGCGCCGCCGCCG CCAAAAGCACATATAAAACAATAGCGCTTAG	*Gp64* signal sequence linker	Cloning	II
GTTCATGCCATTCAATTTTTTGTGCTT CAAAGATATCATTCAGGCCCTGCA	BAP linker	Cloning	II
GGGCCTGAATGATATCTTTGAAGCAC AAAAAATTGAATGGCATGAACTGCA	BAP linker	Cloning	II
GGTACCCCCGGGCGGAGCACTGC	*Gp64* 5'forward	Cloning	II
ATAACCCGGGTCTTTAATATTGTCTATTACGG	*Gp64* 3'reverse	Cloning	II
CTTGGCTCTAACGTTGTGGCGTTCATGCCATT CAATTTTTTGTGCTTCAAAGATATCATTCAGG CCCCAAGTGGGCGGCCGCTTC	BAP + compatible ends to *gp64* site 283, 3' reverse	Cloning	II
AATGATAACCATCTCGCA	5'forw. pPolh-cassette	Sequenc.	I,II
GGATGAAGTGGTTCGCATCC	3'rev. pPolh-cassette	Sequenc.	I,II
GCAACGTGCTGGTCTGTGTGC	5'forw. CMV-cassette	Sequenc.	III
CACACCAGCCACCACCTTCTG	3'rev. CMV-cassette	Sequenc.	III
GGAACATGCTGAGAAACTGATGAAG	5'frw.human/rat ferritin	RT-PCR	III
CACAGTCTGGTTTCTTGATATCCTGA	3'rev. human ferritin	RT-PCR	III
CACGGTCAGGTTTCTTTATATCCTGC	3'rev. rat ferritin	RT-PCR	III
TTAACGCGTCGTACACCAGCG	5'forw. *ie-1*	RT-PCR	IV
TTATAATAACTTAAATAGTCGTTGGG	3'rev. *ie-1*	RT-PCR	IV
ATGAGTCGCCAAATCAACGC	5'forw. *ie-2*	RT-PCR	IV
GGCTTCGGGAGATGTTGTAAAG	3'rev. *ie-2*	RT-PCR	IV
TCCGACTTGGGCAAATGG	5'forw. *lef-3*	RT-PCR	IV
GATTGAAATCCGCACATAGCTTT	3'rev. l*ef-3*	RT-PCR	IV

4.3 Vectors

The characteristics of the recombinant vectors developed in the studies are listed in Table 9 and Table 10.

Table 9. Baculoviral vectors constructed in studies I-IV.

Baculoviral vectors (pseudotype)	Promoter-transgene	Surface Modification	Study
VSV-GED	CMV-LacZ	Transmembrane anchor of VSV-G	I
VSV-G	CMV-LacZ	VSV-G	I
BAP-VSV-GED	CMV-LacZ	BAP fused to VSV-GED	II
BAP-N-gp64	CMV-LacZ	BAP fused to N-terminus of gp64	II
BAP-283-gp64	CMV-LacZ	BAP inserted at aa. position 283 of gp64	II
BAP-283+N-gp64	CMV-LacZ	BAP fused to N-terminus and to site 283 of gp64	II

Table 10. Lentiviral vectors constructed in studies I-IV.

Lentiviral vectors (pseudotype)	Promoter-transgene	Surface Modification	Refe-rence
Gp64	hPGK-GFP	Baculovirus envelope glycoprotein, gp64	III
AVD/Gp64	hPGK-GFP	Avidin fused to VSV-GED, coexpressed with gp64	III
SA/Gp64	hPGK-GFP/ CAG-Ferritin/ CAG-LacZ	Streptavidin fused to VSV-GED, coexpressed with gp64	III
MONO/Gp64	hPGK-GFP	Monomeric avidin fused to VSV-GED, coexpressed with gp64	III

4.4 Antibodies and ligands

Table 11. Antibodies used in immunoblotting, immunofluorescence and targeting experiments. (* Conjugated to biotin)

Antibody	Source	Description	Study
Anti-gp64 mAb	Insight Biotechnology, Webley, UK	Gp64 detection	I, II
Anti-vp39 pAb	Loy Volkman, University of California, Berkeley, USA	Vp39 detection	I, IV
Anti-avidin pAb	(Laitinen et al., 2002)	Avidin detection	III
Anti-streptavidin pAb	(Bayer et al., 1986)	Streptavidin detection	III
Anti-VSVG pAb	Bethyl Laboratories Inc., Montgomery, TX, USA	VSV-GED detection	I- III
Streptavidin-HRP	Vector laboratories, Burlingame, CA, USA	Biotin detection	II
Anti-lamin	Novocastra Laboratories Ltd, Newcastle, UK	Nuclear membrane detection	IV
Anti-TfR mAb*	Ancell Corporation, Bayport, MN, USA	Targeting to transferrin receptor	II, III
Cetuximab mAb*	Merck, Darmstadt, Germany	Targeting to EGFR receptor	II, III
Anti-CD46 mAb*	Exbio, Prague, Czech Republic	Targeting to CD46 receptor	II, III
Transferrin*	Molecular Probes, Invitrogen, Carlsbad, CA, USA	Targeting to transferrin receptor	II, III
EGF*	Molecular Probes, Invitrogen, Carlsbad, CA, USA	Targeting to EGFR receptor	III
Anti-Ferritin rH02	Paolo Arosio, University of Brescia, Milan, Italy	Detection of human heavy chain ferritin	III
Anti-IE-2	Knebel-Mörsdorf,Max-Planck-Institute for Neurological Research, Germany	Baculovirus immediate early protein 2 detection	IV
Anti-GFP	Molecular Probes, Invitrogen, Carlsbad, CA, USA	Green fluorescent protein detection	IV
Anti-SC-35	Abcam, Cambridge, UK	Marker for nuclear speckles	IV
Anti-PML	Abcam, Cambridge, UK	Promyelotic leukaemia protein detection	IV
Anti-p80	A.Lamond, University of Dundee, Scotland, UK	Marker for Cajal bodies	IV
Anti-rabbit IgG AP	Bio-Rad, Richmond, CA, USA	Detection of rabbit antibodies	I-III
Anti-rabbit IgG-HRP	Sigma-Aldrich,St Louis,MO,USA	Detection of rabbit antibodies	IV
Anti-rabbit IgG-alexa 488	Molecular Probes, Invitrogen, Carlsbad, CA, USA	Detection of rabbit antibodies	IV
Anti-rabbit IgG-alexa 555	Molecular Probes, Invitrogen, Carlsbad, CA, USA	Detection of rabbit antibodies	IV
Anti-mouse IgG AP	Bio-Rad, Richmond, CA, USA	Detection of mouse Abs	I-III
Anti-mouse IgG-HRP	Silenus Laboratories, Hawthorn, Australia	Detection of mouse Abs	II, III
Anti-mouse IgG-alexa 555	Molecular Probes, Invitrogen, Carlsbad, CA, USA	Detection of mouse antibodies	IV

4.5 Cell lines

Cell lines used for *in vitro* studies are presented in Table 12. All the mammalian cell lines were grown in +37 ºC under 5% CO_2 in their recommended medium, whereas Sf9-insect cells were grown in +28 ºC. All the cell culture protocols are described with more detail in the cited articles.

Table 12. Cell lines used in studies I-IV.

Cell line	Source	Description	Study
293	ATCC: CRL-1573	Fetal human kidney cell line	IV
293T	ATCC: CRL-11268	Fetal human kidney cell line	I, III
A549	ATCC: CCL-185	Human lung adenocarcinoma	III
D54	(Bigner et al., 1981)	Human glioblastoma	III
BT4C	(Laerum et al., 1977; Sandmair et al., 1999)	Rat glioma cell line	I- III
EAHY-926	University of North-Carolina, Department of Pathology, NC, USA	Hybridoma of human airway epithelium and human umbilical vein endothelial cells	I
HeLa	ATCC: CCL-2	Human cervical carcinoma	I, III
HepG2	ATCC: HB-8065	Human hepatocarcinoma	I- IV
Sf9	Invitrogen, Carlsbad, CA, USA	*Spodoptera frugiperda* IPLB-Sf-21-AE cells	I, II, IV
SKOV-3	ATCC: HTB-77	Human ovarian carcinoma	I- III
U118MG	ATCC: HTB-15	Human glioblastoma	III

4.6 *In vivo* experiments

Table 13. Animals used in the studies and the treatments they received.

Animal	*n*	Virus	Dose	Admin. route	Sacri-fice	Study
BDIX female rat	10	VSV-GED/Ctrl baculovirus	20 µl ($2x10^8$ pfu/ml)	Intra-cranial	d4	I
New Zealand white female rabbit	3	VSV-GED/Ctrl baculovirus	10 x 50 µl ($1x10^9$ pfu/ml)	Intra-muscular	d6	I
Wistar male rat	22	SA/Gp64-lentivirus	15 µl ($2 x10^8$ pg/ml)	Intra-cranial	d5-d63	III

The rats were anestetized with fentanyl-fluanisone-midazolam (Study I) or ketamine-medetomine (Study III). For intracranial injections, the virus was injected with Hamilton syringe and a 27-gaude needle into the right lateral ventricle with the following coordinates: 1.0 mm caudal to bregma, 1.5 mm right to sutura sagittalis, and depth of 3-3.5 mm using stereotaxic apparatus (Figure 13).

The New Zealand white rabbits were anestetized with ketamine-metetomidine before the baculovirus vectors were injected in a volume of 50 µl into M. *semimembranosus* by 10 injections (Figure 14). All the performed studies were approved by the Animal Care and Use Committee of University of Kuopio.

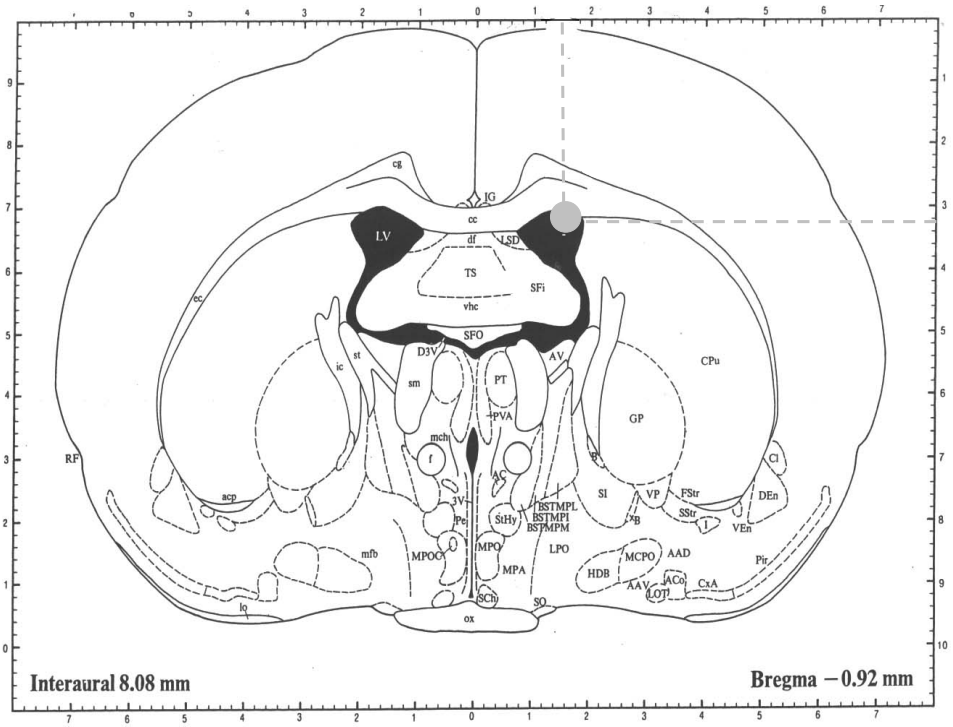

Figure 13. The rat brain showing the stereotaxic coordinates for virus injections in studies I and III (gray dot) [modified from (Paxinos and Watson, 1986)].

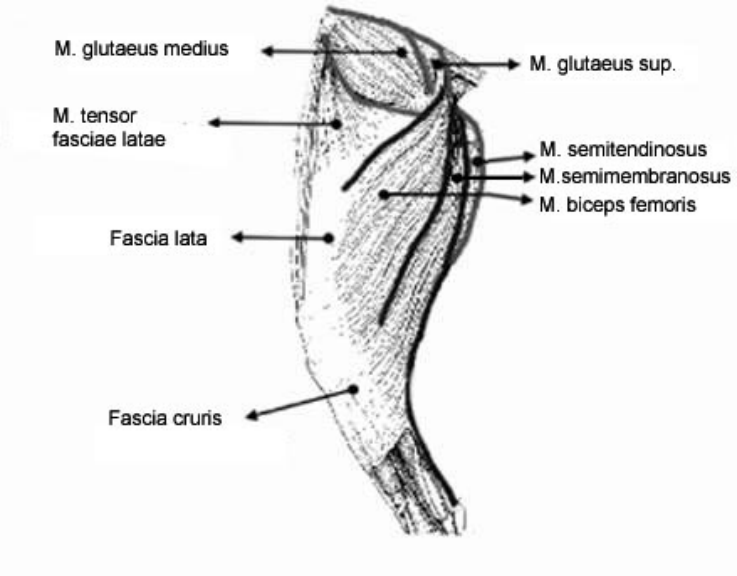

Figure 14. Lateral view of the muscles of a rabbit thigh. The virus injections were done to *musculus semimembranosus*.[(www.hundezeitung.de/Ana11-Dateien/image004.jpg) with modification]

5. RESULTS AND DISCUSSION

5.1 VSV-GED-displaying baculovirus for improved gene delivery (I)

Baculoviruses are gaining popularity as potential vectors for gene transfer technology. Despite many promising aspects, some limitations regarding the efficacy and specificity of these vectors have slowed their widespread use. Although *Ac*MNPV can enter to almost any cell from any origin, they are unable to reach the nucleus of many of these cells (Kukkonen et al., 2003; Volkman and Goldsmith, 1983). This might be due to the inability of the virus to escape from endosomes (Barsoum et al., 1997; Park et al., 2001) or blockage of the transport or entry into the nucleus (Kukkonen et al., 2003; van Loo et al., 2001). To enhance the escape of the baculovirus vectors from endosomes, a novel vector displaying a truncated form of VSV-G was designed.

5.1.1 Generation and characterization of VSV-GED pseudotyped virus

To improve the efficacy of baculovirus vectors as a gene delivery tool, we developed surface-modified baculovirus displaying a truncated form of *vesicular stomatitis* virus G protein, (VSV-G). The truncated VSV-G construct contained an *Ac*MNPV polyhedrin promoter, gp64 signal peptide, 21 amino acids from the C terminus of the VSV-G EctoDomain (positions 442 to 463), and the predicted TM domain and CTD of VSV-G (VSV-GED; I/Fig. 1).

The incorporation of VSV-GED into the virions was studied by immunoblotting using a VSV-G antibody that recognizes 15 carboxy-terminal amino acids of the VSV-G cytoplasmic tail. Bands positive for the VSV-G antibody, representing mainly monomeric (8.6 kDa) and trimeric forms (Robison and Whitt, 2000) of VSV-GED were identified (I/Fig.2). Results demonstrate that VSV-GED can be efficiently incorporated into the virions, in a manner similar to VSV-GED fusion proteins (Chapple and Jones, 2002; Makela et al., 2006; Ojala et al., 2004). A striking characteristic of VSV-G membrane anchor based fusions is their ability to incorporate also on the lateral virion surfaces (Chapple and Jones, 2002; Ojala et al., 2004), which is not possible with gp64-fusions (Boublik et al., 1995). This feature, together with the small size of VSV-GED, might contribute to the high-level of incorporation into the viral particles.

VSV-G is routinely used to pseudotype retroviruses, lentiviruses and baculoviruses (Cronin et al., 2005; Makela and Oker-Blom, 2006; Sandrin et al., 2003). Despite its wide application, there are also drawbacks limiting the production and use of VSV-G pseudotyped vectors. The most well-known problem is the cytotoxicity of VSV-G in packaging cell lines (Burns et al., 1993). In insect cell culture VSV-G causes large cell-cell fusions [I/Fig. 5b; (Park et al., 2001)], thus reducing the virus yields. In addition, incorporation of VSV-G into the viral envelope increases vector toxicity (Facciabene et al., 2004; Park et al., 2000; Watson et al., 2002). To study the effects of VSV-GED expression on the baculovirus production, we determined the ratio of the total particle number (TP) versus the number of infectious virus particles (IP) by immunoblotting. An equal amount of plaque

forming units (PFUs) was probed with gp64 and vp39 antibodies, revealing a similar TP/IP ratio between the control virus and the VSV-GED virus (I/Fig. 2). In line with this, the titers of VSV-GED virus stocks were repeatedly high (after 300 x concentration, a typical titer was 2.5 x 10^{10} PFU/ml), suggesting no adverse effects on the viral replication associated with the expression of VSV-GED. To evaluate the cytotoxicity of VSV-GED vectors, we performed an MTT assay to measure cellular proliferation after viral transduction. No cytotoxicity caused by the VSV-GED or the control virus was detected (I/Table 1). Altogether, these results suggest that VSV-GED-display circumvents the problems associated with the production and cytotoxicity of VSV-G pseudotyped vectors.

5.1.2 Improved transduction efficiency and serum stability *in vitro*

To study if VSV-GED had an effect on the gp64-mediated gene delivery, the transduction efficiency of HeLa, SKOV-3, BT4C, HepG2, 293T and EAHY cells was determined by using CMV promoter-driven *β-galactosidase* as the reporter gene. The gene delivery efficiency of VSV-GED virus was compared to the non-surface-modified control virus using multiplicity of infection (MOI) ranging from 10 to 5000. The transduction efficiencies were assessed by counting the percentage of β-galactosidase positive cells (I/Fig. 3a) and by measuring the β-galactosidase enzyme activity from the cell lysates (I/Fig. 3b) 48 hours post-transduction. VSV-GED resulted in significant increase in gene transfer efficiency in all the cell lines except EAHY, where only negligible gene expression was detected (data not shown). The most notable increase in the transduction efficiency of VSV-GED was observed in BT4C cells transduced at MOI 10, where a 13-fold increase in the percentage of transduced cells was achieved corresponding to an almost 40-fold increase in β-galactosidase enzyme activity (I/Fig. 3). In other cell lines, the β-galactosidase enzyme activity was enhanced by 2-15-fold. In most cells, the highest increase in transduction efficiency was seen at low MOIs, indicating saturation of the virus uptake pathway with increasing MOIs (I/Fig. 4). In HepG2 cells, however, no saturation was detected even at MOI 1000, a phenomenon possibly characteristic to cell lines highly susceptible to baculovirus-mediated gene delivery. Taken together, VSV-GED was able to enhance the transduction of numerous cell lines, except the human endothelial cell line EAHY. This could be explained by a theory that transduction of EAHY cells is blocked at a stage after ensodomal release, during cytoplasmic trafficking or nuclear import (Kukkonen et al., 2003), whereas in other cell lines the escape from the endosomes is the limiting step for transduction (Barsoum et al., 1997; Park et al., 2001).

VSV-G pseudotyping being the most widely adopted strategy to enhance baculovirus transduction; we next wanted to set the positive characteristics of VSV-G against those of VSV-GED. Since VSV-G baculovirus has also been reported to exhibit an enhanced ability to transduce mammalian cells, we first compared the transduction efficiency of these two vectors. The β-galactosidase enzyme activities measured from the transduced BT4C, 293T and HeLa cells (MOI 200) showed no significant differences between the viruses (Figure 15, unpublished results). This implies that VSV-GED functions in a manner similar to VSV-G in enhancing the gene delivery efficiency of baculovirus vectors.

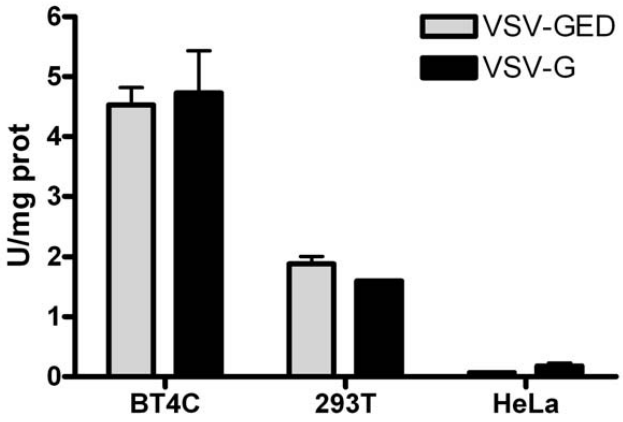

Figure 15. Comparison of the transduction efficiencies of VSV-GED and VSV-G displaying baculoviruses in BT4C, 293T and HeLa cells measured by the β-galactosidase enzyme activity 48 hours p.t. No significant differences were detected between the viruses (n=3; Means ± SD).

Another advantage of the VSVG-modified baculovirus is suggested to be its greater resistance to animal serum inactivation compared to the unmodified control baculovirus (Tani et al., 2003). Barsoum *et al.* hypothesized that VSV-G pseudotyped baculovirus conferred resistance to complement, imparting the ability to perform gene transduction into mouse hepatocytes following tail vein injection (Barsoum et al., 1997). Pieroni *et al.* demonstrated increased gene delivery into mouse quadriceps after direct intramuscular injection of VSVG-modified baculovirus, partially bypassing the complement system (Pieroni et al., 2001). To test if the VSV-GED pseudotyping could confer protection from the complement response, we incubated baculoviruses with either untreated or heat-inactivated serum from mouse and rabbit (Figure 16, unpublished results). Residual infectivity was determined following inoculation into HepG2 cells. Significant reduction in LacZ expression following the control virus transduction was observed after incubation with serum representing 4-8 % residual infectivity. More moderate reductions in infectivity were observed following the VSV-G and VSV-GED virus transductions and the residual infectivities were 4-5-fold higher compared to the control virus. These results suggest that, similarly to VSV-G, VSV-GED exhibits resistance to complement inactivation, thereby enabling more efficient gene delivery also *in vivo*. In conclusion, the display of VSV-GED seems to circumvent the problems associated with the use of VSV-G, while retaining high transduction capacity and resistance to serum inactivation.

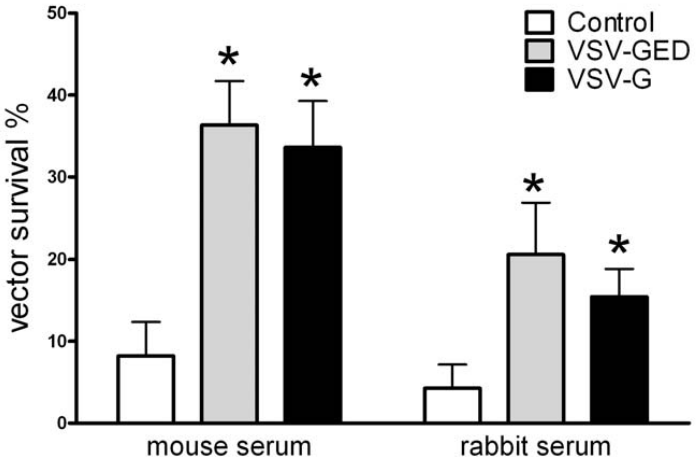

Figure 16. Survival of baculovirus variants determined as the percentage of β-galactosidase levels resulting from vector preincubation with untreated compared with heat-treated sera. The survival percentages were compared to the control virus using the unpaired *t* test with a two-tailed P value, *P<0.05 (n=3; Means ± SD).

5.1.3 Improved transduction efficiency *in vivo*

To examine the ability of the VSV-GED baculovirus to mediate more efficient gene transduction *in vivo*, we injected VSV-GED and the control virus directly into the right ventricle of the rat brain. β-galactosidase expression was examined 4 days after injection. In line with the previous results, the control virus injection led to β-galactosidase expression in cuboidal epithelial cells of the choroids plexus, in endothelial cells of the microvessels and in the subarachnoidal space [I/Fig. 7a and 7b; (Lehtolainen et al., 2002b)]. In addition to this expected transduction pattern the VSV-GED virus showed a strong marker gene expression also in the epithelial lining of the lateral ventricles, epithelial lining of the cerebral aqueduct and subarachnoidal membrane (I/Fig. 7c-7f). The observed change in the tropism of VSV-GED vector resembles that of VSV-G pseudotyped lentiviruses (Watson et al., 2005), confirming the *in vitro* results.

We also inoculated VSV-GED and control virus into the rabbit *semimembranosus* muscle (Figure 13). Interestingly, while the control virus expression was mainly observed in non-muscle cells, for example pericytes, the VSV-GED virus showed an enhanced transduction of muscle cells (Figure 17). However, only modest transduction efficiency was detected. This could be due to the exposure of the viruses to the complement system or rabbit skeletal muscle being a poor target for baculovirus-mediated gene delivery.

Altogether, it seems that VSV-GED possesses a similar transduction pattern as the full-length VSV-G *in vitro* and *in vivo* and VSV-GED pseudotyping provides thus a simple means to increase the baculovirus transduction efficiency.

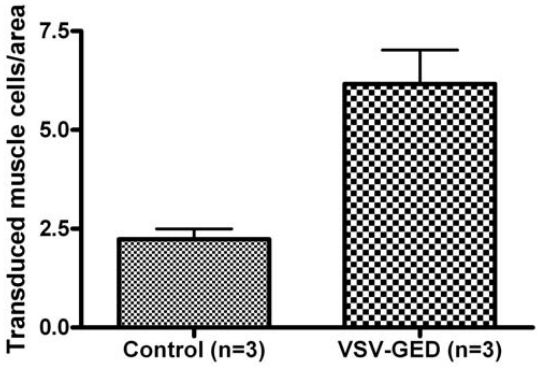

Figure 17. Gene delivery efficiency of VSV-GED virus or control baculovirus after direct injection to rabbit M. *semimembranosus.* The average amount of β-galactosidase expressing muscle cells was counted from 15-20 muscle sections from each animal (n=3; Means ± SD).

5.1.4 Mechanism of action of VSV-GED

Previous studies with truncated forms of VSV-G have shown the role of membrane proximal stem domain in mediating efficient VSV budding with only 12 or more stem region residues together with the TM and CTD domains being sufficient for the production of near wild type levels of virus particles (Robison and Whitt, 2000). A recent report by Zhou and Blissard, demonstrated that the expression of VSV G-stem construct was able to rescue the budding deficient phenotype of gp64-null baculovirus thus resulting in an efficient production of virions (Zhou and Blissard, 2008). Another study by Jeetendra *et al*, revealed that 14 or more membrane proximal residues (G-stem; I/Fig. 1) can potentiate the membrane fusion activities of several heterologous viral glycoproteins, such as simian virus F protein, HIV-1 envelope proteins, when coexpressed (Jeetendra et al., 2002). The mechanism for this action was explained by the ability of VSV-G ectodomain to induce the formation of a hemifusion diaphragm, thus to reduce the energy barrier for membrane fusion. These results suggest that VSV-GED might potentiate the fusion activity of gp64 on the baculoviral membrane. To further study this hypothesis, we performed a series of experiments to examine the viral fusion process.

To study if VSV-GED could relieve the normal low-pH activation step required for gp64-mediated membrane fusion as in the context of wild-type VSV-G, we determined the pH requirement for viral membrane fusion by a syncytium formation assay (I/Fig. 5). Wild-type baculovirus and VSV-G pseudotyped baculoviruses were used as controls. In line with the previous studies, a pH ≤ 5.5 was required to induce the gp64-mediated fusion, whereas the expression of VSV-G resulted in large syncytia formation in the pH 6.2 of the insect cell medium (I/Fig. 5b and 5c) (Blissard and Wenz, 1992; Carneiro et al., 2001; White et al., 1981). VSV-GED mediated infection resulted in

significant syncytia formation at pH under 5.5 (I/Fig 5f), indicating that VSV-GED does not relieve the requirement for gp64 to attain a low-pH trigger to obtain fusion-competent conformation.

To study the efficiency of endosomal release of VSV-GED pseudotyped baculovirus compared to the wild-type virus, monensin and increasing concentrations of ammonium chloride were used to prevent endosomal acidification. Treatment of BT4C and HepG2 cells with monensin prevented the transduction by the control virus whereas the gene delivery of VSV-GED was partly retained (Figure 18; unpublished results). A progressive decrease in transduction efficiency was observed with increasing concentrations of ammonium chloride for both viruses (I/Fig. 6). The transgene delivery by both viruses was completely abolished at 8 mM ammonium chloride, as expected for viruses sharing the same pH for fusion.

Altogether these results suggest that the VSV-GED augments baculovirus transduction by enhancing endosomal escape although the pH requirement for fusion remains unaltered. Proposed model for the fusion potentiation by VSV-GED is depicted in Figure 19 (Jeetendra et al., 2002). The pathway II of this model suggests that the VSV-GED –mediated membrane destabilization can cause outer leaflet mixing, which might result in the establishment of a hydrophobic environment leading to conformational changes in the fusion protein. In line with this hypothesis, synthetic peptides corresponding to the transmembrane domain of VSV-GED have been shown to promote PEG-mediated fusion of liposomes by enhancing the rate of formation of the initial lipid-mixed fusion intermediate and its subsequent conversion into a stable fusion pore (Dennison et al., 2002).

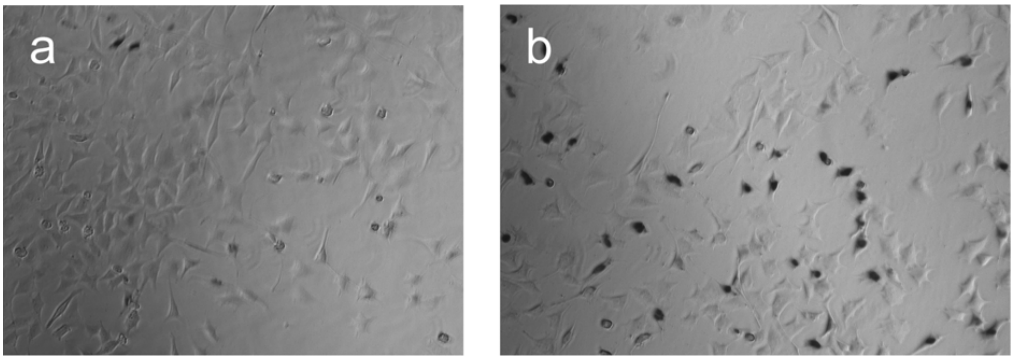

Figure 18. Effect of monensin treatment (0.5 μM) on baculovirus transduction efficiency (unpublished results) cells transduced with LacZ control virus (a) and VSV-GED virus (b) at MOI 200.

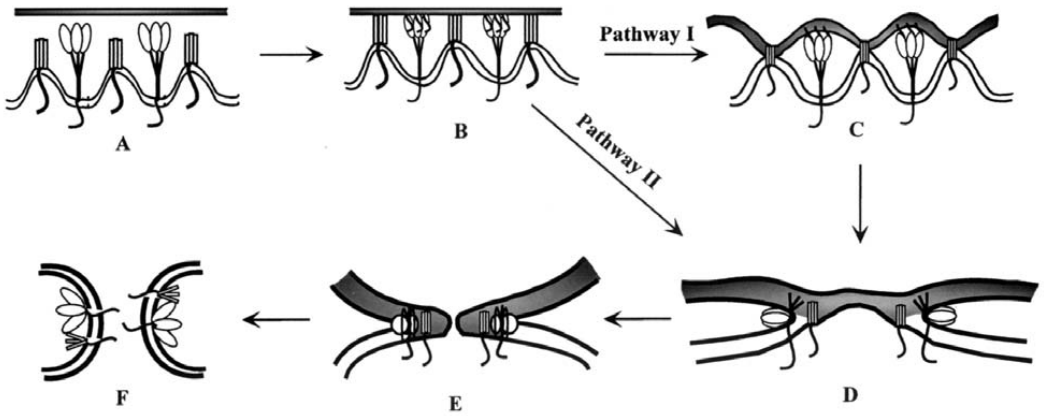

Figure 19. Model for fusion potentiation of heterologous viral glycoptoteins by VSV-GED (Jeetendra et al., 2002). (A) Two separate membranes before the initiation of fusion. (B) Fusion protein and VSV-GED or VSV-GED alone binds to the cell membrane. (C) In pathway I, VSV-GED establishes multiple sites of contact with the cell membrane leading to tighter binding which subsequently triggers the exposure of the fusion peptide in the fusion protein. (D) In pathway II, the VSV-GED mediated membrane destabilization causes lipid mixing which results in the establishment of a hydrophobic environment that drives conformational changes in the fusion protein and thus leads to the formation of a hemifusion diaphragm. (E-F) Formation of the fusion pore leading to the completion of the fusion reaction.

Alternatively, the transduction-enhancing activity of VSV-GED could be entirely or partly due its ability to bind membranes in a manner similar to wild-type VSV-G leading to tighter binding to the membranes (Jeetendra et al., 2002; Ojala et al., 2004). This may be sufficient to trigger conformational changes in the fusion protein resulting in the formation of hemifusion diaphragm (Figure 19, pathway I) (Jeetendra et al., 2002). According to the latest knowledge the binding of VSV-G to the cell membrane is mediated by electrostatic interactions with the negatively charged phospholipid phosphatidylserine through a positively charged p2-like peptide (Carneiro et al., 2003; Coll, 1997). However, p2-like peptide is located between residues 134 and 161 and is thus deleted from VSV-GED. Despite this, the *in vitro* and *in vivo* transduction experiments indicated that VSV-GED virus possesses a tropism similar to VSV-G virus, suggesting that additional interactions between VSV-G and the cell membrane may exist. In the aggregate, both theories support the idea that VSV-GED reduces the energy barrier required to trigger the membrane fusion. In order to elucidate the exact molecular mechanisms responsible for the enhanced transduction potential of VSV-GED, additional experiments are needed.

In conclusion, VSV-GED pseudotyping is an efficient means to enhance baculovirus-mediated gene transfer into mammalian cells *in vitro* and *in vivo* while offering several advantages over VSV-G pseudotyping. This strategy might also be applied to other vectors.

5.2 Targeting of metabolically biotinylated baculoviruses (II)

Limitations regarding the efficacy and specificity of baculovirus-mediated transduction have slowed their widespread application. In this study we tested the utility of metabolical biotinylation of baculovirus for vector targeting. Given the exceptional affinity of (strept)avidin-biotin interactions (Kd~10^{13}- 10^{15} M^{-1}), this system qualifies for *in vivo* application where the stability of the vector-targeting molecule is of utmost importance.

5.2.1 Biotin display on the surface of baculoviruses

To produce recombinant baculoviruses displaying biotin, the biotin acceptor peptide (BAP) was cloned either to different sites in the gp64 or to the N-terminus of VSV-GED (II/Fig. 1). In gp64-based constructs the BAP was either inserted after the signal sequence (BAP-N-gp64), at amino-acid position 283 (BAP-283-gp64) or both (BAP-283+N-gp64) (II/Fig. 1B). Given that BAP is not a substrate for enzymatic biotinylation in *Spodoptera frugiperda* (Sf9) cells (Duffy et al., 1998) and that the biotinylation of the secreted proteins does not naturally occur (Parrott and Barry, 2001), an *Escherichia coli* biotin holoenzyme synthetase, BirA, with gp64 signal sequence was coexpressed from baculovirus genome during the production of recombinant viruses to place its activity within the ER lumen where gp64 and VSV-GED are synthesized.

To verify the expression of the biotin on the surface of baculovirus particles, an equivalent number of PFUs of the gradient purified viruses were subjected to western blot analysis with anti-gp64, anti-VSV-G or streptavidin-HRP. Incorporation of gp64 protein was detected in all the virions, confirming efficient production of viruses (II/Fig. 2A). The amount of gp64 was similar to that of control virus, indicating a normal ratio of total particle number versus infectious virus particles (TP/IP). Expression of BAP-VSV-GED (10 kDa) was identified with VSV-G antibodies, confirming successful incorporation (II/Fig. 2B). The incorporation of biotin was detected in all BAP-displaying virus samples using streptavidin-HRP (II/Fig. 2C). Of BAP-gp64 viruses, BAP-283-gp64 showed the highest level of biotin incorporation, BAP-N-gp64 the lowest, whereas BAP-283+N-gp64 settled between the two. As expected, no biotin was detected in the control virus sample.

To assess the availability of biotin for streptavidin binding in solution and thus to assure of the accessibility of biotin for conjugation applications, a competition ELISA was performed. Binding of the biotinylated baculoviruses (1.5 x 10^9 PFUs) on streptavidin coated plates was inhibited by increasing concentrations of free streptavidin and the amount of virus bound was detected with gp64 antibody. BAP-283-gp64 showed the highest binding to streptavidin and in the absence of free streptavidin the difference was 10-, 4- and 1.5-fold compared to BAP-VSV-GED, BAP-N-gp64 and BAP-283+N-gp64 viruses, respectively (II/Fig. 3). Of note, however, the ELISA underestimates the binding of BAP-VSV-GED since the bound virus is probed with gp64 antibody thus leading to increase of signal for BAP-gp64 vectors expressing two copies of gp64. Complete inhibition by

streptavidin was observed at 1µg/ml concentration of streptavidin. No obvious binding of the control virus to ELISA plates was observed.

Together these experiments verify the incorporation of biotin on the surface of baculovirus vectors but also highlight the role of peptide insertion site in determining the efficiency of biotin display. These results are in line with a previous report where 283-site was described for the successful surface display of peptides ranging from 6 to 23 amino acids (Spenger et al., 2002). In the same study it was shown that the N-terminal insertions lead to weaker peptide display. It is hypothesized that hetero-oligomer formation is the basis of N-terminal fusion protein incorporation into virions, this being responsible for the low level of target protein display (Boublik et al., 1995). On this basis, the incorporation of peptides at site 283 is suggested to allow incorporation through homo-oligomerization of the fusion proteins thus allowing more efficient display (Spenger et al., 2002).

To investigate the effects of BAP and biotin incorporation on the efficiency of baculovirus-mediated transduction, the gene delivery efficiency to HepG2, BT4C and SKOV-3 cells was studied using MOI 200. The transduction efficiency was assessed by measuring the β-galactosidase enzyme activity from the cell lysates 48h post-transduction (II/Fig. 4). As shown in Fig. 4, the BAP-VSV-GED transduced HepG2 and SKOV-3 cells with a similar efficiency to control virus, whereas a 4-fold increase in gene delivery efficiency to BT4C cells was detected. Interestingly, gene delivery by BAP-N-gp64 led to levels of β-galactosidase that were 5-, 6- and 100-fold lower in SKOV3, BT4C, and HepG2 cells, respectively, than those of the unmodified control virus. The most interesting finding was that transduction by BAP-283-gp64 and BAP-283+N-gp64 resulted in enhanced transduction efficiency in all the cell lines tested. BAP-283-gp64 showed 6 - 13- fold enhancement in transduction whereas for BAP-283+N-gp64 an increase of 12 - 25-fold was observed. Correlating with our results, previous reports have shown that N-terminal insertions to gp64 can in some cases affect the functionality of the vector (Huser et al., 2001; Riikonen et al., 2005; Spenger et al., 2002). We hypothesize that hetero-oligomerization of BAP-N-gp64 protein with the native gp64 impairs the assembly of stable gp64 trimers into multiprotein aggregates required for membrane fusion (Markovic et al., 1998; Guibinga et al., 2008) thus leading to poor transduction. On the other hand, the enhanced transduction efficiency by BAP-283-gp64 and BAP-283+N-gp64 viruses implies that the fusion proteins on these vectors could act as functional second copies of the gp64 thus eliciting a positive effect on the transduction efficiency as has been proposed for baculoviruses displaying an extra copy of wild-type gp64 (Tani et al., 2001). However, without further studies we cannot rule out the role of biotin or conformational changes caused by BAP insertion in increasing viral transduction.

BAP-VSV-GED should not affect the functionality of the gp64 since no hetero-oligomerization with gp64 occurs and this was confirmed by the transduction experiments. In the study I we showed that VSV-GED alone is able to augment the transduction efficiency of baculovirus vector probably by potentiating the membrane fusion activity of baculovirus envelope protein gp64. However, VSV-GED fusions do not necessarily share this feature (Chapple and Jones, 2002; Ojala et al., 2004). Indeed, no significant differences were seen in the transduction efficiencies in HepG2 and SKOV3 cells although some increase was seen in BT4C cells.

5.2.2 Vector retargeting by biotinylated ligands and antibodies *in vitro*

To determine whether we could redirect the baculoviral tropism, we tested biotinylated ligands and antibodies selectively binding to transferrin-, epidermal growth factor- and CD46- receptors expressed at high levels on tumor cells (BT4C, SKOV3). No increase of β-galactosidase expression was observed as a result of BAP-VSV-GED and BAP-N-gp64 retargeting to the receptors (II/Fig. 5). This may be due to less efficient biotin display subsequently reducing the extent of ligand binding. The tropism mediated by the wild-type gp64 can thus be predominant in these constructs.

The targeted transduction of BAP-283-gp64 by conjugation of targeting molecules through streptavidin-biotin linker resulted in 15 - 40 % enhancement of transduction efficiency whereas for BAP-283+N-gp64 an increase of 30 - 60 % was attained (II/Fig. 5). This most probably resulted from the augmentation of virus binding to the target cell membrane since it was achieved after very short (15 min) exposure of the cells to the virus. In line with this, specific receptor-mediated binding (Kd ~ 10^{-7}-10^{-11}) and especially streptavidin-biotin binding (Kd = 2.5 x 10^{-13}) have been shown to be faster than the attachment via multiple, low affinity bonds (Kd ~ 10^{-4}-10^{-6}) responsible for the binding of gp64 on the mammalian cell surface (Chilkoti and Stayton, 1995; Duisit et al., 1999; Tani et al., 2001). At present, however, the domains of gp64 involved in cell binding have not been elucidated (Duisit et al., 1999; Tani et al., 2001). The successful targeting of BAP-283-gp64 and BAP-283+N-gp64 led us to speculate that site 283 might be at the proximity of one such determinant, allowing redirection of the viral tropism when conjugated to targeting moieties. This is supported by recent reports suggesting that insertion of a hepatitis B peptide and RGD-motif at amino acid positions 278 and 283, respectively, of the single copy of gp64 alters the domains involved in cell recognition and uptake (Markusic et al., 2007; Riikonen et al., 2005). To further enhance the specificity of viral binding, it might be beneficial to generate baculoviruses lacking the wild-type gp64.

Interestingly, the retargeting capacity of BAP-283-gp64 and BAP-283+N-gp64 was significantly lower compared to retargeted biotinylated adenovirus that showed 80-300-fold increase in reporter gene expression (Parrott et al., 2003). This might be due to the background binding mediated by the wild-type gp64 or other viral membrane proteins, whereas the tropism of adenovirus vectors is more strictly dependent on the initial recognition of coxsackie- and adenovirus receptor (CAR) on cells (Bergelson et al., 1997). However, differences in the used targeting protocols and in the sensitivity of the reporter gene assays, prevents further comparison. Nevertheless, targeting of this vector together with its overall increase in transduction efficiency has several advantages over non-targetable unmodified vectors.

5.2.3 Magnetic targeting

In addition to conjugate-based targeting, the gene delivery location can be controlled spatially by the use of magnetic force. Magnetic targeting has already been used to target biotinylated retro- and adenoviruses (Campos et al., 2004; Hughes et al., 2001; Pandori et al., 2002). In order to examine the magnetic targeting of biotinylated baculoviruses, we conjugated the viruses to streptavidin conjugated

paramagnetic particles (SA-PMP) and transduced monolayer BT4C cells (II/Fig. 7). The results demonstrated successful guidance of PMP-conjugated metabolically biotinylated baculoviruses with local magnetic field indicating that magnetofection could become a choice for local gene delivery *in vivo* (Scherer et al., 2002). In addition, the PMP-conjugated vector was able to potentiate the gene delivery efficiency of metabolically biotinylated baculovirus vectors compared to sucrose gradient purified virus (II/Fig. 6C). This could be explained by the rapid settling vector-PMP complexes onto target cells, promotion of additional virus-cell interactions or enhanced endocytosis, as has been proposed for other vectors (Chan et al., 2005; Hughes et al., 2001; Pandori et al., 2002; Scherer et al., 2002). Indeed, due to their higher density, vector-particle complexes may allow safer local gene delivery by resisting the forces of diffusion in solutions (Pandori et al., 2002). In addition to physical targeting, these virus-PMP complexes can also be, by virtue of free binding sites on streptavidin, conjugated to targeting molecules and this way be directed to any cell, tissue or tumor in the body providing that the tissue specific markers have been identified (Gupta et al., 2007). Finally, PMPs or PMP-conjugated radionuclides can also be traced *in vivo* to allow imaging of vector biodistribution (Raty et al., 2006; Raty et al., 2007).

5.3 Purification of metabolically biotinylated baculoviruses (II)

For gene therapy studies and future clinical need, a simple and efficient purification method for baculovirus is also necessary. Conventional purification techniques for baculovirus involves a series of sucrose gradient ultracentrifugation steps, which is tedious, time-consuming and limited by the small volume capacity and loss of viral activity due to hydrodynamic stress and aggregation of viral particles (Barsoum, 1999; O'Reilly et al., 1994). More simple, effective and scalable methods based on chromatography have been developed for baculovirus purification and are represented in Table 14. One of the objectives of this work was to develop a novel method for baculovirus purification based on magnetic particle-dependent capture of biotinylated baculoviruses.

Since biotin was incorporated and displayed on the viral surface, we used streptavidin conjugated paramagnetic particles for baculovirus purification. The efficiency of the method for 200-fold concentration of baculoviral stocks was evaluated by processing 10 ml of secondary viral preparations (Table 14). The collected PMP-virus pellet was washed and resuspended in 50µl of PBS. The total virus recoveries after end-point dilution titering were 30-100- fold higher compared to the starting preparation, demonstrating 15-50 % virus recovery, respectively (II/Fig. 6A).

To test the functionality of the vector after PMP-purification we transduced HepG2 cells with equal volumes of the starting preparation and the concentrated virus (II/Fig. 6B). PMP-conjugated BAP-VSV-GED showed 200-fold increase in β-galactosidase expression upon transduction corresponding to the concentration factor. For BAP-N-gp64 the resultant gene delivery efficiency was only 20-fold, suggesting some loss of the transduction efficiency. This might be due to the overall decrease in transduction efficiency by BAP-N-gp64 virus (II/Fig. 4). The most surprising finding was that transduction by BAP-283-gp64 and BAP-283+N-gp64 resulted in 400-900- fold increase in β-

galactosidase expression, respectively, as compared to the crude supernatant. As these vectors had showed the lowest recovery in infectious virus amounts (15-20%) after PMP-concentration, we hypothesized that the end-point dilution underestimates the actual titer by being unable to separate between one virus particle and one PMP-vector complex which could bind several virions. To put our theory to the test, we determined the residual titers from the viral supernatant after PMP-based capture and indeed, the results showed that 50-80% of the viruses were recovered by concentration.

To examine the purity of the viral preparations, the SDS-PAGE profile of the concentrated virus-PMP was compared to the original crude supernatant and to the sucrose gradient purified viruses using silver staining (II/Fig. 6D). On contrary to the crude supernatant, only the major bands of budded *Ac*MNPV were present in the PMP- and gradient ultracentrigugation- purified virus samples, indicating highly purified virus (Volkman, 1983). The two major bands corresponded to the baculovirus major envelope protein gp64 and the major capsid protein vp39 as confirmed by immunoblotting (data not shown). The identities of the other minor proteins of 45, 54 and 89 kDa are not clear but all these proteins have been observed in previous studies from the purified *Ac*MNPV (Braunagel and Summers, 1994; O'Reilly and Miller, 1988; Tani et al., 2001).

To summarize, biotin display was demonstrated to allow ready one-step purification and concentration of baculovirus. This method could become a powerful alternative for chromatography-based methods as the virus enrichment and recovery was altogether more effective (Table 14). The suitability of this approach for large-scale manufacturing of viral stocks is currently under investigation. Also, alternative magnetic particle-based concentration methods are being explored for applications where the irreversible attachment of baculoviruses to PMPs is not feasible.

Table 14. Comparison of the virus enrichment and final recoveries obtained in our study to baculovirus chromatography-based purification methods.

Purification method	Start. volume	Start. titer	Conc. volume	Conc. titer	Vol. Conc	Recovery (% IP*)	Reference
PMP- based purification	10 ml	2-5x10^8 PFU/ml	50 µl	1-3x10^{10} PFU/ml	200 x	50-80 %	Article II
Cation exchange chromatography	40 ml	1.4x10^8 PFU/ml	0,6 ml	6 x10^9 PFU/ml	66 x	65 %	(Barsoum, 1999)
Cation exchange chromatography	40 ml	2 x 10^8 PFU/ml	2 x 0.5 ml	6 x 10^9 PFU/ml	40 x	78 %	(Wu et al., 2007)
Size exclusion chromatography	1350 ml	3 x 10^7 PFU/ml	9 ml	1 x 10^9 PFU/ml	150 x	24 %	(Transfiguracion et al., 2007)
Metal affinity chromatography	100 ml		3 ml		33 x	2-3 %	(Hu et al., 2003)

*Percent infectious particles

5.4 (Strept)avidin-displaying lentiviruses for vector targeting (III)

One of the problems limiting the efficiency of lentiviral gene therapy is the lack of specificity in viral particle binding. More selective gene delivery would reduce the viral dose and consequently decrease the vector related side effects and ease clinical applications. In this study we describe the development of a flexible lentiviral vector platform for virus targeting based on (strept)avidin-display.

5.4.1 (Strept)avidin-VSV-GED incorporation on lentivirus surface

To develop lentivirus vectors with targetable gene delivery, we designed novel gp64- pseudotyped vectors coexpressing avidin or streptavidin fused to the transmembrane anchor of VSV-G on the virus envelope. Separation of the targeting moiety and the envelope protein was hoped to leave the fusion protein intact for endosomal escape thus avoiding the common problem of decrease in virus infectivity by the modified envelope proteins (Martin et al., 1999; Zhao et al., 1999b). By transient transfection of five plasmids, we produced lentiviral vectors that efficiently incorporated gp64 and (strept)avidin fusion proteins into virus particles (III/Fig. 1). As the viral amounts were normalized by p24 antigen levels the SA/GP64 virions showed a higher level of fusion protein incorporation. Results of the titering and quantification of the p24 gag protein demonstrated no major differences in the infectivities of GP64 and AVD/GP64 lentiviruses, indicating a normal ratio of total particle number versus infectious virus particles (TP/IP) (III/Fig. 2a and b). Little decrease in the infectivity of SA/GP64 was detected which might be due to high level of streptavidin-VSV-GED incorporation. Optimization of the transfection conditions could help to avoid this. Nevertheless, we conclude that no major adverse effects were associated on (strept)avidin display on lentivirus surface.

In line with the previous studies, SA/GP64 and AVD/GP64 were able to transduce a variety of cell types (BT4C, HeLa, HepG2, SKOV-3, D54, A549, U118MG; III/Fig. 2b and 4), indicating that these viruses share the wide cell tropism of gp64-pseudotyped viruses (Kumar et al., 2003; Schauber et al., 2004). However, the transduction of HeLa cells was several-fold lower compared to the results by Kumar *et al* (Kumar et al., 2003). This discrepancy is most likely due to differences in the old HeLa cell lines between the labs and to variation in the method of titering (Hughes et al., 2007).

Next we wanted to assess if the (strept)avidin displaying lentiviruses were able to bind biotin and thereby assure of the ability of the virions to bind molecular conjugates. Increasing amount of viruses were allowed to attach to the biotin coated ELISA plates and the binding of the virus was detected by gp64 antibody. Results showed that AVD/GP64 and SA/GP64 viruses bound to ELISA plates in a dose-dependent manner but the extent of binding was markedly 3-times higher for SA/GP64 as measured from the slopes (III/ Fig. 3). This is in line with the western blot results demonstrating more efficient display of streptavidin-VSV-GED compared to avidin-VSV-GED.

GP64 control virus showed some unspecific binding to ELISA plates which was, however, significantly weaker compared to AVD/GP64 and SA/GP64.

During this study, alternative vectors displaying streptavidin/avidin-VSV-GED together with VSV-G or avidin fused to the N-terminus of gp64 were also created (data not shown). In both cases, however, efficient (stept)avidin display was not achieved without compromising virus infectivity. In the former case, this might be due to heteromerization of streptavidin/avidin-VSV-GED fusion with the wild type VSV-G molecules leading to display of nonfunctional VSV-G molecules. The low infectivity of N-terminal gp64-fusions is likely explained by similar mechanisms as discussed for baculoviruses in study II.

5.4.2 Vector retargeting to tumor cells *in vitro*

To redirect the specificity of infection of SA/GP64 and AVD/GP64, we used biotinylated ligands and antibodies selectively targeting receptors expressed at high levels on tumor cells. The efficiency of cell targeting was analyzed with BT4C, D54, SKOV-3, HepG2, A549 and U118MG cells by EGFP expression (III/Fig. 4). Biotinylated targeting molecules were incubated with the cells followed by washes and addition of pseudotyped viral particles for 15 minutes. The short virus incubation time was used to favor ligand specific binding events mediated by the high affinity streptavidin-biotin interaction. Targeting to transferrin receptor overexpressed on BT4C and D54 glioma cells led to 20-30% enhancement in transduction efficiency of AVD/GP64 whereas an increase of 50-60% was achieved with SA/GP64 (III/Fig. 4a). Redirection to EGFR led at best to 40% and 100% increase in percentage of EGFP-positive cells by AVD/GP64 and SA/GP64, respectively (III/Fig. 4b). The most successful retargeting was achieved to CD46 receptor-overexpressing U118MG cells as 2-3-fold increase in transduction efficiency was attained with (strept)avidin displaying viruses (III/Fig. 4c). The more efficient targeting of SA/GP64 was probably due to its more efficient biotin binding capacity but it has also been shown that biotin conjugates bind more stably to streptavidin than to avidin (Pazy et al., 2002). It remains to be studied if an optimization of the transfection conditions could further increase the level of avidin-VSV-GED display thus enhancing the retargeting capacity of AVD/GP64. No increase in EGFP expression was observed when the non-biotinylated targeting molecules were used. Targeting of GP64 control virus did not confer enhanced transduction of the cell lines.

Altogether, the results demonstrate that target cell-specific transduction of SA/GP64 and AVD/GP64 can be increased by the use of biotinylated ligands and antibodies *in vitro*. It remains to be studied if precomplexed virus-ligand/Ab particles could favor faster and more efficient binding and internalization of the retargeted vector translating to more prominent transduction efficiencies. In addition, we need to consider the fact that SA/GP64 and AVD/GP64 still exhibit a similar host range to GP64 which limits the targeting efficiency. For applications aiming at transduction of liver, lung, skin or endothelium the presence of wild-type gp64 poses no problem but to achieve more stringent targeting this matter should be addressed (Schauber et al., 2004). In order to overcome this problem,

we could attempt to remove or greatly reduce the natural binding activity of gp64, without disturbing the fusion, by mutagenesis. Alternatively, pseudotyping with other binding-defective mutants, such as hemagglutinin (HA) of influenza A (Lin et al., 2001) and Sindbis virus (Morizono and Chen, 2005; Morizono et al., 2001; Yang et al., 2006) envelope proteins, could be considered.

The approach described here for specific targeting of cells by lentiviral vector transduction overcomes many of the limitations of previous targeting strategies. The preparation of targeting vector is not limited by the introduction of modifications into the envelope proteins (VSV-G, gp64) that might result in substantial decreases in infectivity. This strategy could be applicable to other viruses e.g. to murine retroviral vectors. Finally, the approach described here should in theory be generally applicable to any cell surface molecule for which there are specific reagents that bind.

5.5 Imaging of streptavidin-displaying lentivirus (III)

One of the current limitations of preclinical trials in gene therapy is the difficulty in detecting virus location and gene expression upon administration of the delivery vector. Ideally, this detection should be noninvasive and repeatable over time to provide information about the location and magnitude of gene expression. In this study, we took advantage of (strept)avidin-display and MRI reporter gene, ferritin, to achieve non-invasive multi-modality imaging of virus biodistribution and transduction *in vivo*.

5.5.1 SPECT/CT imaging of virus biodistribution

In vivo imaging techniques have the potential to provide critical information about the safety and kinetics of viral administration. Compared to the traditional immunological and histological methods, non-invasive imaging methods can follow virus localization with anatomical accuracy in real-time. Consequently, these techniques are crucial for elucidation of the specificity of new targeted vectors. In this regard, SPECT imaging has been previously utilized to measure changes in the liver tropism of adenovirus knob domain (Awasthi et al., 2004; Zinn et al., 1998), to image the biodistribution of herpes simplex viruses (Schellingerhout et al., 1998; Schellingerhout et al., 2000) and baculoviruses (Raty et al., 2007).

In this study, we examined the biodistribution of SA/GP64 lentivirus in rats following injection into rat corpus callosum. This was achieved by radiolabeling SA/GP64 lentivirus with ^{111}In labeled biotin-poly-Lys-DTPA and imaging the biodistribution with a dual modality SPECT/CT during three days after injection. Results from the day two when planar and 3D images were performed are shown in III/Fig. 6. In most of the virus injected rats the concentration of ^{111}Indium was observed solely in the brain, indicating no viral leakage (III/Fig. 6a and 6d). This was further confirmed by RT-PCR, showing no transgene expression in the tissues which show lentivirus accumulation after systemic administration i.e. liver and spleen (data not shown) (Pan et al., 2002).

However, a signal from one rat, showing leakage to the circulation during the injection, was also seen in the abdominal area (III/Fig. 6b and 6e). Interestingly, no transgene expression was detected from the liver or the spleen of this rat either, suggesting a specific tropism of the labeled virus to the peritoneum. As expected, the small molecular weight ^{111}In labeled biotin-poly-Lys-DTPA alone showed an accumulation to the brain followed by elimination through the kidneys and bladder (III/Fig. 6c and 6f).

In conclusion, the SPECT/CT imaging provides an efficient means to measure specific targeting of streptavidin-displaying lentivirus vectors for gene therapy applications. Furthermore, the translation of this methodology into clinical applications should be feasible.

5.5.2 MRI imaging of viral gene delivery

In addition to the knowledge about virus biodistribution, the information of the transgene expression is vital for the success of gene therapy. To achieve this, we generated SA/GP64 virus encoding the human heavy chain ferritin (hHF) cDNA. The underlying principle of visualizing ferritin expression on MRI is that this iron storage protein has a superparamagnetic core which disrupts the magnetic field and produces a lower (darker) signal on T2 weighted images (Drayer et al., 1986). As the transduced cells attain iron from the blood transferrin and subsequently transfer it to ferritin, no external contrast agents are needed. To date, ferritin has been utilized in gene therapy to image adenovirus-mediated gene delivery to rat brain (Genove et al., 2005) and to detect transcriptional regulation of gene expression in glioma tumors (Cohen et al., 2005) and in transgenic animals (Cohen et al., 2007).

To investigate the efficiency of iron accumulation after SA/GP64-hHF virus transduction, we conducted *in vitro* studies using a HepG2 cell line. At 48 h post-transduction, the pelleted cells were analyzed by MRI (III/ Fig. 5). Transduced cells grown in their normal growth medium showed little increase in the relaxation rate R2 (=1/T2) compared to the control cells. Incubation of the cells in the iron supplemented medium (ferric ammonium citrate) resulted in 2-fold increase in the R2 of the virus transduced cells compared to the control, indicating that ferritin-transduced cells have an enhanced iron loading capacity (III/Fig. 5). These results are in line with a previous study, showing that the increased iron storage capacity can only be filled under iron supplemented conditions *in vitro* (Genove et al., 2005).

To evaluate the potential of ferritin expression for the imaging of lentivirus-mediated gene delivery, we injected SA/GP64-hHF to the corpus callosum of the rat brain. To monitor the transgene expression, the animals underwent MRI scans at 4.7 T at days 3, 14, 28, 52 and 63 after SA/GP64-ferritin injection. Using gradient echo contrast, T2*-shortening was noted at the inoculation site, demonstrating a stable transduction of the target area by SA/GP64 lentivirus (III/Fig. 7a). As expected, the signal loss became more notable at later time points indicative of the increase in the cell's ability to store iron. Some negative contrast was also detected in the needle track (data not shown). Control animals receiving ^{111}In labeled biotin-poly-Lys-DTPA alone or SA/GP64-LacZ showed minor signal loss at the injection site at day 3 (III/Fig. 7b and c). This, however, resolved

before later measurements and was attributed to small hemorrhage caused by injection (Bradley, 1994). Histology and immunohistochemistry validated the MRI results showing transduction along the needle track, in the corpus callosum and in choroid plexus (III/Fig. 8). In concordance to the previous studies performed with baculovirus the transgene expression was also detected the cuboidal epithelial cells of the choroid plexus cells suggests that GP64 pseudotyped retain the same tropism (Lehtolainen et al., 2002b). However, the transduction of corpus callosum has not been seen with baculovirus. This may be explained by the differences in the membrane composition of these vectors. Nevertheless, these results provide evidence of the potential applicability of Gp64-pseudotyped lentivirus vectors as gene delivery vehicles for the treatment of CNS disorders.

The molecular imaging technology presented in this study can provide unique information about the success of virus administration and the resulting transduction efficiency. Further development of bicistronic vectors leading to coexpression of the therapeutic gene and ferritin may enable long-term monitoring of the status of therapeutic gene expression. Moreover, several reports have indicated that H subunit of ferritin may protect cells from the oxidative effects of iron (Cozzi et al., 2000; Epsztejn et al., 1999; Orino et al., 2001). In humans, oxidative cell and tissue damage has been linked to carcinogenesis, neurodegenerative disorders, autoimmune diseases, and atherosclerosis, among others (Berg and Youdim, 2006; Chau, 2000; Ong and Halliwell, 2004; Valko et al., 2006). Thus, the ferritin gene itself may have some therapeutic potential which should be taken into account when developing new therapies.

5.6 Characterization of baculovirus transduction in mammalian cells (IV)

The fact that the baculovirus *Ac*MNPV is used as a vector for many gene therapy studies makes research of the effects of viral transduction on the cellular machinery a high priority. In this study we investigated the expression profile of baculoviral genes in mammalian cells and the virus-induced alterations in the nuclear organization.

5.6.1 Baculovirus-mediated immediate early gene expression

In general, it appears that baculoviruses are able to enter a phylogenetically broad range of insect cells but the expression of baculovirus genes appears to be blocked at an early gene expression stage during or after viral DNA replication (Morris and Miller, 1993). Similarly, *Ac*MNPV can enter a myriad of vertebrate cells but appear to be unable to reach the nucleus of these cells, a notable exception being mammalian hepatocytes and osteosarcoma cells (Kukkonen et al., 2003; Song et al., 2003; Volkman and Goldsmith, 1983). In this work, we show that in HepG2 and 293 cells the virions accumulate in the nucleus as early as 4 hours p.t reaching the maximum at 8 hours p.t (IV/Fig. 1A-1C). In line with the previous reports, we also demonstrate that the nuclear entry of baculovirus is not dependent upon the disintegration of the nuclear membrane i.e. upon cell division (IV/Fig. 1D and 1E) (Lee et al., 2007; van Loo et al., 2001).

Recently, it was demonstrated that *Ac*MNPV is able to transcribe at least few viral early genes in mammalian cells which are implicated in viral replication, namely *ie-0*, *ie-1*, *pe-38*, *gp64*, *p35 and p6.9* (Fujita et al., 2006; Kitajima et al., 2006). Of these the *ie-1* is the only gene essential for viral replication encoding the principal early transregulator protein IE-1 (Kool et al., 1994; Lu and Miller, 1995). DNA microarray analysis has also suggested the transcription of another essential gene, *lef-3*, and a stimulatory *ie-2* gene but this has not been confirmed by RT-PCR studies (Fujita et al., 2006). IE-2 protein stimulates the expression of *ie-1* (Yoo and Guarino, 1994) and *pe-38* (Lu and Carstens, 1993) whereas LEF-3 is a single-strand binding protein which improves the strand displacement ability of viral DNA polymerase (Hang et al., 1995; McDougal and Guarino, 1999).

In this study, we investigated the transcription of *ie-1* and *ie-2* in human HepG2 and 293 cells. Quantitative RT-PCR showed that both genes were expressed in a time-dependent manner, transcription starting at 4 h p.t and increasing at the last time point studied i.e. 48 h p.t. (IV/Fig 2A and 2B, data not shown at 48h). Both genes were expressed at higher levels in HepG2 cells than in 293 cells (IV/Fig. 2C), probably due to the differences in the transduction efficiency (data not shown). Furthermore, we demonstrated the expression of IE-2 protein first appearing at 4 h p.t. and continuing up to 48 h p.t. (IV/Fig. 3). In additional experiments, the transcription of *lef-3* was also confirmed (Figure 20; unpublished results). Since IE-1, IE-2 and LEF-3 are found colocalized at viral replication sites in the nucleus of infected insect cells (Mainz et al., 2002; Okano et al., 1999), further studies regarding the localization of these proteins in baculovirus-transduced mammalian cells would be of great interest.

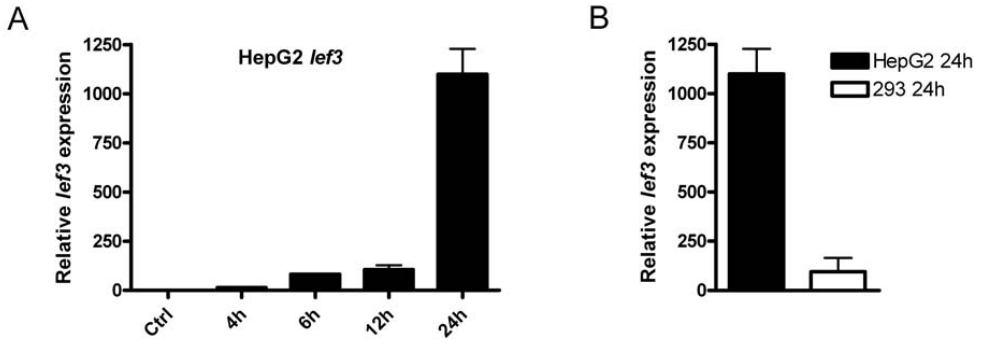

Figure 20. Transcription of baculoviral *lef-3* gene measured by quantitative RT-PCR. (A) Relative gene expression values of control and transduced HepG2 cells 4-24 h p.t. (B) Comparison of the relative *lef-3* expression in transduced HepG2 and 293 cells at 24h p.t.

Taken together, these results confirm that AcMNPV is capable of expressing some viral genes in mammalian cells at the transcriptional and translational level. This is somewhat not surprising since the immediate early and delayed-early genes are transcribed by host RNA polymerase II, transcription mechanism of which is highly conserved among eukaryotes (Kornberg, 1999). The transcription initiation site in mammalian cells, however, may differ from the early viral transcription

site as demonstrated for *pe-38* and *p6.9*, even though the transcription of *ie-0*, *ie-1* and *gp64* is shown to be initiated at the same site as in Sf9 cells (Fujita et al., 2006). Thus, the host RNA polymerase II and associated transcription factors dictate the outcome of early viral transcription in different species. On the contrary, late and very late genes are transcribed by viral RNA polymerase and thus it is unlikely that a late viral promoter would be activated in human cells.

5.6.2 Baculovirus induced nuclear reorganization

All viruses have to interact with the cell nucleus consisting of different nuclear bodies (NBs) including cajal bodies, the nucleolus, perinucleolar and perichromatin regions, nuclear speckles and promyelotic nuclear bodies (PML NBs) (Zimber et al., 2004). In this work we investigated the interaction of baculovirus with PML NBs, Cajal bodies, nuclear speckles and chromatin after transduction of mammalian HepG2 and 293 cells.

PML NBs are distinct subnuclear structures which appear as dense spherical particles, 0.3 to 0.5 μm in diameter, that are tightly associated with the nuclear matrix (Hodges et al., 1998). Although a number of proteins seem to transiently localize to PML NBs, two nuclear body antigens, PML and Sp100, are considered to build the framework of these structures (Sternsdorf et al., 1997). PML-NBs have been suggested to participate in transcriptional regulation, DNA damage response, regulation of apoptosis, senescence and neoangiogenesis (Bernardi and Pandolfi, 2007). The integrity of PML NBs is compromised in certain human diseases, including leukemia and neurodegenerative disorders but also during infection by a number of DNA viruses such as adenovirus (Carvalho et al., 1995; Doucas et al., 1996), herpes simplex virus (Everett and Maul, 1994; Everett et al., 1995), and cytomegalovirus (Ahn and Hayward, 1997; Kelly et al., 1995). Indeed, it appears to be a general tendency for DNA viruses to establish replication centers on the periphery of the PML NBs and first evidence of *Ac*MNPV replication center association at close proximity of human PMLs has been provided by transient transfection experiments (Mainz et al., 2002). To address this issue in baculovirus transduced mammalian cells, we measured the colocalization of baculovirus with PML proteins and sp100 at 6h p.t. (IV/Fig. 4A and 5A). Consistent with the previous results, the virus foci was situated at the close proximity of PML NBs but no significant colocalization was detected at 6-24 h p.t. (data not shown) (Mainz et al., 2002). However, following viral transduction the size of PML NBs was increased by almost 2-fold together with an overall decrease in the number of PML NBs per cell (IV/ Fig. 4B and 4C). This may be a result of virus-induced cellular response or rearrangement of these structures into virus transcription or disassembly sites.

An increased size of PML NBs has previously been shown to be involved in cell cycle, cellular stress and virus induced interferon response (IFN) (Buonamici et al., 2005; Djavani et al., 2001). PML NBs together with nucleophosmin are likely to play an important role, perhaps as sensors for cellular stress, during the DNA damage response (Dellaire and Bazett-Jones, 2004; Gjerset, 2006a; Wu and Yung, 2002). Several studies suggest that they function by regulating p53 stability (Coutts and La Thangue, 2005). Interestingly, the translocation of NPM from the nucleolus to nucleoplasm is indicative of cellular stress (Gjerset, 2006b). To determine the effects of baculovirus

transduction on cellular stress response, we monitored the localization of NPM-EGFP at 6-24 h p.t. No translocation of NPM was detected even at MOIs 1000-2000 (unpublished data), suggesting no evidence of the cytopathic effects in AcMNPV transduced cells. However, it remains to be studied if the reorganization of PML NBs was induced by IFN response as baculoviruses are shown to stimulate the expression of IFN-α/β *in vitro* and *in vivo* (Abe et al., 2005; Gronowski et al., 1999).

Many viruses have also been found to interact with cajal bodies (CBs) and nuclear speckles; T-cell leukemia virus accumulated into nuclear speckles, whereas influenza virus alters their nuclear localization and adenoviral infection leads to the disruption of CBs. To address this matter in baculovirus transduced cells, we monitored the distribution of nuclear speckles markers (PAB2-EGFP and SC-35 Ab) and CB marker (p80coilin Ab) in relation to baculoviral capsids at 6 to 24 h p.t (IV/Fig. 5B-5C). Together, these data showed that baculovirus virions do not associate with nuclear speckles and CBs.

Condensation, marginalization or dispersion of the chromatin, increase of the nucleoli and disruption of the nuclear lamina, has all been observed during infection of viruses. Similarly, baculovirus *Ac*MNPV has been shown to disperse host cell chromatin of insect cells during infection. Here, we used chromatin label Drag5™ and human histone plasmid H2B-EYFP to study changes in host cell chromatin in HepG2 and 293 cells. In control cells the chromatin was detected around the periphery of the nuclear lamina and nucleoli (IV/Fig. 6A). In transduced cells, the chromatin label showed a more dispersed pattern resulting in less detectable lining of the nuclear lamina and nucleolus (IV/Fig. 6B). Similar results were obtained from aphidicolin synchronized G_1/S-phase arrested cells (unpublished data). The altered chromatin distribution increased significantly over time (24-48 h p.t.) and with increasing viral load (MOI 200-1000) (IV/Fig. 6C). The effect of labeling of baculovirus genomes with Drag5™ in transduced cells was ruled out by flow cytometry studies which showed no difference in the chromatin fluorescence intensities in cells transduced at MOIs ranging from 10-1000. We also noticed that the peripheral heterochromatin lining the nucleoli was dispersed gradually during transduction, effect being more evident at higher MOIs (IV/Fig. 6D). These results were confirmed by monitoring changes in H2B-EYFP distribution which was significantly altered (IV/Fig. 7). Taken together, these results demonstrate that baculovirus virions or the products of early viral genes are able to induce alteration in the distribution of host cell chromatin. This chromatin remodeling may be mediated by viral interactions with nuclear actin, actin-related proteins or other histone-modifying factors but more work is required before this issue can be clarified (Chen and Shen, 2007; Lachner and Jenuwein, 2002; Simpson-Holley et al., 2005; Volkman, 2007).

Baculoviruses have been in contact with humans since the emergence of species and there is no evidence that baculoviruses influence human health in any manner. The data provided here suggests that baculoviruses can induce viral gene expression and nuclear alterations in mammalian cells. Whether the expression of baculoviral proteins could induce immune responses or other physiological changes requires further investigation.

6. SUMMARY AND CONCLUSIONS

On the basis of the presented thesis studies, the following conclusions can be made.

1. VSV-GED pseudotyping augments baculovirus transduction *in vitro* and *in vivo* probably by reducing the energy barrier required to trigger the membrane fusion. In addition, VSV-GED display confers enhanced serum stability and offers several advantages over VSV-G pseudotyping such as low cytotoxicity.

2. Metabolic biotinylation of baculovirus membrane proteins allows viral targeting by an easy exchange of surface molecules while enabling efficient capture, purification and concentration of baculovirus vectors. Significant differences in the extent of biotin incorporation and transduction efficiencies were revealed with respect to the site of biotin acceptor peptide on the envelope proteins; most efficient display was achieved by incorporation of BAP at site 283 on gp64 as this is suggested to allow incorporation through homo-oligomerization of the fusion proteins.

3. Targetable gp64-pseudotyped lentivirus vectors were developed by displaying streptavidin or avidin fused to VSV-GED on the virus envelope. This strategy allows ligand- and antibody-mediated targeting of lentivirus to several tumor cell lines *in vitro*. Furthermore, streptavidin displaying lentivirus carrying a ferritin reporter gene enabled long-term non-invasive imaging of virus biodistribution and gene delivery by SPECT/CT and MRI. This study also provided pioneering information about the transduction pattern of gp64-pseudotyped lentiviruses in the central nervous system.

4. Baculovirus nuclear entry into mammalian cells was shown to be followed by the transcription of early viral genes *ie-1*, *ie-2* and *lef-3*. Moreover, the transduction was demonstrated to affect the size and number of PML bodies, which are often implicated in replication and transcription of viruses. Another effect was manifested by the remodeling of the peripheral host chromatin into disperse patterns. To address the safety issues of employing baculoviruses in gene therapy, expression of baculovirus endogenous genes and virus induced nuclear alterations, further studies are needed.

These results encourage further studies in characterization of the enhanced transduction capacity of VSV-GED and targeting potential of biotinylated baculoviruses and (strept)avidin-displaying lentiviruses *in vivo*. Also the use of streptavidin display for noninvasive imaging should allow the monitoring of virus biodistribution and gene delivery in the context of a therapeutic regimen. Finally, we provided significant new knowledge about the effects of baculovirus-mediated gene delivery in mammalian cells which poses new challenges for the future engineering of baculovirus vectors.

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