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Improvement of retroviral vectors for efficient gene transfer

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PhD thesis

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Contents

INTRODUCTION

1. Retroviruses and Their Replication Cycle

The family Retroviridae is represented by small enveloped viruses whose genome is constituted by a single-stranded RNA. The typical feature of retroviruses that makes them very different from other RNA viruses is the ability to transcribe their ssRNA genome into double-stranded DNA. Another special capability is the very efficient integration of their DNA genome into the host genomic DNA by action of the virus-specific integrase.

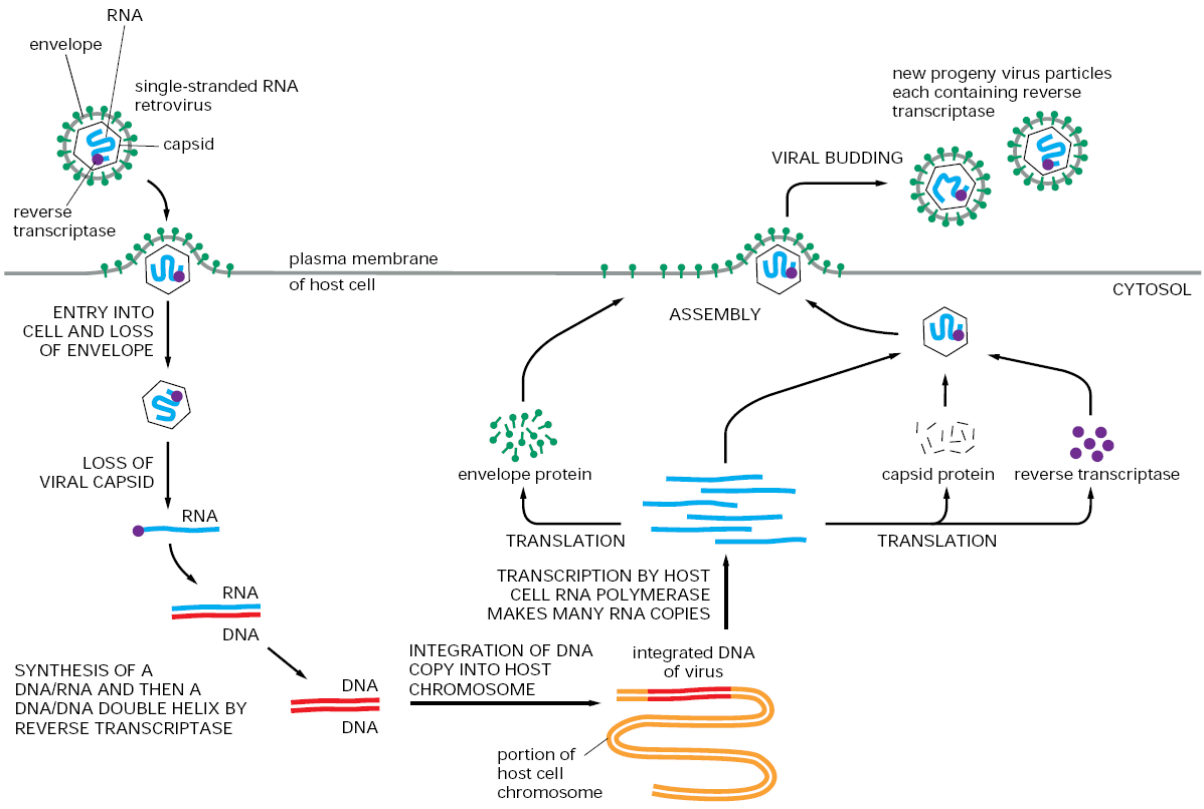

Figure 1: The life cycle of a retrovirus. Reproduced from Alberts et al., 1998.

The retroviral extracellular particles consist of two copies of viral RNA, viral enzymatic proteins all enclosed in the structural proteins forming nucleocapsid, capsid and envelope. The entry of the particle to the host cells is initiated by the interaction of the viral envelope

protein and specific receptor on the cell surface. The envelope protein-receptor interaction triggers conformational changes of the envelope that result in fusion of viral and cellular membranes. This process ensures penetration of the viral capsid into the cytoplasm of the host cell. Once the viral capsid is inside the host cell, the reverse transcription is usually started and the viral genome is transcribed into double-stranded DNA. Several exceptions concerning the timing of reverse transcription exist. A typical example are foamyviruses, which exhibit several unique features and the reverse transcription of their genomic RNA is completed at the end of the viral replication cycle when the virions leave the host cell (Moebes et al., 1997).

A further important step in the retroviral life is decoating, which results in escape of the viral genome complexed with certain proteins into the cytosol. The viral dsDNA-protein preintegration complex consists of viral dsDNA genome, viral integrase, and several viral and cellular proteins. The task of this nucleoprotein complex is to reach the host cell genomic DNA and integrate the viral genome into it. This is achieved by various mechanisms depending on the group of retroviruses. The gammaretroviruses represented by model Moloney mouse leukaemia virus are not able to efficiently penetrate through the nuclear membrane and have to wait until the M-phase of the cell cycle and disintegration of the nuclear membrane. The alpharetroviruses have a nuclear localization signal present in the integrase molecule. Thanks to it they can enter an interphase nucleus and perform their replication cycle even in nondividing cells. The most effective in infection of nondividing cells are lentiviruses, which are able to very efficiently transfer their genetic information into the interphase nucleus and integrate. It is partially due to the accessory viral protein Vpr, which facilitates transfer through the nuclear membrane (Suzuki & Craigie, 2007).

The ability of retroviruses to integrate into the host cell genome is a very important feature that gives them major advantage and is responsible for many consequences of retrovirus infection. The viral integrase inserts the viral genome into the host DNA in an almost random manner and no sequence specificity of the integration was observed. However, the integration is not really random. There are various integration preferences among retroviruses. The HIV-1 integrates preferentially into genes, especially into their coding sequences. The MLV has a slightly lower preference for the genes, but strongly favours the transcription start sites. The lowest preference for the genes is observed in avian sarcoma and leucosis viruses and foamyviruses (see chapter 2.1 Integration preference). The viral genome becomes a stable part of the host cell genome and is

replicated along with it. The integrated form of the retrovirus is called provirus and results in the persistence of the retrovirus in the host cell. The provirus is transcribed by the host cell machinery and the transcripts are spliced to form subgenomic RNAs, which encode additional viral proteins. All viral transcripts are transported into the cytoplasm. The unspliced RNAs are used translated or packed into the nascent viral particles, but the spliced subgenomic RNAs serve only as templates for the translation and production of the viral proteins. The envelope proteins are integrated into the host cell plasma membrane and form zones where the viral particles gain their envelope by budding through this modified cellular membrane. Usually, after the virion release, the precursors of the viral nucleocapsid are cleaved by the viral protease and the particles become mature and able to infect further host cells and start a new replication cycle.

2. Retroviral vectors

Retroviruses are simple and very effective gene transmitters. It makes them very attractive for scientists searching for an effective gene transfer vehicle. Their 10 kb genome consists of regulation sequences at both ends of the genome, which represent approximately 10% of the sequence. The majority of their genome can be replaced by nonviral sequences used for various purposes and the viral proteins are provided in *trans*.

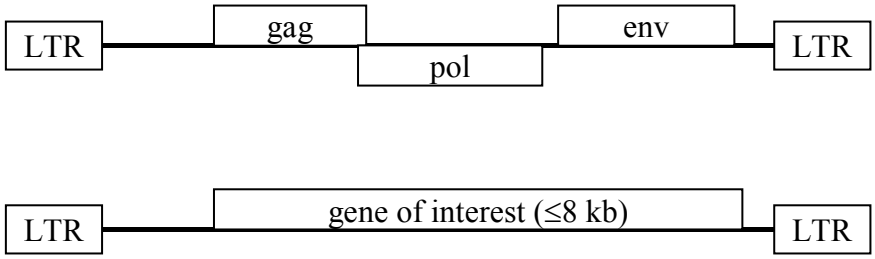

Figure 2. Comparison of a retrovirus and a retroviral vector.

The most common strategy is the preparation of packaging cells, which contain coding sequences of all viral proteins under the control of a constitutive promoter. These cells are transfected with a plasmid that encodes the retroviral vector. The transcribed mRNA is packed into viral proteins present in the packaging cells and the formed viral particles are released into the culture medium. The culture medium is collected and used for transduction of the target cells.

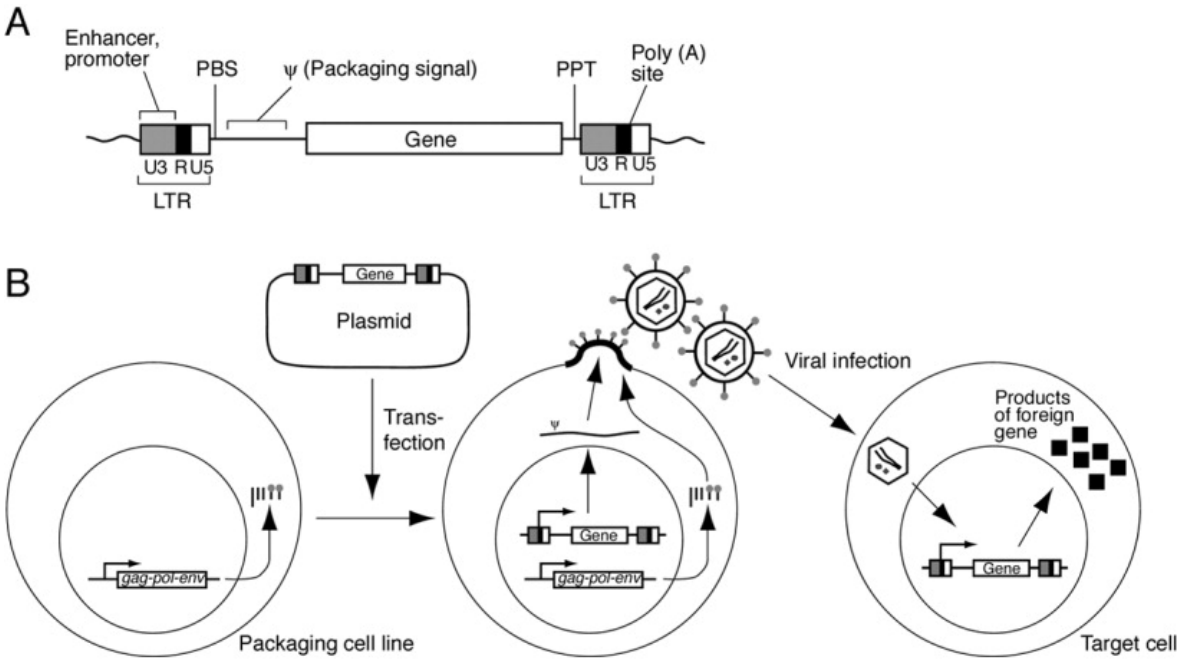

Figure 3: General principle of production of a replication-defective virus. (A) Minimal *cis*-acting elements required for replication and transcription of replication-defective retroviral vectors. (B) Life cycle of replication-defective retroviruses. Reproduced from Ishii et al., 2004.

2.1. Safety issues - Integration preference

There is no sequence-specific integration of retroviruses recorded and for a long time it was thought that retroviruses integrate randomly into the host cell genome. The first clinical trial revealed that the integration would be probably biased. It was due to a surprisingly frequent integration of the retroviral vector in the vicinity of cellular protooncogenes that resulted in cellular transformation and adverse consequences of the gene therapy. The integration patterns were scrutinized and it did not take long to find out that the integration was only seemingly random. The retroviruses used for clinical trials in gene therapy exhibited a certain preference for their integration into the host genome. The preference differs among groups of retroviruses. As it appeared, the first retroviral vectors used in clinical trials that were derived from Moloney murine leukemia virus (MMLV) were the most dangerous. They have a very strong preference for integration in the vicinity of transcription start sites (Wu et al., 2003, Laufs et al., 2004, Hematti et al., 2004). This preference is very dangerous due to the presence of strong enhancers in the viral LTRs that

can activate the adjacent promoter. In case the retroviral vector integrates close to the cellular protooncogene, it can constitutively activate normally finely controlled expression of the cell cycle regulator and cause uncontrolled proliferation of the transduced cell. This had serious adverse consequences in a clinical trial performed by Alain Fischers`s team in Paris in 1999 (Cavazzana-Calvo et al., 2000, 2001, Fischer et al., 2001). To date, four of the eight patients of this trial who have experienced long-term correction of their disease by the infusion of vector-modified autologous bone marrow cells have developed a malignant complication, which, at present, was lethal in one case. The analysis of proviruses present in the malignant cells revealed vector insertion to a known cellular proto-oncogene LMO2. These complications launched an intensive effort to characterize retrovirus integration in detail. Special focus was on the determination of integration preference and finding more suitable retroviral vectors for gene therapy. The integration preference identification revealed a safer pattern of lentiviruses that integrate preferentially into the coding sequences of transcription units (Wu et al., 2003, Mitchell et al., 2004, Elleder et al., 2002). It is less dangerous as the disruption of a gene functions as a recessive mutation. The factors involved in the integration site selection were intensively investigated. The integration preference of HIV-1 is the most scrutinized. Analysis of the integration pattern of HIV vectors with specific sequences replaced with corresponding MLV sequences revealed the role of the pol gene in the integration part selection (as expected, since it codes for the viral integrase), and also the gag modulates the integration preference (Lewinski et al., 2006). The most surprising was the finding that cellular factor LEDGF/p75 is responsible for the HIV-1 preference to the highly expressed genes. The LEDGF/p75 binds the HIV-1 preintegration complex and tethers it to the highly transcriptionaly active loci (Ciuffi et al., 2006, Maele et al., 2006, Vandekerckhove et al., 2006, Shun et al., 2007). Recent studies revealed that other groups of retroviruses exhibit even a more safe integration pattern. The foamyviruses do not integrate preferentially within genes, despite a modest preference for integration near transcription start sites and a significant preference for CpG islands (Trobridge et al., 2006). The alpharetroviruses represented by avian sarcoma and leucosis virus (ASLV) have only a very weak bias for integration into transcription units (Mitchell et al., 2004, Narezkina et al. 2004, Reinisova et al., 2008), and the most recent study on the betaretrovirus MMTV (mouse mammary tumour virus) found random integration of this retrovirus (Faschinger et al., 2007).

Due to the inadvertent risk of gene disruptions, which can only be minimised but not eliminated, several attempts to construct nonintegrating retroviral vectors were done. It

is possible thanks to the ability of retroviral DNA to generate transcriptionally active stable circular form. When the integrase mutants were used, the number of circular forms increases compared to the integrase competent vectors and no integration was detected. However, the nonintegrating retroviral vectors have limited application. Since they are not integrated in the host cell genome, they are gradually lost during cell proliferation. Therefore, their possible utilization is only for differentiated nondividing cells. (Saenz et al., 2004, Cara & Klotman, 2006, Apolonia et al., 2007, Philpott et al., 2007, Wu et al., 2004).

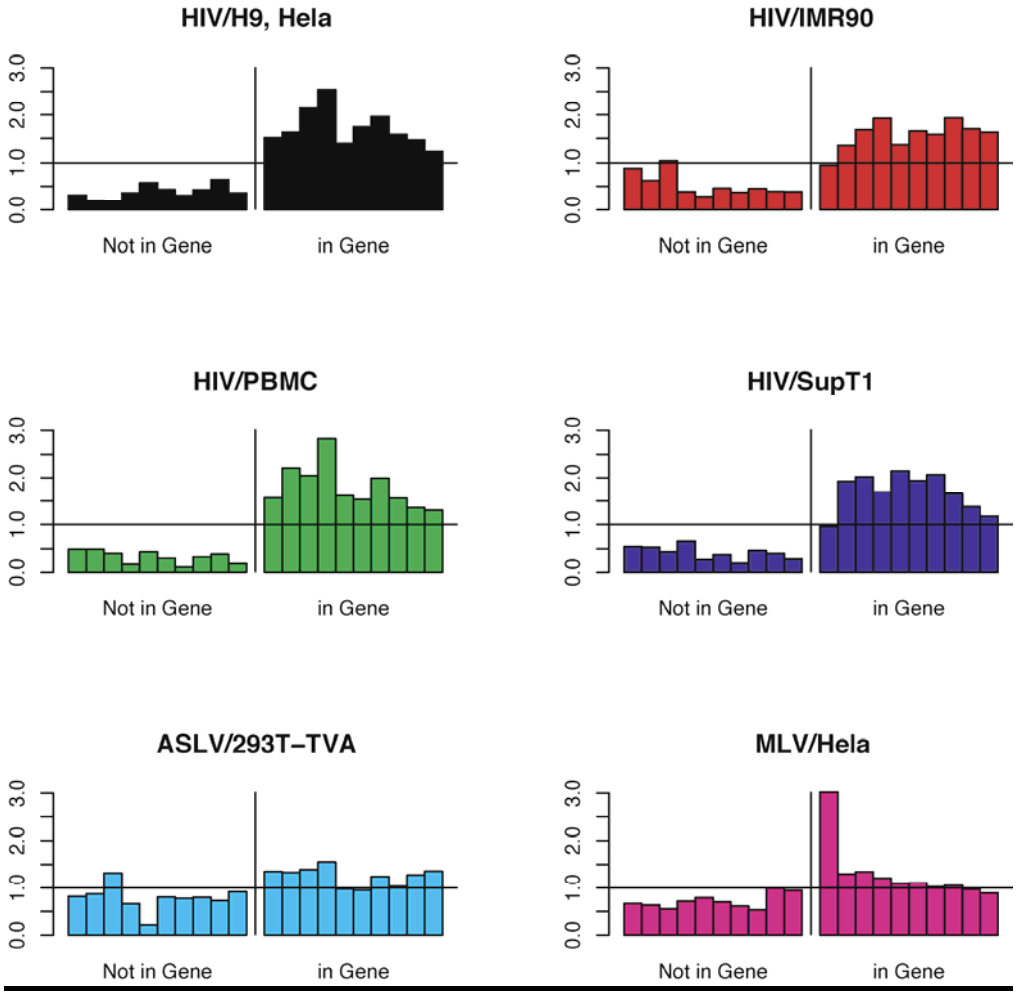

Figure 4. The integration preference of HIV, MLV and ASLV viruses. Reproduced from Mitchel et al., 2004.

2.2. Safety issues - Risk of transactivation

As mentioned above, the use of retroviral vectors constitutes a certain level of risk due to their strong viral enhancers. Several strategies how to cope with this danger were

suggested. The simplest strategy is the design of self-inactivating (SIN) vectors. The SIN vectors have deleted the majority of their 3` U3 regions containing enhancers. After the reverse transcription both LTRs lack their enhancers. The expression of the gene of interest is driven from inner promoter that usually contains weaker and therefore less dangerous enhancers. This strategy significantly lowers the risk of protooncogene transactivation by the vector, but the inner promoter`s enhancers still retain a potentially dangerous transactivation capacity.

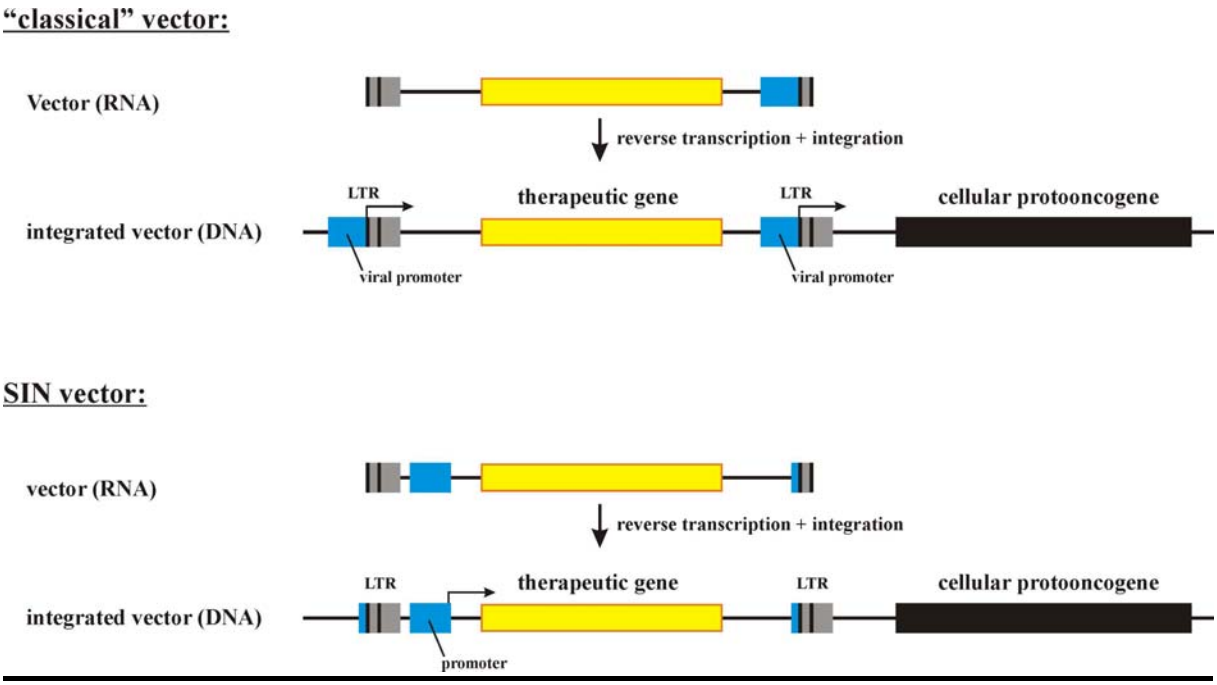

Figure 5. Comparison of "classical" and self-inactivating (SIN) vectors and the principle of the transactivation risk.

The further safety improvement includes incorporation of *cis*-acting elements that are able to prevent action of the enhancers out of the range of the vector. The genome-wide studies searching for such elements discovered a sequence that serves as a chromatin insulator. This element placed between the enhancer and the promoter can prevent the influence of the enhancer on the promoter. Placed at the extremities of the vector, it would prevent the enhancer action outside the vector. The first efficient and still most widely used chromatin insulator is the cHS4 element of the chicken β-globin region. This element was already used in various vectors (Rivella et al., 2000, Emery et al., 2000, Aker et al., 2007, Yannaki et al., 2002). There are also other potential chromatin insulators found in the sea urchin genome (Hino et al., 2004, Yajima et al., 2007). The human CD2 locus control

region also serves as a chromatin insulator **(Indraccolo et al., 2001)**. These elements can very significantly decrease the risk of cellular gene transactivation by the inserted retroviral vector, especially when used in relatively safe SIN vectors.

3. Retrovirus entry

The first step in the retrovirus life cycle is the entry to the cell. It is mediated by a specific cellular receptor, which is indispensable for the retrovirus entry. The viral particle binds to the specific surface molecule of the host cell and this binding triggers several typical conformational changes, which lead to the fusion of viral and cellular envelope and penetration of the viral nucleocapsid into the cytoplasm. The entry is followed by processes described above.

All retroviruses use very specific receptors. A wide variety of surface molecules can serve as viral receptors and the presence of the molecule often strictly determines the susceptibility of the cell to the individual retrovirus infection. For example, the CD4 molecule, which is present on the surface of the T helper cells, serves as the HIV-1 virus receptor. The virus (except for rare mutants) is not able to infect other a CD4 positive cells. However, HIV is not a typical example because it also requires presence of a coreceptor molecule for successful entry into the CD4+ cell. The situation is simpler in the case of alfaretroviruses represented by ASLV. It will be described in the next chapter.

The receptor molecules of avian sarcoma and leucosis viruses

The avian sarcoma and leucosis viruses evolved their envelope glycoproteins to bind ubiquitous surface molecules on avian cells. However, not all ASLV viruses utilize the same receptor molecules. On the basis of interference among ASLV viruses there are characterized six highly related envelope subgroups of ASLVs (A, B, C, D, E and J) thought to have evolved in the chicken population from a common viral ancestor (Barnard et al., 2003, 2006).

The cellular receptors to ASLVs have not been molecularly identified for a long time and were studied indirectly according to their interaction with corresponding viral subgroups or using techniques of cross-neutralization by antibodies or infection interference (reviewed in Weiss 1993). Three autosomal tumour virus (*tv*) loci *tva*, *tvb* and

tvc were described, carrying either dominant susceptibility alleles (e.g. tva^S, tvc^S) or recessive resistance alleles (e.g. tva^R, tvc^R). The susceptibility alleles code for functional virus receptors, whereas the resistance alleles represent the lack of receptor or expression of mutated non-functional receptor. The host range variation reflects the expression of subgroup-specific cellular receptors. Because viral subgroups A and B were discovered first, the loci *tva* and *tvb* were also first to be characterised (Payne and Biggs 1964, Crittenden et al., 1964, Rubin 1965, Vogt and Ishizaki 1965, Payne and Biggs 1966). The genetics of the *tvb* locus is more complex, with alleles coding for susceptibility to subgroups B, D and E (Crittenden et al., 1973, Adkins et al., 2001). The *tvc* locus was defined shortly after the first description of viral subgroup C (Duff and Vogt 1969, Payne and Biggs 1970). The subgroup A uses the *tva* locus, which encodes a low-density lipoprotein receptor. The subgroups B, D and E use the same molecule, which is encoded by the *tvb* locus and is closely related to tumour necrosis factor receptor (TNFR) family proteins. The subgroup C requires a different receptor molecule than the A and B subgroups, but the receptor has not yet been identified.

The genetic locus encoding the receptor for the recently discovered subgroup J has not been identified due to the fact that there is no cell line resistant to the infection by this subgroup (Payne 1992), which precludes mapping of the receptor locus.

3.1. The subgroup A ASLV receptor, Tva

The subgroup A receptor was identified as the first one (Young et al., 1993, Bates et al., 1993). Two alternatively spliced transcripts were identified in the quail. They were designated Tva800 and Tva950 according to their mRNA length. The Tva950 is a transmembrane protein which consists of a 19 AA long signal peptide, followed by an extracellular domain (residues 20-102), a transmembrane region (residues 103-125) and a short cytoplasmic tail (residues 126-157). The splice form Tva800 has an identical N-terminal part of the molecule (signal peptide and extracellular domain), but lacks the transmembrane domain. The molecule employs the glycosyl phosphatidylinositol (GPI) anchor as the membrane attachment structure. Both splice forms of the Tva can serve as ASLV(A) receptors, thanks to the fact that the extracellular domain is crucial for the interaction with the envelope of the ASLV(A) virion and for the function of the Tva as the virus receptor molecule.

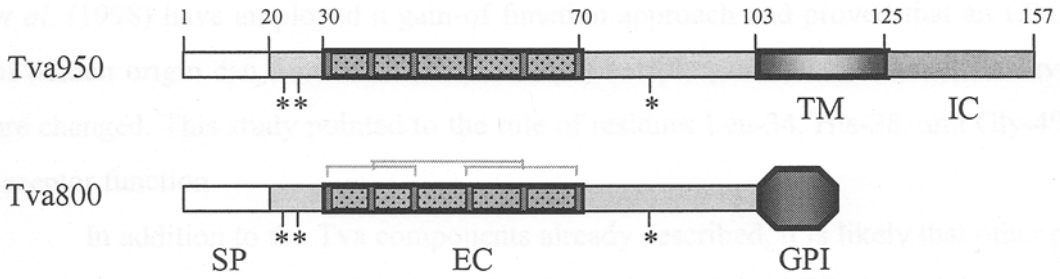

Figure 6: Schematic representation of the two Tva isoforms that are generated by alternative splicing. The numbers indicate amino acids of the precursor protein. The LDL-A module is stippled and the position of the six conserved cysteine residues is shown with the pattern of the disulfide bonds. The asterisks indicate potential N-glycosylation sites. TM, transmembrane domain; IC, intracellular domain; SP, signal peptide; EC, extracellular domain; GPI, GPI anchor

The extracellular part of the Tva contains a single 40-amino acid domain, which is highly related to the ligand-binding repeat of low density lipoprotein (LDL) receptors, termed the LDL-A module (Bates et al., 1993). The LDL-A module binds the ASLV envelope with high affinity and the module alone is sufficient to ensure the viral entry (Rong and Bates, 1995). The Tva molecule is subjected to N-linked glycosylation at three sites, but the presence or absence of this posttranslational modification has only very weak impact on the binding affinity (Balliet et al., 1999).

The mechanism of Tva-mediated ASLV entry has not been sufficiently elucidated and is still a matter of discussions. It was initially predicted that the ASLV uses a pH independent mechanism of entry (Gilbert et al., 1990, Mothes et al., 2000, Narayan et al., 2003, Earp et al., 2003). Most retroviruses employ this type of mechanism, where the receptor-Env interaction is sufficient to mediate fusion and entry of the virus core into the host cell cytoplasm. On the other hand, many other enveloped viruses (e.g. influenza virus, vesicular stomatitis virus) only anchor the receptor molecules by their envelope and are encytosed. In the endosome, they are exposed to low pH, which activates fusion activity of their envelope and enable the viral components to escape into the host cell cytoplasm. Mothes et al. (2000) provided evidence for a further type of entry in case of ASLV, which combines both pH-dependent and independent steps. The authors predict that the receptor-Env interaction is necessary, but it only primes the viral fusion protein for a second crucial pH-dependent step.

Despite the extensive knowledge of the Tva molecule the original role of this molecule in the avian cell is not known.

3.2. The subgroup B, D and E ASLV receptor, Tvb

The Tvb receptor serves as an entry mediator of ASLV subgroups B, D and E (reviewed in Barnard and Young 2003). Similarly to Tva, the Tvb is a single path transmembrane protein, but it is the only similarity of Tva and Tvb proteins. They are evolutionally unrelated. The Tvb protein sequence starts with a 21 amino acid long signal peptide which is followed by an extracellular domain (residues 22-155), transmembrane region (residues 156-177) and a long cytoplasmic domain (residues 178-361). The extracellular region contains three cysteine-rich domains and the cytoplasmic region contains a death domain (Brojatsch et al., 1996). According to the homology with the mammalian proteins, Tvb is most similar to tumour necrosis factor related apoptosis-inducing ligand (TRAIL) receptors DR4 and DR5 (Adkins et al.,2001). The fact that Tvb is a member of the tumour necrosis factor receptor (TNFR) family and the presence of the cytoplasmic death domain implicate involvement of the receptor in the cytopathicity of the subgroup B viruses. The determinants for cytopathogenicity of ASLV(B) colocalize with the determinants of receptor recognition (Dorner and Coffin 1986). The binding of soluble ASLV(B) envelope protein to the receptor can induce cell death in the presence of cycloheximide, which blocks protein synthesis and prevents expression of the presumed protective factors (Brojatsch et al., 2000). Viruses belonging to subgroup E are not cytopathic, although they use the same receptor. The reason for this fact is still unclear.

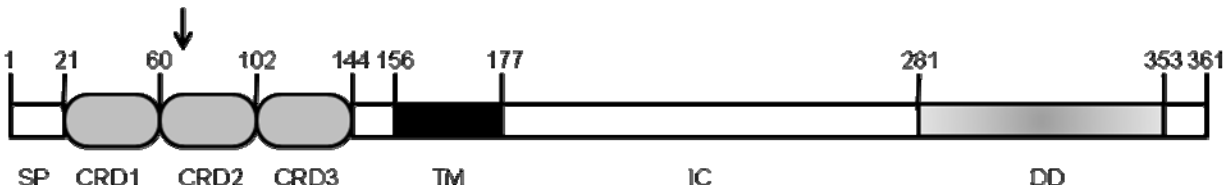

Figure 7: Schematic representation of Tvb. The numbers indicate amino acids of the precursor protein. The asterisks indicate potential N-glycosylation sites. The arrow points at the position of cysteine 62, which is the site of the single amino acid difference between *tvb*^{S3} and *tvb*^{S1}. SP, signal peptide; CRD, cysteine-rich domains; TM, transmembrane domain; IC, intracellular domain; DD, death domain (part of IC).

There are two alleles encoding functional subgroup B receptors identified. The *tvb*^{S1} allele confers susceptibility to subgroups B, D and E, while the *tvb*^{S3} allele confers susceptibility to only subgroup B and D ASLVs. These two alleles differ only in a single amino acid residue in the cysteine-rich domain 2 (CRD2). The serine in position 62 of the *tvb*^{S3} allele is substituted with cysteine in the *tvb*^{S1} allele and this mutation presumably alters the structure of CRD2, resulting in the loss of ASLV(E) binding and entry. The cysteine in position 62 is also conserved in a turkey homolog, *tvb*^{t}, which serves only as a receptor for the subgroup E ASLV. It indicates that the Cys-62 is crucial for the ASLV(E) envelope binding (Adkins et al., 1997). The Cys-62 is not the only cysteine required for ASLV(E) binding. The mutational analysis of the *tvb* also revealed the role of Cys-46 and Cys-59 in the proper ASLV(E) binding capacity.

There is only one *tvb* allele, which confers resistance to all three subgroups. The allele, *tvb*^{r}, containing an in-frame premature stop codon at residue 57, was identified and characterised from inbred line 7_2 chickens (Klucking et al., 2002). The premature codon leads to the production of a severely truncated protein and explains the nature of the receptor defect.

The Tvb binding viral subgroups (B, D and E) differ in the elements on the receptor that they recognize. The ASLV(E) probably bind a structural element of the receptor as they require correct disulfide bond pattern of the CRD2 domain. The ASLV(B) and ASLV(D) bind to the CRD1 domain and probably recognize a linear motif of Tvb. In agreement with this, the ASLV(B) Env can interact with Tvb even when the receptor is deglycosylated or denatured (Adkins et al., 2001). Additionally, the 15-amino acid peptide derived from residue 32-46 of Tvb CRD1 domain appears to be sufficient for ASLV(B) and ASLV(D) binding and entry (Knauss and Young et al., 2002). It appears that the major subgroup B and D viral interaction determinants are present in the short peptide. The aforementioned studies did not demonstrate any significant role of the CRD3 domain in the binding and entry of ASLV.

3.3. The subgroup C ASLV receptor, Tvc

The subgroup C receptor has not been identified yet. The *tvc* locus was reported to be in genetic linkage with *tva* (Pani 1974). This implicates that both *tva* and *tvc* lie on the

same chromosome. The *tva* and *tvc* genes were mapped to a specific region of chicken chromosome 28 (Elleder et al., 2004). The cloning and identification of the Tvc receptor is the subject of the first part of this work.

4. Pseudotyping of retroviral vectors

There is also an opposite strategy enabling a retroviral vector to enter a non-permissive cell. Pseudotyping of retroviral particles with the envelope of a different virus can extend the range of the virus tropism. The virus that provides the envelope is very often an unrelated virus. This is possible thanks to the ability of retroviruses to integrate various envelope proteins into their particle coat. There are many viruses whose envelope is used to modulate cell tropism of retroviral vectors. Very frequently used envelope proteins are the glycoproteins of Rhabdoviruses. The most widely used envelope is the G glycoprotein of vesicular stomatitis virus (VSV-G). HIV-1 was known to develop pseudotypes by the incorporation of heterologous glycoproteins, and it is subsequently able to extend its host range from CD4 receptor possessing T lymphocytes to a broad range of the cells. There are several reports that address human T cells co-infected with HIV-1 and MLV (Canivet et al., 1990, Lusso et al., 1990a, b) or herpes simplex virus (Zhu et al., 1990), thus resulting in cells with phenotypically mixed virions and expansion of the host range, demonstrating that virions pseudotyped with each virus envelope were produced. Furthermore, HIV-1 and vesicular stomatitis virus glycoproteins were reported to phenotypically mix in co-infected cells (Zhu et al., 1990). The VSV-G does not require a cellular receptor protein for the plasma membrane binding and entry. Until recently it was thought that phosphatidylserine serves as a receptor for the VSV-G protein (Schlegel et al., 1983), which is a very common compound of cellular membranes, but there are indirect evidences that the phosphatidylserine does not function as a receptor molecule (Coil & Miller, 2004) and the cell surface receptor molecule remains unknown. Vectors enveloped with this glycoprotein are able to infect a wide range of cells including the majority of cells from insects to mammals. The VSV-G has one additional advantage for vectors and it is their stability during concentration by ultracentrifugation to obtain high titres of the vector solution.

As the G glycoprotein is toxic for producing cells, it is very difficult to prepare a replication competent vector coding for this envelope protein. For experiments where the

use of replication competent vectors is desirable, the original envelope is replaced with the amphotropic envelope protein of mouse leukaemia virus (Barsov & Hughes, 1996).

There is also a range of other envelope glycoproteins that are used for the retroviral vector pseudotyping. One of the examples is Lyssavirus envelope. This includes rabies virus and rabies-related virus including Mokola virus. Rabies causes acute encephalitis in mammals. Therefore, neurotropism of such pseudotype is expected to infect the central nervous system. There is a report in which Lyssavirus pseudotypes of a lentivirus vector were compared to VSV-G pseudotypes by assessing the titre to human osteosarcoma (HOS) cells and Rat-2 fibroblasts. In HOAS cells, the titres of lyssavirus-enveloped vector were five times lower than those of VSV-G based vectors, and four times lower in Rat-2 cells. However, in an *in vivo* study, the transduction efficiencies of Mokola virus-enveloped vector to the striatum and the white matter were more extensive than that of VSV-G-based vector (Watson et al., 2002). Furthermore, intramuscular injection of a vector pseudotyped with rabies virus glycoprotein could transduce axonal projections and undergo retrograde transport in spinal neurons (Mazarakis et al., 2001). This ability can be employed for retrograde transport of therapeutic vectors pseudotyped with the rabies envelope from peripheral muscle to spinal neurons for therapy of the animal model of amyotrophic lateral sclerosis (ALS) (Azzouz et al., 2004). Vectors pseudotyped with the rabies virus envelope are expected to have the ability to express the gene in inaccessible neurons by retrograde transportation along the axons.

Other promising envelope glycoproteins are of filoviruses. The well-known representatives of this deadly virus family are viruses Ebola and Marburg. The primary targets for filoviruses are mononuclear phagocytic cells and endothelial cells. The efficiency of a lentivirus vector pseudotyped with Ebola Zaire (EboZ) glycoprotein was investigated by airway application for the treatment of cystic fibrosis (Kobinger et al., 2001). Vectors pseudotyped with amphotropic MLV, Mokola, influenza-haemaglutinin and EboZ glycoproteins were transduced to cultured airway epithelial cells. EboZ envelope showed high transduction efficiency. Furthermore, explants of human trachea were infected with lentivirus-based vector pseudotypes of VSV-G and EboZ glycoproteins. The EboZ-pseudotyped vector showed high transduction efficiency, but VSV-G was not effective in the airway epithelium. Treatment for cystic fibrosis was limited by poor transduction efficiency to the apical surface of airway epithelia by almost all of the methods including viral vectors. The pseudotypes of filovirus glycoprotein will be useful for gene delivery in the airway application for cystic fibrosis.

The last envelope protein used for the pseudotyping of retroviral vectors is the glycoprotein of lymphocytic choriomeningitis virus (LCMV). Retrovirus-based vectors pseudotyped with LCMV glycoprotein were reported to show efficient transduction to various cells and cell lines including fibroblasts, haematopoietic cells, epithelial cells, as well as hepatoma, neuroblastoma and glioma cell lines (Miletic et al., 1999). Furthermore, various kinds of species including human, rodents and dog were observed to be efficiently transduced (Romanowski et al., 1985). Lentivirus-based vectors pseudotyped with LCMV envelope were shown to yield similar titres compared with those of VSV-G (Beyer et al., 2002). Insulin-secreting pancreatic islet cells are known to exhibit poor transduction efficiency for gene transfer by various methods including virus vectors. Lentiviral vectors pseudotyped with LMCV glycoprotein transduced to these cells with higher efficiency than those with various viral glycoproteins. In addition, the toxicity to these cells was lower when pseudotyped with LCMV envelope than with other various viral glycoproteins including VSV-G (Kobinger et al., 2004). Miletic et al. (2004) examined the transduction efficiency of a lentiviral vector pseudotyped with LCMV glycoprotein to malignant glioma cells. Malignant glioma was known to be the most frequent brain tumour and also to be one with a poor prognosis using the present medical treatment. In an *in vivo* malignant glial tumour model of rat, the lentivirus-based vector pseudotyped with LCMV glycoprotein exhibited satisfactory and efficient transduction to glioma tumour cells. However, vectors pseudotyped with VSV-G transduced the surrounding cells of glioma for the most part and showed low transduction efficiency for the glioma itself.

In other reports, the transduction efficiency of vectors pseudotyped with the glycoprotein of Alphavirus including Ross River virus (Kang et al., 2002) and Sindbis virus (Morizono et al., 2001, Morizono et al., 2005), and those pseudotyped with Baculovirus GP64 (Kumar et al., 2003) were investigated. More selective tropisms could be accomplished by taking advantage of the natural tropism of glycoproteins from specific membrane-enveloped viruses. Many gene therapy approaches require tissue-specific expression of the therapeutic gene, and modulating the vector tropism by selection of the appropriate envelope is the most straightforward solution. That is why the search for the most efficient and selective vector envelope is of special interest.

5. Retroviral vectors for basic research applications

The retroviruses naturally serve as gene transmitters. The first discovered retrovirus, Rous sarcoma virus (RSV), transmits the viral variant of cellular protooncogene the v-*src*. This implied the possibility to utilize retroviruses as vehicles for stable and efficient gene transfer and occupying researchers for the last twenty years, since it provides a robust tool for efficient and stable gene transfer. The retroviral vectors can be divided into two basic groups according to their ability to replicate in the target cell or tissue to replication-competent and replication-defective.

5.1. Replication-competent vectors

The most popular are the vectors derived from avian Rous sarcoma virus (RSV). RSV serves as a natural vector carrying a mutated constitutively active analogue of cellular protooncogene c-*src* (v-*src*). The inspiration with this virus led to the design of the RCAS (replication-competent ALV LTR with a splice acceptor) vector system (reviewed in Hughes, 2004). The RCAS vectors correspond to the original RSV, but have deleted the v-*src* gene and one of the direct repeats flanking v-*src* that could destabilize the vector. The replication-competent vectors have several advantages in comparison with the replication-defective ones. They do not need any helper sequences for their preparation, which avoids the risk of recombination with the helper sequences. Another advantage of these vectors is high efficiency in permissive (avian) cells due to replication and spread of the virus in a cell population.

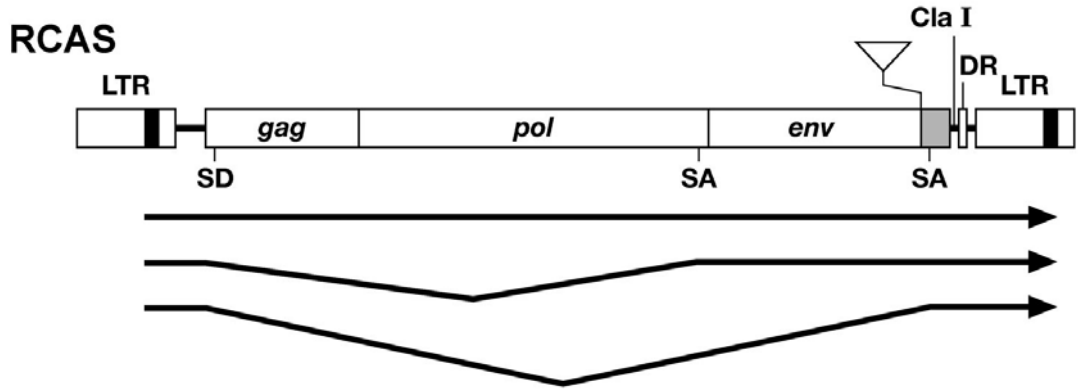

Figure 8: The RCAS vector. The splice variants of the mRNA are depicted. Reproduced from Hughes, 2004.

The receptors for entry of the RCAS vectors are not available on the surface of mammalian cells. However, this does not mean that the use of these vectors is restricted only to the avian cells. It is possible to replace the avian envelope protein gene (*env*) with a chimeric *env* gene that consists of signal peptide derived from the RSV envelope and gene coding for the amphotropic envelope protein of a specific strain of murine leukaemia virus (ampho MLV gp70) (Barsov & Hughes, 1996). This vector can be generated by transient expression in cell lines of avian origin and subsequently used for infection of mammalian cells. Thus adapted vector was successfully used for transduction of murine NIH 3T3, human HeLa and dog D17 cell lines. However, even this virus vector does not confer the advantage of virus spread in the target cell population when used for infection of mammalian cells. It is due to replication blocks at several steps of the replication cycle. The replication is negatively influenced at the RNA level by anomalous splicing (usage of cryptic splicing sites within the *env* gene) and low-efficiency export of viral RNA from the nucleus. There are also blocks involved at the protein level, which include processing of viral precursor protein (Gag) and assembly of virus particles (reviewed in Svoboda, 1998).

The replication deficiency of the RCAS vectors in the mammalian cells lowers the efficiency of infection but on the other hand enables more precise control of the spread of the vector in the organism and also increases the safety of the vector for clinical purposes. For example, the RCAS vectors were used for gene therapy experiments in macaques. Hu et al. (2007) transduced rhesus macaque haematopoietic progenitors and long-term repopulating cells in an autologous transplantation model. RCAS vectors efficiently and stably transduced rhesus macaque CD34+ haematopoietic progenitor cells with an efficiency of transduction of up to 34% *ex vivo*.

5.2. Replication-defective vectors

The aforementioned RCAS vector system represent a robust tool for gene transfer but has several limitations. One of the limits is the capacity of such vectors. The maximum length of the insert is about 2.5 kb in the RCAS vectors. The replication competence can also cause certain complications. The first is the impossibility of precise control of the vector spread upon infection of a target cell of avian origin. A further limitation is the necessity of certain vector integrity and limited space for vector modification. The vector has to be very close to the original virus and has to retain many specific features, which are

indispensable for the virus replication (e.g. splice sites, integrity of *gag*, *pol* and *env* genes, etc.). That is why the replication-defective vectors are preferable for many experiments and especially clinical trials consider only replication-defective retroviral vectors because of a lower risk of adverse effects caused by the retroviral infection. The replication-defective retrovectors provide a platform for a wide range of vector modifications and one of the important advantages is also the possibility to use toxic envelope proteins (e.g. VSV-G) for their envelope modification and change of the vector tropism.

In the nineties of the last century, the most popular replication-defective vectors were derived from Moloney mouse leukaemia virus. The vectors were thought to be very safe and this led to preparation of vectors carrying therapeutic genes and their use for confident clinical trials. The first clinical trial on the SCID patient cured by the gamma C chain delivered by a retroviral vector and the harmful consequences stressed the need for safer and more efficient vectors for gene therapy (Cavazzana-Calvo et al., 2000, Baum et al., 2006, Bushman, 2007, Wilson et al., 2008).

The effort for the retroviral vector improvement is oriented in several directions. The discovery of high-risk integration preference of MLV-derived vectors led to investigation of the preference of other retroviruses and to search for retroviruses with a safer and advantageous integration pattern. The consequence is gradual switch of the interest to lentiviral vectors and vectors derived from foamyviruses or avian retroviruses, which exhibit safer integration (Mitchell et al., 2004, Narezkina et al. 2004, Reinisova et al., 2008, Trobridge et al., 2006).

The change of the integration pattern is not the only possibility how to obtain a vector suitable for efficient and safe application in basic research or clinical trial. The replication-defective viruses often achieve lower titres and lower transduction efficiencies than their replication-competent counterparts.

Vast effort was conducted to the modification of the vectors leading to higher titres and increased transduction efficiencies. While the understanding of the retroviral replication cycle extended, the modification tightly followed the newest discoveries especially concerning the need of particular *cis*-acting sequences to obtain highly infectious retroviral or retrovector particles.

There are several approaches how to increase the vector transduction efficiency. The first possibility is to facilitate export of the transcribed vector RNA into the cytoplasm, which leads to more efficient translation or packaging of genomic vector RNA. It can be achieved first by the lentiviral specific machinery, which requires insertion of the Rev

response element (RRE) into the vector sequence (Delenda, 2004). This approach necessitates the presence of the Rev coding sequence. Which can be in *cis* (inserted in the vector) or in *trans* (expression vector present separately in the packaging cells). A more versatile approach is insertion of the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) in the 3` untranslated region. This modification does not require for its function the presence of the exogenic coding sequence and facilitates the nuclear export of the RNA. It increases either the titre of the vector or the level of expression of the transgene in the target cell (Zufferey et al., 1999, Klein et al., 2006). The second possibility is improvement of the target cell infection. Besides adaptation of the virus particle envelope leading to the improvements of the cell penetration (described in chapter 4. Pseudotyping of retroviral vectors) it is also possible to increase the efficiency of nuclear import of the viral cDNAor respectively, the preintegration complex (PIC). The central polypurine tract (cPPT) of HIV has the ability to promote PIC transport into the nucleus. This cPPT can be inserted in a retroviral vector and increase efficiency of the viral cDNA integration (Sirven et al., 2000, Zennou et al., 2000, Follenzi et al., 2000).

Retroviral vectors, thanks to their simplicity, provide a versatile tool for various modifications of target cells of differing origin. Their application is not restricted to tissue cultures, but their use is aimed to use in animal models or human gene therapy. For many purposes it is sufficient to design a vector that can efficiently transfer the desired gene into the target cells or organism and provide expression of the gene of interest. However, in many applications concerning even basic research or gene therapy it is necessary to target the transgene expression more specifically or have a possibility to control the transgene expression. For this purpose it is necessary to use other than viral promoters. As described above, the SIN vectors lack the U3 region representing the viral promoter. The expression from these vectors is dependent on the inserted inner promoter, which can be of various origin. The frequently used constitutive promoters (e.g. CMV-IE or EF1 promoter) can be replaced with a tissue-specific promoter (Petropoulos et al., 1992, Ellis & Yao, 2005).

One of the first experiments concerning the possibility of tissue-specific expression of a transgene delivered by a retroviral vector was conducted by Petropoulos et al., 1992. The promoter regions of the chicken skeletal muscle α-actin (α_{sk}-actin) and the cytoplasmic β-actin genes were linked to the bacterial chloramphenicol acetyltransferase (CAT) gene. Replication-competent retroviral vectors were used to introduce these two actin/CAT cassettes into the chicken genome. Chickens infected with retroviruses containing the α_{sk}-actin promoter expressed high levels of CAT activity in striated muscle (skeletal muscle

and heart); much lower levels of CAT activity were produced in the other non-muscle tissues. In contrast, chickens infected with retroviruses containing the β-actin promoter linked to the CAT gene expressed low levels of CAT activity in many different tissue types and with no discernible tissue specificity.

In erythroid lineage-specific gene expression, different erythroid-specific promoters including ankyrin-1, α-spectrin, β-globin or γ-globin, and enhancers including the GATA-1 autoregulatory element, α-globin LCR, intron 18 from the 5-aminolevulinate synthase gene or α-globin HS40 were employed to compare the efficiency of transgene expression inserted into a lentivirus vector in human and mouse haematopoietic stem cells (Moreau-Gaudry et al., 2001). The ankyrin-1 promoter and GATA-1/HSP40 or I18/HS40 enhancer combination has been shown to yield the highest level of expression. Furthermore, the enhancement of gene expression using ferrochelatase cDNA for erythropoietic protoporphyric treatment driven by the chimeric promoter/enhancer element ankyrin-1/α-globin HS40 by lentivirus vector-mediated gene transfer was also reported to be effective (Richard et al., 2001). In vascular endothelial cells, the promoter/enhancer sequences of vascular endothelial growth factor receptor Flk-1, endothelial receptor tyrosine kinase Tie1, angiopoietin receptor Tie2, vascular endothelial adhesion molecule cadherin and intracellular adhesion molecule 2 was reported to be applied for SIN vectors *in vivo* and *in vitro* (De Palma et al., 2003). In the central nervous system, enolase promoter increased eGFP positive cells over 30% more than CMV promoter in the mouse striatum and hippocampus (Lai et al., 2002). Furthermore, lentivirus vectors driven by the Rhodopsin promoter have shown photoreceptor-specific transgene expression in retinal cells, and also higher expression than with the CMV and human elongation factor-1 promoter (Miyoshi et al., 1997, Kostic et al., 2003). These results are promising for the patients with inherited retinal degenerative diseases. Phosphoglycerate kinase (PGK) and albumin promoters were reported to exhibit liver-specific gene expression (Akiyama et al., 1994, Pandya et al., 2001).

The approach mentioned above is not the only strategy how to obtain expression of a transgene restricted to one particular tissue. Another strategy is tissue targeting. This strategy is a very useful tool in basic research but is not applicable for gene therapy application due to the need of establishment of a transgenic organism preceding the transduction by the retroviral vector. Tissue targeting exploits the fact that avian retroviruses are unable to infect mammals due to the lack of the appropriate viral receptor on the surface of the target cell. It is possible to prepare transgenic animals expressing a

specific viral receptor for avian retrovirus driven by a tissue-specific promoter. These animals can be infected with a retroviral vector of avian origin belonging to the appropriate subgroup, which utilizes the receptor used for the animal modification. The vector infects the animals but the infection pattern tracks the specific expression of the viral receptor. This approach was tested on mice that were modified by the *tva* locus encoding viral receptor for subgroup A ASLV under the control of the α_{sk}-actin promoter. The skeletal muscles of these transgenic animals were susceptible to efficient infection by subgroup A ASLV (Federspiel et al., 1994). This approach provides more possibilities for a research model than the previous one, because the transgene carried by the retroviral vector doesn`t need to be under the control of tissue-specific promoters, which provide a limited range of the expression level or other desired properties (Du et al., 2006). In this type of gene transfer it is possible to place the trangene under the control of a wide range of promoters in the retroviral vector. For example, it is possible to use an inducible promoter and combine the tissue-specific and inducible transgene expression. This is a robust tool to study an organ function or to establish disease models.

A novel approach has been also developed for tissue-specific expression or tissue-specific exclusion of expression in recent years. Brown et al. (2006) prepared vectors utilizing for their expression profile tissue-specific miRNA mir-142-3p, which is specific for cells of haematopoietic lineage. By challenging mice with lentiviral vectors encoding target sequences of endogenous miRNAs, they showed the efficiency of miRNAs in sharply segregating gene expression among different tissues. Transgene expression from vectors incorporating target sequences for mir-142-3p was effectively suppressed in intravascular and extravascular haematopoietic lineages, whereas expression was maintained in non-haematopoietic cells. This expression profile, which could not be attained until now, enabled stable gene transfer in immunocompetent mice, thus overcoming a major hurdle to successful gene therapy concerning immune response against the transgene. Omitting the expression of the transgene in haematopoietic cells can very efficiently restrict the transgene-specific immune response.

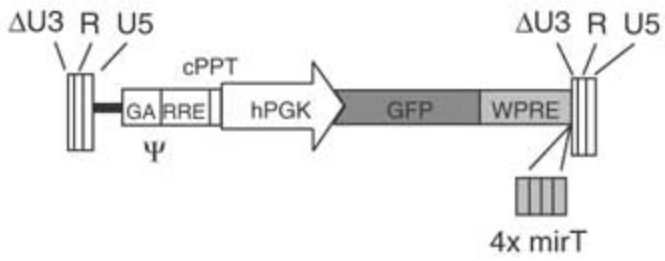

Figure 9. Lentiviral vector with the miRNA recognition site. Reproduced from Brown et al., 2006.

Other modifications of retrovirus vectors include the use of inducible promoters that can be turned on or off. Such vectors allow precise control of the transgene expression both *in vivo* and *in vitro*. In many applications concerning both the basic research and gene therapy it could be necessary to have the possibility to regulate the transgene expression. Several systems were established to control expression of a transgene by an exogenous drug, both at the transcriptional (Gossen, 1995) and at the translational level (Baron & Bujard, 2000). A widely standardized and commonly used inducible promoter is the tetracycline-inducible system (Vigna et al., 2002). The induction of a Tet-regulated gene expression system into recipient cells to attain stable genetic modification was performed by transfection, mouse transgenesis or retroviral transduction (Furth, 1994, Paulus et al., 1996, Lindemann et al., 1997). These approaches have been reported as successful. However, there are disadvantages including the low transfection efficiency and the necessity of clonal selection of integrants to enable optimal regulation (Paulus et al., 2000). The lentivirus-mediated introduction of the Tet-dependent system was shown using first generation (Kafri et al., 2000) and third generation lentiviral vectors (Vigna et al., 2002). Utilization of these methods will improve not only the ability to analyse the function of the gene of interest, but also the safety of gene therapy.

5.2.1. Application of retroviral vectors in basic research

The retroviral vectors are one of the most frequently utilized gene transfer vehicles in basic research. The properties described above make the retroviral vectors very versatile and efficient. Except for the major application in the gene transfer experiments, there are also many other applications. The knocking-down of gene expression is one of them. The vectors were adapted for transfer of an expression cassette coding for short hairpin RNA

which target cellular gene transcripts and are able to downregulate the gene expression (Bromberg-White et al., 2004).

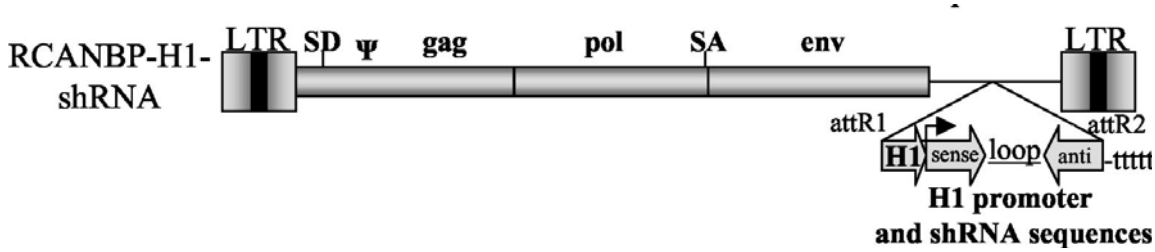

Figure 10. Retroviral vector with an shRNA expression cassette. Reproduced from Bromberg-White et al., 2004.

Another possibility is utilization of the vectors for searching for novel genes. One of the typical examples is a gene trap vector based on the ASLV-derived retroviral vector RCASBP-M2C, which has been modified so that it uses the envelope gene from an amphotropic murine leukaemia virus (Barsov & Hughes, 1996). The vector replicates efficiently in avian cells and infects, but does not replicate in, mammalian cells. The gene trap vector pGT-GFP was developed as a derivative of RCASBP-M2C, which can be used in gene trap experiments in mammalian cells. The gene trap vector pGT-GFP contains a green fluorescent protein (GFP) reporter gene. Appropriate insertion of the vector into genes causes GFP expression; this facilitates the rapid enrichment and cloning of the trapped cells and provides an opportunity to select subpopulations of trapped cells based on the subcellular localization of GFP. About 90 gene-trapped lines were generated using D17 and NIH 3T3 cells with this vector. Five trapped NIH 3T3 lines were selected based on the distribution of GFP in cells. The cellular genes disrupted by viral integration have been identified in four of these lines by using a 5` rapid amplification of cDNA ends protocol (Zheng & Hughes, 1999).

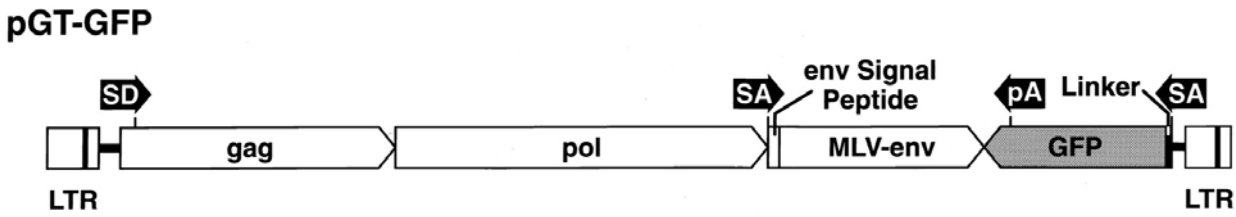

Figure 11: Retroviral vector used for gene trapping. Reproduced from Zheng & Hughes, 1999.

5.2.2. Application of retroviral vectors in gene therapy

Vast effort is directed to development of retroviral vectors suitable for gene therapy. The application of retroviral vectors is very various and often far from its natural role of gene transmitter. The vectors are used not only for therapeutic gene transfer, but also in the opposite role to suppress expression of a cellular gene responsible for the disease. The possible application also includes various strategies for HIV therapy or repair of harmfully mutated genes.

The widest use of retroviral vectors in gene therapy applications is therapeutic gene transfer. The first clinical application was performed in SCID patients. The first trials were performed already in nineties of 20th century. The subjects were patients with the ADA-SCID variant of the SCID syndrome. The patient`s lymphocytes were isolated and modified with retroviral vectors carrying the functional adenosine deaminase gene. The therapy was successful and the patient`s immune system recovered, but this approach had a major disadvantage the necessity to regularly repeat the therapy due to the loss of corrected lymphocytes, which were replaced with new non-functional ones. The extended knowledge of stem cell technologies provided the possibility to correct the haematopoietic stem cells that would produce corrected, fully functional lymphocytes. This strategy was utilised in clinical trials conducted by Alan Fischer and later by Adrian Thrasher in the gene therapy of (SCID)-X1 disease (Cavazzana-Calvo et al., 2000, Gaspar et al., 2004). The patient`s hematopoietic stem cells were isolated and transduced *in vitro* with a gammaretroviral vector carrying a functional common γC chain coding sequence. The corrected cells were returned to the patient where they colonized the bone marrow and gave birth to functional blood cells. The trial appeared to be very successful and the patients recovered well. Their lymphocytes parameters reached almost the level of healthy individuals. The result of this trial provided promise for patients suffering from SCID and other haematopoietic stem cell-involving diseases. However, in a few years the first enthusiasm was spoiled by the appearance of leukaemia in a subset of patients. This drawback led to the temporary interruption of these clinical trials and intensive investigation of adverse effects of retroviral vector integration and improvement of the vector design (Hacein-Bey-Abina et al., 2003a,b, Baum et al., 2006, Bushman, 2007, Wilson et al., 2008, see press release of December 18, 2007 [Thrasher and Gaspar, 2007]).

The use of retroviral vectors doesn`t need to be only the gene transmitter but can be used for many other applications. One possibility is to use the vectors as gene correctors.

First attempts were done by the group of Luigi Naldini. They designed an integrase-defective lentiviral vector, which carried an expression cassette of zinc-finger nuclease (ZFN) specific to IL-2 receptor common γ-chain gene (IL2RG) locus (affected in the SCID-X1 patients) and a sequence homologous to the mutated region of the IL2RG, but with correct sequence of the exon. The mechanism of this system is that the specific ZFN create a double-strand break close to the mutation of genomic sequence. This double-strand break triggers the DNA damage machinery of the cell, which tends to repair this damage by homologous recombination. At this point the rest of the lentiviral vector provides the appropriate template for the homologous recombination that leads to the correction of the mutated region. The authors observed high rates (13–39%) of the gene editing, which makes this strategy promising for use in gene therapy, but after careful assessment of the therapeutic risk of double-strand break generation in the transduced cells (Lombardo et al., 2007).

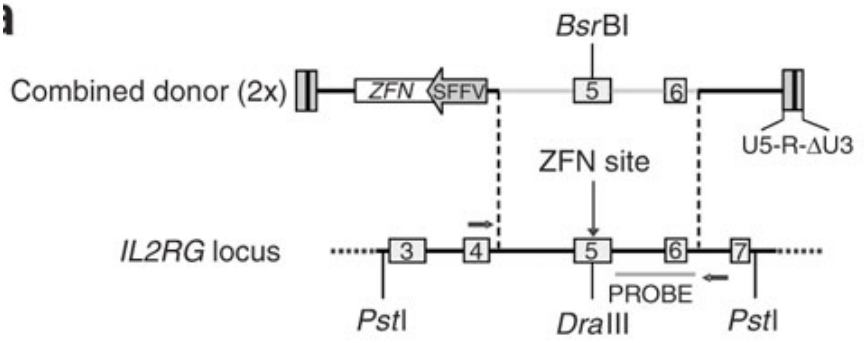

Figure 12: The lentiviral vector used for editing the IL2RG locus. Reproduced from Lombardo et al., 2007.

Many interesting examples of possible use of retroviral vectors for gene therapy lie in the field of HIV-positive patient`s treatment. The strategy can be called as a fight of lentiviruses against lentiviruses. The first example is the lentiviral HIV-1-based vector VRX496 coding for an antisense RNA directed against the envelope of HIV. The vector exhibited high transduction efficiency to $CD4^+$ T lymphocytes, and subsequently inhibited the HIV replication more than 1000-fold in *in vitro* studies (Lu et al., 2004). A similar strategy was developed by Berkhout and his collaborators in the therapy aiming at a decrease of HIV-1 expression via RNA interference. These authors designed a lentiviral vector carrying several expression cassettes for shRNA, which were specific to conserved sequences of HIV-1 genome. This specificity provided the best result of the RNA interference-mediated suppression of the HIV-1 expression. The combination of several target sites in one vector is necessary for successful antiHIV therapy, since the use of only

one effective shRNA led to appearance of escape mutants, which were not affected by the particular shRNA. When combining three or four cassettes, no escape mutants were observed (Brake et al., 2008).

Direct suppression of the HIV-1 transcription is not the only strategy ameliorating the HIV infection. A conditionally replicating HIV-1 (crHIV-1) vector was designed. The crHIV-1 vectors contain only the *cis* and none of the *trans* elements necessary for viral packaging and instead carry an antiviral gene that inhibits any of numerous wild-type HIV-1 functions. If a cell carrying an integrated copy of the crHIV-1 becomes infected with the wild-type virus, the antiviral vector payload acts to limit the production of HIV-1. Also, a fraction of virions produced from these dually infected cells carry crHIV-1 genomes, leading to further propagation of the therapy vector. crHIV-1 vectors carrying HIV-1-induced promoter have the benefit of being expressed only in HIV-1- infected cells, thereby reducing vector toxicity concerns. Importantly, crHIV-1 vectors have the potential to encode inhibitors of cellular factors necessary for HIV-1 replication. These inhibitors should be significantly less susceptible to mutational escape by HIV-1, since the virus would need to evolve away from its dependency on this cellular factor (Dropulic et al. in 1996, Perelson et al., 1996, Weinberger et al., 2003).

The most recent strategy of the HIV-1 virus treatment is focused on the eradication of integrated proviruses from the genome of the infected cell. The suggested therapy if based on the fact that Cre recombinase is able to excise sequences that are flanked by canonical loxP sites. The HIV-1 genome was scanned for sequences similar to the recognition sites for Cre recombinase. A similar site was found in the U3 region of the HIV-1 LTR and this sequence was inserted into the evolution vector, and Cre and an archive of mutagenized Cre libraries were tested for recombination activity. The Cre recombinase specific to the HIV-1 U3 region was developed and was able to efficiently excise the proviral DNA from the host genomic DNA. This recombinase (termed Tre) can be delivered into the infected $CD4^+$ cells by nonintegrating HIV-1 vectors and sweep out the HIV-1 genome from the patient`s cells. This strategy is still far from being used in clinical trials, but first steps were already done and the appropriate recombinase is already available (Sarkar et al., 2007).

6. Transcriptional silencing of retroviral vectors

A large body of evidence suggests that in every host, the machinery that is designed for biogenesis of endogenous gene products has the potential to survey its genome for the presence of exogenous genes. This surveillance system is likely to be most competent in germ cell lines. Therefore, understanding the molecular mechanisms that support the host cell ontogeny or discrimination between self and non-self genes by the host machinery is essential to understanding host retrovirus interactions. The retrovirus integrates into the host cell chromosome as a provirus, which functions as a genetic unit and is transmitted like endogenous genes to progeny cells. Therefore, retroviruses depend on the host cell machinery for their gene expression, whereas the host cell struggles to eliminate the expression of non-self genes. In recent years, the understanding of epigenetic regulation has increased dramatically, and molecular mechanisms such as DNA methylation, histone modifications, chromatin remodelling and post-transcriptional gene silencing have been reported to be important in several organisms. DNA methylation is an epigenetic modification that occurs in conjugation with provirus down-regulation (Bednarik et al., 1990, Hoeben et al., 1991) and its importance has been stressed repeatedly (Cherry et al., 2000, Yoder et al., 1997, Lorincz et al., 2000). In several cell types that exhibit retrovirus gene silencing, however, it is often unclear whether provirus methylation is the primary silencing mechanism or whether it simply reflects transcriptional repression. Furthermore, involvement of several other molecular mechanisms has been suggested (Cherry et al., 2000, Chen et al., 1997, 2000). The efficiency of retroviral vector-mediated transduction is determined by several factors. The efficiency of the host cell penetration by the vector can be significantly influenced by modification of the virus particle envelope. The other crucial step is the transport of the preintegration complex (PIC) into the nucleus and reaching the host cell genomic DNA. The PIC translocation can be promoted by the modification of the viral genome (e.g. insertion of the cPPT). However, solving the problems concerning inefficient host cell entry and integration doesn`t mean that we obtain a highly efficient retroviral vector capable to provide stable and high expression of transgenes. The majority of retroviral vectors are successfully integrated, but they are not transcribed (Mok et al., 2007). Even the vectors that establish transcription after their genome integration into the host cell DNA are frequently gradually switched off and the transgene expression is diminishing in the cell population. This phenomenon differs according to cell type, ontogenetic state of the organism, and it is also influenced by the vector used for the

transduction. The design of retroviral vectors has a major influence on the stability of expression in the target cell that is why the vector design is of special interest. Importantly, the retroviral gene silencing was detected as a reduction in the ratio of cells expressing the exogenous gene rather than a reduction in the average expression level.

6.1. Mechanisms influencing the retroviral vector transduction efficiency

There are many mechanisms that the cells use as a defence against insertion and expression of heterologous elements in the genome. They act at various levels of the transduction process. The transcriptional silencing machineries are the most important with regard to the efficient gene transduction by retroviral vectors.

The first front line of the cell defence is represented by the factor APOBEC3G (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G). This machinery does not involve epigenetic changes, but aim the retroviral genome in order to deaminate the cytosines of the nascent DNA strand. The deamination causes changes of the genetic code by conversion of cytosine to uracil, which leads to mutation or direct degradation of the DNA containing uraciles (Goff, 2003). This defence seems to be smart and very efficient, but coevolution of this mechanism with retroviruses leads to the development of the protein (Vif of HIV) that prevents integration of APOBEC3G to virus particles and also inhibits its expression (Mangeat & Trono, 2005, Sierra et al., 2005).

Other mechanisms concern factors that negatively influence the vector expression by epigenetic mechanisms and lead to the retroviral vector silencing. The proper mechanism of the vector silencing is still an issue of intensive investigation, which still resists to the scientist`s effort. Very little is known about the way how the host cell recognizes a heterologous element (retroviral vector) and specifically aim the epigenetic pathways in order to silence the element expression. The host cell struggles to eliminate the expression of non-self genes.

One of the mechanisms described is recognition of the Pro^{tRNA} primer binding site of Moloney murine leukaemia virus (MMLV) by the TRIM28 corepressor complex in embryonic carcinoma and embryonic stem cells. The binding of TRIM28 to the sequence triggers dimethylation of the lysine 9 of histone H3. This methylated histone is further recognized by heterochromatin protein 1. These events have a consequence of

heterochromatinization of the region and then loss of the retrovirus expression (Ellis et al., 2007, Wolf & Goff, 2007). The primer binding site of the MMLV was described as a negative regulatory element or repressor binding site in these cells previously (Vernet & Cebrian, 1996, Haas et al., 2003). This mechanism resolved at least part of the mechanism responsible for the MMLV silencing in embryonic carcinoma and embryonic stem cells.

Another part of the retrovirus silencing mosaic comes from analysis of ASLV-derived vector expression in mammalian cells. The ASLV-derived vectors are prone to very efficient silencing in heterologous mammalian host cells. One of the proteins suspected of having an important role in the ASLV-derived vector silencing in these cells is Daxx. Recently, the interaction of Daxx with ASLV integrase was described (Greger et al., 2005). The Daxx protein is a very important transcriptional repressor in mammalian cells (Shih et al., 2007) and the repressive effect of Daxx on the ASLV-based vector expression was also described three days postinfection (Poleshko et al., 2008). Nothing is known about the Daxx protein in avian cells. Greger et al. (2005) reported immunoprecipitation of proteins by anti-human Daxx antibodies in avian cells that exhibited sizes similar to those of mammalian Daxx. They also observed its interaction with components of the ASLV preintegration complex. Since Daxx is a highly conserved protein, it is very probable that avian cells also express Daxx. It can`t be concluded that its role in the retroviral life cycle is the same as in mammalian cells, since the avian cells do not exhibit obvious repression of the ASLV-based vector expression. As Daxx represses gene expression by recruitment of histone deacetylases 1 and 2 (Hollenbach et al., 2002) and interacts with the ASLV integrase, it seems to be a very probable candidate for a protein responsible for transcriptional silencing, which occurs immediately or very soon after the retroviral vector integration in mammalian cells. It is not clear whether Daxx protein also influences the expression of non-ASLV retroviruses.

Another possible mechanism responsible for the retroviral vector silencing in mammalian cells is DNA methylation. It was reported that endogenous retroviruses and also silenced retroviral vectors are extensively methylated (Hoeben et al., 1991, Chalita et al., 1994, Lorincz et al., 2000, He et al., 2005). The methylation pattern is not set at one distinct moment, but the genome of the silenced provirus is subjected to DNA methylation for weeks and the density of methylated CpG dinucleotides gradually increases (He et al., 2005). It implies that the DNA methylation machinery will be more probably involved in the decrease of the stability of expression and gradual silencing than in the early silencing events following integration. The aforementioned data concern the silencing of retroviral

vectors in mammalian cells. The expression of retroviral vectors of even avian or mammalian origin is very stable in avian cells. It was reported that exogenous retroviruses in avian cells are not methylated (Blazkova et al., in preparation). This corresponds to a generally low level of avian genomic DNA methylation. These data suggest that avian cells probably dispose of inefficient DNA methyltransferases or DNA methylation machinery, which is not able to reliably recognize heterologous elements and inactivate them by methylation of their genomic DNA. It also suggests that the ASLV was not subjected to selection pressure from an efficient DNA methylation antiretroviral barrier in the permissive avian cells and therefore have not evolved strategies that make them able to escape this host cell defence occurring in mammalian cells. It corresponds to the fact that the ASLV-based vectors are very efficiently methylated and silenced in mammalian cells. The progression of the silencing is much faster than in vectors of mammalian origin.

DNA methylation is not the only mark for DNA sequences that should not be expressed. The configuration of chromatin is also very important. It is partially connected to the DNA methylation, but it is also influenced by a variety of mechanisms. Very important is the so-called histone code. The histones influence the expression state of chromatin and determine the accessibility of the DNA to transcription factors and possibility to be transcribed. The acetylation of histones is the best described modification, which is responsible for decondesation of chromatin and accessibility to the transcription machinery. It was described that the locus corresponding to the silenced provirus exhibited a decreased level of histone acetylation (Lorincz et al., 2001, Yao et al., 2004, Katz et al., 2007). The predominant histone marks of silenced proviruses are deacetylation of histone H3 and dimethylation of lysines 4 and 9 of histone H3 (He et al., 2005). Association of heterochromatin protein 1 was described in silenced proviruses (Poleshko et al., 2008). Several studies described an important role of the SWI/SNF complex in retrovirus expression (reviewed in Iba et al., 2003). The silencing of retroviral vectors is a very complex issue and also additional mechanisms take part in this phenomenon. Probably all mechanisms responsible for regulation and modulation of the expression pattern of cellular genes also influence the expression of exogenously delivered retroviral sequences.

6.2. Strategies for antisilencing protection of retroviral vectors

The data mentioned in the previous chapter clearly describe the complexity of the retroviral vector silencing. The gene therapy and also many other applications need very efficient gene transfer by retroviral vectors. The phenomenon of the vector silencing dramatically reduces the gene transfer efficiency (Mok et al., 2007). This led to extensive effort towards protection of the retroviral vectors from these undesirable transcriptional silencing.

The first retroviral vectors were based on the strategy where the viral genes were replaced by the gene or genes of interest. These vectors exhibited very efficient transduction, but the integrated vectors were frequently silenced and only a small proportion of transduced cells provided a reasonable level of expression of the gene of interest. The most popular vectors were derived from the Moloney murine leukaemia virus. A certain progress in ameliorating the vector efficiency was facilitated by the discovery of a so-called “negative regulatory element” in the MMLV sequence overlapping the primer binding site (PBS). Deletion of this region increased the level of the marker gene expression (Vernet & Cebrian, 1996). Moreover, replacement of the PBS with the one of an endogenous murine retrovirus, dl587rev, which was shown to escape the PBS-associated repression by utilizing a variant PBS that has multiple base pairs divergent from MMLV and eliminates the PBS-associated repressor-binding site, increased the frequency of transcriptionally active proviruses in embryonic stem cells (Robbins et al., 1997).

One possible approach is to eliminate sequences that are able to bind repressive factors. This approach was successfully used in the MMLV vector system. However, there are major limitations arising from the need of the vector integrity, where only a limited range of sequences can be deleted or replaced by other less repressive elements. The fact that DNA methylation is one of the major factors influencing the vector transcriptional silencing led to experiments which were based on mutation of CpG dinucleotides that serve as substrates for DNA methylation. It resulted in partial stabilization of the vector expression, but this approach can`t be widely used due to the necessity of extensive sequence change, which is frequently not desirable (Chevalier-Mariette et al., 2003, Swindle et al., 2004).

It has been shown that viral promoters are very frequently prone to transcriptional silencing and are at least in part responsible for the transcriptional instability upon integration into the host genome. The promoter of retroviruses resides in the U3 region of

the LTR. The majority of the U3 region can be deleted without significant effect on the efficiency of the reverse transcription and integration of the vector. The so-called self-inactivating (SIN) vectors were constructed and the genes incorporated in the vector were driven from an inner promoter, which was usually of cellular origin. The vectors were originally constructed with the purpose of decreasing the risk of the cellular gene transactivation (see chapter 2.2. Risk of transactivation), but further experiments with these vectors revealed their increased transcriptional stability, which is due to lack of the problematic viral promoter (Mohamedali et al., 2004).

All the aforementioned strategies led to significant improvement of the efficiency of the gene transfer via retroviral vectors, but the progress was rather incremental and a distinct breakthrough was needed in the vector design. One of the most significant factors responsible for the vector silencing is the influence of the chromatin flanking the integration site. The investigation effort was focused predominantly on the possibility to protect the integrated retroviral vectors from the repressive influence of the surrounding chromatin. A promising protective element was provided from the investigation of the chicken β-globin locus. The DNA sequence at the 5` end of the chicken β -globin locus can function as an insulator. It is capable of shielding a reporter gene from the activating effects of a nearby mouse β-globin locus control region element in the human erythroleukaemic cell line K562 (Chung et al., 1997, Zhao & Dean, 2004). As it is able to protect from the activating effect, it is also probable that the element would be able to shield from the negative effect of surrounding chromatin. The element responsible for the insulator effect (cHS4) was incorporated into the retroviral vector and tested the frequency of transcritionally active proviruses upon infection both *in vitro* and *in vivo*. As expected, the frequency of active proviruses was higher in case of vectors modified with the cHS4 element (Rivella et al., 2000, Emery et al., 2000, Aker et al., 2007) and the extent of the protective effect was highly dependent on the placement of the element into the retroviral vector (Yannaki et al., 2002).

The human CD2 locus control region was also analysed and exhibited a similar favourable effect on transgene expression delivered by the retroviral vector (Indraccolo et al., 2001). A chromatin insulator from the sea urchin arylsulfatase (Ars) gene locus (Ars insulator, ArsI), which has been shown to epigenetically regulate gene expression across species, was used for protection of retroviral vectors from silencing. ArsI was able to prevent silencing of the transgene in a myeloid cell line, HL-60, and a murine embryonic stem cell line, CCE, in an orientation-dependent manner, but not in Huh-7, K562 and

MCF-7 cells, indicating that the effect of ArsI on gene silencing was cell-type dependent. However, the anti-silencing effect of ArsI was almost equivalent to that of chicken β-globin insulator (Hino et al., 2004, Yajima et al., 2007).

The search for sequence elements with potential of providing the vector with autonomous stable expression also led to the utilization of scaffold or matrix attachment regions (S/MAR). The immunoglobulin heavy chain enhancer with associated matrix attachment regions was incorporated into a lentiviral vector and the expression characteristics were tested in B lymphocytes. A position-independent transgene expression was observed in B cells transduced with the modified vector and also the level of expression was 2- to 10-fold increased (Lutzko et al., 2003). The combination of the human interferon-β scaffold attachment region (IFN-SAR) and the chicken β-globin 5` DNase I hypersensitive site 4 (5`HS4) insulator were inserted into a series of self-inactivating (SIN) lentiviral vectors. The combination of these two elements provided promising results. The 5`HS4/IFN-SAR combination was particularly effective in maintaining open chromatin domains permissive for high-level transgene expression at early and late stages of haematopoietic development, and thus could be of utility in haematopoietic stem cell-directed retroviral vector–mediated gene transfer applications (Ramezani et al., 2003).

The chromatin insulators and matrix attachment regions serve predominantly as a shield against the influence of surrounding chromatin. Another strategy arises from the stable expression of housekeeping genes, which are resistant to the silencing and DNA methylation probably due to the presence of specific sequences in CpG islands that are typical for housekeeping gene promoters. The ubiquitously acting chromatin opening elements (UCOEs) were used for antisilencing protection. The elements consist of methylation-free CpG islands encompassing dual divergently transcribed promoters of housekeeping genes that have been shown to confer resistance to transcriptional silencing and to produce consistent and stable transgene expression in tissue culture systems. To develop improved strategies for haematopoietic cell gene therapy, the potential of the human HNRPA2B1-CBX3 UCOE (A2UCOE) was assessed within the context of a self-inactivating (SIN) lentiviral vector. Unlike viral promoters, the enhancer-less A2UCOE gave rise to populations of cells that expressed a reporter transgene at a highly reproducible level. The efficiency of expression per vector genome was also markedly increased *in vivo* compared with vectors incorporating either spleen focus-forming virus (SFFV) or cytomegalovirus (CMV) promoters, suggesting a relative resistance to silencing.

Furthermore, an A2UCOE-IL2RG vector fully restored the IL-2 signalling pathway within IL2RG-deficient human cells *in vitro* and successfully rescued the X-linked severe combined immunodeficiency (SCID-X1) phenotype in a mouse model of this disease. These data indicate that the A2UCOE displays highly reliable transcriptional activity within a lentiviral vector, largely overcoming insertion-site position effects and giving rise to therapeutically relevant levels of gene expression (Zhang et al., 2007).

Similar experiments were performed by Hejnar et al., 2001. They placed an *aprt* CpG island in the proximity of the ASLV-derived vector and followed the extent of silencing upon infection of mammalian cells. The CpG island very significantly increased the number of expressing vectors and enabled stable long-term expression upon transfection. The CpG island contains three Sp1 binding sites. Their mutation markedly decreased the protective capacity of the CpG island. It points to the role of Sp1 sites in the antisilencing properties of CpG islands. Their role is also supported by the observation that the myeloproliferative sarcoma virus, a derivative of MMSV, has several point mutations in the LTR, which creates an Sp1 binding site, and is transcribed more efficiently to allow productive infection of F9 embryonic carcinoma cells. The MMSV, lacking the Sp1 binding site, is not efficiently transcribed in F9 cells (Prince & Rigby, 1991). The role of Sp1 binding sites as elements protecting from DNA methylation of CpG islands was also described (Brandeis et al., 1994).

In spite of the large progress in the retroviral vector design, driven by the need of efficient vectors for gene therapy, many questions have still to be answered and many problems solved. The areas where the debt is the largest are safety of the vectors and stability of their expression. My thesis in majority concerns the latter and contributes to the solution of this problem.

AIMS

The applications utilizing retroviral vectors for stable gene transfer still face many obstacles, which significantly decrease the efficiency of stable gene transfer via retrovectors and compromise the desired aim of the experiments or gene therapy.

My studies were focused on the efficiency of gene transfer mediated by retroviral vectors and factors that both negatively and positively influence this efficiency. The final aim was to contribute to the improvements in vector design and retrovirus-mediated gene transfer protocols.

The main theme of my PhD project was the modification of retroviral vectors to be protected from transcriptional silencing. The retroviral vectors are subject to progressive transcriptional silencing upon integration into the host genome. It dramatically decreases the efficiency of stable gene transfer especially in long-term experiments and gene therapy. Chromatin insulators and also other elements were used to improve the vector`s stability of expression, but all the modifications resulted only in partial stabilization. The ASLV-derived vectors are the most affected by the silencing machinery in mammalian cells. We have scrutinized the possibility of the ASLV-derived vector protection from the silencing by insertion of the inner element of the *aprt* CpG island. A set of eleven modified vectors were prepared and their stability has been tested under different conditions.

In the last few years, the ASLV-derived vectors have been considered as a prospective tool for gene therapy applications and also for basic research thanks to their special properties concerning use of different receptors and unique integration pattern (see Introduction). The ASLV utilise three different genetic loci for entry to the host cell (*tva, tvb, tvc*). The *tva* and *tvb* loci have already been characterised and cloned and they are already available for further experimental design, e.g. gene targeting or cellular tropism studies. The *tvc* is still missing in this list and needs to be characterised. The *tvc* locus was finely localised in the chicken genome by Elleder et al., 2004 and that is why we decided to localise and clone the particular gene responsible for the ASLV subgroup C binding.

As the *tvb* locus and corresponding receptor molecule has already been characterised, a number of questions involving its receptor function have to be elucidated. We have available an inbred chicken line (line M) that exhibits partial resistance to subgroup B, D and E ASLVs. It suggests a possible mutation that alters the virus binding

site of the molecule and influences the efficiency of infection. As the entry is one of the critical steps in the retroviral life cycle, it is very interesting to study the factors that can influence the kinetics of this process. We aimed to analyse the *tvb* locus of the chicken line M cells and find the cause of the altered efficiency of the viral entry.

The replication-defective retroviral vectors request a relatively complicated mode of their stock preparation. It is necessary to establish an appropriate packaging cell line and transfect the cell line with plasmid containing the desired provirus. I intended to establish a packaging system that would be able to produce a high-titre retroviral vector. After the establishment of the method, I aimed to construct and prepare a retroviral vector expressing the *v-src* oncogene and its inactive variant, prepare cell lines that stably express both *src* variants, and test the *src* properties in this system.

Preparation of transgenic animals is very important for basic research and also for biotechnologies. The chicken provides a very versatile tool for biotechnological production of certain proteins, which can be expressed in eggs and subsequently easily isolated. However, preparation of transgenic chickens is quite complicated due to the inaccessibility of early embryo (the laid egg contains thousands of cells). One possibility is injection of the transfection plasmid or viral vector in the subgerminal cavity. This approach produce chimeras that have to be backcrossed and rare transgenic progeny has to be searched. We investigated the possibility of gene transfer via a retroviral vector into the spermatogonia, which would be transferred into a recipient cockerel. This cockerel would produce heterozygous progeny in F1 generation and it would be much easier to find homozygous individuals in the F2 generation.

COMMENTARY TO THE RESULTS

The retroviral vectors, which are able to efficiently transfer genetic information into target cells and integrate into the host cell DNA, are very useful for many experiment settings, but even the stable gene transfer may not be sufficient. The epigenetic mechanisms play a role in the efficiency of the transferred gene expression. The expression can be stopped already at the moment of the vector DNA integration or in long-term experiments, gradual loss of expression occurs. Retroviral vectors differ in their stability of expression, but all vectors exhibit a certain extent of transcriptional silencing. That is why it is necessary to modify retroviral vectors in order to stabilize their expression in target cells. The high stability is important especially in the gene therapy applications where repeated transduction is often impossible or very complicated.

The retroviral vectors derived from ASLV are very efficiently silenced in mammalian cells. We analysed the possibility to stabilize the expression of ASLV-derived retroviral vector by an insertion of the CpG island inner element (IE). CpG islands are known to maintain adjacent promoters transcriptionally active. We constructed a 120-bp inner element of the CpG island of the hamster adenosine phosphoribosyltransferase housekeeping gene. This element was inserted into the LTR of the vector in three different positions in both sense and antisense orientation (at the beginning of the LTR, between the enhancers and the promoter, and at the beginning of the U5 region, i.e. downstream from the transcription start site). The vectors exhibited various extents of the antisilencing protection. The least stabilized were vectors with the IE element at the beginning of the LTR. The most stabilized were vectors with the IE element between the enhancers and the promoter. They exhibited only very weak silencing after three months of cultivation. We also tried to insert a tandem of two IEs between the enhancer and the promoter in the antisense orientation and we found even a more prominent anti-silencing effect. This vector provided very stable expression and exhibited almost no silencing after four months of cultivation. The orientation of the IE did not have a profound effect on the stability.

The IE comprises two highly affine Sp1 binding sites. We introduced point mutations into the Sp1 binding sites that abolished the protein binding capacity of the DNA sequence. All Sp1-mutated vectors exhibited a significantly decreased protective effect on GFP expression in mammalian cells compared to their non-mutated counterparts. Our

results show that the presence of Sp1 binding sites is important for the protective effect of the IE but at least a part of the entire anti-silencing capacity is maintained in the IE with mutated Sp1 sites. We also analysed the capacity of the silenced vectors to be reactivated by histone deacetylase and DNA methyltransferase inhibitors 5-azacytidine (5azaC) and trichostatin A (TSA), respectively. Both 5-azaC and TSA applied separately reactivated GFP expression, but the combination of both inhibitors provided the strongest effect. The majority of silenced vectors were reactivated by 5-azaC and TSA a few weeks after the infection. However, the effect of these drugs gradually decreased and after four months of cultivation, only 2 % of the vectors were reactivated by the treatment. It is probably due to the onset of an additional epigenetic mechanism keeping the silenced state of the vector. We also analysed the methylation status of the vectors and found the silenced vectors heavily methylated long time after the infection. On the contrary, the modified stable vectors, which are not subjected to silencing, exhibited only negligible DNA methylation. The situation was different soon after the infection. Even the silenced vectors were less extensively methylated than in the later passages. We therefore conclude that the silencing of integrated proviruses is associated with CpG methylation of the promoter sequence; however, there are other mechanisms of silencing that precede the onset of heavy methylation of proviral LTRs. (For more details see Senigl et al., 2008 in section "Publications")

In the second part of the project we focused on the entry process of the ASLV or ASLV-based vectors bearing their original envelope proteins. The key step of the virus entry is the binding of the viral particle to the cellular receptor. We identified the gene encoding the cellular receptor for the subgroup C of ASLV. The *tvc* locus was localised previously (Elleder et al., 2004) and we tested the BACs corresponding to the identified region. After the identification of the BAC bearing the *tvc* locus, we identified the locus by positional cloning and subsequently cloned the cDNA encoding the putative Tvc receptor. The *tvc* mRNA is 1,875 nucleotides long with a single open reading frame encoding 488 amino acids. The deduced amino acid sequence of Tvc was used in a protein-protein BLAST search for homologous protein sequences in the National Centre for Biotechnology Information protein databases, which found as the most similar proteins the human and bovine butyrophilin BTN1A1 and mouse butyrophilin BTN1A1. After the identification of the mRNA sequence, we concentrated on the thorough confirmation of the identified gene as the Tvc receptor. The cDNA was cloned to the expression plasmid under the control of the SV40 early promoter. We transfected the line L15 CEFs, which are

resistant to ASLV(C) infection, with the expression plasmid and challenged them with RCASBP(C)GFP, a replication competent ASLV-derived vector bearing the envelope of subgroup C ASLV and GFP reporter gene. The transfected cells were GFP positive. The transfection of mammalian cells also provided cells susceptible to infection with the RCASBP(C)GFP vector. The previous experiments provided sufficient data confirming the Tvc receptor function of the identified gene. However, the previous data did not provide a clear and unambiguous proof that the identified locus is the only gene encoding the functional Tvc receptor. That is why we decided to knock out the coding sequence from the genome of a susceptible avian cell line and test the susceptibility of the cells lacking the Tvc receptor gene. The chicken DT40 cell line is susceptible to the infection with subgroup C ASLV and due to the high rates of homologous recombination is suitable for targeted gene deletion. The 5` and 3` genomic regions that flank the *tvc* gene in DT40 cells were cloned and used for homologous recombination to target integration and completely delete the *tvc* coding sequence. A DT40 $tvc^{-/-}$ cell clone with a cell morphology, viability, and growth rate similar to those of parental DT40 cells was chosen for further study. Parental DT40 cells and DT40 $tvc^{-/-}$ cells were challenged with RCASBP(C)GFP (subgroup C envelope) and RCASBP(B)GFP (subgroup B envelope) ASLV-based vectors and analysed by fluorescence microscopy. Parental DT40 cells were susceptible to infection with both RCASBP(C)GFP and RCASBP(B)GFP. In contrast, DT40 $tvc^{-/-}$ cells were highly resistant to RCASBP(C)GFP but still susceptible to infection with RCASBP(B)GFP. These data confirm that the identified *tvc* is the ASLV(C) receptor. (For more details see Elleder et al., 2005 in section “Publications”)

Susceptibility to ASLV infection is not only bimodal (susceptible or resistant); it can also be quantitative. Some mutations can change the binding site of the viral receptor in a manner resulting in decreased affinity and semi-resistant phenotype. Characterization of such a semi-resistant allele of the viral receptor could provide important data concerning binding of the viral particle to the receptor molecule. Thanks to the availability of various chicken lines we have the opportunity to search lines with altered susceptibility to infection with ASLV. Detailed analysis of their receptor locuses can help us to better understand the process of the virion binding to the cellular receptor. We have found the line M to be partially resistant to the infection with subgroup B, D and E ASLVs. We analysed the sequence of the *tvb* locus and found the C125S substitution in the cysteine-rich domain 3 (CRD3). To determine the effect of the C125S substitution in the chicken Tvb receptor on susceptibility to ASLV, line M and BL CEFs were infected at low multiplicity with the

RCASBPGFP replication-competent ASLV-derived vector encoding GFP. Subsequently, the time course of infection was followed by quantifying the percentage of green fluorescent cells by flow cytometry. Line BL CEFs are susceptible to subgroup A, B, C, and D ASLVs and were used as a positive control. As expected, both line M and line BL CEFs were efficiently infected by the subgroup C vector, with almost one-half of the cells infected at one day postinfection and virtually all cells infected by day 3. A very different result was obtained when the CEFs were infected with the subgroup B vector. As expected, line BL CEFs were efficiently infected with the subgroup B vector at a rate similar to that of subgroup C vector infection. The line M CEFs were infected with the subgroup B vector much less efficiently and the virus spread through the cells very slowly. In a separate experiment, line M CEFs were infected with vectors of subgroups C, D or E and the infected cells were quantified the next day. As in the first experiment, 50% of the line M CEFs were infected with the control subgroup C vector, but only a few line M CEFs were infected with the subgroup D vector and virtually no line M CEFs were infected with the subgroup E vector. These data clearly demonstrate, at least in cultured cells, that the C125S substitution in the TvbS1 receptor resulted in lower susceptibility of line M CEFs to ASLV(B) infection, including a significant decrease in the rate of virus spread, perhaps an even lower level of susceptibility to ASLV(D) infection and virus spread, and almost complete resistance to infection with ASLV(E).

The affinity assay confirmed the previous data. Binding of the line M Tvb protein to all three ASLV glycoproteins could be detected, but with significantly lower affinities than for the wild-type Tvb: ASLV(B) at 10- to 25-fold lower affinity, ASLV(D) at 25- to 50-fold lower affinity, and ASLV(E) at barely detectable levels.

A detailed discussion concerning the possible structural and functional consequences of the C125S substitution is available in the article Reinisova et al., 2008 (included in section “Publications”).

Besides the study of the entry process and the viral receptor structure and function we were also concerned with the preparation of retroviral vectors suitable for long-term expression of the gene of interest. These vectors are very useful for certain experiments demanding long-term expression, transgenic animal preparation and also for gene therapy. We prepared vectors that expressed the v-*src* gene or its kinase-dead double Y416F-K295N mutant along with the resistance marker gene. By infection of hamster fibroblasts with these two vectors and subsequent selection with an antibiotic we prepared lines stably expressing either v-*src* or mutant v-*src*. We used these lines to study the v-*src* influence on

the c-*src* activity. Expression of the active v-*src*-induced activation of endogenous c-s*rc* and increased general protein-tyrosine phosphorylation in the infected cells. Expression of the kinase-dead mutant induced hypophosphorylation of Tyr416 of the endogenous c-s*rc*. Both activation and inactivation of c-s*rc* may be explained by direct interaction of the v-s*rc* and c-*src* that may either facilitate transphosphorylation of the regulatory Tyr416 in the activation loop, or prevent it by formation of transient dead-end complexes of the Y416F-K295N mutant with c-s*rc*. (For more details see Vojtechova et al., 2006 in section "Publications")

The aforementioned vectors were suitable for long term expression in cell lines that provide enough time for selection of transduced cells and also reselection in case of the loss of expression. These vectors are not usable for the preparation of transgenic animals via transduction of spermatogonial stem cells (SSCs) and their transplantation into the cockerels sterilized by repeated γ-irradiation. This is due to the problematic maintenance of SSCs *in vitro* and the impossibility of selection, because the prolonged cultivation can impair their ability to repopulate the seminiferous epithelium and resume the exogenous spermatogenesis. The explanted SSCs can be cultivated only few hours *in vitro*, which provides enough time only for an efficient infection. For this purpose it was necessary to prepare high-titre virus with an envelope able to infect chicken SSCs. We prepared a MLV-based retroviral vector, which is highly active in avian cells, and this vector was enveloped with the G glycoprotein of the vesicular stomatitis virus (VSV-G). This vector could be produced in a concentration higher than 10^5 FFU/ml. Thanks to the VSV-G envelope and the stability provided by the envelope, we were able to concentrate the vector particles by ultracentrifugation to the concentrations reaching almost 10^8 FFU/ml. These virus concentrations were sufficient for an efficient transduction of the explanted SSCs. We infected the short-term culture of dispersed testicular cells with the concentrated VSV-G-pseudotyped vector carrying the enhanced green fluorescent protein reported gene. The efficiency of infection and the viability of infected cells were analysed by flow cytometry. No significant CpG methylation was detected in the infected testicular cells, suggesting that epigenetic silencing events do not play a role at this stage of germ-line development. After transplantation into sterilized recipient cockerels, these retrovirus-infected testicular cells restored exogenous spermatogenesis within nine weeks with approximately the same efficiency as non-infected cells. Transduction of the reporter gene encoding the green fluorescent protein was detected in the sperms of recipient cockerels with restored spermatogenesis. Our data demonstrate that, similarly as in mouse and rat, the

transplantation of retrovirus-infected spermatogonia provides an efficient system to introduce genes into the chicken male germ line and generate transgenic chickens. (For more details see Kalina et al., 2007 in section “Publications”)

Our results demonstrate the vast versatility of retroviral vectors in the gene transfer applications, but there are still many fields for investigation and improvements. The stability of the vector expression especially in stem cells is still a matter of intensive investigation.

CONCLUSIONS

Retroviral vectors are transcriptionally unstable in mammalian cells. The ASLV-derived vectors are the most affected by silencing. We modified the vector by insertion of the CpG island inner element (IE) into the vector LTR in three different positions in either sense or antisense orientation. Each vector variant exhibited a certain extent of stabilization. The vector with insertion of a tandem of two IEs between the enhancer and the promoter was the most stable and exhibited almost no silencing after four months of cultivation in rodent and human cells. The IE comprises two high-affinity Sp1 binding sites. The presence of Sp1 binding sites is important for the protective effect of IE, but at least a part of the entire anti-silencing capacity is maintained in IE with mutated Sp1 sites.

We identified the Tvc receptor of ASLV. The *tvc* gene encodes a 488-amino-acid protein most closely related to mammalian butyrophilins, members of the immunoglobulin protein family. To confirm the identification of the Tvc receptor, we disrupted both *tvc* alleles in normally susceptible DT40 cells. The DT40 $tvc^{-/-}$ clone was resistant to the ASLV(C) infection.

We identified the mutation that results in decreased susceptibility to infection by ASLV subgroups B and D and resistance to ASLV subgroup E of line M chickens. The *tvb* gene in line M, tvb^{r2}, encodes a mutant Tvb^{S1} receptor protein with substitution of a serine for a cysteine at position 125 (C125S). The C125S substitution significantly reduces the binding affinity of the Tvb^{S1} receptor for the subgroup B, D, and E ASLV envelope glycoproteins. These are the first results that demonstrate a possible role of the cysteine-rich domain 3 in the function of the Tvb receptors.

The MLV-based retroviral vectors expressing the v-*src* and kinase-dead double Y416F-K295N mutant were constructed. They were used for the preparation of stable cell lines expressing one of the variants of the *src* gene in order to assess the effect of these mutants on the c-*src* activity. We found that expression of the active v-*src* induced activation of endogenous c-s*rc* and increased general protein-tyrosine phosphorylation in the infected cells. Expression of the kinase-dead mutant induced hypophosphorylation of Tyr416 of the endogenous c-s*rc*.

We prepared a MLV-based VSV-G pseudotyped vector capable of efficiently transducing spermatogonial stem cells (SSC). The explanted SSCs were infected with the

vector *in vitro* and subsequently transplanted into the testes of recipient cockerels sterilized by repeated γ-irradiation. The transduced reporter gene encoding the green fluorescent protein was detected in the sperms of recipient cockerels with restored spermatogenesis.

REFERENCES

Adkins, H., S. Blacklow, et al. (2001). "Two functionally distinct forms of a retroviral receptor explain the nonreciprocal receptor interference among subgroups B, D, and E avian leukosis viruses." J Virol **75**(8): 3520-6.

Adkins, H., J. Brojatsch, et al. (1997). "Identification of a cellular receptor for subgroup E avian leukosis virus." Proc Natl Acad Sci U S A **94**(21): 11617-22.

Aker, M., J. Tubb, et al. (2007). "Extended core sequences from the cHS4 insulator are necessary for protecting retroviral vectors from silencing position effects." Hum Gene Ther **18**(4): 333-43.

Akiyama, T., B. Whitaker, et al. (1994). "Tissue-specific expression of mouse tyrosinase gene in cultured chicken cells." Exp Cell Res **214**(1): 154-62.

Apolonia, L., S. Waddington, et al. (2007). "Stable gene transfer to muscle using non-integrating lentiviral vectors." Mol Ther **15**(11): 1947-54.

Azzouz, M., G. Ralph, et al. (2004). "VEGF delivery with retrogradely transported lentivector prolongs survival in a mouse ALS model." Nature **429**(6990): 413-7.

Balliet, J., J. Berson, et al. (1999). "Production and characterization of a soluble, active form of Tva, the subgroup A avian sarcoma and leukosis virus receptor." J Virol **73**(4): 3054-61.

Barnard, R. and J. Young (2003). "Alpharetrovirus envelope-receptor interactions." Curr Top Microbiol Immunol **281**: 107-36.

Baron, U. and H. Bujard (2000). "Tet repressor-based system for regulated gene expression in eukaryotic cells: principles and advances." Methods Enzymol **327**: 401-21.

Barsov, E. and S. Hughes (1996). "Gene transfer into mammalian cells by a Rous sarcoma virus-based retroviral vector with the host range of the amphotropic murine leukemia virus." J Virol **70**(6): 3922-9.

Bates, P., J. Young, et al. (1993). "A receptor for subgroup A Rous sarcoma virus is related to the low density lipoprotein receptor." Cell **74**(6): 1043-51.

Baum, C., O. Kustikova, et al. (2006). "Mutagenesis and oncogenesis by chromosomal insertion of gene transfer vectors." Hum Gene Ther **17**(3): 253-63.

Bednarik, D., J. Cook, et al. (1990). "Inactivation of the HIV LTR by DNA CpG methylation: evidence for a role in latency." EMBO J **9**(4): 1157-64.

Beyer, W., M. Westphal, et al. (2002). "Oncoretrovirus and lentivirus vectors pseudotyped with lymphocytic choriomeningitis virus glycoprotein: generation, concentration, and broad host range." J Virol **76**(3): 1488-95.

Brake, O., K. Hooft, et al. (2008). "Lentiviral vector design for multiple shRNA expression and durable HIV-1 inhibition." Mol Ther **16**(3): 557-64.

Brandeis, M., D. Frank, et al. (1994). "Sp1 elements protect a CpG island from de novo methylation." Nature **371**(6496): 435-8.

Brojatsch, J., J. Naughton, et al. (2000). "TVB receptors for cytopathic and noncytopathic subgroups of avian leukosis viruses are functional death receptors." J Virol **74**(24): 11490-4.

Brojatsch, J., J. Naughton, et al. (1996). "CAR1, a TNFR-related protein, is a cellular receptor for cytopathic avian leukosis-sarcoma viruses and mediates apoptosis." Cell **87**(5): 845-55.

Brown, B., M. Venneri, et al. (2006). "Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer." Nat Med **12**(5): 585-91.

Bushman, F. (2007). "Retroviral integration and human gene therapy." J Clin Invest **117**(8): 2083-6.

Canivet, M., A. Hoffman, et al. (1990). "Replication of HIV-1 in a wide variety of animal cells following phenotypic mixing with murine retroviruses." Virology **178**(2): 543-51.

Cara, A. and M. Klotman (2006). "Retroviral E-DNA: persistence and gene expression in nondividing immune cells." J Leukoc Biol **80**(5): 1013-7.

Cavazzana-Calvo, M., S. Hacein-Bey, et al. (2000). "Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease." Science **288**(5466): 669-72.

Cavazzana-Calvo, M., S. Hacein-Bey, et al. (2001). "Gene therapy of severe combined immunodeficiencies." J Gene Med **3**(3): 201-6.

Ciuffi, A. and F. Bushman (2006). "Retroviral DNA integration: HIV and the role of LEDGF/p75." Trends Genet **22**(7): 388-95.

Coil, D. and A. Miller (2004). "Phosphatidylserine is not the cell surface receptor for vesicular stomatitis virus." J Virol **78**(20): 10920-6.

Crittenden, L., L. Johnson, et al. (1964). "Histocompatibility and erythrocyte antigen variability within highly inbred lines of white leghorns." Transplantation **2**: 362-74.

Crittenden, L., E. Wendel, et al. (1973). "Interaction of genes controlling resistance to RSV(RAV-O)." Virology **52**(2): 373-84.

De Palma, M., M. Venneri, et al. (2003). "In vivo targeting of tumor endothelial cells by systemic delivery of lentiviral vectors." Hum Gene Ther **14**(12): 1193-206.

Delenda, C. (2004). "Lentiviral vectors: optimization of packaging, transduction and gene expression." J Gene Med **6 Suppl 1**: S125-38.

Dorner, A. and J. Coffin (1986). "Determinants for receptor interaction and cell killing on the avian retrovirus glycoprotein gp85." Cell **45**(3): 365-74.

Dropulić, B., M. Hěrmánková, et al. (1996). "A conditionally replicating HIV-1 vector interferes with wild-type HIV-1 replication and spread." Proc Natl Acad Sci U S A **93**(20): 11103-8.

Du, Z., K. Podsypanina, et al. (2006). "Introduction of oncogenes into mammary glands in vivo with an avian retroviral vector initiates and promotes carcinogenesis in mouse models." Proc Natl Acad Sci U S A **103**(46): 17396-401.

Duff, R. and P. Vogt (1969). "Characteristics of two new avian tumor virus subgroups." Virology **39**(1): 18-30.

Earp, L., S. Delos, et al. (2003). "The avian retrovirus avian sarcoma/leukosis virus subtype A reaches the lipid mixing stage of fusion at neutral pH." J Virol **77**(5): 3058-66.

Elleder, D., A. Pavlícek, et al. (2002). "Preferential integration of human immunodeficiency virus type 1 into genes, cytogenetic R bands and GC-rich DNA regions: insight from the human genome sequence." FEBS Lett **517**(1-3): 285-6.

Elleder, D., J. Plachý, et al. (2004). "Close linkage of genes encoding receptors for subgroups A and C of avian sarcoma/leucosis virus on chicken chromosome 28." Anim Genet **35**(3): 176-81.

Ellis, J., A. Hotta, et al. (2007). "Retrovirus silencing by an epigenetic TRIM." Cell **131**(1): 13-4.

Ellis, J. and S. Yao (2005). "Retrovirus silencing and vector design: relevance to normal and cancer stem cells?" Curr Gene Ther **5**(4): 367-73.

Emery, D., E. Yannaki, et al. (2000). "A chromatin insulator protects retrovirus vectors from chromosomal position effects." Proc Natl Acad Sci U S A **97**(16): 9150-5.

Faschinger, A., F. Rouault, et al. (2008). "Mouse mammary tumor virus integration site selection in human and mouse genomes." J Virol **82**(3): 1360-7.

Federspiel, M., P. Bates, et al. (1994). "A system for tissue-specific gene targeting: transgenic mice susceptible to subgroup A avian leukosis virus-based retroviral vectors." Proc Natl Acad Sci U S A **91**(23): 11241-5.

Fischer, A., S. Hacein-Bey, et al. (2001). "Gene therapy for human severe combined immunodeficiencies." Immunity **15**(1): 1-4.

Follenzi, A., L. Ailles, et al. (2000). "Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences." Nat Genet **25**(2): 217-22.

Furth, P., L. St Onge, et al. (1994). "Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter." Proc Natl Acad Sci U S A **91**(20): 9302-6.

Gaspar, H., K. Parsley, et al. (2004). "Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector." Lancet **364**(9452): 2181-7.

Gilbert, J., D. Mason, et al. (1990). "Fusion of Rous sarcoma virus with host cells does not require exposure to low pH." J Virol **64**(10): 5106-13.

Goff, S. (2003). "Death by deamination: a novel host restriction system for HIV-1." Cell **114**(3): 281-3.

Gossen, M., S. Freundlieb, et al. (1995). "Transcriptional activation by tetracyclines in mammalian cells." Science **268**(5218): 1766-9.

Greger, J., R. Katz, et al. (2005). "The cellular protein daxx interacts with avian sarcoma virus integrase and viral DNA to repress viral transcription." J Virol **79**(8): 4610-8.

Haas, D., C. Lutzko, et al. (2003). "The Moloney murine leukemia virus repressor binding site represses expression in murine and human hematopoietic stem cells." J Virol **77**(17): 9439-50.

Hacein-Bey-Abina, S., C. von Kalle, et al. (2003). "A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency." N Engl J Med **348**(3): 255-6.

Hacein-Bey-Abina, S., C. Von Kalle, et al. (2003). "LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1." Science **302**(5644): 415-9.

He, J., Q. Yang, et al. (2005). "Dynamic DNA methylation and histone modifications contribute to lentiviral transgene silencing in murine embryonic carcinoma cells." J Virol **79**(21): 13497-508.

Hejnar, J., P. Hájková, et al. (2001). "CpG island protects Rous sarcoma virus-derived vectors integrated into nonpermissive cells from DNA methylation and transcriptional suppression." Proc Natl Acad Sci U S A **98**(2): 565-9.

Hino, S., J. Fan, et al. (2004). "Sea urchin insulator protects lentiviral vector from silencing by maintaining active chromatin structure." Gene Ther **11**(10): 819-28.

Hoeben, R., A. Migchielsen, et al. (1991). "Inactivation of the Moloney murine leukemia virus long terminal repeat in murine fibroblast cell lines is associated with methylation and dependent on its chromosomal position." J Virol **65**(2): 904-12.

Hollenbach, A., C. McPherson, et al. (2002). "Daxx and histone deacetylase II associate with chromatin through an interaction with core histones and the chromatin-associated protein Dek." J Cell Sci **115**(Pt 16): 3319-30.

Hu, J., A. Ferris, et al. (2007). "Transduction of rhesus macaque hematopoietic stem and progenitor cells with avian sarcoma and leukosis virus vectors." Hum Gene Ther **18**(8): 691-700.

Hughes, S. (2004). "The RCAS vector system." Folia Biol (Praha) **50**(3-4): 107-19.

Chen, W., E. Bailey, et al. (1997). "Reactivation of silenced, virally transduced genes by inhibitors of histone deacetylase." Proc Natl Acad Sci U S A **94**(11): 5798-803.

Chen, W. and T. Townes (2000). "Molecular mechanism for silencing virally transduced genes involves histone deacetylation and chromatin condensation." Proc Natl Acad Sci U S A **97**(1): 377-82.

Cherry, S., D. Biniszkiewicz, et al. (2000). "Retroviral expression in embryonic stem cells and hematopoietic stem cells." Mol Cell Biol **20**(20): 7419-26.

Chevalier-Mariette, C., I. Henry, et al. (2003). "CpG content affects gene silencing in mice: evidence from novel transgenes." Genome Biol **4**(9): R53.

Chung, J., A. Bell, et al. (1997). "Characterization of the chicken beta-globin insulator." Proc Natl Acad Sci U S A **94**(2): 575-80.

Iba, H., T. Mizutani, et al. (2003). "SWI/SNF chromatin remodelling complex and retroviral gene silencing." Rev Med Virol **13**(2): 99-110.

Indraccolo, S., S. Minuzzo, et al. (2001). "Effects of CD2 locus control region sequences on gene expression by retroviral and lentiviral vectors." Blood **98**(13): 3607-17.

Kafri, T., H. van Praag, et al. (2000). "Lentiviral vectors: regulated gene expression." Mol Ther **1**(6): 516-21.

Kang, Y., C. Stein, et al. (2002). "In vivo gene transfer using a nonprimate lentiviral vector pseudotyped with Ross River Virus glycoproteins." J Virol **76**(18): 9378-88.

Katz, R., E. Jack-Scott, et al. (2007). "High-frequency epigenetic repression and silencing of retroviruses can be antagonized by histone deacetylase inhibitors and transcriptional activators, but uniform reactivation in cell clones is restricted by additional mechanisms." J Virol **81**(6): 2592-604.

Klein, R., B. Ruttkowski, et al. (2006). "WPRE-mediated enhancement of gene expression is promoter and cell line specific." Gene **372**: 153-61.

Klucking, S., H. Adkins, et al. (2002). "Resistance to infection by subgroups B, D, and E avian sarcoma and leukosis viruses is explained by a premature stop codon within a resistance allele of the tvb receptor gene." J Virol **76**(15): 7918-21.

Knauss, D. and J. Young (2002). "A fifteen-amino-acid TVB peptide serves as a minimal soluble receptor for subgroup B avian leukosis and sarcoma viruses." J Virol **76**(11): 5404-10.

Kobinger, G., S. Deng, et al. (2004). "Transduction of human islets with pseudotyped lentiviral vectors." Hum Gene Ther **15**(2): 211-9.

Kobinger, G., D. Weiner, et al. (2001). "Filovirus-pseudotyped lentiviral vector can efficiently and stably transduce airway epithelia in vivo." Nat Biotechnol **19**(3): 225-30.

Kostic, C., F. Chiodini, et al. (2003). "Activity analysis of housekeeping promoters using self-inactivating lentiviral vector delivery into the mouse retina." Gene Ther **10**(9): 818-21.

Kumar, M., B. Bradow, et al. (2003). "Large-scale production of pseudotyped lentiviral vectors using baculovirus GP64." Hum Gene Ther **14**(1): 67-77.

Lai, Z. and R. Brady (2002). "Gene transfer into the central nervous system in vivo using a recombinanat lentivirus vector." J Neurosci Res **67**(3): 363-71.

Lewinski, M., M. Yamashita, et al. (2006). "Retroviral DNA integration: viral and cellular determinants of target-site selection." PLoS Pathog **2**(6): e60.

Lindemann, D., E. Patriquin, et al. (1997). "Versatile retrovirus vector systems for regulated gene expression in vitro and in vivo." Mol Med **3**(7): 466-76.

Lombardo, A., P. Genovese, et al. (2007). "Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery." Nat Biotechnol **25**(11): 1298-306.

Lorincz, M., D. Schübeler, et al. (2000). "Dynamic analysis of proviral induction and De Novo methylation: implications for a histone deacetylase-independent, methylation

density-dependent mechanism of transcriptional repression." Mol Cell Biol **20**(3): 842-50.

Lorincz, M., D. Schübeler, et al. (2001). "Methylation-mediated proviral silencing is associated with MeCP2 recruitment and localized histone H3 deacetylation." Mol Cell Biol **21**(23): 7913-22.

Lu, X., Q. Yu, et al. (2004). "Antisense-mediated inhibition of human immunodeficiency virus (HIV) replication by use of an HIV type 1-based vector results in severely attenuated mutants incapable of developing resistance." J Virol **78**(13): 7079-88.

Lusso, P., F. Di Marzo Veronese, et al. (1990). "In Reply: Pseudotypes in HIV-Infected Mice." Science **250**(4984): 1153-1154.

Lusso, P., F. Lori, et al. (1990). "CD4-independent infection by human immunodeficiency virus type 1 after phenotypic mixing with human T-cell leukemia viruses." J Virol **64**(12): 6341-4.

Lutzko, C., D. Senadheera, et al. (2003). "Lentivirus vectors incorporating the immunoglobulin heavy chain enhancer and matrix attachment regions provide position-independent expression in B lymphocytes." J Virol **77**(13): 7341-51.

Mangeat, B. and D. Trono (2005). "Lentiviral vectors and antiretroviral intrinsic immunity." Hum Gene Ther **16**(8): 913-20.

Mazarakis, N., M. Azzouz, et al. (2001). "Rabies virus glycoprotein pseudotyping of lentiviral vectors enables retrograde axonal transport and access to the nervous system after peripheral delivery." Hum Mol Genet **10**(19): 2109-21.

Miletic, H., M. Bruns, et al. (1999). "Retroviral vectors pseudotyped with lymphocytic choriomeningitis virus." J Virol **73**(7): 6114-6.

Miletic, H., Y. Fischer, et al. (2004). "Selective transduction of malignant glioma by lentiviral vectors pseudotyped with lymphocytic choriomeningitis virus glycoproteins." Hum Gene Ther **15**(11): 1091-100.

Mitchell, R., B. Beitzel, et al. (2004). "Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences." PLoS Biol **2**(8): E234.

Miyoshi, H., M. Takahashi, et al. (1997). "Stable and efficient gene transfer into the retina using an HIV-based lentiviral vector." Proc Natl Acad Sci U S A **94**(19): 10319-23.

Moebes, A., J. Enssle, et al. (1997). "Human foamy virus reverse transcription that occurs late in the viral replication cycle." J Virol **71**(10): 7305-11.

Mohamedali, A., F. Moreau-Gaudry, et al. (2004). "Self-inactivating lentiviral vectors resist proviral methylation but do not confer position-independent expression in hematopoietic stem cells." Mol Ther **10**(2): 249-59.

Mok, H., S. Javed, et al. (2007). "Stable gene expression occurs from a minority of integrated HIV-1-based vectors: transcriptional silencing is present in the majority." Gene Ther **14**(9): 741-51.

Moreau-Gaudry, F., P. Xia, et al. (2001). "High-level erythroid-specific gene expression in primary human and murine hematopoietic cells with self-inactivating lentiviral vectors." Blood **98**(9): 2664-72.

Morizono, K., G. Bristol, et al. (2001). "Antibody-directed targeting of retroviral vectors via cell surface antigens." J Virol **75**(17): 8016-20.

Morizono, K., Y. Xie, et al. (2005). "Lentiviral vector retargeting to P-glycoprotein on metastatic melanoma through intravenous injection." Nat Med **11**(3): 346-52.

Mothes, W., A. Boerger, et al. (2000). "Retroviral entry mediated by receptor priming and low pH triggering of an envelope glycoprotein." Cell **103**(4): 679-89.

Narayan, S., R. Barnard, et al. (2003). "Two retroviral entry pathways distinguished by lipid raft association of the viral receptor and differences in viral infectivity." J Virol **77**(3): 1977-83.

Narezkina, A., K. Taganov, et al. (2004). "Genome-wide analyses of avian sarcoma virus integration sites." J Virol **78**(21): 11656-63.

Pandya, S., K. Boris-Lawrie, et al. (2001). "Development of an Rev-independent, minimal simian immunodeficiency virus-derived vector system." Hum Gene Ther **12**(7): 847-57.

Pani, P. (1974). "Close linkage between genetic loci controlling response of fowl to subgroup A and subgroup C sarcoma viruses." J Gen Virol **22**(2): 187-95.

Paulus, W., I. Baur, et al. (1996). "Self-contained, tetracycline-regulated retroviral vector system for gene delivery to mammalian cells." J Virol **70**(1): 62-7.

Paulus, W., I. Baur, et al. (2000). "Variability of transcriptional regulation after gene transfer with the retroviral tetracycline system." J Biotechnol **81**(2-3): 159-65.

Payne, L. and P. Biggs (1964). "Differences between highly inbred lines of chickens in the response to Rous sarcoma virus of the chorioallantoic membrane and of embryonic cells in tissue culture." Virology **24**: 610-6.

Payne, L. and P. Biggs (1966). "Genetic basis of cellular susceptibility to the Schmidt-Ruppin and Harris strains of Rous sarcoma virus." Virology **29**(2): 190-8.

Payne, L. and P. Biggs (1970). "Genetic resistance of fowl to MH2 reticuloendothelioma virus." J Gen Virol **7**(3): 177-85.

Payne LN. (1992) “Biology of avian retroviruses”, p. 299-404. In J. A. Levy (ed.), The retroviruses, vol. 1. Plenum Press, New York, N.Y.

Perelson, A., A. Neumann, et al. (1996). "HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time." Science **271**(5255): 1582-6.

Petropoulos, C., W. Payne, et al. (1992). "Appropriate in vivo expression of a muscle-specific promoter by using avian retroviral vectors for gene transfer [corrected]." J Virol **66**(6): 3391-7.

Philpott, N. and A. Thrasher (2007). "Use of nonintegrating lentiviral vectors for gene therapy." Hum Gene Ther **18**(6): 483-9.

Poleshko, A., I. Palagin, et al. (2008). "Identification of cellular proteins that maintain retroviral epigenetic silencing: evidence for an antiviral response." J Virol **82**(5): 2313-23.

Prince, V. and P. Rigby (1991). "Derivatives of Moloney murine sarcoma virus capable of being transcribed in embryonal carcinoma stem cells have gained a functional Sp1 binding site." J Virol **65**(4): 1803-11.

Ramezani, A., T. Hawley, et al. (2003). "Performance- and safety-enhanced lentiviral vectors containing the human interferon-beta scaffold attachment region and the chicken beta-globin insulator." Blood **101**(12): 4717-24.

Reinisova M, Pavlicek A, Divina P, Geryk J, Plachy J, Hejnar J (2008) “Target Site Preferences of Subgroup C Rous Sarcoma Virus Integration into the Chicken DNA.” The Open Genomics Journal **1**: 6-12

Richard, E., M. Mendez, et al. (2001). "Gene therapy of a mouse model of protoporphyria with a self-inactivating erythroid-specific lentiviral vector without preselection." Mol Ther **4**(4): 331-8.

Rivella, S., J. Callegari, et al. (2000). "The cHS4 insulator increases the probability of retroviral expression at random chromosomal integration sites." J Virol **74**(10): 4679-87.

Robbins, P., X. Yu, et al. (1997). "Increased probability of expression from modified retroviral vectors in embryonal stem cells and embryonal carcinoma cells." J Virol **71**(12): 9466-74.

Romanowski, V., Y. Matsuura, et al. (1985). "Complete sequence of the S RNA of lymphocytic choriomeningitis virus (WE strain) compared to that of Pichinde arenavirus." Virus Res **3**(2): 101-14.

Rong, L. and P. Bates (1995). "Analysis of the subgroup A avian sarcoma and leukosis virus receptor: the 40-residue, cysteine-rich, low-density lipoprotein receptor repeat motif of Tva is sufficient to mediate viral entry." J Virol **69**(8): 4847-53.

Rubin, H. (1965). "Genetic control of cellular susceptibility to pseudotypes of Rous sarcoma virus." Virology **26**: 270-6.

Saenz, D., N. Loewen, et al. (2004). "Unintegrated lentivirus DNA persistence and accessibility to expression in nondividing cells: analysis with class I integrase mutants." J Virol **78**(6): 2906-20.

Sarkar, I., I. Hauber, et al. (2007). "HIV-1 proviral DNA excision using an evolved recombinase." Science **316**(5833): 1912-5.

Shih, H., C. Chang, et al. (2007). "Daxx mediates SUMO-dependent transcriptional control and subnuclear compartmentalization." Biochem Soc Trans **35**(Pt 6): 1397-400.

Shun, M., N. Raghavendra, et al. (2007). "LEDGF/p75 functions downstream from preintegration complex formation to effect gene-specific HIV-1 integration." Genes Dev **21**(14): 1767-78.

Schlegel, R., T. Tralka, et al. (1983). "Inhibition of VSV binding and infectivity by phosphatidylserine: is phosphatidylserine a VSV-binding site?" Cell **32**(2): 639-46.

Sierra, S., B. Kupfer, et al. (2005). "Basics of the virology of HIV-1 and its replication." J Clin Virol **34**(4): 233-44.

Sirven, A., F. Pflumio, et al. (2000). "The human immunodeficiency virus type-1 central DNA flap is a crucial determinant for lentiviral vector nuclear import and gene transduction of human hematopoietic stem cells." Blood **96**(13): 4103-10.

Suzuki, Y. and R. Craigie (2007). "The road to chromatin - nuclear entry of retroviruses." Nat Rev Microbiol **5**(3): 187-96.

Svoboda, J. (1998). "Molecular biology of cell nonpermissiveness to retroviruses. Has the time come?" Gene **206**(2): 153-63.

Swindle, C., H. Kim, et al. (2004). "Mutation of CpGs in the murine stem cell virus retroviral vector long terminal repeat represses silencing in embryonic stem cells." J Biol Chem **279**(1): 34-41.

Thrasher, A., and Gaspar, B. (2007). Press release dated December 18, 2007; available at http://www.esgct.org/upload/ESGCT %20Commentary-UK-SCID-Leukaemia-18DEC2007%20(GDFINAL). pdf (accessed December 2007).

Trobridge, G., D. Miller, et al. (2006). "Foamy virus vector integration sites in normal human cells." Proc Natl Acad Sci U S A **103**(5): 1498-503.

Van Maele, B., K. Busschots, et al. (2006). "Cellular co-factors of HIV-1 integration." Trends Biochem Sci **31**(2): 98-105.

Vandekerckhove, L., F. Christ, et al. (2006). "Transient and stable knockdown of the integrase cofactor LEDGF/p75 reveals its role in the replication cycle of human immunodeficiency virus." J Virol **80**(4): 1886-96.

Vernet, M. and J. Cebrian (1996). "cis-acting elements that mediate the negative regulation of Moloney murine leukemia virus in mouse early embryos." J Virol **70**(8): 5630-3.

Vigna, E., S. Cavalieri, et al. (2002). "Robust and efficient regulation of transgene expression in vivo by improved tetracycline-dependent lentiviral vectors." Mol Ther **5**(3): 252-61.

Vogt, P. and R. Ishizaki (1965). "Reciprocal patterns of genetic resistance to avian tumor viruses in two lines of chickens." Virology **26**(4): 664-72.

Watson, D., G. Kobinger, et al. (2002). "Targeted transduction patterns in the mouse brain by lentivirus vectors pseudotyped with VSV, Ebola, Mokola, LCMV, or MuLV envelope proteins." Mol Ther **5**(5 Pt 1): 528-37.

Weinberger, L., D. Schaffer, et al. (2003). "Theoretical design of a gene therapy to prevent AIDS but not human immunodeficiency virus type 1 infection." J Virol **77**(18): 10028-36.

Weiss RA (1993) “Cellular receptors and viral glycoproteins involved in retrovirus entry”, p.1-108, In J. A. Levy (ed.) , The retroviruses, vol. 2, Plenum Press, New York, N.Y.

Wilson, J. (2008). "Adverse events in gene transfer trials and an agenda for the new year." Hum Gene Ther **19**(1): 1-2.

Wolf, D. and S. Goff (2007). "TRIM28 mediates primer binding site-targeted silencing of murine leukemia virus in embryonic cells." Cell **131**(1): 46-57.

Wu, X., Y. Li, et al. (2003). "Transcription start regions in the human genome are favored targets for MLV integration." Science **300**(5626): 1749-51.

Wu, Y. (2004). "HIV-1 gene expression: lessons from provirus and non-integrated DNA." Retrovirology **1**: 13.

Yajima, M., M. Kiyomoto, et al. (2007). "Ars insulator protects transgenes from long-term silencing in sea urchin larva." Dev Genes Evol **217**(4): 331-6.

Yannaki, E., J. Tubb, et al. (2002). "Topological constraints governing the use of the chicken HS4 chromatin insulator in oncoretrovirus vectors." Mol Ther **5**(5 Pt 1): 589-98.

Yao, S., T. Sukonnik, et al. (2004). "Retrovirus silencing, variegation, extinction, and memory are controlled by a dynamic interplay of multiple epigenetic modifications." Mol Ther **10**(1): 27-36.

Yoder, J., C. Walsh, et al. (1997). "Cytosine methylation and the ecology of intragenomic parasites." Trends Genet **13**(8): 335-40.

Young, J., P. Bates, et al. (1993). "Isolation of a chicken gene that confers susceptibility to infection by subgroup A avian leukosis and sarcoma viruses." J Virol **67**(4): 1811-6.

Zennou, V., C. Petit, et al. (2000). "HIV-1 genome nuclear import is mediated by a central DNA flap." Cell **101**(2): 173-85.

Zhang, F., S. Thornhill, et al. (2007). "Lentiviral vectors containing an enhancer-less ubiquitously acting chromatin opening element (UCOE) provide highly reproducible and stable transgene expression in hematopoietic cells." Blood **110**(5): 1448-57.

Zhao, H. and A. Dean (2004). "An insulator blocks spreading of histone acetylation and interferes with RNA polymerase II transfer between an enhancer and gene." Nucleic Acids Res **32**(16): 4903-19.

Zheng, X. and S. Hughes (1999). "An avian sarcoma/leukosis virus-based gene trap vector for mammalian cells." J Virol **73**(8): 6946-52.

Zhu, Z., S. Chen, et al. (1990). "Phenotypic mixing between human immunodeficiency virus and vesicular stomatitis virus or herpes simplex virus." J Acquir Immune Defic Syndr **3**(3): 215-9.

Zufferey, R., J. Donello, et al. (1999). "Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors." J Virol **73**(4): 2886-92.

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