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Lentiviral Gene Therapy Vectors: Challenges and Future Directions

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1. Introduction

Lentiviral vectors (LV) are efficient vehicles for gene transfer in mammalian cells due to their capacity to stably express a gene of interest in non-dividing and dividing cells. Their use has exponentially grown in the last years both in research and in gene therapy protocols, reaching 12% of the viral vector based clinical trials in 2011 [1]. This chapter reviews and discusses the state of the art on the production of HIV-1- based lentiviral vectors.

1.1. Lentiviruses

Lentiviruses are human and animal pathogens that are known to have long incubation periods and persistent infection. The time between the initial infection and the appearance of the first symptoms can reach several months or years [2]. Nowadays lentiviruses are classified as one of the seven genus of *Retroviridae* family. *Lentivirus* genus is composed by nine virus species that include primate and non-primate retroviruses (Figure 1) [3].

All Retroviruses share similarities in structure, genomic organization and replicative properties. Retroviruses are spherical viruses of around 80-120 nm in diameter [4] and are characterized by a genome comprising two positive-sense single stranded RNA. Also, they have a unique replicative strategy where the viral RNA is reverse transcribed into double stranded DNA that is integrated in the cellular genome [5]. Together with the RNA strands, the enzymes necessary for replication and the structural proteins form the nucleocapsid. The later is inside a proteic capsid that is surrounded by a double lipidic membrane [6]. Connecting the lipidic membrane and the capsid there are the matrix proteins. The lipidic membrane has its origin in the host cells and presents at surface the envelope viral glycoproteins (Env) (Figure 2).

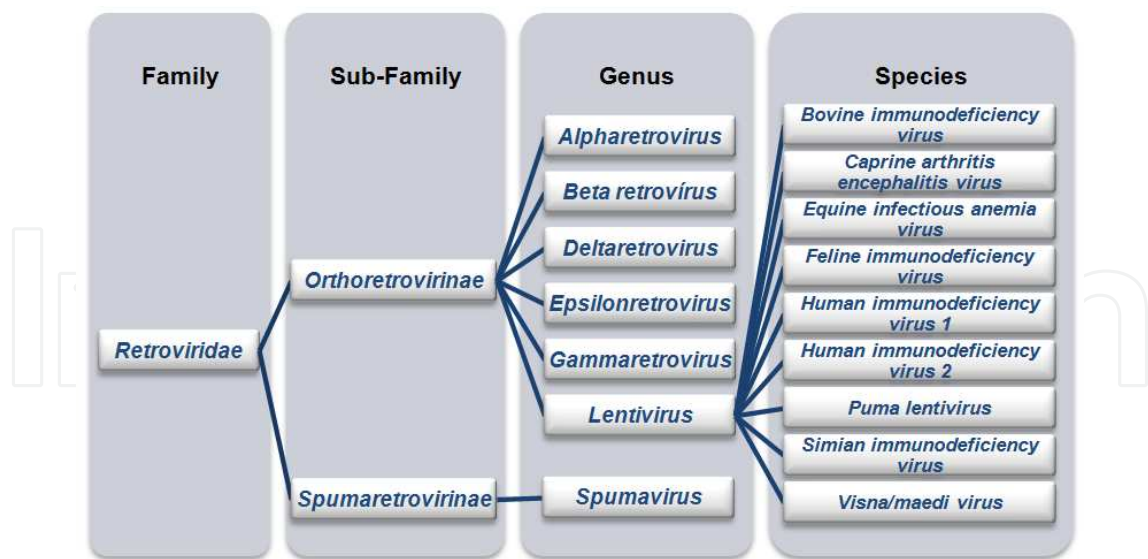


Figure 1. Lentiviruses taxonomy by the International Committee on Taxonomy of Viruses (ICTV).

Within the *Retroviridae* family, retroviruses can be classified as simple or complex. The complex retroviruses include the lentiviruses and spumaviruses presenting a more complex genome with additional regulation steps in their life cycle.

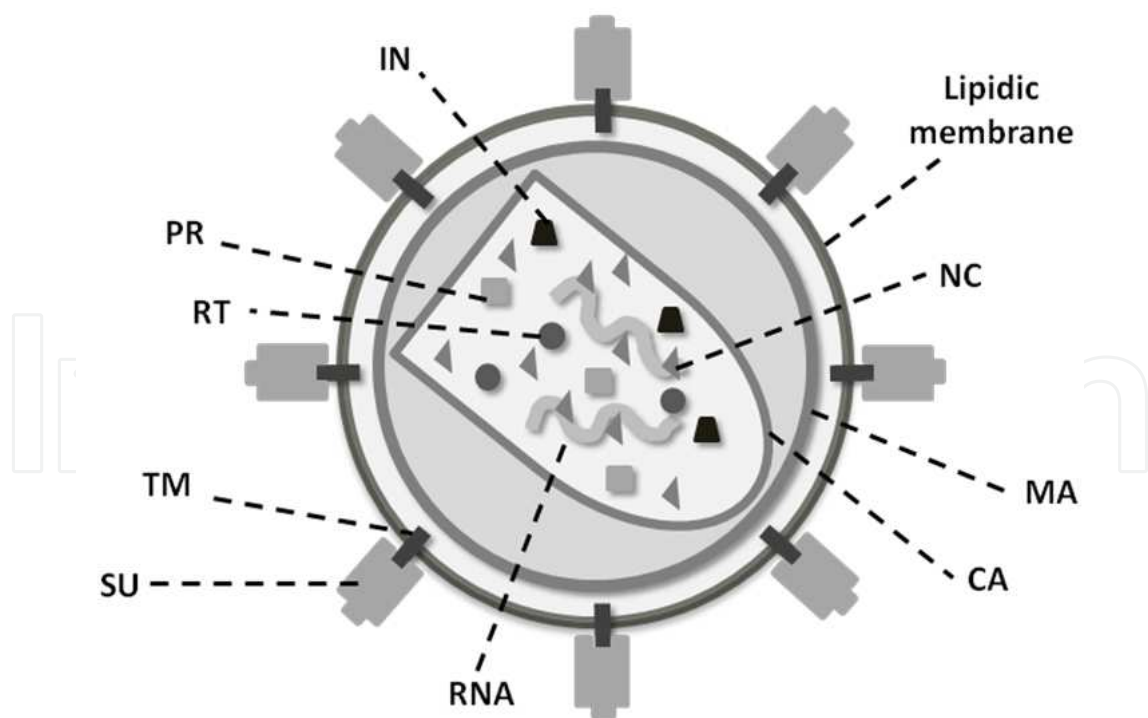


Figure 2. Schematic representation of a retrovirus particle. Abbreviations: NC – nucleocapsid; MA – matrix; CA – capsid; SU – surface subunit of Env protein; TM – transmembrane subunit of Env protein; RT – reverse transcriptase; PR – protease; IN – integrase.

1.2. HIV-1 genome

HIV-1 genome has about 9-10 kb and is constituted by several non-coding sequences that control gene expression and protein synthesis, and genes that code for regulatory and accessory proteins in addition to the structural and enzymatic genes *gag*, *pol* and *env*, common to all retroviruses (Figure 3).

The *gag* gene codes for a polypeptide that is proteolytic cleaved by the viral protease (PR) originating three main structural proteins: matrix (MA), capsid (CA) and nucleocapsid (NC). The *pro* gene codes for a polypeptide that after cleavage by PR, during the virus maturation, originates PR, reverse transcriptase (RT) and integrase (IN). These enzymes play critical roles in the life cycle of retroviruses since their functions are the cleavage of viral polypeptides (also involved in virus maturation), the reverse transcription of viral RNA to double-stranded DNA (provirus) and the integration of the provirus into the cellular genome [7]. Finally the *env* gene encodes a polypeptide that is cleaved by cellular proteases in two proteins, the SU (surface) and TM (transmembrane) subunits. Together, these two proteins are the structural units of the Env protein that will interact with cellular receptors of the host cell allowing for virus entrance into the cell [10].

Flanking the retroviral provirus there are the 5' and 3' Long Terminal Repeats (LTRs) composed by the 3' untranslated region (U3), repeat elements (R) and 5' untranslated region (U5). The LTRs contain the enhancer/promoter sequence that allows for gene expression, the att repeats important for provirus integration and the polyadenylation signal (polyA).

The HIV-1 genome also has other six genes that code for two regulatory proteins (Tat and Rev), and four accessory proteins: Vif, Nef, Vpr, and Vpu. Tat protein interacts with cellular proteins and the mRNA TAR sequence acting by increasing the viral transcription hundreds of times. Rev interacts with Rev Responsive Element (RRE), a cis-acting sequence located in the middle of the *env* gene allowing the efficient nuclear export of unspliced or singly spliced messenger RNA. The functions of accessory proteins are related with pathogenesis of the virus and they are not crucial for the viral replication *in-vitro*.

The function of all HIV-1 proteins and their interactions with the host cells are not yet clearly understood but it is already reported that there are 2589 unique HIV-1-to-human protein interactions that are formed by 1448 human proteins [8,9].

Additionally to the coding sequences, the lentivirus genome also has several non-coding cis-acting sequences that play important roles in viral replication. The LTRs contains the Transactivator Response element (TAR) for the interactions of the complex formed by the Tat protein and transcriptional factors. After the 5' LTR there are the primer binding site (PBS), where the reverse transcription starts, and the packaging signal (Ψ). Within the *pol* sequence there are also the central polypurine tract (cPPT) and the central termination sequence (CTS) contributing both for the efficient reverse transcription. Further there are the RRE in the middle of *env* gene and near the beginning of the 3'LTR the polypurine tract (PPT), a purine rich region where the synthesis of the plus strand DNA during the reverse transcription starts [10].

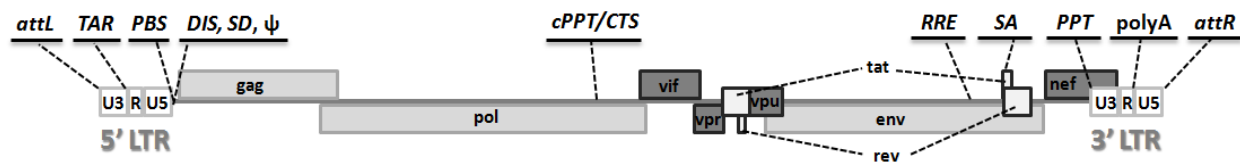


Figure 3. Schematic representation of HIV-1 provirus genome. Abbreviations: LTR - long terminal repeat; attL and attR - left and right attachment sites; U3 - 3' untranslated region; R - repeat element; U5 - 5' untranslated; TAR - transactivation response element; PBS - primer binding site; DIS - dimerization signal; SD - splice donor site; SA - splice acceptor site; ψ - packaging signal; cPPT - central polypurine tract; CTS - central termination sequence; RRE - Rev response element; PPT - 3' polypurine tract; polyA - polyadenylation signal.

1.3. HIV-1 life cycle

The HIV-1 Life cycle starts when the Env glycoproteins GP120 located at surface of the viral envelope bind the CD4 cellular receptor and co-receptor CCR5, CXCR4 or both. This binding induces conformational changes of Env glycoproteins that allows for the fusion of the viral envelope with the cell membrane and the consequent entry of the viral core into the cell. Once inside the cell the capsid starts to disintegrate and the RT enzyme begins the reverse transcription where a double-stranded proviral DNA is synthesized using one of the positive single-strand viral RNA molecules as template. When reverse transcription is completed the double-strand DNA now called provirus forms a complex with viral proteins (RT, IN, NC, Vpr and MA) and cellular proteins termed pre-integration complex (PIC) that is imported to the cell nucleus by an ATP-dependent manner. It is this energy-dependent mechanism that allows the transduction of non-dividing cells by lentiviruses, unlike γ -retrovirus.

In the nucleus the linear provirus is integrated into the cellular genome by the integrase. Now all the requisites to produce new viruses are filled and the proviral DNA is transcribed into mRNA by the cellular RNA polymerase II. Still inside the nucleus some transcripts suffer a splicing event. The mRNA transcripts are exported from the nucleus to cytoplasm to be transcribed and to start to form the viral particles; two full-length RNA transcripts will be packaged in the viral particles.

The assembly of the viral proteins and the viral RNA occurs near the cellular membrane, in specific regions called lipid-rafts that are rich in cholesterol and sphingolipids. The immature viral particles are released from cells by budding. After leaving the cells, the viral protease cleaves the Gag and Pol proteins precursors to finally generate a mature infectious virion (reviewed by [5,10]).

2. Lentiviral vector development

The development of lentiviral vectors (LVs) started in 1989 when an HIV-1 provirus with *achloramphenicol acetyltransferase* (*cat*) reporter gene in place of the non-essential *nef* gene was constructed. The transfection of Jurkat cells with this modified provirus plasmid produced infectious replicative competent viruses, very similar with wild-type HIV-1, that could be used

as a tool for study HIV infection [11]. Few months after, the same group presented the first replication-defective HIV-1 vector. In a trans-complementation assay for measuring the replicative potential of HIV-1 envelope glycoprotein mutants they used an identical HIV-1 provirus construction but with a deletion in the *env* gene. The Env glycoproteins were supplied by an independent expression plasmid. The co-transfection of these two plasmids allowed for the production of replication-defective viruses [12]. These vectors were structural identical to the wild-type virus, but lacked in their genome the *env* gene. They could only perform a single cycle of replication because their host cells, after infection, did not have the *env* gene to produce infectious virus. Although the principal aim of these studies was not the creation of viral vectors, they were the basis of lentiviral vector development, suggesting that lentiviruses could be adapted as a tool for genetic material transfer and permanent modification of animal cells.

Other preliminary studies were being conducted and several important discoveries or innovations had also contributed for the development of LVs. The introduction of the resistance marker gene *hypoxanthine-guanine phosphoribosyl-transferase* (*gpt*) under the expression control of SV40 promoter in the place of *env* gene deletion allowed the first quantification of infectious LVs produced [13]. Like it had been observed for other γ -retroviral vectors (γ -RVs) it was possible to produce infectious lentiviral particles with Env glycoproteins from other viruses (pseudotyping); for example the Moloney Murine Leukemia Virus amphotrophic envelope 4070A (A-MoMLV) [13], and Human T-cell Leukemia Virus Type I (HTLV-I) envelope [14] were successfully used suggesting that *env* gene was not necessary for virion particle formation. The localization and sequence of packaging signal was identified as the main responsible for the packaging of viral RNA [15] suggesting that modified RNAs with Ψ could also be packaged into virions. The discovery of the great stability conferred to LV pseudotyped with Vesicular Stomatitis Virus-G protein protein (VSV-G) allowed to concentrate the LV up to 10^9 by ultracentrifugation or ultrafiltration without significant loss of infectivity [16,17]. It was shown that LVs can transduce efficiently non-dividing cells, their principal advantage over the oncoretroviral vectors [16,18,19].

All these steps showed the potential of using modified lentiviruses as vectors, stimulating the iterative studies and the evolution of LVs in the next years. Their further development was based in safety principles (most of them already used in the development of oncoretroviral vectors) such as the splitting of the genome into several independent expression cassettes: the packaging cassette with the structural and enzymatic elements, the transfer cassettes with the gene of interest and the envelope expression cassette. In addition, the elimination of non-essential viral elements and the homology reduction among the expression cassettes also contributed to avoid the possibility of recombination, vector mobilization and the generation of replicative competent lentiviral vectors (RCLVs).

2.1. Four generations of packaging constructs

Four generations of LVs are currently considered; these differ from each other in terms of the number of genetic constructs used to drive the expression of the viral components, the number of wild-type genes retained as well as the number and type of heterologous *cis*-elements used to increase vector titers and promote vector safety.

The system of three expression cassettes developed in 1996 by Naldini *et al.* [16] is considered the first generation of LVs. In this system the packaging cassette has all structural proteins, with exception of Env glycoproteins, and all accessory and regulatory proteins. Later the 5' LTR was substituted by a strong promoter (CMV or RSV) and the 3' LTR by an SV40 or insulin poly(A) site to reduce the homology between the cassettes. To prevent the packaging of viral elements the Ψ and PBS were deleted. In the *env* expression cassette the gp120 from HIV-1 was replaced by other *env* genes as VSV-G or amphotrophic MLV envelope (Figure 4). Finally the transgene cassette was composed by the 5' LTR, the ψ with a truncated *gag* gene, the RRE cis-acting region and the gene of interest under the control of a heterologous promoter (usually CMV) and the 3'LTR [16,20]. This system allowed in an easy way to achieve good titers but its level of safety was not very high. RCL could be generated just with three recombination events by homologous recombination between the viral sequences in all cassettes or endogenous retroviral sequences in cells. In order to improve the safety and decrease the cytotoxicity of LVs, the three plasmid system was maintained, but all accessory genes not required for viral replication *in vitro* (*vif*, *vpr*, *vpu*, and *nef*) [21] were removed without negative effects on vector yield or infectivity. And in this way the second generation of LVs was developed (Figure 4) [22–25]. In the second generation, if by chance some RCL was generated, it would be unlikely to be pathogenic [26]. However the number of homologous events to generate RCL was the same as in the previous generation.

Reducing the lentiviral sequences by eliminating the *tat* and place the *rev* in an independent plasmid was the further step that originated the third generation of LV [27]. The *tar* sequence was replaced by a strong heterologous promoter. Therefore Tat protein was no longer necessary to increase the transgene transcription and *tat* gene was eliminated. This contributed for the reduction of lentiviral elements in the constructs. Rev was placed in an independent non-overlapping plasmid increasing the safety since now four events of homologous recombination were required for RCL formation [27]. With these new features, the vectors of third generation (Figure 4) presented a higher level of biosafety and, as the titers did not decreased, their use was widespread. Today they are the most commonly used LVs.

Although the formation of RCL was improbable, homologous recombination between the constructs was still possible since RRE sequence and part of packaging sequence in *gag* gene was in both transfer and structural packaging constructs. To solve these problems other solutions were developed originating the fourth generation of LV. The first approach used consisted in the replacement of the RRE sequences by heterologous sequences with similar functions that do not need the Rev protein. Some of these sequences were the Mason-Pfizer monkey virus constitutive transport element (CTE), the posttranscriptional control element (PCE) of the spleen necrosis virus and the human nuclear protein Sam68 [28–31]. The heterologous sequences increased the stability of the transcripts allowing their nuclear export. However the titers have decreased.

In 2000 a different approach based on codon optimization was implemented in lentiviral vector design [33]. This approach consists in perform silent mutations, changing the codon that codes for a certain aminoacid for other that codes for the same aminoacid, in principle, with a higher intracellular availability [32]. Applying this to the packaging and transfer con-

structs the homology between them was eliminated. These changes also allowed an independent expression of Rev since the sequences with suboptimal codon usage in HIV-1 genome, that conferred RNA instability and consequently lower expression, disappeared [32]. In the fourth generation (Figure 4) the homology between constructs were severely reduced but the titers had also been affected comparing with systems with the Rev/RRE [32]. Also, with the independence of Rev/RRE system, the level of biosafety decreased since the number of homologous recombination events for RCL formation was again three. Maybe due to these drawbacks the fourth generation has not been extensively used. However the codon optimization technology had been used to decrease the homology between sequences, improve the expression of viral components and viral titers [33].

Regarding the biosafety concerns about RNA mobilization and the possibility of generating RCLs, other improvements in packaging constructs were used and tested in transient LVs productions. These improvements relied on the concept of *split-genome* used for retroviral and lentiviral vector development but this time applied to the packaging construct. The *gag-pol* sequences were divided by two or three independent expression cassettes, disarming the functional *gag-pol* structure that is essential for vector mobilization [34]. In these systems additional recombination events between the several expression cassettes are necessary to generate RCLs which seems to contribute to a significant decrease of recombinant vectors formation with a functional *gag-pol* structure [35,36]. Although this increased LV safety the transduction efficiency and the LV production are challenged by the higher number of plasmids required [37].

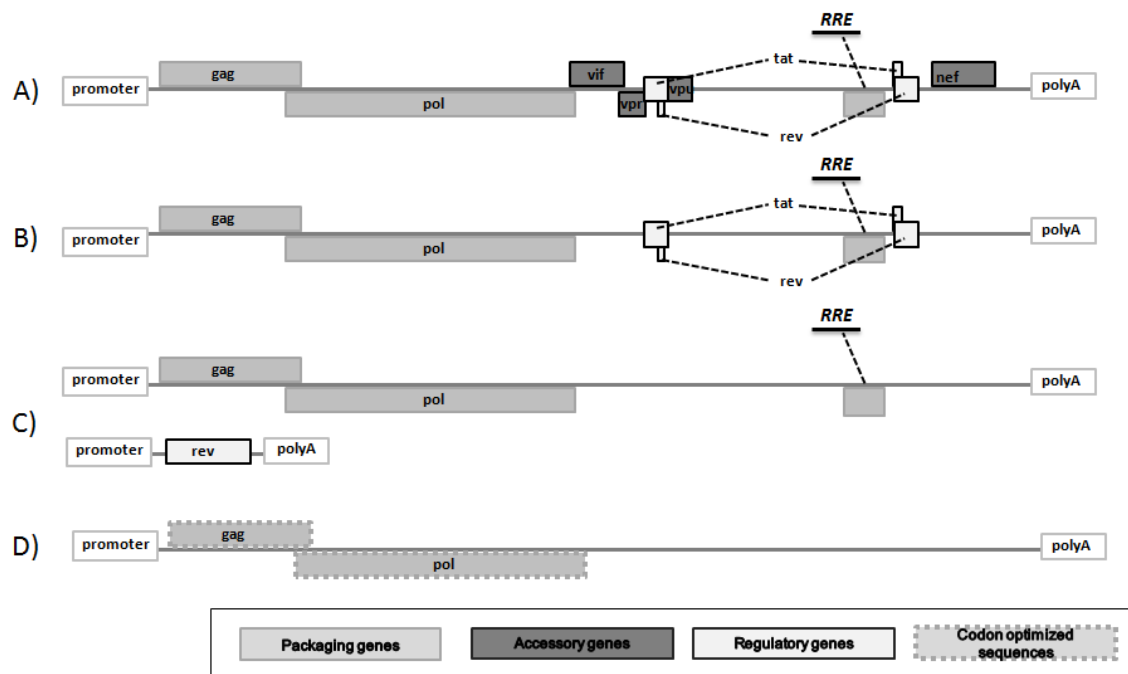


Figure 4. Schematic representation of the four generations of lentiviral packaging constructs: A) First generation packaging vector. B) Second generation packaging vector. C) Third generation packaging vector. D) Fourth generation packaging vector.

2.2. Transfer vector

The transfer vector is the expression cassette of the transgene that will be packaged into the viral vector and integrated in the cellular genome of the target cells. Besides the gene of interest and the commonly heterologous promoter for transgene expression, the transfer expression cassette must have: the sequences responsible for the expression of the full-length transcript and its packaging into the newly formed virions in the producer cells; the sequences that will interact with viral and cellular proteins to allow an efficient reverse transcription, transport into the cellular nucleus and proviral integration into target cells genome. Despite the simple design and the lack of sequences that code for viral proteins, the transfer vector also evolved over the time. This evolution was primarily focused on safety by reducing and replacing the viral sequences by heterologous elements and in optimizing both safety and efficiency by the addition of several *cis*-acting elements to the transfer cassette [10].

The transfer vectors usually used in the first and second generation of packaging constructs LVs were composed by the 5' LTR which include the TAR sequence, the PBS, the SD, the Ψ , the 5' part of *gag* gene, the RRE sequence, the SA, an heterologous promoter followed by the gene of interest, the PPT and polyA within the 3' LTR. The first hundreds of base pairs of *gag* are included after the packaging signal to increase the packaging efficiency (Figure 5). To avoid *gag* translation the initiation codon is usually mutated or cloned out of frame [16,20]. However, like it was previously found for γ -RVs, this transfer vector design with both wild-type LTRs can lead to integration genotoxicity and facilitates the mobilization of the transgene in the case of posterior infection of transduced cells [38]. To overcome these biosafety problems the LTRs of the transfer vector suffered additional changes. One of the first modifications was the replacement of the enhancer/promoter and Tar sequence of the 5' LTR by a strong heterologous promoter allowing the transcription of the full-length viral RNA in a Tat-independent manner [25]. In addition the wild-type enhancer/promoter sequences in the U3 region of the 3' LTR were deleted originating the self-inactivating (SIN) LVs [27,39,40], as it had already been done for γ -RVs [41].

The SIN design (Figure 5) generates in the target cells a proviral vector without enhancer/promoter sequences in both LTRs. In producer cells the packaged RNA transcript does have the heterologous promoter in the 5' end. Afterwards, in the target cells, during the reverse transcription, the U3 region of 3' LTR is copied and transferred to the 5' LTR. This transcriptional inactivation offered by the SIN design presents several safety advantages: prevents the formation of potentially packageable viral transcripts from the 5' LTR and consequently prevents vector mobilization by prior infection with a replicative retrovirus [39,42]; reduces the risk of insertional mutagenesis induced by the transcriptional interference of the LTRs in the neighboring sequences that can lead to the activation or up-regulation of oncogenes [43]; and lowers the risk of RCL formation by the reduction of the sequences with homology with wild-type virus.

The adoption of SIN design did not affected LV production as it happened with γ -RVs [27,39,40,44]. However both LVs and γ -RVs displayed high frequencies of read-through of the 3' polyadenylation signal which can lead to the transcription of cellular sequences as oncogenes. This inefficient termination of transcription could suggest that some of the enhanc-

er/promoter sequences deleted can have a role in an efficient transcription termination [45]. In this context several improvements were done by the addition of heterologous elements to increase safety, expression and efficacy of LVs: heterologous polyadenylation signals in the 3'LTR could increase the efficiency of LVs and are particularly beneficial in the case of SIN LVs avoiding the read-through of cellular genes [40,46]; the chromatin insulators as the chicken hypersensitive site 4 (cHS4) sequence core from the β -globin locus control region (LCR) can reduce the interference from the neighboring regions in the vector and transgene expression [48]. Also these can improve the LV safety avoiding the full-length vector transcription or reducing long-distance effects of the integrated transgene promoter on neighboring cellular genes in the target cells. Additionally to the increased safety, insulators can help to maintain the gene expression over time preventing transcriptional silencing events in both producer and target cells [47–49]; incorporation of certain post-transcriptional regulatory elements (PRE) like the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) near the 3' untranslated region can also decrease the read-through in SIN vectors increasing the transgene expression and viral titers, [50–53]. The firsts WPRE sequences used contained part of a sequence that codes for a protein (WHV X) that has been reported a few times as related with carcinoma formation, posing safety concerns. However a further improved WPRE was created without this potential harmful sequence [54]; The cPPT sequence contributes for efficient reverse transcription and the proviral nuclear import processes. Although this non-essential sequence was not used in the firsts transfer vectors, its re-insertion increased the gene transfer efficiency [55–57].

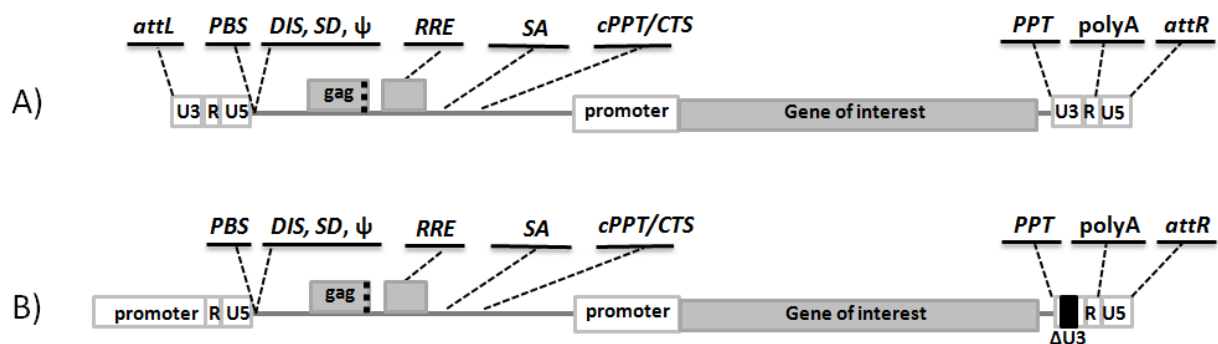


Figure 5. Schematic representation of a non-SIN transfer vector (A) and a SIN transfer vector (B).

3. Pseudotyping

LVs, as other retroviral vectors, can incorporate in their viral particles Env glycoproteins from other enveloped viruses, a feature denominated pseudotyping. This was firstly demonstrated for the HIV-1-based lentiviral vector using a Moloney Murine Leukemia Virus amphotropic envelope 4070A (A-MoMLV) [13] and an Human T-cell Leukemia Virus Type I (HTLV-I) envelope [14].

In general the pseudotyped LVs have the tropism of the virus where glycoproteins are derived from, but there are some exceptions such as the glycoprotein of the Mokola virus, where the pseudotyped vectors did not presented the specific neurotropism of the parental virus [58]. This ability of LVs to be pseudotyped showed to be advantageous since several glycoproteins could be tested to improve the transduction of cells with different receptors. As an example, HIV-based LVs pseudotyped with glycoprotein derived from the Rabies virus PV strain exhibited a great efficiency and neuronal tropism among the tested envelopes [59].

In addition to the tropism of LVs, the Env glycoproteins also affect vector structure and stability, the interactions with the target cells and the LV behavior during the infection. One example is LVs pseudotyped with rabies virus glycoprotein which allow for the retrograde axonal transport and access to the nervous system after peripheral infection [60]. Another example is the stability conferred to LVs by the VSV-G glycoproteins. The VSV-G glycoproteins are one of the most used Env proteins due to their wide tropism, with high titers achieved, great stability and resistance conferred to the LVs that allows for their concentration by ultracentrifugation. In addition they resist to freeze-thaw cycles, an important factor for storage of the vectors [16,18,19,61]. Despite these positive characteristics, the VSV-G protein is toxic for producer cells if expressed constitutively [17] and is inactivated by human serum complement [62], although this inactivation can be minimized using VSV-G conjugated with poly(ethylene glycol) [63].

Up to the present, several glycoproteins were used to pseudotype LVs (Table 1) each one presenting specific advantages and disadvantages that also depend on the LV application.

Although LVs pseudotyped with different Env glycoproteins present different tropisms, being some tropisms more selective than others, in general these are not specific for a particular cell type as happens with HIV-1 glycoproteins [80,81]. For instance, the Ebola Zaire (EboZ) glycoprotein seems to be superior to other glycoproteins in the transduction of apical airway epithelia [72]. However also has been shown to transduce liver, heart, and muscle tissues [82].

This lack of specificity is not ideal from a clinical point of view, especially for *in vivo* gene therapy applications since it can lead to the infection of cells that do not need to be transduced [83].

Several strategies have been used to increase the specificity of infection in order to retarget the LVs to a cell of interest. These strategies consisted in genetic engineering of virus envelopes by deletion of some domains or fusing molecule-ligands (growth factors, hormones, peptides or single-chain antibodies) in several locations of the viral glycoproteins. The purpose is to choose cellular receptors specifically expressed on the target cells that will interact with the chimeric glycoproteins, restricting this way the vector tropism. A successful example was the removal of the heparan sulfate binding domain from the Sindbis virus envelope protein which effectively restricted the tropism of pseudotyped LVs to dendritic cells. This genetic modified Env protein interacts solely with the C-type lectin-like receptor almost exclusively on primary dendritic cells unlike its natural counterpart [84].

Species/Envelope	Vectors	Comments	References
Vesicular stomatitis virus (VSV-G)	HIV-1	Very wide tropism. Presents resistance to high-speed centrifugation.	[16][64][65][66][66][66][67 - 67][67 – 69]
	HIV-2		
	FIV	Cytotoxic for producer cells if expressed constitutively.	
	EIAV		
	SIV	Susceptible to complement-mediated degradation which can be minimized by PEGylation	
	BIV		
	JDV		
CAEV			
Feline endogenous retrovirus (RD114)	HIV-1	More efficient and less toxic than VSV-G in cells of the hematopoietic system	[70][71]
	SIV		
Ebola	HIV-1	Efficiently transduces airway epithelium	[72]
Lymphocytic choriomeningitis virus (LCMV)	SIV	Low toxicity	[73]
	HIV-1		
	FIV		
Rabies	HIV-1	Rabies confers retrograde transport in neuronal axons	[24]
Mokola	EIAV	Mokola selectively transduces RPE upon subretinal injection	[24][74]
Ross River virus	HIV-1	Transduces hepatocytes, glia cells and neurons	[75][76]
	FIV		
Sindbis virus	HIV-1	pH-dependentendosomal entry. Useful for vector targeting	[77]
Influenza virus hemagglutinin	HIV-1	Transduces airway epithelium	[72]
Moloney murine leukemia virus 4070 envelope	HIV-1	Able to transduce most cells	[18][16]
	SIV		

Table 1. Lentiviral Vectors pseudotyped with various heterologous viral glycoproteins. Adapted from [78,79].

The envelope proteins engineered by fusion of natural ligands were in general able to bind to target cells. However the fusion domain of Env resulted generally in low vector titers since the ligand inhibits the fusogenic properties of the Env protein that allows for viral entry [85]. This approach seems to be more challenging but there are already improvements. One example is the LV pseudotyped with a chimeric glycoprotein of Sindbis virus covalently linked with mouse/human chimerical CD20-specific antibody which resulted in specific and stable transduction of CD20+ human lymphoid B cells. In this case the membrane fusion is triggered by the glycoprotein, in a pH-dependent manner, and it happens inside endocytic vesicles formed after antibody binding [86].

Other glycoproteins and ligands are being tested and used as well as alternative strategies to improve infection specificity of LVs [87–91].

4. Lentiviral vector production

The continuous research in LV development in the last twenty years and the acquired knowledge from the previous development of γ -RVs allowed the production of LVs with a significant biosafety level. However to apply LVs to clinical use they need to be easily and inexpensively produced and purified at a large-scale since, high concentrations of lentiviral particles are usually needed for efficient gene transfer in primary cells and the treatment of a single patient may require several liters of viral supernatant [92,93]

For large-scale and clinical-grade LV productions, a stable LV producer cell line seems to be the best approach for increased safety and well characterized production process. However, unlike γ -RVs, the development of LV packaging cell lines has been more challenging because of the cytotoxicity of some viral proteins like Tat, Nef, Vpr, Rev and PR [94]. Also VSV-G envelope, the typically envelope of choice for LV production because of its wide tropism and stability conferred to viral particles, is toxic for the producer cells. The VSV-G envelope can however be replaced by other non-toxic envelopes as the feline endogenous virus RD114 or the amphotropic MLV 4070A Env glycoproteins [33,95] and thus among the cytotoxic lentiviral proteins just the protease is still necessary for lentiviral vector production with the current packaging systems [93].

HIV protease mediates its toxicity *in vitro* and *in vivo* by cleaving procaspase 8, originating the casp8p41 fragment. This fragment induces mitochondrial depolarization leading to mitochondrial release of cytochrome C, activation of the downstream caspases 9 and 3 and nuclear fragmentation [96–98]. This cytotoxicity has hampered the development of stable cell lines.

The most used cells for LV production are the human embryonic kidney HEK-293 cell line and its genetic derivatives the 293T (expressing the SV40 large-T antigen) and 293E (expressing the Epstein-Barr virus nuclear antigen-1, EBNA-1) cell lines. For clinical application human 293 and 293T cells have been the exclusive cell substrates [93]. Both cell lines can be used to produce LV in adherent systems and both can be easily adapted to serum-free suspension cultures. The 293T cells are most widely used because presents superior LV productivities compared with HEK-293 cells. However the HEK-293 cell line may have an advantage in terms of safety as it lacks the SV40 large T antigen encoding gene (expressed in 293T cells) which is oncogenic [93,99,100]. In some research works other human or monkey derived cells have been used (other 293 derived clones, HeLa, HT1080, TE671, COS-1, COS-7, CV-1), although most of them showed lower LV titers [101]. However, COS-1 cells have shown to be capable of producing 3-4 times improved vector quality (expressed in infectious vector titer per ng of CA protein, p24), comparing with 293T cells, under serum-containing conditions [102].

4.1. Transient lentiviral vector production

Commonly LVs are produced by co-transfecting cells with the several expression cassettes harboring the transgene and the viral elements using chemical agents (e.g. calcium phos-

phate or polyethylenimine) and after 24 to 72 hours the LV are harvested [93]. This production system is fast and can be easily adapted to produce LVs with new genes of interest or with other Env glycoproteins. It is a simple process to apply at small scales commonly used in research, unlike the laborious development of a packaging cell line. However transient production is not the ideal choice for large and clinical LV productions since it is difficult to scale-up and requires large amounts of Good Manufacturing Practices (GMP) grade plasmid expressions cassettes turning the production more expensive [93,103]. In addition, transient LV production brings some biosafety problems like recombination between expression cassettes that could originate or facilitate the RCL formation. The recombination can occur in the mixture of transfection, inside the producer cells or during reverse transcription in the target cells since, generally after transfection cells have several copies of the plasmids which can contribute for the co-packaging of RNA transcripts [33,104]. Also batch to batch variability is common in transient productions since a population of transfected cells that expresses viral elements from episomal cassettes is generated. This can further complicate biosafety validation steps.

Nevertheless transient LV production is commonly used and recently it was shown that high titers of HIV-based LVs for clinical applications can be obtained by transient calcium phosphate transfection at large-scale under GMP conditions (Table 2) [99].

Cell origin	Vector	Packaging generation	Envelope	Maximal titers (I.P./ml)	Observations	Reference
293 E	SIN HIV-1 based	3 rd	VSV-G	1x10 ⁶	PEI-mediated transfection	[107]
HEK293	HIV-1 based	3 rd	VSV-G	1x10 ⁸	PEI-mediated transfection	[101]
293T	HIV-1 based	3 rd	VSV-G	2x10 ⁹	Transfection with calcium phosphate	[99]
293T	HIV-1 based	3 rd	VSV-G	1x10 ⁸	Transfection by Flow Electroporation	[105]

Table 2. Transient LV productions. In this table they are presented several features of recent lentiviral productions in a transient manner.

There are several transfection agents that can be used to transfect mammalian cells as calcium phosphate, polyethylenimine (PEI) and cationic molecules (such as LipofectAMINE® and FuGENE®). For large scale only Ca-phosphate and PEI are used since the others are much more expensive. Both reagents are efficient but PEI is usually preferred since Ca-phosphate efficiency is highly sensitive to pH variations and can require serum or albumin to reduce Ca-phosphate cytotoxicity, unlike PEI [93]. However their use can raise some purity problems and can be cost-ineffective. Recently a method that does not use chemicals for transfection, flow electroporation, was used for transiently LV production at

large-scale [105]. The electroporation systems are normally used to transfect small volumes but flow electroporation addresses this limitation by continuously passing the desired volume of a cell and DNA suspension between two electrodes [106]. The procedure can be effectively scaled up for large bioprocessing avoiding additional costs and purification problems (Table 2) [105].

4.2. Stable lentiviral vector production

To overcome the biosafety problems in LV transient productions, inducible packaging cell lines have been developed (Table 3). The development of these systems is more time-consuming since after insertion of each expression cassette the population of stably transfected cells is usually screened for the best producer clone, like for γ -RVs, to maximize the LV production. However, these packaging cell lines are derived from one clone, therefore all the cells have the same growth and LV production behavior being the LV productions reproducible. This allows the generation of GMP cell banks, increasing safety conditions.

Cell origin	Vector	Packaging generation	Envelope	Maximal titers (I.P./ml)	Observations	Reference
293T	HIV-1 based	2 nd	VSV-G	1x10 ⁷	Tet-off	[108]
293T	HIV-1 based	3 rd	VSV-G	1.8x10 ⁵	Ecdysone inducible system. Codon-optimized gag-pol	[109]
293T	SIV-based	3 rd	VSV-G	1x10 ⁵	Ponasterone inducible system. Codon-optimized gag-pol	[110]
293T	HIV-1 based	2 nd	VSV-G	3x10 ⁵	Tet-off. Codon-optimized gag-pol	[103]
293T	HIV-1 based	3 rd	VSV-G	3.4x10 ⁷	Tet-on	[111]
293T	EIAV based	3 rd	VSV-G	7.4x10 ⁵	Tet-on	[112]
293T	SIV-based	3 rd	VSV-G	5x10 ⁷	Introduction of vector by concatemeric array transfection. Tet-off	[113]
293T	HIV-1 based	2 nd	Ampho GaLV RDpro	1.2x10 ⁷ 1.6x10 ⁶ 8.5x10 ⁶	Continuous system. Codon-optimized gag-pol	[33]

Table 3. Lentiviral vector packaging cell lines. In this table they are presented several features of available packaging cell lines for LV production.

In conditional packaging cell lines the expression of cytotoxic proteins is under control of inducible promoters and the number of cells and growth conditions can be controlled, starting the LV production at a defined moment by adding an inducer or removing the suppressor from the culture medium. Originally the titers were low but further improvements in the expression cassettes design and optimization of the induction parameters led to similar levels of transient productions. However, such systems can only produce LV for a few days because of the activity of the cytotoxic viral proteins. In addition these packaging cells have often shown to be instable due to leaky expression of the cytotoxic viral elements that are under control of the inducible promoters and the need to add an inducer to the medium in some systems can add further difficulties to the purification process [93].

In 2003 Ikeda and co-workers have reported the development of a non-inducible packaging cell line that continuously produces LV for three months in culture (Table 3). However, significant titers could only be obtained after MLV-based vector transduction. This procedure raises serious problems from the biosafety point of view, since it increases the chances of RCL by homologous recombination, posing further concerns of co-packaging [37]. Nevertheless it was shown that it is possible to establish a cell line that can continuously produce LV although, until now no additional reports for this system appeared.

5. Lentiviral vector applications

Lentiviral vectors have emerged as powerful and versatile vectors for *ex vivo* and *in vivo* gene transfer into dividing and non-dividing cells. The particular characteristics of LVs allied to their marked development during the last years have triggered the attention of different fields, consequently a vast range of applications for these vectors, from fundamental biological research to human gene therapy have appeared. One of the applications of LVs is in genome-wide functional studies. The combination of synthetic siRNAs (small interfering RNA) or shRNAs (short hairpin RNAs) that can suppress the expression of genes of interest in mammalian cells [114], with engineered LVs allowed the formation of libraries like the Netherlands Cancer Institute (NKI) libraries, the RNAi consortium (TRC) libraries, the Hannon-Elledge libraries, and the System Biosciences (SystemBio) libraries for high-throughput loss-of-function screens in a wide range of mammalian cells [115]. For example, the TRC shRNA library has nearly 300,000 shRNAs targeting for 60,000 human and mouse genes [116]. The ability of LVs to achieve stable high-efficiency gene silencing in a wide variety of cells including primary cells, that are difficult to transduce, or non-dividing cells such as neurons thus greatly expanded the possibility of the RNAi screens [117].

Other application for LVs is in animal transgenesis. Genetic-modified animals can be created by infection of fertilized or unfertilized oocytes, single-cell embryos, early blastocysts, embryonic stem cells or by transduction of cells that are used as donors of nucleus for somatic cell nuclear transfer (SCNT) [10]. These animals (transgenic mice, rats, pigs, cows, chicken, monkeys) are used to understand gene function or biological processes, for validation of drug targets, for production of human therapeutic proteins and as preclinical models for human diseases [118].

Lentiviral vectors are being increasingly used for the cell genetic modification leading to cell-engineering applications. Stable gene transduction can be used for *in vivo* imaging of vector infected cells. *In vivo* imaging studies of cells, including stem cells, have become increasingly important to understand cell distribution, differentiation, migration, function, and transgene expression in animal models. As an example, LVs expressing the firefly luciferase gene were used to monitor human embryonic stem cell (hESC) engraftment and proliferation in live mice after transplantation [119]. LVs can also be used to cellular reprogramming of somatic cells. More specifically, the promising induced pluripotent stem cells (iPS) can be generated from a somatic cell by transduction of four key transcription factors, Oct4, Sox2, Klf4, and c-Myc, using LVs [120,121]. iPS can be used to study stem cell biology, as a cellular platform for pharmacological and toxicological [122] and are considered a possible source of autologous stem cells for use in regenerative medicine. LVs also have been used in biotechnology to engineer cell lines for the production of proteins of interest [123].

The main goal of LV technology is their use in clinical gene therapy applications. Within this purpose considerable efforts have been made to increase the safety and efficacy of LVs. Proof-of-concept has been established in preclinical animal models since several research groups have reported that LVs could treat or cure a disease including β -thalassaemia [124], sickle cell anemia [125], hemophilia B [126] and ζ -chain-associated protein kinase of 70 kDa immunodeficiency [127]. Moreover, improvements in other genetic disorders like Parkinson's disease [128], cystic fibrosis [129] and spinal muscular atrophy [130] have been reported.

LVs have more recently moved beyond the preclinical stage into the clinical arena. The first human clinical trial using LVs was initiated in 2003. In this, a VSV-G-pseudotyped HIV-based vector was engineered to conditionally express an antisense RNA against envelope glycoprotein in the presence of regulatory proteins provided by wild-type virus. Five subjects with chronic HIV infection received a single dose of gene-modified autologous CD4⁺ T cells which resulted in an increase of CD4⁺ T cells (in four out of the five subjects) and decrease in the viral load (in all five participants) after 1 year. Further studies over 2 years have not detected any adverse clinical events [131].

Since this first gene therapy clinical trial until June 2012, about 54 gene therapy clinical trial using LVs are ongoing or have been approved. Among them there are 12 trials for the treatment of HIV infection, 22 for the treatment of monogenic diseases (X linked cerebral adrenoleukodystrophy, Sickle cell anemia, Wiskott-Aldrich Syndrome, Metachromatic Leukodystrophy, X-Linked Chronic Granulomatous Disease, Inherited Skin Disease Netherton Syndrome, mucopolysaccharidosis type VII, β -thalassemia, Fanconi Anemia Complement Group A, X-Linked Severe Combined Deficiency, Adenosine Deaminase Deficient Severe Combined Immunodeficiency, Hemophilia A), 15 against various cancers, 2 for Parkinson's disease, 3 for ocular diseases and 1 for patients with Stargardt Macular Degeneration [1].

6. Conclusions and outlook

The major concerns associated with the use of all retroviral vectors are the formation of replication competent retroviral vectors (RCR), the mutational integration of the provirus into the host cellular genome and mobilization of structural viral genes to target cells. In addition, the majority of developed LVs are HIV-derived raising further safety concerns since this is a well known human pathogen. Significant efforts have been made to develop LVs with improved biosafety and increased transduction efficiency. Some of those biosafety features include the splitting of viral elements by several expression cassettes, the use of self-inactivating vectors (SIN), decreasing to a minimum the number of viral elements and reducing homology between them.

Lentiviral vectors have already won its place as valuable and flexible tool for gene delivery, being used in several applications but further research is still ongoing towards the development of a lentiviral vector providing higher titers, higher robustness, lower toxicity and higher biosafety.

Lentiviral vector gene therapy is becoming a real alternative vector for therapy with dozens of clinical trials either been already performed or ongoing. These, together with the future incoming clinical trials, will enable to assess overall the pros and cons of the newcomer lentiviral vectors and will provide insights to further vector innovations that will be important to increase their productivity, quality and safety.

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References

- [1] The Journal of Gene Medicine: Gene Therapy Clinical Trials Worldwide www.wiley.com/legacy/wileychi/genmed/clinical/ (accessed 1 August 2012)
- [2] Campbell RS, Robinson WF. The comparative pathology of the lentiviruses. *Journal of comparative pathology*. 1998; 119(4):333–95.
- [3] International Committee on Taxonomy of Viruses (ICTV) <http://ictvonline.org/virusTaxonomy.asp?version=2011> (accessed 1 August 2012)
- [4] Vogt VM, Simon MN. Mass determination of rous sarcoma virus virions by scanning transmission electron microscopy. *Journal of virology*. 1999;73(8):7050–5. jvi.asm.org/content/73/8/7050.full.pdf+html (accessed 1 August 2012)
- [5] Coffin JM, Hughes SH, Varmus H. 1997. *Retroviruses*: Cold Spring Harbor. www.ncbi.nlm.nih.gov/books/NBK19376/ (accessed 1 August 2012)
- [6] Adamson CS, Jones IM. The molecular basis of HIV capsid assembly--five years of progress. *Reviews in medical virology*. ;14(2):107–21. onlinelibrary.wiley.com/doi/10.1002/rmv.418/citedby (accessed 1 August 2012)
- [7] Katz RA, Skalka AM. The retroviral enzymes. *Annual review of biochemistry*. 1994;63:133–73. www.annualreviews.org/doi/abs/10.1146/annurev.bi.63.070194.001025 (accessed 1 August 2012)
- [8] Ptak RG, Fu W, Sanders-Beer BE, Dickerson JE, Pinney JW, Robertson DL, et al. Cataloging the HIV type 1 human protein interaction network. *AIDS research and human retroviruses*. 2008;24(12):1497–502. online.liebertpub.com/doi/abs/10.1089/aid.2008.0113 (accessed 1 August 2012)
- [9] Fu W, Sanders-Beer BE, Katz KS, Maglott DR, Pruitt KD, Ptak RG. Human immunodeficiency virus type 1, human protein interaction database at NCBI. *Nucleic acids research*. 2009;37 (Database issue) :D417–22. nar.oxfordjournals.org/content/37/suppl_1/D417 (accessed 1 August 2012)
- [10] Pluta K, Kacprzak MM. Use of HIV as a gene transfer vector. *Acta biochimica Polonica*. 2009;56(4):531–95. www.actabp.pl/pdf/4_2009/531.pdf (accessed 1 August 2012)
- [11] Terwilliger EF, Godin B, Sodroski JG, Haseltine W a. Construction and use of a replication-competent human immunodeficiency virus (HIV-1) that expresses the chloramphenicol acetyltransferase enzyme. *Proceedings of the National Academy of Sciences of the United States of America*. 1989 May;86(10):3857–61. <http://www.pnas.org/content/86/10/3857.full.pdf+html> (accessed 1 August 2012)
- [12] Helseth E, Kowalski M, Gabuzda D, Olshevsky UDY, Haseltine W, Sodroski J. Rapid complementation assays measuring replicative potential of human immunodeficiency virus type 1 envelope glycoprotein mutants. *Rapid Complementation Assays Measuring Replicative Potential of Human Immunodeficiency Virus Type 1 Enve-*

- lope Glycoprotein M. *Journal of Virology*. 1990;64(5):2416–20. jvi.asm.org/content/64/5/2416.full.pdf+html (accessed 1 August 2012)
- [13] Page K a, Landau NR, Littman DR. Construction and use of a human immunodeficiency virus vector for analysis of virus infectivity. *Journal of virology*. 1990;64(11):5270–6. jvi.asm.org/content/64/11/5270.full.pdf+html (accessed 1 August 2012)
- [14] Landau NR, Page K a, Littman DR. Pseudotyping with human T-cell leukemia virus type I broadens the human immunodeficiency virus host range. *Journal of virology*. 1991;65(1):162–9. jvi.asm.org/content/65/1/162 (accessed 1 August 2012)
- [15] Clever J, Sassetti C, Parslow TG. RNA secondary structure and binding sites for gag gene products in the 5' packaging signal of human immunodeficiency virus type 1. *Journal of Virology*. 1995;69(4):2101–9. jvi.asm.org/content/69/4/2101.full.pdf (accessed 1 August 2012)
- [16] Naldini L, Blömer U, Gally P, Ory D, Mulligan R, Gage FH, et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science (New York, N.Y.)*. 1996;272(5259):263–7. www.sciencemag.org/content/272/5259/263.long (accessed 1 August 2012)
- [17] Burns JC, Friedmann T, Driever W, Burrascano M, Yee JK. Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proceedings of the National Academy of Sciences of the United States of America*. 1993;90(17):8033–7. www.pnas.org/content/90/17/8033.full.pdf (accessed 1 August 2012)
- [18] Reiser J, Harmison G, Kluepfel-Stahl S, Brady RO, Karlsson S, Schubert M. Transduction of nondividing cells using pseudotyped defective high-titer HIV type 1 particles. *Proceedings of the National Academy of Sciences of the United States of America*. 1996;93(26):15266–71. www.pnas.org/content/93/26/15266.long (accessed 1 August 2012)
- [19] Akkina RK, Walton RM, Chen ML, Li QX, Planelles V, Chen IS. High-efficiency gene transfer into CD34+ cells with a human immunodeficiency virus type 1-based retroviral vector pseudotyped with vesicular stomatitis virus envelope glycoprotein G. *Journal of virology*. 1996;70(4):2581–5. jvi.asm.org/content/70/4/2581.long (accessed 1 August 2012)
- [20] Naldini L, Blömer U, Gage FH, Trono D, Verma IM. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proceedings of the National Academy of Sciences of the United States of America*. 1996;93(21):11382–8. www.pnas.org/content/93/21/11382.full.pdf (accessed 1 August 2012)
- [21] Gibbs JS, Regier DA, Desrosiers RC. Construction and in vitro properties of HIV-1 mutants with deletions in “nonessential” genes. *AIDS research and human retroviruses*. 1994;10(4):343–50. <http://www.ncbi.nlm.nih.gov/pubmed/8068414> (accessed 1 August 2012)

- [22] Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nature biotechnology*. 1997;15(9):871–5. www.nature.com/nbt/journal/v15/n9/full/nbt0997-871.html (accessed 1 August 2012)
- [23] Kafri T, Blömer U, Peterson DA, Gage FH, Verma IM. Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. *Nature genetics*. 1997;17(3):314–7. www.nature.com/ng/journal/v17/n3/abs/ng1197-314.html (accessed 1 August 2012)
- [24] Mochizuki H, Schwartz JP, Tanaka K, Brady RO, Reiser J. High-titer human immune deficiency virus type 1-based vector systems for gene delivery into nondividing cells. *Journal of virology*. 1998;72(11):8873–83. jvi.asm.org/content/72/11/8873.long (accessed 1 August 2012)
- [25] Kim VN, Mitrophanous K, Kingsman SM, Kingsman a J. Minimal requirement for a lentivirus vector based on human immunodeficiency virus type 1. *Journal of virology*. 1998 Jan;72(1):811–6. jvi.asm.org/content/72/1/811(accessed 1 August 2012)
- [26] Naldini L, Verma IM. Lentiviral vectors. *Advances in virus research*. 2000;55:599–609. <http://www.ncbi.nlm.nih.gov/pubmed/11050959>(accessed 1 August 2012)
- [27] Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, et al. A third-generation lentivirus vector with a conditional packaging system. *Journal of virology*. 1998;72(11):8463–71. jvi.asm.org/content/72/11/8463.long (accessed 1 August 2012)
- [28] Bray M, Prasad S, Dubay JW, Hunter E, Jeang KT, Rekosh D, et al. A small element from the Mason-Pfizer monkey virus genome makes human immunodeficiency virus type 1 expression and replication Rev-independent. *Proceedings of the National Academy of Sciences of the United States of America*. 1994;91(4):1256–60. www.pnas.org/content/91/4/1256.full.pdf+html?sid=f828b8ae-496c-476c-bace-e85aeee47e57(accessed 1 August 2012)
- [29] Reddy TR, Xu W, Mau JK, Goodwin CD, Suhasini M, Tang H, et al. Inhibition of HIV replication by dominant negative mutants of Sam68, a functional homolog of HIV-1 Rev. *Nature medicine*. 1999;5(6):635–42. www.nature.com/nm/journal/v5/n7/full/nm0799_849c.html (accessed 1 August 2012)
- [30] Roberts TM, Boris-Lawrie K. The 5' RNA terminus of spleen necrosis virus stimulates translation of nonviral mRNA. *Journal of virology*. 2000;74(17):8111–8. jvi.asm.org/content/74/17/8111.long (accessed 1 August 2012)
- [31] Pandya S, Boris-Lawrie K, Leung NJ, Akkina R, Planelles V. Development of an Rev-independent, minimal simian immunodeficiency virus-derived vector system. *Human gene therapy*. 2001 May 1;12(7):847–57. online.liebertpub.com/doi/pdfplus/10.1089/104303401750148847 (accessed 1 August 2012)
- [32] Kotsopoulou E, Kim VN, Kingsman AJ, Kingsman SM, Mitrophanous KA. A Rev-independent human immunodeficiency virus type 1 (HIV-1)-based vector that exploits

- a codon-optimized HIV-1 gag-pol gene. *Journal of virology*. 2000;74(10):4839–52. jvi.asm.org/content/74/10/4839.long (accessed 1 August 2012)
- [33] Ikeda Y, Takeuchi Y, Martin F, Cosset F-L, Mitrophanous K, Collins M. Continuous high-titer HIV-1 vector production. 2003. www.nature.com/nbt/journal/v21/n5/full/nbt815.html (accessed 1 August 2012)
- [34] Kappes JC, Wu X. Safety considerations in vector development. *Somatic cell and molecular genetics*. 2001;26(1-6):147–58. www.springerlink.com/content/w93t23118464r17k/?MUD=MP (accessed 1 August 2012)
- [35] Wu X, Wakefield JK, Liu H, Xiao H, Kralovics R, Prchal JT, et al. Development of a novel trans-lentiviral vector that affords predictable safety. *Molecular therapy: the journal of the American Society of Gene Therapy*. 2000;2(1):47–55 www.nature.com/mt/journal/v2/n1/pdf/mt2000140a.pdf (accessed 1 August 2012)
- [36] Westerman KA, Ao Z, Cohen EA, Leboulch P. Design of a trans protease lentiviral packaging system that produces high titer virus. *Retrovirology*. 2007;4(96):1–14. www.retrovirology.com/content/pdf/1742-4690-4-96.pdf (accessed 1 August 2012)
- [37] Pauwels K, Gijsbers R, Toelen J, Schambach A, Willard-Gallo K, Verheust C, et al. State-of-the-art Lentiviral Vectors for Research Use: Risk Assessment and Biosafety Recommendations. *Current gene therapy*. 2009;9(6):459–74. www.benthamdirect.org/pages/content.php?CGT/2009/00000009/00000006/0002Q.SGM (accessed 1 August 2012)
- [38] Montini E, Cesana D, Schmidt M, Sanvito F, Bartholomae CC, Ranzani M, et al. The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy. *The Journal of clinical investigation*. 2009;119(4):964–75. www.jci.org/articles/view/37630 (accessed 1 August 2012)
- [39] Zufferey R, Dull T, Mandel RJ, Bukovsky A, Quiroz D, Naldini L, et al. Self-Inactivating Lentivirus Vector for Safe and Efficient In Vivo Gene Delivery. *J. Virol.*. 1998;72(12):9873–80. jvi.asm.org/cgi/content/abstract/72/12/9873(accessed 1 August 2012)
- [40] Iwakuma T, Cui Y, Chang LJ. Self-inactivating lentiviral vectors with U3 and U5 modifications. *Virology*. 1999;261(1):120–32. www.sciencedirect.com/science/article/pii/S004268229998501(accessed 1 August 2012)
- [41] Yu S-F. Self-Inactivating Retroviral Vectors Designed for Transfer of Whole Genes into Mammalian Cells. *Proceedings of the National Academy of Sciences*. 1986;83(10):3194–8. www.pnas.org/cgi/content/abstract/83/10/3194(accessed 1 August 2012)
- [42] Bukovsky AA, Song JP, Naldini L. Interaction of human immunodeficiency virus-derived vectors with wild-type virus in transduced cells. *Journal of virology*. 1999;73(8):7087–92. jvi.asm.org/content/73/8/7087.long (accessed 1 August 2012)

- [43] Bokhoven M, Stephen SL, Knight S, Gevers EF, Robinson IC, Takeuchi Y, et al. Insertional gene activation by lentiviral and gammaretroviral vectors. *Journal of virology*. 2009;83(1):283–94. jvi.asm.org/content/83/1/283 (accessed 1 August 2012)
- [44] Miyoshi H, Blomer U, Takahashi M, Gage FH, Verma IM. Development of a Self-Inactivating Lentivirus Vector. *J. Virol.* 1998;72(10):8150–7. jvi.asm.org/cgi/content/abstract/72/10/8150(accessed 1 August 2012)
- [45] Yang Q, Lucas A, Son S, Chang L-J. Overlapping enhancer/promoter and transcriptional termination signals in the lentiviral long terminal repeat. *Retrovirology*. 2007;4:4. www.retrovirology.com/content/4/1/4 (accessed 1 August 2012)
- [46] Schambach A, Galla M, Maetzig T, Loew R, Baum C. Improving transcriptional termination of self-inactivating gamma-retroviral and lentiviral vectors. *Molecular therapy: the journal of the American Society of Gene Therapy*. 2007;15(6):1167–73. www.nature.com/mt/journal/v15/n6/full/6300152a.html (accessed 1 August 2012)
- [47] Hanawa H, Persons DA, Nienhuis AW. Mobilization and mechanism of transcription of integrated self-inactivating lentiviral vectors. *Journal of virology*. 2005;79(13):8410–21. jvi.asm.org/content/79/13/8410.long (accessed 1 August 2012)
- [48] Hino S, Fan J, Taguwa S, Akasaka K, Matsuoka M. Sea urchin insulator protects lentiviral vector from silencing by maintaining active chromatin structure. *Gene therapy*. 2004;11(10):819–28. www.nature.com/gt/journal/v11/n10/full/3302227a.html (accessed 1 August 2012)
- [49] Arumugam PI, Scholes J, Perelman N, Xia P, Yee J-K, Malik P. Improved human beta-globin expression from self-inactivating lentiviral vectors carrying the chicken hypersensitive site-4 (cHS4) insulator element. *Molecular therapy: the journal of the American Society of Gene Therapy*. 2007;15(10):1863–71. www.nature.com/mt/journal/v15/n10/full/6300259a.html (accessed 1 August 2012)
- [50] Zufferey R, Donello JE, Trono D, Hope TJ. Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element Enhances Expression of Transgenes Delivered by Retroviral Vectors. *J. Virol.* 1999;73(4):2886–92. jvi.asm.org/cgi/content/abstract/73/4/2886(accessed 1 August 2012)
- [51] Oh T, Bajwa A, Jia G, Park F. Lentiviral vector design using alternative RNA export elements. *Retrovirology*. 2007;4(1):38. www.retrovirology.com/content/4/1/38 (accessed 1 August 2012)
- [52] Pistello M, Vannucci L, Ravani A, Bonci F, Chiuppesi F, del Santo B, et al. Streamlined design of a self-inactivating feline immunodeficiency virus vector for transducing ex vivo dendritic cells and T lymphocytes. *Genetic vaccines and therapy*. 2007;5(1):8. www.gvt-journal.com/content/5/1/8(accessed 1 August 2012)
- [53] Salmon P, Kindler V, Ducrey O, Chapuis B, Zubler RH, Trono D. High-level transgene expression in human hematopoietic progenitors and differentiated blood lineages after transduction with improved lentiviral vectors. *Blood*. 2000;96(10):3392–8.

bloodjournal.hematologylibrary.org/content/96/10/3392.long (accessed 1 August 2012)

- [54] Schambach A, Bohne J, Baum C, Hermann FG, Egerer L, von Laer D, et al. Woodchuck hepatitis virus post-transcriptional regulatory element deleted from X protein and promoter sequences enhances retroviral vector titer and expression. *Gene therapy*. 2006;13(7):641–5. www.nature.com/gt/journal/v13/n7/full/3302698a.html (accessed 1 August 2012)
- [55] Manganini M, Serafini M, Bambacioni F, Casati C, Erba E, Follenzi A, et al. A human immunodeficiency virus type 1 pol gene-derived sequence (cPPT/CTS) increases the efficiency of transduction of human nondividing monocytes and T lymphocytes by lentiviral vectors. *Human gene therapy*. 2002;13(15):1793–807. online.liebertpub.com/doi/abs/10.1089/104303402760372909 (accessed 1 August 2012)
- [56] Sirven A, Pflumio F, Zennou V, Titeux M, Vainchenker W, Coulombel L, et al. 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*. 2000;96(13):4103–10. bloodjournal.hematologylibrary.org/content/96/13/4103.long (accessed 1 August 2012)
- [57] Logan AC, Nightingale SJ, Haas DL, Cho GJ, Pepper K a, Kohn DB. Factors influencing the titer and infectivity of lentiviral vectors. *Human gene therapy*. 2004;15(10):976–88. online.liebertpub.com/doi/abs/10.1089/hum.2004.15.976 (accessed 1 August 2012)
- [58] Desmaris N, Bosch A, Salaün C, Petit C, Prévost MC, Tordo N, et al. Production and neurotropism of lentivirus vectors pseudotyped with lyssavirus envelope glycoproteins. *Molecular therapy: the journal of the American Society of Gene Therapy*. 4(2):149–56. www.nature.com/mt/journal/v4/n2/abs/mt2001210a.html (accessed 1 August 2012)
- [59] Federici T, Kutner R, Zhang X-Y, Kuroda H, Tordo N, Boulis NM, et al. Comparative analysis of HIV-1-based lentiviral vectors bearing lyssavirus glycoproteins for neuronal gene transfer. *Genetic vaccines and therapy*. 2009;7(1):1. www.gvt-journal.com/content/7/1/1(accessed 1 August 2012)
- [60] Mazarakis ND. Rabies virus glycoprotein pseudotyping of lentiviral vectors enables retrograde axonal transport and access to the nervous system after peripheral delivery. *Human Molecular Genetics*. 2001;10(19):2109–21. hmg.oxfordjournals.org/cgi/content/abstract/10/19/2109(accessed 1 August 2012)
- [61] Bartz SR, Rogel ME, Emerman M. Human immunodeficiency virus type 1 cell cycle control: Vpr is cytostatic and mediates G2 accumulation by a mechanism which differs from DNA damage checkpoint control. *Journal of virology*. 1996;70(4):2324–31. jvi.asm.org/content/70/4/2324.full.pdf+html?sid=9792589f-078b-4868-a8a5-f0926b6b2dee (accessed 1 August 2012)

- [62] DePolo NJ, Reed JD, Sheridan PL, Townsend K, Sauter SL, Jolly DJ, et al. VSV-G pseudotyped lentiviral vector particles produced in human cells are inactivated by human serum. *Molecular therapy: the journal of the American Society of Gene Therapy*. 2000;2(3):218–22. www.nature.com/mt/journal/v2/n3/abs/mt2000164a.html (accessed 1 August 2012)
- [63] Croyle MA, Callahan SM, Auricchio A, Schumer G, Linse KD, Wilson JM, et al. PE-Gylation of a vesicular stomatitis virus G pseudotyped lentivirus vector prevents inactivation in serum. *Journal of virology*. 2004;78(2):912–21. jvi.asm.org/content/78/2/912 (accessed 1 August 2012)
- [64] Berkowitz R, Ilves H, Lin WY, Eckert K, Coward A, Tamaki S, et al. Construction and molecular analysis of gene transfer systems derived from bovine immunodeficiency virus. *Journal of virology*. 2001;75(7):3371–82. jvi.asm.org/content/75/7/3371. long (accessed 1 August 2012)
- [65] Metharom P, Takyar S, Xia HQ, Ellem KA, Wilcox GE, Wei MQ. Development of disabled, replication-defective gene transfer vectors from the Jembrana disease virus, a new infectious agent of cattle. *Veterinary microbiology*. 2001;80(1):9–22. www.sciencedirect.com/science/article/pii/S037811350000376X (accessed 1 August 2012)
- [66] Mselli-Lakhal L, Guiguen F, Greenland T, Mornex J-F, Chebloune Y. Gene transfer system derived from the caprine arthritis-encephalitis lentivirus. *Journal of virological methods*. 2006;136(1-2):177–84. <http://www.sciencedirect.com/science/article/pii/S0166093406001571> (accessed 1 August 2012)
- [67] Duisit G, Conrath H, Saleun S, Folliot S, Provost N, Cosset F-L, et al. Five recombinant simian immunodeficiency virus pseudotypes lead to exclusive transduction of retinal pigmented epithelium in rat. *Molecular therapy: the journal of the American Society of Gene Therapy*. 2002;6(4):446–54. www.nature.com/mt/journal/v6/n4/pdf/mt2002197a.pdf (accessed 1 August 2012)
- [68] Kobinger GP, Deng S, Louboutin J-P, Vatamaniuk M, Matschinsky F, Markmann JF, et al. Transduction of human islets with pseudotyped lentiviral vectors. *Human gene therapy*. 2004;15(2):211–9. online.liebertpub.com/doi/abs/10.1089/104303404772680010 (accessed 1 August 2012)
- [69] Mochizuki H, Schwartz JP, Tanaka K, Brady RO, Reiser J. High-Titer Human Immunodeficiency Virus Type 1-Based Vector Systems for Gene Delivery into Nondividing Cells. *J. Virol.* 1998;72(11):8873–83. jvi.asm.org/cgi/content/abstract/72/11/8873 (accessed 1 August 2012)
- [70] Hanawa H, Kelly PF, Nathwani AC, Persons DA, Vandergriff JA, Hargrove P, et al. Comparison of various envelope proteins for their ability to pseudotype lentiviral vectors and transduce primitive hematopoietic cells from human blood. *Molecular therapy: the journal of the American Society of Gene Therapy*. 2002;5(3):242–51. www.nature.com/mt/journal/v5/n3/full/mt200238a.html (accessed 1 August 2012)

- [71] Zhang X-Y, La Russa VF, Reiser J. Transduction of bone-marrow-derived mesenchymal stem cells by using lentivirus vectors pseudotyped with modified RD114 envelope glycoproteins. *Journal of virology*. 2004;78(3):1219–29 jvi.asm.org/content/78/3/1219.long (accessed 1 August 2012)
- [72] Kobinger GP, Weiner DJ, Yu QC, Wilson JM. Filovirus-pseudotyped lentiviral vector can efficiently and stably transduce airway epithelia in vivo. *Nature biotechnology*. 2001;19(3):225–30. www.nature.com/nbt/journal/v19/n3/full/nbt0301_225.html (accessed 1 August 2012)
- [73] Beyer WR, Westphal M, Ostertag W, von Laer D. Oncoretrovirus and lentivirus vectors pseudotyped with lymphocytic choriomeningitis virus glycoprotein: generation, concentration, and broad host range. *Journal of virology*. 2002;76(3):1488–95. jvi.asm.org/content/76/3/1488.long (accessed 1 August 2012)
- [74] Wong L-F, Azzouz M, Walmsley LE, Askham Z, Wilkes FJ, Mitrophanous KA, et al. Transduction patterns of pseudotyped lentiviral vectors in the nervous system. *Molecular therapy: the journal of the American Society of Gene Therapy*.;9(1):101–11. www.nature.com/mt/journal/v9/n1/full/mt200415a.html (accessed 1 August 2012)
- [75] Kahl CA, Marsh J, Fyffe J, Sanders DA, Cornetta K. Human immunodeficiency virus type 1-derived lentivirus vectors pseudotyped with envelope glycoproteins derived from Ross River virus and Semliki Forest virus. *Journal of virology*. 2004;78(3):1421–30. jvi.asm.org/content/78/3/1421.long (accessed 1 August 2012)
- [76] Kang Y, Stein CS, Heth JA, Sinn PL, Penisten AK, Staber PD, et al. In vivo gene transfer using a nonprimate lentiviral vector pseudotyped with Ross River Virus glycoproteins. *Journal of virology*. 2002;76(18):9378–88. jvi.asm.org/content/76/18/9378.long (accessed 1 August 2012)
- [77] Morizono K, Bristol G, Xie YM, Kung SK, Chen IS. Antibody-directed targeting of retroviral vectors via cell surface antigens. *Journal of virology*. 2001;75(17):8016–20. jvi.asm.org/content/75/17/8016 (accessed 1 August 2012)
- [78] Cronin J, Zhang X-Y, Reiser J. Altering the Tropism of Lentiviral Vectors through Pseudotyping. *Current gene therapy*. 2005;5(4):387–98. www.benthamdirect.org/pages/content.php?CGT/2005/00000005/00000004/0003Q.SGM (accessed 1 August 2012)
- [79] Felder JM, Sutton RE. Lentiviral Vectors. In: *Gene and Cell Therapy: Therapeutic Mechanisms and Strategies*. 2009.
- [80] Lodge R, Subbramanian R a, Forget J, Lemay G, Cohen E a. MuLV-based vectors pseudotyped with truncated HIV glycoproteins mediate specific gene transfer in CD4⁺ peripheral blood lymphocytes. *Gene therapy*. 1998;5(5):655–64. www.nature.com/gt/journal/v5/n5/abs/3300646a.html (accessed 1 August 2012)
- [81] Thaler S, Schnierle BS. A packaging cell line generating CD4-specific retroviral vectors for efficient gene transfer into primary human T-helper lymphocytes. *Molecular*

- therapy: the journal of the American Society of Gene Therapy. 2001;4(3):273–9. www.nature.com/mt/journal/v4/n3/abs/mt2001227a.html (accessed 1 August 2012)
- [82] MacKenzie TC, Kobinger GP, Kootstra NA, Radu A, Sena-Esteves M, Bouchard S, et al. Efficient transduction of liver and muscle after in utero injection of lentiviral vectors with different pseudotypes. *Molecular therapy: the journal of the American Society of Gene Therapy*. 2002;6(3):349–58. www.nature.com/mt/journal/v6/n3/pdf/mt2002180a.pdf (accessed 1 August 2012)
- [83] Peng KW, Pham L, Ye H, Zufferey R, Trono D, Cosset FL, et al. Organ distribution of gene expression after intravenous infusion of targeted and untargeted lentiviral vectors. *Gene therapy*. 2001;8(19):1456–63. www.nature.com/gt/journal/v8/n19/full/3301552a.html (accessed 1 August 2012)
- [84] Yang L, Yang H, Rideout K, Cho T, Joo KI, Ziegler L, et al. Engineered lentivector targeting of dendritic cells for in vivo immunization. *Nature biotechnology*. 2008;26(3):326–34. www.nature.com/nbt/journal/v26/n3/full/nbt1390.html (accessed 1 August 2012)
- [85] Escors D, Breckpot K. Lentiviral vectors in gene therapy: their current status and future potential. *Archivum immunologiae et therapeuticae experimentalis*. 2010;58(2):107–19. www.springerlink.com/content/725574uvq7552u2g/ (accessed 1 August 2012)
- [86] Ziegler L, Yang L, Joo K il, Yang H, Baltimore D, Wang P. Targeting lentiviral vectors to antigen-specific immunoglobulins. *Human gene therapy*. 2008;19(9):861–72. online.liebertpub.com/doi/abs/10.1089/hgt.2007.149 (accessed 1 August 2012)
- [87] Frecha C, Szécsi J, Cosset F-L, Verhoeven E. Strategies for targeting lentiviral vectors. *Current gene therapy*. 2008;8(6):449–60. www.benthamdirect.org/pages/content.php?CGT/2008/00000008/00000006/0005Q.SGM (accessed 1 August 2012)
- [88] Trimby C. STRATEGIES FOR TARGETING LENTIVIRAL VECTORS. http://uknowledge.uky.edu/gradschool_diss/157 (accessed 1 August 2012)
- [89] Zhang X-Y, Kutner RH, Bialkowska A, Marino MP, Klimstra WB, Reiser J. Cell-specific targeting of lentiviral vectors mediated by fusion proteins derived from Sindbis virus, vesicular stomatitis virus, or avian sarcoma/leukosis virus. *Retrovirology*. 2010;7(1):3. <http://www.retrovirology.com/content/7/1/3> (accessed 1 August 2012)
- [90] Yang L, Bailey L, Baltimore D, Wang P. Targeting lentiviral vectors to specific cell types in vivo. *Proceedings of the National Academy of Sciences of the United States of America*. 2006;103(31):11479–84. www.pnas.org/cgi/content/abstract/103/31/11479 (accessed 1 August 2012)
- [91] Lei Y, Joo K-I, Zarzar J, Wong C, Wang P. Targeting lentiviral vector to specific cell types through surface displayed single chain antibody and fusogenic molecule. *Virology journal*. 2010;7:35. www.virologyj.com/content/7/1/35 (accessed 1 August 2012)

- [92] Haas DL, Case SS, Crooks GM, Kohn DB. Critical factors influencing stable transduction of human CD34(+) cells with HIV-1-derived lentiviral vectors. *Molecular therapy*: the journal of the American Society of Gene Therapy. 2000;2(1):71–80. www.nature.com/mt/journal/v2/n1/abs/mt2000143a.html (accessed 1 August 2012)
- [93] Schweizer M, Merten O-W. Large-scale production means for the manufacturing of lentiviral vectors. *Current gene therapy*. 2010;10(6):474–86. www.benthamdirect.org/pages/content.php?CGT/2010/00000010/00000006/0006Q.SGM (accessed 1 August 2012)
- [94] Gougeon M-L. Apoptosis as an HIV strategy to escape immune attack. *Nature reviews. Immunology*. 2003;3(5):392–404. www.nature.com/nri/journal/v3/n5/full/nri1087.html (accessed 1 August 2012)
- [95] Bell AJ, Fegen D, Ward M, Bank A. RD114 envelope proteins provide an effective and versatile approach to pseudotypelentiviral vectors. *Experimental biology and medicine* (Maywood, N.J.). 2010;235(10):1269–76. ebm.rsmjournals.com/content/235/10/1269.long (accessed 1 August 2012)
- [96] Sainski AM, Natesampillai S, Cummins NW, Bren GD, Taylor J, Saenz DT, et al. The HIV-1-specific protein Casp8p41 induces death of infected cells through Bax/Bak. *Journal of virology*. 2011;85(16):7965–75. jvi.asm.org/content/85/16/7965.long (accessed 1 August 2012)
- [97] Algeciras-Schimmich A, Belzacq-Casagrande A-S, Bren GD, Nie Z, Taylor J a, Rizza S a, et al. Analysis of HIV Protease Killing Through Caspase 8 Reveals a Novel Interaction Between Caspase 8 and Mitochondria. *The open virology journal*. 2007;1:39–46. benthamscience.com/open/openaccess.php?tovj/articles/V001/39TOVJ.htm (accessed 1 August 2012)
- [98] Nie Z, Phenix BN, Lum JJ, Alam a, Lynch DH, Beckett B, et al. HIV-1 protease processes procaspase 8 to cause mitochondrial release of cytochrome c, caspase cleavage and nuclear fragmentation. *Cell death and differentiation*. 2002;9(11):1172–84. www.nature.com/cdd/journal/v9/n11/full/4401094a.html (accessed 1 August 2012)
- [99] Merten O-W, Charrier S, Laroudie N, Fauchille S, Dugué C, Jenny C, et al. Large-scale manufacture and characterization of a lentiviral vector produced for clinical ex vivo gene therapy application. *Human gene therapy*. 2011;22(3):343–56 online.liebertpub.com/doi/abs/10.1089/hum.2010.060 (accessed 1 August 2012)
- [100] Gama-Norton L, Botezatu L, Herrmann S, Schweizer M, Alves PM, Hauser H, et al. Lentivirus production is influenced by SV40 large T-antigen and chromosomal integration of the vector in HEK293 cells. *Human gene therapy*. 2011;22(10):1269–79. online.liebertpub.com/doi/abs/10.1089/hum.2010.143 (accessed 1 August 2012)
- [101] Ansorge S, Lanthier S, Transfiguracion J, Durocher Y, Henry O, Kamen A. Development of a scalable process for high-yield lentiviral vector production by transient transfection of HEK293 suspension cultures. *The journal of gene medicine*.

- 2009;11(10):868–76. onlinelibrary.wiley.com/doi/10.1002/jgm.1370/abstract (accessed 1 August 2012)
- [102] Smith SL, Shioda T. Advantages of COS-1 monkey kidney epithelial cells as packaging host for small-volume production of high-quality recombinant lentiviruses. *Journal of virological methods*. 2009;157(1):47–54. www.sciencedirect.com/science/article/pii/S0166093408004412 (accessed 1 August 2012)
- [103] Ni Y, Sun S, Oparaocha I, Humeau L, Davis B, Cohen R, et al. Generation of a packaging cell line for prolonged large-scale production of high-titer HIV-1-based lentiviral vector. *The journal of gene medicine*. 2005;7(6):818–34. onlinelibrary.wiley.com/doi/10.1002/jgm.726/abstract (accessed 1 August 2012)
- [104] Mann R, Mulligan RC, Baltimore D. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell*. 1983;33(1):153–9. onlinelibrary.wiley.com/doi/10.1002/jgm.726/abstract (accessed 1 August 2012)
- [105] Witting SR, Li L-H, Jasti A, Allen C, Cornetta K, Brady J, et al. Efficient large volume lentiviral vector production using flow electroporation. *Human gene therapy*. 2012;23(2):243–9. online.liebertpub.com/doi/abs/10.1089/hum.2011.088 (accessed 1 August 2012)
- [106] Rols MP, Coulet D, Teissié J. Highly efficient transfection of mammalian cells by electric field pulses. Application to large volumes of cell culture by using a flow system. *European journal of biochemistry / FEBS*. 1992;206(1):115–21. onlinelibrary.wiley.com/doi/10.1111/j.1432-1033.1992.tb16908.x/abstract (accessed 1 August 2012)
- [107] Segura M, Garnier A, Durocher Y, Coelho H. Production of Lentiviral Vectors by Large-Scale Transient Transfection of Suspension Cultures and Affinity Chromatography Purification. 2007;98(4):789–99. onlinelibrary.wiley.com/doi/10.1002/bit.21467/pdf (accessed 1 August 2012)
- [108] Kafri T, van Praag H, Ouyang L, Gage FH, Verma IM. A Packaging Cell Line for Lentivirus Vectors. *Journal of Virology*. 1999;73(1):576–84. jvi.asm.org/cgi/content/abstract/73/1/576 (accessed 1 August 2012)
- [109] Pacchiaa L, Adelson ME, Kaul M, Ron Y, Dougherty JP. An inducible packaging cell system for safe, efficient lentiviral vector production in the absence of HIV-1 accessory proteins. *Virology*. 2001 Mar 30;282(1):77–86. www.sciencedirect.com/science/article/pii/S0042682200907876 (accessed 1 August 2012)
- [110] Kuate S, Wagner R, Uberla K. Development and characterization of a minimal inducible packaging cell line for simian immunodeficiency virus-based lentiviral vectors. *The journal of gene medicine*;4(4):347–55. onlinelibrary.wiley.com/doi/10.1002/jgm.290/abstract (accessed 1 August 2012)
- [111] Broussau S, Jabbour N, Lachapelle G, Durocher Y, Tom R, Transfiguracion J, et al. Inducible packaging cells for large-scale production of lentiviral vectors in serum-free suspension culture. *Molecular therapy®: the journal of the American Society of Gene*

Therapy. 2008;16(3):500–7. www.nature.com/mt/journal/v16/n3/full/6300383a.html (accessed 1 August 2012)

- [112] Stewart HJ, Leroux-Carlucci MA, Sion CJM, Mitrophanous KA, Radcliffe PA. Development of inducible EIAV-based lentiviral vector packaging and producer cell lines. *Gene therapy*. 2009;16(6):805–14. www.nature.com/gt/journal/v16/n6/full/gt200920a.html (accessed 1 August 2012)
- [113] Throm RE, Ouma A a, Zhou S, Chandrasekaran A, Lockey T, Greene M, et al. Efficient construction of producer cell lines for a SIN lentiviral vector for SCID-X1 gene therapy by concatemeric array transfection. *Blood*. 2009;113(21):5104–10 bloodjournal.hematologylibrary.org/content/113/21/5104.long (accessed 1 August 2012)
- [114] Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, Conklin DS. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes & development*. 2002;16(8):948–58. genesdev.cshlp.org/content/16/8/948.long (accessed 1 August 2012)
- [115] Hu G, Luo J. A primer on using pooled shRNA libraries for functional genomic screens. *Acta biochimic et biophysica Sinica*. 2012 ;44(2):103–12. abbs.oxford-journals.org/cgi/content/abstract/44/2/103(accessed 1 August 2012)
- [116] Moffat J, Grueneberg DA, Yang X, Kim SY, Kloepper AM, Hinkle G, et al. A lentiviralRNAi library for human and mouse genes applied to an arrayed viral high-content screen. *Cell*. 2006;124(6):1283–98. www.cell.com/retrieve/pii/S0092867406002388 (accessed 1 August 2012)
- [117] Singer O, Verma IM. Applications of lentiviral vectors for shRNA delivery and transgenesis. *Current gene therapy*. 2008;8(6):483–8. www.benthamdirect.org/pages/content.php?CGT/2008/00000008/00000006/0008Q.SGM (accessed 1 August 2012)
- [118] Pfeifer A. Lentiviral transgenesis--a versatile tool for basic research and gene therapy. *Current gene therapy*. 2006;6(4):535–42. www.benthamdirect.org/pages/content.php?CGT/2006/00000006/00000004/0006Q.SGM (accessed 1 August 2012)
- [119] Pomper MG, Hammond H, Yu X, Ye Z, Foss CA, Lin DD, et al. Serial imaging of human embryonic stem-cell engraftment and teratoma formation in live mouse models. *Cell research*. 2009;19(3):370–9. www.nature.com/cr/journal/v19/n3/full/cr2008329a.html (accessed 1 August 2012)
- [120] Welstead GG, Brambrink T, Jaenisch R. Generating iPS cells from MEFS through forced expression of Sox-2, Oct-4, c-Myc, and Klf4. *Journal of visualized experiments®: JoVE*.2008;(14). www.jove.com/video/734/generating-ips-cells-from-mefs-through-forced-expression-sox-2-oct-4 (accessed 1 August 2012)
- [121] Brambrink T, Foreman R, Welstead GG, Lengner CJ, Wernig M, Suh H, et al. Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells. *Cell stem cell*. 2008;2(2):151–9. [www.cell.com/cell-stem-cell/abstract/S1934-5909\(08\)00005-2](http://www.cell.com/cell-stem-cell/abstract/S1934-5909(08)00005-2) (accessed 1 August 2012)

- [122] Lian Q, Chow Y, Esteban MA, Pei D, Tse H-F. Future perspective of induced pluripotent stem cells for diagnosis, drug screening and treatment of human diseases. *Thrombosis and haemostasis*. 2010;104(1):39–44. www.schattauer.de/en/magazine/subject-areas/journals-a-z/thrombosis-and-haemostasis/contents/archive/issue/1093/manuscript/13188.html (accessed 1 August 2012)
- [123] Spencer HT, Denning G, Gautney RE, Dropulic B, Roy AJ, Baranyi L, et al. Lentiviral vector platform for production of bioengineered recombinant coagulation factor VIII. *Molecular therapy: the journal of the American Society of Gene Therapy*. 2011;19(2):302–9. www.nature.com/mt/journal/v19/n2/full/mt2010239a.html (accessed 1 August 2012)
- [124] Malik P, Arumugam PI, Yee J-K, Puthenveetil G. Successful correction of the human Cooley's anemia beta-thalassemia major phenotype using a lentiviral vector flanked by the chicken hypersensitive site 4 chromatin insulator. *Annals of the New York Academy of Sciences*. 2005;1054:238–49. onlinelibrary.wiley.com/doi/10.1196/annals.1345.030/abstract (accessed 1 August 2012)
- [125] Pawliuk R, Westerman KA, Fabry ME, Payen E, Tighe R, Bouhassira EE, et al. Correction of sickle cell disease in transgenic mouse models by gene therapy. *Science (New York, N.Y.)*. 2001;294(5550):2368–71. www.sciencemag.org/content/294/5550/2368.abstract (accessed 1 August 2012)
- [126] Brown BD, Cantore A, Annoni A, Sergi LS, Lombardo A, Della Valle P, et al. A microRNA-regulated lentiviral vector mediates stable correction of hemophilia B mice. *Blood*. 2007;110(13):4144–52. bloodjournal.hematologylibrary.org/content/110/13/4144.long (accessed 1 August 2012)
- [127] Adjali O, Marodon G, Steinberg M, Mongellaz C, Thomas-Vaslin V, Jacquet C, et al. In vivo correction of ZAP-70 immunodeficiency by intrathymic gene transfer. *The Journal of clinical investigation*. 2005;115(8):2287–95. www.jci.org/articles/view/23966 (accessed 1 August 2012)
- [128] Lo Bianco C, Schneider BL, Bauer M, Sajadi A, Brice A, Iwatsubo T, et al. Lentiviral vector delivery of parkin prevents dopaminergic degeneration in an alpha-synuclein rat model of Parkinson's disease. *Proceedings of the National Academy of Sciences of the United States of America*. 2004;101(50):17510–5. www.pnas.org/cgi/content/abstract/101/50/17510(accessed 1 August 2012)
- [129] Wang G, Slepushkin V, Zabner J, Keshavjee S, Johnston JC, Sauter SL, et al. Feline immunodeficiency virus vectors persistently transduce nondividing airway epithelia and correct the cystic fibrosis defect. *The Journal of clinical investigation*. 1999;104(11):R55–62. www.jci.org/articles/view/8390 (accessed 1 August 2012)
- [130] Azzouz M, Le T, Ralph GS, Walmsley L, Monani UR, Lee DCP, et al. Lentivector-mediated SMN replacement in a mouse model of spinal muscular atrophy. *The Journal of clinical investigation*. 2004;114(12):1726–31. www.jci.org/articles/view/22922(accessed 1 August 2012)

- [131] Levine BL, Humeau LM, Boyer J, MacGregor R-R, Rebello T, Lu X, et al. Gene transfer in humans using a conditionally replicating lentiviral vector. *Proceedings of the National Academy of Sciences of the United States of America*. 2006;103(46):17372–7. www.pnas.org/cgi/content/abstract/103/46/17372 (accessed 1 August 2012)

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