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Lentiviral Delivery of Proteins for Genome Engineering

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Abstract: Viruses have evolved to traverse cellular barriers and travel to the nucleus by mechanisms that involve active transport through the cytoplasm and viral quirks to resist cellular restriction factors and innate immune responses. Virus-derived vector systems exploit the capacity of viruses to ferry genetic information into cells, and now - more than three decades after the discovery of HIV - lentiviral vectors based on HIV-1 have become instrumental in biomedical research and gene therapies that require genomic insertion of transgenes. By now, the efficacy of lentiviral gene delivery to stem cells, cells of the immune system including T cells, hepatic cells, and many other therapeutically relevant cell types is well established. Along with nucleic acids, HIV-1 virions carry the enzymatic tools that are essential for early steps of infection. Such capacity to package enzymes, even proteins of nonviral origin, has unveiled new ways of exploiting cellular intrusion of HIV-1. Based on early findings demonstrating the packaging of heterologous proteins into virus particles as part of the Gag and GagPol polypeptides, we have established lentiviral protein transduction for delivery of DNA transposases and designer nucleases. This strategy for delivering genome-engineering proteins facilitates high enzymatic activity within a short time frame and may potentially improve the safety of genome editing. Exploiting the full potential of lentiviral vectors, incorporation of foreign protein can be combined with the delivery of DNA transposons or a donor sequence for homology-directed repair in so-called 'all-in-one' lentiviral vectors. Here, we briefly describe intracellular restrictions that may affect lentiviral gene and protein delivery and review the current status of lentiviral particles as carriers of tool kits for genome engineering.

Keywords: Protein transduction, zinc-finger nucleases, DNA transposase, gene therapy, lentiviral vector.

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INTRODUCTION

As opposed to many conventional medicines, gene therapy provides a potential 'cure' rather than a 'treatment' and remains an attractive therapeutic goal for combatting inherited diseases. To permanently keep genetic diseases at bay, integration of a therapeutic gene into the patient genome is usually a prerequisite. Nonetheless, such manipulation may disrupt endogenous genes and cause severe side effects. In clinical trials aiming at curing patients with SCID-X1, integrating vectors derived from gammaretroviruses were found to induce oncogene activation, resulting in leukemia in some of the patients treated with engineered stem cells [1, 2]. Since then, lentiviral vectors have come to the stage and have been applied in several clinical trials. Thus far, no adverse event has been reported [3]. Although encouraging, safety aspects of lentiviral vectors should still be looked at with caution due to a relatively small number of subjects in the trials and short trial follow-up periods. Therefore, new and improved – and potentially safer - strategies to exploit the gene-delivering capacity of lentiviral vectors are still desired.

The most frequently used lentiviral vectors, developed in the late 1990s [4-6], are derived from human immunodeficiency virus type 1 (HIV-1). State-of-the-art lentiviral vectors are referred to as self-inactivating (SIN) third-generation lentiviral vectors. As opposed to earlier generation vectors, these vectors are devoid of accessory proteins that are not crucial for vector transfer and are produced *via* co-transfection of separate plasmids encoding structural proteins (GagPol), Rev protein, the envelope (often VSV-G glycoprotein), and vector RNA, respectively [4]. Early on, the safety of these vectors was further improved by the introduction of the SIN deletion of the HIV-1 promoter sequence in the U3-region of the LTR, allowing in transduced cells production of a proviral vector sequence that does not carry the HIV-1 promoter [5].

HIV-1 infection can be sensed by host cells and is under the scrutiny of host restriction factors during replication and

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the traverse through the intracellular environment from cell entry to provirus formation in the nucleus. Usually, HIV-1 is able to overcome restrictive cellular mechanisms at least partially due to the activity of its accessory proteins [7]. Accessory proteins are excluded in third-generation HIV-1-derived lentiviral vector systems and, thus, are not present in the virus particles [8]. As a consequence, intruding lentiviral vectors may potentially be more vulnerable to cellular restrictions, leading to lower levels of gene transfer. Despite the widespread use and broad applications of lentiviral gene transfer, it is not yet well documented how nucleic acids sensing mechanisms and other innate responses mounted by the host cell influence vector performance and safety.

Viruses are natural carriers of genetic information, and HIV-1-based lentiviral vectors are primarily used as carriers of genetic cargo for gene delivery. Now, these vectors are standard tools in conventional gene transfer. However, the structural properties of lentiviruses and the capacity of these viruses to assemble a few thousand virus-derived polypeptides in a single virus particle budding from the cell surface unveil a potential as a tool for protein encapsidation and delivery. Viruses are composed of structural proteins (in HIV-1 encoded by the *gag* gene), which represent the bricks and mortar of the virion. However, each virion also carries its own tools, in HIV-1 proteins like protease, reverse transcriptase, and integrase (encoded by the *pol* gene), which are required for establishing an infection in host cells. This sheds light on the protein-ferrying capacity of HIV-1-derived virions and has inspired efforts to develop and exploit lentiviral particles as carriers of foreign proteins. Since such efforts are most frequently based on engineering the protein content of lentiviral vectors, we refer to lentivirus-based protein incorporation and transfer as 'lentiviral protein transduction'. Initial efforts to include fluorescent proteins in HIV-1 virions were made to track the virus and assist in visualizing biological aspects of viral infection [9, 10]. Alternative applications include vaccine development and incorporation of antivirus reagents [11, 12]. More recently, others and we have found ways to exploit lentiviral particles for delivery of enzymes and tool kits for genome engineering [13-19].

Protein transduction seems particularly useful for delivery of cellular tools, which preferably are active minutes after delivery and should be active for only a limited time to limit potential toxicity and reduce the risk of evoking immune responses. Hence, as opposed to most applications of conventional gene transfer strategies, short-lived rather than sustained activity of the transferred agent is a key ambition. Along with high activity, we have met this ambition for delivery of transposases and programmable nucleases. In this review, we recapitulate these findings and describe the general aspects of lentiviral particles as carriers of foreign protein. For further details on incorporating protein into HIV-1-derived particles, the reader is referred to previous reviews [20-22]. Successful lentiviral delivery of genome repair tools or gene-inserting proteins may benefit from the inherent capacity of intruding viruses to ferry cargo through the cytoplasm and over membranes. Hence, the capacities to protect the content from cellular antiviral mechanisms and subsequently drop off the consignment in a suitable genomic neighborhood are attractive properties of virus-based protein carriers. Initially, therefore, we discuss some basics of HIV-1 biology and describe some of the cellular restriction pathways that are fought during protein delivery using lentiviral freight carriers.

PROTEIN TRANSPORT AND PROCESSING DURING THE HIV-1 LIFE CYCLE

HIV-1 predominantly infects $CD4^+$ T cells and, to a smaller extent, macrophages and dendritic cells. The HIV-1 replication cycle can be divided into assembly, maturation, infection, reverse transcription and integration (Fig. **1**). We briefly review the life cycle by highlighting aspects of lentiviral replication related to protein incorporation and processing.

Assembly of the HIV-1 virion occurs prior to budding when the two primary translation products, the Gag and GagPol polypeptides, traffic to the plasma membrane. Movement towards the cell surface and docking at the intracellular surface of the membrane is facilitated by the myristoylation (myr) signal located at N-terminal domain of the polypeptides [23]. Notably, the native myr signal can be replaced with an alternative motif like the phospholipase δ-1 pleckstrin homology (PH) domain or a Lyn-derived myristoylation signal, two well-characterized membrane trafficking domains, without compromising virus production [24, 25]. In fact, insertion of a heterologous myr signal may even in some cases boost the production of viruses [25]. For successful assembly, Gag-Gag multimerization is crucial. Hence, myristoylated monomeric Gag fails to form viral particles, although it is capable of trafficking to the plasma membrane [26]. Low order oligomers can form in the cytoplasm, whereas high order multimerization occurs at the cell membrane [27]. The successful formation of an infectious virus also requires incorporation of genomic RNA, which is transcribed in the nucleus and transported to cytoplasm as part of a nuclear exporting complex consisting of the viral protein Rev, bound to the Rev response element (RRE) RNA secondary structure, and the host protein Crm1 [28]. Two copies of viral RNA are packaged as a dimer into each virion through interaction between the nucleocapsid domain of Gag and RNA motifs in the 5'-untranslated region [29]. In parallel, approximately 20 molecules of cellular $tRNA^{Lys}$, one of which serves as a primer for initiation of reverse transcription, are incorporated *via* interactions between Lysyl-tRNA synthetase, $tRNA^{Lys}$ and GagPol polyproteins [30].

During or immediately after budding, the proteases present in the Pol region of GagPol polypeptides are activated. Firstly, proteases liberate themself from polypeptides *via* autocatalysis, a process believed to depend on GagPol dimerization based on the finding that proteases are effective as a dimer [31]. Once released, proteases chop Gag and GagPol into the final products in an ordered manner [32]. The cleavage sites of proteases are typically octapeptides of which classical forms appear at twelve distinct positions in the HIV-1 precursor proteins [33]. If a foreign protein is fused to Gag and GagPol, adding a HIV-1 cleavage site at the junction will allow release of the cargo during virus maturation. Notably, however, the HIV-1 protease is not very specific and can cut at protein motifs that are different from the HIV-1 cleavage site present in HIV-1 Gag/GagPol [14, 34, 35]. This means that co-packaged foreign proteins,

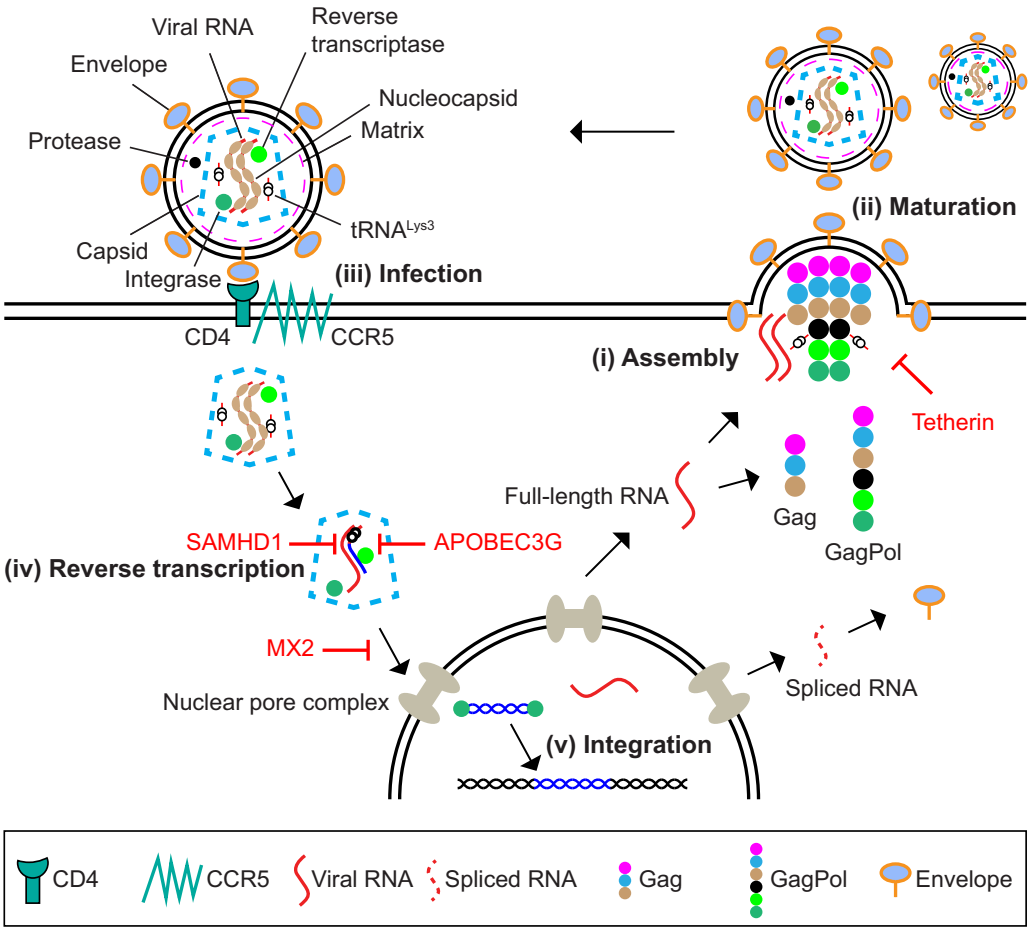

Fig. (1). The life cycle of HIV-1 and host restriction factors. Budding of HIV-1 particles from the cell surface is facilitated by the assembly of the virus at the intracellular side of the membrane where Gag and GagPol assemble into higher-order multimers. Genomic RNA and tRNA^{Lys} are incorporated into the virion through interaction with Gag and GagPol. The budding process can be blocked by Tetherin. Maturation of HIV-1 virions is initiated by activation of the viral protease, leading to subsequent processing of the polypeptides. HIV-1 entry into a new host cell is mediated by step-wise fusion of the cell and virus membranes. First, the envelope protein binds to CD4 receptor exposing the core of envelope allowing subsequent binding to the *CCR5* co-receptor. The capsid core is released from the early endosome in a pH-independent fashion. Reverse transcription most likely takes place inside the core, but it is still unclear when uncoating takes place. Reverse transcription is restricted by APOBEC3G and SAMHD1 by introducing hypermutations and depleting dNTPs, respectively, whereas uncoating is blocked by MX2, which binds to the capsid core. HIV-1 cDNA integrates preferentially at open chromatin near nuclear pores in the presence of integrase and the cellular proteins LEDGF and Nup153. Viral RNAs transcribed from proviral DNA exit the nucleus by interacting with Rev protein (through the Rev response element) and Crm1. Unspliced RNA encodes Gag and GagPol, while envelope proteins and accessory proteins are produced from spliced RNA variants. Different symbols used to illustrate important components of the HIV life cycle are explained in the box at the bottom of the figure.

like Vpr-fused Cre recombinase, zinc-finger nucleases (ZFNs), and TAL-effector nucleases (TALENs), may potentially be cleaved at internal positions [14, 34, 35].

HIV-1 entry follows a sequential route [36]. Firstly, the envelope protein complex, a trimer composed of gp120 and gp41 heterodimers, binds to the CD4 primary receptor. Secondly, the interaction between the trimer and CD4 triggers a conformational change, resulting in exposure of the co-receptor binding domain. Thirdly, the exposed co-receptor binding motif interacts with either CCR5 or CXCR4, which drives fusion of the cell and virus membranes, although this is still debatable through formation of an early endosome [37, 38]. Notably, lentiviral vectors pseudotyped with different envelope proteins use distinct routes for cell entry. For instance, VSV-G-pseudotyped lentiviral vectors access cells through binding to the LDL receptor (LDLR) or, alternatively, to other members of the LDLR family [39, 40]. The virus is then internalized by clathrin-mediated endocytosis and released to the cytoplasm in a pH-dependent manner [41].

Following fusion of the viral and cellular membrane, HIV-1 releases an intact conical core consisting of capsid monomers into the cytoplasm [42]. Although the details of how the capsid core disassembles after entry are still not clear, a recent report showed that capsid protein remains associated with the reverse transcription complex (RTC) in the cytoplasm as well as the pre-integration complex (PIC) in the nucleus, supporting that the capsid core traffics to the

nuclear membrane (as part of RTC/PIC) before being disassembled during or after nuclear entry [43]. The viral RNA is reverse-transcribed into double-stranded DNA by reverse transcriptase using $tRNA^{Lys3}$ as the primer in a process that involves two strand transfers [44]. Catalyzed by the integrase, which is released from GagPol during virus maturation, HIV-1 DNA is inserted into the genome preferentially into areas with open chromatin. Notably, integration occurs primarily in the outer shell of the nucleus at genomic locations that are associated with the nuclear pores [45]. Hence the nuclear architecture as well as cellular helper proteins, like Nup153 and LEDGF, play key roles in modulating the integration pattern of HIV-1 and derived vectors [45]. From the point of establishing a genomically integrated provirus, HIV-1 relies on the cellular transcription machinery for production of full-length RNA molecules that may serve as new viral genomes or encode Gag and GagPol [30]. Spliced transcripts allow production of the viral envelope proteins and accessory proteins [30], paving the way for the assembly of a new generation of viruses.

IMMUNE SENSING OF HIV-1- AND VECTOR-DERIVED NUCLEIC ACIDS

Successful entry of HIV-1 and establishment of infection is at different levels challenged by intracellular defense machineries in the host cell [46]. Virally delivered nucleic acids play a central role in the antiviral responses evoked by the host cell, and innate immune responses may therefore depend on the genetic content of viruses and may be absent or strongly reduced in cells treated with lentiviral particles devoid of genomic RNA and loaded only with foreign proteins. As mentioned above, viral single-stranded RNA (ssRNA) is reverse-transcribed to a final double-stranded DNA (dsDNA) product. This process, however, will also produce intermediate products like DNA:RNA duplexes and single-stranded DNA (ssDNA) [44]. Viral RNA and DNA intermediates serve as strong triggers of pattern recognition receptors (PRRs) like Toll-like receptors (TLRs), retinoic acids-inducible gene 1 (RIG-I) like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and cytosolic DNA sensors. In general, activation of PRRs initiates a proinflammatory and anti-microbial response, leading to production of proinflammatory cytokines, chemokines and type I IFN through gene-regulatory pathways involving nuclear factor (NF)-κB, mitogen-activated protein kinase (MAPK) and IFN regulatory factor (IRF) [47]. Here, we just briefly touch on the topic of HIV-1-specific immune sensing (Fig. **2**) and refer to excellent previous reviews for further information [7, 47, 48].

Among PRRs, TLRs are the most characterized. TLRs 1, 2, 4, 5, 6, and 10 are localized at the cell surface and mainly recognize hydrophobic molecules unique to microbes, whereas TLR3, TLR7/8, and TLR9 are endosome-located sensors of dsRNA, ssRNA, and unmethylated CpG dsDNA, respectively [7, 49]. In the case of HIV-1, TLR2 and TLR4 are involved in sensing the HIV-1 gp120/gp41 envelope at the cell surface, whereas TLR7 and TLR8 participate in sensing viral genomic RNA [50, 51]. TLR9 senses the reverse transcription products of HIV-1, but its function is impaired by HIV-1 gp120 [52]. Cyclic GMP-AMP synthase (cGAS) and IFN inducible protein 16 (IFI16) are cytosolic sensors of both ssDNA and dsDNA [46, 53]. Interestingly, cGAS generally interacts weakly with ssDNA, but was recently reported to sense HIV-1 ssDNA in a sequence-specific manner [53, 54]. RIG-I normally recognizes dsRNA, which is not produced during the HIV-1 life cycle. Still RIG-I is involved in sensing of HIV-1 [55] potentially through interactions related to formation of secondary structures (and hence shorter stretches of dsRNA) of genomic HIV-1 RNA or mRNA transcribed from proviral HIV-1 [56]. The secondary structures of ssRNA may also play a role in triggering other dsRNA sensors, including endosomal TLR3 and cytoplasmic melanoma differentiation-associated protein 5 (MDA5), although reports on roles of these proteins in HIV-1 sensing are rare [57, 58]. Of note, the VSV glycoprotein G present on the surface of VSV-G-pseudotyped lentiviral vectors is sensed by TLR4 [59].

Since detection of HIV-1 by sensors restricts virus replication, it is likely that interference with these pathways may potentially promote higher levels of lentiviral transduction. Indeed, blocking inflammatory response by pretreatment of mice with dexamethasone was found to boost lentiviral vector transduction in liver dramatically [60]. It has also been documented that shRNA knockdown of IFI16 enhances the transduction of both conventional and integrase-defective lentiviral vectors [46]. However, it should be noted that HIV-1 and lentiviral vectors might mount different innate immune responses, due to different routes of cell entry, the lack of HIV-1-associated proteins in lentiviral vectors, and the potential contamination of plasmid DNA in lentiviral vector preparations. It is unclear how lentiviral protein transfer in particles devoid of genomic RNA may be affected by innate cellular responses.

FIGHTING HIV-1 INFECTION WITH RESTRICTION FACTOR PROTEINS

HIV-1 is subjected to various host restriction factors that target different stages of the virus life cycle. A schematic representation of such restriction factors is provided in (Fig. **1**). APOBEC3G, initially referred to as CEM15, is a deaminase, which is co-packaged into the virion and acts during new DNA synthesis by introducing G-to-A hypermutations [61]. HIV-1 counteracts this type of restriction through the action of the viral accessory protein Vif, leading to ubiquitination of APOBEC3G and targeting of the restriction factor for proteasomal degradation [62].

SAMHD1 inhibits the reverse transcription step by depleting the pool of dNTPs used for new DNA synthesis [63, 64]. Although HIV-1 has not evolved a mechanism to counteract SAMHD1, HIV-2, SIVsm and SIVmac encode an auxiliary protein Vpx, which induces proteasomal degradation of SAMHD1 [63, 65]. Packaging of Vpx into HIV-1-based lentiviral vectors by adapting the Vpx-interacting motif in the p6 domain of SIVmac boosts the titer by two logs in myeloid cells, which are known to be refractory to conventional lentiviral vector transduction [66].

Tetherin (also known as BST2), a surface protein, restricts enveloped viruses by inhibiting the release of virion from the cell membrane. HIV-1 combats this cellular action through the activity of the accessory protein Vpu [67]. To block Tetherin from membrane localization, Vpu hijacks a membrane trafficking pathway that depends on clathrin adaptor protein complex [68].

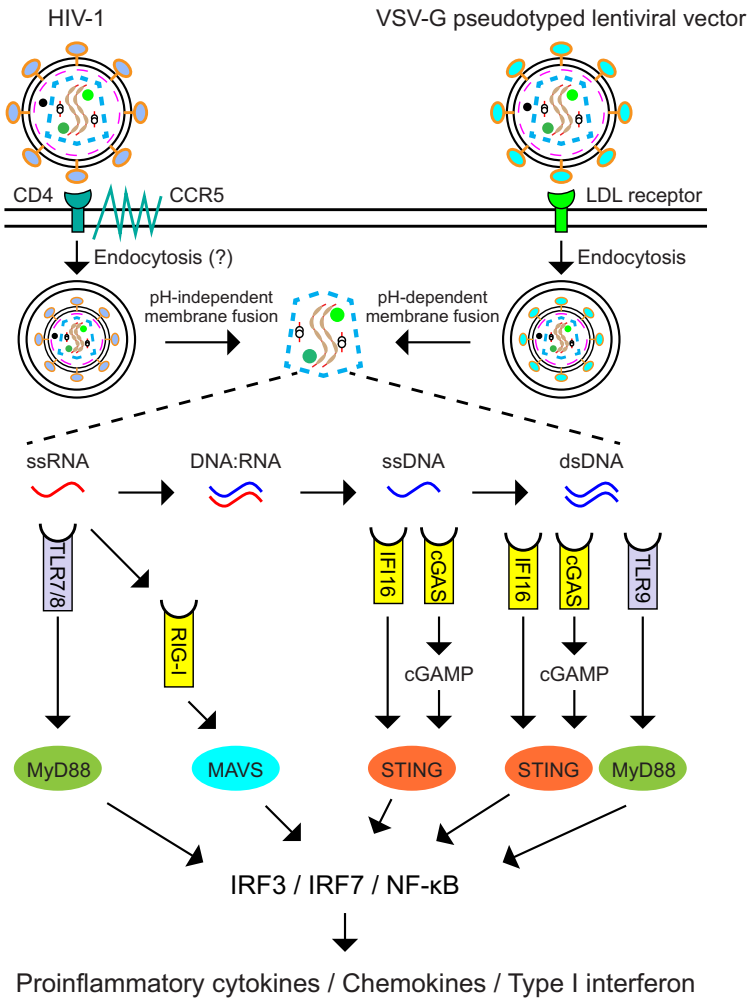

Fig. (2). Innate immunity response against RNA and DNA intermediates sensed during HIV-1 infection and lentiviral vector transduction. HIV-1 and VSV-G-pseudotyped lentiviral vectors enter cells through endocytosis mediated by interactions with CD4-CCR5 and the LDL receptor, respectively. Escape from the endosome occurs *via* PH-independent and PH-dependent processes depending on the port of entry. Genomic ssRNA, intermediate products of reverse transcription including the DNA:RNA duplex and ssDNA, and the final dsDNA products are under the scrutiny of the innate immune sensing system. The cytoplasmic sensor proteins (yellow) RIG-I, IFI16 and cGAS as well as the endosomal sensors (purple) TLR7/8 and TLR9 have been reported to sense HIV-1 nucleic acids. cGAS produces cGAMP as a second messenger molecule that binds STING, whereas IFI-16 interacts with STING directly. MyD88 is activated by TLR7/8 and TLR9, and MAVS by RIG-I. The activation of MyD88, MAVS and STING drives the transcription factors IRF3, IRF7 and NF-κB to initiate the expression of proinflammatory cytokines, chemokines and type I interferon. MyD88, Myeloid differentiation primary response 88; MAVS, mitochondrial antiviral signaling; STING, stimulator of interferon genes; IRF3 and 7, IFN regulatory factors 3 and 7; TLR, Toll-like receptor; cGAS, cyclic GMP-AMP synthase; cGAMP, cyclic guanosine monophosphate–adenosine monophosphate. It is still debated whether virus-host membrane fusion occurs at the cell membrane or in the endosome. This aspect is indicated by the question mark placed next to the part of the figure showing HIV-1 entry through endocytosis.

The action of the human antiviral factor MX2 (also known as MXB), leading to inhibited HIV-1 infection at a late post-entry step, is manifested as failed nuclear accumulation of viral cDNA [69]. The mechanistic details are still unclear, but a recent study indicated that MX2 targets the capsid core [70]. The binding of MX2 to the capsid core involves 20 N-terminal amino acids and requires oligomerization of MX2 [71].

ENGINEERING LENTIVIRAL VECTORS FOR DELIVERY OF TRANSPOSASE PROTEIN

Viral vector transduction is based on the capacity of viruses to traverse cellular barriers and carry genetic information into the nucleus of infected cells. Years of accumulated insight into the biology of HIV-1 have led to effective lentiviral vector technologies and to genetic therapies based on lentiviral gene transfer [72]. Although lentiviruses rely on and exploit many cellular properties, viral proteins transported into the cells as a viral tool kit orchestrate key steps of infection. The ability to carry and deliver protein cargo inside the nucleus of infected cells has inspired others and us to investigate the capacity of lentiviral particles to transport foreign proteins. Such protein delivery may involve production of particles that are devoid of the viral RNA genome, and may in this context fly under the radar of cellular immune responses triggered by sensing of nucleic acids. For some purposes, however, it may be relevant to incorporate

both foreign protein and a viral vector genome in virus particles.

Delivery of proteins to cells results in only transient protein activity, and repeated administration may therefore be necessary to achieve long-term efficacy. For genome engineering purposes, however, short-term activity of protein tools may be sufficient and perhaps even desired to reduce safety risks by limiting off-target protein activities [15, 73, 74]. One could envision that enzymes fused to destabilization domains, like the degradation domain of mouse ornithine decarboxylase (MODC) used for generation of the destabilized d2EGFP reporter protein, [75], could further narrow the window of enzyme activity due to increased protein turn-over. Overall, lentiviral protein transduction seems an attractive option for delivery of genome-modifying enzymes like recombinases, transposases, and tailored endonucleases.

Our endeavors to develop lentiviral particles for protein delivery initially focused on delivering DNA transposases. The *Sleeping Beauty* and *piggyBac* DNA transposon systems are the two most important vector systems based on cut-and-paste DNA transposons. Typically, such systems are based on co-delivery of two plasmids carrying the DNA transposon vector and a DNA transposase expression cassette, respectively. Such integrating, non-viral vectors have a variety of applications, including generation of iPSCs [76, 77] and transgenic animals [78, 79] as well as gene therapy [80, 81] and cancer immunotherapy [82]. Typically based on plasmid DNA, the efficacy of these vectors relies on DNA transfection or electroporation, or alternatively, viral vectors to ferry transposon and transposase across the cell membrane and into the nucleus.

In our early studies, we demonstrated that the gene encoding the *Sleeping Beauty* transposase and the DNA transposon vector itself could be co-delivered by integrase-defective lentiviral vectors (IDLVs) to human cells, resulting in high levels of DNA transposition [83]. As opposed to conventional lentiviral vectors, which are capable of inserting vector DNA into the genome of treated cells, IDLVs carry defective integrase proteins (typically due to a D64V mutation in the integrase catalytic domain) and are not able to insert into the genome in an integrase-dependent manner. Such vectors can successfully support stable transgene expression in postmitotic tissues [84]. By using IDLVs as a platform for DNA transposon delivery, we provided proof-of-principle that transposases can get access to reverse-transcribed dsDNA and catalyze transposon mobilization from lentivirally delivered substrates. We also found that the integration profile of such vectors was markedly altered from the profile of conventional lentiviral vectors [85], suggesting that transposase-directed gene insertion was less prone for insertion into genes and thus potentially safer. From these findings, we moved on to investigate the capacity of lentiviral particles to accommodate transposase protein. Based on discoveries made by the group of Jun Komano, showing incorporation of various proteins, including β-lactamase, eGFP, and CASP3, into lentiviruses [12, 86], we envisioned that a similar approach, exploiting protein-carrying properties of Gag and GagPol, could be attractive for delivery of genome engineering tools. An overview of proteins that have been successfully incorporated in lentiviral, gamma-retroviral, and alpha-retroviral particles is provided in (Table **1**).

We fused the *Sleeping Beauty* and *piggyBac* transposases to the N-terminus of GagPol (between a Lyn-derived myristoylation signal and HIV matrix protein). This resulted in efficient production of transposase-loaded lentiviral particles (Fig. **3**) [13]. Remarkably, the transposase proteins were precisely released from viral GagPol polyprotein upon virus maturation, as determined by Western blot analyses, just like other viral components such as the matrix and capsid proteins [13]. The released *Sleeping Beauty* transposase, however, turned out to be inactive. Control experiments did not give indications that this protein was not transferred to cells but rather that the few amino-acid residues that were left at the C-terminus after protease cleavage rendered the enzyme inactive. Until now, we have not been able achieve activity of the *Sleeping Beauty* transposase after delivery by lentiviral protein transduction. *PiggyBac* transpoase, on the other hand, was not affected by these additional C-terminal residues and has proven very active after lentiviral delivery. In fact, DNA transposition after protein delivery was more potent than we observed after standard plasmid transfections [13]. We found this quite remarkable considering the fact that only a relatively small total amount of transposase protein was delivered to cells (as evaluated by Western blotting and confocal microscopy analyses of virus-treated cells) and that the protein is most likely gone - degraded or diluted - around 24 hours after exposure of the target cells to VSV-G-pseudotyped viral particles [22]. Hence, although a single lentivirus particle harbors up to a few thousand copies of the transposase protein (see Table **1**), the overall level after lentiviral transduction is far from the level of protein achieved after plasmid transfection, which results in overexpression of transposase for several days.

The successful engineering of particles carrying DNA transposases led to the idea of co-delivering transposase proteins and DNA transposon donor in lentiviral particles carrying both components of the system (Fig. **3**) [13, 22]. We refer to such vectors as 'all-in-one' IDLVs. Notably, transposase-fused GagPol harboring the integrase D64V mutation by itself failed to produce reverse transcription products for yet unknown reasons, but this function could be rescued by supplementing with GagPol which did not carry the transposase domain (but still harbored the D64V integrase mutation) during virus production, leading to production of particles consisting of both normal-sized and transposase-fused GagPol [13]. Using such chimeric particles, efficient DNA transposition was observed, leading to integration into TTAA tetranucleotide sequences as would be expected for *piggyBac* transposase-mediated gene insertion [13, 87].

GENOME EDITING BY LENTIVIRAL PROTEIN DELIVERY

ZFNs represent a group of artificial nucleases, initially developed almost 20 years ago [88], consisting of a designed DNA-binding domain fused to an endonuclease, typically FokI. FokI cuts DNA only as a dimer, and ZFNs therefore work together in pairs that bind to DNA, form FokI dimers, and cleave the DNA between the ZFN binding sites.

Table 1. Retroviral protein delivery of genome-modifying enzymes.

Vector System	Protein of Interest and Delivery Strategy	Estimated Units Per Virion	Target Cells	Reference(s)
LV	Vpr-Cre	700	HT1080 reporter cell line containing loxP sites	[35]
LV	Vpr-I-SceI	700	CHO-K1 reporter cell line with the I-SceI recognition sequence	[103]
LV	Myr^{lyn}-PB-Gag-Pol, *Myr^{lyn}-SB-Gag-Pol	5000	HeLa, F9, HaCaT, HEK293, HT1080, CHANG, HepG2, ARPE-19, primary human keratinocytes and NHDFs	[13]
LV	ZFN(gfp)-PH-Gag-Pol, ZFN(AAVS1)-PH-Gag-Pol, ZFN(CCR5)-PH-Gag-Pol	5000	HEK293, NHDFs, primary human keratinocytes, human cord blood $CD34^+$ cells and human iPSCs	[14, 15]
LV	TALEN-PH-Gag-Pol	5000	HEK293 reporter cell line containing a mutant *egfp* gene.	[14]
LV	SpCas9-PH-Gag-Pol	5000	Primary $CD4^+$ T cells, TZM-BL cells, Jurkat cells	[16]
LV	Meganuclease-CypA	500	HEK293, primary T cells	[111]
LV	IN-I-PpoI	100-200	HeLa, MRC-5, A549	[19]
γ-RV	NC-Flp and MA-Flp	3000-5000	Murine SC1, murine iPSCs and human HT1080 with Flp-indicator cassette	[112]
γ-RV	NC-ZFN(gfp), MA- ZFN(gfp)	3000-5000	U2OS, K562 and murine ESC reporter cell line containing *egfp*	[104]
α-RV	Gag-Cre	2000-5000	293T reporter cell line with loxP sites	[113]

Notes to table: Retroviral vector systems include vehicles based on lentiviral vectors (LV), gamma-retroviral vectors (γ-RV), and alpha-retroviral vectors (α-RV); Myr^{lyn}, a heterologous myristoylation signal derived from the Lyn kinase; PH, a heterologous phospholipase C-δ1 pleckstrin homology domain; NC, nucleocapsid; MA, matrix; IN, integrase; PB, hyperactive piggyBac transposase; SB, codon-optimized SB100X. Asterisk (*) indicates that SB was incorporated into virions, but no catalytic activity was observed.

Exploiting cellular repair pathways involving homologous recombination, this technology paved the way for establishment of genome editing at predetermined loci, as previously covered in many reviews [89, 90]. However, construction of functional ZFNs with optimal DNA-binding properties turned out to be technically challenging, and huge costs related to production, or purchase, of ZFNs targeting a genomic region of interest have impeded widespread use of ZFNs. Later, TAL-effector nucleases (TALENs) [91] and now obviously RNA-guided gene knockout and editing strategies based on CRISPR systems [92-94] have revolutionized many areas of life science due to the availability and relative simplicity of these systems. Based on a combination of two tools, an easily designed guide RNA and the Cas9 nuclease, the CRISPR/Cas9 system, in particular, has changed agendas of genetic research, and many efforts will be made to bring this promising technology to the clinic [95]. At present, however, the community has accumulated a vast amount of knowledge related to specific pairs of ZFNs, including those targeting the human *AAVS1* and *CCR5* loci, and ZFNs are currently being investigated for clinical translation [96, 97].

ZFNs are relatively small proteins with a mass of around 40 kDa, depending on the number of zinc-fingers, corresponding to a gene size of approximately 1 kb. This simplifies their delivery by existing vector technologies. IDLVs were the first viral vectors adapted for ZFN delivery, and these vectors have been successfully used in therapeutically relevant cell types like $CD34^+$ cells and embryonic stem cells [98]. The gene expression from IDLV, however, can be markedly restricted in primary cells, possibly due to epigenetic silencing [99], and IDLVs may thus be less attractive as carriers of ZFN expression cassettes [100]. Moreover, lentiviral delivery of ZFN and TALEN genes can be further compromised by potential recombination events between homologous regions occurring during reverse transcription in transduced cells [101, 102]. In a clinical trial, ZFNs were delivered with an adenoviral vector to T cells to knockout the *CCR5* gene encoding a co-receptor for HIV-1 entry [97].

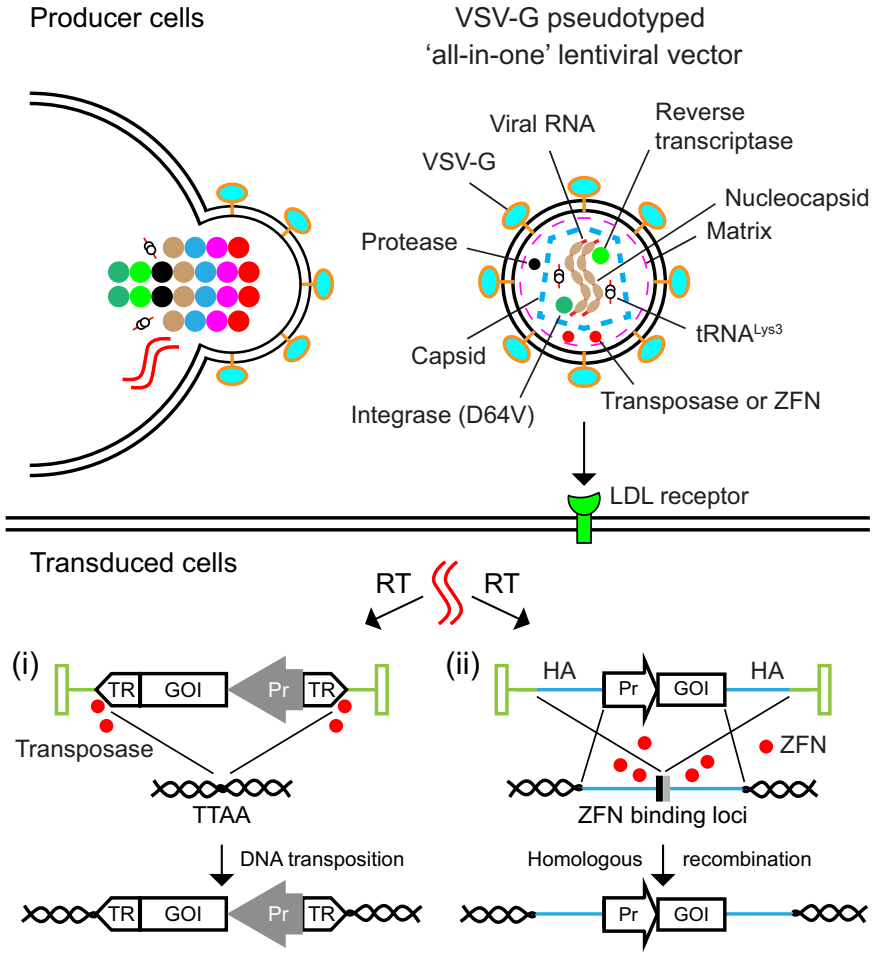

Fig. (3). DNA transposition and genome editing by lentiviral protein transduction. The figure shows a schematic representation of the production of 'all-in-one' lentiviral vectors leading to (i) DNA transposition and (ii) targeted gene insertion in cells treated with vectors carrying DNA transposases and ZFNs, respectively. *PiggyBac* DNA transposase and ZFNs (indicated in red) are fused to the N-terminus of lentiviral GagPol leading to production of lentiviral particles containing the protein of interest. To produce 'all-in-one' IDLVs, containing both proteins (transposases or ZFNs) and viral RNAs carrying the donor (either transposon or homology sequence facilitating homologous recombination), wild-type GagPol is included in the virus particles, as IDLVs carrying the fusion GagPol alone do not produce cDNA products in transduced cells. Following cell entry, the viral genomic RNA is reverse transcribed to generate dsDNA that serves as donor for DNA transposition or homology-directed repair. Pr, promoter; GOI, gene of interest; RT, reverse transcription; TR, terminal region of *piggybac* transposon; HA, homology arm.

The decline of HIV-1 loads in the majority of patients demonstrated the feasibility and therapeutic potential of site-specific nuclease for HIV therapy. Based on its strong liver tropism, AAV8 has been used to deliver a pair of ZFNs targeting the *albumin* gene for insertion of transgenes into this genomic locus. Using this approach long-term expression of human factors VIII and IX at therapeutic levels was achieved in hemophilia mice models [96]. Persistent production of ZFNs from potent viral vectors, like AAV8, may represent a significant challenge in relation to safety in cells that are not actively proliferating. Without stringent regulation of gene expression, such viral vectors may therefore not be clinically applicable.

With the goal of delivering site-directed endonucleases in a 'hit-and-run' fashion, allowing only short-term nuclease activity, we and other groups have investigated gammaretroviral and lentiviral vectors as carriers of nuclease proteins [14, 103, 104]. Izmiryan *et al.* showed lentiviral incorporation of I-SceI, a prototypic meganuclease derived from yeast, by fusing it to the Vpr protein [103]. Co-packaging of I-SceI proteins and donor (as RNA) into lentiviral virions led to successful gene modification in a reporter cell line. However, the lack of genuine I-SceI cutting sites in the human genome as well as the low efficiency has limited the applicability of this method. In our work, we fused ZFNs to the N-terminus of GagPol based on the assumption that this would result in the incorporation of a large number of ZFN proteins into each lentiviral particle, increasing the chance of achieving DNA cleavage at the desired genomic site [14, 105] (Fig. **3**).

By using packaging constructs carrying each of two ZFNs for production of VSV-G-pseudotyped lentiviral particles, we could knock out up to 24% of the target alleles (*e.g. CCR5*) in primary cells transduced with the resulting parti-

cles. To verify that delivery of a limited amount of ZFN protein by this approach could potentially reduce off-target events, we focused on the *CCR2* locus, in which the ZFN target sequence varies from the *CCR5* recognition site in only two nucleotide positions and therefore is considered a highly plausible off-target site. By comparing with the actions of ZFNs delivered by standard plasmid transfection, lentiviral protein transduction showed reduced cleavage at the *CCR2* locus under conditions resulting in similar levels of on-target cutting [14]. By next-generation sequencing of high-ranked predicted off-target sites, we have recently confirmed reduced off-target cleavage after ZFN protein delivery leading to on-target cleavage [15]. In a recent report, a gammaretroviral vector system was adapted for delivery of *egfp*-targeting ZFN proteins [104]. The rate of gene disruption was in this case relatively low (below 15%), but these studies showed that the treatment had little effect on the cell cycle in mouse embryonic stem cells, indicating potentially minimal side effects by protein transduction of nucleases [104].

Incorporation of foreign proteins into lentiviral particles is challenged by the actions of the viral protease, which may cleave in unforeseen regions of the protein-fused Gag and GagPol polypeptides. It is difficult to predict such cleavage, which may be affected by structural properties of the virus particle undergoing maturation. Despite the fact that longer proteins are more likely to undergo such internal cleavage, we showed that TALEN proteins could be delivered by lentiviral transduction [14]. Also, using the same packaging strategy recent findings have demonstrated incorporation of full-length *streptococcus pyogenes* Cas9 (spCas9) into lentiviral particles, resulting in *CCR5* gene knockout in transduced primary T-cells [16]. Notably, this strategy could be combined with expression of guide RNAs from a co-delivered expression cassette.

For potential gene therapy applications of genome editing, DNA cleavage and creation of double-strand DNA breaks is the first step of the process, inducing increased levels of homologous recombination for targeted gene repair or gene integration as the final goal. Using lentiviral protein transduction, a donor sequence can be delivered simultaneously with the ZFN proteins as part of the vector RNA. In a reporter cell line that contains a mutated *egfp* gene, eGFP expression could be restored in 8% of the cells by transduction of the cells with 'all-in-one' IDLVs containing ZFNs and a donor sequence flanked by homology arms. In a follow-up study, we adapted 'all-in-one' IDLVs for targeted gene insertion by co-packaging of a donor vector containing a whole gene expression cassette and ZFNs targeting safe genomic loci [15]. Using this approach for delivering genes to induced pluripotent stem cells, we found that the far majority of the analyzed clones harbored a site-directed lentiviral insertion, which could not be detected in cells treated with conventional IDLVs. These findings demonstrated that site-directed genomic insertion of transgenes cassettes could be achieved by homology-directed gene insertion into DNA breaks generated by co-delivered ZFNs.

CONCLUDING REMARKS

It is estimated that each HIV-1 particle consists of 5000 Gag polypeptides that are assembled in a manner that allows subsequent virion maturation and establishment of a fixed and conserved viral structure. Considering structural constraints and the selection of a powerful viral architecture during evolution, it may seem surprising that the viral structure is sufficiently flexible to allow incorporation of foreign proteins. Here, we have discussed strategies based on fusing heterologous proteins to the matrix protein near the N-terminus of Gag/GagPol, but in ongoing work we have seen that fusion of foreign protein to the integrase protein in the C-terminus of GagPol may equally well lead to protein delivery. This is an interesting finding that deserves further attention in light of the fact that virions contain only relatively few full-length GagPol polypeptides and that vectors carrying such GagPol fusion proteins have retained the capacity to transfer and reverse-transcribe viral RNA. Notably, our efforts so far also suggest that some proteins, at least when introduced in the N-terminal part of Gag, may to some degree affect virus assembly and thus the total viral yield. Hence, the size of the protein may have importance, although this is not formally confirmed by experimentation.

The ultimate goal of conventional gene delivery-based therapies is life-long transgene expression. Delivery of proteins using lentiviral strategies confers only short-term protein activity and, therefore, is not a viable alternative in applications that require high steady-state levels of foreign, therapeutic proteins. The technique seems very well suited, however, for delivery of tool kits for genome maintenance or construction work, for which short-term activity of the tools is desired. So far, successful genome editing has been achieved mainly by methods that involve massive intracellular production of nucleases, either from DNA or RNA, but protein delivery, of Cas9 in particular, is currently showing great promise [106-108]. Delivery of recombinant protein appears to be an attractive approach for *ex vivo* applications, for example in relation to the introduction of genetic modifications in stem cells. For this approach Cas9 and guide RNA can be assembled in ready-to-go and short-lived ribonucleoprotein (RNP) complexes, which can be used for safe editing in cultured cells [73, 109]. For *in vitro* purposes, RNP delivery complexes can be easily produced and delivered leading to robust levels of DNA cleavage [109]. For *in vivo* purposes, however, it is still unclear how protein is most effectively delivered. One could easily imagine that IDLVs loaded with both nuclease proteins and a vector genome carrying the donor sequence for homology-directed repair represent a both effective and gentle option for *in vivo* delivery of tool kits for genome editing. Interestingly, a recent publication shows efficient mRNA delivery using chimeric MS2-lentiviral particles taking advantages of the affinity between MS2-Coat protein and MS2-RNA genome [110]. With preliminary data showing the possibility to deliver Cas9 mRNA, further scrutiny will address the robustness of this method for delivery of different designer nucleases. Interestingly, lentiviral protein transduction offers the possibility of targeting the molecular tools to specific cell types through modification of the

vector tropism, but this concept of cell-directed protein delivery remains to be demonstrated experimentally. It is worth mentioning also that virus-based protein delivery does not overload treated cells with foreign protein. As opposed to other delivery strategies, including transfection of plasmid DNA, RNA, and RNP complexes, lentiviral protein transduction does not lead to exceptional intracellular levels of nucleases and transposases. Still, the activity of delivered nucleases and transposase often matches the activity after conventional DNA or RNA transfection [13]. One possible explanation for this observation is that hitchhiking proteins are ferried into the nucleus by the lentiviral pre-integration complex, but this remains to be experimentally verified.

In this review, we also briefly covered some of sensing mechanisms and restriction factors that together constitute a cellular defense against HIV-1 infection. However, it is far from clear how such cellular defense mechanisms, which vary between cell types and between tissues, may affect lentiviral gene transfer and to what extent sensing properties leading to an innate immune response restrict gene delivery. Nevertheless, in the gene therapy and genome editing communities there is increased focus not only on restriction mechanisms, but also on the potential inhibitory role of innate responses to loads of foreign DNA and RNA delivered to cells with the purpose of editing the genome. Delivering proteins in lentiviral vehicles may to some extent bypass restrictive cellular responses, but small RNAs, either expressed or *in vitro*-transcribed variants, may still elicit responses that influence efficacy of the genome engineering process. It should be noted also that protein delivery strategies using viral vehicles might be advantageous in cell types that are refractory or very sensitive to transfection.

Every future attempt to bring genome editing to the clinic will focus on the safety of the procedure and on potential side effects. Protection of cargo within the virus core that travels through the cell and the capacity of the virus to drop off the protein consignment inside the nucleus in a desired genomic neighborhood are key features of lentiviral protein transduction that may increase safety of genome editing procedures. As the genome editing field keeps moving ahead with unprecedented speed, there are still many reasons to evaluate aspects related to delivery, and the development of protein delivery strategies, including lentiviral protein transduction, deserves continued scrutiny.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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