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ORIGINAL PAPER

Gene delivery by lentivirus vectors

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Abstract The capacity to efficiently transduce nondividing cells, shuttle large genetic payloads, and maintain stable long-term transgene expression are attributes that have brought lentiviral vectors to the forefront of gene delivery vehicles for research and therapeutic applications in a clinical setting. Our discussion initiates with advances in lentiviral vector development and how these sophisticated lentiviral vectors reflect improvements in safety, regarding the prevention of replication competent lentiviruses (RCLs), vector mobilization, and insertional mutagenesis. Additionally, we describe conventional molecular regulatory systems to manage gene expression levels in a spatial and temporal fashion in the context of a lentiviral vector. State of the art technology for lentiviral vector production by transient transfection and packaging cell lines are explicitly presented with current practices used for concentration, purification, titering, and determining the safety of a vector stock. We summarize lentiviral vector applications that have received a great deal of attention in recent years including the generation of transgenic animals and the stable delivery of RNA interference molecules. Concluding remarks address some of the successes in preclinical animals, and the recent transition of lentiviral vectors to human clinical trials as therapy for a variety of infectious and genetic diseases.

Keywords Lentiviral vector · Gene therapy · RNA interference · Clinical · Animal model · Envelope · Packaging · Biosafety · Mobilization · Insertional mutagenesis

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Introduction

For more than two decades retroviral biology has been the most intensely studied field in virology. The retroviral genome is encoded by a 7–12 kb positive sense single stranded RNA molecule, two of which homodimerize and package in lipid-enveloped viral particles. Following attachment and receptor-mediated entry into host cells, viral reverse transcriptase and integrase enzymes mediate reverse transcription and integration of the virus genome into the host cell chromatin. The ability of a replication competent retrovirus to incorporate a herpes simplex virus thymidine kinase (*tk*) gene into the genome of a mouse cell and to convert NIH-3T3 TK-cells into TK+ transformants was first described in 1981 [1, 2]. These studies established the basis of using retroviruses as vehicles for efficient therapeutic gene delivery into mammalian cells. More than 20 years of extensive research of retrovirus vector biology resulted in major improvements in vector design and retrovirus vector production. High titer concentrated retrovirus vectors (>10^9 IU/ml) can be generated by several retrovirus vector stable producer lines. The ability to pseudotype retrovirus vectors with a variety of envelope proteins, including the vesicular stomatitis virus G glycoprotein (VSV-G), significantly broadens the tropism of replication defective retrovirus vectors. In addition, combinations of synthetic and tissue-specific promoters, which were incorporated into retrovirus vectors, allowed long term and regulated gene expression in vector transduced cells. These vector modifications have paved the way for the initiation of more than 250 gene therapy clinical trials employing simple retroviruses over

the last decade (www.wiley.co.uk/genetherapy/clinical/). The inability of simple retroviral based vectors to transduce non-dividing cells has limited their potential utility for gene therapy to predominantly the treatment of hematopoietic disorders and various cancers. Successful correction of three hematopoietic disorders in humans was achieved with simple retroviral based vectors (ADA-SCID, SCID-X1, and X-linked CGD [3–7]); nonetheless, excitement was tempered by the finding that insertional mutagenesis had resulted in leukemogenesis in three patients from a SCID-X1 clinical trial [8–10]. As a result, safety considerations, as well as the inability to transduce non-dividing cells, have rendered simple retroviruses inefficient as an in vivo gene delivery system.

In contrast with simple retroviruses, lentiviruses have evolved the ability to infect non-dividing cells [11], an attribute that significantly broadens the utility of lentiviral vectors to numerous target tissue and cell types. HIV-1 is a member of the lentiviradae genus also comprising HIV-2, simian immunodeficiency virus (SIV) and non-primate lentiviruses, such as visna virus, equine infectious anemia virus (EIAV), caprine arthritis-encephalitis virus (CAEV) and the feline and bovine immunodeficiency viruses (FIV and BIV). Even though the first lentiviral vectors developed were based on HIV-1, researchers have since engineered each of the aforementioned lentiviruses into vectors to mediate gene delivery [12–19]. Several lentiviral vectors exhibit proficient transduction of non-dividing cells in target organs in vivo including the central nervous system, liver, eye, heart, hematopoietic stem cells, and pancreas, reviewed in Wiznerowicz et al. [20] and Cockrell et al. [21]. A number of ongoing studies to elucidate the mechanism by which lentiviruses infect non-dividing cells, primarily with HIV-1, have indicated that an interaction between the host cell nuclear import machinery, and nuclear localization signals within gene products of the *gag*, *vpr*, and *pol* genes, and the cPPT (central polypurine tract) *cis*-element may each mediate an active transfer of HIV-1 preintegration complexes into nuclei of non-dividing cells [22–26]. Nonetheless, recent evidence supports an alternative theory; posing that the efficiency of uncoating, due to the capsid protein association with intracellular retroviral complexes, may account for the disparity between lentiviruses and simple retroviruses in transduction of quiescent cells, reviewed in Yamashita et al. [27]. Effective transduction of non-dividing cells with lentiviral vectors has led to their wide-spread use in both research and clinical environments.

Pursuant to transduction of non-dividing cells stable, long-term transgene expression is a desirable attribute for any research/clinical application involving transgene delivery via lentiviral vectors. Lentiviral vectors have the demonstrated capacity to mediate long-term transgene expression in vivo [28–31]. In the course of vector development there are two factors that can be manipulated to adjust the level and duration of transgene expression (1) *cis*-elements within the vector and (2) the specific transgene being delivered, the choice of which will depend upon the application being addressed. Both the vector *cis*-elements and transgene can influence the degree to which transgene expression may be repressed by target cell silencing machinery, reviewed in Ellis [32], or completely eliminated by the innate and/or adaptive immune responses. Initial development of HIV-1 vectors exhibited efficient transduction of non-dividing cells while retaining the ability to integrate transgenes into the target cell genome in the absence of an inflammatory response [12, 28, 33, 34]. Ensuing studies have since demonstrated the lack of innate and cellular immune responses against vector associated proteins [35]; nonetheless, the gene of therapeutic, or research, interest (transgene) being delivered should be carefully assessed for its potential to elicit a cellular immune response in vivo [35–37], an obstacle that afflicts all gene delivery systems. Although gene silencing and immune response are important factors to consider in developing lentiviral vectors, safety has been the underpinning issue guiding progress with lentiviral vectors.

As therapeutic applications for lentiviral vectors are now beginning to transition from preclinical animal models to corrective therapy in humans it is imperative that lentiviral vector design and production obviate all putative risks. In most cases, the objective for transgene delivery with a lentiviral vector is to obtain a single transduction event into the target host cell, without perturbing normal function of the host cell genome. Insertional mutagenesis, vector mobilization, generation of replication competent lentiviral vectors (RCLs), and germ-line transmission of vector sequences are all potential hazards that could arise as a consequence of the design and production of lentiviral vectors [38]. These topics will be addressed further in the following sections, however in light of recent evidence regarding insertional mutagenesis of simple retroviral vectors from a SCID-X1 clinical trial it is important to note that HIV-1 based vectors, as well as HIV viruses, have not been associated with neoplastic transformations as a consequence of insertion into the host genome. The initial design of replication defective HIV-1 vectors is based on the strategy of segregation of the *cis*-acting elements in the HIV-1 genome (non-coding sequence elements that are required for vector RNA synthesis, packaging, reverse transcription, and integration) from the *trans*-elements encoding enzymatic, structural, and accessory proteins [12]. As an additional measure of safety, envelope-encoding sequences are usually separated from the rest of the HIV-1 packaging and vector cassettes. In some cases, the rev gene has also been placed on an individual cassette, thereby totaling four cassettes. Together, separation of these three or four components minimizes the likelihood of generating RCLs, requiring at least two recombination events. Based on

this approach, the components required for the production of HIV-1 based vectors are supplied in producer cells from three, or a fourth (rev), separate expression cassettes: envelope, packaging, and vector cassettes.

The envelope cassette

Substituting the parental HIV-1 envelope with the vesicular stomatitis virus G protein (VSV-G) was a major breakthrough in lentivirus vector development [12, 34] (Fig. 1). The VSV-G envelope confers three novel features on lentivirus vector particles: (1) dramatically broadening vector tropism by facilitating transduction of numerous tissue and cell types of various species in vitro and in vivo, (2) stabilization of vector particles from shear forces during centrifugation, thereby supporting vector concentration by ultracentrifugation and (3) directing lentiviral vector entry to an endocytic pathway, which reduces the requirements for viral accessory proteins for full infectivity [39]. Although VSV-G pseudotyped lentivirus vectors were found efficient at transducing non-dividing cells in various animal models, results from at least four studies have indicated that systemic administration to humans can be

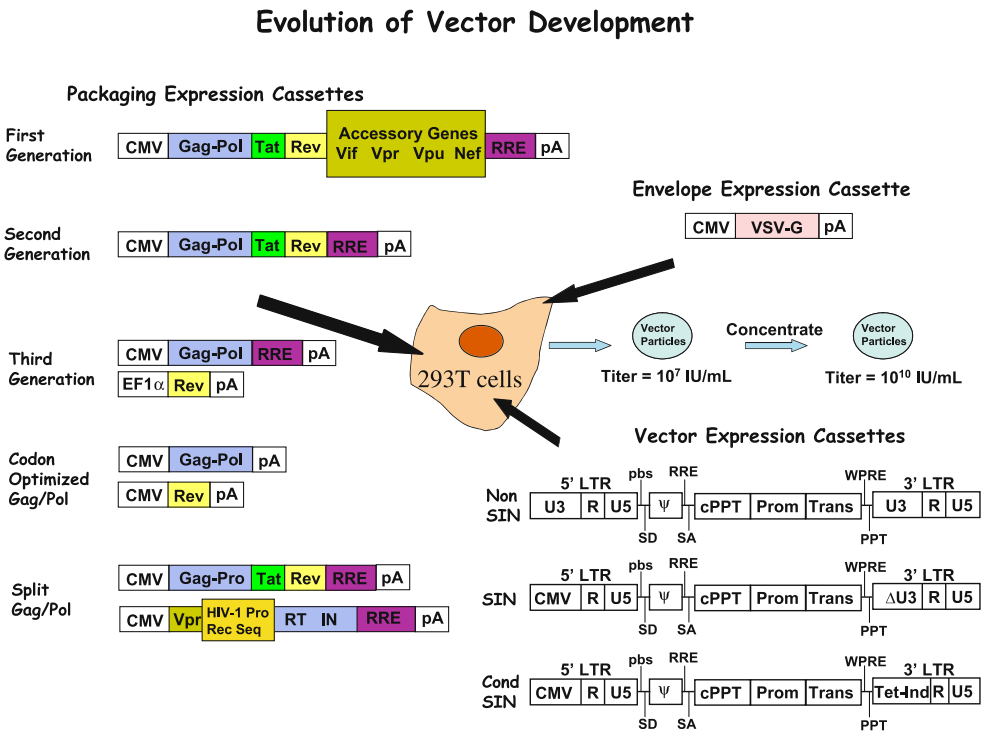

Fig. 1 The evolution of HIV-1 vector development and transient vector production. The calcium phosphate technique is used to transfect 293T cells with individual plasmids containing: (1) a packaging cassette system that may comprise one or two plamid(s) depending upon the system used; (2) a vector expression cassette; and, (3) an envelope expression cassette. Titers are typically about 10^7 IU/ml prior to concentration and 10^{10} IU/ml after concentration. Information regarding the relevance of each cassette in the evolution of vector development can be obtained from the body of the manuscript. All expression cassettes are presented as they would be in the context of a transfected plasmid. The following key explains the genetic elements in each cassette. *Packaging expression cassettes*: CMV and EF1α are constitutive promoters driving expression of the packaging elements; *gag* and *pol* genes encode the structural and enzymatic proteins required to form functional vector particles; *tat* gene encodes the Tat transactivator required to promote expression of full-length vector RNA from non-SIN vectors; *rev* gene encodes the Rev protein required for nucleocytoplasmic transport of full-length vector RNA and packaging RNAs that are not codon optimized; RRE (Rev response element) is a *cis* element bound by Rev to mediate nucleocytoplasmic export; and pA is a polyadenylation signal. Three types of *vector expression cassettes* are depicted: (1) Non SIN vectors are those which require Tat protein for full-length vector RNA expression; (2) SIN vectors are made Tat-independent through the use of a CMV promoter to drive expression of the full-length vector RNA, and contain a deletion in the promoter/enhancer region of the U3 in the 3′ LTR which is transposed onto the 5′ LTR following transduction of a target cell; and, (3) the conditional SIN vector is similar to a SIN vector, however the promoter/enhancer region of the U3 was replaced with a Tet-inducible promoter. The following elements are common to all the vector cassettes: the primer binding site (pbs) facilitates the initiation of reverse transcription; SD and SA represent splice donor and splice acceptor sites, respectively; ψ represents the *cis* packaging signal; cPPT is the central polypurine tract; the promoter/transgene cassette (Prom/Trans) comprise the promoter and transgene of interest for vector-mediated delivery; WPRE is the woodchuck hepatitis virus posttranscriptional regulatory element; and PPT is the polypurine tract. *Envelope expression cassette*: depicts the CMV promoter driving expression of the vesicular stomatitis virus glycoprotein (VSV-G), and a polyadenylation site (pA). Although, other envelope proteins may substitute for VSV-G

hampered by complement and antibody mediated immune responses directed against the VSV-G envelope [40–42]. Importantly, Croyle et al. describe studies demonstrating the in vivo pharmacokinetic properties of VSV-G pseudotyped lentiviral vectors following intravenous administration of C57BL/6 mice [42]. Vector activity decreased ~2 logs within 45 min after systemic administration, and was nearly undetectable by 6 h after injection. Croyle et al. were able to dramatically extend the in vivo vector half-life (~5-fold) by conjugating the VSV-G envelope protein with poly(ethylene) glycol (PEG), without a concomitant loss in vector titer. Moreover, PEGylated vector was highly resistant to inactivation by normal human serum in vitro, and exhibited increased transduction efficiency in numerous murine tissues including bone marrow, liver, and muscle. Although PEGylation successfully ameliorated vector susceptibility to the complement-mediated immune response, pre-existing neutralizing antibodies to VSV-G in human sera, or a requirement for vector re-administration, may render vectors incompetent for transduction and mediate vector elimination.

Pseudotyping lentiviral vectors with envelope proteins other than VSV-G may facilitate evasion of neutralizing antibodies to VSV-G; nevertheless, the predominant objective for pursuing alternate envelope proteins was to restrict lentiviral vector tropism in a cell- and/or tissue-specific manner. Lentiviral vectors have been pseudotyped with a plethora of envelope proteins other than VSV-G, reviewed in Cronin et al. [43]. Additionally, the VSV-G envelope from the Chandipura strain of vesicular stomatitis virus can substitute for the commonly used VSV-G from the Indiana strain without sacrificing titer or specific activity [44]. Despite the use of various envelope proteins, limiting vector entry to specific cell- and/or tissue types following systemic in vivo administration continues to be a significant challenge. Progress in this area was recently demonstrated using antibodies to direct lentiviral vectors to specific tissue/cell types through systemic delivery of preclinical murine xenograft models [45, 46]. Morizono et al. employed a modified Sindbis virus envelope (m168) protein to pseudotype lentiviral vectors combined with soluble antibody to demonstrate increased specificity for metastatic melanoma cells in populated murine lungs with an ancillary decrease in liver and spleen transduction, when compared to VSV-G pseudotyped lentiviral vectors [45]. In a different approach Yang et al. incorporated a viral envelope fusogenic domain and an antibody to human CD20 into the lipid bilayer of the lentiviral vector to demonstrate specific transduction of B cells in vivo; nonetheless, specificity was not assessed relative to tissues outside the peripheral blood compartment [46]. Although both studies show encouraging alternatives to VSV-G, these methodologies will require further testing in immunocompetent animal models. Concerning VSV-G pseudotyped lentiviral vectors, an interesting study by Torashima et al. established that minor changes in pH (from 7.2 to ~7.0) during vector production can modify the tropism profile with a shift from Purkinje cells to primarily Bergmann glial cells following in vivo administration into the murine cerebellum [47]. These data indicate that steps within the production process may alter the biophysical properties of VSV-G, and consequently the tropism of VSV-G pseudotyped lentiviral vectors. In general, titers of VSV-G pseudotyped lentivirus vectors are more than ten-fold higher than the typical titers of non-VSV-G pseudotyped lentivirus vectors and VSV-G pseudotyped vectors are typically more stable than non-VSV-G envelope proteins upon concentration by ultracentrifugation. Therefore, VSV-G continues to be the envelope of choice for many lentiviral vector researchers. Clearly, further studies are required to optimize envelope protein usage in lentiviral vector based gene therapy models. In this regard, the ability to specifically direct lentiviral vectors to specific target organs in vivo, while circumventing an immune response, is most desirable.

The packaging cassette

Excluding the envelope protein, the packaging cassette encodes all lentiviral vector *trans*-elements (expressed from *gag* and *pol* genes), which comprise structural and enzymatic proteins required for vector particle production and efficient transduction of target cells. Lentiviruses encode additional *trans*-elements termed accessory proteins that are required for productive infection/replication of the wild-type virus in a host organism. The initial lentivirus vector packaging constructs, derived from HIV-1, were developed by Naldini et al. to be devoid of Env expression, while retaining the accessory proteins (Vpu, Vpr, Vif, Nef, Rev, and Tat) as functional components of the packaging cassette [12, 30]. Additional engineering included removal of *cis*-elements (packaging signal, LTRs, and primer binding site) from the packaging cassette to curtail the generation of RCLs by preventing the packaging of the full-length mRNA encoding *trans*-elements into vector particles, and their subsequent transfer to target cells [12, 30]. *Cis*-elements [Rev response element (RRE) and parental splice donor site] pertinent to post-transcriptional mRNA processing events are maintained, and full-length mRNA synthesis of the packaging cassette is typically achieved using heterologous constitutive promoters (CMV or RSV) and polyadenylation signals (SV40 and insulin gene). These initial packaging cassettes are termed first generation packaging constructs (Fig. 1). In order to augment the biosafety of first generation HIV-1 packaging

cassettes, several groups systematically deleted the accessory genes [28, 29, 33, 48]. Deletion of all accessory genes except Tat and Rev led to a second generation packaging cassette [29] (Fig. 1), which was shortly followed by a third generation packaging cassette whereby the *tat* gene was deleted, and the *gag/pol* and *rev* genes were placed on two separate expression cassettes [48] (Fig. 1). Partitioning the remaining packaging components improved lentiviral vector biosafety by increasing the minimal number of recombination events to three (between vector, envelope, gag/pol, and rev cassettes). Furthermore, should RCLs be inadvertently generated by the new packaging system, they will lack all the accessory proteins that afford HIV-1 its pathological capabilities. Notably, improvements in the packaging cassette did not compromise vector titers or tissue/cellular transduction profiles.

Modifications of the *gag* and *pol* Genes to Prevent Generation of RCLs

Developments succeeding the third generation packaging cassette included efforts to mitigate the synthesis of RCLs through direct modifications of the *gag* and *pol* genes within the packaging construct. The exceptionally high content of AU bases in the HIV-1 genes results in a biased codon usage and the generation of unstable transcripts. Instability sequences within HIV-1 transcripts render Gag/Pol protein expression Rev-dependent. The Rev accessory protein interacts with the RRE *cis*-element in the *gag/pol* mRNA transcript to confer nucleocytoplasmic transport and subsequent expression. The potential for production of RCLs arises from the sequence homology shared between the packaging and vector cassettes, both of which contain the RRE *cis*-element and a portion of the *gag* gene. Kotsopoulou et al. altered the codon bias of the HIV-1 *gag/pol* sequences to those more commonly found in the human genome (humanization) rendering a highly efficient, Rev-independent *gag/pol* gene expression cassette that was not hampered by deletion of the RRE sequence [49] (Fig. 1). Humanization eliminated the sequence homology between the packaging and vector cassettes through deletion of the RRE and altering the *gag* sequence, thereby alleviating concerns regarding RCLs. Furthermore, humanization supports efficient production of vectors expressing anti-HIV-1 gene products, such as *trans*-dominant proteins, ribozymes, and shRNA/siRNAs directed against *gag/pol* gene sequences. In consonance with this approach ter Brake et al. recently utilized a humanized *gag/pol* cassette to package a lentiviral vector harboring multiple shRNAs directed against conserved regions of the *gag* and *pol* genes of wild-type HIV-1 virus [50]. Compared to a single shRNA incorporation of three shRNAs exhibited an additive affect in reducing wild-type HIV-1 virus production.

According to a novel, sensitive assay for the detection of RCLs employed by Wu et al. RCLs can also be generated through non-homologous recombination events [51]. RCLs were eliminated through the development of a sophisticated HIV-1 *trans*-lentiviral vector packaging construct, in which the *gag/pro/pol* coding region is split into two expression cassettes: the *gag/pro* cassette and a *vpr/pol* fusion cassette [51, 52] (Fig. 1). Vpr binding to the Gag precursor protein p6 mediates efficient incorporation of the Vpr/Pol fusion protein into vector particles. A protease cleavage site was introduced between the *vpr* and *pol* genes to ensure removal of the Vpr protein and appropriate processing of Pol into reverse transcriptase and integrase proteins. Using the new packaging system, it was possible to completely prevent vector-mediated transfer of functional *gag/pol* sequences into target cells. Thus, further improvements in the biosafety of the lentiviral vectors were achieved by splitting the entire system into four constructs from which the vector, envelope, Gag/Protease, and Vpr/Pol are independently expressed. Presumably, it may be possible to place the *rev* gene on an individual expression cassette, thereby further enhancing safety by separating the entire system onto five distinct constructs. Efforts to enhance biosafety of lentiviral vectors with regard to preventing the generation of RCLs have included a combination of deleting *cis*-elements from the packaging cassette, removing accessory genes, increasing the number of recombination events to form RCLs by partitioning relevant packaging genes onto separate expression cassettes, and minimizing the homology between packaging, vector, and envelope cassettes.

Modifications of the *pol* genes to prevent insertional mutagenesis

Modifications in the packaging cassette have primarily focused on forestalling the generation of RCLs. A new phase in packaging cassette development, and consequently vector development, is underway to palliate the consequences of insertional oncogenesis. The development of T cell leukemia in three patients from a clinical trial that employed simple retroviral vectors to successfully treat nine patients for SCID-X1 disease shifted conventional concerns regarding insertional mutagenesis from a potential threat to a real outcome to be averted when considering retroviral vectors for gene therapy protocols [8, 9, 53]. Insertional mutagenesis can lead to genotoxicity as a result of insertion of a retroviral vector into, or near, genes that confer a survival/growth advantage upon the transduced target cells, reviewed in [10]. A growth advantage imparted to only a few transduced target cells may gradually change the cellular profile from polyclonal (numerous cells with myriad integration sites) to monoclonal (numerous cells

with few integration sites) cell populations, a putative precursor to oncogenesis [7, 8]. Moving to the use of lentiviral vectors has been one way to temper concerns regarding insertional oncogenesis, as is evident from recent reports describing preclinical studies correcting ADA-SCID mice through HIV-1 vector mediated delivery of adenosine deaminase [54, 55]. These preclinical studies represent a transition away from the use of simple retroviral vectors, which were previously demonstrated to correct ADA-SCID in humans [3]. Nevertheless, lentiviral vectors are not completely detached from the potential for insertional oncogenesis. Recently, EIAV vectors were shown to be associated with the formation of tumors in the livers of mice following *in utero* and neonatal vector administration [56]. A causal relationship between EIAV vectors and tumorigenesis has yet to be established; however it is most important to note that in this same study the use of various HIV-1 based vectors were not associated with the formation of any detectable tumors. Despite the evidence from this study and the lack of any precendent for HIV-1 vectors to be associated with tumor formation, lentiviral vectors that would obviate insertional mutagenesis are most desirable.

Two strategies to modify the lentiviral vector packaging cassette are being pursued to avert insertional mutagenesis: (1) directing lentiviral vector insertion into specific sites within the host genome, and (2) exploiting non-integrating HIV-1 vector episomes to deliver therapeutic transgenes. Amending either the carboxy or amino termini of the HIV-1 integrase gene by fusing a polydactyl zinc finger could enhance site-specific integration of HIV-1 vectors by 7–10-fold, compared to integration over the entire genome [57]. The synthetic polydactyl zinc fingers are modular with regard to preference of the genomic recognition site, an advantage over more primitive targeting systems, reviewed in Bushman [58] and Sandmeyer [59]. In another study, a polydactyl zinc finger fused to a heterologous nuclease was delivered to B cells from an X-SCID patient by an integrase deficient lentiviral vector, demonstrating 8% correction of an IL2Rγ locus mutation by promoting homologous recombination [60]. Although promising, progress in site-directed technology will require a more profound knowledge of the cellular mechanisms that govern retroviral integration.

During the natural course of HIV-1 infection, at least four forms of non-integrated DNA have been detected in tissue reservoirs and tissue culture comprising the majority (>90%) of HIV-1 DNA forms present in infected cells: linear, 2-LTR, 1-LTR, and autointegrant (mutant) episomal forms [61–64]. Originally thought of as unstable, transient entities, the 2-LTR episomal forms were used as markers of ongoing HIV-1 infection; however, recent evidence has demonstrated that these forms are stable, especially in non-dividing cells, and are diluted only upon cell division [65–67]. In addition to the stability of non-integrating DNA forms in non-dividing cells, accumulating evidence indicates that they can support gene expression [68]. Moreover, class I integrase mutations (mutations in the core catalytic domain) in the integrase genes of lentiviruses dramatically abate the catalyzed reaction of cleaving and rejoining during the integration process in target cell genomes and results in increased levels of 2-LTR and 1-LTR circles [69–72]. Although not pertinent to the discussion of packaging cassettes, it is important to mention that integration defective vectors can also be generated by mutating positions in the attachment (*att*) sites in the vector LTR, which are required for processing by the integrase prior to integration [73]. Through the use of class I integrase mutations in packaging cassettes and *att* mutations in the vector, researchers have begun to exploit the properties of non-integrating DNA forms for gene delivery to non-dividing cells, a critical advancement in vector development for evading the consequences of insertional mutagenesis in the course of gene therapy protocols.

Non-integrating lentiviral vectors are primarily gene delivery vehicles for diseases with pathologies in non-dividing cellular compartments including the eye and brain, and in slowly dividing tissues such as the liver. Initial studies with class I integrase defective lentiviral vectors exhibited little transgene expression following in vivo administration [30, 72, 74], yet stable in vitro transduction could be acquired [72, 75]. Most recently, however, two groups employed packaging cassettes harboring distinct mutations to demonstrate efficient in vivo transgene delivery in ocular and brain tissues [76, 77]. In addition to long-term reporter transgene expression in the eye (9 months) and brain (30 days), Yanez-Munoz et al. described the first effective use of a non-integrating lentiviral vector for gene therapy in a preclinical murine model of retinitis pigmentosa [76]. The disparity in in vivo transduction between the most recent studies, and earlier reports, are yet to be reconciled.

Modifications in the packaging cassette have yielded significant advances in lentiviral vector mediated gene delivery through the enhancement of biosafety by limiting the potential risk of RCLs, and, most recently, insertional mutagenesis. Knowledge of the cellular mechanisms underlying lentiviral integration and formation of episomal DNA forms will afford further manipulation of lentiviral vectors for gene delivery.

The vector cassette

The lentiviral vector cassette expresses the full-length vector RNA, containing all the *cis*-acting elements (required for efficient packaging, reverse transcription,

nuclear import and integration) and the transgene expression cassette (internal promoter and transgene sequence). The archetype for subsequent lentiviral vectors was an HIV-1 Tat-dependent vector expressing full-length vector mRNA from the 5′ LTR (long terminal repeat) and terminating in the 3′ LTR [12, 30] (Fig. 1). Packageable full-length vector mRNA contained critical *cis*-elements: 1) a 5′ UTR comprised of the primer binding site (PBS), splice donor site, packaging signal, rev-response element (RRE), and splice acceptor; (2) an expression cassette constituting a heterologous promoter driving expression of a transgene; and, (3) a 3′ UTR harboring a polypurine tract (PPT) and the 3′ LTR. Replacement of the U3 in the 5′ LTR with a potent heterologous promoter rendered the vector Tat-independent, and, thus, enabled vector production with a third generation packaging construct [33, 78, 79]. A further increase in vector titer was obtained by substituting the bovine growth hormone polyadenylation signal for the 3′ U5 [80].

The self-inactivating (SIN) lentiviral vector and vector mobilization

A critical improvement in vector safety was the deletion of the enhancer/promoter sequences in the U3 region of the 3′ LTR, which defines the vectors as self-inactivating (SIN) [78–80] (Fig. 1), as adapted from simple retroviruses [81]. Subsequent to reverse transcription in a host target cell, the SIN deletion in the U3 region of the 3′ LTR is transposed to the U3 region of the 5′ LTR in the proviral DNA and prevents expression of full-length vector RNA. The SIN deletion mutation imparts enhancement of lentiviral vector safety profiles with regard to RCL formation (elimination of sequence homologous to wild-type HIV) and insertional mutagenesis (prevents interference with promoter/enhancer elements of the host genome following integration). The most substantial improvement, however, is the dramatic reduction in expression of full-length packageable vector transcripts, which mitigate the potential for vector mobilization upon superinfection with wild-type HIV virus. Inadvertant mobilization of vector *cis* and *trans*-elements may have unforeseen consequences with respect to insertional mutagenesis and the immune response. The most severe fallout, however, could be the spread of vector elements among individuals within a population by traditional routes of HIV transmission; a consequence that must not be overlooked when considering novel vector designs for clinical applications. Evidence from a number of researchers clearly demonstrates rescue and mobilization of non-SIN, Tat-dependent lentiviral vectors upon superinfection with wild-type HIV-1 in PBMCs, T cell lines, and cell lines that are CD4 receptor and coreceptor positive [82–85]. Concomitant experiments within some of the same reports verified that SIN lentiviral vectors were not mobilized upon superinfection with wild-type HIV-1 [83–85]. Nonetheless, expression of full-length SIN lentiviral vector transcripts have been detected in SIN vector transduced 293T cells, T cells, and myeloid cells [86, 87]. As might be expected, SIN vectors were rescued and mobilized upon transfection of 293T cells previously transduced with a SIN vector, or following transduction of a packaging cell line with a SIN vector yielding titers in the range of 10^2–10^3 TU/ml [86–88]. Even more profound are the results of Ma et al., whereby standard vector production via transient transfection of 293T cells with a single-LTR vector containing a SIN mutation yielded vector particles with titers (>10^6 TU/ml) comparable to those of traditional vectors [88]. Implicit from these results is the fact that 1-LTR episomal forms of SIN vectors following transduction may be a highly significant source of packageable vector for subsequent mobilization. Presumably, episomal SIN vector forms can be diluted upon cell passaging, however without molecular evidence to demonstrate the absence of non-integrated episomes, it is possible that the mobilized SIN vectors in the aforementioned studies [86–88] are at least partly derived from non-integrated episomal forms. As previously discussed, episomes are stable entities in quiescent cells, and therefore may significantly contribute to the availability of full-length vector transcripts competent for packaging and subsequent mobilization. Toward this end, recent data from our laboratory indicate that episomal SIN vectors formed shortly after transduction do, in fact, contribute quite significantly (~10^6 TU/ml) to full-length vector RNA competent for mobilization (manuscript in preparation). Indeed, the potential for mobilization of SIN vectors is a true concern, especially with regard to episomal SIN vector forms; however, *bona fide* mobilization of a SIN vector following wild-type HIV superinfection in T cells, or otherwise, has yet to be confirmed.

Notwithstanding a scarcity of data showing SIN vector mobilization with HIV virus, anticipation of the potential for SIN vector mobilization begets the need for a resolution. One approach is use of chromatin insulators that are derived from chromatin boundaries and are, inherently able to protect adjacent chromatin regions from influencing each others' promoters/enhancers, and from silencing effects that may spread from region to region [89]. Therefore, inclusion of insulators upstream of the promoter that initiates transcription from the R region may prevent expression of the potential full-length SIN vector transcripts competent for packaging, reverse transcription, and integration. In fact, inclusion of the chicken β-globin insulator (cHS4) into the U3 region of the LTRs may mitigate vector RNA expression from integrated SIN vectors [87]. Futhermore, incorporation of insulators into lentiviral vectors can help maintain long-term transgene expression by precluding chromosomal position effects

following integration into host chromatin [90, 91]. A similar vector design may be drawn upon to preclude putative affects of vector *cis* elements on the chromatin environment, thereby heading off insertional mutagenesis.

The WPRE and cPPT *cis* elements

Improvements upon transgene expression and transduction efficiency of lentiviral vectors were accomplished through the incorporation of the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) [92] and the central polypurine tract (cPPT) [26], respectively. When placed in the sense orientation in the 3′ untranslated region of a transgene, the WPRE sequence increases overall transgene expression by more than five fold. Recently, it was proposed that the WPRE *cis*-element might incite oncogenic activity due to the fact that the sequence harbors enhancer/promoter elements and encodes the first 60 amino acids of the woodchuck hepatitis virus X protein, which may be associated with hepatocellular oncogenesis [93]. Conceivably, inadvertent expression of the truncated X protein could also elicit a cellular immune response. Currently there is no evidence demonstrating expression of the 60 amino acid protein, however efforts to curb these presumed detrimental consequences resulted in the development of modified WPREs that lack sequences encoding the truncated X protein and promoter elements, and exhibit no loss in vector titers or its ability to enhance transgene expression [94].

The efficacy of HIV-1 based lentiviral vectors could be enhanced further through the addition of a central polypurine tract (cPPT) and central termination sequence (CTS) [26]. As the negative DNA strand of HIV-1 is reverse transcribed from the viral RNA, much of the RNA is degraded by the RNase H activity of reverse transcriptase. RNA from the polypurine tract (PPT), located adjacent to the 3′ LTR, and the cPPT, located centrally in the *pol* gene of the viral genome, are not degraded and prime concurrent synthesis of the plus strand proviral DNA from two locations. Termination of synthesis from the PPT primer at the CTS generates a DNA flap in the middle of the genome. The central DNA flap has been determined to be essential for efficient wild-type HIV-1 replication, and presumably lentiviral vectors, in various cell types at a step that apparently involves nuclear import [26, 95–99]. Routine inclusion of the cPPT-CTS *cis*-element into HIV-1 vectors enhances transduction efficiency 2–10-fold in dividing and non-dividing cells [26, 100–104)]. Despite these improvements in transduction efficiency, a large body of evidence, including initial reports using HIV-1 vectors [12, 28, 29, 33, 34, 48], indicates that the DNA flap is not absolutely required for vector transduction, or even for wild-type HIV-1 replication [105, 106]. Recent evidence, however, establishes that there is a dose-dependent effect, wherein low MOI (<0.01) of wild-type HIV-1 was dramatically inhibited in the absence of the DNA flap (10–100-fold) [99]. This may help explain the discrepancies observed with lentiviral vectors in which higher MOIs are typically used, thereby overriding a putative block at the point of nuclear import. Furthermore, to acquire the highest transduction efficiency of HIV-1 vectors, the cPPT-CTS should be oriented in the sense direction with respect to the vector, and it should be incorporated into the vector in a central or 5′ vector location [99, 107].

Transgene regulation

The desire to therapeutically regulate gene expression in a time and dose-dependent manner in vivo prompted the incorporation of the tetracycline (Tet) inducible system into HIV-1 based vectors [108, 109]. Other systems, such as that derived from the ecdysone receptor [110], have been adapted for lentiviral vectors; nonetheless, the Tet-inducible systems are most widely used. The system is derived from two components: (1) the Tet repressor protein which is fused to a transactivator or transrepressor, and (2) the *tet* operator (tetO), also referred to as the *tet* response element (TRE), which is a *cis*-element comprised of a series of seven 18 base pair repeats fused to a minimal promoter. The Tet inducible system comes in two flavors: (1) The original ‘‘Tet-Off ‘‘ whereby the presence of tetracycline, or its more biologically robust analog doxycycline (Dox), interacts with the ligand binding domain of the Tet transactivator (tTA) precluding its interaction with the TRE, and consequent transcriptional activation to maintain the system in an ‘‘Off state’’ [111]; and, (2) the modified ‘‘Tet-On’’ system, in which Dox interacts with the ligand binding domain of the reverse Tet transactivator (rtTA) to activate transcription through its interaction with the TRE [112]. Initial inducible HIV-1 vector designs were comprised of either the Tet-Off [109] or Tet-On [108] inducible systems, wherein the CMV-tTA/rtTA constitutive cassette was placed upstream of the TRE-transgene inducible cassette in a single vector construct. Inducible HIV-1 vector systems were efficiently generated using standard transient transfection procedures; however, the titers were typically 5–10-fold lower than those obtained for non-inducible vector systems. HIV-1 vectors harboring the Tet-Off system exhibited the most efficient inducible transgene expression of greater than 500-fold over basal levels, with demonstrated in vivo transgene regulation following vector administration to rat brain [109].

Even though the original Tet-based vector systems exhibited high inducibility, there was an apparent level of basal expression due to leakiness of the TRE minimal promoter. Efforts to reduce the level of basal expression

included separation of the constitutive (CMV-tTA/rtTA) and inducible (TRE-transgene) expression cassettes onto two separate vector constructs, thereby requiring the use of a binary vector system [113–115]; and changing the position of the TRE minimal promoter within the context of the single vector system [113, 115]. A detailed assessment of the vector components responsible for inadvertent basal expression revealed that *cis* elements within the vector (WPRE and internal CMV promoter) were culpable for the apparent basal expression [115]. Separation of the internal CMV promoter from the minimal TRE promoter through use of a binary vector system resulted in lower basal transgene expression levels and higher maximal expression levels. Despite these obvious advantages, the binary vector system may prove inefficient for applications involving in vivo systemic administration in which two vectors would be required to transduce the same target cell. Furthermore, to avert potential detrimental consequences associated with insertional mutagenesis it may be prudent to limit the number of vectors inserted into a target cell genome through the use of a single vector delivery system. Accordingly, second generation HIV-1 based single vector delivery systems were developed in which the TRE was repositioned to the U3 of the 3′ LTR to drive expression of the transgene of interest introduced upstream of the constitutive CMV-tTA cassette [113, 115]. Incorporation of the minimal TRE promoter into the U3 of the 3′ LTR was derived from the conditional SIN HIV-1 vectors (Fig. 1), from which the SIN phenotype is maintained in transduced target cells in the absence of the tTA transactivator [116]. Reduced basal expression was characteristic of these modified vectors; however, there was an ancillary decrease in maximal transgene expression, thus the achievable inducible levels were slightly lower than those obtained with a binary vector system [115]. Further improvements in regulated vector design have included: 1) the common employment of modified tTA and rtTA transactivators ($tTA2^S$, $rtTA2^S$-M2, and $rtTA2^S$-S2), initially isolated by Urlinger et al. and shown to have one or more of the attributes including lower background expression, higher affinity for doxycycline, and increased stability in mammalian cells [113, 114, 117–121]; and, (2) incorporation of improved TRE minimal promoters into HIV-1 vectors, originally characterized by Agha-Mohammadi et al. and demonstrated to enhance maximal transgene expression levels and lower basal expression levels [118, 122].

Traditional applications of Tet-inducible systems include temporal- and dose-dependent regulation of transgene expression from type II RNA polymerase (RNA Pol II) promoters for research and therapeutic applications. The recent advent of short-hairpin RNAs (shRNAs) and microRNAs (miRNAs) to manipulate endogenous gene expression in mammalian cells manifested an expansion of the Tet-inducible systems to confer the reversible regulation of shRNAs and miRNAs following stable delivery with HIV-1 based lentiviral vectors [117, 121, 123–125]. Progress in Tet-dependent expression of shRNAs and miRNAs in the context of a single lentiviral vector has been realized through the control of both RNA Pol II [123, 125] and RNA Pol III [117, 121, 124] promoters. The recent breadth of literature encompassing the use of shRNAs and miRNAs in the context of lentiviral vectors demands a more elaborate discourse on this topic, as discussed below.

The broad applications of Tet-inducible systems to regulate RNA expression have made it a staple in molecular biology laboratories for research and therapeutic applications in preclinical animal models. However, the tTA and rtTA transactivators are comprised of bacterial and viral components shown to elicit humoral and cellular immunological responses [126–129], and may ultimately preclude their incorporation into vector designs for therapeutic regimens in the clinical setting. Although these studies caution the use of the Tet system for clinical purposes, the mode of transactivator delivery in all the aforementioned studies was via plasmid, adenovirus, or adeno-associated virus through an intramuscular route of administration in non-human primates or mice. As of yet it is unknown what immunological response, if any, is invoked to the tTA or rtTA transactivator following lentiviral vector delivery intramuscular, or otherwise.

Production of lentivirus vectors

The routine method of HIV-1 and other lentivirus vector production is based on a transient transfection of 3–4 plasmids into 293T cells (Fig. 1). In addition, several lentivirus vector packaging cell lines were recently developed as an alternative method of vector production. The following sections summarize the methods for producing lentiviral vectors by these two techniques.

Vector production by transient transfection

Cells

Lentivirus vectors are commonly generated in human embryonic kidney (HEK) 293T cells, which are SV40 large-T antigen expressing 293 cells. These cells are highly transfectable, thus at least 60% of the cells should be successfully transfected by the calcium-phosphate transfection method. Prior to transfection, the cells are plated on 10 cm, poly-L-lysine coated plates at a density such that they are 60–70% confluent at the time of transfection. Covering the plates with 0.001% poly-L-lysine (Sigma) in 1X PBS for 5 min should be sufficient.

DNA

A total of 30–40 μg of endotoxin free DNA should be transfected into 4–5 × 10^6 cells in a 10 cm plate. The transfection mix should include 15, 10, and 5 μg of DNA of the vector, packaging cassette (typically first- or second-generation) and the VSV-G envelope expression cassette, respectively. The amount of envelope DNA should be increased to 10 μg when a non-VSV-G envelope is being used. For third-generation HIV-1 vector packaging systems use 10 μg of the Gag/Pol expression cassette DNA and 3 μg of the Rev expression cassette DNA [48]. Similar ratios of the packaging constructs are used for transient production of the *trans*-lentiviral packaging system: 10 μg *gag/pro* cassette and 3 μg of the *vpr/pol* cassette [130].

Transfection

The conventional calcium phosphate transient transfection method (either HBS or BBS) is the most efficient technique for HIV-1 vector production. Other methods have included the use of commercially available lipid based reagents; however, upon direct comparison, the use of BES buffer (BBS) has proven most effective [131]. Eight to twelve hours after the addition of DNA, the transfected cells are washed and fresh media is added.

Harvesting vector particles

Collect vector particles by harvesting the media 60–72 h post transfection. Vector particles can be separated from cellular debris by centrifugation of the media at 4000g for 5 min, followed by filtration through a 0.45-micron filter.

Concentration/purification of vector particles

Concentration of vector particles can be achieved by centrifugation, precipitation, and filtration, reviewed in Segura et al. [132]. The standard concentration of small volumes (<1 l) of VSV-G pseudotyped lentiviral vector particles can be executed by ultrcenrifugation at 50,000*g* for 2 h [12, 133]. Following centrifugation the vector pellets can be resuspended in PBS, HBSS or DMEM, and frozen at –80°C for later use. Vectors are conventionally concentrated 100–1000-fold yielding titers of 10^9–10^{10} TU/ml. Purification of vector is critical for the elimination of cellular and serum products that may elicit immune responses. The conventional purification method for lentiviral vectors is through a sucrose gradient comprised of 20–70% sucrose, which reduced immune responses in the brain [134]. Retrovirus particles have a density of ~1.16 g/ml, corresponding to 35% w/w sucrose [135]. A wide variety of chromatography methods have been employed to achieve a greater level of purity, reviewed in Segura et al. [132]. More recently, Yu et al. employed a novel purification method to reduce protein impurities by 14,000-fold with no loss in vector titers; nonetheless, the starting titers were ~10-fold lower than conventional titers [136].

Large-scale production of lentiviral vectors needed for large animal/clinical studies may require production in multiple tissue culture plates, or in cell factories or bioreactors, and the subsequent concentration of large volumes of vector (>1 l). Concentration of large volumes may be achieved through the use of tangential flow filtration (TFF) and ultracentrifugation [137]. The combination of TFF and ultracentrifugation yielded a vector recovery of 90–100% with a nearly 2000-fold concentration in vector titers.

Titering vector stocks

Typical titers of VSV-G pseudotyped HIV-1 vectors are in the range of 10^6–10^7 TU/ml prior to concentration and 10^9–10^{10} TU/ml following concentration. Vectors expressing reporter genes (GFP or LacZ) or selection markers (genes that confer resistance to puromycin, neomycin, etc.) can be titered by serial dilution on target cells (293T or HeLa cells). The number of positive cells can be scored by microscopy for transgene expressing cells or colonies, or FAC scanning analysis. Titers for vectors that do not express reporter genes can be estimated by assays that measure either the amount or activity of proteins incorporated into the vector particles. These include the commercially available $p24^{gag}$ ELISA (Perkin-Elmer) and the reverse transcriptase assay (Roche). Extrapolating from the $p24^{gag}$ concentration to vector titers is not accurate, and the ratio between $p24^{gag}$ and the number of infectious particles may vary significantly between different vectors, laboratories and vector stocks. Typically, ~1 × 10^4 infectious vector particles contain 1ng of $p24^{gag}$ [48]. The $p24^{gag}$ ELISA assay is commonly used in combination with reporter assays to express the specific activity of a vector as TU/ng $p24^{gag}$.

Within the last 6 years the use of real time PCR-based methods to determine vector titers has become commonplace, reviewed in Delenda and Gaillard [138]. Direct comparison of integrated vector DNA, vector particle RNA, and marker expression assays demonstrated that quantitative PCR to measure integrated vector DNA in transduced cells is the most reliable method [139]. Various researchers have employed a number of primers, probes, and standards to measure integrated vector DNA, as well as vector episomal forms, many of which are described in Delenda et al. and references therein [138]. Moreover, quantitative RT-PCR techniques were recently employed to measure transgene mRNA expression from an integrated

lentiviral vector [140, 141]. Although the use of RT-PCR to assess titer by transgene expression may be applicable under certain conditions, the type of promoter (constitutive or tissue specific) driving transgene expression, as well as the promoter strength (two different constitutive promoters, e.g. PGK and CMV) may influence the results of the assay, and therefore the titers. Quantitative PCR to measure integrated, and episomal, vector forms may be the most universal method for measuring titers of different vectors.

Vector production by stable packaging cell lines.

Production of lentiviral vectors by transient transfection allows for rapid flexibility in testing different vectors in the laboratory setting; however, as applications shift to pre-clinical testing in multiple animal models, and eventually human gene therapy trials, so to will the requirement for large, reproducible stocks of lentiviral vectors that are safe. Accommodating such a requirement may necessitate a shift from the standard production of lentiviral vectors by transient transfection to a method amenable to low-cost and large-scale production, namely the use of packaging cell lines. There are a number of drawbacks that may preclude the use of plasmid-based transient transfection methods for generating pre-clinical and clinical grade vectors for in vivo administration: (1) the end-product must be free of bacterial products, such as endotoxins, that can co-purify with plasmid DNA; (2) residual plasmid DNA must be eliminated through treatment with benzonase or DNase I; (3) variation in transfection efficiency, as well as plasmid preparation, quality, and yield, may all impact the reproducibility of vector stocks; (4) ease of transfection makes transformed cell lines (293T cells) amenable to transient vector production, however transformed cell lines may not be desirable for production of clinical grade vector stocks since they would have to be certifiably free of any putative transforming agents such as large T antigen; (5) there may be difficulties scaling-up transient vector production due to the large quantities of reagents required and technical limitations; (6) exorbitant costs may be incurred to continuously produce the reagents, and execute the tests, required for transient transfections; and (7) most importantly, large quantities of transfected plasmid DNA may exacerbate the risk of RCLs by increasing the potential for recombination between cotransfected plasmids. Through the use of lentiviral vector packaging cell lines safe, reproducible vector stocks may be routinely generated subsequent only to the initial investment of cost and time to generate a therapeutically useful packaging cell line.

Considering these reasons a number of lentiviral vector packaging cell lines have been developed. A stable packaging cell line contains the envelope, packaging, and vector cassettes stably incorporated into the genome of a cell line. Cytotoxic and cytostatic affects associated with constitutive expression of the VSV-G [142], protease [143], and Vpr [144] proteins dictated the use of inducible expression systems to regulate expression of the envelope and packaging components. Tetracycline and ecdysone inducible systems were used to regulate these components in the initial packaging cell lines [116, 145–149]. Each of the envelope, packaging, and vector cassettes are introduced sequentially to avoid unintended recombination events. Typically, the vector cassette is introduced as the last step in packaging cell line development to allow for flexibility in introducing different vectors. Introducing a vector by transfection may be more time-consuming and can form stable concatamers that may result in silencing of full-length vector mRNA expression. Early packaging cell lines based upon first and second generation packaging cassettes alleviated these concerns by supporting stable introduction of vector through transduction [146, 149]. Nevertheless, SIN vectors could not be introduced by transduction, thereby restricting these packaging cell lines to the use of non-SIN, Tat-dependent vectors. Development of a Tat-independent, third generation packaging cell line was accomplished by incorporation of the constitutive CMV promoter into U3 region of the 3′ LTR [148]. Although the CMV promoter facilitated Tat-independent expression of the vector, the SIN phenotype of the vector was not retained since vector mRNA would be constitutively expressed in target cells. Moreover, a strong promoter such as CMV is a grave safety concern since it may perturb gene expression following integration into a target cell genome, the consequence being insertional mutagenesis. These concerns were allayed through the development of a conditional SIN vector (cSIN) [116]. Incorporating the tetracycline response element (TRE) into the U3 of the 3′ LTR supported introduction of the vector into packaging cells by transduction and ensuing vector production in packaging cells that constitutively express the tTA transactivator, while concomitantly maintaining the SIN phenotype in target cells which lack the tTA transactivator. Additionally, packaging cell lines, in which all the components are expressed only upon induction ensure that the packaging cell line can be expanded in the ‘‘Off state’’ to eliminate risk of vector superinfection, which may compound any risk of RCL formation. Vector particles produced from some of these earlier packaging cell lines met the standard of in vivo transduction in the brain [116, 146, 149], yet the typical titers for many of these early cell lines were ~10^6 TU/ml, about 10–50-fold lower than those commonly achieved by transient transfection.

Further developments in packaging cell lines have exploited the sophisticated advancements acquired through the evolution of the packaging cassette, as discussed earlier [130, 150, 151]. Packaging cell lines derived from codon

optimized *gag/pol* packaging cassettes facilitate the elimination of the RRE, thus reducing the risk of RCL formation through recombination with the vector [150, 151]. The STAR packaging cell line described by Ikeda et al. conferred high plasticity with regard to pseudotyping HIV-1 vectors with envelope proteins other than VSV-G [150, 152, 153]. Similar to packaging cell lines derived from codon optimized *gag/pol* genes, splitting the *gag* and *pol* genes for development of the SODk3 packaging cell line should afford a greater level of safety by reducing the risk of RCL formation [130]. Futhermore, Cockrell et al. demonstrated that reporter gene expression in the ''Off state'' from producer cells correlated with increases in vector yield upon induction; therefore, reporter gene expression could be used as a rapid method to acquire high producer packaging cells by FAC sorting [130].

Using the SODk3 packaging cell line

Generally, the procedure of vector production by stable packaging cell lines can be divided into two stages: (i) the induction stage and (ii) the vector collection/concentration stage. Both stages vary slightly between the different packaging cell lines. Here, we describe a method of producing lentiviral vectors pseudotyped with the VSV-G envelope by the SODk3 packaging cell line [130]. Similar methods have been used successfully by research groups at different institutes to produce vectors from both the SODk1 and SODk3 cell lines [116, 130, 146, 154]. The SODk3 cell line is based on tetracycline inducible (Tet-off) HEK293 cells, which constitutively express the tTA transactivator, and are termed SODk0 cells. The SODk3 cell line contains a split *gag/pol* genome in which the *gag/pro* genes are expressed from one cassette and the *pol* genes are expressed from another. A *vpr-pol* fusion was made to direct proper assembly, since the Vpr protein interacts with the p6 region of the Gag protein. Upon maturation of the vector particles, Vpr is cleaved from the Pol protein products by the HIV-1 protease, which acts at a protease cleavage site inserted between the *vpr* and *pol* genes. Both the *gag/pro* and *vpr/pol* expression cassettes were placed under the control of a Tet-inducible promoter. The cells also express the VSV-G envelope gene and the GFP gene from a Tet-inducible bi-directional promoter.

Packaging cell line maintenance

Culture the cells in medium containing DMEM (high glucose), 10% fetal calf serum (FCS) and 1 μg/ml doxycycline. Split the cells at 1:4 dilutions every 2–3 days during which they will reach 80–90% confluence.

Generation of a producer line

An HIV-1 vector should be introduced into the SODk3 packaging cell line to generate a stable vector producer line. The vector can be introduced either by stable transfection and clonal selection using a selection marker such as Hygromycin B, or by transduction with rescuable vectors (non-SIN, and conditional-SIN vectors). Provided the inclusion of a fluorescent reporter (e.g. GFP behind an IRES [internal ribosomal entry site]) the cells may be sorted for high producers by FAC sorting for those with the highest reporter expression levels.

Induction of vector production

Remove the doxycycline by washing the cells with PBS. Split the cells at 1:4 dilution in medium containing DMEM and 10% tetracycline free FCS (Clontech). Change media daily. When reaching 90% confluence (in 2–3 days) split the cells at a 1:4 dilution onto poly-l-lysine precoated plates as described previously. After ~12 h add 5–10 mM sodium butyrate (Sigma). The media should be replaced the following day with fresh tetracycline free media containing sodium butyrate. Induction of gene expression from the bi-directional tetracycline inducible promoter should result in GFP and VSV-G gene expression. The induction efficiency can be determined by fluorescence microscopy or FACS analysis if a transgene other than GFP is delivered by the vector. At day one after the addition of sodium butyrate, ~80% of the cells should be green.

Vector collection and concentration

Harvest media containing vector particles daily, starting from day two to day four after the addition of sodium butyrate. Maximal vector titer ($>10^6$ TU/ml) will be reached at day three after changing the media containing sodium butyrate. The vector can then be harvested, concentrated, purified, and titered by one or more of the methods discussed above.

Biosafety standards and assays

The advances discussed above in lentiviral vector development have ameliorated some of the safety concerns associated with the generation of replication competent lentiviruses (RCLs), vector mobilization, and insertional mutagenesis. An appreciation for progress in vector development can only be discerned from assays designed to assess the safety of these sophisticated vectors. Other than pre-clinical testing in animal models, there are

currently no direct assays to predictably ascertain information that would identify a vector as having the potential for insertional mutagenesis. Moreover, genotoxicities associated with lentiviral vectors may go undetected in preclinical animal models. Instead, the FDA's Center for Biologics Evaluation and Research (CBER) has compiled a set of guidelines for long-term follow-up of patients enrolled in human gene transfer studies (www.asgt.org/regulatoryissues.shtml). These guidelines were born out of a workshop on long-term follow-up of patients at the 2004 Meeting of the American Society of Gene Therapy (ASGT) in which the potential long-term risks were recognized as ''...persistence of vector sequences, integration of the vector into host genomic DNA, and transgene-specific effects'' [155]. CBER guidelines to test vector stocks for RCLs can also be acquired through the indicated website.

As indicated by the CBER guidelines the ability to reliably screen vector stocks for RCLs is a prerequisite for using lentiviral vectors in clinical trials. Thus, it is not surprising that several assays have been developed to assess vector stocks for the emergence of RCLs and the transfer of viral genes into the genome of target cells. Most of the currently used assays are based on cell lines genetically engineered to report on the transfer of functional viral genes, such as the HeLa P4.2 cell line, which constitutively expresses CD4 and contains the *lacZ* gene under the control of the HIV-1 LTR [96]. The HeLa P4.2 cell line has been used to measure both the transfer of functional *tat* (tat transfer assay) and *gag* (gag transfer assay) genes as a consequence of recombination between the vector and packaging cassettes [12, 146]. Further modification of the HeLa P4.2 cell line by stably introducing a non-SIN vector containing a GFP expression cassette allowed for the generation of the HeLa4G cell line, which facilitates detection of functional RCLs by rescue of the GFP vector [146]. All three assays (*tat*-transfer, *gag*-transfer, and GFP marker rescue) can be performed on the single HeLa4G cell line [146]. These assays are executed by transducing the HeLa4G cell line with an aliquot of the vector stock for 3–4 weeks (>5 passages) to amplify putative RCLs within the vector stocks. Following the final passage, an aliquot of the media is transferred to a GFP negative cell line (293T cells) to test for the transfer of GFP positive vector (marker rescue assay) by FACS and immunofluorescence microscopy. GFP positive cells indicate the presence of all functional components of a lentiviral vector (transfer of *gag*, *pol*, and *env* genes) required to package and mobilize full-length vector mRNA. A separate aliquot of media is tested for $p24^{GAG}$ (Gag-transfer) using a commercially available ELISA assay (Perkin-Elmer). The transfer of the *gag* gene to HeLa4G cells will result in the expression of detectable p24 capsid protein in the media (sensitivity of >1pg/ml, or ~1–2 TU/ml), and detectable increases of p24 after each passage of cells would be indicative of RCLs competent to amplify the signal through repeated infection of the cells. In a concurrent assay, cells can be assessed for the transfer of the *tat* gene by staining the HeLa4G cell line for β-galactosidase activity (detection limit of 20 tat-transducing units per ml of test media) after the final cell passage. Since the β-galactosidase gene is only expressed in the presence of the Tat protein, cells that stain blue are indicative of *tat* gene transfer. Clearly, this assay is most relevant for vectors produced with first- and second-generation packaging constructs, which contain the *tat* gene.

Recombination events that only occur between the *gag/pol* and vector cassettes may result in the production of envelope-less vector particles unable to be detected by the aforementioned assays. Modifications of *gag*-transfer [149] and *tat*-transfer [51] assays supported increased sensitivity by facilitating the detection of envelope-less vector particles generated from homologous and non-homologous recombination between the *gag/pol* and vector cassettes. Both assays require supplying the VSV-G envelope protein in *trans* to support efficient target cell transduction, and amplification of the signal. Regarding the *gag*-transfer assay, femtogram quantities of p24 were detectable in vector stocks [149]. Additional augmentation of the *gag*-transfer assay demonstrated that 1 RCL infectious unit in a stock of $>10^8$ transducing units could be ascertained by utilizing the highly permissive T cell line, C8166–45, for the amplification step [156]. Enhanced sensitivity of the improved assays may provide efficient quality control for large clinical grade vector stocks to detect *bona fide* RCLs.

The vast majority of vectors currently in use are SIN vectors. Following transient production of SIN lentiviral vector stocks using a third generation packaging construct, recombinants were detected in 7/7 transductions from two separate vector stocks using a quantitative PCR assay [157]. *Gag* sequence elements common to both the vector and packaging cassettes were determined to facilitate recombination. However, *VSV-G* sequence elements were detected in only 1/7 transduction, demonstrating a low level of recombination between the vector and envelope construct. None of the recombinants were mobilized to fresh cells, and no RCLs were detectable by the $p24^{GAG}$ ELISA assay [157]. These data substantiate that sequence homology between packaging and SIN vector cassettes can support efficient recombination. Codon optimized packaging cassettes were designed to circumvent such recombination events, but there is a dearth of evidence to corroborate this claim.

Research applications

Under the auspices of safety, prevailing efforts have guided lentiviral vector development toward corrective gene

therapy for a number of incurable infectious and genetic maladies. Throughout the course of this maturation lentiviral vectors have also facilitated advances in transgenic animal development and stable delivery of molecules (shRNA and miRNA) for RNA interference (RNAi) to establish post-transcriptional control of gene expression. Intracellular expression of shRNA molecules from the H1 and U6 type III RNA polymerase (RNA pol III) promoters' conferred highly efficient post-transcriptional gene suppression following transient plasmid transfection [158–160] or adenoviral gene delivery [161], to mammalian cells. Short hairpin RNAs are synthetic structures designed to emulate the hairpin structures commonly found as excised products of primary miRNA (pri-mRNA), such that they requisition the RNAi machinery for processing and subsequent targeting of specific mRNAs for degradation, reviewed in Du and Zamore [162]. The H1 and U6 Pol III promoters yield precise 5′ and 3′ ends of the shRNA, similar to the hairpin structures commonly found as the excised products of pri-miRNA. The advent of this technology afforded the incorporation of pol III-shRNA hairpin cassettes into simple retroviral [163], and lentiviral [164–167], vectors for stable insertion into mammalian target cell genomes. In contrast to simple retroviral vectors, lentiviral vectors can confer stable transfer of shRNA to non dividing cells [167], and impart the capacity to generate transgenic animals expressing shRNA cassettes to knock down specific genes, essentially mimicking knockout animals [166, 167]. Furthermore, Tiscornia et al. demonstrated that this effect was passed to the F1 progeny. The therapeutic efficacy of lentiviral vector mediated delivery of shRNA cassettes has been realized in preclinical animal models of amyotrophic lateral sclerosis [168, 169] and Alzheimer diseases [170], whereby RNAi was used to ameliorate the neurodegenerative effects associated with both diseases. Moreover, it is anticipated that RNAi will be beneficial for antiviral treatment [171].

Heretofore, all shRNAs described were expressed from constitutive H1 or U6 pol III promoters. Depending upon the target gene being retarded, constitutive expression of shRNAs from pol III promoters may hamper development of transgenic animals and potentially evoke unwanted cytotoxic effects. The desire to regulate shRNA expression in a temporal and spatial manner ushered-in a new phase in lentiviral vector design for delivery of shRNA. Initial efforts were intended to acquire conditional control over the expression of shRNA from a pol III promoter [117, 121, 124, 172, 173]. Reconfiguring the shRNA expression cassette such that the shRNA is expressed upon exposure to the Cre recombinase supported conditional knock-down of gene expression in tissue culture [172, 173], and, in a temporal and spatial fashion, in transgenic shRNA mice bred to transgenic mice expressing the Cre recombinase under a tissue-specific promoter [173]. A clever conditional tetracycline-inducible expression and knockdown system was designed with the combined benefits of expressing a transgene cassette, a shRNA cassette, and regulating the expression of both from a single vector construct [121]. Fusing the KRAB domain to the Tet repressor in either the tTR (Tet On) or rtTR (Tet Off) format supported reversible and conditional control over the entire system in tissue culture, in vivo following rat brain injections, in a tumor model in nude mice, and in transgenic mice [121]. Note that when bound to the TRE, the KRAB based system represses transcription from all promoters through modifications that lead to heterochromatin formation; thus, this system works in a manner opposite to the Tet systems described previously, as indicated by the terminology. These technologies are promising advancements, but recent progress in understanding miRNA expression offers a more direct alternative in achieving control over shRNA expression.

Recent data indicate that RNA pol II promoters can drive expression of authentic miRNAs that have been manipulated to yield specified siRNAs for targeted gene suppression in tissue culture, described by Zeng et al. and references therein [174]. Replacement of the stem structure, within the context of the pri-miRNA, with an artificially derived stem can redirect the siRNA product to knock-down expression of a specific gene. Synthetic shRNAs were incorporated into the miR-30 miRNA backbone and shown to suppress gene expression from simple retroviral [175] and lentiviral [123, 125] vector platforms. All systems exhibited Tet-dependent regulation in tissue culture. Moreover, Shin et al. used a single lentiviral vector to reversibly control the concomitant expression of two synthetic miRNAs placed in tandem, as well as a transgene positioned directly upstream of the synthetic miRNA. Employing pol II promoters for expression of synthetic miRNAs will presumably support temporal and spatial control of RNAi through the utilization of established regulatory systems and tissue-specific promoters in vivo.

The merging of shRNA expression cassettes into simple retroviral [176, 177] and lentiviral [178, 179] vectors provided a foundation to support the generation of large-scale RNAi libraries for functional screening of human and mouse genomes. Even more, second generation simple retroviral vector-based libraries have been developed which incorporate >140,000 synthetic shRNAs into the miR-30 backbone [180]. Conceivably, generating knock-downs in transgenic mice following positive identification from a functional library would be a suitable follow-up study, making lentiviral vectors preferable for generating large-scale synthetic miRNA libraries.

As mentioned above the use of lentiviral vectors to generate transgenic animals in a time and cost effective

manner will support large-scale genetics experiments in mammals. Attempts to generate transgenic animals with gamma retroviruses have not yet been possible due to the silencing effects of transgene expression following introduction into mouse embryos and embryonic stem cells [32]. As initially demonstrated through transduction of murine embryonic stem cells and preimplantation embryos, lentiviral vectors can be used to efficiently generate transgenic animals [181, 182]. Although mice have been the predominant species used to generate transgenics, lentiviral vectors have also mediated the production of transgenic rats [183], pigs [184, 185], cattle [186], birds [187], and chickens [188]. In addition to large-scale genetic approaches, lentiviral vectors may be designed to facilitate the generation of transgenic animals with human genes that more readily mimic disease states for developing preclinical animal models.

Preclinical/clinical progress

The extensive range of target tissues/cell types transducible by lentiviral vectors in vitro and in vivo makes them an attractive modality for treating a number of genetic disorders. The spectrum of target tissues comprises cell lineages of the hematopoietic system, central nervous system, ocular tissues, liver, pancreas, heart, skin, synovium, kidney, and muscle. Considering that many of these topics warrant extensive reviews, the authors highlight successes associated with the CNS, ocular, and hematopoietic system, referring to more comprehensive reviews when possible. Early studies demonstrating efficient transduction of neurons in the rat brain, following in vivo administration, established this as a standard in the field for assessing the efficacy of a lentiviral vector to transduce non-dividing cells [12, 29–31]. Recognizing the benefit of lentiviral vectors for stable, long-term transgene expression in the central nervous system (CNS) prompted numerous investigators to appropriate these attributes for treating neurological disorders in preclinical animal models, including Alzheimer's, Parkinson's, and Huntington's diseases, reviewed in Ralph et al. [189] and Wong et al. [190]. Prefaced by an initial investigation describing efficient transgene expression in the eye [191], the correction of genetic ocular disorders in preclinical animal models has garnered much success [76, 192, 193]. Extending host survival and reversing disease pathologies have been accomplished in preclinical animal models of neurological and ocular diseases; however, translation into the clinic awaits further evaluation of the long-term consequences that may be associated with in vivo vector administration including pharmacokinetics, inadvertent biodistribution, germ-line transmission, vector mobilization, genotoxicity, and immunogenicity.

Unlike the CNS, therapeutic intervention for genetic disorders of the hematopoietic system does not require in vivo administration of lentiviral vectors; but rather, can be accomplished by an ex vivo route of administration to purified autologous hematopoietic stem cells (HSCs), reviewed in Chang et al. [194]. Notwithstanding the potential for genotoxicity, vector mobilization and transgene-elicited immune responses, some of the risks associated with in vivo administration may be thwarted. The pluripotential of HSCs makes them amenable to corrective therapy with lentiviral vectors prior to differentiation into various cell lineages. Since the initial report by Myoshi et al. demonstrating efficient transduction of human HSCs with lentiviral vectors [195], several researchers have reported successful correction of genetic disorders in preclinical animal models, including immunodeficiencies and various hemoglobin disorders [54, 196–203]. Clinical trials to correct immunodeficiencies have received much attention in recent years, due to the potential for insertional oncogenesis following integration of oncoretroviral vectors into the genome of HSCs, reviewed in Nienhuis et al. [10]. In contrast to oncoretroviral vectors, the large body of preclinical studies with HIV-1 based lentiviral vectors indicates that there is no predilection for insertional oncogenesis following transduction of HSCs. Enhanced safety, transduction of non-dividing cells, and accommodation of large genetic payloads are all attributes that poise HIV-1 based lentiviral vectors to supplant oncoretroviral vectors as a primary vector for therapeutic gene delivery in HSCs.

Accordingly, the first phase I clinical trial employing a HIV-1 based lentiviral vector to treat five HIV-1 positive patients with an antisense RNA against the HIV-1 envelope was well tolerated after 21–36 months [204]. The trial was initiated through ex vivo transduction of autologous $CD4^+$ T cells. The safety profile revealed an absence of RCLs, a lack of clonal outgrowths as precursors to insertional oncogenesis, integration preferences commensurate with wild-type HIV-1, and limited mobilization. A conditional lentiviral vector was used to expand the effect through limited mobilization to additional T cells upon expression of wild-type HIV-1 proteins, and subsequent packaging by wild-type HIV-1. An acceptable safety evaluation and improved T cell responses in four of five patients render this inaugural phase I clinical trial a success. Ensuing lentiviral vector based clinical trials under consideration include a phase I/II trial for β-thalassemia [205], malignant melanoma, leukemia/lymphoma, and mucopolysaccharidosis type VII (www.wiley.co.uk/genetherapy/clinical/), all of which involve ex vivo gene delivery to autologous HSCs or T cells.

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