Library Selection Approaches to Engineering Enhanced Retroviral and Lentiviral Vectors

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Abstract: Retroviral and lentiviral based gene delivery vectors have been used in numerous pre-clinical studies and clinical trials due to their advantages, including stable and prolonged expression of therapeutic transgenes and minimal immune responses against the vector. Despite such advantages, however, retroviral vectors also have several limitations for gene therapy applications. For example, they can suffer from a lack of efficient or targeted gene delivery to key cell types. In addition, retroviral vector stability can be compromised by their envelope proteins. This review briefly describes how such limitations have been overcome by recently developed library selection approaches that borrow a lesson from nature: the ability of evolution to generate biomolecules with novel function. These library selection approaches are based on the construction of retroviral libraries where the sequences encoding natural viral components are partially randomized using a variety of methods in order to generate diverse libraries that can be selected to create improved or novel functions. These high throughput, library-based approaches provide a strong complement to rational engineering of viral components for the rapid development of efficient and safe retroviral and lentiviral vector systems for gene therapy.

Keywords: Gene therapy, retroviral library, library selection, cell targeting, viral vector engineering, protein engineering.

INTRODUCTION

Retroviruses, including lentiviruses as a subgroup, are enveloped viruses containing two copies of positive-sense single stranded genomic RNA [1, 2]. Due to their ability to stably transfer their genetic information into host chromosomes, retroviruses have been broadly utilized as platforms for efficient gene delivery vectors [1, 2]. Retroviruses first bind to cells *via* interactions between their envelope proteins and cell surface receptors, a key determinant of their cell and tissue specificity, or tropism [1, 2]. These interactions induce conformational changes in the surface and transmembrane units of the viral envelope protein, leading to initiation of fusion between viral membrane and host membrane. In some situations, the fusion initiation requires an additional trigger, the exposure of virus particles to a low pH condition in endosomes after receptor-mediated endocytosis, as for ecotropic murine leukemia virus (MLV) and avian leucosis virus (ALV) [3, 4]. After fusion, the viral nucleoprotein complex is released into the cytoplasm, and viral RNA genomes are reverse transcribed into a double stranded DNA form. The viral genomic DNA in the form of a preintegration complex (PIC), which contains both viral and host proteins, is then transported into the nucleus and integrated into host chromosomes [1, 2]. The resulting provirus, which contains a functional RNA polymerase II promoter within the 5' viral long terminal repeat (LTR), is then transcribed by host machinery *via* the same mechanisms as endogenous genes [2].

To convert a retrovirus into a retroviral vector, the viral genes are excised from the genome, and the shortened genome is placed on a piece of DNA, which both renders the virus replication incompetent and liberates space for the insertion of exogenous genetic cargo. The resulting vector construct is then introduced into packaging cells, and key viral protein components necessary for virion assembly are provided in trans *via* separate DNA constructs, either through transient transfection or stable integration in a producer cell [5-10]. These encode *gag* for structural proteins, *pol* for enzymatic proteins, *env* for envelope proteins, and other key genes (including *rev* and in some cases *tat*) in the case of lentiviral vectors [6-10].

Based on their intrinsic cell specificity or tropism, retroviruses mediate gene delivery to different cell types *in vitro* and tissues *in vivo* [1]. However, this tropism can be altered or extended to other cells and tissues by substituting the native retroviral envelope proteins with heterologous envelopes from foreign viruses, a process known as pseudotyping [11-16]. As an example, lentiviral vectors pseudotyped with Ebola virus envelope proteins transfer genes to airway epithelial cells *in vivo* [15]. Various naturally occurring envelope proteins can be packaged into and thereby generate infectious retroviral vector particles, but they often have several limitations. For example, in the majority of cases, no natural viral envelope protein is capable of highly specific gene delivery to a therapeutically relevant cell type of interest. In addition, natural envelope proteins can be inactivated by immune responses [17]. Furthermore, some natural viral envelope proteins are unstable to harsh steps required for vector purification [18].

To improve the properties of retroviral vectors, their components have been rationally engineered *via* genetic and chemical methods. For example, insertion of targeting peptides, ligands and antibodies, into envelope proteins has enabled selective gene delivery to target cells or tissues [19-21]. In addition, PEGylation of surface envelope proteins of lentiviral vectors has significantly reduced inactivation of the vectors by serum [22]. However, rational design can require

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detailed mechanistic knowledge of protein structure-function relationships, information that is largely unavailable for entities with highly complex structures and functions such as viruses. Furthermore, rational design for a new function can, in some cases, result in distinct, unintended functions [23]. Recently, library-based approaches have emerged as a complementary approach to either improve existing properties of retroviral vectors or to generate novel functions not attainable by conventional approaches such as pseudotyping and rational engineering. Library-based approaches involve construction of genetic libraries, where genes or regulatory sequences are diversified using a variety of potential technologies (Fig. **1**) followed by selections (Fig. **2**) for improved or novel functions (Fig. **3**). In some cases, the resulting selected viral pool can be further mutagenized and subjected to additional selection, an iterative process referred to as directed evolution that mimics the ability of natural evolution to achieve successive improvements in function [24]. However, this review will present only the development and application of library selection approaches for retroviral vector engineering, which have to date involved only a single round of library generation and selections.

ENGINEERING VECTORS FOR CELL SPECIFICITY

Receptor Binding Peptides

Phage display libraries, in which short random peptides are displayed from the surface proteins of bacteriophage particles containing genomes encoding the peptides fused to a viral protein, have for some time been successfully utilized and screened to identify functional peptide molecules for various biotechnology applications [25]. This concept was extended to generate retroviral display libraries in which

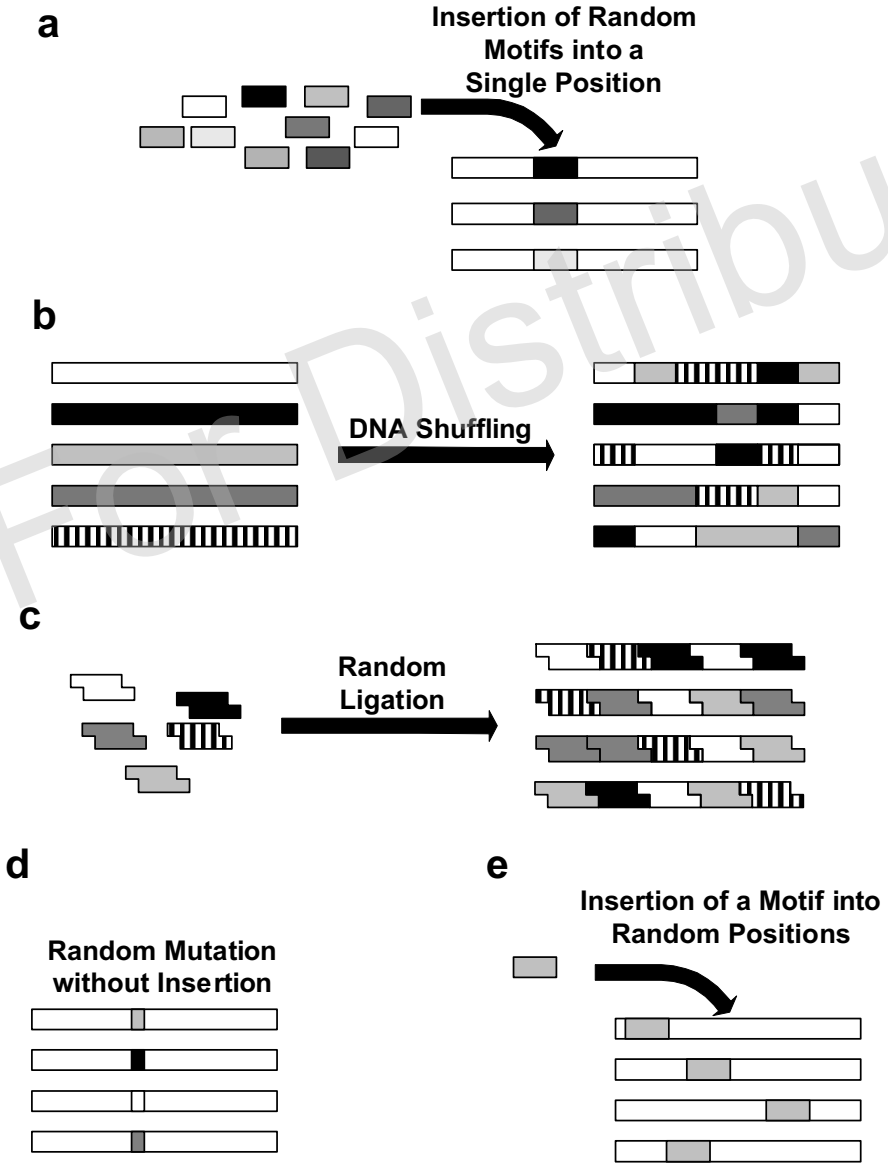

Fig. (1). Schemes for library construction. Various techniques have been applied to construction of retroviral libraries: (**a**) Insertion of short random targeting motifs such as ligand peptides at a single position of envelope proteins; (**b**) Shuffling of envelope gene templates; (**c**) Random ligation of promoter components with cohesive ends; (**d**) Random mutagenesis of protein residues at specific locations; (**e**) Insertion of hexa-histidine tag and NLS into random positions of envelope proteins and gag-pol proteins, respectively.

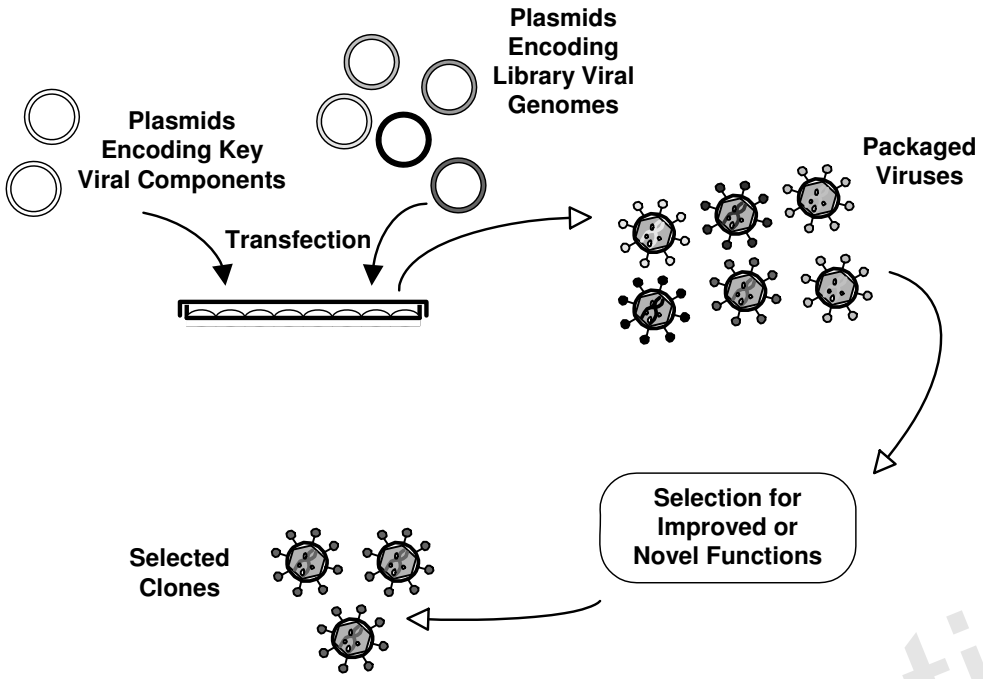

Fig. (2). Schematic for packaging of retroviral library and selection. Retroviral libraries have been in general packaged by transfection of producer cells with plasmids encoding base viral proteins and library viral genomes containing mutant genes or regulatory sequences. In one example process, particles are generated such that each is composed of mutant envelope protein surrounding the genome encoding that protein. Packaged retroviral libraries are then selected for improved or novel functions for one or more rounds, and selected clones are analyzed by sequencing of the viral genome and validated in functional assays.

random peptides are presented from the envelope proteins of retroviruses, and selection was implemented to isolate peptides that can mediate targeted retroviral gene delivery and thereby modify viral tropism (Fig. **3**). Studies by Bupp *et al.* employed libraries where 10 amino acid randomized peptides were substituted into the variable region A (VRA) of subgroup A feline leukemia virus (FeLV-A) envelope proteins [26-28]. Unlike amphotropic MLV envelopes, which require changes in both the VRA and VRB domains to alter tropism, it has been shown that modification of only the VRA domain of the FeLV-A envelope is sufficient to alter tropism, thereby simplifying the engineering of novel specificity [27, 29]. Library selection on AH927 feline fibroblasts yielded a unique clone conferring a novel viral tropism highly specific to D17 canine osteosarcoma cells [27]. Interestingly, the selected clone had a receptor usage pattern distinct from its parental FeLV-A and FeLV-C envelope proteins; however, it is unclear how selecting the library on one cell type yielded a clone conferring a tropism to the other cell type. In a subsequent study, selection of a library, constructed in the same manner as above but on a larger scale, on *ras*-transformed 143B human osteosarcoma (HOS) cells yielded a FeLV-A envelope protein variant selective for 143B cells and 293T cells among more than 10 different cell lines [28]. By contrast, retroviruses displaying the parental wild type (wt) FeLV-A envelope proteins showed a minimal infectivity for 143B cells. Interference assays based on the cellular expression of FeLV-A, -B and –C envelope proteins indirectly indicated that viruses displaying the selected protein mutants did not rely on any FeLV receptors for infection of 143B cells [28]. More interestingly, the selected clones mediated retroviral infection of *ras*-transformed 143B cells at levels up to 50-fold higher than the parental cell line, untransformed HOS cells [28].

A subsequent study examined the effects of using different cell types during library selection on the properties of the resulting clones [26]. When the peptide display libraries [27, 28] were selected on feline AH927 cells, isolated clones exhibited a broad host tropism and utilized a non-FeLV receptor [26]. In contrast, when the libraries were selected on D17 canine cells, D17-specific variants that utilized FeLV-C receptors were isolated [26]. However, library selections did not always deterministically yield clones with the same properties, as library selection on AH927 cells yielded clones with broad tropism in one trial [26] but D17 canine cell specific tropism in another trial [27].

In addition to cell surface receptors, expression patterns of proteases that are cell type or disease state specific can be harnessed for targeted gene delivery [30, 31]. The detailed concepts and relevant studies are well described in the accompanying review by Buchholz and coworkers.

DNA Shuffling for Novel Envelopes

In addition to engineering single envelope proteins, it is possible to generate novel function by melding the properties of several envelopes. This generation of mutant or chimeric envelope proteins offers additional opportunities for modulating vector tropism. In one study, envelope genes from six different ecotropic murine leukemia viruses (MLV) that were each unable to infect a target cell type, Chinese Hamster Ovary (CHOK1) cells, were randomly shuffled to generate a library of infectious Moloney MLVs displaying chimeric envelope proteins [32]. The library construction involved PCR amplification of six parent envelope gene templates,

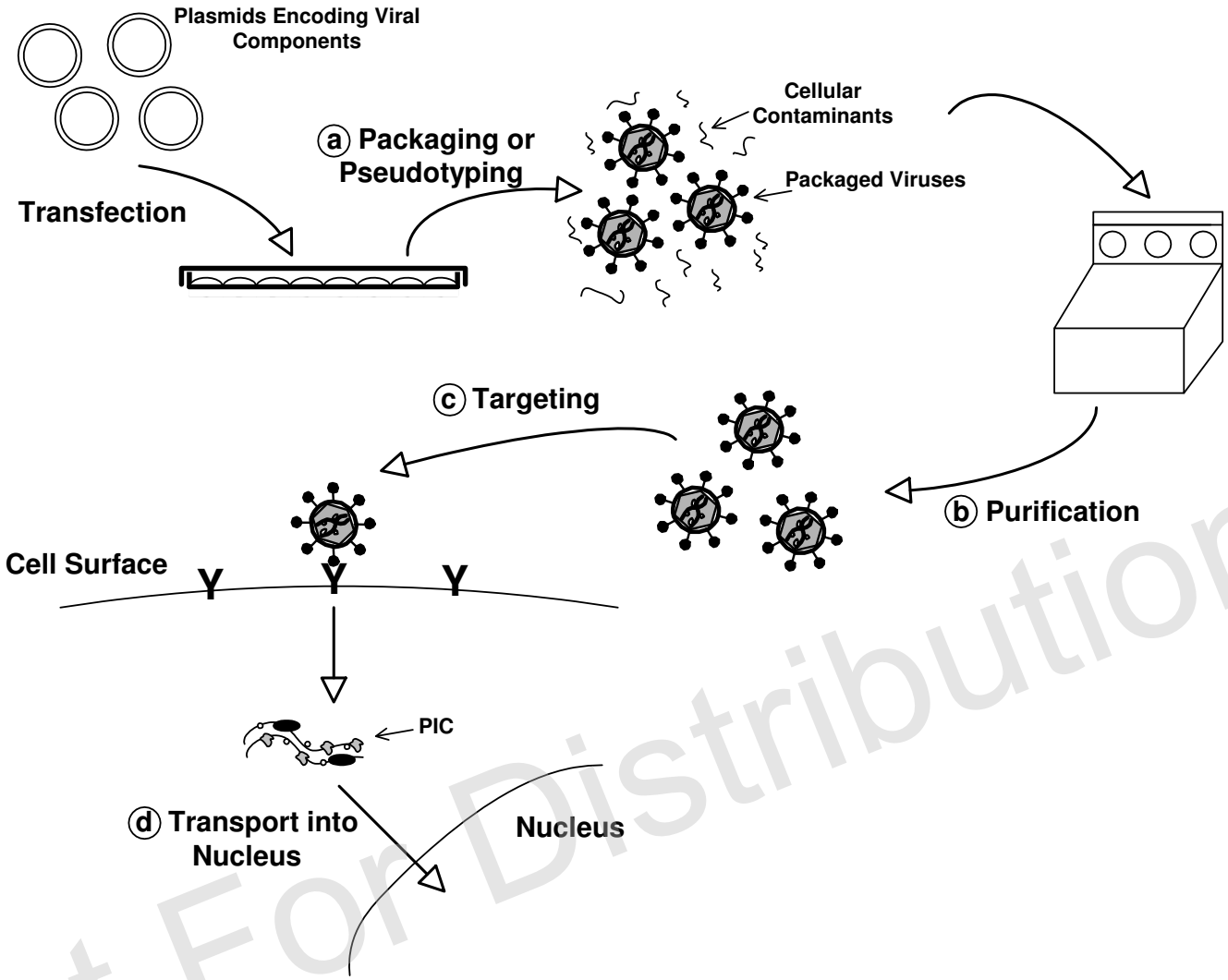

Fig. (3). Key steps in which retroviral vectors with naturally occurring viral components have suffered due to limited stability or low delivery efficiency: (**a**) Packaging or pseudotyping of capsidated viral genome with foreign envelope proteins; (**b**) Purification or concentration based on harsh conditions; (**c**) Targeting to key cell types; and (**d**) Transduction of non-dividing cells, limited by low efficiency of transport of viral DNA into the cell nucleus.

DNase I fragmentation of the PCR product, and PCR based assembly of the DNA fragments. The MLV library was selected several rounds on co-cultures of target CHOK1 cells and Lec8 cells, a derivative from CHOK1 cells, artificially made permissive to MLV infection by inhibition of glycosylation pathways. Small fractions of Lec8 were included in the co-cultures to accelerate amplification of mutants able to infect CHOK1 cells at early rounds of selection. An envelope protein clone isolated after several rounds of selection was found to be composed of segments from three out of the six parental envelope proteins, and it conferred MLV with the new capability of infecting CHOK1 cells [32]. Mutant proteins with a novel property can therefore be generated by shuffling several templates that individually lack this property.

Transcriptional Targeting *via* Promoter Engineering

Naturally occurring tissue- and cell-specific promoters [33, 34] can facilitate the development of tissue- or cell-targeted retroviral vectors. However, at times the weak transcriptional activity of such natural promoters can be problematic for their use in expressing therapeutic levels of delivered genes [35, 36]. To generate strong and cell-specific promoters, library techniques have been applied. Li *et al.* generated synthetic promoter libraries, where several elements from myogenic promoters, including the proximal serum response element (SRE), E-box, MEF-2 and TEF-1 sites, were randomly annealed and then ligated into plasmids having a luciferase gene [35]. The libraries were screened based on the level of luciferase activity in transfected chicken myoblast cells. Interestingly, a synthetic promoter clone identified from the selection offered at least several fold higher transcriptional activity on myogenic cells relative to the natural skeletal alpha-actin 448 promoter and the cytomegalovirus (CMV) promoter *in vitro* and *in vivo*. In addition, the selected promoter exhibited a high level of specificity, leading to gene expression only in myogenic organs such as muscle and heart [35]. The selected promoter consisted of

multiple copies of each natural promoter element, suggesting that combining existing natural promoter elements is sufficient to generate synthetic promoters with an improved transcription activity as well as cell selectivity [35]. This approach can readily be extended to constructing tissue targeted retroviral vector systems relying on synthetic promoters. In subsequent work by Dai *et al.*, a similar approach was applied to generate endothelial cell-specific promoters. Five binding sites for endothelial cell specific and general transcription factors were randomly assembled and inserted into the upstream of a reporter eGFP gene inside HIV-1 based lentiviral vectors, and the resulting library was selected on a monkey endothelial cell line [36]. Several isolated promoters mediated highly active and endothelial cell specific (up to more than 100 fold compared to CMV promoter) transcription [36]. Although this transcriptional targeting approach can improve the specificity of retroviral vector mediated gene expression, one limitation is that the specificity occurs at a post-binding step, which may require the addition of large amounts of vector to ensure that sufficient numbers arrive in the nucleus of the target cells.

INCREASING PSEUDOTYPING EFFICIENCY

Pseudotyping with heterologous envelope proteins has not only extended the tropism of retroviral vectors but also improved their stability [11-16]. However, not all foreign envelope proteins are able to support functional pseudotyping of retroviruses. For example, HIV-1 envelope proteins apparently cannot pseudotype MLV retroviral vectors because their long cytoplasmic tails hinder interactions with MLV core complex during virus assembly and budding [37]. In addition, Gibbon ape leukemia virus (GaLV) envelope proteins are unable to functionally pseudotype HIV-1 based lentiviral vectors [38, 39]. Substitution of the three amino acids in the envelope protein cytoplasmic tail with the corresponding residues of MLV was previously known to allow the resulting envelope protein mutant (called as RTM variant) to pseudotype HIV-1 [38, 39]. In order to further develop a GaLV envelope protein variant capable of efficiently pseudotyping lentiviral vectors, a library in which three amino acids in the envelope cytoplasmic tail (residues 618, 619 and 623) were randomized was selected for the ability to pseudotype lentiviral vectors and thereby mediate multiple rounds of infection on HT1080 cells [38]. Several selected variants showed increased expression on the cell surface compared to wild type GaLV envelope proteins, and they could ultimately support lentiviral vector pseudotyping at a significant level [38]. This improvement was apparently related to the efficient cleavage of the R peptide, known to inhibit fusion, from the novel envelope variants by HIV-1 proteases, as shown for the RTM variant. In other cases pseudotyping efficiency has been increased by rational removal of cytoplasmic regions of envelope proteins [37]. However, library-based selection methods will be still useful to create envelope protein variants having a high pseudotyping efficiency, especially when insufficient mechanistic information is available to empower rational engineering approaches.

IMPROVEMENT OF VECTOR STABILITY

Retroviruses suffer from other limitations in addition to targeting. For example, one property requiring improvement is viral stability during various centrifugation and/or purification processes required to generate vector for preclinical and clinical studies. Although retroviral and lentiviral vectors are very often pseudotyped with structurally stable heterologous envelopes, such as the vesicular stomatitis virus glycoprotein (VSV-G), at times vectors utilize retroviral envelopes that are composed of surface and transmembrane subunits with unstable associations [18, 40, 41]. To find stable mutant retroviral envelope proteins, a retroviral library in which six ecotropic MLV envelope proteins were recombined by DNA shuffling was selected for the ability to withstand harsh ultracentrifugation conditions commonly employed in vector concentration and purification [18]. In contrast to the parental envelope proteins, which suffered from 30- to 100- fold losses of titer upon ultracentrifugation, clones isolated from the selected library yielded viral vectors very resistant to the ultracentrifugation steps with no or insignificant loss of titer [18]. Selected clones contained sequence contributions from three of the six parental envelope proteins used to generate the library. Interestingly, although the exact mechanism was not determined, the improved stability of retroviral vectors was apparently not due to a more stable association of the surface units of the selected envelope protein variants with viruses [18]. This example further demonstrates that shuffling of parental templates, each of which may individually lack the relevant desirable traits, may still yield improved properties through a novel combination of domains from their parents [18, 32].

IMPROVING VECTOR PURIFICATION

Vector concentration, such as by ultracentrifugation, provides a way to reduce cellular contaminants after vector production, but further purification is often required for clinical use to minimize vector toxicity or immunogenicity. Affinity chromatography based purification can remove many such contaminants from concentrated virus [42]. VSV-G is broadly used for retroviral and lentiviral vectors, but efforts to engineer it have met with limited success since the insertion of exogenous sequence into most sites results in compromised titers. To adapt VSV-G for nickel nitrilotriacetic acid (Ni-NTA) based column purification, we recently developed a novel random peptide insertion approach [43]. In this library, a hexa-histidine (His_6) tag that has high affinity for Ni-NTA was randomly inserted into likely every position of VSV-G using a transposon system. The resulting variants were inserted into a retroviral vector and packaged so that each member of the resulting virion library was composed of a particle displaying a mutant VSV-G encompassing a genome encoding that mutant. Subsequent selections of the library first for infectivity and then for the ability to bind to a Ni-NTA column led to the identification of three viable insertion sites for hexa-histidine [43]. These insertions, close to the VSV-G N-terminus, were revealed to be exposed to the protein surface when subsequent VSV-G crystal structures were reported [44, 45]. The mutant VSV-G variants were used to generate retroviral and lentiviral vectors that could be purified *via* Ni-NTA column chromatography to yield virus stocks with undetectable contaminating proteins and DNA [43]. Furthermore, the highly purified virus stocks led to efficient transduction and weaker immune responses after injection into the rat brain compared to conventionally purified viruses.

EXTENSION OF RETROVIRAL INFECTION TO NON-DIVIDING CELLS

Another limitation of gammaretroviruses (such as MLV) as gene delivery vehicles is that, unlike lentiviruses, they are unable to infect non-dividing cells [1]. This inability may be due to lack of nucleus localization signals (NLS) in the viral capsid that mediate active transport of lentiviral PICs into the host nucleus, though evidence for functional NLS sequences in HIV is debated (reviewed elsewhere [46]). However, insertion of canonical NLS sequences into appropriate locations within viral proteins associated with the retroviral PIC could potentially facilitate retroviral gene transfer to non-dividing cells. This approach hinges upon the ability to identify viable and functional insertion sites within the viral *gag-pol* genes, so we recently applied a library-based, high throughput scanning of the MLV gag-pol to create retroviral vectors able to transfer genes to non-dividing cells *in vitro* and *in vivo* [47]. Two libraries, in which functional NLS sequences from simian virus 40 (SV40) and the matrix protein of HIV were randomly inserted with the aid of a transposon system into likely every position of MLV *gag-pol*, were selected for variants that mediated infection of non-dividing cells. One isolated clone contained a HIV NLS insertion into the viral p12 protein. When mixed with wild type *gag-pol*, this variant enabled significant retroviral infection of cells growth-arrested by various drugs, such as mitomycin C, hydroxyurea and aphidicolin [47]. In addition, selected clones showed significant levels of infection of neurons *in vitro* and *in vivo* [47].

CONCLUDING REMARKS

In conclusion, retroviral libraries have been generated and screened in numerous ways to improve the properties of retroviral vectors. As summarized in Fig. **1**, libraries can be constructed by inserting random motifs into a single position of viral components [26-28], shuffling parental DNA templates through PCR [18, 32], randomly ligating pools of DNA templates having cohesive ends [36], randomizing specific residues through PCR without insertions [38], and inserting a single type of motif into various positions of viral components [43, 47]. Several rounds of selection of such libraries (Fig. **2**) have successfully and rapidly yielded various viral mutant components with improved properties such as transductional targeting, transcriptional targeting, purification, and intracellular transport, thereby highlighting the power of library selection approaches for engineering efficient and enhanced retroviral vector systems. Furthermore, the inherent ability of viruses to physically link genotype to phenotype offers the capacity to generate and select extremely large libraries in a high throughput fashion. Moreover, because they do not rely on substantial mechanistic knowledge in order to yield results, library-based approaches are particularly suited to engineering novel function into entities with a high degree of structural and functional complexity, such as viruses. Finally, in addition to generating enhanced gene carriers, analysis of the fruits of these efforts can yield novel insights into basic virology [48, 49].

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ABBREVIATIONS

ALS	=	Avian leucosis virus
CHO	=	Chinese Hamster Ovary
CMV	=	Cytomegalovirus
FeLV-A	=	Subgroup A feline leukemia virus
FeLV-B	=	Subgroup B feline leukemia virus
FeLV-C	=	Subgroup C feline leukemia virus
GaLV	=	Gibbon ape leukemia virus
His_6	=	Hexa-histidine
HOS	=	Human osteosarcoma
LTR	=	Long terminal repeat
MLV	=	Murine leukemia virus
Ni-NTA	=	Nickel nitrilotriacetic acid
NLS	=	Nucleus localization signals
PIC	=	Preintegration complex
SRE	=	Serum response element
SV40	=	Simian virus 40
VRA	=	Variable region A
VRB	=	Variable region B
VSV-G	=	Vesicular stomatitis virus glycoprotein

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