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Library screening and receptor-directed targeting of gammaretroviral vectors

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Abstract

Gene- and cell-based therapies hold great potential for the advancement of the personalized medicine movement. Gene therapy vectors have made dramatic leaps forward since their inception. Retroviral-based vectors were the first to gain clinical attention and still offer the best hope for the long-term correction of many disorders. The fear of nonspecific transduction makes targeting a necessary feature for most clinical applications. However, this remains a difficult feature to optimize, with specificity often coming at the expense of efficiency. The aim of this article is to discuss the various methods employed to retarget retroviral entry. Our focus will lie on the modification of gammaretroviral envelope proteins with an in-depth discussion of the creation and screening of envelope libraries.

Keywords

DNA shuffling; FeLV; library screening; MLV; murine leukemia virus; pseudotyping; retroviral entry; sindbis Env; viral envelopes; viral receptors; viral retargeting

Gene therapy holds the potential to treat a host of both genetic and acquired disorders through the selectable expression or repression of a target gene. Delivery of a therapeutic transgene allows for proper gene expression in those with an inherited deficiency or selective RNA silencing in those with a dominant-negative phenotype. Just as therapeutic genes can be delivered to restore normal cellular function, toxic genes can also be delivered to cancerous cells.

Various methods have been employed to deliver these transgenes using both viral and nonviral vectors. Viral vectors have been derived from adenovirus [1], adeno-associated virus [2] and vaccinia [3], among others. However, the earliest clinically effective vectors were those derived from retroviruses [4]. There are several features that make retroviral vectors particularly attractive for use in clinical applications: their genome can be stably integrated, providing long-term transgene expression; they can accommodate large transgenes, up to 10 kb [5]; and lentiviruses provide the additional advantage of transducing nondividing cells.

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Ex vivo transduction has shown promise in treating certain disorders, primarily blood-borne in nature [4,6–8]. In these settings, the target cells are removed and purified prior to exposure to the virus. The cells are transduced *in vitro* and only then are reintroduced into the patient. In this scenario, the vector only needs to provide efficient indiscriminate transduction.

However, for delivery into cancerous cells or solid organs, an *in vivo* delivery system is necessary. *In vivo* delivery adds the additional level of complexity that the vector must be specifically targeted to the organ or pathology in question in order to avoid harmful side effects from errant transduction into nontarget cells. As retroviral entry is tightly regulated by the interactions between the retroviral envelope (Env) glycoprotein and its host receptor, artificial manipulation of this viral protein can create retargeted viruses with novel tropisms.

In this article we will discuss various methods that have been employed to retarget retroviral Envs, with a special focus on the creation and screening of randomized Env libraries and *in vitro* evolution. These methods have developed novel Envs with potential therapeutic applications, and have provided us with a deeper understanding of retroviral entry and approaches to manipulating it for both research and clinical applications.

The Env protein & retroviral entry

The retroviral Env is translated as a polyprotein before being cleaved into two separate subunits; a surface subunit (SU) and a transmembrane subunit (TM) [9]. The N-terminus of a SU contains one or more hypervariable regions, which show little homology between species and contain sequences that specifically recognize the target receptor [10–12]. All gammaretroviruses have at least two of these hypervariable regions, although they differ in size and function. Studies have demonstrated that mutations in these variable regions are able to alter viral tropism, although the amount of alteration required varies from species to species. The amphotropic 10A1 murine leukemia virus (MLV) Env, for example, requires mutations in two regions (variable region A [VRA] and variable region B) in order to alter viral tropism [13]. In the feline leukemia virus (FeLV)-A these regions are both significantly shorter than their MLV homologs [14] and mutations in VRA alone can alter the viral tropism [15].

For MLV Envs, linking the N- and C-termini of SU is a flexible hinge domain known as the proline-rich region. The C-terminus of SU forms disulfide bonds with the N-terminal ectodomain of TM, covalently linking the two subunits [16,17]. The remainder of TM is comprised of a transmembrane domain and an intracellular C-terminus. In lentiviruses, identification of second-site mutations in TM that compensate for mutations in the viral structural matrix protein (MA) implicate an interaction that anchors the Env with the rest of the virion [18]. This interaction, however, has not been fully established in gammaretroviruses. The ectodomain of TM also contains a fusion peptide that, when triggered by SU, inserts into the target cell membrane prompting fusion and viral entry [19]. The binding of SU to its host receptor produces conformational changes within the SU, which are subsequently transmitted to the TM, through isomerization of the aforementioned disulfide linkages, activating the fusion process [17,20].

Utilizing host receptors that are ubiquitously expressed at high levels provides a survival advantage to the virus. As one would expect, most of the naturally occurring retroviral Env proteins follow such a pattern (Table 1). However, in addition to its expression profile, there are other factors that are common amongst retroviral receptors.

One well-conserved feature is an apparent preference, particularly by gammaretroviruses, for multipass transmembrane proteins. All known gammaretroviruses utilize this class of

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protein for entry (see Table 1 for an exemplary listing). The use of this class of proteins as viral receptors also extends beyond gammaretroviruses. Alpharetroviruses, including avian sarcoma leucosis virus, which utilize single pass glycosylphosphatidylinositol-linked transmembrane proteins [21,22], are an exception to this rule. Even retroviruses with more complex entry processes utilizing coreceptors, including HIV and human T-lymphotropic virus type 1, require a multipass protein to mediate the actual process of fusion and entry (CXCR4 or CCR5 for HIV [23,24] and GLUT-1 for human T-lymphotropic virus type 1 [25]). Perhaps even stronger evidence comes from the observation that this class of receptor is also selected for and remains advantageous to artificially targeted Envs [26,27].

There are various reasons why multipass transmembrane receptors may be so vital to this process. It may be a matter of proximity to the actual lipid membrane. Multipass transmembrane proteins have smaller extracellular domains than many single-pass transmembrane proteins. As the fusion peptide of TM is of limited length, attachment at a site that is relatively far from the cell membrane may not allow for effective contact with the cell membrane. Alternatively, there is evidence to support the hypothesis that receptor clustering takes place after the initial binding event [28]. This class of receptor may be preferentially found in highly fluid membrane regions such as lipid rafts, which would allow for movement and clustering. Another possible explanation is that this class of proteins may utilize the proper endocytic pathway to mediate efficient internalization of the virus. As noted in Table 1, the majority of the multipass transmembrane proteins that are utilized as retroviral receptors, function as transport proteins for a variety of biochemicals (vitamins and amino acids, among others). This innate function requires conformational flexibility, which may also aid in mediating viral entry.

In studies aiming to retarget entry, the features of multipass transmembrane proteins should be considered, as should the need to maintain the necessary interactions between SU and TM. Many of the early retargeting methods were hampered by poor fusion despite strong SU binding [29–31]. Many of these cases involved large modifications of SU and the targeting of a gammaretroviral Env to a single-pass transmembrane protein. Similar attempts have led to specific and efficient transduction *in vitro*; however, the presence of both the retargeted Env and its parental wild-type (WT) Env was required to gain efficient entry through the WT receptor [32]. These studies will be discussed further in subsequent sections.

Pseudotyping

The earliest and simplest form of viral retargeting was pseudotyping, a method by which the Env of one virus was expressed on the surface of a different viral particle (Figure 1B). This was originally discovered through the observation that one virus could adopt the interference pattern of another when they were grown in coculture [33]. Later, this process was simplified through molecular cloning and replacement of the natural *Env* gene with that of another virus. Pseudotyping has been accomplished both within the same genus, from one gammaretrovirus to another [34], or across genera, in other words, lentivirus to gammaretrovirus [34,35]. Pseudotyping can even be further extended between families of viruses. Surface glycoproteins from a host of viruses, including vesicular stomatitis virus [36], Semliki Forest virus [37], rabies [38], measles [39], sindbis and ebola [40] have all been successfully pseudotyped onto retroviral particles.

The ability to pseudotype one retroviral Env onto another retrovirus is largely governed by the compatibility of their TM and MA proteins [41]. The cytoplasmic domain of the gammaretroviral TM is relatively small, 16 amino acids in the mature form of ecotropic MLV Env [42], and the gammaretroviral Envs, including gibbon–ape leukemia virus (GALV) and amphotropic MLV, can be pseudotyped onto HIV-based viral particles without

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modification [43,44]. However, the HIV Env, with its large 150-amino acid cytoplasmic domain, cannot be incorporated onto MLV-based particles without modifying this region [35]. Further support for the importance of this interaction comes from the observation that Envs that do not easily pseudotype onto certain particles can be rescued if their cytoplasmic tail is exchanged with that of a virus that efficiently pseudotypes. Normally, the Env glycoprotein from the feline endogenous retrovirus, RD114, and GALV, do not pseudotype efficiently onto lentiviral particles. However, when the cytoplasmic tails of these TM subunits were replaced with that of the amphotropic MLV Env, the new constructs readily incorporate onto lentiviral particles [44–47]. Furthermore, while the surface glycoproteins of the measles virus do not make known contacts with MA, truncation of their cytoplasmic domains is required to prevent steric impedance [39].

Pseudotyping, however, can only extend viral tropism to that of other naturally occurring viruses. While this method has been used to create safe and efficient retroviral vectors, and lentiviral vectors with GALV pseudotypes [48] have been used for *ex vivo* transfection, the majority of naturally occurring Envs do not provide the tissue specificity necessary for *in vivo* gene therapy. One possible exception is RD114. This Env utilizes a neutral amino acid transporter, which is highly enriched on the surface of hematopoietic stem cells (HSCs), as its host receptor [49,50]. However, the expression of this receptor is not limited to HSCs and, while important, HSCs are only one of a large number of potential clinical targets for gene therapy.

While it does not provide an ultimate solution, pseudotyping does provide a broad base from which a number of retargeting techniques can emerge. Different structural features of known Envs make them each suited for specific retargeting methods. The ability to pseudotype these modified Envs onto an array of retroviral and lentiviral particles increases the therapeutic potential of these vectors. Particularly, the ability to pseudotype gammaretroviral Envs onto the surface of lentiviruses allows targeted Envs to be expressed on particles that can infect nondividing cells.

Targeting with chimeric Env proteins

Antibody-based targeting

Antibodies provide astounding specificity and have been cloned and well classified for some time now. This makes antibodies attractive prospects as potential retargeting domains (Figure 1G). Single-chain antibodies (scFv) have been inserted into a host of retroviral backbones, including the ecotropic [32,51] and amphotropic MLV Envs [52], and the spleen necrosis virus Env [53]. This was first successfully demonstrated by inserting a scFv against the low-density lipoprotein receptor into MLV ecotropic Env [32]. Additional scFv have proven effective at targeting, and this technology has incorporated the use of phage display libraries to discover novel scFv domains that can provide alternative targeting [54].

Two common obstacles, however, often hampered the success of scFv Envs. In some circumstances, the Envs were synthesized but failed to incorporate into virions [55]. In others, the modified Envs were often highly effective at mediating strong viral binding to the target receptor, but were compromised for fusion and proper Env function [31,56]. Binding in the absence of fusion could often be overcome by coexpression with the WT Env [32,57]. This solution, however, decoupled targeting and entry by binding via the nonfunctional retargeted Env and allowed fusion and entry through the function of WT Env and its abundantly expressed receptor [51]. Furthermore, this feature requires that the retargeting take place in an Env with high titers on the target cell prior to modification. This approach was not universally successful and it remained difficult to predict which Envs could remain

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functional. Using a lentiviral delivery system, success with scFvs fused to modified measles virus Envs has been obtained on pseudotyped particles [58].

An alternate approach to circumvent these obstacles was to insert the immunoglobin-binding domain from staphylococcal protein A [57], creating a rapidly adaptable means of targeting. The Fc region of an IgG noncovalently binds to the modified Env and presents the variable domains for targeting. The Env can be incubated with any IgG and would then, in theory, retarget to any surface-exposed antibody target. This method continues to show promise and has also been expanded into the sindbis virus glycoprotein (Figure 1C) [59–61]. While this technology continues to improve, it is often hampered by high levels of background transduction. The cause of this nonspecific transduction was recently identified as being the result of entry via a novel surface receptor, Axl. Remarkably, the receptor was not recognized by the viral glycoproteins, but rather by a soluble serum protein (Gas6) that bridges Axl with phosphatidylserine in the viral lipid envelope [62].

Targeting peptides

Similar to scFv, the insertion of known binding domains and peptide ligands has also been used to create retargeted chimeric Envs. The first published report used erythropoietin inserted into the receptor-binding domain to retarget ecotropic MLV to the erythropoietin receptor [63]. Mutational studies [64–66] complemented by structural studies [67] revealed several areas within the surface glycoproteins of ecotropic MLV Env that could present targeting peptides. Sites tolerant of insertion have included the extreme N-terminus [29,68], within the receptor-binding domain [64,66] and within the proline-rich region [66,69]. Targeting domains have ranged from short peptide ligands [70,71] to large subunits [29,72], and to entire proteins [63].

Early targets were known tumor antigens including HER-2 [73] and the EGF receptor [29], and based on the initial results the insertion of other receptor targeting domains was attempted, including those directed towards gastrin-releasing protein [71], c-kit [72] and the IGF-1 receptor [68]. While some attempts were successful, others were met with impaired Env function. Similar to scFv chimeras, these domains mediated strong receptor binding, but were bulky and often impaired the fusogenic properties of the Env [29]. Much like the antibody-targeted Envs mentioned above, coexpression of the targeted Env with its WT counterpart was often able to reconstitute the infectious potential of the virus [63], however, this decoupling has the same limitations. Additional studies revealed that titers could be partially restored by presenting the targeting domain on a hinged linker that presumably provided the conformational flexibility necessary for fusion [74].

When the targeting peptide is inserted outside of the receptor-binding domain, the WT infectivity of the Env may be preserved (Figure 1F). This is not a concern if the parental Env does not infect human cells. However, with the use of Env glycoproteins, including amphotropic MLV, background entry may eliminate the benefits of targeting. Alternatively, complementation with WT MLV has been exploited to rescue retargeted entry-defective Envs. The insertion of the collagen-binding domain from von Willibrand's factor into the MLV ecotropic Env glycoprotein provided strong binding to collagen matrices, and *in vivo* transduction in mice [75]. However, since the ecotropic receptor is not expressed on human cells, another backbone would be necessary. When the same binding ligand was inserted into a variety of positions within the 4070A isolate of the MLV amphotropic Env, a chimera was identified that provided *in vivo* targeting to sites of exposed extracellular matrix (damaged vasculature and solid tumors), while mediating entry into the surrounding cells at levels near to that of WT amphotropic MLV [76]. Interestingly, the target in this scenario is collagen within the extracellular matrix, not a cell-surface protein. This 'pathotropic' construct targets cells to the pathologic microenvironment and entry is then conducted

nonspecifically through the cognate receptors for MLV amphotropic Env. *In vivo* studies have demonstrated targeting to areas of exposed basement membrane, and additionally, when combined with a cytotoxic cyclin D mutant, targeted tumor killing [77,78]. This vector, known as rexin-G, has entered clinical trials for treatment of certain advanced-stage cancers [79–81].

Complementation studies have also been extended to combinations of novel receptor-binding cell-surface proteins with binding-defective fusogenic Env proteins. These systems have been effective for CD20 antigen with fusogenic sindbis Env [82] and MLV–Flt-3 chimeras with binding-defective, but fusion-competent, influenza hemagglutinin molecules [83].

Newer attempts have employed the use of structural data and molecular modeling to predict efficient targeting peptides. Through structural data of the ecotropic MLV glycoprotein and molecular modeling software, Li and colleagues were able to predict the successful insertion of somatostatin, a small peptide hormone, into the receptor-binding domain of ecotropic MLV SU in a manner that would preserve the majority of the Env structure, and, therefore, function (Figure 1E) [84]. A separate group has utilized structural data to incorporate the small peptide apelin into the FeLV-B related Env, SL3-2, and retarget entry to the apelin receptor, APJ [85]. These studies improve on an earlier peptide-retargeted Env, in which insertion of the SDF-1α peptide into the ecotropic MLV VRA directed entry to the CXCR4 receptor, albeit with a low titer [86]. In the future, this technology may be able to be incorporated with the screening of phage display libraries to generate targeting sequences to receptors that do not have naturally occurring peptide ligands. It must be taken into account that both the somatostatin and apelin receptors are multipass transmembrane proteins, the natural receptor class of gammaretroviruses. The ability to expand this method to other receptor classes has yet to be studied.

Blocking peptides

An alternative approach has been developed that utilizes blocking domains to prevent binding to the WT receptor until they are removed at the target site (Figure 1F). By linking these to the Env backbone by a protease-cleavable linker, the blocking peptide is specifically removed at the surface of the cells of interest, revealing the WT receptor-binding site preferentially at the surface of the target cell. The specificity of these Envs comes from the upregulation of certain surface proteases on the surface of certain cell and tumor types. In this scenario, the blocking agent must be attached to the N-terminus of an Env that mediates efficient, nonspecific, high-titer infection, such as the amphotropic MLV Env.

This targeting scheme can be separated into two tracts, both of which have been demonstrated to provide specific infection within a mixed population *in vitro* [52,87], as well as in tumor xenografts [87]. The first involves the inclusion of bulky blocking agents, such as trimeric CD40L [88], attached by a linker that is cleavable by a specific cell-surface protease. A variety of proteases have been exploited in this manner, including matrix metalloproteases [52], urokinase [89], plasmin [90], factor Xa [68] and intracellular proprotein convertases [91]. Owing to the fact that these cleavage sites are not entirely specific, libraries have been generated to optimize the sequence and flanking regions of these linkers [90].

In this scenario, the ‘targeting’ is mediated solely by the presence of the protease on the cell surface. The second tract, adds additional complexity and potential specificity. Instead of a bulky blocking group that simply inhibits Env function by steric impedance, a strongly binding receptor ligand, such as EGF [91], is attached to the N-terminus of the Env protein. This domain mediates strong binding to its host receptor, but is unable to mediate the

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subsequent steps of entry. However, this process highly enriches the virus at the surface of the target cell and, when attached by a cleavable linker, the WT virus is then preferentially released in this region. Similar to the ‘pathotropic’ targeting discussed above, entry is then mediated by the WT Env function, however, owing to the high degree of enrichment in the target microenvironment, specificity is achieved. This technique allows the use of a ubiquitously expressed protease because of the ligand-binding specificity, and provides a second layer of specificity to further reduce aberrant infection.

Retroviral library screening

One drawback common to all of the aforementioned retargeting methods is that modifications are made to the virus with the assumptions that the virus will maintain functionality and the receptor will mediate viral entry. In order to circumvent these issues researchers have exploited the virus’ natural means of receptor selection and optimization: evolution. As mentioned in previous sections, small libraries have been generated to improve linker regions within previously retargeted viruses [91]. Additionally, mutational libraries that randomize individual residues have improved species tropism and expanded our knowledge of Env function [92]. However, while these methods have provided technical advances towards retargeting libraries, they themselves do not truly retarget entry, and are beyond the scope of this review.

By creating complex libraries of Envs and screening them for functional isolates, rapid, *in vitro* evolution has uncovered novel retargeted Envs (Figure 1). Libraries have been generated through DNA shuffling [93] and through the randomization of the receptor-binding domain [14]. These methods of *in vitro* evolution isolate a single evolutionary technique and expand it to screen all (or as many as technically possible) potential mutants in one large-scale screening, as opposed to over the course of many generations.

DNA shuffling

DNA shuffling is a laboratory technique that promotes high-rate homologous recombination *in vitro* in order to expand viral tropism (Figure 1). In a study by Soong *et al.*, six strains of ecotropic MLV were recombined by this process and their tropism was extended to the normally non-permissive Chinese hamster ovary (CHO) cells [93]. Ecotropic MLV is normally unable to infect CHO cells, not because of receptor expression, but rather owing to extensive glycosylation of the receptor that blocks Env binding [94]. However, in this study, the recombinant Env was able to bind to and infect CHO cells at appreciable levels.

To accomplish this *in vitro* recombination, the *Env* genes of these six related viruses were partially digested with DNAse I and amplified through PCR without the presence of internal primers. This created an overlapping PCR in which *Envs* with significant homology could recombine at any of the digestion sites. When the library was screened, isolates were identified that were able to infect CHO cells, despite heavy receptor glycosylation. Interestingly, it took several rounds of passage for an isolate to become predominant. In addition, to allow the virus to efficiently spread, the target CHO cells had to be cocultured with Lec8 cells, which are a glycosylation-deficient CHO strain that some WT ecotropic MLV strains are capable of infecting. These were included to serve as a safe harbor for poorly infectious chimeric strains to gain a foothold during early passages. While this method did not direct binding to an alternate receptor, it did allow for usage of receptors that were otherwise inaccessible by WT viruses, and may provide a means by which to optimize other artificially retargeted Envs.

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Random targeting sequences

By randomizing the receptor-binding site, libraries can be generated from which truly retargeted Envs can be isolated (Figure 1E) [14,27]. In this method, the binding domain of an Env is randomized, essentially introducing all of the possible receptor ligands into the Env backbone and selecting based on the Envs ability to mediate efficient transgene delivery. Owing to the fact that the retargeting is not driven towards a specific receptor, after an isolate is identified, its receptor must be cloned before its clinical applicability can be determined. However, because the isolates with the highest titer on the cell type on which they are being screened spread during passage in culture, the isolates are guaranteed to efficiently mediate viral entry and not be hindered by impaired translation, membrane fusion or transgene delivery.

In order for this method to be successful, the Env chosen must contain a single, small, receptor-binding domain. Many Envs contain multiple variable regions that participate in receptor binding [13,95]. Furthermore, in many cases small regions or single amino acids outside of the true receptor-binding domain are required for strong binding and maximal titers [96,97]. For the FeLV-A Env, it is known that substitution of a single variable region near its N terminus can alter viral tropism [15]. This region contains a proposed cysteine loop and libraries have been generated by replacing an 11-amino acid region within this loop. Although this Env has been the one studied thus far, as previously discussed, replacement of a single 12-amino acid sequence within ecotropic MLV glycoprotein can also alter tropism, suggesting that other Envs may be suited for similar adaptation [84].

A schematic representation of the generation and screening of randomized Env libraries is shown in Figure 2. To generate the library, a region within the *env* gene that encodes 14 amino acids within the FeLV-A receptor-binding domain is excised and replaced with back-to-back recognition sites for BbsI, a type II restriction enzyme that cuts outside of the enzyme recognition sequence. Initial constructs included a stop codon between the BbsI sites to eliminate any *env* gene products from the parental vector. Later versions included a stuffer region plus stop codons to better separate the fully cleaved vector from its singly cleaved counterpart prior to generation of the library. The region randomized encodes 11 amino acids and creates libraries with a potential complexity of 10^{14} isolates.

To generate the random library, a combination of three oligonucleotides is used that hybridize to the BbsI restriction sites in a directional manner. In order to reduce the number of stop codons, and thus improve the proportion of open reading frames in the library, primers in which the third residue of every codon is randomized to only T, C or G have been employed. This reduces the incidence of stop codons by 66%.

The bacterial colonies are pooled and maintained as a population of plasmid DNAs. This library then needs to be expressed in mammalian cells and mobilized into viral particles. The Env is expressed within a bicistronic retroviral vector that also encodes a selectable marker. FeLV Envs readily pseudotype onto MLV-based virions and all work can be performed under biological safety level 2 conditions. The library is either transduced (via vesicular stomatitis virus) or transfected into a packaging cell line, and then the assembled virions are screened on the cell line of choice. Library screening has been performed on renal cell carcinoma cell lines (Caki-1) [27], osteosarcoma (143B and D17) [98,99], prostate (PC-3) [100] and feline A H927 cells [14,99]. While the Envs all produced titers on the cell lines from which they were screened, titers on 293T cells, the cell line used in the initial mobilization of the library, were observed. The library virions were collected 48 h after the Env plasmids had been introduced into the packaging cells; however, this left enough time for the Envs to assemble and propagate through at least one round of infection prior to collection. Studies are currently underway to determine if the library was being enriched and

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undergoing an initial round of selection in the packaging cell line and whether more efficient targeting can take place when the library is mobilized and screened within the same cell line. Maintaining the library within the same cell line during all stages of the screening process could allow for enrichment during multiple steps within the screening process, thus allowing isolates with low initial titers to gain a foothold.

Once functional isolates are obtained, the cognate receptors need to be identified to define the potential clinical applications. To date, two such pairs of FeLV Env library isolates with their cognate receptors have been identified. Both of the Envs utilized multipass transmembrane receptors, the same class utilized by WT FeLV strains and all other known gammaretroviruses. The first of these Envs, designated A5, utilizes SLC35F2, a putative nucleotide sugar transporter, as its host receptor [26]. This family of receptors is predominantly found on the endoplasmic reticulum–Golgi membrane [101,102]. While, its function as a viral receptor proves that SLC35F2 must exist on the cell surface, its presence in the endoplasmic reticulum membrane suggests that it may follow an endocytic pathway that is favorable for retroviral entry, a variable that needs to elucidated further for better directed Env targeting. More significantly, SLC35F2 has not been previously reported to function as a known retroviral receptor, validating the use of randomized Env libraries to direct viral entry through novel host cell-surface proteins.

The second of these Envs, CP, utilizes two closely related riboflavin transporters, SLC52A2 and SLC52A1 (previously named GPCR-172A and -172B, respectively) [27,103]. These receptors also serve as the receptors for porcine endogenous retrovirus A (PERV-A), and are thus referred to as human PERV-A receptor 1 (HuPAR-1) and -2 [104]. This similar receptor usage provided a unique opportunity to study the *in vitro* evolution that occurs during library selection, by comparing the receptor usage of CP and the naturally evolved PERV-A. Studies in this regard revealed that the primary binding site for both CP and PERV-A Envs mapped to the same nine-amino acid extracellular loop [103]. This was astounding, given that these Envs contain only 32% identity; 46% homology within SU [103]. Furthermore, while FeLV-A is proposed to rely on a single receptor-binding domain, PERV-A relies on additional receptor contacts for efficient binding [95,96]. These data suggest that even though natural evolution has the additional selective pressures of immune evasion and environmental persistence, the library screening, which selects solely for efficient gene transfer, may still select for similar receptor factors.

The receptor-binding domain of FeLV (the region that is randomized during library generation), is located within a putative cysteine loop [67]. Interestingly, the randomized region of CP within this loop contained two additional cysteine residues that are essential to Env binding and cannot be replaced by the conservative cysteine–serine mutations [27]. It is possible that these residues create a novel conformation within the receptor-binding domain by creating a new disulfide-bonding pattern within the Env backbone.

The clinical significance of these envelopes is currently under investigation. HuPAR-1 is ubiquitously expressed but is known to be upregulated in solid tumors [105]. Recent studies have analyzed infection with virus bearing the CP Env in animal studies and have proven it to be effective in targeting mouse tumor xenografts with known HuPAR-1 overexpression [Zhang X & Roth MJ, Unpublished Data]. The A5 receptor, SLC35F2, was recently discovered to be overexpressed in non-small-cell lung cancer, also implicating the therapeutic potential of this isolate [106]. Owing to the fact that the receptor is unknown at the time of Env isolation, a large panel of isolates needs to be obtained in the hope of identifying one with the specificity required for gene therapy. In addition, it would seem probable that since these libraries are being screened for efficient transgene delivery, those isolates directed at the most heavily expressed receptors would have a selection advantage.

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However, expression levels do not necessarily correlate to specificity. One way to overcome this may be to artificially overexpress a receptor in the target cell line in order to bias library selection towards the newly enriched receptor.

The full potential of this method has not yet been achieved, and many more isolates are yet to be discovered. While the theoretical complexity of these libraries is in the range of 10^{14}, even large-scale cloning has so far yielded libraries closer to the 10^7 range. Further limitation of the library complexity comes during the screening process. The library is introduced into the packaging cells at a very low multiplicity of infection in order to avoid chimeric strains that package a different Env than that which is displayed on the cell surface. Under normal circumstances, gammaretroviral glycoproteins form trimers on the viral surface. Mixed trimers are known to alter viral tropism [100]. Introducing the library at a low multiplicity of infection also reduces the possibility of mixed trimers resulting from the simultaneous transfection of multiple library plasmids.

Further studies on the CP Env have raised some interesting points regarding library screening. In nature, more than encoding the genes necessary for propagation, the viral genome is optimized for appropriate splicing and expression of each gene product and/or splice variant at the appropriate proportions. By introducing an outside sequence into the vector backbone, novel splice sites and expression levels are generated. Work with the CP Env has demonstrated that the expression, and splice sites vary widely when the Env is expressed with a different promoter, selectable marker combinations and with the addition of stabilizing elements such as woodchuck hepatitis virus post-transcriptional regulatory element [Zhang X & Roth MJ, Unpublished Data]. The next generation of retroviral Env libraries have been minimized for aberrant splice sites and maximized for packaging of the full-length vRNA to increase the identification of functional Env isolates.

Future perspective

As retroviral vector technology continues to improve, targeting must follow in parallel with this. Pseudotyping continues to expand to more and more distantly related viruses. The pseudotyping of rabies virus glycoprotein offers the hope of neuron targeting [38]. The sindbis virus surface protein incorporates efficiently onto lentiviral vectors, and has been modified for rapidly adaptable antibody-based targeting [59], similar to that described in the aforementioned section on antibody targeting. It has also been adapted for targeting with avidin-/biotin-based bridging peptides [107]. These methods may provide rapidly adaptable means to target a broad range of clinical targets and have already proven effective in the transduction of HSCs, human embryonic stem cells and melanoma cells [60,61,108,109].

As more structural data is gathered, molecular modeling may play a larger role in vector design. This has already been applied to the insertion of small peptides as discussed earlier, and could be expanded to additional Envs and additional targeting sequences. The ability to predict Env structure prior to design would be of great benefit in vector targeting, providing tremendous savings in both time and cost.

Library screening is still in its early stages, and advances in screening methods may allow for more isolates to be identified. As FeLV has been the only prototypical Env studied thus far, the expansion of these techniques into additional Envs may prove beneficial. In addition, as Envs that have already been isolated are expanded into animal models, their full clinical potential will be determined.

As new techniques arise to retarget retroviral glycoproteins and new targets are discovered, the next step needs to be the leap from bench to bedside. Many of the retargeted Envs have proven successful in targeting xenografts without aberrant transduction, however, human

studies have thus far been lacking. One construct, rexin-G, which utilizes the matrix targeting described previously, has shown promise and has been moved into late-phase studies in advanced sarcoma, osteosarcoma, breast and pancreatic cancer [79–81]. Should these trials prove successful, this may pave the way for more retargeted Envs to gain approval.

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Executive summary

The Env protein & retroviral entry

- Families of viruses use a similar class of proteins as their receptors.
- Gammaretroviruses utilize multipass transmembrane proteins as receptors.

Pseudotyping

- Pseudotyping is the process in which noncognate Env proteins are assembled into virion particles.
- Pseudotyping is an efficient means of altering the receptor usage of a known virus.
- Domains affecting the ability of an alternative Env to pseudotype frequently lie in the cytoplasmic tail of the transmembrane Env protein.

Targeting with chimeric envelope proteins

- Insertion/replacement of receptor-binding domain with alternative recognition domains, such as using single-chain antibody, can result in altered binding; however, they face the bottleneck of decreased Env fusion.
- Insertion of IgG-binding domains (ZZ domains) has proven successful in a sindbis Env-based pseudotype system.
- Background entry in the sindbis Env system occurs through a Gas6–Axl-dependent pathway.
- Smaller targeting peptides, including the somatostatin peptide hormone, have been successfully engineered into retroviral Env receptor-binding domains.
- Blocking peptides have been engineered to mask the wild-type Env receptor-binding domain. Specificity is provided though the introduction of tissue-specific proteases to release the blocking peptide at the targeted tissue.

Retroviral library screening

- DNA shuffling utilizes PCR to recombine multiple divergent Env proteins followed by selection of isolates with improved or changed properties.
- Using DNA shuffling, isolates of ecotropic murine leukemia viruses capable of infecting Chinese hamster ovary cells were identified.
- Libraries of feline leukemia virus Env have been generated that randomize 11 amino acids of the receptor-binding domain.
- Screening of the feline leukemia virus Env library identifies isolates capable of binding and fusion, scoring for productive infection.
- One novel Env isolate (A5) has been identified that utilizes a protein not previously identified as a viral receptor (SLC35F2).
- The cognate receptor for second Env isolate (CP) was identified as the SLC52A2 and A1 proteins, the receptors for porcine endogenous retrovirus A.

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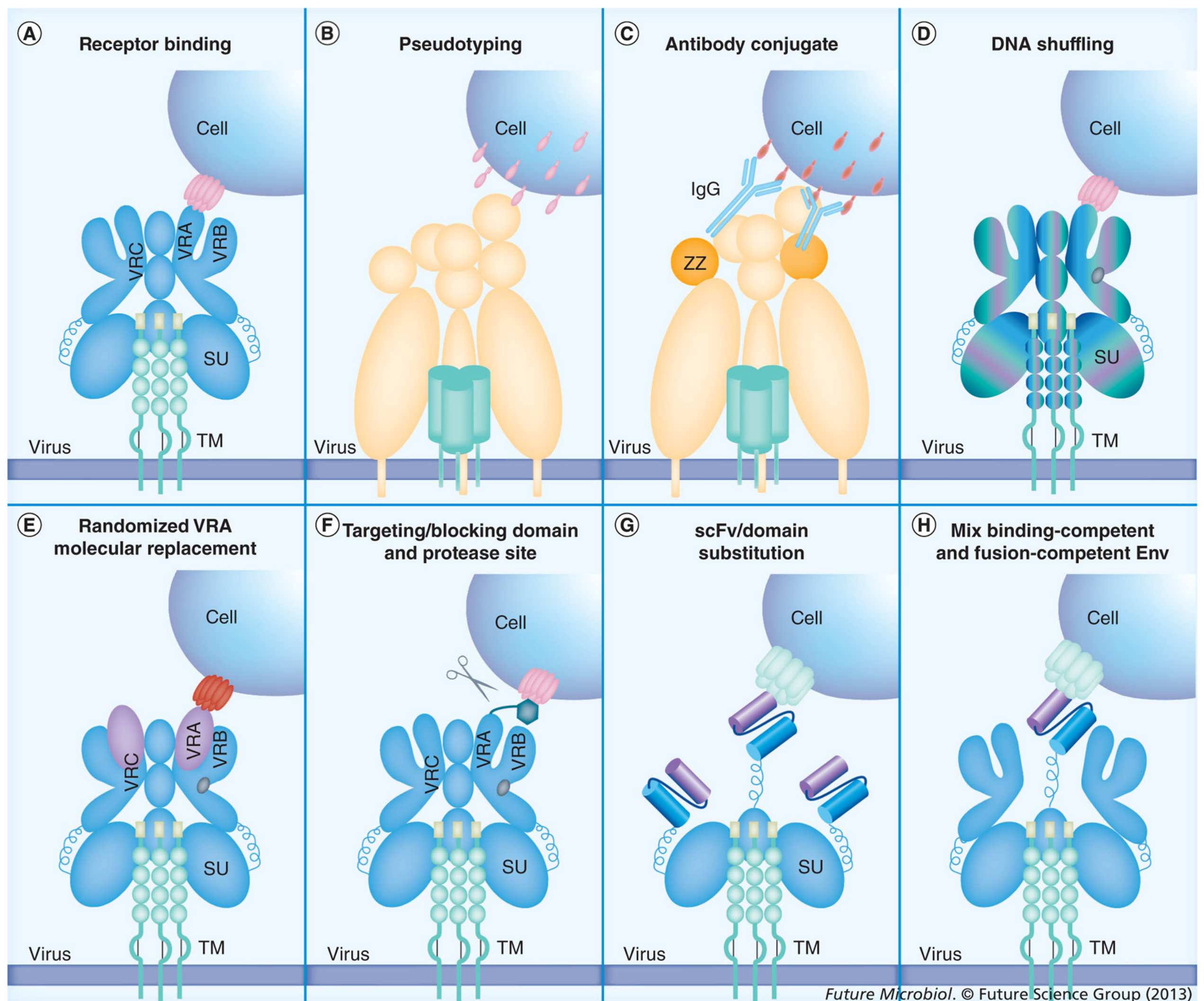

Figure 1. Approaches to alter retroviral receptor usage

(A) Entry of a gammaretrovirus. The envelope (Env) protein consists of a trimer of the SU and TM proteins. The VRA and VRB function in receptor binding. The host cell receptor is depicted as a multiple transmembrane protein (Table 1). (B) Viral pseudotyping. The Env protein from an alternative virus (schematically depicted as sindbis virus) can associate with retroviral particles. The pseudotyped Env will bind to its cognate receptor, which is not limited to multiple transmembrane proteins [36–40]. **(C)** Antibody conjugation systems. Antibodies can be nonspecifically bound to modified Env proteins to direct viral entry. For sindbis virus, the mutation of the receptor-binding domains and insertion of the protein A ZZ domain allows for association with IgG molecules. Antibody binding to antigen delivers viral particles to cells [59–61]. **(D)** DNA shuffling. Combinations of related viral species (schematically shown using a six-color gradient) are mixed during PCR, allowing for the generation of complex chimeras derived from mixed portions of all of the parental sequences. These chimeras are then selected for properties including altered receptor recognition or protein stability [93]. **(E)** Randomized VRA or molecular replacements. For feline leukemia virus, substitutions within the VRA region are known to alter receptor usage. By randomizing 11 amino acids within the VRA, libraries of random Env proteins are generated and have been screened for functional entry into cells. The cognate receptors need

to be identified. This method has identified novel Env/receptor pairs [14,26,27,98–100,103]. Alternatively, through the use of molecular modeling, specific substrates have been engineered into the VRA region of murine leukemia virus, allowing entry through the somatostatin receptor [84]. **(F)** Insertion of additional domains to either target or block the wild-type Env receptor-binding domain. For blocking domains, the cleavage by a host cell protease results in the release of the virus in the vicinity of the targeted cell [85–89]. Additional binding domains function to bind alternative receptors, but can allow the wild-type Env to function as a trigger for membrane fusion. **(G)** Domain substitutions. Binding domains can be used to substitute for a large section of the surface subunit protein. Examples of these types of substitutions include single-chain antibodies [32,51–55]. **(H)** Complementation studies. Viral entry involves more than receptor binding and requires a complex series of conformational changes to allow for membrane fusion. Viruses capable of binding, but not fusion, can be complemented with alternative fusogenic Env proteins [57,81,82].

SU: Surface subunit; TM: Transmembrane subunit; VRA: Variable region A; VRB: Variable region B; VRC: Variable region C; ZZ: IgG binding domain.

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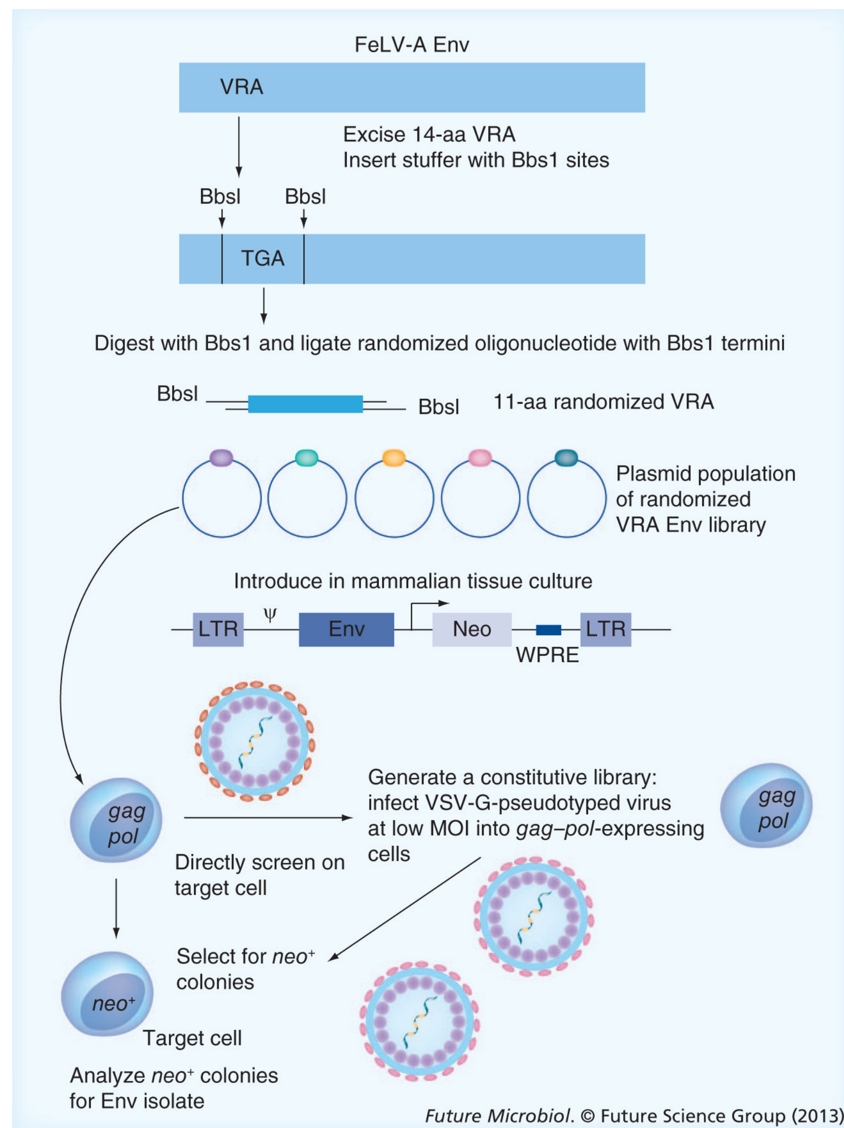

Figure 2. Generation of feline leukemia virus envelope variable region A 11-amino acid randomized library and the selection of isolates in tissue culture for functional entry

Individual steps are shown schematically. The FeLV-A Env is first modified to remove a 14-amino acid region encoding the receptor-binding domain and replaced with a stuffer fragment encoding back-to-back BbsI type 2 restriction sites. This modified vector is used to create the library, randomizing 11 amino acids of the receptor-binding domain through ligation of three oligonucleotides that regenerate the BbsI overhangs. The expression vector is murine leukemia virus based, and expresses both the *env* gene and a selectable marker (*neo*). Viral particles are assembled through introduction into producer cells expressing the *gag* and *pol* genes. Viruses released can be used directly to screen for viral entry. Alternatively, the genes can be transferred using VSV-G-pseudotyped virus at a low MOI to establish a constitutive producing library. Viruses released from the constitutive library can be used for screening gene delivery on the target cells of interest. Productive infection is scored by the transfer of neo^R, after selection in G418. Env isolates are subsequently identified through PCR analysis of the *env* gene and further characterized for tropism and receptor usage.

FeLV-A: Feline leukemia virus-A; LTR: Long terminal repeat; MOI: Multiplicity of infection; TGA: Stop codon; VRA: Variable region A; VSV-G: Vesicular stomatitis virus; WPRE: Woodchuck post-transcriptional regulatory element.

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Table 1

Examples of envelope/retroviral pairs.

Virus	Subtype	Receptor	Structure/function	Ref.
Alpharetroviruses				
ALV	A	Tva	Single pass – low-density lipoprotein receptor related	[110]
ALV	B, D, E	Car1	Single pass – TNF receptor related	[21,111,112]
ALV	C	Tvc	Single pass – butyrophilin related (Ig superfamily)	[113]
Deltaretroviruses				
HTLV	1	Glut-1 †	Multipass – glucose transporter	[25]
Gammaretroviruses				
MLV	Ecotropic	MCAT	Multipass – cationic amino acid transporter	[114–116]
	Amphotropic 10A1	SLC20A1; SLC20A2	Multipass – Na^+-dependent phosphate symporter	[117–120]
	Amphotropic 4070A	SLC20A2	Multipass – Na^+-dependent phosphate symporter	[121]
	Xenotropic	XPR1	Multipass – G-protein-coupled receptor	[122–125]
GALV	B	SLC20A1	Multipass – Na^+-dependent phosphate symporter	[126,127]
PERV	A	SLC52A1; SLC52A2	Multipass – riboflavin transporter	[128–130]
FeLV	A	THTR	Multipass – thiamine transporter	[131]
	B	SLC20A1	Multipass – Na^+-dependent phosphate symporter	[132]
	C	FLVCR	Multipass – heme transporter	[133,134]
RD114		SLC1A5	Multipass – neutral amino acid transporter	[49,135]
SNV		SLC1A5	Multipass – neutral amino acid transporter	[49]
Lentiviruses				
HIV	1	CXCR4; CCR5 †	Multipass – chemokine receptors	[23,24]

† HIV and HTLV utilize multiple coreceptors; however, fusion is mediated through the receptors listed in the table.

ALV: Avian leukosis-sarcoma virus; FeLV: Feline leukemia virus; GALV: Gibbon–ape leukemia virus; HTLV: Human T-lymphotropic virus; MLV: Murine leukemia virus; PERV: Porcine endogenous retrovirus; SNV: Spleen necrosis virus.