Receptor-mediated gene transfer by phage-display vectors: applications in functional genomics and gene therapy

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Recent studies have demonstrated targeted gene-delivery to mammalian cells using modified phage-display vectors. Specificity is determined by the choice of the genetically displayed targeting ligand. Without targeting, phage particles have virtually no tropism for mammalian cells. Thus, novel ligands can be selected from phage libraries by their ability to deliver a reporter gene to targeted cells. Together with advances in cDNA display technologies, these findings offer new opportunities for the use of phage-display technology in functional genomics. In addition, targeted phage particles have potential as alternative gene therapy vectors that can be further improved using directed evolution.

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▼ In 1985, George Smith demonstrated that foreign proteins could be displayed on the surface of filamentous bacteriophage as genetic fusions with the minor coat protein, pIII (Ref. 1). Because the displayed peptide forms a structural association with its encoding DNA, phage-display was immediately recognized as a powerful tool for selecting and evolving ligands from large combinatorial libraries[2,3]. Peptides were most commonly selected from phage-display libraries by virtue of their affinity for a given target. Recently, however, several functional selection strategies have been developed[4] including genetic selection. For example, it has been demonstrated that an appropriately engineered phage can deliver functional genes to mammalian cells through receptor-mediated endocytosis[5–8]. This led to the concept of using genetic selection to identify novel ligands and the prospect of using phage vectors for gene therapy[5,6,9]. These results have been confirmed by independent studies with phage particles targeted with an antibody or viral capsid protein[10,11]. This review will discuss how these new findings are leading to novel applications of phage-display for both gene discovery and evolving phage particles capable of therapeutic gene delivery.

Phage-display technology

Filamentous phage, unlike lytic phage, are assembled as particles of protein and single-stranded circular DNA that are created by extrusion from the bacterial cell wall. The wildtype phage particle is ~6.5 nm in diameter and 900 nm in length and consists of DNA surrounded by a protein sheath of approximately 2700 copies of the major coat-protein, pVIII. By contrast, there are only 3–5 copies of the minor coat-protein (pIII), which caps one end of the particle. Historically, peptide display has relied on creating amino-terminal fusion peptides with either pIII or pVIII. Small peptides (6–8 amino acids) can be displayed on all copies of pVIII in the coat to create phage particles that are 'landscaped' with altered peptide sequences[12]. Larger proteins, however, require a mosaic display with the inclusion of some wildtype pVIII in the phage coat so as not to disrupt assembly[13,14]. More recently, alternative display systems using C-terminal fusions to pVI, pVIII and pIII have been described[15–17], suggesting greater flexibility in genetically modifying phage particles than previously thought.

Not all proteins are efficiently displayed on phage, yet a wide range of peptides and

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proteins can be used, and most retain at least some of their biological activity[18]. In addition, various forms of antibodies, including Fabs and single-chain antibodies (ScFvs), have been displayed. Indeed, when antibody repertoires are prepared from immunized or naive animals, they serve as a rich source of molecular diversity[19]. Once candidate proteins have been selected and identified, mutation libraries from their individually cloned genes are an additional source of diversity. For example, improved ligands have been selected from mutation libraries of displayed cytokines and growth factors[20–24].

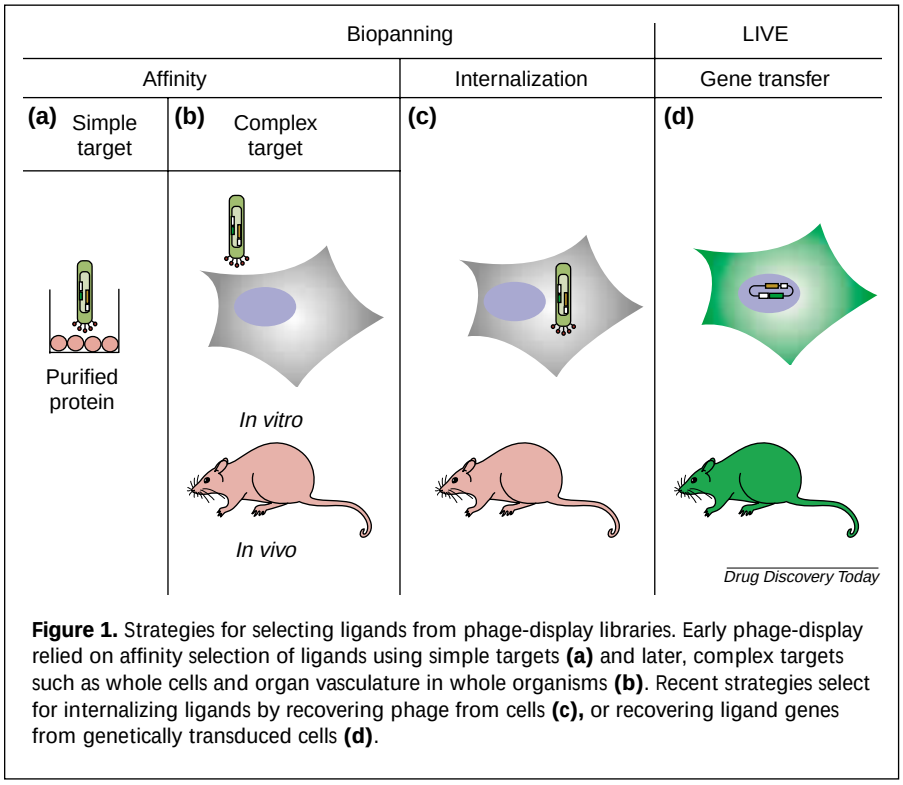

Figure 1. Strategies for selecting ligands from phage-display libraries. Early phage-display relied on affinity selection of ligands using simple targets **(a)** and later, complex targets such as whole cells and organ vasculature in whole organisms **(b)**. Recent strategies select for internalizing ligands by recovering phage from cells **(c),** or recovering ligand genes from genetically transduced cells **(d)**.

Selection of ligands from phage-display libraries

Generally, the selection of ligands from phage-display libraries relies on peptide affinity and avidity. The number of phage recovered is determined by the complexity of the library, the target protein and the selection stringency. Accordingly, four types of selection have been evaluated over the past few years: (1) affinity selection against simple targets, such as immobilized proteins; (2) affinity selection against complex targets, such as the cell surface; (3) selection after phage processing, such as their internalization by cells; and (4) functional selection, such as phage-mediated gene transfer (Fig. 1).

Affinity selection

Peptide selection from phage libraries uses the same principles of selection and biological amplification found in natural evolution. Selection pressure is determined by a peptide's affinity for a target protein attached to a solid support. Unbound phage are washed away with buffers of different stringencies and the remaining attached phage particles are recovered, propagated in bacteria, and then further enriched by repeated rounds of adsorption and recovery. In early rounds of selection, specific-binding phage could be present among millions, if not billions, of other phage particles depending on the complexity of the library. However, because filamentous phage are extremely stable, they can be selected under a variety of harsh conditions including low pH, chaotropic (protein denaturing) agents and protease cleavage. Although the phage recovered might be present in extremely low concentrations, they are readily amplified by infecting host bacteria. As the selection is repeated, the library is significantly reduced in complexity and phage encoding the binding ligands can then be characterized by DNA sequencing.

Selection using complex targets

With the success of ligand selection using phage libraries screened against immobilized proteins, investigators then began to select against whole cells[25–28]. A clear advantage of this kind of 'biopanning' is that little, or no, previous knowledge of the target molecule (i.e. a receptor) is needed and it can be in its native form on the cell surface. However, the fact that the target protein might be present in low concentrations, relative to other cell surface proteins, presents a significant disadvantage to selection and non-specifically adherent phage can give false positive signals. A low concentration of non-specific phage can interfere in the early rounds of selection when the specific phage are in low concentration.

Despite these issues, the selection of peptides on complex targets has been remarkably successful. Recent studies by Pasqualini's laboratory have extended this approach even further by demonstrating that organ homing-peptides can be selected from libraries that are 'biopanned' *in vivo*[29,30]. By applying standard phage-display selection techniques to mice *in vivo*, they identified peptides capable of selectively targeting phage to the vasculature of different organs, including brain, prostate and kidney[29].

Selection of internalizing ligands

In an effort to increase selection stringency and to overcome the problems of non-specific adsorption that are associated with biopanning against whole cells, alternative strategies have been explored. Hart and coworkers initially demonstrated that phage targeted to RGD (an integrin-binding peptide sequence) are internalized through receptor mediated endocytosis[31]. Subsequently, Barry and coworkers showed that cell-specific internalizing peptides can be selected from large, diverse libraries of displayed peptides by washing phage off the cell surface at low pH and recovering internalized phage from cell lysates[32]. Ivanenkov and colleagues, and Poul and coworkers, used a similar approach to identify internalizing ligands from peptide libraries displayed on gene VIII (Ref. 33) and to select tumor-specific internalizing antibodies[34], respectively.

When selecting peptides by phage internalization it is useful to control the number of ligands displayed per phage (valency). Internalization by cells is increased by multivalent ligand display, either on pVIII (Ref. 35) or pIII (Ref. 36). Under controlled conditions, the internalization of multivalent phage particles displaying epidermal growth factor (EGF) is more efficient than monovalent particles produced by an equivalent vector[8]. This increased internalization probably results from increased avidity and/or the stimulation of receptor dimerization or clustering and subsequent internalization. Indeed, peptides displayed as multiple copies on phage, or by artificial dimerization of the peptide, are often found to be more active than the free synthetic peptide[37,38]. It is clear, therefore, that cellular internalization selects for a function (receptor activation) that requires higher order structure and not simply primary sequence.

Genetic selection of ligands that mediate gene transfer

The original rationale behind selection by internalization was simply to increase the stringency of selection and, therefore, increase the ratio of signal (useful ligands) to background (false positives). Therefore, we reasoned that receptor activation and gene transfer should increase selectivity and specificity even further. However, filamentous phages were thought to be too large to pass through an endosomal pathway[31] and if they did, it seemed unlikely that the particles could traffic appropriately through the endosomal environment and uncoat and express their single-stranded DNA in a mammalian cell. Remarkably, however, significant levels of gene transfer are obtained when phage particles are targeted to mammalian cells[5–8]. Figure 2 illustrates how mammalian cell tropism is conferred to a phage-display vector. The phage genome is altered by the addition of: (1) a reporter gene (e.g. green fluorescent protein, GFP) that is regulated by a mammalian promoter (e.g. cytomegalovirus, CMV) and (2) a ligand gene (e.g. EGF) fused to the pIII coat-protein gene. The engineered phage particle has a new tropism for mammalian cells that express the ligand's cognate receptor. Little or no transduction is obtained when cells are transfected with an identical vector that lacks the targeting ligand. Phage-mediated gene delivery is dose- and time-dependent. Specificity for the targeted receptor has been demonstrated by several studies including competition experiments with free ligand[5–7]. The targeting ligand displayed on the phage can also be a peptide, protein or antibody. For example, Poul and coworkers[10] delivered a GFP gene to cells bearing the HER2 receptor (a member of the EGF receptor family) using phage targeted to a single-chain antibody and Di Giovane *et al.*[11] recently obtained similar results using an adenoviral penton-base-targeted bacteriophage. Although the percentage of GFP-expressing cells was low in these early studies (~1–4%), transduction levels of up to 10% are obtained using a multivalent phage-based vector[8] and >45% transduction is achieved after genotoxic treatment[39].

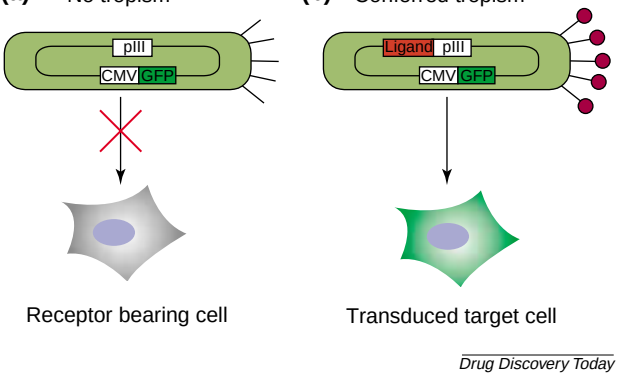

Figure 2. Filamentous bacteriophage particles are genetically modified to transfect mammalian cells. Phage particles are targeted to cell surface receptors by fusing a ligand to the pIII coat protein, and carry a reporter gene, such as green fluorescent protein (GFP), which is capable of expression in mammalian cells **(a)**. Transduction occurs only when the appropriate cell-targeting ligand is expressed on the phage coat, and its receptor is expressed on target cells **(b)**.

The discovery that targeted bacteriophage can deliver genes to cells using receptor-mediated endocytosis (RME) led to the development of an alternative phage-display technique based on functional ligand selection[5]. Ligand identification via expression (LIVE) is a selection strategy designed to select phage that have a functional effect on cells (Fig. 3). The end-point in LIVE selection is phage-mediated gene delivery and genetic transduction of the target cells. Cells that have internalized phage by RME are identified by expression of a reporter gene (e.g. GFP,

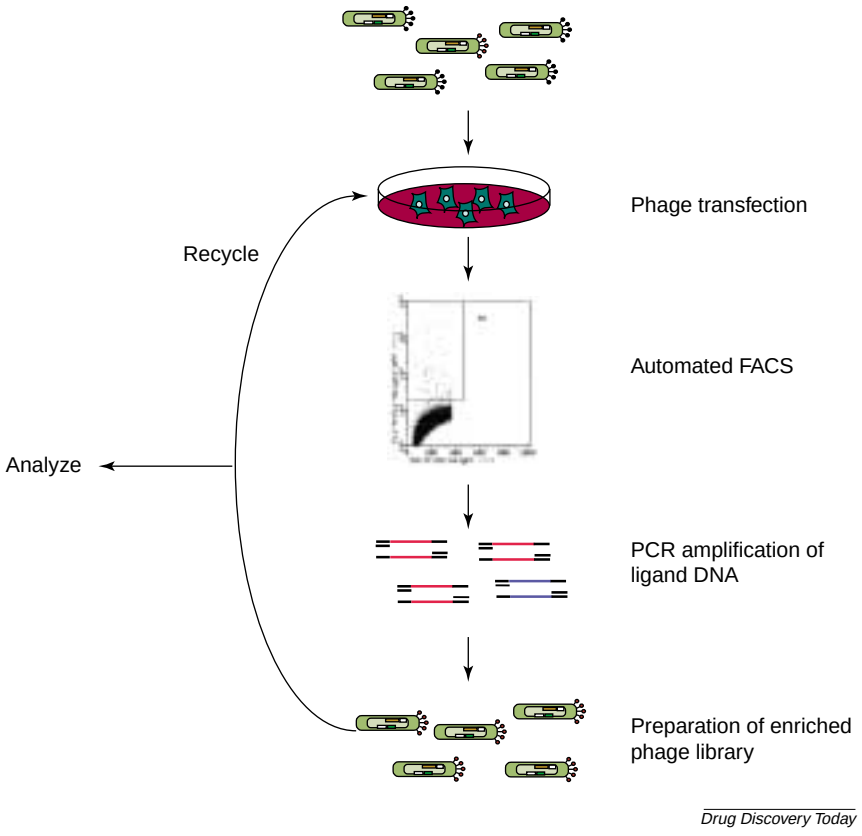

Figure 3. Ligand identification via expression (LIVE) selection strategy. A phage display library that carries a reporter gene, such as green fluorescent protein (GFP), is contacted with target cells. Phage particles that display ligands enter cells and deliver the reporter gene. Automated fluorescence activated cell sorting (FACS) is used to isolate GFP-positive cells from which the internalized phage particles are reconstituted using PCR amplification. The reconstituted phage particles are then used as input phage for the next cycle of enrichment. When the complexity of the library is sufficiently reduced, the ligands are characterized by DNA sequencing.

β-galactosidase or luciferase) or drug resistance gene [e.g. neomycin phosphotransferase (*neo*)]. Autofluorescent GFP-expressing cells are then isolated by fluorescence-activated cell sorting (FACS) or, alternatively, drug resistant cells expressing the *neo* gene are isolated by growth in the presence of the antibiotic G418. PCR amplification is performed on DNA from the selected cells to recover the sequences of phage DNA responsible for phage cell-targeting (and subsequent transduction). These sequences are then inserted into the phage vector, new phage are prepared and the cycle of transfection and selection is repeated. Each round of transfection, transduction and phage isolation enriches for phage that display ligands capable of targeting transducing phage. Using EGF-targeted phage to test this system, it has been shown that the targeted phage can be enriched over one-millionfold after 3–4 rounds of LIVE selection against EGF-receptor bearing cells[5].

In principle, the genetic selection of functional ligands by LIVE represents a significant departure from traditional biopanning (Table 1) because it increases the stringency of selection by requiring the displayed ligand to bind, internalize and deliver a selectable genetic marker. This increased stringency decreases background binding from phage-displaying simple binding-proteins. In addition, biopanning relies on the recovery of infective phage, whereas LIVE selection recovers phage DNA sequences (i.e. by PCR) instead. Therefore, LIVE would be expected to recover phage that are subjected to proteolytic cleavage after internalization, which would otherwise be lost during biopanning. Moreover, because selection by LIVE is genetic, a stable inherited change in the cell (i.e. drug resistance) can be used as the basis for selection. Thus, for example, it is feasible that stable drug-resistant cell colonies could be used to directly identify rare phage-internalization events in one round of screening. Although the full potential of LIVE and other functional selection strategies remains to be determined, initial results suggest that it will be useful for identifying novel gene-targeting peptides and antibodies. Identification of cDNAs that encode ligands of therapeutic value, however, could be the most significant application of LIVE selection for drug discovery.

Applying phage selection strategies to functional genomics

The human genome is widely recognized as a rich source of biopharmaceuticals. Now that the first drafts of the human genome sequence are completed, it will be a formidable task to elucidate the function of the estimated 30,000 genes. The challenge for drug discovery is to identify the sequences encoding therapeutic proteins or therapeutic drug targets. LIVE phage-display technology has the potential to be a powerful tool for identifying natural ligands because it combines phage combinatorial biology with functional selection of altered cell function through gene transfer. In this case, it is the cell surface itself that selects the 'most fit' ligand by its ability to stimulate RME

Table 1. A comparison of phage selection on cells using biopanning versus ligand identification via expression (LIVE)

Biopanning	LIVE
Selects by affinity or internalization	Selects by internalization and gene transfer
Requires recovery of infective phage	Phage sequences recovered by PCR
High background from adherent phage	Low background from adherent phage
Selection transitory	Can select cells having stable genetic change (e.g. drug resistant)

and subsequent phage transduction. If LIVE and other selection methods are successful, the identification of novel ligands and their receptors is likely to lead to new drugs and drug targets, because cell-surface interacting ligands affect crucial cellular processes, such as cell growth and differentiation. After all, natural ligands having direct clinical use are the leading therapeutic products in biotechnology (e.g. erythropoietin, growth hormone, interleukin-2 and granulocyte–macrophage colony-stimulating factor). However, they are the most difficult to mine from published genomic databases because they often exist as small fragments contained in much larger genes, which are processed in a cell-specific fashion.

A significant advantage of the LIVE strategy is that it selects for those ligands that not only bind and internalize but also traffic appropriately for gene delivery. Modifications of LIVE could be used to 'cast a wider net' and select for ligands that bind and internalize but that are not necessarily trafficked on their own for gene delivery. For example, a less stringent LIVE genetic selection would include trafficking signals on the phage particle for endosomal escape and nuclear localization. In this case, the goal would be to identify a wider variety of ligands that are internalized by the transduced target cells.

Phage-display of cDNA libraries

The potential of phage-display as a tool to directly dissect cDNA libraries has been limited for several reasons. First, the efficiency with which the host bacteria secrete the cDNA–pIII fusion protein is highly dependent on the cDNA sequence, resulting in a disproportionate representation of certain sequences in the library. Second, cDNAs must be in the same reading frame as both the pIII signal-peptide-encoding sequences and the pIII structural gene for the natural peptide to be displayed. Also, the gene fusion must not contain any in-frame stop codons that would prematurely terminate the fusion protein. Finally, ligands often require processing from larger precursors to be active and, therefore, fusion to full-length cDNAs might result in no activity. For example, the pro-opiomelanocortin (POMC) gene encodes adrenocorticotropic hormone (ACTH), α-melanocyte-stimulating hormone (MSH), γ-MSH, β-endorphin and β-lipotropic hormone (LPH) but these products display none of their hormonal activities without post-translational cleavage of the POMC precursor into smaller, active peptides.

Early approaches to displaying cDNAs used C-terminal fusions to eliminate the problem of premature termination by stop codons. For example, Crameri and coworkers[40] described a system where cDNA is displayed on pIII indirectly through the association of a C-terminal cDNA–Fos fusion protein with a Jun–pIII fusion protein during phage assembly. This system was used to identify allergenic proteins by probing *Aspergillus fumigatus* phage cDNA libraries with patient sera[41]. Another alternative cDNA display approach is fusion of the cDNA library to the C-terminus of pVI protein. Jespers and colleagues[15] used this approach recently to identify tumor-associated antigens[42]. Although both methods use C-terminal fusion to allow the display of cDNAs with stop codons, inefficient display of certain cDNAs, particularly those with longer sequences, and the inactivity of some full-length genes, limit the application of these approaches.

Displaying protein domain libraries on phage

A promising alternative approach to the problem of ligand discovery is to display protein domains instead of full-length cDNAs by fragmenting the cDNAs to an average size that would encode proteins ranging from 50 to 900 amino acids in length (~150–2700 bp). It is estimated that 80% of all active protein domains fall within this size class. In addition to revealing active domains, fragmentation could allow more sequences to be displayed because the smaller, active peptide domains would be separated from sequences that inhibit display. Fragmentation is achieved either by using random primers and PCR amplification during cDNA synthesis[43], or by DNase-digestion of full-length cDNA[44]. Santini and coworkers used the former approach to select specific cDNAs from a fragmented hepatitis C viral cDNA library[45] and, recently, more complex human brain and mouse embryo cDNA libraries[46]. Cochrane and colleagues demonstrated the feasibility of the latter approach by selecting platelet-endothelial cell adhesion molecule-1, CD31 (PECAM-1), a natural ligand for SHP-2, from a fragmented human leukocyte library[44]. Recently, a modification of the random primer method described by Santi and coworkers[46] was used to construct a fragmented cDNA

display library from human placental mRNA, from which novel cDNAs that target phage to prostate carcinoma cells for gene delivery will be selected using LIVE technology. Taken together, these studies suggest that LIVE selection will be useful for identifying cell-targeting ligands from cDNAs derived from various cell types. The use of cDNA display libraries for gene discovery is likely to become more widespread as these, and other, techniques are developed to effectively display representative cDNA repertoires from various cell-types.

Phage as vectors for gene therapy

Effective gene therapy depends on the development of vectors that can specifically deliver a therapeutic gene to target cells in the body with minimal toxicity from non-specific uptake of the vector[47,48]. The prevalent vectors in use today are modified animal viruses (i.e. adenovirus, retrovirus and lentivirus) and non-viral DNA conjugates[49]. There are significant advantages to each approach but also serious drawbacks (Table 2). Certainly, recombinant animal viruses are the most efficient vehicles for gene delivery, having evolved optimal mechanisms for cell entry, trafficking and foreign gene expression. However, there are problems associated with animal viral vectors, such as intrinsic toxicity from viral protein expression, non-specific uptake and immunogenicity. From a practical viewpoint, the commercial production of recombinant viruses in animal cells is costly, being complicated by the reversion to replication-competent virus and difficulties with quality control. By contrast, synthetic DNA conjugates are much simpler than viral vectors[50–52]. However, the efficiency of targeted gene-transfer is much lower than viral vectors and production of homogeneous preparations can be problematic. For these reasons, we explored whether the combinatorial biology of phage-display could be combined with biopanning and genetic selection (LIVE), to direct the evolution of phage particles into a vector for therapeutic gene delivery.

Phage-mediated gene delivery

Although still in the early research stage, bacteriophage vectors offer an attractive alternative to both viral and non-viral vectors because they can potentially overcome the drawbacks of either approach (Table 2). For example, a major advantage of bacteriophage over animal viral vectors is their lack of intrinsic infectivity in animal cells. This allows phage to be genetically targeted without the need to eliminate native tropism, a concern for current animal viral-vector targeting. Even if non-specific internalization of phage were to occur, the production of phage proteins or replicative phage is unlikely in the foreign milieu of an animal cell. In addition, phage vector production is expected to be simpler and more cost effective than existing vectors because phage can be produced to high titer in the supernatant of bacterial cultures and easily purified at a large scale.

Table 2. A comparison of features of phage vectors with animal and synthetic vectors

	Animal virus	Synthetic vector	Phage
Potential toxicity	High	Low	Low
Generates replication competent virus (RCV)	Yes	No	No
Viral proteins expressed	Yes	No	No
Complexity	High	Low	Low
Cost	High	Low	Low
Gene transfer efficiency	High	Low	Low
Reproducibility	High	Low	High
Genetic targeting	Limited	Limited	Yes
Directed evolution	No	No	Yes

The efficiency of phage-mediated gene delivery is low compared with typical viral vectors, however, it now surpasses the levels described for targeted DNA conjugates. Although, under normal conditions, targeted phagemid-mediated cell transduction levels are similar to antibody-[53] or ligand-targeted[54] DNA-complex gene delivery (1–10%), the amount of DNA used is 2–3 orders of magnitude lower with phagemid gene delivery (~30 ng), than with DNA complexes (1–10 μg) Moreover, phagemid particle gene-transfer efficiencies of up to 45% have recently been achieved in cells undergoing genotoxic stress[39]. These are the same conditions (e.g. heat-shock, UV irradiation, topoisomerase 1 inhibition) that enhance gene transfer by another single-stranded DNA vector, adeno-associated virus, and are thought to enhance the conversion of single-stranded to double-stranded DNA. Accordingly, it might be possible to engineer targeted phage vectors with similar gene transfer efficiencies to viral vectors. Moreover, because phage-display can be directly applied to vector engineering, gene delivery might be improved using molecular evolution in addition to rational design.

To test this possibility, we evaluated the steps required for transduction. Efficient targeted gene delivery, by any gene therapy vehicle, requires passing of the vector through many of the same processes that occur in early viral infection. After cell-surface binding and internalization, the vector must be released from the endosomal compartment and DNA trafficked to the nucleus. At some point during this process the vector DNA must be separated from coat proteins and replicated. Recent studies have shown that

the binding and internalization of filamentous phage can be optimized by increasing the valency of the targeting ligand, whether it be a peptide[8] or an antibody[36]. However, the processes underlying phage gene-transfer after internalization are less well understood. Internalization of targeted phage particles appears to be much more efficient than gene transfer: nearly 100% of cells are transfected by phage provided they have sufficient receptors[8], yet, under ordinary conditions, <10% are transduced. It is not surprising that endosomal escape, and subsequent trafficking, is less efficient in phage because they lack the specialized protein sequences that animal viruses have evolved for intracellular trafficking in infected cells. However, it might be possible to adapt phage vectors for more efficient gene transfer, for example, by genetically grafting viral endosome escape sequences, trafficking and replication sequences into the phage genome.

Evolving phage vectors for gene delivery

Although it is possible to modify vectors, both chemically and genetically, for more efficient gene transfer, the choice of each enhancing element must be determined by trial and error. However, with phage it becomes possible to apply the power of phage-display and genetic selection to the evolution of more efficient vectors (Fig. 4) and thus bypass the more tedious and time-consuming process of rational design. Indeed, along these lines, we have demonstrated that it is possible to selectively enrich for specific phage by their ability to introduce a reporter gene into the target cells[5]. Thus, novel sequences, unanticipated by rational design, might then be selected from libraries of highly diverse peptides or cDNAs using a functional selection, such as LIVE.

This directed evolution could also be used to create phage that are more suitable for *in vivo* gene delivery having, for example, increased serum half-life, selective tissue-targeting and decreased immunogenicity. A recent example of this is the selection of long-lived phage by repeated rounds of injection of phage libraries into animals and selection of surviving phage using either lambda[55] or T7[56] phage. Sokoloff and coworkers[56] have identified sequences that increase the half-life of T7 phage by protecting against complement activation. Perhaps even more importantly, these peptides could be used as 'stealthing' agents to protect other gene- or drug-delivery vectors from clearance. The work of Pasqualini and Ruoslahti[29,30] also demonstrates the ability to use directed evolution to enable phage to home to specific tissues or tumors *in vivo*. Recently, Samoylova and colleagues[57] applied *in vivo* panning to identify phage able to target muscle, indicating that phage can be developed that penetrate the vasculature to target tissues *in vivo*. In addition, targeted bacteriophage vectors have now been used to deliver a foreign gene to tumour cells *in vivo*[58]. Taken together, these studies point to the potential of evolving phage vectors for the delivery of therapeutic genes *in vivo*.

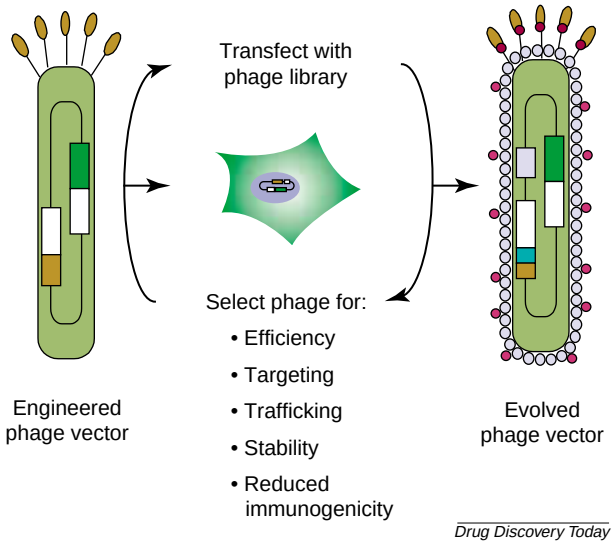

Figure 4. Evolving phage display vectors for therapeutic gene transfer. The principles of directed evolution used in phage display could be applied to phage vectors to tailor them for gene delivery to specific target cells *in vivo*. Reiterative transfection and genetic selection is used to engineer phage with favorable stability, targeting and immunogenicity, and so on, for *in vivo* gene transfer.

Conclusions

Phage-display is a powerful and widely used drug discovery technology that uses the darwinian principles of natural evolution to identify novel interacting peptides, proteins and antibodies. The discovery that targeted display phage can deliver genes to cells through RME has created new opportunities for expanding the use of phage-display in gene discovery and now gene therapy. Accordingly, new selection methods, such as LIVE and related techniques, are being employed to functionally select novel ligands. Although further investigation will determine the overall applicability of genetic selection, the fact that selection requires active internalization and gene delivery gives it the potential to identify new ligands that might otherwise be missed by affinity selection alone. It is reasonable to predict that recent advances in cDNA display, which allow for the display of functional domains in a cDNA repertoire, will be useful for identifying new ligands both by affinity and genetic selection. To this end, current phage-display technology and future improvements are likely to play an increasingly important role in the post-genomic era; whether

it be in mining the human genome for novel ligands or engineering phage for gene delivery. It is now feasible to apply phage-display technology to the directed evolution of phage particles, such that unique phage can be genetically tailored for different gene therapy applications.

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