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Review Article

Phage display technology - Applications and innovations

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Abstract

The expression of exogenous peptides on the surface of filamentous bacteriophage was initially described by Smith in 1985. Since his first study, different molecules such as small peptides and antibodies have been displayed on coat proteins of phage, greatly expanding the applications of the technology. The past decade has seen considerable progress in the techniques and applications of phage libraries. In addition, different screening methods have allowed isolation and characterization of peptides binding to several molecules in vitro, in the context of living cells, in animals and in humans. Here we review the applications, recent innovations, and future directions of phage display technology.

Key words: phage display, applications.

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Introduction

Phage display technology was first introduced in 1985 by George Smith . It was used as an expression vector, capable of presenting a foreign amino acid sequence accessible to binding an antibody. Since then, a large number of phage displayed peptide and protein libraries have been constructed (Bass *et al.* 1990, McCafferty *et al.* 1990, Barbas *et al.* 1991, Smith 1991, Smith and Scott 1993, Hoogenboom 2002, Szardenings 2003), leading to various techniques for screening such libraries. This technology has had a major influence on the work and discoveries done in the fields of immunology, cell biology, pharmacology and drug discovery.

Phage display allows the presentation of large peptide and protein libraries on the surface of filamentous phage, which leads to the selection of peptides and proteins, including antibodies, with high affinity and specificity to almost any target. The technology involves the introduction of exogenous peptide sequences into a location in the genome of the phage capsid proteins. The encoded peptides are expressed or "displayed" on the phage surface as a fusion product with one of the phage coat proteins. This way, instead of having to genetically engineer different proteins or peptides one at a time and then express, purify, and analyze each variant, phage display libraries containing up to 10^{10} variants can be constructed simultaneously. Phage particles withstand very harsh conditions, such as low pH and low temperatures, without losing bacterial infectivity. Thus, protocols using low pH and high concentration urea have been used to dissociate bound phage from a target. In addition, bound phage does not need to be eluted from a microtiter well or animal tissue before bacterial infection. Instead, infection can proceed after addition of bacteria directly into the well or to the homogenized organ or tissue.

The strength of phage technology is its ability to identify interactive regions of proteins and other molecules without preexisting notions about the nature of the interaction. The past decade has seen considerable progress in the applications of phage display technology. Different screening methods have allowed isolation and characterization of peptides binding to several molecules in vitro, in the context of living cells, in animals and in humans (Arap 2002b). Here we review the applications, as well as recent innovations and future directions of phage display technology.

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Bacteriophage - Structure and biology

The bacteriophage (or simply phage) mostly used in phage display technology, are single-stranded DNA viruses that infect a number of gram-negative bacteria. The filamentous phage particles mostly used for display purposes are known as Ff and include strains M13, f1, Fd and ft. Fd phage particles consist of a long cylindrical protein capsid, 930 nm in length and 6.5 nm in diameter, enclosing a single-stranded DNA genome of about 6400 nucleotides, consisting of 11 genes. The viral mass is approximately

16.3 MDa, and consists mainly of about 2700 copies of the pVIII, a 50 aa residue protein encoded by *gene VIII*. On one side of the phage particle there are 3 to 5 copies of the proteins pVII and pXIX (genes VII and XIX) and on the other side there are 3 to 5 copies of the proteins pIII and pVI (Figure 1) (Webster 2001). In most display applications, pIII, a 406 aa adsorption protein, is the protein used for peptide expression. The pIII protein appears to have two functional domains: an exposed N-terminal domain that binds the F pilus, but is not required for phage particle assembly, and a C-terminal domain that is buried in the particle and is an integral part of the capsid structure. The C-terminal portion of pVIII is inside the phage particle, close to the DNA, while the N-terminal part is exposed to the surroundings.

Fd particles are able to infect a variety of Gram-negative bacteria, including *E. coli,* using pili (F pilus in *E. coli*) as receptors. Filamentous phage infection does not produce lytic infection in *E coli.*, but rather induces a state in which the infected bacteria produce and secrete phage particles into the growing medium. Infection begins by the attachment of phage pIII to the F pilus of a male *E. coli*. The circular single-stranded DNA enters the bacteria where it is converted by the host DNA replication machinery into double-stranded plasmid replicative form. By rolling circle replication, the replicative form makes single-stranded DNA and the templates for expression of proteins pIII and pVIII are formed. Phage descendants are assembled by packaging of the single-stranded DNA into protein coats and extruded through the bacterial membrane (Russel 1991).

Most of the currently used phage display vectors use the N-terminus of pIII protein or pVIII protein to display the foreign peptide or protein (Smith and Scott 1993). The pIII libraries display 3-5 copies of each individual peptide (Scott and Smith 1990), whereas pVIII libraries can display up to 2700 copies of small (up to six amino acids) peptides (Greenwood *et al.* 1991). The pIII and pVIII proteins can display peptides of various lengths and cysteine residues can be introduced to the fusion peptide to create conformational constraints by the formation of "loops" between disulfide bridged cysteine residues. Furthermore, the exogenous peptides are well exposed, facilitating the insert-target interactions. Large peptide inserts of up to 38 amino acids can be introduced into the amino terminus of pIII protein without the loss of phage infectivity or particle assembly.

Working with phage display technology

Detailed description of the materials, methods and space needed for those who want to start phage work may be found in specialized textbooks (Barbas *et al.* 2000, Pasqualini and Arap 2002). The basic protocols used with phage studies can also be obtained in these books, whereas its variations are found in published studies. Initial investment is relatively small, as most of the fundamental materials are common laboratory devices, such as Petri dishes, Falcon tubes and centrifuges. However, one cannot forget the most important tool for phage work: k91kan E. coli and peptide or antibody phage libraries. The construction of a peptide phage library involves a detailed protocol and, for those who are not experienced with phage work, one way to obtain reliable phage libraries and start a "bio-panning" is to establish a collaboration with a more experienced laboratory. This is a very important step, as the panning results depend directly on the quality of the library. After the "bio-panning" is started, the most expensive step is to sequence the clones obtained after a few rounds of selection, as hundreds of clones are usually sequenced in each panning.

Figure 1 - Phage structure. PIII, pVI, pVII, pVIII and pXIX represent phage proteins. Exogenous peptides are expressed or "displayed" usually on pIII or pVIII.

Peptide libraries offer the possibility of characterizing peptide binding specificity of important targets, such as proteins, cells and vascular endothelium. In general, the affinity selection of ligands from phage display random peptide libraries involves 5 fundamental steps:

i) preparation of a primary library or amplification of an existing library,

ii) exposure of the phage particles to a target (immobilized protein/cell surface protein/vascular endothelium) for which specific ligands are planned to be identified,

iii) removal of non-specific binders (washing/perfusion),

iv) recovery of the target bound phage by elution or direct bacterial infection and amplification of the recovered phage,

v) back to step i two to four times.

This "bio-panning" can be repeated several times until a population of best binders is enriched. By sequencing the phage genome encoding the displayed peptide, one can determine and reproduce its sequence as recombinant or synthetic peptides, and finally determine specific and selective ligands to target receptors (Koivunen *et al.* 1999).

It is a common observation that the binding motif of a targeting peptide is a tripeptide motif appearing several times in different sequence contexts. Three amino-acid residues seem to provide the minimal framework for structural formation and protein-protein interaction (Vendruscolo *et al.* 2001). This is therefore the minimal searched framework, although it is obvious that repeated peptides consisting of more residues may mean a stronger interaction.

Applications

Identification of receptor-ligands *in vitro*

Peptide libraries have been used to determine the epitope to which an antibody binds. Antibodies recognize peptide motifs based on only three or four conserved residues. Therefore, it is possible to define the region of a protein recognized by an antibody based on the motif revealed by phage display (Scott and Smith 1990). Folgori and colleagues selected antigenic mimics (mimotopes) of two different epitopes from the human hepatitis B virus envelope protein (HBsAg) and showed that a humoral response to these mimotopes was widespread in the immunized population, suggesting that the strategy identifies displayed peptides with a potential role as diagnostic reagents (Folgori *et al.* 1994).

Phage libraries were used to define peptide structures recognized by major histocompatibility (MHC) molecules. Peptides with binding specificity for lymphoblastoid-derived DR1 molecules, determined by phage display libraries (Hammer *et al.* 1992), may have important applications as MHC-specific antagonists. Other examples of binding of ligand motifs to their receptors discovered by phage display technology include a family of cell surface integrins that recognize the tripeptide RGD (Koivunen *et al.* 2001, Ruoslahti 1996). These integrins mediate cell attachment to many types of extracellular matrix proteins that contain the RGD motif, such as fibronectin, vitronectin and fibrinogen (Ruoslahti 1991, Ruoslahti 1996). Phage display peptide libraries have also been used to find ligands to the SH2, a common domain in protein kinases and signaling proteins. The SH2 domain recognizes peptides containing a phosphorylated tyrosine residue (Dente *et al.* 1997, Gram *et al.* 1997).

Using phage display in combination with other methods, potent mimetics of proteins and minimal active peptides have been found. A dimmer of the 14-amino acid cyclic thrombopoietin mimetic, for example, was found to be as active as the parental polypeptide (Cwirla *et al.* 1997). More recently, Cardo-Vila *et al.* introduced an approach based on phage display technology to identify molecules that specifically interact with the cytoplasmic domain of the beta 5 integrin subunit. Initially, they isolated beta 5 specific peptides by screening a phage library on a recombinant fusion protein containing the beta 5 cytoplasmic domain. Next, they designed and synthesized an internalizing version of the peptide by using the penetratin system for intracellular delivery, and showed that it triggered cell apoptosis in a caspase-dependent manner, suggesting a functional link between the alpha v beta 5 integrin, annexin V, and programmed cell death (Cardo-Vila *et al.* 2003).

Besides proteins, peptides affecting biologically significant protein-DNA interactions (Cheng *et al.* 1996), peptides binding to carbohydrates (Matsubara *et al.* 1999, Noda *et al.* 2001, Peletskaya *et al.* 1996), to carbon nanotubes (Wang *et al.* 2003) and to small chemical compounds such as taxol (Rodi *et al.* 1999) have been isolated from phage display random peptide libraries.

Phage display has also been applied as a tool for directed evolution for more than a decade. In enzymology, it has been applied for mechanistic-based studies and to generate enzyme variants with new or improved properties. Enzymes with a broader range of substrates, for instance, would have improved function and allow certain advantages to its host. A strategy described by Petersen and colleagues makes possible the *in vitro* isolation of enzymes for almost any reaction. Moreover, this strategy theoretically allows one to functionally clone natural enzymes based on their ability to catalyze specific reactions rather than their structure or binding ability (Pedersen *et al.* 1998). Using a combination of phage display selection and high-throughput screening methods, Wirsching and colleagues generated variants of hirudin, a thrombin-specific inhibitor, with increased protease resistance that may prove useful for hematologic disorders (Wirsching *et al.* 2003). Phage display technology has also allowed the development of DNA-binding proteins with novel specificities, energetics of protein folding and directed evolution of antibodies (O'Neil and Hoess 1995).

Selection of ligand-receptors in complex biological systems

Direct selection against molecules expressed on living cells and mouse and human tissues has been carried out

during recent years. When using such complex targets, enhancement of specific binding above the background phage adherence is usually necessary, as unspecific binding to common molecules such as albumin is expected and may interfere with the panning results.

Selection on living cells can be done on either monolayers of adherent cells or on cells in suspension. Unbound phage must be washed away and phage recovery is done by bacterial infection. Goodson and colleagues isolated ligands to the urokinase receptor after transfecting cells with the gene for the same receptor (Goodson *et al.* 1994). Human platelets have been used as targets for the selection of a peptide antagonist of the thrombin receptor (Doorbar and Winter 1994). Another group isolated two antibody clones that recognize melanoma cells by subjecting an antibody phage library to human melanoma cells (Kupsch *et al.* 1999). More recently, Ardelt and colleagues screened 2 phage display random peptide libraries on human urothelium and peptides. Those selected in this bio-panning were tested for their binding abilities to normal and malignant urothelial cells. Two classes of peptide motifs shared the same amino acid sequence bound to normal urothelium and to 2 transitional carcinoma cells, and were therefore suitable for translation into targeted intravesical therapy (Ardelt *et al.* 2003).

In a very elegant study, Giordano and colleagues described a new approach for the screening, selection and sorting of cell-surface binding peptides from phage libraries. The method, termed bio-panning and rapid analysis of selective interactive ligands (BRASIL), allows separation of complexes formed by the cells and bound phage from the remaining unbound phage still in the suspension. This technique is based on a differential centrifugation of the aqueous phage/cell suspension through a non-miscible organic lower phase. Centrifugation will drive the cells from a hydrophilic phase into a non-miscible (hydrophobic) organic phase. The passage of cells from a hydrophilic to a hydrophobic setting will separate water-soluble materials, such as the unbound phage. The cell/phage pellet is then recovered from the bottom of the tube after immediate freezing in liquid nitrogen. Next, the cell pellet is thawed and bound phage are recovered by bacterial infection. As the method involves one centrifugation and does not require repeated washes, it allows a simpler and more convenient phage recovery from cell membranes than other cell-panning techniques. As a proof of the principle, they screened human endothelial cells stimulated with vascular endothelial growth factor (VEGF), constructed a peptide-based ligand-receptor map of the VEGF family and validated a chimeric ligand-mimic that binds specifically to VEGF receptor-1 and neuropilin-1 (Giordano *et al.* 2001).

One of the most important tumor targets identified using bio-panning on cultured cells is the Glucose-regulated protein-78 (GRP78) (Mintz *et al.* 2003), a chaperone protein initially found to be expressed in the endoplasmic reticulum of various cell types (Munro and Pelham 1986, Lee 1992 and Morimoto 1993). GRP78 induction is markedly increased in a variety of cellular stress conditions, such as glucose starvation, oxygen deprivation and accumulation of unglycosylated proteins (Lee 1987, Li *et al.* 1993). This induction is a cellular protective response against stress (Li *et al.* 1992, Sugawara *et al.* 1993, Jamora *et al.* 1996) and prevents apoptosis (Myiake *et al.* 2000).

The relatively hypoxic environment found in some solid tumors is one of the possible mechanisms involved in the over-expression of GRP78 in human cancers. GRP78 over-expression triggers an immune response against the protein that has proven to be related to androgen-independent prostate cancer and reduced overall patient survival (Mintz *et al.* 2003). Our group is now conducting in vivo studies aiming to develop targeted therapies for human cancers via GRP78. Initial results showed that the peptides used for targeted delivery are capable of killing cells in vitro, based on the GRP78 surface expression. In addition, in vivo assays with the same peptides showed that the protein was successfully used as a molecular target for directed therapy against breast and prostate cancers.

In vivo selection

In this technique phage libraries are injected intravenously into animals and then organs or tissues are collected and examined for phage bound to tissue-specific endothelial cell markers (Figure 2). Pasqualini was the first to describe this method and to isolate peptides that home to renal and cerebral vascular endothelium in vivo (Pasqualini and Ruoslahti 1996). Since Pasqualini's study, identification of receptor-ligand pairs has been described for several organs such as lung, kidney, pancreas, adrenal gland, muscles, intestines and uterus. Selection in vivo has several advantages: first, peptides displayed on the phage particles are identified and tested functionally and must overcome natural mechanisms of degradation. Second, peptides recognizing unspecific molecules are depleted from circulation. Finally, in vivo selection proved to be able to identify receptors expressed selectively on tumor endothelium (Arap *et al.* 2002). These receptors may serve as molecular targets for the development of diagnostic techniques and targeted therapies.

In vivo use of phage display has yielded the identification of many receptor-ligand pairs in malignant (Koivunen *et al.* 1999, Mintz *et al.* 2003) and benign diseases (Kolonin *et al.* 2002, Kolonin *et al.* 2004). An in vivo selection of phage display peptide libraries revealed a prostate homing peptide that specifically binds to the prostate vasculature and parenchyma. After coupling this peptide to a pro-apoptotic peptide and injecting the chimera intravenously in mice, Arap and colleagues obtained not only selective tissue destruction, but also delayed development of tumors in prostate cancer-prone transgenic mice (Arap *et*

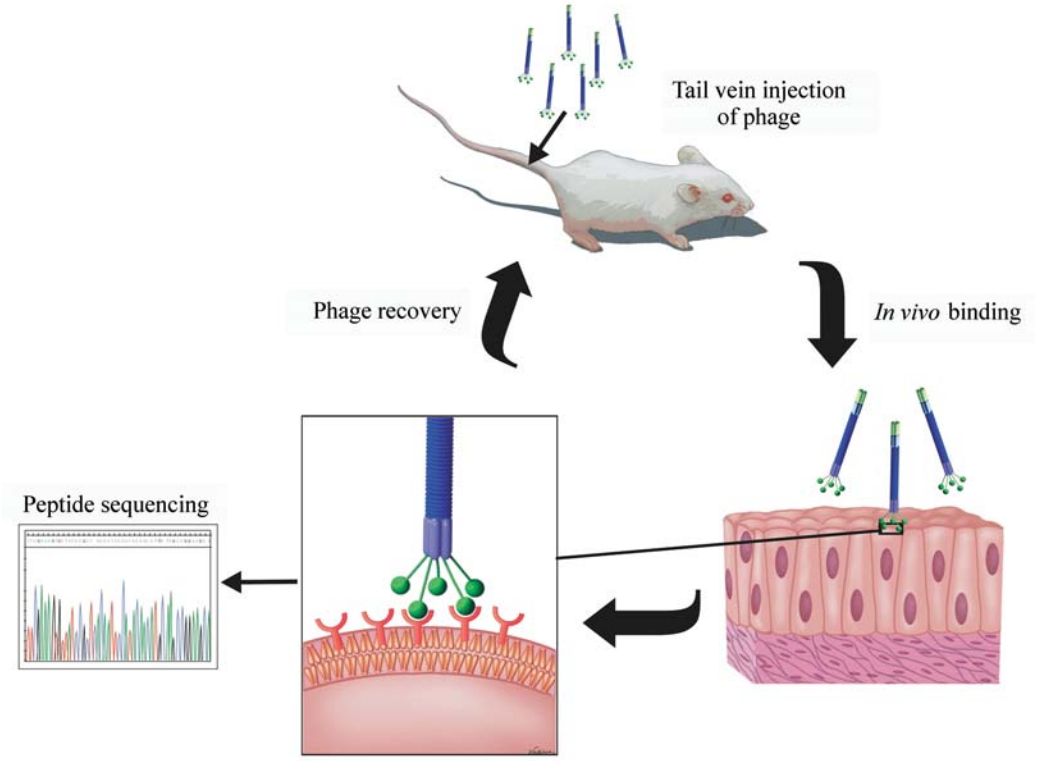

Figure 2 - *In vivo* use of phage libraries. Initially the phage library is injected in the circulation. Next, phage is allowed to circulate for minutes or hours (in this case when internalized phages are to be recovered). Finally, after deep anesthesia, mice are euthanized and the organs are dissected for phage recovery and peptide sequencing.

al. 2002). This may mean a reduction in prostate cancer risk, as well as alternatives to surgical prostate ablation.

Recently, a novel breast-homing peptide was selected from a phage library, and Aminopeptidase P was identified as the peptide receptor (Essler *et al.* 2002). As it is expressed in normal and malignant breast tissue, this aminopeptidase-P-binding peptide may be used for the development of drugs directed to prevent and treat breast cancer. Perhaps the most striking and promising contribution of in vivo use of phage display comes with the work of Kolonin and colleagues, who recently published a study in which a fat-targeting peptide was used to cure high-calorie related obesity in living animals (Kolonin *et al.* 2004). These findings not only mean hope for the treatment of several high-incidence diseases, but also demonstrate the versatility of phage display technology.

Molecular diversity of receptors in human blood vessels remains largely unexplored due to the lack of safe techniques applicable for human research. The first report of in vivo screening of a phage peptide library in humans was published in 2002 (Arap *et al.* 2002b). They developed a selection method in which peptides that home to specific human vascular beds are identified after intravenous administration of a peptide library. Moreover, they selected and isolated a mimic motif of interleukin 11 (IL-11) from prostate biopsies after the administration of phage. Analysis of the selected motifs revealed similarities to ligands for differentially expressed cell-surface proteins. Recently, the same group showed that IL-11 receptor alpha is a candidate target for translational clinical trials against advanced and metastatic prostate cancer (Zurita *et al.* 2004). Table 1 represent some of the peptides with therapeutic potential identified by phage display.

Recent innovations in phage display technology

Selective infective phage (SIP)

Enrichment for phage displaying high-affinity molecules over non-specific binders is one of the most difficult tasks in phage display technology. In SIP technology (Krebber *et al.* 1997), the N-terminal domains of pIII are replaced by the gene for a peptide or a protein leading to the generation of noninfective phage particles. The missing N-terminal domains, necessary for phage infectivity, are supplied within adapter molecules consisting of the ligand coupled covalently to these N-terminal domains. Infectivity is restored when noninfective phage and adapter molecules are mixed only to phage particles displaying peptides that are capable of binding the ligand with the latter, providing the missing N-terminal domains of pIII to the phage. This is a method that eliminates the need for physical separation of specific and unspecific binders, therefore providing an efficient and rapid procedure for selection of high-affinity interactions (Krebber *et al.* 1997).

Table 1 - Peptides with potential therapeutic implications identified by phage display.

Organ/tissue/ molecule	Phage sequence recovered	Reference
αvβ5 Integrin	VVISYSMPD	Cardo-Vila *et al.*(2003)
Aminopeptidase N	CNGRCVSGCAGRC	Pasqualini *et al.* (2000)
Mouse lung	CGFECVRQCPERC	Rajotte *et al.* (1998)
Mouse brain	CSSRLDAC	Pasqualini and Ruoslahti (1996)
Mouse kidney	CLPVASC	Pasqualini and Ruoslahti (1996)
Mouse retina	CSCFRDVCC	Rajotte *et al.* (1998)
Mouse pancreas	SWCEPGWCR	Rajotte *et al.* (1998)
Mouse skin	CVALCREACGEGC	Rajotte *et al.* (1998)
Mouse white fat	CKGGRAKDC	Kolonin *et al.* (2004)
Mouse placenta	TPKTSVT	Kolonin *et al.* (2002)
Human prostate	CGRRAGGSC	Arap *et al.* (2002b)

Single letter abbreviations of amino acids: A = Ala; C = Cys; D = Asp; E = Glu; F = Phe; G = Gly; H = His; I = Ile; K = Lys; L = Leu; M = Met; N = Asp; P = Pro; Q = Gln; R = Arg; S = Ser; T - Thr; V = Val; W = Trp; Y = Tyr.

Landscape phage

Phage particles with amino acids 2 to 4 on every wild-type pVIII coat protein replaced with random octamers are called landscape phage (Petrenko *et al.* 1996). This substitution leads to a fixed peptide framework that allows phage to have properties dependent on the introduced variable peptides. Moreover, additional properties may arise owing to the global architecture of the phage particle. Clones that can bind to dioxin, streptavidin, avidin, and beta-galactosidase with nanomolar affinity were selected from landscape phage libraries against immobilized targets and were named "substitute-antibody filaments" (Petrenko *et al.* 1996 and Petrenko and Smith 2000). Due to their novel surface properties, these filaments may have binding advantages over their immunoglobulin counterparts.

Isolation of allergens by phage display

Rhyner and colleagues described the isolation of allergens that induce IgE production using the association of robotic-based high-throughput screening technology and the selective enrichment of cDNA libraries expressed on phage surface with serum IgE from allergic individuals. Fusion proteins created by the principle of linking the phage phenotype (expressed as a gene product displayed on its coat) to its genetic information are covalently associated with the phage particle. Therefore, cDNA libraries displayed on phage surface can be screened for the presence of specific clones by affinity purification. Phage clones that bind to IgE may be selected by screening and enrichment of phage libraries against serum IgE immobilized in a solid phase. The amino acid sequence of surface-expressed allergens can be clarified by sequencing the DNA of the integrated section of the phage, as there is a physical linkage between its genotype and phenotype (Rhyner *et al.* 2004).

Use of phage display for gene delivery

Gene delivery to mammalian cells has also been accomplished by the use of single- (Yokoyama-Kobayashi and Kato 1993) and double-stranded phage (Ishiura *et al.* 1982, Okayama and Berg 1985). The modification of existing gene therapy vectors by selecting alternative ligands may increase their selectivity and therefore improve efficacy and reduce toxicity of gene delivery. In order to increase vector potency and selectivity, growth factors such as fibroblast growth factor (FGF) and other ligands that bind to cell surface receptors have been used for targeting viral and non-viral vectors (Goldman *et al.* 1997, Rogers *et al.* 1998). Larocca and colleagues successfully displayed FGF2, a growth factor ligand, on the phage surface as a pIII fusion (Larocca *et al.* 1999). It was the first report to demonstrate gene transfer to mammalian cells by genetically targeted filamentous phage. It also supported previous suggestions that peptide-displaying phage can be used themselves as gene delivery vectors (Barry *et al.* 1996 and Larocca *et al.* 1998). Therefore, the combination of gene delivery techniques and the power of combinatorial phage libraries allowed the creation of genetically altered phage, displaying a known gene targeting ligand. This may result in safer and more efficient gene transfer into mammalian cells.

Tumor targeting

Tumor targeting peptide ligands found by phage display technology have also been used for delivery of cytotoxic chemotherapy (Arap *et al.* 1998), proapoptotic peptides (Arap *et al.* 2002, Ellerby *et al.* 1999), and cytokines (Curnis *et al.* 2000) to receptors in the angiogenic vasculature showing marked therapeutic efficacy in tumor-bearing mouse models. Tumor targeting peptide ligands can also deliver imaging agents to tumor vasculature (Hong and Clayman 2000).

Final Remarks

Here we reviewed basic concepts of the structure and use of phage display technology. The applications of the technology are being actively explored, yielding different peptides that may prove useful for basic and clinical research. Recombinant antibodies, new diagnostic procedures, targeted therapies and several other phage display-based applications have arisen, facilitating diagnosis and treatment of benign and malignant conditions. Some of the peptides described here will probably be part of different therapies directed towards human diseases. In this context, human phage screenings must be continued so that other treatment options for conditions such as cancer and atherosclerosis may take place.

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