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Landscape phage ligands for PC3 prostate carcinoma cells

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Tumor-specific cytotoxicity of drugs can be enhanced by targeting them to tumor receptors using tumor-specific ligands. Phage display technology with its high throughput capacity for the analysis of targeting ligands possessing specific binding properties represents a very attractive tool in the quest for molecular ligands. Also, current phage nanobiotechnology concepts allow the use of intact phage particles and isolated phage coat proteins *per se* as components of nanomedicines. Herein, we describe the use of two landscape phage libraries to obtain phage ligands against PC3 prostate carcinoma cells. Following a very stringent selection scheme, we were able to identify three phage ligands, bearing the fusion peptides, DTDSHVNL, DTPYDLTG and DVVYALSDD that demonstrated specificity and selectivity to PC3 cells based on target-association assays, microscopy and flow cytometry. The phage ligands and their fusion coat proteins can be used as navigating modules in both therapeutic and diagnostic approaches to prostate carcinoma.
Keywords: affinity selection/landscape phage/PC3 cells/phage display

Introduction

The narrow therapeutic windows of most anticancer chemotherapeutic agents represent a prohibitive factor in their clinical use. This leads to a clinical threshold dose which cannot be exceeded even if it is required as in the case of tumor resistance to drugs. Conceptually, preferential delivery of drugs to pathologic sites should increase the local concentration of the therapeutic in the tumor milieu leading to a higher cytotoxic effect while simultaneously decreasing the side effects. One way to realize this concept is by targeting drugs to their sites of action by the way of ligands specific for cancer cells. Ligands with specific binding properties can be discovered using phage display technology which represents a high-throughput combinatorial technique for screening billions of random fusion peptide ligands against multiple targets on the surface of cancer cells, or located within them. The distinctive advantages of this technique are that targets may be unknown and non-immunogenic yet may serve as a delineating character for a particular cell type or tumor type. In particular, the concept of landscape phage has emerged in which phage particle serves as new nanomaterial in which the foreign peptide is expressed on all copies of the major coat protein pVIII (Petrenko, 2008). Wild-type phage usually expresses ~2700 copies of pVIII protein. The extra genomic inserts in landscape phage are accommodated by adding more pVIII subunits during phage assembly so that landscape phage expresses ~4000 copies of pVIII coat protein (Hunter *et al*., 1987). Landscape phage libraries have been used to isolate phage ligands for malignant glial cells (Samoylova *et al*., 2003) and breast cancer cells (Fagbohun *et al*., 2008). Romanov *et al*. (2001) isolated landscape phages that blocked spreading of LNCaP cells as well as their derivatives, C4-2 and C4-2b. Apart from cancer-related targets, landscape phage has also been used in the development of diagnostic probes for bacteria and spores (Brigati *et al*., 2004; Sorokulova *et al*., 2005), as a molecular recognition interface in detection devices and in gene, and drug delivery systems (Petrenko, 2008) demonstrating the enormous flexibility of this technology. As a relevant example, we have exploited the 'membranophilic' properties of the phage fusion coat protein to harness them as targeting ligands for liposomes (Jayanna *et al*., 2009).

We describe here the selection of landscape phage ligands for PC3 prostate carcinoma cells. Selected phage ligands, after four rounds of selection, were analyzed for their specificity and selectivity toward the target cells using cell-association assays. We further characterized specificity and selectivity by fluorescence microscopy and flow cytometry. Such selective phage ligands may provide an impetus for the development of diagnostic or therapeutic reagents specific for prostate cancer.

Materials and methods

Cells

Cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The PC3 (CRL-1435) cells were derived from a bone metastasis of grade IV prostatic adenocarcinoma. HepG2 (HB-8065) cells derived from a human hepatocellular carcinoma and HEK293 (CRL-1573) cells derived from fetal kidneys were used as controls. WI-38 (CCL-75) normal human lung fibroblasts were used for depletion of phage libraries. All cells were grown as recommended by ATCC and incubated at 37°C, 5% CO_2. For use in phage display selection protocols, all cell types were grown in 25 cm^2 polystyrene flasks for 48 h or in 24/96-well cell culture plates for 24 h until they reached subconfluent or confluent monolayers.

Landscape phage display libraries

Two landscape phage display libraries, f8/8 (Petrenko *et al*., 1996) and f8/9 (Kuzmicheva *et al*., 2009), constructed based on the type 8 phage display system were used in selection

procedures. In this system, the guest peptide is displayed as an extension of each major coat protein unit due to an in-frame random oligonucleotide insertion in the gene for the major coat protein, *gpVIII* resulting in the display of up to 4000 guest peptide units on the surface of a phage particle. The f8/8 library had random octapeptide inserts and a size of 1.4×10^9 particles, whereas the f8/9 library had random non-apeptide inserts and a size of 1.2×10^9 particles. All general methods of handling phage, including propagation, purification, titering, production of pure phage clone and isolation of phage DNA, have been described previously (Barbas *et al*., 2001; Brigati *et al*., 2008).

Selection of landscape phage ligands

The selection protocol previously described was used to identify phage clones homing to PC3 cells (Samoylova *et al*., 2003; Brigati *et al*., 2008). In an effort to minimize the selection of peptides binding non-specifically and maximize the affinity of target binding ligands, we implemented a rigorous library depletion regimen wherein the naïve library was sequentially exposed to culture flasks, serum treated culture flasks and non-target cells (fibroblasts) before being incubated with target PC3 cells. An aliquot of the primary library (100 copies of each phage clone) in 2 ml of blocking buffer (0.5% BSA in serum-free RPMI 1640) was added to an empty cell culture flask (depletion flask) and incubated for 1 h at room temperature. Following incubation, phage that did not bind to plastic was transferred from the depletion flask to serum-treated culture flasks. At the same time, the WI-38 fibroblast cells were washed twice with serum-free Leibovitz L-15 media and incubated for 1 h at 37°C, 5% CO_2 in serum-free Leibovitz L-15 media that was removed immediately before application of the phage. Phage that did not bind to serum-treated culture flasks was transferred to the fibroblast cell culture flask and incubated for 1 h at room temperature. At the same time, the PC3 cells were washed twice with serum-free RPMI 1640 media and incubated for 1 h at 37°C, 5% CO_2 in serum-free RPMI 1640 media that was removed immediately before application of the phage. Phage that did not bind to fibroblasts was transferred to the PC3 cell culture flask and incubated for 1 h at room temperature. Following the final incubation with PC3 cells, phage not associated with tumor cells was washed away by extensive washing with washing buffer (0.5% BSA, 0.1% Tween 20 in serum-free RPMI 1640). Phage bound to the cell surface was collected by treating the PC3 cells with 2 ml of elution buffer (200 mM glycine–HCl with 1 mg/ml BSA and 0.1 mg/ml phenol red, pH 2.2) for 10 min on ice. After collection, the eluate was neutralized with 376 μl of 1 M Tris (pH 9.1). To recover cell internalized phage, after further washing, PC3 cells in the flask were scraped in 5 ml of serum-free RPMI 1640 and pelleted by centrifugation at 130*g* for 10 min. RPMI 1640 was removed and the cell pellet was lysed with 200 μl of lysis buffer [2% deoxycholic acid (sodium salt), 10 mM Tris, 2 mM EDTA (pH 8.0)]. Phage in eluate was concentrated by centrifugal concentrators (Centricone 100 kDa, Fisher Scientific, Pittsburgh, PA, USA) to an approximate volume of 80 μl. The phage input and output were titered in bacteria as described previously (Brigati *et al*., 2008), and the results of the selection were expressed as the percentage of the ratio of output to input phage. The phage fractions, cell-surface bound phage and cell-internalized phage were amplified separately in bacteria and used in subsequent rounds of selection which were similar to the procedure described above except for the lack of the extensive depletion regimen. Following four rounds of selection, phage DNAs of randomly selected phage particles were PCR amplified, isolated and sequenced to reveal the peptide sequences responsible for target association. For convenience, all phages are designated by the sequence of the guest peptide that they bear.

Specificity and selectivity of phage ligands for PC3 cells

Individual phage clones identified by sequencing were propagated and purified to be used in cell-association assays. Owing to the large number of possible candidates, an initial semi-quantitative screening assay was employed where the association of a selected clone with the target PC3 cells compared with serum alone was monitored. The protocol essentially followed that of selection with phage particles ($\sim 10^6$ cfu/well) being incubated with target as well as serum-incubated control wells in a 24-well cell culture plate. Following washing, cell- or serum-associated phage was collected by lysing the cells, titered in bacteria and presented as a ratio of output to input phage. A panel of phage ligands identified based on these screening assays was further characterized in assays to determine their selectivity. PC3, HEK293 and HepG2 cells were grown to confluence in separate wells of a 96-well cell culture plate. As a control, selected wells were treated with media alone. Before application of phage, media in the wells were replaced with serum-free media for 1 h. Each phage probe ($\sim 10^6$ cfu/well) was added to the designated wells in 100 μl of blocking buffer and incubated for 1 h at room temperature. The buffer containing unbound phage was carefully removed and the cells were washed eight times with 100 μl of cold washing buffer. To collect cell-associated phage, 25 μl of lysis buffer (2.5% CHAPS in serum-free media) was then added to each of the wells and incubated for 10 min on a shaker (20 cycles/min). To each well 125 μl of starved host bacteria was added and incubated for 15 min at room temperature. Following this, 180 μl NZY containing 0.4 μg/ml tetracycline was added to the mixture and further incubated for 45 min at 37°C. The entire mixture was finally spread on NZY microbiological plates containing 20 μg/ml tetracycline and incubated overnight. Phage titers were presented as a ratio of output to input phage to determine recovery %. Phage bearing un-related peptide was used as a control, and all assays were performed in triplicate.

Labeling of PC3-specific phage with fluorophores

PC3 cell-binding phage probe harboring the peptide DTDSHVNL (designated as 8-3) was labeled with Alexa Fluor 488 (Molecular Probes, Carlsbad, CA, USA) according to manufacturer's instructions. Briefly, $\sim 1 \times 10^{13}$ virions in 500 μl of 1× PBS was incubated with the Alexa Fluor® 488 carboxylic acid TFP ester in the presence of 50 μl of 1 M sodium bicarbonate (pH ~8.3) for 1 h. Following incubation, phage particles were collected by a double PEG precipitation technique as described previously (Brigati *et al*., 2008). The number of fluorophores on each phage particle was

calculated as per the instructions of the manufacturer as follows:

$$\text{moles dye per mole phage} = \frac{\text{A494} \times \text{dilution factor}}{71\,000 \times \text{phage concentration (M)}}$$

where 71 000 cm^{-1} M^{-1} is the approximate molar extinction coefficient of the Alexa Fluor 488 dye at 494 nm. As a control, wild-type phage particles were also labeled with AF488 reagent.

Evaluation of targeting ability of fluorescently labeled PC3-specific phage

PC3 and HEK293 cells were grown to subconfluence in 25 cm^2 cell culture flasks, collected by trypsinization and counted. Equal numbers of each line were then re-suspended in 1 ml of GIBCO™ Improved MEM Zn++ Option (Richter's Modification) liquid (Invitrogen, Carlsbad, CA, USA). CellTracker Orange CMTMR (Molecular Probes) was used to stain HEK293 cells using a 1:40 dilution of CellTracker Orange CMTMR stock (5 mM) in sterile clear 1× Hanks buffer and then 1 μl of diluted dye per 1×10^6 cells for 30 min at 37°C. The cell suspension was pelleted by centrifugation, resuspended in Improved MEM and incubated for 30 min at 37°C followed by centrifugation. The pellet was washed three times with Improved MEM. Following labeling, equal numbers of PC3 and labeled HEK293 cells were mixed together. The cell mixtures in different tubes were then treated with either PC-specific AF488 labeled phage or with different control phage preparations at equivalent fluorophore levels for 90 min at 37°C. The cells were then washed five times in Improved MEM at room temperature followed by reconstitution in 500 μl of the same media and then used for fluorescence microscopy as well as flow cytometry.

For microscopy, aliquots of the cell mixtures were applied onto microscopic slides (Fisher Scientific), covered with coverslips (Fisher Scientific) and visualized with a Cytoviva® microscope system (Auburn, AL, USA) using FITC, Texas Red and multiple band-pass filters. Images were captured using DAGE® software.

For flow cytometry, the samples were further filtered through a 50-μm CellTrics filter (Partec GmbH, Germany) before being analyzed on a MoFlo flow cytometer in the green (530 ± 20 nm) and red (700 ± 15 nm) channels. The data were analyzed using Summit 4.3 software (Dako, Carpinteria, CA, USA). Data shown were derived from three separate experiments.

Results

Selection of phage ligands against PC3 cells

We chose to use PC3 cells as our target cells as they are androgen-independent and thus are representative of advanced prostate tumors (Culig *et al*., 1994). We decided to evaluate two landscape libraries, f8/8 (8-mer) and f8/9 (9-mer), in our selection scheme to increase the chances of finding peptides with optimal target-association properties. Also, since we were interested in isolating phage peptides with high selectivity, we adopted a rigorous library depletion protocol, in which the naïve library was progressively depleted against plastic, serum and normal fibroblast cells before being allowed to interact with the target cells. Furthermore, a very extensive washing procedure was followed after incubation to eliminate unbound phage as well as weak binders. Following incubation and washing, phage associated with the cells was recovered using two different approaches (Fig. 1). Phage bound to the tumor cell surface was eluted using an acidic elution buffer of pH 2.1. Following elution, cells were washed two times (post-elution wash) and internalized phage were recovered by detergent lysis of the tumor cells. Both phage fractions were separately amplified in bacteria and used as input phage in subsequent rounds of selection. In the next round of selection, phage purified from the eluate fraction and phage purified from the cell-lysate fraction were added to different flasks containing target cells so that a parallel two-channel selection scheme could be followed, one for isolation of cell-binding phage and the other for isolation of cell-internalized phage. The numbers of infective phage particles in input, washes as well as the phage associated with the cells were titered in bacteria and results of each round presented as a ratio of output phage particles to the input phage particles expressed as a percentage (defined as Yield). Enrichment with target-specific clones was manifested as a progressive increase in yield through consecutive rounds of selection and was indicative of successful selection for tumor cell-specific clones from both libraries (Fig. 2). For both libraries, following four rounds of selection, 40 phage clones each were randomly chosen from the eluate, cell-lysate and post-elution wash fractions, respectively, for PCR amplification and sequencing (Table I).

Specificity and selectivity of phage ligands for PC3 cells

In characterizing phage ligands, specificity is defined as the ability of a phage probe to associate with its target due to the presence of a specific peptide sequence displayed on the surface of the phage, whereas selectivity is the ability of a phage probe to identify its cognate target from a mixture of targets. The cell-association assays that we adopted were designed to evaluate both selectivity and specificity. Selected phage clones ($\sim 10^6$ cfu/well) were added to designated wells of a 96-well cell culture plate containing target cells, control cells or cell-free media. As we were most interested in evaluating the targeting potential of each selected probe rather than cellular mode of interaction of the probe, we collected phage associated with cells in a single fraction by lysing the cells using a newly optimized lysis buffer (2.5% CHAPS in serum-free medium). Collected phage was titered in bacteria and a ratio of output to input phage calculated for both libraries (Fig. 3). We identified two clones from the 8-mer library, DTDSHVNL and DTPYDLTG, which were specific and selective for PC3 cells. The association of DTDSHVNL with PC3 cells was ~9 times higher than with either of the control cells and 32 times higher than with serum (cell-free media), whereas the association of DTPYLDTG to PC3 cells was ~8 times higher than with either of the control cells and 15 times higher than with serum. The other clones analyzed showed high association with target cells as well as control cancer cells but not with normal epithelial cells or serum leading us to postulate that these peptides may be directed against a more universal/shared cancer receptor. A single clone from the 9-mer library was identified, DVVYALSDD, which associated with PC3

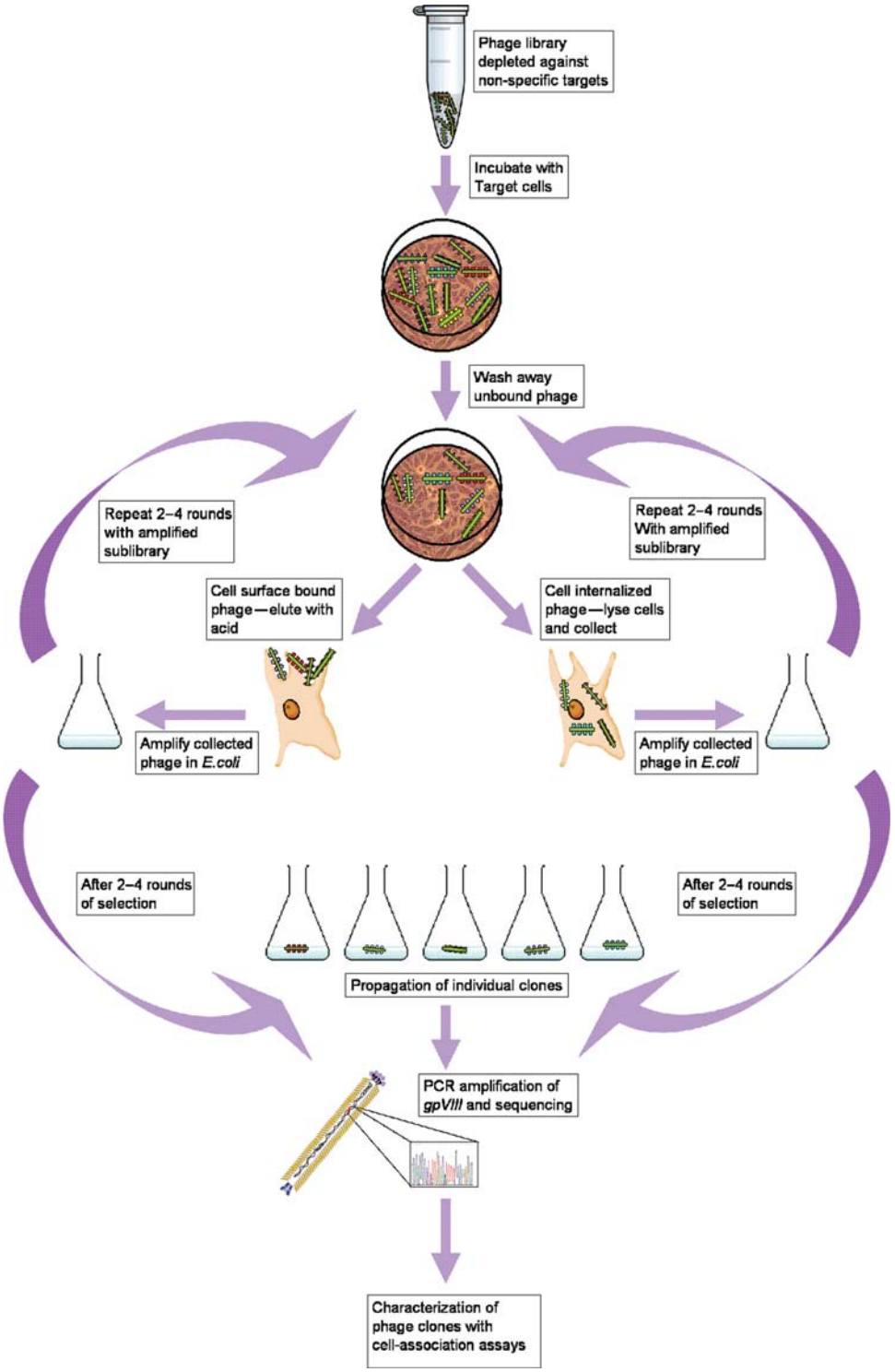

Fig. 1. Scheme for affinity selection of landscape phage ligands against tumor cells demonstrating the isolation of cell-surface bound phage as well as cell-internalized phage as described in detail in text.

cells ~80 times better than with control cells and 600 times better than with serum (cell-free media). Surprisingly, the other clones analyzed showed a high association to target as well as normal epithelial cells but not to control cancer cells or serum indicating that they may directed toward a marker that is common to both tumor and normal cells. A phage bearing an un-related streptavidin-specific peptide (VPEGAFSS) was used as a control to demonstrate that specificity of selected phage peptides was due to the peptide insert and not other phage components.

Evaluation of targeting ability of fluorescently labeled PC3-specific phage

The average number of fluorescent moieties on phage particles was calculated to be ~200. The targeting efficiency of fluorescently labeled PC3-specific phage peptides was then

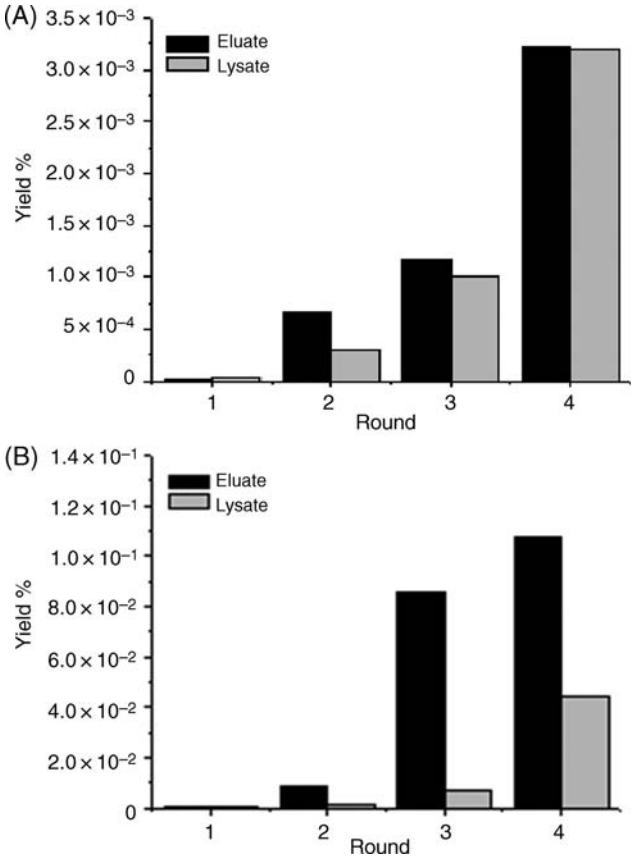

Fig. 2. Phage output during successive rounds of selection. *x*-axis, round of selection; *y*-axis, ratio of phage output to input expressed as percentage and termed as Yield. A progressive increase in the yield across successive rounds indicates selective enrichment of the library for target specific clones. (**A**) f8/8 library and (**B**) f8/9 library.

Table I. Phage peptides identified after four rounds of selection of landscape phage libraries against PC3 tumor cells

8-mer	9-mer
DTDSHVNL[a] (9)	DVVYALSDD[abc] (14)
GDNSHVNL[a] (5)	AEYGERGNA[a] (6)
VSDNTDYS[a] (3)	AEYGESGNA[a] (5)
VGQDSDYS[bc] (4)	AEYGESVLI[a] (1)
ERAPLSVE[abc] (6)	AEYGESVNA[abc] (12)
GPDSTWAG[ab] (2)	GAYDVNVND[a] (1)
DSSNKPTG[b] (1)	DSDVGWVND[a] (1)
DSSRLERV[b] (1)	EAAGANIAP[a] (2)
EGMMYTDV[bc] (2)	GPNWAEGDS[a] (1)
DTPYDLTG[ab] (15)	VADDRDYSD[a] (1)
AHALTTEE[c] (1)	VDVSEQMSL[a] (1)
APLPTNGE[c] (1)	VGDNVDYMD[a] (1)
ATDHAAPQ[c] (1)	VGDYDVVDS[a] (1)
ATPTTPDP[c] (1)	EGLVWIGMD[bc] (3)
DLTYVNSQ[c] (2)	
EKFASNST[c] (1)	
EVSMYTDV[c] (1)	

Superscripted alphabets indicate the presence of a particular phage peptide in one of the three fractions collected during the course of the selection protocol; a, eluate; b, post-elution wash; c, lysate. Numbers in parentheses indicate the number of times a clone was identified in the group of sequenced phages.

evaluated based on their preferential association with PC3 cells in a mixture of target and control cells. Control HEK293 cells, representative of a non-cancer cell line, were

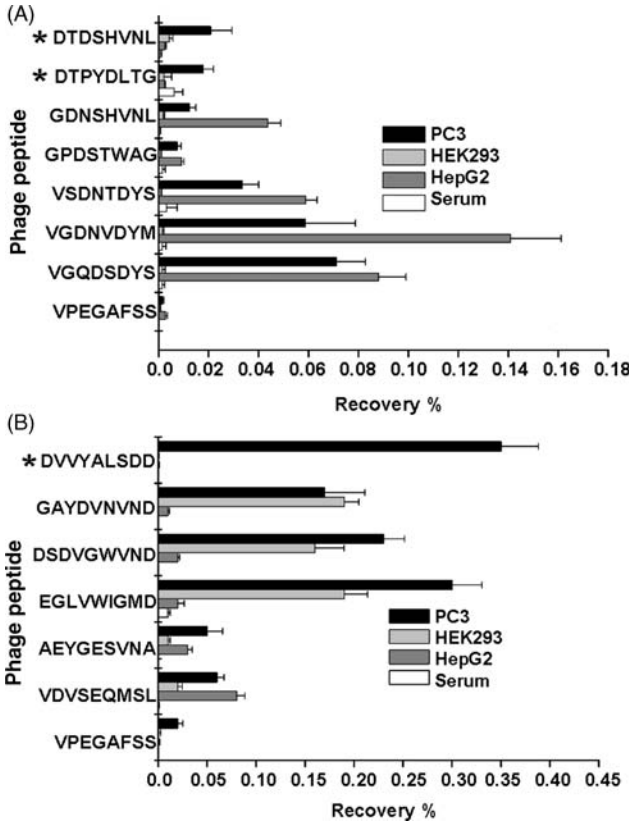

Fig. 3. Selectivity and specificity of phage ligands. Phage selected from preliminary screening assays was incubated with target PC3 cells, control cells (HEK293 and HepG2) or serum-treated wells of a 96-well cell culture plate. Phage associated with cells or serum was titered in bacteria, and the ratio of phage output to phage input was expressed as a percentage (recovery %) to obtain a measure of the relative affinity of a particular phage toward individual cell types used in the assay. The relative recovery % of a probe in relation to the control phage bearing an unrelated peptide was an indicative of probe's specificity. Results are an average of three replicates. (**A**) f8/8 library and (**B**) f8/9 library. Clones selected for further characterization are noted (*).

labeled with a vital dye, CellTracker Orange CMTMR to allow visualization by microscopy as well separation as a discrete population by flow cytometry, whereas the target cells were unlabeled prior to phage addition. Only specific labeling of PC3 cells by targeted fluorescent phage peptides would result in fluorescent visualization of PC3 cells. A representative field of cells observed under three different fluorescent filters is shown (Fig. 4). Under the red filter, only control HEK293 cells stained red with the vital dye were evident, whereas the same field under the green filter allowed visualization of cells stained as a result of AF488-conjugated phage probe binding. Comparison of these two images allowed clear discrimination between target PC3 cells and control HEK293 cells. PC3-specific phage peptides show an increased avidity for PC3 cells as substantiated by the intense staining of the surface of only PC3 cells. The last panel used a triple band filter and allowed visualization of both cell types creating a synoptic image demonstrating that phage only bound to PC3 cells as no double labeled cells were detected.

Flow cytometry analysis also revealed that the mixture of cells prior to labeled phage treatment resolved into two distinct populations based on the fluorescence intensity in the

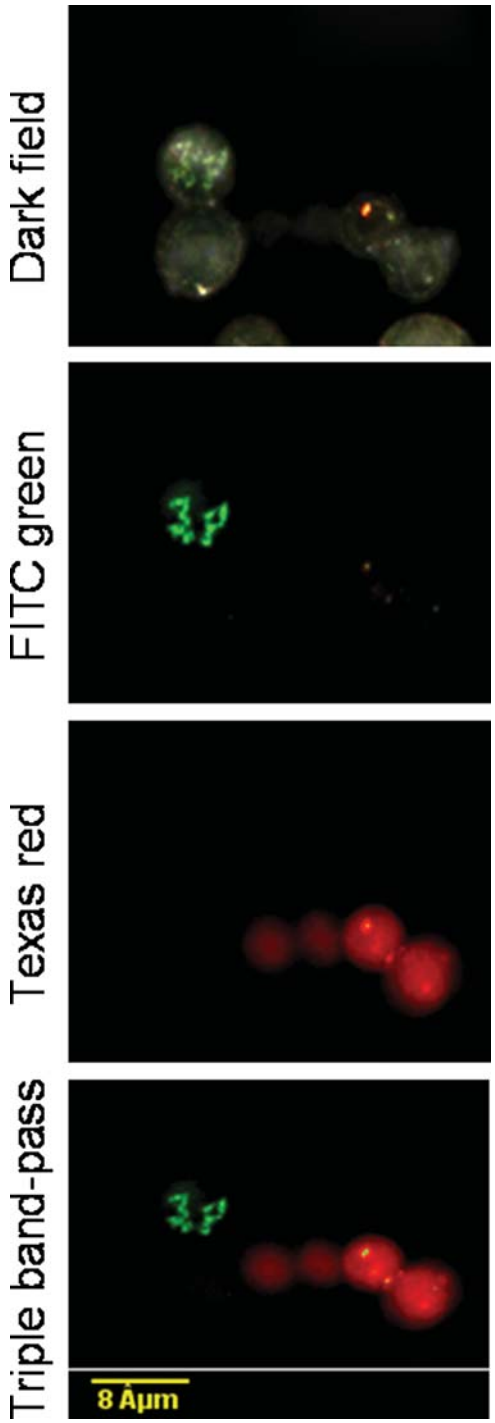

Fig. 4. Cancer cell specific association of AF488-conjugated PC3-specific phage. Fluorescent microscopy of a mixture of unlabeled target PC3 cells and red-labeled control HEK293 cells following incubation with PC3-specific AF488-conjugated PC3-specific phage DTDSHVNL (8-3). Selective association of the green fluorescent phage particles with PC3 cells enabled their visualization under relevant filters. Panels depict a representative field of observation under different filters.

red channel. One population consisted of labeled control HEK cells and the other population of unlabeled PC3 target cells. Increase in the AF488 mean channel fluorescence (MCF) of each cell population after treatment with AF488-labeled PC3-specific or wild-type phage particles was monitored to obtain a measure of the target selectivity

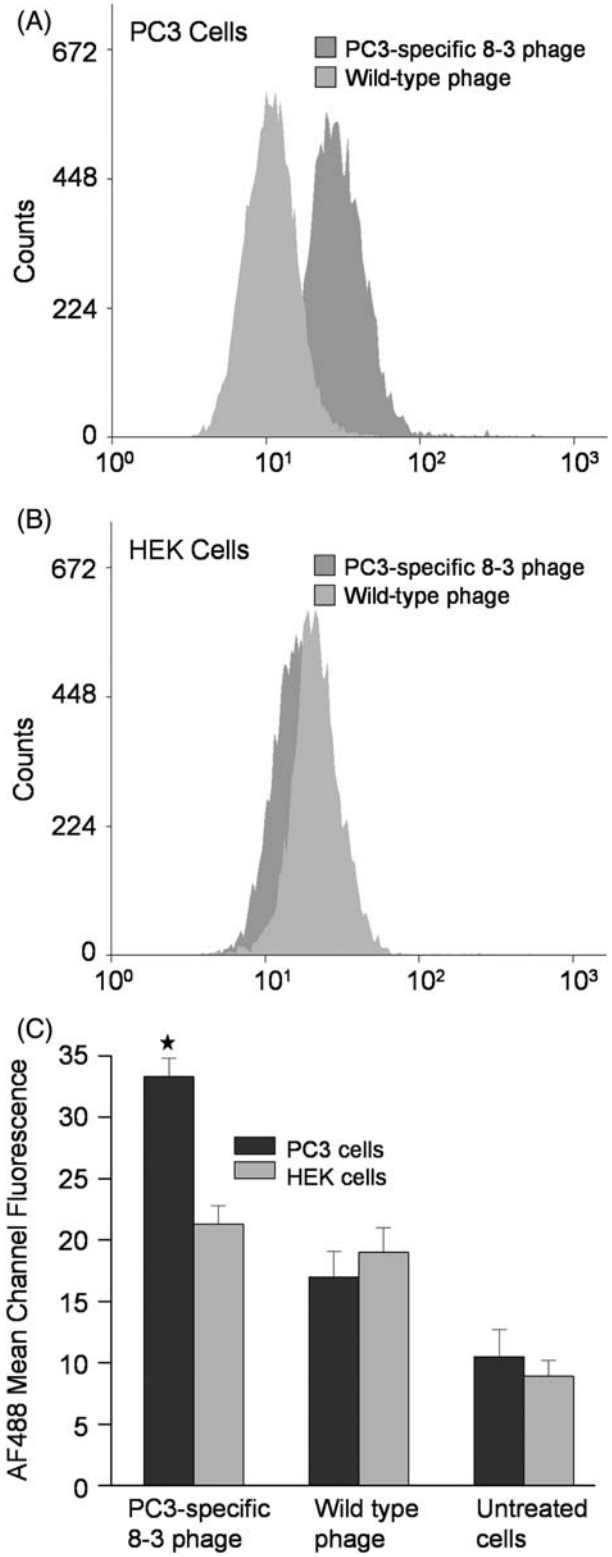

Fig. 5. Cancer cell specific association of labeled PC3-specific phage. Flow cytometry of a mixture of unlabeled target PC3 cells and red-labeled control HEK293 cells without any labeled-phage treatment created two distinct populations, based on the fluorescence intensity in the red channel, one consisting of labeled control cells and the other of unlabeled target cells. Thus, changes in the AF488 MCF for each population could be monitored after treatment with green-labeled PC3-specific phage. An appreciable shift in the target PC3 cells (**A**) but not HEK cells (**B**) along the green channel was observed after treatment with PC3-specific phage but was not detected with the control phage. AF488 MCF of the PC3 cells was 2-fold higher when target-specific phage was employed when compared with control phages (**C**) whereas it did not change appreciably with HEK cells. $*P < 0.05$, one-way ANOVA with Tukey's honestly significant difference *post hoc* test.

(Fig. 5A and B). AF488 MCF of PC3 cells was nearly 2-fold higher after treatment with PC3-specific phage than wild-type phage, whereas for HEK cells, it did not alter appreciably between the target-specific and wild-type phage (Fig. 5C, $P < 0.05$ according to Tukey's HSD).

Discussion

Phage display, since its conception by Smith (1985) has emerged as a premier tool in molecular biology with widespread applications and far-reaching implications. The potential of phage display lies in the self-assemblage and self-perpetuation of the query molecules on the viral surface through the expression of the viral life cycle. Additional features are the stability and longevity of phage particles and their encoded peptides (Brigati and Petrenko, 2005). The surface display of random peptides allows high throughput screening to discover ligands to a host of targets. Also, the unique binding properties of peptides allow selection for high affinity, specificity and selectivity toward the target. Such characteristics make this technology an ideal molecular mining scheme to extract probes directed against complex targets such as tumor cells. Indeed, several studies have not only selected tumor-specific phage particles and their corresponding peptides but also have adapted the whole fusion phage as nanocarriers for diagnostic and therapeutic moieties (Kelly *et al*., 2006; Newton *et al*., 2006; Bar *et al*., 2008; Newton-Northup *et al*., 2009). In particular, landscape phage with their intensive surface array of guest peptides represents a potential source of cell-specific probes which can be harnessed in varied drug/gene delivery platforms (Petrenko, 2008). Also, the huge copy number of the pVIII fusion coat protein units and their unique properties enable their use as targeting ligands for nanoparticles (Jayanna *et al*., 2009).

To identify phage ligands with high specificity for PC3 cells, we adopted increased stringency in our selection scheme. Also, we were interested in characterizing the cellular localization of phage ligands and hence we collected separately the surface-bound phage, the phage internalized into the cells as well as the wash obtained after acid elution. There was considerable redundancy in phage peptides isolated between the different fractions though there were some notable exceptions such as DTDSHVNL and GDNSHVNL which were characteristically found only in the eluate fraction. This redundancy can be attributed to dual properties (binding and penetrating) of a phage probe in some cases (DTPYDLTG was recovered 9 times in the eluate fraction and 10 times in the lysate fraction) or may be an artifact of isolation in other cases (DVVYALSDD was found 12 times in the lysate fraction but just once in the eluate fraction) arising due to the cross-contamination between washes. Also, phage ligands were found in post-elution washes which led us to the hypothesis that changes in pH during elution and subsequent washings may cause membrane perturbations leading to the release of non-eluted membrane-bound phage to the media. This follows from a phenomenon previously described in cell injury studies as the 'pH paradox' (Currin *et al*., 1991). Here, a drop in pH is observed after cellular injury and serves to stabilize the plasma membrane of cells by inhibiting the activity of phospholipases whereas a sudden return to normal pH, as occurs following reperfusion, induces phospholipase activity which in turn damages the cell membrane cleaving substrate phospholipid molecules (Harrison *et al*., 1991). Our experimental setup closely mimics this physiological situation in that cells were treated with acid elution buffer followed by treatment with washing buffer the pH of which is close to the physiological pH. This sudden change in pH may lead to phospholipase activation and subsequent membrane damage allowing release of membrane bound components like peptides and phage particles to the medium thus explaining their presence in the post-elution washes as well as in the lysate fraction.

As a means to narrow down the number of clones to be analyzed, we conducted a screening assay in which relative affinity of each recovered phage probe for PC3 cells was measured against its relative affinity for serum. This assay is semi-quantitative and provides an estimate of the apparent affinity of each phage probe. Accordingly, 13 clones from both libraries were chosen for further analysis which involved assessment of selectivity and specificity. Hepatocellular carcinoma cell line, HepG2, was used as a control transformed cell of non-prostatic origin, and human embryonic kidney cell line HEK293 were chosen as being representative of normal non-transformed control cells to compare selectivity. A control phage bearing an unrelated guest peptide was also used to verify specificity of each phage probe. Despite the stringency of the selection procedure, our results indicate a selection of some ligands which were directed toward markers shared between normal cells and cancer cells as well as markers shared by both cancer types. This may be related to the relative abundance of different receptors/markers on tumor cell surface and may require further development of selection protocols with landscape libraries containing different repertoires of guest peptide as has been described previously (Kuzmicheva *et al*., 2009).

Tumors have considerable heterogeneity both between and within tumors (Lleonart *et al*., 2000), which has been a major barrier in the development of tumor diagnostics and therapy but could be overcome if precisely targeted vectors for therapeutics and diagnostics can be developed to detect and treat cancer. Such advancements would enable not only a tumor-specific but a patient-specific profile of diagnosis and treatment (Samoylova *et al*., 2006). The successful selection and subsequent use of a PC3-cell surface binding phage in targeting studies proved the validity of phage selection technique as well as utilizing phage particles for imaging applications. Extrapolating these results, landscape phage with target-specific peptides may be used as efficient nanocarriers to deliver drugs and genes themselves, a postulate further strengthened by considering other studies that have used similar vehicles (Wu *et al*., 2005; Yacoby *et al*., 2006, 2007; Bar *et al*., 2008) or as an inexpensive source of targeted fusion proteins. For example, phage fusion proteins from the phage ligands identified in this study were isolated and incorporated into labeled and therapeutic liposomes. Targeting of labeled liposomes was demonstrated using fluorescence microscopy as well as flow cytometry. Targeting of doxorubicin-loaded liposomes enhanced their cytotoxic effect against PC3 cells *in vitro* indicating a possible therapeutic advantage (submitted to Nanomedicine). Thus, landscape phage technology can be harnessed not only as a source of target-specific ligands but also as a source of novel nanomaterials.

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