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# Identification of FGF receptor-binding peptides for cancer gene therapy

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Linear FGF receptor-binding heptapeptides were identified by phage display using sequential rounds of biopanning against cells with displacement of phage by FGF2. The consensus motif MXXP was iterated after four to five rounds and the peptide MQLPLAT was studied in depth. Phage bearing MQLPLAT showed high levels of binding to FGF receptor positive cells, with over 90% of phage bound being eluted competitively by adding free FGF2. MQLPLAT phage showed only limited binding to Cos7 cells deficient in receptors for FGF. MQLPLAT phage bound to SKOV3 cells with a $K_d$ of $2.51 \times 10^{-10}$ M. Although binding could be blocked by preincubation with free FGF2, heparin could not displace the phage. Use of MQLPLAT to target polyelectrolyte gene delivery vectors *in vitro* in the presence of serum achieved up to 40-fold greater transgene transduction than nontargeted vectors. MQLPLAT phage were administered into gastric carcinomas via the tumor-feeding artery immediately following resection from patients. The phage showed up to 9-fold more accumulation in the tumor than in adjacent regions of normal tissue, whereas control phage showed less than 2-fold. These peptides should provide useful ligands for specific delivery of gene therapy vectors to clinically relevant targets.
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Gene therapy has great therapeutic potential in cancer, combining powerful action with the possibility for selectivity against tumor cells. This combination makes it particularly promising for treatment of multiple metastatic disease. Therapeutic systems are already well advanced, often involving immunostimulatory approaches, enzyme-based chemotherapy such as gene-directed enzyme-prodrug therapy (GDEPT), or supplementation of key mutated genes. A critical problem that currently restricts progress in cancer gene therapy is poor target selectivity of the vectors, resulting in unwanted infection of many non–target cells. This can be addressed in part by using tumor-associated promoters to regulate transgene expression but such promoters are often too weak for effective GDEPT and do not provide the proper physiological regulation of expression that is desirable for gene supplementation. In addition, uptake into non–target cells depletes the pool of vector available for targeting the tumor and simultaneously can promote unwanted toxicities that could be dose limiting. Consequently, several groups have focused on the investigation of methods to confer tumor tropism on the vector mainly employing targeting systems based on tumor-selective ligands.[1-5]

One ligand that has received considerable attention is fibroblast growth factor 2 (FGF2), which is known to bind to many cancer cells[6,7] and which has receptors up-regulated in endothelial cells proliferating during angiogenesis.[7,8] It has been studied previously as a ligand for receptor-mediated gene delivery, using a range of approaches to incorporate the molecule onto the surface of both viral[1,3,4] and synthetic[2] gene delivery vectors. However, FGF2 has complex biological properties,[9] is mitogenic, shows limited stability in serum, and has a tendency to bind heparans and proteins in the plasma. It is also relatively expensive to produce in bulk. Elucidation of simple molecules that can bind specifically to receptors for FGF would have considerable usefulness in the development of gene delivery systems targeted to cells bearing appropriate receptors.

Barry et al[10] introduced peptide-presenting phage libraries to enable selection of peptides that bind and enter several different cell types. Since then, phage display libraries have been used to identify peptides that target specific organs[11] and tumors,[12] providing important tools for a range of therapeutic developments.[13,14]

In this study we have used a phage panning technique to identify peptides that bind specifically to FGF receptors. The peptide-presenting phage library used is based on a

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combinatorial library of random peptide heptamers fused to a minor coat protein (pIII) of M13 phage and contains $\sim 2.8 \times 10^9$ different sequences.[15] Panning with the library against FGF receptors on cells permits identification of binding peptide sequences by extrapolation from the corresponding DNA sequences of phage eluted from the cells following the addition of FGF2. Five consecutive rounds of panning in each of triplicate experiments were performed to identify any consensus. Four heptapeptide sequences, derived from the fifth round of biopanning, were evaluated for their FGF receptor-binding ability and a single sequence (MQLPLAT) was selected. This sequence was synthesized and used in simple polyelectrolyte complexes to test whether *in vitro* targeted transfection of 911 and B16F10 cells could be enhanced. M13 phage bearing MQLPLAT were administered directly into the tumor-feeding artery of recently excised clinical human gastric carcinomas to investigate whether greater localization within tumor, than within equivalent regions of normal gastric mucosa, could be achieved. The identification of a nonmitogenic FGF receptor-binding heptapeptide sequence should provide important opportunities for retargeting drugs and genes selectively to tumors, using a range of vectors. In fact, given the current development of phage themselves as vectors for gene delivery,[2,16-18] even peptide-modified phage could provide a useful means for applying gene therapy against primary and metastatic cancer.

## Materials and methods

### Tissue culture and reagents

The cell lines SKOV3 (human ovarian carcinoma), PC3 (human prostate carcinoma) and Cos7 (monkey kidney epithelium) were obtained from American Type Culture Collection (ATCC, Manassas, VA). B16F10 murine melanoma cells were a kind gift from Professor Ernst Wagner (IMC, Vienna, Austria), and 911 human embryonic retinoblasts[19] were from Leiden University Medical Centre (Leiden, Netherlands). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 1% penicillin/streptomycin (Sigma Chemical, Dorset, UK) at 37°C and 5% $CO_2$. Human umbilical vein endothelial (HUVE) cells were isolated from cords donated to the Birmingham Women's Hospital, maintained in medium M199 (Sigma) plus 20% FCS and used before passage 3. FGF2 was a kind gift from Selective Genetics, San Diego, CA.

### Iteration of FGF receptor-binding peptides

The Ph.D-7 M13 heptapeptide phage display library (New England BioLabs, Beverly, MA) was used for biopanning. 911 cells were grown to confluence in six well plates and acclimatized to 4°C for 30 minutes. They were washed briefly in Dulbecco's phosphate-buffered saline (PBS, Life Technologies) before addition of phage library ($2 \times 10^{11}$ plaque-forming units [pfu]/well) diluted in 1 mL/well DMEM containing 1% (wt/vol) bovine serum albumin (BSA; Sigma). Phage were allowed to bind to cellular receptors for 1 hour at 4°C with gentle agitation, then medium containing unbound phage was removed and discarded. Cells were washed four times for 5 minutes in PBS containing 1% (wt/vol) BSA. Finally, phage bound to FGF-receptors were specifically eluted by the addition of 10 $\mu$g/mL FGF2 (in PBS) to each well for 1 hour at 4°C with gentle agitation. Numbers of eluted phage were established by titering on X-gal (5-bromo-4-chloro-3-indolyl-$\beta$-D-galactopyranoside; Bioline, London, UK), IPTG (isopropyl-$\beta$-thiogalactopyranoside; Bioline) agar plates containing *Escherichia coli* strain ER2537. Remaining phage were amplified by addition to a 20-mL early log phase culture of ER2537 for 5 hours at 37°C with vigorous shaking (200 rpm). Amplified phage were isolated from the resulting culture by manufacturer's recommended protocol, concentrated, titered, and used for subsequent rounds of biopanning. In total, five rounds of biopanning were performed in triplicate.

### Isolation and sequencing of phage DNA

Individual phage clones were isolated from each replicate for each of five rounds of biopanning and total DNA was isolated according to the sequencing kit manufacturer's recommended protocol (Biosystems, Perkin Elmer, Foster, CA). The resulting DNA was used for sequencing using the $-96$ primer together with the BigDye terminator cycle sequencing kit (Biosystems, Perkin Elmer). DNA sequences were determined using an automated ABI PRISM 377 sequencer (Perkin Elmer).

### Evaluation of cell-binding activities of selected phage clones

To evaluate the binding of selected phage clones for cell surface receptors, cells were grown to confluence in six-well plates. Cells were acclimatized to 4°C for 30 minutes, then washed briefly in PBS before the addition to each well of $5 \times 10^7$ pfu of each selected phage clone diluted into 1 mL DMEM, 1% BSA. Phage were allowed to bind to cells for 1 hour at 4°C with gentle agitation. Media containing unbound phage were discarded and the cells washed four times for 5 minutes in PBS containing 1% BSA. Bound phage were subsequently eluted from the cell surface by the addition of 0.01–10 $\mu$g/mL FGF2 for 1 hour at 4°C with gentle agitation. In some experiments, residual phage that remained bound to the cells following elution with FGF2 were quantified by scraping the cells from the six-well plate. Recovery was determined by plaque infection assay of multiple dilutions of eluted phage on bacterial lawns grown overnight on Xgal IPTG agar plates at 37°C, with plaques counted the following morning. To discount the possibility that selected phage clones might be binding to cell surface heparans, heparin (0.4–400 $\mu$g/mL; Sigma) was added to the elution medium, cells were incubated for 1 hour, and phage recovery was determined by titration as described above.

*Competitive inhibition of selected phage clones binding FGF receptors*

Selective inhibition of phage binding was achieved by preincubation of cells with FGF2 (0.5 $\mu$M), MQLPLAT peptide or VRWEMNL peptide (each 0.5 $\mu$M, synthesized by Alta Biosciences, Birmingham, UK) for 1 hour at 4°C. Medium was discarded and the cells were washed briefly with PBS before addition of $5\times10^7$ pfu of selected phage (diluted into 1 mL DMEM, 1% BSA). Phage were allowed to bind to cellular receptors for 1 hour at 4°C with gentle agitation. Medium containing unbound phage was discarded, the cells washed four times for 5 minutes in PBS, 1% BSA, and bound phage eluted using 2 $\mu$g/mL FGF2. Eluted phage were quantified by titering, as described earlier.

*Evaluation of mitogenicity of MQLPLAT peptide in HUVE cells*

HUVE cells were plated at a density of $1.5\times10^4$ per well in 12-well plates and incubated at 37°C in medium M199 containing 10% FCS, either in the presence or absence of 1 nM of FGF2, MQLPLAT peptide, or VRWEMNL peptide (an irrelevant peptide control). After 48, 72, and 96 hours, viability of the HUVE cell culture was assessed using the MTS assay, as described previously.[20] Media were replaced with 600 $\mu$l of FCS-free M199 containing 100 $\mu$l of CellTiter 96 AQueous One solution reagent (Promega, Madison, WI). Culture plates were incubated at 37°C for 2 hours. Following incubation, 100 $\mu$l of medium was transferred to new 96-well plates and the quantity of formazan product present was determined by measuring the absorbance at 490 nm using a Microplate Autoreader (Bio-Tek Instruments, Winooski, VT).

*Immunofluorescence analysis of binding of phage clones to SKOV3 cells*

SKOV3 cells were grown to confluence in six-well plates in DMEM containing 10% FCS. Cells were subsequently washed with PBS and incubated for 1 hour at 37°C in 1 mL DMEM (containing 2% heat-inactivated goat serum, HINGS, and 2% FCS) containing $2\times10^{11}$ pfu of either MQLPLAT-bearing phage or insertless phage. Medium was then discarded and the cells were washed three times for 5 minutes with DMEM (2% HINGS and 2% FCS), followed by incubation for 1 hour at room temperature with an anti-M13 monoclonal antibody (Amersham Pharmacia, Piscataway, NJ) at a dilution of 1:100 in 1 mL DMEM (2% HINGS and 2% FCS). Antibody containing medium was then discarded and the cells washed twice for 5 minutes with 1 mL DMEM (2% HINGS and 2% FCS), before the addition of a 1:100 dilution of FITC-conjugated $F(ab')_2$ fragment of goat anti-mouse immunoglobulin (DAKO, Ely, UK) in DMEM (2% HINGS and 2% FCS) for 1 hour at room temperature. Cells were then washed twice for 5 minutes in DMEM (2% HINGS and 2% FCS). Finally, 3 mL PBS was added to each well and the cells analyzed and photographed using a Zeiss Axiovert 25 UV fluorescent microscope ($\lambda_{ex}$=495 nm, $\lambda_{em}$=520 nm) (Carl Zeiss, Herts, UK).

In vitro *gene expression of polylysine/DNA complexes targeted with MQLPLAT*

To investigate the *in vitro* transfection efficiency of simple polyelectrolyte-based complexes targeted with the MQLP LAT peptide, the DNA-condensing peptide MQLPLATGG GS$(K)_{16}$ was synthesized (Severn Biotech, Kidderminster, UK) and mixed with plasmid DNA (20 $\mu$g/mL in 10 mM HEPES, pH 7.4) encoding the luciferase reporter gene under the control of the SV40 promoter (pGL3) to an N:P ratio (defined as the number of amino groups in the cationic polymer divided by the number of DNA phosphates) of four. Control complexes were formed using untargeted 4.0-kDa poly(L-lysine) (representing an average of 19 positively charged amino groups per polycation). Complexes were allowed to form for 15 minutes and then complexed DNA (0.5 $\mu$g DNA/25 $\mu$l/well) was added to cells ($2\times10^4$ cells/well in a 48-well plate) in a total volume of 200 $\mu$l of medium. Transfections were performed in the presence and absence of 10% FCS, and in the presence of 100 $\mu$M chloroquine. Cells were then incubated for 5 hours at 37°C in a 5% $CO_2$ humidified environment before medium was discarded and replaced with 500 $\mu$l fresh DMEM containing 10% FCS. Luciferase expression was evaluated 24 hours post transfection. Culture medium was discarded and the cell lysates harvested after incubation of cells for 30 minutes at room temperature in 200 $\mu$l of 1× lysis reagent (Promega). Fifty microliters of the resulting lysate was diluted in 100 $\mu$l of luciferase reaction buffer (in mM: 20 glycoglycine, 1 $MgCl_2$, 0.1 EDTA, 3.3 DTT, 0.5 ATP, 0.27 coenzyme A lithium salt) and the luminescence integrated over 10 seconds in a Lumat LB 9507 (EG & G Berthold, Bundoora, Australia). Results are expressed as relative light units (RLU) per milligram of cell protein, determined by BCA assay (Pierce, Chester, UK).

*Phage-binding assay on surgically resected human specimens*

We have developed an *ex vivo* human organ system using surgically resected specimens to assess binding of phage to human tumors in a clinically relevant setting. Patients selected for this study were suffering from operable gastric adenocarcinoma. Immediately following surgical resection of the stomach, cannulae were inserted into the left gastric artery and vein. The vessels were then washed with 100 mL of PBS. A total of $2\times10^9$ pfu of selected phage, diluted in 5 mL PBS, were then injected into the left gastric artery through the cannula and allowed to bind for 5 minutes. Unbound phage were washed away using 100 mL of PBS injected via the cannula into the left gastric artery. The stomach was then opened along the greater curvature and samples of tumor and adjacent normal gastric tissue were collected. Samples were weighed and homogenized using a motor-driven Teflon-on-glass homogenizer. Phage were quantified by titering multiple dilutions of the homogenate, as described above. Results are presented as plaque-forming units of associated phage per milligram tissue. The informed consent of patients and approval of the South Birmingham

Local Research Ethics Committee were obtained before experimentation.

### *Statistics*

Results are represented as the mean and standard deviation of the data from three independent experiments, with significance of differences evaluated using Student's *t* test.

## Results

### *Iteration of consensus oligopeptide sequence binding FGF receptors*

Five rounds of biopanning were performed using a phage library of clones, which each exhibit a random linear heptapeptide fused with the pIII coat protein, to identify peptide sequences capable of binding to FGF receptors. The phage library was incubated with cells for 1 hour at 4°C, washed, and subsequently eluted by competition with excess free FGF2. After five rounds of selection, eight times more phage were recovered from each cell than when using the naive library.

Several individual phage plaques were picked, their DNA isolated and sequenced after each round of panning. The corresponding amino acid sequences for the displayed heptapeptides were deduced from the DNA sequences. The derived sequences from the first and second round displayed no discernible similarity, whereas those isolated after the third, fourth, and fifth rounds displayed significant consensus (Fig 1). The most common consensus sequence showed enrichment of a methionine and a proline residue at the N-terminal and fourth position (from N-terminus), respectively. This motif (MXXP, where X represents any amino acid) was found in 27% of sequences in round three, 36% in round four, and 50% in round five. Notably, the consensus sequence was iterated independently in all three replicate series of biopanning. In fifth round MXXP sequences, glutamine occupied the second position in 40% of cases, whereas leucine occupied the third position in 47% of cases.

### *Quantification of binding of phage clones to FGF receptors on cells*

To assess the binding of selected phage clones to FGF receptors, each clone was incubated at 4°C with 911, SKOV3, B16F10, PC3, and Cos7 cells. Cells were washed, and phage were eluted using excess FGF2, collected, and titered. The phage clone displaying the sequence MQLPLAT showed the greatest FGF2-displaceable binding on 911, SKOV3, and B16F10 cells (Fig 2), with the other phage clones (MQPPGSI and MQTPLAP) showing substantial, but less, binding in each case. MQLPLAT phage bound to 911 cells 66 and 36 times better than insertless and VRWEMNL phage, respectively. In SKOV3, B16F10, and PC3 cells, MQLPLAT phage bound 343, 65, and 24 times more than insertless phage, respectively. Binding was also evaluated in Cos7 cells, a cell line known to be deficient in FGF receptors.[21] Interestingly, none of the phage showed any significant binding, all showing levels of association that were similar to insertless phage (Fig 2E).

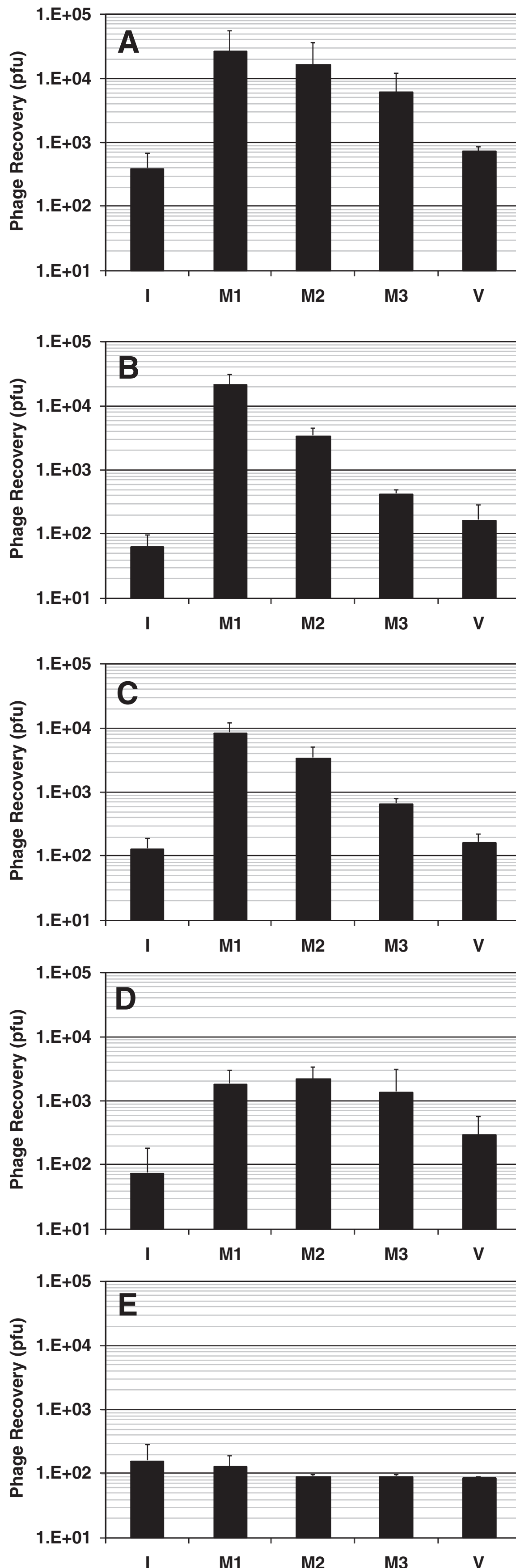
These data were compatible with phage bearing the MXXP motif binding to receptors for FGF. The sequence MQLPLAT was selected for further investigation.

### *Measurement of affinity of binding of MQLPLAT phage*

The binding affinity of the MQLPLAT phage clone was determined by incubating SKOV cells with MQLPLAT phage concentrations ranging from $10^7$ to $10^{12}$ pfu/mL. Cells were incubated at 4°C for 30 minutes, then washed (4×5 minutes) and cell-associated phage were titered. The number of phage binding increased with concentration and appeared to plateau when the incubation concentration was

| | Ex 1 | Ex 2 | Ex 3 |
|---|---|---|---|
| 1st Round | Q L T M R L Q | Q N L R G D S | T R L P H N P |
| | Y G R E T S T | E A P A Q L P | R Y L V E S P |
| | S A E S R H Y | A G P T W A H | W P I R P L S |
| 2nd Round | R P L P Y P D | M A T P R N S | M M L H P Y A |
| | L A S P N G H | M A A R A P M | L P S K L L D |
| | G A A S T R Y | A A A H A L F | L V T A P R L |
| 3rd Round | M A N P G R A | M Q L P K S L | M A A A P F S |
| | M Q P S L V H | M G K P L H S | M A T A S Q L |
| | M T T Y P L S | M S T P S K V | A A M P I I P |
| | M T T T M Q L | L A Q F P S P | L T T V P P Y |
| | M A V D P L R | A P F T H L R | F A K D Y S R |
| 4th Round | M Q S P P F H | M T L P M L Q | D N P R G F S |
| | M T P P A S I | M S Q P P L A | F H P L N L S |
| | M T Y P R E F | M W P T I T P | N N M A P H L |
| | S A R P V P L | T A L S R P P | V N R L S E N |
| | S P R P Y S T | S A T N Q A P | |
| 5th Round | M Q L P P D T | M Q L P V Y P | M Q L P L A T |
| | M Q T P L A P | M Q M P A V N | M G L P L G K |
| | M S L P L L P | M Q P P G S I | M G L P P S P |
| | M T A P P A R | M A T P G L G | M T L P A A S |
| | M G N P I N H | M A V P Q P I | M T S P P T P |
| | M Q I S G H P | M S A R F P P | M S L S P P Q |
| | L T R P V S P | L T R P P W G | M G T L P T T |
| | L A A P P K A | F T K T T S P | L T L P P S P |
| | D Q S R M P T | G P T R I L K | L S P P P T H |
| | L A K V P P Q | V R W E M N L | S T H P L Q T |

**Figure 1** Identification of candidate peptides binding FGF receptors. DNA was isolated from individual plaques from each round of selection. The isolated DNA was sequenced across the insert region of pIII and the displayed peptide deduced. Binding peptides were iterated from each of five rounds of selection and from each of triplicate experiments run in parallel (Exp. 1–3). The appearance of M, Q, L, and P at first, second, third, and fourth amino acid positions from N-terminus are highlighted.




about $10^{12}$ pfu/mL (Fig 3). The data were analyzed by fitting the general logistic equation: $[\text{bound}]=B_{max}\cdot[\text{free}]^n/([\text{free}]^n+K_d{}^n)$. Analysis gave $K_d=1.51\times10^{11}$ pfu/mL (equivalent to $2.51\times10^{-10}$ M phage), $B_{max}$ (the number of binding sites)$=7.01\times10^8$/well and $n$ (the pseudo Hill coefficient)=2.32, suggesting a component of positive cooperativity in the binding. The binding affinity for MQLPLAT phage is quite high, although substantially lower than that reported for FGF2 itself.[6] Each phage presents five MQLPLAT peptides;[15] hence, the binding measured is likely to contain a significant component of avidity, with individual peptides showing lower binding strengths. Nevertheless, many therapeutic targeting studies will involve multiple peptides on each vector, and this should enable very high binding affinities to be attained.

### *Influence of the concentration of FGF2 added during elution on the efficiency of phage recovery*

The influence of FGF2 added during elution on the numbers of phage displaced was evaluated. Following incubation of 911 cells with MQLPLAT phage, cells were washed with a range of FGF2 concentrations (0–10 $\mu$g/mL) and eluted phage were titered. Low concentrations of FGF2 mediated release of only small numbers of phage, although release was strongly dose dependent (Fig 4A). Increasing the FGF2 concentration from 1 to 10 $\mu$g/mL led to a 2.4-fold increase in the numbers of phage released. Although all phage within the clone are identical, it is feasible that they bind with different strengths depending on how many of their peptides can bind simultaneously to FGF receptors on the cell surface, providing variable binding avidity.

### *Evaluation of specificity of phage clones for binding FGF receptors*

The feasibility for competitive displacement of phage from 911 cells by adding FGF2 was compared for MQLPLAT, MQPPGSI, and MQTPLAP phage. Cells were incubated with phage clones, washed with FGF2 (2 $\mu$g/mL), and released phage were titered, along with phage remaining cell-associated. All three phage clones were displaced over 90% by treatment of the cells with FGF2 (data not shown). This suggests that each of these peptides binds to regions on FGF receptors.

The specificity of the MQLPLAT phage binding to FGF receptors was evaluated by blockading cell binding using FGF2 or free peptides. Accordingly, 911 cells were

**Figure 2** Phage-binding assay on cell lines. To assess the affinity of selected phage clones for binding FGF receptors, $5\times10^7$ pfu of selected phage clones displaying the consensus motifs MQLPLAT (M1), MQPPGSI (M2), MQTPLAP (M3), nonconsensus peptide insert VRWEMNL (V) or no peptide insert (insertless, I) were incubated on the cell lines 911 **(A)**, SKOV3 **(B)**, B16F10 **(C)**, PC3 **(D)**, and Cos7 **(E)** for 1 hour at 4°C. Following extensive washing, remaining bound phage were eluted with 10 $\mu$g/mL FGF2 and quantified by plaque assay.

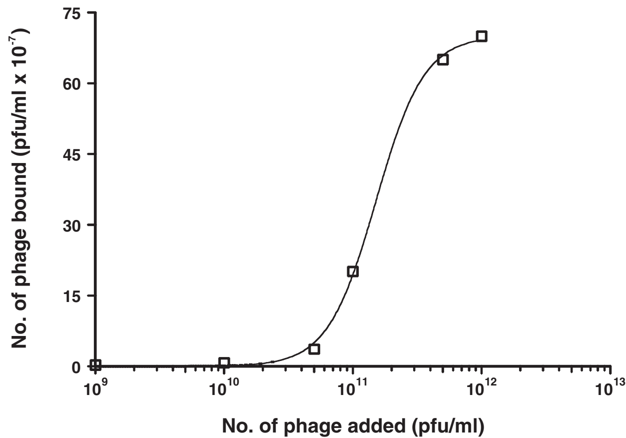



**Figure 3** Determination of binding affinity of MQLPLAT phage. MQLPLAT phage were incubated with SKOV cells at concentrations ranging from $10^7$ to $10^{12}$ pfu/mL. Cells were incubated at 4°C for 30 minutes, then washed (4×5 minutes) and cell-associated phage were titered. The data were analyzed by fitting the general logistic equation: $[\text{bound}]=B_{\max}\cdot[\text{free}]^n/([\text{free}]^n+K_d{}^n)$. Analysis gave $K_d=1.51\times10^{11}$ pfu/mL (equivalent to $2.51\times10^{-10}$ M phage), $B_{\max}$ (the number of binding sites)$=7.01\times10^8$/well, and $n$ (the pseudo Hill coefficient)=2.32.

preincubated with either FGF2 or with the peptides MQLPLAT or VRWEMNL. MQLPLAT phage were then added, cells incubated for a further 1 hour, and washed. Phage were eluted by adding FGF2 and titered. It was found that preincubation of cells with FGF2 or MQLPLAT peptide significantly diminished the number of MQLPLAT phage binding to the cells ($P<.05$), with phage numbers recovered falling to 6% and 12% that of nonincubated controls, respectively (Fig 4B). Preincubation with the VRWEMNL peptide had no significant influence on phage recovery.

The possibility that phage clones are binding to heparin binding sites on the cell surface, rather than to specific FGF receptors, was evaluated by using heparin to elute phage from the cell surface. Incubation of MQLPLAT phage with 911 cells, followed by washing with heparin (up to 50 $\mu$M) led to release of less than $1\times10^3$ pfu, compared with $2.6\times10^4$ pfu eluted using 0.5 $\mu$M FGF2. Heparin had only a very limited ability to release MQLPLAT phage from the cells, suggesting the peptides are not binding significantly to heparin-binding sites.

*Examination of cell association of phage by fluorescence microscopy*

Phage binding to SKOV3 cells was assessed using immunofluorescence. Cells were incubated for 1 hour at 37°C with MQLPLAT phage, or insertless phage as a control. Examination by microscopy showed strong fluorescence for MQLPLAT phage, associated with the majority of the cells. In contrast, the insertless phage gave no discernible signal (Fig 5), verifying the ability of MQLPLAT to mediate attachment of phage to SKOV3 cells *in vitro* and suggesting that the peptide interacts with a component of the cell surface as proposed.

*Assessment of possible mitogenicity of MQLPLAT peptide*

The possibility that MQLPLAT may activate FGF receptors and mediate a mitogenic response was evaluated by quantifying its stimulation of growth in HUVE cells. Cells were incubated with 1 nM MQLPLAT, VRWEMNL peptides, and FGF2. Cell viability (reflecting total cell number) was monitored after 48, 72, and 96 hours using the MTS assay. The presence of FGF2 caused a large increase in cell growth after 48–96 hours, compared with untreated cells, whereas the peptides MQLPLAT and VRWEMNL showed no discernible stimulation of cell growth (Fig 6). This lack of a mitogenic effect for MQLPLAT is important as it makes its administration to cancer patients more justifiable.

*Enhancement of gene expression by using MQLPLAT to target polyelectrolyte DNA complexes*

The ability of MQLPLAT peptide to promote transfection of cells was assessed *in vitro* using a conjugate of MQLPLAT linked, via a GGGS spacer, to a 16-mer of L-lysine

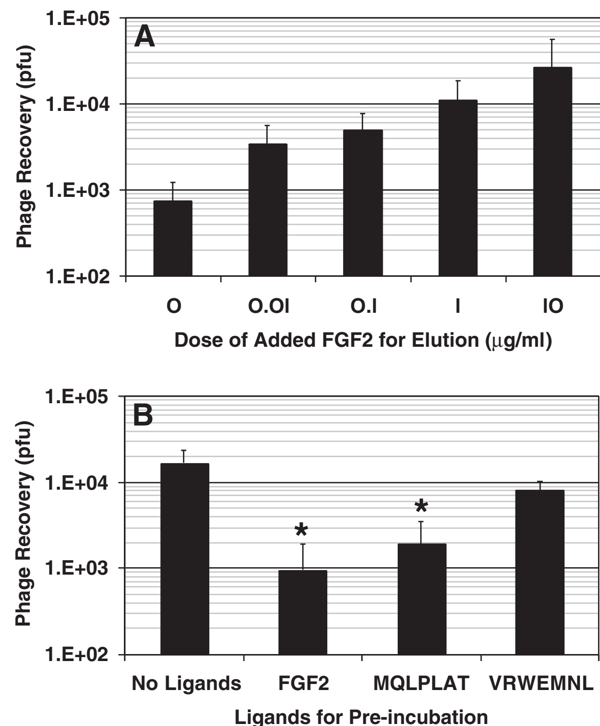



**Figure 4** Evaluation of the binding specificity of MQLPLAT phage to 911 cells. MQLPLAT phage ($5\times10^7$ pfu) were allowed to bind to 911 cells for 1 hour at 4°C. Following extensive washing, residual binding phage were eluted using a range of concentrations of FGF2 (0–10 $\mu$g/mL) and eluted phage were quantified by plaque assay **(A)**. To investigate whether this binding could be competitively inhibited, 911 cells were preincubated with either 0.5 $\mu$M FGF2, or 0.5 $\mu$M of the synthesized peptides MQLPLATGGGS, or VRWEMNLGGGS. Media were subsequently discarded and $5\times10^7$ pfu of MQLPLAT phage were allowed to bind to the cells for 1 hour at 4°C. After washing with PBS (1% BSA), bound phage were eluted with 2 $\mu$g/mL FGF2 and quantified by plaque assay **(B)**. (*$P<.05$ compared to preincubation with no ligands.)

(MQLPLATGGGS$(K)_{16}$). The resulting conjugate showed good activity to bind DNA electrostatically and, following application to 911 and B16F10 cells, it mediated high levels of reporter transgene expression (Fig 7). In the absence of serum, the MQLPLAT-containing DNA complexes achieved $4.0\times10^4$ and $2.6\times10^6$ RLU/mg protein, equivalent to 80- and 3000-fold increases over simple $K_{16}$/DNA complexes (without MQLPLAT), on the two cell types, respectively. Less activity was seen in the presence of 10% FCS, although the MQLPLAT complexes still achieved 20- and 40-times greater transfection, than the control complexes, in the two cell types.

### *Evaluation of MQLPLAT phage binding to human tumors in surgically resected gastric cancer*

MQLPLAT phage ($2\times10^9$ pfu) were injected via the tumor feeding artery into clinical cancer specimens immediately following their surgical resection. Case 1 was a 74-year-old man receiving total gastrectomy for advanced gastric carcinoma (T2N2M0) in the upper third of the lesser curvature of the stomach. The tumor was an intestinal-type adenocarcinoma, 40 mm in diameter. Case 2 was a 67-year-old man who received esophagogastrectomy for advanced gastric carcinoma (T3N1M0) in the esophagogastric junction. The tumor was an intestinal-type adenocarcinoma, 40 mm in diameter. Pieces of the tumor, and adjacent normal tissues, were isolated and contained phage were titered. The MQLPLAT phage were found to accumulate in tumor tissue approximately nine and three times more than in adjacent normal gastric mucosa in cases 1 and 2, respectively (Fig 8). A third tumor, isolated from a 69-year-old female patient who received total gastrectomy for advanced gastric carcinoma (T3N2M0) in the upper third of the lesser curvature of the stomach, was used for evaluation of the distribution of insertless phage, as a control. Here the insertless phage also showed increased accumulation in the tumor, but only reached 1.7 times that in the adjacent normal tissue. This slight accumulation of insertless phage in tumor tissue is likely to represent greater vascular density in the tumor, and may also result from increased leakiness of capillaries within the tumor. The 3- to 9-fold greater accumulation of MQLPLAT phage in tumor, compared with adjacent normal tissue, is likely to reflect a component of this nonspecific accumulation, coupled with the effect of MQLPLAT phage in binding directly to tumor-associated FGF receptors.

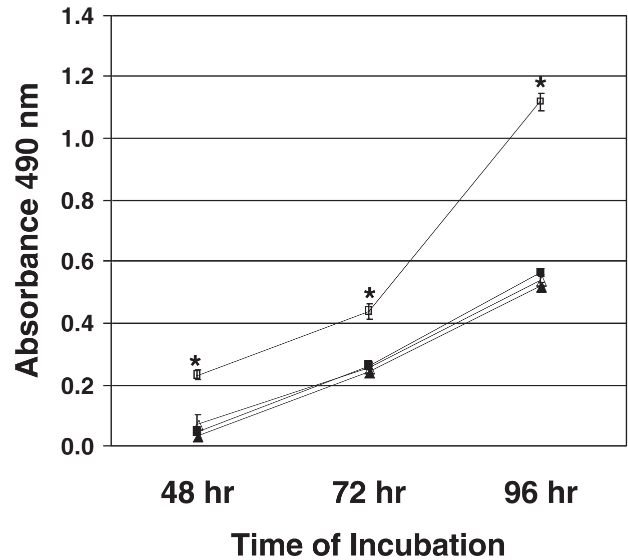



**Figure 6** Evaluation of mitogenicity of MQLPLAT peptide in HUVE cells. HUVE cells were plated at a density of $1.5\times10^4$ per well in 12-well plates and incubated in medium M199 containing 10% FCS, either in the presence or absence of 1 nM of FGF2 (□), MQLPLAT peptide (■) or VRWEMNL peptide (▲), an irrelevant peptide control). Results with untreated cells are also shown (△). After 48, 72, and 96 hours, viability of the HUVE cell culture was assessed using the MTS assay, as described in *Materials and methods*. (*$P<.01$ compared to untreated cells.)

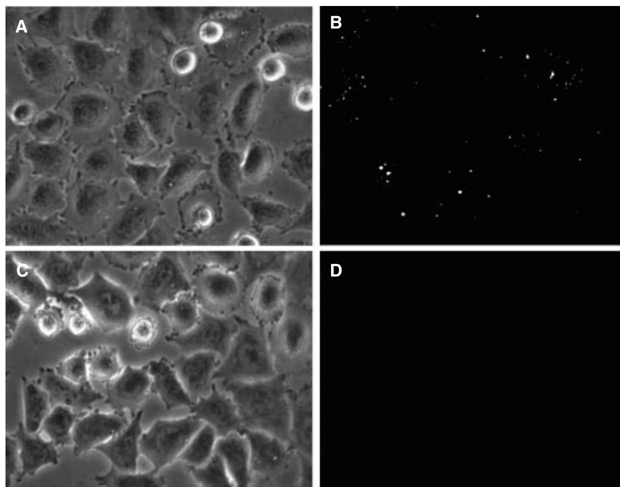



**Figure 5** Immunofluorescence analysis of MQLPLAT phage binding to SKOV3 cells. SKOV3 cells were incubated with $2\times10^{11}$ pfu of either MQLPLAT phage or insertless phage for 1 hour at 37°C. Following extensive washing of the cells, bound phage were detected *in situ* using an anti-M13 monoclonal antibody and an FITC-conjugated $F(ab')_2$ fragment of goat anti-mouse immunoglobulin. **Left panel:** Depicts phase contrast images. **Right panel:** Shows immunofluorescence images of the same samples (magnification: ×320). Significant signals were detected when cells were incubated with MQLPLAT displaying phage **(A and B)** whereas no significant signal was detected when cells were incubated with insertless phage **(C and D)**.

## Discussion

Peptide phage display libraries represent a powerful means to identify sequences binding specific targets, without the complexities of rational peptide design. Repeated enrichment of the library by target-based selection provides an opportunity to identify consensus sequences mediating the most effective interaction with the target. In this study, we have used phage display to identify peptide sequences capable of binding to receptors for FGF, aiming to enable the use of simple, serum-stable ligands for delivery of agents, including

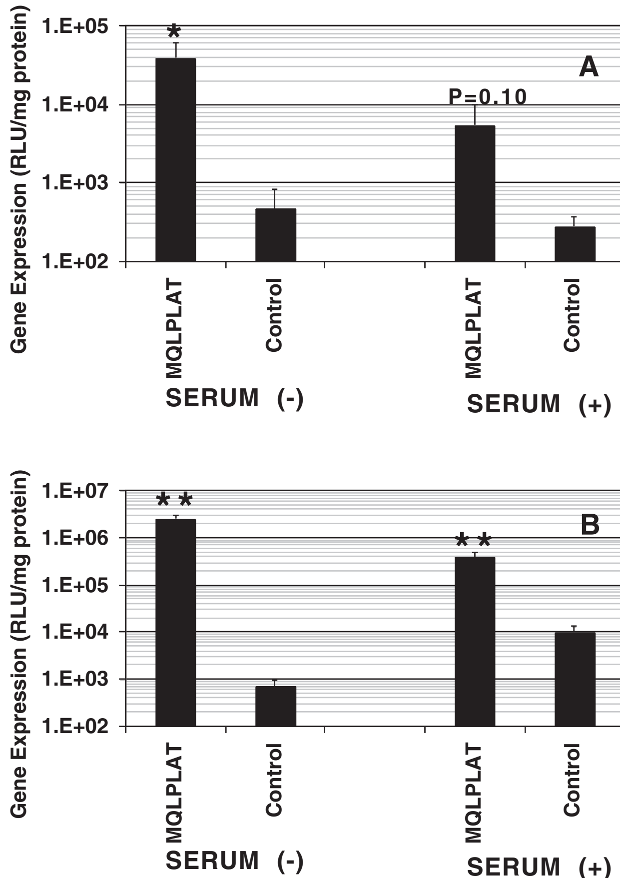



**Figure 7** *In vitro* transfection efficiency of poly(L-lysine)/DNA complexes targeted with MQLPLAT. The peptide MQLPLAT-GGGS(K)$_{16}$ was used to condense DNA encoding the luciferase reporter gene. Poly(L-lysine) (4.0-kDa) was also used to condense the same DNA as a nontargeted control containing approximately the same number of amino groups as the synthesized peptide. Cells were transfected with 0.5 $\mu$g DNA/well for 5 hours at 37°C in the presence and absence of serum and in the presence of 100 $\mu$M chloroquine. After 5 hours, the media were discarded and replaced with fresh media containing 10% FCS. Reporter gene expression was analyzed 24 hours later and corrected for the total protein content. Results are expressed as relative light units (RLU) per milligram of cellular protein. **A:** 911 cells. **B:** B16F10 cells. (*$P<.05$ and **$P<.01$ compared to respective controls.)

genes, to FGF receptors. Over five rounds of selection the consensus sequence MXXP was identified and the lead peptide sequence MQLPLAT was examined in depth.

MQLPLAT phage showed a remarkable ability to bind to a range of cells-bearing receptors for FGF,[4,6,7] achieving over 300 times the binding of insertless phage. The ability of free FGF2 to displace over 90% of cell-bound MQLPLAT phage supported the interpretation that the peptide is binding to FGF receptors. Preincubation of 911 cells with FGF2 or free MQLPLAT peptides all gave significant inhibition of binding. In contrast, the smaller number of MQLPLAT phage binding to PC3 cells was consistent with known low levels of receptors for FGF in these cells,[7] and the absence of binding to Cos7 cells was also consistent with known deficiencies in receptor expression.[21]

FGF2 is known to bind to both low-affinity receptors (heparan sulfate proteoglycans or heparin glycosaminoglycans) and high-affinity receptors (FGF receptor family tyrosine kinases),[6,22] and ligand activation of FGF receptors requires formation of a complex between the ligand, the receptor, and cell surface heparan.[22] Therefore, it was important to ascertain whether MQLPLAT phage might be binding to cell surface heparans. In fact, heparin showed no significant ability to displace bound MQLPLAT phage, indicating this is not an important means of attachment. The nonbasic nature of MQLPLAT would anyway seem to render this an unlikely possibility. Together these data suggest that MQLPLAT is binding to high-affinity transmembrane receptors for FGF.

Molecules previously identified that bind FGF receptors include members of the FGF family, peptide fragments of FGF2,[23,24] peptide sequences with homology to fragments of FGF2,[25] and completely synthetic ligands.[22,26] Sequences of FGF1–23 were obtained from the database of the National Center for Biotechnology Information (Bethesda, MD.) Sequence analysis showed no homology of MQLPLAT with any known FGF molecules or known alternative ligands. Nevertheless, the ability of FGF2 to block attachment of MQLPLAT phage suggests either that

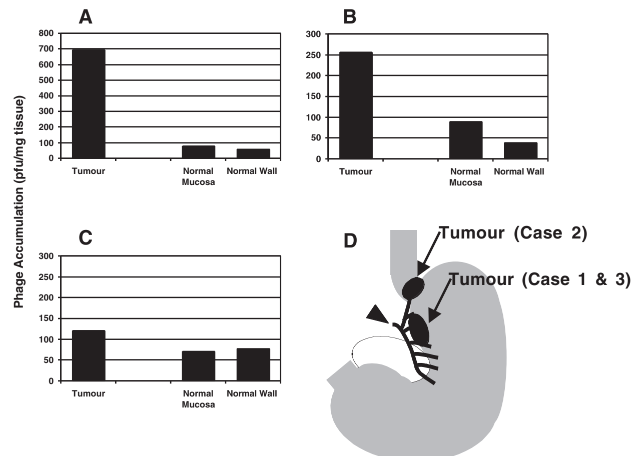



**Figure 8** Evaluation of MQLPLAT phage binding to human tumor in surgically resected gastric cancers. To assess binding of MQLPLAT-inserted phage to human tumor, we have developed an *ex vivo* human organ system using surgically resected specimens. Immediately following resection of stomach from patients, cannulae were inserted into the left gastric artery (arrow) and washed out with PBS. A total of $2\times10^9$ pfu of MQLPLAT-phage (case 1 and 2) or insertless phage (as a control, case 3) in PBS were injected into the left gastric artery and incubated for 5 minutes to allow binding. The stomach was then opened and sections of tumor and normal gastric tissue were collected for assessment of phage binding. Case 1 **(A)** received a total gastrectomy for advanced gastric carcinoma (moderately differentiated adenocarcinoma) in the upper third of the lesser curvature of the stomach, whereas case 2 **(B)** received esophagogastrectomy for advanced gastric carcinoma (well-differentiated adenocarcinoma) in the esophagogastric junction. Case 3 **(C)** received a total gastrectomy for advanced gastric carcinoma (moderately differentiated adenocarcinoma) in the upper third of the lesser curvature of the stomach. **D:** Shows the anatomical location of the tumors in each case.

the peptide is binding to the ligand-binding domain of the receptor, or that its binding site is occluded by allosteric effects following binding of FGF2.

Two peptides that bind to FGF receptors but have no homology with FGFs have been reported previously.[22,26] Both were identified using phage display, and phage binding to FGF receptors were identified by binding to beads bearing the extracellular domain of FGF receptors or to Sf9 cells overexpressing full-length, transmembrane FGF receptors. In our study, binding phage were eluted from 911 cells by adding an excess of FGF2. Our studies employed a linear heptapeptide insert library, whereas the other studies used linear 26-residue[22] or 13- to 43-residue[26] peptide libraries. Both of these differences and the existence of many receptor subtypes for FGF[27] may contribute to the different sequences obtained in each study.

MQLPLAT peptide showed no significant ability to mediate signaling (data not shown) or mitogenesis *in vitro* following binding to FGF receptors. This is as expected because the peptide is monomeric and unable to promote receptor cross-linking. Indeed, it may be interesting to speculate that appropriate peptide dimers might be able to mediate receptor cross-linking, although these experiments have not been performed.

MQLPLAT peptide, synthesized as part of a cationic DNA-binding construct, showed good activity to deliver plasmid DNA into cells in culture and enable transgene expression. Receptors for FGF are known to be suitable targets for gene delivery, using both viral and synthetic vectors; hence, the result obtained is compatible with MQLPLAT binding cell surface FGF receptors. Presentation of several MQLPLAT peptides as part of the same complex may enable binding to several receptors simultaneously, increasing the strength of the interaction and also increasing the likelihood of internalization into the cell by endocytosis.

When MQLPLAT phage were injected into the tumor-feeding artery in human patients, the phage were recovered in tumor approximately nine and three times more than in adjacent normal tissue. There may be several reasons for this difference; for example, tumor may have a denser and more complicated vasculature than normal tissue. A control experiment was therefore performed, where insertless phage was injected into a comparable tumor. This phage showed a slight (1.7-fold) accumulation in tumor tissue compared with adjacent normal tissue, probably due to nonspecific accumulation in tumor-associated vasculature. The greater tumor accumulation of the MQLPLAT phage is likely to reflect its binding to FGF receptors within the tumor, either present on tumor cells invading inside the vascular lumen, or expressed selectively on tumor-associated endothelium. Such selective expression could be due to up-regulation of the receptors during proliferation of tumor-associated endothelial cells,[28] or might reflect increased anatomical availability due to decreased tight junctions between the endothelial cells.

It is likely that the small size of the MXXP-containing heptapeptides restricts their binding affinity. The corresponding phage can show higher strength binding due to simultaneous binding of several peptides leading to a high binding avidity, and the $K_d$ for binding of the MQLPLAT phage to FGF receptors on SKOV cells was measured as $2.51 \times 10^{-10}$ M. This is a high binding strength and illustrates that even relatively low-affinity ligands can be very useful in targeted delivery of pharmaceuticals, provided several copies of the targeting ligand can be presented simultaneously. Whereas high-affinity ligands will bind strongly to individual receptors, vectors bearing clusters of low affinity ligands will only bind to surfaces that carry an appropriate density of targets. Because many tumor-associated target molecules (including receptors for FGF, carcinoembryonic antigen, etc.) have expression that is essentially up-regulated from that of normal cells, it is essential that vectors can distinguish between cells bearing high- and low-density targets to avoid delivery to the "sink" of normal cells. MQLPLAT, and other peptides iterated by phage display, may be ideal for this purpose. Indeed, it is possible that the high level of phage recovered from tumor following intra-arterial infusion into resected clinical specimens, compared with normal tissue, may reflect this type of selectivity.

FGF2 has been used as a targeting ligand for delivery of both synthetic[2] and viral vectors[3,4] to increase selectivity and potency of the vectors. However, FGF2 is a relatively large protein[9] and has some pharmacological limitations, notably a short plasma half-life, unwanted interactions with serum components and high costs of manufacture. In contrast, MQLPLAT is a simple peptide with FGF receptor-binding activity and may show several pharmacological advantages. Apart from all the issues of delivery, stability, and cost, MQLPLAT should have a superior safety profile because, unlike FGF2, it is not mitogenic. It seems undesirable to administer potent mitogens to patients with cancer. Several well-defined systems are gradually being developed to enable target-selective delivery of genes to cells. Apart from fully defined synthetic systems, polymer-coated "stealth" adenovirus can now be used for target-selective infection.[1] The MQLPLAT peptide identified here seems ideal for incorporation onto the surface of a stealth adenovirus to gain selective retargeting to receptor-positive cells. The density of ligand incorporation could be manipulated to maximize selectivity for the target cells.

Finally, the clinical arterial perfusion system employed here can make an important contribution to the search for vectors enabling cancer-selective gene therapies. Methods enabling assessment of the activity of vectors in human organs have not been developed before, except during clinical trials, and reliable data using relevant human tumor tissues have been difficult to obtain. Use of tissues immediately post clinical resection provides a simple and useful means to gain clinically relevant information with no experimental implications for the patient.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## References

1. Fisher KD, Stallwood Y, Green NK, et al. Polymer-coated adenovirus permits efficient retargeting and evades neutralising antibodies. *Gene Ther.* 2001;8:341–348.
2. Larocca D, Kassner PD, Witte A, et al. Gene transfer to mammalian cells using genetically targeted filamentous bacteriophage. *FASEB J.* 1999;13:727–734.
3. Goldman CK, Rogers BE, Douglas JT, et al. Targeted gene delivery to Kaposi's sarcoma cells via the fibroblast growth factor receptor. *Cancer Res.* 1997;57:1447–1451.
4. Gu DL, Gonzalez AM, Printz MA, et al. Fibroblast growth factor 2 retargeted adenovirus has redirected cellular tropism: evidence for reduced toxicity and enhanced antitumor activity in mice. *Cancer Res.* 1999;59:2608–2614.
5. Kasahara N, Dozy AM, Kan YW. Tissue-specific targeting of retroviral vectors through ligand–receptor interactions. *Science.* 1994;266:1373–1376.
6. Sosnowski BA, Gu DL, D'Andrea M, et al. FGF2-targeted adenoviral vectors for systemic and local disease. *Curr Opin Mol Ther.* 1999;1:573–579.
7. Chandler LA, Sosnowski BA, Greenlees L, et al. Prevalent expression of fibroblast growth factor (FGF) receptors and FGF2 in human tumor cell lines. *Int J Cancer.* 1999;81:451–458.
8. Basilico C, Moscatelli D. The FGF family of growth factors and oncogenes. *Adv Cancer Res.* 1992;59:115–165.
9. Kurokawa T, Sasada R, Iwane M, et al. Cloning and expression of cDNA encoding human basic fibroblast growth factor. *FEBS Lett.* 1987;213:189–194.
10. Barry MA, Dower WJ, Johnston SA. Toward cell-targeting gene therapy vectors: selection of cell-binding peptides from random peptide-presenting phage libraries. *Nat Med.* 1996;2:299–305.
11. Pasqualini R, Ruoslahti E. Organ targeting *in vivo* using phage display peptide libraries. *Nature.* 1996;380:364–366.
12. Arap W, Pasqualini R, Ruoslahti E. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. *Science.* 1998;279:377–380.
13. Trepel M, Grifman M, Weitzman MD, et al. Molecular adaptors for vascular-targeted adenoviral gene delivery. *Hum Gene Ther.* 2000;11:1971–1981.
14. Pasqualini R, Koivunen E, Ruoslahti E. Alpha v integrins as receptors for tumor targeting by circulating ligands. *Nat Biotechnol.* 1997;15:542–546.
15. New England Biolabs Inc. Ph.D-7 Phage Display Peptide Library Kit: Instruction Manual. 1999 Version 2.5.
16. Monaci P, Urbanelli L, Fontana L. Phage as gene delivery vectors. *Curr Opin Mol Ther.* 2001;3:159–169.
17. Larocca D, Witte A, Johnson W, et al. Targeting bacteriophage to mammalian cell surface receptors for gene delivery. *Hum Gene Ther.* 1998;9:2393–2399.
18. Poul MA, Marks JD. Targeted gene delivery to mammalian cells by filamentous bacteriophage. *J Mol Biol.* 1999;288:203–211.
19. Fallaux FJ, Kranenburg O, Cramer SJ, et al. Characterization of 911: a new helper cell line for the titration and propagation of early region 1–deleted adenoviral vectors. *Hum Gene Ther.* 1996;7:215–222.
20. Wong JK, Kennedy PR, Belcher SM. Simplified serum- and steroid-free culture conditions for high-throughput viability analysis of primary cultures of cerebellar granule neurons. *J Neurosci Methods.* 2001;110:45–55.
21. Johnston CL, Cox HC, Gomm JJ, et al. bFGF and aFGF induce membrane ruffling in breast cancer cells but not in normal breast epithelial cells: FGFR-4 involvement. *Biochem J.* 1995;306:609–616.
22. Ballinger MD, Shyamala V, Forrest LD, et al. Semirational design of a potent, artificial agonist of fibroblast growth factor receptors. *Nat Biotechnol.* 1999;17:1199–1204.
23. Baird A, Schubert D, Ling N, et al. Receptor- and heparin-binding domains of basic fibroblast growth factor. *Proc Natl Acad Sci USA.* 1988;85:2324–2328.
24. Ray J, Baird A, Gage FH. A 10-amino acid sequence of fibroblast growth factor 2 is sufficient for its mitogenic activity on neural progenitor cells. *Proc Natl Acad Sci USA.* 1997;94:7047–7052.
25. Yayon A, Aviezer D, Safran M, et al. Isolation of peptides that inhibit binding of basic fibroblast growth factor to its receptor from a random phage-epitope library. *Proc Natl Acad Sci USA.* 1993;90:10643–10647.
26. McConnell SJ TV, Spinella DG. Isolation of fibroblast growth factor receptor binding sequences using evolved phage libraries. *Comb Chem High Throughput Screening.* 1999;2:155–163.
27. Ornitz DM, Xu J, Colvin JS, et al. Receptor specificity of the fibroblast growth factor family. *J Biol Chem.* 1996;271:15292–15297.
28. Ueki T, Koji T, Tamiya S, et al. Expression of basic fibroblast growth factor and fibroblast growth factor receptor in advanced gastric carcinoma. *J Pathol.* 1995;177:353–361.