Journal of Drug Targeting, January 2005; 13(1): 7–18

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Phage display selection for cell-specific ligands: Development of a screening procedure suitable for small tumor specimens

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(Received 9 July 2004; revised 12 October 2004; accepted 12 October 2004)

Abstract
Phage display technology has been widely used for developing tumor-targeting agents. Most of the efforts were directed towards identifying phage-displayed ligands against cancer-relevant purified targets and cancer cell lines. Whole cell screening procedures typically use a relatively large sample size and are not ideally suited for complex tumor tissues. We describe here a screening protocol that is suitable for non-adherent tumor cells from biopsy specimens. It requires only ~20,000 cells/round for biopanning and ~10,000 cells/well for subsequent clone binding assessment by ELISA. We standardized the newly developed protocol using erbB2-overexpressing SKBR3 breast cancer cells and compared the results with conventional protocols employing about 10-times more plate-adhered fixed or live cells. The selection rate of SKBR3-binding clones from biopanning ~20,000 non-adherent SKBR3 cells by our filter cup protocol was comparable to that obtained from using ~200,000 plate-adhered cells. Assessment of clones selected from different phage libraries showed that clones from fixed or live cells, adherent or non-adherent cells, either biopanned in filter cup or plate share specific motifs and binding properties. Some of the clones from each biopanning protocol bound to purified erbB2 and shared motifs with erbB family of receptors and their known ligands. These results demonstrated that the protocol developed in this study was capable of selecting cell-specific ligands using relatively small numbers of cells. Screening cells from a fresh human breast cancer specimen using our protocol showed enrichment of tumor binding clones at successive rounds of selection and some of the selected clones were tumor-specific in comparison to normal breast cells. These protocols have direct application to screen for tumor-binding ligands with small tumor tissue specimens.

Keywords: *Targeted therapy, tumor, phage display, ligands*

Introduction

Developing medications that can target cancer cells without harming the rest of the body is one of the most important challenges for cancer researchers today. It is believed that an ability to home in on cancer cells makes targeted therapy both more effective and less likely to cause side effects (Arap et al. 1998, Gibbs 2000, Oh et al. 2004). Identifying tumor-specific ligands that can be used for directed delivery of drug and gene therapies is a critical step in the success of this approach (Nilsson et al. 2000, Yip and Ward 2002, Aina et al. 2002, Guillemard and Saragovi 2004, Siemann et al. 2004). The screening and selection of bioactive peptides and antibodies by phage display technology is increasingly used to identify sequences that interact specifically and with high affinity with a particular target. The successful application of phage displayed libraries has been used to identify peptides and antibodies against an incredibly diverse population of targets (Smith and Petrenko 1997).

A common selection strategy is to screen for phage displayed ligands to a target comprised of a relevant purified subcellular component anchored to a well or a bead. We have previously used this strategy to identify a series of novel ligands for Grb2, Grb7 and erbB2 by screening purified proteins with phage displayed libraries (Oligino et al. 1997, Pero et al. 2002, Pero et al. 2004). However, the approach of

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ISSN 1061-186X print/ISSN 1029-2330 online © 2005 Taylor & Francis Group Ltd
DOI: 10.1080/10611860400020464

developing ligands only to known targets leaves out a variety of other possible targets including novel ones that may uniquely be present on abnormal cells. Whole cell panning or panning on more complex targets is an alternative strategy to biopanning only known subcellular targets proteins. The cell surface is a landscape of numerous macromolecules that are specific to type and the functional state of the cell (Brown 2000). This may be particularly important for cancer cells that have complex and possibly changing cell-surface landscapes.

Most whole cell panning has been performed on the cell lines cells and blood cancer cells using a larger sample size, typically ranging from 100,000 to 1,000,000 cells each round (Barry et al. 1996, Ridgway et al. 1999, Poul et al. 2000, Giordano et al. 2001, Oyama et al. 2003, Takahashi et al. 2003, Heitner et al. 2003). These experiments were possible because of the enormous amount of cell supply from blood and cell lines. A few reports have screening tissue in an intact state and have isolated cell-specific ligands from human bladder mucosa, and from an *ex vivo* infusion of a 6 inch long piece of human umbilical cord (Ardelt et al. 2003, Maruta et al. 2003). The feasibility of considerably reducing the panning sample size has been recently demonstrated (Tanaka et al. 2002). However, there is no satisfactory phage library screening procedure against biopsies or small samples of human cancer tissue obtained directly from patients. Biopanning tumor tissue can be particularly difficult because of limited sample material. In addition, such cells are not reliably adherent, which would otherwise facilitate important separation steps in the biopanning process. Once the screening process is complete, evaluation of candidate tumor-binding clones is also problematic due to severe restrictions on the amount of target material available. The available procedures typically require 100,000–1,000,000 cells for each clone assessment (Barry et al. 1996, Ridgway et al. 1999, Poul et al. 2000, Oyama et al. 2003, Takahashi et al. 2003, Heitner et al. 2003). If the binding studies of each clone are performed in triplicate and 50–100 clones are assessed, a huge amount of cells are required with existing methods.

Our goal was to develop a reliable method of obtaining tumor binding clones to cancer cells from freshly obtained surgical biopsies of cancer patients. This has required miniaturizing the phage library screening process, adapting the separation steps to cells that are non-adherent and not in perfect single cell suspension, and importantly developing binding assay for candidate clones that requires smaller numbers of target tumor cells. We present here our data on development of these procedures first with cell line cells and then on cells obtained from surgical biopsy breast cancer specimens.

Materials and methods

Phage-displayed libraries

We used phage-displayed two random peptide libraries (RPLs) and one single chain antibody (scFv) library for our experiments. The peptide/scFv sequences in these libraries were expressed as N-terminus fusion to minor coat pIII protein. The RPLs that vary in the number of total amino acids and their cyclic portion were constructed in our laboratory using the half-site cloning method of Cwirla et al. (1990) in the fd-tet-derived fUSE5 vector that we received as a gift from George Smith (1985). One RPL displays 12 random amino acids with two cysteine residues fixed at positions one and 14 attached to a linker ($CX_{12}C$-$G_4SG_3A_2$, where X = any amino acid) and the other 20-mer with cysteines fixed at positions 5 and 16 ($X_4CX_{10}CX_4$). The cysteines allow the formation of a disulfide bond that results in the display of a constrained loop. Peptides that are conformationally constrained often possess a higher affinity for a target than their linear counterpart. While each phage particle displays only one specific peptide, the complexities of the random 14- and 20-mer libraries were 1.26×10^7 and 1.8×10^7 different peptides, respectively. The human single fold scFv Tomlinson I library (de Wildt et al. 2000) cloned in ampicillin resistant phagemid vector pIT2 was obtained from MRC, HGMP Resource Centre (Hinxton, Cambridge, UK). The scFv fragments comprise a single polypeptide with the VH and VL domains attached to one other by a flexible glycine–serine linker. The scFv library has a diversity of 1.47×10^8.

Cell culture

Human breast carcinoma SKBR3 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were cultured in McCoy 5A medium supplemented with 10% (v/v) fetal bovine serum and 2 mm l-glutamine. Cells were shifted to serum-free medium for 2 h before use.

Collection and processing of human tumor and normal breast tissue

Tumor and normal breast tissue were obtained from patient specimens immediately following therapeutic surgical resection with a protocol approved by the University of Vermont Committees on Human Research. Normal human breast tissue used in the present study was predominantly glandular tissue that was collected from breast reduction surgery. The sample was rinsed and divided into two pieces, one placed over ice for cell preparation and the other fixed in formalin for histological analysis. For cell preparation, tumor and normal breast tissues were cut into small pieces in the presence of protease

inhibitors and rinsed to remove blood. The tissue pieces were minced finely with the help of a sharp razor blade in a Petri dish with chilled RPMI medium with protease inhibitors. The minced tissue was rinsed with medium and the liberated cells were collected in a chilled tube. The residual tissue was transferred along with 1 ml medium for further disaggregation into a 35 μm Medicon (Medimachine, Dako, Denmark). The pooled cell preparation from both processes was passed through 100 μm and 70 μm nylon mesh filters (Filcon, Dako, Denmark) and collected cells were washed twice with RPMI medium containing protease inhibitors (Essler and Ruoslahti 2002). The cell preparations were counted for cell density and fixed in 2% paraformaldehyde for 1 h at room temperature (RT) for biopanning and binding assessment studies.

Biopanning protocol for fixed cells in suspension

We conducted biopanning of non-adherent cells in a 0.5 ml centrifugal filter device with 0.65 μm low binding Durapore® PVDF membrane (Millipore, Bedford, MA). SKBR3 cells grown to 80–90% confluency were detached, washed and fixed in 2% paraformaldehyde made in PBS. The cells were washed 5 times, 3 min each, with TBST (TBS 25 mm Tris-HCl, 150 mm NaCl, pH 7.4; with 0.05% Tween-20). About 20,000 SKBR3 cells suspended in 1% casein blocker (Pierce, Rockford, IL) were transferred to a filter cup and to another cup RPL ($X_4CX_{10}CX_4$ or $CX_{12}C$, ~100 copies) in biopanning buffer (0.2% casein, 0.05% Tween-20 and 10% goat serum in TBS) was taken. Both filter cups were incubated overnight in cold to block cells and to deplete plastic/membrane-binding phage-peptides in RPL. The next day, both filter cups mounted on their filtrate-collecting tubes were centrifuged (600 g, 30 s) and filtered RPL was transferred to a filter cup containing casein blocked tumor cells. Cells were incubated with RPL for 2 h at RT with slow shaking. The filter cup was mounted on a 2-ml eppendorf tube and the tumor cells were washed 10 times with 0.6 ml TBST and once with TBS. Though the filter cups used were designated to be of 0.5 ml in size, they accommodated 0.7 ml fluid. The bound phage particles were eluted twice (3 min each) with 100 μl 50 mm glycine–HCl buffer–saline, pH 2.2 and once with 100 μl 50 mm carbonate buffer–saline, pH 12. The remaining cell-associated phages were rescued by infecting K91/Kan cells in a filter cup. The eluted phage fractions were pooled and amplified in K91/Kan cells with tetracycline selection (Oligino et al. 1997, Pero et al. 2002) for the next round of panning. The phages eluted after the 4th round of panning were plated on tetracycline plates for isolation, binding assessment and identification of individual clones.

For breast tumor biopanning, ~20,000 washed breast tumor cells and ~100,000 normal breast cells were taken separately into filter cups and blocked overnight with 1% casein blocker at 4°C. The cup containing normal breast cells was centrifuged and an aliquot (~2500 copies of primary clones) of Tomlinson I scFv library in biopanning buffer was added. The cells were incubated for 2 h with slow shaking at RT. This negative subtraction step depletes scFv-phages that bind to non-malignant tissue, filter plastic and membrane. The filter cups with their collecting tubes were centrifuged and subtracted scFv library was transferred to other casein-blocked filter cup containing ~20,000 tumor cells. Tumor cells were incubated for 2 h with slow shaking at RT. The tumor cells were washed 10 times with 0.6 ml TBST and once with TBS. The elution, plating and amplification of phages were done as described earlier (Griffiths et al. 1994, de Wildt et al. 2000), using KM13 helper phage (Kristensen and Winter, 1998) and TG1 *E. coli* bacteria.

Biopanning protocol for plate-adhered fixed and live cells

SKBR3 cells grown to 80–90% confluency in a 24-well plate were fixed with 2% paraformaldehyde in PBS for 1 h at RT. After washing cells 5 times, 3 min each, with TBST, the well was filled with 1% casein blocker. In an empty well, 0.5 ml biopanning buffer containing about 200 copies of RPL (CX12C) was added. The plate was incubated overnight at 4°C. The next day, the casein blocker was aspirated out from the SKBR3 containing well and plastic-cleared RPL from the other well was added for biopanning. The cells were incubated with phage for 2 h with slow shaking at RT. Cells were washed 9 times with TBST and once with TBS. The bound phages were eluted 2 times (3 min each) with 333 μl 50 mm glycine–HCl buffer–saline, pH 2.2 and once (5 min) with 333 μl 50 mm carbonate buffer–saline, pH 12. The cells were scraped after 5 min incubation in alkaline buffer and pooled with acid-eluted fraction. This neutralized eluted phage pool was amplified (Oligino et al. 1997, Pero et al. 2002, Pero et al. 2004) and used as input for the next round of panning. The eluted phages, following the 4th round of selection, were plated on tetracycline plates for isolation, binding assessment and identification of individual clone binders.

For biopanning with live cells, an aliquot of RPL ($CX_{12}C$) was mixed with 0.5 ml McCoy 5A medium and protease inhibitors in a microplate well and incubated overnight at 4°C for subtracting plastic binders. The next day, near confluent SKBR3 cells in a well of 24-well plate were washed twice with cold medium and incubated with plastic-cleared RPL for 1 h with slow shaking at 4°C. The binding assessment of live cells was conducted in the cold to avoid endocytosis of the phage. Cells were washed 5 times

with glutamine free medium, 4 times with TBST and once with TBS. Following elution of cell surface-bound phages, the cells were lysed in 25 mm Tris–HCl buffer, pH 7.4 for 30 min in cold. The cells were scraped and added to other eluted phage fractions. The subsequent steps were the same as described above for fixed cells panning.

Chemiluminiscence ELISA for phage supernatant normalization

In order to use equal amounts of phage particles for binding assessment of different clones, the relative concentrations of phages in supernatants were determined by our recently developed chemiluminescence ELISA method. The modifications made to the conventional method enhanced the detection sensitivity to about 1000 TUs/well of filamentous phage (Shukla and Krag, in preparation). White F96 MaxisorbTM Nunc Immuno plate wells (Nulge Nunc International, Roskilde, Denmark) were coated for 2 h at RT with 100 µl of rabbit anti-fd bacteriophage (Sigma Chemical Co., St. Louis, MO), diluted 1000 times in carbonate buffer (15 mmol/l carbonate and 35 mmol/l bicarbonate), pH 9.6. After washing 4 times with TBST, wells were blocked for 1 h with 1% casein blocker in TBS. Wells were washed twice with TBST and once with TBS. Phage supernatants were diluted and 100 µl of each dilution was added to wells for an overnight incubation in cold. The next day, wells were washed 4 times with TBST and 100 µl of HRP/anti-M13 conjugate (Amersham Pharmacia Biotech, Little Chalfont, UK), diluted 2000 times, were added and incubated for 1 h at RT. Wells were washed 5 times with TBST and once with TBS. One hundred microlitres SuperSignal ELISA femto substrate (Pierce, Rockford, IL), constituted with equal volumes of Luminol/Enhancer and Stable Peroxide Buffer, were added and chemiluminescence was read immediately using GloRunner microplate luminometer (Turner Biosystems, Sunnyvale, CA).

Cell-ELISA for binding assessment of selected phage-peptides

About 40,000 SKBR3 cells/well were plated in a white 96-well tissue culture treated plate with a clear flat bottom (Corning Inc., Corning, NY) and allowed to adhere overnight at 37°C. The next day, the medium was removed and cells rinsed with PBS. The cells were fixed in 100 µl of 2% paraformaldehyde (made in PBS) at RT for 30 min. After washing cells 3 times with TBST (5 min each) and once with TBS, wells were blocked overnight with 1% casein blocker at 4°C. Twenty-five microlitres normalized phage supernatants from individual clones and 25 µl of binding buffer (2× TBS, 0.1% Tween-20, 20% goat serum and 0.4% casein blocker) were added to wells and incubated for 2 h at RT. Wells were washed 4 times with TBST and cells were incubated with 50 µl of 1:600 diluted anti-M13/HRP conjugate (Amersham Pharmacia Biotech, Little Chalfont, UK) for 1 h at RT. Wells were washed 5 times with TBST and once with TBS. The chemiluminescence was developed with 100 µl/well SuperSignal ELISA femto substrate and read immediately using GloRunner microplate luminometer.

For non-adherent tumor and normal breast cells, we modified our above described cell-ELISA procedure. The binding assessment was standardized using 1000, 10,000 and 40,000 non-adherent fixed SKBR3 cells in filter-plate wells. Different amounts (3 and 25 µl of phage supernatant) of a known SKBR3-binding phage-peptide (EC1), developed earlier in our laboratory (Pero et al. 2004), was added to determine the sensitivity and quantitative nature of the binding procedure when using a cell suspension in a filter-plate. The 96-well white filter-plates (Multiscreen$_{HTS}$ Plates, Millipore, Bedford, MA), used in a present study, had low binding 0.65 µm hydrophilic PVDF membrane that allowed fluid to pass only under applied vacuum. The data was analysed for linear regression analysis by using GraphPad Prism version 3 for Windows (GraphPad Software, San Diego, CA).

An amount of 10,000 washed tumor and normal cells were added to each well and incubated overnight in cold with 1% casein blocker containing 1% polyvinylpyrrolidone-10 (PVP-10; ICN Biochemical Inc., Aurora, OH). Next, endogenous peroxidase was blocked with 0.3% H_2O_2 in TBS for 30 min and the cells were washed 4 times with TBST. The phage incubation and other subsequent steps were the same, as described above for adhered SKBR3 cells. The binding assessment of each selected phage to both tumor, as well as normal cells, was done in three wells, so as to get a representation of heterogeneity of the cell population in the wells.

Immunofluorescence method for binding assessment of selected phage-peptides

SKBR3 cells were grown and treated as described for phage ELISA of adherent cells. However, after anti-M13/HRP treatment and washing step, cells were incubated in the dark with 50 µl of 75 times diluted Alexa Fluor® 488 tyramide reagent (Molecular Probes, Eugene, OR). The cells were washed 3 times with TBST. Fifty microlitres PBS was added to each well and the cells were observed under fluorescence microscope.

Phage ELISA for erbB2 binding

SKBR3-binding phage clones were evaluated for their binding to purified preparation of extracellular domain (ECD) of ErbB2 (HER2/neu; Creative Biomolecules Inc., Hopkinton, MA) according to

the method described earlier (Pero et al. 2004). Briefly, polystyrene microplate wells (F96 Maxisorb^{TM} Immuno plate; Nulge Nunc International, Roskilde, Denmark) were coated overnight in cold with 100 µl (250 ng)/well of ECD-ErbB2 or BSA. Wells were blocked for 30 min at RT with casein blocker and the phage supernatants of individual clones were added. Phage-peptides were incubated for 2 h at RT. An HRP conjugated anti-M13 monoclonal antibody (Amersham Pharmacia, Piscataway, NJ) was used to identify bound peptide-phage clones. The bound HRP was assayed at $A_{405\,nm}$ with ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) substrate (Sigma Chemical Co., St. Loius, MO).

DNA sequencing and bioinformatics

The vector DNA of each selected clone was purified with the help of QIAprep M13 kit (Qiagen, Valencia, CA). Following cycle sequencing reactions performed using BigDye® Terminator version 3.1 kit (Applied Biosystems, Foster City, CA) and appropriate primer (Oligino et al. 1997, Pero et al. 2002), Vermont Cancer Center DNA Analysis Facility at the University of Vermont carried out automated DNA sequencing using ABI Prism® 3100 Genetic Analyser. The translated amino acid sequences were searched for significant motifs using IBM Sequence Pattern Discovery Tool software program based on TEIRESIAS algorithm (Rigoutsos and Floratos 1998). The similarities between the selected peptides and the known biological proteins were determined a using receptor ligands contacts program (Mandava et al. 2004).

Results

Binding assessment of selected phage clones to SKBR3 cells

The data presented in Figure 1 shows the binding of different amounts (3 and 25 µl of phage supernatant) of a known SKBR3-binding phage-peptide (EC1), developed earlier in our laboratory, to 1000, 10,000 and 40,000 SKBR3 cells present in filter-plate wells. A linear increase in the binding, as assessed by linear regression analysis, is evident from high coefficient correlation (r^2) values with increasing cell numbers ($r^2 = 0.979$ for 3 µl; 0.943 for 25 µl supernatant) in the assay system.

About 40 clones after the 4th round of each panning protocol were randomly selected and their binding to SKBR3 cells was assessed following phage amplification and concentration normalization. The clones showing a binding 4-fold or more over the background (non-specific binding by phage clone having no peptide insert) were considered to be SKBR3-binders. The binding assessment of clones from panning with 14-mer ($CX_{12}C$) RPL identified 13 SKBR3-binders

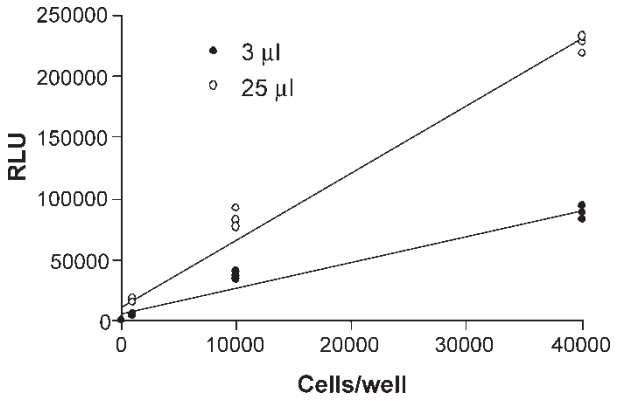


Figure 1. Effects of increasing numbers of non-adherent SKBR3 cells and EC1 phage concentrations on binding by cell-ELISA using filter-plate. EC1 phage, a known binder of SKBR3 cells/erbB2, was used as a positive control. The individual values obtained at each cell count were plotted ($n = 3$). Three microlitres (open circles) and 25 µl (closed circles) EC1 phage supernatants were added in the incubation mixture. RLU = relative light units represent chemiluminiscence readings.

(8 unique) in the filter cup protocol using ~20,000 non-adherent fixed cells, 9 (5 unique) binders from using plate-adhered fixed ~200,000 cells and 10 (8 unique) binders from using plate-adhered live ~200,000 cells. The panning of ~20,000 non-adherent fixed SKBR3 cells in a filter cup with 20-mer ($X_4CX_{10}CX_4$) RPL identified 23 clones (17 unique) that bind to SKBR3 cells. The binding assessment data of the clones from one of the four panning experiments are presented as a bar diagram in Figure 2. The first two bars in Figure 2 show the values from Lurea broth medium (no phage) and an equal amount of the phage without insert, which served as negative controls.

A parallel method of immunofluorescence detection of phage binding to SKBR3 cells was also employed for some of the clones tested positive by chemiluminescence ELISA method. The microscopic examination showed fluorescence staining of SKBR3 cells. A previously identified SKBR3-binding EC-1 clone (Pero et al. 2004) was used as a positive control (data not presented).

Binding assessment of SKBR3-binding phage clones to purified ectodomain of erbB2 receptor

Since SKBR3 cells are known to overexpress erbB2 (HER2/neu) receptor, all the clones, which bound to SKBR3 cell surface were assessed for their binding to purified extra-cellular domain of erbB2 also. The results presented in Figure 3 show the binding of SKBR3-binding clones to purified erbB2-ectodomain protein and to BSA (as a non-specific protein control). The specific erbB2 binding for each clone was calculated by subtracting the value of non-specific binding from erbB2 binding

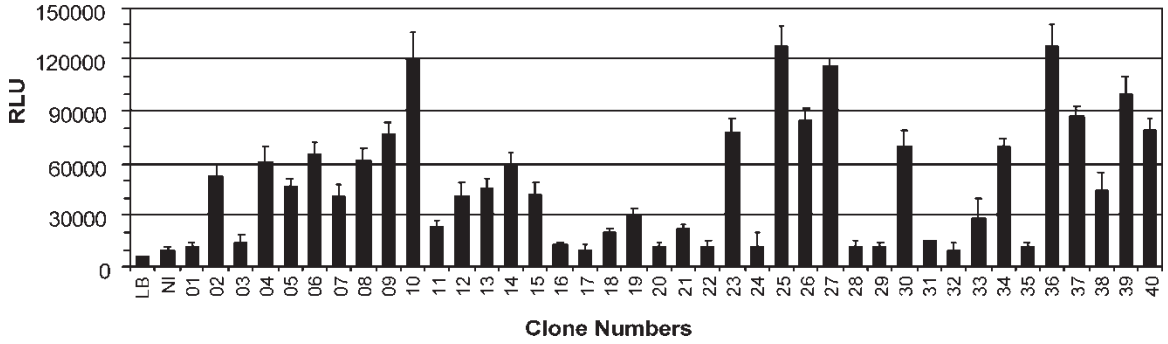


Figure 2. Binding assessment of phage-peptides to SKBR3 cells. The clones randomly selected following the last round of biopanning non-adhrent ~20,000 fixed SKBR3 cells with 20-mer RPL, using filter cup protocol, were assessed for their binding to fixed SKBR3 cells in 96-well culture plate. Values represent mean ± SE ($n = 3$). The first two bars represent negative controls; LB = Lurea broth without phage, NI = Phage having no insert.

(erbB2-ECD binding—BSA binding). The clones showing 4-fold or more specific binding than insert-less clone (first set of bars) were considered to be positive binders. Out of 23 SKBR3-binding clones (Nos. 2–40) selected from filter cup biopanning of non-adherent ~20,000 fixed cells with 20-mer RPL, 13 (8 unique) showed positive binding to purified erbB2 ectodomain. The testing of 13 SKBR3-binding clones (Nos. 41–53) from filter biopanning with 14-mer RPL identified five clones (2 unique) that bind to purified erbB2. The binding assessment of SKBR3-binding clones derived from the biopanning of plate-adhered fixed (Nos. 54–62) and live cells (Nos. 63–72) showed 5 (2 unique) and 4 (2 unique) clones, respectively, as erbB2 binders. Positive controls with known erbB2-binding clones, EC1 and EC1.1, were run in parallel.

Insert amino acid sequences, motif analysis and similarities with relevant proteins

Table I shows the insert amino acid sequences of clones that bound to SKBR3 cells. The motif analysis shows that the sequences from SKBR3-binding clones selected from different biopanning experiments using different libraries, plate-adhered, non-adherent, fixed and live cells share significant motifs. Two known SKBR3-binders EC1 and EC1.1 were also included in the analysis so as to determine if they share motifs with the clones derived from the present study. They share TXGFX[K/R]X[V/L]S, TWG, WTG motifs with 3 newly identified sequences.

The insert sequences from the clones that bound to purified ECD of erbB2 were aligned with the amino acid sequences of known ligands of erbB (HER) family members (Table II). The amino acid sequences of human Pro-EGF precursor, Heparin-binding EGF-like growth factor precursor, Epiregulin, β-cellulin precursor, Neuregulin-1 (NRG1), NRG2, NRG3 and NRG4 as given in Table II, are from Swiss–Prot databank. It is evident that some of the insert sequences of erbB2-binding clones share significant motifs with known ligands. The insert sequences did not align significantly with two other known ligands of EGFR, TGF-α and amphiregulin.

Since ErbB2 is known to heterodimerize with ECD of other erbB family members (Roskoski 2004), the insert amino acid sequences of erbB2-binding clones

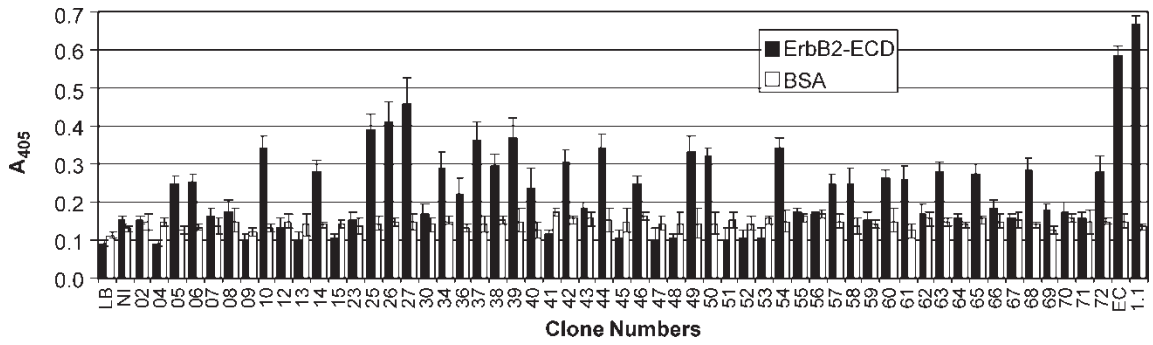


Figure 3. Binding assessment of SKBR3-binding clones to extracellular domain of erbB2. All the clones that showed binding to SKBR3 cells were assessed for their binding to ECD-erbB2 protein. The data (mean ± SE, $n = 3$) represent binding of clones from filter cup protocol, biopanned non-adherent ~20,000 fixed cells with 20-mer RPL (clones 02–40) or 14-mer RPL (clones 41–53), and those from conventional protocol, biopanned plate-adhered ~200,000 fixed (clones 54–62) or live (clones 63–72) cells with 14-mer RPL. BSA was used as a control for assessing non-specific binding. The first two sets of bars represent negative controls; LB = Lurea broth without phage, NI = Phage having no insert. EC(EC1) and 1.1 (EC1.1) are known erbB2-binding clones that were used as positive controls. A_{405} represents absorbance at 405 nm.

Table I. Motif-sharing among insert peptides of SKBR3-binding clones, selected from biopanning fixed, live, plate-adhered, and non-adherent SKBR3 cells with different RPLs using filter cup and conventional plate protocols.

DYDLCTSRSPAMAPLCISAN	**14:**F,X,1,EB
GVRLCTVPGPATKHTCDSSH	**34:**F,X,1,EB
GLARCNSDLHDGNSWCGVRQ	**23:**F,X,1
VPPVCKSMLYSKDTACGVLP	**30:**F,X,1
GTGSCGYGKLHTGYWCSYFP	**02:**F,X,1
RSHSCGNYQHKGFGECEYAP	**09:**F,X,1
TRWGCNVLNVRHSCSCQAL	**07:**F,X,1
CTGHLCCVGHSCPC	**62:**P,X,1
GKGRCHSCRTDHRLPCVNVS	**12:**F,X,1
DQDACNSRGSSDPLRCYERV	**06:**F,X,1,EB
GLARCNSDLHDGNSWCGVRQ	**23:**F,X,1
CNSNFRISARQSPC	**44,49,50:**F,X,3,EB
DAWRCTTGRRTQMHECTGVQ	**05:**F,X,1,EB
FDRWCTGGKSPVAPQCSGYE	**27:**F,X,1,EB
DQDACNSRGSSDPLRCYERV	**06:**F,X,1,EB
DYDLCTSRSPAMAPLCISAN	**14:**F,X,1,EB
DAWRCTTGRRTQMHECTGVQ	**05:**F,X,1,EB
FDRWCTGGKSPVAPQCSGYE	**27:**F,X,1,EB
NANICGENKDVSLATCYFYA	**38:**F,X,1,EB
WTGWCLNPEESIWGFCRGLS	**EC1.1:**Known-EB
CRSDRALTGMLFYC	**59:**P,X,1
CDKALRQYELLYFC	**56:**P,L,1
CWDNYRDVIFWGC	**57,58,60,61:**P,X,4,EB
CFKRMSFWGSPARC	**54:**P,X,1,EB
DYDLCTSRSPAMAPLCISAN	**14:**F,X,1,EB
CTFSINTTVFPTTC	**63,68:**P,L,2,EB
GTGSCGYGKLHTGYWCSYFP	**02:**F,X,1
TRWGCNVLNVRHSGSCQALP	**07:**F,X,1
CTLPIDGPNSDFVC	**4,66,67,71:**P,L,4
GVRLCTVPGPATKHTCDSSH	**34:**F,X,1,EB
HDAFCAQHVRVASRYCQRGF	**13:**F,X,1
CDRVADHFKEVSC	**70:**P,L,1
CFKRMSFWGSPARC	**54:**P,X,1,EB
DYDLCTSRSPAMAPLCISAN	**14:**F,X,1,EB
CNSNFRISARQSPC	**44,49,50:**F,X,3,EB
ITWGCSARTGEFPSKCSPFG	**10,25,26,37,39:**F,X,5,EB
RSHSCGNYQHKGFGECEYAP	**09:**F,X,1
CGSHSVSQELIVLC	**55:**P,L,1
CTLPIDGPNSDFVC	**64,66,69,71:**P,L,4
GLARCNSDLHDGNSWCGVRQ	**23:**F,X,1
TRWGCNVLNVRHSGSCQALP	**07:**F,X,1
ITWGCSARTGEFPSKCSPFG	**10,25,26,37,39(A):**F,X,5,EB
ITWGCSARTGEFPSKCSPFG	**(A):**F,X,5,EB
WTGWCLNPEESTWCFCRGLS	**EC1.1:**Known-EB
WTGWCLNPEESTWGFCTGSF	**EC1:**Known-EB
WTGWCLNPEESTWGFCTGSF	**EC1:**Known-EB
CWDNYRDVIFWTGC	**57,58,60,61:**P,X,4,EB
WTGWCLNPEESTWGFCRGLS	**EC1.1:**Known-EB
CSKKEVDLFNLGVC	**41,52:**F,X,2
RDEGCRDMKRSSWFQCKEVG	**08,15:**F,X,2
CDRVADHFKEVSC	**70:**P,L,1
DAWRCTTGRRTQMHECTGVQ	**05:**F,X,1,EB
FDRWCTGGKSPVAPQCSGYE	**27:**F,X,1,EB
FDRWCTGGKSPVAPQCSGYE	**27:**F,X,1,EB
CNSNFRISARQSPC	**44,49,50:**F,X,3,EB
DYDLCTSRSPAMAPLCISAN	**14:**F,X,1,EB
CFKRMSFWGSPARC	**54:**P,X,1,EB
GLARCNSDLHDGNSWCGVRQ	**23:**F,X,1
DYDLCTSRSPAMAPLCISAN	**14:**F,X,1,EB
CDFSAMAYASHLSC	**65,72:**P,L,1
ITWGCSARTGEFPSKCSPFG	**(A):**F,X,5,EB
LRASCARMESCNNSGCSNGM	**36,40:**F,X,2,EB
CWDNYRDVIFWTGC	**57,58,60,61:**P,X,4,EB
CNSNFRISARQSPC	**44,49,50:**F,X,3,EB

Amino acids sharing consensus motifs are highlighted; white letters = exactly the same, black letters = conservative substitution. Clone ID nos. are given in bold, (A) = clone represents ID nos. 10,25,26,37,39; F = Filter-panning with ~20,000 non-adherent cells; P = Plate-panning with ~200,000 adherent cells; X = Fixed cells; L = Live cells, followed by digit showing frequency out of 40 clones sequenced; EB = Binds to purified extra-cellular domain of erbB2; EC1 and EC1.1 are (known-EB) positive SKBR3/erbB2-binding clones developed earlier in our lab.

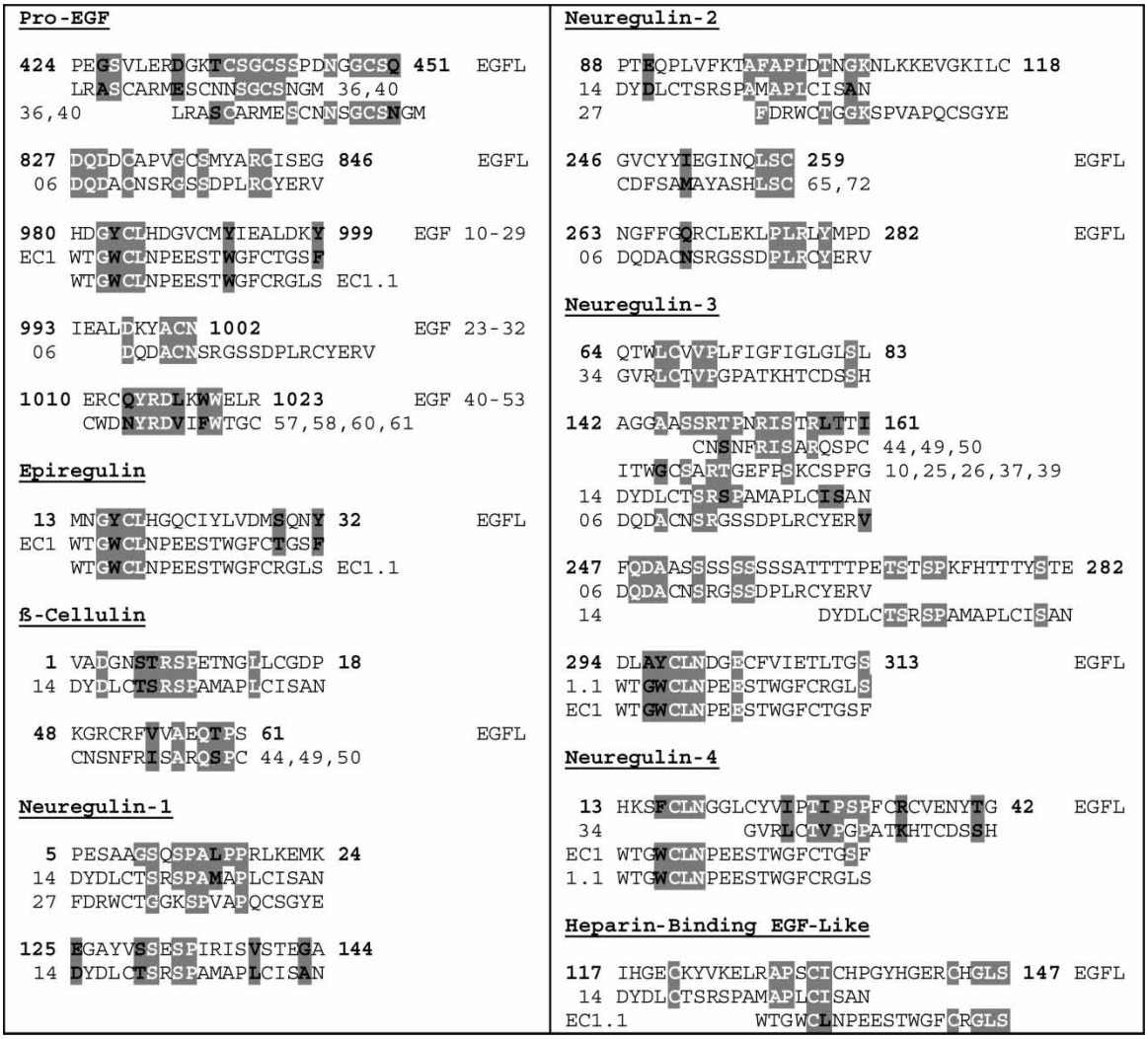
were aligned with these receptors. The alignments presented in Table III show that some insert sequences from erbB2-binding clones share significant motifs with erbB family of receptors. The amino acid sequences of ECD of erbB family members presented in the Table III are according to Swiss–Prot databank.

Selection of tumor-specific ligands using filter cup panning protocol

The data presented in Figure 4 shows the enrichment of human breast tumor cells binding clones with successive rounds of biopanning non-adherent ~20,000 fixed cells with Tomlinson 'I' scFv library subjected to negative subtraction on normal human breast cells. The tumor-binding clones eluted (outputs) following each round of selections were amplified, and their concentrations were normalized before conducting binding studies. The results demonstrate successful biopanning of ~20,000 tumor cells using filter cup protocol.

The data regarding binding assessment of individual clones selected following the final round of biopanning of tumor cells are presented in Figure 5. The binding studies were conducted following normalization of phage concentrations of clones tested, so that each filter-well received the same amount of phage-peptide. It is evident from the data

Table II. The alignments of insert peptide sequences from all erbB2-binding clones with known ligands of erbB family members of receptors.



The first line in every set shows the biological ligand sequence (flanked with amino acid nos.) that matches with insert peptides. The following lines show the insert peptide sequences with clone ID nos. from filter cup protocol, biopanning non-adherent ~20,000 fixed cells with 20-mer RPL (02–40) or 14-mer RPL (41–53), and those from conventional protocol, biopanning plate-adhered ~200,000 fixed (54–62) or live (63–72) cells with 14-mer RPL. EGF = from the portion designated as EGF in pro-EGF sequence. EGFL = from a portion designated as EGF-like domain within a ligand sequence. EC1 and EC1.1 are known erbB2-binding clones developed earlier in our lab. Amino acids sharing consensus motifs are highlighted; white letters = exactly the same, black letters = conservative substitution. The amino acid sequences of biological ligands were taken from the Swiss–Pro database.

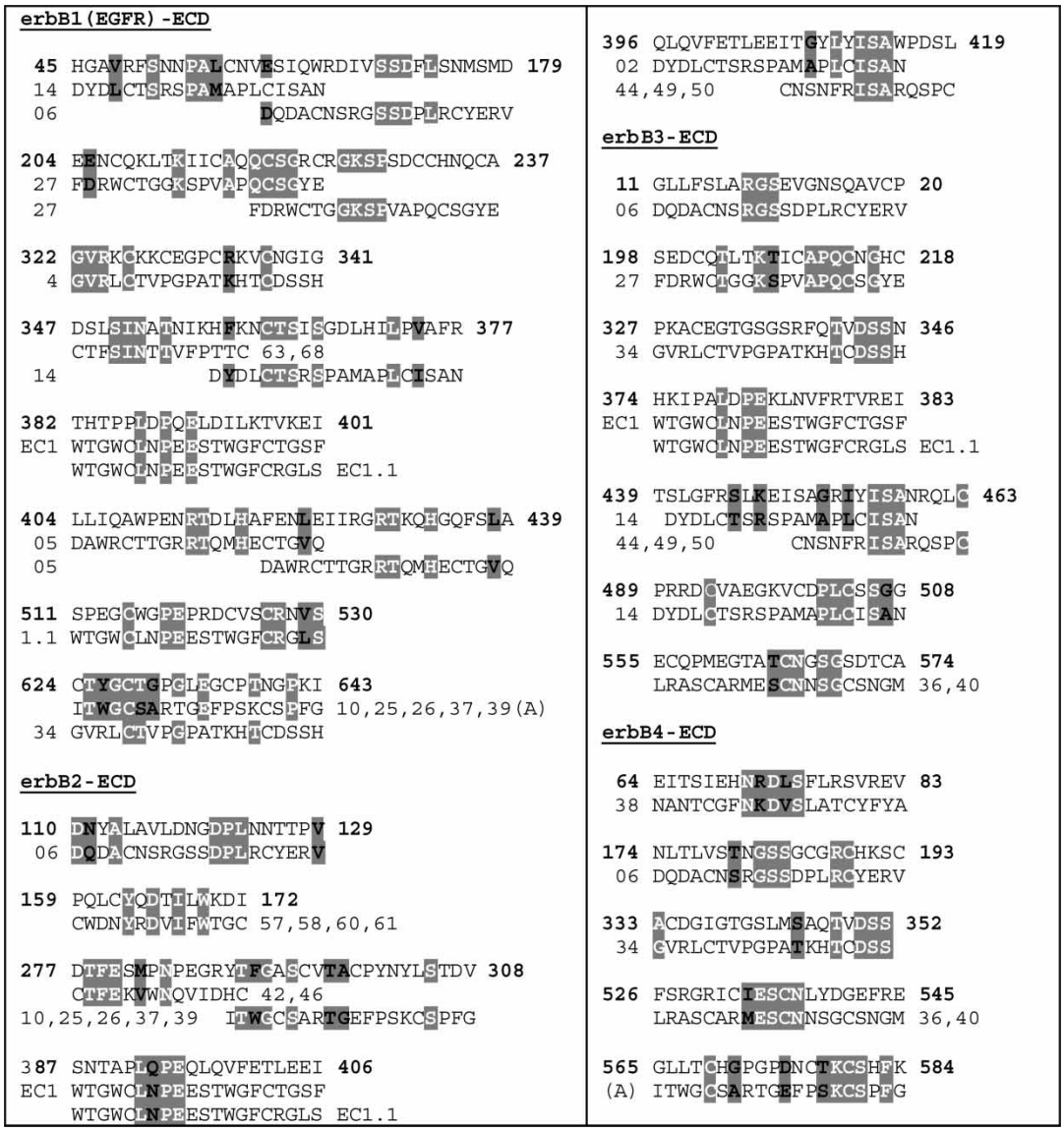
that some of the clones selected at the final round of biopanning are tumor-specific. These clones showed either exclusive or higher binding to tumor cells in comparison to normal human breast cells.

Discussion

The methods presented here address some very important problems encountered in developing tumor-specific small ligands from fresh tumors, using phage displayed random libraries. We have successfully developed an effective biopanning procedure to select tumor-specific ligands. In addition, we have applied an improved ELISA method employing chemiluminescence detection for binding assessment of selected phage clones to tumor targets. The methods we developed are very sensitive, and require only small samples of tumor tissue specimens. This is important because availability of fresh tumor tissue is frequently very small.

Using both random peptide and scFv libraries we have successfully conducted biopanning experiments with ~20,000 mildly fixed SKBR3 cancer cells and fresh human breast tumor tissue. The benefit of using freshly harvested tissue is that cells are in a state most closely resembling the *in vivo* condition. Rapid processing and fixation of cells will prevent degradation of cells and other changes related to stress and growth in an artificial *ex vivo* environment. Cells fixed in this manner allow archiving of sufficient cells in a stable condition. The stability of fixed cells offers considerable advantages for conducting several

Table III. The alignments of insert peptide sequences from all erbB2-binding clones with extracellular domains of erbB family members of receptors.



The first line in every set shows the erbB sequence (flanked with amino acid nos.) that matches with insert peptides. The following lines show the insert peptide sequences with clone ID nos. from filter cup protocol, biopanning non-adherent ~20,000 fixed cells with 20-mer RPL (02–40) or 14-mer RPL (41–53), and those from conventional protocol, biopanning plate-adhered ~200,000 fixed (54–62) or live (63–72) cells with 14-mer RPL. (A) = clone ID nos. 10, 25, 26, 37, 39. EC1 and EC1.1 are known erbB2-binding clones developed earlier in our laboratory. Amino acids sharing consensus motifs are highlighted; white letters = exactly the same, black letters = conservative substitution. The amino acid sequences of erbB receptors were taken from the Swiss–Pro database.

rounds of panning and subsequent cell-binding assessment of selected clones, which require several days. The trade off is that fixation introduces changes that may be undesirable. However, this did not appear to be a limiting factor in our experiments comparing fixed and live cells.

Out of 40 clones randomly selected from the biopanning of mildly fixed non-adherent SKBR3 cells in the filter cup protocol, multiple clones from both the 20- and 14-mer RPLs showed binding to SKBR3 cells. The parallel experiments with biopanning plate-adhered live and fixed cells also identified multiple clones that showed positive binding to SKBR3 cells. Considering the results of all biopanning protocols employing the 14-mer RPL, biopanning using the filter cup protocol produced an equally good or slightly better success rate in comparison to conventional protocol with adherent cells. This is true even though the filter cup protocol used 10-fold less cells for the panning step. The finding of a higher number of hits with the 20-mer RPL in comparison to the 14-mer is consistent with earlier report that longer amino acid- libraries may have advantages for binding cells in some systems (Barry et al. 1996).

The comparison of clones selected from different RPLs using SKBR3 cell-line as a model showed that

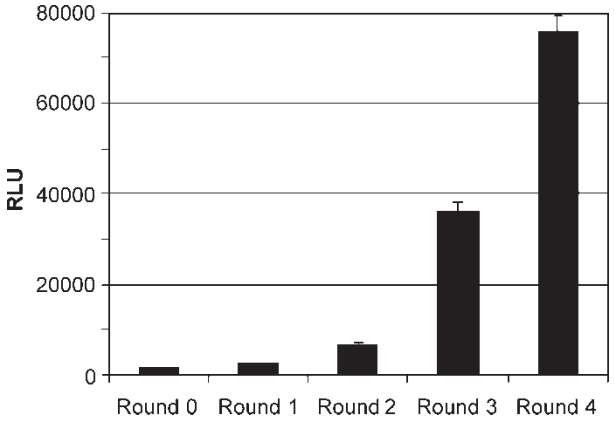


Figure 4. Enrichment of clones that bind to tumor cells with successive rounds of biopanning of non-adherent ~20,000 fixed human breast tumor cells with scFv library, using filter cup protocol. Values represent mean ± SE ($n = 3$). The first bar labeled as "Round 0" depicts the binding of naïve scFv library without any biopanning.

clones from fixed, live, adherent and non-adherent cells either biopanned in filter cup or plate share specific motifs and binding properties. These results indicate that the selection was non-random. The data from SKBR3 studies provide proof-in-principle that the filter cup biopanning protocol with ~20,000 fixed cells is capable of selecting targeted- ligands relevant to the cells. The cell-ELISA used for binding assessment in the present study is very sensitive and requires only 10,000 non-adherent target cells in filter-plate well. The quantitative nature of this binding assessment protocol has been demonstrated by a linear increase in binding with increasing target cell-numbers. This cell-ELISA considerably reduces the total amount of cells required for binding assessment of individual clones following biopanning steps. This procedure was optimized from our recently developed chemiluminescence-based phage ELISA titration method (Shukla and Krag, in preparation) that can detect as low as 1000 TUs of filamentous Phage.

Since SKBR3 cells are known to overexpress cell-surface erbB2, SKBR3-binding clones were tested for their binding to purified ECD of erbB2 receptor. The results showed that some of the clones from each biopanning protocol bound to purified erbB2 ectodomain. It is a remarkable finding considering that no negative selection was employed. It shows that the selection of ligands against whole cells with no directive pressures on the selection scheme allows for the isolation of targeting ligands for cell types. Since erbB2 does not have a known biological ligand to date, we compared the erbB2-binding sequences with known ligands to other erbB family receptors (Roskoski 2004). The alignments analyses revealed that selected peptides share significant motifs with EGF, Epiregulin, β-Cellulin, Heparin-binding EGF, Neuregulin-1, -2, -3 and -4. However, none of the sequences align significantly with TGF-α and Amphiregulin. It is interesting to note that the majority of the shared motifs are within EGF or EGF-like portions of these biological ligands. The sequences of two known erbB2 binders, EC1 and EC1.1, which were earlier developed and investigated in detail in our laboratory (Pero et al. 2004), align with a conserved motif [F/Y]CLN present in both Neuregulin-3 and -4. They align very well with a similar conserved motif [A/G]YCL present in EGF, Epiregulin and Neuregulin-3. The importance of these amino acids in EC-1 binding to erbB2 ectodomain has been shown in our earlier studies with EC1-biased libraries (Pero et al. 2004). The extracelluler domain of erbB2 has been reported to homodimerize and preferentially heterodimerize with the ectodomains of other erbB family of receptors (Roskoski 2004), indicating the presence of certain amino acid motifs on erbBs that may bind to ECD of erbB2. The peptide mimetics derived from ECD of erbB receptors have been reported to specifically bind erbB ectodomains, block inter-receptor interactions and inhibit signaling (Berezov et al. 2002). Therefore, the amino acid sequences in selected

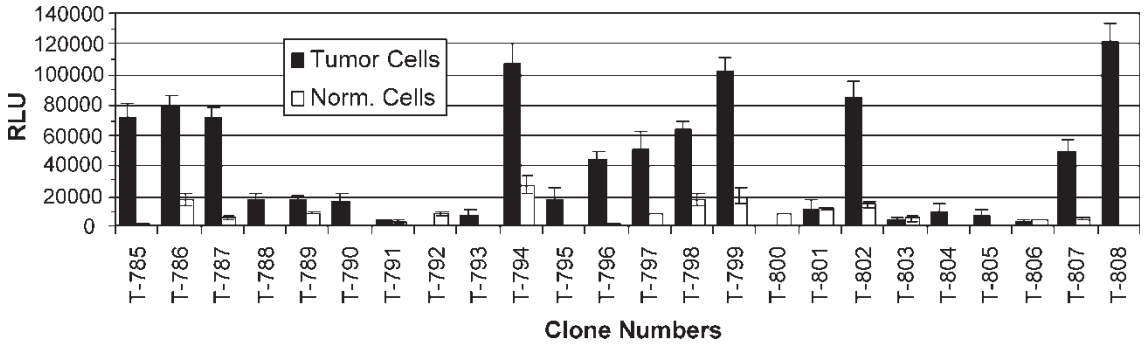


Figure 5. Binding assessment of individual clones to tumor and normal human breast cells. These clones were randomly selected following the final round of biopanning of non-adherent ~20,000 fixed human breast tumor cells with scFv library, using filter cup protocol. Values represent mean ± SE ($n = 3$) of specific binding following background subtraction. Solid bars = binding to tumor cells; Hollow bars = binding to normal human breast cells.

peptide-clones that share motifs with erbB family members could possibly be involved in the peptide binding. Alignment analyses showed that the selected peptide sequences share significant motifs with the erbB family of receptors. Of particular interest, motifs to mention here are those conserved in more than one erbB family member. The sequence TVDSS is present in both ECD-erbB3 and -erbB4 and shares TXDSS motif with peptide-clone 34. The peptide on clone 27 shares motifs with erbB1 (AXQCSG) and erbB3 (APQCXG) at the conserved portion (AXQCXG) in these two receptors. Another motif GX[I/L]YISA that is conserved in erbB2 and erbB3 was found in the insert sequence of clone 14 (AXLYISA). The relevance of these motifs in relation to target binding may be further studied, using deletion and site-directed mutagenesis. However, these results appear to be in with the line of conclusion drawn from binding data that the isolated peptides are target-selective.

The methods standardized using SKBR3 model experiments were also applied to the biopanning of fixed human breast tumor cells with phage displayed scFv library. In these experiments, a negative subtraction of library was performed with cells prepared from normal human breast tissue prior to biopanning with tumor cells. The successful biopanning was indicated by an enrichment of tumor binding clones at successive rounds of selection. The binding assessment of individual clones following final round of selection identified several clones that bind to tumor cells. Comparative binding to the cells from normal human breast cells indicated the presence of tumor-specific clones. These clones either bound to the tumor exclusively or with higher magnitude as compared to normal breast cells. It is remarkable to note that selected clones show specificity for the cell type that they were panned against when compared to another cell type. This specificity suggests that one negative subtraction of naive library on normal breast cells successfully removed the majority of clones that had binding affinities to abundant targets common to both cell preparations. The selection of tumor-specific ligands in the present investigation using the numbers of cells that can be obtained from a good quality fine needle aspiration or core needle biopsy is encouraging. This capability would allow panning of cells obtained from tumor tissue from almost any location in the body through minimally invasive methods that can even be repeated over time, if needed. The development of methodologies for ligand selections against whole tumor cells is important as the possible set of targets on tumors is large, complex, and consists of many different cell types. For example, the breast tumor targets are not limited to just carcinoma cells, but are also present on the modified epithelial cell types, stromal cells, adipose cells, endothelial cells and infiltrating lymphocytes (Tavassoli and Schnitt 1992). Furthermore, breast carcinoma cells themselves are morphologically and genetically diverse (Ronnov-Jessen et al. 1996).

The model studies with SKBR3 cells and later with human breast tumor cells showed the capability of the methods presented here in selecting cell-specific ligands using a relatively small number of non-adherent target cells. This biopanning procedure can be rapidly adapted to a 96-well format using appropriate filter-plates. The development of high throughput methods for identifying ligands for cell-surface receptors would facilitate the generation of a panel of specific ligands that may be exploited for targeted cancer therapies (Aina et al. 2002) and may also possibly help in classifying tumor types based on their ligand profiles (Oyama et al. 2003).

Acknowledgements

The work was supported by The National Cancer Institute R01 CA80790, NIH CA22435, and in part by SD Ireland Professorship of Oncology for Research and Vermont Cancer Center core grant.

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