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Internalizing cancer antibodies from phage libraries selected on tumor cells and yeast displayed tumor antigens

Yu Zhou, Hao Zou, Shaoyi Zhang, and James D. Marks*

Department of Anesthesia and Pharmaceutical Chemistry, University of California, San Francisco
Rm 3C-38, San Francisco General Hospital, 1001 Potrero Ave, San Francisco, CA 94110

Abstract

A number of approaches have been utilized to generate antibodies to cancer cell surface receptors which can be used as potential therapeutics. A number of these therapeutic approaches, including antibody-drug conjugates, immunotoxins, and targeted nucleic acid delivery, require antibodies that not only bind receptor, but that also undergo internalization into the cell upon binding. We previously reported the ability to generate cancer cell binding and internalizing antibodies directly from human phage antibody libraries selected for internalization into cancer cell lines. While a number of useful antibodies have been generated using this approach, limitations include the inability to direct the selections to specific antigens and identifying the antigen bound by the antibodies. Here we show that these limitation can be overcome by using yeast displayed antigens known to be associated with a cell type to select the phage antibody output after several rounds of selection on a mammalian cell line. We used this approach to generate several human phage antibodies to yeast displayed EphA2 and CD44. The antibodies bound both yeast displayed and mammalian cell surface antigen and were endocytosed upon binding to mammalian cells. This approach is generalizable to many mammalian cell surface proteins, results in the generation of functional internalizing antibodies, and does not require antigen expression and purification for antibody generation.

Keywords

asialoid breast cancer; single chain Fv antibody; phage display; yeast display

Introduction

Antibodies specific to cell surface receptors overexpressed on a number of cancers have been utilized for development of targeted immunotherapeutics. For example, HER2, CD20, and EGFR are overexpressed on a number of tumors and antibodies recognizing these

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*Corresponding author: Department of Anesthesia and Pharmaceutical Chemistry, University of California, San Francisco Rm 3C-38, San Francisco General Hospital, 1001 Potrero Ave, San Francisco, CA 94110 USA, tel: 415-206-3256, FAX: 415-206-3253, marksj@anesthesia.ucsf.edu.

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receptors have been developed to treat metastatic breast cancer (Trastuzumab)¹, lymphoma (Rituximab)², and colorectal cancer (Cetuximab)³. As such, identification of overexpressed cell surface receptors and antibodies which bind them provide a route to the development of cancer therapies, especially for those cancer subtypes with poor prognosis and resistance to traditional therapies.

Extensive studies of cancer transcriptional patterns have led to the discovery of many potential molecular targets to distinguish the malignant from the benign and the most aggressive cancers from those that are less aggressive^{4; 5; 6}. However, multiple steps are required, from the identification of genes by transcriptional pattern to the development of antibodies that can be used to treat humans. These steps include validation of protein expression, tumor associated antigen (TAA) expression and purification, generation of antibodies specific to the TAA, characterization of antibodies to identify therapeutic candidates, and further modification of antibodies which are therapeutic candidates including humanization. As an alternative, human antibodies binding TAA have been generated by direct selection of phage antibody libraries on live cancer cell lines^{7; 8}. In a modification of this approach, it has proven possible to select not only for cell binding, but also for binding and internalization into cancer cell lines^{9; 10; 11; 12; 13}. Such antibodies may prove especially useful for the development of antibody-drug or antibody-toxin conjugates, where the therapeutic molecule must enter the cell for efficacy^{14; 15; 16; 17}. Identification of the TAA bound by phage antibodies resulting from cell selections is a challenge which requires multiple steps, including the identification of specific phage antibodies, immunoprecipitation of the TAA using the phage antibody, and indentifying the TAA, for example by using mass spectrometry^{11; 13; 18}. Since this approach requires the isolation of the TAA by immunoprecipitation, it is not successful when the TAA is not abundant or easily immunoprecipitated by the antibody. Moreover, this approach is applied one antibody at a time to identify the antigen bound.

For this work, we sought to develop a general method to direct tumor cell selection of internalizing phage antibodies to a single tumor antigen. In this approach, the phage antibody library is subjected to several rounds of selection for internalization on a cancer cell line to enrich for internalizing phage antibodies binding native cell surface receptors. Next, the polyclonal phage output is subjected to several rounds of selection on yeast displaying a specific TAA thought to be associated with that cancer. Using this approach, we generated several internalizing human single chain Fv (scFv) phage antibodies to two TAA (CD44 and EphA2) known from transcriptional profiling and proteomic analysis to be overexpressed in basal breast cancers^{19; 20; 21; 22; 23; 24}.

Results

Display of tumor associated antigens on the surface of yeast

Two TAA (CD44 and EphA2) known to be overexpressed in basal breast cancers^{19; 20; 21; 22; 23; 24} were selected for display on the surface of *Sachromyces cerevisiae*. For yeast surface display, the full-length extracellular domain (ECD) of EphA2 (aa 1-510) and the link domain of CD44 (aa 1-149) (domain 1) were cloned into the yeast display vector pYD2 for (C-terminal) fusion to Aga2²⁵. Vector DNA was used to transform

EBY100, and cell surface display was induced. Both extracellular domains of CD44 and EphA2 were well displayed on the yeast surface as quantitated by a monoclonal antibody to a C-terminal epitope tag (Figure 1). Specific binding to yeast displayed EphA2 and CD44 extracellular domains of the EphA2 natural ligand Ephrin A1, and antibodies to EphA2 and CD44 suggests that the domains are not only displayed but displayed in a form that can be specifically recognized by antibodies (Figure 1).

Efficient recovery of antigen specific phage antibodies from yeast cell surface displayed antigen

A scFv phage antibody that specifically bound to EphA2 was used to study the ability to select phage antibodies on yeast displayed antigen. Approximately 10^{11} phage particles displaying anti-EphA2 human scFv 2D6 (YZ and JDM, unpublished) were incubated with 10^8 yeast cells displaying the target antigen EphA2 ECD (Y-EphA2). As a control, an identical number of anti-EphA2 phage antibodies were incubated with 10^8 yeast cells displaying an irrelevant scFv (Y-CON). The recovery of anti-EphA2 phage antibody from yeast displaying the EphA2 ECD was more than 10^4 fold higher than the recovery of anti-EphA2 phage antibody from yeast displaying the scFv (Figure 2a). To determine the optimal buffer to elute phage antibodies from the yeast surface, different buffers (PBS, 2-MEA, DTT, triethylamine (TEA) and glycine) were evaluated. Although yeast surface display of TAA results from the disulfide linkage between the Aga2 and Aga1 proteins on the yeast surface, reducing agents, including 2-MEA and DTT, resulted in poorer recovery of viable phage antibodies than spontaneous dissociation of phage by incubation in PBS (Figure 2b). In contrast, elution with high pH TEA buffer or low pH glycine buffer increased the number of viable phage recovered approximately two fold, with low pH glycine being the optimal elution buffer of those studied. This elution buffer was used for subsequent studies.

To determine the minimum frequency of a specific antibody within a library that can be enriched and selected, phage displayed anti-EphA2 antibodies were serially diluted from 10^9 to 10^0 cfu and then mixed with 10^9 helper phage VCSM13. Phage mixtures were incubated with 10^7 yeast cells displaying the EphA2 ECD or with yeast displaying the CD44 domain 1, followed by washing, elution and titration of the recovered phages. With an input of 10^2 specific phage particles, about 12 phage were recovered from yeast cells displaying EphA2, while an input of at least 10^5 phage were required before phage were present in the output when selected on CD44 (Figure 2c). The average recovery of phage antibodies was 6.5×10^{-2} for the specific antigen-antibody pair, and 6.5×10^{-5} for the mismatched pair (Table 1), respectively. This high recovery ratio for specific compared to non-specific phage suggested that it would be possible to enrich and select phage antibodies on yeast displayed antigen.

Selection of antigen specific phage antibodies on yeast cells displaying tumor antigens

The strategy used to select internalizing phage antibodies to specific tumor antigens is shown in Figure 3. To ensure that phage antibodies bound tumor antigens as presented on the surface of human tumor cells and could be internalized upon antigen binding, we used as a starting phage library the polyclonal phage output after the second round of selection of a non-immune human scFv phage library²⁶ selected for endocytosis into MDAMB231 tumor

cells. Based on the fact that basal subtype breast cancer cells over-express EphA2⁶ and CD44²¹, the phage output of selection on the basal subtype breast cancer cell line MDAMB231 was selected independently on yeast displayed EphA2 ECD (aa 25-534) and CD44 domain 1 (aa 21-169). To remove phage antibodies that bound to irrelevant proteins on the yeast surface, 2.5×10^{11} phage from the second round output of the MDAMB231 selection were incubated first with 1×10^9 yeast cells displaying the scFv 4E17 (Y-CON). Then, the depleted phage library was incubated with 2×10^7 yeast cells displaying the relevant antigen (Y-Ag) (Figure 3a).

For both EphA2 ECD and CD44 domain 1 selections, the number of phage recovered from each yeast cell in the second round of selection increased over 40 fold compared to the first round (Table 2), suggesting enrichment for phage binding yeast displayed tumor antigens. This was verified by using polyclonal phage to stain the yeast displayed antigens. From both the first and second round of selections, polyclonal phage antibodies showed specific binding to the antigen domain that was used for selection, with stronger staining after the second round of selection (Figure 3b). In contrast, prior to selection, the input phage antibody library gave no signal above background on yeast cells displaying either EphA2 or CD44. Binding was specific for the yeast displayed antigen, since the polyclonal phage did not bind yeast cells displaying an irrelevant protein (the scFv 4E17) (Figure 3b). To determine the frequency of binding phage antibodies, 96 individual clones were picked, phage produced, and the phage analyzed for binding to the yeast displayed tumor antigen. After one round and two rounds of selection, 31/96 (32.2%) and 39/96 (40.6%) of the clones from EphA2 selection bound yeast cells displaying EphA2 ECD, and 11/96 (11.7%) and 21/96 (21.9%) of the clones from CD44 selection bound yeast displayed CD44, respectively (Figure 3c).

Phage antibody characterization

Individual phage antibodies from the second round of selection that bound the yeast displayed tumor antigen were analyzed by PCR fingerprinting and DNA sequencing of the scFv genes. Three unique human scFv antibodies (D2-1A7, D2-1A9 and D2-1B1) were identified which bound to EphA2, and two unique scFv (F2-1A6 and F2-1H9) were identified which bound to CD44 ECD domain 1. Characterization of each of these scFv on yeast displayed EphA2-ECD (Y-EphA2 ECD), CD44 ECD domain 1 (Y-CD44 ECD D1) and full-length ECD (Y-CD44 ECD), and scFv 4E17 (Y-CON) indicated that each scFv was specific for its target antigen (Figure 4a). Each unique phage antibody was also analyzed for its ability to bind the original selecting tumor cell line MDAMB231 by flow cytometry. Each phage antibody strongly stained MDAMB231 cells (Figure 4b). Since the initial selection of the phage antibody library aimed to target cell surface receptors specific to basal subtype breast cancer cells, we studied the binding of the selected mAbs to basal and luminal breast cancer cell lines. Each mAb was relatively specific for basal breast cancer cell lines compared to luminal breast cancer cell lines (Figure 4c).

Binding specificity of phage antibodies

The binding of the identified EphA2 and CD44 antibodies to native antigens was also confirmed by immunoprecipitation of the receptors from cell extracts of MDAMB231 cells

followed by Western blotting with murine monoclonal antibodies specific to EphA2 and CD44 (Figure 5a). The specificity of the EphA2 mAbs was further studied. Each anti-EphA2 scFv was evaluated for its ability to compete with the natural ligand, Ephrin A1 for binding to EphA2 on the surface of MDAMB231 cells. Although the IC₅₀ of Ephrin A1 for phage antibody D2-1A7 and D2-1A9 at the given concentration differed by nine fold, 1 µg/ml of Ephrin A1 can fully block the cell binding of both D2-1A7 and D2-1A9 phage antibodies (Figure 5b), indicating that these two antibodies bind epitopes which overlap with Ephrin A1.

Phage antibodies are internalized by MDAMB231 cells

Since the phage antibodies identified by yeast display antigen biopanning were originally selected for the ability to be endocytosed into MDAMB231 cells (Fig 3a), it was anticipated that they would be internalized efficiently. To determine whether D2-1A7, D2-1A9 and F2-1A6 phage antibodies were endocytosed, the phage antibodies were incubated with MDAMB231 cells at 37°C to allow receptor mediated endocytosis, the surface bound phage removed by stripping with low pH buffer, and the internalized phage stained with anti-fd antibody and visualized using confocal microscopy (Fig 6). D2-1A7 and D2-1A9 anti-EphA2 antibodies gave strong intracellular staining while the control phage gave no staining. F2-1A6 anti-CD44 antibody also gave intracellular staining, most clearly visible in the higher magnification panel E. The staining pattern was more diffuse than the classic endosomal pattern seen for the EphA2 phage antibodies. These results indicate that all three phage antibodies were endocytosed by MDAMB231 cells.

Discussion

Breast cancers have been characterized by expression profile into several subtypes including luminal, basal, and HER2 positive, among which the basal subtype is related to reduced disease free survival, resistance to multiple drugs, and poor prognosis. Although multiple genes have been identified from gene expression arrays that are associated with this aggressive breast cancer subtype, generation of specific antibodies to these targets remains challenging, especially for cell surface receptors. Relatively large proteins must be expressed and purified in their native conformation and used for antibody generation. Traditional antibody generation approaches, such as hybridomas or direct selection from antibody libraries using recombinant protein, may generate antibodies that do not bind the receptor as expressed on cells. As an alternative, we developed an approach where phage antibody libraries can be directly selected on the target cell type not only for binding but for binding in a manner that results in antibody internalization^{9; 27}. We and others have used this approach to make cell targeting antibodies binding HER2, EGFR, transferrin receptor, integrin $\alpha\beta$ 1, CD9P-1, CD166, CD146, and other antigens^{9; 10; 11; 13; 18; 28; 29}. One useful aspect of such antibodies is that they are internalized upon cell binding and thus can be used for targeted drug, toxin or nucleic acid therapeutic approaches^{14; 15; 30; 31; 32; 33; 34; 35; 36}. Major limitations of this approach include the fact that one cannot direct the selection to specific antigens and identification of the antigen bound by what appear to be useful antibodies can be quite challenging using approaches such as immunoprecipitation and mass spectrometry sequencing^{11; 13; 18}.

Here we have shown that these limitations can be overcome by using yeast displayed antigens known to be associated with a cell type to select the phage output after several rounds of selection on a mammalian cell line. The antigen bound by the resulting antibodies is thus known and the antibodies bind the mammalian cell surface protein and undergo receptor mediated endocytosis. Expression and purification of the antigen used for selection is not necessary, simplifying antibody generation. We did not attempt to generate antibodies to TAA by directly selecting the primary phage antibody library on yeast displayed TAA. While this approach may prove successful, potential problems include generation of non-internalizing antibodies, generation of antibodies binding yeast displayed but not mammalian TAA, and dominance of antibodies to yeast antigens other than the displayed TAA.

While bacteria have been used to display antigens for phage antibody selection³⁷, the use of a simple eukaryote for display may result in a greater proportion of antigens being displayed in their proper conformation³⁸. Besides the two antigens described here, EGFR³⁹, HER2 domains (YZ and JDM, unpublished), cancer-testis antigen NY-ESO-1⁴⁰ and breast cancer-related antigens⁴¹ have been successfully displayed on the surface of yeast. Thus it appears that this approach may be generalizable to many mammalian cell surface proteins, with the caveat that there would be a low probability of generating antibodies to specific post-translational modifications, such as glycosylation, as these are quite different in yeast compared to mammalian cells. Finally, it may be possible to apply this approach in a more parallel manner, selecting for antibodies to multiple antigens at the same time. Bowley et al have proposed that a library of scFv antibodies on yeast can be panned against a library of cDNA displayed on phage to generate cognate pairs⁴². Reversing this approach, so that antibodies are displayed on phage and antigens on yeast would have two potential advantages. First, a greater probability of successful antigen display in the eukaryotic yeast compared to bacteria, and second the ability to display antigen as a C-terminal fusion protein, eliminating issues with the stop codon in cDNA. Such yeast cDNA display has been reported by Bidlingmaier as a means to isolate proteins binding phosphatidyl inositols⁴³. The possibility of such highly parallel approaches remains to be proved. In the meantime, the use of yeast displayed mammalian surface receptors appears to offer a simple means of generating internalizing antibodies to cell surface receptors from phage antibody libraries.

Materials and Methods

Cell lines, media, antibodies and full-length cDNA clones

Breast cancer cell lines MCF7, T47D, MDAMB453, MDAMB231, human mammary epithelial cell (HMEC), and SUM52PE were obtained from the ATCC and Clontech (HMEC), or from collections developed in the laboratories of Dr. Steve Ethier (SUM52PE). The cell lines were cultured using conditions described previously⁶. Yeast strain EBY100 was grown in YPD medium (*Current Protocols in Molecular Biology, John Wiley and Sons, Chapter 13.1.2*). EBY100 transfected with expression vector pYD2²⁵ was selected on SD-CAA medium (*Current Protocols, Chapter 13*). The Aga2p antigen fusion was expressed on the yeast surface by induction in SG-CAA medium (identical to SD-CAA medium except the glucose is replaced by galactose) at 20°C for 24~48 hr as described previously⁴⁴.

Bacteria strain *E. coli* DH5 α and TG1 were used for the preparation of plasmid DNA and the expression of soluble scFv antibodies respectively. SV5 antibody was purified from hybridoma supernatant using Protein G and directly labeled with Alexa-488 or Alexa-647 using a kit provided by the manufacturer (Invitrogen; Carlsbad, CA). Biotin conjugated rabbit anti-fd bacteriophage was purchased from Sigma and used to detect phage antibodies. Monoclonal antibody D7 against EphA2 ECD was purchased from Upstate Biotech, polyclonal goat anti-EphA2 and recombinant mouse Ephrin A1 with human Fc fusion protein from R&D Systems, anti-CD44 antibody for Western Blotting from NeoMarkers, and monoclonal anti-CD44 recognizing link domain from Abcam. The full-length cDNA of EphA2 and CD44 was obtained from the ATCC.

Antigen and antigen domains displayed on the yeast surface

Primers annealing to antigen cDNA and having a 25-mer overlapping sequence with pYD2/NcoI-NotI-digested vector were designed to amplify antigen domains by PCR using Pfu polymerase. After gel purification, the amplified antigen fragment and NcoI-NotI digested pYD2 vector were used to transform LiAc-treated EBY100 cells by gap repair^{45; 46}. The transformation mixes were cultured and subcultured in SD-CAA, and induced by culturing in SG-CAA medium for 24–48 hours at 18°C. To validate antigen display, anti-EphA2 (R&D) and recombinant mouse Ephrin A1 (R&D) were analyzed for binding to yeast displayed EphA2 ECD, and anti-CD44 antibody (Abcam) was analyzed for binding to CD44 domain 1 by flow cytometry. Briefly, the induced yeast cells (10^6 cells) with specific displayed antigen domains were incubated with monoclonal or polyclonal antibodies (1 μ g/ml) for 1 h at 4°C, detected using anti-goat PE conjugate for anti-EphA2, anti-human (Fc specific) for rEphrinA1-human Fc fusion protein, and anti-rabbit PE for anti-CD44 respectively, and co-stained with SV5-Alexa-647.

Optimization of elution buffer for phage antibody selection

Different elution buffer including phosphate buffered saline, pH 7.4 (PBS), 40 mM 2-mercaptoethylamine (2-MEA), 1 mM dithiothreitol (DTT), 100 mM triethylamine (TEA) and 100 mM Glycine/150 mM NaCl/0.1% BSA/0.5% Tween 20 were evaluated for their ability to elute bound phage from the yeast surface. The elution time was 1 hour at 37°C for PBS, 2-MEA and DTT, and 2 minutes at RT for TEA and glycine. After neutralizing with 10 mM cysteine for 2-MEA and DTT elution, and ½ volume of 1M Tris-HCl (pH 7.4) for TEA and glycine elutions, the eluted mixture was used to infect exponentially growing *E. coli* TG1 cells, and the titer of phage determined by serial dilution and plating on tetracycline resistant media.

Selection of phage antibodies specific to yeast displayed antigen domains

Human mammary epithelial cell (HMEC), luminal breast cancer cell line SUM52PE, T47D, and MDAMB453 were used to deplete the phage library of nonspecific binders by incubating 10^{12} phage particles^{47; 48} with 10^8 cells for 4 h at 4°C. The depleted phage library was then incubated with 5×10^6 basal breast cancer cell line MDAMB231 cells for 1 h at 4°C, followed by washing with cold PBS and incubation with 37°C-prewarmed medium/10% FBS for 30 min at 37°C to enable the receptor mediated endocytosis of phage

particles. The cell surface was stripped by three incubations of five minutes with 4 ml of glycine buffer (150 mM NaCl, 0.1 M glycine, pH 2.5). The cells were then trypsinized, washed with PBS, lysed with 1 ml of 100 mM TEA for four minutes at 4°C and neutralized with 0.5 ml of 1M Tris (pH 7.4). Internalized phage (TEA lysate) was amplified for further selections.

After two rounds of selection on MDAMB231 cells, the polyclonal phage antibodies were used to select phage antibodies specific to yeast displayed antigens EphA2 (Y-EphA2) and CD44 link domain (Y-CD44 D1). The induced yeast cells displaying an irrelevant protein were used to deplete the non-specific binders by incubating 2.5×10^{11} phage particles with 10^9 yeast cells for 2 h at 4°C. The filtered supernatant containing the depleted phage library was then incubated with 2×10^7 yeast-cells displaying specific antigen domain for 1 h at 4°C. Yeast cells were washed with cold PBS ten times and pelleted by centrifugation. The bound phage antibodies were eluted by incubating yeast cells with 1 ml of 100 mM Glycine/150 mM NaCl/0.1% BSA/0.5% Tween 20, neutralized with 0.5 ml of 1M Tris-HCl (pH 7.4), and amplified for another round of selection. In the second round of selection, 2×10^7 yeast cells were used for both antigens, while 2.1×10^{12} phage particles from the first round selection were used for CD44 domain 1 compared to 3.4×10^{11} used for EphA2. Two rounds of selection were performed.

Characterization of phage antibodies

After two rounds of selection, individual phage antibodies were prepared by growing single colonies in 96-well microtiter plates as described²⁶. Binding of each phage antibody to yeast displayed antigen was determined by incubation of 10^5 yeast cells with 100 µl phage supernatant diluted in FACS buffer (PBS with 1 mM MgCl₂, 0.1 mM CaCl₂ and 0.3% BSA) for 2 h at 4°C in conical 96-well microtiter plates, followed by incubation with biotinylated anti-fd antibody and streptavidin-phycoerythrin conjugate (PE) (Jackson), and analyzed using a FACS LSRII (Becton Dickinson). The number of unique phage antibodies was determined by patterns of BstNI digestion of 18 scFv genes amplified by PCR from phage-infected bacteria⁴⁹ and confirmed by DNA sequencing.

For binding to breast cancer cells and Ephrin A1 competition experiments, 5×10^4 MDAMB231 cells were incubated with 10^9 phage antibodies in the presence of recombinant mouse Ephrin A1 (R&D) at concentration ranging from 0 to 1000 ng/ml for 2 h at 4°C. The bound phage antibodies were detected by incubating cells with biotin conjugated anti-fd antibody (1 µg/ml) (Sigma) for 30 min at 4°C and streptavidin-PE (Jackson) followed by flow cytometry analysis.

Immunoprecipitation and Western blot using scFv antibodies

MDAMB231 cell extracts were prepared using 1 ml of lysis buffer per T75 culture flask, containing 0.5 % NP40, 50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM DTT, and protease inhibitor cocktail (Sigma). Soluble scFv antibodies with a (His)₆ tag at the C-terminal were generated by subcloning scFv genes from the phage vector into the expression vector pUC119mycHis⁵⁰, followed by purification from the periplasmic fraction of *E. coli* TG1 by IMAC⁵¹ using a Ni-NTA column (Qiagen) and gel filtration⁵². Cell extracts were incubated

with scFv at 26 µg/ml for 2 h at 4°C before the immune complexes were captured on Ni-NTA agarose. The agarose captured immune complexes were then washed 5 times in lysis buffer and heated to 94 °C for 4 min in non-reducing protein loading buffer. Immunoprecipitates were resolved by SDS-PAGE and analyzed by Western Blotting using anti-EphA2 (Upstate) and anti-CD44 (NeoMarkers) antibodies.

Immunofluorescence

MDAMB231 cells were grown on coverslips to 70% of confluence in 12 well-plates and incubated with 10¹¹ phage antibodies for three hours at 37°C. The coverslips were washed once with PBS, three times for five minutes with glycine buffer (50 mM glycine (pH 2.5), 150 mM NaCl), neutralized with PEM (80 mM Potassium PIPES (pH 6.8), 5 mM EGTA (pH 7), 2 mM MgCl₂), and fixed with PEM containing 4% (W/V) paraformaldehyde for 30 min on ice. Cells were quenched with 0.1 M NH₄Cl, permeabilized with 0.5 % Triton X-100, and blocked with 5% non-fat dry milk in TBS-T buffer overnight at 4°C. After blocking endogenous biotin with Avidin-Biotin Kit (Lab Vision), intracellular phages were detected with biotinylated anti-fd polyclonal antibody (Sigma) and streptavidin Texas Red (Amersham). Coverslips were inverted on a slide on mounting medium and microscopic images were taken with a Zeiss LSM 510 laser scanning microscope (Zeiss, Germany).

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Abbreviations used

TAA	tumor associated antigen
EGFR	epidermal growth factor receptor
ECD	extracellular domain
FACS	fluorescent activated cell sorting
IMAC	immobilized metal affinity chromatography
IgG	immunoglobulin G
ILs	immunoliposomes
IPTG	Isopropyl-β-D-thiogalactopyranosid
K_D	dissociation equilibrium constant
2-MEA	2-Mercaptoethylamine
DTT	Dithiothreitol
TEA	triethylamine
PBS	phosphate buffered saline
TBS-T	Tris-buffered saline Tween-20

Ni-NTA	Nickel-nitrilotriacetic acid
PE	phycoerythrin
scFv	single chain Fv
mAb	monoclonal antibody
HMEC	human mammary epithelial cell

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Figure 1. Display of antigen domains on the surface of yeast

(a) The extracellular domain (ECD) of receptor EphA2 was displayed on yeast surface and recognized by anti-EphA2 antibody and recombinant mouse Ephrin A1 (R&D) as determined by flow cytometry analysis. (b) The link domain of CD44 (domain 1, or D1) was displayed on the yeast surface and recognized by anti-CD44 rabbit monoclonal antibody as determined by flow cytometry analysis. Both anti-EphA2 and anti-CD44 antibodies did not recognize an irrelevant protein displayed on the yeast surface.

Figure 2. Recovery of phage antibodies from yeast displayed antigens

(a) Comparison of the recovery of anti-EphA2 phage antibody 2D6 from yeast displaying EphA2 ECD (Y-EphA2) versus yeast displaying an irrelevant protein (Y-CON). A total of 10^{11} anti-EphA2 phage antibody 2D6 were incubated with each of the yeast displayed antigens. (b) Impact of the elution buffer on the titer of EphA2 phage antibody eluted from the surface of yeast displaying EphA2 ECD. A total of 10^{11} anti-EphA2 phage antibody 2D6 were incubated with each of the yeast displayed antigen proteins prior to elution. (c) The impact of input phage titer on eluted phage titer. The indicated titer of anti-EphA2 phage

antibody 2D6 was incubated with yeast displayed EphA2 ECD or an irrelevant yeast displayed protein and the titer of eluted phage determined.

Figure 3. Strategy for selecting internalizing antigen specific phage antibodies

(a) After two rounds of selection for internalization on the basal breast cancer cell line MDAMB231, the pool of phage antibodies was first incubated with irrelevant control yeast to remove any yeast binding antibodies followed by panning on yeast displaying either EphA2 ECD or CD44 domain 1. (b) The binding signal of the polyclonal phage antibody pool to EphA2 ECD or CD44 domain 1 after 2 rounds of panning was measured by using flow cytometry. The irrelevant control yeast was stained with unselected phage antibody library (R0), round 1 (R1) and round 2 (R2) polyclonal phage. (c) Frequency of antigen

specific phage antibodies after one and two rounds of selection on yeast displayed antigen. Binding frequency was determined by analyzing 96 randomly picked phage antibodies for binding to yeast displayed antigen by flow cytometry. The induced yeast cells displaying an irrelevant protein, EphA2-ECD and CD44 domain 1 were stained with un-selected phage library (R0), polyclonal phages from R1 and R2 respectively. The un-induced yeast cells (yeast only), irrelevant phage antibody (phage control) and the un-selected phage antibody library were used as control.

Figure 4. Binding specificity of monoclonal phage antibodies from yeast antigen biopanning
 (a) Binding of monoclonal phage antibodies to yeast displayed antigen domains as determined by flow cytometry. The induced yeast cells displaying an irrelevant protein (Y-CON), EphA2-ECD (Y-EphA2 ECD), CD44 link domain (Y-CD44 ECD D1) and CD44 full length ECD (Y-CD44 ECD) were stained with monoclonal phage antibodies isolated from Y-EphA2 and Y-CD44 D1 selections. (b) Binding of monoclonal phage antibodies to MDAMB231 cells as determined by flow cytometry, (c) differential binding of monoclonal

phage antibodies to breast cancer cell lines including luminal breast cancer cell lines SUM52PE and MCF7, and basal breast cancer cell line MDAMB231.

Figure 5. Characterization of scFv antibodies by western-blot and flow cytometry
 (a) Anti-EphA2 scFv 2D6 or D2-1A7 and anti-CD44 scFv F2-1A6 were used to immunoprecipitate their target antigen from MDAMB231 cells. Antigen was detected by Western blotting using either anti-EphA2 antibody D7 or anti-CD44 antibody Ab-4. (b) EphA2 antibodies D2-1A7 (□), D2-1A9 (◇), and 2D6 (○) compete with ephrin A1 for binding to MDAMB231 cells. Ability of phage antibodies D2-1A7, D2-1A9, and 2D6 binding to MDAMB231 cells in the presence of increasing concentrations of EphA2 ligand, Ephrin A1 was determined by flow cytometry.

Figure 6. Phage antibodies specific to EphA2 and CD44 are endocytosed by MDAMB231 cells
Cultured cells were incubated with irrelevant phage (A), anti-EphA2 phage D2-1A7 (B) and D2-1A9 (C), anti-CD44 phage F2-1A6 (D, E) for 3 hr at 37°C followed by glycine buffer wash. Panels D and E are from the same experiment, but E is imaged at higher magnification. Endocytosis was visualized by detection of intracellular phage with anti-fd antibody, and analyzing by confocal microscopy.

Table 1
Recovery of specific phage antibody from yeast displayed antigens

The indicated yeast displayed antigen was incubated with 10^9 phage and the titer of bound phage determined. Results are expressed as the ratio of output/input phage.

Ag : Ab	PhAb Output / Input
Y-EphA2 ECD : PhAb-EphA2	6×10^{-2}
Y-CD44 D1 : PhAb-CD44	7×10^{-2}
Y-EphA2 ECD : PhAb-CD44	3×10^{-5}
Y-CD44 D1 : PhAb-EphA2	1×10^{-6}

Y = yeast displayed antigen; PhAb = phage displayed antibody.

Table 2
Phage display scFv antibody selection on yeast displayed antigens

Phage input and output ratios during the first and second rounds of selection on yeast displayed antigens.

Antigen	Round 1 selection				Round 2 selection			
	Input (cfu)	Output (cfu)	Phage		Input (cfu)	Output (cfu)	Phage	
			Input	Yeast			Input	Yeast
EphA2-ECD	2.5×10^{11}	3.6×10^6	1.3×10^{-5}	0.18	3.4×10^{11}	2.4×10^8	7×10^{-4}	12
CD44-ECD D1	2.5×10^{11}	9.8×10^6	3.9×10^{-5}	0.49	2.1×10^{12}	4.3×10^8	2×10^{-4}	20