Identification of Single Chain Antibodies to Breast Cancer Stem Cells Using Phage Display

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Recent evidence suggests that most malignancies are driven by "cancer stem cells" sharing the signature characteristics of adult stem cells: the ability to self renew and to differentiate. Furthermore these cells are thought to be quiescent, infrequently dividing cells with a natural resistance to chemotherapeutic agents. These studies theorize that therapies, which effectively treat the majority of tumor cells but 'miss' the stem cell population, will fail, while therapies directed at stern cells can potentially eradicate tumors. In breast cancer, researchers have isolated 'breast cancer stem cells' capable of recreating the tumor in vivo *and* in vitro. *Generated new tumors contained both additional numbers of cancer stem cells and diverse mixed populations of cells present in the initial tumor, supporting the intriguing self-renewal and differentiation characteristics. In the present study, an antibody phage library has been used to search for phage displayed-single chain antibodies (scFv) with selective affinity to specific targets on breast cancer stem cells. We demonstrate evidence of two clones binding specifically to a cancer stem cell population isolated from the SUM159 breast cancer cell line. These clones had selective affinity for cancer stem cells and they were able to select cancer stem cells among a large population of non-stem cancer cells in paraffin-embedded sections. The applicability of these clones to paraffin sections and frozen tissue specimens made them good candidates to be used as diagnostic and prognostic markers in breast cancer patient samples taking into consideration the cancer stern cell concept in tumor biology.* © 2009 American Institute of Chemical Engineers *Biotechnol. Prog.,* 25: 1780–1787, 2009
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Introduction

Recently, there is accumulating evidence supporting the "cancer stem cell hypothesis" stating that a tumor is hierarchically organized with its own stem cell compartment called "cancer stem cells." These cancer stem cells drive tumorigenesis and give rise to a large population of differentiated progeny that makes up the bulk of the tumor. This model, first developed in human myeloid leukemia, is now extended to solid tumors including breast, brain, prostate, gastrointestinal cancers, head and neck tumors, and melanoma.[1,2] In addition, it has been reported that established cancer cell lines also retain a small population of cells mimicking cancer stem cell behavior.[3,4]

Cancer stem cells with their self renewal and differentiation capacity and their relative resistance to chemo and radiotherapy may be the only cells in a malignancy with the ability to promote tumor growth, distant organ metastasis, and multidrug resistance, leading to failure of treatment and ultimately returning of the disease. Therefore, to achieve better antineoplastic therapies with decreased morbidity and mortality, we need novel therapeutic regimens targeting cancer stem cells.

Additional Supporting Information may be found in the online version of this article.

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Our goal is to use bacteriophage display technology to develop a set of ligands binding specifically to cancer stem cells. We believe that identification of clinically relevant ligands with selective affinity to specific targets on breast cancer stem cells may be used as novel diagnostic and therapeutic tools in breast cancer management.

In this study, we demonstrate evidence of two unique clones binding specifically to "cancer stem cells." These clones have been isolated from a scFv phage library after a series of panning on "cancer stem cells." Cancer stem cell population has been sorted from SUM159 breast cancer cell line by utilizing Aldefluor assay, which detects cells expressing stem cell marker aldehyde dehydrogenase. It has been shown by Wicha and coworkers[5] that Aldefluor positive cells isolated from human breast tumors contain cancer stem cell population able to self renew and recapitulate the heterogeneity of the parental tumor. In addition, aldehyde dehydrogenase 1 (ALDH1) expression is found to be a predictor of poor clinical outcome for breast cancer patients.[5] Our two clones showed specific binding to Aldefluor positive cancer stem cell population and they were able to select ALDH1 positive cells among a large population of ALDH1 negative cells in paraffin-embedded tissue sections.

Material and Methods

Cell line and tissue culture

SUM159 breast cancer cell line developed from a primary anaplastic breast carcinoma was obtained from Dr. Stephen Ethier (Kramanos Institute, MI). The cells were cultured in Ham's F12 media containing glutamine with 5% fetal bovine serum, insulin (5 μg/mL), and hydrocortisone (2 μg/mL). It was grown at 37°C and 5% CO_2.

Xenotransplantation and harvesting of tumor from NOD/SCID mice

Five female NOD/SCID mice at 7–9 weeks of age were purchased from Charles River Laboratories (Wilmington, MA) and maintained under specific pathogen free conditions at the University of Vermont. Animals were used in accordance with the Institutional Animal Care and Use Committee (IACUC) at the University of Vermont. The mammary fat pads of mice were cleared as described previously.[5] On the day of transplantation, SUM159 cells were trypsinized, washed, and cells were resuspended in matrigel (BD Biosciences). A total of 4×10^6 cells in 40 μL matrigel were subcutaneously transplanted in the cleared mammary fat pads of mice. The mice were monitored every week and when the tumor size reached 1 cm in diameter, animals were euthanized in CO_2 chamber and tumor tissue was harvested.

Aldefluor assay and separation of ALDH positive cells by FACS

The ALDEFLUOR kit (StemCell Technologies, Durham, NC) was used to isolate the population with a high ALDH enzymatic activity. Cells were trypsinized when they were 80% confluent and were suspended in ALDEFLUOR assay buffer containing ALDH substrate (BAAA, 1 mmol/L per 1×10^6 cells) and incubated for 40 min at 37°C. As negative control, for each sample of cells, an aliquot was treated with 50 mmol/L diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor. These cells were used as negative controls to establish the sorting gates. Propium iodide (PI) (1 μg/mL) (Sigma Aldrich) was used to assess cell viability.

Phage library

A single-chain antibody (scFv, Tomlinson I) library was used for panning on Aldefluor positive cancer stem cells. scFv sequences in these libraries were expressed as NH2 terminus fusion to minor coat pIII protein of filamentous bacteriophage. The trypsin cleavable human single-fold scFv Tomlinson I library, cloned in ampicillin-resistant phagemid vector pIT2 and transformed into TG1 *E. coli* cells, was obtained from MRC, HGMP Resource Centre (Hinxton, Cambridge, United Kingdom). The scFv fragments compose a single polypeptide with the VH and VL domains attached to each other by flexible glycine–serine linker. scFv library has diversity of 1.47×10^8. The scFv library amplification, titration, elution of cell-bound phage, and their plating were done as previously described using KM13 helper phage.[6–8]

Biopanning on Aldefluor positive cancer stem cells

We conducted biopanning on FACS sorted Aldefluor positive cancer stem cells in a 0.5 mL centrifugal filter cup with 0.65 μm low binding Durapore PVDF membrane (Millipore, Bedford, MA). FACS sorted Aldefluor positive and negative SUM159 cells were washed and fixed in 4% paraformaldehyde made in PBS. After washing paraformaldehyde away with PBS, 50,000 Aldefluor negative and 20,000 Aldefluor positive cells suspended in 1% casein blocker (Pierce, Rockford, IL) transferred into two separate filter cups. Both filter cups were incubated overnight at 4°C to block cells. The cup containing Aldefluor negative breast cancer cells was centrifuged and an aliquot (1×10^{12} TU) of Tomlinson I scFv library in biopanning buffer (0.2% casein, 0.05% Tween-20, and 10% goat serum in TBS) was added. The cells were incubated for 2 h with slow shaking at RT. This negative subtraction step depletes scFv-phages that bind to Aldefluor negative cell population, filter plastic, and membrane. The filter cups with their collecting tubes were centrifuged and subtracted scFv library was transferred to the other casein-blocked filter cup containing 20,000 Aldefluor positive cancer stem cells. Cancer stem cells were incubated for 2 h with slow shaking at RT. The tumor cells were washed 10 times with 0.6 mL TBST (TBS 25 mm Tris-HCl, 150 mm NaCl, pH 7.4; with 0.05%Tween-20) and once with TBS. The trypsin elution, plating, and amplification of phages were done as described earlier.[6–8] The amplified, eluted, phage pool was used as input for the next round of panning. Panning was repeated five rounds. The eluted phages from fifth round of selection were plated on ampicillin plates for isolation and identification of individual clone binders.

Binding assessment of phage monoclones to Aldefluor positive cells

Binding assessment of individual clones isolated from fifth round of panning was done in a 96-well plate with a 5.0 μm pore size polycarbonate membrane support. (Corning, NY). One thousand Aldefluor positive and negative cells were fixed with 4% paraformaldehyde made in PBS, washed, and put into each filtered well. Before incubation with cells, phage supernatants from individual clones were normalized by chemiluminescence ELISA method developed in our

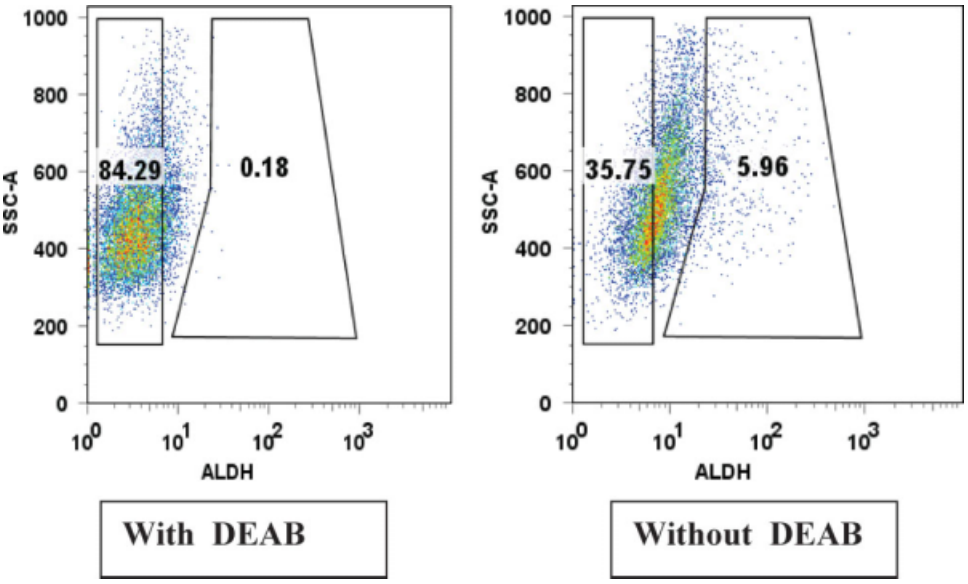

Figure 1. Representative FACS analysis of SUM159 cells using Aldefluor assay.

Cells incubated with Aldefluor substrate (BAAA) and the specific inhibitor of ALDH, DEAB. Cells incubated with DEAB were used to establish the baseline fluorescence and to define aldefluor positive region. Incubation of cells with Aldefluor substrate in the absence of DEAB induces a shift in defining the Aldefluor positive population (5.96%).

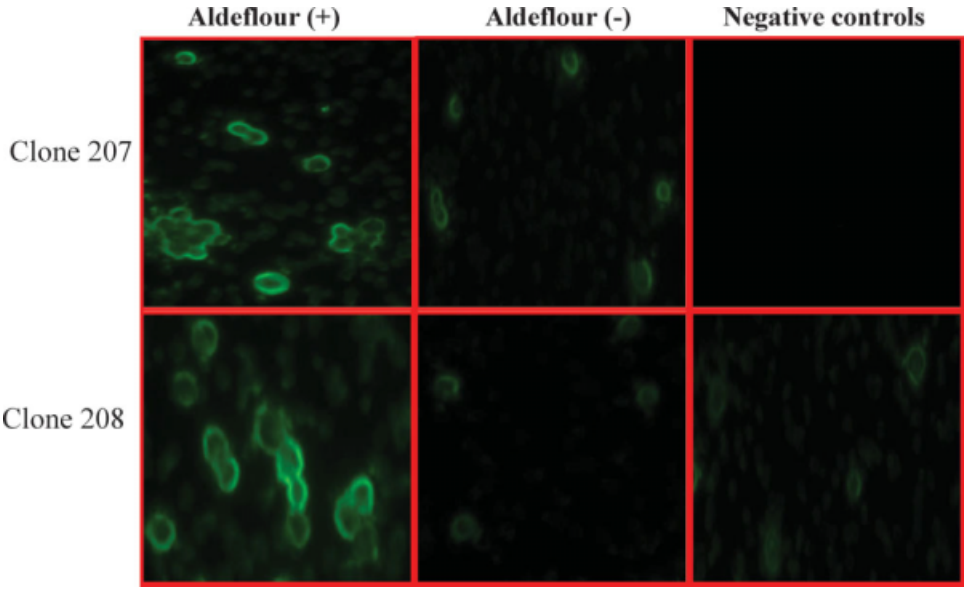

Figure 2. Immunofluorescence staining or clone 207 and clone 208 on Aldefluor positive and negative cell populations.

Unselected scFv library was used as a negative control. Both clones had higher binding to Aldefluor positive cells compared with aldefluor negative population.

laboratory.[9] Normalized phage supernatant (50 μL) were mixed with 50 μL of binding buffer (2× TBS, 0.1%Tween-20, 20% goat serum, and 0.4% casein blocker) and incubated with cells overnight at 4°C, on a slow rotator. Unselected scFv library was used as a negative control. The next day cells were washed four times with TBS containing 0.05% Igepal (TBS-Igepal) and incubated with polyclonal rabbit anti M13 antibody (Sigma-Aldrich; 1:100 dilutions) in TBS-Igepal for 1 h at room temperature. After washing, the cells were incubated with chicken anti-rabbit/Alexa Fluor 488 conjugate (Molecular Probes, Carlsbad, CA) in TBS-Igepal (1:400). Cells were washed six times with TBS-Igepal before immunofluorescence or confocal microscopy.

For ALDH1 immunostaining, ALDH1 antibody (BD biosciences) used at 1:25 dilution and goat antimouse/Alexa-fluor 568 conjugate was used as the secondary antibody (1:400).

Binding assessment of individual clones to cancer stem cells

Either trypsinized, unsorted-SUM159 cells or paraffin-embedded sections of SUM159 cells or frozen xenograft tumors from mice were used. Trypsinized cells were fixed with 100% ice cold methanol. Frozen tissue sections of xenograft tumors were mildly fixed with 2% paraformaldehyde. Paraffin sections were deparaffinized in xylene and rehydrated in graded alcohol and followed by antigen retrieval by microwaving (Sharp Carousel) sections in citrate buffer (pH 6) at low power for 5 min. Both cells and tissue sections

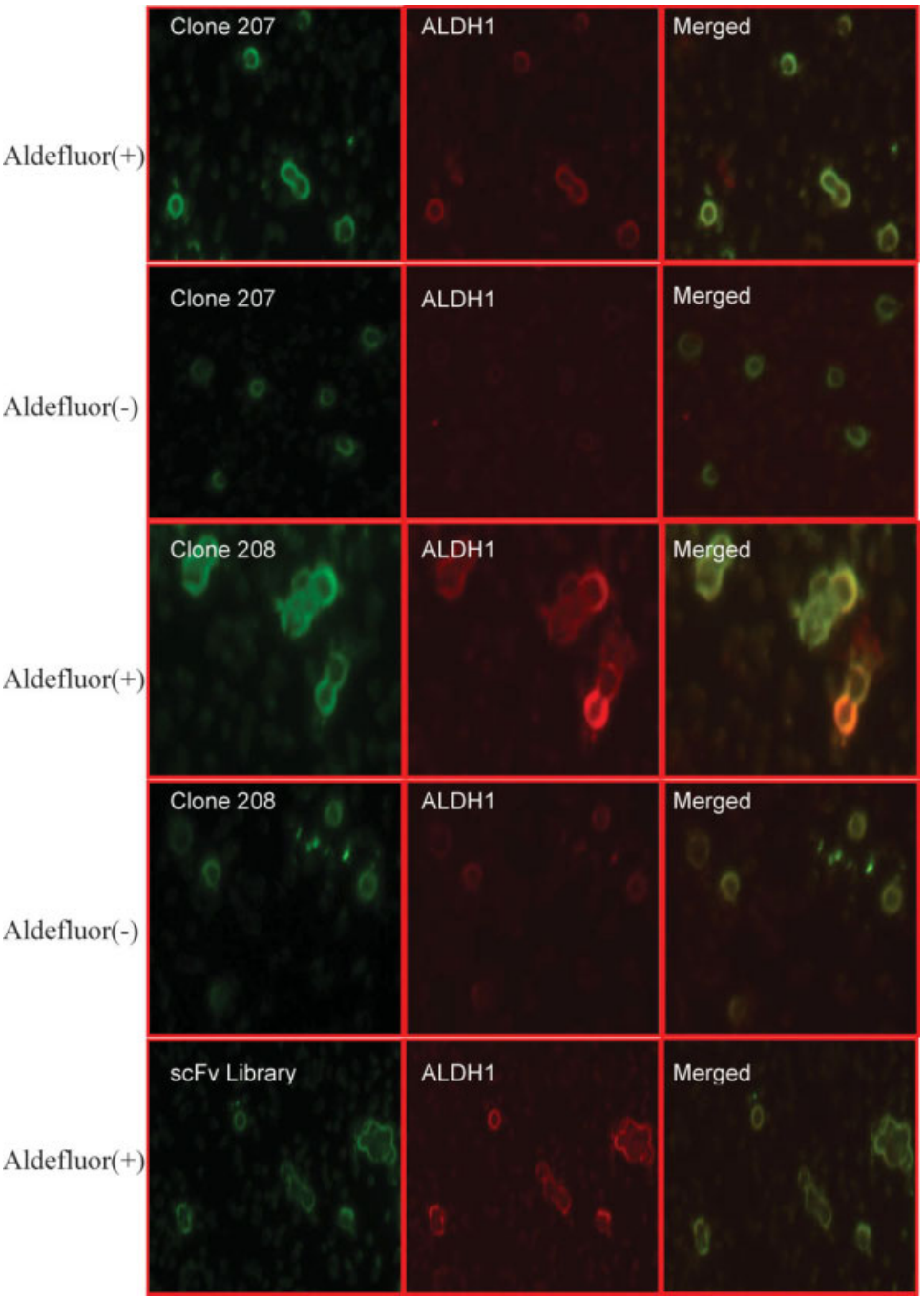

Figure 3. Immunostaining of FACS sorted cells with ALDH1 and candidate clones.

All the aldefluor positive cells were expressing higher levels of ALDH1 compared with aldefluor negative cell population. Both clone 207 and 208 had higher binding to ALDH1 positive cells compared with ALDH1 negative cells. Unselected scFV library was used as a negative control. It showed much lesser binding on ALDH1 positive cell population compared with both clone 207 and 208.

were incubated with selected phage clones as described previously and immunostaining was done with antibody cocktails of ALDH1 and anti-M13 and appropriate secondary antibodies as stated previously. Tissue sections of tumors harvested from mice were stained with anti-M13 after phage incubation.

Results

Isolation of Aldefluor positive cell population from SUM159 cancer cell line

SUM159 cells were removed from tissue culture plates by trypsinization when they became 80% confluent and ALDEFLUOR assay was utilized to detect and sort out the cancer cells with high-ALDH enzymatic activity. The average percentage of Aldefluor positive cells in the unsorted population was 5.6% (5.6 ± 1.8) and these Aldefluor positive cells were used both in panning and screening experiments (Figure 1).

Isolation of scFv expressing phage clones binding to Aldefluor positive cancer cells

The Tomlinson I scFv phage library was used to select cancer stem cell binding phages. For each round of selection, the scFv library was first preabsorbed with Aldefluor negative cells. 1×10^{12} TU of scFv phage was incubated with

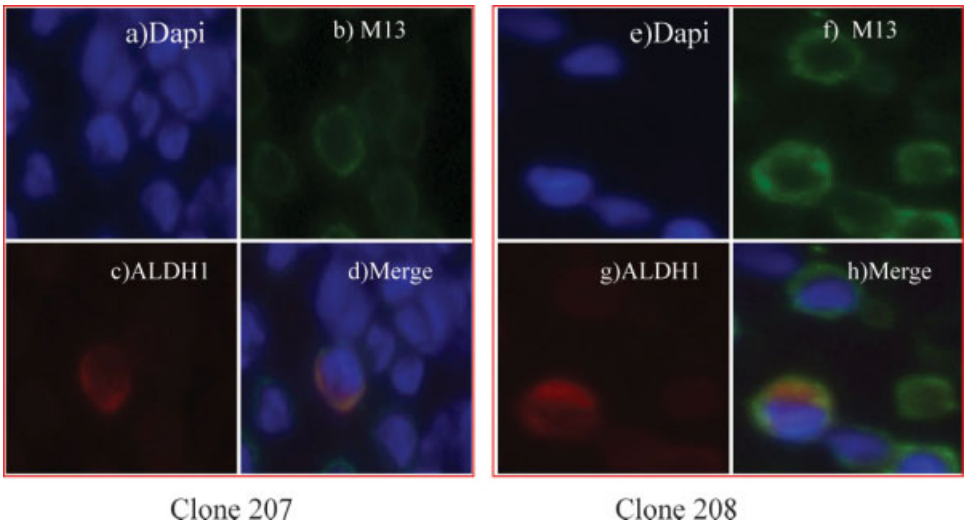

Figure 4. Immunostaining on unsorted SUM159 cell line showing the selective binding of clone 207 and clone 208 to ALDH1 positive cells.

(a–d) Cells treated with page clone 207 with the following staining: (a) dapi staining of nuclei, (b) Anti-M13, (c) Anti-ALDH1, (d) Merged image of a–c. (e–h) Cells treated with clone 208 with the following staining: (e) Dapi staining of nuclei, (f) Anti-M13, (g) Anti-ALDH1, (h) Merged image of e–g.

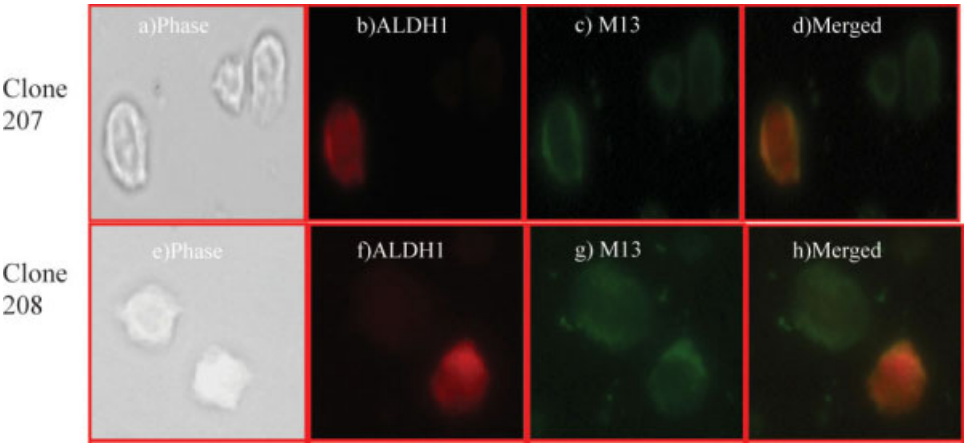

Figure 5. Immunostaining on paraffin embedded tissue sections of SUM159 cell line showing the selective binding of clone 207 and 208 to ALDH1 positive cells.

(a–d) Cells treated with phage clone 207 with the following staining: (a) Phase contrast, (b) Anti-ALDH1, (c) Anti-M13, (d) Merged image of b–c. (e–h) Cells treated with clone 208 with the following staining: (e) Phase contrast, (f) Anti-ALDH1, (g) Anti-M13, (h) Merged image of f–g.

5×10^4 Aldefluor negative cells for 2 h. This subtraction step removed not only the binders of Aldefluor negative cell population but also the phage clones binding to the filter cup. Subsequently, the depleted library was panned with 2×10^4 Aldefluor positive cells and phage eluted from each step were amplified and used as the input of the next round of panning. After five rounds of subtractive selection, isolated clones from fifth panning output were further analyzed for their specific binding ability to Aldefluor positive cells.

Binding assessment of scFv expressing phage monoclones to Aldefluor positive cell population

Clones randomly isolated from the last round of biopanning were amplified and after normalization of their concentration, they were assessed for their binding to Aldefluor positive cells by immunofluorescence imaging in 96-well plates with a permeable polycarbonate membrane. The use of 96-well filter plates miniaturized the binding assay, so that as few as, 1,000 Aldefluor positive cells/well were enough to analyze the individual clones. Individual clones (171) were analyzed and two of them, clone 207 and clone 208, clearly showed higher binding to Aldefluor positive cell population, compared with Aldefluor negative cells (Figure 2).

Next, we investigated whether ALDH1 could be used as a marker for detecting cancer stem cells. All the Aldefluor sorted cells were fixed and stained with an ALDH1 monoclonal antibody. Only the Aldefluor positive cells expressed ALDH1, whereas Aldefluor negative population contained no ALDH1 positive cells. Double staining with ALDH1 and a polyclonal antibody against the M13 filamentous phage showed that clone 207 and 208 were specifically binding to ALDH1 positive cells (Figure 3, Suppl Figure 1).

Selective binding of clone 207 and 208 to ALDH1 positive cells

To further investigate the selective binding of clone 207 and 208 to ALDH1 positive cancer stem cells, we performed double staining with ALDH1 and anti-M13 phage antibody on unsorted SUM159 cell line. Cells trypsinized from

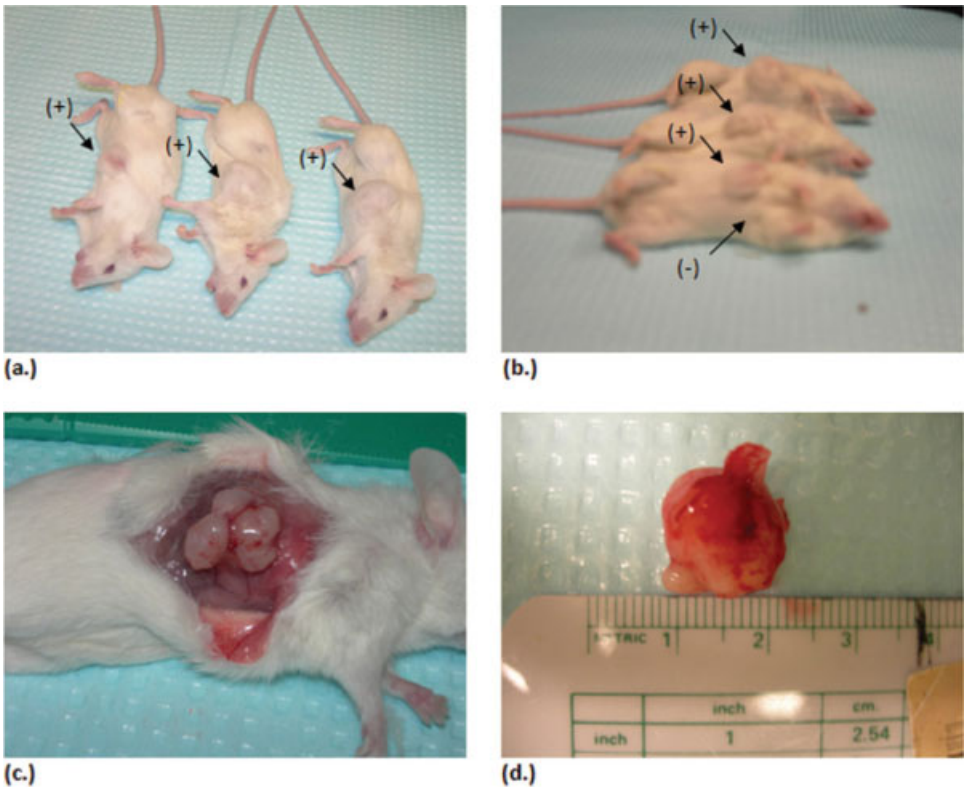

Figure 6. SUM159 tumor xenografts in NOD/SCID mice.

(a) Tumor injection site and successful tumorigenesis. (b) Tumor growth on the injected site and no tumor growth on the opposite mammary gland fat pad. (c) Well demarcated, multilobulated tumor nodule. (d) Excised tumor nodule, 1 cm in diameter.

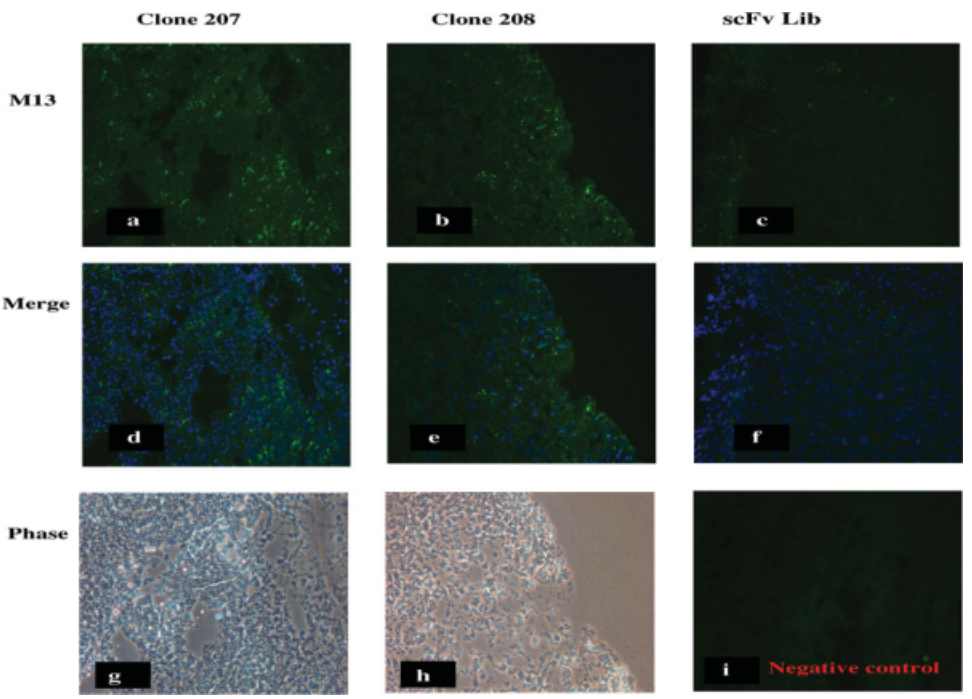

Figure 7. Paraffin-embedded tissue sections from SUM159 tumor xenografts grown in NOD/SCID mice.

(a) Tissue incubated with clone 207 and stained with anti M13. (b) Tissue incubated with clone 208 and stained with anti M13. (c) Tissue incubated with unselected scFV library and stained with anti M13. (d–f) Merged images of tissues stained with anti M13 and dapi. (g–h) Phase contast. (i) Negative control.

tissue culture plates were fixed, incubated with clone 207 and 208, and then stained for detection of cancer stem cells and binding of phage monoclones. Among the unsorted SUM159 cells, both clones showed significantly higher binding to the ALDH1 positive cells when compared with the unselected library clones. More importantly, both clones could select and bind to ALDH1 positive cells with higher intensity than the ALDH1 negative population (Figure 4).

Similar immunostaining pattern was also observed when paraffin-embedded tissue sections of unsorted SUM159 cells were used (Figure 5).

Xenograft tumor growth in NOD/SCID mice and binding of clone 207 and 208 to tumor tissue

NOD/SCID mice were injected with 4×10^6 SUM159 cells subcutaneously into the mammary gland fat pads. Palpable tumor nodules first appeared 3 weeks after tumor injection and they rapidly grew and reached 1 cm in diameter in 2 weeks (Figure 6). Frozen tissue section prepared from xenograft tumors were mildly fixed, incubated with clone 207 and 208, and then stained for detection of cancer stem cells and binding of phage monoclones. Both clones showed positive binding to tumor cells, whereas no positive fluorescence signal was detected on tissue sections incubated with unselected scFv library (Figure 7).

Discussion

The "Cancer stem cell hypothesis" stemmed from the idea that tumors are composed of functionally heterogeneous population of cells and among them only a minority of tumor cells is able to regenerate the tumor and sustain its growth. These cancer stem cells have the ability to self renew and give rise to differentiated progeny. There is growing evidence supporting that cancer stem cells are the cells responsible for tumor chemoresistance and recurrence by virtue of their slow cell cycle kinetics, transporter proteins, and antiapoptotic mechanisms.[10] Like many other investigators, these features directed our efforts toward development of novel therapeutic and diagnostic strategies targeting cancer stem cells.

Our goal is to provide a set of breast cancer stem cell specific ligands which will accelerate understanding the molecular biology of cancer stem cells, as well as lead to generation of reagents useful for diagnosis and therapy. We have chosen phage display technology to identify "cancer stem cell" specific ligands because it is a relatively rapid and high throughput process. Modifications previously developed in our laboratory[11] to miniaturize the biopanning and binding assays enabled us to do experiments with very limited number of cells which is one of the main obstacles of working with cancer stem cells.

Recently, in addition to the presence of cancer stem cells in solid human tumors, it has been shown that established cancer cell lines also retain the cellular hierarchy characteristic of primary breast tumors and contain a small population of cells mimicking cancer stem cell behavior.[3,4] Therefore, we worked with SUM159 breast cancer cell line to isolate cancer stem cells. This approach overcame the problems of not only procuring and using primary human tissues but also the difficulty of control because of the heterogeneity of their cellular, genetic, and epigenetic composition.

Aldehyde dehydrogenase 1 (ALDH1), which is a detoxifying enzyme responsible for the oxidation of intracellular aldehydes has a role in early differentiation of stem cells. It has been shown to be one of the markers used to isolate cancer stem cells from both primary human breast tumors and breast cancer cell lines.[5,12]

We used the Aldefluor kit from Stem Cell Technologies (Durham, NC) which isolates cancer stem cells based on their high-aldehyde dehydrogenase activity. Aldefluor positive and negative cell populations sorted by FACS were used in panning and clone analysis. After five rounds of panning and screening on cancer stem cells, we were able to identify two unique clones—clone 207 and 208—binding specifically to Aldefluor positive cells. Both clones had higher binding compared with unselected library clones and they also had significantly higher signal on Aldefluor positive cells than on Aldefluor negative population. More importantly, both clones were able to select and bind to ALDH1 positive cancer stem cells from unsorted SUM159 cell line ~90% of which is composed of ALDH1 negative non-stem cancer cells. When the clones were incubated with unsorted SUM159 cell line, they also showed some degree of immunostaining with the ALDH1 negative cells. Although the binding was higher than the unselected library clones, it was much lower than the real positive signal detected on ALDH1 positive cancer stem cells. Similar binding features were observed both with the paraffin-embedded sections of SUM159 cell line and tumor sections of xenografts grown in NOD/SCID mice which made these clones good candidates for analysis of archived breast cancer patient specimens. This is important because it should allow definition of the relationship of breast cancer stem cells to vascular and stromal elements. This will also be an important tool to guide laser capture of cancer stem cell in clinical specimens for genomic and proteomic analysis which will help in understanding the molecular biology behind cancer stem cells.

The applicability of clone 207 and 208 to paraffin-embedded and frozen-tissue specimens may enable us to use them as prognostic markers. Recent evidence demonstrates that the percentage of cancer stem cells may dictate the aggressiveness and relapse potential of the tumor.[5,12–14] In addition, these ligands may be used as treatment follow-up markers in select clinical situations. Most of the chemotherapeutic agents used today for cancer treatment preferentially kill the non-stem cancer cells that make up the bulk of the tumor. Unfortunately, these treatments are sparing the cancer stem cell population which is thought to be responsible for drug resistance, failure of treatment, and recurrence of disease. As a consequence of this type of therapeutic regimen, the traditional response criteria are the measurement of shrinkage in tumor bulk. This approach may not reflect the changes in smaller cancer stem cell population. Therefore, a dramatic response may overestimate the effect of therapy. Clone 207 and 208 can select for the cancer stem cells and reflect the effect of therapy on stem cell population. This may help the development of new clinical approaches taking into account the fate of cancer stem cells and help to modify the traditional measures of clinical response.

Acknowledgments

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Literature Cited

1. Ailles LE, Weissman IL. Cancer stem cells in solid tumors. *Curr Opin Biotechnol*. 2007;18:460–466.
2. Dalerba PR, Cho W, Clarke MF. Cancer stem cells: models and concepts. *Annu Rev Med*. 2007;58:267–284.

3. Fillmore CM, Kuperwasser C. Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. *Breast Cancer Res*. 2008;10:R25.
4. Setoguchi T, Taga T, Kondo T. Cancer stem cells persist in many cancer cell lines. *Cell Cycle*. 2004;3:414–415.
5. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, Jacquemier J, Viens P, Kleer CO, Liu S, Schott A, Hayes D, Bimbatun D, Wicha MS, Dontu G. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell*. 2007; 1:555–567.
6. de Wildt RM, Mundy CR, Gorick ED, Tomlinson IM. Antibody arrays for high-throughput screening of antibody-antigen interactions. *Nat Biotechnol*. 2000;18:989–994.
7. Griffiths AD, Williams SC, Hartley O, Tomlinson IM, Waterhouse P, Crosby WL, Kontcrmann RE, Jones PT, Low NM, Allison TJ. Isolation of high affinity human antibodies directly from large synthetic repertoires. *EMBO J*. 1994;13: 3245–3260.
8. Kristensen P, Winter G. Proteolytic selection for protein folding using filamentous bacteriophages. *Fold Des*. 1998;3:321–328.
9. Shukla GS, Krag DN. A sensitive and rapid chemiluminescence ELISA for filamentous bacteriophages. *J Immunoassay Immunochem*. 2005;26:89–95.
10. Liu S, Dontu G, Wicha MS. Mammary stem cells, self-renewal pathways, and carcinogenesis. *Breast Cancer Res*. 2005;7:86–95.
11. Shukla GS, Krag DN. Phage display selection for cell-specific ligands: development of a screening procedure suitable for small tumor specimens. *J Drug Target*. 2005;13:7–18.
12. Liu S, Ginestier C, Charafe-Jauffret E, Foco H, Kleer CG, Merajver SD, Dontu G, Wicha MS. BRCA1 regulates human mammary stem/progenitor cell fate. *Proc Natl Acad Sci USA*. 2008;105:1680–1685.
13. Ginestier C, Wicha MS. Mammary stem cell number as a determinate of breast cancer risk. *Breast Cancer Res*. 2007;9: 109.
14. Abraham BK, Fritz P, McClellan M, Hauptvogel P, Athelogou M, Brauch H. Prevalence of CD44+/CD24-/low cells in breast cancer may not be associated with clinical outcome but may favor distant metastasis. *Clin Cancer Res*. 2005;11:1154–1159.

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