CD44\textsuperscript{pos}CD49f\textsuperscript{hi}CD133/2\textsuperscript{hi} Defines Xenograft-Initiating Cells in Estrogen Receptor–Negative Breast Cancer


Abstract

Defining the populations of tumor-initiating cells that are present in tumors is a first step in developing therapeutics to target these cells. We show here that both CD44\textsuperscript{pos}CD24\textsuperscript{neg} and CD44\textsuperscript{pos}CD24\textsuperscript{pos} cell populations in estrogen receptor (ER) α-negative breast tumors are tumorigenic in murine xenograft models. We also describe a third population of xenograft-initiating cells (XIC) enriched in CD44\textsuperscript{pos}CD49f\textsuperscript{hi}CD133/2\textsuperscript{hi} cells that display heightened tumorigenicity, self-renewal in vivo, and the capacity to give rise to functional and molecular heterogeneity. Consistent with their capacity for self-renewal, these cells express elevated levels of Sox2, Bmi-1, and/or Nanog and their CpG islands are hypermethylated relative to nontumorigenic cells. These differences in methylene regulation may be responsible for the dramatic functional differences between the two populations. The identification of CD44\textsuperscript{pos}CD49f\textsuperscript{hi}CD133/2\textsuperscript{hi} XIC in ER-negative tumors may lead to expanded understanding of these tumors and ultimately the development of therapeutics designed to specifically target the cells. Cancer Res; 70(11); 4624–33. ©2010 AACR.

Introduction

The cancer stem cell hypothesis states that tumors consist of a cellular hierarchy. Cancer stem cells reside at the peak of this hierarchy, and the root of the cancer, and give rise to nontumorigenic, differentiated progeny that make up the tumor bulk (1). Initially identified in leukemia (2), the identification of solid tumor cancer stem cells was first described in 2003 by Al-Hajj and coworkers in breast cancer (3). In the ensuing years, cancer stem cells have also been identified in a wide range of solid tumors, including brain, colon, lung, skin, ovary, pancreas, and head and neck (4). Herein, we refer to these cells as xenograft-initiating cells (XIC) in a deliberate effort to emphasize their primary functional characteristic, the ability to yield xenografts in immunocompromised mice.

CD49f (α6 integrin) homodimerizes with CD29 (β1 integrin) or CD104 (β4 integrin) and binds laminin, facilitating attachment of epithelial cells to the extracellular matrix. In addition to this mechanical role, CD49f cooperates with receptor tyrosine kinases to communicate, bidirectionally, between the cell and the extracellular matrix. In the normal mouse (5) and human (6) mammary gland, only CD49f-positive cells possess in vivo repopulating ability. In breast cancer, elevated CD49f expression is associated with reduced survival (7) and knockdown of its partner CD104 retards in vivo tumorigenicity (8). Although a functional role of CD133 in the stem cell phenotype seems lacking (9), this protein has been shown to mark normal stem cells of the hematopoietic system, kidney, neural tissue, skin, prostate, and pancreas (10). Furthermore, CD133 is expressed by XIC in the prostate, colon, brain, and kidney (10). In the mammary gland, however, CD133 localizes to estrogen receptor (ER)–positive luminal cells that lack in vivo repopulating capabilities (6, 11).

Herein, we show that CD44\textsuperscript{pos}CD49f\textsuperscript{hi}CD133/2\textsuperscript{hi} cells, isolated from four independent ER-negative patient tumors and xenografts, are enriched for XIC capable of giving rise to triple-negative tumors [those lacking ER, progesterone receptor (PR), and HER2] and ER-negative/HER2-positive tumors. These cells have elevated expression of genes known to play key roles in both stem cell self-renewal and cancer cell tumorigenesis. Furthermore, the methylome of CD44\textsuperscript{pos}CD49f\textsuperscript{hi}CD133/2\textsuperscript{hi} cells is divergent from that of the tumor bulk, providing a possible means by which their functional heterogeneity exists. Whereas targeted therapies for ER- and HER2-positive breast tumors have...
been successful (12, 13), nontargeted, cytotoxic therapies are currently the primary means of treating triple-negative tumors. The identification of triple-negative and ER-negative/HER2-positive tumor CD44posCD49fhiCD133/2hi XIC may lead to the improved understanding of these tumors and ultimately the development of targeted therapies.

Materials and Methods

Patient samples
Primary breast specimens and pleural effusion samples were collected from breast cancer patients in accordance with the National Cancer Institute (NCI) Review Board. Cells from pleural effusions were collected by centrifugation, washed twice with HBSS (Invitrogen), frozen viably in DM50 freeze media (Millipore), and frozen in liquid nitrogen until used. Tumor fragments were cut into 1-mm pieces and placed immediately into the intact abdominal fat pads of nonobese diabetic–severe combined immunodeficient (NOD-SCID) mice. Sorted or unsorted cells from pleural effusions were similarly injected.

In vivo tumorigenicity and processing of xenografts
In vivo tumorigenicity was assessed by both frequency and latency of tumor formation in the abdominal mammary gland fat pad of 4- to 8-week-old NOD-SCID mice obtained from the NCI colony (APA, Frederick, MD). All animal experiments were conducted in accord with accepted standards of humane animal care and approved by the Animal Care and Use Committee at the NIH. Five days before injection of cells, mice were administered etoposide i.p. (30 mg/kg body weight; Calbiochem) and estrogen via a subcutaneous pellet (>95% release; Innovative Research of America). Mice were anesthetized by an i.p. injection of ketamine/xylazine (750 and 50 mg/kg body weight; Arkam). Mice were administered etoposide i.p. (30 mg/kg body weight; Calbiochem) and estrogen via a subcutaneous pellet (90-d release; Innovative Research of America). Mice were anesthetized by an i.p. injection of ketamine/xylazine (750 and 50 mg/kg body weight, respectively) in 200 μL HBSS before surgically exposing the gland for injection. Cells were suspended in an F12 (Invitrogen)/Matrigel (high concentration; BD Biosciences) mixture (4:1) and injected into the mammary fat pad in a 50 μL volume. Tumor sizes were monitored weekly for 90 days and were not permitted to exceed 1 cm³. Xenografts were minced into <1-mm pieces and dissociated in F12 media containing collagenase type 3 (100 units/mL; Worthington Biochemical Corp.), dispase (0.8 units/mL; Invitrogen), and penicillin-streptomycin (100 units/mL; Invitrogen); incubated at 37°C with gentle agitation (250 rpm) for 90 to 120 minutes; and collected. Single cells were generated by an additional incubation in 0.05% trysin-EDTA (Invitrogen) for 5 minutes at 37°C. H&E-stained sections of mammary glands devoid of frank tumors were examined for the presence of macroscopic lesions.

Flow cytometric analysis and sorting
Antibodies were purchased from BD Pharmingen unless otherwise noted. Anti-human CD44 conjugated to allophycocyanin or phycoerythrin-cyanine 7 (PE-Cy7; eBioscience), anti-human CD24 conjugated to PE, anti-human CD49f-PE-Cy5, and anti-human CD133/2-PE (Miltenyi Biotech) were used for both analysis and live cell sorting. 7-Aminoactinomycin D (1 μg/mL final concentration; BD Pharmingen) or 4',6-diamidino-2-phenylindole (DAPI; 0.1 μg/mL final concentration; Invitrogen) was used for live/dead cell distinction. Antibodies used for depletion of nontumor cells in pleural effusions (FITC-conjugated anti-human CD2, CD3, CD10, CD16, CD18, CD31, CD64, and CD140b) were similar to those described by Al Hajj and colleagues (3). For analysis of xenograft tumor cells, FITC-conjugated anti-mouse Ter119, CD31, CD45, and H-2Kd were used to exclude cells of mouse origin.

For flow cytometric analysis, cells were stained with antibodies in a 1× PBS solution containing 0.1% bovine serum albumin (BSA) and 0.1% sodium azide (Sigma) for 25 minutes at 4°C. Analyses were performed on either a BD FACSCalibur or BD LSR II. For live sorting, cells were stained with antibodies in a 1× PBS solution containing 0.1% fetal bovine serum and 100 units/mL penicillin-streptomycin for 25 minutes at 4°C. For dissociated xenografts, gates were established after compensation with lineage pos cells (Ter119neg, CD31neg, CD45neg, and H-2Kdpos cells) that were not exposed to anti-CD44, anti-CD49f, or anti-CD133/2 antibodies. This was done because lineage pos cells were found to have different background autofluorescence than lineage neg cells (Supplementary Fig. S1). Cell sorting was performed on a BD FACSARia operating at low pressure (20 p.s.i.) using a 100-μm nozzle. Cell clusters and doublets were electronically gated out. Post-sort analysis routinely indicated purities of >95%. Fluorescence-activated cell sorting data were analyzed using FlowJo v8.7.3 (Tree Star).

Tumor sphere formation assay
Xenografts were sorted as described above directly into 96-well, ultralow attachment plates (Corning Life Sciences) containing 50 μL of DMEM/F12 (Invitrogen) supplemented with 20 ng/mL human epidermal growth factor (PeproTech), 20 ng/mL basic fibroblast growth factor (PeproTech), 2% B27 (Invitrogen), 100 units/mL penicillin-streptomycin, and 1 μg/mL fungizone (Invitrogen). Cells were sorted in the following dilutions: 50, 250, and 500 cells per well. Cells were cultured at 37°C for 14 days. Growth factors were replenished 7 days after initiation of experiments. Tumor sphere growth was quantified microscopically under ×20 magnification.

Cytosine extension assay
Alterations in DNA methylation were evaluated via a modified cytosine extension assay (14). Briefly, duplicate tubes containing 50 ng of genomic DNA [isolated via Trizol (Invitrogen) from live sorted xenografts] were digested overnight with a 10-fold excess of HpaII or BssHII endonuclease (New England Biolabs); in addition, one tube was incubated without restriction enzyme addition and served as a non-specific background control. The 35-μL nucleotide extension reaction [containing 50 ng of DNA, 1x PCR buffer (Invitrogen), 1.0 mmol/L MgCl2, 0.35 unit of Taq DNA polymerase (Invitrogen), and 8.0 μCi [32P]dCTP (Perkin-Elmer)] was incubated at 72°C for 2 hours. Triplicate 10-μL aliquots from
each reaction were applied to DE-81 ion-exchange filters (Whatman). Unincorporated [\(^{32}\)P]dCTP was removed with three 10-minute washes of 1.0 mol/L sodium phosphate buffer (pH 7.0). Incorporated [\(^{32}\)P]dCTP was quantified with a Beckman LS600iC liquid scintillation counter (Beckman Coulter). [\(^{32}\)P]dCTP that bound nonspecifically to uncut DNA was subtracted from enzyme-treated samples, and the results were expressed as relative [\(^{32}\)P]dCTP incorporation. With this methodology, reduced [\(^{32}\)P]dCTP incorporation is associated with hypermethylation.

**Real-time reverse transcription-PCR**

Total RNA was isolated from cells using the Trizol reagent and treated with amplification-grade DNase I (Invitrogen) to remove genomic contamination. RNA was then reverse transcribed using SuperScript III reverse transcriptase (Invitrogen) primed with oligo(dT) and random hexamers. cDNA was subjected to real-time PCR amplification using gene-specific primers and 2× Brilliant II SYBR Green QPCR Mastermix (Roche Applied Science). Primer sequences and PCR conditions are given in Supplementary Table S1. Data are presented using the mean \(\Delta \Delta C_t\) method.

**Immunohistochemistry**

Cross-sections of xenografts were formalin fixed and paraffin embedded. Five-micrometer-thick sections were deparaffinized with xylene and rehydrated with decreasing concentrations of ethanol. Antigen retrieval was achieved with citrate buffer (Dako). Endogenous peroxidase activity was blocked by a 10-minute incubation in 3% hydrogen peroxide followed by a 10-minute wash in 1× PBS. Immunostaining was performed using Vectastain ABC kit (Vector) according to the manufacturer’s instruction. Color was developed with diaminobenzidine peroxidase substrate kit (Vector), and sections were counterstained with hematoxylin (Sigma). Antibodies were obtained from the following sources: cytokeratin 5 (CK5), CK8, ER, PR (Novacastra, Leica Microsystems), p63 (Abcam), and HER2 (Dako). A breast tissue sample that had been previously determined to express high levels of the protein of interest was used as a positive control. A serial section of each sample that received all staining steps, with the exception of the primary antibody, was used as a negative control.

**Immunofluorescence and confocal microscopy**

Two human breast tumor tissue microarrays (U.S. Biomax, Inc.) were used for analysis; each contained 2 invasive lobular and 35 invasive ductal carcinoma specimens and 3 normal breast samples. One array was stained with the antibodies of interest, whereas the other was processed appropriately as a control. Arrays were blocked in 1× PBS containing 10% goat serum and 2% BSA (blocking buffer) for 30 minutes at room temperature. Arrays were then incubated with a 1:50 dilution of the primary antibodies in 2% BSA and 2% goat serum sequentially as follows: overnight at 4°C with anti-CD44 (rabbit polyclonal; Abcam), 1 hour at room temperature with anti-CD49f (rat monoclonal; Affinity BioReagents, Thermo Fisher Scientific), and 1 hour at room temperature with anti-CD133/2 (mouse monoclonal; Miltenyi Biotech). Each slide was subsequently washed with 1× PBS and then incubated with appropriate secondary antibody (anti-rabbit Alexa Fluor 594, anti-rat Alexa Fluor 647, or anti-mouse Alexa Fluor 488; Invitrogen) at a 1:1,000 dilution in blocking buffer for 30 minutes at room temperature. Coverslips were mounted using Prolong Gold antifade reagent with DAPI (Invitrogen). Imaging was performed using the Carl Zeiss LSM510 confocal imaging system (Carl Zeiss MicroImaging) at ×63 magnification.

**Statistics**

ANOVA was performed using StatView 5.0.1 (SAS Institute).

**Results**

**Tumorigenicity of CD44\(^{+}\)CD24\(^{−}\) and CD44\(^{+}\)CD24\(^{−/\text{low}}\) cells from ER-negative tumors**

Breast XICs were previously identified as CD44\(^{+}\)CD24\(^{−/\text{low}}\) (3). We set out to expand on this seminal work by identifying novel markers capable of further enriching XIC from the CD44\(^{+}\)CD24\(^{−/\text{low}}\) population of ER-negative breast tumors. Xenografts were established with pleural effusions by injecting sorted or unsorted cells into the fat pad of NOD-SCID mice. Ten of 14 pleural effusions with unknown ER status failed to engraft (data not shown). Xenografts were characterized for the expression of ER, PR, HER2, CK5, CK8, and p63 by immunohistochemistry. All xenografts possessed basal/myoepithelial markers (CK5 and p63) and were ER/PR negative. Two expressed elevated levels of the HER2 oncoprotein (Supplementary Table S2).

Whereas CD44\(^{−}\) cells were largely nontumorigenic, experiments evaluating the tumorigenicity of CD44\(^{+}\)CD24\(^{−}\) and CD44\(^{+}\)CD24\(^{−/\text{low}}\) cells sorted from ER-negative tumors indicated that both populations were tumorigenic (Table 1). Specifically, 250 cells (both CD44\(^{+}\)CD24\(^{−}\) and CD44\(^{+}\)CD24\(^{−/\text{low}}\)) were frequently able to form tumors. Furthermore, CD44\(^{+}\)CD24\(^{−/\text{low}}\) cells readily gave rise to CD44\(^{+}\)CD24\(^{−/\text{low}}\) progeny and vice versa (Supplementary Fig. S2A). Pleural effusions SH1 and SH12 were the notable deviations from this observation. In the case of pleural effusion SH1, 1,000 CD44\(^{+}\)CD24\(^{−}\) pleural cells were tumorigenic, whereas an equal number of CD44\(^{+}\)CD24\(^{−/\text{low}}\) cells were not (Table 1; Supplementary Fig. S2B). SH12 CD44\(^{+}\)CD24\(^{−/\text{low}}\) pleural cells were tumorigenic, whereas all other cells failed to yield a xenograft, consistent with observations made by Al-Hajj and colleagues (3). However, after one passage in vivo, SH12 XIC acquired CD24 expression. Specifically, both CD44\(^{+}\)CD24\(^{−/\text{low}}\) and CD44\(^{+}\)CD24\(^{−}\) cells from the passage 1 SH12 xenograft were tumorigenic (Table 1; Supplementary Fig. S2C). The purity of post-sorted cells routinely approached or exceeded 95% (Supplementary Fig. S3). Taken together, these data strongly suggest that in ER/PR-negative tumors, XICs are not always restricted to the CD44\(^{+}\)CD24\(^{−/\text{low}}\) population.
CD44posCD49fhiCD133/2hi cells are enriched for tumor-forming ability

After confirming that CD44neg cells are largely nontumorogenic, CD44pos cells were evaluated to further segregate this population into a tumorigenic and nontumorigenic fraction. With the tumors described above, CD44pos cells were evaluated for the expression of CD29, SSEA4, CD49f, CD133/2, TDGF1, CD200, CD117, CD135, or CD205. All markers, with the exception of CD205, were initially selected because they had previously been associated with normal stem cells and/or XIC (6, 10, 15 – 19). CD205 was chosen based on unpublished data suggesting it may decorate XIC. These populations were then sorted and injected into the abdominal mammary fat pad of NOD-SCID mice to evaluate the ability of each marker to segregate out a tumorigenic and nontumorigenic fraction from the CD44pos population. The expression of CD29, SSEA4, and CD205 was chosen based on unpublished data suggesting it may decorate XIC. These populations were then sorted and injected into the abdominal mammary fat pad of NOD-SCID mice to evaluate the ability of each marker to segregate out a tumorigenic and nontumorigenic fraction from the CD44pos population. The expression of CD29, SSEA4, and CD205 was chosen based on unpublished data suggesting it may decorate XIC. These populations were then sorted and injected into the abdominal mammary fat pad of NOD-SCID mice to evaluate the ability of each marker to segregate out a tumorigenic and nontumorigenic fraction from the CD44pos population. The expression of CD29, SSEA4, and CD205 was chosen based on unpublished data suggesting it may decorate XIC. These populations were then sorted and injected into the abdominal mammary fat pad of NOD-SCID mice to evaluate the ability of each marker to segregate out a tumorigenic and nontumorigenic fraction from the CD44pos population.

Table 1. Summary of in vivo tumorigenicity of six pleural effusions and xenografts sorted into CD44posCD24neg/dim and CD44posCD24pos populations

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Tumors/injection</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>250 500 1,000</td>
</tr>
<tr>
<td>SH5 xenograft</td>
<td></td>
</tr>
<tr>
<td>CD44posCD24pos</td>
<td>2/4 2/2</td>
</tr>
<tr>
<td>CD44posCD24neg/dim</td>
<td>3/4 4/4</td>
</tr>
<tr>
<td>CD44neg</td>
<td>0/2 0/3</td>
</tr>
<tr>
<td>SH12 pleural effusion</td>
<td></td>
</tr>
<tr>
<td>CD44posCD24pos</td>
<td>0/4 0/6</td>
</tr>
<tr>
<td>CD44posCD24neg/dim</td>
<td>2/6 2/4</td>
</tr>
<tr>
<td>SH12 xenograft</td>
<td></td>
</tr>
<tr>
<td>CD44posCD24pos</td>
<td>1/4 4/4</td>
</tr>
<tr>
<td>CD44posCD24neg/dim</td>
<td>3/5 1/2</td>
</tr>
<tr>
<td>SH1 pleural effusion</td>
<td></td>
</tr>
<tr>
<td>CD44posCD24pos</td>
<td>4/4 0/3</td>
</tr>
<tr>
<td>CD44posCD24neg/dim</td>
<td>0/5 0/5</td>
</tr>
<tr>
<td>SH1 xenograft</td>
<td></td>
</tr>
<tr>
<td>CD44posCD24pos</td>
<td>1/5 1/5</td>
</tr>
<tr>
<td>CD44neg</td>
<td>1/5 1/5</td>
</tr>
<tr>
<td>012 xenograft</td>
<td></td>
</tr>
<tr>
<td>CD44posCD24pos</td>
<td>1/5 1/5</td>
</tr>
<tr>
<td>CD44posCD24neg/dim</td>
<td>0/5 0/5</td>
</tr>
<tr>
<td>CD44neg</td>
<td>0/5 0/5</td>
</tr>
</tbody>
</table>

CD44posCD49fhiCD133/2hi cells give rise to immunophenotypic and functional heterogeneity

Xenografts initiated with CD44posCD49fhiCD133/2hi cells were dissociated and evaluated for their expression of CD44posCD49neg/lowCD133/2neg/low populations were sorted and injected into the mammary fat pad. Adding a third marker had mixed results in further enriching XIC activity, but CD44posCD49neg/lowCD133/2neg/low cells were consistently less tumorigenic than CD44posCD49neg/low or CD44posCD133/2neg/low cells (Table 2). Across all tumors evaluated, CD44posCD49fhiCD133/2hi cells were enriched for XIC relative to CD44posCD49neg/lowCD133/2neg/low cells, which were largely depleted of this population. Despite this commonality, variation in the tumorigenicity of CD44posCD49fhiCD133/2hi cells exists across samples tested, with SH12 being most tumorigenic. Likewise, SH12 CD44posCD49neg/lowCD133/2neg/low cells retained some capacity for forming xenografts, whereas this population sorted from other tumors was nontumorigenic.

Table 2. Tumor-initiating ability of CD44pos-CD49fhiCD133/2hi and CD44posCD49neg/low-CD133/2neg/low cells

<table>
<thead>
<tr>
<th>Tumor, population</th>
<th>Tumors/injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 250 2,500</td>
</tr>
<tr>
<td>012 xenograft</td>
<td></td>
</tr>
<tr>
<td>CD44posCD49fhiCD133/2hi</td>
<td>0/5 2/5</td>
</tr>
<tr>
<td>CD44posCD49neg/lowCD133/2neg/low</td>
<td>0/5 0/5</td>
</tr>
<tr>
<td>CD44posCD49fhi</td>
<td>1/3 1/3</td>
</tr>
<tr>
<td>CD44posCD49neg/low</td>
<td>0/5 0/5</td>
</tr>
<tr>
<td>CD44posCD133/2hi</td>
<td>0/5 0/5</td>
</tr>
<tr>
<td>CD44posCD133/2neg/low</td>
<td>1/5 1/5</td>
</tr>
<tr>
<td>SH5 xenograft</td>
<td></td>
</tr>
<tr>
<td>CD44posCD49fhiCD133/2hi</td>
<td>0/5 7/10 3/4</td>
</tr>
<tr>
<td>CD44posCD49neg/lowCD133/2neg/low</td>
<td>0/5 0/5</td>
</tr>
<tr>
<td>CD44posCD49fhi</td>
<td>1/3 1/3</td>
</tr>
<tr>
<td>CD44posCD49neg/low</td>
<td>0/5 0/5</td>
</tr>
<tr>
<td>CD44posCD133/2hi</td>
<td>0/5 0/5</td>
</tr>
<tr>
<td>CD44posCD133/2neg/low</td>
<td>1/5 0/5</td>
</tr>
<tr>
<td>SH1 xenograft</td>
<td></td>
</tr>
<tr>
<td>CD44posCD49fhiCD133/2hi</td>
<td>3/5 4/5</td>
</tr>
<tr>
<td>CD44posCD49neg/lowCD133/2neg/low</td>
<td>0/5 0/5</td>
</tr>
<tr>
<td>SH12 xenograft</td>
<td></td>
</tr>
<tr>
<td>CD44posCD49fhiCD133/2hi</td>
<td>3/6 4/4</td>
</tr>
<tr>
<td>CD44posCD49neg/lowCD133/2neg/low</td>
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</tr>
<tr>
<td>CD44posCD49fhi</td>
<td>2/5 2/5</td>
</tr>
<tr>
<td>CD44posCD49neg/low</td>
<td>4/5 4/5</td>
</tr>
<tr>
<td>CD44posCD133/2hi</td>
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</tr>
<tr>
<td>CD44posCD133/2neg/low</td>
<td>9/10 4/9</td>
</tr>
</tbody>
</table>
CD44, CD49f, and CD133/2. In all cases, the immunophenotype of the xenograft was similar to that of the parental tumor (Fig. 2A; Supplementary Figs. S4 and S5; unstained gating controls in Supplementary Fig. S6). This was observed in both passage 1 xenografts initiated with sorted pleural cells and in xenografts passaged as many as six times. Xenografts resulting from CD44posCD49fiCD133/2hi cells were also the same molecular subtype as the parental tumor. Specifically, the expression level and distribution of CK8, CK5, and p63 (Fig. 2B) as well as ER, PR, and HER2 (data not shown) were similar between the parental tumor from which CD44posCD49fiCD133/2hi cells were derived and the resulting xenografts.

CD44posCD49fiCD133/2hi cells also expanded in vivo, giving rise to functional heterogeneity. Specifically, these XICs expanded via self-renewal and gave rise to nontumorigenic progeny. Furthermore, this ability was maintained over multiple in vivo passages. In the case of passage 5 xenograft SH5, CD44posCD49fiCD133/2hi cells were nontumorigenic, whereas CD44posCD49fhiCD133/2hi cells gave rise to passage 6 xenografts. Likewise, passage 6 CD44posCD49fhiCD133/2hi cells formed passage 7 xenografts that contained nontumorigenic CD44posCD49fhiCD133/2neg/low cells (Supplementary Table S4).

In addition to giving rise to immunophenotypic and functional heterogeneity, CD44posCD49fhiCD133/2hi cells were enriched for the ability to grow in suspension culture. With all three xenografts evaluated, CD44posCD49fhiCD133/2hi cells were more efficient at forming tumor spheres than CD44posCD49fhiCD133/2neg/low cells (P < 0.05; Supplementary Fig. S7). The number of cells sorted into each well did not affect the frequency of tumor sphere formation (data not shown). Despite differences between the populations within a xenograft, there were marked differences among xenografts in the efficiency with which CD44posCD49fhiCD133/2hi cells formed tumor spheres. Whereas ∼2% of SH5 CD44posCD49fhiCD133/2hi cells had the capacity to grow in suspension, <0.5% of SH1 and SH12 CD44posCD49fhiCD133/2hi had this ability. Whereas some CD44posCD49fhiCD133/2neg/low cells grew in suspension, the resulting spheres were typically smaller than those formed by CD44posCD49fhiCD133/2neg/low cells (Supplementary Fig. S7).

**Molecular heterogeneity in ER-negative tumors**

Data presented above show that CD44posCD49fhiCD133/2hi cells represent a functionally unique population. Quantitative real-time reverse transcription-PCR was used to determine if these cells also possessed divergent transcript abundance of specific genes. Relative to CD44negCD49fneg/lowCD133/2neg/low cells and CD44posCD49fneg/lowCD133/2neg/low cells (hereon referred to as CD44pos and negCD49fneg/lowCD133/2neg/low cells), CD44posCD49fhiCD133/2hi cells had elevated levels of the luminal marker K18 and the basal marker K14 (Fig. 3A). Additionally, these cells expressed elevated levels of genes critical for self-renewal (Bmi-1, Nanog, and Sox2; Fig. 3B). These data show that key transcripts associated with a primitive cell phenotype were upregulated in the CD44posCD49fhiCD133/2hi population. Differences in cell function and transcript abundance between CD44posCD49fhiCD133/2hi and CD44pos and negCD49fhiCD133/2neg/low cells suggest that differential transcriptional regulation may exist between these two populations. A means by which these differences could arise is through divergent CpG methylation patterns. The cytosine extension assay (14) was used to evaluate the methylation status of CpG dinucleotides of sorted cells and unfractonated...
xenografts. This assay takes advantage of the methylation-sensitive restriction enzymes HpaII and BssHII and the incorporation of \[^{32}P\]dCTP at the guanine overhang resulting from restriction enzyme activity. With this methodology, reduced \[^{32}P\]dCTP incorporation is associated with hypermethylation. Whereas HpaII cleaves unmethylated CpG sites scattered randomly throughout the genome, BssHII sites are enriched in CpG islands (14).

Among all four xenografts, the ratio of CpG island methylation to global CpG methylation (BssHII to HpaII ratio; hereon referred to as island to global CpG methylation index) was lower for CD44\(^{\text{pos}}\)CD49f\(^{\text{hi}}\)CD133/2\(^{\text{hi}}\) cells than both CD44\(^{\text{pos}}\) and negCD49f\(^{\text{neg/low}}\)CD133/2\(^{\text{neg/low}}\) cells and the unfractionated xenograft (\(P < 0.05\); Fig. 3C). The lower island to global CpG methylation index was attributed largely to hypermethylation (\(P = 0.054\)) of CpG islands of CD44\(^{\text{pos}}\)CD49f\(^{\text{hi}}\)CD133/2\(^{\text{hi}}\) cells relative to CD44\(^{\text{pos}}\) and negCD49f\(^{\text{neg/low}}\)CD133/2\(^{\text{neg/low}}\) cells (data not shown). These data indicate that the CD44\(^{\text{pos}}\)CD49f\(^{\text{hi}}\)CD133/2\(^{\text{hi}}\) DNA methy- lome is unique from that of nontumorigenic cancer cells.

**Localization of cells positive for CD44, CD49f, and CD133/2 in normal and cancerous breast tissue.**

We showed above that CD44\(^{\text{pos}}\)CD49f\(^{\text{hi}}\)CD133/2\(^{\text{hi}}\) cells represent a unique, highly tumorigenic population of cells. Next, we set out to evaluate the localization of these cells in primary, ER-negative breast tumors and normal breast tissue using confocal microscopy. A high percentage of tumor cells expressed CD44 (red) and had some degree of reactivity to CD49f (Fig. 4, purple). A smaller fraction of primary tumor cells stained positive for CD133 (green). Merging of confocal images permitted visualization of cells positive for all three antigens (white). In tumors, CD44\(^{\text{pos}}\)CD49f\(^{\text{hi}}\)CD133/2\(^{\text{hi}}\) cells were primarily observed in unorganized clusters and only rarely as a single cell. In the normal breast, CD44\(^{\text{pos}}\)CD49f\(^{\text{hi}}\)CD133/2\(^{\text{hi}}\) cells were very abundant and were frequently found to be the polarized cells that make up the lobular/alveolar subunit, with CD133/2 marking the apical side and CD49f marking the basolateral side of the cells (Fig. 4; Supplementary Fig. S8A). This highly organized localization pattern is in stark contrast to that observed in primary ER-negative breast tumor tissue. In an effort to confirm whether the structures evaluated in Fig. 4 were indeed mammary epithelium, reduction mammoplasties were queried for the expression of CK8 and CK5. As seen in Supplementary Fig. S8B, normal epithelial structures reminiscent of those seen in Fig. 4 and Supplementary Fig. S8A stained positive for the mammary epithelial keratins CK8 and CK5. Negative controls for confocal images are provided in Supplementary Fig. S9.

**Discussion**

The cancer stem cell hypothesis suggests that cancers are made up of a cellular hierarchy, with the tumor bulk consisting of differentiated cells that lack self-renewal mechanisms.
Instead, the ability to self-renew (and conceivably seed distant metastases) resides exclusively in a population of cancer stem cells. The first characterization of solid tumor XIC was described by Al-Hajj and colleagues (3). Subsequently, the prospective identification of XIC has been described in a broad range of solid tumors (reviewed in ref. 4).

Here, we provide the first evidence that the XIC immunophenotype of ER-negative breast cancer is rarely CD44\textsuperscript{pos}\textsuperscript{CD24\textsuperscript{neg}}. Specifically, using four triple-negative and ER-negative/HER2-positive tumors, we found that XICs were equally dispersed between the CD44\textsuperscript{pos}\textsuperscript{CD24\textsuperscript{neg}} and CD44\textsuperscript{pos}\textsuperscript{CD24\textsuperscript{pos}} populations (Table 1). Similarly, we recently reported that CD44\textsuperscript{pos}\textsuperscript{CD24\textsuperscript{pos}} and CD44\textsuperscript{pos}\textsuperscript{CD24\textsuperscript{neg}} populations sorted from breast cancer cell lines are equally tumorigenic (20).

Whereas XICs reside in both CD44\textsuperscript{pos}\textsuperscript{CD24\textsuperscript{neg}} and CD44\textsuperscript{pos}\textsuperscript{CD24\textsuperscript{pos}} populations in ER-negative tumors, we found that CD44\textsuperscript{neg} cells are largely nontumorigenic (Table 1), a finding consistent with that reported by Al Hajj and colleagues (3). CD44 expression is known to support survival, invasiveness, and tumorigenicity of cancer cells, and depletion of the protein ablates these phenotypes (21–25). Given the dominant phenotype associated with CD44 negativity, we chose to identify markers that could segregate CD44\textsuperscript{pos} cells into a tumorigenic and nontumorigenic population. This work led to our observation that the tumorigenic capacity of ER-negative breast cancer is enriched in the CD44\textsuperscript{pos}\textsuperscript{CD49\textsuperscript{hi}}CD133/2\textsuperscript{hi} population relative to CD44\textsuperscript{pos}\textsuperscript{CD49\textsuperscript{neg/low}}CD133/2\textsuperscript{neg/low} cells, which were largely nontumorigenic. In addition to enriching for tumorigenic cells, this population also possesses other hallmarks of XIC, including the capacity to form tumors in vivo and give rise to functional and molecular heterogeneity. Consistent with their ability to self-renew in vivo, CD44\textsuperscript{pos}\textsuperscript{CD49\textsuperscript{hi}}CD133/2\textsuperscript{hi} cells expressed elevated levels of Sox2, Bmi-1, and Nanog, transcription factors known to play key roles in the self-renewal process. The relationships between these factors and transforming growth factor \(\beta\) (TGF\(\beta\)) signaling (the latter is upregulated in CD44\textsuperscript{pos}\textsuperscript{CD24\textsuperscript{neg/dim}} breast cancer cells; ref. 26) are inconsistent and seem to be cell type dependent (27–29).

The CD44\textsuperscript{pos}\textsuperscript{CD49\textsuperscript{hi}}CD133/2\textsuperscript{hi} population obtained from two pleural effusions and four xenografts consistently enriched for cells capable of initiating xenografts in mouse mammary fat pads. Across pleural effusions and xenografts, however, there were marked differences in the tumorigenicity of the CD44\textsuperscript{pos}\textsuperscript{CD49\textsuperscript{hi}}CD133/2\textsuperscript{hi} population. Specifically, SH12 CD44\textsuperscript{pos}\textsuperscript{CD49\textsuperscript{hi}}CD133/2\textsuperscript{hi} cells were between 5- and 6-fold more tumorigenic than those cells sorted from SH5, SH1, and 012 xenografts. Similar differences were
observed in the frequency of tumor sphere–forming efficiency. These data show that CD44posCD49fhiCD133/2hi cells remain a highly heterogeneous population. Similar heterogeneity in tumorigenicity across xenografts was observed in the CD44posCD49fneg/lowCD133/2neg/low population. Specifically, these cells from SH5, SH1, and 012 xenografts were nontumorigenic, whereas those from SH12 pleural effusion and xenograft possessed a very limited capacity to form xenografts.

Identification of the cell population targeted for transforming mutations and ultimately becoming a XIC is of great interest. Given their shared capacity for unlimited self-renewal, fewer accumulated mutations would presumably be required to transform a normal stem cell into a cancer stem cell or XIC than would be required for a differentiated cell. Indeed, in the case of most acute myeloid leukemias, hematopoietic progenitors, as opposed to more differentiated cells, are the target of mutations leading to the disease (2). CD49f positivity has been associated with primitive cells uniquely possessing in vivo mammary gland repopulating ability in both mice (5) and humans (6). Stem cells of multiple mouse and human organs express CD133 (10). In the breasts of both mice (11) and humans (6), however, its CD133 expression is limited to ER-positive, differentiated luminal cells devoid of in vivo repopulating capability (6, 11). Indeed, in the normal human mammary gland, we report that CD133-positive cells are frequently polarized and reside in the luminal/alveolar structure (Fig. 4). Given its localization in the normal breast and the fact that the ER-negative XICs described herein express CD133 support the hypothesis that they may originate from luminal-committed cells (6).

The observation that a differentiated cell can be the target of mutations yielding a XIC phenotype is not unprecedented. Specifically, differentiated populations are targets of transformation in both the hematopoietic system (30) and prostate (31). More recently, Lim and colleagues (6) showed that basal-like breast tumors likely originate from luminal progenitors as opposed to the more primitive population enriched for in vivo repopulating ability. These findings support the hypothesis that normal CD44posCD49fhiCD133/2hi cells, once transformed, become enriched with a XIC population. In addition to the acquisition of unlimited self-renewal capabilities, the normal, luminal CD44posCD49fhiCD133/2hi cell must lose ER expression on its journey to becoming a XIC capable of giving rise to ER-negative tumors. Indeed, the fact that the ER promoter becomes hypermethylated in ER-negative breast cancer is well established (32). An alternative hypothesis is that normal cells acquire the CD44posCD49fhiCD133/2hi immunophenotype on transformation, independent of their previous marker profile.

It is understood that cancer has both a genetic and epigenetic underpinning. Relative to normal tissue, CpG islands of tumor cells tend to be hypermethylated (33). We show that a similar relationship exists between nontumorigenic cells and XIC isolated from the same tumor. Specifically, relative to the tumor bulk and CD44pos and negCD49fneg/lowCD133/2neg/low cells, the island to global CpG methylation ratio was lower in CD44posCD49fhiCD133/2hi cells due to hypermethylation in the CpG islands of XIC. It is plausible that these differences in the epigenetic landscape between XIC and nontumorigenic cells lie at the heart of their functional differences. This is supported by the role DNA methylation plays in

![Figure 4. Localization of CD44, CD49f, and CD133/2 in primary ER-negative breast tissue and normal breast tissue.](image)
plays in human embryonic stem cell differentiation (34). Furthermore, TGFβ signaling in ER-positive breast XIC is under epigenetic control (35) and tumor suppressor expression is frequently repressed in cancer by CpG island methylation (33, 36). We are currently comparing the methyolmes of CD44posCD49fhiCD133/2hi cells. would be consistent with the primitive nature of transformed the coexpression of both luminal and basal lineage markers postulated to occur in mammary stem cells (43). Therefore, been observed in hematopoietic stem cells (42) and has been expression of two or more markers of divergent lineages has simultaneous expression of both markers. The simultaneous CD44posCD49fhiCD133/2hi cells have elevated levels of Nanog, CD44 pos and neg CD49f neg/low CD133/2 neg/low and CD44 pos -(33, 36). We are currently comparing the methylomes of is frequently repressed in cancer by CpG island methylation der epigenetic control (35) and tumor suppressor expression awaits a larger cohort of ER-negative tumors with full clinical data and follow-up. However, the CD44posCD49fhiCD133/2hi marker profile may lead to the improved understanding of these tumors and ultimately the development of drugs designed to specifically target them.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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