Supporting Information

Chang *et al.* 10.1073/pnas.0804979105

Supporting Information corrected 10/28/2008

SI Materials and Methods

Reagents. Collagenase, hyaluronidase, DNase I, and bovine insulin were purchased from Sigma–Aldrich. RNeasy kit was purchased from Qiagen. Diethyl pyrocarbonate (DEPC)-treated water was purchased from Invitrogen. The fluorochrome-conjugated antibodies used for cell surface markers analyses, including anti-CD24-phycoerythrin (PE), anti-CD44-allophycocyanin (APC), and anti-CD45-peridinin chlorophyll protein-Cy5.5 (PerCP-Cy5.5), were obtained from BD Biosciences. Fluorescein isothiocyanate (FITC)-conjugated anti-H2K^d antibody and Alexa-488 conjugated anti-SSEA3 antibody were obtained from eBioscience. Hybridoma clone VK-9, which produces IgG3 monoclonal antibody, was kindly provided by Dr. Phillip Livingston (Memorial Sloan–Kettering Cancer Center, New York, NY). Purified VK-9 was conjugated to AlexaFluor-488 by using the commercial kit purchased from Molecular Probes. Anti-ER and anti-PR antibodies were purchased from Dako. The antibody against HER-2 was obtained from Biocare Medical. Reagents required for cDNA conversion and q-PCR analysis were purchased from Applied Biosystems; these include High-Capacity cDNA Reverse Transcription Kits, TaqMan Universal PCR Master Mix, as well as primers and probes for *FUT1* (Hs00355741_m1) and *FUT2* (Hs00382834_m1) and the internal control gene hypoxanthine ribosyltransferase 1 (*HPRT1*; Hs99999909_m1) from the TaqMan Gene Expression Assays product line.

Cell Lines. The human embryonic kidney cell-line 293T and breast cancer cell lines T-47D, MB157, MCF-7 were obtained from American Type Culture Collection. 293T cells and MB157 cells were cultured in DMEM and T-47 D cells in RPMI1640 medium, and both were supplemented with 10% heat-inactivated FBS and Glutamax (2 mM). MCF-7 cells were cultured in MEM supplemented with 10% FBS, bovine insulin (0.1 mg/ml), sodium pyruvate (1 mM), and Glutamax (2 mM). The cells were maintained in a 5% CO_2 air-humidified atmosphere at 37°C.

Globo H-KLH Vaccination Procedure and Detection of the Production of Anti-Globo H or Anti-SSEA3 Antibodies. Groups of two mice (6-week-old female BALB/c mice, BioLASCO) were immunized s.c. with Globo-H-KLH (Optimer Pharmaceuticals) weekly for 3 weeks. Each vaccination contained 0.6 μg Globo-H with or without 2 μg α-Galactosylceramide as an adjuvant. Control mice were injected with PBS. The mouse sera were obtained before the first immunization and 10 days after the third immunization. Anti-Globo H or anti-SSEA3 antibody was detected with glycochip assay as previous reported (28). Briefly, mice sera were diluted 1:25 with 3% BSA/PBS buffer, and 50 μl of diluted sera were applied to the Globo H analogs glycoarrays and incubated in a humidifying chamber for 1 h. The slides were washed three times with 0.05% Tween 20/ PBS (PBS-T) buffer, followed by incubation with 100 μl of Cy5-conjugated goat anti-mouse IgG antibody (1:200) in a humidifying chamber for 1 h. The slide was air-dried after being washed three times with PBS-T and three times with H_2O. Resulting fluorescence was measured at 635 nm with microarray scanner (GenePix 4000B; Molecular Devices) and analyzed with GenePix Pro software.

Immunohistochemistry. For Globo H and SSEA3 expression on normal tissues, tissue microarray slides (Biomax) that contained 20 different organs, with each organ derived from five individuals, were used. Slides were dried overnight at 56°C, dewaxed in xylene, and rehydrated according to the standard histopathologic procedures, followed by antigen retrival with AR-10 solution pH 9.0 (BioGenex Laboratories). Globo H or SSEA3 expression was determined with the use of VK-9 or anti-SSEA3 antibody (eBioscience), respectively. Staining for Globo H was detected with the use of BioGenex Non-Biotin Polymer HRP kit. SSEA3 was detected by using anti-rat IgM as a secondary antibody and was developed by DAB substrate. Slides were counterstained with hematoxylin. Primary breast tumor BC0145 and tumor xenografts from NOD/SCID mice were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Paraffin sections were cut at a thickness of 2 μM, mounted on SuperFrost Plus microscopy slides (Menzel-Gläser), and dried overnight at 55°C. The sections were dewaxed in xylene and rehydrated according to the standard histopathologic procedures, followed by staining with hematoxylin and eosin (H&E). Before immunostaining, the slides were first placed in the solution of 10 mmol/L citrate buffer (pH 6.0) and microwaved for 15 min. The slides were then incubated overnight with anti-ER, anti-PR, or anti-HER-2 antibody. Immunodetection was performed with the Super Sensitive Polymer-HRP IHC Detection System (BioGenex).

Lentivirus Production and Transduction. The sequence corresponding to siFUT1 is located from 1,435 to 1,455 (CGCGGACTTGAGAGATCCTTT), and the one for siFUT2 is from 723 to 743 (CTA TGTCC ATGTCAYGCCAAA), as verified by DNA sequencing. VSV-G-pseudotyped lentiviruses were produced by cotransfecting 293T cells with siFUT1 or siFUT2 clone, as well as two packaging plasmids: pMD.G and pCMVΔR8.91. Infectious lentiviruses were harvested at 48 and 72 h after transfection and were concentrated by ultracentrifugation (82,705 $\times$ *g*, 90 min). T-47D and MB157 cells were plated at 2 $\times$ 10^5 cells per well in six-well plates and transiently transduced with lentivirus in the presence of 8 μg/ml polybrene (Sigma–Aldrich). The cells were harvested at 96 h posttransduction for subsequent analyses.

***In Vivo* Propagation of BC0145 Tumor Cells in NOD/SCID Mice.** Before inoculation of BC0145 tumor cells, 8-week-old female NOD/SCID mice (Tzu Chi University, Hualien, Taiwan) received a sublethal dose of gamma irradiation. Each mouse was injected s.c. with 1 $\times$ 10^7 (for first inoculation) or 1 $\times$ 10^6 (for serial passages) primary human breast tumor cells per mammary fat pad, and the animals were monitored weekly for tumor growth. At 8 weeks after the inoculation, tumor cells from the engrafted NOD/SCID mice were harvested in a similar fashion by enzymatic digestion and filtration.

Cell Sorting. The cells harvested from human breast tumor engrafted in mice were stained with anti-CD24-PE, anti-CD44-APC, and anti-H2K^d-FITC antibody mixtures (BD Biosciences). Fluorescence activated sorting of antibody-labeled cells was carried out on a FACSAria cell sorter (Becton Dickinson). H2Kd$^-$/CD24$^-$/CD44$^+$ cells were sorted as BCSCs, and other populations of H2Kd$^-$ cells were sorted as non-BCSCs. The typical purities of BCSCs and non-BCSCs were >85% and >90%, respectively.

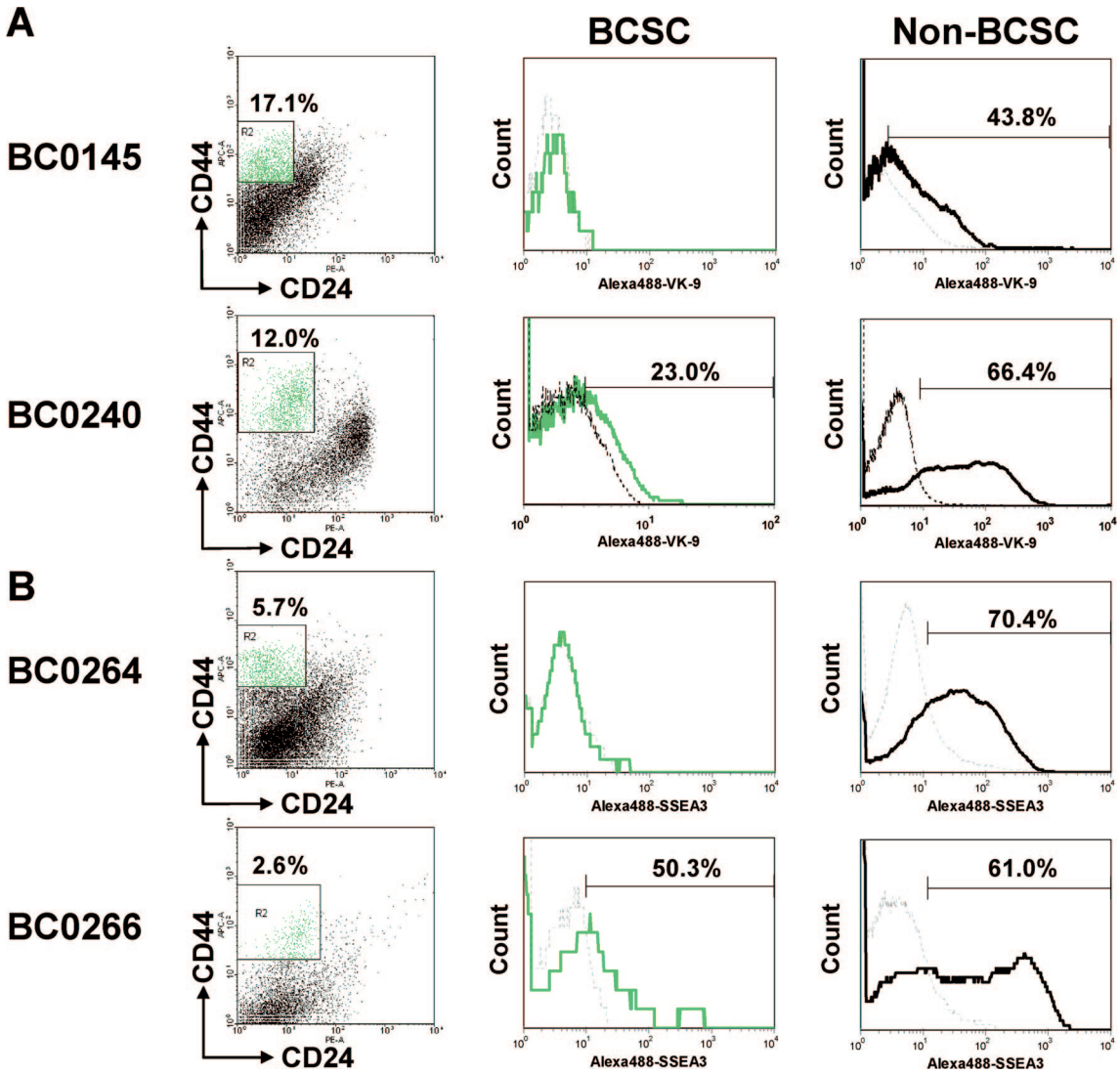


Fig. S1. Globo H and SSEA3 staining of human primary breast cancer cells isolated after enzymatic digestion of tumor tissues, as described in *Materials and Methods*. Expression of Globo H on the surface of BCSCs and non-BCSCs was evaluated with four-color immunofluorescence staining and subsequent flow cytometric analysis. BCSCs were defined as $CD45^{-}/CD24^{-}/CD44^{+}$ cells, and non-BCSCs were defined as other populations of $CD45^{-}$ cells, as shown in left panel. Expression of antigens of interest on BCSCs and non-BCSCs is shown in the middle and right panel, respectively. The dotted line represents isotype control, and the numbers represent the percentage of positive cells. (*A*) Globo H expression on BCSCs and non-BCSCs. Cells from tumor specimen BC0145 displayed Globo H expression on non-BCSCs, but not BCSCs. BC0240 cells showed Globo H expression on BCSCs, but at a lower level than that on non-BCSCs. (*B*) SSEA3 expression on BCSCs and non-BCSCs. Cells from tumor specimen BC0264 showed expression of SSEA3 on non-BCSCs but not on BCSCs. BC0266 cells showed Globo H expression on both BCSCs and non-BCSCs.

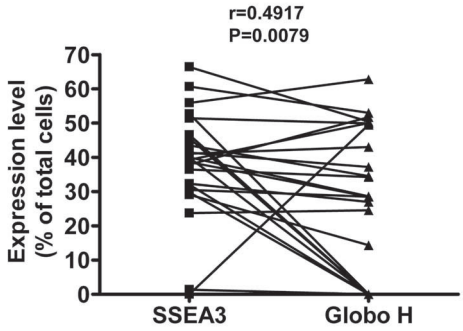


Fig. S2. The correlation of expression level of SSEA3 and Globo H in primary breast cancer. The expression level of SSEA3 and Globo H of 28 human primary breast cancer specimens was determined by flow cytometric analysis and represented as percentage of positive cells within total isolated tumor cells. Data were calculated with Pearson correlation coefficient analysis and showed a significant positive correlation between the expression of SSEA3 and Globo H.

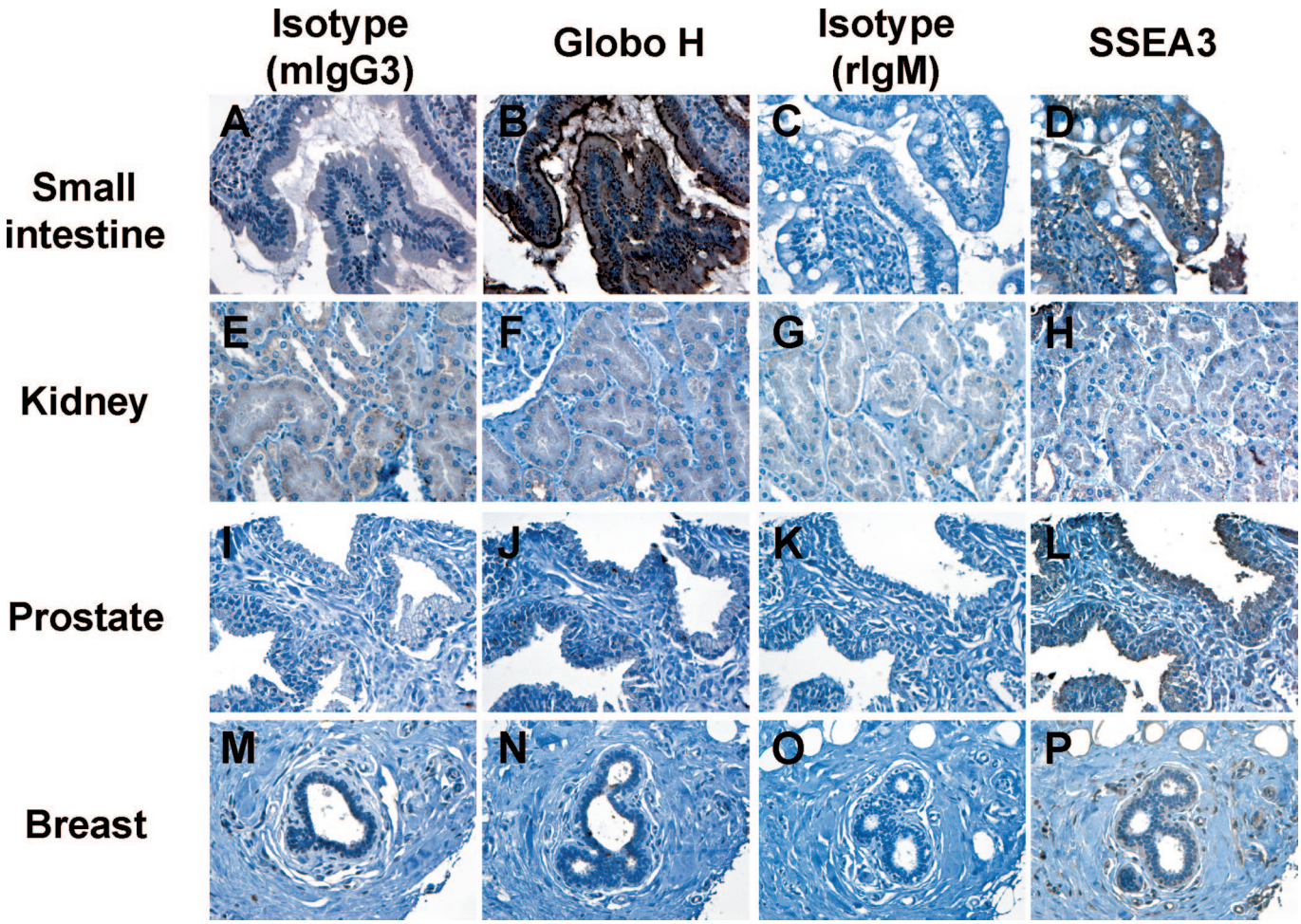


Fig. S3. Restricted expression of Globo H and SSEA3 in normal tissues. Immunohistochemical staining of normal tissue arrays was used to examine the expression of Globo H and SSEA3. (*A–D*), small intestine; (*E–H*), kidney; (*I–L*), prostate; (*M–P*), breast. Positive staining for Globo H in small intestine, prostate, and breast was restricted to the apical surface of epithelial cells. Positive staining for SSEA3 in small intestine, kidney, and prostate was located in cytoplasm and in the apical surface of epithelial cells. The brown signals for Globo H staining of kidney in (*F*) were considered false positive because isotype control showed similar signals (*E*). The brown signals of breast in SSEA3 staining (*P*) were not located on cells and also considered as negative for SSEA3 expression. (20× objective)

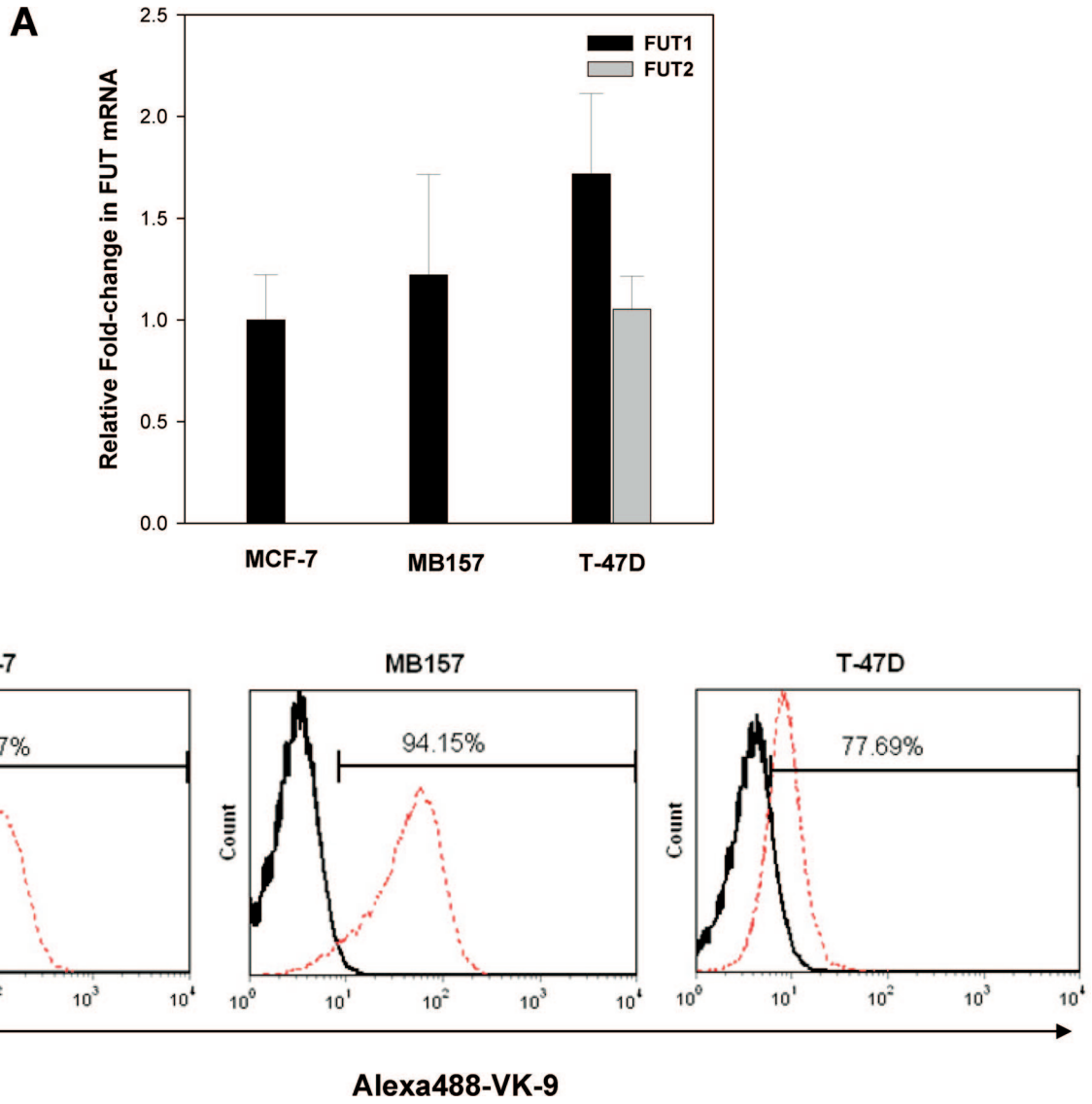
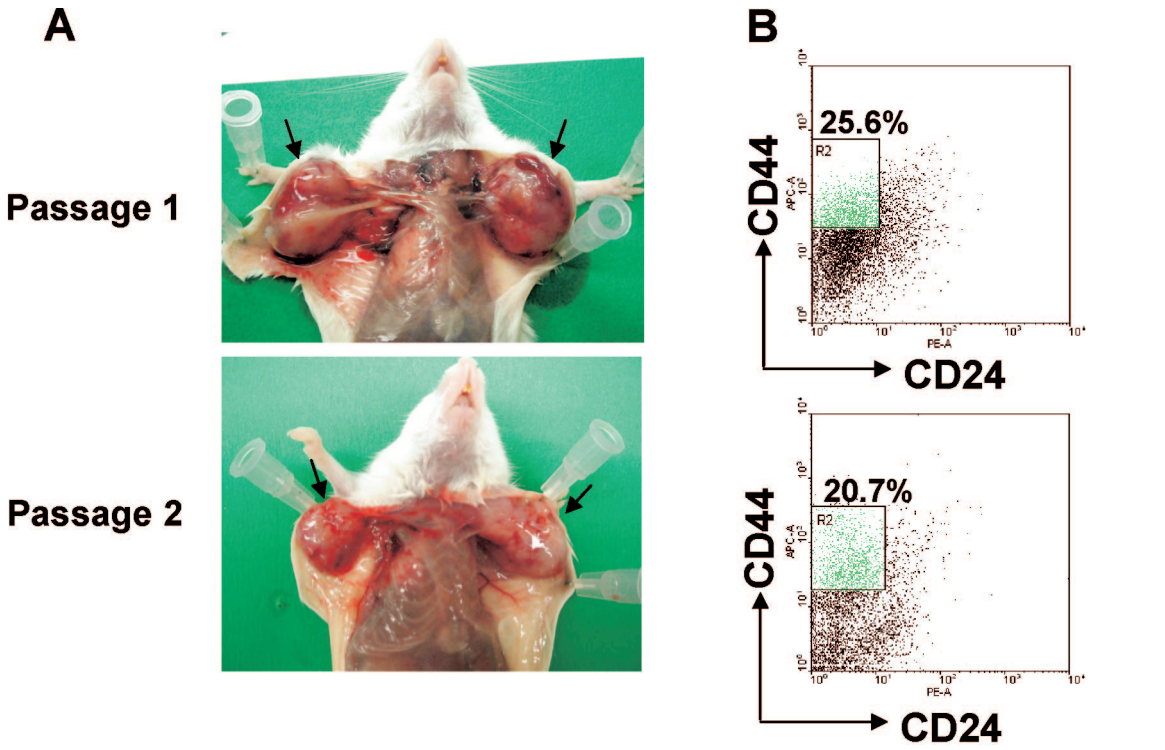


Fig. S4. Differential expressions of *FUT1* and *FUT2* mRNA in breast cancer cell lines and Globo H antigen. (*A*) The expression of *FUT1* and *FUT2* genes was determined by qRT-PCR in breast cancer cell lines MCF-7, MB157, and T-47D. Each measurement was done in triplicates, and the average ΔCt value of FUT1 mRNA in MCF-7 was used as a reference point for normalization. The fold-change in mRNA level was calculated from ΔCt values with the formula: $2^{-[\Delta Ct(\text{target gene}) - \Delta Ct(\text{FUT1 in MCF-7})]}$. (*B*) Staining of surface Globo H antigen was carried out by labeling the cells with AlexaFluor-488-conjugated VK-9 antibodies (Alexa488-VK-9) and analyzed by flow cytometry.



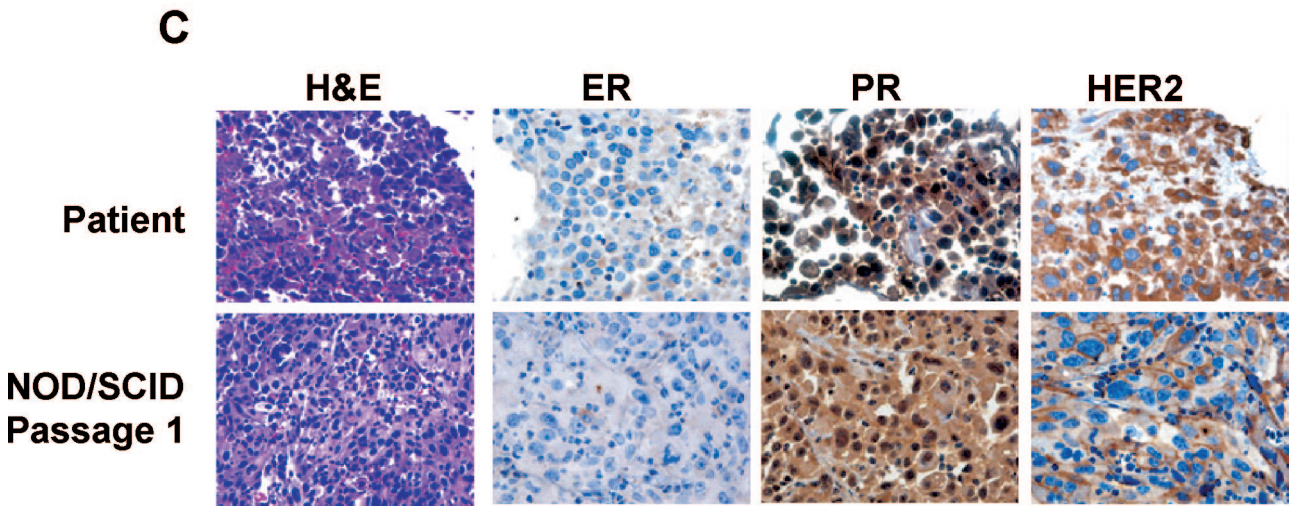


Fig. S5. BC0145 cancer cells could be engrafted in the mammary fat pads of recipient NOD/SCID mice. (*A*) Primary BC0145 cancer cells were injected at a density of 1×10^7 cells into the mammary fat pads of NOD/SCID mouse (passage 1), and tumor engraftment was noted on Day 37 postinjection (*Top*). The cancer cells from NOD/SCID mouse engrafted tumors could be further passaged in another NOD/SCID mouse (passage 2) and harvested on Day 28 after a single injection of 1×10^6 cells per fat pad (*Bottom*). The arrows indicate the location of the engrafted tumors. (*B*) Immunofluorescent staining of CD24 and CD44 was performed by using the cancer cells isolated from NOD/SCID mouse engrafted with human primary breast tumors. *Top*: Cancer cells from passage 1. *Bottom:* Cancer cells from passage 2. Fluorescent signals were detected by flow cytometry. (*C*) Histopathology and the status of ER, PR, or HER2 in primary tumor specimen BC0145 and the xenografted tumors from NOD/SCID mouse were evaluated by H&E and immunohistochemical staining. *Top*: Sections from patient's primary tumor specimen BC0145. *Bottom:* Sections from xenograft tumors in NOD/SCID mouse of passage 1. Development of brown color indicated positive staining. (40× objective)

Table S1. Clinical characteristics of the patients with breast cancer

Characteristic	Number	Globo H expression range, %*	% of total	*P* value†
Patients enrolled	53		100	
Age, years				
Median		48		
Range		31–81		
Tumor type				
Infiltrating ductal carcinoma	41		77.3	
Infiltrating lobular carcinoma	4		7.5	
Ductal carcinoma in situ	1		1.9	
Medullary carcinoma	1		1.9	
Atypical medullary carcinoma	2		3.8	
Metaplastic carcinoma	2		3.8	
Inflammatory carcinoma	1		1.9	
Sarcoma	1		1.9	
Stage				0.3498
0	1	0	1.9	
I	17	0–75.2	32.1	
II	26	0–65.8	49.0	
III	8	0–37.2	15.1	
Unknown	1	0	1.9	
ER‡				0.075
Negative	23	0–51.7	43.4	
Positive	30	0–75.2	56.6	
HER-2‡				0.6468
Negative	27	0–75.2	50.9	
Positive	26	0–65.8	49.1	

*Range of Globo H expression was represented by percentage of positive cells within total cancer cells.
†A *t* test was used for statistical analysis of Globo H expression relative to ER or HER-2 status (negative vs. positive), and one-way ANOVA was used for Globo H expression in different clinical stages (I, II, or III).
‡Status of ER or HER-2 expression was determined by immunohistochemistry. ER, estrogen receptor; HER-2, human epidermal growth factor receptor 2.

Table S2. The expression level of Globo H on non-BCSCs in tumor specimens containing Globo H$^+$ BCSCs

	Expression level, % (MFI)*	
Patient ID	Non-BCSCs	BCSCs
BC0230	60.0 (11.9)	21.5 (6.3)
BC0231	51.4 (107.9)	20.0 (20.8)
BC0240	66.4 (53.2)	23.0 (6.3)
BC0270	37.2 (30.7)	9.7 (43.3)
BC0273	51.1 (55.8)	33.2 (12.9)
BC0277	52.5 (22.1)	37.3 (13.9)
BC0297	44.3 (11.0)	18.5 (30.9)
BC0304	49.1 (49.6)	71 (45.9)

Globo H expression was determined by flow cytometry as described in *Materials and Methods*. BCSCs were defined as CD45$^-$/CD24$^-$/CD44$^+$ cells, and non-BCSCs were defined as the remaining population of CD45$^-$ cells.
*MFI, mean fluorescence intensity.