

Stage-specific embryonic antigen-3 (SSEA-3) and β 3GalT5 are cancer specific and significant markers for breast cancer stem cells

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The discovery of cancer stem cells (CSCs), which are responsible for self-renewal and tumor growth in heterogeneous cancer tissues, has stimulated interests in developing new cancer therapies and early diagnosis. However, the markers currently used for isolation of CSCs are often not selective enough to enrich CSCs for the study of this special cell population. Here we show that the breast CSCs isolated with CD44⁺CD24^{-/lo}SSEA-3⁺ or ESA^{hi}PROCR^{hi}SSEA-3⁺ markers had higher tumorigenicity than those with conventional markers in vitro and in vivo. As few as 10 cells with CD44⁺CD24^{-/lo}SSEA-3⁺ formed tumor in mice, compared with more than 100 cells with CD44⁺CD24^{-/lo}. Suppression of SSEA-3 expression by knockdown of the gene encoding β -1,3-galactosyltransferase 5 (β 3GalT5) in the globo-series pathway, led to apoptosis in cancer cells specifically but had no effect on normal cells. This finding is further supported by the analysis of SSEA-3 and the two related globo-series epitopes SSEA4 and globo-H in stem cells (embryonic stem cells and induced pluripotent stem cells) and various normal and cancer cells, and by the antibody approach to target the globo-series glycans and the late-stage clinical trials of a breast cancer vaccine.

globo-series | glycolipids | cancer-specific

Cancer stem cells (CSCs), which are rare cells with the ability of self-renewal and tumor initiation, are closely related to cancer progression and specific targets for effective therapy and early diagnosis (1–5). To date, many CSCs have been identified and characterized by protein markers. Breast CSCs (BCSCs) were first discovered in 2003 by Al-Hajj et al. (6); it was demonstrated that breast cancer cells with CD44⁺CD24^{-/lo} expression have higher level of tumorigenicity than others and can form tumor in animals with ~100 of such cells. In addition, other proteins such as ALDH-1, CD133, CD326 (ESA), CD201 (PROCR), and their combinations, are also reported as BCSCs biomarkers (7–9). However, the BCSCs obtained from the enrichment process based on these markers still contain a large number of noncancer stem cells, and study of such cells would provide nonspecific characteristics of CSCs. Therefore, new markers are required to enrich and obtain better-defined BCSCs for analysis and study.

Glycolipids are known to be altered during cancer development (10–15). In our previous study, the globo-series glycans SSEA-3 (Gb5), SSEA-4 (sialyl-Gb5), and globo-H (fucosyl-Gb5) are found exclusively on the cell surface of many cancers, including breast cancer and BCSCs (16–18). We also reported that BCSCs carrying either ESA^{hi}PROCR^{hi} or CD44⁺CD24^{-/lo} showed high expression of these globo-series epitopes (19). SSEA-3 is synthesized from Gb4 by β 3GalT5 (20), and globo-H and SSEA-4 are synthesized from SSEA-3 by fucosyltransferases 1 and 2 (FUT1, FUT2) (21, 22) and ST3 β -galactoside α -2,3-sialyltransferase 2 (ST3Gal2) (23), respectively. These reports prompt us to investigate whether SSEA-3 and the related glycans and enzymes in the globo-series pathway are cancer specific and are BCSC markers.

Results and Discussion

To study the tumorigenic ability of cells, cancer cells were stained with corresponding antibodies against the glycolipid molecules SSEA-3, SSEA-4, and globo-H and the known marker sets CD44/CD24 and ESA/PROCR in breast cancer cell lines MCF-7 and MDA-MB-231, respectively, for the cell sorting (Fig. S1, sorting 1). The isolated cell populations were next examined by both in vitro (24) and in vivo (8) assays (Fig. 1). In MCF-7, cancer cells expressing CD44⁺CD24^{-/lo}SSEA-3⁺ formed a higher percentage of mammospheres than those expressing CD44⁺CD24^{-/lo}SSEA-3⁻ or CD44⁺CD24^{-/lo} (Fig. 1A, Left). Similarly, in MDA-MB-231, the ESA^{hi}PROCR^{hi}SSEA-3⁺ subpopulation formed a higher percentage of cell colonies than ESA^{hi}PROCR^{hi}SSEA-3⁻ or ESA^{hi}PROCR^{hi} cells in the soft agar assay (Fig. 1B, Left). However, there were no significant differences in the formation of cell colony and mammosphere using the cells isolated by the known marker sets along with the glycolipid epitopes SSEA-4 or globo-H (Fig. S2). To show the tumorigenicity in cells carrying known BCSC markers and SSEA-3, different subpopulations were inoculated into the mammary glands of NOD-SCID mice for tumor-growth. The result showed that both CD44⁺CD24^{-/lo}SSEA-3⁺ and ESA^{hi}PROCR^{hi}SSEA-3⁻ effectively generated tumor in vivo with a low cell number, compared with other corresponding subpopulations (Fig. 1C and D). Particularly for cells expressing CD44⁺CD24^{-/lo}SSEA-3⁺, as few as 10 cells were able to form tumor in mice (Fig. 1C). In terms of

Significance

Cancer stem cells are cancer cells with self-renewal and tumor-growth properties and are important targets for development of anticancer therapy. We have found breast cancer stem cells can be enriched by stage-specific embryonic antigen 3 (SSEA-3) and known protein markers (CD24 and CD44), and as few as 10 such enriched cells can develop tumor in mice. Also, the enzyme galactosyltransferase (β 3GalT5) for the biosynthesis of SSEA-3 is expressed in breast cancer stem cells and cancer cells but not in normal cells, and both SSEA-3 and β 3GalT5 are shown to be essential for cancer cell survival. These findings have led to the development of a new anticancer strategy with a proof of principle shown in this and previous studies.

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The authors declare no conflict of interest.

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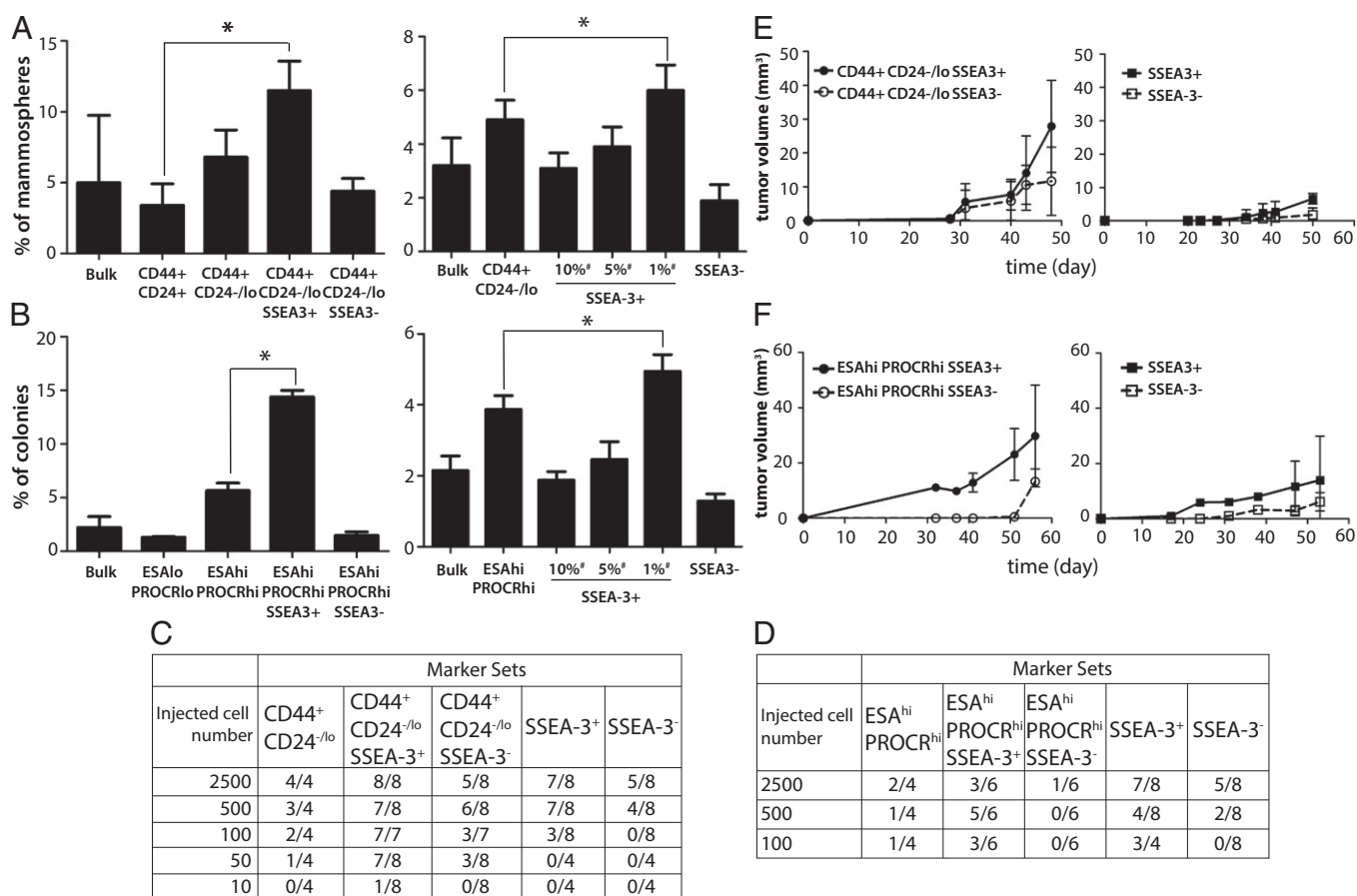


Fig. 1. The tumorigenicity of cells carrying conventional markers and SSEA-3 was higher than other subpopulations. (*A* and *B*) Percentage of cell colony or mammosphere formation of the subpopulation isolated by selected marker(s) for suspension culture or soft agar assay in MCF-7 or MDA-MB-231 respectively. Graphs are the triple samples from one representative experiment. (*C* and *D*) The number of tumor formed in mammary gland of NS injected with selected marker-expressing cell subpopulations from breast cancer cell lines MDA-MB-231 and MCF-7. The corresponding limiting dilution assay was done in vivo. (*E* and *F*) The tumor volume from different subpopulations of MDA-MB-231 (2500 cells/injection), and MCF-7 (500 cells per injection) was monitored and compared ($n = 4$ tumors per group). #, The percentage of SSEA-3⁺ cells sorted in the total cell population. Data represent the mean and SD. Asterisks indicate statistical significance, $P < 0.05$.

tumor growth, the tumor volume of CD44⁺CD24^{-/lo}SSEA-3⁺ cells was twice larger than that of CD44⁺CD24^{-/lo}SSEA-3⁻ cells (Fig. 1*E*, *Left*). In addition, ESA^{hi}PROCR^{hi}SSEA-3⁺ cells developed tumors earlier and formed tumors in a greater average volume than ESA^{hi}PROCR^{hi}SSEA-3⁻ cells (Fig. 1*F*, *Left*). These results indicate that SSEA-3 is a specific marker for the enrichment of BCSCs in different breast cancer cell models. Among these glycan molecules, only cells carrying SSEA-3 and known BCSC markers had a higher tumorigenicity than other subpopulations.

We next compared the stem-like properties of cancer cells with highly expressed SSEA-3 and those without SSEA-3 (Fig. S1, sorting 2). In SSEA-3⁺ MCF-7 cells, the top 1% of cells expressing a high level of SSEA-3 within the total population formed a higher percentage of mammosphere than the bulk population and those without SSEA-3 and CD44⁺CD24^{-/lo} (Fig. 1*A*, *Right*). In addition, the top 1% of MDA-MB-231 cells with the highest SSEA-3 expression within the bulk population also formed more cell colonies than the bulk population and other subpopulations (Fig. 1*B*, *Right*). In the animal study, results showed that the cells with top 1% SSEA-3 expression had a higher potential to form tumor than SSEA-3⁻ cells (Fig. 1*C* and *D*), and the average tumor volume of SSEA-3⁺ cells was greater than that of SSEA-3⁻ cells (Fig. 1*E* and *F*, *Right*). Thus, cancer cells expressing a high level of SSEA-3 had a higher tumorigenicity than those without SSEA-3 on cell surface, indicating that SSEA-3 is also an independent CSCs marker for breast cancer.

To understand the function of SSEA-3, the gene of β 3GalT5 responsible for SSEA-3 biosynthesis (Fig. S3) was overexpressed or knocked down for further study. Overexpression of β 3GalT5 increased the expression level of surface SSEA-3 in both MCF-7 and MDA-MB-231 cells (Fig. 2). Notably, in MCF-7 cells, the percentage of CD44⁺CD24^{-/lo} cell population showed fivefold increase comparing to control (Fig. 2*A*); in MDA-MB-231 cells, there was no change in the percentage of ESA^{hi}PROCR^{hi} when β 3GalT5 was overexpressed (Fig. 2*B*). In MCF-7 cells with β 3GalT5 knockdown, comparing with control cells, the expression level of surface CD44 was reduced, and therefore the CD44⁺CD24^{-/lo} cell population increased 10 folds (Fig. 2*A*). In MDA-MB-231 cells with β 3GalT5 knockdown, the level of surface PROCR decreased and the ESA^{hi}PROCR^{hi} BCSC subpopulation reduced (Fig. 2*B*). These findings further confirmed that SSEA-3 is a critical glycan molecule associated with BCSCs. It is, however, not clear regarding the relationship between the expression of β 3GalT5 and that of CD44 and PROCR, and this issue remains to be investigated.

To gain insights into the role of SSEA-3 in breast cancer and normal cells, the cellular phenotypes were examined in the β 3GalT5 knockdown cells. In both MDA-MB-231 and MCF-7 cells, knockdown of β 3GalT5 suppressed cell growth (Fig. 3*A* and *B*), along with the appearance of cell apoptosis, especially in MDA-MB-231 cells that >60% of cells underwent apoptosis on day 4 (Fig. 4*B* and *C*). In contrast, in normal breast cells

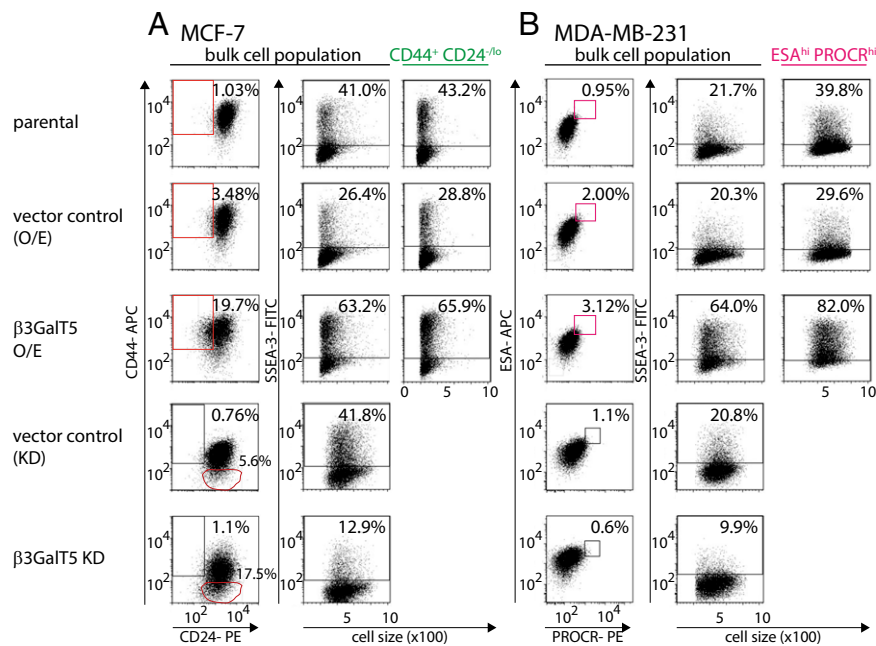


Fig. 2. Knockdown or overexpression of β 3GalT5 in MCF-7 and MDA-MB-231 cell culture reduced or increased the level of SSEA-3 on cell surface and stemness properties by FACS analysis. The expression of CSC markers and SSEA-3 in parental cells. The level of SSEA-3 in subpopulations CD44⁺CD24^{-/lo} and ESA^{hi} PROCR^{hi} gated in overexpressed β 3GalT5 group was also determined. (A) The expression of CD24, CD44, and SSEA-3 in MCF-7 with overexpression, knockdown of β 3GalT5 or their corresponding vector control. (B) The expression of ESA, PROCR and SSEA-3 in MDA-MB-231 with overexpression, knockdown of β 3GalT5 or their corresponding vector control. All of experiments are the representative sample from triplicate.

MCF-10A and human telomerase reverse transcriptase (hTERT)-immortalized human mammary epithelial cells, hTERT-HME1, the same growth rate and no apoptosis were observed with knockdown of β 3GalT5 (Figs. 3 C and D and 4 B and C). However, knockdown of FUT1 and FUT2 for the synthesis of globo-H from SSEA-3 or ST3Gal2 for the synthesis of SSEA-4 from SSEA-3 did not induce cell apoptosis in MDA-MB-231 cells (Fig. 4 A and D).

To further investigate whether the apoptosis induced by β 3GalT5 knockdown is associated with the activation of caspase-3, the most effector caspase for the downstream execution of apoptosis. Results showed that caspase-3 was activated in MDA-MB-231 cells with knockdown of β 3GalT5 (Fig. 3E). When the inhibitor for caspase 3, Z-DEVD, was added, the percentage of apoptosis induced by β 3GalT5 knockdown reduced (Fig. 3F). The involvement of caspase-3 in the apoptosis induced by β 3GalT5 knockdown was also confirmed in MCF-7, a caspase-3-deficient cell line. Although the growth rate of MCF-7 was significantly reduced by knockdown of β 3GalT5, only a low level of apoptosis was shown when the expression of SSEA-3 was suppressed (Fig. 4 B and C). Further investigation of the upstream caspases (caspase-8, -9, and -12) was then studied by testing with specific inhibitors, and the result illustrated that caspase-8 also reduced the percentage of cell apoptosis in MDA-MB-231 cells with β 3GalT5 knockdown (Fig. 3G). These results suggest that SSEA-3, the immediate enzymatic product of β 3GalT5, is an important glycolipid for growth and survival in cancer.

To confirm whether SSEA-3 or any of the three globo-series glycans was only found in cancer cells, the glycolipids from embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs, Fig. S4), MCF-7 and MDA-MB-231 cells, and normal cell lines, including MCF-10A and hTERT-HME1, were extracted and the glycans were released, tagged and examined by LC-MS analysis (Fig. 5A). The data were compared with the results of flow cytometric analysis, in which same antibodies used for cell sorting were used to detect the expression levels of SSEA-3, SSEA-4,

and globo-H (Fig. 5). It was found that ESCs, iPSCs, and cancer cell lines but not normal cell lines expressed SSEA-3, SSEA-4, and globo-H. The result from this study was also supported by quantitative RT-PCR (qPCR) of β 3GalT5 gene expression in normal and cancer cell lines (Fig. S5).

The expression level of SSEA-3 in MCF-7 cells detected by flow cytometry was relatively higher than that by the LC-MS analysis, whereas the level of SSEA-3 in MDA-MB-231 detected by LC-MS was much higher than that by flow cytometry. The variation between the LC-MS and flow cytometry data could be due to the specificity of antibody and the distribution of the glycans on the cell surface (25). Due to the cross-reaction of anti-SSEA-3 antibody (MC-631) toward SSEA-4 and to a lesser extent, Gb4 (14), it is possible to overestimate the level of SSEA-3 detected by flow cytometry when there is a high expression level of SSEA-4. On the other hand, the level of SSEA-3 could be underestimated because of hindrance caused by other biomolecules on cell surface and thus SSEA-3 on the cells may not be reached in antibody staining (26, 27). Therefore, we believe that the LC-MS result, which is supported by the qPCR detection of β 3GalT5 gene expression (Fig. S5), more accurately reflects the expression of these glycolipids.

In the process of BCSC isolation, it is possible that some cells with a high level of SSEA-4 expression but carry no SSEA-3 are enriched when sorted based on MC-631 staining. Because we proved that both SSEA-3 and its synthetic enzyme β 3GalT5 are BCSCs markers, SSEA-3 negative cells are low tumorigenic. The cell population is not purified enough and thus the tumorigenicity of the cells sorted based on anti-SSEA-3 staining may be underestimated. We suggest that an antibody or molecule, which is highly specific to SSEA-3, should be generated for the enrichment of BCSC. On the other hand, if SSEA-3 on the cell surface can be specifically detected and sorted by flow cytometry, the results of both antibody staining and LC-MS analysis should be consistent.

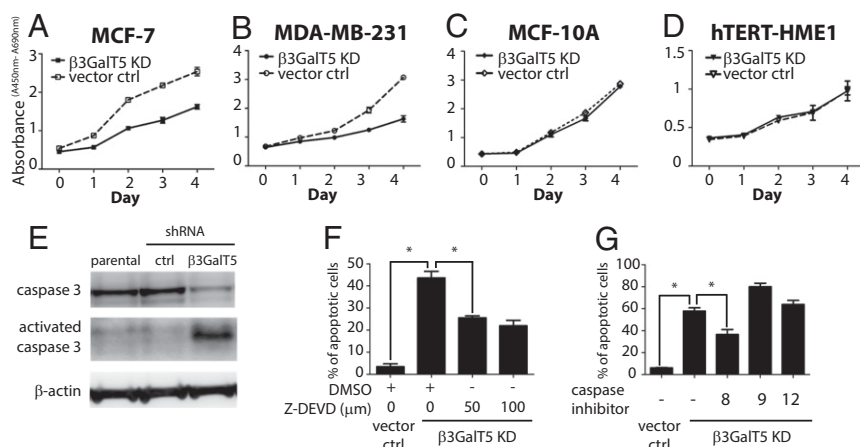


Fig. 3. Knockdown of β 3GalT5 caused reduced proliferation rate and increased apoptosis in cancer cell culture but no effect on normal breast cell culture. (A–D) The rate of proliferation in cancer cell culture MCF-7 and MDA-231, as well as breast normal cell culture MCF-10A and hTERT-HME1. Proliferation rate, in terms of absorbance (A450nm–A690nm), is the triplicate from a representative sample. (E) MDA-MB-231 cells infected with shRNA β 3GalT5 or shRNA vector were lysed and whole-cell extract, cytoplasmic and nuclear fractions were prepared. Top, Western blot analysis of anti-caspase-3 antibody; middle, that of cleaved caspase-3 antibody; bottom, that of β -actin (served as a loading control). (F and G) The percentage of apoptotic MDA-MB-231 cells with β 3GalT5 knockdown. MDA-MB-231 cells were treated with caspase-3 inhibitors Z-DEVD in different concentrations or the inhibitors for caspase-8, -9, or -12. Data represent here is the mean and SD from triplicated sample. Asterisks indicate statistical significance, $P < 0.05$; n.s., not significant.

It appears that SSEA-3 is a BCSC marker both apoptosis and inhibition of cell proliferation through different mechanisms, as MCF-7, a caspase-3 null cell line, underwent a limited level of apoptosis and profound suppression of cell growth after knockdown of β 3GalT5. In contrast, in normal mammary epithelial cells, which lack SSEA-3 expression, knockdown of β 3GalT5 did not affect these phenotypes.

In summary, this study reveals that SSEA-3 is a previously unidentified glycan marker useful for the enrichment of BCSCs, and both SSEA-3 and β 3GalT5 are potential new targets for the development of breast cancer therapeutics. In addition to their specific expression on most CSCs and cancer cells, the globo-series glycolipids SSEA-3, SSEA-4, and globo-H are also highly expressed on the surface of ESCs and iPSCs, but they disappear

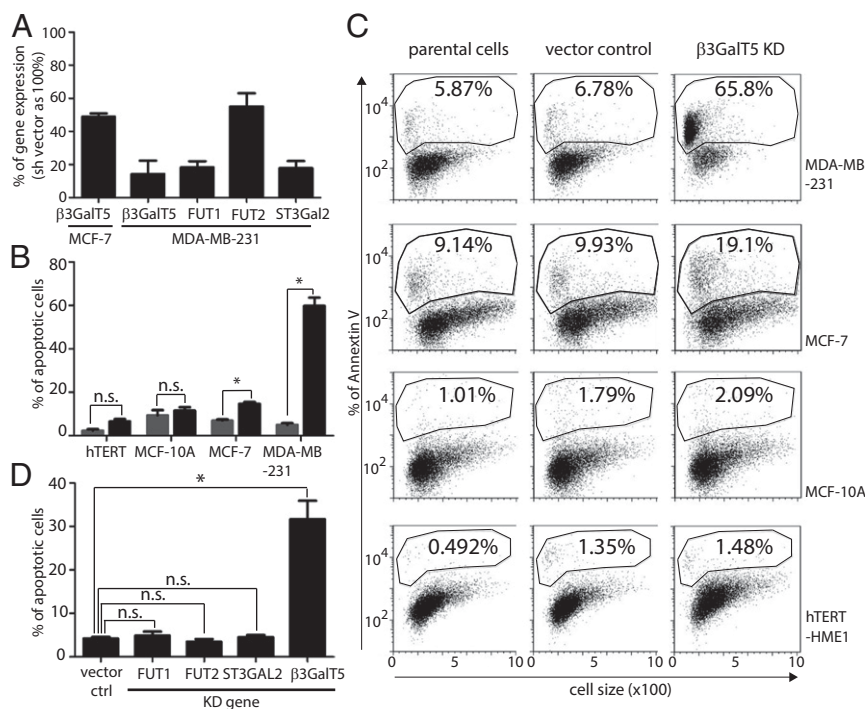


Fig. 4. The induction of apoptosis in β 3GalT5 knockdown cell lines. (A) The relative percentage of gene expression (β 3GalT5 in MCF-7, β 3GalT5, FUT1, FUT2, and ST3Gal2 in MDA-MB-231) after knocking down the respective genes. The percentage of gene expression in vector control cells were normalized to 100. (B) The mean percentage of apoptosis in breast normal (hTERT-HME1, MCF-10A) and cancer (MCF-7, MDA-MB-231) cell lines from three experiments. (C) Flow cytometric analysis of the apoptosis percentage in breast cancer cell lines MDA-MB-231, MCF-7, and breast noncancer lines hTERT-HME1 and MCF-10A was examined after knockdown of β 3GalT5 for 4 d. The apoptotic cells were compared with unstained cells and gated. (D) The percentage of apoptosis in MDA-MB-231 cells with knockdown of gene FUT1, FUT2, ST3Gal2, or β 3GalT5 and vector control. The mean of apoptotic cells is from three experiments. Asterisks indicate statistical significance, $P < 0.05$; n.s., not significant.

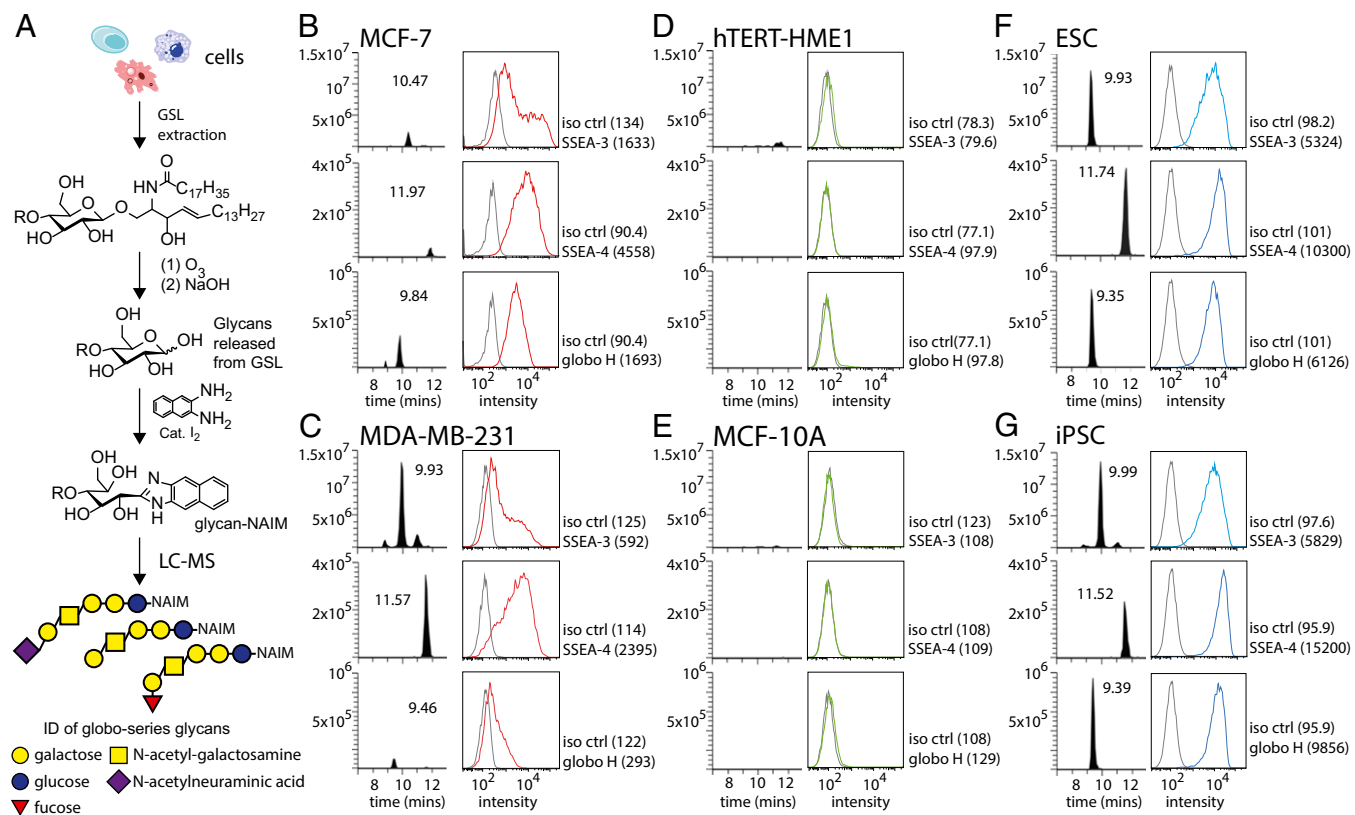


Fig. 5. The comparison of the abundance of globo-series epitopes in cell lines by flow cytometry and mass spectrometry. (A) The scheme of extraction of glycan from glycolipid for fluorescent labeling and LC-MS analysis. (B–G) The relative abundance of globo-series epitopes SSEA-3, SSEA-4, and globo-H in breast cancer cell lines MCF-7 and MDA-MB-231, normal cell lines hTERT-HME1 and MCF-10A, ESCs, as well as iPSCs was detected by FACS and mass spectrometry. For figures of flow cytometry, histograms of cells stained with anti-glycan antibodies (in red, blue or green) and their corresponding antibody isotype controls (in gray) were shown. Geometric mean of fluorescence was shown in the bracket. For MS, the mass chromatograms of *m/z* for glycans (SSEA-3 = 1008.3667; SSEA-4 = 1299.4621; and globo-H = 1154.4246) are shown in graphs.

after differentiation of ESCs. It would be interesting to understand the fate of the globo-series glycolipids after differentiation of iPSCs for use in regenerative medicine. Nevertheless, it appears that, unlike other tumor-associated glycolipids, these three globo-series glycans are cancer specific and could be considered as nonself epitopes for vaccine development. These findings are further supported by the study of antibodies designed to target the globo-series glycans (13–18), and by the development of globo-H cancer vaccines (19), especially the one in the late-stage clinical trials for the treatment of metastatic breast cancer (28).

Materials and Methods

Cell Culture. Breast cancer cell lines MDA-MB-231, MCF-7, and human breast cancer associated fibroblast (CAF) were obtained from American Type Culture Collection (ATCC). The culture of MDA-MB-231 was in DMEM supplemented with 10% (vol/vol) heat-inactivated FBS and antibiotic-antimycotic whereas that of MCF-7 culture was in RPMI supplemented with 10% (vol/vol) heat-inactivated FBS, nonessential amino acids and antibiotic-antimycotic. For the culture of CAF, it was in DMEM/F12 supplemented with 10% (vol/vol) heat-inactivated FBS, nonessential amino acids, sodium pyruvate, glutamine, penicillin, and streptomycin. They were incubated at 37 °C incubator with 5% (vol/vol) CO₂ and humidified atmosphere control. All of the cell culture media and supplements were purchased from Life Technologies. Human ESC H9 and iPSCs were maintained and cultured on mitomycin C treated-mouse embryonic fibroblasts (MEFs) in human ES medium (*Knockout DMEM* with *Knockout Serum Replacement*, GlutaMAX, nonessential amino acids,

2-Mercaptoethanol, Penicillin/Streptomycin and bFGF) and were passaged weekly using collagenase IV.

Derivation of iPSCs from Dermal Fibroblasts. Fibroblasts derived from dermal biopsies were reprogrammed into pluripotent stem cells using the CytoTune-iPS Sendai Reprogramming Kit (Life Technologies). Briefly, 5×10^4 fibroblasts were seeded per well in a six-well dish at passage 3 for recovery overnight. The next day, Sendai viruses expressing human transcription factors OCT4, SOX2, Klf4, and c-Myc were mixed in fibroblast medium to infect fibroblast cells according to the manufacturer's instructions. After 2 d, the medium was exchanged with human ES medium supplemented with the ALK5 inhibitor SB431542 (2 μM; Stemgent), the MEK inhibitor PD0325901 (0.5 μM; Stemgent), and thiazovivin (0.5 μM; Stemgent). Day 7–10 after infection, cells were detached using TrypLE (Life Technologies) and passaged onto feeder cells. Individual colonies of iPSCs were picked between days 21 and 28 after infection, and each iPSC line was expanded from a single colony. All iPSC lines were cultured on mouse embryonic fibroblast cells in human ES medium.

Karyotyping was performed by Cell Line Genetics. In teratoma analysis, $1-2 \times 10^7$ from each iPSC line were detached and collected after TrypLE treatment. They were suspended in 0.5 mL of human ES media. Followed by mixing with 0.5 mL Matrigel (BD Biosciences), cells were injected s.c. into dorsal flanks of an immunodeficient mouse (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ, stock no. 005557, The Jackson Laboratory). Eight weeks after injection, teratomas were harvested, fixed overnight with 4% (vol/vol) paraformaldehyde, and processed according to standard procedures for paraffin embedding. The samples were then sectioned and H&E stained.

Flow Cytometry and Cell Sorting. Cell labeling was done by staining with antibodies in buffer composed of PBS supplemented with 1% FBS. Accutase

(eBioscience) detached cells were incubated with antibodies (using antibody titration suggested by the supplier) for 30 min on ice in the dark. Antibodies used in this study were PE-conjugated anti-PROCR (RCR-252; BD Biosciences), APC-conjugated anti-ESA (1B7; eBioscience), PE-conjugated anti-CD24 (SN3 A5-2H10; eBioscience), APC-conjugated anti-CD44 (IM-7; eBioscience), along with biotinylated anti-SSEA-3 (MC-631; eBioscience) at 4 °C for 30 min in the dark. After washing twice, the cells were stained with Alexa Fluor 488-conjugated streptavidin at 4 °C for 30 min in the dark. Proper isotype controls were used for each cell labeling experiment. The same antibodies were used in all staining and sorting experiments in this study. Live cell sorting was done using a BD FACSAriaU with a 100- μ m nozzle following the manufacturer's instructions. For MDA-MB-231 cells, the sorted cells were incubated with DMEM/10% (vol/vol) FBS/antibiotics/antimycotics to recover in ultra-low attachment surface plates overnight in a humidified 37 °C incubator before further analyses. For MCF-7 cells, they were subject to further experiments readily after sorting (Fig. S1). The percentage of cells in different marker populations was evaluated using the software FlowJo.

Soft Agar Assay. Soft agar colony formation assay was performed by seeding cells in a layer of 0.35% SeaPlaque agarose with DMEM/FBS over a basal layer of 0.5% SeaPlaque agarose/DMEM/FBS. Cultures were maintained in a humidified 37 °C incubator. Additional media was added every 2–3 d to continuously supply growth supplements to the cells. On day 21 after seeding, cells were fixed with pure ethanol containing 0.05% crystal violet and colony forming efficiency quantified by light microscopy.

Mammosphere Formation. In the mammosphere formation assay, cells were incubated in DMEM/F12 with supplement B27 (Life Technologies) and 10 ng/mL EGF on 96-well low-attachment plates in the density of 100 cells per well. Culture was maintained in a humidified 37 °C incubator. After 14 d, the number of mammospheres was counted under a light microscope.

Mouse Tumorigenicity Assay. NOD-SCID (NS) mice were used to evaluate the stem cell properties of sorted cells expressing potential stem cell markers from the human breast cancer cell lines. Animal care and experiments were approved by the Institutional Animal Care and Utilization Committee of Academia Sinica (IACUC 130-09-575). Four-week-old NS mice were injected with sorted cancer cells mixed with CAF (1:1) and Matrigel (BD Biosciences) (1:1) in fat pads. For MCF-7, mice were additionally injected with estrogen pellets (0.18 mg per pellet, 90-d release; Innovative Research of America) before the day of experiment. Tumor volumes were evaluated every 5 d after initial detection. The tumor formation efficiency was determined on day 50 after cell injection.

Overexpression and Knockdown of β 3GalT5. To establish human β 3GalT5 overexpression stable lines, full-length cDNA that encodes human β 3GalT5 was PCR amplified (forward primer, GCAGATCTATGGCTTCCGAAGATG; reverse primer, GTCTCGAGTCAGACA GGCGACAAT), and subcloned into BglII/XhoI cut pMSCVpuro vector (Clontech). Murine stem cell virus (MSCV)-control and MSCV- β 3GalT5 vesicular stomatitis virus G glycoprotein (VSV-G) pseudotyped retrovirus were then generated in GP2-293 cells (Clontech) and used to infect MCF-7 and MDA-MB-231 cells. Two days after viral infection, control and β 3GalT5 stable pools were selected with puromycin (2 μ g/mL). To establish β 3GalT5 knockdown cells, the lentivirus-shRNA systems for human β 3GalT5 were purchased from National RNAi Core Facility Platform, Academia Sinica, and the β 3GalT5-short hairpin sequence is 5'-CCGGGCAAGTGGTTTGTCAAGAAATCTCGAGATTACTGACAAACCACTTGCTTTTGG-3'. Briefly, sh β 3GalT5 and shControl lentiviruses were incubated with MCF7 and MDA-MB-231 cells according to the manufacturer's instructions. Infected cells were harvested 48 h postinfection or selected with puromycin (2 μ g/mL) and the knockdown efficiency was determined by qPCR.

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