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Investigation of SSEA-4 Binding Protein in Breast Cancer Cells

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Supporting Information

ABSTRACT: SSEA-4, a sialyl-glycolipid, has been commonly used as a pluripotent human embryonic stem cell marker, and its expression is correlated with the metastasis of some malignant tumors. However, there is no in-depth functional study related to the receptor and the role of this glycolipid. Here, we report the identification of an SSEA-4-binding protein in a breast cancer cell line, MCF-7. By using affinity capture and glycan microarray techniques, the intracellular FK-506 binding protein 4 (FKBP4) was identified to bind directly to SSEA-4. The biological significance of SSEA-4/FKBP4 interaction was investigated.

Aberrant glycosylation is characteristic of cancers.[1] Glycosylation-promoting or -inhibiting cancerous progression is of crucial importance in current cancer research. Nevertheless, there are only limited studies in this field, mainly because the functional roles of glycans in cancer are difficult to delineate.[2] To evaluate the functional role of aberrant glycans in cancers, one essential step is to identify specific glycan-binding proteins (GBPs), which may mediate cell−cell interactions, signaling, and immune responses.[3] However, owing to the low affinity of GBPs toward glycans and the lack of efficient tools available for investigation, this study therefore remains one of the major challenges in the field.[4]

Glycolipids are a type of glycoconjugates with their carbohydrate moieties covalently bound to lipids; they are widely found in the cell membrane of eukaryotic cells.[5] Aberrant glycolipids, especially the sialylated ones, give the most characteristic patterns of a given cancer. Much evidence has shown their involvement in cellular adhesion, proliferation, receptor activation, cellular recognition, differentiation, and oncogenesis, all of which are related to metastasis of cancer.[6] SSEA-4 (stage-specific embryonic antigen-4), a sialyl-glycolipid, has long been used as a cell surface marker for pluripotent human embryonic stem cells.[7] Recent studies show that SSEA-4 is implicated in the malignancy of cancers, such as invasion and metastasis of cancer cells.[8] However, due to the lack of well-defined SSEA-4 binding protein, a definite functional role of SSEA-4 has not been demonstrated. In this study, we report the identification of an SSEA-4 binding protein for functional study, using two strategies that rely on their inherent surface multivalency:[9] one using SSEA-4-conjugated magnetic beads (SSEA-4-MBs),[10] and the other based on high-throughput glycan microarray.[9,11]

To apply SSEA-4-MBs for protein capturing in cell lysates, we synthesized SSEA-4-MBs in two steps (Figure 1). First, a polyethylene glycol (PEG) reagent (carboxylate-PEG_{12}-amine) was attached to beads to reduce nonspecific binding on the bead surface.[12] SSEA-4 previously synthesized in our group[13] was then conjugated onto PEG-modified beads. All the coupling reactions were carried out in mild conditions for amide bond formation. Methyl-PEG_4 (MEG)-conjugated MB was synthesized as a glycan-free control. The results showed that anti-SSEA4 antibody (MC-813-70) bound specifically to SSEA-4-MBs but not MEG-MBs in flow cytometry, indicating the success of SSEA4 conjugation (Figure 1).

Affinity capture of SSEA-4-binding proteins in the cell lysate of a breast cancer cell line, MCF-7, was performed. The assay was conducted by mixing SSEA-4-MBs (100 μg) with various amounts (ranging from 20 to 180 μg) of cell lysate. After incubation, the protein−bead complexes were precipitated by a magnet, and the supernatant was removed. The captured proteins were then subjected to SDS-PAGE and silver staining (Figure 2). Compared with MEG-MB control, SSEA-4-MBs pulled down a unique protein band (~60 kDa) in a dose-dependent manner. This protein band was then excised, trypsin-digested, and analyzed by liquid chromatography−mass spectrometry (LC-MS).

LC-MS revealed that this protein was FK-506 binding protein 4 (FKBP4) (Supporting Information, Table S1), which is a cytoplasmic protein and is highly expressed in breast cancer cell lines MCF-7 and T-47D.[14] This protein belongs to a subfamily of immunophilin with peptidyl−prolyl *cis*−*trans* isomerase (PPIase) activity, which is inhibited when the protein is complexed with FK-506. Although the FKBP12-like consensus sequence in FKBP4 indicates an immunosuppressant binding site, FKBP4 does not mediate the immunosuppressive action of FK-506. FKBP4 contains numerous functional domains (e.g., PPIase-like and TPR domains), which enable it to play diverse roles in cellular processes including protein folding, trafficking, and immunoregulation.[14] However, its glycan-binding ability has never been reported.

We next profiled the glycan-binding specificity of FKBP4 by glycan microarray. A glycan microarray of 63 glycans (Supporting Information, Figure S1) was probed with recombinant FKBP4 (Figure 3). The result showed that FKBP4 bound to all the glycans with Neu5Acα2-3Gal terminus (glycans **8**, **10**, and **11**), in addition to SSEA-4 (glycan **12**). Although glycan **1** contained a Neu5Acα2-3Gal epitope, its binding intensity to FKBP4 was relatively weak, which could be due to its short glycan sequence.[11] Further binding analysis

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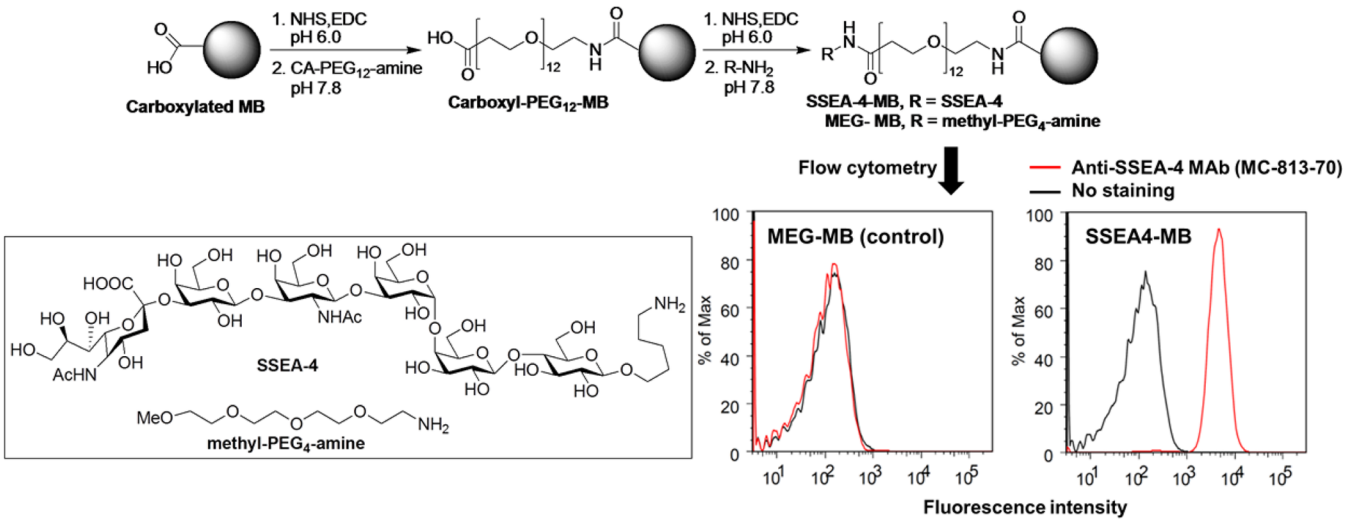

Figure 1. Conjugation of SSEA-4 onto magnetic beads and detection of SSEA-4 via flow cytometry.

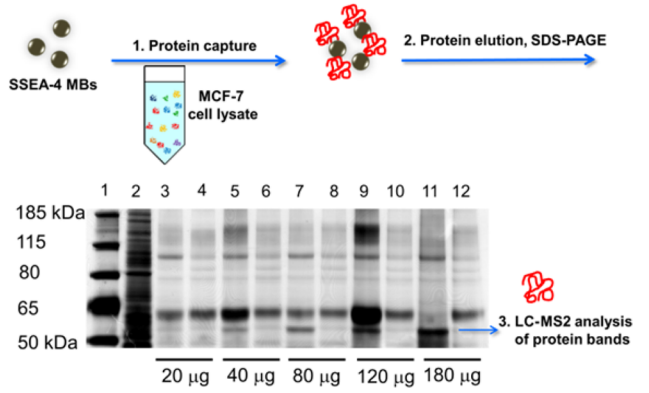

Figure 2. Magnetic bead-based capture for the identification of SSEA-4-binding proteins. Different amounts (20, 40, 80, 120, 180 μg) of MCF-7 total cell lysate were mixed with SSEA-4-MBs or MEG-MBs (100 μg each), and the bound proteins were eluted, separated by SDS-PAGE, and visualized after silver staining. Lane 1, marker; lane 2, MCF-7 total cell lysate (0.5 μg); lanes 3, 5, 7, 9, and 11, protein eluted from SSEA-4-MBs; lanes 4, 6, 8, 10, and 12, protein eluted from MEG-MBs for comparison.

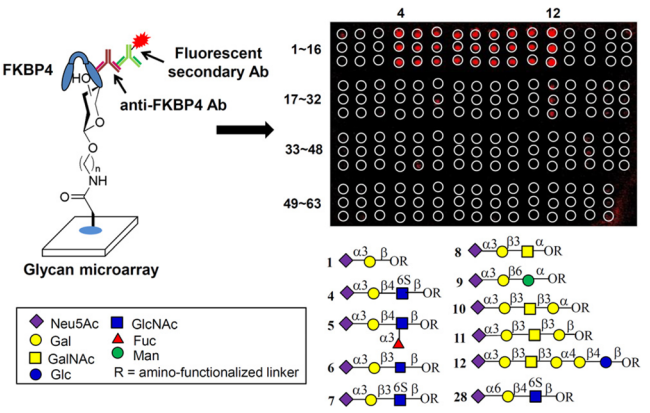

Figure 3. Binding analysis of FKBP4 in a printed glycan array with 63 glycans. Glycans (100 μM) were printed in triplicate and probed with FKBP4 as described in the Supporting Information. The structures of FKBP4-binding glycans are shown.

showed that glycans **8**, **10**, and **11** bound to FKBP4 with $K_{d,surface}$ (surface-dissociation constant) values ranging from 150 to 300 nM (Figure S3), and SSEA-4 (glycan **12**) exhibited the strongest interaction with FKBP4 ($K_{d,surface}$ = 150 nM).[15]

Since targeting FKBPs by FK-506 can inhibit the rotamase activity of FKBPs (K_i = 0.5−10 nM) and change the folding of FKBPs,[14] we next examined if FK-506 could influence the binding of FKBP4 to SSEA-4 and glycans **4**−**11**. Printed glycan microarray was incubated with FKBP4 in the absence or presence of FK-506. After detection by anti-FKBP4 antibody and fluorochrome-conjugated secondary antibody, we concluded that FK-506 could inhibit the interaction of FKBP4 with SSEA-4 and the glycans **4**−**11** (Figures 4 and S5).

We next examined whether the inhibition of FKBP4 in MCF-7 could influence the expression of SSEA-4, or other glycans. After treating MCF-7 with various concentrations of FK-506 for 36 h, the expression of two SSEA-4-related globo series glycans (SSEA-3 and Globo H, and the glycans containing the

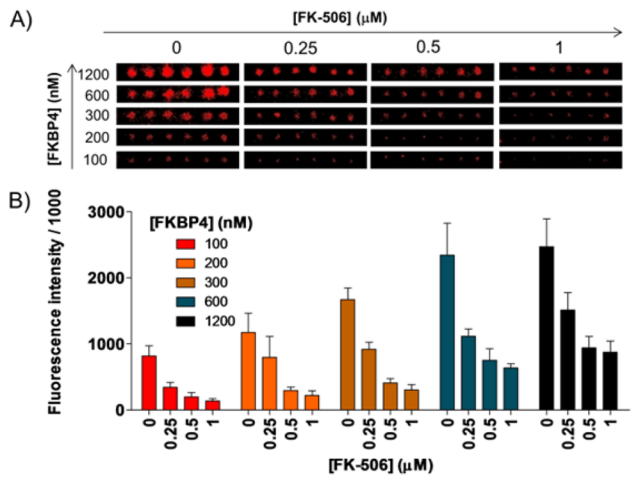

Figure 4. Competitive inhibition of SSEA-4/FKBP4 interaction by FK-506. Different concentrations of FKBP4 (100 nM to 1.2 μM) and FK-506 (0−1 μM) were tested in this competition assay. SSEA-4 (100 μM) was printed with six replicates on the array. Glycan array images (A) and the relative geometric mean of fluorescence intensity (B) in each experimental set are shown.

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Neu5Acα2-3Gal sugar unit: GM2, GD3, and GD2) was detected by specific antibodies. As shown in Figures 5 and

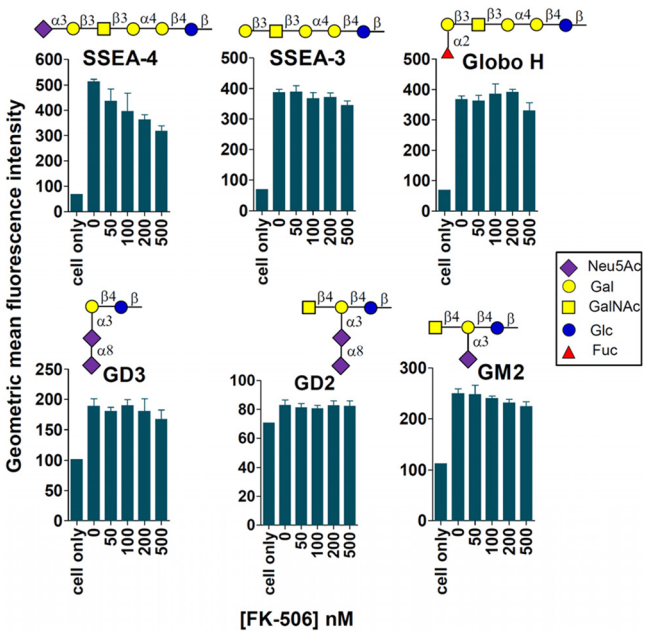

Figure 5. Surface expression of the glycan markers of MCF-7 cells analyzed by flow cytometry after FK-506 treatment."Cell only" means no staining performed, indicating the fluorescence background of the cells.

S4A, the level of cell surface SSEA-4 diminished after treatment with FK-506, while other glycan markers showed no changes of surface expression (Globo H, SSEA-3, GD3, and GM2) or lower expression level (GD2). Therefore, inhibition of FKBP4 could specifically reduce the expression level of surface SSEA-4, which suggested that FKBP4 might possibly exert a regulatory function for transporting SSEA-4 from cytoplasm to cell membrane, via direct interaction. To confirm the observation, we fixed the permeabilized MCF-7 cells and detected both surface and cytoplasmic SSEA-4 (Figure S4B). It showed that there was no difference of the total SSEA-4 expression level in FK-506-treated and untreated cells, indicating that FK-506 inhibited the surface expression but not the production of SSEA-4. Previous studies have shown that the treatment of FK-506 can suppress the malignancy processes of tumor cells via inhibiting the activity of FKBPs.[16] We thus speculated that the down-regulated expression of SSEA-4 by FK-506 could be linked to the suppression of malignant processes of cancers.

In conclusion, we have identified FKBP4, a cytoplasmic protein, as a specific binding protein for SSEA-4, a cell surface marker, by bead-based affinity capture and glycan microarray techniques. Using glycan microarray, we also investigated the glycan binding specificity of FKBP4 and observed that FK-506 could inhibit FKBP4/SSEA-4 interaction. The surface expression of SSEA-4, but not SSEA-3, globo H, GM2, or GD3, was down-regulated by FK-506 via inhibiting the activity of FKBP4 and the interaction with SSEA-4. Further studies of SSEA-4 and FKBP4, including their interaction, the trafficking of SSEA-4 from intracellular compartment to cell surface, the specificity of FKBP family for glycans, the effect of rapamycin, another small-molecule drug that also affects the activity of FKBPs, and the role of SSEA-4 in malignancy, are underway.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary schemes, synthetic methods, and experimental protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

⊥T.-C.H. and C.-W.L. contributed equally.

Notes

The authors declare no competing financial interest.

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