A SPECIFIC MICRODOMAIN ("GLYCOSYNAPSE 3") CONTROLS PHENOTYPIC CONVERSION AND REVERSION OF BLADDER CANCER CELLS THROUGH GM3-MEDIATED INTERACTION OF α3β1 INTEGRIN WITH CD9
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Running title: GM3/CD9/α3 complex controlling tumor cell motility

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Cell motility is highly dependent on organization and function of microdomains composed of integrin, proteolipid/ tetraspanin CD9, and ganglioside (Ono M, Handa K, Sonnino S, et al, Biochemistry 40: 6414-21, 2001; Kawakami Y, Kawakami K, Steelant W, et al, JBC 277: 34349-58, 2002), later termed "glycosynapse 3" (Hakomori S, Handa K, FEBS Lett 531: 88-92, 2002). Human bladder cancer cell lines KK47 (non-invasive, non-metastatic) and YTS1 (highly invasive, metastatic), both derived from transitional bladder epithelia, are very similar in terms of integrin composition, and levels of tetraspanin CD9. Tetraspanin CD82 is absent in both. The major difference is in level of ganglioside GM3, which is several times higher in KK47 than in YTS1. We now report that GM3 level reflects glycosynapse function, as shown by: (i) a stronger interaction of integrin α3 with CD9 in KK47 than in YTS1, (ii) conversion of benign, low-motility KK47 to invasive, high-motility cells by depletion of GM3 by P4 (D-threo-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol) treatment, or by knockdown of CD9 by RNAi method; (iii) reversion of high-motility YTS1 to low-motility phenotype like that of KK47 by exogenous GM3 addition, whereby the α3-to-CD9 interaction was enhanced; (iv) low GM3 level activated cSrc in YTS1 in P4-treated KK47; high GM3 level by exogenous addition caused Csk translocation into glycosynapse, with subsequent inhibition of cSrc activation; (v) inhibition of cSrc by "PP2" in YTS1 greatly reduced cell motility. Thus, GM3 in glycosynapse 3 plays a dual role in defining glycosynapse 3 function. One is modulating interaction of α3 with CD9; the other is activating or inhibiting cSrc activity, possibly through Csk translocation. High GM3 level decreases tumor cell motility/ invasiveness. Oncogenic transformation and its reversion can be explained through difference in glycosynapse organization.

Interaction of tumor cells with their microenvironment may define direction of tumor development and degree of malignancy (1). Such interaction is likely based on structure and function of microdomain at the tumor cell surface interfacing with normal cell microdomain, and with extracellular matrix components, particularly at basement membrane. A crucial event for progression of many types of tumor cells of epithelial origin is their adhesion/ motility/ invasion on basement membrane underlying epithelial cells. In this process, specific microdomains of tumor cells are considered to interact with laminin-5 ("epiligrin") (2) or laminin-10/11 (3), which are major components of epithelial basement membrane and ligands of integrin α3 or α3β1 (4). Such microdomains, having proteolipid/ tetraspanin (PLP/ TSP) CD9, integrin α3β1, and ganglioside GM3 (5-7), are capable of controlling cell adhesion and motility (6,8-10), in contrast to "caveolar membrane" or "raft", which is cholesterol-dependent (11), has no integrins (12), and is not involved in cell adhesion and motility. GM3/ TSP/ integrin microdomains have therefore been termed "glycosynapse" (8,13), in analogy to the microdomain involved in immunocyte adhesion/ antigen presentation with concurrent signaling, termed immunosynapse (14). Among glycosynapses, GM3/ CD9/ integrin complex termed "glycosynapse 3" (8) was previously suggested to play a role in regulation of cell motility (see Discussion).

GSLs, including GM3, have been implicated as inhibitors of signal transduction, since various signaling molecules such as cSrc and phospholipase C-γ (15,16), and growth factor receptor tyrosine kinases (17), are activated when
GSLs are depleted by P4, a GlcCer transferase inhibitor (18).

The goal of the present study was to clarify contrasting composition of GM3, α3, and CD9 in glycosynapse 3, and their interaction, to define motility/ invasive properties of two closely-related human bladder cancer cell lines: KK47 with non-invasive, low-motility phenotype, and YTS1 with invasive, high-motility phenotype. Our results make clear the dual functional role of GM3 in glycosynapse 3: (i) high, or low, GM3 level promotes, or inhibits, interaction of α3 with CD9, to stabilize, or de-stabilize, α3/CD9/GM3 complex within the microdomain; (ii) high, or low, GM3 level activates, or inhibits, cSrc through association, or dissociation, with Csk within the same microdomain. Through either process (i) or (ii), GM3 level regulates tumor cell motility/ invasiveness. Oncogenic conversion, or reversion to normal cell phenotype, as suggested in previous studies of bladder cancer (7,19,20), colorectal cancer (5,6,21), and Jun-transformed (22).

The present study may help explain the mechanism by which glycosynapse organization and composition define oncogenic conversion, or reversion to normal cell phenotype, as suggested in previous studies of bladder cancer (7,19,20), colorectal cancer (5,6,21), and Jun-transformed cells (22).

MATERIALS AND METHODS

Cells, antibodies, and reagents

Cells: YTS1 cell line was established from invasive human urinary bladder cancer (23), and was donated by H. Kakizaki (Dept. of Urology, Yamagata Univ., Yamagata, Japan). KK47 cell line was established from non-invasive, superficial bladder cancer (24), and was donated by T. Masuko (Dept. of Hygienic Chemistry, Faculty of Pharmaceutical Science, Tohoku Univ., Sendai, Japan). Both cell lines were grown in RPMI 1640 containing 10% fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37 °C, 5% CO2.

Antibodies: Mouse anti-GM3 IgG, mAb DH2 (25) was established in this laboratory. Mouse anti-CD9 IgG, mAb was from Pharmingen (San Diego, CA). Rabbit anti-α3, α5, and mouse anti-β1 were from Chemicon Intl., Inc. (Temecula, CA). Rabbit anti-Src, -P-Src (Tyr416), -P-Src (Tyr527), and -Csk were from Cell Signaling Technologies (Beverly, MA). Anti-β-actin and anti-γ-tubulin mouse IgG were from Sigma (St. Louis, MO). Goat anti-mouse IgG labeled with HRP and goat anti-mouse IgG, labeled with Texas Red were from Southern Biotech (Birmingham, AL). Goat anti-rabbit IgG labeled with HRP and goat anti-rabbit IgG labeled with FITC were from Santa Cruz Scientific (Santa Cruz, CA). Goat anti-mouse IgM+IgG labeled with FITC was from Biosource Intl. (Camarillo, CA).

Reagents: Gangliosides GM3 and GM1 were from Matreya (Pleasant Gap, PA). D-threo-1-phenyl-2-palmitoylaminono-3-pyrrolidino-1-propanol (P4) (18) was originally established and kindly donated by J.A. Shayman (Univ. of Michigan). Cholera toxin subunit B was from Sigma. 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-d] pyrimidine (PP2), a Src family kinase inhibitor (26), was from Biomol (Plymouth Meeting, PA). Micro-BCA protein assay reagent kit was from Pierce (Lockford, IL), Immunostain HRP-1000 Kit was from Konica (Tokyo, Japan), Vecstain ABC kit was from Vector (Burlingame, CA), and Protein A/G agarose was from Santa Cruz. Other reagents were from Sigma unless described otherwise.

Determination of total levels of TSPs and integrins by SDS-PAGE and Western blot – Total cell lysate was prepared as described previously (6). Briefly, ~1×107 cells were collected and the cell pellet was suspended in 1 ml RIPA buffer (1% Triton X-100, 150 mM NaCl, 25mM Tris pH 7.5, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM pyrophosphate, 50 mM NaF) containing 75 units of aprotinin and 2 mM phenylmethanesulfonyl fluoride. The suspension was kept on ice for 30 min and Dounce homogenized (10-15 strokes). The lysate was centrifuged at 15,000 rpm for 15 min at 4 °C, and the supernatant was subjected to SDS-PAGE and Western blot after determination of protein concentration. The membranes were reblotted with anti-β-actin antibody after stripping with Re-Blot Plus solution (Chemicon). Intensity of Western blot was determined by densitometry using Scion image program.

Determination of total GSL and ganglioside levels by thin-layer chromatography and immunostaining – GSLs were extracted and analyzed as described previously (27,28). Briefly, YTS1 and KK47 cells were grown until ~90% confluence in 15 cm dishes and washed three times with PBS. ~2×107 cells were collected and
extracted twice with C/M (2:1). The extracts were dried under N\textsubscript{2} stream. To remove phospholipids, the dried residue was dissolved in 2 ml of 0.1 M NaOH in methanol and incubated at 40 °C for 2 h. After neutralization with 1 N HCl, fatty acids were extracted twice with 2 ml hexane. GSLs in the lower phase were evaporated, dissolved in water, and applied to 3 ml BondElut C18 columns (Varian, Harbor City, CA) to remove salt. Columns were washed with water, and GSLs were eluted with C/M (2:1). Solvents were evaporated and equal aliquots of GSLs dissolved in C/M (1:1) were subjected to HPTLC plate (Merck, Darmstadt, Germany), developed with C/M/ 0.2% CaCl\textsubscript{2}-H\textsubscript{2}O (50:40:10), and stained with 0.5% orcinol in 2 N sulfuric acid to visualize GSLs, or immunostained with DH2 using Vecstain ABC Kit and Immunostain HRP-1000 Kit according to the manufacturer’s instructions. For analysis of GSLs in GEM, after fractionation of postnuclear fractions (PNF) through sucrose density gradient ultracentrifugation, low-density fractions and high-density fractions were applied to C18 column and processed as described above.

**PNF and GEM preparations for determination of GEM components** – PNF was prepared as described previously (6). Briefly, ~4x10\textsuperscript{7} cells were collected and the pellet was suspended in 1 ml Brij 98 lysis buffer (1% Brij 98, 25 mM HEPES buffer pH 7.5, 150 mM NaCl, 5 mM EDTA) containing 75 units of aprotinin and 2 mM phenylmethylsulfonyl fluoride. The suspension was kept on ice for 30 min and Dounce homogenized (10~15 strokes). The lysate was centrifuged at 2500 rpm for 7 min at 4 °C to remove nuclei and debris. PNF was subjected to sucrose density gradient ultracentrifugation to separate low density membrane fraction as described previously (6). Fractions were separated and numbered 1-12 from top to bottom. Aliquots of each fraction, containing equal protein content (~2.5 µg), were analyzed by SDS-PAGE and Western blot.

**Phagokinetic gold sol assay for cell motility** – Cell motility was studied by an improved method (29) based on (30). Briefly, 24-well plates were incubated with 1% bovine serum albumin for 24 h at 37 °C, washed with 100% ethanol, and dried. Gold sol suspension was prepared by adding 11 ml H\textsubscript{2}O and 6 ml 36.5 mM Na\textsubscript{2}CO\textsubscript{3} to 1.8 ml 14.5 mM AuHCl\textsubscript{4}. The mixture was boiled, and 1.8 ml freshly prepared 0.1% formaldehyde solution was added. Gold sol suspension was put in each well and incubated for 40 min at RT, and the plate was washed with culture medium. Cells in culture medium (1x10\textsuperscript{7}/ well) were plated onto gold sol-coated wells and incubated for 16 h at 37 °C. Migratory cells were observed and photographed under light microscope (Nikon) Migratory areas of 20 cells of each well were measured by Scion image program and expressed as square pixels.

**Determination of interaction between CD9 and integrin by co-IP** – Interaction between CD9 and integrins was analyzed by co-IP as described previously (6). Briefly, PNF (400 µl, containing 400 µg protein) was prepared as described above and mixed with protein A/G agarose beads (~30 µl bed volume). The mixture was placed on rotator at 4 °C for 3 h and centrifuged at 1000 rpm to collect supernatant. ~3 µg antibody were added to the supernatant and rotated at 4 °C overnight; antibodies used were rabbit anti-α3 and α5, and mouse anti-β1. Protein A/G agarose beads was added, rotated at 4 °C for 3 h, and centrifuged to collect the beads. After washing twice with lysis buffer, the immunoprecipitates were resolved in SDS-PAGE sample buffer, and subjected to SDS-PAGE and Western blot with mouse anti-CD9 IgG\textsubscript{1} antibody.

**Interaction of CD9 with α3 analyzed by laser scanning confocal microscopy** – Interaction between CD9 and α3 was also analyzed by confocal microscopy as described previously (6). Briefly, YTS1 and KK47 cells were grown for 24 h on cover glass (12 mm diameter) placed in 24-well plates. Cells on cover glass were washed three times with PBS, and fixed with 3.7% paraformaldehyde/ PBS for 15 min. Fixed cells were washed three times with PBS, incubated with 1% bovine serum albumin/0.1% Na\textsubscript{2}O\textsubscript{2} PBS for 30 min, and incubated with mouse anti-CD9 IgG\textsubscript{1} mAb for 1 h at RT. After washing and permeabilization with 0.05% Triton X-100 in PBS for 5 min, rabbit anti-α3 was added and incubated for 1 h at RT. Cells were incubated with mixture of goat anti-mouse IgG\textsubscript{1} labeled with Texas Red and goat anti-rabbit IgG labeled with FITC for 1 h at RT, washed, mounted with a drop of Glycergel mounting medium (Dako, Carpinteria, CA), and observed by laser scanning confocal microscopy (FluoView™, Olympus, Tokyo) using appropriate filter set.

**Effect of exogenous GM3 on interaction of CD9 with α3 and cell motility in YTS1 cells** – YTS1 cells were grown in a 10-cm dish for 24 h, and
growth medium was changed to serum-free medium. GM3 was dissolved in serum-free medium by sonication and standing for 24 h at RT, added to cells in serum-free medium at concentrations of 10, 20, and 50 µM, and incubated at 37 °C for 24 h. GM1 was exogenously added in the same way, in a separate experiment. Cells were analyzed by co-IP and confocal microscopy for interaction between CD9 and α3, and by phagokinetic gold sol assay for cell motility.

**Effect of P4 on GM3 and ceramide levels, interaction of CD9 with α3, and motility of KK47 cells** – KK47 cells were grown in 10-cm dish for 24 h at 37 °C. The medium was changed to fresh growth medium with or without P4 (1 µM) and further incubated for 72 h. The cells were analyzed by co-IP and confocal microscopy for interaction between CD9 and α3, and by phagokinetic gold sol assay for cell motility. Ceramide in P4-treated cells was analyzed by HPTLC with C/M/water (65: 25: 4) and charring in 3% cupric acetate and 10% phosphoric acid for 20 min at 130 °C, on 400 µg cellular protein basis, as described previously (31,32).

**Knockdown of CD9 expression through RNAi in KK47 cells**

**Plasmid construct**: A plasmid-based system for production of RNAi were made in pSUPER vector (Oligoengine, Seattle, WA) according to the manufacturer’s instructions, as described previously (33,34). Four oligonucleotides (68 nt) encoding CD9 specific sequences and one oligonucleotide (68 nt) encoding control scrambled sequence were constructed (CD9 specific sequences or control scrambled sequences shown in italic characters):

(A) 5'-GATCCCCgctgttccgatattaacttcacTTCAAGAGA atgaaatuaatacgacacgt TTGTTGAAAA-3', 3'-GGGcgaacagaaccccataaaagttgaagaagtaAAGTTCTCTtactt aatttagctttgcaAAAAACCTTTTCGA-5',

(B) 5'-GATCCCCCagcaggtctataatctgcTTCAAGAGA Agatacaaatatatcaacctgt TTTTTTGAAA-3', 3'-GGGgcgtctctttttctgataaatag AAGTTCTCTctagtctttatctctagagagaaaaAAAACCTTTTCGA-5',

(C) 5'-GATCCCCCcaagacagatcttcgacggctggTTCAAGAGA Gaactgctgaagatgctttgg TTGTTGAAAA-3', 3'-GGGgtctcttattctgacactgccte AAGTTCTCTctgagagagctctactcagagaaaaAAAACCTTTTCGA-5',

(D) 5'-GATCCCCggattgtctcttctccattgTTCAAGAGA caatggcaagggcgaagctt TTGTTGAAAA-3', 3'-GGGcgaacagaacccctaatataaagttgaagaagtaAAGTTCTCTgttacctgttctgtaaggAAAAACCTTTTCGA-5',

**Control**

5'-GATCCCCggtgaacttatttctccagTTCAAGAGA Agacttaaatatacgacgt TTTTTTGAAA-3', 3'-GGGcgaacagaacccctaatataaagttgaagaagtaAAGTTCTCTtacttactacagagaaaaAAAACCTTTTCGA-5'.

**Transfection**: These plasmids were co-transfected with pPUR vector (BD Bioscience Clontech, Mountain View, CA) into KK47 cells by electroporation. Two days after transfection, puromycin (0.5 µg/ml) was added for selection. After cloning, CD9 mRNA level was measured by RT-PCR, and CD9 expression was measured by flow cytometry and Western blot.

**RT-PCR**: Total RNA was isolated from transfecants by RNeasy MiniKit (QIAGEN, Valencia, CA). cDNA was prepared from 1 µg total RNA using Superscript II kit (Invitrogen, Carlsbad, CA). PCR was performed using Taq DNA polymerase (Promega, Madison, WI) according to the manufacturer’s instruction, with CD9 primers (sense: 5'-TTGGACTATGGCTCCGATTC-3', antisense: 5'-AGCATGCACTGGGACTCCT-3'), yielding a 175bp product, and with β-actin primers (sense: 5'-AACCACCAGGAGATGACCCAG-3', antisense: 5'-CTCCTGCTTGCTGATCCACAT-3'), yielding a 721bp product.

**Flow cytometry**: Cells were detached by trypsin-EDTA and washed with PBS. Aliquots of cells (1x10^5) were incubated with mouse anti-CD9 IgG, for 1 h on ice, washed with PBS, incubated with goat anti-mouse IgM+G labeled with FITC for 40 min on ice, fixed in 2% paraformaldehyde/ PBS, and analyzed using a Coulter EPICS XL flow cytometer (Beckman Coulter, Fullerton, CA).

**Effect of GM3 level on cSrc and Csk in glycosynapse 3** – cSrc, and its phosphorylation at Tyr416 (for activation) and at Tyr527 (for inhibition), were determined by Western blot analysis in KK47 vs. YTS1 cells, using phosphorylation site-specific antibodies. PNF, low-density membrane fractions (Fr. 4, 5, 6), and high-density membrane fractions (Fr. 10, 11, 12) separated through sucrose density gradient ultracentrifugation were analyzed. Csk was determined simultaneously in these fractions. To test GM3 effect on cSrc activity, KK47 cells were pre-treated with P4 for 72 h (see "Effect of P4 on
GM3 and ceramide levels ..."), followed by Western blot analysis of cSrc, Tyr416, Tyr527, and Csk. Intensity of γ-tubulin in each fraction was used as loading control. YTS1 cells were incubated with 50 µM GM3 in serum-free medium for various durations (1, 3, 16 h) (see "Effect of exogenous GM3 on interaction of ..."); followed by sucrose density gradient ultracentrifugation to separate low-density and high-density fractions, and Western blot analysis of each fraction for determination of cSrc phosphorylation status and Csk translocation into low-density fractions (GEM).

Effect of cSrc activity on tumor cell motility – YTS1 cells were seeded on a 15-cm dish and grown in RPMI with 10% fetal bovine serum. PP2, a Src tyrosine kinase inhibitor, was added to the medium at 0, 5, 10, and 20 µM, and incubated at 37 °C for 24 h. Cells were subjected to phagokinetic gold sol assay as described above.

RESULTS

Components, and their interactions, characteristic of KK47 vs. YTS1 cells

Composition of KK47 vs. YTS1 microdomain. At first, we compared major components of glycosynapse 3 (integrins α3, α5, and β1; PLP/ TSP CD9 and CD82; gangliosides) in total cell lysate prepared in RIPA buffer from KK47 and YTS1 as shown in Figs. 1A, B, C. Levels of the integrins were essentially the same in the two cell lines (Fig. 1A). CD9 level was similar and CD82 was absent in both cell lines (Fig. 1B). Ganglioside expressed in YTS1 was mainly GM2, while that in KK47 was mainly GM3, as revealed by TLC with orcinol/ sulfuric acid staining. GM3 level in KK47 was ~12 ng/ 30 µg protein and <2 ng/ 30 µg protein, respectively. GM2 concentration in the same fractions from YTS1 and KK47 was ~12 ng/ 30 µg protein and <2 ng/ 30 µg protein, respectively. Neither GM3 nor GM2 was detectable in Fr. 1-3 or Fr. 7-9 from YTS1 or KK47 (data not shown).

Interactions of components in KK47 vs. YTS1 microdomain. Interactions were determined by co-IP and by confocal microscopy. Co-IP of α3 with CD9, and of β1 with CD9, was performed by addition of anti-α3 or anti-β1 antibody to PNF, followed by Western blot with anti-CD9, as described in M&M. Co-IP of α3 with CD9 was much higher in KK47 than in YTS1, whereas co-IP of β1 with CD9 was weak (and similar) in the two cell lines. α5 was not co-IPd with CD9 from either cell line (Fig. 2A). Co-localization of α3 with CD9 was significantly higher in KK47 than in YTS1, as indicated by enhanced merge image in confocal microscopy (Fig. 2B).

Difference in motility of KK47 vs. YTS1, by phagokinetic gold sol assay. Transwell membrane motility through thick Matrigel was much higher for YTS1 than for KK47 (7). This difference in cell motility was confirmed in the present study by phagokinetic gold sol assay (Fig. 3A, B).

Phenotypic conversion of non-invasive KK47 cells to invasive variants by knockdown of CD9 through RNAi method, or by P4-induced GM3 depletion

Knockdown of CD9 in KK47 cells through RNAi causes increased motility. Preliminary experiments with transient transfectants of KK47 using four different CD9 specific sequences (A-D) showed that sequence B was the most effective. Therefore, permanent transfectants with this sequence were cloned as described in M&M. Four clones (B201, B201, B254, B255) were analyzed with control transfectant (S17), which was transfected with control scrambled sequence based on CD9 sequence B. All four clones showed significant reduction of CD9 expression -- particularly B255, in which CD9 expression was depleted (Fig. 4A,B,C) compared to the control, while expression of α3 and β1, and GM3, was essentially the same in the four clones and control clone (Fig. 4D). Phagokinetic motility was assayed using gold sol-coated plates as described in M&M, and compared among the four clones and control. Motility of B255 and B254 was significantly higher than that of control (p < 0.001.
and p< 0.01 respectively). Motility of the other two clones (B201, B202) was almost the same as control (Fig. 4E).

GM3 depletion by P4 in KK47 cells causes reduced α3-to-CD9 interaction and increased motility. The high GM3 level characteristic of KK47 was nearly depleted by incubation of cells with 1 µM P4 (Fig. 5A), whereby significant change of ceramide level was not detected (data not shown). GM3-depleted KK47 showed clear reduction of α3-to-CD9 interaction, as indicated by reduced co-IP between α3 and CD9 (Fig. 5B), and by reduced merge image in confocal microscopy (Fig. 5C). Phagokinetic motility of KK47 was greatly enhanced following depletion of GM3 with P4 (Fig. 5D).

Phenotypic reversion of high-motility YTS1 cells to low-motility variant by exogenous GM3 addition – GM3 synthase gene transfection to YTS1 did not sufficiently increase GM3 level, because GM3 could be converted to GM2 in these cells (data not shown). We therefore tried exogenous addition of GM3 in culture medium to observe possible effect on α3-to-CD9 interaction and cell motility. Incubation of YTS1 with GM3 caused significant increase of GM3 level, as determined by flow cytometry (Fig. 6A, left). Increased GM3 level caused enhanced co-IP with α3 and CD9. i.e., more CD9 was co-IP’d with α3 in PNF from GM3-preincubated YTS1 than from control non-treated cells, as detected by Western blot analysis (Fig. 6B, left) and densitometry of co-IP’d bands (Fig. 6B, right). Exogenous addition of GM3 also enhanced co-localization of α3 and CD9 in YTS1, as indicated by enhanced merge image observed by confocal microscopy (Fig. 6C), whereby significant reduction of phagokinetic cell motility was observed, particularly when cells were incubated with 20 or 50 µM GM3 (Fig. 6D). GM2 is present in significant amount in YTS1, so exogenous GM2 addition experiment was not relevant.

GM1 is absent in YTS1, and exogenous GM1 is incorporated efficiently at the cell surface, as revealed with FITC-labeled cholera toxin subunit B (Fig. 6A, right). However, GM1 addition did not significantly affect α3/CD9 interaction as determined by co-IP (Fig. 6B, left and right) or by confocal microscopy (Fig. 6C). Motility of 50 µM GM1-added cells was much higher than that of 50 µM GM3-added cells, but not significantly different from that of control cells (Fig. 6E).

Expression level of cSrc, activated cSrc, and Csk in YTS1 vs. KK47 cells – cSrc level in PNF was lower in KK47 than in YTS1, and this difference was greater in low-density Fr. 4 and 5. The activated form of cSrc, with phosphorylation at Tyr416, was clearly present in PNF from YTS1, and nearly absent in PNF from KK47. In contrast, the inactive form of cSrc, with phosphorylation at Tyr527, showed similar levels in PNF of YTS1 and KK47. These comparisons were based on the same level of loading control γ-tubulin (Fig. 7A). Distribution pattern of cSrc and Csk in low-density Fr. 4-6 and high-density Fr. 10-12 from PNF, separated by sucrose density gradient centrifugation, was determined by Western blot. cSrc level was much higher in Fr. 4-6 of YTS1 compared to KK47. Csk was virtually absent in Fr. 4-6 of YTS1, but clearly present in Fr. 5 of KK47 (Fig. 7B). cSrc and Csk were not detected in Fr. 1-3 or Fr. 7-9 from either YTS1 or KK47 (data not shown).

Effect of GM3 level on cSrc activation status, Csk translocation, and tumor cell motility – The results shown in Fig. 7 suggest that low GM3 level activates cSrc in YTS1 cells, while high GM3 level inactivates cSrc in KK47 cells. We examined this possibility by depletion of GM3 with P4 in KK47 cells, and by exogenous GM3 addition to YTS1 cells.

Low GM3 level activates cSrc. KK47 cells were incubated with 1 µM P4 for 72 h, followed by Western blot for detection of cSrc phosphorylation status and Csk expression in PNF. P4 treatment caused increased Tyr416 phosphorylation and decreased Tyr527 phosphorylation, while total cSrc and Csk levels did not change (Fig. 8A, left). The ratio of phosphorylation level (Tyr416 divided by Tyr527) did not cause significant change of cSrc phosphorylation status and Csk in PNF (Fig. 8B, left). However, cSrc with Tyr527 phosphorylation increased with GM3 incubation (maximum at 3 h), with clear translocation of Csk to GEM (Fr.4-6) This was closely associated with a decrease of activated cSrc with Tyr416 phosphorylation after prolonged incubation with GM3 (Fig.8B, middle).
Phosphorylation pattern in cSrc, and Csk status, were essentially unchanged in high-density Fr. 10-12 of YTS1 cells (Fig. 8B, right).

Effect of Src inhibitor "PP2" on YTS1 cell motility. The possibility that cSrc activity is correlated with GM3-mediated changes of cell motility in YTS1 was further assessed using Src tyrosine kinase inhibitor PP2. Motility of YTS1, associated with high cSrc activity, was decreased significantly in a dose-dependent manner in PP2-incubated cells (Fig. 8C).

DISCUSSION

Cell adhesion and motility are controlled by types of integrin receptor expressed at the cell surface, in combination with various cytoplasmic signaling molecules, some of which connect to cytoskeletal components (35). Integrin functions are modulated by three other factors: (i) N-glycosylation status of integrin (36,37); for review see (38); (ii) interaction with TSP (6,21,39,40); for review see (9); (iii) ganglioside association that may mediate integrin interaction with TSP (41,42).

TSPs CD9 and CD82 were originally identified as cell motility inhibitory factors highly expressed in normal cells or non-metastatic tumor cells, but down-regulated in metastatic deposits (43-45). Decreased expression of CD9 (46) and CD82 (47) in urothelial cancer was recently reported to be associated with recurrence and increased metastasis. However, detailed study on CD9 indicates that CD9 alone does not inhibit tumor cell motility or invasiveness; rather, such inhibition requires GM3 and complete N-glycosylation, including that of integrin. This concept arose from studies (5) with mutant ldlD cells defective in UDP-Gal 4-epimerase, in which such glycosylation does not occur unless galactose is added to growth medium (48). CD9 was found to be soluble in C/M, to display properties of PLPs (49,50), and to be closely associated with GSLs (6,51). Studies with various colorectal cancer cell lines expressing different levels of GM3 and CD9, aided by application of photoactivatable GM3, showed that GM3 is a co-factor of CD9 for inhibition of tumor cell motility (21).

Several lines of studies, including those above, indicate that microdomains controlling glycosylation-dependent adhesion and concurrent signal transduction are cholesterol-independent, soluble in 1% Triton X-100, and provide adhesion sites. These properties of GM3/ TSP/ integrin microdomains are distinguishable from those of moving, signaling platforms, "rafts", that do not contain TSP/ integrin, are not soluble in Triton X-100, and are not involved in cell adhesion, but display clear cholesterol-dependent signaling function. We therefore applied the term "glycosynapse" to such glycosyl microdomains (8,13,52), in analogy to "immunological synapse" (14), the microdomain involved in immunocyte adhesion, and antigen presentation to T-cell receptor. Among various glycosynapses, those having GM3/ TSP/ integrin complex are termed "glycosynapse 3" (8).

Thus, the "classic" concept of integrin-dependent motility and invasiveness of tumor cells is now re-formulated in terms of a particular interaction and organization of integrin with PLP/ TSP and GM3 in glycosynapse 3. This concept has been extended to phenotypic conversion or reversion induced by deletion or addition of a single component in glycosynapse 3, as described in the present study.

Phenotypic conversion from low-motility bladder cancer cells, KK47, to high-motility cells was caused by knockdown of CD9 by RNAi, whereby association of α3 with CD9 was disrupted. Similarly, depletion of GM3 by P4 treatment of KK47 gave rise to a high-motility variant characterized by dissociation of α3 from CD9. Level of ceramide, a well-established, versatile signaling molecule (53,54), was not significantly changed in P4-treated KK47, similarly to the case of WI38 cells (17), MDCK cells (18), and 3T3 cells (32). Thus, the conversion of KK47 to high-motility variant by P4 treatment was due to GM3 depletion, not to ceramide change.

In contrast, phenotypic reversion from high-motility bladder cancer cells, YTS1, to low-motility cells was caused by exogenous addition of GM3, whereby significant increase of α3-to-CD9 interaction and reduction of cell motility occurred. We attempted to enhance GM3 expression in YTS1 by transfection of GM3 synthase gene, but without success -- presumably because GM3 produced in this way is immediately converted to GM2 or other higher gangliosides, and not accumulated. These findings indicate that GM3 mediates and stabilizes α3-to-CD9 interaction in glycosynapse 3. In addition, high GM3 level in KK47 and/or low GM3 level in YTS1 affect not only interaction of α3 with CD9, but also cSrc
activation state, in glycosynapse. High GM3 level inhibits cSrc activation due to translocation of Csk, which promotes Tyr527 phosphorylation in cSrc (55). In contrast, low GM3 level in YTS1 causes cSrc activation, with enhanced Tyr416 phosphorylation. This process is promoted by Csk translocation out of glycosynapse 3 in YTS1. Exogenous GM3 addition to YTS1 promotes Csk translocation into glycosynapse, resulting in inhibition of cSrc activation. GM3 effect on YTS1 cannot be replaced by GM2 or GM1, since a significant amount of GM2 pre-exists, and exogenous GM1 addition had no effect on α3/CD9 interaction or on cell motility.

Reversion and conversion of phenotype can be manipulated by GM3 addition or depletion, via well-organized, stable framework vs. disorganized, unstable framework of glycosynapse 3. In addition, high GM3 level in KK47 may inhibit cSrc activation due to the presence of Csk, which promotes phosphorylation of Tyr527 in cSrc (55). In contrast, low GM3 level in YTS1 causes cSrc activation, with phosphorylation at Tyr416. This process is promoted by the absence of Csk in glycosynapse 3 of YTS1. In our previous study of human fibroblast WI38, GM3 was implicated as an inhibitor of signal transduction, since various signaling processes initiated by cSrc activation following Akt/MAPK were promoted by P4-induced depletion of GM3 (17). Effects of GSLs on signal transduction and cellular functions, e.g., cell cycle arrest (61), and phospholipase C activation with bradykinin stimulation (62), have been studied using the GlcCer synthase inhibitors PDMP (61,62) and, more recently, P4 (20).

The survival signals PI3K, Akt, and Rac, occurring through activation of α3β1 integrin, induce adhesion-mediated cell growth and motility (3). Such signaling may be blocked when GM3/CD9/integrin complex is formed, as in glycosynapse 3. Reversion from malignant to benign phenotype induced by high GM3 level is based on not only stabilized glycosynapse framework, but also inhibition of cSrc activation, which causes reduced cell motility, growth, and invasiveness. Similarly, low GM3 level not only disorganizes glycosynapse framework, but also induces cSrc activation, with consequent enhancement of cell motility, growth, and invasiveness. However, cSrc activation status may not control GM3 level, since low GM3 level in YTS1, which show high cSrc activation, is not increased by treatment of cells with cSrc inhibitor PP2 (data not shown).

It is not yet clear how GM3 level in glycosynapse 3 is controlled. A mechanism involving GM3 synthase promoter region, which is affected by the transformation process, must be crucial. Down-regulation or depletion of GM3 is often observed associated with oncogenic transformation, e.g., in polyoma virus-transformed BHK cells (56), Rous sarcoma virus-transformed chicken embryonic fibroblasts (57), and more recently vJun-transformed chicken and mouse fibroblasts, in which reversion of oncogenic to normal phenotype is caused by enhanced GM3 synthesis through GM3 synthase gene transfection (22). In the latter case, a possible role of GM3 in glycosynapse was suggested.

Current trends in cell biology and molecular oncology are based on genomic or transcriptomic analysis by microarray assay. If this approach were performed on either KK47, YTS1, or various breast cancer cell lines, activation or inhibition of many genes would be detected. However, such changes cannot easily identify a single or few crucial molecules, such as GM3, CD9, integrin, or their organization in glycosynapse, involved in defining oncogenic transformation or its reversion. Obviously, GM3, CD9, α3, and β1 are neither oncogene nor anti-oncogene products. Therefore, genomic or transcriptomic analysis by itself has limited usefulness in molecular cell biology and oncology research. We are facing the task to look for the essential mechanism defining organization of a few crucial molecules in glycosynapse on one hand; on the other, to find out whether an epigenetic mechanism (58-61) can be applied for expression of the crucial molecules.

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FOOTNOTES

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1 The abbreviations used are: C/M, chloroform/ methanol; Csk, C-terminal Src kinase; co-IP, co-immunoprecipitation; FITC, fluorescein isothiocyanate; GEM, GSL-enriched microdomain; GSL, glycosphingolipid; HPTLC, high-performance thin-layer chromatography; PBS, phosphate-buffered saline (137 mM NaCl, 8.1 mM Na₂HPO₄, 2.68 mM KCl, 1.47 mM KH₂PO₄, pH 7.4); PLP, proteolipid; PNF, postnuclear fraction; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-d] pyrimidine; P4, D-threo-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TSP, tetraspanin.

FIGURE LEGENDS

Fig. 1. Comparison of expression of integrins, TSPs, and GSLs in highly-malignant YTS1 vs. less-malignant KK47 bladder cancer cell lines

Panel A: Western blot analysis of α3, α5, and β1 expression. Top: 20 μg protein of cell lysate prepared with RIPA buffer was analyzed in triplicate. The membranes were rebotted with anti-β-actin antibody after stripping. Bottom: Scion image densitometry analysis of each band, normalized with actin level.

Panel B: Western blot analysis of CD9 and CD82 expression as described in Panel A. CD82-expressing HCV29 cells were used as positive control for CD82.
Panel C: HPTLC analysis of gangliosides and other GSLs. GSLs were extracted with C/M. Phospholipids were removed by alkaline treatment, and GSLs from equal cell number (~2x10^7 cells) were loaded in each lane on HPTLC plate. After developing, GSLs were visualized by orcinol staining as described in M&M.

Panel D: Top: Western blot analysis of α3 and CD9 levels in PNF, and in Fr. 4, 5, 6, 10, 11, 12 separated by sucrose density gradient ultracentrifugation from ~4x10^7 cells. ~2.5 μg protein of each fraction and 6 μg protein of PNF were analyzed. Bottom: HPTLC analysis of GM3 and GM2 in GEM. Fr. 4-6 and Fr. 10-12 were combined, respectively. GSLs were prepared using C18 columns, analyzed by HPTLC, and visualized by orcinol staining as described in M&M.

Fig. 2. Association of α3, α5, and β1 with CD9 in YTS1 vs. KK47 cells
Panel A: Different levels of α3/CD9, α5/CD9 and β1/CD9 association in YTS1 vs. KK47. PNF of YTS1 or KK47 was IP'd with α3, α5, or β1. Level of CD9 IP'd with α3, α5, or β1 was determined by Western blot (WB) with anti-CD9 antibody. As control, PNF was IP'd with CD9 and Western blotted with anti-CD9.
Panel B: Different level of α3/CD9 association in YTS1 vs. KK47 studied by confocal microscopy. The cells were double-stained with anti-α3 (green) and anti-CD9 (red). In KK47, α3 was co-localized with CD9 (yellow) more extensively than in YTS1.

Fig. 3. Phagokinetic cell motility of YTS1 vs. KK47 cells, on gold sol-coated plate
Panel A: Motility tracks of YTS1 and KK47. Cells were seeded on gold sol-coated plates and incubated at 37 °C for 16 h. Representative photos are shown.
Panel B: Cleared area on gold sol-coated plate produced by cell movement as shown in Panel A were calculated by Scion image program and indicated as square pixels. Motility tracks of 20 cells in each well were analyzed.

Fig. 4. Knockdown of CD9 expression by RNAi, and effect of the knockdown on cell motility. KK47 cells were transfected with CD9 specific oligonucleotide or a scrambled control oligonucleotide in pSUPER, together with pPUR. Transfectants were cloned after puromycin selection and analyzed as described in M&M. Four knockdown clones (B201, B202, B254, B255) were compared to control clone (S17)
Panel A: CD9 mRNA level measured by RT-PCR. Reduced level of CD9 mRNA in four knockdown clones compared to control clone. As control, mRNA level of actin was measured by RT-PCR.
Panel B: Flow cytometry analysis of CD9 expression at the cell surface of each transfectant. Expression was reduced in four knockdown clones (shades lines) compared to control clone (solid line).
Panel C: Left: Western blot analysis of CD9 level in four knockdown clones compared to control clone. β-actin was rebotted as control after stripping. Right: Scion image densitometry analysis of each band, normalized with actin level.
Panel D: α3, β1, and GM3 expression in four CD9 knockdown clones compared to control clone. α3 and β1 levels were measured by Western blot. GM3 level was revealed by orcinol staining and DH2 immunostaining.
Panel E: Phagokinetic cell motility of four CD9 knockdown clones compared to control clone. Cleared areas on gold sol-coated plate produced by cell movement were calculated by Scion image program and indicated as square pixels as described in M&M.

Fig. 5. Effect of GM3 depletion on α3-to-CD9 interaction and motility of KK47 cells
Panel A: GSLs extracted from control and P4-treated KK47, revealed by HPTLC with orcinol staining as described in M&M.
Panel B: Reduced interaction of α3 and CD9 assessed by co-IP in P4-treated KK47. Top: Cell lysates were IP'd with anti-α3 or anti-CD9 as control, and analyzed by Western blot with anti-CD9. Bottom: Densitometry (Scion image) of α3 band co-IP'd with CD9 in P4-nontreated and -treated KK47. Values are expressed in arbitrary units.
Panel C: Confocal microscopic analysis of α3 and CD9 localization. Reduced co-localization (shown by reduced merge image) of α3 and CD9 in P4-treated KK47.

Panel D: Effect of P4 treatment on cell motility was analyzed on gold sol-coated plate as described in M&M. Cell motility increase in P4-treated KK47 is significant at p<0.001.

Fig. 6. Effect of exogenous GM3 or GM1 addition on α3-to-CD9 interaction and motility of YTS1 cells

Panel A: GM3 or GM1 expression on cell surface of YTS1 after incubation with GM3 or GM1, analyzed by flow cytometry using DH2 and FITC-labeled anti-mouse IgG (for GM3), or FITC-labeled cholera toxin subunit B (for GM1). Dotted line, 0 µM. Solid line, 10 µM. Shaded area, 50 µM.

Panel B: Enhanced interaction of α3 and CD9 assessed by co-IP was observed only in YTS1 incubated with 50 µM GM3. Left: Cell lysates were IP'd with anti-α3 or anti-CD9 as control, and analyzed by Western blot with anti-CD9. Right: Densitometry (Scion image) of α3 band co-IP'd with CD9 in control and GM3- or GM1-treated YTS1. Values are expressed in arbitrary units.

Panel C: Confocal microscopic analysis of α3 and CD9 localization. Enhanced co-localization (shown by increased merge image) of α3 and CD9 was observed in YTS1 incubated with 50 µM GM3, but not with 50 µM GM1.

Panel D: Effect of exogenous addition of GM3 on cell motility was analyzed on gold sol-coated plate as described in M&M. Cell motility decreased significantly in YTS1 incubated with 50 or 20 µM GM3 (p<0.001).

Panel E: Effect of exogenous addition of 50 µM GM1, as compared to 50 µM GM3, on cell motility was analyzed as in Panel D.

Fig. 7. Comparison of expression of cSrc, Csk, and cSrc phosphorylated at Tyr416 or Tyr527, in YTS1 vs. KK47 cells

Panel A: Western blot analysis of cSrc, activated cSrc (P-Src, Tyr416), inactivated cSrc (P-Src, Tyr527), and Csk. 20 µg protein of PNF was analyzed. The membranes were reblotted with anti-γ-tubulin antibody after stripping.

Panel B: Western blot analysis of cSrc and Csk in Fr. 4, 5, 6, 10, 11, 12, and PNF separated by sucrose density gradient ultracentrifugation from 4x10^7 cells. ~2.5 µg protein of each fraction and 6 µg protein of PNF were analyzed.

Fig. 8. Effect of GM3 depletion (KK47) or addition (YTS1) on cSrc activation or inhibition, and inhibitory effect of Src tyrosine kinase inhibitor PP2 on YTS1 cell motility

Panel A: Effect of GM3 depletion by P4 in KK47 cells on cSrc phosphorylation status and Csk in PNF, analyzed by Western blot. Left: Enhanced Tyr416 phosphorylation and decreased Tyr527 phosphorylation in P4-treated KK47. Right: P-Src (Tyr416)/ P-Src (Tyr527) ratio calculated from densitometric analysis (Scion image) of each band.

Panel B: Effect of exogenous GM3 addition to YTS1 cells on cSrc phosphorylation status and Csk localization. Cells were incubated with GM3 for 1, 3, or 16 h, harvested, and separated into PNF, low-density Fr. 4-6, and high-density Fr. 10-12, as described in M&M. Aliquots of PNF, low-, and high-density fractions were analyzed by Western blot. Note that Csk translocation to Fr. 4-6 was maximal at 3 h after GM3 addition, associated with maximal phosphorylation at Tyr527. Thereafter, activated cSrc signal (Tyr416 phosphorylation) declined significantly.

Panel C: Inhibitory effect of Src family kinase inhibitor PP2 on YTS1 cell motility. Phagokinetic motility of cells pretreated with PP2 at 5, 10, or 20 µM concentration was compared to that of non-treated cells. Motility was decreased by PP2 in a dose-dependent manner, confirming involvement of cSrc activation in enhanced YTS1 cell motility.
Figure 1A

**A**

|    | YTS1 | KK47 |
|----|------|------|
| α3 | ![Image](image1.png) | ![Image](image2.png) |
| actin | ![Image](image3.png) | ![Image](image4.png) |

P = 0.391

|    | YTS1 | KK47 |
|----|------|------|
| α5 | ![Image](image5.png) | ![Image](image6.png) |
| actin | ![Image](image7.png) | ![Image](image8.png) |

P = 0.080

|    | YTS1 | KK47 |
|----|------|------|
| β1 | ![Image](image9.png) | ![Image](image10.png) |
| actin | ![Image](image11.png) | ![Image](image12.png) |

P = 0.191
Mitsuzuka, Fig. 1B

B

|        | YTS1 | KK47 |
|--------|------|------|
| CD9    |      |      |
| actin  |      |      |

P = 0.369

|        | YTS1 | KK47 |
|--------|------|------|
| CD82   |      |      |
| actin  |      |      |

Downloaded from http://www.jbc.org/ by guest on 3/27/20
Mitsuzuka, Fig. 2

A

| IP  | YTS1 | KK47 |
|-----|------|------|
| α3  |      |      |
| α5  |      |      |
| β1  |      |      |
| CD9 |      |      |

B

| α3 | CD9 | merge |
|----|-----|-------|
| YTS1 |      |       |
| KK47 |      |       |
Mitsuzuka, Fig. 3

A

YTS1  
KK47

B

square pixels ($\times 10^{-3}$)

|       | YTS1 | KK47 |
|-------|------|------|
|       | 12.5 | 2.5  |

$p < 0.001$
Mitsuzuka, Fig. 4A,B,C

A

|       | S17 | B201 | B202 | B254 | B255 |
|-------|-----|------|------|------|------|
| CD9   |     |      |      |      |      |
| β-actin |    |      |      |      |      |

B

Cell number

Fluorescence intensity

C

|       | S17 | B201 | B202 | B254 | B255 |
|-------|-----|------|------|------|------|
| CD9   |     |      |      |      |      |
| β-actin |    |      |      |      |      |

Intensity (Arbitrary units)
Mitsuzuka, Fig. 4D, E

D

\[ \beta\text{-actin} \]

\[ \alpha_3 \]

\[ \beta\text{-actin} \]

\[ \beta_1 \]

\[ \beta\text{-actin} \]

GM3

orcinol

DH2

Western blot

E

TLC

\[ \text{square pixels (x}10^{-3}\text{)} \]

S17 B201 B202 B254 B255

\[ p < 0.001 \]

\[ p < 0.01 \]
A

B

Mitsuzuka, Fig. 5A,B

A

B

GM3

GM2

IP

α3

CD9

CD9

WB

- P4   + P4

- P4   + P4

Intensity (arbitrary units)

- P4   + P4
Mitsuzuka, Fig. 5C,D

C

\( \alpha_3 \)  CD9  merge

- P4

+ P4

D

|          | square pixels \( \times 10^{-3} \) |
|----------|----------------------------------|
| - P4     | ![Bar Graph](http://www.jbc.org) |
| + P4     | ![Bar Graph](http://www.jbc.org) |

p \(< 0.001 \)
Mitsuzuka, Fig. 6A,B

A

Cell number

Fluorescence intensity

GM3

0 μM

10 μM

50 μM

GM1

0 μM

50 μM

B

IP

α3

CD9

Control   GM3   GM1

WB

CD9

Intensity (arbitrary units)

Control   GM3   GM1
Mitsuzuka, Fig. 6D,E

D

E

square pixels ($\times 10^{-3}$)

square pixels ($\times 10^{-3}$)

GM3 (µM)

Control  GM3     GM1

p < 0.001

p < 0.01

p < 0.001  p < 0.001
Mitsuzuka, Fig. 7A,B

A

- cSrc
- P-Src (Tyr416)
- P-Src (Tyr527)
- Csk
- tubulin

YTS1  KK47

B

| Fraction # | YTS1 | KK47 |
|------------|------|------|
| 4          |      |      |
| 5          |      |      |
| 6          |      |      |
| 10         |      |      |
| 11         |      |      |
| 12         |      |      |
| PNF        |      |      |

- cSrc
- Csk
A

| Protein          | cSrc | P-Src (Tyr416) | P-Src (Tyr527) | Csk | Tubulin |
|------------------|------|---------------|---------------|-----|---------|
|          | ![cSrc](image) | ![P-Src (Tyr416)](image) | ![P-Src (Tyr527)](image) | ![Csk](image) | ![tubulin](image) |

![Intensity Graph](image)

Mitsuzuka, Fig. 8A
B

|          | PNF | Fr. 4-6 | Fr. 10-12 |
|----------|-----|---------|-----------|
| cSrc     |     |         |           |
| P-Src (Tyr416) |     |         |           |
| P-Src (Tyr527) |     |         |           |
| Csk      |     |         |           |
| control  | 1   | 3  16 h | GM3       |
| GM3      |     |         | control   |
| GM3      |     |         | GM3       |

C

![Graph showing square pixels (x10^3) for PP2 (μM)]

- p < 0.001
- p < 0.001
- p < 0.05

Mitsuzuka, Fig. 8B,C
A specific microdomain ("Glycosynapse 3") controls phenotypic conversion and reversion of bladder cancer cells through GM3-mediated interaction of α3β1 integrin with CD9

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