Review

Organization and function of glycosphingolipids in membrane

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Abstract: We have characterized novel glycosphingolipids (GSLs) of antigenic or functional importance, including type 3 and type 4 blood group ABH antigens, globo-series gangliosides, sialosyl dimeric Le^x, myeloglycan, β1-4GalNAc disialyl-Lc_4Cer, etc. Many GSLs have been identified as developmentally-regulated, tumor-associated antigens, suggesting their role in defining stage of development, and tumor cell phenotype. Out of the many types of GSLs, relatively few have been studied and shown to control cellular functions. Our studies indicate that functional effects of these GSLs are based on their interaction with specific target molecules in membrane, including (i) signal transducers (*e.g.*, cSrc, Src family kinases, small G-proteins), to initiate signal transduction; (ii) integrin receptors (*e.g.*, $\alpha 3\beta 1$), to modulate cell adhesion and motility; (iii) growth factor receptors (*e.g.*, for FGF, EGF), to modulate cell growth; (iv) tetraspanins (*e.g.*, proteolipid CD9, CD81, CD82), to affect complex formation with integrin or with growth factor receptor; (v) GSL itself, through GSL-to-GSL interaction; (vi) microbial "adhesin". In many cases, these GSL interactions take place through GSL clusters at GSL-enriched microdomain (GEM). Some GEM show properties similar to those of "lipid rafts", whereas others, particularly those highly enriched in proteolipid/tetraspanin and involved in cell adhesion and cell growth, are distinguishable from "lipid rafts" since they are independent of cholesterol but are non-resistant to (soluble in) 1% Triton X-100. Such microdomains, showing GSL-dependent or -modulated cell adhesion and growth, are termed "glycosynapse". Further studies on GSL structure and function through glycosynapse will help clarify cell social behavior and various disease processes based on malfunction of cellular interaction, or of adhesion with concurrent signaling.

Key words: Growth factor receptor; integrin receptor; tetraspanin; microdomain; signal transduction; carbohydrate-to-carbohydrate interaction; oncofetal antigen; phenotypic reversion; tyrosine kinase.

Introduction: Glycosphingolipids with novel structures and biological implications. When I started learning about the world of glycosphingolipids (GSLs), only a few were known: cerebrosides (discovered by J. L. W. Thudichum), sulfatide (established by G. Blix), gangliosides and lactosylceramide (discovered by E. Klenk), hematoside (discovered by T. Yamakawa), and globoside (presence indicated by E. Klenk; composition and structure elaborated, and so named, by T. Yamakawa).

I followed the work of these pioneers. Studies were enormously difficult in the late 1950s to 1960s, since methods of isolation and characterization were not fully developed. We were involved in improvement of isolation procedures, sequence analysis using exoglycosidases (thanks to the help of Yu-teh Li), and determination of carbohydrate sequence and linkage analysis by mass spectrometry of permethylated derivatives (I spent some time in Bengt Lindberg's lab). We overcame various difficulties step by step. Thus, novel GSLs were isolated, and their structures characterized, in our lab. In

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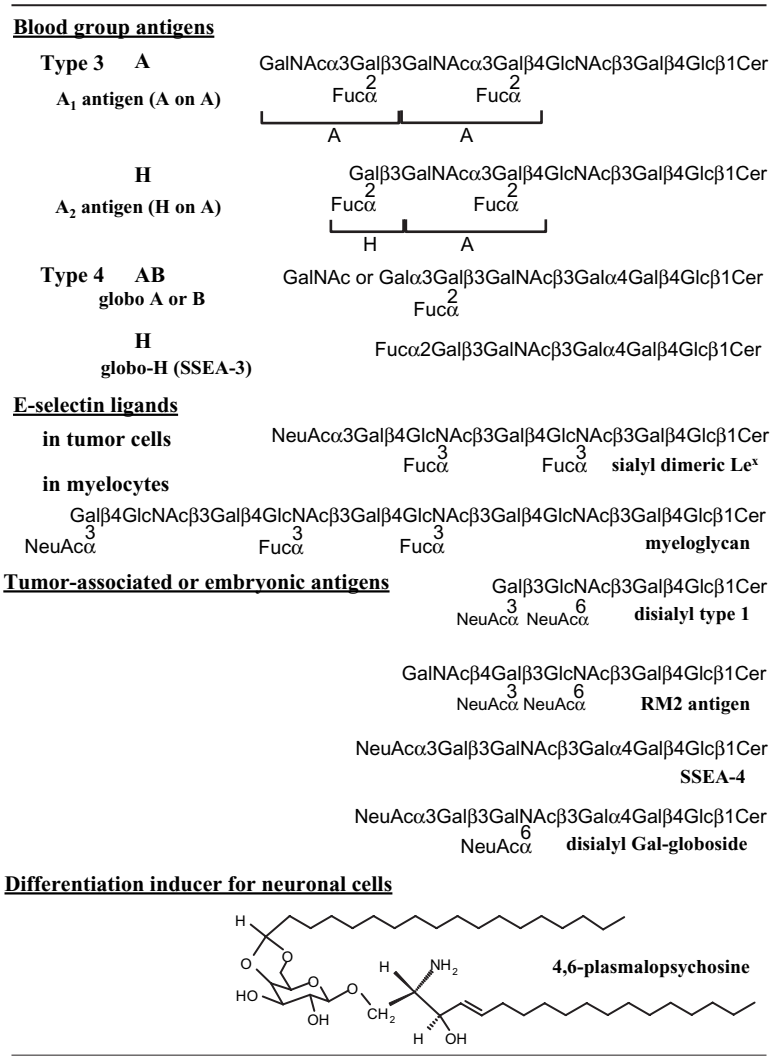
Abbreviations used: Cer, ceramide; ECM, extracellular matrix; EGF(R), epidermal growth factor (receptor); FGF(R), fibroblast growth factor (receptor); FN, fibronectin; Gal, galactose; GFR, growth factor receptor; GSL, glycosphingolipid; LN, laminin; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PL, proteolipid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SPG, sialosylparagloboside (sialyl-lactoneotetraosylceramide); TSP, tetraspanin.

Table I. Novel GSL structures established by our research group



my previous review, ~25 new structures were listed.[1] Here, in Table I, I list several novel structures of antigenic or functional importance. Blood group ABH antigens are known to be carried by type 1 (Galβ1-3GlcNAcβ1-3Galβ1R) or type 2 (Galβ1-4GlcNAcβ1-3Galβ1R) with various chain lengths and degrees of branching.[2]

More recently, novel carriers showing different distribution patterns were discovered. Type 3 chain A or H are A-on-A (A_1-specific antigen)[3] and H-on-A (A_2-specific antigen)[4] respectively. Type 4 chain A or B are those carried by Gb5. Type 4 chain H is α1-2FucGb5, *i.e.*, globo-H. Both type 3 and type 4 chain ABH antigens are found exclusively as GSLs, not as glycoproteins. Sialyl-Le^x (SLe^x) and sialyl-dimeric Le^x, which bind strongly to E-selectin,[5] are accumulated in various human cancers,[6] and provide a basis for tumor cell metastasis.[7] It was expected that SLe^x epitope might also play a role in neutrophil recruitment during inflammatory response. Surprisingly, however, neither SLe^x nor sialyl-dimeric Le^x are found in human neutrophils or myelogenous leukemia HL60 cells — rather, these cells highly express unbranched, long-chain polylactosamines having terminal 2-3 sialylation and internal fucosylation.[8] These are collectively termed "myeloglycan", and play an important role in E-selectin binding under dynamic flow conditions.[9]

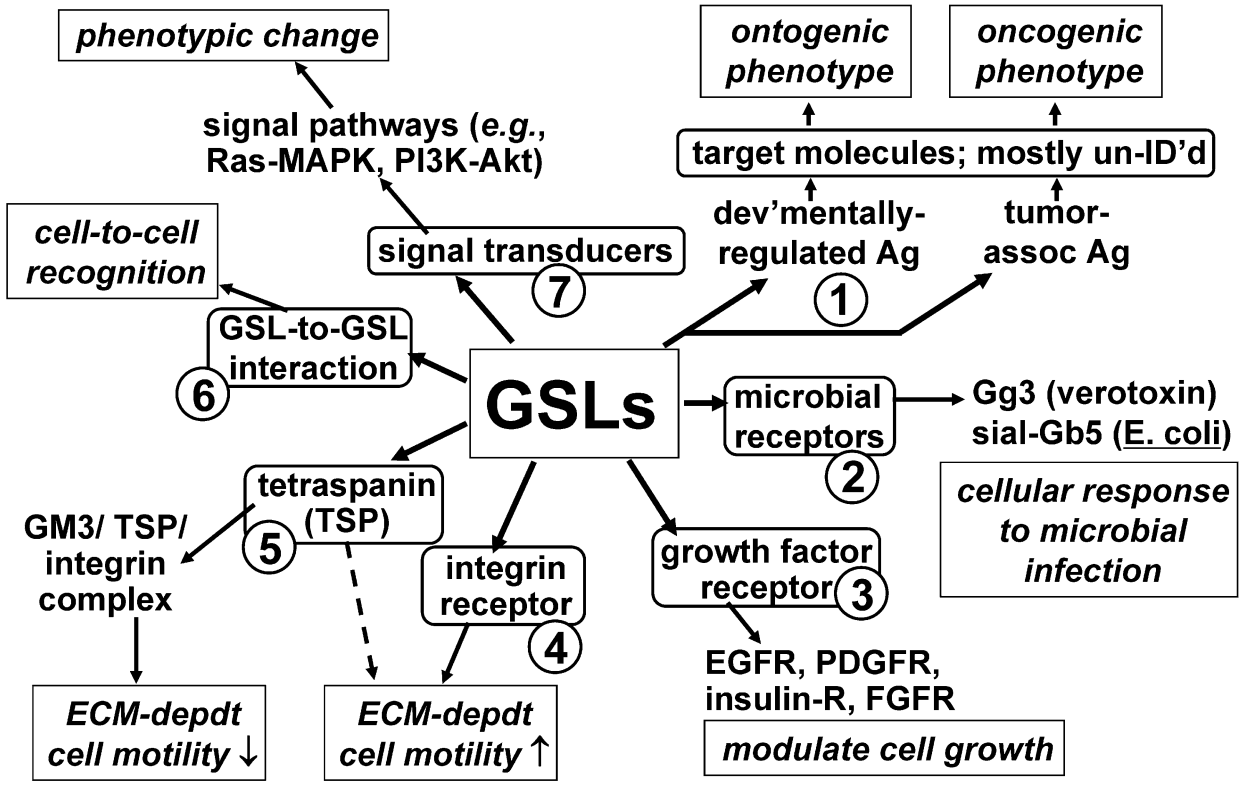


Fig. 1. Functional roles of GSLs, classified according to GSL interaction with target molecule. GSLs interact with bacterial toxins or "adhesins" (Process 2), with GFRs (Process 3), with integrin receptor (Process 4), or with integrin/TSP (PL) complex (Process 5). Cell adhesion is mediated through interaction of GSLs in interfacing microdomains (Process 6). GSLs interact with signal transducers in microdomains to initiate signal pathways (Process 7). Target molecules in Processes 2 through 7 are clear. However, various GSLs that display dramatic changes during ontogenic development or oncogenic transformation may interact with different target molecules at different stages of development or transformation to change cellular phenotype (Process 1). In most of these cases, the target molecule has not been identified.

Disialyl-Lc_4Cer, defined by mAb FH9,[10] was originally found in colorectal cancer. Its related structure β1-4GalNAc-disialyl-Lc_4Cer, defined by mAb RM2,[11] was originally found as renal cell carcinoma-associated antigen. We found recently that RM2 antigen is expressed highly in prostate cancer, but not in benign prostate hypertrophy or normal prostate tissue.[12]

Novel cationic GSLs having plasmal (myristal or palmital) conjugated with psychosine through 4,6 or 3,4 cyclic acetal linkage were found in brain white matter, and termed "plasmalopsychosine". 4,6-plasmalopsychosine displayed strong neurotrophic effect through activation of tyrosine kinase associated with Trk A (NGF receptor), and consequent long-term activation of Erk, inducing neuritogenesis.[13]

GSL structures, and their changes associated with biological functions, have been the central focus of our studies, since structural change is the starting point for understanding biological significance, and enzymatic/genetic mechanisms.

Functional interaction of GSLs through GSL-enriched microdomain. During the 1980s and 1990s, the following lines of study evolved on functional roles of GSLs, as summarized in Fig. 1. These studies were greatly accelerated by application of monoclonal antibody (mAb) approach and by cloning of key glycosyltransferase genes. (i) GSLs were identified as cell type-specific, developmentally-regulated antigens, as well as tumor-associated antigens, suggesting that they play essential roles in defining differentiation, development, and oncogenesis[14] — although target molecules with which GSLs interact during these processes have not been clearly identified. (ii) GSLs were identified as receptors for bacterial toxins, or shown to mediate

interaction of microbes with host cells during infectious processes.[15] (iii) GSLs modulate function of growth factor receptors (GFRs) through their effect on receptor-associated tyrosine kinase.[16] (iv) GSLs modulate function of integrin receptors, particularly through complex formation with TSPs.[17] (v) GSLs directly mediate cell-to-cell adhesion through GSL-to-GSL *trans* interaction (mainly carbohydrate-to-carbohydrate interaction).[18] (vi) GSLs associate directly or indirectly with major signal transducers such as cSrc, Src family kinases, small G-proteins (RhoA, Ras, Cas), or Fak, and thereby activate or inhibit signal transduction pathways through change of GSL organization in membrane.[19),20]

Recent studies indicate that GSL functions, categorized as 1 through 7 in Fig. 1, are based on interaction with specific target molecules present within a certain type of GSL-enriched microdomain (GEM) (see below).

The cell surface membrane does not consist of homogeneous lipid bilayer as proposed in the classic Singer/Nicolson model.[21] Rather, it may have multiple, physically-distinguishable "plates" constituted by phospholipids having fatty acids with different degrees of unsaturation.[22] It was further proposed that plates consisting of sphingolipid may be distinguishable from those consisting of phospholipid, since sphingolipid has CONH or OH group which provides hydrogen bond donor as well as acceptor, whereas phospholipid has no such group and acts only as hydrogen bond acceptor.[23] These ideas were based on conformations of sphingolipid structures, but no clear supporting data from cell membranes were available. The concept that plasma membrane consists of heterogeneous GSL assemblies was first introduced as an "annular model" of GSLs.[24),25] According to this model, GSLs are arranged in annular (ring-like) fashion surrounding micropores or membrane proteins.[25]

We found originally that GSLs are enriched at cell adhesion sites as detergent-insoluble form, termed "detergent-insoluble matrix (DIM)".[26] This is assumed to correspond to clustered form of GSL, as observed by scanning electron microscopy of "freeze-fractured" cell surface membrane,[27] or of GSL-phospholipid liposome membrane without cholesterol.[28]

Based on these and many other studies, the microdomain concept has developed slowly but steadily during the past 20 years. It was greatly advanced by analysis of caveolae or caveolar membrane,[29] proposal of "lipid raft",[30] and detailed documentation of physical properties of "detergent-resistant membrane (DRM)".[31] Some GEM display properties similar to those of lipid raft or "caveolar membrane", which are regarded as constantly-moving, signaling platforms involved in endo- or exocytosis, but not involved in cell adhesion, and are characterized by: (i) high dependence on cholesterol, *i.e.*, disruption by cholesterol-binding reagent; (ii) resistance to 1% Triton X-100. The diameter of lipid raft was estimated as 50-100 nm, sometimes as small as 10 nm.[32),33]

In contrast, other types of GEM involved in GSL-dependent or GSL-modulated cell adhesion and cell growth, particularly those highly enriched in PL/TSP, are distinguishable from lipid raft or caveolar membrane since they are independent of cholesterol but are non-resistant to (soluble in) 1% Triton X-100. Such GEM, are termed "glycosynapses"[17),34] (see Fig. 2A), in analogy to "immunological synapse" that controls immunocyte adhesion to T-cell receptor, and antigen presentation.[35),36] GSL clusters at adhesion site, corresponding to glycosynapses, can be seen by light microscopy with fluorescent immunostaining, and their size is at least 1 μm. Contrasting properties of lipid rafts vs. glycosynapses are summarized in Table II.

Our recent studies suggest that glycosynapses control adhesion-dependent cell growth, differentiation, and development, as well as oncogenic transformation and reversion (see "Expression of oncogenic phenotype, and its reversion...", p. 198).

GSLs as tumor-associated, and developmentally-regulated, antigens (Process 1 in Fig. 1). *Tumor-associated antigens*. Tumor cells are generally characterized by (i) down-regulated expression, or depletion, of a defined GSL, and (ii) accumulation of a defined GSL, recognized as a "tumor-associated antigen". The process (ii) results from precursor accumulation or activation of neosynthesis.[41]

Our original studies on accumulation of Le^x pentasaccharide Cer,[42] dimeric or trimeric Le^x in colorectal cancer,[43] and Gg3 in KIMSV tumors in Balb/c mice,[44] were subsequently extended using the mAb approach. This led to discovery of many tumor-associated GSL antigens, *e.g.*, Gb3 in Burkitt lymphoma[45]; GD3 in melanoma[46]; sialyl-Le^x and sialyl-dimeric-Le^x,[6] disialyl-Lc_4,[10] and trifucosyl-Le^y[47] in colorectal cancer; globo-H in breast cancer[48]; and β4GalNAc disialyl-Lc_4 in renal cell cancer.[11] These and other antigens have been utilized for diagnosis and treatment of human cancers. Of special interest, immunization with synthetic globo-H/KLH conjugate significantly suppressed prostate cancer progression.[49] This research area has been reviewed

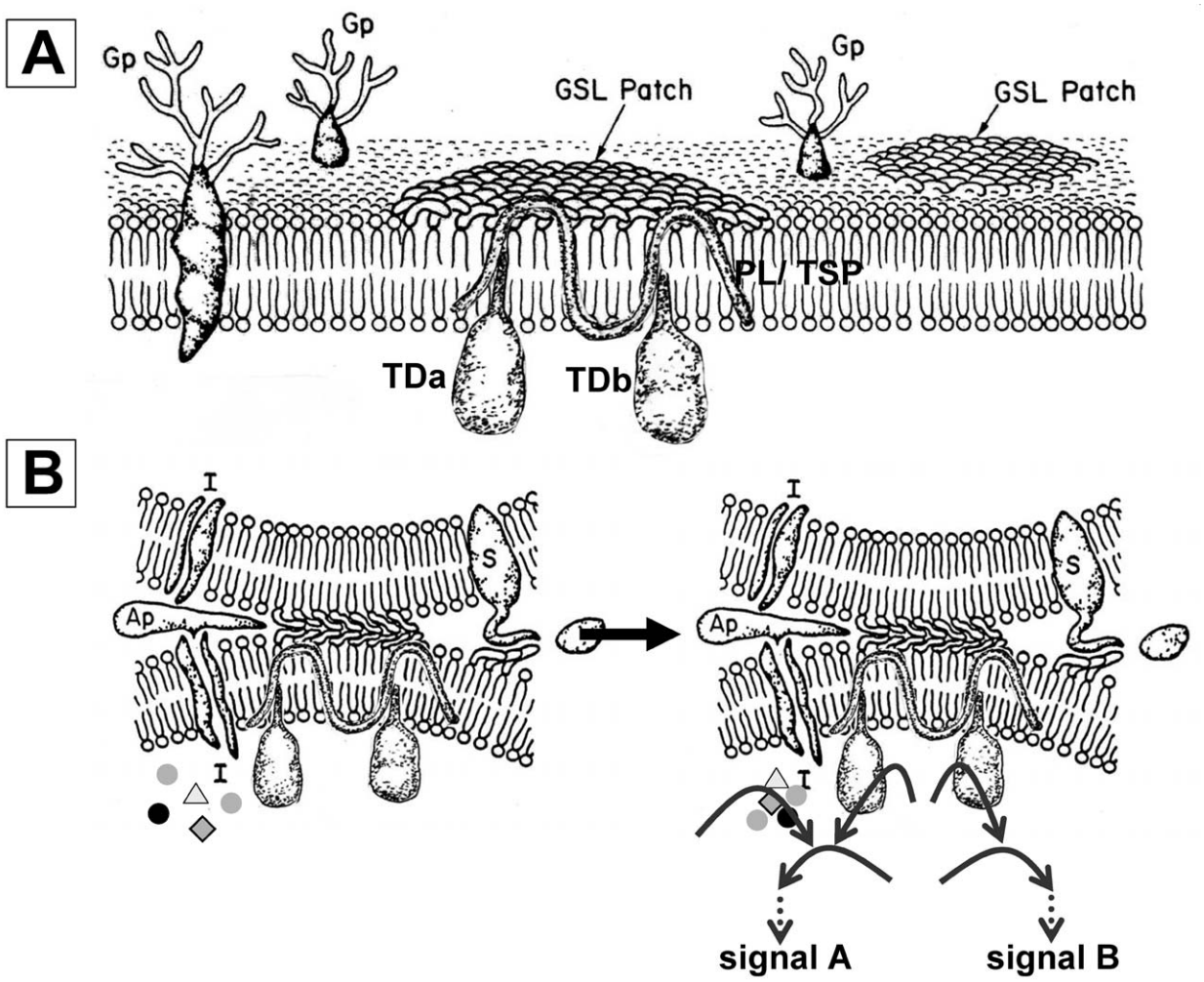


Fig. 2. Essential components of GSL-enriched microdomain (GEM) at the cell surface. **Panel A**: GSLs at the cell surface interact with each other through hydrogen bonds to form clusters, and interact with PL/TSP and signal transducers (TDa, TDb) having acyl group, to form GEM. **Panel B**: GEM as in Panel A, interfacing between two adjacent cells, mediates cell-to-cell adhesion (**left**). The adhesion process induces activation of TDa or TDb, in terms of tyrosine phosphorylation or GTP binding, to initiate signal pathway (**right**). GEM containing high level of PL/TSP, involved in GSL-mediated cell adhesion, is termed "glycosynapse", and is distinguishable from moving, signaling platform ("raft") (see text). Cell adhesion through GSL-to-GSL interaction at GEM occurs in synergy with adhesion process based on interaction of integrin (I) with extracellular adhesive protein (Ap) such as FN or LN,[96] or on sugar-binding protein (S).

repeatedly (*e.g.*, Refs. 50, 51).

Developmentally-regulated GSL antigens. The process of development consists of a series of stage-specific cell adhesion events, which may generate signaling to define phenotypic change. In mouse embryogenesis, the first adhesion event, termed "compaction", is mediated by cooperative effect between Le^x-to-Le^x interaction[52),53)] and E-cadherin.[54)] A model of compaction was provided by autoaggregation of mouse teratocarcinoma F9 cells, which is also mediated by cooperative effect between Le^x and E-cadherin.[55)] Mice with knockout of E-cadherin alone,[56)] or FucT-IX alone,[57)] exhibit normal compaction. FucT-IX may control synthesis of stage-specific embryonic antigen-1 (SSEA-1), which is Le^x carried by long-chain poly-LacNAc.[58)] Other types of Le^x (carried by short-chain or branched-chain LacNAc) are not controlled by FucT-IX, rather by FucT-IV. Therefore, compaction may not be blocked unless the three genes for FucT-IV, -IX, and E-cadherin are knocked out simultaneously.

Table II. Contrasting properties of "lipid raft" vs. glycosynapse

	Lipid raft[a]	Glycosynapes[b]
General functional concept	constantly-moving signaling platform	cell adhesion/motility with concurrent signaling to affect cellular phenotype
Exo-/endocytosis	involved	not involved
Cell adhesion	not involved	involved
GSL/ganglioside	++	++
Cholesterol dependence[c]	++	±
Size (diameter)	10-100 nm	500-1000 nm
Tetraspanin proteolipid (CD9, CD81, CD82)	–[d]	++
Integrin receptor	–	++
Growth factor receptor	– or +	++
Resistance to 1% Triton X-100	++	–
cSrc, Src family, small G-protein	++	++

[a] Refs 30, 37; [b] Refs 17, 20, 38-40; [c] structure and function are disrupted by cholesterol-binding reagents (β-cyclodextrin, filipin); [d] levels are low or undetectable (not well studied)

In primates (and presumably humans), compaction may be mediated by Gb4 and SSEA-3/4 (extended globo) in cooperation with E-cadherin, based on studies of the mechanism for autoaggregation of human teratocarcinoma 2102 or TERA-2. Several lines of evidence indicate that 2102 autoaggregation, mediated by Gb4, Gb5, and nLc_4, induces signaling to activate transcription factors AP-1 and CREB.[59)]

Roles of GSLs in later stages of cell adhesion and development have not been studied. In GlcCer synthase gene (*Ugcg* gene) knockout mouse, embryogenesis proceeded normally until formation of primitive germ layers, but further development into mesoderm, endoderm, and ectoderm was halted,[60)] indicating an essential role of GSLs in developmental processes.

Functional role of oncodevelopmental antigens. Only a few target molecules of these antigens are known. Le^x and its analogues Gb4 and Gb5, expressed in early embryogenesis, are adhesion molecules that induce differentiation, as described above. Among tumor antigens, only SLe^x and SLe^a are known to mediate tumor cell adhesion to activated microvascular endothelial cells expressing E-selectin,[5),61),62)] thereby promoting blood-borne tumor metastasis.[7)] Several other antigens, *e.g.*, Le^y and sialyl-Tn, are well known to inhibit 5-year survival rate of patients (for review see Ref. 51) but their target molecules and mechanisms to enhance invasiveness are not clear. Depletion or down-regulation of GM3 in many transformed cells and human tumors causes enhanced motility and invasiveness, through the mechanism described in "Expression of oncogenic phenotype, and its reversion..." (p. 198).

GSLs as microbial receptors (Process 2 in Fig. 1). Our group has made little contribution in this area, except for our (i) identification of Shigella toxin receptor as Gb3, in collaboration with G.T. Keusch's group,[63)] and (ii) characterization of receptor for *E. coli* infection in urogenital epithelia as 2-3 sialyl-Gb5, in collaboration with A. Stapleton and W. E. Stamm. Sialyl-Gb5 is expressed only in blood group A/B non-secretor urogenital epithelia.[64)] Secretor urogenital epithelia express globo-A or -B (Table I). Three clinical isolates of pathogenic *E. coli*, having different types of "adhesin", showed consistently higher binding affinity to 2-3 sialyl-Gb5 (SGb5) than to other globo-series structures (Figs. 3A, B). This provides a rationale for the finding that non-secretor women have much higher incidence of recurrent *E. coli* infection than secretors. *E. coli* infectivity to vaginal epithelial cells was abolished by treatment of cells with PDMP, which greatly suppresses synthesis of all GSLs. Interestingly, carrying of A/B epitope by globo-series structure is unique to urogenital epithelia. In other types of epithelia (bronchopulmonary, gastrointestinal, salivary), A/B epitope is carried by lacto-series structure.

Ganglioside effect on GFR function (Process 3 in Fig. 1). Many growth factors and their receptors (GFRs) are characterized by the presence of cytoplasmic

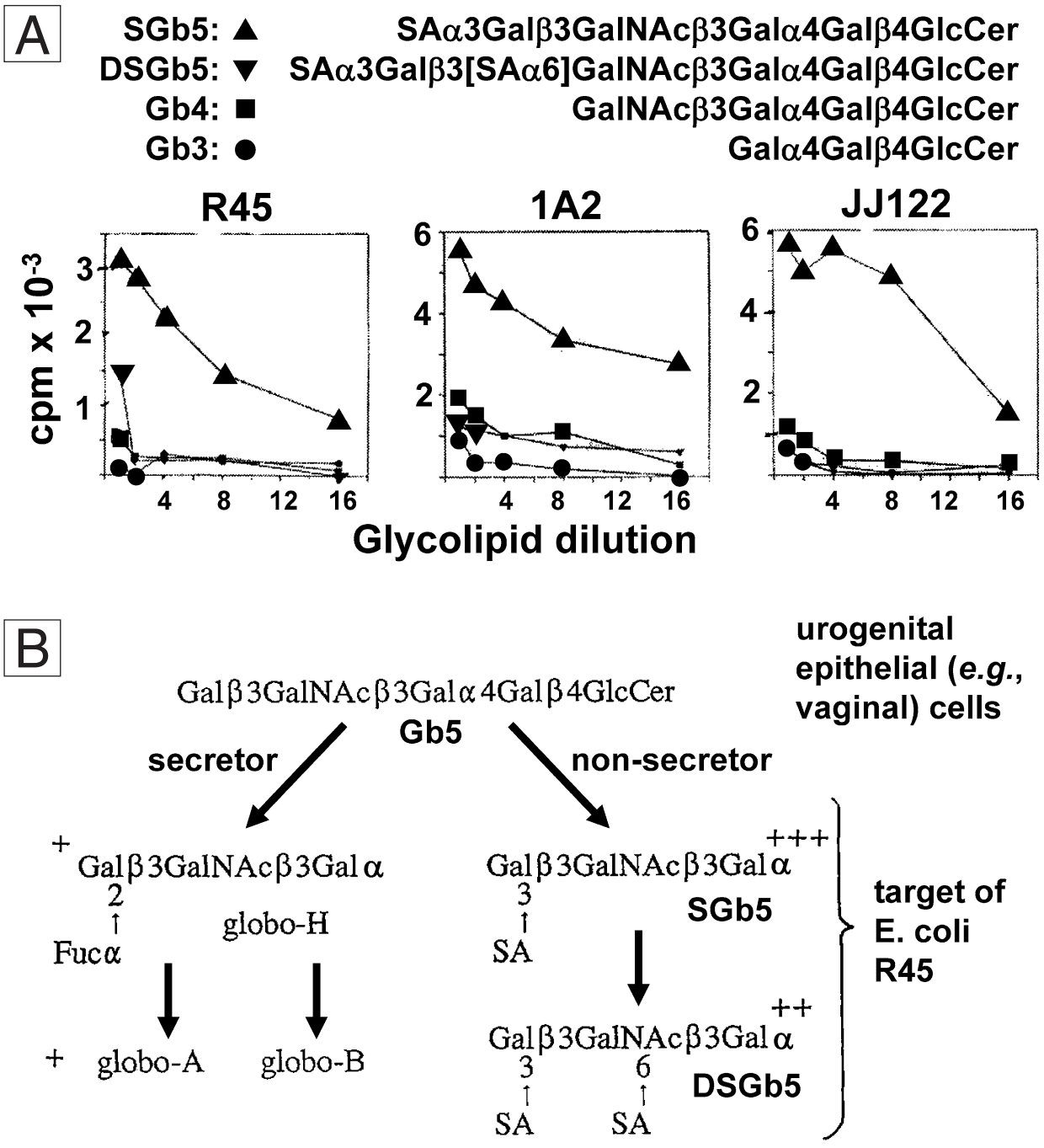


Fig. 3. Globo-series structures as receptors for *E. coli* infection in urogenital epithelia. **Panel A**: The infection process depends on types of "adhesin", at fimbriae of *E. coli*, that bind to host cell GSLs. Blood group non-secretor women have a high rate of recurrent *E. coli* infection at urogenital epithelia, causing urethritis or cystitis. Such infection is based on the binding ability of *E. coli* to 2-3 sialyl-Gb5 (SGb5) expressed at urogenital epithelia. Three pathogenic *E. coli* clinical isolates (R45, 1A2, JJ122) showed higher binding affinity to SGb5 than to other globo-series structures. This property is common to all pathogenic isolates. **Panel B**: Globo-series GSLs expressed in secretor vs. non-secretor epithelia. In secretor women, GalGb4 (Gb5) is converted to globo-H, which is further converted to blood group globo-A or globo-B present in urogenital secretions (**left**). In contrast, in non-secretor women, GalGb4 is converted to SGb5 or DSGb5, which are targets of *E. coli* infection (**right**). Results of a collaborative study with A. Stapleton and W. E. Stamm (Dept. of Medicine, Div. of Allergy & Infectious Diseases, Univ. of Washington).

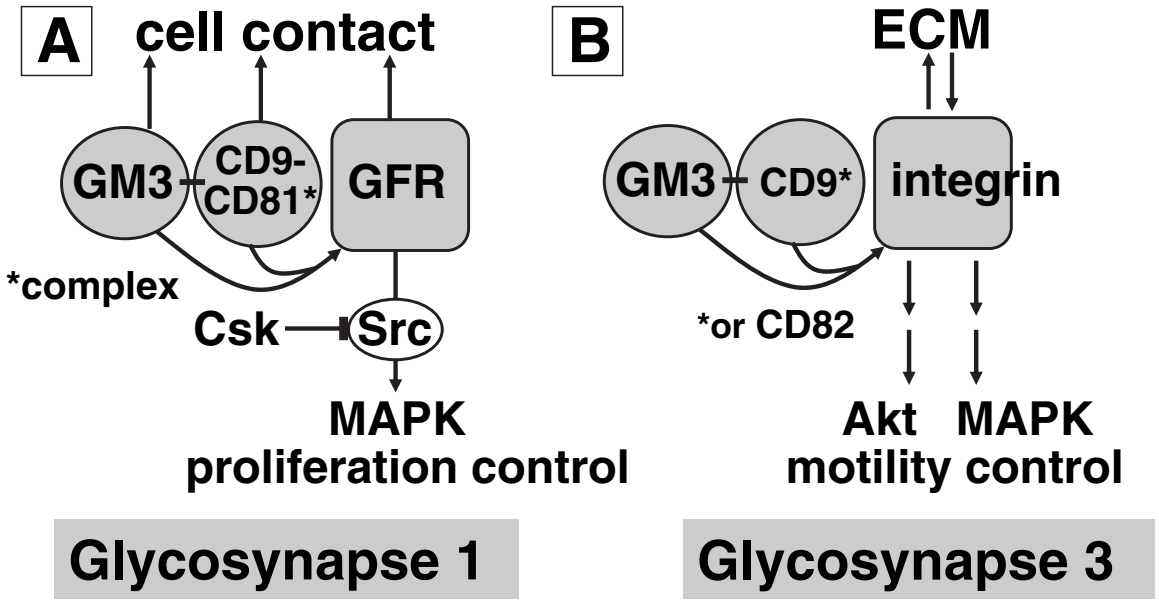


Fig. 4. Components and functions of two closely-related microdomains, glycosynapse 1 and glycosynapse 3. Components of glycosynapse 1 and glycosynapse 3 are similar, but function is very different. **Panel A**: Glycosynapse 1 major components are GM3, CD9-CD81, and GFR. Glycosynapse 1 may mediate interaction of cell with adjacent cell, and thereby control cell growth. Cell-to-cell interaction may occur via contact between GM3 and FGFR, or GM3 and CD9-CD81. Within such microdomain, (i) GM3/CD9 complex inhibits FGFR activation, and (ii) the presence of Csk inhibits cSrc. Processes i and ii together inhibit MAPK signaling, resulting in contact inhibition of cell growth.[39] **Panel B**: Glycosynapse 3 major components are GM3, CD9, and integrin $\alpha 3\beta 1$. ECM-mediated integrin activation through Akt/MAPK pathway is inhibited by GM3/CD9 complex that inhibits integrin function, leading to suppression of cell motility.[38),89]

tyrosine kinases which are activated by binding of ligand growth factor to its receptor.[65),66] A concept arose that cell proliferation is induced and regulated by a common mechanism based on cytoplasmic tyrosine phosphorylation (for review see Ref. 67).

In some non-transformed cells having high GM3 level, FGF-induced FGFR function[68] and FGFR tyrosine phosphorylation[39] are inhibited by GM3, whereas FGFR tyrosine phosphorylation in transformed cells is not inhibited by GM3.[39] Similarly, EGF-dependent tyrosine phosphorylation is inhibited by GM3.[69] In contrast, PDGF-induced tyrosine phosphorylation and cell growth are not inhibited by GM3, but are inhibited by gangliosides with more complex structures, such as GM1, GD1a, GD1b, and GT1b.[70),71]

The major question remains: how do gangliosides inhibit receptor-associated tyrosine kinase? The well-accepted hypothesis is that receptor-associated kinase is activated through receptor-to-receptor interaction that occurs upon ligand binding.[72] A reasonable assumption is that GM3 may inhibit such interaction. However, quantitative inhibition of receptor-to-receptor dimer formation by GM3 is not clear.[73] Results of such experiments depend greatly on quality of antibody to detect such dimer. Further studies are necessary.

Two factors modulate the effect of GM3 on EGF function. One is TSP (*e.g.*, CD9, CD81), which promotes the inhibitory effect of GM3 on FGFR.[39] Similarly, CD82 mediates interaction of ganglioside GD1a with EGFR, thereby inhibiting EGF-induced signaling.[74] These findings indicate that association of GFR with GSL clusters in GEM is promoted by TSP displaying PL properties (Fig. 4A). The other factor is N-glycosylation status of the receptor, as indicated by inhibition of binding of fluorescent "GM3 nanospheres" to EGFR at the cell surface when receptor N-glycosylation was blocked by tunicamycin.[75] EGFR tyrosine kinase activity in A431 cells was significantly reduced when cells were treated with N-glycosylation processing inhibitor N-methyl deoxynojirimycin or swainsonine, suggesting that conversion from high-mannose-type to complex-type receptor may inhibit interaction of GM3 with

EGFR (Hikita, T., Handa, K., Hakomori, S., unpubl. data).

Insulin-dependent cell growth in human lymphoid cell line IM9, and in promyelocytic leukemia cell line HL60 was inhibited strongly by 2-3 sialylparagloboside, weakly by GM3, and was not affected by 2-6 sialylparagloboside. The degree of cell growth inhibition was correlated with inhibition of tyrosine kinase associated with insulin receptor.[76)]

Mouse mammary carcinoma mutant cells FUA169 contain high level of GM3, whereas parental F28-7 cells contain high LacCer and no GM3. F28-7 cells display strong insulin-dependent cell growth, whereas FUA169 do not. GM3 strongly inhibited insulin-dependent growth of F28-7 cells.[77)] GM3 in mice, therefore, may regulate insulin-dependent metabolism. In fact, recent studies show that GM3 knockout mice display various symptoms of type 2 diabetes, due to loss of insulin resistance.[78)] Similarly, GM3 is accumulated in 3T3-L1 adipocytes when TNFα is added, whereby insulin resistance is induced.[79)]

Ganglioside effect on integrins and their complex with PL/TSP (Processes 4 and 5 in Fig. 1). Types of integrin subunit α/β combination define cellular interaction with ECM components.[80),81)] Recent studies have revealed a few additional factors that promote or inhibit integrin-mediated adhesion and motility: (i) various types of TSP (some TSP display PL properties); (ii) gangliosides that mediate interaction of integrin with PL/TSP; (iii) N-glycosylation status of integrin and TSP. These factors are organized to form ganglioside/integrin/TSP complex that mediates inhibition of cell motility and concurrent signaling (Fig. 4B).

TSPs CD9 and CD82 as motility-inhibitory factors. In studies using motility-inhibitory mAbs, we found a few factors controlling cell motility. One was H and/or Le^y epitope[82)]; another was motility-regulatory protein-1 (MRP-1), having the same sequence as previously-known lymphocyte-differentiation factor CD9.[83)] CD82 ("KAI-1") was originally discovered as anti-metastasis gene product based on subtraction of mRNA of high- vs. low-metastatic tumor.[84)] Both CD9 and CD82 were characterized as typical TSP having four transmembrane regions. Expression of both TSPs was down-regulated in metastatic deposits of various cancers, *e.g.*, Ref. 85. In many cases, their expression was inversely correlated with patient survival rate, *e.g.*, Ref. 86.

Ganglioside (particularly GM3), and N-glycosylation, are required for TSP/integrin-mediated motility-inhibitory effect. Surprisingly, the motility-inhibitory effect of CD9 and CD82 was not observed unless GM3 synthesis did not occur, or N-glycosylation was complete, as demonstrated in ldlD cells transfected with CD9 or CD82. ldlD cells are a UDP-Gal 4-epimerase-defective mutant, incapable of synthesizing GM3 or of complete N-glycosylation unless Gal is added to specific serum-free medium.[87)] In many cases, interaction of TSP with integrin activates adhesion and motility (*e.g.*, Ref. 88). However, α3/ CD9 interaction inhibits laminin-induced cell motility. This inhibitory effect requires the presence of GM3, and presumably N-glycosylation of α3.[38)] The role of GM3 as co-factor of CD9 function was also demonstrated in various colorectal cancer cell lines with or without CD9 transfection. This concept was verified by direct interaction of photoactivatable GM3 with CD9 — but not with integrins α3, α5, α6, or β1.[89)]

The important role of N-glycans in integrin function is indicated by a series of studies by N. Taniguchi and colleagues. The GlcNAcβ1-6Manα6 side chain (which depends on GlcNAc T-V) enhances integrin-dependent cell motility, whereas bisecting GlcNAcβ1-4Man (which depends on GlcNAc T-III) inhibits such motility (for review see Ref. 90).

Effect of GSL on "cross-talk" between FGFR and TSP/integrin $\alpha3\beta1$ (Processes 3, 4, and 5 in Fig. 1). Functional interaction ("cross-talk") of integrins with GFRs has become increasingly clear as a basic mechanism in cell biology, defining cell growth, adhesion, and motility.[91)] Our recent studies indicate that cross-talk of FGFR with $\alpha3\beta1$ or $\alpha5\beta1$ in WI38 cells is strongly influenced by GM3 and TSP. GM3 depletion by P4 enhanced signaling by FGFR and Akt, and enhanced co-IP of FGFR with integrins. LN5- or FN-dependent proliferation of WI38 was enhanced by GM3 depletion, and by CD9/CD81 co-knockdown by siRNA.[40)] Thus, integrin-FGFR cross-talk is inhibited by GM3 and/or TSPs within GEM.

Cell adhesion with concurrent signaling to affect cellular phenotype, through GSL-to-GSL interaction based on glycosynapses (Processes 6 and 7 in Fig. 1). The basic mechanism of cell-to-cell adhesion is through (i) protein-to-protein interaction, *e.g.*, between Ig adhesion receptors, cadherins, and ECM/integrins; (ii) carbohydrate-to-protein interaction, *e.g.*, selectins, siglecs, galectins, and perhaps many other carbohydrate-binding proteins; (iii) carbohydrate-to-carbohydrate interaction (CCI), particularly well-established for GSL-to-GSL interaction from our studies, and for multiple oligosaccharides assembled in proteoglycans for species-specific sponge cell adhe-

sion.[92] Specific carbohydrate structures known to be involved in cell recognition through CCI are limited at this time. Exact affinities and atomic forces acting between them have been elaborated in detail in recent studies (for review see Ref. 18).

Many lines of evidence indicate that cell adhesion/recognition based on GSL-to-GSL interaction takes place through GEM containing chloroform/methanol-soluble PL such as CD9, as well as various signal transducer molecules as described above. We hypothesize that some tumor cells, or cells at defined stages of development, adhere together through glycosynapse 1, leading to activation of signal transducers to enhance cell growth, motility, and/or invasiveness (Fig. 2B).[19),20)]

Another type of adhesion system was found in which mucin-type glycans play an essential role in cell adhesion through a microdomain termed "glycosynapse 2".[17),93)] A third adhesion system was found more recently in which ganglioside, particularly GM3, modulates cell adhesion and motility through CD9 (or CD82)/integrin complex. Such microdomain is termed "glycosynapse 3".[17),20),38)-40)] These glycosynapse complexes are dissociated and solubilized in 1% Triton X-100. However, the complexes are maintained in 1% Brij 98, and are resistant to cholesterol-binding reagent. Glycosynapse 3 is involved in cell-to-cell or cell-to-ECM adhesion, and is relatively stationary. These properties are distinctive from those of moving, signaling platform ("lipid raft"), which is insoluble in 1% Triton X-100 but disrupted by cholesterol-binding reagent (see Table II).

Expression of oncogenic phenotype, and its reversion, by depletion or enhancement of GM3 synthesis in glycosynapse 3. Jun-induced oncogenic phenotypes in C3H mouse fibroblast 10T1/2 and chicken fibroblast DF1 cell lines have been well-established, as enhanced cell growth and colony formation in soft agar. Numerous changes of gene expression induced by Jun are known. However, on a membrane molecular level, GM3 is down-regulated or depleted in both mouse and chicken fibroblasts. Transfection of GM3 synthase gene to increase GM3 level in these transformants interferes with or blocks expression of transformed phenotype; *i.e.*, oncogenic phenotype is "reverted" to normal phenotype. Membrane organization in terms of CD9/α5 or CD9/β1 interaction is thereby significantly enhanced.[94)]

Haptotactic motility of various colorectal cancer cell lines (SW480, SW620, HRT18) having high CD9 level was strongly suppressed by exogenous GM3 addition. In contrast, motility of gastric cancer cell line MKN74 having low CD9 level was not affected by GM3 addition. When CD9 was increased in MKN74 through gene transfection, motility was suppressed by GM3 addition.[89)] Similarly, motility of highly-invasive bladder cancer cell line YTS-1, having low GM3 but high CD9 level, was strongly suppressed by brefeldin A-induced enhancement of GM3 synthesis. Bladder cancer cell line KK47, having high GM3 and high CD9 level, is not invasive.[95)] Thus, reversion from invasive/malignant phenotype to nearly-normal phenotype is possible through increase of GM3 and CD9 in glycosynapse 3 of bladder cancer cells. Conversion from non-malignant to highly-malignant phenotype is possible through decrease of GM3 (Mitsuzuka, K., Handa, K., Hakomori, S., unpubl. data). The process of oncogenic transformation, and its reversion, can be correlated with levels of GM3, PL/TSP, and integrin receptor, as shown schematically in Fig. 5.

Conclusion and perspectives. Our initial, major effort in GSL studies was characterization of structures, and their changes during ontogenic development and oncogenic transformation. We identified many GSLs as antigens or receptors. We also identified gangliosides, particularly GM3, as modulators of cell growth, or of cell-to-cell or cell-to-ECM interaction. Some of the target molecules with which GSLs interact, such as GFRs (with PL/TSP), and integrins (with PL/TSP), have been identified. However, target molecules of many tumor-associated GSL antigens, and of GSLs that show changes during development or transformation, remain to be studied.

Some types of GEM function as moving, signaling platforms characteristic of "raft", while others are involved in integrin/TSP-dependent cell adhesion or GFR/TSP-dependent cell growth. Functional interaction ("cross-talk") between integrin and GFR, mediated by GM3/TSP complex within microdomain, was clarified recently.[40)] There may be many other microdomains, depending on type of cell and location within cell. Cross-talk between rafts, glycosynapses, and other microdomains is an important topic for future study, since many functions of GSLs are based on their interaction with key membrane molecules at respective microdomains. These studies will clarify the mechanism by which GSLs control differentiation, development, oncogenesis, and apoptosis.

Much commentary has been directed to tumor-associated antigens and their functional concepts. Reversion from transformed to normal cell phenotype, and conversion from normal to transformed phenotype, reflects ganglioside-dependent stability or its loss in

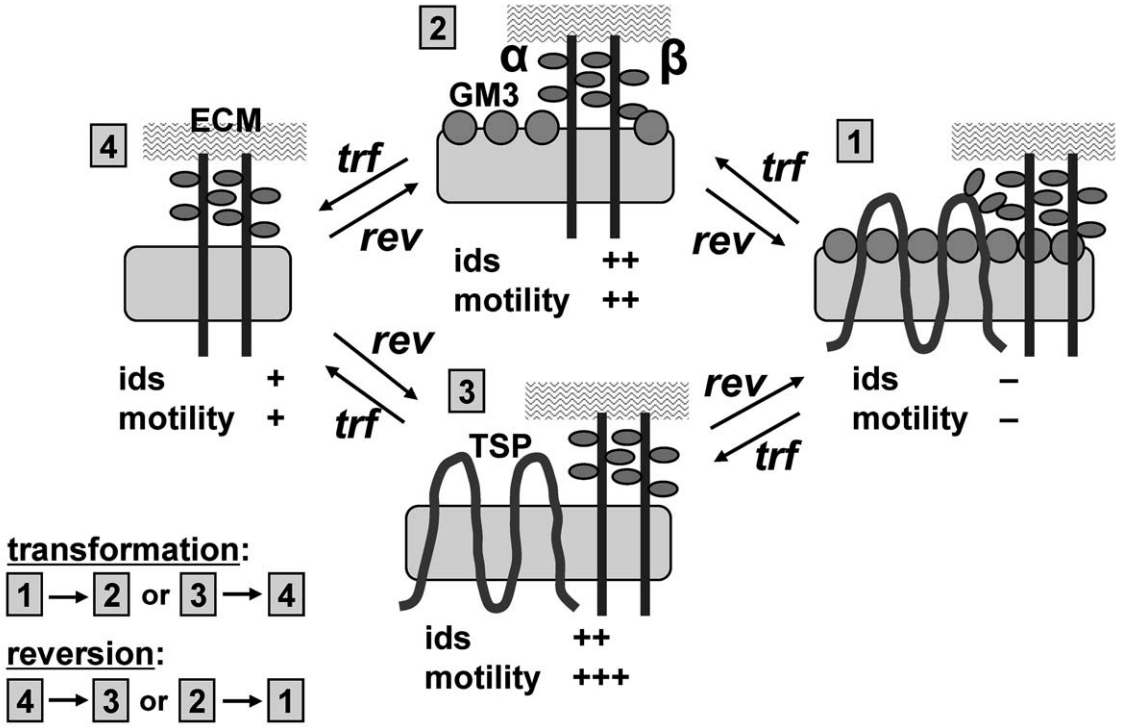


Fig. 5. Integrin receptor status at microdomain: Changes associated with malignant transformation and with reversion. **1.** When four factors (N-glycosylation, integrin $\alpha\beta$, TSP, Gg) are present together in complex, integrin-dependent signals ("ids") and motility are down-regulated, giving "benign" or "normal" phenotype, as observed in non-malignant bladder cancer cells (*e.g.*, KK47), and in model ldlD/CD9 or /CD82 cells under +Gal condition. **2.** Integrin with N-linked glycan is surrounded by Gg (GM3); ids and motility are enhanced. **3.** When TSP is present and complexed with integrin without Gg, it strongly facilitates ids and motility, as observed in ldlD cells transfected with CD9 or CD82 and grown under -Gal condition. **4.** N-glycosylated integrin with α and β subunits attains proper configuration, causing ids and cell motility. The process 1→2→4 or 1→3→4 is malignant transformation (***trf***). In contrast, 4→2→1 or 4→3→1 is reversion from malignant to normal cell phenotype (***rev***).

glycosynapse 3. A wide variety of other diseases such as diabetes, inflammatory processes, and infection have been explained in terms of structure, composition, and organization of microdomains (including glycosynapses). Further studies along this line will not only clarify the role of GSLs, but also reveal secrets of various disease processes.

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Profile

Senitiroh Hakomori was born in 1929, and started his research career in glycoscience at the Dept. of Biochemistry, Tohoku Univ. School of Medicine (mentor, the late Prof. Hajime Masamune). He earned his Dr. Med. Sci. at the same institution in 1956, and conducted post-doctoral research as a Fulbright Fellow at Harvard Medical School, 1956-58 (mentor, Prof. Roger W. Jeanloz). Since 1970, he has been Prof. of Pathobiology, Univ. of Washington. He is also Head, Division of Biomembrane Research, Pacific Northwest Research Institute, Seattle, WA (1996-present). His scientific career has been devoted to studies on structure, and function of glycosphingolipids (GSLs). His initial studies were characterization of novel extended lacto-series and globo-series structures, including A_1, A_2, H, I/i, Le^x, Le^y, sialyl-Le^x, and globo-ABH, some of which were identified as developmentally-regulated or tumor-associated antigens. His group was the first to identify tumor-associated antigens as GSLs, providing the basis for diagnosis and treatment of human cancer. More recently, they have been studying functional roles of GSLs in defining cell adhesion and signal transduction. For example, they were the first to characterize the effect of gangliosides on growth factor receptor function, in terms of inhibitory or promoting effect on receptor-associated tyrosine phosphorylation. They pioneered the elucidation of GSLs as adhesion molecules based on carbohydrate-to-carbohydrate interaction. They provided clear evidence that GSLs or other glycoconjugates, organized in microdomains, define cell adhesion coupled with signal transduction. Subsidiary studies, such as discovery of cell surface fibronectin and its deletion during transformation, and defining of ABO gene structure, also resulted from their studies. Hakomori's major honors are the Philip Levine Immunohematology Award (1984), Asahi Prize for Culture (1990), Morton Award (British Biochemical Society, 1994), Honorary Doctor of Philosophy Degree (Univ. of Helsinki, Finland, 1994), and Karl Meyer Award (Society of Glycobiology, 1995). He was elected to the U.S. National Academy of Sciences, and named as Fellow of the American Academy of Microbiology, in 2000.