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MINI-REVIEW

GM3 and cancer

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Abstract Our studies during the early 1970s showed that expression of GM3, the simplest ganglioside and an abundant animal cell membrane component, is reduced during malignant transformation of cells by oncogenic viruses. Levels of mRNA for GM3 synthase were reduced in avian and mammalian cells transformed by oncoprotein "v-Jun", and overexpression of GM3 synthase in the transformed cells caused reversion from transformed to normal cell-like phenotype. GM3 has a well-documented inhibitory effect on activation of growth factor receptors (GFRs), particularly epidermal GFR (EGFR). De-N-acetyl GM3, which is expressed in some invasive human cancer cells, has an enhancing effect on EGFR activation. The important role of the sialosyl group of GM3 was demonstrated using NEU3, a plasma membrane-associated sialidase that selectively remove sialic acids from gangliosides GM3 and GD1a and is up-regulated in many human cancer cells. GM3 is highly enriched in a type of membrane microdomain termed "glycosynapse", and forms complexes with co-localized cell signaling molecules, including Src family kinases, certain tetraspanins (*e.g.*, CD9, CD81, CD82), integrins, and GFRs (*e.g.*, fibroblast growth factor receptor and hepatocyte growth factor receptor c-Met). Studies by our group and others indicate that GM3 modulates cell adhesion, growth, and motility by altering molecular organization in glycosynaptic microdomains and the activation levels of co-localized signaling molecules that are involved in cancer pathogenesis.

Keywords Glycosphingolipids · GM3 · Cancer · Growth factor receptors · Membrane microdomains · Tetraspanins · Cell signaling

Abbreviations

GFR	Growth factor receptor
EGFR	Epidermal growth factor receptor
GSL	Glycosphingolipid
TS	Tetraspanin
GPI	Glycosylphosphatidylinositol
GEM	Glycolipid-enriched microdomain

Introduction

Glycosphingolipids (GSLs), including the simplest ganglioside GM3, are ubiquitous components of animal cell membranes. The basic structures of most GSLs were identified by the 1970s, and many studies since then have focused on the functional/ biological roles of GSLs in cells. Some of the important functions of GSLs are as allogeneic histo-blood group antigens, heterophile antigens, receptors for bacteria and their toxins, developmentally regulated antigens, and tumor-associated antigens [1, 2]. This minireview describes studies by our group and others on the role of GM3 in suppression of cancer development and progression.

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GM3 expression and cell phenotype

In our initial study of GSL expression in oncogenically transformed cells, we found that polyoma virus-transformed baby hamster kidney (BHK) cells showed significantly reduced GM3 expression and enhanced expression of the GM3 precursor lactosyl-Cer (LacCer) [3]. Similarly, Mora *et al*. observed reduced expression of mono- and di-sialogangliosides in mouse 3 T3 cells transformed by oncogenic RNA viruses [4]. These findings led to the concept of "aberrant glycosylation in cancer cells" and further extensive research along this line. In subsequent studies, we showed that GM3 expression was reduced in chicken embryo fibroblast cells transformed by Rous sarcoma virus [5], and that such reduction was closely associated with the transformed phenotype using a temperature-sensitive mutant of the virus. At a "permissive temperature" (35 °C), GM3 expression was reduced, whereas at a "non-permissive temperature" (41 °C) GM3 expression was not reduced and the cells did not display a transformed phenotype [6].

We investigated the functional significance of reduced GM3 expression using the viral oncoprotein v-Jun, which induces transformation in avian and mammalian cells [7]. Transfection of v-Jun induced transformed phenotypes in mouse fibroblast cell line 10 T1/2 and chicken fibroblast cell line DF1 and enhanced their anchorage-independent growth in soft agar. GM3 was a major GSL component in both cell lines, and expression of GM3 and GM3 synthase (LacCer α2, 3-sialyltransferase) mRNA were greatly reduced following v-Jun transformation. Transfection of mouse and chicken GM3 synthase into v-Jun-transformed 10 T1/2 and DF1 cells, respectively, resulted in increased GM3 expression and reduced anchorage-independent cell growth. These findings indicated the functional involvement of GM3 in transformed cell phenotypes, and the possibility of reverting transformed to normal cell phenotype by manipulating GM3 expression (Fig. 1). In non-transformed (control) 10 T1/2 and DF1 cells and reverted cells, more integrin α5β1 was co-immunoprecipitated with tetraspanin (TS) CD9 than in transformed cells, although CD9 expression was similar in the three types of cells [8]. Consistent findings were obtained in our earlier study of human colon cancer cell lines [9].

We also studied the functional role of GM3 in regulation of cell population density. Early studies by Abercrombie *et al*. demonstrated "contact inhibition" of cell growth and motility of various types of normal cells following cell-to-cell contact, and the loss of this phenomenon in transformed cells [10, 11]. In a study of hamster cell lines NIL and BHK, which display clear contact inhibition, we showed that expression of GSLs GM3, Gb3, and GD3 was significantly enhanced at high cell density (with contact inhibition) in comparison with low cell density (without contact inhibition). The enhanced expression

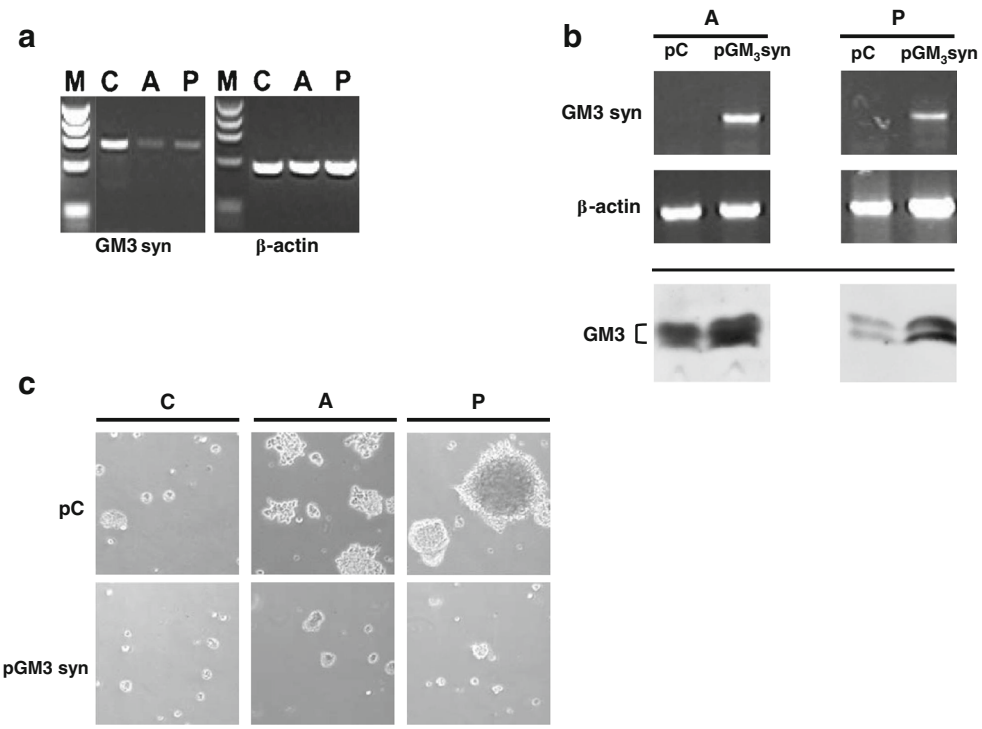

Fig. 1 Decrease of GM3 synthase mRNA level in v-Jun transformed cells, and phenotypic reversion by overexpression of GM3 synthase. **a** RT-PCR analysis of GM3 synthase (GM3 syn) and β-actin in control mouse 10 T1/2 cells (*C*), 10 T1/2 ASV17 v-Jun cells (*A*), and 10 T1/2 pBabe v-Jun cells (*P*). M: DNA size markers. **b** RT-PCR analysis of GM3 synthase (GM3 syn) and β-actin following transfection of control vector (pC) or GM3 synthase-containing vector (pGM3 syn). *Bottom*: GM3 level detected by immunostaining with mAb DH2 after TLC separation of GSLs extracted from the cells. **c** Colony formation assay in soft agar

of these GSLs was lost in transformed cells [12]. Studies by Robbins *et al*. showed similar association of GSL expression changes with contact inhibition and oncogenic transformation in other cell types [13, 14].

Inhibitory effect of GM3 on growth factor receptors

Growth factor receptors (GFRs) are involved in many cellular functions. Binding of a growth factor to its receptor activates the receptor tyrosine kinase that subsequently autophosphorylates certain tyrosine residues on the receptor itself and/or certain signal transducers involved in downstream cell signaling pathways. Overactivation of several GFRs, including epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR), and platelet-derived growth factor receptor (PDGFR), is well known to be associated with cancer development and progression. These GFRs are therefore promising targets for cancer therapy. Following our initial demonstration of GSL effects on cell growth [15], we found that exogenously added GM3 and GM1 were incorporated equally well by BHK cells, but that only GM3-treated (not GM1-treated) cells showed significant reduction in FGF-induced cell growth. Radiolabeled FGF was accumulated on the surface of GM3-treated but not GM1-treated cells. Neither GM3 nor GM1 showed direct interaction with FGF [16]. In human epidermoid carcinoma A431 cells, exogenous addition of GM3 (but not GM1) inhibited EGF-induced autophosphorylation of EGFR [17]. Similar effects of endogenous GM3 were observed in ldlD cells, a UDP-Gal-4-epimerase-deficient mutant of Chinese Hamster Ovary (CHO) cells that express GM3 only when cultured in galactose (Gal)-containing medium [18]. The order of binding intensity with various gangliosides using the extracellular domain of recombinant human EGFR prepared in insect cells was GM3 >> GM2, GD3, GM4 > GM1, GD1a, GD1b, GT1b, GD2, GQ1b > LacCer, consistent with the inhibitory effect of these gangliosides on EGFR activation [19].

We examined the effects on EGFR activation of chemically synthesized GM3 derivatives, including de-N-acetyl-lyso-GM3, lyso-GM3, de-N-acetyl-GM3 with N-acetyl-sphingosine, GM3 with N-acetyl-sphingosine, and mono-chloro- and di-chloro-GM3 derivatives [20–25]. Using mAb DH5 directed to de-N-acetyl-GM3, which has a neuraminosyl-lactosyl group instead of the sialosyl-lactosyl group in GM3 [26], we detected small amounts of de-N-acetyl-GM3 in A431 cells and mouse melanoma B16 cells. De-N-acetyl-GM3 enhanced both EGFR kinase activity and EGF-induced growth of A431, Swiss 3 T3, and B16 cells. GM3 inhibited tyrosine phosphorylation, whereas de-N-acetyl-GM3 enhanced serine phosphorylation of EGFR [27]. Subsequently, Wang and colleagues reported that de-N-acetyl-GM3 is highly expressed in metastatic/ invasive human melanoma cells and related to their high cell migration activity [28]. We found recently that mono-chloro-GM3 has a stronger inhibitory effect than GM3 on EGFR activation, and that mono-chloro-GM3, but not GM3, inhibits EGF-independent activation of a mutant EGFR, ΔEGFR, often detected in human glioblastoma cells [23]. These findings indicate the therapeutic potential of such derivatives in the future.

Studies during the 1980s suggested that the N-acetyl group of the sialosyl residue and the N-fatty-acyl group of the ceramide moiety of gangliosides can modify the essential functional organization of GSLs in cell membrane. This idea was more clearly formulated in the 1990s and subsequently extended to concepts of membrane microdomains in which GSLs (*e.g.*, GM3), GFRs, and other signaling molecules (*e.g.*, TSs and integrins) are localized and function as clusters [29] (see following section).

The importance of the sialosyl group of GM3 for its inhibitory effect was demonstrated by transfecting A431 cells with a gene encoding a soluble Mr 42,000 sialidase. In comparison with control cells, the transfected cells showed reduced surface expression of lipid-bound sialic acids, faster growth, and enhanced EGFR autophosphorylation, although the level of EGF binding was similar [30]. NEU3, a plasma membrane-associated sialidase whose selective substrates are GM3 and GD1a, was cloned by Miyagi's group in 2000 [31, 32]. The group demonstrated that the enhanced expression of *NEU3* results in suppression of apoptosis and that knockdown of *NEU3* expression inhibited Ras activation. Ras activation by NEU3 was blocked by the EGFR kinase inhibitor AG1478, and *NEU3* overexpression enhanced EGF-induced EGFR autophosphorylation [33]. Increased EGFR phosphorylation was also detected in *NEU3* transgenic mice, suggesting the involvement of *NEU3* up-regulation in cancer development [34]. *NEU3* expression was found to be up-regulated in renal cell carcinoma relative to adjacent normal kidney tissue, and the sialidase appeared to play an important role in the cancer progression by suppressing apoptosis and promoting invasive potential [35, 36]. Up-regulation of *NEU3* expression was also reported in colonic cancer [34] and melanoma [37]. Localization of NEU3 in glycolipid-enriched microdomains (GEM; see following section) was reported recently [38].

N-glycolyl-GM3, which contains N-glycolylneuraminic acid instead of N-acetylneuraminic acid, is widely expressed in most mammals, but not in humans, due to an inactivating mutation in the human cytidine monophospho-N-acetylneuraminic acid hydroxylase gene.

However, N-glycolyl-GM3 is detected in various human cancers including colon carcinomas and breast cancers, and is considered as one of potential target molecules in cancer therapy; although the mechanism of its expression and its functional role in human cancer still remain to be elucidated [39–42].

GM3 facilitates activity of tetraspanins in glycolipid-enriched microdomains

Various lines of study support the idea that molecules expressed at the cell membrane are compartmentalized rather than the fluid mosaic model in which the molecules are distributed homogeneously. The physical properties and specialized biological functions of the compartmentalized domains are long-standing major topics in cell biology. Scanning electron microscopic examination with "freeze-fracture" technique in 1983 revealed clustered GSLs and glycoproteins in erythrocytes [43], and similar clustered structures were observed in a liposome membrane model [44]. We observed clustering of GSLs with cell matrix proteins and microfilaments at adhesion sites of cultured fibroblast cells in the early 1980s [45, 46]. Simons & van Meer demonstrated differential composition of sphingolipids and glycerophospholipids in apical vs. basolateral membranes of polarized kidney epithelial cells [47]. Brown's group selectively detected glycosylphosphatidylinositol (GPI)-anchored proteins and GSLs in non-ionic detergent-insoluble, low-density membrane fractions isolated from epithelial cell lysate, and termed such fractions as detergent-resistant membrane (DRM). Their further studies indicated a close association of detergent resistance with the presence in cell membrane of an "ordered liquid phase" with restricted fluidity [48]. DRM fractions isolated from epithelial cells were found to contain caveolin, a hydrophobic membrane protein characteristically present in caveolae (flask-shaped invaginations in plasma membrane long known on a morphological basis), and to play an important role in endocytosis [49]. Similar DRM fractions were found in various other cell types, including hematopoietic cells which lack caveolae. Horejsi's group reported an association of Src family kinases with GPI-anchored membrane proteins [50], and later detected GM3 in kinase/ GPI-anchored protein complexes in human T-cell and myeloid cell lines [51]. In numerous subsequent studies, such membrane microdomains, which were also termed detergent-insoluble membrane (DIM) or glycolipid-enriched microdomain (GEM), were shown to be involved in cell interaction, cell adhesion, and related signal transduction. These findings led to the "lipid raft" concept in the late 1990s [52].

We applied the GEM concept to our earlier finding that B16 cells, which express very high GM3 levels, adhere to plates coated with asialo-GM2 (Gg3) or LacCer through GM3 on the cell surface, based on carbohydrate-to-carbohydrate interaction, a novel type of molecular interaction [53–55]. GEM fraction was isolated from B16 cells as a low-density fraction by sucrose density gradient centrifugation, from cell lysate prepared with a widely-used lysis buffer containing 1 % Triton X-100. Over 90 % of total GM3 was found in the GEM fraction, along with several signal transducer molecules (c-Src, RhoA, H-Ras, FAK). When GEM fraction was immunoprecipitated with anti-GM3 mAb DH2, RhoA and c-Src were co-precipitated with GM3 [56]. Tyrosine phosphorylation of FAK was greatly enhanced in B16 cells placed on Gg3-coated plates, but not on control GM3- or GlcCer-coated plates. The enhanced FAK phosphorylation was inhibited by DH2 or anti-Gg3 mAb 2D4, indicating that the phosphorylation was induced by GM3/ Gg3 interaction. Along with FAK activation, activation of Ras and Rho was also enhanced [57]. Our further studies of B16 cells revealed the presence of "GSL signaling domains" (GSD), which differed from caveolin-containing membrane domains [57]. In mouse neuroblastoma Neuro2a cells, we found that GM3-enriched microdomains were involved in signal transduction during neurite formation [58].

The above studies focused on functional roles of GSLs in GEM led us a concept of "glycosynapse", named in analogy to "immunological synapse" [59, 60]. Glycosynapses are membrane microdomains isolated from cell lysates prepared with milder detergents (*e.g.*, Brij 98) instead of Triton X-100 (commonly used for GEM preparation). Relative to GEM, glycosynapses are highly enriched in TSs (a family of membrane proteins carrying 4 transmembrane domains and 2 extracellular domains), hydrophobic proteolipids, integrins, and a wider range of signaling molecules and GFRs. Our subsequent studies demonstrated the involvement of glycosynapses in various cellular functions, particularly cell growth and motility, which are typically enhanced during cancer progression [61].

In studies with ldlD cells, we found that increased GM3 expression enhanced the anti-metastatic functions of TSs CD82 and CD9 [62], and that the effect of GM3 on CD82 function involved N-glycosylation status of CD82 [63]. We detected CD9 cross-linked with a photoactivatable GM3 analogue incorporated in HRT18, a human colon cancer cell line that highly expresses CD9 and is susceptible to exogenously added GM3 (Fig. 2) [9]. We showed that CD9 is a proteolipid [64] based on its solubility in chloroform/ methanol, and that endogenous or exogenous increase of cell surface GM3 expression resulted in increased interaction of CD9 with integrin α3 and the decreased cell migration through laminin-5-coated membranes [65]. In human diploid embryonic lung fibroblast WI38 cells, which display clear contact inhibition of cell growth, GM3 depletion by preincubation with the GlcCer synthase inhibitor D-threo-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4) [66] suppressed the contact inhibition of cell growth. At high cell density, FGF-induced cell

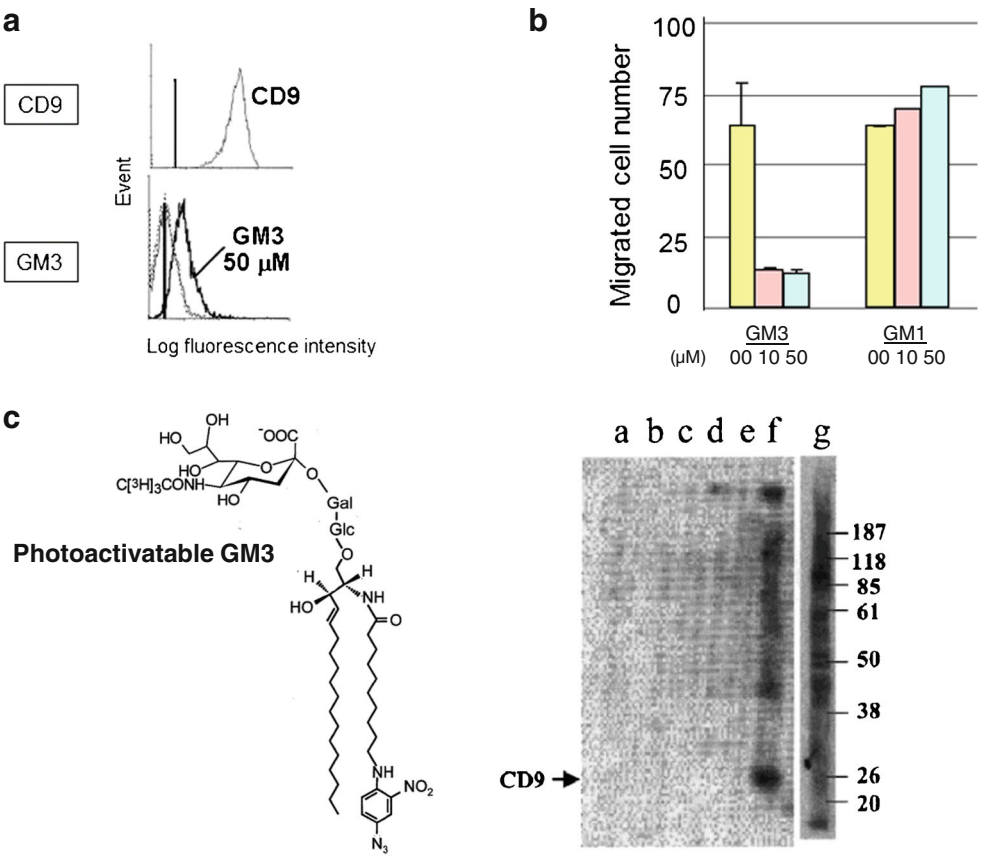

Fig. 2 Inhibition of cell motility by exogenously added GM3 in human colonic cancer cell line HRT18, and cross-linking of CD9 with photoactivatable GM3 analogue. **a** Flow cytometric analysis of HRT18 cells. *Top*: CD9 expression. *Bottom*: GM3 expression in control cells (*thin line*) and cells preincubated with 50 μM GM3 (*thick line*). *Vertical lines*: peak positions with control antibody staining. **b** Transwell migration assay with HRT18 cells preincubated with 0 μM (yellow), 10 μM (pink), and 50 μM (blue) GM3 or GM1. **c** *Left*: structure of ^{3}H-labeled photoactivatable GM3 analogue used in cross-linking assay. *Right*: HRT18 cells were treated with the photoactivatable GM3 analogue, cell lysate was immunoprecipitated with control mouse IgG (*a*), anti-integrin β1 (*b*), anti-integrin α6 (*c*), anti-integrin α4 (*d*), anti-integrin α3 (*e*), or anti-CD9 mAb (*f*), and precipitates were separated by SDS-PAGE, transferred to a PVDF membrane, and analyzed by a Beta-Imager. *Arrow*: location of CD9

growth was observed in P4-treated cells but not in control cells [67].

Another series of our studies focused on three human bladder cancer cell lines differing in their GM3 expression and malignant potential. The YTS1 cell line was established from invasive human urinary bladder, KK47 from non-invasive superficial bladder cancer, and HCV29 from non-malignant transitional epithelial cells of the ureter. In comparison with KK47, YTS1 showed higher cell motility in *in vitro* assay and lower GM3 expression. We analyzed TS, integrin, and c-Src levels in glycosynaptic microdomains of YTS1 and KK47. Neither cell line expressed CD82. They expressed similar levels of integrin α3 and CD9, but α3/ CD9 interaction was weaker in YTS1. Depletion of GM3 in KK47 by P4 treatment resulted in weaker α3/ CD9 interaction, and endogenous addition of GM3 to YTS1 resulted in stronger α3/ CD9 interaction and reduced cell motility [68].

Next, we compared non-malignant HCV29 cells, which express GM2, GM3, and CD82, with invasive YTS1 cells, which express GM2 and GM3 but not CD82. CD82 was initially identified as a protein depleted in metastatic prostate cancer cells, and later shown to suppress cell motility and invasiveness by blocking interaction of integrins with the receptor tyrosine kinase c-Met (the hepatocyte growth factor (HGF) receptor), which has been indicated to be involved in cancer cell motility and invasiveness [69]. Interaction of c-Met with GM2/ CD82 complex in HCV29 cells blocked interaction of c-Met with integrin α3β1 and consequently reduced HGF-induced cell motility. GM2/ GM3 complex bound more strongly to CD82 than did GM2 or GM3 alone, and GM2/ GM3 complexes were detected on the cell surface by a specific mAb [70, 71]. The above series of studies on human bladder cell lines indicate that GM3 suppresses cell motility by modulating functions of other molecules co-localized in membrane microdomains (Fig. 3). Sonnino's group reported that GM3 synthase overexpression in human ovarian cancer cells reduces cell motility by modulating caveolin expression [72] and regulating c-Src kinase activity [73].

Concluding remarks

Several lines of study summarized in this minireview indicate that GM3 has an inhibitory or suppressive effect on cancer development and progression. The inhibitory effect of GM3 on activation of GFRs, particularly EGFR, is well documented. Many studies have demonstrated the involvement of various GFRs in cancer formation and metastasis. GM3 is

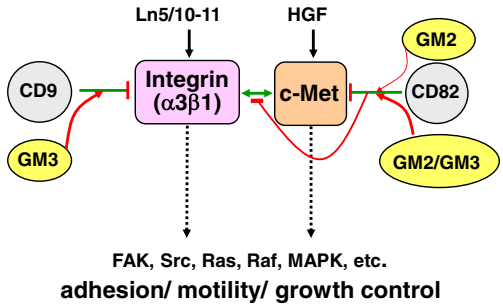

Fig. 3 Schematic representation of the inhibitory/ suppressive effects of GM3 and GM2 on cell motility and growth through modulation of TSs, integrins, and growth factor receptors co-localized in glycosynaptic microdomain. Ln, laminin. (i) GM3 enhances interaction of CD9 with integrin α3 and suppresses c-Src activation following the Ln- induced integrin activation. (ii) GM2 (weakly) and GM2/GM3 complex (strongly) bind with CD82 and the CD 82/ganglioside(s) complexes inhibit HGF-induced activation of c-Met and also the cross-talk between c-Met and integrin α3β1. (iii) Signaling (i) and (ii) involve various signal transducer molecules such as focal adhesion kinase (FAK), Src family kinases (Src), Ras, Raf, and mitogen-activated protein kinase (MAPK); and subsequently control cell adhesion, motility or growth

localized in membrane microdomains, forms complexes with TSs, GFRs, and signaling molecules, and modulates the functions of TSs, GFRs, and integrins. Further systematic searches for novel synthetic GM3 derivatives or analogues that show greater inhibitory effects on EGFR activation or cell motility in combination with TSs will lead to novel approaches to block cancer formation and progression.

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