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Changing the Nature of Melanoma Cells by Manipulation of Ganglioside Expression

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1. Introduction

Gangliosides, GSLs that contain sialic acid residues, are components of all animal cell membranes. It was first found by Klenk in 1935. He extracted something of new that was called substance X from the brain of a Niemann–Pick disease patient (Klenk, 1939b). In the following years, he understood (Klenk, 1939a) that substance X was a mixture of compounds and he named them "gangliosides". Gangliosides attracted immediately the interest of many investigators, but in spite of this, progresses in elucidating their structures were slow. In 1947, the structure of sphingosine was elucidated (Carter et al., 1947) and in 1955 that of sialic acid (Gottschalk, 1955). Finally, in 1963, the first ganglioside structure was described (Kuhn and Wiegandt, 1963). Following studies were extensively devoted to fully understand the ganglioside structural complexity, metabolism, cellular topology, biological functions, and pathobiological implications (Macher and Sweeley, 1978; Miller-Podraza et al., 1992; Sandhoff and Christomanou, 1979; Sandhoff and Conzelmann, 1984; Svennerholm et al., 1994). This research is still far to be considered concluded, but today there is a general agreement to consider gangliosides as functional molecules involved in the modulation of tumor metastasis and of cell signaling, cell invasive proliferation, adhesion, and motility (Bassi et al., 1991; Bremer et al., 1984; Caputto et al., 1977; Chan, 1988; Chan, 1989; Davis and Daly, 1980; Facci et al., 1984; Glebov and Nichols, 2004a; Glebov and Nichols, 2004b; Goldenring et al., 1985; Kim et al., 1986; Kreutter et al., 1987; Leon et al., 1981; Lin and Shaw, 2005; Morgan and Seifert, 1979; Partington and Daly, 1979; Roisen et al., 1981; Rybak et al., 1983; Tsuji et al., 1983; Yates et al., 1989).

In particular, systematic analysis of ganglioside antigens in various types of cancer was carried out. In these studies, ganglioside changes were observed based on the comparison of tumor tissues with corresponding normal tissues. Dramatic changes of ganglioside composition and metabolism were first shown using a cultured cell population after viral transformation (Hakomori and Murakami, 1968; Mora et al., 1969). In Balb/c 3T3 cells transformed with Kirsten strain of murine sarcoma virus (the tumor is called 3T3KiMSV), asialo-GM2 (Gg3) is greatly accumulated, with deletion of higher gangliosides. Rabbit

antibodies directed to Gg3 specifically stained 3T3KiMSV tumor grown in Balb/c mice. The antibodies did not stain various normal tissues of Balb/c mice, except for a small population in spleen (Rosenfelder et al., 1977). In more critical experiments, rats and mice were immunized with tumors derived from genetically identical (syngeneic) animals. For example, mAb M2590 was established after immunization of C57/BL mice with syngeneic B16 melanoma cells followed by selection of hybridoma clones showing specific reactivity with melanoma. Thus, the mAb reacted only with melanoma cells (human and hamster as well as mouse) but not with normal mouse, hamster, or human tissues (Taniguchi and Wakabayashi, 1984). Surprisingly, the epitope structure was identified as GM3, which is widely distributed in normal cells and tissues (Hirabayashi et al., 1985). Further studies revealed that M2590 reacted only with GM3 with density above a threshold value (Nores et al., 1987), that is the mAb recognized not only GM3 but also density of GM3. In line with the above cases, metastatic and invasive abilities of mouse melanoma B16 cell variants, in the order BL6>F10>F1>>WA4, are closely correlated with level of GM3 surface expression (Otsuji et al., 1995), and also with degree of adhesion to cultured endothelial cells (ECs) (mouse SPE1l human umbilical vein ECs) in vitro (Kojima et al., 1992; Otsuji et al., 1995). In addition, GM3 as the dominant GSL in B16 cells (Vedralova et al., 1995), has also been implicated involving in differentiation (Nojiri et al., 1986) and growth regulation (Bremer et al., 1986). These results suggested that ganglioside, GM3, organized in B16 cell membrane differ from the same antigens present in normal cell membrane of B16 cells, involved in changing the nature of melanoma cells via modulating the characteristics of melanoma cells in growth, differentiation, adhesion, invasion and metastasis.

Taken the advantage of recent success in the molecular cloning of glycosyltransferase genes responsible for the synthesis of gangliosides (Lloyd and Furukawa, 1998; Nagata et al., 1992) has enabled us to modify the expression profiles of gangliosides in cultured cells and experimental animals by manipulating the cloned genes (Furukawa et al., 2001). Although many studies have been performed to clarify the roles of gangliosides with various approaches such as usage of metabolic inhibitors, glycosidase treatment, carbohydrate probes including lectins and antibodies, and carbohydrate mutant cells and animals, results obtained with the manipulation of glycosyltransferase genes are providing us with much more exciting and novel information on the biological function of individual enzyme products. Although glycol-remodeling experiments revealed novel and unexpected functions of complex carbohydrates (Furukawa et al., 2001), molecular mechanisms for the roles of gangliosides remain to be investigated in many cases.

This chapter reviews experimental aspects of GM3-mediated invasive growth, motility and adhesion, which in turn resulting in metastasis of melanoma cells. The biological functions of GM3 would be further focused in modulating the nature of melanoma, especially in the process of metastasis. Relationship between the gene manipulation to modify GM3 expression and B16 cell function was extended to be discussed in order to understand how GM3 regulates molecular signals, leading to the change of melanoma B16 cell phenotype. We conclude by discussing the *in vitro* model of melanoma, B16 cells, that gangliosdes expression changed the nature of melanoma cells.

2. Biological functions of gangliosides

Gangliosides are classified as acidic glycosphingolipids containing sialic acid. Gangliosides occur not only as well known ganglio-series but also as globo-series or lacto-series

gangliosides. Each ganglioside series shows distinctive cell type or tissue type specificity, and they may play different functional roles in adhesion or signaling characteristics of cell types (Hakomori, 2003). Many subsequent extensive studies clarified functional roles of gangliosides as the following ways: 1) intracellular membrane trafficking, sorting, targeting and shedding; 2) functional receptors; 3) cell adhesion; 4) modulation of cell membranes to form gangliosides enriched microdomains (GSMs); 5) mediators or modulators of signal transduction.

In the view of the biological functions and given the strong amphiphilic characteristics of gangliosides, theoretical considerations and experimental data from artificial membranes suggest that gangliosides can cooperate in governing the membrane domain formation, existence, and organization according to the gangliosides physical-chemical properties, such as the lipid transition temperature, the hydrogen-bond network at the lipid-water interface, the geometry of the hydrophilic headgroups, and the carbohydrate-water interactions. This kind of interaction not only includes ganglioside itself but also recruit signal tranducer molecues to form glycosphingolipid enriched microdomains (GEMs), through which exerts its biological functions. The interest for GEMs, zones of the membrane with a peculiar composition different from that of the majority of bilayer, became very strong in the last 15 years. The concept of "GEMs" evolved, based on detergent-resistant properties (Brown and London, 1997; Okada et al., 1984) and three models of GEMs have been established after extensive experiments: 1) unique caveolar structures, which are also enriched in characteristic hydrophobic membrane protein caveolin (Anderson, 1998; Rothberg et al., 1992) are firstly identified by transmission electron microscopy with anti-GSL antibodies (Rahmann et al., 1994; Sorice et al., 1997); 2) similar composition, detergent-resistantce, and cholesterol-dependent properties (e.g. structure and function are disrupted by cholesterol-binding reagents β-cyclodextrin, filipin, and nystatin) were further found in not only caveolar but also non-caveolar region, the term "lipid raft" was proposed, representing "floating signaling platform" (Simons and Ikonen, 1997). 3) recently, a different microdomain was proposed, termed "immunological synapse" by the size, dynamic status, and detergent-resistance properties (Krummel and Davis, 2002) are different from the above two cell membrane microdomains.

Based on the above model of GEMs, some tumor-associated gangliosides antigens have been recovered as detergent-insoluble, low-density membrane fractions organized closely with various transducer molecules such as c-Src, Ras, Rho, and focal adhesion kinase (FAK). For example, >90% of c-Src, >90% of Ras, ~50% of Rho, and ~25% of FAK are enriched in GM3 microdomains of B16 cells (Iwabuchi et al., 1998). These observations indicate the possible presence of gangliosides enriched microdomains in cells and their involvement in signal transduction.

Upon thse findings, we have tried to construct a conceptual view with a focus on how the mechanistic process of GM3 is converted to signaling impulses affecting cellular phenotype, especially in influencing melanoma B16 cell metastasis, such as adhesion, invasive proliferation and motility.

3. GM3 changes the nature of melanoma B16 cells

A significant role of GM3 in defining membrane-based cell functions is indicated by quantitative and qualitative changes of GM3 associated genes exression, as shown in Table 1. Besides the "classic" function of gangliosides as antigens and toxin receptors, it is also

Regulation Manner by GM3	Gene Name	GM3(+)	GM3(-)	GM3(-)	Biological Functions
Positive	Caveolin-1	1.378	0.321	0.146	(1) (Felicetti et al., 2009), (4) (Felicetti et al., 2009), (5) (Felicetti et al., 2009)
	Ly-GDI	2.156	0.423	0.387	(5) (Seftor et al., 2002)
	PKN-1	1.658	0.626	0.495	(4) (Wang et al., 2006)
	E-cadherein	1.875	0.695	0.721	(1) (Lau et al., 2011), (3) (Tang et al., 1994), (5) (Wong and Gumbiner, 2003), (6) (Semb and Christofori, 1998)
	Gelsolin	1.841	0.543	0.502	(4) (Fujita et al., 2001)
	PTEN	2.482	0.290	0.153	(1) (Stahl et al., 2003)
	MMP-9	1.915	0.174	0.282	(4) (Desai and Chellaiah, 2006), (5) (Wang et al., 2010)
	MMP-2	1.532	0.534	0.472	(4) (Leotlela et al., 2007), (5) (Denkert et al., 2002)
	Apaf1	1.350	0.608	0.509	(2) (Rockmann and Schadendorf, 2005)
	RhoB	2.247	0.427	0.318	(5) (Jiang et al., 2004), (6) (Jiang et al., 2004), (5) (Jiang et al., 2004)
	Midkine	1.403	0.518	0.417	(1) (Escalante et al., 2000)
	Lymphotoxin α	2.245	0.475	0.497	(6) (Dobrzanski et al., 2004)
	Tnf α	2.188	0.349	0.292	(4) (Katerinaki et al., 2003), (5) (Katerinaki et al., 2003)
	Plau	1.453	0.397	0.750	(5) (Lee et al., 2006), (6) (Lee et al., 2006)
	Plaur	2.209	0.543	0.720	(2) (Besch et al., 2007)
Negative	Integrin β5	0.783	1.465	1.754	(1) (Taverna et al., 2005; Taverna et al., 2004),(2) (Cardo-Vila et al., 2003), (3) (Niu et al., 2007), (4) (Zhang et al., 2002)
	Vimentin	0.111	1.984	2.089	(5) (Leader et al., 1987)
	TGF β1	0.571	2.124	3.309	(1) (Paterson et al., 2002), (4) (Xu et al., 2003), (5) (Xu et al., 2003)
	TGFBR 2	0.716	1.453	1.903	(1) (Li et al., 2008)
	N-Cam	0.282	2.901	2.223	(3) (Anastassiou et al., 2000)
	Src	0.639	1.347	1.925	(1) (Frame, 2002), (3) (Frame, 2002), (4) (Bourguignon et al., 2001), (5) (Frame, 2002)

Table 1. GM3 regulated tumor related genes expression in melanoma B16 cells. The numbers represent the fold changes of the corresponding genes in GM3 modulating cells compared with that of control cells. The biological functions of the genes in the process of metastasis are shown as (1) Invasive Proliferation; (2) Apoptosis; (3)Adhesion; (4) Motility; (5) Invasion; (6) Metastasis

responsible for the processes of tumor cell phenotype including invasive proliferation, apoptosis, adhesion, motility, invasion and metastasis coupled with signal transduction. To keep the discussion focused, we would respectively elucidate the mechanisms of GM3 regulating melanoma B16 cells adhesion, invasive proliferation and motility, which in turn mediate metastasis of melanoma B16 cells.

3.1 Adhesion

There may be many cell adhesion/recognition systems in which GSLs play an essential role. However, only the initiation of B16 melanoma metastasis has been elucidated to an appreciable extent. Adhesion of mouse B16 melanoma cells to LacCer, Gb4 or Gg3 coated plates is mediated by interaction of GM3 (expressed highly on B16 melanoma cells) with the above GSLs (Kojima and Hakomori, 1989; Kojima and Hakomori, 1991a; Kojima and Hakomori, 1991b). Since GM3 dependent adhesion of B16 cells to nonactivated mouse endothelial cells (which express LacCer, Gb4, and Gg3) is regarded as the initial step in metastasis of B16 cells (Kojima et al., 1992; Otsuji et al., 1995), GM3 dependent adhesion has been extensively investigated. In detail, the adhesion system based on carbohydrate-carbohydrate interaction has the following characteristics: 1) adhesion process is rapid (within <10 min, compared to >30 min for integrin-dependent adhesion); 2) specificity is high in some cases, low in others; 3) most require bivalent cation such as Ca^{2+}., but a few do not; 4) synergistic with other adhesion systems, e.g., integrins; 5) negative interaction (repulsion) occurs between certain pairs of carbohydrates, e.g., GM3-GM3.

In addition, our results demonstrated that GM3 is able to regulate the expression of adhesive genes, such as E-cadherin, N-Cam and Src, which in turn modulate the adhesion of melanoma B16 cells (Table 1). Although we could not provide further evidence to show that these signaling molecules reside in GEMs, it will be of great interest to see the results of further studies along this line.

3.2 Proliferation

Studies performed during the early 1970s suggested that GSLs may interact with unidentified functional membrane components, which in turn may cause changes in cellular proliferation. However, at that time, no realistic information on such functional components was available. It took almost 20 years for the development of the current concept of growth factor receptors with tyrosine kinases. For understanding GM3 effects on B16 cell growth in culture, basic knowledge on types of growth factors required for culturing specific types of cells was needed.

3.2.1 Fibroblast growth factor receptor

Thus, the first experiment was undertaken to determine the effects of GM3 on BHK cell growth. Given the reason that BHK cells require fibroblast growth factor (FGF) but not epithelial growth factor (EGF) or platelet derived growth factor (PDGF), fibroblast growth factor (FGF) was used to observe the inhibitory effects of GM3 on BHK cell growth. Curiously, GM3-enriched BHK cells became refractive to growth stimulation by FGF, and internalization of FGF was completed blocked (Bremer and Hakomori, 1982). It was assumed that high GM3 level blocked function of FGFR (Bremer and Hakomori, 1982). However, at that time, there was no knowledge on tyrosine kinase associated with FGFR; studies along this line were not performed until 20 years later (Toledo et al., 2004).

3.2.2 EGF receptor

This line of studies was further extended to effects of gangliosides on EGF-dependent A431 cell growth, and on tyrosine kinase associated with epithelial growth factor receptor (EGFR). GM3, but no other GSLs, strongly inhibited EGF-dependent cell growth, and EGFR tyrosine kinase (Bremer et al., 1986). Since EGFR is highly expressed in various epidermal cancers, and its tyrosine kinase activity is closely associated with cancer malignancy, a possibility was investigated whether any ganglioside could have better inhibitory effect than GM3 (see below).

Hanai et al. (Hanai et al., 1988b) further found that lyso-GM3 showed much stronger inhibitory effect than GM3 on EGFR tyrosine kinase in vivo as well as in membrane extract *in vitro*. Furthermore, lyso-GM3 was detected in normal A431 cells. In contrast, exogenously added "de-N-acetyl-GM3" (GM3 having de-N-acetyl sialic acid) strongly promoted EGFR tyrosine kinase and promoted growth of A431 cells (Hanai et al., 1988a). Thus, effect of gangliosides on EGFR tyrosine kinase is more complicated than originally considered, i.e., 1) tyrosine kinase is modulated by GM3 when EGFR is activated by EGF under normal conditions; 2) trace quantity of lyso-GM3 present, which may result from GM3 by de-N-acylation, strongly inhibits receptor function; 3) de-N-acetylation of GM3 in resting A431 cells may promote cell growth, possibly through a channel different from simple activation of EGFR. Exogenous lyso-GM3 is highly cytotoxic, whereas lyso-GM3 dimer is not cytotoxic, but inhibits EGFR tyrosine kinase as strongly as lyso-GM3. Therefore, synthetic lyso-GM3 dimer has been studied for inhibition of EGFR activity and A431 cell growth, for the purpose of developing pharmacologically effective inhibitors of epidermal tumor cell growth (Murozuka et al., 2007).

3.2.3 GM3/Ly-GDI Arhgdib inhibits cell proliferation through modulation of phosphotidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR)/regulatory associated protein of mTOR (Raptor) pathway under rigorous environment.

3.2.3.1 GM3 suppresses B16 invasive proliferation

Given the key role of GM3 in regulating cell growth as the above discussion, several lines of evidence have shown that GM3 invovled in tumor cell invasive proliferation Anchorage-independent growth experiments were effective *in vitro* experiments to determine the characteristics of tumor cell invasive proliferation. For example, reduced expression of GM3 and GM3 synthase as a result of v-Jun transformation resulted in enhanced ability of anchorage-independent growth and re-expression of GM3 by introducing GM3 gene to the transfectants correlated with a reduced ability of the cells to form colonies in nutrient agar (Miura et al., 2004). Contrary to this observation, expression of GM3 in 3LL Lewis lung carcinoma cells endowed cells with ability of anchorage-independent growth (Uemura et al., 2003). Thus, the effects of GM3 expression on anchorage-independent growth are controversial in different cell lines and the mechanism still remained unknown.

Our recent results demonstrated that GM3 modulates B16 invasive growth under rigorous environment, such as serum free or anchorage-independent growth. A close association of GM3 with B16 invasive proliferation was found in the following series of studies, which will be discussed in more detail below: 1) in melanoma B16 cells, GM3 suppression cell lines CAH-2 and CAH-3 showed remarkably enhancing anchorage-independent growth in soft agar medium. This observation demonstrates that the cells seemed easier to proliferate in

rigorous environment once knocking down the expression of GM3. 2) in this context, GM3 knocking down by siRNA targeting St3gal5 resulted in highly activated cell proliferation under serum free and soft agar medium. These results give further support to the notion that GM3 reduction enhances invasive proliferating ability of B16 cells in rigorous condition. It is also the characteristic of tumor cells that the proliferation was deregulated and the cells can escape the rigorous environment (Wang et al., 2011).

3.2.3.2 GM3 inhibits B16 invasive proliferation via PI3K/Akt/mTOR/Raptor pathway

In many contexts, the proliferation of mammalian cells depends upon PI3K activity. The strongest influences are probably exerted through activation of Akt (Vivanco and Sawyers, 2002). Although some growth factors do not directly activate PI3Ks, stimulation of Ras, an extremely potent mitogenic signal, leads directly to activation of phosphotidylinositol 3-kinases (PI3Ks) (Rodriguez-Viciana et al., 2004) and, in some cases, it is clear that PI3Ks, and not the MEK/ERK pathway, are the most important mediators of the transforming activity of oncogenic Ras (Li et al., 2004). Furthermore, it is a prevalently accepted notion that PI3K transduces signals via mammalian target of rapamycin (mTOR)/S6K pathway which directly regulates the synthesis of proteins and has intrinsic relationship with translation. Therefore, it is no doubt that cell proliferation is regulated by PI3K. In addition, several lines of evidence show that GM3 signals are transferred to downstream molecules via PI3K pathway. In human keratinocyte-derived squamous carcinoma cell line (SCC12F2), GM3 depletion concretely stimulates the phosphorylation of Akt at Ser473 and Thr308 sites (Sun et al., 2002). Treatment with GM3 antibody is able to increase phosphorylation of the Thr308 site, but not the Ser473 (Sun et al., 2002) site, indicating that GM3 is able to module PI3K activity. These findings are also consistent with the known concept that GM3 is capable to regulate PI3K activity by inhibiting EGF receptor phosphorylation (Bremer et al., 1986). On the other hand, GM3 also showed ability to modulate phosphatase and tensin homolog (PTEN) activity, a dual-specificity phosphatase that antagonizes PI3K/Akt signaling (Choi et al., 2006). Thus, PI3K is an important molecule that is responsible for GM3 signal transduction. However, although PI3K has shown its presence in GEMs (Liu et al., 1996), it is yet unclarified if it is located downstream of GM3 to mediate cell proliferation, especially under rigorous environment.

As a first step, we have to introduce the components of PI3K signaling pathway (Fig. 1). In the PI3K/Akt/mTOR pathway, Akt is flanked by two tumor suppressors: PTEN, which antagonizes PI3K and therefore inhibits Akt, and tuberous sclerosis complex (TSC)1/TSC2 heterodimer, which inhibits mTOR by inhibiting the activity of Rheb. Akt activates mTOR via direct phosphorylation of TSC2 and by the inhibition of AMP-activated protein kinase (AMPK), thereby activating Rheb and mTOR-Raptor activity. Upon activation, mTOR-Raptor (regulatory associated protein of mTOR) activates S6K and inhibits eIF4E binding protein (4E-BP1) to accelerate mRNA translation, and also initiates feedback inhibition of Akt, which is at least in part mediated by S6K.

Next, we established a different concept to explain the involvement of PI3K pathway in mediating GM3 signals to abnormal melanoma proliferation under rigorous environment. Just as described above, PI3K/Akt, 3-phosphoinositide dependent protein kinase-1 (PDK1, Pdpk1), Raptor and rapamycin-insensitive companion of mTOR (Rictor) play important role in cell proliferation. Our data further demonstrated that they are the key molecules in mediating GM3 signals to the invasive proliferation of B16 cell. 1) That GM3 suppression specifically decreased the expression of Pdpk1 and Raptor indicated that Pdpk1 and Raptor

are involved in the invasive proliferating pathway of melanoma B16 cells in soft agar or serum-free medium. 2) Pdpk1 and Raptor siRNA silencing cells had a similar growth rate to B16 control or parental cells under serum-containing conditions; however, the growing rate of Pdpk1 and Raptor knocking down cells, but not Rictor knocking down cells, was faster compared with B16 control or parental cells under serum-free conditions. 3) Raptor or Pdpk1 knocking down cells, but not Rictor knocking down cells, resulted in the formation of colony in soft agar. Collectively, these results further confirmed that GM3 regulates B16 cell invasive proliferation via Pdpk1 and Raptor in soft agar or serum deprived medium (Wang et al., 2011).

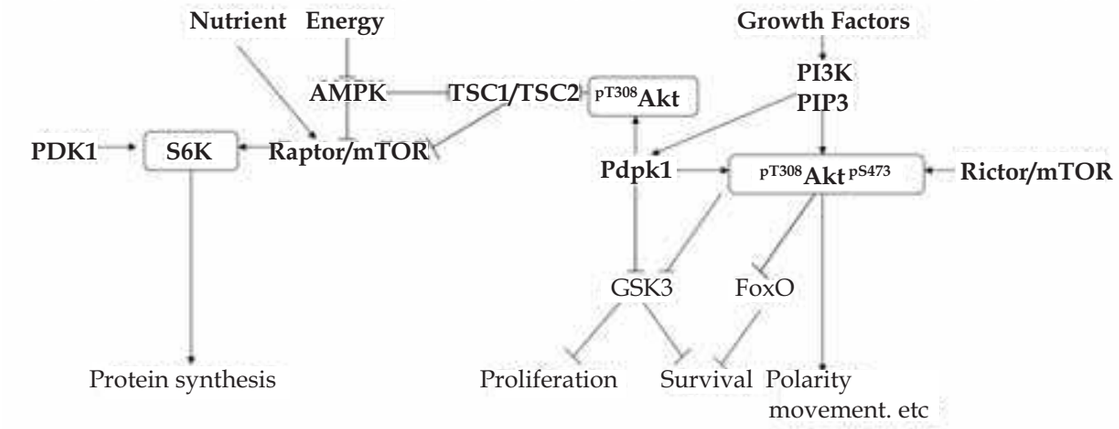


Fig. 1. PI3K signal transduction model. General concept of PI3K signaling pathway was summarized which involves in protein synthesis, proliferation, survival and polarity movement

3.2.3.3 Ly-GDI played a key role in mediating GM3 signals to inhibit B16 cell growth

Although it is confirmed that GM3 is capable to inhibit B16 melanoma cells proliferation via PI3K signaling pathway, it still seems to be conflicted with the universal accepted concept that PI3K is always hyper-activated in cancers, which drive the cells proliferation and avoid apoptosis (Luo et al., 2003). This controversy could not be resolved until we identified the Ly-GDI, which is located downstream of GM3 and acts as an effector of GM3 to change the nature of melanoma B16 cells. The proliferating characteristics would be changed once Ly-GDI expression was altered. Thus, it is not conflicting with the previously accepted concept since Ly-GDI would play a key role in mediating GM3 signals to inhibit B16 cell growth.

1. GM3 has been shown to regulate Ly-GDI expression at the transcriptional level in murine melanoma B16 cells. Ly-GDI expression was increased by addition of GM3 to the B16 transfectants and decreased after treatment with D-PDMP, an inhibitor of glucosyl-ceramide synthesis. These results clearly indicate that GM3 positively regulates Ly-GDI expression in B16 cells.
2. Phosphoinositide 3-kinase inhibitor, LY294002, suppressed the Ly-GDI expression that is stimulated by GM3 in B16 cells, suggesting that the GM3 signal is located upstream of the PI3K-Akt pathway. GM3 was shown to increase phosphorylation of Akt. Treatment of B16 cells with small interfering RNA (siRNA) targeted to Akt1/2 resulted in Ly-GDI suppression, indicating that Akt plays an important role in regulation of Ly-GDI expression. Suppression of Akt1/2 rendered cells insensitive to GM3, suggesting that

the GM3 signal may be transduced via Akt in view of the above reason, we further demonstrated that GM3 is located upstream of PI3K pathway to regulate Ly-GDI, by incubating B16 cells with GM3 in the presence or absence of PI3K inhibitors. As a result, PI3K inhibitor treatment thoroughly blocked the effects of GM3 in stimulating PI3K pathway, leading to overexpression of Ly-GDI. These results strongly demonstrated that GM3 regulates Ly-GDI expression via PI3K/Akt pathway, and Akt^{Thr308} was identified as a key active form of Akt to mediate this process by Pdpk1 or Raptor knocking down.

3. Most importantly, Ly-GDI silenced B16 cells showed markedly enhanced invasive proliferation in soft agar or serum-free medium.

These results clearly revealed the important role of Ly-GDI in regulating the abnormal proliferation of melanoma B16 cells (Fig. 2) (Wang et al., 2011b) and provide a noteworthy theory to explain the effects of GM3 on melanoma invasive proliferation, though it is different from the previous theory that GM3 inhibits tumor cell proliferation via modulating different receptors.

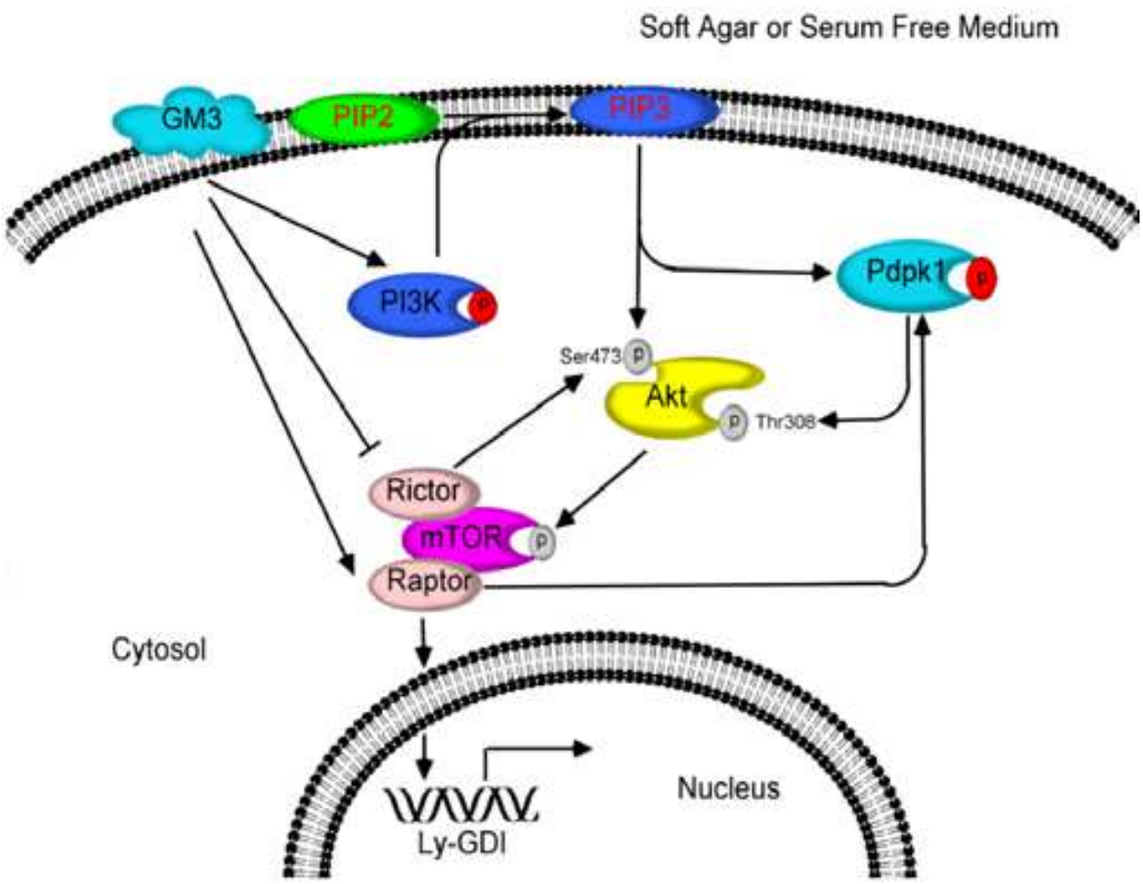


Fig. 2. Proposed cascade of signaling events regulating Ly-GDI expression by GM3, which in turn inhibits B16 cells proliferation under rigorous environment. GM3 signals are transduced in B16 cells through PI3K, Pdpk1, Akt, mTOR/Raptor pathway, leading to the enhanced expression of Ly-GDI mRNA, which in turn suppresses melanoma B16 cells proliferation under rigorous environment

3.3 Motility

Although there are several systems of GM3 mediated tumor cell motility in which GM3 plays an essential role, such as GSP/tetraspannin (TSP)/integrin and GM2/GM3/CD82 to explain the mechanism of cancer cell motility, there is no relative evidence to show the effects of GM3 on the motility of B16 cells. Based on the established theory, we found a new signal transduction pathway to mediate GM3 signals to the motility of B16 cells.

3.3.1 GM3/TSP CD9 complex inhibits integrin-dependent cell motility

Both gangliosides and TSP are reported to locate at GEMs in association with integrins (Kawakami et al., 2002; Ono et al., 2001; Ono et al., 1999). Integrins have been implicated in regulating cellular processes such as adhesion, mobility, signaling, for review see (Hehlgans et al., 2007). Integrin function, including α/β-subunit interaction, is affected by N-glycosylation status (for review see (Gu and Taniguchi, 2004)) and by interaction with TSP and/or gangliosides (Hakomori and Handa, 2002; Ono et al., 2001). TSP are palmitoylated and N-glycosylated and associate with integrin receptors, gangliosides and signaling molecules forming a membrane multi molecular complex referred as tetraspanin web (Ono et al., 2001); for review see (Hemler, 2005).

Since TSP CD9 inhibits cell motility and its expression is down-regulated in various human cancers (Cajot et al., 1997; Miyake et al., 1991), a possibility was opened that CD9 function was affected by glycosylation. ldlD mutant of Chinese Hamster Ovary cells, defective in UDPGlc: 4-epimerase, has been utilized for study of glycosylation of functional proteins (Kingsley et al., 1986; Krieger et al., 1989). ldlD cells with high CD9 expression were cloned after CD9 gene transfection. Motility of these ldlD/CD9 cells was greatly inhibited when cells were grown in serum-free medium (ITS: insulin/transferrin/selenium) containing galactose (Ono et al., 1999), allowing glycoproteins to be fully glycosylated and GM3 to be synthesized. A close association of GM3 with CD9 function was found in the following series of further studies, which will be discussed in more detail below:

1. CD9 and integrin α3 were co-immunoprecipitated in ldlD/CD9 cells when GM3 was synthesized (+Gal condition), but not when GM3 synthesis did not occur (–Gal condition). Interaction of GM3 with CD9, and CD9 with α3, were demonstrated by confocal microscopy. GM3/CD9/α3 is associated in the same microdomain, which is resistant to 1% Brij 98 but soluble in Triton X-100 (Kawakami et al., 2002). Since CD9 is chloroform/methanol soluble, its complex with GM3 or other gangliosides was expected, similarly to proteolipid protein (Folch and Lees, 1951).
2. Various colorectal tumor cell lines whose motility was clearly inhibited by exogenous GM3 addition were all characterized by high CD9 expression. Motility of a CD9-non-expressing tumor cell line was unaffected by GM3 addition, but became inhabitable by GM3 when CD9 was expressed by its gene transfection (Ono et al., 2001).
3. Addition of ^{3}H-labeled photoactivatable GM3 having ω-phenylazido acyl group to HRT18 cells, followed by UV irradiation, caused specific ^{3}H-labeling of CD9 but not other glycosynaptic proteins (α3, α5, or β1 integrin). However, other proteins were labeled by the probe (Ono et al., 2001).
4. Down regulation of GM3 synthesis is associated with oncogenesis in v-Jun transformation. Transfection of GM3 synthase gene resulted in reversion of oncogenic to normal phenotype in v-Jun-transformed chicken and mouse fibroblasts and inhibition of motility and invasiveness through formation of GM3/CD9/α5β1 complex (Miura et al., 2004).

5. Human diploid embryonal lung WI38 fibroblasts are highly contact-inhibitable cells. They are biochemically unusual in having high level of CD9 and CD81, which are complexed with FGFR. GM3, the major ganglioside in these cells, interacts specifically with FGFR, whereas other gangliosides and glycophingolipids do not. Since FGFR is closely associated with c-Src and GM3, cell contact induced by interaction of GM3 with FGFR may inhibit tyrosine kinase associated with FGFR as well as c-Src (Toledo et al., 2004). The exact mechanism for GM3 interaction with FGFR remains to be elucidated.
6. In a typical case with bladder cancer cells, decrease or depletion of GM3 by D-threo-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4) suppresses interaction of CD9 with integrin α3β1, leading to enhanced motility and invasiveness (Mitsuzuka et al., 2005). Such conversion of less malignant to highly malignant cell phenotype was also caused by decrease of CD9 by RNAi. Besides, exogenous addition of GM3 resulted in inhibition of motility in YTS1 cells. These results suggest that integrin/CD9/GM3 organized in membrane, "glycosynapse 3" (for review see (Hakomori, 2002)), may define tumor cell invasiveness. This is also consistent with previous observations that highly invasive YTS1 is reverted to less-invasive phenotype by enhanced GM3 expression induced by brefeldin A (Satoh et al., 2001). Moreover, Mitsuzuka and coworkers (Mitsuzuka et al., 2005) demonstrated that GM3 levels, in bladder cancer cells, define glycosynapse function by controlling the interaction of CD9 with integrin α3; and by modulating c-Src activity. Enhanced levels of GM3 induce csk translocation into glycosynapse resulting in phosphorylation on Tyr 527 of c-Src with consequent inhibition of c-Src activity and cell motility (Regina Todeschini and Hakomori, 2008).

3.3.2 GM2/GM3 complexed with CD82 inhibits cell motility

TSP CD82 was originally found as product of metastasis suppressing gene KAL-1, highly expressed in normal epithelial cells such as prostate, bladder, or colorectal epithelia and downregulated or depleted in their metastatic deposits (Adachi et al., 1996; Dong et al., 1995; Dong et al., 1996). CD82 is known to suppress cell invasiveness by inhibiting functional interaction of integrin with tyrosine kinase receptor for hypatocyte growth factor (HGF), hypatocyte growth factor receptor (Met) (Sridhar and Miranti, 2006). Met has been implicated in promotion of cancer cell motility and invasiveness; for review see (Birchmeier et al., 2003). In analogy with CD9, it is expected to observe an effect of glycosylation on CD82-dependent motility inhibition (Ono et al., 1999).

1. It is initially observed that GM2, but not GM3 or Gb4, specifically interacted with CD82 in normal bladder epithelial cell line HCV29, while GM3 showed specificity for CD9.
2. GM2/CD82 complex physically interacted with Met inhibiting functional interaction of integrin α3 or β1 with Met, whereby HGF-induced Met tyrosine phosphorylation was strongly suppressed.
3. Treating normal cells with P4, which depleted GM2, or abrogating CD82 expression by RNAi method, greatly enhanced HGF-induced Met phosphorylation and cell motility. In contrast, highly invasive bladder cancer cells, YTS1 (lacking CD82), were characterized by HGF-independent Met activation and cell motility. Met activation and cell motility were inhibited by co-expression and mutual interaction of GM2 with CD82, as observed in YTS1 cells transfected with CD82 gene; or by the exogenous addition of GM2 (Illmensee and Mintz, 1976).
4. YTS-1 cells, when adhered on LN5-coated plate, showed strong activation of Met phosphorylation without stimulation by HGF, and this process was promoted when

gangliosides were depleted by P4 treatment of YTS-1 cells. These results indicated that highly malignant cells are characterized by enhanced cross-talk between integrin and Met kinase. Such cross-talk in normal cells is minimal, but was greatly enhanced when GM2 was depleted by P4; i.e., CD82/GM2 complex plays a major role in inhibiting not only HGF-induced Met kinase activity but also LN5-induced cross-talk between integrin and Met (Todeschini et al., 2007).

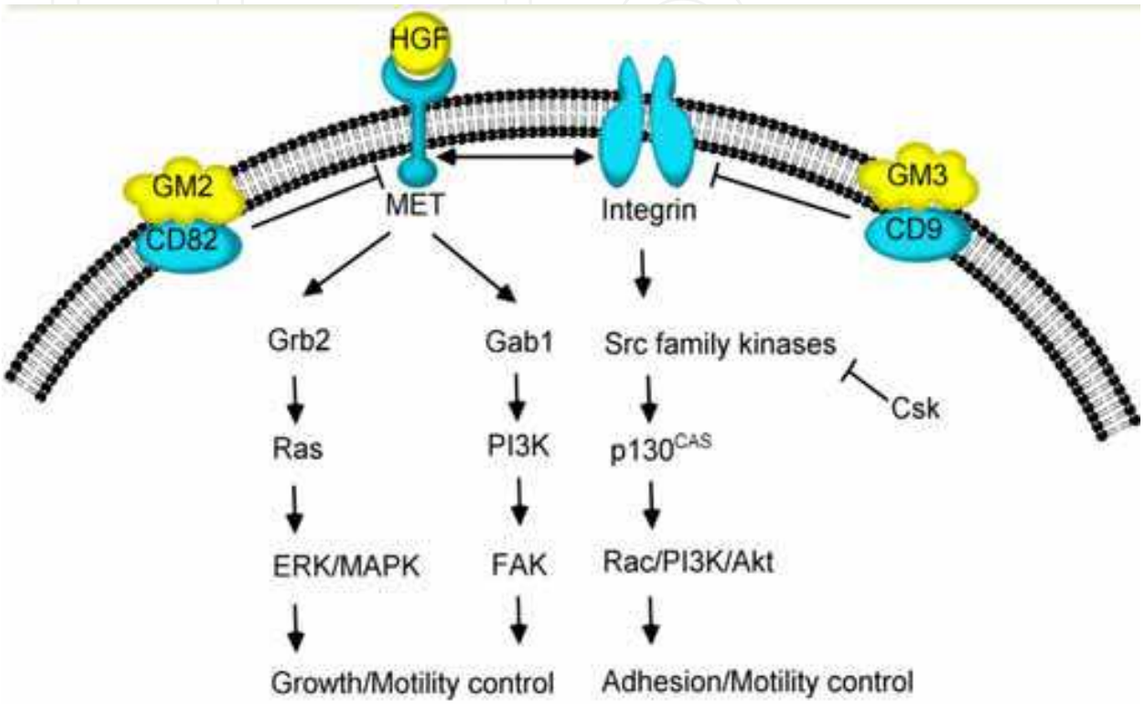


Fig. 3. Hypothetical associations among components of glycosynapse from bladder epithelial cells. Bladder epithelial cells express two major receptors as follows: 1) HGF receptor Met and its kinase (shown at left), which is inhibited by GM2-CD82 complex; 2) integrin receptor α3β1, which binds to extra cellular matrix component LN5/10-11 upon cell adhesion (shown at right). α3β1 activation is blocked by GM3-CD9 complex in bladder epithelial cells (Mitsuzuka et al., 2005). The functional interaction between systems 1 and 2 is blocked by GM2-CD82 complex. Signaling shown for both systems is arbitrary, based on a few previous reviews or studies by others and by our group (Birchmeier et al., 2003; Mitsuzuka et al., 2005). Grb2 and Gab1 are initial signaling molecules that may lead to activation of extra cellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK), PI3K, or FAK (Birchmeier et al., 2003), controlling cell growth and motility. α3β1 may act through Src family kinases (which are inhibitable by Csk) (Mitsuzuka et al., 2005; Toledo et al., 2004), and lead to Rak/PI3K/Akt signaling (Gu and Taniguchi, 2004), controlling cell adhesion and motility. From Todeschini et al.(Todeschini et al., 2007)

The molecular mechanism of GM2 inhibition of the HGF-Met signaling pathway leading to cell motility may be controlling the distribution of CD82 in- and outside of the glycosynapse; and interacting with CD82 in the glycosynapse forming the GM2/CD82 complex which acts as a functional constituent of the microdomain. Fig. 3 shows a

hypothetical scheme for this mechanism. Besides, inhibition of GM2/CD82 complex on Met activation, or on α3-to-Met interaction, may involve cis-carbonhydrate-carbonhydrate interaction (cis-CCI) between GM2 and N-linked glycan of CD82, since partial deletion of three N-linked glycans (at Asn129, 157, and 198) from mutant CD82 caused remarkable change in interaction with α3 and α5 integrins (Todeschini et al., 2007).

Further studies on effects of various gangliosides, and their combinations, on HCV29 cell motility, clearly indicate that GM2 together with GM3 (but not other gangliosides or GSLs, or their combinations) show stronger binding to CD82, compared to GM2 or GM3 alone, and based on the following observations:

1. GM2 binding to CD82 was greatly enhanced by addition of GM3, although GM3 per se did not bind to CD82 (Ono et al., 2000).
2. Cells expressing CD82, when cultured with silica nanospheres co-coated with GM2 and GM3, displayed much stronger inhibition of cell motility than those cultured with silica nanospheres coated with GM2 alone.
3. GM2/GM3 combination in the above process strongly inhibited phosphorylation of Src and MAPK.
4. ldlD mutant cells transfected with GM2 synthase gene showed greatly reduced motility when endogenous synthesis of both GM2 and GM3 occurred, as compared with cells grown under conditions in which only one of these gangliosides was synthesized.

In addition to functional changes 1) to 4) as above, a physical and chemical basis for interaction of GM2 and GM3 was provided by (a) electrospray ionization mass spectrometry (Ono et al., 2000), and (b) in situ cross-linking of cell surface GM2 and GM3 by periodate oxidation followed by succinyl dihydrazide (data not shown). Taken together, these results suggest the existence of heterotypic cis carbohydrate-to-carbohydrate interaction of GM2 and GM3, providing a basis for control of cell motility through inhibition of signal transduction (Regina Todeschini and Hakomori, 2008).

3.3.3 GM3 promotes cell motility via inducing matrix metalloproteinase (MMP-9) expression in melanoma B16 cells

As we know, the murine melanoma B16 cell line is characterized by its highly invasive and metastatic capacity. Growth factors, adhesion molecules, proteases, and other components are involved in the process of metastasis (Herlyn et al., 2002). MMP family members have been clearly shown to play an important role in this process (Hamilton et al., 1993; Tsuchida et al., 1987). Among the MMPs thus far studied, MMP-9 (gelatinase B) appears to have an important role in a wide array of physiological and pathophysiological processes, including pacental development, wound healing, angiogenesis, inflammation, tumor invasion, and metastasis (Van den Steen et al., 2002). Thus, studies of the mechanism(s) regulating the expression of MMP-9 are also important to the understanding of mechanisms underlying tumor metastasis.

MMP-9 secretion can be stimulated by interleukin 1β (IL-1B) (Librach et al., 1994), tumor necrosis factor (TNF) α (Meisser et al., 1999), HGF (Zhou and Wong, 2006), and EGF (Qiu et al., 2004). MMP-9 is stimulated in several cell lines via the PI3K/Akt signaling pathway (Shukla et al., 2007). Hyperactivated PI3K results in the activation of several transcriptional factors, such as nuclear factor (NF)-κB and activator protein (AP)-1, further leading to promotion of MMP-9 gene expression (Bancroft et al., 2002). Restoration of phosphatase and tensin homolog to hyperactivated PI3K cell lines reversibly suppresses MMP-9 expression. S6K located downstream of PI3K is involved in the regulation of MMP-9 expression

following stimulation with hepatocyte growth factor (Zhou and Wong, 2006). These lines of evidence clearly show that PI3K signaling pathway plays an important role in MMP-9 regulation.

Reports from several laboratories have concluded that MMP-9 expression is modulated not only by cytokines but also by gangliosides (Hu et al., 2007; Moon et al., 2004; Zhang et al., 2006). GM1, present in the glycolipid-enriched microdomain, is one of the crucial factors regulating cancer metastatic potential via the modulation of MMP-9 localization and secretion, as well as suppression of tumor invasion potential (Zhang et al., 2006). Overexpression of the GD3 synthase gene suppresses MMP-9 expression by inhibiting the combination between the MMP-9 promoter and transcription factors (NF-κB and AP-1) in vascular smooth muscle cells (Moon et al., 2004). In murine FBJ cells, GD1a is found to suppress MMP-9 expression at the transcriptional level (Hu et al., 2007). On the other hand, overexpression of plasma membrane-expressed sialidase Neu3 inhibits MMP-9 expression in vascular smooth muscle cells; implying gangliosides promote MMP-9 (Moon et al., 2007). Thus, there is no definite concept as to whether gangliosides positively or negatively regulate MMP-9 expression.

Among tumor-associated glycolipids, ganglioside GM3 is the simplest ganglioside in structure that resides in the membrane of murine melanoma B16 cells (Iwabuchi et al., 1998).

1. GM3 has been shown to regulate TNF α both at the transcriptional and translational levels in murine melanoma B16 cells (Wang et al., 2007b; Wang et al., 2007c). TNF α expression was increased by addition of GM3 to the B16 transfectants and decreased after treatment with D-PDMP, an inhibitor of glucosyl-ceramide synthesis. These results clearly indicate that GM3 positively regulates TNF α expression in B16 cells.
2. PI3K inhibitors, wortmannin and LY294002, suppressed the TNF α expression that is stimulated by GM3 in B16 cells, suggesting that the GM3 signal is located upstream of the PI3K-Akt pathway. GM3 was shown to increase phosphorylation of Akt. Treatment of B16 cells with small interfering RNA (siRNA) targeted to Akt1/2 resulted in TNF α suppression, indicating that Akt plays an important role in regulation of TNF α expression. Suppression of Akt1/2 rendered cells insensitive to GM3, suggesting that the GM3 signal may be transduced via Akt (Wang et al., 2007a).
3. Rapamycin suppressed TNF α expression, indicating mammalian target of rapamycin (mTOR) to be involved in the pathway. Either siRNA Raptor or siRNA Rictor suppressed TNF α expression, but the latter suppressed the effects of GM3 on TNF α expression and Akt phosphorylation at Ser473, indicating the GM3 signal to be transduced via mTOR-Rictor and Akt (Ser473), leading to TNF α stimulation. Finally, Ly-GDI, the tumor suppressor gene, whose expression is associated with GM3, was shown to be upstream of TNF α (Wang et al., 2007b). Thus, the GM3 signal is transduced in B16 cells through a PI3K, mTOR-Rictor, Akt, Ly-GDI pathway, leading to stimulated expression of TNF α.
4. Since TNF α is known to stimulate MMP-9 synthesis, which is highly involved in tumor cell metastasis, we investigated the possibility that MMP-9 is regulated by GM3. In the present study, MMP-9, but not MMP-2, messenger RNA (mRNA) expression was found to be consistent with GM3 levels in every B16-derived cell variant. GM3 has been suggested to stimulate the PI3K/Akt signaling pathway in previous investigations (Bremer et al., 1986; Choi et al., 2006). GM3 signals are thus transduced via the PI3K/Akt pathway, leading to the regulation of MMP-9 expression.

5. Most importantly, cell migration tested by transwell experiments showed that the numbers of cells migrating were consistent with MMP-9 expression (Wu et al., 2011). These data strongly suggest that capacity of cell migration in B16 cells is proportional to MMP-9 expression, which is under the positive control of GM3 (Fig. 4).

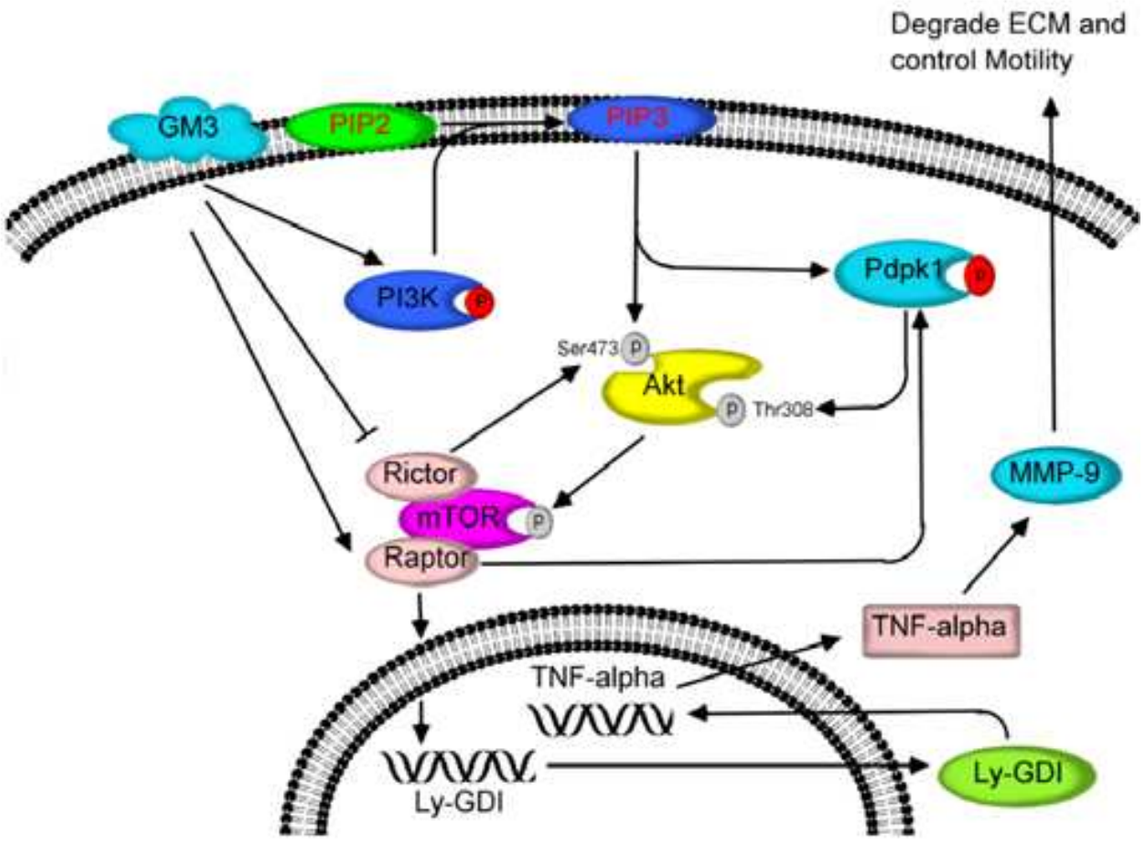


Fig. 4. Proposed cascade of signaling events regulating MMP-9 expression by GM3, which in turn promotes B16 cells motility via Ly-GDI. GM3 signals are transduced in B16 cells through PI3K, Pdk1, Akt, mTOR Raptor pathway, leading to the enhanced expression of Ly-GDI mRNA. Further data demonstrated that Ly-GDI located upstream of TNF α, which in turn regulate melanoma B16 cells motility via inducing MMP-9 secretion

3.4 Metastasis

Melanoma cells break the most basic rules of behavior by which multicellular organisms are built and maintained, and they exploit every kind of opportunity to do so. In studying the transgressions, we discover what the normal rules are and how they are enforced. Thus, in the context of cell biology, melanoma has a unique importance, and the emphasis given to melanoma research has profoundly benefited a much wider area of medical knowledge than that of melanoma alone.

Melanoma cells are defined by two heritable properties: they and their progeny (Hakomori, 1996) reproduce in defiance of the normal restraints on cell division and (Hakomori et al., 1998) invade and colonize territories normally reserved for other cells. It is the combination of these actions that makes cancers peculiarly dangerous. An isolated abnormal cell that

does not proliferate more than its normal neighbors does not significant damage, no matter what other disagreeable properties it may have; but if its proliferation is out of control, it will give rise to a tumor, a relentlessly growing mass of abnormal cells. As long as the tumor cells remain clustered together in a single mass, however, the tumor is said to be benign. At this stage, a complete cure can usually be achieved by removing the mass surgically. A tumor is considered a cancer only if it is malignant, that is, only if its cells have acquired the ability to invade surrounding tissue. Invasiveness usually implies an ability to break loose, enter the bloodstream or lymphatic vessels, and form secondary tumors, called metastasis, at other sites in the body. The more widely a cancer spreads, the harder it becomes to eradicate.

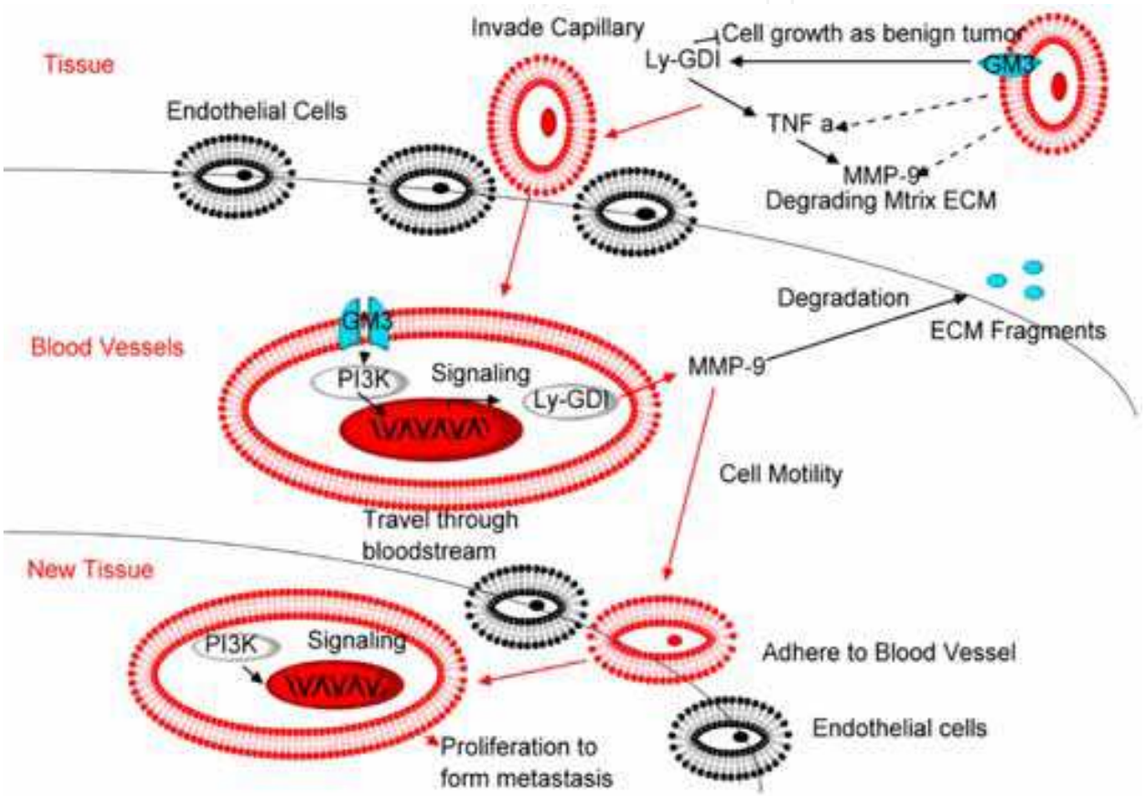


Fig. 5. Steps in the process of melanoma metastasis. This example illustrates the spread of a melanoma from an organ such as the lung or bladder to the skin. Tumor cells may invasively proliferate in the original tissue with inhibiting Ly-GDI expression. Then, tumor cells will enter the bloodstream directly by crossing the wall of a blood vessel, as diagrammed here, or, more commonly perhaps, by crossing the wall of a lymphatic vessel that ultimately discharges its contents (lymph) into the bloodstream. The motility of melanoma cells would be triggered by MMP-9 activation during this process. Finally, tumor cells that have entered a blood or lymphatic vessel will proliferate in a new tissue (skin) and finish the circle of metastasis

As discussed in this chapter, ganglioside GM3 is involved in every aspects of melanoma metastasis. Ly-GDI mediated melanoma invasive proliferation under rigorous conditions, which in turn benign tumor would form in tissues. At this stage, GM3 would concurrently

modulate melanoma cell adhesion via gangliosides interaction or modulating adhesive genes expression, through which mediate melanoma cells getting loose from orignional tisse or adhere to the new locations. Once the activity of MMP-9 was stimulated by GM3 in melanoma cells, MMP-9 will trigger the motility of melanoma cells throughout the bloodstream or lymphatic vessels, and form secondary tumors (Fig. 5). That means a tumor is considered as a cancer with metastasis. Although these steps are not separate and are always combination of these actions, our *in vitro* experiments have partially revealed the metastatic mechanism of melanoma B16 cells. These results clearly indicated that GM3 changed the nature of melanoma B16 cells. In addition, elucidation of the molecular mechanism of gangliosides modulating tumor phenotype will help to find new therapeutic targets or critical genes in cancer therapy.

4. Conclusion

Our results along with others' investigations have shown that GM3 is invovled in each step of metastasis in melanoma B16 cells. 1) GM3 regulatea B16 cell adhesion via gangliosides interaction or modulating adhesive gene expression, such as E-cadherin, N-Cam and Src. 2) GM3 is able to inhibit B16 cells invasive proliferation under soft agar or serum deprived medium via stimulating Ly-GDI expression. 3) MMP-9 is identified to mediate B16 cell motility via Tnf α. Therefore, GM3, predominantly expressed ganglioside in B16 cells, is the key molecule responsible for the phenotype or nature of melanoma cells.

5. Abbreviations

Abbreviations	Full Name
AMPK	AMP-activated protein kinase
AP-1	activator protein-1
CCI	carbonhydrate carbonhydrate interaction
ECs	endothelial cells
EGF	epithelial growth factor
EGFR	epithelial growth factor receptor
ERK/MAPK	Extracellular signal-regulated kinase/mitogen-activated protein kinase
4E-BP1	eIF4E binding protein
FAK	focal adhesion kinase
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GEMs	glycosphingolipids enriched microdomains
HGF	hypatocyte growth factor
IL-1B	Interleukin-1β
MET	hypatocyte growth factor receptor
MMP-9	matrix metalloproteinase 9
mTOR	mammalian target of rapamycin
PDGF	platelet derived growth factor
Pdk1	pyruvate dehydrogenase kinase
PI3K	phosphotidylinositol 3-kinase

PTEN	phosphatase and tensin homolog
P4	D-threo-1-phenyl-2-palmitoylamino-3-pyrolidino-1-propanol
Raptor	regulatory associated protein of mTOR
Rictor	rapamycin-insensitive companion of mTOR
SCC12F2	squamous carcinoma cell line
TNF α	Tumor necrosis factor alpha
TSC	tuberous sclerosis complex
TSP	tetraspannins

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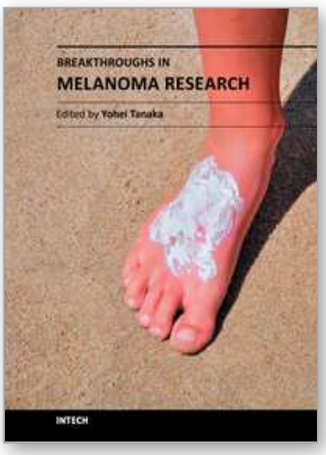
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Breakthroughs in Melanoma Research

Edited by Dr Yohei Tanaka

ISBN 978-953-307-291-3
Hard cover, 628 pages
Publisher InTech
Published online 30, June, 2011
Published in print edition June, 2011

Melanoma is considered to be one of the most aggressive forms of skin neoplasms. Despite aggressive researches towards finding treatments, no effective therapy exists to inhibit the metastatic spread of malignant melanoma. The 5-year survival rate of metastatic melanoma is still significantly low, and there has been an earnest need to develop more effective therapies with greater anti-melanoma activity. Through the accomplishment of over 100 distinguished and respected researchers from 19 different countries, this book covers a wide range of aspects from various standpoints and issues related to melanoma. These include the biology of melanoma, pigmentations, pathways, receptors and diagnosis, and the latest treatments and therapies to make potential new therapies. Not only will this be beneficial for readers, but it will also contribute to scientists making further breakthroughs in melanoma research.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Pu Wang, Sadako Yamagata and Tatsuya Yamagata (2011). Changing the Nature of Melanoma Cells by Manipulation of Ganglioside Expression, Breakthroughs in Melanoma Research, Dr Yohei Tanaka (Ed.), ISBN: 978-953-307-291-3, InTech, Available from: http://www.intechopen.com/books/breakthroughs-in-melanoma-research/changing-the-nature-of-melanoma-cells-by-manipulation-of-ganglioside-expression

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