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REVIEW

Gangliosides in Breast Cancer: New Perspectives

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Abstract—Gangliosides are essential compounds of the plasma membrane involved in cell adhesion, proliferation, and recognition processes, as well as in the modulation of signal transduction pathways. These functions are mainly supported by the glycan moiety, and changes in the structure of gangliosides occur under pathological conditions including cancers. With progress in mass spectrometric analysis of gangliosides, the role of gangliosides in breast cancer progression was recently demonstrated. In this review, we summarize current knowledge on the biosynthesis of gangliosides and of the role of disialogangliosides in triple-negative breast cancer progression and metastasis. New perspectives in breast cancer therapy targeting gangliosides are also discussed.

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Gangliosides define a subclass of glycosphingolipids (GSLs) characterized by the presence of one or several sialic acid residues in the carbohydrate moiety. In mammals, they are essential compounds of the outer leaflet of the plasma membrane, where they interact with other sphingolipids, cholesterol, and transmembrane proteins including receptors or signal transducers, forming lipid rafts. Gangliosides were demonstrated to be central molecules in the plasma membrane involved in cell adhesion, proliferation, and recognition processes, as well as in the modulation of signal transduction pathways [1, 2]. These different functions are mainly supported by the glycan moiety, and changes in the structure of gangliosides can occur under pathological conditions, including atherosclerosis, neurodegenerative disorders, and cancers [3-5]. In particular, the neo-expression of disialogangliosides has been demonstrated in several neuroectoderm-derived tumors in which they play a key role in invasion and metastasis [6], making disialogangliosides attractive target molecules for cancer immunotherapy [7, 8].

GANGLIOSIDES STRUCTURE AND BIOSYNTHESIS

Most of the structural variability of GSLs is borne by the carbohydrate domain that exhibits staggering structural diversity. As a result, GSLs are classified in a number of series defined by their monosaccharide sequences that include (iso)globo, (iso)ganglio, (neo)lacto, (neo)gala, and muco for the most abundant series in vertebrates (table) [9]. Although some gangliosides derive from lacto-, neolacto-, and globo-series, most belong to the ganglio-series GSLs. Gangliosides display carbohydrate core sequence of variable length that can be differently sialylated quantitatively (number of sialic acid residues) and qualitatively (position of sialic acid residues) (Fig. 1). The main core sequence is the tetrasaccharide Galβ1-3GalNAcβ1-4Galβ1-4Glcβ (Gg_4), but the di-, tri-, and tetrasaccharide versions usually coexist within a single cell type, whereas the total number of substituting sialic acids varies from 0 (which should formally not qualify as gangliosides) to 5.

The biosynthesis of gangliosides starts by the transfer in the *cis*-Golgi of a glucose residue onto ceramide (Cer) by the UDP-Glc:N-acylsphingosine β-D-gluco-

Abbreviations: DHB, 2,5-dihydroxybenzoic acid; ER, estrogen receptor; ESI, electrospray ionization; GSLs, glycosphingolipids; GTs, glycosyltransferases; HPLC, high-performance liquid chromatography; IMS, imaging mass spectrometry; mAb, monoclonal antibodies; MALDI, matrix-associated laser desorption-ionization; MS, mass spectrometry; shRNA, short hairpin RNA; siRNA, small interfering RNA; TLC, thin layer chromatography.

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Major series of glycosphingolipids in animals

Series	Structure	Abbreviation
Globo/isoglobo	Galα1-4Galβ1-4/3Glcβ1-Cer	Gb/iGb
Lacto/neolacto	Galβ1-3/4GlcNAcβ1-3Galβ1-4Glcβ1-Cer	Lc/nLc
Ganglio/isoganglio	GalNAcβ1-4/3Galβ1-4Glcβ1-Cer	Gg/iGg
Muco	Galβ1-4Galβ1-4Glcβ1-Cer	Mc
Gala	Galα1-4Glcβ1-Cer	Ga

syltransferase (GlcCer synthase) encoded by the *UGCG* gene [10]. The glucosylceramide synthase is highly specific for ceramide and can be inhibited by D,L-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) or D,L-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP), blocking the synthesis of almost all GSLs [11]. The next step is the galactosylation of glucosylceramide (GlcCer) to lactosylceramide (LacCer) by the UDP-Gal:GlcCer β1,4-galactosyltransferase (LacCer synthase) [12, 13]. The transfer of sialic acid residues to LacCer is then catalyzed by the sialyltransferases ST3Gal V (G_{M3} synthase), ST8Sia I (G_{D3} synthase), and ST8Sia V (G_{T3} synthase), all showing high specificity toward glycolipid substrates [14]. The human ST3Gal V was shown to use only LacCer as an acceptor substrate to synthesize G_{M3} ($II^3Neu5Ac_1$-Gg_2Cer) [15]. The G_{D3} synthase (GD3S) ST8Sia I is also highly specific to G_{M3} [16], but the human enzyme was also shown to use G_{D3} ($II^3Neu5Ac_2$-Gg_2Cer) to synthesize G_{T3} ($II^3Neu5Ac_3$-Gg_2Cer) [17]. The human ST8Sia V exhibits broader enzymatic activity toward gangliosides, using G_{D3}, but also G_{M1b} ($IV^3Neu5Ac_1$-Gg_4Cer), G_{D1a} ($IV^3Neu5Ac_1II^3Neu5Ac_1$-Gg_4Cer), or G_{T1b} ($IV^3Neu5Ac_1II^3Neu5Ac_2$-Gg_4Cer) as acceptors [18]. Thus, LacCer, G_{M3}, G_{D3}, and G_{T3} are the precursors for the 0-, a-, b-, and c-series gangliosides, respectively [19], and the biosynthesis of these compounds determines the relative proportion of gangliosides in each series (Fig. 1).

Afterwards, GalNAc, Gal, and Neu5Ac residues can be transferred in a stepwise manner by the β1,4-N-acetylgalactosaminyltransferase I (G_{M2}/G_{D2} synthase) [20], the β1,3-galactosyltransferase IV [21], and different sialyltransferases (Fig. 1). The β1,4-N-acetyl-galactosaminyltransferase I is active on the four series of gangliosides and converts LacCer, G_{M3}, G_{D3}, and G_{T3} into G_{A2} (asialo-G_{M2}), G_{M2}, G_{D2}, and G_{T2}, respectively [22, 23]. Similarly, the β1,3-galactosyltransferase IV equally uses G_{A2}, G_{M2}, G_{D2}, and G_{T2} as acceptor substrates [22]. The terminal Gal residue can be further used as acceptor substrate by an α2,3-sialyltransferase. Both ST3Gal I and ST3Gal II were shown to transfer a sialic acid residue onto Galβ1-3GalNAc disaccharide sequence [24, 25]. However, based on kinetic parameters and tissue distribution, it appears that ST3Gal II is largely responsible for ganglioside terminal α2,3-sialylation [26]. Finally, the terminal trisaccharide Neu5Acα2-3Galβ1-3GalNAc can be further substituted by another sialic acid residue in α2,8-linkage by ST8Sia V [18] or in α2,6-linkage to the GalNAc residue of G_{M1b}, G_{D1a}, or G_{T1b} (Fig. 1) to form α-series gangliosides. Three members of the CMP-Neu5Ac:β-N-acetylgalactosaminide α2,6-sialyltransferase family (ST6GalNAc III, V, and VI) were shown to catalyze *in vitro* the transfer of a sialic acid residue onto G_{M1b} ($IV^3Neu5Ac_1Gg_4$-Cer) to form $G_{D1\alpha}$ [27]. However, according to its substrate specificity and expression pattern, ST6GalNAc V is generally considered as the main enzyme forming the α-series gangliosides [28].

Normal human tissues mainly express "simple" gangliosides from the 0- and a-series, whereas "complex" gangliosides from the b- and c-series are essentially restricted to the nervous system of healthy adults but can be re-expressed in several types of cancer, including melanoma and brain tumors [29]. The enzymes of ganglioside biosynthesis are typical type II membrane-anchored glycosyltransferases (GTs) showing a gradient distribution within the Golgi apparatus and forming functional complexes, such as those shown for LacCer synthase, G_{M3} synthase, and G_{D3} synthase that are associated in multi-enzymatic complexes in the *cis*-Golgi [30]. These complexes are thought to act without releasing intermediate structures, ensuring the biosynthesis of a clearly defined ganglioside end-product. The regulation of GTs involved in the synthesis of gangliosides is mainly achieved at the transcriptional level, and GT gene expression is also tissue-specific. For example, the *B4GALNACT1* gene is essentially expressed in human brain, lung, and testis, whereas *ST3GAL5* is expressed in almost all human tissues [15, 31].

RECENT PROGRESS IN MASS SPECTROMETRIC ANALYSIS OF GSLs

Despite their intrinsic structural variability, GSLs can be easily extracted and separated by a wide panel of chromatographic techniques. Most of the purification protocols developed through the years since the isolation of homogenous ganglioside fractions in the forties are based on organic solvent extractions, and they have not significantly evolved recently. They have been comprehensively presented by R. L. Schnaar and by S. I. Hakomori [32, 33]. The majority of protocols used today, such as the Svennerholm and Fredman method [34], take advantage of the amphiphilic nature of glycosphingolipids

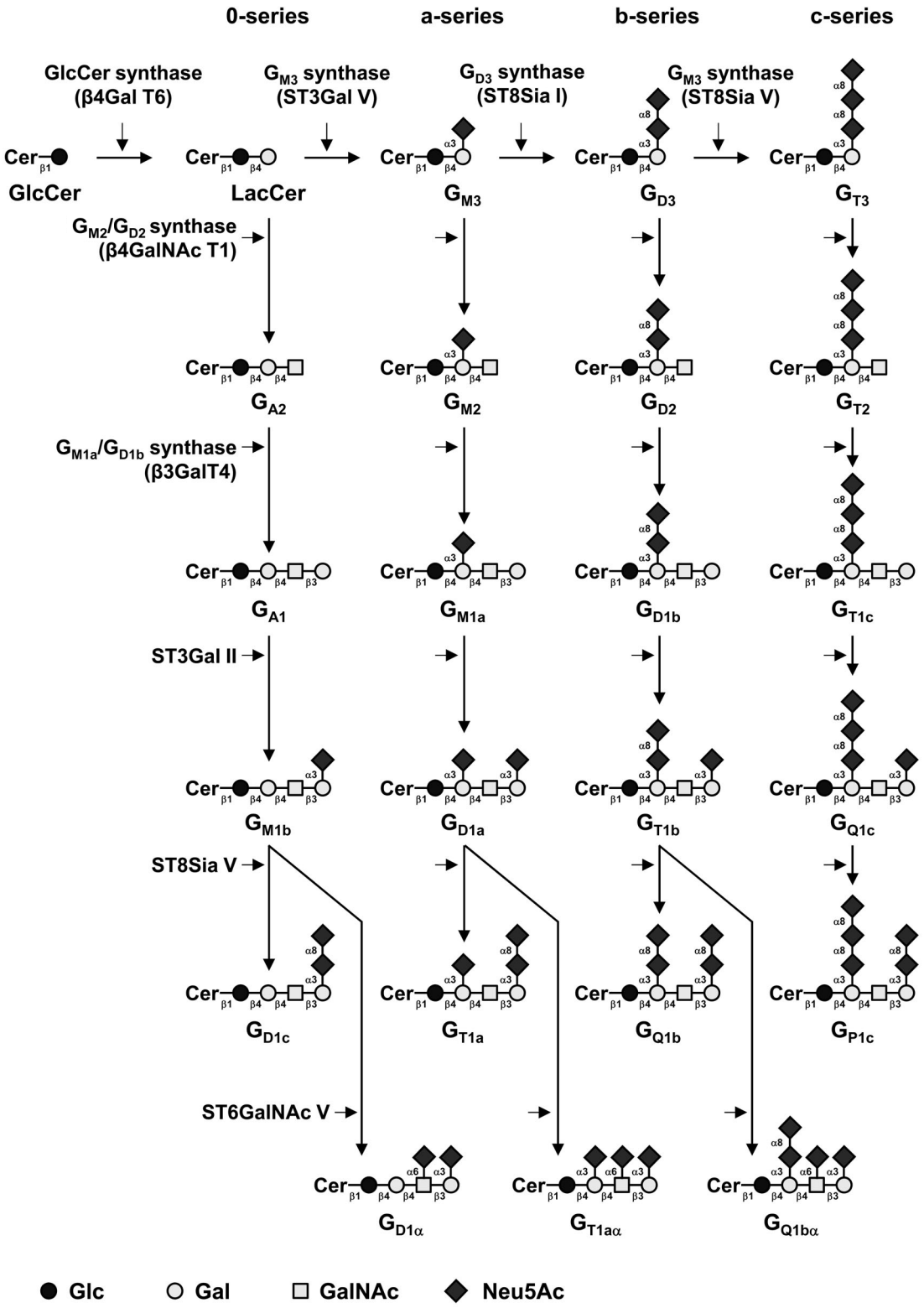

Fig. 1. Biosynthesis pathways for gangliosides. Gangliosides are synthesized by stepwise addition of monosaccharides to ceramide. Ceramide (Cer) is the acceptor for UDP-Glc:ceramide β-D-glucosyltransferase. Extension of GlcCer occurs by the action of UDP-Gal:GlcCer β1,4-galactosyltransferase to make lactosylceramide (G_{A3}). The action of ST3Gal V (G_{M3} synthase), ST8Sia I (G_{D3} synthase), and ST8Sia V (G_{T3} synthase) leads to the biosynthesis of the precursors of a-, b-, and c-series gangliosides, respectively. The 0-series gangliosides are directly synthesized from lactosylceramide. Elongation is performed by the sequential action of N-acetyl-galactosaminyltransferase (β4GalNAc T1), galactosyltransferase (β3Gal T4), and sialyltransferases (ST3Gal II and ST8Sia V). α-Series gangliosides derive from the action of ST6GalNAc V on G_{M1b}, G_{D1a}, or G_{T1b}. The code names of gangliosides are according to Svennerholm [19]. Cer, ceramide; LacCer, lactosylceramide.

through the extraction of tissues or cells by monophasic or diphasic mixtures of chloroform, methanol, and water. Other solvents such as tetrahydrofuran, ether, or butanol have also been used. To these, salts (KCl or phosphate salts usually) can be added to improve the extraction yield of the most polar compounds such as sulfated or sialylated glycolipids [35]. In addition, a number of chromatography media can be used to further purify and separate bulk glycosphingolipids, including anion-exchange (DEAE-Sephadex, Q-Sepharose, etc.), salicylic acid, or reverse-phase. Bulk separation can be coupled to mild-alkaline saponification to clean up triglycerides, but at the expense of alkali-labile functions such as acetyl groups.

Most of the recent progress has been observed in the analytical field and has been driven by the rapid pace of development and dissemination of mass spectrometry (MS)-associated technologies in research laboratories. Not only that, the constant improvement of mass spectrometers in terms of resolution, sensitivity, and analysis speed has dramatically improved the quality of robustness of the structural analyses, but it has also provided novel opportunities. The main technical shortcomings of MS for the analysis of GSLs are their intrinsic diversity borne by both the lipid and the sugar moieties and their amphiphilic properties, which render their handling difficult. Within the scope of this review, we will focus on recent technical advances that have helped to tackle these two aspects.

MALDI-MS. Compared with liquid sources, MALDI sources neutralized all the problems linked to the handling of organic solvents by co-crystallization of the compounds with a matrix, and thus they rapidly appeared as a method of choice for the analysis of GSLs. However, severe losses of sialic acid during the laser-induced desorption from gangliosides and other sialylated GSLs has been a nagging problem for analysts. One solution is to stabilize the sialic acids by specific carboxymethyl esterification [36, 37] or permethylation of the molecules [38]. In particular, permethylation not only provides robust simultaneous semiquantitative profiles of both neutral and negatively charged sialylated species without loss of sialic acid [39], but also yields more sequence-informative fragmentation patterns in CID MS/MS than non-permethylated glycans. In parallel, intense efforts have been made to minimize the cleavage of very labile glycosidic bonds without chemically modifying GSLs by testing a vast panel of so-called "cold" matrixes. Doing this, numerous dry droplet and ionic liquid matrixes were shown to produce less fragmentation for ganglioside than 2,5-dihydroxybenzoic acid (DHB) does. This is the case for 5-methoxysalicylic acid (MSA), matrix preparation of 2,9-dihydroxyacetophenone (DHA)/ammonium sulfate/heptafluorobutyric acid (HFBA), or 2,5-dihydroxybenzoic acid butylamine (DHBB) that appear to generate better quality spectra of gangliosides than DHB [40-42].

Tissue imaging. Imaging mass spectrometry (IMS) using MALDI-MS technology has recently become a powerful tool for label-free visualization of biomolecules, including glycosphingolipids, on tissue slides. Most MALDI imaging mass spectrometers presently exhibit lateral resolution between 10 and 50 μm. Compared with the classical histochemical approach, it not only distinguishes gangliosides by their oligosaccharide moieties, but also by the nature of their ceramides. Furthermore, the use of tandem mass spectrometry can provide detailed information on the ganglioside structures. As for classical MALDI-MS, experimental efforts have been made to substitute the initially used DHB [43] by colder matrixes to minimize the loss of sialic acids from gangliosides [41, 44, 45]. Alternatively, a softer ionization method than MALDI, the newly developed Laserspray Ionization Inlet (LSII) demonstrated its usefulness in the field of IMS by providing imaging of disialylated GD1 without any visible fragmentation event [46]. These technological developments enabled several groups to convincingly demonstrate that gangliosides were differentially distributed in mouse brain in diverse pathological or environmental conditions [43, 47]. Finally, further studies recently demonstrated that the lateral resolution of ganglioside MALDI imaging could be enhanced down to 10 μm by using an oversampling method, which will open the possibility to achieve cellular resolution in the near future [48, 49].

TLC-MS coupling. No analytical technique other than thin-layer chromatography has had such long-lasting success in the field of structural analysis of GSLs. Compared with HPLC, it shows many advantages such as cost effectiveness, simplicity, single-run comparison of samples, and quantification by the colorimetric method. Indeed, although very simple to put into practice, neutral and sialylated GSLs can be reproductively separated with high resolution on silica gel 60 HPTLC plates after development in chloroform–methanol–water (e.g. 65 : 25 : 4 v/v) or chloroform–methanol–0.2% $CaCl_2$ (e.g. 60 : 42 : 10 v/v). Thus, for decades it has been used both for analytical purposes using standard GSLs and for preparative purposes. Considering the wide distribution of this method, ways to combine TLC and MS analysis were developed early, but the technical challenge lies in the efficient extraction of GSLs from the stationary phase to introduce them into the mass spectrometer. The readers can refer to a recent publication that summaries most of the technological development of TLC-MS interfacing [50].

The earlier process for collecting GSLs for MS analysis relies on scraping the stationary phase (most often silica gel for GSLs) and eluting the molecules with organic solvents. Although presenting many drawbacks (low yield, labor intensiveness, restriction to major components), this technique is still effectively used to produce high quality structural data [51]. Direct sampling of GSLs

from TLC in the MALDI source was developed early but was mostly restricted to neutral GSLs [52, 53] because of the high risk of sialic acid loss during laser-induced desorption. An alternative procedure using blotted gangliosides on PVDF membranes following TLC separation, although promising, exhibited similar desialylation [54]. Loss of sialic acids could, however, be minimized when using specific devices such as orthogonal or vibrational cooling MALDI sources [55, 56]. However, these high-end equipment requirements drastically reduce the widespread application of this method for the analysis of gangliosides to a handful of very specialized laboratories.

What seems to be the most promising recent development of TLC-MS interfacing in the field of gangliosides was achieved by coupling commercially available liquid extraction surface automated devices to an electrospray ionization MS source [57]. Indeed, the two surface extraction systems, namely the TLC-MS interface from CAMAG and the Liquid Extraction Surface Analysis (LESA®; CAMAG, Switzerland), showed reliable semi-automated extraction abilities toward gangliosides that could be injected online into a Q-q-TOF MS spectrometer to be analyzed by MS and MS/MS. Owing to the softer ionization procedure than MALDI-MS, gangliosides could be observed with minimal fragmentation, which opens up broad application for TLC-ESI-MS analysis of gangliosides.

LC-ESI-MS. As mentioned above, ESI-MS appears as the method of choice for ionizing gangliosides under soft conditions, and it has been heavily used for structural study of GSLs. Although it offers improved sensitivity and selectivity over TLC-based technologies, the application of LC-ESI-MS to the study of gangliosides lagged behind other biomolecules (peptides, oligosaccharides, etc.) because of the lack of resolution of liquid chromatography columns for gangliosides. Nonetheless, several studies have reported the use of LC-MS for the identification and quantification of gangliosides from complex biological matrixes such as dairy products [58], human plasma [59], or rat brains [60] using either reverse- or normal-phase columns. Recent developments in LC-MS analysis of gangliosides have focused on the use of nano-HPLC chips that can deliver high retention time and abundance reproducibility [61] and on rapid ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS) [62].

ST8Sia I IS THE KEY ENZYME FOR BIOSYNTHESIS OF b- AND c-SERIES GANGLIOSIDES

ST8Sia I is the only sialyltransferase catalyzing the transfer of a sialic acid residue onto G_{M3} through an α2,8-linkage to synthesize G_{D3}. Whereas ST8Sia I mainly sialylates G_{M3}, its ability to synthesize G_{T3} from G_{D3}, as well as the unusual tetra- and pentasialylated lactosylceramide derivatives G_{Q3} ($II^3Neu5Ac_4$-Gg_2Cer) and G_{P3} ($II^3Neu5Ac_5$-Gg_2Cer) has also been demonstrated [17, 63]. ST8Sia I was also shown to use G_{M1b}, G_{D1a}, or G_{T1b} as acceptor substrates to synthesize G_{D1c}, G_{T1a}, or G_{Q1b}, respectively, both *in vitro* and *in vivo* [64]. However, the α2,8-sialyltransferase ST8Sia V is a much better candidate for G_{T1a}/G_{Q1b} synthase activity [18], and no ST8Sia V activity was detected toward G_{M3}. ST8Sia I is therefore considered as the only GD3S that controls the biosynthesis of b- and c-series gangliosides.

The human GD3S cDNA was simultaneously isolated by expression cloning by three research groups [16, 65, 66]. The *ST8SIA1* gene is located on chromosome 12, in p12.1-p11.2, and consists of five coding exons (E1 to E5) spanning 135 kb of genomic DNA [67]. The *ST8SIA1* gene encodes a typical type II 341 amino-acid protein having a 12-amino-acid cytoplasmic tail, a transmembrane domain of about 20 residues, and a Golgi catalytic domain containing the conserved sialyl motifs involved in substrate binding and transfer [27]. GD3S is expressed in fetal brain at an early developmental stage, where gangliosides play a key role in cell–cell interactions, cell differentiation, and proliferation [68, 69]. In adult human tissues, GD3S transcripts are significantly detected in brain [17]. GD3S has also been shown to be overexpressed in neuroectoderm-derived malignant tumors such as melanoma, glioblastoma, and neuroblastoma [29]. The promoter region controlling *ST8SIA1* gene expression lacks a TATA or CCAAT box, as commonly observed for *GT* genes, but contains several SP1 sites. Electrophoretic mobility shift assay (EMSA) and mutagenesis experiments have demonstrated the key role of NFκB in activating the expression of *ST8SIA1* in melanoma cells [70], and the essential role of AREB6 and Elk-1 transcription factors was also demonstrated for in *ST8SIA1* expression in glioblastoma cells [71].

DISIALOGANGLIOSIDES AND BREAST CANCER

Breast tumor tissues were shown to be distinct from normal mammary tissues in terms of ganglioside composition. The gangliosides G_{M3}, G_{D3}, and their derivatives 9-O-acetyl-G_{D3} (CDw60 antigen) and 9-O-acetyl-G_{T3}, which show a very restricted expression in normal breast tissues, are overexpressed in about 50% of invasive ductal carcinoma [72]. N-Glycolyl-G_{M3} is also detected in 100% of stage II breast cancers [73]. Neu5Gc is normally absent in humans due to the irreversible inactivation of the *CMAH* gene encoding the CMP-Neu5Ac hydroxylase. The lack of the enzyme responsible for the conversion of CMP-Neu5Ac into CMP-Neu5Gc results in the total absence of Neu5Gc in healthy human tissues. In contrast, Neu5Gc was shown to be expressed on glycoproteins and gangliosides in melanoma and colon, retinoblastoma, and breast cancers.

The expression of GTs implicated in ganglioside biosynthesis is also altered in breast cancer tumors. Microarray analysis of more than one thousand tumor samples in different subtypes of breast cancer has shown that GD3S displayed higher expression among estrogen receptor (ER)-negative breast cancer tumors [74]. *ST8SIA1* overexpression was associated with poor pathohistological grading in ER-negative tumors and lower survival rate of patients [75]. In contrast, better prognosis for ER-positive samples exhibiting high expression of *ST8SIA1* was described [75]. GD3S was also shown to be five-fold increased in a variant of MDA-MB-231 breast cancer cell line that colonizes bone [76]. It has been shown that G_{M3} synthase silencing in mammary murine 4T1 cells significantly inhibited cell migration, invasion, and anchorage-independent growth *in vitro*, as well as lung metastasis *in vivo*. In parallel, overexpression of G_{M3} synthase in non-metastatic 67NR cells significantly restored the malignant phenotype [77].

Recent data have shed light on the important role of disialoganglioside G_{D2} in breast cancer. The expression of the GD3S in MDA-MB-231 breast cancer cells induced the accumulation of b- and c-series gangliosides (G_{D3}, G_{D2}, and G_{T3}) at the cell surface together with the acquisition of a proliferative phenotype in the absence of serum or exogenous growth factors [78]. GD3S expressing cells bypass the need for growth factors for cell growth by specific and constitutive activation of the c-Met receptor in the absence of its ligand, hepatocyte growth factor/scatter factor (HGF/SF), and the activation of the phosphoinositide 3-kinase (PI3K)/Akt and extracellular signal-regulated kinase (Erk)/mitogen-activated protein kinase (MAPK) pathways [79]. GD3S expression also enhanced tumor growth in severe combined immunodeficient (SCID) mice, and a higher expression of *ST8SIA1* and *MET* in the "basal-like" subtype of human breast tumors was observed [79]. The decrease of G_{D2} expression by small interfering RNA (siRNA) silencing of the G_{M2}/G_{D2} synthase reversed the proliferative phenotype as well as c-Met phosphorylation, and competition assays using anti-G_{D2} monoclonal antibodies also inhibited cell proliferation and c-Met phosphorylation [80] (Fig. 2), demonstrating the involvement of the disialoganglioside G_{D2} in MDA-MB-231 cell proliferation *via* the constitutive activation of c-Met. The accumulation of G_{D2} in c-Met expressing cells

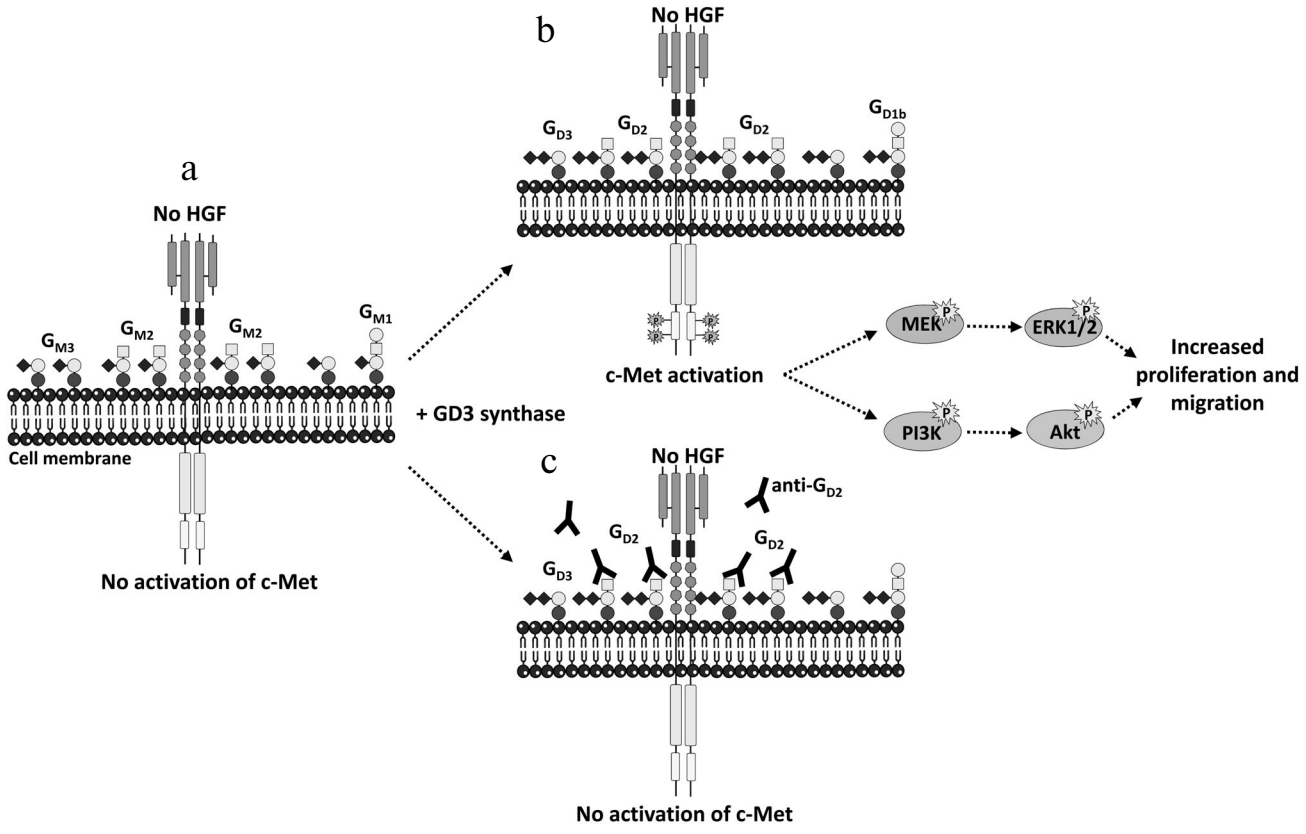

Fig. 2. Activation of c-Met by G_{D2} ganglioside. a) MDA-MB-231 breast cancer cells express mainly G_{M3} and G_{M2}. b) The expression of GD3S induces the accumulation of b- and c-series gangliosides, mainly G_{D2}. This leads to the activation of c-Met in the absence of HGF and increases proliferation and migration through the PI3K/Akt and MEK/Erk pathways. c) Anti-G_{D2} mAb used in competition assays inhibits c-Met phosphorylation and cell proliferation [79, 80].

could therefore reinforce the tumorigenicity and aggressiveness of breast cancer tumors.

Ganglioside G_{D2} was recently identified as a new specific cell surface marker of $CD44^{hi}CD24^{lo}$ breast cancer stem cells (CSC) from human breast cancer cell lines and patient samples that are capable of forming mammospheres and initiating tumors [81]. Gene expression analysis revealed that several GT genes involved in G_{D2} biosynthesis (*ST3GAL5*, *B4GALNT1*, and *ST8SIA1*) are highly expressed in CSC [81, 82]. The reduction of G_{D2} expression by *ST8SIA1* knockdown reduced mammosphere formation and cell motility and completely abrogated tumor formation *in vivo*, changing the phenotype from CSC to non-CSC [81, 82]. Moreover, the induction of epithelial–mesenchymal transition (EMT) in transformed human mammary epithelial cells dramatically increased GD3S as well as G_{D2} expression, whereas the inhibition of GD3S compromised EMT initiation and maintenance and prevented metastasis [83]. Since GD3S expression correlated with the constitutive activation of the c-Met signaling pathway leading to increased stem cell properties and metastatic competence, the GD3S/c-Met axis could serve as an effective target for the treatment of metastatic breast cancer.

ALPHA-SERIES GANGLIOSIDES IN BREAST CANCER

Alpha-series gangliosides define a particular sub-class of GSL containing Neu5Ac residue α2,6-linked to the GalNAc of the gangliopentaosyl backbone Neu5Acα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ ($IV^3Neu5Ac_1Gg_4$). The typical α-series ganglioside $G_{D1\alpha}$ ($IV^3Neu5Ac_1$, $III^6Neu5Ac_1Gg_4$-Cer) was first isolated as a minor compound from rat ascites hepatoma AH 7974F cells [84] and from bovine brain [85], with expression restricted to particular cell populations of the forebrain, the midbrain, and the cerebellum [86]. Amongst the ST6GalNAc sialyltransferases, ST6GalNAc V is generally considered as the main $G_{D1\alpha}$ synthase catalyzing the transfer of a sialic acid residue onto G_{M1b} ($IV^3Neu5Ac_1Gg_4$-Cer) to form $G_{D1\alpha}$. ST6GalNAc V cDNA was cloned from mouse brain [28, 87], and the *st6galnac5* gene is specifically expressed in brain tissues, mostly in forebrain and cerebellum [87]. When expressed as a soluble recombinant protein, the mouse ST6GalNAc V showed α2,6-sialyltransferase activity almost exclusively for G_{M1b}, while being inactive toward glycoproteins [28]. However, it was shown that transfection of human ST6GalNAc V into U373MG glioma cells produced the unusual α2,6-monosialoganglioside $G_{M2\alpha}$ (Neu5Acα2-6GalNAcβ1-4Galβ1-4Glcβ1-Cer, $III^6Neu5Ac_1Gg_3$-Cer) instead of $G_{D1\alpha}$ [88].

To date, little is known concerning the specific function of α-series gangliosides, but recent evidence indicates a possible function in the adhesion of cancer cells to endothelium. In highly metastatic murine lymphosarcoma cell line RAW117-H10, GD1α serves as an adhesion molecule to hepatic sinusoidal endothelial cells (HSE) [89]. Incubation with $G_{D1\alpha}$ oligosaccharide or with anti-$G_{D1\alpha}$ monoclonal antibody inhibited the adhesion between the RAW117-H10 and HSE cells. Recently, *ST6GALNAC5* was identified as one of the genes overexpressed in breast cancer cell populations selected for their ability to produce brain metastasis [90]. Short hairpin RNA (shRNA) inhibition of *ST6GALNAC5* decreased the brain metastatic capacity of breast cancer cells, whereas the expression of *ST6GALNAC5* in parental cell lines mediated brain metastasis [90]. It was also demonstrated that ST6GalNAc V expression enhanced the adhesion of breast cancer cells to brain endothelium and passage through the blood–brain barrier, Moreover, *ST6GALNAC5* is the only gene specifically correlated with brain metastasis of breast cancer and is upregulated in human brain metastasis samples. However, the capacity of human breast cancer cells expressing *ST6GALNAC5* to produce α-series gangliosides remains to be clearly demonstrated.

THERAPEUTIC PERSPECTIVES

Innovative therapeutic tools based on the use of anti-tumor-associated ganglioside mAbs have been developed and are currently under investigation in preclinical or clinical studies, especially in the field of neuroectoderm-related cancers (melanoma, neuroblastoma, small cell lung cancer) [7, 8]. As an example, anti-G_{D2} clinical trials for neuroblastoma have confirmed the efficacy of anti-G_{D2} antibody immunotherapy for this rare but often lethal childhood cancer [91]. Racotumomab, an anti-idiotypic antibody registered under the trade name Vaxira [92], and Neu5Gc-G_{M3}/VSSP, a Neu5Gc-G_{M3} ganglioside conjugated to proteoliposomes, have shown efficacy for patients with advanced melanoma [93] and non-small-cell lung cancer [94]. To date, no clinical trial has addressed the possible use of anti-ganglioside antibodies in breast cancer treatment. Only a phase I clinical trial has shown that the 14F7 mAb specific for Neu5Gc-G_{M3} could target human breast carcinoma [73]. The recently demonstrated involvement of the G_{D2}/Met axis in ER-negative breast cancer aggressiveness strongly supports that anti-G_{D2} immunotherapeutic approaches could be useful in breast cancer treatment. In parallel, recent findings strongly suggest that c-Met itself may be a valuable therapeutic target in triple negative breast cancers, since high expression of c-Met in breast cancer was correlated with poor overall survival ($p = 0.001$) and disease-free survival ($p = 0.01$) [95]. In addition, c-Met expression was relatively high in triple negative breast cancers cell lines, and its silencing using siRNA reduced cell proliferation and migration [95]. Other results demonstrated that miR-185, a micro RNA whose expression is downregulated in breast cancer

tissues, inhibited the proliferation of breast cancer cells by regulating the expression of c-Met, confirming its potential as a therapeutic target for breast cancer [96]. As mentioned before, GD3S expression and generated G_{D2} ganglioside correlated with the constitutive activation of the c-Met signaling pathway leading to increased metastatic competence of breast cancer cells. G_{D2} overexpression enhanced stem cell properties and might be responsible for the aggressive nature of triple negative breast cancers and the poor clinical outcome observed, suggesting that GD3S and the c-Met axis could be synergically targeted for the treatment of metastatic breast cancer [83] using, for example, anti-Met and anti-G_{D2} mAbs. Because of its restricted normal tissue distribution, ganglioside G_{D2} has been proven safe for mAb targeting, and anti-G_{D2} mAb treatment is now part of the standard care for the treatment of high-risk metastatic neuroblastoma [97]. Since both G_{D2} and c-Met are independently expressed in a range of healthy cells with few overlaps in the body, one can consider their combination to be a cancer-specific event. Pending appropriate demonstration of this, the G_{D2}/c-Met couple might therefore also qualify as an interesting target for anticancer bispecific mAb [98]. Another strategy to target the GD3S/c-Met axis could be the use of specific inhibitors of both targets.

Finally, pharmacological approaches targeting ganglioside biosynthesis could also be applied for breast cancer therapy. As an example, Triptolide is an inhibitor of GD3S expression that exhibits a strong cytotoxicity against in human breast cancer stem cells and primary breast cancer cells *in vitro* and *in vivo*, suggesting that this natural diterpenoid triepoxide compound from *Tripterygium wilfordii* might have clinical applications for the suppression of breast tumor growth [99]. In addition, recent data by Sarkar and coworkers showed that inhibition of GD3S, using shRNA or Triptolide, reduced metastatic burden in mice and primary tumor growth, suggesting again that this molecule could be of great interest for the treatment of triple negative breast cancers and for targeting cancer stem cell-enriched tumors [83]. The ongoing clinical trials for Triptolide and the development of new specific inhibitors of GD3S seem highly promising for breast cancer therapy.

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