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Expression of G_{D3} synthase modifies ganglioside profile and increases migration of MCF-7 breast cancer cells

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ABSTRACT

B- and c-series of gangliosides are over-expressed in neuro-ectoderm-related cancers, including breast cancer. It has been shown that G_{D3} ganglioside is over-expressed in about 50% of invasive ductal breast carcinoma and the G_{D3} synthase (GD3S) gene displays higher expression among estrogen receptor (ER) negative breast tumors. We previously showed that GD3S expression in MDA-MB-231 breast cancer cells induces the expression of G_{D2} and increased cell proliferation and migration *via* a G_{D2}-dependent activation of c-Met receptor. Here, we show that in ER-positive MCF-7 breast cancer cells, GD3S expression resulted in an increase of G_{D1b}, which was associated with a decrease of G_{M1a} and G_{M2}. Meanwhile, GD3S expressing MCF-7 cells exhibited an increased migration without any modification of proliferation rate. Therefore, GD3S expression can result in different modifications of both ganglioside profiles and cell phenotypes depending on breast cell types.

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RÉSUMÉ

Les gangliosides des séries b- et c- sont surexprimés dans différents cancers d'origine neuro-ectodermique, dont le cancer du sein. Il a été montré que le ganglioside G_{D3} est surexprimé dans environ 50 % des carcinomes canalaires infiltrants et le gène de la G_{D3} synthétase (GD3S) est surexprimé dans les tumeurs estrogène-récepteur négatives. Nous avons précédemment démontré que l'expression de la GD3S dans les cellules cancéreuses mammaires MDA-MB-231 induisait l'expression du G_{D2} et augmentait la

Abbreviations: β3Gal T4, GM1a/GD1b synthase; β4GalNAc T1, GM2/GD2 synthase; BSA, Bovine Serum Albumin; Cer, ceramide; DMEM, Dulbecco's Modified Eagle's Medium; EDTA, Ethylene Diamine Tetra-acetic Acid; EGFR, Epithelial Growth Factor Receptor; ER, Estrogen Receptor; ERK, Extracellular Signal-Regulated Kinase; FBS, Fetal Bovine Serum; FITC, Fluorescein Isothiocyanate; GD3S, GD3 synthase; GSL, glycosphingolipid; HPRT, Hypoxanthine Phosphoribosyltransferase; LacCer, Lactosylceramide; mAb, monoclonal Antibody; MALDI-TOF, matrix assisted laser desorption-ionization time-of-flight; MEK, Mitogen-Activated Protein (MAP) Kinase/ERK kinase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PBS, Phosphate Buffered Saline; PI3K, Phosphoinositide-3 Kinase; PR, Progesterone Receptor; QPCR, Quantitative real-time Polymerase Chain Reaction; RTK, Receptor Tyrosine-Kinase; SDS-PAGE, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; ST3Gal V, GM3 synthase; ST8Sia I, GD3 synthase; ST8Sia V, GT3 synthase.

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prolifération et la migration cellulaires par l'activation G_{D2}-dépendante du récepteur c-Met. Nous montrons ici que l'expression de la GD3S dans les cellules MCF-7 provoque une accumulation du G_{D1b} associée à une diminution du G_{M1a} et du G_{M2}. Parallèlement, les cellules MCF-7 GD3S positives montrent une augmentation de migration sans modification de la prolifération. Ce résultat montre que les modifications du profil gangliosidique et du phénotype induites par l'expression de la GD3S sont dépendantes du type cellulaire.

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1. Introduction

Gangliosides, the GSL carrying one or more sialic acid residues, are essentially located in the outer part of the plasma membrane in microdomains named "glycosynapses", where they can interact with transmembrane receptors or signal transducers involved in cell proliferation and intracellular signaling [1,2].

GSL from ganglio-series are usually classified in four series according to the presence of 0 to 3 sialic acid residues linked to LacCer: β-Gal*p*-(1→4)-β-Glc*p*-Cer (LacCer, G_{A3}). The transfer of sialic acid residues is catalyzed in the Golgi apparatus by specific sialyltransferases (namely G_{M3} synthase, GD3S and G_{T3} synthase, respectively) that exhibit a high specificity toward their respective glycolipid substrates [3]. Thus, G_{A3}, G_{M3} (α-Neu*p*5Ac-(2→3)-β-Gal*p*-(1→4)-β-Glc*p*-Cer), G_{D3} (α-Neu*p*5Ac-(2→8)-α-Neu*p*5Ac-(2→3)-β-Gal*p*-(1→4)-β-Glc*p*-Cer) and G_{T3} (α-Neu*p*5Ac-(2→8)-α-Neu*p*5Ac-(2→8)-α-Neu*p*5Ac-(2→3)-β-Gal*p*-(1→4)-β-Glc*p*-Cer) initiate 0-, a-, b- and c-series gangliosides and the biosynthesis of these compounds determines the relative proportion of gangliosides in each series (Fig. 1). The steady state level of membrane-associated gangliosides is therefore dependent on the activity of corresponding sialyltransferases, and the expression of b- and c- series gangliosides is strictly controlled by ST8Sia I, also named GD3S. Afterwards, further monosaccharides, including Gal*p*NAc, Gal*p* and Neu*p*5Ac, can be transferred in a stepwise manner by other glycosyltransferases [4].

Normal human tissues usually express a-series gangliosides, mainly G_{M1a}, whereas complex gangliosides from b- and c-series are essentially found during embryogenesis, in developing tissues and are mostly restricted to the nervous system in adult [5]. Complex gangliosides expression also increases in different pathological conditions including cancers, such as melanoma [6], neuroblastoma [7], or small cell lung carcinoma [8]. In particular, disialogangliosides (G_{D3} and G_{D2}) play a key role in proliferation and migration of neuro-ectoderm-derived tumor cells [9] and are used as targets for development of anti-cancer vaccines [10]. In breast cancer, G_{D3} and 9-O-acetyl-G_{D3} are over-expressed in about 50% of invasive ductal carcinoma [11] and *ST8SIA1*, the gene that encodes GD3S, displays higher expression among ER negative breast cancer tumors [12,13]. We have previously shown that GD3S expression in the ER-negative MDA-MB-231 cells results in the accumulation of b- and c- series gangliosides [14] and mass spectrometry analysis of permethylated GSL has shown that G_{D2} is the main ganglioside expressed at the cell surface of MDA-MB-231 GD3S+ clones (unpublished data). The expression of G_{D2} is associated with an increased cell migration and with a proliferative phenotype of MDA-MB-231 GD3S+ clones in absence of serum or exogenous growth factors [14]. We have also shown that the proliferative capacities of MDA-MB-231 GD3S+ clones in serum-free conditions directly proceed from the specific and constitutive activation of c-Met receptor and the downstream MEK/ERK and PI3 K/Akt signaling pathways [15]. Moreover, GD3S expression also stimulates primary tumor growth in severe immunodeficient mice [15].Here, we describe the effect of GD3S expression in MCF-7 cells, a usual model of ER-positive breast cancer cells in culture. We show by mass spectrometry that the MCF-7 GD3S+ cells accumulate di- and trisialogangliosides, mainly G_{D1b}. Moreover, the MCF-7 GD3S+ clones showed an increased migration without any change in proliferation compared to control cells.

2. Material and methods

2.1. Antibodies and reagents

Anti-G_{M3} GMR6 (mouse IgM) and anti-G_{D2} (mouse IgG3) S220-51 mAbs were purchased from Seikagaku Corp. (Tokyo, Japan). Anti-G_{D3} R24 mAb was purchased from AbCam (Cambridge, UK). Anti-G_{D1b} GGR12 mAb (mouse IgG2b) [16] was kindly provided by Pr. Ronald L. Schnaar (Depart. of Pharmacology and Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, USA). Anti-G_{T3} A2B5 mAb [17] was kindly provided by Pr. Jacques Portoukalian (Depart. of Transplantation and Clinical Immunology, Claude Bernard University and Edouard Herriot Hospital, Lyon, France). FITC-conjugated cholera toxin B-subunit from *Vibrio cholerae* was from Sigma-Aldrich (Lyon, France) and FITC-conjugated sheep anti-mouse IgG was from GE Healthcare (Templemars, France). Alexa Fluor 488 donkey anti-mouse IgG (H + L), Alexa Fluor 488 donkey anti-mouse IgM and FITC-conjugated anti-mouse IgM were purchased from Molecular Probes (Invitrogen, Carlsbad, CA, USA). Mouse mAb directed against the cytoplasmic region of human c-Met was purchased from Invitrogen, and rabbit polyclonal antibody against phosphorylated tyrosine 1234 and 1235 of the c-Met kinase domain was purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-rabbit and anti-mouse IgG conjugated with horseradish peroxidase were purchased from GE Healthcare.

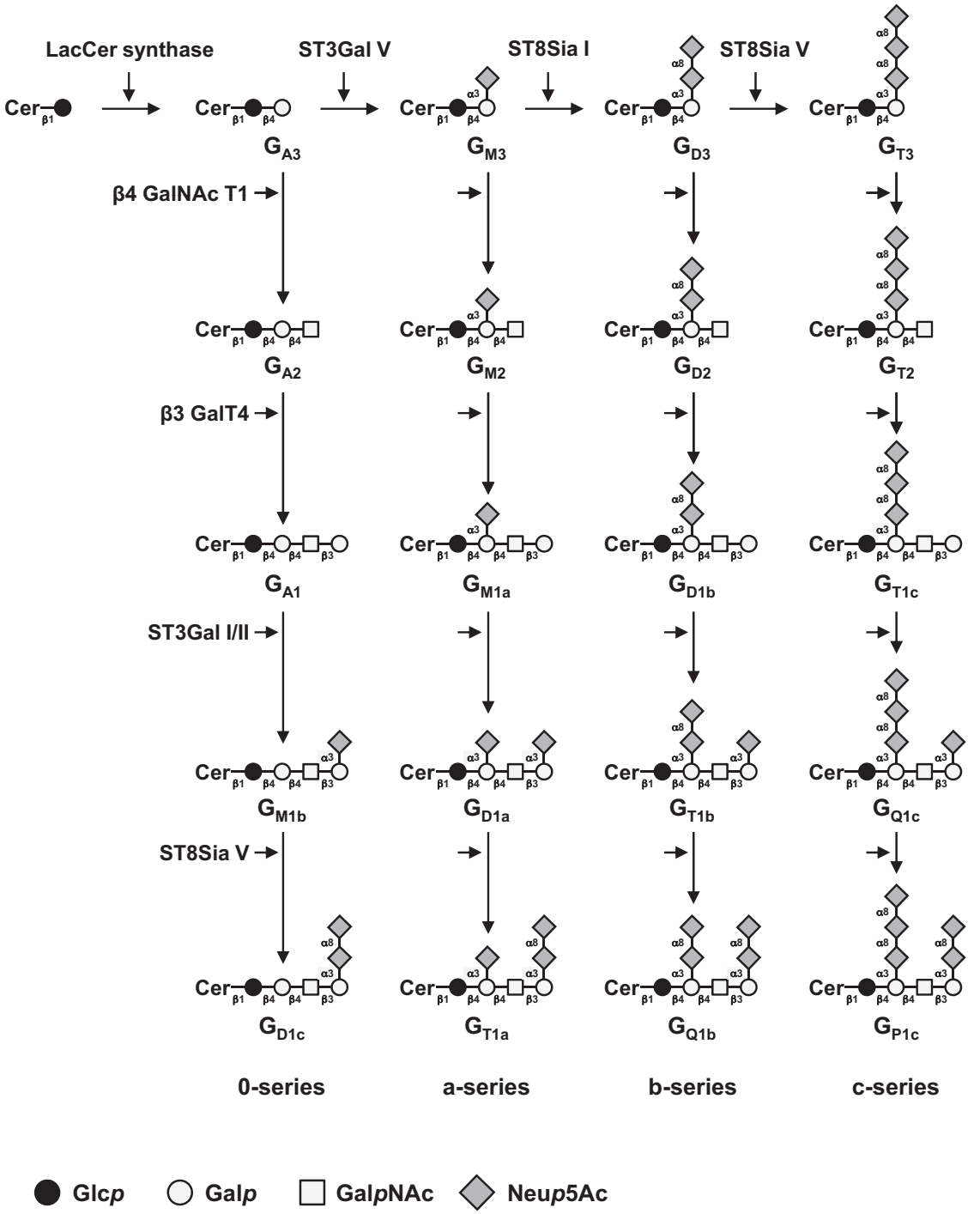

Fig. 1. Biosynthesis of gangliosides. The action of ST3 Gal V (G_{M3} synthase), ST8Sia I (G_{D3} synthase) and ST8Sia V (G_{T3} synthase) leads to the biosynthesis of the precursors of a-, b- and c-series gangliosides, respectively. The 0-series gangliosides are directly synthesized from lactosylceramide (G_{A3}). Elongation is performed by the sequential action of N-acetyl-galactosaminyltransferase (β4GalNAc T1), galactosyltransferase (β3 Gal T4) and sialyltransferases (ST3 Gal I, ST3 Gal II and ST8Sia V). Cer: ceramide; LacCer: lactosylceramide.

2.2. Cell culture

The breast cancer cell line MCF-7 and the melanoma cell line SK-Mel 28 were obtained from the American Type Cell Culture Collection. Cell culture reagents were purchased from Lonza (Levallois-Perret, France). Cells were routinely grown in monolayer and maintained at 37 °C in an atmosphere of 5% CO_2, in DMEM supplemented with

10% FBS, 2 mM L-glutamine, and 100 units/mL penicillin-streptomycin. MCF-7 control (empty vector transfected) and MCF-7 GD3S+ clones #1 and #4 were cultured in the presence of 1 mg/mL G418 (Invitrogen).

2.3. Stable transfection

The pcDNA3-GD3S expression vector encoding the full-length human G_{D3} synthase [18] was obtained from Pr. C.H. Kim (Molecular and Cellular Glycobiology Unit, Department of Biological Science, SungKyunKwan University, Suwon, Korea). Transfections were performed by electroporation using the Nucleofection technology according to Amaxa Biosystem protocol (Lonza). Briefly, 2×10^6 cells were resuspended in 100 μl of Cell Line Nucleofector™ Solution V and cell suspension was mixed with 2 μg of pcDNA3 or pcDNA3-GD3S vector. The sample was transferred into an electroporation cuvette and transfection was performed using the program E-014 according to manufacturer's instructions. After nucleofection, cells were transferred into prewarmed complete DMEM medium and maintained at 37 °C in an atmosphere of 5% CO_2. After 48 h, the transfected cells were cultured in the presence of 1 mg/mL G418 (Invitrogen). After 21 days of culture in the selective medium, individual G418-resistant colonies were isolated by limit dilution. Two positive clones (#1 and #4), expressing GD3S and complex gangliosides, as determined by real-time PCR and flow cytometry analysis, respectively, were used for further study.

2.4. Extraction and preparation of glycolipids

20 dishes (10 cm diameter) of cultured cells were washed twice with ice-cold PBS and cells were scraped and homogenized. Cells were suspended in 200 μL of water and sonicated on ice. The resulting material was dried under vacuum and sequentially extracted by $CHCl_3/CH_3OH$ (2:1, v/v), $CHCl_3/CH_3OH$ (1:1, v/v) and $CHCl_3/CH_3OH/H_2O$ (1:2:0.8, v/v/v). Supernatants were pooled, dried and subjected to a mild saponification in 0.1 M NaOH in $CHCl_3/CH_3OH$ (1:1) at 37 °C for 2 h and then evaporated to dryness [19]. Samples were reconstituted in CH_3OH/H_2O (1:1, v/v) and applied to a reverse phase C_{18} cartridge (Waters, Milford, MA) equilibrated in the same solvent. After washing with CH_3OH/H_2O (1:1, v/v), GSLs were eluted by CH_3OH, $CHCl_3/CH_3OH$ (1:1, v/v) and $CHCl_3/CH_3OH$ (2:1, v/v).

2.5. Mass spectrometry analysis of GSL

Prior to mass spectrometry analysis, GSL were permethylated according to Ciucanu and Kerek [20]. Briefly, compounds were incubated 2 h in a suspension of 200 mg/mL NaOH in dry DMSO (300 μL) and CH_3I (200 μL). The methylated derivatives were extracted in $CHCl_3$ and washed several times with water. The reagents were evaporated and the sample was dissolved in $CHCl_3$ in the appropriate dilution. MALDI-MS and MS/MS analyses of permethylated GSL were performed on 4800 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA) mass spectrometer, operated in the reflectron mode. For MS acquisition, 5 μL of diluted permethylated samples in $CHCl_3$ were mixed with 5 μL of 2,5-dihydroxybenzoic acid matrix solution (10 mg/mL dissolved in $CHCl_3/CH_3OH$ (1:1, v/v)). The mixtures (2 μL) were then spotted on the target plate and air-dried. MS survey data comprises a total of 50 subspectra of 1500 laser shots. Peaks observed in the MS spectra were selected for further MS/MS. CID MS/MS data comprises a total of 100 subspectra of 3000 laser shots. Two or more spectra can be combined postacquisition with mass tolerance set at 0.1 Da to improve S/N ratio. The potential difference between the source acceleration voltage and the collision cell was set to 1 kV and argon was used as collision gas.

2.6. Analysis of cell surface ganglioside by flow cytometry

Cells were washed in cold PBS and detached by EDTA 4 mM. Cells were incubated at 4 °C during 1 h with anti-ganglioside mAbs: anti-G_{M3} GMR6 (1:75), anti-G_{D3} R24 (1:100), anti-G_{D2} S220-51 (1:100), anti-G_{D1b} GGR12 (1:100) and anti-G_{T3} A2B5 (1:10), diluted in PBS containing 0.5% PBS-BSA (Sigma-Aldrich). After washing with PBS-BSA, cells were incubated on ice during 1 h with FITC-conjugated anti-mouse IgM or IgG. To analyze G_{M1a} expression, cells were incubated with a FITC-conjugated cholera toxin B-subunit (1:1000). Control experiments were performed using secondary antibody alone. Cells were analyzed by flow cytometry (FACScalibur, Becton Dickinson).

2.7. Analysis of ganglioside by confocal microscopy

Cells were cultured on glass coverslips in DMEM supplemented with 10% FBS. After 24 h, cells were washed twice in PBS and fixed in 4% paraformaldehyde (Sigma-Aldrich) for 20 min at room temperature. After washing with PBS, cells were blocked in PBS-BSA 0.2% for 30 min. Cells were incubated for 1 h at room temperature with anti-G_{D3} R24 (1:100), anti-G_{D2} S220-51 (1:100), anti-G_{D1b} GGR12 (1:100) and anti-G_{T3} A2B5 (1:10), diluted in PBS-BSA. Cells were then washed three times in PBS-BSA and incubated with Alexa Fluor® 488 anti-IgG or anti-IgM (1 h at room temperature; dilution 1:2000 in PBS-BSA). After three washes in PBS-BSA, PBS and deionised water, cells were mounted in Mowiol. Stained slides were examined under a Leica SP5 spectral microscope (IRI CNRS USR 3078, Villeneuve d'Ascq, France) with a 63X oil immersion lens at room temperature. Data were therefore collected using the LAS 6000 AF software.

2.8. Quantitative real-time-PCR (QPCR) analysis of G_{D3} synthase

Total RNA was extracted using the Nucleospin RNA II kit (Macherey Nagel, Hoerdt, France), quantified using a NanoDrop spectrophotometer (Thermo Scientifics, Wilmington, USA) and the purity of the preparation was checked by ratio of the absorbance at 260 and 280 nm. The cDNA was synthesized using 2 μg of RNA (GE Healthcare). PCR primers for GD3S and HPRT were previously described

[14,21] and synthesized by Eurogentec (Seraing, Belgium). PCR reactions (25 μL) were performed using 2X SYBR® Green Universal QPCR Master Mix (Stratagene, Amsterdam, The Netherlands), with 2 μL of cDNA solution and 300 nM final concentration of each primer. PCR conditions were as follows: 95 °C for 30 s, 51 °C for 45 s, 72 °C for 30 s (40 cycles). Assays were performed in triplicate and GD3S transcript expression level was normalized to HPRT using the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen [22]. Serial dilutions of the appropriate positive control cDNA sample were used to create standard curves for relative quantification and negative control reactions were performed by replacing cDNA templates by sterile water.

2.9. Proliferation assays

Control and clones cells (2×10^3) were seeded in 96-well plates (Thermo Fisher Scientific, Rockford, IL, USA) and grown in DMEM containing 5, 1, 0.5% FBS, or in FBS-free medium. Cell growth was analyzed at different times using the MTS reagent (Promega, Charbonnières-les-Bains, France) according to the manufacturer's instructions.

2.10. Cell migration analysis

Cells (5×10^4) were seeded in the upper surface of Transwell 12 plates (BD Biosciences, Le-Pont-de-Claix, France) and cultured for 24 h in 10% FBS-containing medium. After incubation, cells were fixed in 4% paraformaldehyde (20 min at room temperature) and stained with Hoescht 33528 (Sigma-Aldrich) during 20 min in the dark at room temperature. Then, the upper chamber of the well's porous membrane was removed by scraping with a cotton swab and washed several times with PBS. The membrane was mounted on slide with Glycergel mounting medium (Dako, Trappes, France). The migrating cells were counted on the overall membranes under × 200 magnification and the mean number of cells was evaluated in three independent experiments.

2.11. Immunodetection of c-Met and phospho-c-Met

Cell pellets were treated with lysis buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 1% Triton X-100, 1 mM sodium orthovanadate, and protease inhibitor cocktail tablet). The supernatants were assessed for protein concentration using the Bio-Rad DC protein assay kit II. One hundred microgram of total proteins were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were saturated in TBS containing 5% BSA and 0.05% Tween-20. Membranes were then incubated with blocking buffer (0.2% casein, 0.1% Tween-20 in PBS) for 1 h and probed for 1 h at room temperature with appropriate antibodies diluted in blocking buffer according to the manufacturer's recommendations. After washing in PBS-Tween 0.2%, immune complexes were detected with specific secondary antiserum conjugated with alkaline phosphatase followed by an enhanced chemiluminescence detection system (Amersham ECL Western Blotting Detection Reagents).

2.12. Statistical analyses

Student's *t*-test was used for statistical analysis. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Establishment of GD3S+ MCF-7 breast cancer cells

MCF-7 cells were transfected with the pcDNA3-GD3S expression vector containing the full-length cDNA of human GD3S or the empty pcDNA3 vector as control. Transfected cells were cultured 21 days in the presence of 1 mg/mL G418. As previously shown [14], QPCR analysis of GD3S expression (Fig. 2A) indicates that GD3S mRNA is expressed at a very low level in wild-type and control (empty vector transfected) MCF-7 cells compared to SK-Mel 28 melanoma cells used as positive control for GD3S expression [23]. Meanwhile, GD3S expression was significantly increased in pcDNA3-GD3S transfected MCF-7 cell population. Flow cytometry analysis of gangliosides in both control and pcDNA3-GD3S transfected cells shows that GD3S stable transfectants do not expressed G_{D3} or G_{D2} and only a low percentage of cells (about 15%) expresses G_{D1b} concomitantly with a decrease of G_{M1a} expression, a larger proportion of cells being recognized by the anti-G_{T3} A2B5 mAb (Fig. 2B).

Given the fact that the pcDNA3-GD3S transfected cell population was heterogenous, individual G418-resistant colonies were isolated by limiting dilution cloning. Forty-three different clones were obtained and analyzed for the expression of GD3S and gangliosides. Positive clones for G_{D1b} and G_{T3} expression were selected and two of them, clones #1 and #4, were used for this study. The pattern of gangliosides was monitored in the two clones by flow cytometry using different anti-ganglioside mAbs. As shown in Fig. 3A, the two selected clones expressed high levels of G_{D1b} and G_{T3}. In contrast, G_{M1a} expression level was reduced (Fig. 3A). G_{D1b} expression appeared to be slightly higher in clone #1 than in clone #4. Control cells showed no change in the ganglioside profile compared with wild-type MCF-7 (data not shown). Finally, immunofluorescence and confocal microscopy confirmed that G_{D1b} and G_{T3} were expressed at the cell surface of both clones (Fig. 3B).

3.2. Mass spectrometry analysis of GD3S+ MCF-7 cells GSL composition

In order to confirm the modifications in gangliosides composition observed by flow cytometry analysis in MCF-7 GD3S+ cells, we compared the GSLs profiles from control and GD3S+ MCF-7 cells by MALDI-TOF-MS and MALDI-TOF-MS/MS sequencing (Figs. 4 and 5). The total glycolipids were extracted and subjected to mild alkalinolysis. GSLs were further purified on reversed-phase C_{18} column and analyzed by mass spectrometry as permethylated derivatives. As shown in Fig. 4, the MALDI-MS analysis revealed that MCF-7 cells expressed a complex pattern of GSLs. Individual signals were identified by calculating

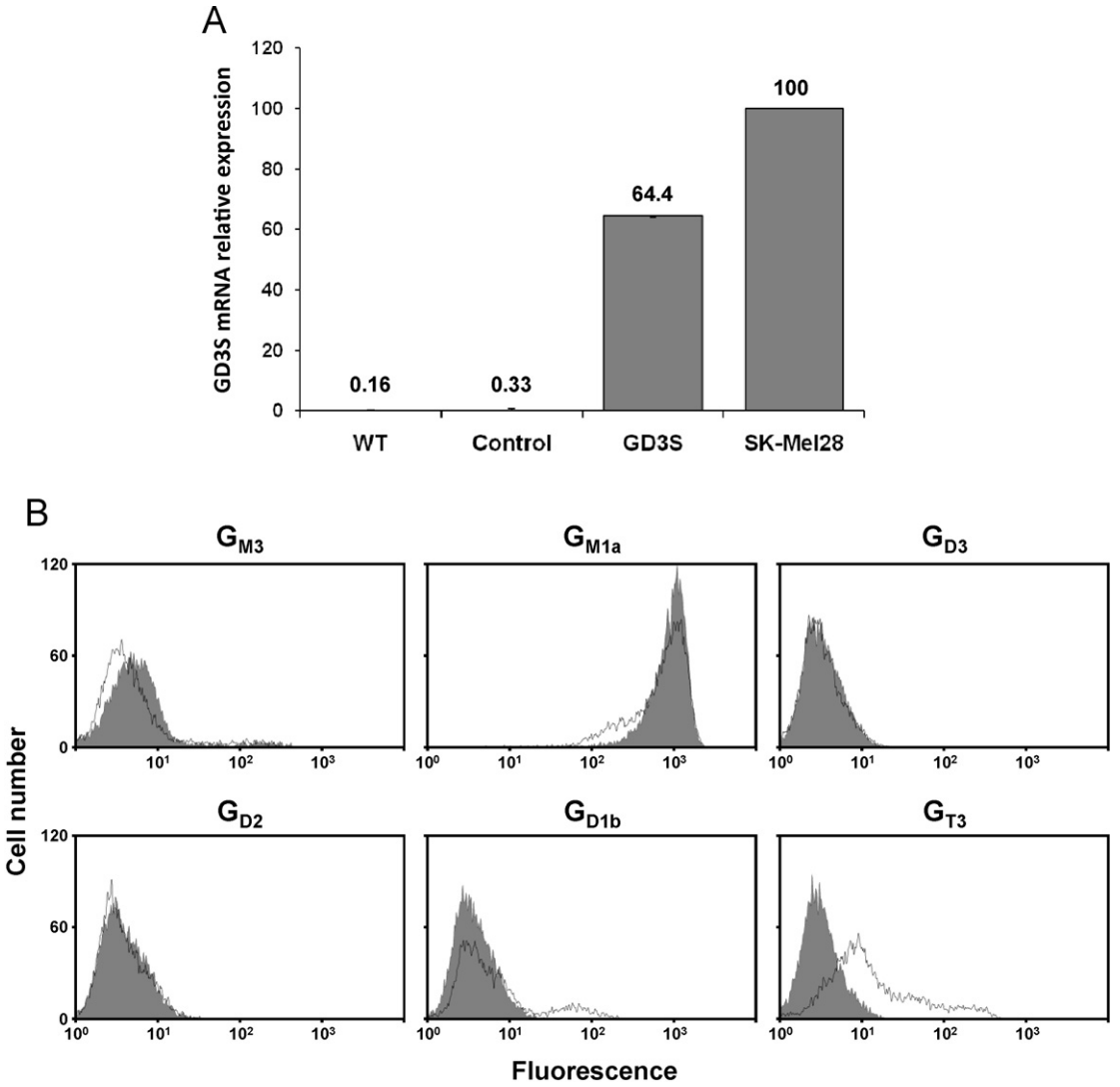

Fig. 2. A: QPCR analysis of GD3S mRNA in pcDNA3-GD3S transfected MCF-7 cell population. Results were normalized to the expression of HPRT mRNA and expressed relative to GDS3 mRNA in SK-Mel 28 melanoma cells, which was regarded as 100%. The quantification was performed by the method described by Livak and Schmittgen [22]. WT: MCF-7 wild-type; Control: empty vector transfected MCF-7; GD3S: pcDNA3-GD3S transfected MCF-7. B: Flow cytometry analysis of gangliosides expression in pcDNA3-GD3S transfected MCF-7 cell population. The immunodetection of G_{M3}, G_{D3}, G_{D2}, G_{D1b}, G_{T3} and G_{M1a} was performed using GMR6, R24, S220-51, GGR12, A2B5 mAbs and cholera toxin B-subunit from *Vibrio cholerae*, respectively. The grey peaks correspond to control MCF-7 cells (empty vector transfected) and black lines correspond to pcDNA3-GD3S transfected MCF-7 cells.

monosaccharide and Cer compositions and by establishing monosaccharide sequences by MALDI-TOF-MS/MS fragmentation. Then, the final attribution of individual GSLs to existing series was based on the known biosynthetic pathways of GSLs in human cells. Altogether, the data established that MCF-7 cells expressed GSLs from the globo- and the ganglio-series. Each GSL, irrespective of their series, were identified as two distinct signals exhibiting 112 mu differences, which established that they are substituted by two Cer moieties differing by eight $-CH_2-$ groups. Indeed, two Cer isoforms are usually expressed in human tissues due to the substitution of the sphingosine by palmitic acid C16:0 or lignoceric acid C24:0. Globo-series GSLs were characterized by the presence of simple G_{b3} and G_{b4} at *m/z* 1215/1327 and 1460/1572, respectively. Ganglio-series GSLs were characterized by the presence of a single neutral G_{A1} at *m/z* 1215/1327, monosialylated G_{M2} and G_{M1} at *m/z* 1616/1728 and 1821/1933, as well as disialylated at *m/z* 2182/2294. MS/MS fragmentation of individual signals allows one to differentiate isobaric molecules such as G_{b4}/G_{A1} and G_{M1a}/G_{M1b} (data not shown). It is noteworthy that the MS/MS sequencing established that the disialylated G_{D1} expressed in empty vector-transfected MCF-7 cells was exclusively constituted by the a-series ganglioside. Indeed, the intense B- and C-cleavage ions at *m/z* $[M+Na]^+$ 847 and 620 unambiguously established that the terminal Gal residue of the Hex-HexNAc motif was monosialylated (Fig. 5B). Accordingly to the attribution as G_{D1a}, Y-cleavage ion at *m/z* $[M+Na]^+$ 1469 and the Y2α/C5 double cleavage ion at *m/z*

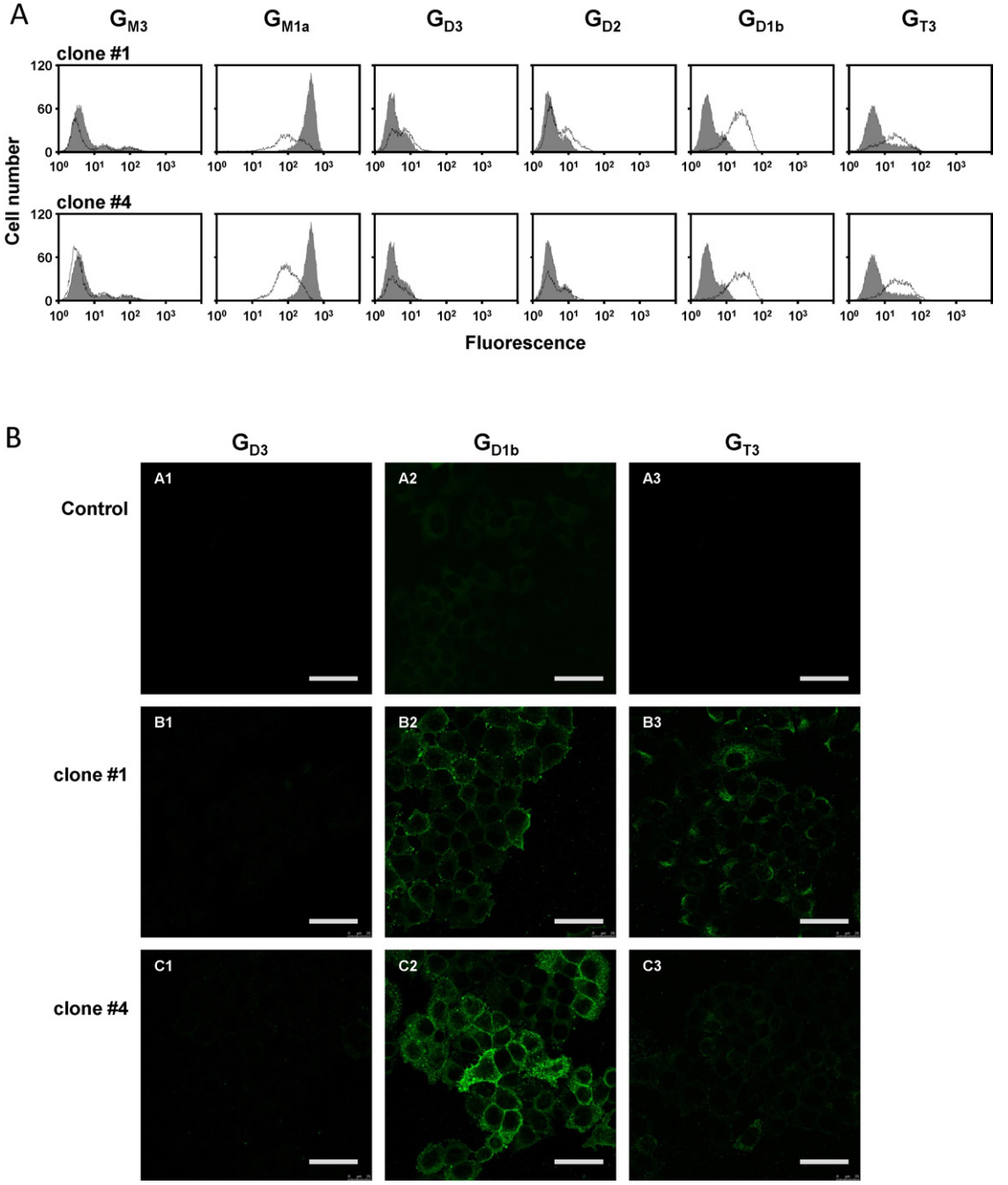

Fig. 3. Analysis of the expression of gangliosides in MCF-7 GD3S+ clones. A: flow cytometry analysis of ganglioside expression. Gangliosides G_{M3}, G_{D3}, G_{D2}, G_{D1b}, and G_{T3} were revealed with anti-G_{M3} GMR6, anti-G_{D3} R24, anti-G_{D2} S220-51, anti-G_{D1b} GGR12, and anti-G_{T3} A2B5 mAbs, respectively. G_{M1a} expression was analyzed with FITC-labeled cholera toxin B from *Vibrio cholerae*. The grey peaks correspond to control MCF-7 cells, whereas black lines correspond to MCF-7 GD3S+ cells. Results are representative of three independent experiments. B: Analysis of ganglioside expression at the membrane surface MCF-7 cells by immunocytochemistry and confocal microscopy. Ganglioside were revealed with anti-G_{D3} R24 (A1, B1, C1), anti-G_{D1b} GGR12 (A2, B2, C2) or anti-G_{T3} A2B5 (A3, B3, C3) mAbs and anti-mouse IgM or anti-mouse IgG labeled with Alexa 488. Bars: 50 μm.

810 $[M+Na]^+$ confirmed that Gal*p*-(1→4)-β-Glc*p* disaccharide was also mono-sialylated.

As expected, the gangliosides profiles of MCF-7 GD3S+ clones established by MALDI-MS significantly differed from those of the wild-type (data not shown) and empty vector-transfected MCF-7 (control) cells (Fig. 4). Indeed, whereas MCF-7 cells expressed simple a-series gangliosides including G_{M2}, G_{M1a} and G_{D1a}, GD3S+ clones accumulated di-, tri- and tetra-sialylated gangliosides of higher complexity including G_{D3}, G_{D2}, G_{D1}, G_{T2}, G_{T1} and G_{Q1}. In parallel, monosialylated gangliosides totally disappeared in MCF-7 GD3S+ cells compared to control cells.

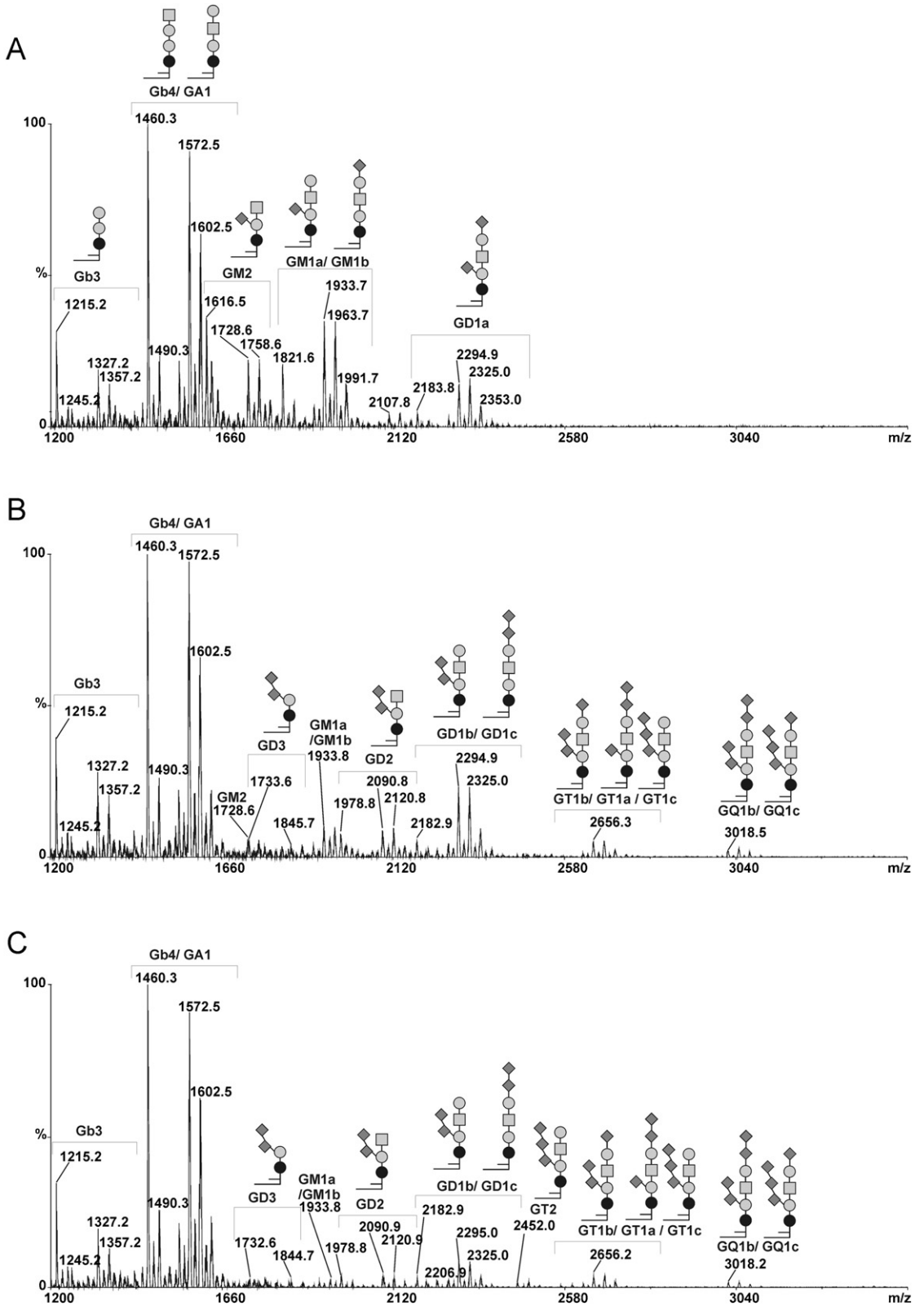

Fig. 4. MALDI-TOF-MS profiles of permethylated GSLs from control and GD3S+ MCF-7 cells. Mass Spectra of permethylated GSLs isolated from control MCF-7 transfected with empty vector (A), GD3S+ MCF-7 cells clone #1 (B) and clone #4 (C). Gangliosides from b- or c-series were not detected in control cells, whereas complex gangliosides containing 2 to 4 sialic acids residues (i.e. G_{D3}, G_{D2}, G_{D1b}, G_{T2}, G_{D3}, $G_{T1b/c}$, $G_{Q1b/c}$) were detected in GD3S+ MCF-7 cells. ● Glc*p*; ○ Gal*p*; □ Gal*p*NAc; ◆ Neu*p*5Ac; ⌐| Ceramide.

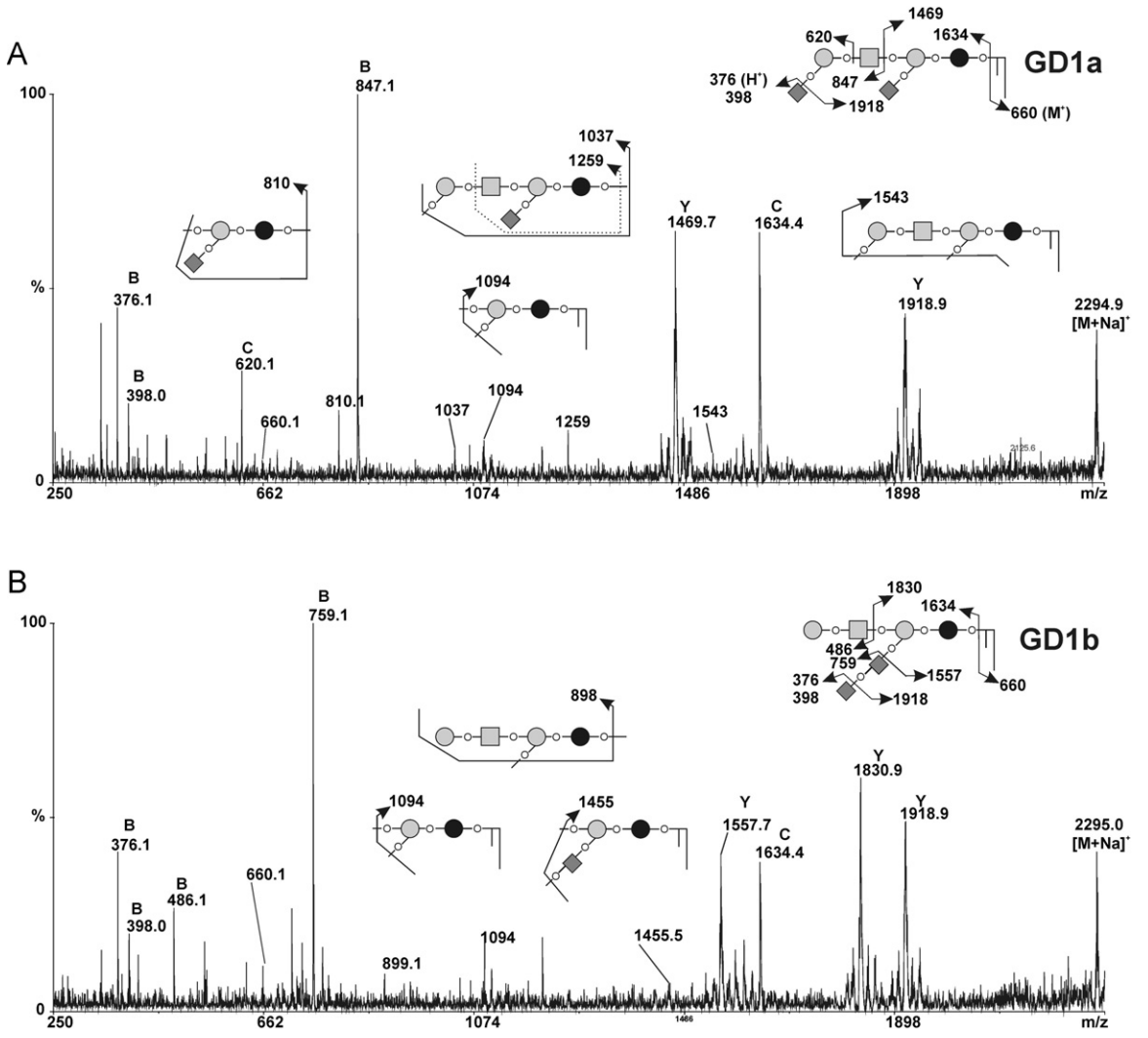

Fig. 5. MALDI-MS/MS sequencing of the permethylated gangliosides from control and GD3S+ MCF-7 cells. MALDI-MS/MS spectra of the $[M + Na]^+$ molecular ion *m/z* 2495 corresponding to permethylated G_{D1} gangliosides in MCF-7 transfected with empty vector (**A**) and GD3S+ MCF-7 clone #1 (**B**). Fragment ions were annotated according to nomenclature of Domon and Costello [31]. All molecules are detected as $[M + Na]^+$ ions with the exception of those into brackets {}. The fragmentation profiles of permethylated G_{D1} ganglioside typifies G_{D1a} in control MCF-7 cells and G_{D1b} in GD3S+ MCF-7 clones. The C/Z couple fragment ions at *m/z* 1634 $[M + Na]^+$ and 660 $[M + H]^+$ typify the ceramide moiety as sphingosine C18:1 substituted by lignoceric acid C24:0. ● Glc*p*; ○ Gal*p*; □ Gal*p*NAc; ◆ Neu*p*5Ac; ⌉ Ceramide.

Then, MS/MS fragmentation analysis of individual compounds in MCF-7 GD3S+ cells established that all GSLs are b- and c-series, which permitted to precisely identify G_{D1b}, $G_{T1b/c}$ and $G_{Q1b/c}$. In particular, comparison of the fragmentation patterns of G_{D1} signals from control MCF-7 cells and GD3S+ clones revealed that G_{D1} isolated from GD3S+ cells exclusively contained the b-series isomer whereas control MCF-7 cells only contained the a-series isomer (Fig. 5B). The presence of the di-sialylated motif on the lactose moiety typifying b-series was unambiguously established by the presence of Y/B couple cleavage ions at *m/z* 1830/486 and B ion at *m/z* 759.

3.3. Growth and migration of GD3S+ MCF-7 cells

Cell growth was determined by MTS assay in culture media containing decreasing concentrations of FBS. As shown in Fig. 6A, no difference in cell growth was observed between control and GD3S+ cells. Similar result was obtained with wild-type MCF-7 cells (data not shown). In all cases, cell proliferation decreased with serum concentration. Similar result was obtained by direct cell counting experiments (data not shown). Interestingly, cell migration assay using Transwell Boyden chambers showed that GD3S expression induced twice as much cell migration to the bottom chamber compared to control cells (Fig. 6B).

3.4. Immunodetection of c-Met and phospho-c-Met in MCF-7 GD3S+ clones

The expression and phosphorylation of c-Met were analyzed in control and MCF-7 GD3S+ clones by Western blotting and immunodetection using anti-c-Met and anti–phospho-c-Met antibodies directed against phosphorylat-

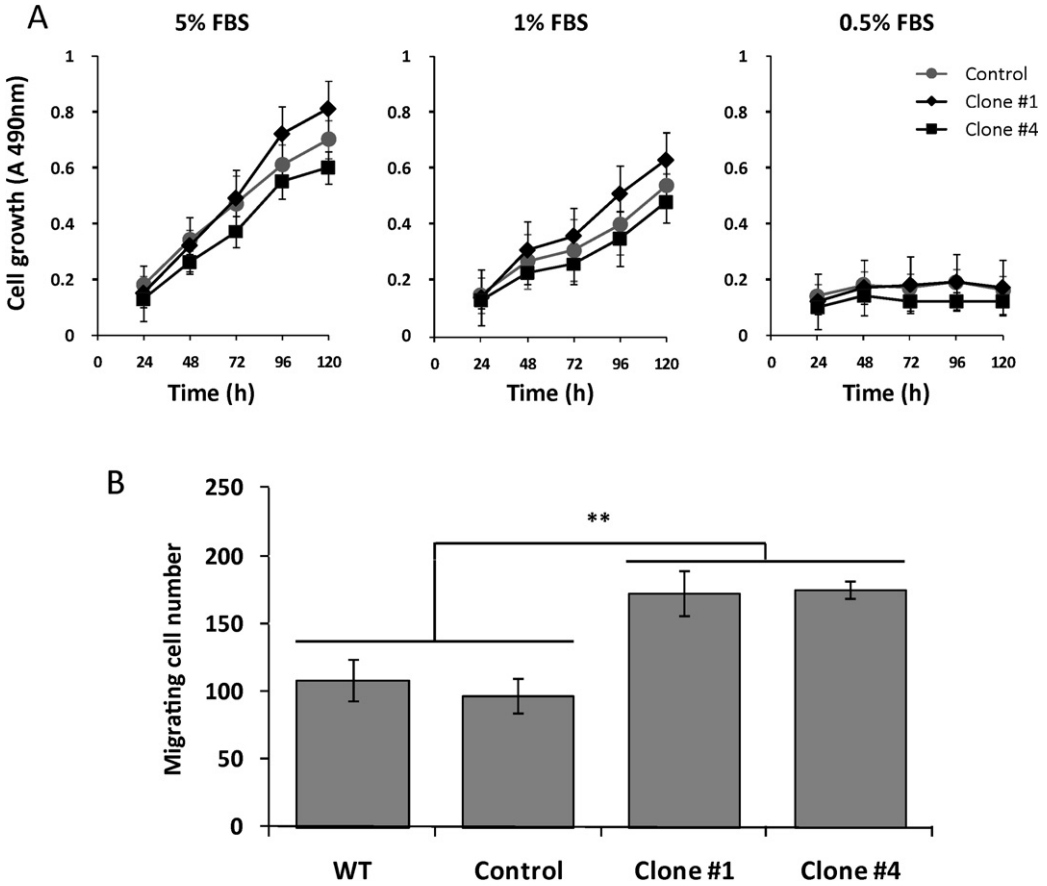

Fig. 6. Analysis of the effect of GD3S expression on MCF-7 cell proliferation and migration. A: Proliferation assays were performed by MTS in 96-wells plates and cells were grown in DMEM supplemented with 5, 1 or 0.5% FBS. Cells were counted after 24, 48, 72, 96 and 120 h. Each measure was performed in 16 wells and data are mean of four independent manipulations. ●: control cells; ◆: clone #1; ■: clone #4. B: Transwell cell migration analysis. Cells were counted after 24 h of culture in DMEM medium supplemented with 1% FBS. ** $p < 0.02$.

ed tyrosine residues 1234 and 1235 of the receptor. As shown in Fig. 7, c-Met was detected at the same expression level in both control and GD3S+ clones (Fig. 7). Phosphorylation of c-Met was revealed neither in control nor in untreated GD3S+ clones, while HGF/SF induced efficient Met phosphorylation in MCF7 cells (Fig. 7).

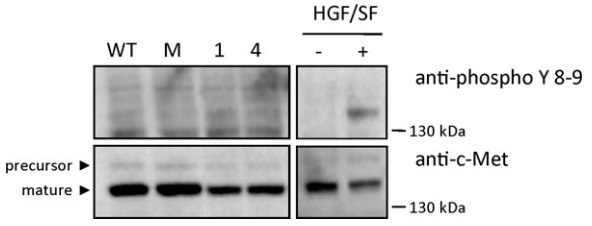

Fig. 7. Western blot analysis using specific c-Met and phospho-c-Met antibodies. Untransfected MCF-7 (WT), control MCF-7 transfected with empty vector, GD3S+ MCF-7 cells clone #1 and clone #4 were cultured in serum free condition. Untransfected MCF-7 (WT) were treated or not 15 min with 30 ng/ml HGF/SF. Cell lysates were analyzed by Western blotting with antibodies directed against human c-Met and the phosphorylated tyrosine residues of the c-Met kinase domain. The positions of molecular weight markers are indicated. Arrowheads indicate the positions of precursor and mature c-Met.

4. Discussion

Recent evidences have proposed that GD3S could be associated with breast cancer development and aggressiveness. Clinical studies using breast tumors microarray datasets have shown that the GD3S gene (*ST8SIA1*) displayed higher expression among ER negative tumors [12]. *ST8SIA1* expression is associated with poor histopathological grading and tumor size in ER negative tumors [12,13]. We have also recently showed that *ST8SIA1* expression is significantly higher in the basal-subtype [24] breast cancer tumors [15], indicating that GD3S could be over-expressed in aggressive breast cancer tumors. However, in comparison with tumors, breast cancer cells do not express complex gangliosides from b- and c-series in culture conditions [14]. To analyze the effect of GD3S expression in breast cancer, we have established and characterized cellular models deriving from two breast cancer cell lines: the triple negative (ER-, PR- and Her2-) basal-subtype MDA-MB-231 and the luminal-A subtype ER+ MCF-7 breast cancer cells, that both express GD3S. We have shown that MDA-MB-231 GD3S+ clones exhibit a proliferative phenotype in absence of serum or growth factors, and an increased tumor growth in severe

immunodeficiency mice. This phenotype results from the constitutive activation of c-Met receptor and subsequent activation of MEK/ERK and PI3 K/Akt pathways [15]. Here, we reported the phenotype of MCF-7 GD3S+ clones.

MALDI-TOF analysis has confirmed that wild-type MCF-7 and MDA-MB-231 have different GSL composition. GSL composition of MDA-MB-231 and MCF-7 cell lines was previously examined [25], showing that there is twice as much neutral GSL in MCF-7 as in MDA-MB-231 cells and that ganglioside content is four-fold higher in MDA-MB-231 cells compared to MCF-7 cells. Our data confirm that MCF-7 express large amount of globosides such as G_{b3} and G_{b4} and lower amount of gangliosides compared to MDA-MB-231 [14]. This difference in GSL composition may be explained by the lowest expression of the G_{M3} synthase (ST3 Gal V) in MCF-7, the enzyme that regulates the first step of gangliosides biosynthesis [14]. Similarly to MDA-MB-231 cells, wild-type MCF-7 cells only express a-series gangliosides, but mainly accumulate G_{M1a} instead of G_{M2} in MDA-MB-231, probably due to the 5-fold higher expression of the β3 Gal T4 in MCF-7 cells (data not shown). In MCF-7 clones, GD3S expression resulted in the conversion of a-series in b- and c-series gangliosides absent from control cells, G_{D1b} being the main compound instead of G_{D2} in MDA-MB-231 GD3S+ clones (unpublished data). These data are in good agreement with ganglioside profiles of wild-type MCF-7 and with the expression of related enzymes, G_{D2} being efficiently converted in G_{D1b} by the β3 Gal T4 in MCF-7 cells. It is noteworthy that GD3S+ clones also express c-series gangliosides whereas the G_{T3} synthase ST8Sia V is weakly expressed in both MDA-MB-231 and MCF-7 cells. This may be due to the G_{T3} synthase activity of GD3S that also uses G_{D3} as acceptor substrate to synthesize G_{T3} [26].

As previously shown, GD3S expression in MDA-MB-231 induces a proliferative phenotype in serum-free conditions, resulting from the constitutive activation of c-Met receptor and subsequent activation of MEK/ERK and PI3 K/Akt transduction pathways [15]. Moreover, the silencing of the G_{M2}/G_{D2} synthase (β4GalNAc T1) reverses the proliferative phenotype and c-Met phosphorylation, underlying the role of G_{D2} in c-Met activation. It is commonly known that ganglioside expression can have important effects on RTKs-mediated signaling and regulates cell growth through activation or inhibition of intracellular transduction pathways [27]. For example, EGFR tyrosine kinase activity is inhibited by G_{M3} through interactions of G_{M3} with terminal β-Glc*p*NAc of EGFR *N*-glycans [28]. However, no change in the proliferative capacity of GD3S+ MCF-7 clones was observed and no activation of c-Met was detected in these cells. The absence of phenotype of GD3S+ MCF-7 clones can be related to the expression of G_{D1b} instead of G_{D2}, and reinforces the role of G_{D2} in c-Met activation. In parallel, MCF-7 GD3S+ cells show an increased migration as observed for MDA-MB-231 clones. This seems to indicate that proliferation and migration are activated by different mechanisms in GD3S+ cells.

In conclusion, the present data together with our previous results show that the expression of GD3S in breast cancer cells results in different ganglioside profiles and phenotypes according to the cell type and the cell-dependent expression of other glycosyltransferases involved in ganglioside biosynthesis (i.e. ST3 Gal V, β4GalNAc T1, and β3 Gal T4). Recently, it has been shown that breast cancer cell gangliosides can serve as functional E-selectin ligands and be involved in cell adhesion to vascular endothelium, favoring metastasis [29]. Moreover, the expression of ST6GalNAc V, a sialyltransferase catalyzing α-ganglioside biosynthesis, has been associated to brain metastasis of breast cancer. Normally restricted to the brain, the expression of ST6GalNAc V in breast cancer cells enhances their adhesion to brain endothelial cells and their passage through the blood-brain barrier [30]. We have also previously shown that *ST8SIA1* expression is significantly higher in the basal-subtype breast cancer tumors and that G_{D2} expression induces a growth factor independent proliferative phenotype *via* the constitutive activation of c-Met [15]. Nevertheless, *ST8SIA1* alone is not sufficient to induce G_{D2} accumulation and c-Met activation, as low expression of β3 Gal T4 is also required. Altogether, these data indicate that the fine analysis of ganglioside glycosyltransferase genes expression should give new insights on proliferation capacity and tissue-specific metastasis targeting of basal-subtype breast cancer tumors.

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