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Molecular Cancer Research

G_{D3} Synthase Expression Enhances Proliferation and Tumor Growth of MDA-MB-231 Breast Cancer Cells through c-Met Activation

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Abstract

The disialoganglioside G_{D3} is overexpressed in ~50% of invasive ductal breast carcinoma, and the G_{D3} synthase gene (*ST8SIA1*) displays higher expression among estrogen receptor–negative breast cancer tumors, associated with a decreased overall survival of breast cancer patients. However, no relationship between ganglioside expression and breast cancer development and aggressiveness has been reported. We have previously shown that overexpression of G_{D3} synthase induces the accumulation of b- and c-series gangliosides (G_{D3}, G_{D2}, and G_{T3}) at the cell surface of MDA-MB-231 breast cancer cells together with the acquisition of a proliferative phenotype in the absence of serum. Here, we show that phosphoinositide 3-kinase/Akt and mitogen-activated protein kinase/extracellular signal-regulated kinase pathways are constitutively activated in G_{D3} synthase–expressing cells. Analysis of phosphorylation of tyrosine kinase receptors shows a specific c-Met constitutive activation in G_{D3} synthase–expressing cells, in the absence of its ligand, hepatocyte growth factor/scatter factor. In addition, inhibition of c-Met or downstream signaling pathways reverses the proliferative phenotype. We also show that G_{D3} synthase expression enhances tumor growth in severe combined immunodeficient mice. Finally, a higher expression of *ST8SIA1* and *MET* in the basal subtype of human breast tumors are observed. Altogether, our results show that G_{D3} synthase expression is sufficient to enhance the tumorigenicity of MDA-MB-231 breast cancer cells through a ganglioside-dependent activation of the c-Met receptor. *Mol Cancer Res; 8(11); 1526–35. ©2010 AACR.*

Introduction

Gangliosides, the glycosphingolipids carrying one or more sialic acid residues, are essentially located at the outer leaflet of the plasma membrane in microdomains named "glycosynapse," where tyrosine kinase receptors (RTK) are also located (1). They are often found in growing and developing tissues and complex gangliosides from b- and c-series, initiated by G_{D3} or G_{T3}, respectively; are mainly expressed during embryogenesis; and are restricted to the central nervous system in adults (2). In mammalian cells, the expression of di- and trisialogangliosides increases in pathologic conditions such as cancer. G_{D3}, G_{D2}, and G_{T3} are considered as oncofetal markers in animal and human tumors such as melanoma and neuroblastoma. They play a key role in invasion and metastasis by mediating cell proliferation, migration, adhesion, and angiogenesis (3-5). The restrictive expression in pathologic conditions and the accessibility of gangliosides on the cell surface make them good targets for cancer immunotherapy, as it was well studied in melanoma (6).

Little is known about ganglioside expression in breast cancer. Total ganglioside levels seem to be significantly higher in breast tumors than in normal breast tissues (7). The gangliosides G_{D3}, 9-*O*-acetyl-G_{D3}, and 9-*O*-acetyl-G_{T3} are absent or expressed at a very low levels in normal breast tissues, but are overexpressed in ~50% of invasive ductal breast carcinoma (7). Recently, two clinical studies have shown that the G_{D3} synthase (GD3S) gene (*ST8SIA1*), the key enzyme for the biosynthesis of b- and c-series gangliosides, displayed higher expression among estrogen receptor (ER)–negative breast cancer tumors (8) and had a prognostic impact in breast cancer dependent on ER status: *ST8SIA1* gene expression was associated with poor pathohistologic grading in ER-negative tumors and a reduced

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overall survival of the patients (9). In contrast, a better prognosis for ER-positive samples with high expression of *ST8SIA1* was noticed (9). However, no functional relationship between ganglioside expression and breast cancer development and aggressiveness has been reported. We have recently developed a cellular model derived from MDA-MB-231 breast cancer cells expressing the human GD3S. GD3S-expressing cells accumulate gangliosides of b- and c-series (i.e., G_{D3}, G_{D2}, and G_{T3}) at the cell surface. The change in cell membrane ganglioside composition is associated with morphologic changes, and increased migration and proliferation in the absence of serum or exogenous growth factors. Here, we show that proliferative capacity of MDA-MB-231 GD3S$^+$ clones in serum-free medium is directly correlated to the constitutive activation of c-Met receptor and downstream signaling pathways phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK/ERK). Inhibition of either c-Met activity or downstream signaling abrogates the proliferation of GD3S-expressing cells. We also show that GD3S expression induces an increased tumor growth in severe combined immunodeficient (SCID) mice.

Materials and Methods

Antibodies and reagents

Antiphosphorylated extracellular signal-regulated kinase ERK (E-4), anti-ERK (K-23), anti-Akt (H-136), and rabbit polyclonal IgG anti-actin antibodies were purchased from Santa Cruz Biotechnology. Anti-phosphorylated Akt S473, mouse monoclonal antibody directed against the COOH-terminal region of human Met, and rabbit polyclonal antibody against phosphorylated tyrosine 1234 and 1235 of the Met kinase domain were purchased from Cell Signaling Technology. Anti-rabbit and anti-mouse IgG conjugated with horseradish peroxidase were purchased from GE Healthcare. Alexa Fluor 488 donkey anti-mouse IgG (H+L) was purchased from Molecular Probes Invitrogen.

U0126, Akt inhibitor VIII, SU11274, and PHA665752 were purchased from Calbiochem Merck Chemicals Ltd. and dissolved in dimethyl sulfoxide (DMSO). Human recombinant hepatocyte growth factor/scatter factor (HGF/SF) was purchased from Peprotech.

Cell culture

Cell culture reagents were purchased from Lonza. The breast cancer cell line MDA-MB-231 was obtained from the American Type Cell Culture Collection. These cells were routinely grown in monolayer cultures and maintained at 37°C in an atmosphere of 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, and 100 units/mL penicillin-streptomycin. MDA-MB-231 control (empty vector transfected) and MDA-MB-231 GD3S$^+$ clones 4 and 11 were obtained as previously described (10). The transfected cells were cultured in the presence of 1 mg/mL G418 (Invitrogen).

RNA interference assays

Cells were transfected with 20 pmol siRNA (Invitrogen) targeting Met [*Met7* (stealth) 5′-CCAUUUCAACUGAGUUUGCUGUUAA-3′; *Met8* (stealth) 5′-UCCAGAAGAUCAGUUUCCUAAUUCA-3′] or a scramble sequence using 5 μL Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. Cells were transfected with the corresponding siRNAs and were collected for cell growth assay or Western blot analysis 24 hours later.

Proliferation assays

Cell growth was analyzed using the MTS reagent (Promega) according to the manufacturer's directions. For pharmacologic inhibition, cells (2×10^3) were seeded in 96-well plates (Thermo Fisher Scientific) and grown in DMEM containing 0.1% FBS. After 12 hours, the medium was replaced with serum-free DMEM containing specific inhibitors: 1 μmol/L U0126, 1 μmol/L Akt inhibitor VIII, 500 nmol/L SU11274, or 100 nmol/L PHA665752. Control cells were treated with DMSO at a 1:1,000 dilution. The medium was changed every 2 days. For inhibition with neutralizing antibody against c-Met, cells were cultured during 5 days in DMEM serum-free medium containing 10 μg/mL of 5D5 Fab or control IgG.

For RNA interference assay, 2×10^3 cells were seeded in 96-well plates in DMEM serum-free medium 1 day after the transfection with siRNA sequences, then cultured at 2 days before the assessment of cell growth.

Migration assays

Cells (5×10^4) were seeded and cultured in the upper surface of Transwell 12-well plates (BD Bioscience) for 6 hours, as described previously (10). Specific inhibition was done with 1 μmol/L SU11274 or 200 nmol/L PHA665752. Cells treated with DMSO (1:1,000 dilution) were used as controls.

Phospho-RTK array analysis

Cells were cultured for 48 hours in six-well plates in serum-free DMEM or in DMEM containing 10% FBS. Cells were then lysed in NP40 lysis buffer [1% NP40, 20 mmol/L Tris-HCl (pH 8.0), 137 mmol/L NaCl, 10% glycerol, 2 mmol/L EDTA, 1 mmol/L sodium orthovanadate, and protease inhibitor cocktail tablet (Roche)]. The human Phospho-RTK array kit (R&D Systems) was used according to the manufacturer's protocol.

Immunoblotting

Cell pellets were treated with lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), 1% Triton X-100, 1 mmol/L sodium orthovanadate, and protease inhibitor cocktail tablet]. The supernatants were assessed for protein concentration using the Bio-Rad DC protein assay kit II.

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For the studies of the MEK/ERK and PI3K/Akt pathways, 20 μg of proteins from cell lysate were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were saturated in TBS–0.05% Tween 20 containing either 5% (w/v) nonfat dry milk or 5% bovine serum albumin (Sigma-Aldrich). Membranes were then incubated overnight at 4°C with the primary antibody, and then incubated at room temperature for 1 hour with a horseradish peroxidase–conjugated anti-mouse or anti-rabbit secondary antibody. Analysis was done by chemiluminescence using the ECL-Plus Western blotting detection reagent (GE Healthcare) with Kodak film.

For the phospho-Met and Met detection, 100 μg of total proteins were subjected to SDS-PAGE. The membranes were then incubated with blocking buffer [0.2% (v/v) casein, 0.1% (v/v) Tween 20 dissolved in PBS] for 1 hour and probed for 1 hour 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

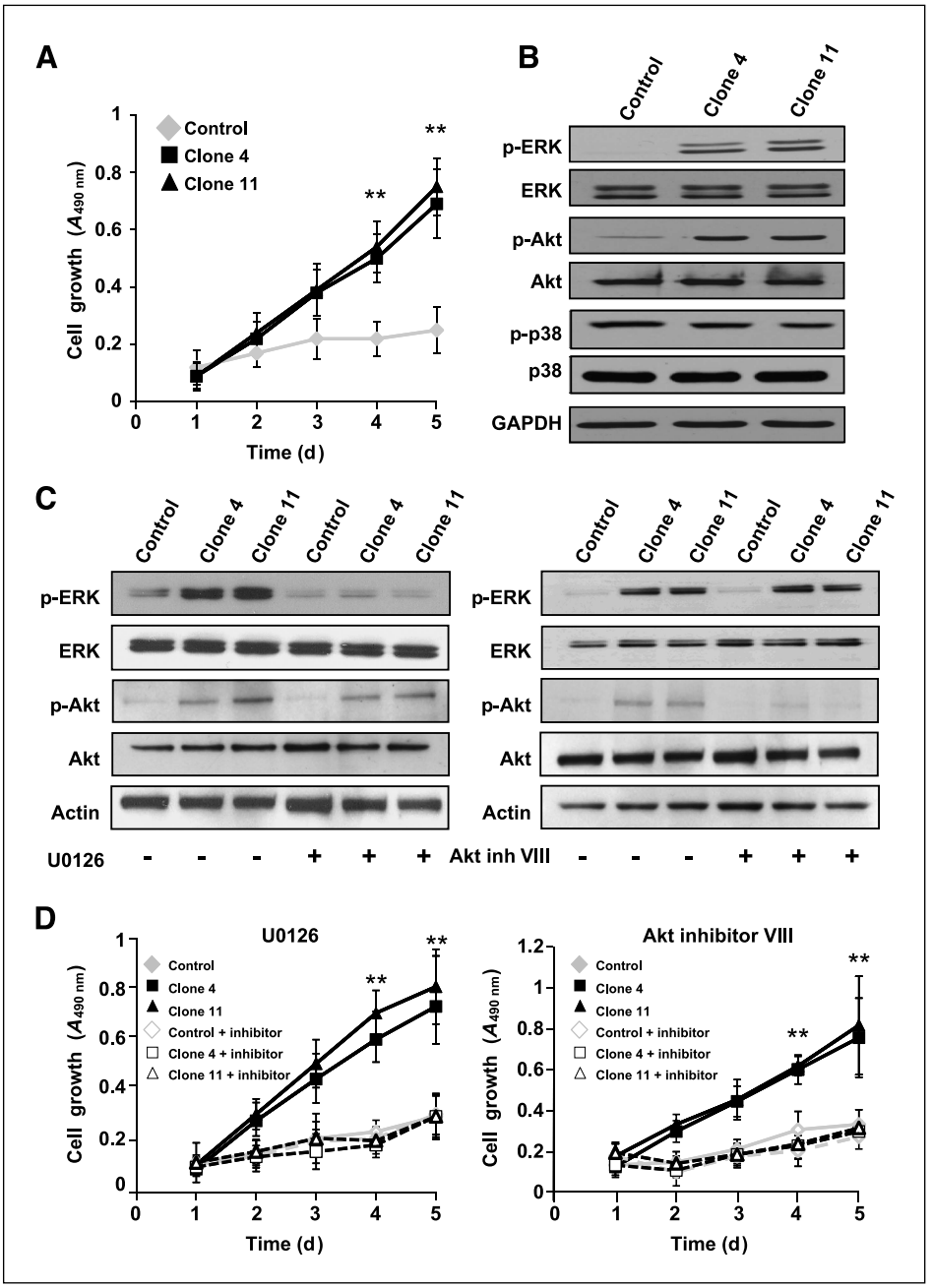

FIGURE 1. Involvement of MEK/ERK and PI3K/Akt pathways in the proliferation of GD3S$^+$ clones. A, cell proliferation assay on standard monolayer plates. GD3S$^+$ clones 4 and 11 and control cells were cultured in serum-free conditions for 5 d. Cell growth was determined by the MTS assay. Each measurement was done in 16 wells, and data are the means of four independent manipulations. **, $P < 0.01$, GD3S$^+$ cells versus control cells. B, Western blot analysis of ERK, Akt, and p38, and their phosphorylated forms in control and GD3S$^+$ MDA-MB-231 clones after 48 h of culture in serum-free conditions. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control for protein loading. C, cells were treated in serum-free medium for 48 h with 1 μmol/L U0126 or 1 μmol/L Akt inhibitor VIII. Lysates were subjected to immunoblotting to determine the decrease in pERK and pAkt proteins. D. Proliferation assay using MTS. GD3S$^+$clones 4 (■) and 11 (▲) and control cells (♦) were cultured in serum-free medium with the indicated concentration of DMSO as control. In parallel, clones 4 (□) and 11 (△) and control cells (◇) were cultured in the presence of U0126 or Akt inhibitor VIII for 5 d. **, $P < 0.01$, inhibitor-treated versus untreated GD3S$^+$ cells.

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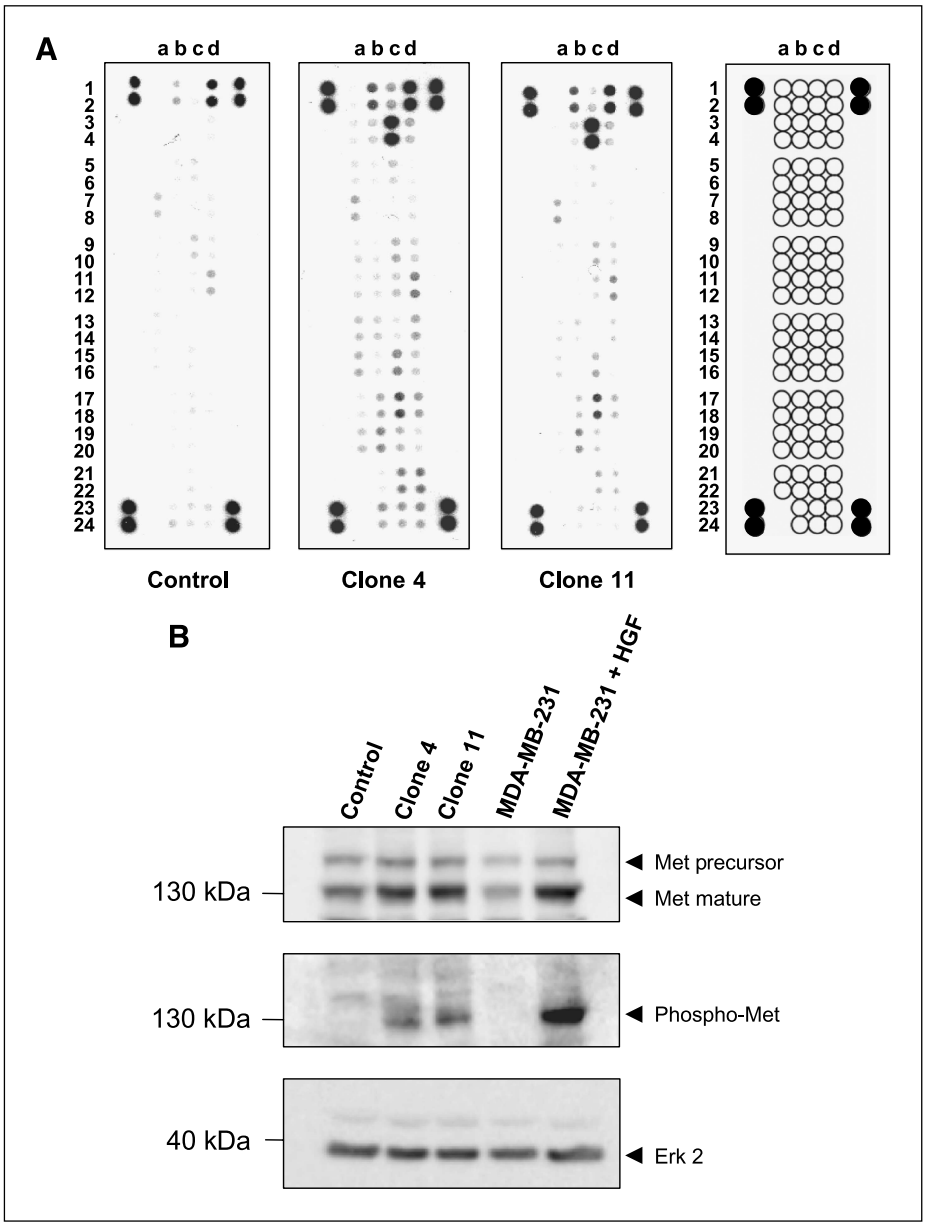

FIGURE 2. Activation of c-Met in GD3S$^+$ clones in serum-free conditions. A, total cell lysates from control, clone 4, and clone 11, cultured for 48 h under serum-free conditions, were subjected to phospho-RTK array. The phospho-RTK array coordinates are given on the right side of the figure. Black dots represent phospho-tyrosine positive controls: a1, a2: EphA6; a3, a4: EphA7; a5, a6: EphB1; a7, a8: EphB2; a9, a10: EphB4; a11, a12: EphB6; a13, a14: mouse IgG1 negative control; a15, a16: mouse IgG2A negative control; a17, a18: mouse IgG2B negative control; a19, a20: goat IgG negative control; a21, a22: PBS negative control; b1, b2: Tie-2; b3, b4: TrkA; b5, b6: TrkB; b7, b8: TrkC; b9, b10: VEGFR1; b11, b12: VEGFR2; b13, b14: VEGFR3; b15, b16: MuSK; b17, b18: EphA1; b19, b20: EphA2; b21, b22: EphA3; b23, b24: EphA4; c1, c2: Mer; c3, c4: c-Met; c5, c6: MSPR; c7, c8: PDGFRα; c9, c10: PDGFRβ; c11, c12: SCFR; c13, c14: Flt-3; c15, c16: M-CSFR; c17, c18: c-Ret; c19, c20: ROR1; c21, c22: ROR2; c23, c24: Tie-1; d1, d2: EGFR; d3, d4: ErbB2; d5, d6: ErbB3; d7, d8: ErbB4; d9, d10: FGFR1; d11, d12: FGFR2α; d13, d14: FGFR3; d15, d16: FGFR4; d17, d18: insulin R; d19, d20: IGF-IR; d21, d22: Axl; d23, d24: Dtk. B, Western blot analysis using specific c-Met and phospho-c-Met antibodies. Control, GD3S$^+$ MDA-MB-231, and wild-type MDA-MB-231 cultured in serum-free conditions were treated or not for 15 min with 30 ng/mL HGF/SF. Cell lysates were analyzed by Western blotting with antibody directed against human Met and the phosphorylated tyrosine residues of the kinase domain. Reprobing with anti-ERK2 antibody was done to assess loading.

detected with specific secondary antiserum conjugated with alkaline phosphatase followed by an enhanced chemiluminescence detection system (Amersham ECL Western Blotting Detection Reagents).

Tumor growth in SCID mice

Female SCID mice were purchased from the Pasteur Institute and acclimatized for at least 2 weeks. Mice were maintained under a 12 hours light/dark cycle at

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a temperature of 20°C to 22°C. Food and water were available *ad libitum*. Mice were maintained in accordance with the Institutional Animal Care and Use Committee procedures and guidelines. The local ethics committee approved the protocol used. MDA-MB-231 control and GD3S$^+$ cells were harvested and resuspended in phosphate-buffered solution before s.c. injection into the flanks of mice (10 mice per group) as previously described (11).

Statistical analysis

Microarray data from two datasets previously published by van de Vijver et al. (ref. 12; Agilent platform) and Chin et al. (ref. 13; Affymetrix platform) were analyzed using Prism 5 (GraphPad Software). The probes considered for the analysis were UGID Hs.408614 (Agilent) and A.210073_at (Affymetrix) for *ST8SIA1* and A.203510_at (Affymetrix) for *MET*. The levels of expression of these genes were compared from one group to another by using the one-way ANOVA test. Correlation of gene expression was tested using Pearson's test. Statistical analysis of other results was done using Student's *t* test. $P < 0.05$ was considered statistically significant.

Results

G_{D3} synthase expression promotes MDA-MB-231 proliferation through the activation of MEK/ERK and PI3K/Akt pathways

To determine the functional importance of the GD3S expression in breast cancer development and aggressiveness, we previously established a cellular model derived from MDA-MB-231 breast cancer cells expressing GD3S. The selected clones (named clones 4 and 11)

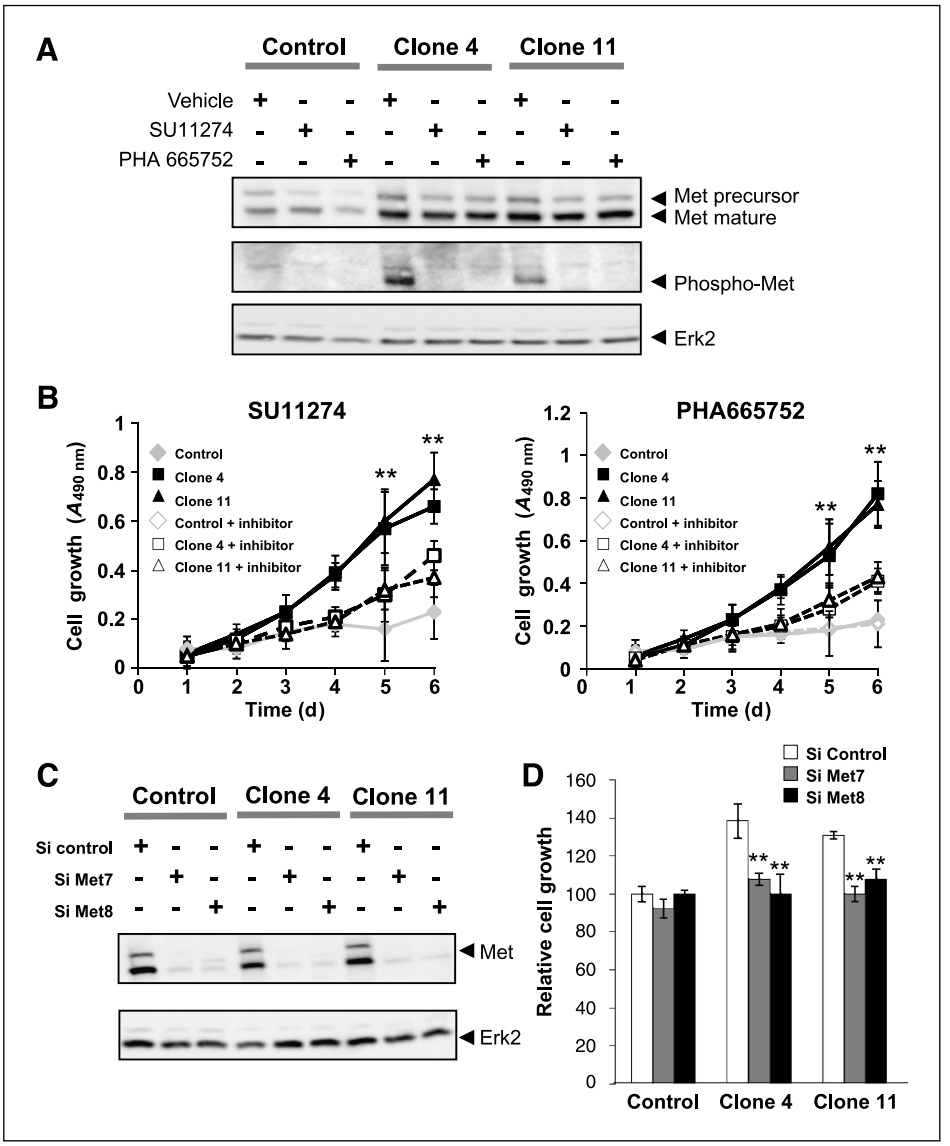

FIGURE 3. Reduction of the proliferation of the GD3S$^+$ clones on c-Met inhibition. A, inhibition of c-Met phosphorylation by pharmacologic inhibitors of c-Met. Control cells and clones 4 and 11 were cultured in the presence of 500 nmol/L SU11274 or 100 nmol/L of PHA 665752 for 48 h before lysis and Western blot analysis. Expression of Erk2 was used as control for protein loading. B, cell proliferation assays. Clones 4 (■) and 11 (▲) and control cells (♦) were cultured for 5 d in serum-free medium containing 500 nmol/L SU11274, 100 nmol/L PHA 665752, or the indicated dilution of DMSO as the negative control (□, clone 4; △, clone 11; ◇, MDA-MB-231 control). **, $P < 0.01$, inhibitor-treated versus untreated GD3S$^+$ cells. C, immunoblotting analysis of c-Met after siRNA transfection. Total cell lysates were extracted after one round of transfection with siRNA control or with siRNA targeting c-Met (named *Met7* and *Met8*). D, proliferation assay after c-Met siRNA transfection. One day after the transfection, cells were seeded in 96-well plates and cell growth was then analyzed after 48 h as described in Materials and Methods. Absorbance values were normalized to the control cells transfected with a scramble sequence, which was arbitrarily set as 100%. **, $P < 0.01$, GD3S$^+$ cells versus control cells.

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express b- and c- series gangliosides such as G_{D3}, G_{D2}, and G_{T3} at the cell surface (10). As shown in Fig. 1A, the modification of the ganglioside pattern of MDA-MB-231 cells was correlated with an increase of cell proliferation capacity in serum-free conditions. After 5 days of culture, the relative number of GD3S$^+$ cells is ~4-fold of control cells. The activation of the main signaling pathways involved in MDA-MB-231 proliferation (i.e., MEK/ERK, PI3K/Akt, and p38/mitogen-activated protein kinase pathways; ref. 14) was then determined by Western blot analysis. As shown in Fig. 1B, after 48 hours of culture in the absence of serum, higher levels of phospho-Akt (pAkt) and phospho-ERK (pERK) were observed in GD3S$^+$ cells compared with control.

In contrast, no difference in phospho-p38 was observed between clones and control cells. The involvement of MEK/ERK and PI3K/Akt pathways in cell proliferation was then determined using selective inhibitors against MEK (1 μmol/L U0126) and Akt (1 μmol/L Akt inhibitor VIII). The concentration used was based on the absence of toxicity in MDA-MB-231 cells, as determined by cell proliferation assay in serum-free medium for 48 hours (data not shown). Efficient inhibition of MEK or Akt (Fig. 1C) did not modify proliferation of control cells but strongly reduced that of GD3S$^+$ cells, with the relative number of GD3S$^+$ cells being similar to that of control ones (Fig. 1D). Taken together, these results indicate an essential function of MEK/ERK and PI3K/Akt pathways in the proliferative phenotype of GD3S$^+$ MDA-MB-231 cells.

c-Met receptor is constitutively activated in G_{D3} synthase–expressing cells

Several studies have shown that cellular ganglioside composition can modulate the activity of growth factor receptors, and consequently induce the activation of intracellular molecular pathways (15-17). In this context, the phosphorylation status of 42 RTKs was simultaneously examined using a phospho-RTK array in MDA-MB-231 cells. As shown in Fig. 2A, GD3S$^+$ MDA-MB-231 cells displayed a strong phosphorylation of c-Met receptor compared with control cells cultured in serum-free or complete medium (Supplementary Fig. S1). A weak increase of epidermal growth factor receptor (EGFR), Tie-2, and Ret phosphorylation was also observed. To confirm c-Met activation, we performed Western blot analysis using anti–phospho-c-Met antibodies directed against phosphorylated tyrosine residues 1234 and 1235 of c-Met. As shown in Fig. 2B, phospho-c-Met was detected only in GD3S$^+$ cells but not in control cells. As expected, HGF/SF stimulation induced efficient phosphorylation of c-Met in control MDA-MB-231 cells. In addition, confocal microscopy analysis of c-Met showed no significant difference in c-Met distribution between control and MDA-MB-231 GD3S$^+$ cells (Supplementary Fig. S2). Treatment of GD3S$^+$ MDA-MB-231 cells with 5D5 Fab, which inhibits ligand-dependent activation of c-Met through inhibition of HGF/SF-Met association (refs. 18, 19; Supplementary Fig. S3A), does not affect cell proliferation, excluding autocrine activation of c-Met by HGF/SF (Supplementary Fig. S3B). A slight increase of c-Met expression was also observed in both clones, in comparison with control cells. However, quantitative PCR analysis did not reveal modification of *MET* transcriptional expression in GD3S$^+$ MDA-MB-231 clones (Supplementary Fig. S4).

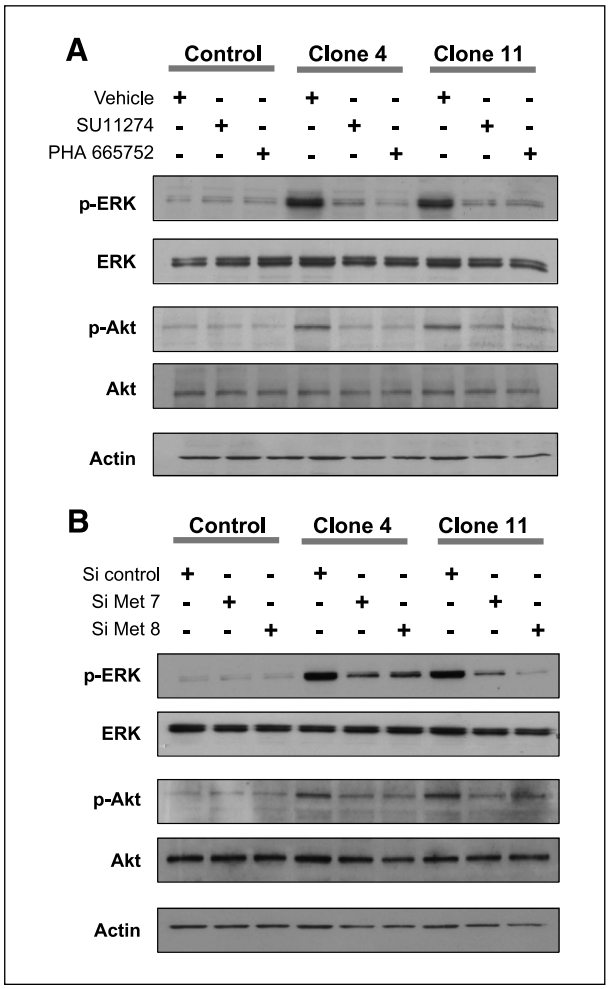

FIGURE 4. Western blot analysis of p-ERK and p-Akt on c-Met inhibition. A, Western blot analysis of pERK and pAkt in control cells and GD3S$^+$ clones after treatment with c-Met inhibitors: 500 nmol/L SU11274 or 100 nmol/L PHA 665752. B, Western blot analysis of MEK/ERK and PI3K/Akt pathways in control cells and GD3S$^+$ clones after treatment with 20 pmol of specific siRNA.

c-Met activation is involved in the increased proliferation of GD3S$^+$ MDA-MB-231 cells

To determine the potential involvement of c-Met in the increased proliferation of GD3S$^+$ MDA-MB-231 cells, we first inhibited c-Met activation using two selective ATP-competitive inhibitors of the c-Met kinase, SU11274 and PHA665752 (20, 21). Both inhibitors abrogated constitutive c-Met phosphorylation (Fig. 3A) and significantly

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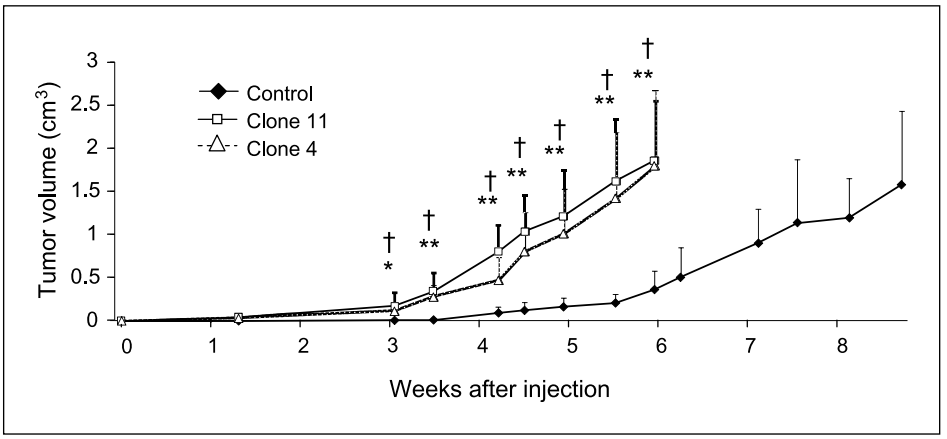

FIGURE 5. Xenograft tumor growth in SCID mice. Growth curve of tumors. Control and GD3S$^+$ MDA-MB-231 cells were s.c. injected into SCID mice. Ten animals were used in each group. Tumor volume was monitored every 2 or 3 d until tumor volume approached 2 cm^3. Student's *t* test was done between control and clone 11 (*, $P < 0.05$; **, $P < 0.01$), and between control and clone 4 (†, $P < 0.05$).

decreased the proliferation of both GD3S$^+$ clones (Fig. 3B). In addition, both inhibitors of the c-Met kinase activity reduced the migration capacity of both clones to the level of control cells (Supplementary Fig. S5). We then targeted c-Met with specific siRNA, which efficiently silenced c-Met expression in control and GD3S$^+$ MDA-MB-231 cells (Fig. 3C). Inhibition of c-Met expression prevented increased proliferation of clones 4 and 11, thus demonstrating the involvement of c-Met receptor in the high proliferative property of GD3S$^+$ cells in deprivation conditions. Moreover, both pharmacologic agents and c-Met siRNA also strongly decreased the phosphorylation of ERK and Akt in GD3S$^+$ clones (Fig. 4A and B). Altogether, these results show that the enhanced proliferation of GD3S$^+$ MDA-MB-231 cells is essentially due to c-Met activation, which in turn activates the downstream MEK/ERK and PI3K/Akt signaling pathways.

G_{D3} synthase overexpression increases tumor growth in SCID mice

MDA-MB-231 cells were s.c. injected into SCID mice. All animals formed a tumor at the injection site. However, tumor growth of GD3S$^+$ cells were significantly increased because tumors were palpable 1 week after injection and attained a size of 1.75 cm^3 in ~6 weeks. In contrast, empty vector–transfected cells formed palpable tumors with a latency of 3 weeks and formed tumors of similar size in ~8 weeks (Fig. 5).

Statistical analyses of microarray data

ST8SIA1 expression in breast cancer primary tumors was investigated by exploring two microarray datasets previously published by van de Vijver et al. (12) and Chin et al. (13). The first dataset consisted of 295 stage I or II breast cancers (12), and the second consisted of 118 stage I-III (mostly I-II) tumors (13). As previously reported (9), we found a significant association of *ST8SIA1* mRNA expression with ER negativity in both datasets (data not shown). To refine this result, we used the established subtype classification based on the 534 genes signature defined by Sorlie et al. (22). For both datasets, each tumor has been categorized as one of the five defined subtypes: basal, erbB2, normal-like, luminal A, and luminal B. This classification has been shown to reflect the clinical outcome of the patients with both basal and erbB2 subtypes being associated with poorer prognosis (22). We have analyzed the level of expression of *ST8SIA1* in the different subtypes and found it significantly higher in the basal subtypes than in any other group in both datasets (Fig. 6A and B). Interestingly, higher expression of *MET* was also observed in the basal subgroup (Fig. 6B). We also found that the expression levels of *ST8SIA1* and *MET* were significantly correlated ($P < 0.001$ Pearson's correlation test) in the Chin et al. dataset. Taken together, these results suggest that *ST8SIA1* is overexpressed in poor-prognosis breast cancers (basal type), possibly in conjunction with *MET*.

Discussion

In the present study, we showed that the expression of GD3S and complex gangliosides in MDA-MB-231 cells leads to a proliferative phenotype in the absence of serum or growth factors. This phenotype is induced by the constitutive activation of c-Met and subsequent signal transduction MEK/ERK and PI3K/Akt pathways. GD3S expression not only promotes cell growth *in vitro* but also stimulates primary tumor growth in SCID mice and is associated with *MET* gene expression in the basal subtype of human breast tumors.

We previously shown that GD3S expression renders MDA-MB-231 cells independent of serum (10). The ability to survive and proliferate under serum-free conditions is one of the well-known features of aggressive cancer cells *in vitro* that can be mediated by a constitutive phosphorylation of RTKs, the key regulators of critical cellular processes such as cell growth and survival. Dysregulated activation of RTKs is implicated in the genesis and progression of a variety of cancers, including breast cancer (23). By using phospho-RTK array, we analyzed the activation pattern of various RTKs in GD3S$^+$ clones. We observed a strong and specific constitutive activation of

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the c-Met receptor despite the absence of ligand-dependent stimulation. Moreover, the downregulation of c-Met induced a significant reversion of the phenotype, demonstrating the crucial function of c-Met in the proliferative capacity of GD3S$^+$ clones. It has been well shown that during oncogenesis, abnormal c-Met activation results in a complex program called "invasive growth," consisting of the activation of a series of cellular processes such as proliferation, scattering, invasion, and survival (24). Thus, c-Met activation through HGF/SF stimulation induced MDA-MB-231 growth, motility, and invasiveness (25). c-Met is also known to induce survival of bladder carcinoma cells submitted to serum starvation (26); however, to our knowledge, nothing is described concerning the implication of c-Met in breast cancer cell proliferation in serum-free conditions.

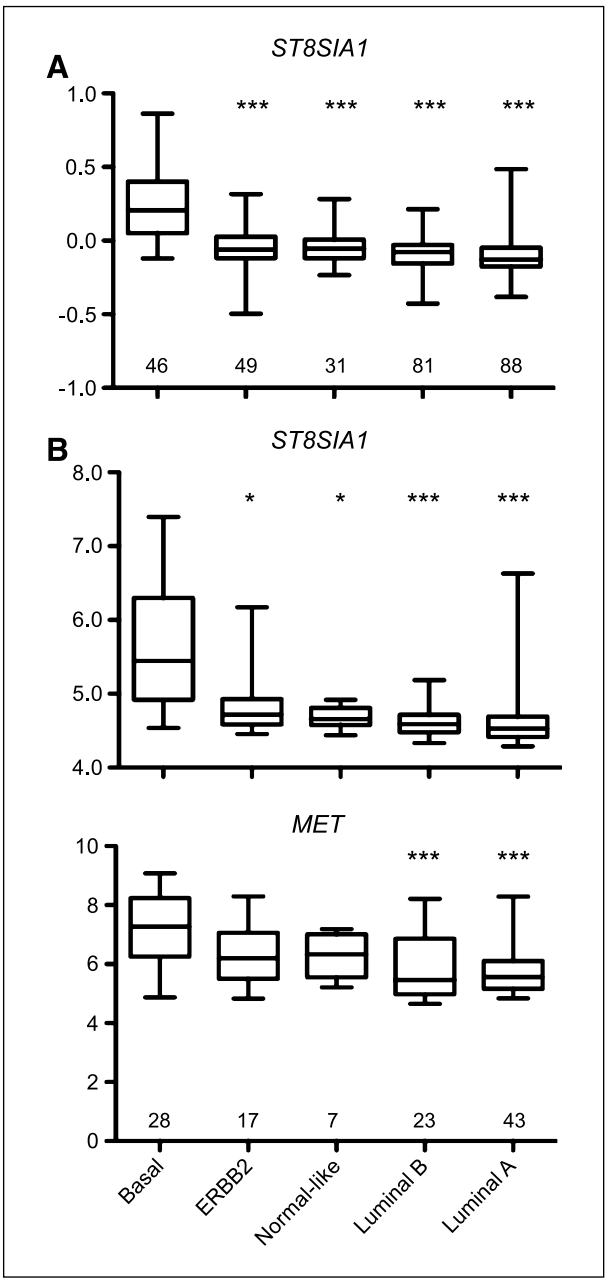

FIGURE 6. *ST8SIA1* and *MET* expression in breast cancer subtypes. A, *ST8SIA1* RNA expression is expressed as the difference to the median signal detected on Agilent microarray (12). B, *ST8SIA1* and *MET* expression is expressed as normalized signal detected on the Affymetrix microarray (13). For both dataset, the number of tumors in each subtype group is reported next to the *X* axis. Significant differences between basal and other subtypes are indicated: *, $P < 0.05$; ***, $P < 0.001$.

Perturbation of the HGF/Met signaling axis leads to enhanced signaling that occurs in a wide range of aggressive human cancers, correlated with poor prognosis. As previously described (27), the two intracellular pathways, MEK/ERK and PI3K/Akt, promoted by c-Met *trans*-phosphorylation serve as key downstream integrators of growth and survival signals in MDA-MB-231 cells (14). Signaling for mitogenesis and growth essentially occurs through the MEK/ERK pathway, whereas PI3K-Akt signaling has been shown to play a central role in the antiapoptotic responses induced by activated c-Met. Interestingly, in this context, we showed using specific pharmacologic inhibitors that the simultaneous activation of both pathways is necessary to maintain the proliferative phenotype of GD3S$^+$ cells in serum-free conditions.

The GD3S expression leads to the accumulation of complex gangliosides from b- and c-series at the plasma membrane of MDA-MB-231 cells. It is commonly known that gangliosides are associated or complexed with signal transducers such as small G-proteins, Src family kinases, tetraspanins, and integrins (28). The modulation of ganglioside expression can therefore have deep effects on receptor-mediated signaling, notably RTKs, and regulate cell growth through inhibition/activation of signal transduction pathways (15-17). For example, tyrosine kinase activity of EGFR is inhibited by the monosialoganglioside G_{M3} through carbohydrate-carbohydrate interactions of G_{M3} with GlcNAc terminated *N*-glycan on EGFR, without interfering with EGF binding (29, 30). Moreover, G_{M3} has been described as an angiogenesis suppressor by regulating vascular endothelial growth factor receptor-2 phosphorylation (31, 32). Previously, it has been shown that a-series gangliosides act as negative regulators of c-Met. G_{D1a} inhibits HGF-induced motility and scattering of cancer cells through suppression of tyrosine phosphorylation of c-Met (33). G_{M3} and G_{M2} form heterodimers that specifically interact with tetraspanin (CD82) in glycosynapses, and such complexes inhibit c-Met activation and integrin cross talk (34, 35). However, to our knowledge, it is the first demonstration of ganglioside-induced c-Met constitutive activation in cancer cells.

Dysregulation of c-Met signaling through mutation, overexpression, or autocrine/paracrine loops has been well documented and is an important feature in various human cancers, especially in terms of metastasis development (36). To date, no mutation has been found either in human breast cancer biopsies or in breast cancer cell lines, including MDA-MB-231 (http://www.vai.org/met/). A slight

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increase of c-Met expression was observed in both GD3S$^+$ clones, but was not correlated to an increase of *MET* transcription, as revealed by quantitative PCR analysis. A majority of breast tumors aberrantly expresses HGF and its receptor (37, 38), which may activate c-Met in an autocrine manner. However, it has been shown that the autocrine activation of c-Met does not occur in MDA-MB-231 (39). Proliferation assay of GD3S$^+$ clones with 5D5 Fab, which specifically blocks HGF binding and subsequent pathway activation, has confirmed ligand-independent activation of c-Met on GD3S expression. Thus, we described the original mechanism of c-Met activation independently of gene amplification or autocrine activation induced by complex gangliosides in MDA-MB-231 GD3S$^+$ cells.

As shown in Fig. 3, downregulation of c-Met activation did not completely abolish the proliferative capacities of these cells after 5 days of culture in deprivation conditions. It is possible that other RTKs could contribute to the enhanced proliferation, such as EGFR, Ret, and Tie-2, which are also found to be slightly activated in GD3S$^+$ cells. Consistent with this hypothesis, recent evidence shows that c-Met cross talk with other cell surface proteins, including RTKs, contributes to tumorigenesis (40, 41). For example, the synergistic action of c-Met and EGFR has been shown to enhance the malignant properties of cancer cells by promoting the resistance of tumors to therapy (42, 43).

In vivo experiments reveal that GD3S expression enhances the tumorigenicity of MDA-MB-231 cells in SCID mice. This suggests that modification of complex ganglioside patterns may provide some advantage for cancer cells to grow in the cellular environment of host tissues. Previous studies have shown that stable inhibition of G_{D3} and G_{D2} expression in human lung cancer cells by GD3S siRNA significantly suppressed cell growth in SCID mice (44). In parallel, *ST8SIA1* overexpression was associated with poor pathohistologic grading in ER-negative tumors and reduced overall survival of patients (8, 9). We report here a higher expression of *ST8SIA1* in the basal subtype of breast tumors compared with others. Interestingly, a similar pattern was found for *MET*, in accordance with previous reports (38, 45, 46). c-Met is also associated with poor clinical outcome and considered as a possible marker for earlier recurrence and shorter survival in breast cancer patients (47-49). Our results support the idea that the products of these two genes may cooperate within the same subtype of breast cancers: the basal type, which consists mainly of the triple-negative breast cancers (ER, progesterone receptor, and erbB2 negative). These cancers are of poor prognosis and cannot benefit from available targeted therapies (i.e., hormone therapy and Herceptin treatment; ref. 22).

It has been recently shown that the GD3S expression renders human hepatocarcinoma cells susceptible to hypoxia, which mediates ROS generation and subsequently cell death (50). Hepatocarcinoma GD3S$^+$ cells display enhanced G_{D3} levels both at the cell surface and in internal organelles, most likely including mitochondria. This specific subcellular localization is consistent with the proapoptotic role of G_{D3} described in a variety of cell types. Therefore, the biological outcome of G_{D3} synthase expression in cancer cell biology also depends on the subsequent cellular localization and the relative amounts of complex gangliosides that localize to the mitochondria.

To conclude, our results show that GD3S and complex ganglioside expression may contribute to increase the malignant properties of breast cancer cells, by activating RTKs, especially c-Met. This mechanism underlies a new way for c-Met constitutive activation. Molecular targeted therapies have been developed for over a decade to specifically inhibit consistently activated oncogene products, including c-Met (25). However, their efficiency is limited to some patients because of the development of intrinsic or acquired resistance. The present results suggest that complex gangliosides are not only antigenic markers but could be also valid therapeutic targets in basal-type/triple-negative breast cancers. Although further studies are needed to precisely determine which type of gangliosides is responsible for the specific activation of c-Met, our results suggest that targeting both gangliosides and c-Met would lead to a more favorable clinical outcome than targeting RTKs alone.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Hakomori SI. Inaugural article: the glycosynapse. Proc Natl Acad Sci U S A 2002;99:225–32.
2. Tettamanti G. Ganglioside/glycosphingolipid turnover: new concepts. Glycoconj J 2004;20:301–7.
3. Hakomori SI. Tumor-associated carbohydrate antigens defining tumor malignancy: basis for development of anti-cancer vaccines. Adv Exp Med Biol 2001;491:369–402.
4. Birklé S, Zeng G, Gao L, Yu RK, Aubry J. Role of tumor-associated gangliosides in cancer progression. Biochimie 2003;85:455–63.
5. Dyatlovitskaya EV, Kandyba AG. Role of biologically active sphingolipids in tumor growth. Biochemistry 2006;71:10–7.
6. Furukawa K, Hamamura K, Nakashima H, Furukawa K. Molecules in the signaling pathway activated by gangliosides can be targets of therapeutics for malignant melanomas. Proteomics 2008;8:3312–6.

Published OnlineFirst October 1, 2010; DOI: 10.1158/1541-7786.MCR-10-0302

7. Marquina G, Waki H, Fernandez LE, et al. Gangliosides expressed in human breast cancer. Cancer Res 1996;56:5165–71.
8. Ruckhäberle E, Karn T, Rody A, et al. Gene expression of ceramide kinase, galactosyl ceramide synthase and ganglioside G_{D3} synthase is associated with prognosis in breast cancer. J Cancer Res Clin Oncol 2009;135:1005–13.
9. Ruckhäberle E, Rody A, Engels K, et al. Microarray analysis of altered sphingolipid metabolism reveals prognostic significance of sphingosine kinase 1 in breast cancer. Breast Cancer Res Treat 2008;112:41–52.
10. Cazet A, Groux-Degroote S, Teylaert B, et al. GD3 synthase overexpression enhances proliferation and migration of MDA-MB-231 breast cancer cells. Biol Chem 2009;390:601–9.
11. Lagadec C, Meignan S, Adriaenssens E, et al. TrkA overexpression enhances growth and metastasis of breast cancer cells. Oncogene 2009;28:1960–70.
12. Van de Vijver MJ, He YD, van't Veer LJ, et al. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 2002; 347:1999–2009.
13. Chin K, DeVries S, Fridlyand J, et al. Genomic and transcriptional aberrations linked to breast cancer pathophysiologies. Cancer Cell 2006;10:529–41.
14. Ripple MO, Kalmadi S, Eastman A. Inhibition of either phosphatidylinositol 3-kinase/Akt or the mitogen/extracellular-regulated kinase, MEK/ERK, signaling pathways suppress growth of breast cancer cell lines, but MEK/ERK signaling is critical for cell survival. Breast Cancer Res Treat 2005;93:177–88.
15. Miljan EA, Bremer EG. Regulation of growth factor receptors by gangliosides. Sci STKE 2002;2002:re15.
16. Lopez PH, Schnaar RL. Gangliosides in cell recognition and membrane protein regulation. Curr Opin Struct Biol 2009;19:549–57.
17. Kaucic K, Liu Y, Ladisch S. Modulation of growth factor signaling by gangliosides: positive or negative? Methods Enzymol 2006;417: 168–85.
18. Martens T, Schmidt NO, Eckerich C, et al. A novel one-armed anti-c-Met antibody inhibits glioblastoma growth *in vivo*. Clin Cancer Res 2006;12:6144–52.
19. Jin H, Yang R, Zheng Z, et al. MetMAb, the one-armed 5D5 anti-c-Met antibody, inhibits orthotopic pancreatic tumor growth and improves survival. Cancer Res 2008;68:4360–8.
20. Berthou S, Aebersold DM, Schmidt LS, et al. The Met kinase inhibitor SU11274 exhibits a selective inhibition pattern toward different receptor mutated variants. Oncogene 2004;23:5387–93.
21. Christensen JG, Schreck R, Burrows J, et al. A selective small molecule inhibitor of c-Met kinase inhibits c-Met-dependent phenotypes *in vitro* and exhibits cytoreductive antitumor activity *in vivo*. Cancer Res 2003;63:7345–55.
22. Sorlie T, Tibshirani R, Parker J, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A 2003;100:8418–23.
23. Gschwind A, Fischer OM, Ullrich A. The discovery of receptor tyrosine kinases: targets for cancer therapy. Nat Rev Cancer 2004;4:361–70.
24. Trusolino L, Comoglio PM. Scatter-factor and semaphorin receptors: cell signalling for invasive growth. Nat Rev Cancer 2002;2:289–300.
25. Beviglia L, Matsumoto K, Lin CS, Ziober BL, Kramer RH. Expression of the c-Met/HGF receptor in human breast carcinoma: correlation with tumor progression. Int J Cancer 1997;74:301–9.
26. Yamamoto N, Mammadova G, Song RX, Fukami Y, Sato K. Tyrosine phosphorylation of p145met mediated by EGFR and Src is required for serum-independent survival of human bladder carcinoma cells. J Cell Sci 2006;119:4623–33.
27. Eder JP, Vande Woude GF, Boerner SA, LoRusso PM. Novel therapeutic inhibitors of the c-Met signaling pathway in cancer. Clin Cancer Res 2009;15:2207–14.
28. Kasahara K, Watanabe Y, Yamamoto T, Sanai Y. Association of Src family tyrosine kinase Lyn with ganglioside GD3 in rat brain. Possible regulation of Lyn by glycosphingolipid in caveolae-like domains. J Biol Chem 1997;272:29947–53.
29. Miljan EA, Meuillet EJ, Mania-Farnell B, et al. Interaction of the extracellular domain of the epidermal growth factor receptor with gangliosides. J Biol Chem 2002;277:10108–13.
30. Kawashima N, Yoon SJ, Itoh K, Nakayama K. Tyrosine kinase activity of epidermal growth factor receptor is regulated by GM3 binding through carbohydrate to carbohydrate interactions. J Biol Chem 2009;284:6147–55.
31. Mukherjee P, Faber AC, Shelton LM, Baek RC, Chiles TC, Seyfried TN. Ganglioside GM3 suppresses the pro-angiogenic effects of vascular endothelial growth factor and ganglioside GD1A. J Lipid Res 2008;49: 929–38.
32. Chung TW, Kim SJ, Choi HJ, et al. Ganglioside GM3 inhibits VEGF/VEGFR-2-mediated angiogenesis: direct interaction of GM3 with VEGFR-2. Glycobiology 2009;19:229–39.
33. Hyuga S, Kawasaki N, Hyuga M, et al. Ganglioside GD1a inhibits HGF-induced motility and scattering of cancer cells through suppression of tyrosine phosphorylation of c-Met. Int J Cancer 2001; 94:328–34.
34. Todeschini AR, Dos Santos JN, Handa K, Hakomori SI. Ganglioside GM2-tetraspanin CD82 complex inhibits met and its cross-talk with integrins, providing a basis for control of cell motility through glycosynapse. J Biol Chem 2007;282:8123–33.
35. Todeschini AR, Dos Santos JN, Handa K, Hakomori SI. Ganglioside GM2/GM3 complex affixed on silica nanospheres strongly inhibits cell motility through CD82/cMet-mediated pathway. Proc Natl Acad Sci U S A 2008;105:1925–30.
36. Corso S, Comoglio PM, Giordano S. Cancer therapy: can the challenge be MET? Trends Mol Med 2005;11:284–92.
37. Edakuni G, Sasatomi E, Satoh T, Tokunaga O, Miyazaki K. Expression of the hepatocyte growth factor/c-Met pathway is increased at the cancer front in breast carcinoma. Pathol Int 2001;51:172–8.
38. Kang JY, Dolled-Filhart M, Ocal IT, et al. Tissue microarray analysis of hepatocyte growth factor/Met pathway components reveals a role for Met, matriptase, and hepatocyte growth factor activator inhibitor 1 in the progression of node-negative breast cancer. Cancer Res 2003;63:1101–5.
39. Parr C, Jiang WG. Expression of hepatocyte growth factor/scatter factor, its activator, inhibitors and the c-Met receptor in human cancer cells. Int J Oncol 2001;19:857–63.
40. Lai AZ, Abella JV, Park M. Crosstalk in Met receptor oncogenesis. Trends Cell Biol 2009;19:542–51.
41. Xu AM, Huang PH. Receptor tyrosine kinase coactivation networks in cancer. Cancer Res 2010;70:3857–60.
42. Shattuck DL, Miller JK, Carraway KL III, Sweeney C. Met receptor contributes to trastuzumab resistance of Her2-overexpressing breast cancer cells. Cancer Res 2008;68:1471–7.
43. Karamouzis MV, Argiris A. Update on the role of EGFR inhibitors in cancer therapeutics. Cancer Treat Res 2007;135:257–75.
44. Ko K, Furukawa K, Takahashi T, et al. Fundamental study of small interfering RNAs for ganglioside GD3 synthase gene as a therapeutic target of lung cancers. Oncogene 2006;25:6924–35.
45. Ponzo MG, Lesurf R, Petkiewicz S, et al. Met induces mammary tumors with diverse histologies and is associated with poor outcome and human basal breast cancer. Proc Natl Acad Sci U S A 2009;106:12903–8.
46. Graveel CR, DeGroot JD, Su Y, et al. Met induces diverse mammary carcinomas in mice and is associated with human basal breast cancer. Proc Natl Acad Sci U S A 2009;106:12909–14.
47. Tolgay Ocal I, Dolled-Filhart M, D'Aquila TG, Camp RL, Rimm DL. Tissue microarray-based studies of patients with lymph node negative breast carcinoma show that met expression is associated with worse outcome but is not correlated with epidermal growth factor family receptors. Cancer 2003;97:1841–8.
48. Lengyel E, Prechtel D, Resau JH, et al. C-Met overexpression in node-positive breast cancer identifies patients with poor clinical outcome independent of Her2/neu. Int J Cancer 2005;113:678–82.
49. Garcia S, Dalès JP, Charafe-Jauffret E, et al. Poor prognosis in breast carcinomas correlates with increased expression of targetable CD146 and c-Met and with proteomic basal-like phenotype. Hum Pathol 2007;38:830–41.
50. Lluis JM, Llacuna L, von Montfort C, et al. GD3 synthase overexpression sensitizes hepatocarcinoma cells to hypoxia and reduces tumor growth by suppressing the cSrc/NF-κB survival pathway. PLoS One 2009;4:e8059.

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