Introduction

Glycosphingolipids (GSLs) are conjugates of ceramide (Cer) and carbohydrates that have been shown to participate in a variety of biological functions at cell surfaces during mammalian embryonic development and cell differentiation (Hakomori 1981; Hakomori and Kannagi 1984; Yamashita et al. 1999; Ngamukote et al. 2007). Lactosylceramide (Lac-Cer), a GSL, is synthesized from glucosylceramide (Glc-Cer) by the transfer of galactose from UDP-Gal by Lac-Cer synthase (Basu et al. 1968). Recent studies have shown that there are at least two Lac-Cer synthases, β4-galactosyltransferases 5 (β4GalT5) and 6 (β4GalT6), in mammalian tissues (Nomura et al. 1998; Sato, Shirane, et al. 2000; Kumagai et al. 2010; Nishie et al. 2010). The β4GalT5 is expressed constitutively while β4GalT6 is expressed in a tissue-specific manner (Lo et al. 1998; Nomura et al. 1998; Sato et al. 1998). Lacto-, globo- and ganglio-series of GSLs are synthesized in the Golgi apparatus by further glycosylation of Lac-Cer (reviewed in Nagui and Iwamori 1995). Changes in the composition of GSLs have been reported in a variety of tumors and of malignantly transformed cells where there is an enhanced expression of gangliosides, sialic acid-containing GSLs (Mora et al. 1969; Brady and Fishman 1974; Siddiqui et al. 1978; Baumann et al. 1979; Ye et al. 1990). In particular, gangliosides GM3 and GD3 derived directly from Lac-Cer are characteristic of many aggressive tumors such as melanomas and neuroblastomas (Furukawa and Lloyd 1990; Dong et al. 2011).

We have recently shown that β4GalT5 is solely involved in the biosynthesis of Lac-Cer in vivo (Kumagai et al. 2010), although it galactosylates N- and O-glycans in vitro (Sato et al. 1998; Van Die et al. 1999). Quite interestingly, we also found that the expression of the β4GalT5 gene increases significantly upon malignant transformation of NIH3T3 cells (Shirane et al. 1999). Analysis of the expression levels of the β4GalT5 gene in several human cancer cell lines, although the levels of their normal counterparts are unavailable, revealed them to correlate with those of the N-acetylglucosaminyltransferase V gene (Sato, Shirane, et al. 2000) whose high expression level is associated with malignant transformation of cells (Dennis et al. 1987; Lu and...
In support of this finding, the expression of the β4GalT5 gene has been shown to increase in human colon cancer-derived epithelial cell when compared with their normal counterpart, and in the progression of human glioma (Jiang et al. 2006; Kolmakova et al. 2009). Although β4GalT6 has been isolated as a Lac-Cer synthase from rat brain (Nomura et al. 1998), its involvement in the malignant transformation of cells has not been demonstrated.

In the present study, the biological significance of the increased expression of the β4GalT5 gene in tumors and malignant transformed cells (Shirane et al. 1999; Sato, Shirane, et al. 2000; Jiang et al. 2006; Kolmakova et al. 2009) was investigated by reducing the expression level of the β4GalT5 gene by transfecting its antisense cDNA into B16-F10 mouse melanoma cells, a highly tumorigenic and metastatic cell line that shows little β4GalT6 gene expression, and examining their malignant potentials in vivo.

**Results**

In order to reduce the expression level of the β4GalT5 gene, the siRNA sequence was initially designed using the siRNA Target Finder for the pSilencer Vectors (Ambion Inc., Austin), and cloned into the pSilencer. The vector was then transfected into B16-F10 cells, and several positive clones were obtained together with control clones. However, the expression level of the β4GalT5 gene was also reduced to some extents in the control clones with the pSilencer containing a random sequence. Replacement of the random sequence with others also resulted in the reduction. Therefore, in the present study, we decided to use not siRNA but rather its antisense cDNA to reduce the expression level of the β4GalT5 gene.

**Cloning of stable gene transfectants**

The antisense nucleotide sequence against mouse β4GalT5 cDNA as described in the Materials and methods section of this paper was cloned into the pcDNA3.1 vector (pcDNA3.1/ GT5-AS), and it was transfected into B16-F10 cells in the present study. Five clones, named E1–E5, were obtained after selection with geneticin, and the gene expression levels were determined by quantitative real-time reverse-transcription-polymerase chain reaction (RT-PCR) analysis using total RNA preparations from each clone. The expression levels in clones E4 and E5 were suppressed by 23 and 65%, respectively, while those in clones E1, E2 and E3 were suppressed by 10–15% when compared with those of two control clones, C1 and C2 (Figure 1). The expression levels in clones C1 and C2 appeared to be the same (Figure 1) and were similar to that of the parental B16-F10 cells (data not shown). The expression level of the β4GalT6 gene was very low both in clones C1 and E5 (data not shown). Therefore, in the present study, we used clone C1 as a control and clones E4 and E5 with the expression level of the β4GalT5 gene reduced by 23 and 65%, respectively.

**Cellular properties of clones C1 and E5**

When the growth rates of clones C1 and E5 were determined with a Cell Proliferation Assay kit (Promega, Madison, WI), they showed similar growth rates. However, after reaching the confluence, clone E5 cells were immediately eliminated probably due to the overcrowd of cells while clone C1 cells grew further for a few days after reaching the confluence and then eliminated (Figure 2A). The results indicate that clone E5 cells lose partially an overgrowth potential of clone C1 cells. During the growth phase, there was no difference in morphological appearance (data not shown), but at sub-confluence, a partial interaction among cells appeared in clone E5 that was not observed in clone C1 (Figure 2B). These results suggest that the cell surface properties of clone E5 are changed by reducing the expression level of the β4GalT5 gene in B16-F10 cells. Similar changes, although slight when compared with those of clone E5, were also observed in clone E4 (data not shown).

**Analysis of GSL composition of clones C1 and E5**

Since β4GalT5 has been shown to be a Lac-Cer synthase (Kumagai et al. 2010), neutral and acidic GSLs extracted from the membrane fractions of clones C1 and E5 were subjected to thin-layer chromatography (TLC) analysis. The results showed that the amount of Lac-Cer is decreased and that of Glc-Cer increased significantly in clone E5 when compared with those of clone C1 (left panel of Figure 2C). Similarly, the amount of GM3, a Lac-Cer derivative and a major GSL in B16 cells (Nozue et al. 1988; Wang et al. 2011), is decreased in clone E5 when compared with that of clone C1 (right panel of Figure 2C). The ratios of amounts of Lac-Cer and GM3 between clones C1 and E5 are indicated at the bottoms of the TLC plates (Figure 2C). When the amount of Cer was determined, no change was detected between clones C1 and E5 (Figure 2D).

**Tumorigenic potentials of clones C1, E4 and E5**

The tumorigenic potential of B16-F10 cells is very high when the cells are transplanted into C57BL/6 mice (Fidler and Nicolson 1976; Nozue et al. 1988; Wang et al. 2011). In order to examine whether the reduced expression level of the β4GalT5 gene affects the tumorigenic potential, clone C1, E4 and E5 cells showing >95% viability were each transplanted subcutaneously into 10 C57BL/6 mice. After 2 weeks, the tumors formed were excised from the animals (representative clone C1- and E5-derived tumors shown in Figure 3A), and diameters of the tumors were measured. The results showed that the average diameters of the tumors formed with clones C1, E4 and E5 to be 13.3 ± 0.5 mm, 10.4 ± 0.6 mm (P < 0.05)
and 5.9 ± 0.7 mm ($P < 0.01$), respectively (Table I). It is noteworthy that one out of 10 animals failed to develop tumors following transplantation of clone E5. In fact, RT-PCR analysis showed that the β4GalT5 gene expression is maintained at roughly 50% that of control tumors (data not shown). Furthermore, in the case of clone E4 whose β4GalT5 gene expression was suppressed by 23%, the average tumor size was ≈10 mm under the present conditions. Other clones with the expression levels of the β4GalT5 gene similar to clone E5 showed inhibition of tumor growth by the similar extents to that of clone E5 when they were transplanted into mice (data not shown). These results indicate that the tumorigenic potential of B16-F10 cells is suppressed according to the reduced expression levels of the β4GalT5 gene.

**Fig. 2.** (A) Growth curves of clone C1 and E5 cells. Values indicate means ± SD of triplicate assays. Closed and open circles indicate clones C1 and E5, respectively. (B) Morphological appearances of clones C1 and E5. Cells were grown to sub-confluence in DMEM containing 10% FCS (×100). (C) GSL compositions of clones C1 and E5. Neutral (left side) and acidic (right side) GSL fractions were prepared and analyzed by TLC, and detected with anthrone-thiourea reagent (left side) or with resorcinol-HCl reagent (right side). Migration positions of authentic Glc-Cer, Lac-Cer, Gb3-Cer, Gb4-Cer, SM and GM3 are indicated. The ratios of amounts of Lac-Cer and GM3 between clones C1 and E5 are shown at the bottom. (D) Cer in clones C1 and E5. Cer was separated by TLC and detected with a reagent containing 3% cupric acetate and 8% phosphoric acid. The ratio of amounts of Cer between clones C1 and E5 is shown at the bottom.

**Fig. 3.** Tumors formed with clones C1 and E5 in mice. (A) Clone C1 and E5 cells were transplanted subcutaneously into C57BL/6 mice and the tumors formed after 2 weeks are shown. (B) Paraffin sections of tumor tissues stained with haematoxylin-eosin reagent. Bar indicates 100 μm. (C) GSL compositions of tumors formed with clones C1 and E5. Neutral (left side) and acidic (right side) GSL fractions were prepared and analyzed by TLC, and detected with anthrone-thiourea reagent (left side) or with resorcinol-HCl reagent (right side). Migration positions of authentic Glc-Cer, Lac-Cer, Gb3-Cer, Gb4-Cer, SM and GM3 are indicated. The ratios of amounts of Lac-Cer and GM3 between clone C1- and E5-derived tumors are shown at the bottom. (D) Cer in clone C1- and E5-derived tumors. Cer was separated by TLC and detected with a reagent containing 3% cupric acetate and 8% phosphoric acid. The ratio of amounts of Cer between clone C1- and E5-derived tumors is shown at the bottom.
Histological study of tumors

Paraffin preparations obtained from solid tumors formed with clones C1 and E5 were stained with haematoxylin-eosin solution. As shown in Figure 3B, the tumors formed with clone C1 comprised cells distributed randomly with many intercellular cavities of different sizes (panel C1), whereas those formed with clone E5 comprised cells arranged compactly with fewer intercellular cavities (panel E5). This indicates that the cellular properties of clone E5 are changed markedly upon reducing the expression level of the β4GalT5 gene.

Analysis of GSL composition of tumors

Neutral and acidic GSL fractions were prepared from the solid tumors formed with clones C1 and E5, and subjected to TLC analysis. The results showed a decrease in the amount of Lac-Cer and no accumulation of Glc-Cer are observed in clone E5-derived tumors when compared with clone C1-derived tumors (left panel of Figure 3C). In the case of acidic GSLs, the amount of GM3 was significantly less in clone E5-derived tumors when compared with clone C1-derived tumors (right panel of Figure 3C). The ratios of amounts of Lac-Cer and GM3 between clone C1- and E5-derived tumors are indicated at the bottoms of the TLC plates (Figure 3C). When the amount of Cer was determined, a slight but not a significant decrease in the amount was observed in clone E5-derived tumors compared with clone C1-derived tumors (Figure 3D).

Metastatic potentials of clones C1, E4 and E5

B16-F10 cells possess a high metastatic potential to colonize to the lung when they are injected intravenously into animals (Fidler and Nicolson 1976; Bhavanandan and Furukawa 1995; Shirane et al. 1995). In the present study, 1 × 10^5 cells of clones C1, E4 and E5 showing >95% viability were injected intravenously into C57BL/6 mice and allowed to colonize to the lung for 3 weeks, after which the numbers of nodules formed were counted. Representative lung tissues with nodules formed with clones C1 and E4, and with clones C1 and E5, respectively. The numbers of nodules formed with clones C1, E4 and E5 were 101 ± 2.7, 79 ± 2.4 (P < 0.01) and 49 ± 4.0 (P < 0.01), respectively (Table I). Other clones with the expression levels of the β4GalT5 gene similar to clone E5 showed reduced number of nodules formed in the lung which is similar to that of clone E5 when they were injected intravenously into animals (data not shown). These results indicate that the metastatic potential of B16-F10 cells is also suppressed by reducing the expression level of the β4GalT5 gene.

Table I. Tumorigenic potentials of B16-F10 mouse melanoma cells transfected with the antisense β4GalT5 cDNA

<table>
<thead>
<tr>
<th>Clone</th>
<th>Tumors formed (%) (no. of animals)</th>
<th>Tumor size (mm) (no. of animals)</th>
<th>P-valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>100% (10)</td>
<td>13.3 ± 0.5 (10)b</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>E4</td>
<td>100% (10)</td>
<td>10.4 ± 0.6 (10)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>E5</td>
<td>90% (10)</td>
<td>5.9 ± 0.7 (10)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

P-values refer to comparisons between the average sizes of tumors formed with clones C1 and E4, and with clones C1 and E5, respectively.

Analysis of signal transducing molecules involved in the MAPK pathway

Because the Ras–MAPK pathway has been shown to be involved in tumor growth and progression (Marshall 1995; Coulombe and Meloche 2007), we looked for changes in the activation status of the molecules involved in this pathway by western blot analysis using antibodies against the respective molecules. The results showed that total amounts of Ras (panel a), c-Raf (panel c), MEK (panel e), ERK (panel g) and G3PDH as an internal control (panel i) are similar between clone C1- and E5-derived tumors as determined by measuring individual band intensities by NIH image analysis (Figure 5A). However, a significant decrease of the amount of GTP-bound Ras, a 25K active form (Figure 5A-b and B-b), a significant increase in the amount of GDP-bound Ras and a 25K inactive form (Figure 5A-b’ and B-b’) were detected in clone E5-derived tumors when compared with clone C1-derived tumors. A significant decrease in the phosphorylation level of c-Raf (75K) was observed in clone E5-derived tumors (Figure 5A-d and B-d). In the case of MEK (45K), a slight but significant decrease in the phosphorylation level of MEK was observed in clone E5-derived tumors when compared with that of clone C1-derived tumors (Figure 5A-f and B-f). A marked decrease in the phosphorylation level of the 42 and 44K ERK proteins, ERK1 and ERK2, respectively, was observed in clone E5-derived tumors when compared with those of clone C1-derived tumors (Figure 5A-h and B-h). These results indicate that tumor growth signals mediated by the MAPK pathway are attenuated due to the decreased amount of Lac-Cer in the E5-derived tumors by the reduced expression of the β4GalT5 gene in B16-F10 cells since Lac-Cer has been shown to activate the
MAPK signaling in human arterial smooth muscle cells (Bhunia et al. 1996; Mu et al. 2009).

Malignant transformation of mouse embryonic fibroblast cells with polyoma virus oncogene

Although clones E1–E5 showed variable degrees of inhibition of the β4GalT5 gene expression, it was hard to determine the relationship between its gene expression levels and the
tumorigenic potentials of malignantly transformed cells. For this purpose, mouse embryonic fibroblast (MEF) cells were prepared from B4galts5+/+, B4galts5+/− and B4galts5−/−-mice, respectively, and transformed with the viral oncogene. *P<0.05 referred to that of B4galts5−/−-MEF cells.

Fig. 5. Immunoblot analysis of proteins involved in the MAPK pathway. (A) Blotts were incubated with antibody against Ras to detect Ras (a), GTP-bound Ras (b) and GDP-bound Ras (b’), with anti-c-Raf antibody (c), antiphospho-c-Raf antibody (d), anti-MEK antibody (e), antiphospho-MEK antibody (f), anti-ERK antibody (g), antiphospho-ERK antibody (h) and anti-G3PDH antibody (i). The representative results are shown. (B) Densitometric analysis of protein bands detected with respective antibodies shown in (A-h, b, c, d, f and h). In each case, they were initially normalized to their respective proteins shown in (A-a, c, e and g), and then the band intensity of clone E5 was estimated by taking the band intensity of clone C1 as 1.00. The graph shows results ± SD of three independent experiments. Solid and open bars indicate clones C1 and E5, respectively. *P<0.05 and ***P<0.001 referred to that of clone C1-derived tumors.

Fig. 6. Tumorigenic potentials of oncogene-transformed MEF cells. (A) Numbers of colonies formed with the transformed MEF cells in the soft agarose gels. (B) Sizes of colonies formed with the transformed MEF cells in soft agarose gels. (C) Volumes of tumors formed with the transformed MEF cells in athymic mice 3 weeks after transplantation. (A)–(C) indicate MEF cells isolated from B4galts5−/−- mice, respectively, and transformed with the viral oncogene.

Fig. 5. Immunoblot analysis of proteins involved in the MAPK pathway. (A) Blotts were incubated with antibody against Ras to detect Ras (a), GTP-bound Ras (b) and GDP-bound Ras (b’), with anti-c-Raf antibody (c), antiphospho-c-Raf antibody (d), anti-MEK antibody (e), antiphospho-MEK antibody (f), anti-ERK antibody (g), antiphospho-ERK antibody (h) and anti-G3PDH antibody (i). The representative results are shown. (B) Densitometric analysis of protein bands detected with respective antibodies shown in (A-h, b, c, d, f and h). In each case, they were initially normalized to their respective proteins shown in (A-a, c, e and g), and then the band intensity of clone E5 was estimated by taking the band intensity of clone C1 as 1.00. The graph shows results ± SD of three independent experiments. Solid and open bars indicate clones C1 and E5, respectively. *P<0.05 and ***P<0.001 referred to that of clone C1-derived tumors.

Malignant transformation of mouse embryonic fibroblast cells with polyoma virus oncogene

Although clones E1–E5 showed variable degrees of inhibition of the β4GalT5 gene expression, it was hard to determine the relationship between its gene expression levels and the
were 115 ± 9.8, 87 ± 6.4 (P < 0.05) and 42 ± 2.2 (P < 0.05) μm (Figure 6B), respectively, while no colonies were formed with control MEF clones. Next, the transformed MEF clones (2 × 10^5 cells/each) were transplanted subcutaneously into athymic mice and tumors were allowed to develop for 3 weeks. The average volumes of the tumors formed with transformed B4galT5+/−, B4galT5+/− and B4galT5−/−-derived MEF clones were 34.8 ± 1.0, 25.6 ± 1.8 (P < 0.05) and 11.2 ± 2.0 (P < 0.05) mm^3 (Figure 6C), respectively. These results indicate that the tumorigenic potentials of transformed MEF cells correlate with the gene dosage of β4GalT5, namely with the amounts of Lac-Cer synthesized in cells.

Discussion

The present study clearly demonstrates that the tumorigenic and metastatic potentials of B16-F10 cells are suppressed by reducing the expression level of the β4GalT5 gene by transfection of its antisense cDNA. As mouse β4GalT5 has been shown to be a Lac-Cer synthase (Kumagai et al. 2010), the amounts of Lac-Cer decreased significantly in clones E4 and E5 cells and their tumors in which the β4GalT5 gene expression levels were reduced. Similarly, the amount of GM3, a major ganglioside in B16-F10 cells that is derived from Lac-Cer, also decreased significantly in the cells and their tumors. Lac-Cer and GM3 are considered to localize in GSL-enriched domains where various transmembrane signals are modulated (Prinetti et al. 1999; Li et al. 2001; Iwabuchi and Nagaoa 2002; Hashiramoto et al. 2006). In fact, Lac-Cer can stimulate the expression of integrin molecules and the phosphorylation of ERK proteins in the MAPK pathway (Bhunia et al. 1996; Mu et al. 2009), and GM3 is involved in the activation of tyrosine phosphatase RPTPσ (Suarez Pestana et al. 1999). Therefore, changes in the amounts of Lac-Cer and GM3 at the cell surface can alter the cell adhesion properties, and transmembrane signals that lead to the suppression of the original tumorigenic and metastatic potentials in clone E4- and E5-derived tumors. Indeed, the present study showed that cellular interactions occur partially in clone E5 cells when they are cultured in vitro to sub-confluence, and tumors formed with clone E5 have compactly arranged cells with fewer cavities than clone C1-derived tumors whose mechanisms remain to be elucidated. Furthermore, the expression level of GTP-bound Ras decreased and that of GDP-bound Ras increased in clone E5-derived tumors without altering the expression of total Ras between two types of tumors. This inactivation of Ras followed the decreased expression levels of phosphorylated c-Raf, phosphorylated MEK and phosphorylated ERK proteins without altering expressions of their respective total proteins, which clearly demonstrates the inactivation of the MAPK pathway involved in the transformation-dependent growth of cells (reviewed in Marshall 1995; Coulombe and Meloche 2007) by the reduced expressions of Lac-Cer and GM3 in clone E5-derived tumors. It could be also possible that the reduced expression of Lac-Cer attenuates the angiogenesis of the tumors since the ablation of the β4GalT5 gene reduces the vascular endothelial growth factor-mediated angiogenesis in human endothelial cells (Rajesh et al. 2005). Therefore, reducing the expression level of Lac-Cer in tumors by enzymatic or genetic tool or by the use of its biosynthetic inhibitors may be useful for a suppression of tumor growth and metastasis not only in B16-F10 cells but also in many other types of cancer cells and tumors.

Since a normal counterpart of mouse melanoma cells is not available, we do not know the expressions of the β4GalT5 and β4GalT6 genes, the latter of which was isolated as a Lac-Cer synthase from rat brain (Nomura et al. 1998), in the normal cells such as melanocytes. However, our previous studies and those of others suggest that the expression level of the β4GalT5 gene but not the β4GalT6 gene increases upon malignant transformation of cells (Shirane et al. 1999; Sato, Shirane, et al. 2000; Jiang et al. 2006). In the case of the human colorectal cancer-derived epithelial cells, the expression level of the β4GalT5 transcript has been shown to increase by 4.5-fold when compared with its normal counterpart without changing the expression level of the β4GalT6 transcript (Kolmazkova et al. 2009). These studies indicate that β4GalT5 is solely involved in the malignant process of cancer cells and tumors.

Lac-Cer is a common precursor for a series of GSLs including gangliosides. An enhanced expression of gangliosides such as GM3, GM2, GD3 and GD2 has been reported in a variety of cancer cells and tumors, and such gangliosides may be involved in their malignant properties (Mora et al. 1969; Brady and Fishman 1974; Siddiqui et al. 1978; Baumann et al. 1979; Furukawa and Lloyd 1990; Ye et al. 1990; Inokuchi et al. 2004; Dong et al. 2011). Furthermore, MEF cells prepared from mice whose gangliosides are completely depleted by abrogation of the GM3 synthase/GM2 synthase genes have been shown to impair tumor growth when they were transformed with an oncogene (Liu et al. 2010). These studies indicate that certain types of gangliosides are important for expressing the malignant properties of cancer cells and tumors. However, even when gangliosides are completely removed from malignantly transformed cells, such cells still possess tumorigenic potentials (Liu et al. 2010). Our present study shows that MEF cells isolated from B4gal5−/−-mice show the lowest susceptibility to malignant transformation, while those from B4gal5+/−- and B4gal5+/+ -mice show intermediate and high susceptibilities towards malignant transformation upon transfection with the viral oncoprotein based on the results of their sizes and numbers of colonies formed in soft agarose gels, and volumes of their tumors formed in vivo, indicating that Lac-Cer itself is important for the malignant transformation of cells aside from just being the precursor molecule for gangliosides because a certain amount of Lac-Cer is expressed constantly in cells. Lac-Cer has been shown to interact with GM3 by carbohydrate–carbohydrate interaction, which affects cell adhesion, spreading and motility of some types of cells (Kojima and Hakomori 1991). Lac-Cer may also serve as a ligand for galectins to form galectin-lattice on the cell surface, and such lattice modulates or alters functions of cell surface molecules such as cell adhesion molecules and growth factor receptors. Although such a binding has not been found yet, there is one study describing an interaction between Gal-Cer and galectin-3 (Lukyanov et al. 2005). More directly, Lac-Cer itself may be contributing to membrane dynamics such as microdomain formation at the cell surface. Further study is required for establishing intrinsic functions of Lac-Cer at the cell surface, although some of Lac-Cer localizes in cytoplasmic vesicles in cells (Chatterjee et al. 1983; Symington et al. 1987).
Materials and methods

Animals and chemicals

B16-F10 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS) and 50 units/mL penicillin and 50 µg/mL streptomycin at 37°C in a humidified 5% CO2 incubator. Male 6-week-old C57BL/6 mice and Balb/c Slc-nu/nu athymic mice were obtained from a local animal supplier (Japan SLC Co., Hamamatsu). Glc-Cer, Lac-Cer, GM3, sphingomyelin, Cer and TLC silica gel plates were obtained from Sigma–Aldrich Inc. (St. Louis, MO) and Merck KGaA (Darmstadt, Germany), respectively.

Construct of pcDNA3.1/GT5-AS expression vector

The mouse βGalT5-antisense cDNA fragment (505 bp; −5 to +500 bp) was obtained by PCR using a plasmid containing the full-length mouse βGalT5 cDNA as a template. The PCR product was amplified with a pair of oligonucleotide primers, TS72 (5′-CGGGATCCCTACGATCCTGGCCC-3′) and TS73 (5′-CCAGCTTGGGATACACCTTC-3′), both of which contained a newly created BamHI-restriction site at the 5′-end and a HindIII-restriction site at the 3′-end as indicated by underlines. The resulting fragment, named βGalT5fr, was cloned into pGEM-T Easy vector (Promega, Madison, WI) and sequenced. The BamHI–HindIII digested fragment obtained from pGEM-T Easy/βGalT5fr was inserted in the HindIII–BamHI site of a mammalian expression vector pcDNA3.1(+) (Invitrogen, Carlsbad, CA) in an antisense orientation.

Construct of pSilencer/GT5-KD expression vector

The plasmid used for RNAi was constructed as described previously (Tadokoro et al. 2009). In brief, the target sequences were selected from the mouse βGalT5 gene (GenBank™ accession number: AB004786) by siRNA Target Finder (Ambion, Austin, TX). The candidate target sequences were blasted by BLAST search to ensure that they were unique to the βGalT5 mRNA. The following target oligonucleotides were used to generate the double-stranded oligonucleotides: TSMGT5F, 5′-GATCCCA CCTACCTCTCATGTTGTTCAAGAGCACCTAGAAGAG GTAGGTTGTTA-3′; and TSMGT5R, 5′-AGCTTACACCTAC CTCTTACGTTGCTCTTTGAACACCATGAAAG-AGGTAGG TGG-3′. The oligonucleotides were annealed and cloned into the BamHI/HindIII sites of the pSilencer4.1-hygro vector (Ambion). The correct orientation and sequence of the plasmid constructs were verified by sequencing. Stable transfectants were selected with 0.5 µg/mL hygromycin B (Calbiochem, Darmstadt, Germany). Negative control-short hairpin RNA plasmid (Ambion) was used as a control.

Cloning of gene-transfected B16-F10 cells

B16-F10 cells (2 × 10⁵ cells) were plated in a 35 mm dish a day before transfection. Two milligrams of vector pcDNA3.1/GT5-AS or pcDNA3.1(+) (mock) was co-transfected with FuGENE™ 6 Transfection Reagent (Roche Diagnostics, Manheim, Germany) into the plated cells. Transfection was performed according to the manufacturer’s protocol. After incubation for 48 h, the medium was changed to DMEM containing 1 mg/mL geneticin (G418 sulfate, Sigma–Aldrich Inc.) and the cells were cultured continuously for 2 weeks. Geneticin-resistant colonies were isolated by limited dilution. Growth rates of clone C1 and E5 cells were determined with CellTiter 96® AQ One Solution for Cell Proliferation Assay (Promega) according to the manufacture’s instructions. In brief, clone C1, E4 and E5 cells were seeded at 1 × 10⁴ cells/well in a 96-well plate, and cells were incubated at 37°C for 1–10 days. Finally, 20 µL of CellTiter 96® AQ One Solution was added to each well (3 wells for each assay), incubated at 37°C for 1 h, and then, absorbance at 490 nm was measured with a microplate reader (ImmunoMini NJ-2300, Naigle Nunc International, Rochester, NY) by using wells containing cells without a reagent as blanks.

Analysis of the gene expression by quantitative real-time RT-PCR

In order to screen for cloned cells showing lower expression of the βGalT5 transcript than control cells, the expression of the βGalT5 gene was analyzed by RT-PCR using a Quick Master Mix (Toyobo Co., Ltd., Osaka) according to the manufacturer’s instructions. In brief, total RNA fractions were prepared from cloned cells with Sepasol RNA I total RNA Isolation Reagent (Nacalai Tesque Inc., Kyoto). RT-PCR analysis was conducted using total RNAs and oligonucleotide primers specific to the mouse βGalT5 gene. The nucleotide sequences of the forward and reverse primers for the βGalT5 gene and G3PDH gene used were described previously (Kumagai et al. 2010). Conditions for RT-PCR were as follows: 1 cycle at 90°C for 30 s, 60°C for 30 min, 94°C for 1 min, and then 26 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 1 min. Quantitative real-time RT-PCR was performed according to the method described previously (Tadokoro et al. 2006). PCR thermocycling parameters were as follows: 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 1 min. Gene-specific PCR was performed in duplicate on each cDNA sample. The gene-specific primers were as follows: βGalT5F, 5′-AGATCTACACCTACCTACCTCTTACGTTGTTCAAGAGCACCTAGAAGAG GTAGGTTGTTA-3′; and βGalT5R, 5′-GATCCCA CCTACCTCTCATGTTGTTCAAGAGACACCTAGAAGAG GTAGGTTGTTA-3′. The PCR products were analyzed by agarose gel electrophoresis as described previously (Shirane et al. 1999).

Analyses of tumorigenic and metastatic potentials of cloned cells

Clone C1, E4 and E5 cells cultured in DMEM containing 0.5 mg/mL geneticin at 37°C were harvested by 0.25% trypsin treatment, and 2 × 10⁵ cells showing >95% viability were suspended in 50 µL of serum-free DMEM and transplanted subcutaneously into 7-week-old male C57BL/6 mice (10 animals/group). The diameters of the tumors formed were measured periodically until 2 weeks after inoculation. Tumors were removed from the animals and fixed in phosphate-buffered saline (pH 7.4) containing 4% formaldehyde. Paraffin sections, 10 µm in thickness, were stained with haematoxylin-eosin solution. For metastatic assays, 1 × 10⁵ cells of each clone suspended in 50 µL of serum-free DMEM were injected intravenously into 7-week-old male C57BL/6 mice. After 3 weeks, the lungs were
removed from the animals and fixed in phosphate-buffered saline (pH 7.4) containing 4% formaldehyde. The numbers of nodules formed in the lung were determined with a cell counter under a microscope.

Analysis of GSLs
GSLs were analyzed as described previously (Ando et al. 1987; Kumagai et al. 2010). In brief, the total lipid fraction was extracted from cells or tumors with a mixture of chloroform: methanol:water (1:2:0.8, v/v), and centrifuged at 12,000 rpm for 5 min. The supernatant containing lipids was dried by centrifugation using a SpeedVac concentrator (Savant Instruments Inc., Farmingdale, NY). Lipid samples were subjected to mild alkaline hydrolysis with 0.1 M NaOH/methanol solution at 40°C for 1 h, and then neutralized with 1 M acetic acid/methanol solution. For neutral glycolipid analysis, lipid samples whose amounts were equivalent to 2 mg of cellular proteins were analyzed on high-performance TLC silica gel 60 plates (Merck KGaA). The plates were developed in a solvent containing chloroform:methanol:water (60:25:4, v/v), and stained with an antrone-thiourea reagent by heating at 120°C to detect neutral GSLs. In the case of gangliosides, lipid samples whose amounts were equivalent to 500 µg of cellular proteins were subjected to TLC as described before (Sato, Guo, et al. 2000; Kolmakova et al. 2009). The plates were developed in a solvent containing chloroform:methanol:0.5% CaCl₂ (11:9:2, v/v), and stained with a resorcinol-HCl reagent by heating at 120°C to detect gangliosides. For Cer analysis, lipid samples equivalent to 2 mg of cellular proteins were subjected to TLC in a solvent containing chloroform:methanol:acetic acid (90:2.8: v/v), and lipids were detected with a reagent containing 3% cupric acetate and 8% phosphoric acid according to the method published previously (Selvam and Radin 1981). The assay was repeated three times. Each band intensity was determined by NIH image analysis, and ratios between those of clones C1 and E5 were calculated.

Western blot analysis of proteins involved in the MAPK pathway
Western blot analysis was conducted according to the method described previously (Sato et al. 1993). In brief, proteins prepared from cells and tumors were subjected to SDS–polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes. After blocking, the blots were incubated with anti-c-Raf, antiphospho-c-Raf, anti-MEK, antiphospho-MEK, anti-ERK and antiphospho-ERK antibodies (Cell Signaling Technology, Beverly, MA), followed by alkaline phosphatase/horseradish peroxidase-conjugated secondary antibodies. Complexes were detected using a BCIP/NBT Phosphatase Substrate kit (KPL; Caithersburg, MD) or an enhanced chemiluminescence detection reagent. In order to detect activated and inactivated Ras proteins, the Ras proteins were immunoprecipitated from samples using a Ras-activation/inactivation kit (Enzo Life Science, Plymouth Meeting, PA), and then subjected to SDS–polyacrylamide gel electrophoresis followed by western blot analysis using anti-Ras antibody. In all experiments, G3PDH was used as an internal control.

Preparation and transformation of MEF cells
MEF cells were prepared from B4gal5+/–, B4gal5+/- and B4galt5−/– (βGalT5-null mutant) mouse embryos as described previously (Kumagai et al. 2010). Transformation of MEF cells with polyoma virus oncogene (pSRneo/MTAg) was performed as described previously (Segawa and Yamaguchi 1986; Asada et al. 1997), and the transformation of the cells was examined by soft agarose assay (Gunnur Dikmen et al. 2005). In brief, a mixture (1 mL) of DMEM containing 10% FCS and 1.6% agarose solution (1:1, v/v) was distributed into 6-well plates. In each well, 5 × 10⁵ MEF cells suspended in 1.5 mL of a mixture of DMEM containing 10% FCS and 0.8% agarose solution (1:1, v/v) were placed, and the plates were incubated for 20 days. Numbers and sizes of colonies formed were determined. Transformed MEF cells (2 × 10⁵ cells suspended in 50 µL) were transplanted subcutaneously into 7-week-old male Balb/c Slc-nu/nu athymic mice (5 animals/one group). Three weeks later, the tumors formed were excised from the animals and analyzed.

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Conflict of interest
None declared.

Abbreviations
Cer, ceramide; DMEM, Dulbecco’s Modified Eagle’s Medium; FCS, fetal calf serum; βGalT, β4-galactosyltransferase; Glc-Cer, glucosylceramide; GSL, glycosphingolipid; Lac-Cer, lactosylceramide; MEF, mouse embryonic fibroblast; RT-PCR, reverse-transcription-polymerase chain reaction; TLC, thin-layer chromatography. Ganglioside nomenclature used in the present study is described by Svennerholm (1994).

References


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