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Transcription of human β4-galactosyltransferase 3 is regulated by differential DNA binding of Sp1/Sp3 in SH-SY5Y human neuroblastoma and A549 human lung cancer cell lines

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Abstract

Poor prognosis of neuroblastoma patients has been shown to be associated with increased expression of β4-galactosyltransferase (β4GalT) 3. To address the underlying mechanism of the increased expression of β4GalT3, the transcriptional regulation of the human β4GalT3 gene was investigated in SH-SY5Y human neuroblastoma cell line comparing with A549 human lung cancer cell line, in which the β4GalT3 gene expression was the lowest among four cancer cell lines examined. The core promoter region was identified between nucleotides −69 and −6 relative to the transcriptional start site, and the same region was utilized in both cell lines. The promoter region contained two Specificity protein (Sp)1/3-binding sites at nucleotide positions −39/−30 and −19/−10, and the sites were crucial for the promoter activity. Although the gene expression of Sp family transcription factors Sp1 and Sp3 was comparable in each cell line, Sp3 bound to the promoter region in SH-SY5Y cells whereas Sp1 bound to the region in A549 cells. The promoter activities were enhanced by Sp1 and Sp3 in SH-SY5Y cells. In contrast, the promoter activities were enhanced by Sp1 but reduced by Sp3 in A549 cells. Furthermore, the function of each Sp1/3-binding site differed between SH-SY5Y and A549 cells due to the differential binding of Sp1/Sp3. These findings suggest that the transcription of the β4GalT3 gene is regulated by differential DNA binding of Sp3 and Sp1 in neuroblastoma and lung cancer. The increased expression of β4GalT3 in neuroblastoma may be ascribed to the enhanced expression of Sp3, which is observed for various cancers.

Key words: lung cancer, neuroblastoma, Specificity protein, transcriptional mechanism, β4-galactosyltransferase 3

Introduction

Glycosylation is one of the important modifications of functional molecules (Hakomori 2002; Ohtsubo and Marth 2006). Drastic changes in glycosylation of glycans linked to proteins and lipids are observed by malignant transformation of cells (Yamashita et al. 1985; Asada et al. 1997; Lau and Dennis 2008). The changes modulate the function of cell adhesion molecules and receptors in cancer cells (Guo et al. 2002, 2003; Hoja-Łukowicz et al. 2017). The altered expression of the glycosyltransferase genes is considered to be the causes of changes in glycosylation. Glycans usually consist of monosaccharides such as *N*-acetylglucosamine (GlcNAc), galactose (Gal), glucose (Glc), fucose, mannose and sialic acid. The

Galβ1→4GlcNAc/Glc groups, which serve as the backbone structures for expressing a number of carbohydrate antigens, are synthesized by the β4-galactosyltransferase (β4GalT) family (Furukawa and Sato 1999; Furukawa et al. 2014).

Our previous study showed that the expression of the β4GalT5 gene increases whereas that of the β4GalT2 gene decreases by malignant transformation of cells (Shirane et al. 1999). When the expression of the β4GalT5 gene was reduced in B16-F10 mouse melanoma cells, tumorigenic and metastatic potentials were suppressed significantly (Shirane et al. 2014). In contrast, tumorigenic potentials of B16-F10 cells suppressed by increasing the expression of the β4GalT2 gene (Tagawa et al. 2014). Therefore, the galactosylation of glycans is critical for the malignant properties of cancer cells.

Glycosphingolipids compose of carbohydrate and ceramide (Cer) portions. The cDNA encoding β4GalT3 was originally isolated by Clausen's group, and β4GalT3 was shown to transfer Gal from UDP-Gal to GlcNAc residue of lacto-*N*-triaosylceramide (GlcNAcβ1→3Galβ1→4Glc-Cer) (Almeida et al. 1997). Besides the possible involvement in the biosynthesis of lacto-*N*-tetraosylceramide, in vitro analysis of substrate specificity showed that β4GalT3 can be involved in the galactosylation of *N*-glycans, and the synthesis of poly-*N*-acetyllactosamine (Guo et al. 2001; Sato et al. 2001; Ito et al. 2007).

β4GalT3 was shown to be expressed strongly in heart, kidney, ovary, pancreas, placenta, skeletal muscle and testis of human adult tissues (Almeida et al. 1997; Lo et al. 1998). On the other hands, the high expression was observed for brain, kidney, liver, and lung of human fetal tissues (Lo et al. 1998). Previously, we demonstrated that the expression of the β4GalT3 gene increases by malignant transformation of cells (Shirane et al. 1999). In neuroblastoma, β4GalT3 was shown to increase, which was associated with poor prognosis of neuroblastoma patients (Chang et al. 2013). Neuroblastoma, a common malignant tumor in children, shows poor clinical outcome (Maris et al. 2007; Kamijo and Nakagawara 2012). The galactosylated *N*-glycans produced by β4GalT3 were solely involved in the malignant properties of neuroblastoma such as cell migration and invasion (Chang et al. 2013). Among β4GalT family, the regulatory mechanisms of the human β4GalT1, β4GalT2, β4GalT4 and β4GalT5 transcription were reported by others and us so far (Sato and Furukawa 2004; Zhu et al. 2005; Zhou et al. 2008; Sugiyama et al. 2017). However, the transcriptional mechanism of the β4GalT3 gene has not been clarified yet. The understanding of the regulatory mechanism of the β4GalT3 gene expression would provide novel therapeutic strategies for neuroblastoma.

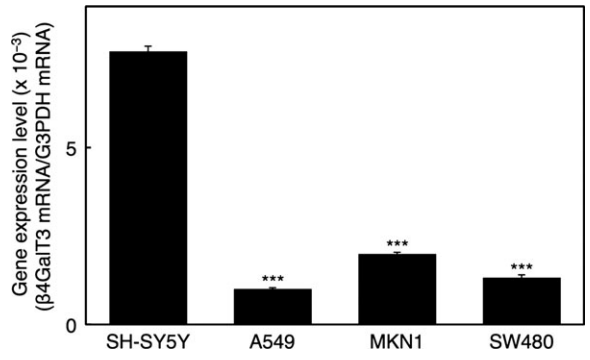

Fig. 1. Expression levels of the β4GalT3 gene in SH-SY5Y, A549, MKN1, and SW480 cells. Values are the mean ± SD for triplicate assays from three different experiments. ***$P < 0.001$ vs. SH-SY5Y cells.

In this study, we isolated the promoter region of the human β4GalT3 gene and determined the cis-acting elements and transcription factors that regulate the β4GalT3 gene expression in SH-SY5Y human neuroblastoma cell line as compared with A549 human lung cancer cell line whose expression level of the β4GalT3 gene was the lowest among four cancer cell lines examined.

Results

Identification of transcriptional start site

First, we examined the expression levels of the β4GalT3 gene in four cancer cell lines by real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. The expression level of the β4GalT3 gene in SH-SY5Y cells showed 7.7-, 3.9- and 5.8-fold higher than those in A549, MKN1 human gastric cancer, and SW480 human colon cancer cells, respectively (Figure 1). Further studies were carried out using SH-SY5Y cells as compared with A549 cells due to the lowest expression level of the β4GalT3 gene among the cell lines examined. The position of the transcriptional start site utilized in SH-SY5Y and A549 cells was identified by two round PCR using the oligonucleotide primer pairs, 5′-Primer and TS25-20, and 5′-Nested Primer and TS25-19 (Figure 2A). As the results of nested PCR, the 350-bp DNA fragment was obtained, and the nucleotide sequencing revealed that RNA Oligo is attached to a cytidine residue at nucleotide position 1440 bp upstream of the translational start site in SH-SY5Y and A549 cells (Figure 2B). The results indicate that the transcription of the β4GalT3 gene starts at this position and the same start site is utilized in both cell lines. In the further studies of this paper, the transcriptional start site was taken as +1.

To obtain information about the regulatory mechanism of the β4GalT3 gene, the genomic structure of the 5′-flanking region of the β4GalT3 gene needs to be elucidated. We searched in the GenBank™ a human genomic clone RP11-297K8 (accession number AL590714) derived from chromosome 1 containing the human β4GalT3 cDNA sequence registered by Clausen's group and us (accession numbers AB024435 and Y12509) (Almeida et al. 1997; Guo et al. 2001). The alignment of the 5′-untranslated region (UTR) of the β4GalT3 gene and the human genomic clone RP11-297K8 allowed us to predict the exon–intron structure as shown in Figure 3A. The 5′-UTR of the β4GalT3 gene was composed of three exons, which was identical between SH-SY5Y and A549 cells (Figure 3B). These findings suggest that the same splicing is occurred in both cell lines.

Identification of β4GalT3 gene core promoter

To identify the core promoter region of the β4GalT3 gene, seven reporter plasmids, pGL(−2190/+89), pGL(−1567/+89), pGL(−1145/+89), pGL(−595/+89), pGL(−140/+89), pGL(−69/+89), and pGL(−5/+89) containing the 2.3 kb, 1.7 kb, 1.2 kb, 0.7 kb, 0.23 kb, 0.16 kb, and 0.1 kb 5′-flanking regions, respectively, were constructed (Figure 4A) and transfected into SH-SY5Y and A549 cells, and then the promoter activities were determined. In both cell lines, pGL(−69/+89) showed relatively high promoter activity whereas almost no promoter activity was observed for pGL(−5/+89), in which the region between nucleotides −2190 and −6 was deleted (Figure 4B). Therefore, the region between nucleotides −69 and −6 is considered to be responsible for promoter activation, and contain the important positive regulatory elements. These results indicate that the same promoter region is utilized for the transcription of the β4GalT3 gene in SH-SY5Y and A549 cells. On the other

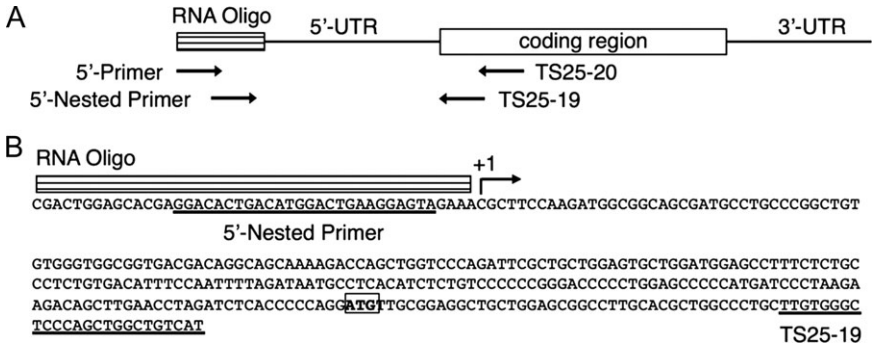

Fig. 2. Transcriptional start site of the human β4GalT3 gene utilized in SH-SY5Y and A549 cells. (**A**) Schematic illustration of the oligonucleotide primers used. (**B**) Nucleotide sequence of the nested PCR product. The overline indicates the sequence of the RNA Oligo linked to the β4GalT3 cDNA. The underlines indicate the 5′-Nested Primer sequence and the complementary sequence for TS25-19 used in the nested PCR. The arrow and box indicate the transcriptional start site (+1) and initiation codon, respectively. The identical sequence was obtained between both cell lines.

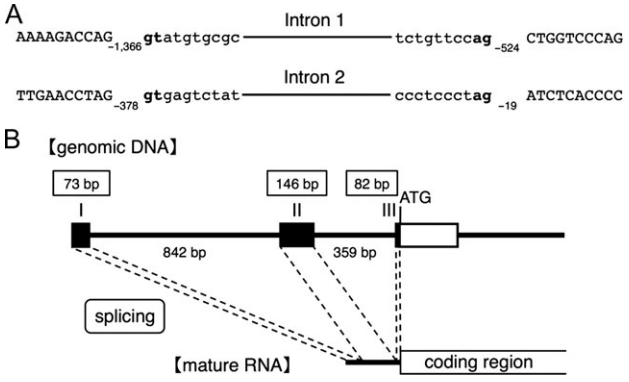

Fig. 3. Genomic structural organization of the 5′-flanking region of the human β4GalT3 gene. (**A**) The exon–intron sequences of the 5′-flanking region. The uppercase letters show the exon sequences with the nucleotide positions from the translational start site as subscripts. The lowercase letters show the flanking intron sequences. The sequences of splice junctions are indicated in bold letters, as confirmed to the GT/AG rule. (**B**) Schematic drawing of the genomic DNA and mRNA structures. The filled and open boxes indicate the untranslated and translated regions of exons, respectively.

hands, when the promoter activity of pGL(−2190/+89) was set at 100%, the promoter activities of pGL(−595/+89) and pGL(−69/+89) in SH-SY5Y cells showed 187% and 199%, respectively (Figure 4B, left panel), suggesting that the regions between nucleotides −595 and −141, and nucleotides −140 and −70 contain negative regulatory elements, which differed in A549 cells (Figure 4B, right panel).

The β4GalT3 gene promoter did not contain canonical TATA or CAAT box but contained GC-rich sequences. The presence of GC-rich sequence is a characteristic feature of the TATA-less genes including the glycosyltransferase genes, and the gene expression is regulated by Specificity protein (Sp) family transcription factors (Li and Davie 2010; Beishline and Azizkhan-Clifford 2015). Among Sp family transcription factors, Sp1, Sp3 and Sp4 have been shown to be closely related members with quite similar structural features, thereby showing similar DNA binding affinity (Kingsley and Winoto 1992; Hagen et al. 1994). The Sp1 and Sp3 genes were expressed ubiquitously, but the Sp4 gene was expressed primarily in brain (Saffer et al. 1991; Hagen et al. 1992). When the promoter region between nucleotides −69 and −6 was analyzed by TFBIND program (Tsunoda and Takagi 1999), two putative Sp1/3-binding sites with high similarity scores were found at nucleotide positions −39/−30 (score: 0.97) and −19/−10 (score: 1.00) relative to the transcriptional start site (Table I). To analyze the importance of the Sp1/3-binding sites, one additional reporter construct, pGL(−30/+89), was made (Figure 5A). Upon transient transfection into SH-SY5Y and A549 cells, the promoter activities of the reporter constructs decreased proportionally depending on the number of the Sp1/3-binding sites in both cell lines (Figure 5B). These results indicate that both Sp1/3-binding sites play key roles for the promoter activity of the β4GalT3 gene, suggesting that Sp1 and/or Sp3 are involved in the transcription of the β4GalT3 gene in SH-SY5Y and A549 cells.

Analysis of transcription factors bound to β4GalT3 gene promoter

The expression of the Sp1 and Sp3 genes was ubiquitously observed in mammalian cells (Saffer et al. 1991; Hagen et al. 1992). Real-time RT-PCR analysis revealed that the expression levels of the Sp1 and Sp3 genes in SH-SY5Y cells are higher than those in A549 cells (Figure 6A and B). However, the levels of both genes were comparable in each cell line (Figure 6A and B).

To examine the transcription factors that bind to the core promoter region of the β4GalT3 gene, chromatin immunoprecipitation (ChIP) assay was carried out. The 245 bp promoter region containing two Sp1/3-binding sites was amplified by PCR using the purified DNA as a template, and TS26-9 and TS26-10 (Figure 7A). The results demonstrated that Sp3 binds to the promoter region in SH-SY5Y cells (Figure 7B-SH-SY5Y), while Sp1 binds to the region in A549 cells (Figure 7B-A549). These results suggest that the expression of the β4GalT3 gene is regulated by Sp3 in SH-SY5Y cells, but by Sp1 in A549 cells.

Effects of Sp1 and Sp3 on promoter activities of β4GalT3 gene

It is well documented that Sp1 acts as an activator whereas Sp3 acts as an activator or as a repressor depending on the cellular context (Li and Davie 2010; Beishline and Azizkhan-Clifford 2015). Since Sp3 and Sp1 bound to the core promoter region of the β4GalT3 gene in SH-SY5Y and A549 cells, respectively (Figure 7B), the effects of Sp1 and Sp3 on the promoter activity were examined using SH-SY5Y and A549 cells. In SH-SY5Y cells, ectopic expression of Sp1 and Sp3 dose-dependently enhanced the promoter activities by 1.3–2.1-fold and 1.7–3.0-fold, respectively, as compared to control

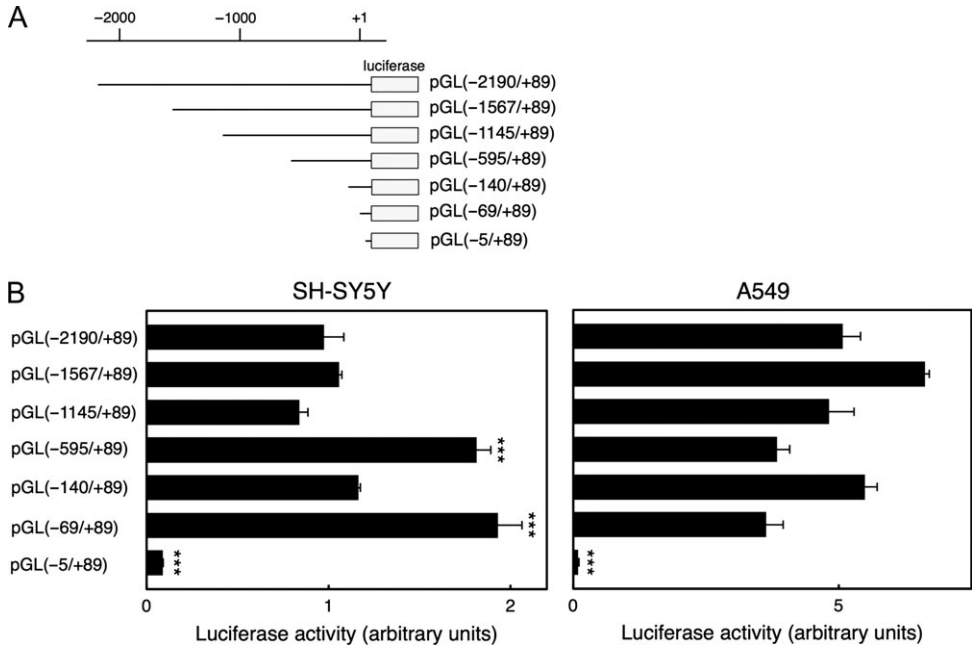

Fig. 4. Promoter activity of the human β4GalT3 gene in SH-SY5Y and A549 cells. (**A**) Schematic of the 5′-deletion constructs of the β4GalT3 gene. The 2.3 kb, 1.7 kb, 1.2 kb, 0.7 kb, 0.23 kb, 0.16 kb and 0.1 kb 5′-flanking regions were inserted into the upstream of the firefly luciferase gene. (**B**) Promoter activities of the 5′-deletion constructs in SH-SY5Y and A549 cells. Values are the mean ± SD for triplicate assays from three different experiments. ***$P < 0.001$ vs. pGL(−2190/+89).

Table I. The Sp1/3-binding sites in the core promoter region of the human β4GalT3 gene.

Consensus core sequence	β4GalT3 sequence (nucleotide position)
GRGGCRGGGW	GCCCCGCCCC (−39/−30) ACCCCGCCCC (−19/−10)

The nucleotide positions are referenced relative to the transcriptional start site.

(Figure 8A-Sp1 and -Sp3), indicating that both Sp1 and Sp3 act as transcriptional activators in SH-SY5Y cells. On the other hands, in A549 cells, ectopic Sp1 expression dose-dependently enhanced the promoter activities by 2.0–4.3-fold as compared to control (Figure 8B-Sp1), while ectopic Sp3 expression reduced the promoter activities by 0.6–0.7-fold as compared to control (Figure 8B-Sp3). The results indicate that Sp1 acts as an activator but Sp3 acts as a repressor in A549 cells. Taken together, these studies demonstrated that Sp3 and Sp1 act as transcriptional activators in SH-SY5Y and A549 cells, respectively.

Mutational analysis of Sp1/3-binding sites

To examine the function of the Sp1/3-binding sites for the promoter activity, the Sp1/3-binding sites at nucleotide positions −39/−30 and −19/−10 were mutated in pGL(−69/+89) to generate the reporter plasmids, m1, m2 and m3 (Figure 9A), the promoter activity was determined using both cell lines. In SH-SY5Y cells, the promoter activities of the mutation constructs with mutation in one Sp1/3-binding site showed 36% (m1) and 42% (m2) of the control, and that of the mutation constructs with mutations in both Sp1/3-binding sites decreased to 6% (m3) of the control (Figure 9B). However, in A549 cells, the promoter activities of the mutation constructs with mutation in one Sp1/3-binding site increased to 108% (m1) and 223% (m2) of the control, and that of the mutation constructs with mutations in both Sp1/3-binding sites decreased to 11% (m3) of the control (Figure 9C). These results suggest that the function of each Sp1/3-binding site differs between SH-SY5Y and A549 cells, depending on which transcription factors bind to the Sp1/3-binding site.

Discussion

The present study revealed the transcriptional start site, 5′-UTR structure, and transcriptional regulation of the β4GalT3 gene in SH-SY5Y human neuroblastoma cell line as compared with A549 human lung cancer cell line. One transcriptional start site at the same nucleotide position was identified in SH-SY5Y and A549 cells. The phylogenic analysis with multiple sequence alignment showed that β4GalT3 shares higher similarity with β4GalT4 than other β4GalT family members at amino acid level (Lo et al. 1998). Our previous study demonstrated that multiple transcriptional start sites of the β4GalT4 gene are identified in SH-SY5Y and A549 cells (Sugiyama et al. 2017), and some of them are different between these cancer cell lines. Therefore, the transcription is considered to be different between human β4GalT3 and β4GalT4 even though the near relatives in the phylogenic tree. The 5′-UTR of the human β4GalT3 gene was composed of three exons, which is similar to the human β4GalT4 gene but different from the human β4GalT1, β4GalT2 and β4GalT5 genes whose 5′-UTRs are composed of a single exon (Sato and Furukawa 2004; Zhu et al. 2005; Zhou et al. 2008). In general, 5′-UTR is involved in the regulation of the tissue-specific expression and the translational efficiency of genes (Smith 2008). The gene expression patterns of human α1,2-fucosyltransferase, α1,3-fucosyltransferase 4, α2,6-sialyltransferase I and α2,3-sialyltransferase IV were shown to be associated with tissue-specific

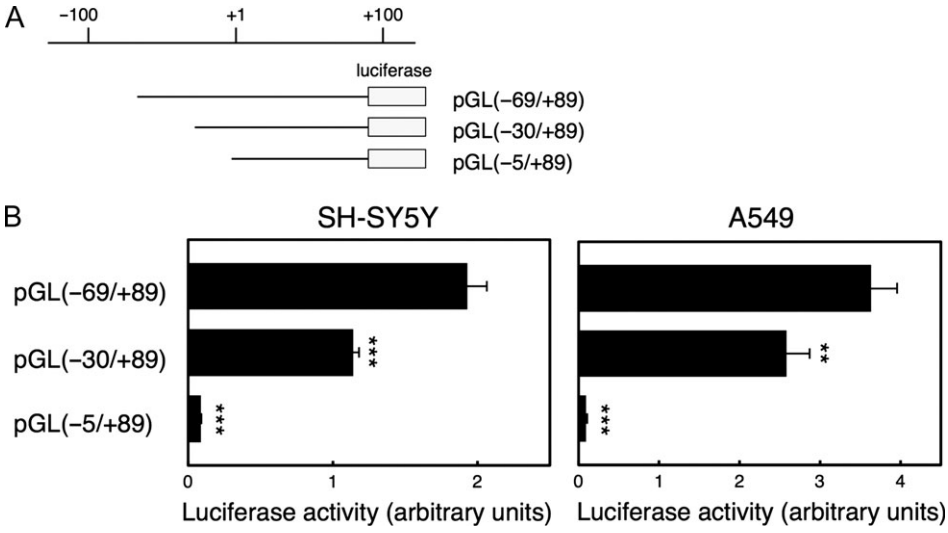

Fig. 5. Identification of the essential elements within the core promoter region of the β4GalT3 gene. (**A**) Schematic of the 5′-deletion constructs of the β4GalT3 gene. The 0.16 kb, 0.12 kb and 0.1 kb 5′-flanking regions were inserted into the upstream of the firefly luciferase gene. (**B**) Promoter activities of the 5′-deletion constructs in SH-SY5Y and A549 cells. Values are the mean ± SD for triplicate assays from three different experiments. $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. pGL (−69/+89).

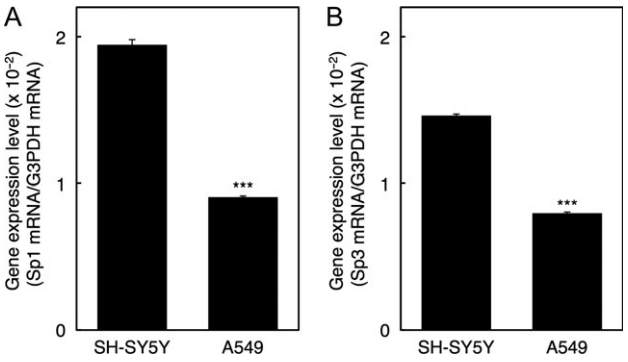

Fig. 6. Expression levels of the Sp1 (**A**) and Sp3 (**B**) genes in SH-SY5Y and A549 cells. The amplification efficiency of the Sp1 and Sp3 mRNAs with the primer pairs was reflected in the results. Values are the mean ± SD for triplicate assays from three different experiments. $^{***}P < 0.001$ vs. SH-SY5Y cells.

glycosylation patterns owing to the different combinations of exons in 5′-UTRs (Koda et al. 1997; Taniguchi et al. 2000, 2001; Xu et al. 2003). However, the same 5′-UTR structure of the β4GalT3 gene was found in SH-SY5Y and A549 cells, suggesting that the same splicing is occurred in both cell lines regardless of the different cell types.

This study clearly demonstrated that two Sp1/3-binding sites in the core promoter region are critical for the promoter activity of the β4GalT3 gene, and the promoter is regulated by different transcription factors Sp3 and Sp1 in SH-SY5Y and A549 cells, respectively. Sp1 and Sp3 belong to Sp family transcription factors with C_2H_2-type zinc finger (Li and Davie 2010; Beishline and Azizkhan-Clifford 2015) and show similar affinity for the Sp1/3-binding site, thereby competing at the site each other (Kingsley and Winoto 1992; Hagen et al. 1994). Quite interestingly, although the same promoter region was utilized for the transcription of the β4GalT3 gene in both cell lines, Sp3 bound to the core promoter region of the β4GalT3 gene in SH-SY5Y cells whereas Sp1 bound to the region in A549 cells. In transcription, Sp1 acts as an activator, while whether Sp3 acts as an activator or as a repressor depends on cellular context (Li and Davie 2010; Beishline and Azizkhan-Clifford 2015). The present study indicates that both Sp1 and Sp3 regulate positively the transcription of the β4GalT3 gene in SH-SY5Y cells, while Sp1 regulates positively and Sp3 regulates negatively the transcription in A549 cells. In SH-SY5Y cells, Sp3 has been shown to act as an activator for the transcription of the zipper protein kinase gene (Itoh et al. 2004), and the NOX4 gene (Katsuyama et al. 2011). By the differential binding of Sp1 and Sp3, the function of each Sp1/3-binding site in the β4GalT3 gene promoter differed between SH-SY5Y and A549 cells. Sp1 possesses a C-terminal multimerization domain that assembly Sp1 multimers in the promoters containing multiple adjacent Sp1-binding sites (Pascal and Tjian 1991). The β4GalT3 gene promoter region between nucleotides −69 and −40 contained multiple Sp1/3-binding sites with lower similarity score (0.80–0.92) as predicted by TFBIND program. Due to the synergistic activation of the promoter by multimeric Sp1, it is considered that the significant promoter activities in A549 cells are still observed for the reporter construct with the mutation in each Sp1/3-binding site, which is totally different from the promoter activities in SH-SY5Y cells. On the other hands, because Sp3 does not contain the multimerization domain unlike Sp1 (Li and Davie 2010; Beishline and Azizkhan-Clifford 2015), the mutation in each Sp1/3-binding site decreased the promoter activities in SH-SY5Y cells proportionally depending on the number of the Sp1/3-binding sites.

Although the expression levels of the Sp1 and Sp3 genes were comparable in each cell line, Sp3 and Sp1 bound to the promoter region in SH-SY5Y and A549 cells, respectively. Accordingly, the fact that different transcription factors bind to the core promoter region of the β4GalT3 gene cannot be explained by the expression levels of the Sp1 and Sp3 genes, and some other reasons may exist in the differential binding of Sp1 and Sp3 to the region. Both Sp1 and Sp3 have been shown to be posttranslationally modified by phosphorylation, glycosylation, sumolylation, and acetylation (Li and Davie 2010; Beishline and Azizkhan-Clifford 2015). These modifications of Sp1 and Sp3 regulated their DNA binding activity, transcriptional activity, stability and localization (Li and Davie 2010; Beishline and

Azizkhan-Clifford 2015). Thus, the different modification of Sp1 and Sp3 between SH-SY5Y and A549 cells may lead to the differential binding of Sp1 and Sp3 to the core promoter region. In Sp1 and Sp3, there are several phosphorylation sites that regulate their DNA binding activity positively and negatively (Li and Davie 2010; Beishline and Azizkhan-Clifford 2015). The phosphorylation of Ser59 in Sp1 increased its DNA binding activity (Fojas de Borja et al. 2001), while that of Thr668 and Thr739 in Sp1 decreased its DNA binding activity (Armstrong et al. 1997; Chuang et al. 2012). Although such a differential binding of Sp1 and Sp3 to the promoter in different cell types has not been found yet, there is one study describing the switch in the binding from Sp1 to Sp3 to the Toll-like receptor 5 gene promoter (Thakur et al. 2016). By the treatment of HT29 human intestinal epithelial cells with butyrate, Sp1 was dephosphorylated by Ser/Thr phosphatase whereas Sp3 was phosphorylated by extracellular-regulated kinase-mitogen-activated protein kinase through activation of two protein kinase C isoforms, thereby switching the binding from Sp1 to Sp3 to the promoter (Thakur et al. 2016). Therefore, the differences in the phosphorylation status of Sp1 and Sp3 between SH-SY5Y and A549 cells may be ascribed to the differential binding of Sp1 and Sp3 to the β4GalT3 gene promoter, which is a matter to be elucidated in a further study.

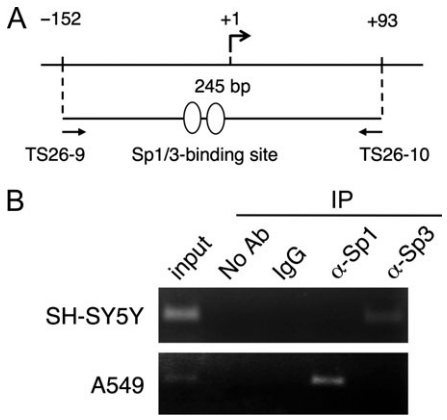

Fig. 7. In vivo binding of Sp1/Sp3 to the Sp1/3-binding sites of the β4GalT3 gene promoter. (**A**) Schematic drawing of the amplified promoter region by PCR using the oligonucleotide primers TS26-9 and TS26-10. (**B**) Binding of Sp1/Sp3 to the β4GalT3 gene promoter in SH-SY5Y and A549 cells. Chromatin fragments were immunoprecipitated (IP) from SH-SY5Y and A549 cells with antibodies that recognize Sp1 and Sp3, or IgG as a negative control. PCR was carried out using the purified DNA as a template, and TS26-9 and TS26-10. The PCR products were separated by 2% agarose gel followed by ethidium bromide staining. Three experiments were conducted, and identical results were obtained each time.

Both Sp1 and Sp3 have been reported to increase in various cancers. For instance, the expression of Sp1 increased in pancreatic cancer, gastric cancer, lung cancer and glioma (Shi et al. 2001; Wang et al. 2003; Chen et al. 2011; Guan et al. 2012). As the expression of the β4GalT3 gene was regulated by Sp1 in A549 cells, the expression of the β4GalT3 gene may increase in lung cancer by the elevated expression of Sp1. On the other hands, the expression of Sp3 increased in breast cancer, neck and head tumor, nasopharyngeal cancer, soft tissue sarcoma, prostate cancer and liver cancer (Mertens-

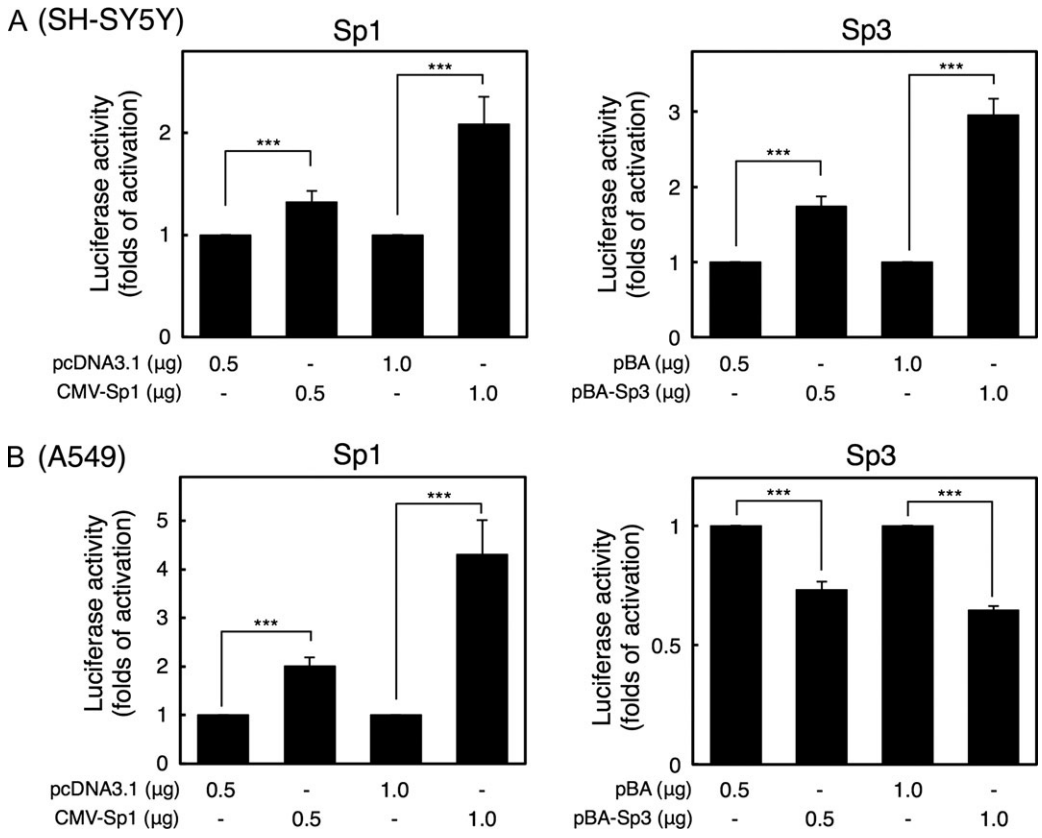

Fig. 8. Effects of the Sp1 and Sp3 expression on the activities of the β4GalT3 gene promoter. SH-SY5Y (**A**) and A549 cells (**B**) were transiently transfected with pGL(−2190/+89) and either CMV-Sp1 or pBA-Sp3. Values are the mean ± SD for triplicate assays from three different experiments. ***$P < 0.001$.

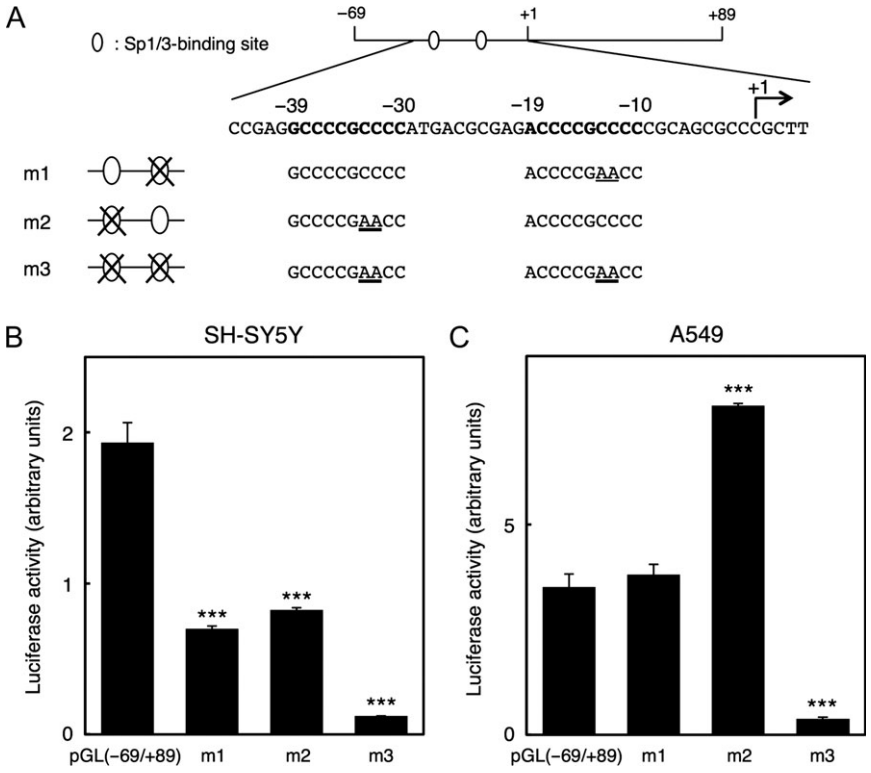

Fig. 9. Function of the Sp1/3-binding sites at nucleotide positions −39/−30 and −19/−10 on the promoter activity of the β4GalT3 gene. (**A**) Schematic drawing of mutations introduced into the Sp1/3-binding sites of pGL(−69/+89). SH-SY5Y (**B**) and A549 cells (**C**) were transiently transfected with pGL(−69/+89), m1, m2 or m3. Values are the mean ± SD for triplicate assays from three different experiments. ***$P < 0.001$ vs. pGL(−69/+89).

Talcott et al. 2007; Essafi-Benkhadir et al. 2009; Zhao et al. 2012; Kajita et al. 2013; Huang et al. 2015a, b). However, whether Sp3 increases in neuroblastoma remains to be clarified. In neuroblastoma, the expression of transcription factor N-myc, which is utilized as a biomarker for prognosis of neuroblastoma (Brodeur et al. 1984; Seeger et al. 1985), was reported to increase according to the amplification of the gene (Brodeur et al. 1984; Seeger et al. 1985; Maris et al. 2007; Kamijo and Nakagawara 2012). Sp3 was involved in the transcriptional regulation of the N-myc gene, and the promoter activity of the N-myc gene was enhanced by Sp3 (Tuthill et al. 2003; Kramps et al. 2004). Since the promoter activity of the β4GalT3 gene increased by Sp3 in SH-SY5Y cells, the enhanced Sp3 expression may increase the expression of the β4GalT3 gene in neuroblastoma. Alternatively, the enhanced N-myc in neuroblastoma may increase the β4GalT3 gene expression in cooperation with Sp3. The detailed mechanism for the increased expression of the β4GalT3 gene in neuroblastoma has to be elucidated further.

This is the first report showing the transcriptional mechanism of the β4GalT3 gene and the involvement of different Sp family transcription factors in the regulation of the β4GalT3 gene in neuroblastoma and lung cancer. In the case of colon cancer, the expression of β4GalT3 decreased, which was correlated with metastasis of colon cancer (Chen et al. 2014). As Sp3 acts as a transcriptional repressor (Li and Davie 2010; Beishline and Azizkhan-Clifford 2015), Sp3 may bind to the core promoter region of the β4GalT3 gene in colon cancer cells, and regulate the transcription negatively. Analysis of the differences in the transcriptional networks of Sp3 between colon cancer and neuroblastoma will provide the further understanding of the regulatory mechanisms of the β4GalT3 gene in a variety of cancers.

Materials and Methods

Cell cultures

SH-SY5Y human neuroblastoma cell line was maintained in a mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium (1:1, v/v) supplemented with 10% fetal calf serum (FCS), 50 μg/ml kanamycin, and 1.0 mg/ml glucose. A549 human lung cancer, MKN1 human gastric cancer, and SW480 human colon cancer cell lines were maintained in DMEM supplemented with 10% FCS, 50 units/ml penicillin and 50 μg/ml streptomycin.

Chemicals

Anti-Sp1 (H-255) and -Sp3 (D-20) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Quantitative real-time RT-PCR analysis

Quantitative real-time RT-PCR analysis was carried out with SYBR Premix Ex Taq II (TaKaRa Bio Inc., Shiga, Japan) as described previously (Shirane et al. 2014; Tagawa et al. 2014; Sugiyama et al. 2017). Total RNA was extracted from SH-SY5Y, A549, MKN1, and SW480 cells using Sepasol RNA I (Nacarai Tesque, Kyoto), and used for the synthesis of the first-strand cDNAs with PrimeScript RT reagent kit (TaKaRa Bio Inc.). The oligonucleotide primers were used as follows: β4GalT3, F: 5′-CTATAAGATGGTGAAGCA CCGAGGA-3′, R: 5′-TCTCGAGCCAGCAACTGGTATG-3′; Sp1, F: 5′-GCCTCCAGACCATTAACCTCAG-3′, R: 5′-TCATGTATT CCATCACCACCAG-3′; Sp3, F: 5′-CTGTCCCAACTGTAAAGAA GGTG-3′, R: 5′-AGAATGCCAACGCAGATGAG-3′; glyceraldehyde 3-phosphate dehydrogenase (G3PDH), F: 5′-GCACCGTCAAGGCT

GAGAAC-3′, R: 5′-TGGTGAAGACGCCAGTGGA-3′. The PCR conditions were: 95°C for 0.5 min; 40 cycles of 95°C for 5 s, 60°C for 0.5 min. The G3PDH transcript was used as an internal standard. In order to compare the gene expression levels between Sp1 and Sp3, the amplification efficiency of the Sp1 and Sp3 mRNAs with the primer pairs was calculated (Sugiyama et al. 2017), and was reflected in the results.

Identification of transcriptional start site

To identify the transcriptional start site, RNA ligase-mediated rapid amplification of the 5′ cDNA end was conducted using GeneRacer kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol (Sato and Furukawa 2004; Sugiyama et al. 2017). Total RNA was extracted from SH-SY5Y cells or A549 cells using RNeasy total RNA system (Qiagen Inc., Hilden, Germany). The total RNA was treated with calf intestinal phosphatase to eliminate the truncated mRNA and non-mRNA. Subsequently, in order to remove the 5′-cap structure from the full length mRNA, the total RNA was treated with tobacco acid pyrophosphatase. The RNA Oligo in the kit was ligated to the 5′-end of the full length mRNA. The first strand cDNAs were synthesized, and used as templates for the first PCR. The gene-specific primer TS25-20:5′-ACTGTAGACA TCACGAGGGTGAGAA-3′ (complementary to nucleotides +168/+144 relative to the translational start site), and the 5′-Primer in the kit were included in the first PCR. The nested PCR was carried out with the first PCR products as templates and the gene-specific primer TS25-19:5′-A TGACAGCCAGCTGGGAGCCCACAA-3′ (complementary to nucleotides +68/+44 relative to the translational start site), and the 5′-Nested Primer in the kit. The nested PCR products were inserted into pGEM-T Easy (Promega Corp., Madison, WI, USA), and the nucleotide sequences were determined.

Isolation of human β4GalT3 gene 5′-flanking region

The 5′-flanking region of the transcriptional start site of the human β4GalT3 gene was isolated as described previously (Sato and Furukawa 2007; Sugiyama et al. 2017). Briefly, PCR was carried out with the human whole blood genomic DNA as a template. The oligonucleotide primers were designed from 5′-flanking sequence of the human β4GalT3 gene (accession number AL590714) and used: TS28-38:5′-GGGGTACCCTAAGGTAGGTGGATCATGAGG-3′ (underline indicates KpnI site) and TS28-41:5′-ATTGAAGGTGG AGGGAAAGACAGCGTCTTC-3′ for the amplification of the region −2190 to −513, and TS24-5:5′-ACAATATGCTAGGCGG TAGTGTTTG-3′ and TS27-35:5′-CCAAGCTTTTCCCAGCGCA CATACCTGGTC-3′ (underline indicates HindIII site) for the amplification of the region −645 to +89, relative to the transcriptional start site. The PCR products were ligated into pGEM-T Easy (Promega Corp.). The nucleotide sequences were analyzed to verify the amplification of the 5′-flanking region of the β4GalT3 gene.

Reporter plasmid construction

The reporter plasmids were constructed by insertion of various 5′-flanking regions of the β4GalT3 gene with different length into the upstream of the firefly luciferase gene in pGL3-Basic (Promega Corp.) as described previously (Sato and Furukawa 2004, 2007; Sugiyama et al. 2017). The strategy for the construction of the reporter plasmids was following and the numbers in the parentheses of the plasmids indicated the nucleotide positions relative to the transcriptional start site. (1) pGL(−2190/+89), the KpnI/HpaI (−2190/−595) and HpaI/HindIII (−595/+89) fragments were released from pGEM(−2190/−513) and pGEM(−645/+89), respectively, and then inserted into the KpnI and HindIII sites of pGL3-Basic. (2) pGL(−1567/+89), the pGL(−2190/+89) was treated with KpnI and BlnI to delete the region −2190 to −1568. The digested KpnI and BlnI sites were blunted using T4 DNA polymerase followed by self-ligation. (3) pGL(−1145/+89), the pGL(−2190/+89) was treated with KpnI and PmaCI to delete the region −2190 to −1146. The digested KpnI site was blunted as described above, followed by self-ligation. (4) pGL(−595/+89), the pGEM(−645/+89) was treated with HpaI and HindIII. The HpaI/HindIII fragment (−595/+89) was inserted into the SmaI and HindIII sites of pGL3-Basic. (5) pGL(−140/+89), the KpnI/HindIII fragment was generated by PCR with TS27-34:5′-GGGGTACCCGTTCCAGGTGGGCA GGCTCAG-3′ (underline indicates KpnI site) and TS27-35:5′-CCAA GCTTTTCCCAGCGCACATACCTGGTC-3′ (underline indicates HindIII site). The KpnI/HindIII fragment (−140/+89) was inserted into the KpnI and HindIII sites of pGL3-Basic. (6) pGL(−69/+89), the KpnI/HindIII fragment was generated by PCR with TS29-5:5′-GGGGTA CCCCTAGTCAGCCCGGGTGGCTCC-3′ (underline indicates KpnI site), and TS27-35. The KpnI/HindIII fragment (−69/+89) was inserted into the KpnI and HindIII sites of pGL3-Basic. (7) pGL(−30/+89), the KpnI/HindIII fragment was generated by PCR with TS29-6:5′-GGGGTA CCCATGACGCGAGACCCCGCCCCC-3′ (underline indicates KpnI site), and TS27-35. The KpnI/HindIII fragment (−30/+89) was inserted into the KpnI and HindIII sites of pGL3-Basic. (8) pGL(−5/+89), the KpnI-HindIII fragment was generated by PCR with TS29-9:5′-GGGGTA CCGCGCCCGCTTCCAAGATGGCGG-3′ (underline indicates KpnI site), and TS27-35. The KpnI/HindIII fragment (−5/+89) was inserted into the KpnI and HindIII sites of pGL3-Basic.

In order to examine the function of the Sp1/3-binding sites at nucleotide positions −39/−30 and −19/−10, the Sp1/3-binding sites were mutated using pGL(−69/+89) as a template with a KOD–Plus–Mutagenesis Kit (TOYOBO, Osaka, Japan) according to the manufacturer's protocol. The mutated nucleotides in the Sp1/3-binding sites were indicated with underline in nucleotides −19 to −10 (ACCCCGAACC in place of the wild type, ACCCCGCCCC) and −39 to −30 (GCCCCGAACC in place of the wild type, GCCCCGCCCC). The plasmids m1 and m2 contained the mutations in the Sp1/3-binding sites at −19/−10 and −39/−30, respectively. The plasmid m3 contained the mutations in both Spl/3-binding sites. The nucleotide sequences of all plasmids were verified by sequencing. pRL-TK (Promega Corp.) containing the *Renilla* luciferase gene under the control of the herpes simplex virus thymidine kinase (TK) promoter was used for adjusting transfection efficiencies.

ChIP assay

ChIP assay was conducted with a ChIP assay kit (Upstate, Temecula, CA, USA) according to the manufacturer's protocol (Sato and Furukawa 2007). Briefly, the cells were crosslinked by the treatment with formaldehyde and harvested. The collected cells were lysed in the lysis buffer supplemented with the kit, and then the cell lysates were sonicated. The sheared chromatin was immunoprecipitated at 4°C overnight with 1 μg of antibodies that recognize Sp1 and Sp3, or IgG (negative control), and subsequently incubated with protein A agarose at 4°C for 2 h. The beads were precipitated by centrifugation and washed sequentially with low salt wash buffer, high salt wash buffer, and LiCl wash buffer supplemented with the kit each twice. Finally, the beads were washed four times with 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA. The

immune complexes were eluted by sodium bicarbonate buffer containing sodium dodecyl sulfate. After adding NaCl, the mixture was incubated at 65°C for 12 h. The remainder of the proteins was digested with proteinase K at 45°C for 1 h. The DNA was recovered by phenol/chloroform extraction and then purified by ethanol precipitation. The purified DNA was analyzed by PCR as described previously (Sato and Furukawa 2007). For amplification of the βGalT3 gene promoter region (−152 to +93, relative to the transcriptional start site), TS26-9:5′-TCGCCTACAACGCGTTCCAG-3′ and TS26-10:5′-CGCATTCCCAGCGCACATAC-3′ were used.

Sp3-expression plasmid

Sp3-expression plasmid, pBA-Sp3, was prepared as described previously (Sato and Furukawa 2007). Briefly, total RNA was extracted from A549 cells using RNeasy total RNA system (Qiagen Inc.). The single-strand cDNAs were synthesized from the total RNA with a hexadeoxyribonucleotide mixture (oligo(dN)$_6$, TaKaRa Bio Inc.) as a primer and SuperScript III reverse transcriptase (Invitrogen). The human Sp3 cDNA with full-length was generated by PCR with Takara Ex Taq (Takara Bio Inc.). The following oligonucleotide primers were designed from sequence of the human Sp3 gene (accession number BC126414) and used: TS28-19:5′-CGGGATCCATGACCGCTCCCGAAAAGCCCG-3′ (underline indicates BamHI site) and TS28-20:5′-CCAAGCTTTTACTCCATTGTCTCATTTCCA-3′ (underline indicates HindIII site). The PCR conditions were: 40 cycles of 94°C for 10 s, 60°C for 0.5 min, and 72°C for 3 min. The PCR product was cloned into the pGEM-T Easy vector, and the nucleotide sequence was analyzed to verify the amplification of the coding region of the Sp3 gene. The plasmid containing the Sp3 cDNA was treated with BamHI and HindIII, and the BamHI/HindIII fragment was inserted into the BamHI and HindIII sites of the mammalian expression vector pBApo-EF1α Pur DNA (Takara Bio Inc.), containing EF1α promoter, to generate pBA-Sp3. Sp1-expression plasmid, CMV-Sp1, was generously provided by Dr. Robert Tjian (University of California, Berkeley).

Luciferase assay

The promoter activities of the reporter plasmids were determined as described previously (Sato and Furukawa 2004, 2007; Sugiyama et al. 2017). In brief, SH-SY5Y and A549 cells were transiently transfected with each reporter plasmid and pRL-TK. After 48 h-transfection, the firefly and *Renilla* luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega corp.). In the case of the co-transfection with CMV-Sp1 and pBA-Sp3, the firefly luciferase activity was normalized to the protein amount and is expressed as fold activation in comparison to cells co-transfected with pcDNA3.1 or pBA.

Statistical analysis

All experiments were repeated three times, and the results are represented as the mean ± SD. Statistical evaluations were conducted using Student's *t*-test or one-way analysis of variance with Tukey's multiple comparison.

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Conflicts of interest statement

None declared.

Abbreviations

Cer, ceramide; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; Gal, galactose; β4GalT, β4-galactosyltransferase; Glc, glucose; GlcNAc, *N*-acetylglucosamine; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction; Sp, Specificity protein; TK, thymidine kinase; UTR, untranslated region.

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