Mechanism for decreased gene expression of β4-galactosyltransferase 5 upon differentiation of 3T3-L1 mouse preadipocytes to adipocytes

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Summary

Upon differentiation of cells, remarkable changes in the structures of glycans linked to lipids on cell surface have been observed. Lactosylceramide (Lac-Cer) serves as a common precursor for a series of glycosphingolipids with diverse structures. In the present study, we examined the underlying mechanism for the biosynthesis of Lac-Cer upon differentiation of 3T3-L1 mouse preadipocytes to adipocytes. TLC analysis showed that the amounts of Lac-Cer decrease in 3T3-L1 adipocytes compared to 3T3-L1 preadipocytes. In accordance with this change, the gene expression level of β4-galactosyltransferase (β4GalT) 5, which was identified as Lac-Cer synthase, decreased drastically upon differentiation of 3T3-L1 preadipocytes. The analysis of the transcriptional mechanism of the β4GalT5 gene demonstrated that the core promoter region is identified between nucleotides -299 and -1 relative to the translational start site. During adipocyte differentiation, the expression levels and promoter activities of the β4GalT5 gene decreased dramatically. Since the Specificity protein 1 (Sp1)-binding sites in the promoter region were critical for the promoter activity, it is suggested that Sp1 plays an important role for the expression of the β4GalT5 gene in 3T3-L1 cells. The gene and protein expression of Sp1 decreased significantly upon differentiation of 3T3-L1 preadipocytes. Taken together, the present study suggest that the expression of the β4GalT5 gene decreases through reduced expression of the Sp1 gene and protein upon differentiation of 3T3-L1 peradipocytes to adipocytes, which may lead to the decreased amounts of Lac-Cer in 3T3-L1 adipocytes.

Keywords: lactosylceramide; β4-galactosyltransferase 5; preadipocytes; adipocytes; differentiation; Specificity protein 1.
INTRODUCTION

The prevalence of obesity is one of the serious problems in modern societies globally. Obesity has been shown to be closely associated with a number of pathological disorders, including hypertension, atherosclerosis, and non-insulin-dependent diabetes.\(^1\) Obesity is ascribed to the excessive accumulation of adipose tissues consisting of adipocytes with hyperplasia and hypertrophy.\(^2\) To develop a novel therapeutic treatment for obesity, the underlying mechanism of the regulation of the molecules related to the adipocyte differentiation needs to be clarified.

In association with the adipocyte differentiation, dramatic changes are induced in the morphology and physiology of the preadipocytes.\(^1\) One of the prominent physiological changes in the adipocyte differentiation model using mouse 3T3-L1 cell line is the increased synthesis of triglycerides and fatty acids in accompany with the elevated expression levels and activities of the enzymes such as glycerophosphate dehydrogenase and lipoprotein lipase, which are involved in the lipogenic capabilities of the cells.\(^3,4\) The gangliosides belonging to the glycosphingolipids (GSLs) consist of carbohydrate and ceramide (Cer) portions, the former of which is generally composed of glucose (Glc), galactose (Gal), N-acetylgalactosamine (GalNAc), and sialic acid (Sia). The gangliosides are involved in essential biological functions at cell surface such as cell growth and differentiation.\(^5,6\) The amounts of gangliosides, including GM3 (Sia\(\alpha_2\rightarrow3\)Gal\(\beta_1\rightarrow4\)Glc-Cer), which was one of the major components in the cells, decreased upon adipocyte differentiation.\(^7\) Lactosylceramide (Lac-Cer, Gal\(\beta_1\rightarrow4\)Glc-Cer), one of GSLs, is synthesized from glucosylceramide (Glc-Cer) by Lac-Cer synthase, which transfers Gal from UDP-Gal to Glc residue of Glc-Cer, and serves as a common precursor for GSLs.\(^8\) In the previous studies, two cDNAs encoding human \(\beta_4\)-galactosyltransferase (\(\beta_4\)GalT) 5 and rat \(\beta_4\)GalT6 were originally isolated,\(^9,10\) and they were identified to be Lac-Cer synthases in mammalian cells.\(^11-13\)

In our laboratory, the human and mouse cDNAs encoding \(\beta_4\)GalT5 were isolated,\(^9,14\) and the relationships between \(\beta_4\)GalT5 and cancer properties were analyzed so far.\(^15-17\) Since
Lac-Cer is an important precursor for expressing a variety of GSLs, it is of interest to examine the relationship between β4GalT5 and diseases except for cancer. Although the amounts of some GSLs have been shown to decrease upon differentiation of 3T3-L1 preadipocytes, whether or not the amounts of Lac-Cer upon adipocyte differentiation change remains unknown. As the first step, we analyzed the amounts of Lac-Cer and the underlying mechanism of Lac-Cer biosynthesis upon adipocyte differentiation using 3T3-L1 mouse cell line, which is a well-characterized model for adipocyte differentiation.
MATERIALS AND METHODS

Chemicals

Bovine insulin, dexamethasone (Dex), 3-isobutyl-1-methylxanthine (IBMX), and oil red O were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Rabbit anti-Specificity protein 1 (Sp1) and anti-β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Cell culture and induction of differentiation

3T3-L1 mouse cell line was obtained from Human Science Research Resources Bank (Osaka, Japan). 3T3-L1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 50 units/ml penicillin and 50 μg/ml streptomycin. 3T3-L1 cells were cultured until confluent, and two days later, the cells were induced to differentiation by the addition of 1 μM Dex, 0.5 mM IBMX, and 10 μg/ml insulin for eight days. The medium containing the above-mentioned differentiation inducers was changed every two days.

Oil red O staining

The 3T3-L1 preadipocytes and adipocytes at Day-8 were washed with 10 mM phosphate-buffered saline (pH 7.4) and fixed with 10% formaldehyde for 1 h. The fixed cells were washed with water and then stained with 0.3% oil red O for 15 min. After staining, the cells were washed with water, and then isopropanol was added to the cells to extract oil red O from the cells. The extracts were transferred to the 96-well microtiter plates, and the absorbance of each well at 540 nm was determined using a microtiter plate reader.

Neutral glycolipid analysis
Total lipid fractions were prepared from 3T3-L1 preadipocytes and adipocytes at Day-8, and TLC analysis was performed as described previously.\textsuperscript{12,17} Lipid preparations whose amounts were equivalent to 800 μg of cellular proteins were applied to a high-performance TLC silica gel 60 plate (Merck KGaA, Darmstadt, Germany), and separated by TLC using chloroform/methanol/water (60:25:4, v/v/v) as a solvent. The neutral GSLs were detected with an anthrone-thiourea reagent by heating at 120°C. The bands of glycolipids were determined by comparison with the positions of authentic Glc-Cer, Lac-Cer, globotriaosylceramide (Gb3-Cer, Galα1→4Galβ1→4Glc-Cer), and globotetraosylceramide (Gb4-Cer, GalNAcβ1→3Galα1→4Galβ1→4Glc-Cer) migrated. Each band intensity of Lac-Cer was determined by ImageJ analysis (ver. 1.46r), and ratios between those of preadipocytes and adipocytes were calculated as described previously.\textsuperscript{12,17}

**Quantitative real-time and conventional RT-PCR analyses**

Quantitative real-time RT-PCR analysis was performed as described previously.\textsuperscript{17-20} In brief, total RNA samples were prepared from 3T3-L1 preadipocytes and adipocytes, and used for first-strand cDNA synthesis. The gene-specific primers were used as follows: β4GalT5, F: 5'-AGATTTCACCTACTTTGCCAACCAC-3', R: 5'-GCTTAATGGCCGGGTCTCTG-3'; β4GalT6, F: 5'-AGACTGCAAGCCCAGATGGAA-3', R: 5'-CATCGGAATCAGGTGCAGGA-3'; Sp1, 5'-TCATGGATCTGTTGGTGATGGG-3', R: 5'-GCTCTTTCCCTCACTGTCTTTTG-3'; aP2, F: 5'-GCGTGGAAATTCGATGAAATCA-3', R: 5'-CCCAGGCATCTAAGGGTTATGA-3'; 18S ribosomal RNA (18S), F: 5'-ACCGCAGCTAGGAATAATGGA-3', R: 5'-GCCTCAGTCCGAAAACCA-3'. The amplification efficiency of the β4GalT5 and β4GalT6 mRNAs using each primer pairs was calculated as described previously.\textsuperscript{20}

Conventional RT-PCR analysis was carried out by the method as described previously.\textsuperscript{12,16} The gene-specific primers were used as follows: β4GalT5, F: 5'-ACTTGGATTGGGATTGTCTGAT-3', R: 5'-GCAGGAGTAGGCCAGGTTG-3'; β4GalT6, F: 5'-CATCAGCTGCTCTTCCAGGGACT-3', R: 5'-ACTCAGTTAGGAATAATGGA-3'; elongation factor (EF)-1α, F: 5'-CCATGAAGCTTTGAGTGAAGCTCT-3', R: 5'-CCCAGCCATCTAAGGGTTATGA-3'.
Isolation of 5'-flanking region of mouse β4GalT5 gene

The DNA fragment with the 5'-flanking and partial coding regions of the mouse β4GalT5 gene was cloned by PCR using the mouse genomic DNA prepared from whole blood cells as a template. Based on the mouse DNA sequence from the clone RP23-465I6 on chromosome 2 (accession no. AL591762), the following 5’- and 3’-primer pairs were designed and used: TS27-57 (5’-CTGGGCGGATCAGGCCTGAGCCATGTCCCTTCCCTTACCAG-3’) and TS137 (5’-TGCCGGGCGGCACATAGACGAATG-3’) for the amplification of the region -1980 to +112 relative to the translational start site.

Reporter plasmid construction

To analyze the promoter activities of the β4GalT5 gene, several deletion constructs of the 5’-flanking regions with different length were generated by insertion of the PCR fragments between NheI and BglII sites of pGL3-Basic vector (Promega, Madison, WI, U.S.A.) containing the firefly luciferase gene as a reporter. The NheI-BglIII fragment was cloned by PCR with the 5’- and 3’-primer pairs, in which NheI and BglIII sites are underlined, respectively, as described below. The numbers indicate the nucleotide positions relative to the translational start site. 1) pGL(-1980/-1), TS27-63 (5’-CGGCTAGCGGCTTGCCATGTCCCTTCTTTCCCTTAC-3’) and TS27-64 (5’-GAAGATCTGCTGAGCCGCGGCGCCGCTCGAG-3’) were used. 2) pGL(-1513/-1), TS27-72 (5’-CGGCTAGCGGATGCGGAAAGTCGAGGCTTGAG-3’) and TS27-64 were used. 3) pGL(-1048/-1), TS27-73 (5’-CGGCTAGCGAGGGCTTGAGGCTTCCCTCCTCAATCTC-3’) and TS27-64 were used. 4) pGL(-480/-1), TS27-74 (5’-CGGCTAGCGGCAAAATGCTTGTTATGCTCCCTG-3’) and TS27-64 were used. 5) pGL(-299/-1), pGL(-480/-1) was digested with KpnI. The plasmid was then self-ligated with
T4 DNA ligase. 6) pGL(-218/-1), TS28-25 (5’-CGGCTAGCCCTAACAGGCCCCGCCCCTCC-3’) and TS27-64 were used. 7) pGL(-132/-1), pGL(-480/-1) was digested with KpnI and SacII. The ends were blunted with T4 DNA polymerase, and the plasmid was then self-ligated. 8) pGL-Sp1m1, two Sp1-binding sites of pGL(-299/-1) were mutated using a KOD –Plus– Mutagenesis Kit (TOYOBO, Osaka, Japan). The mutations are underlined in nucleotides -240 to -231 and -228 to -219 relative to the translational start site (ACCCCGAACC and ACCCCGAACT in place of the wild-type sequences, ACCCCGCCCC and ACCCCGCCCT, respectively). 9) pGL-Sp1m2, one Sp1-binding site of pGL(-218/-1) was mutated as described above. The mutation is underlined in nucleotides -210 to -201 relative to the translational start site (GCCCGAACC in place of the wild-type sequence, GCCCGGCCCT). The correct sequences of the plasmids used in this study were confirmed by nucleotide sequencing.

Luciferase assay

The promoter activities of the reporter plasmid constructs were evaluated by luciferase assay as described previously.18,20-22)

Establishment of 3T3-L1 cells containing β4GalT5 gene promoter

The reporter plasmid pGL4-mGT5-0.3 was prepared by the insertion of the 0.3 kb promoter region (-299/-1) into upstream of the firefly luciferase gene in pGL4.15 vector [luc2p/Hygro] (Promega) containing the hygromycin-resistant gene. To establish the 3T3-L1 cells having the luciferase gene under the control of the mouse β4GalT5 gene promoter, named as mGT5-0.3-sensor cells, pGL4-mGT5-0.3 (4 μg) was transfected by electroporation (960 μF and 160 V) into 3T3-L1 cells (1.0 x 10^6 cells in 0.2 cm cuvette) using a Gene Pulser Xcell CE system (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.). After being cultured for 72 h, the plasmid-transfected cells were selected by the treatment with DMEM containing 5%
FCS and hygromycin B (400 μg/ml) for two weeks. To induce the adipocyte differentiation, the sensor cells were treated with 1 μM Dex, 0.5 mM IBMX, and 10 μg/ml insulin as described above.

**Immunoblot analysis**

The cell lysates were prepared from 3T3-L1 preadipocytes and adipocytes. Immunoblot analysis with the antibodies against Sp1 and β-actin was conducted as described previously.\(^{21,23}\)

**Statistical analysis**

All experiments were conducted three times, and the mean values with standard deviations were shown. A significant difference in the experiments was determined with Student’s t-test for the comparison of the means of two independent, and with one-way ANOVA followed by Bonferroni’s test for the comparison of the means of more than two independent groups.
RESULTS

Differentiation of 3T3-L1 preadipocytes to adipocytes

3T3-L1 mouse cell line is used as a well-characterized model system to study on the adipocyte differentiation.\textsuperscript{24,25} The cells at confluency were cultured in the presence of Dex, IBMX, and insulin for eight days. As shown in Fig. 1A, the cell morphology drastically changed after eight days. The adipocytes at Day-8 accumulated the oil droplets, while the preadipocytes did not (Fig. 1A). In order to confirm the differentiation of 3T3-L1 preadipocytes to adipocytes, oil red O staining was performed. 3T3-L1 preadipocytes were not stained with oil red O, but the cells by the treatment with Dex, IBMX, and insulin were strongly stained with oil red O. When the lipid accumulation in the differentiated 3T3-L1 adipocytes was evaluated quantitatively by measurement of oil red O staining, about two-fold higher accumulation was observed for 3T3-L1 adipocytes compared to 3T3-L1 preadipocytes (Fig. 1B), suggesting that the cells differentiate to adipocytes by the treatment with Dex, IBMX, and insulin. Furthermore, the gene expression of aP2, which is a fatty acid binding protein and one of the markers for adipocytes,\textsuperscript{26} increased dramatically in 3T3-L1 adipocytes when compared with preadipocytes (Fig. 1C). These results indicate that upon treatment with Dex, IBMX, and insulin, 3T3-L1 preadipocytes differentiates into cells showing the morphological and biochemical characteristic of adipocytes.

Neutral glycolipid components of 3T3-L1 preadipocytes and adipocytes

To examine whether or not the amounts of Lac-Cer changed upon differentiation of 3T3-L1 preadipocytes to adipocytes, TLC analysis was performed using total lipid fractions. Upon detection of neutral glycolipids with an anthrone-thiourea reagent, the amounts of Lac-Cer in 3T3-L1 adipocytes decreased when compared with that of 3T3-L1 preadipocytes (Fig. 2). On the other hand, the amounts of Glc-Cer appeared to be similar between
preadipocytes and adipocytes (Fig. 2). These results suggest that the biosynthesis of Lac-Cer is inhibited upon differentiation of 3T3-L1 preadipocytes.

Expression of the \( \beta_4 \text{GalT5} \) gene in 3T3-L1 preadipocytes and adipocytes

Since \( \beta_4 \text{GalT5} \) and \( \beta_4 \text{GalT6} \) are involved in the biosynthesis of Lac-Cer\(^{10-13} \), the expression levels of both genes were analyzed. The results of RT-PCR showed that significant amplification of the \( \beta_4 \text{GalT5} \) gene is detected, but the amplification of the \( \beta_4 \text{GalT6} \) gene can not be detected in 3T3-L1 preadipocytes and adipocytes under the same PCR condition (Fig. 3A), suggesting that \( \beta_4 \text{GalT6} \) is not expressed in 3T3-L1 cells, and \( \beta_4 \text{GalT5} \) is responsible for the biosynthesis of Lac-Cer in 3T3-L1 cells. Quantitative real-time RT-PCR analysis revealed that the expression of the \( \beta_4 \text{GalT5} \) gene decreased to 22% of that of 3T3-L1 preadipocytes (Fig. 3B). The amplification efficiency was 88.9% and 94.5% for the amplicons of the \( \beta_4 \text{GalT5} \) and \( \beta_4 \text{GalT6} \) mRNAs, respectively. However, the expression of the \( \beta_4 \text{GalT6} \) gene could not be also detected by quantitative real-time RT-PCR even though much higher amplification efficiency was associated with the amplification of the \( \beta_4 \text{GalT6} \) gene.

Transcriptional mechanism of the \( \beta_4 \text{GalT5} \) gene in 3T3-L1 preadipocytes

To clarify the mechanism for decreased expression of the \( \beta_4 \text{GalT5} \) gene upon adipocyte differentiation, the 5'‐flanking region of the \( \beta_4 \text{GalT5} \) gene was isolated, and the transcriptional regulation was analyzed using the deletion constructs. Luciferase assay showed that significant promoter activity is associated with pGL(-299/-1) in spite of deleting the region between -1980 and -300 (Fig. 4). Furthermore, the promoter activities of pGL(-218/-1) and pGL(-132/-1) were significantly reduced to 49% and 16% of pGL(-1980/-1) (Fig. 4-lower panel). These results indicate that the core promoter region is identified between nucleotides -299 and -1, and contains the crucial positive regulatory
Expression and Promoter activity of the β4GalT5 gene during adipocyte differentiation

During differentiation of 3T3-L1 preadipocytes, the expression levels of the β4GalT5 gene decreased gradually in time-dependent manner (Fig. 5A). By the treatment with Dex, IBMX, and insulin, the expression levels of the β4GalT5 gene decreased to 64% at Day-2, 22% at Day-4, 16% at Day-6, and 15% at Day-8 when compared with those of the β4GalT5 gene in 3T3-L1 preadipocytes at Day-0 (Fig. 5A). To analyze the promoter activity of the β4GalT5 gene during adipocyte differentiation, the mGT5-0.3-sensor cells having the luciferase reporter driven by the β4GalT5 gene promoter were established. By the treatment with Dex, IBMX, and insulin, the promoter activities decreased to 30% at Day-2, 25% at Day-4, 24% at Day-6, and 20% at Day-8 when compared with those of the mGT5-0.3-sensor cells at Day-0 (Fig. 5B). These results indicate that the promoter activity of the β4GalT5 gene decreases during adipocyte differentiation. Taken together, these results indicated that the expression of the β4GalT5 gene decreases upon differentiation of 3T3-L1 preadipocytes to adipocytes, which may lead to decreased amounts of Lac-Cer.

Involvement of the Sp1-binding sites in the promoter activity of the β4GalT5 gene

In mammalian glycosyltransferase genes, the GC-rich promoter lacking the TATA and CCAAT boxes were typically found, and the consensus Sp1-binding sites were included in the promoter regions of the glycosyltransferase genes. In the previous study, we showed that the expression of the human β4GalT5 gene is regulated by Sp1. These findings suggest that the expression of the mouse β4GalT5 gene is also regulated by Sp1. By using the TFBIND program for prediction of the putative binding sites for transcription factors, three Sp1-binding sites were found to be located at nucleotide positions -240/-231, -228/-219, and -210/-201 in the core promoter region (Table 1). Upon introduction of the mutations in the
Sp1-binding sites, the promoter activities of pGL-Sp1m1 and pGL-Sp1m2 decreased to 50% and 14% of pGL(-299/-1) (Fig. 6). By introducing these mutations in the Sp1-binding sites, the Sp1-binding to the promoter region and promoter activities could be reduced.22) Taken together, these results suggest that Sp1 is involved in the transcriptional regulation of the β4GalT5 gene in 3T3-L1 cells.

**Expression of the Sp1 gene and protein in preadipocytes and adipocytes**

Next, we examined the expression levels of the Sp1 gene in 3T3-L1 preadipocytes and adipocytes by quantitative real-time RT-PCR analysis. By the treatment with Dex, IBMX, and insulin, the expression level of the Sp1 gene decreased to 61% at Day-8 when compared with that of the Sp1 gene in 3T3-L1 preadipocytes at Day-8 (Fig. 7A). Moreover, immunoblot analysis with anti-Sp1 antibody demonstrated that the expression of Sp1 also decreases in adipocytes as compared to that in preadipocytes (Fig. 7B). The results indicate that the expression of the Sp1 gene and protein decreases in 3T3-L1 adipocytes (Figs. 7A and 7B), which is similar to the expression of the β4GalT5 gene (Fig. 3B). These results suggest that the expression of the β4GalT5 gene decreases through reduced expression of the Sp1 gene upon differentiation of 3T3-L1 preadipocytes to adipocytes.
DISCUSSION

Dramatic changes in glycosylation have been observed for the development of a variety of diseases such as cardiovascular disorders and diabetes. For instance, in the retina from diabetic rats, the activities of fucosyltransferase and sialyltransferase decreased, suggesting that the altered glycosylation is associated with diabetic retinopathy. Tumor necrosis factor-α-induced insulin resistance in 3T3-L1 adipocytes lead to elevated GM3 synthesis. On the other hand, the targeted disruption of the gene encoding GM3 synthase, which is Lac-Cer α-2,3-sialyltransferase, in mice showed the protection from high-fat diet-induced insulin resistance. The dysregulation of O-linked β-N-acetylglucosamine modification was involved in the causes of cardiovascular disorders and type-2 diabetes. Furthermore, the expression of the N-acetylglucosaminyltransferase 4a gene has been shown to decrease in the pancreatic β cells from the mice fed a high-fat diet and the human donors with type 2 diabetes. Since these diseases are closely associated with obesity, the glycans, which play important roles in adipocyte differentiation, are also considered to change dramatically upon adipocyte differentiation.

The present study demonstrated that the amounts of Lac-Cer decrease in accordance with the decreased expression of the β4GalT5 gene upon differentiation of 3T3-L1 preadipocytes. Since Lac-Cer plays an important role as a precursor of gangliosides, the amounts of gangliosides were also expected to decrease upon differentiation of 3T3-L1 preadipocytes, which was reported by the previous study. The analysis of the transcriptional mechanism suggests that the promoter activity of the β4GalT5 gene is regulated by Sp1 in 3T3-L1 cells, and the decreased expression of the β4GalT5 gene is brought about by the reduced gene and protein expression of Sp1 upon adipocyte differentiation. In support of this finding, the expression of Sp1 has been shown to decrease upon differentiation of 3T3-L1 preadipocytes. Sp1 belongs to Sp transcription factor family with C2H2-type zinc finger and is involved in the regulation of the housekeeping genes. IBMX, which is a cAMP phosphodiesterase inhibitor and one of the differentiation inducers, suppressed the expression
of Sp1 by increasing the cellular cAMP level.\textsuperscript{35} Since the transcription of the Sp1 gene has been shown to be auto-regulated by Sp1,\textsuperscript{36} the expression of the Sp1 gene and protein decreased synergistically upon differentiation of 3T3-L1 preadipocytes, thereby decreasing the expression of the β4GalT5 gene drastically. As our previous study demonstrated that the transcription of the human β4GalT5 gene is regulated by Sp1,\textsuperscript{22} the transcription of the mouse β4GalT5 gene has been shown to be also regulated by Sp1, suggesting that the transcription of the β4GalT5 gene is regulated by similar mechanism across species. Besides β4GalT5, the reduced expression of Sp1 may affect another glycosyltransferase genes. If the promoter regions of the glycosyltransferase genes contain the Sp1-binding sites, which regulate the promoter activities positively, the expression of the genes decreases due to reduced expression of Sp1 during adipocyte differentiation.

Lipid rafts are plasma membrane microdomains, in which cholesterol and GSLs are enriched, and signaling molecules and growth factor receptors become concentrated.\textsuperscript{37} In adipocytes, the binding of insulin to its receptor stimulates Glc uptake by recruiting Glc transporter (GLUT) 4 to cell surface.\textsuperscript{38} Lipid rafts were shown to be required for the insulin receptor signaling, and the translocation and internalization of GLUT4.\textsuperscript{39,40} By disrupting the integrity of lipid rafts with cholesterol binding reagent, thereby preventing the signaling, the differentiation of 3T3-L1 preadipocytes was inhibited.\textsuperscript{41} These studies indicate that lipid rafts play crucial roles in insulin receptor signaling and adipocyte differentiation. On the other hand, obesity shows the deleterious effects on the structures and functions of hippocampus leading to cognitive impairment.\textsuperscript{42} The analysis of the lipid rafts from hippocampus revealed that significant decreases of insulin receptor and its downstream signaling molecule AKT are observed for obese mice when compared with wild-type mice.\textsuperscript{43} The findings suggest that the obesity-induced changes in lipid rafts cause the dysfunction of hippocampus in obesity.

In adipocyte differentiation, CCAAT/enhancer-binding protein (C/EBP) family transcription factors and peroxisome proliferation-activated receptor γ play key roles in the regulation of the adipogenic genes.\textsuperscript{1} Although the activation of mitogen-activated protein kinase (MAPK) signaling was not necessary for 3T3-L1 adipocyte differentiation,\textsuperscript{44} the
The phosphorylation of C/EBPβ by MAPK has been shown to be critical for the early stage of adipocyte differentiation. Therefore, the suppression of MAPK signaling at early stage is considered to be useful strategy to the regulation of adipocyte differentiation. In fact, the compounds, dioscin and theobromine, both of which are derived from plants, inhibited the differentiation of 3T3-L1 preadipocytes to adipocytes through suppression of the phosphorylation of extracellular-regulated kinase 1/2 (ERK1/2) in MAPK signaling pathway. On the other hand, Lac-Cer in lipid rafts has been shown to be involved in a variety of transmembrane signals and increase the phosphorylation of ERK proteins in MAPK signaling pathway. Our previous study showed that the expression of the β4GalT5 gene increases upon malignant transformation of cells, and a subsequent study demonstrated that by decreasing the expression of the β4GalT5 gene in B16-F10 mouse melanoma cells, tumorigenic potentials were inhibited through attenuation of MAPK signaling as the results of the decreased expression of Lac-Cer. The genetic manipulation of β4GalT5 could affect to the proliferation and differentiation of 3T3-L1 preadipocytes due to changes in the phosphorylation status of MAPK. Quite recently, the gene expression of β-galactoside α2,6-sialyltransferase-1 (ST6Gal1), which is involved in α2,6-sialylation of N-glycans, has been shown to decrease in visceral adipose tissues from obese mice and in 3T3-L1 adipocytes as compared with controls. Furthermore, the differentiation of 3T3-L1 preadipocytes to adipocytes was suppressed by regulating the ST6Gal1 gene.

In summary, the present study reports novel findings that the expression of the β4GalT5 gene decreases through reduced expression of the Sp1 gene and protein upon differentiation of 3T3-L1 preadipocytes to adipocytes, which may lead to the reduced amounts of Lac-Cer in 3T3-L1 adipocytes. During the process of this study, we established the mGT5-0.3-sensor cells having the luciferase reporter driven by the mouse β4GalT5 gene promoter from 3T3-L1 cells. By using the sensor cells, the compounds that regulate the expression of the β4GalT5 gene can be discovered from compound libraries, which may influence the proliferation and differentiation of 3T3-L1 preadipocytes and may apply to the control of obesity.
Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.
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FIGURE LEGENDS

Fig. 1. Confirmation of adipocyte differentiation upon treatment with Dex, IBMX, and insulin. (A) Morphology of 3T3-L1 preadipocytes (P) and adipocytes (A) at Day-8. The oil drops were accumulated in 3T3-L1 adipocytes. (B) Lipid accumulation upon adipocyte differentiation, as quantified by measurement of absorbance at 540 nm after oil red O staining. ***p < 0.001. (C) The expression of the aP2 gene in 3T3-L1 preadipocytes and adipocytes. The 18S was used for internal control. ***p < 0.001.

Fig. 2. Separation of neutral GSL components of 3T3-L1 preadipocytes and adipocytes. TLC analysis of total lipids from preadipocytes (P) and adipocytes (A) at Day-8. Positions of authentic Glc-Cer, Lac-Cer, Gb3-Cer, and Gb4-Cer migrated are indicated at the left side of plate. The arrow indicates the position of Lac-Cer. The ratios of amounts of Lac-Cer between preadipocytes and adipocytes are shown at the bottom. The asterisk indicates phospholipids.

Fig. 3. Expression of the β4GalT5 and β4GalT6 genes between 3T3-L1 preadipocytes and adipocytes. (A) RT-PCR analysis of the β4GalT5 and β4GalT6 genes in preadipocytes (P) and adipocytes (A). The EF-1α gene was used for internal control. (B) Quantitative real-time RT-PCR analysis of the β4GalT5 gene. Open and closed bars indicate preadipocytes (P) and adipocytes (A), respectively. The 18S was used for internal control. ***p < 0.001.

Fig. 4. Identification of the core promoter region of the mouse β4GalT5 gene. The reporter plasmids were transiently transfected into 3T3-L1 cells, and the luciferase assay was conducted. ***p < 0.001.

Fig. 5. Expression and promoter activities of the β4GalT5 gene during differentiation of 3T3-L1 cells. (A) Expression levels of the β4GalT5 gene during adipocyte differentiation. The 18S was used for internal control. ***p < 0.001. (B) Promoter activities of
mGT5-0.3-sensor cells upon treatment with Dex, IBMX, and insulin. ***p < 0.001.

Fig. 6. Role of the Sp1-binding sites in the promoter activities of the β4GalT5 gene. The reporter plasmids were transiently transfected into 3T3-L1 cells, and the luciferase assay was conducted. ***p < 0.001.

Fig. 7. Expression of the Sp1 gene and protein in 3T3-L1 preadipocytes and adipocytes. (A) Quantitative real-time RT-PCR analysis of the Sp1 gene. Open and closed bars indicate preadipocytes (P) and adipocytes (A), respectively. The 18S rRNA was used for internal control. ***p < 0.001. (B) Immunoblot analysis of Sp1 protein in preadipocytes (P) and adipocytes (A). The ratio of amounts of Sp1 against β-actin between preadipocytes and adipocytes is shown at the bottom of the blot.
TABLE 1
Consensus sequences for Sp1-binding sites in the core promoter region of the mouse β4GalT5 gene

<table>
<thead>
<tr>
<th>Consensus core sequence</th>
<th>β4GalT5 sequence (position)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRGGCRGGGW</td>
<td>ACCCCGCCCC (-240/-231)</td>
</tr>
<tr>
<td></td>
<td>ACCCCGCCCT (-228/-219)</td>
</tr>
<tr>
<td></td>
<td>GCCCCGCCCC (-210/-201)</td>
</tr>
</tbody>
</table>

The positions of the nucleotide are indicated relative to the translational start site of the gene.
Fig. 1

A  Preadipocytes  Adipocytes

B

Absorbance (540 nm)

0  0.5  1.0

P  A

C

Gene expression levels (aP2 mRNA/18S x 10^-2)

0  5  10  15

P  A

***

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Fig. 2

Glc-Cer
Lac-Cer
Gb3-Cer
Gb4-Cer

P
A

* ←
* ←

Lac-Cer 1.0 0.6
Fig. 3

A

β4GalT

500 bp

P  A  P  A  P  A

B

Gene expression levels
(β4GalT5 mRNA/18S x 10^-6)

β4GalT5

Gene expression levels
(β4GalT5 mRNA/18S x 10^-6)

0  2.5  5.0

P  A

***
Fig. 4

Luciferase activity (arbitrary units)

-1980 -1000 -1

promoterless
pGL(-1980/-1)
pGL(-1513/-1)
pGL(-1048/-1)

Luciferase activity (arbitrary units)

-1980 -1000 -1

promoterless
pGL(-1980/-1)
pGL(-480/-1)
pGL(-299/-1)
pGL(-218/-1)
pGL(-132/-1)
**Fig. 5**

**A**

Gene expression levels (β4GalT5 mRNA/18S x 10^{-6})

<table>
<thead>
<tr>
<th>Days</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
</tbody>
</table>

**B**

Luciferase activity (arbitrary units)

<table>
<thead>
<tr>
<th>Days</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
</tbody>
</table>
Fig. 6

-299  -150  -1

pGL(-299/-1)

pGL-Sp1m1

pGL(-218/-1)

pGL-Sp1m2

pGL(-132/-1)

Sp1-binding site

Luciferase activity (arbitrary units)
Fig. 7

A

Gene expression levels (Sp1 mRNA/18S x 10^-5)

B

(kDa)

Sp1

β-actin

97

45

P

A

1.0

0.4

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