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Accelerated biosynthesis of neolacto-series glycosphingolipids in differentiated mouse embryonal carcinoma F9 cells detected by using dodecyl N-acetylglucosaminide as a saccharide primer

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Nao Ogasawara[1,2], Yohko U. Katagiri[1,*,†], Nobutaka Kiyokawa[1,†], Tomonori Kaneko[1,2], Ban Sato[1,†], Hideki Nakajima[1], Yoshitaka Miyagawa[1], Yasunori Kushi[3], Hideharu Ishida[4], Makoto Kiso[4], Hajime Okita[1,†], Toshinori Sato[2] and Junichiro Fujimoto[5,†]

[1]Department of Pediatric Hematology and Oncology Research, National Research Institute for Child Health and Development, Setagaya-ku, Tokyo 157-8535; [2]Department of Bioscience and Informatics, Keio University, Yokohama 223-8522; [3]Department of Materials and applied Chemistry, College of Science and Technology, Nihon University, Chiyoda-ku, Tokyo 101-8308; [4]Department of Applied and Bio-organic Chemistry, Gifu University, Gifu 501-1193; and [5]Director, Clinical Research Center, National Center for Child Health and Development, Setagaya-ku, Tokyo 157-8535, Japan

*Yohko U. Katagiri, Department of Pediatric Hematology and Oncology Research, National Research Institute for Child Health and Development, 2-10-1, Okura, Setagaya-ku, Tokyo 157-8535, Japan. Tel/Fax: +81 3 3417 2496, email: kata@nch.go.jp

[†]Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology Corporation (JST), Japan

Using dodecyl N-acetylglucosaminide (GlcNAc-C12) as a saccharide primer, we investigated the biosynthetic changes of neolacto-series glycosphingolipids (GSLs) in mouse embryonal carcinoma F9 cells during differentiation induced by retinoic acid plus dibutyryl cyclic AMP (RA/dbcAMP). In the differentiated cells, the glycosylation of GlcNAc-C12 was greatly enhanced. The sugar compositions of glycosylated primers were assigned as Hex-GlcNAc, $[Hex]_2$-GlcNAc, $[Hex]_2$[HexNAc]-GlcNAc, and [NeuAc][Hex]-GlcNAc by liquid chromatography-tandem mass spectrometry. The detection of augmented biosynthesis of endogenous sialylparagloboside indicated that [NeuAc][Hex]-GlcNAc was predicted to be the non-reducing end trisaccharide of sialylparagloboside. The transcription of B3gnt5, B4galt1, Ggta1, Fut4 and St3gal6, encoding glycosyltransferases involved in the neolacto-series glycosphingolipids biosynthesis, was increased, whereas that of Fut9 and St6galI was decreased after RA/dbcAMP treatment. Furthermore, the sialyltransferase activity of ST3GalVI sialylating paragloboside was enhanced with the increase in St3gal6 expression. Since most stage-specific embryonic antigen-1 (SSEA-1) active determinants are carried by glycoproteins in F9 cells, the changes in glycolipid metabolism do not seem to be closely related to loss of cell surface SSEA-1 expression upon F9 differentiation. These results indicate that RA/dbcAMP treatment activates the biosynthesis of neolacto-series GSL and enhances sialylation of paragloboside in F9 cells with down-regulation of Fut9 expression.

Keywords: F9/Fut9/GlcNAc-C12 primer/sialylparagloboside/St3gal6.

Abbreviations: C/M/W, chloroform/methanol/water; DMEM, Dulbecco's modified Eagle's medium; EC, embryonal carcinoma; EIC, extracted ion chromatogram; ES, embryonic stem; ESI, electro spray ionization; FBS, foetal bovine serum; −F9, undifferentiated F9 cells; +F9, differentiated F9 cells; GlcNAc-C12, dodecyl N-acetylglucosaminide; GSL, glycosphingolipid; HRP, horseradish-peroxidase; KSR, knockout serum replacement; LC–MS/MS, liquid chromatography-tandem mass spectrometry; Mab, monoclonal antibody; RA/dbcAMP, retinoic acid plus dibutyryl cyclic AMP; SPE, solid phase extraction; SSEA-1, stage-specific embryonic antigen-1; TEA, triethylamine.

The nomenclature for glycosphingolipids follows the recommendations (*21*) of the IUPAC-IUB, and the ganglioside nomenclature of Svennerholm (*22*) was used.

Mouse embryonal carcinoma (EC) F9 cells derived from an induced teratocarcinoma are considered a suitable model for studying the mechanism of embryogenesis because their morphological appearance and biochemical properties are similar to those of discrete populations of cells which arise during early embryogenesis, and have the ability to differentiate into primitive endoderm-like cells under treatment with all-trans-RA either alone (*1*) or with dbcAMP (*2*). For example, the monoclonal antibody (Mab) MC480 recognizing SSEA-1 was raised against F9 cells and the expression of SSEA-1 in embryos can specify the certain stages of early development (*3*). SSEA-1, the antigenic determinant of which is Galβ1,4(Fucα1,3)GlcNAcβ1-R (*4*) is intensely expressed in F9 cells, but disappears with their differentiation *in vitro*. Besides the disappearance of SSEA-1, the expression of several carbohydrate antigens dramatically changes during the *in vitro* differentiation of F9 cells. The expression of I/i (*5*) antigens and Forssman antigen (*6*) is also down-regulated significantly, whereas that of SSEA-3 is up-regulated in F9 cells treated with retinoic acid (RA) or RA plus dibutyryl cyclic AMP (dbcAMP) (*7*).

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Inokuchi and his colleagues reported that the overall expression of gangliosides was elevated in differentiated F9 cells (*8*). In this study, we focused on changes in the expression of neolacto-series GSLs during the *in vitro* differentiation of F9 cells. Here we report that the biosynthesis of neolacto-series GSLs was also enhanced in F9 cells treated with RA/dbcAMP using the GlcNAc-C12 primer, a recently developed saccharide primer to synthesize neolacto-series oligosaccharides in cultured mammalian cells (*9*), and that the expression of the genes responsible for the synthesis of neolaco-series GSLs was up-regulated, resulting in elevated levels of sialylparagloboside.

Materials and Methods

Cell culture and antibodies

F9 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (Ibaraki-shi, Osaka-hu, Japan) and were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma Chem, St Louis, MO, USA) containing 10% foetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA). The cells were induced to differentiate into primitive endoderm cells by the addition of 10^{-7} M RA (Wako Pure Chem. Ind., Osaka, Japan) plus 10^{-3} M dbcAMP (Aldrich, Milwaukee, WI, USA) for 4 days. The anti-SSEA-1 Mab and both the anti-Dab2 Mab and anti-Oct3 Mab were purchased from Chemicon International (Temecula, CA, USA) and BD Biosciences (San Jose, CA, USA), respectively. The anti-sialylparagloboside Mab SPS established by Watarai *et al.* (*10*, *11*) was used. The FITC-conjugated goat anti-mouse IgG + M antibody and horseradish-peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin antibody were purchased from Jackson Laboratory (West Grove, PA, USA) and DAKO A/S (Denmark), respectively.

Western analysis

Cells were homogenized in a lysis buffer (250 mM NaCl, 50 mM HEPES, pH 7.4 and 0.1% NP-40) by passing them through a 23-gauge needle and the homogenate was centrifuged at 15,000 rpm for 20 min at 4°C. The supernatant, the protein concentration of which was determined with a Bio-Rad Protein Assay (Bio-Rad Laboratories Inc., Hercules, CA, USA), was stored frozen prior to use. Ten micrograms of protein was separated by SDS–PAGE and transferred to a PVDF membrane (Millipore Corp., Bedford, MA, USA). Non-specific binding sites were blocked with 5% skim milk in PBS. The blots were then incubated with the primary antibodies (diluted 1 : 1000) for 1.5 h, followed by HRP-conjugated goat anti-mouse immunoglobulin antibody (1 : 1000) for 1 h. The antibodies that bound to the membrane were visualized with the ECL Plus Western Blotting Detection system (GE Healthcare UK Ltd, Buckinghamshire, UK).

Flow cytometry

Cells were harvested and incubated with anti-SSEA-1 Mab for 1 h on ice. After being washed, the cells were incubated with FITC-conjugated goat anti-mouse $IgG^{+}M$ (diluted 1 : 50) and *analyse*d by flow cytometry (EPICS-XL, Beckmann-Coulter, Fullerton, CA, USA).

Incubation of F9 cells with the GlcNAc-C12 primer

The GlcNAc-C12 primer was prepared as described (*9*). Cells were incubated with the primer as reported by Yamagata and colleagues (*12*, *13*). Briefly, 2×10^6 differentiated F9 cells (+F9) or undifferentiated F9 cells (−F9) were seeded into a 100-mm culture dish and cultured for 18 h. The cultured cells were washed with DMEM (Gibco, Grand Island, NY, USA) supplemented with D-(+)-glucose (Sigma) and L-glutamine (Gibco) to remove FBS, and then further incubated with 50 mM of GlcNAc-C12 primer in DMEM (Gibco) supplemented with 10% knockout serum replacement (KSR) (Gibco)/D-(+)-glucose/L-glutamine for 24 h. The glycosylated products secreted into the culture medium were collected with a solid phase extraction (SPE) cartridge, OASIS (Waters, Milford, MA, USA). The water-soluble compounds were removed with water, and the lipids were eluted with methanol. The eluates containing the glycosylated products were evapourated and dissolved in methanol for TLC analysis. For the analysis by liquid chromatography–tandem mass spectrometry (LC–MS/MS), the glycosylated products as obtained above were dissolved in 1 ml of chloroform/methanol (C/M) (19/1, v/v) and adsorbed to an aminopropyl SPE cartridge, Discovery DSC-NH2 (SUPELCO, Bellefonte, PA, USA). After washing of the cartridge with chloroform, the products were eluted with 3% acetic acid/4% triethylamine (TEA) in methanol, and then with methanol. The eluates were passed through a cellulose acetate membrane filter, Cosmospin Filter H (pore size; 0.45 mm) (Nakalai Tesque Inc., Kyoto, Japan), evaporated, and dissolved in C/M (9 : 1).

LC–MS/MS

Mass spectrometric analyses were performed using Electrospray ionization (ESI)/an ion trap type mass spectrometer (HCT-Ultra 11S, Bruker Daltonics, Billerica, MA, USA), coupled online by the Agilent 1,100 series Capillary LC system (Agilent Technologies, Wilmington, DE, USA) with ion extraction, separation and detection in both the positive and the negative ion modes. The glycosylated primers were loaded onto a 0.3 × 150 mm silica column (Imtakt, Japan) equilibrated with solvent A [chloroform/methanol/water (C/M/W) containing 50 mM TEA acetate buffer, pH 4.2 (83/16/1, v/v/v)] and eluted with a 0–100% linear gradient of solvent B [M/W containing 50 mM TEA acetate buffer, pH 4.2, (3/1, v/v)] in 45 min at a flow rate of 3 µl/min. The ESI parameters were as follows: nebulizer, 15.0 psi; dry gas (N_2), 6.0 l/min; dry temperature, 320°C; capillary, −4 kV for negative ion mode and 3.5 kV for positive ion mode. The mass recorded in survey acquisitions ranged from 150 to 3,000 m/z.

The endogenous acidic glycolipids were loaded onto a 0.3 × 150 mm NH2 column (NH2P50-M3D, Shodex, Japan) equilibrated with solvent C [5% acetic acid/3% TEA in water], and eluted with a 5–90% gradient of solvent D [2% acetic acid in acetonitrile] in 90 min at a flow rate of 3 µl/min. The mass recorded in survey acquisitions ranged from 150 to 3,000 m/z.

Glycolipid analysis

Lipids were extracted from the packed cell pellets with 2 ml of C/M (2/1, v/v) and then with 2 ml of C/M/W (7/11/2, v/v). Total extracts were combined and evaporated to dryness and then treated with 0.2 M KOH in methanol at 37°C for 2 h to saponify the phospholipids. The extracts were concentrated to 1/10 volume and mixed with 3 ml of water. Fatty acids were removed by mixing with hexane, and the solution was applied to a reverse-phase cartridge, Sep-Pak C18 (Waters). The cartridge was washed with 10 ml of water, and the lipids were eluted with 5 ml of methanol. The eluates were evapourated to dryness. To remove sialic acid residues, lipids were treated with 0.1 U of sialidase from *Clostridium perfringens* (Roche Applied Science, Mannheim Germany) in 50 mM sodium acetate buffer, pH 5.0 for 24 h at 37°C. Desialylated products were isolated using a Sep-Pak C18 cartridge.

TLC and TLC immunostaining

Glycolipids were separated on glass plates pre-coated with Silica Gel 60 (HPTLC plate, Merck, Darmstadt). The developing solvent system was a mixture of C/M/0.2% aqueous $CaCl_2$ (5 : 4 : 1, v/v/v). TLC immunostaining was performed according to a previously described method (*14*). The plates were probed with Mab SPS-20 or anti-SSEA-1 Mab for 2 h, and then the HRP-conjugated rabbit anti-mouse immunoglobulins antibody (diluted 1 : 1000 in 1% BSA/PBS) for 1 h. The antibodies that bound to the plates were visualized with ECL Plus Western Blotting Detection system. NeuAcα2,3-linked paragloboside (IV^3NeuAcα-nLc_4Cer) and NeuAcα2,6-linked paragloboside (IV^6NeuAcα-nLc_4Cer) purified from human O-type erythrocytes and human meconium, respectively, were used as control glycosphingolipids. III^3Fucα-nLc_4Cer was obtained by desialylation of sialylLewis X purchased from Wako Pure Chem. Industries Ltd. (Osaka, Japan).

Real-time RT–PCR

Total RNA was isolated from cultured cells using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. cDNA was synthesized from 5 µg

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of total RNA using a First-Strand cDNA Synthesis Kit (GE Healthcare Bio-Science Corp.). Real-time PCR was performed in 20 μl of a mixture consisting of 10 μl of SYBER Green Master mix (Applied Biosystems, CA, USA) and template cDNA using the ABI Prism 7900HT sequence detection systems. The levels of β-actin transcript were used for normalization of cDNA levels. Primer sequences for the selected genes encoding neolacto-series GSL synthases and β-actin are listed in Table I.

Paragloboside (neolactotetraosylceramide) α2,3sialyltransferase assay

The sialyltransferase assay was performed in a reaction mixture containing 5 mM $MgCl_2$, 0.3% (w/v) Triton CF-54, 100 mM sodium cacodylate buffer, pH 6.0, 3.9 mM CMP-NeuAc (Sigma), 3.7 kBq CMP-[^{14}C]NeuAc (NEC), 50 μg of cell lysates as the enzyme source, and 23.5 nmol of paragloboside analogue as an acceptor in a total volume of 50 μl. Paragloboside analogue was prepared by sialidase digestion of α2,3sialylparagloboside mimetic conjugate, which was synthesized by conjugation of NeuAcα2,3Galβ1,4GlcNAcβ1, 3Galβ1,4Glc (*15*) to 3-branched fatty-alkanol as a ceramide mimetic (*16*), according to the procedure previously described (*17*). The reaction mixture was incubated at 37°C for 3.5 h. The products were isolated using a Discovery DSC-18 cartridge (SUPELCO) and separated by TLC. The plate was exposed to an imaging plate for 3 days and the radioactivity on the plate was visualized with a BAS 2000 image analyser (Fuji film, Tokyo, Japan).

Results

Effect of RA/dcAMP treatment on in vitro *differentiation of F9 cells*

To ascertain that RA/dcAMP treatment induces the differentiation of F9 cells in our culture system, we tested the expression of differentiation-related molecules during the culture in the presence of RA/dbcAMP. Western analysis revealed that a marker of differentiation, Dab2, appeared in F9 cells on Day 2 and increased on Day 4, while a marker of undifferentiation, Oct3, decreased on Day 2 and completely disappeared on Day 4 (Fig. 1A). Flow cytometric analysis showed that the expression of SSEA-1 in F9 cells dramatically decreased over 4 days in the presence of RA/dbcAMP (Fig. 1B). The results indicate that RA/dbcAMP treatment can induce the differentiation of F9 cells in our culture system.

GlcNAc-C12 does not affect RA/dbcAMP-mediated differentiation of F9 cells

The −F9 and +F9 cells were further cultured in the absence or presence of 50 mM GlcNAc-C12 for 24 h. No apparent difference in morphology in the presence and absence of GlcNAc-C12 in the culture was observed in −F9 or +F9 cells (Fig. 2A). The presence of GlcNAc-C12 affected neither the appearance of Dab2 nor disappearance of Oct3 induced by RA/dbcAMP (Fig. 2B). The results clearly show that GlcNAc-C12 does not influence the differentiation of F9 cells mediated by RA/dcAMP treatment.

The glycosylation of GlcNAc-C12 primer in F9 cells

The products elongated from the GlcNAc-C12 primer were recovered from −F9 and +F9 cells, and separated and detected by HPTLC with the orcinol or resorcinol reagent. Only a small part of the primer recovered from the −F9 cells was glycosylated, whereas a large quantity of the primer recovered from the +F9 cells was significantly glycosylated (indicated as G1, G2, G3 and G4 in Fig. 3A left panel). Since G4 was stained to

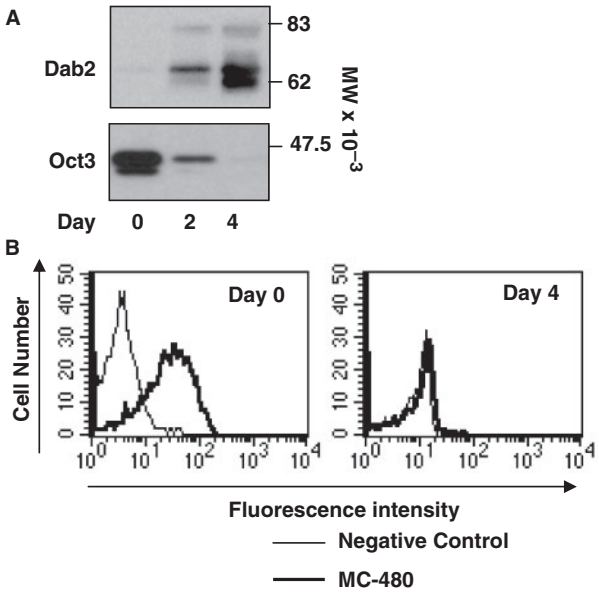

Fig. 1 Effect of treatment with RA/dcAMP on the expression of differentiation markers in F9 cells. (A) The change in expression of Dab2 and Oct3 in F9 cells during the culture in the presence of RA/dbcAMP. Ten micrograms of proteins was separated by SDS–PAGE and transferred onto a PVDF membrane. The blot was cut off between the 47.5 k and 62 k molecular weight markers. The upper and the lower blot were probed with anti-Dab2 and anti-Oct3 Mab, respectively. (B) Histogram of SSEA-1 expression detected by MC-480 in F9 ells. The expression of SSEA-1 in F9 cells cultured on Day 0 (left) and Day 4 (right) in the presence of RA/dbcAMP was analysed by flow cytometry.

Table I. Selected genes and corresponding primers for real-time RT-PCR.

Name	Gene bank number	Forward primer	Reverse primer
B3gnt5	NM_054052	aaaggaaagatagaacctagggta	agacttttattgcttctttgtcttcc
B4galt1	NM_022305	tcgactatggcatctacgtca	gagcagcttagctcgattgaa
B4galt2	NM_017377	caatggctttcccaacga	cgtgagatcttcatcccagtc
B4galt3	NM_020579	agcttgcgtacggcatttat	tttgccctgttaaacgttcc
B4galt4	NM_019804	tcaaggtgaatggattctctaaca	ttttcattttatggagctcaacc
St3gal6	NM_018784	aagccagctttcgccaat	agctctgcacagaaatgggta
St6gal1	NM_145933	gtgtgccgtcgtgtcttct	ttaaacctcaggaccgcatc
Fut9	NM_010243	acctgtacccggctgctat	gatggagatgatcaccgagag
Fut4	NM_010242	caagcaatcttcccactcatt	tcgccatttatgtctcttcca
Ggta1	NM_010283	gaagacctcgcgctctca	gagcttctgtcgagctctgg
Actb	NM_007393	aaggccaaccgtgaaaagat	gtggtacgaccagaggcatac

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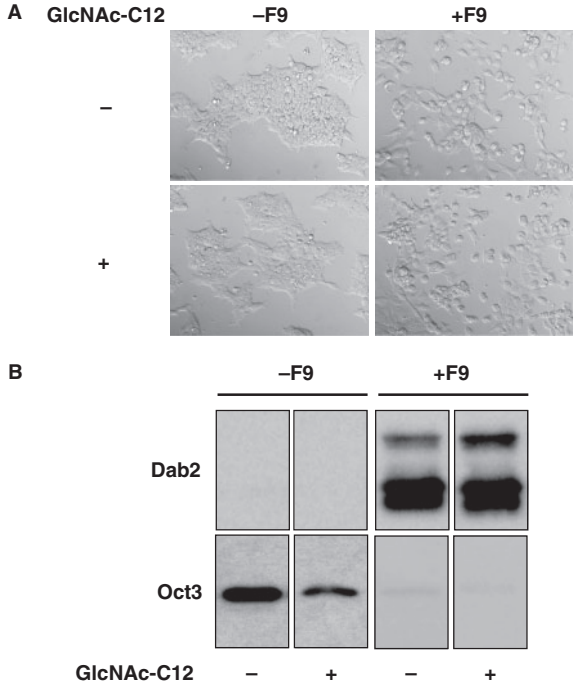

Fig. 2 Effect of GlcNAc-C12 on differentiation of F9 cells. (A) Differential interference contrast image of −F9 and +F9 cells. −F9 and +F9 cells (2.0×10^6) were seeded into 100-mm culture dishes and incubated in the absence (−) or presence (+) of 50 mM GlcNAc-C12 for 24 h. (B) Dab2 and Oct3 expression in −F9 and +F9 cells. See the legend for Fig. 1.

typical blue-purple colour like GM1 and the others were non-specifically stained to non-purple colour with resorcinol reagent, only G4 was thought to be sialylated (Fig. 3A right panel).

A structural analysis of these glycosylated products was performed by LC–MS/MS. The observed mass and the predicted composition of the products are listed in Table II. Figure 3B shows extracted ion chromatogram (EIC) of m/z at 653.8, 815.9 1,019.0 and 945.0 as $[M + TEA + H]^+$ obtained from GlcNAc-C12 products and Fig. 3C shows the LC-ESI MS/MS spectra from the ion pointed by the arrow in each EIC. LC–MS/MS analysis of the other ions did not demonstrate the presence of the characteristic GlcNAc-C12 product fragmentation pattern such as [Hex][GlcNAc] (data not shown). The precursor ion pointed by the arrow in EIC of m/z 653.8 was assigned as Hex-GlcNAc-C12 (G1). That of m/z 815.9 was assigned as Hex-Hex-GlcNAc-C12 (G2) due to the presence of a peak at m/z 366.1 corresponding to Y_2/B_3 $\{[(Hex + anHexNAc) + H]^+\}$. Since the informative data for structural analysis of that of m/z 1.019 could not be obtained from the MS/MS spectrum of $[Hex]_2[HexNAc]_1$GlcNAc-C12 (G3), the definitive branching site of HexNAc could not be deduced. The most probable structure of G3 is shown in Fig. 3C. That of m/z 945.0 was assigned as NeuAc-Hex-GlcNAc-C12 (G4) due to the presence of a peak at m/z 454.1 corresponding to B_2 $\{[(NeuAc + anHex) + H]^+\}$. Since GlcNAc-C12 can be used as a primer for the elongation of lacto/neolacto-series GSLs, the carbohydrate moiety of Hex-Hex-GlcNAc-C12 and NeuAc-Hex-GlcNAc-C12 was thought to be the non-reducing end trisaccharide of $IV^3Gal\alpha$–Lc_4/nLc_4Cer (neolactotetraosylceramide) and $IV^{3/6}NeuAc\alpha$-Lc_4/nLc_4Cer, respectively. Peak area of the EICs of m/z 550.3, 712.4, 915.5 and 841.5 of −F9 and +F9 corresponding to $[M-H]^-$ of G1, G2, G3 and G4, respectively, were measured and normalized with the peak area of m/z 236.9 corresponding to isopropyl-β-D-thiogalactopyranoside, which was simultaneously run as an internal standard. The production of G1, G2, G3 and G4 increased 3.6, 2.2, 4.2 and 8.2-fold after differentiation, respectively (Fig. 3C).

Increase in biosynthesis of endogenous sialylparagloboside in RA/dbcAMP-treated F9 cells

We examined whether RA/dbcAMP treatment enhances the biosynthesis of endogenous sialylparagloboside in F9 cells by LC–MS/MS. The EIC of m/z 1627.0, which corresponds to $[NeuAc]_1[Hex]_3[HexNAc]_1$-Ceramide ($[M-H]^-$), in the negative ion mode, was composed of Peak 1 at 18 min, Peak 2 at 26 min and Peak 3 at 27 min (Fig. 4A). All three peaks were much higher in +F9 cells than −F9 cells. Peak 1 of m/z 1627.0 was assigned as GM1 due to the presence of a product ion at m/z 1261.9 corresponding to [(NeuAc-Hex-Hex-Cer)-H]$^-$, which was not found in the MS/MS spectra of peaks 2 and 3 (data not shown). The MS/MS spectrum for peak 2 is shown in Fig. 4A. Peak 2 was assigned as NeuAc-Hex-HexNAc-Hex-Hex-Cer due to the presence of fragment ions at m/z 1335.8 (Y_4 {(Hex-HexNAc-Hex-Hex-Cer)-H]$^-$}, 1173.7 {Y_3, [(HexNAc-Hex-Hex-Cer)-H]$^-$}, 970.5 {Y_2, [Hex-Hex-Cer]-H)$^-$}, 808.6 {Y_1, [(Hex-Cer)-H]$^-$}, 790.5 {Y_1-H_2O, [(Hex-H_2O-Cer)-H]$^-$ and 646.6 (Y_0, [Cer-H]$^-$}, and deduced to be sialylparagloboside ($IV^{3/6}NeuAc\alpha$-nLc_4Cer), sialyllactotetraosylceramide ($IV^{3/6}NeuAc\alpha$-Lc_4Cer) or GM1b. The MS/MS spectrum of peak 3 also revealed peaks at m/z 1335.8 [(Hex-HexNAc-Hex-Hex-Cer)-H]$^-$, 1173.7 [(HexNAc-Hex-Hex-Cer)-H]$^-$, 970.5 [(Hex-Hex-Cer)-H]$^-$ and 646.6 [Cer-H]$^-$, indicating that peak 3 is similar, but not the same to peak 2 (data not shown).

In order to obtain conclusive evidence for the endogenous expression of sialylparagloboside, we performed TLC immunostaining of mock-incubated and sialidase-digested F9 GSLs with Mab SPS-20. SPS-20 reacted to α2,3linked (Fig. 5 lane 1), and not to α2,6linked sialylparagloboside (lane 2). A small amount of $IV^3NeuAc\alpha$–nLc_4Cer, which could not be removed through purification, was found in lane 2. No detection of GM3 in lane 4 of the orcinol-stained panel indicates the successful digestion of gangliosides with *Clostridium perfringens* sialidase. SPS-20 bound to a slow migrating doublet band with an Rf value similar to GM1 (lane 7, 9), and the binding was greatly diminished by treatment with *C. perfringens* sialidase (lane 6, 8). The intensity of the band of +F9 cells (lane 9) was higher than that of −F9 cells (lane 7). Since F9 cells express GSLs at m/z 1516.8 (d18 : 1/16 : 0) besides 1627 (d18 : 1/24 : 1) corresponding to $[NeuAc]_1[Hex]_3[HexNAc]_1$-Ceramide ($[M-H]^-$) in the negative ion mode (data not shown), the doublet band might be attributed to divergence of ceramide part. These results indicate that F9 cells certainly

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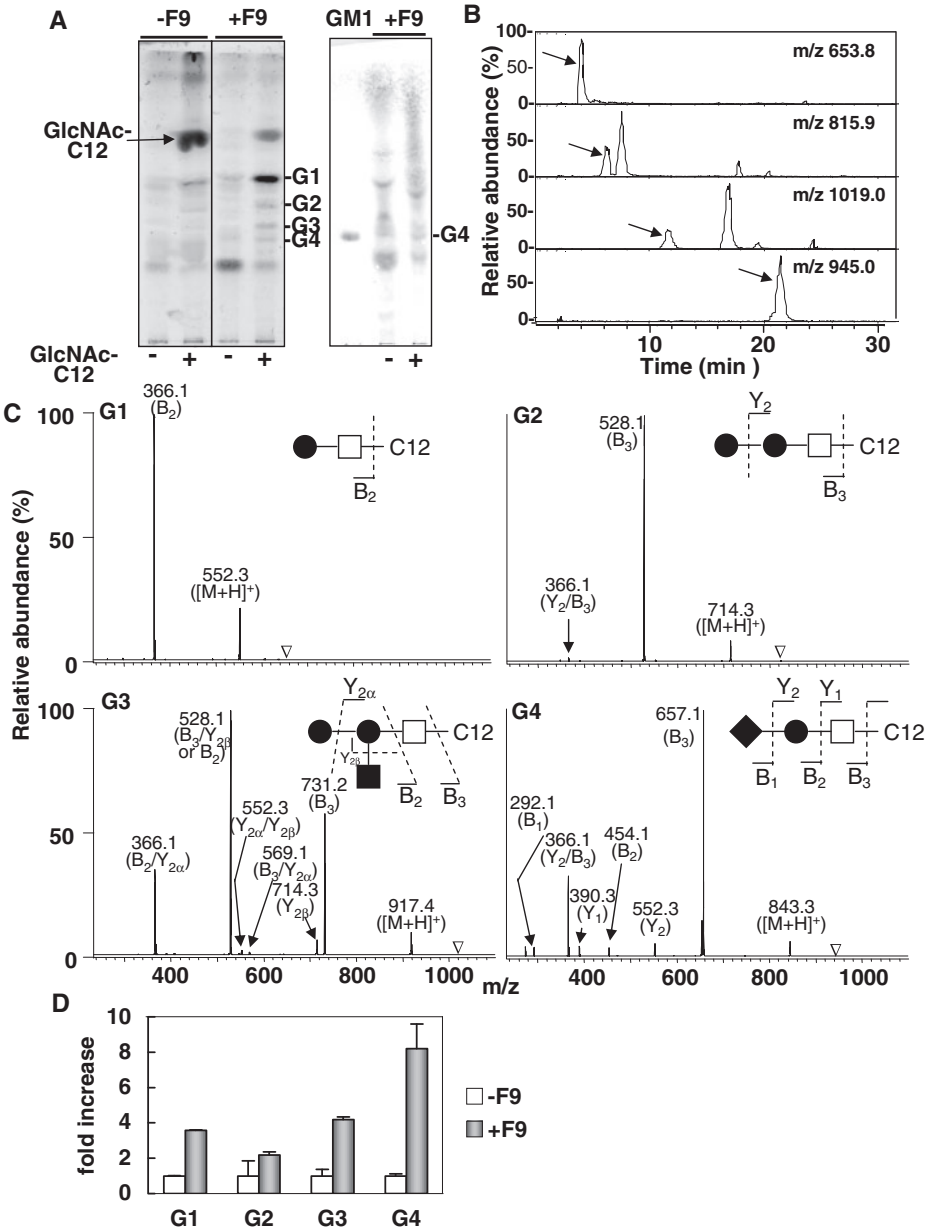

Fig. 3 Analysis of the glycosylated products derived from GlcNAc-C12. (A) HPTLC of the glycosylated products produced by −F9 and +F9. The lipids recovered from 0.58 to 2.3 mg of protein of cells were separated on a TLC plate, and visualized by spraying with the orcinol (left) and resorcinol (right) reagent, respectively. (B) EICs of m/z at 653.8, 815.9, 1019.0 and 945.0 in the positive ion mode. (C) LC-ESI MS/MS spectra of $[M+TEA+H]^+$of G1 (m/z 653.8), G2 (m/z 815.9), G3 (1019.0 m/z) and G4 (m/z 945.0) in the positive ion mode. An open arrowhead indicates $[M+TEA+H]^+$. Open square, GlcNAc; filled circle, Hex; filled square, HexNAc; filled rhombus, NeuAc. (D) Relative amount of GlcNAc-C12 primer products. The peak area of each EIC of G1, G2, G3 and G4 was normalized to that of isopropyl-D-thiogalactopyranoside (0.4 nmol) and the amount of the products produced by −F9 (open column) and +F9 (shaded column) cells were compared.

express sialylparagloboside, and that the expression is augmented with differentiation.

Elevation of mRNA levels of glycosyltransferases involved in the neolacto-series GSL path way in RA/dbcAMP-treated F9 cells

Next, we examined whether the expression of genes involved in the biosynthesis of neolacto-series GSLs is up-regulated in F9 cells after RA/dbcAMP-treatment using real-time RT–PCR (Fig. 6A). The expression of the gene (B3gnt5) encoding β-1,3-N-acetylglucosaminyltransferase 5, *i.e.* lactotriaosylceramide (Lc_3Cer) synthase, which plays a key role in the synthesis of lacto/neolacto-series carbohydrate chains on GSLs, was increased 4-fold (Fig. 6B upper). In the neolacto-series biosynthetic cascade, we examined four genes (B4galt1–4) responsible for the formation of the Gal β1,4GlcNAc linkage. All the genes except B4galt2 were increased and the increase in B4galt1 was most prominent, 8-fold (Fig. 6B middle). The up-regulation of B3gnt5 and B4galt1 expression indicates augmentation of the biosynthesis of neolacto-series GSLs in +F9 cells. The expression of Fut9, esponsible for the fucosylation of paragloboside to produce SSEA-1, and that

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of St6gal1, responsible for α2,6sialylation of paragloboside, was decreased to 1/10 and 1/2, respectively (Fig. 6B lower, right). Fut4 responsible for the fucosylation of LacNAc-R on glycoproteins to produce SSEA-1 was increased 5-fold (data not shown). The expression of St3gal6 gene encoding α2,3sialyltransferase VI for synthesizing IV^3NeuAcα-nLc_4Cer and Ggta1 encoding α1,3galactosyltransferase for synthesizing IV^3Galα-nLc_4Cer was increased 18- and 17-fold, respectively (lower left).

Table II. Deduced composition and mass observed by LC–MS/MS for the glycosylated products from GlcNAc-C12.

Name	Composition	Observed Mass
G1	$(Hex)_1$ GlcNAc-C12	653.8 $[M+TEA+H]^+$
G2	$(Hex)_2$ GlcNAc-C12	815.9 $[M+TEA+H]^+$
G3	$(Hex)_2$ $(HexNAc)_1$ GlcNAc-C12	1019.0 $[M+TEA+H]^+$
G4	$(NeuAc)_1$ $(Hex)_1$ GlcNAc-C12	945.0 $[M+TEA+H]^+$

Increase in α2,3Sialyltransferase activity towards paragloboside in RA/dbcAMP-treated F9 cells

Since the increased production of IV^3NeuAcα-nLc_4Cer seems to be due to the up-regulation of α2,3sialyltransferase (ST3GalVI), we next examined the changes in activity of ST3GalVI during differentiation. The extracts of −F9 and +F9 were assayed for

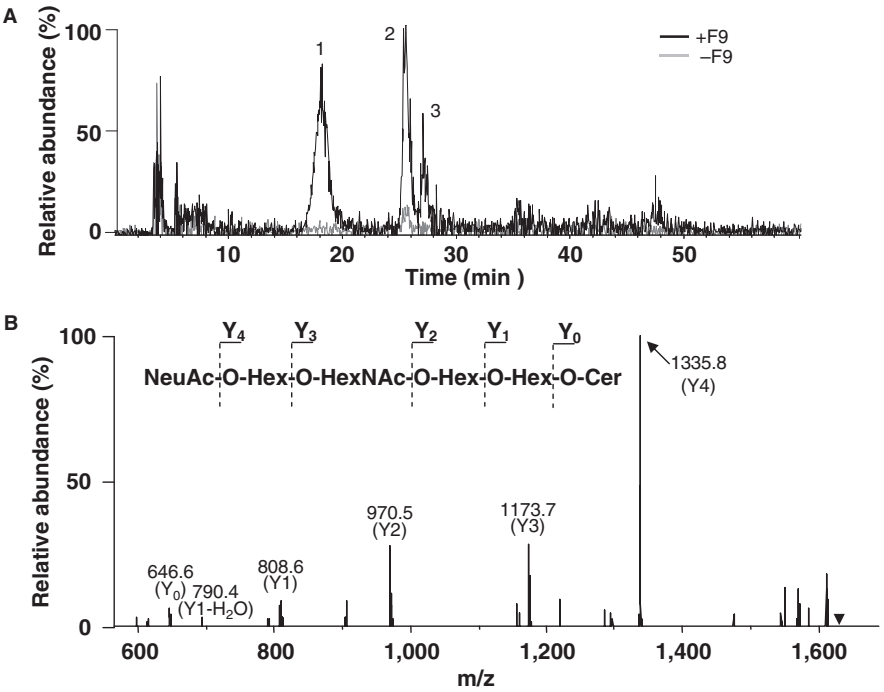

Fig. 4 Analysis of endogenous glycolipids using LC–MS. (A) Overlay of EIC at m/z 1,627.0 of the endogeneous glycolipids produced by −F9 (black) and +F9 (grey) cells. Lipids prepared from the same amount of protein were subjected to analysis. (B) LC-ESI MS/MS spectrum of $[M-H]^-$ (m/z 1,627) in the negative ion mode acquired from peak 2.

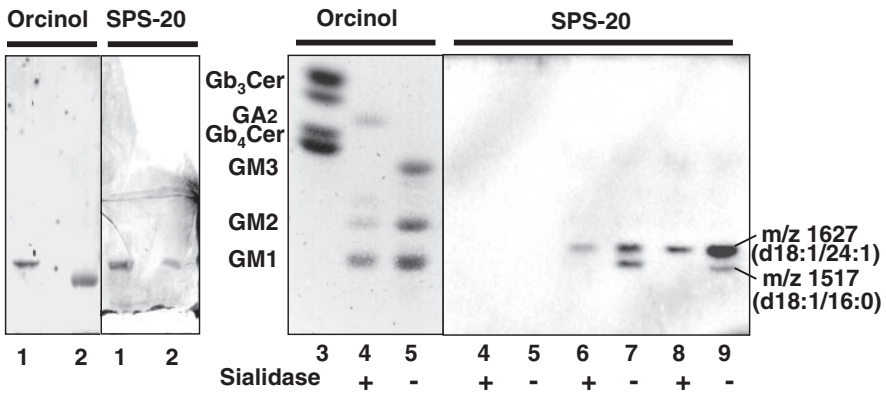

Fig. 5 TLC immunostaining of GSLs produced by −F9 and +F9 cells with anti-sialylparagloboside Mab SPS-20. α2,3-Linked sialylparagloboside (lane 1) and α2,6-linked sialylparagloboside (lane 2) separated by HPTLC were stained with orcinol reagent (left panel) and immunostained with SPS-20 (right panel). GMs (GM3, GM2 and GM1) and GSLs extracted from 4.8 mg of protein of −F9 and +F9 cells were incubated with *C. Perfringens* sialidase (lane 4, 6 and 8) or mock-incubated (5, 7 and 9), and separated by HPTLC. Standard GSLs (left panel) were visualized by spraying with orcinol reagent. The plate (right panel) was immunostained with SPS-20. Lane 1: IV^3NeuAcα-nLc_4Cer; Lane 2: IV^6NeuAcα-nLc_4Cer; Lane 3: Gb_3Cer and Gb_4Cer; Lane 4, 5: GM1, GM2 and GM3; Lane 6, 7: −F9; Lane 8, 9: +F9.

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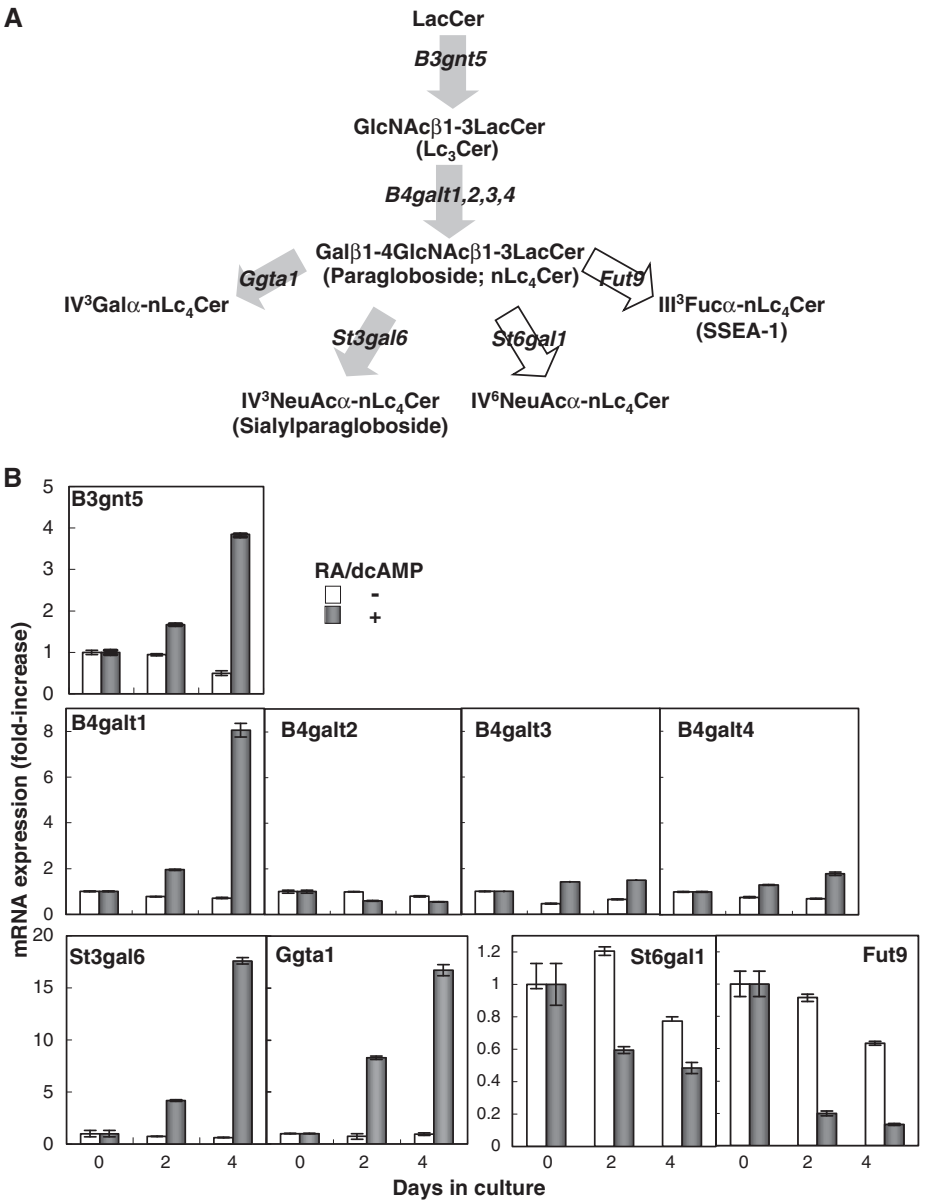

Fig. 6 Real-time RT–PCR analysis of glycosyltransferases during differentiation in F9 cells. (A) Map of genes examined for real-time RT–PCR. Grey solid arrow:up-regulated, open arrow:down-regulated. (B) mRNA expression of glycosyltransferases during differentiation in F9 cells. The mean and SD of three determinations are shown.

sialyltransferase activity using CMP-[^{14}C]NeuAc as a donor and paragloboside mimetic conjugates as acceptors. As shown in Fig. 7A, the lysate of +F9 (lane 6) sialylated the paragloboside analogue and the products co-migrated with the α2,3sialylparagloboside analogue (lane 3), whereas that of −F9 (lane 5) sialylated the paragloboside analogue only a little. The amount of [^{14}C]NeuAc incorporated by +F9 lysates was 10.6-times that incorporated by −F9 cells (Fig. 7B).

Expression of SSEA-1 epitope on glycoproteins and elevation of Fut4 gene expression in F9 cells

Next, we examined whether SSEA-1 epitope is carried by glycoproteins or glycolipids in F9 cells. As presented in Fig. 8A, Western analysis clearly detected SSEA-1 in F9 cells with the 40%-decreased expression after *in vitro* differentiation (Fig. 8A), whereas no SSEA-1^{+} signals by TLC immunostaining of endogenous GSL (Fig. 8B) was observed, indicating that SSEA-1 epitope is mainly carried by glycoproteins, but not glycolipids. We further examined mRNA expression of Fut4 that is responsible for biosynthesis of SSEA-1 epitope on glycoproteins in F9 cells and observed 5-fold-increased transcription of Fut4 in F9 cells after RA/dbcAMP-treatment (Fig. 8C).

Discussion

RA/dbcAMP activates cell metabolism, including protein synthesis and glycosylation, in F9 cells and causes them to differentiate into primitive endodermal like cells. Previously, Sato *et al.* (*8*) reported up-regulation of the expression of genes responsible for the synthesis

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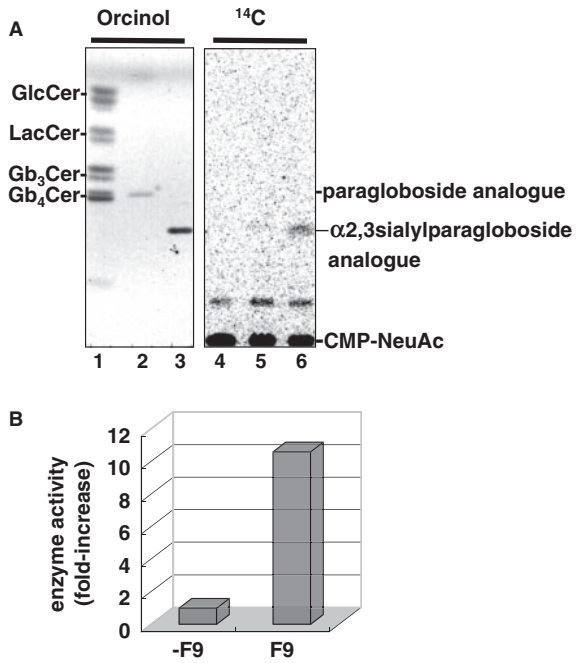

Fig. 7 α2,3-Sialylransferase activity towards paragloboside with −F9 and +F9 cell lysates. Assays were conducted as described in 'Materials and Methods' section. (A) Left panel: orcinol staining of standard glycolipids of neutral GSLs (lane 1), a paragloboside analogue (lane 2), and an α2,3-sialylparagloboside analogue (lane 3), right panel: radioactive bands of the products isolated from the reaction mixture of a lysis buffer (lane 4), the lysates of −F9 (lane 5) and +F9 (lane 6). (B) quantification of radioactive bands corresponding to α2,3-sialylparagloboside of lane 5 (−F9) and lane 6 (+F9) by Bio-Imaging Analyzer BAS2000.

of glycosphingolipids, except Gb3Cer and GlcCer, using their customized cDNA array focusing on genes responsible for the regulation of GSL metabolism, with a major increase in ganglioside content in differentiated F9 cells. Consistent with their observations, using the GlcNAc-C12 primer, we have detected elevated levels of lacto/neolacto-series GSLs, including sialylparagloboside, in F9 cells during RA/dbcAMP-mediated differentiation in this study. The enhanced biosynthesis of endogenous sialylparagloboside has also been confirmed. In parallel, real-time RT–PCR showed an up-regulation of the gene expression for synthases of Lc_3Cer, nLc_4Cer, $IV^3NeuAc\alpha$-nLc_4Cer and $IV^3Gal\alpha$-nLc_4Cer. Furthermore, we confirmed the augmentation of the sialyltransferase activity of ST3GalVI responsible for the synthesis of sialylparagloboside in RA/dbcAMP-treated F9 cells. While most genes encoding glycosphingolipid synthases are up-regulated, the Fut9 and St6gal1 genes responsible for the synthesis of SSEA-1 and $IV^6NeuAc\alpha$-nLc_4Cer, respectively, are down-regulated. We summarized the up-regulated (grey solid arrow) and down-regulated (open arrow) expression of genes encoding neolacto-series glycosyltransferase in F9 cells differentiated by RA/dbcAMP treatment in Fig. 6A. Based on the above observations, we concluded that RA/dbcAMP treatment activates the biosynthetic pathway producing neolacto-series GSLs and enhances sialylation and galactosylation of paragloboside in F9 cells in the absence of Fut9 and St6gal1 up-regulation, resulting in an increase of sialylparagloboside.

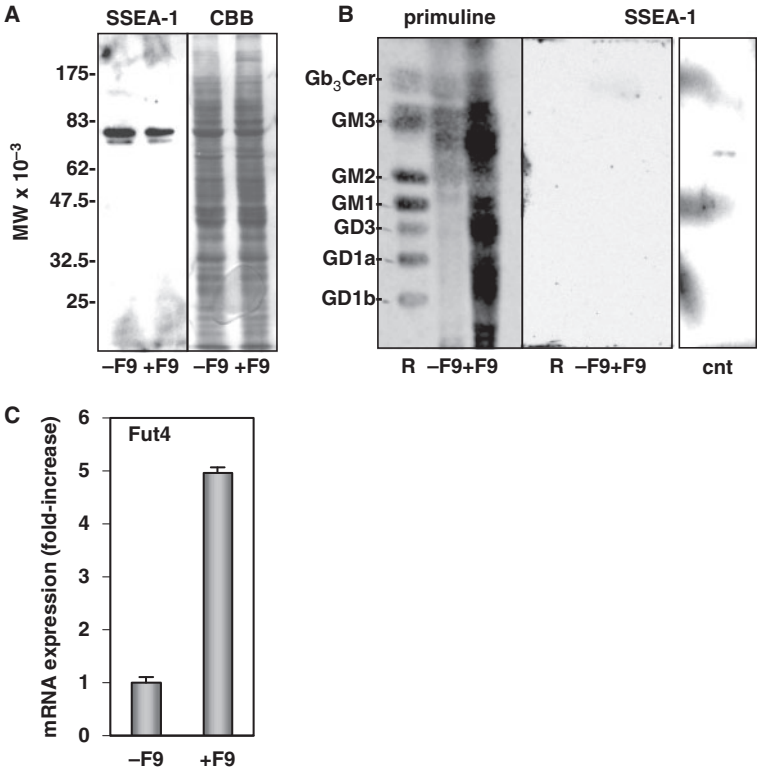

Fig. 8 Detection of SSEA-1 carried by glycoprotein and expression of Fut4 gene in −F9 and +F9 cells. (A) Western analysis using anti-SSEA-1 Mab was performed as in Fig. 1a. The blot was stained with CBB after immunostaining. (B) The HPTLC plate was sprayed with primuline reagent, and then immunostained with anti-SSEA-1 Mab as in Fig. 5. $III^3Fuc\alpha$-nLc_4Cer (designated 'cnt') was used as positive control for staining of SSEA-1. (C) Real-time RT–PCR analysis of Fut4 was performed as in Fig. 6b.

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As we presented in Fig. 7, a single band of [^{14}C]NeuAc-incorporated product was found at the same position to α2,3-sialylparagloboside analogue, which migrates a little bit faster than α2,6-sialylparagloboside analogue, indicating that the sialyltransferase activated in +F9 cells transfers NeuAc via α2,3-linkage, and not α2,6-linkage. Furthermore, since NeuAc-NeuAc-Hex-GlcNAc-C12, of which the internal NeuAc might be α2,3-linked to Hex-GlcNAc-C12, was detected in the primer products of +F9 (data not shown), the augmented production of IV^{3}NeuAcα-nLc$_4$Cer is more probable than that of IV^{6}NeuAcα-nLc$_4$Cer in +F9 cells.

In mice, two functional α1,3 fucosyltransferase genes, Fut4 and Fut9, responsible for SSEA-1 biosynthesis are known to be present (*18*, *19*) and Fut9 exhibited >100-fold strong activity for glycolipid acceptors than that of Fut4 (*20*). As we presented in this study, SSEA-1 was detected in F9 cells only by western immunostaining with the 40%-decreased expression after *in vitro* differentiation, whereas no SSEA-1$^+$ signals by TLC immunostaining of endogenous GSL was observed (Fig. 8). In addition, although the expression of Fut9 was decreased in F9 cells differentiated *in vitro*, Fut4 expression was rather increased after treatment with RA/dbcAMP. These results indicate that most SSEA-1 active determinants in F9 cells are carried by glycoproteins, and therefore any changes in glycolipid metabolism are not much related to loss of cell surface SSEA-1 expression upon F9 differentiation.

Although the precise role of glycosylation in early development is yet to be clarified, the expression of SSEA-1 has been thought to be important for compaction or interaction between blastomeres in preimplantation mouse embryos. It would be, therefore, interesting to examine whether sialylation or α-galactosylation of paragloboside after decreased expression of SSEA-1 is implicated in the differentiation into primitive endoderm.

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Conflict of interest

None declared.

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