Supporting Information

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SI Materials and Methods

hESC Culture. The hESC lines HES5 and H9, purchased from ES Cell International and WiCell Research Institute (1), were maintained in a feeder-free culture on Matrigel-coated plates in mouse embryonic fibroblast (MEF)-conditioned medium. The basal medium consisted of 80% DMEM/F12, 20% Knockout Serum Replacement (Invitrogen), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, and 4 ng/mL FGF-2 (Invitrogen). Before use, the medium was conditioned on mitomycin-C (Sigma-Aldrich) inactivated MEFs for 24 h at a density of 1.2×10^5 cells/mL.

Differentiation of hESCs into Embryoid Body Outgrowth. To induce differentiation, hESCs were treated with 1 mg/mL dispase for approximately 30 min until the cells had completely detached from the plates. Then, the cell suspensions were transferred to conical tubes. After the cells had precipitated by gravity, the medium was removed, and the cells were washed twice with hESC media. Cells were transferred to an ultra-low-attachment cell culture flask (Corning) containing hESC medium without FGF-2. Then, the cells became embryoid bodies (EBs) after approximately 48 h. After EB formation, EBs were grown on gelatin-coated tissue culture dishes or flasks with culture medium consisting of 80% DMEM/F12, 20% FBS, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, and 0.5% penicillin and streptomycin for another 2 wk. The medium was changed every other day.

Antibodies Used in Flow Cytometric Analysis or Immunofluorescence Analysis. ***Flow cytometric analysis.*** For germ layer marker analysis, primary antibodies used were anti-nestin (mAb clone 196908; 1:3,000 dilution; R&D Systems), α-smooth muscle actin (mAb clone 1A4; 1:10,000 dilution; R&D Systems), and α-fetoprotein (mAb clone 189506; 1:1,000 dilution; R&D Systems). For hESC surface glycosphingolipid (GSL) analysis, primary antibodies used were anti-SSEA-3 (mAb clone MC-631; 1:100 dilution; Chemicon), SSEA-4 (mAb clone MC-813–70; 1:1,600 dilution; Chemicon), Globo H (mAb prepared from VK9 hybridoma provided by Dr. Philip Livingston, Memorial Sloan-Kettering Cancer Center, New York; 10 μg/mL), H type1 (mAb clone 17-206; 1:100 dilution; Abcam), GM1 (Vybrant Alexa Fluor 488 lipid raft labeling kit; Invitrogen), GM3 (mAb clone GMR6; 1:100 dilution; Seikagaku), GD3 (mAb clone MB3.6; 1:100 dilution; BD Pharmingen), and Oct3/4 (mAb clone C-10; 1:1,000 dilution; Santa Cruz Biotechnology). The secondary antibodies used were Alexa Fluor 488-conjugated goat anti-mouse IgG, goat anti-mouse IgM, goat anti-human IgG, and goat anti-human IgM (1:1,000 dilutions; Invitrogen). The negative isotype controls, depending on the species and subclasses of the primary antibodies used, were Rat IgM (for SSEA-3), mouse IgG3 (for SSEA-4, VK9, H type1, and GD3), IgG2b (for Oct3/4), or mouse IgM (for GM3), respectively (all from eBioscience).

Immunofluorescence analysis. For germ layer marker analysis, primary antibodies used were as described: anti-nestin (1:1,500 dilution), α-smooth muscle actin (1:4,000 dilution), and α-fetoprotein (1:400 dilution). For hESC surface GSL analysis, primary antibodies used were anti-SSEA-3 (1:50 dilution), SSEA-4 (1:1,600 dilution), Globo H (50 μg/mL), and Oct3/4 (mAb clone C-10; 1:100 dilution; Santa Cruz Biotechnology). The secondary antibodies used were Alexa Fluor 488-conjugated goat anti-mouse IgG, goat anti-mouse IgM, goat anti-human IgG, and goat anti-human IgM (1:1,000 dilutions; Invitrogen).

Purification of GSLs. For each cell line, harvested cells are suspended by 15× volume of chloroform:methanol:water in a ratio of 4:8:3. After centrifugation at $6,000 \times g$ for 5 min, the extracted GSL supernatant was dried under a stream of nitrogen and redissolved in chloroform:methanol at 4:2 (vol/vol) followed by the addition of water to a final ratio of 4:2:1 (vol/vol/vol) of chloroform:methanol:water to produce Folch partitioning. The Folch upper layer containing the GSLs was dried by Speed-Vac and then permethylated by the NaOH/dimethyl sulfoxide slurry method (2) before MS analysis.

Quantitative RT-PCR. Total RNA samples of hESC cell line HES5 were isolated from ES or EB outgrowth cells using TRIzol reagent (Invitrogen). cDNAs were synthesized from total RNAs using SuperScript III reverse transcriptase (Invitrogen) with the oligo-dT primer. A TaqMan real-time PCR assay was performed on an ABI 7300 Detection System and analyzed with ABI Prism 7300 software (Applied Biosystems). Five nanograms of a cDNA sample was used for the quantitative PCR reaction at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min. The threshold cycle numbers (Ct value) and quantity of each analyzed sample were determined using the comparative Ct method. For accurate normalization of quantitative data, multiple housekeeping genes, such as GAPDH, GUSB (glucuronidase, β), and UBC (polyubiquitin), were assayed, and a normalization factor was calculated from the geometric mean of their expression levels by the geNorm program (3). All gene expression values were obtained relative to this normalization factor.

1. Thomson JA, et al. (1998) Embryonic stem cell lines derived from human blastocysts. *Science* 282:1145–1147.
2. Dell A, et al. (1994) Mass spectrometry of carbohydrate-containing biopolymers. *Methods Enzymol* 230:108–132.
3. Vandesompele J, et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3: RESEARCH0034.

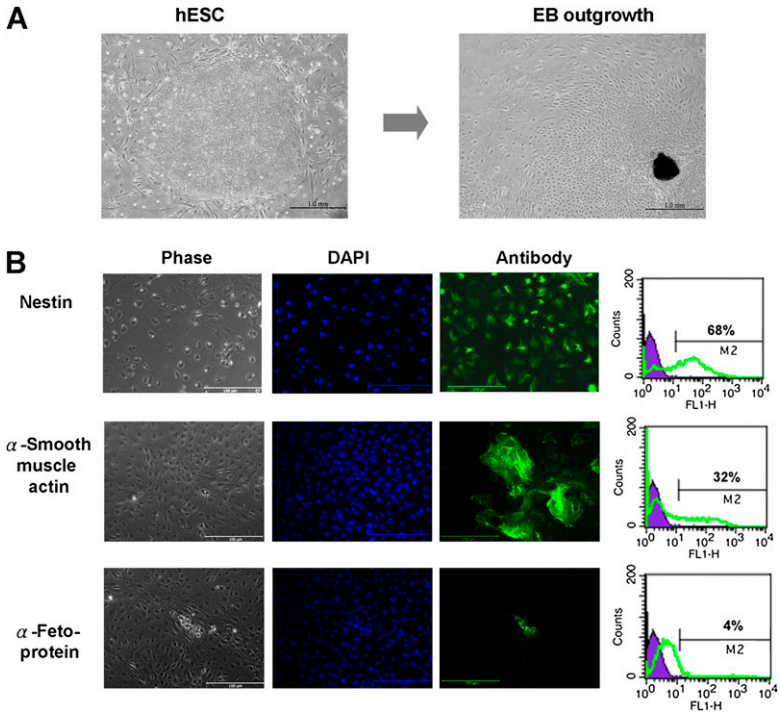

Fig. S1. Characterization of hESCs and EB outgrowth. (*A*) Morphology of hESCs and EB outgrowth. The hES5 hESC line was maintained in an undifferentiated state on inactivated MEF feeder cells. The hESCs differentiated into the monolayer EB outgrowth in differentiation medium for 2 d in vitro, and cells were then transferred to a gelatin-treated plate for 2 wk. (*B*) Immunofluorescence and flow cytometric analysis. Antibodies of three germ layer markers were used to monitor the differentiation of EB outgrowth. Anti-nestin, anti-α–smooth muscle actin, and anti-α–fetoprotein antibodies were respectively used to detect ectodermal, mesodermal, and endodermal cells (green). Nuclei were stained with DAPI (blue). The distribution of the three germ layer markers was estimated by flow cytometry. Viable cells were gated, and the data represent cells from this population. Cells stained with specific antibodies are shown in green; isotype antibodies are shown in black. Values represent the mean of three experiments.

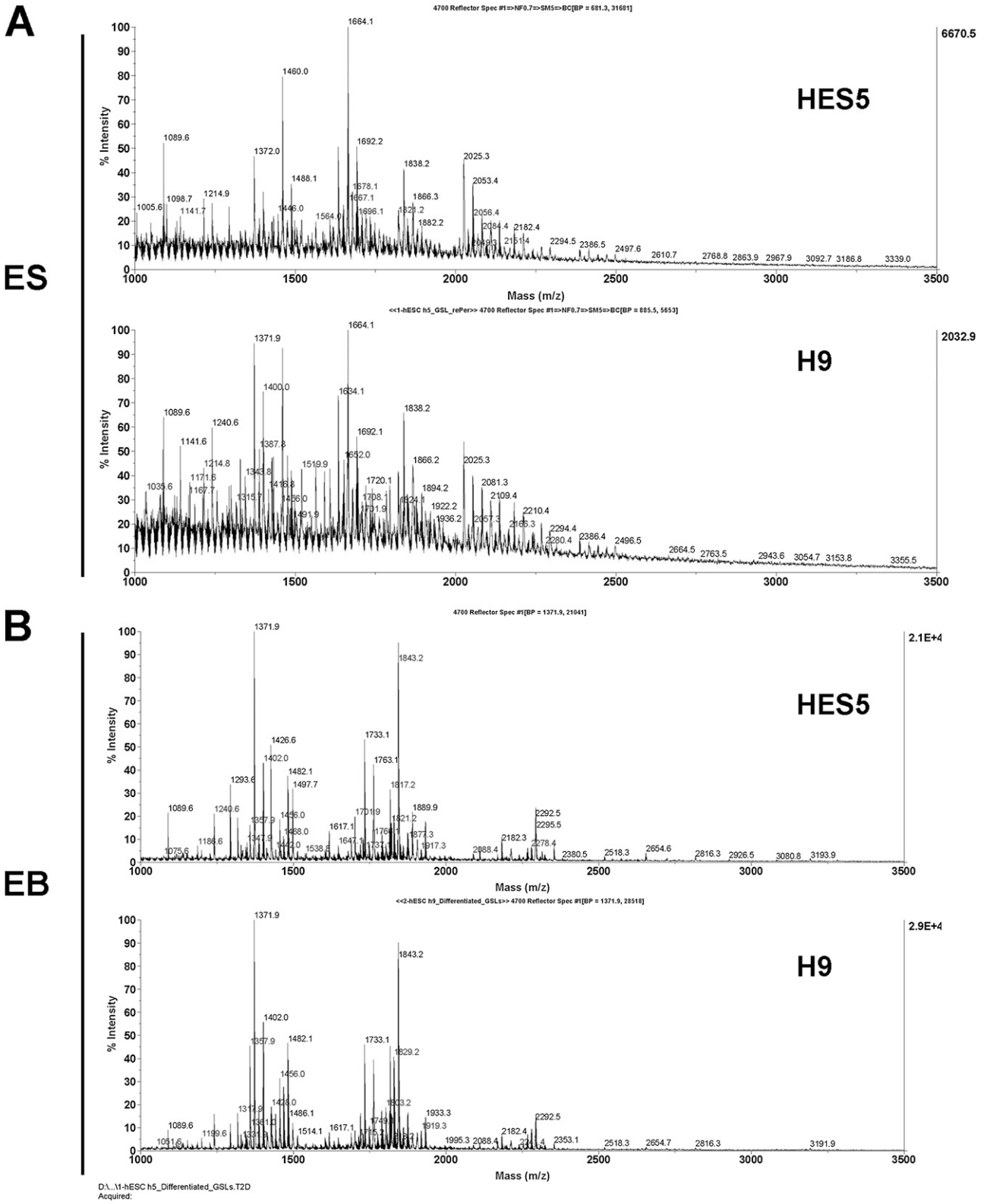

Fig. S2. HES5 and H9 exhibited similar expression patterns of GSLs during differentiation. (*A*) MALDI-MS profiles of undifferentiated hESC from HES5 and H9 cells. (*B*) MALDI-MS profiles of differentiated EB outgrowth cells from HES5 and H9 cells. MS profiling of total GSL from two hESC cell lines showed a similar pattern of GSLs during differentiation.

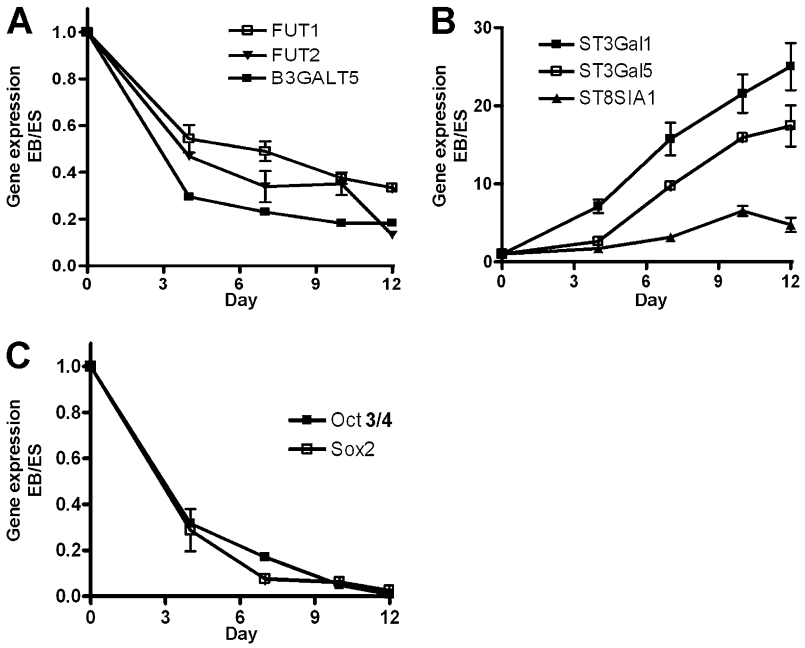

Fig. S3. Time course analysis of gene expressions of glycosyltransferases (GTs) during the differentiation of hESCs. (*A*) Globoside- and lactoside-related GTs such as FUT1, FUT2, and B3GALT5, the gene expressions of which were progressively down-regulated during hESC differentiation. (*B*) Ganglioside-related GTs such as ST3GAL1, ST3GAL5, and ST8SIA1, the gene expressions of which were progressively down-regulated during hESC differentiation. The relative GT gene expression levels of embryoid body (EB)/ESCs were plotted as a straight-line graph. Error bars represent 1 SD from the mean. (*C*) Oct3/4 and Sox2 gene expressions, which were expected to be down-regulated during hESC differentiation, were used as a control.

Table S1. Structures of glycolipids mentioned in this study

Name	Structure
nLc_4Cer	Galβ4GlcNAcβ3Galβ4Glcβ1-1′Cer
Lc_4Cer	Galβ3GlcNAcβ3Galβ4Glcβ1-1′Cer
H type 1 glycolipid	Fucα1-2Galβ3GlcNAcβ3Galβ4Glcβ1-1′Cer
Fucosyl-nLc_4Cer	Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer
Gb_5Cer	Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1′Cer
sialyl-Gb_5Cer	NeuAcα2-3Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1′Cer
Forssman antigen	GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1′Cer
Globo H	Fucα1-2Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1′Cer
disialyl-Gb_5Cer	NeuAcα2-3Galβ1-3(NeuAcα2-6)GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1′Cer
	NeuAcα2-6(NeuAcα2-3)Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1′Cer
	NeuAcα2-8NeuAcα2-3Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1′Cer
GM3	NeuAcα2-3Galβ1-4Glcβ1-1′Cer
GD3	NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-1′Cer
GM2	GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1′Cer
GM1a	Galβ1-3GalNAcβ1-3(NeuAcα2-3)Galβ1-4Glcβ1-1′Cer
GM1b	NeuAcα2-3Galβ1-3GalNAcβ1-3Galβ1-4Glcβ1-1′Cer
GD1a	NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1′Cer
GD1b	Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-1′Cer
GD1c	NeuAcα2-8NeuAcα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1′Cer
GT1a	NeuAcα2-8NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1′Cer
GT1b	NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-1′Cer
GT1c	Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-1′Cer