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Cell surface sialylation and fucosylation are regulated by the cell recognition molecule L1 via PLCγ and cooperate to modulate embryonic stem cell survival and proliferation

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ABSTRACT

Cell surface glycosylation patterns are markers of cell type and status. However, the mechanisms regulating surface glycosylation patterns remain unknown. Using a panel of carbohydrate markers, we have shown that cell surface sialylation and fucosylation are upregulated in L1-transfected embryonic stem cells (L1-ESCs). Consistently, the mRNA levels of sialyltransferase ST6Gal1 and ST3Gal4, and fucosyltransferase FUT9 were significantly increased in L1-transfected ESCs. Activation of L1 signaling promoted cell survival and inhibited cell proliferation. ShRNAs knocking down FUT9, ST6Gal1 and ST3Gal4 blocked these effects. A phospholipase Cγ (PLCγ) inhibitor and shRNA reduced ST6Gal1, ST3Gal4 and FUT9 mRNA levels in the L1-ESCs. Thus, embryonic stem cell surface sialylation and fucosylation are regulated via PLCγ by L1, with which they cooperate to modulate cell survival and proliferation.

1. Introduction

Cell recognition molecules at the cell surface are both donors and acceptors of carbohydrates. These carbohydrates mediate interactions between recognition molecules in cis or trans and thereby modulate their functions as receptors at the cell surface and as signal transducers [1]. For instance, the cell recognition molecule, L1, a member of the immunoglobulin superfamily, is one of many carbohydrate-carrying molecules in the nervous system, where it is widely expressed and involved in many aspects of neural development, regeneration and synaptic plasticity in the adult [2]. Various human genetic disorders with prominent nervous system defects are caused by mutations in L1 [3]. L1 specifically recognizes 2,3-linked sialic acids present on the cell surface mucin, CD24, a heavily glycosylated cell surface molecule [1,4]. Recently, using a murine embryonic stem cell line constitutively expressing L1 at all stages of neural differentiation, we have shown decreased cell proliferation in vitro, enhanced neuronal differentiation in vitro and in vivo, and an increased yield of GABAergic neurons and enhanced migration of embryonic stem cell-derived neural precursor cells into the lesioned striatum in an in vivo animal model [5]. However, the molecular mechanisms by which L1 contributes to these processes remain unknown.

Glycosylation, a crucial event dominating post- or co-translational modifications [6], contributes to the functional diversity required to generate extensive phenotypes from a limited genotype [7]. In most congenital diseases of glycosylation, defects in

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glycosylation are associated with psychomotor/mental retardation or other neuropathological symptoms [1]. Fucosylated carbohydrate structures play important roles in a variety of biological and pathological processes, such as tissue development, angiogenesis, fertilization, selectin-mediated leukocyte-endothelial adhesion, inflammation, host immune response, and tumor metastasis [8]. In mammals, alterations in the expression of fucosylated oligosaccharides occur in several pathological processes, including cancer and atherosclerosis [9]. Sialic acids are one of the most important monosaccharides. They are expressed as terminal sugars with a shared nine-carbon backbone in several classes of cell surface and secreted glycan molecules [10]. In addition to providing negative charge and hydrophilicity to vertebrate cell surfaces, to masking subterminal galactose residues from recognition by certain receptors, and to acting as receptors for pathogens and toxins, sialic acids play an important role during development, including early embryonic development in mice [11].

In the present study, we have demonstrated that the cell recognition molecule L1 modulates both sialylation and fucosylation on embryonic stem cell surfaces through increased mRNA expression of the glycosyltransferases ST6Gal1, ST3Gal4 and FUT9. Glycosyltransferase induction by L1 requires phospholipase Cγ and influences cell survival and proliferation.

2. Materials and methods

2.1. Antibodies, lectins and inhibitors

Goat polyclonal anti-mouse, rat and human FUT9 antibody and goat polyclonal anti-mouse, rat and human ST3Gal4 antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Mouse monoclonal anti-human ST6Gal1 antibody was purchased from Chemicon International Inc. (Temecula, CA, USA). Rabbit polyclonal anti-mouse, rat and human PLCγ antibody was purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). The monoclonal anti-mouse L1 antibody 557 was produced and purified as previously described [12]. Anti-SSEA-1 antibody was purchased from Santa Cruz Biotechnology Inc. Rat anti-mouse IgM-microbeads and LS+ positive selection columns were purchased from Miltenyi Biotec Inc. (Miltenyi Biotec. Inc., Bergisch Gladbach, Germany). L3, L4 and L5 antibodies were produced as previously described [13–16]. R-Phycoerythin (R-PE)-conjugated mouse anti-rat monoclonal, PerCP-CY5.5-conjugated rat anti-mouse IgM monoclonal and R-PE-conjugated rat anti-mouse IgM monoclonal antibodies were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Biotinylated lectins and Texas Red avidin D were purchased from Vector Laboratories, Inc. (Burlingame, CA, USA). Inhibitors were either from Calbiochem, San Diego, CA, USA (U73122, Cdc25 phosphatase inhibitor II, and Erk inhibitor) or from Sigma, St. Louis, MO, USA (LY294002 and KT5720). Leukemia inhibitory factor (LIF) was purchased from Chemicon International Inc.

2.2. Cell culture

All cell culture reagents were purchased from Invitrogen Life Technologies (Merelbeke, Belgium) unless indicated otherwise. Murine embryonic stem cells (ESCs) stably transfected with L1 were generated at the laboratory of M. Schachner as previously described [17]. They were cultured in ES-DMEM supplemented with 15% ES-FBS, 1× non-essential amino acids, 20 mM L-glutamine, 0.5 U/mL penicillin, 0.5 U/mL streptomycin, and 0.1 mM 2-mercaptoethanol. Undifferentiated ES cells were grown on mitomycin C-treated murine embryonic fibroblasts (MEFs) in the presence of 1000 U/mL leukemia inhibitory factor (LIF; Chemicon Inc.). ES cells were passaged every 2 or 3 days using 0.05% trypsin/0.04% EDTA for 2–3 min. The flow cytometry assay was performed using routinely maintained adherent mouse ESC colonies. The NIH3T3 cells were maintained in DMEM supplemented with 10% FBS, 0.5 U/mL penicillin, 0.5 U/mL streptomycin. Cells were passaged every 2 or 3 days using 0.05% trypsin/0.04% EDTA. The single cells obtained were used for flow cytometry assay.

2.3. Enrichment of SSEA-1 positive murine embryonic stem cells (ESCs)

Murine ESCs were grown in 10 cm dishes and passaged into single cell suspensions as described above. Cells were incubated at 4 °C for 30 min in a 1:10 dilution of SSEA-1 antibody in D/G buffer. D/G buffer consisted of 0.5% BSA and 2 mM EDTA in phosphate buffered saline (PBS), pH7.4. After incubation, 10 ml D/G buffer was added and the cells were re-suspended and centrifuged at 300×*g* for 5 min. The supernatant was removed, leaving the cell pellet intact, and cells were washed again in D/G buffer and re-suspended. Following centrifugation, a 1:4 dilution of secondary anti-mouse IgM-beads in D/G buffer was added to the cell pellet, and the re-suspended pellet was incubated at 4 °C for 5 min. After incubation, 10 ml D/G buffer was added to wash the cell pellet, followed by centrifugation (300×*g*) for 5 min. This process was repeated two more times with 5 ml D/G buffer. The cells were finally re-suspended in 500 μl of D/G buffer before being applied to a pre-washed magnetic beads column. The flow-through from the column was collected and re-applied to the magnetic beads column three times. Then, 5 ml D/G buffer was applied to the magnetic beads column to obtain the eluate. The eluate was collected, and the cell number was counted. The SSEA-1 positive cell enriched populations were then used for the subsequent experiments.

2.4. Flow cytometric analysis of surface expression of carbohydrates

Cell surface carbohydrate expression was assessed by indirect immunofluorescence detection using a flow cytometer, FACSCalibur (Becton-Dickinson, San Jose, USA) equipped with an argon laser with emission wavelength at 488 nm. Flow cytometry results presented here are from mESCs that were used immediately after the SSEA-1 positive cell enrichment procedure. CellQuest Pro software (Becton-Dickinson) was used for cell acquisition and analysis. Embryonic stem cells were prepared and cultured as described above. The cells were digested with 0.05% trypsin and washed twice with PBS (Gibco, Long Island, USA). Single cell suspension was prepared in PBS with 10%FBS with the concentration adjusted to 10^7 cells/ml for indirect antibody labeling. Antibody labelings of the cells were carried out using 4 ml sterile tubes (Falcon, Becton-Dickison). In brief, 50 μl of cell suspension (5×10^5 cells) was aliquoted into 4 ml sterile centrifuge tubes (Falcon) and incubated with (amount = 1 μg) primary antibodies against the 14 carbohydrates and lectins in PBS with 10%FBS for 30 min at 4 °C in the darkness. Cells were washed 3 times with chilled PBS and then incubated with secondary antibody conjugated with R-phycoerythin or Peridinin chlorophyll protein (R-PE or Per-CP, 1 μg) in 50 μl of PBS with 10% FBS for further 30 min at 4 °C in the darkness. Cells were washed three times with chilled PBS, fixed in PBS with 1% formaldehyde (Sigma) and kept at 4 °C in the darkness before flow cytometric analysis. 10^4 cells from each tube were acquired for analysis. Unstained cells and cells stained with secondary antibody alone were used as controls for autofluorescence.

2.5. Microarray analysis

Procedures for cDNA synthesis, labeling, and hybridization were carried out in accordance with the manufacturer's protocol (Affymetrix Inc., Santa Clara, CA, USA). All experiments were performed using Affymetrix mouse genome Genechip 430 2.0 arrays.

Briefly, 15 μg total RNA was used for first strand cDNA synthesis with a high-performance liquid chromatography purified T7-(dT) 24 primer. Synthesis of biotinlabeled cRNA was carried out using the ENZO RNA transcript labeling kit (Affymetrix). For hybridization, 15 μg fragmented cRNA were incubated with the chip in 200 μl hybridization solution in a Hybridization Oven 640 (Affymetrix) at 45 °C for 16 h. Genechips were washed and stained with streptavidin–phycoerythrin using the microfluidic workstation™ (Affymetrix) and scanned with Affymetrix Microarray scanner system. Data were analyzed using Spotfire software (Somerville, MA, USA).

2.6. Real-time PCR

To compare the transcript levels, total RNA from embryonic stem cells was isolated using an RNeasy mini kit (Qiagen, Valencia, CA, USA). Total RNA concentration was determined using a spectrophotometer by measuring absorption at 260 nm and 280 nm wavelengths. To check RNA integrity, total RNA was separated in a 1% agarose gel containing formaldehyde and stained with ethidium bromide. Reverse transcription was performed with the SuperScript™ First-strand Synthesis System for RT-PCR Kit (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's protocol. Briefly, 5 μg total RNA was reverse transcribed with random hexamers. First-strand reverse transcribed cDNA was then diluted 1:10 in water before use for real-time PCR. cDNA was subjected to real-time PCR using the 7000 ABI Detection System (Applied Biosystems, Foster City, CA, USA). Real-time PCR reactions (Applied Biosystems) included 1× TaqMan Universal PCR master mix, 1× Assays-on-Demand TM Gene Expression Assay Mix, and 2 μl of template in a 20 μl reaction volume. The following program was run: 50 °C for 30 min, 95 °C for 15 min, then 40 cycles of 94 °C for 15 s, 60 °C for 1 min, followed by plate reading. The assay ID number of Fut9 is Mm01223272_m1; the assay ID number of St6gal1 is Mm00486119_m1; the assay ID number of St3gal4 is Mm00501503_m1. Gapdh was used as reference, and its assay ID number is Mn99999915_g1.

2.7. Cell survival measurement (MTT assay)

The cells were cultured in 96 well plates for 24 h. Afterwards, 10 μl 5 mg/mL MTT solution was then added and cells were cultured at 37 °C for 4 h. After washing with PBS, 20% SDS in PBS was added to lyse the cells and dissolve the dye crystals. The absorbance at 570 nm was determined with an El×800 universal microplate reader (BioTek Instruments Inc., Winooski, VT, USA) and the percentage of surviving cells was calculated [18].

2.8. Cell proliferation assay

2.8.1. 5-Bromo-2′-deoxyuridine incorporation method

Cell proliferation was measured by incorporation of 5-bromo-2′-deoxyuridine (BrdU) (Roche Diagnostics GmbH, Mannheim, Germany) into the genomic DNA during the S phase of the cell cycle. Cells were incubated and processed with the BrdU Labeling and Detection Kit III (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. Cells were stained with BrdU, anti-BrdU-PODS, and peroxidase substrate. Cells from the same population that were not BrdU-labeled were used as negative control. Absorbance at 405 nm was measured and compared using an ELISA plate reader fluorometer (Spectrafluor, TECAN, USA); Magellan software was used for analysis.

2.8.2. mRNA knockdown using short hairpin RNAs (shRNAs)

Isolated ES cells ($(3–4) \times 10^6$) were combined with 2 μg respective shRNA vector or an empty control vector (control group) and 100 μl ES cells-specific Nucleofector solution (Amaxa, Cologne, Germany), then transferred to an Amaxa-certified cuvette. Electroporation was performed according to Amaxa Nucleofection protocol with the Amaxa Nucleofector™ II device and the mouse ESCs A-030 program [19]. After transfection, cells were transferred into culture dishes using the culture medium described above. Twenty-four hours later, cells were used for MTT assay and proliferation assay. For NIH-3T3 cell transfection, 100 μl cell line-specific Nucleofector solution (Amaxa), vector and NIH3T3 cells were mixed. The U-030 program was used for transfection with the Amaxa Nucleofector™ II device as described for the transfection of embryonic stem cells. For culturing, DMEM medium was used as described above.

2.9. Western blot analysis

Immunoblotting was performed as described [5]. Transfected NIH3T3 cells were lysed with RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.25% SDS, 0.15 M NaCl, 0.01 M sodium-phosphate, 2 mM EDTA, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 0.5 mM PMSF, 10 μg/ml aproptinin and leupeptin) [20]. Protein concentration was determined by the Bio-Rad BCA assay. Samples containing equal amounts of protein were resolved by SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore), followed by incubation with primary and secondary antibodies and chemiluminescent detection using the ECL kit (Amersham Biosciences Corp.). The following antibodies were used in this study: FUT9, ST6Gal1 and St3Gal4 (Santa Cruz Biotechnology), PLCγ1 (Cell Signaling Technology Inc., Danvers, MA, USA), and β-actin (Sigma).

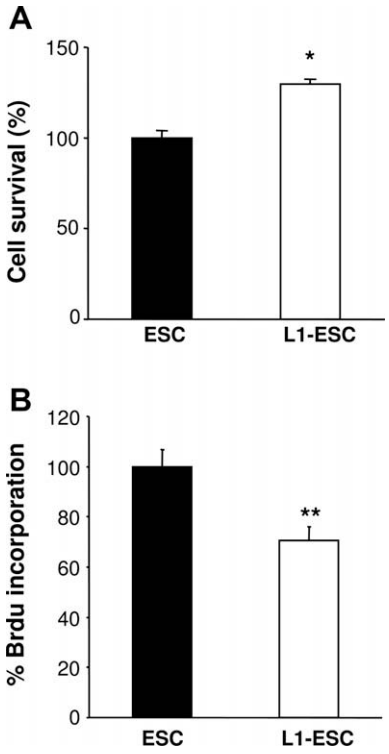
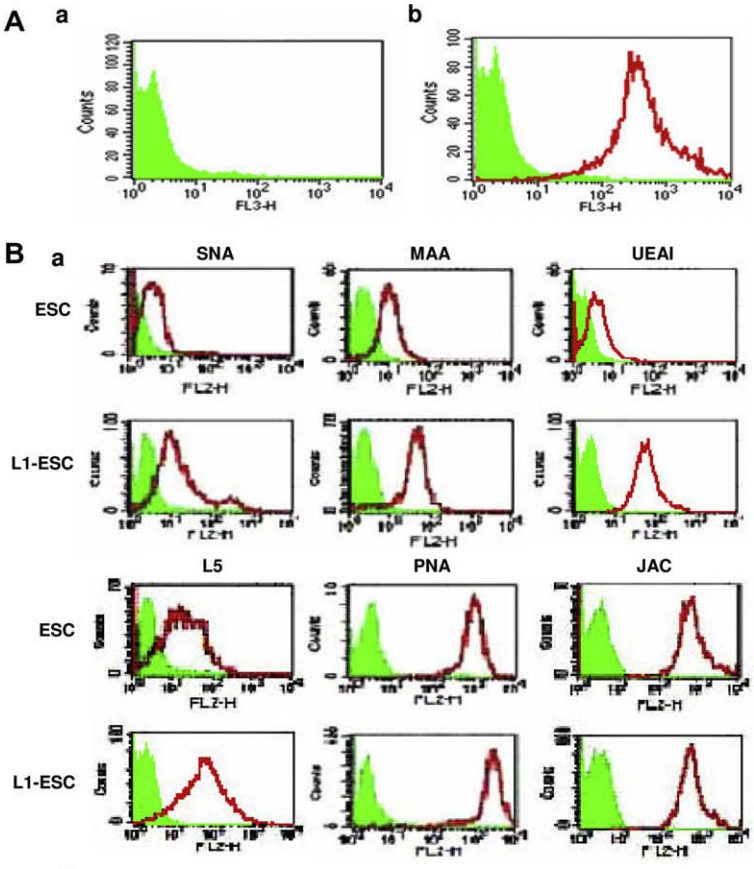


Fig. 1. Activation of L1 promotes embryonic stem cell (ESC) survival and inhibits ESC proliferation. (A) To investigate the role of L1 in ESC survival, MTT analysis was performed. Mouse ESC survival was significantly enhanced in the L1-transfected ESCs (L1-ESCs) versus control ESCs. (B) To investigate the role of L1 in cell proliferation, BrdU incorporation method was performed. ESC proliferation was significantly inhibited in the L1-transfected ESCs (L1-ESCs) versus control ESCs. $^{*}P < 0.05$, by Student's *t*-test.



C

	Structures and transferases of the carbohydrates	Recognized by
a	ST6Gal1: NeuAcα2 → 6Gal/GalNAc	SNA
b	ST3Gal4: NeuAcα2 → 3Galβ1 → 4GlcNAc/Glc; β 1,4 GALT	MAA
c	β 1,4 GALT: Gal β → 4GlcNAc3 ← α1Fuc; FUT9	UEAI lectin L5 Ab

Fig. 2. Glycosylation patterns on the cell surface of ESCs and L1-transfected ESCs. Murine embryonic stem cells (ESCs) and L1-transfected ESCs (L1-ESCs) were assayed by flow cytometry using a panel of carbohydrate surface markers, including lectins and antibodies against carbohydrates. (A) SSEA-1 expression in enriched ESCs determined by flow cytometry. The histogram plots of unstained, control ESCs (left) and cells stained with SSEA-1 antibody after enrichment using magnetic bead sorting at day 0 (right). (B) In the flow cytometry histograms (above), the filled green areas show the number of unstained cells and the areas outlined in red represent cells binding to various lectins (SNA, MAA, UEAI, PNA and JAC) and carbohydrates antibodies (L5). A quantitative analysis (bar graph, below) showed that the expression of carbohydrates recognized by SNA, MAA, UEAI and PNA as well as L5 antibodies was significantly increased in L1-ESCs versus control ESCs. $^{*}P < 0.05$, $^{**}P < 0.001$, by Student's *t*-test. (C) The carbohydrate structures for terminal sialylation (first two rows from top) and fucosylation (bottom row) with related transferases are recognized by SNA, MAA, UEAI, and L5 antibodies on cell surfaces.

2.10. Data analysis

All data were expressed as means ± S.E.M. Statistical evaluations were achieved by one-way analysis of variance and Student's *t*-test. Differences were considered to be significant when $P < 0.05$.

3. Results

3.1. L1. promotes survival and inhibits proliferation of embryonic stem cells (ESCs)

L1 enhances neuronal survival [21,22]. Recently, we have also shown that L1 decreases cell proliferation of embryonic stem cells (ESCs) [5]. Thus, we investigated whether L1 plays a role in ESC survival and proliferation. MTT analysis showed that cell survival was significantly enhanced in murine ESCs stably transfected with L1 (L1-ESCs) (A_{570}: 0.497 ± 0.011) versus untransfected ESCs (A_{570}: 0.351 ± 0.013) (Fig. 1A). Next, using the 5-bromo-2′-deoxyuridine incorporation, we further confirmed that cell proliferation was significantly reduced in L1-transfected ESCs (A_{570}: 0.319 ± 0.031) versus ESCs (A_{570}: 0.521 ± 0.035) (Fig. 1B).

3.2. L1. modulates expression of specific carbohydrates on the cell surface of ESCs

Given that L1 is a carbohydrate-carrying molecule at the cell surface and mediates interactions with itself or other adhesion molecules, carbohydrate expression at the cell surface of ES cells and ES cells transfected with L1 was analyzed by flow cytometry and the cell surface glycosylation patterns were compared. After SSEA-1 (stage specific embryonic antigen 1) enrichment using magnetic bead sorting, 99.92 ± 0.67% of isolated ESCs expressed SSEA-1 (Fig. 2A). In the L1-ESCs, the expression of carbohydrates recognized with SNA, MAA, UEAI and PNA as well as L5 antibody was significantly upregulated (Fig. 2B). Both SNA and MAA recognize terminal sialic acids, UEAI recognizes terminal fucose and L5 antibodies recognize Lewis^X, containing a terminal fucose residue (Fig. 2C). Thus, these results demonstrate that L1 may play a role in modulating the sialylation and fucosylation at ES cell surfaces.

3.3. L1. regulates the expression of the sialyltransferases ST6Gal1 and ST3Gal4 and of the fucosyltransferase FUT9

Next, we investigated whether L1 could regulate the expression of specific sialyltransferases and fucosyltransferases. To identify the genes that respond to L1, cDNA microarray analysis was performed to compare L1-ESCs and ESCs (L1-ESC versus ESC group, Fig. 3A). Of 15 genes involved in the synthesis and hydrolysis of carbohydrates examined, fucosyltransferase 9 (FUT9), galactoside 2, 6 sialyltransferase (ST6Gal1), and galactoside 2, 3 sialyltransferase (ST3Gal4) transcript levels were elevated in the L1-ESC versus ESC group (4.596-fold, 1.231-fold, and 1.486-fold, respectively). mRNA expression of UDP-Gal: GlcNAc 1,4 galactosyltrasferase (B4GalT2) was strongly elevated as well. However, as this enzyme is not involved in formation of the fucosylation or sialylation at the end of carbohydrate structures, we did not focus on it. Real-time PCR analysis confirmed that mRNA levels of ST6Gal1, St3Gal4 and FUT9 were significantly upregulated in L1-ESCs versus ESCs (Fig. 3B). Thus, these results demonstrate that L1 upregulates the expression of ST6Gal1, ST3Gal4 and FUT9 in ESCs.

3.4. ShRNA knocking down FUT9, ST6Gal1 and ST3Gal4 blocks the L1-induced ESC survival and proliferation

To test the hypothesis that sialylation and fucosylation might be involved in the L1-induced ESC survival and proliferation, we

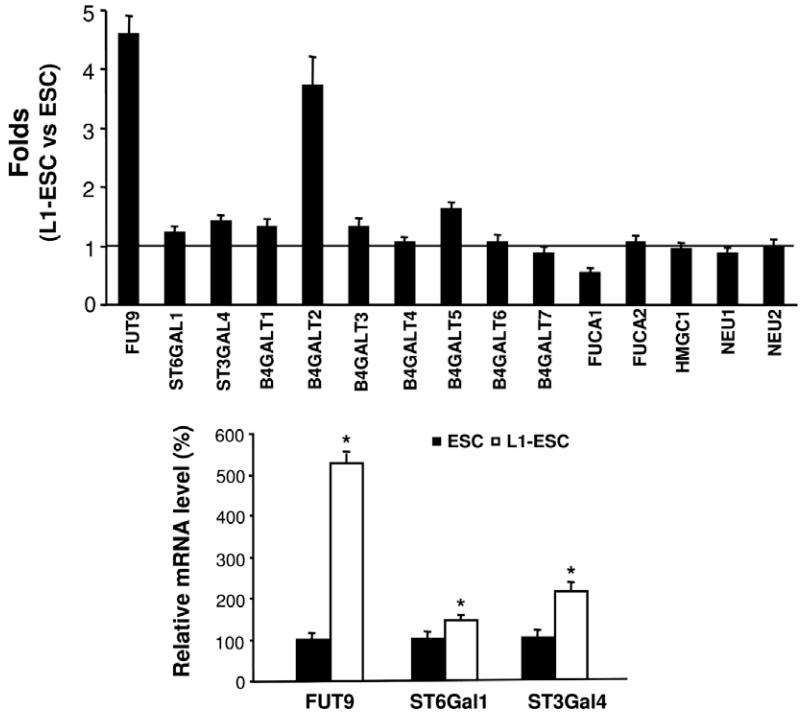


Fig. 3. The mRNA expression of ST6Gal1, ST3Gal4 and FUT9 is modulated by L1. (A) cDNA microarray analysis was performed to investigate the expression of genes involved in the synthesis and hydrolysis of carbohydrates recognized by SNA, MAA, UEAI lectins and L5 antibody by comparing between L1-transfected ESCs and untransfected ESCs (L1-ESC versus ESC group). Per group, two independent samples underwent microarray analysis. The expression of fucosyltransferase 9 (FUT9) was elevated in the L1-ESC versus ESC group (4.596-fold); β galactoside α 2,6 sialyltransferase (ST6Gal1) was elevated in L1-ESC versus ESC group (1.231-fold); and β galactoside α 2,3 sialyltransferase (ST3Gal4) was elevated in L1-ESC versus ESC group (1.486-fold). (B) To verify the results obtained by cDNA microarray analysis, real-time PCR was performed. The expression of ST6Gal1, ST3Gal4 and FUT9 mRNAs was significantly upregulated in the ESCs transfected with L1 (L1-ESC) versus ESCs. $^*P < 0.05$, by Student's *t*-test.

employed shRNAs of FUT9, ST6Gal1 and ST3Gal4, which significantly reduced the expression of either FUT9, ST6Gal1 or ST3Gal4 in transfected cells compared with their expression in empty vector transfected cells (Fig. 4A). To examine the impact of FUT9, ST6Gal1 and ST3Gal4 on L1-enhanced cell survival, we performed a MTT assay, demonstrating that enhancement of ESC survival by L1 was abolished after treatment with shRNA against FUT9 or ST6Gal1 or ST3Gal4 (Fig. 4B). A proliferation assay also showed that ESC proliferation inhibited by L1 transfection was significantly increased after transfection with these shRNAs compared to their respective control empty vector transfections (Fig. 4C). Together, these results demonstrate that sialylation and fucosylation may also contribute to the L1-induced modulation in cell survival and proliferation.

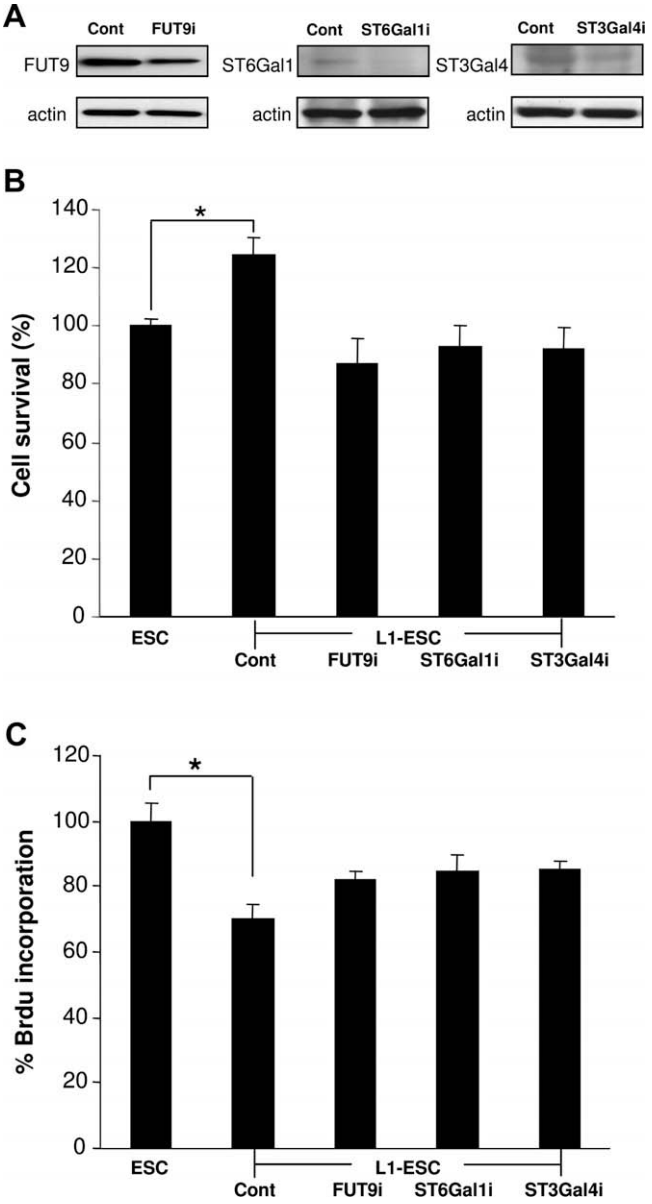


Fig. 4. shRNAs of FUT9, ST6Gal1 and ST3Gal4 reversed ESC survival and proliferation induced by L1. (A) NIH3T3 cells were transfected with empty plasmid (control: Cont) or shRNA of FUT9, ST3Gal4 or ST6Gal1 by electroporation. Twenty-four hours later, cells were lysed and total protein was obtained. Western blotting was used to detect the expression of FUT9, ST3Gal4 and ST6Gal1. (B) ESCs and L1-ESCs were transfected with empty plasmid (control: Cont) or shRNA of FUT9, ST3Gal4 or ST6Gal1, and seeded on coverslips. MTT analysis was performed to detect cell survival. (C) ESC proliferation assay was performed in ESCs and L1-ESCs transfected with empty plasmid (control: Cont) or shRNA of FUT9, ST3Gal4 or ST6Gal1, BrdU incorporation relative to the ESC control group indicates relative cell proliferation. $^{*}P < 0.05$; by Student's *t*-test.

3.5. L1-induced changes in FUT9, ST6Gal1 and ST3Gal4 mRNA levels are dependent on phospholipase Cγ

To understand the mechanism by which L1 induces changes in sialylation and fucosylation, we blocked signal transduction pathways known to be activated by L1 and investigated the effects on FUT9, STGGal1 and ST3Gal4 mRNA levels by real-time PCR. In ESCs transfected with L1, treatment with the phospholipase Cγ (PLCγ) inhibitor U73122 led to reduced FUT9, ST6Gal1 and ST3Gal4 mRNA levels (Fig. 5A). In contrast, Cdc25 phosphatase (Cdc25) inhibitor II, a phosphoinositide-3 kinase (PI3 K) inhibitor (LY294002) and a protein kinase A inhibitor (KT5720) did not have consistent effects on FUT9, STGGal1 and ST3Gal4 mRNA levels in L1-transfected ESCs (Fig. 5A). Extracellular signal-regulated protein kinase (Erk) inhibitor increased FUT9, ST6Gal1 and ST3Gal4 mRNA levels (Fig. 5A). The specificity of the PLCγ inhibitor was not very high, so we used a shRNA directed against PLCγ, which significantly reduced the expression of PLCγ in transfected cells compared with its expression in empty vector transfected cells (Fig. 5B). We found that the shRNA against PLCγ decreased the FUT9, STGGal1 and ST3Gal4 mRNA levels (Fig. 5B). Thus, together these data suggest that L1 regulates sialylation and fucosylation via a PLCγ-dependent pathway.

4. Discussion

We have demonstrated that the cell recognition molecule L1 increases sialylation and fucosylation via a PLCγ-dependent pathway. In turn, L1-dependent enhancement of cell survival and inhibition of cell proliferation can be blocked by shRNAs directed against the sialyltransferases STGGal1 and ST3Gal4 and against the fucosyltransferase FUT9.

Using a murine embryonic stem cell line constitutively expressing L1 at all stages of neural differentiation, decreased cell proliferation in vitro, enhanced neuronal differentiation in vitro and in vivo, and an increased yield of GABAergic neurons and enhanced migration of embryonic stem cell-derived neural precursor cells into the lesioned striatum have been previously shown in an in vivo animal model [5]. In order to explore the mechanisms by which L1 contributes to these processes, we have first shown that the cell surface expression of both sialic acid and fucose are significantly increased in ESCs in which L1 is overexpressed, further suggesting that L1 plays a role in regulation of sialylation and fucosylation on ESC surfaces. Consistently, we have demonstrated that mRNA levels of ST6Gal1 and ST6Gal4, two sialyltransferases, and FUT9, a fucosyltransferase, are significantly upregulated in L1-transfected ESCs using both cDNA microarray and real-time PCR approaches. These findings suggest that L1 induces specific carbohydrate expression patterns on stem cells.

Given that sialylation and fucosylation are enhanced at cell surfaces of the murine embryonic stem cell line constitutively overexpressing L1, the specific pattern of glycosylation induced by L1 may also be an important factor in modulating ES cell survival and proliferation. In agreement with this notion, we have confirmed that L1 promotes ES cell survival, and that this effect can be blocked by shRNA knockdown of enzymes that mediate the sialylation and fucosylation on the ESC surface. Thus, L1 may improve cell survival by modulating cell surface sialylation and fucosylation. Interestingly, sialylation of β1 integrins in colon cancer cell lines is reported to enhance survival by protecting against galectin-3-induced apoptosis [23] and to increase resistance to radiation-induced cell death [24]. It is intriguing to speculate that

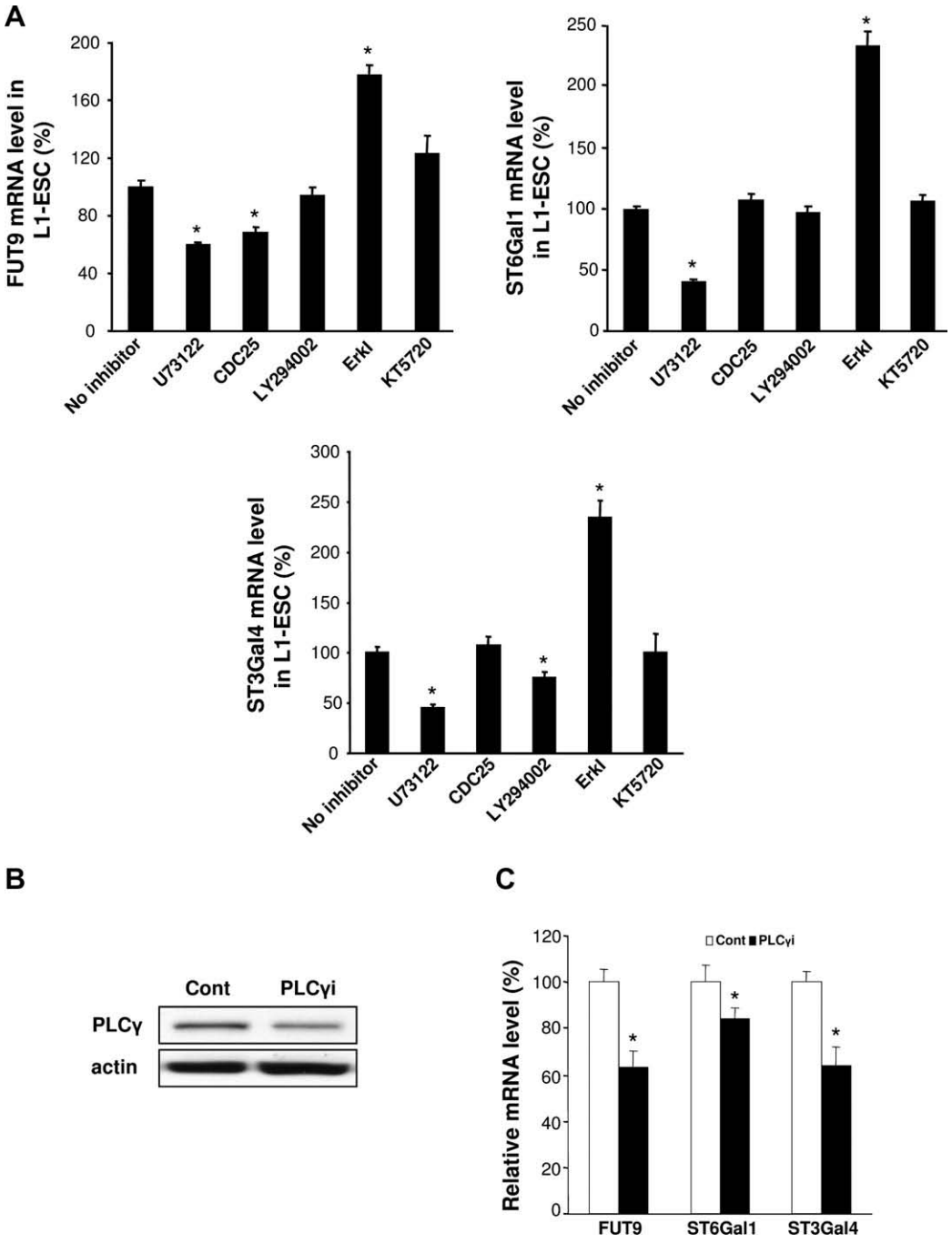


Fig. 5. L1 influences FUT9, ST3Gal4 and ST6Gal1 mRNA levels via various intracellular signaling mediators. (A) One hour after passage of L1-transfected ESCs, inhibitors of PLCγ (U73122, 10.5 μM), CDC25 phosphatase (CDC25 inhibitor II, 1.05 μM), PI3 K (LY294002, 16.5 μM), Erk (Erk inhibitor, 50 μM) or PKA (KT5720, 280 nM) were added into the culture medium and the cells were cultured for a further 24 h. Levels of FUT9, ST3Gal4 and ST6Gal1 mRNA were quantified by real-time PCR. (B) NIH3T3 cells were transfected with empty vector (control: Cont) or shRNA of PLCγ (PLCγi) by electroporation. Twenty-four hours later, cells were lysed with RIPA buffer and total protein was obtained. Western blot was used to detect the expression of PLCγ. (C) The expression levels of ST6Gal1, ST3Gal4 and FUT9 mRNAs were detected with real-time PCR after L1-ESCs were transfected with PLCγ shRNAs (PLCγi). $^{*}P < 0.05$; $^{**}P < 0.001$, by Student's *t*-test.

L1-induced elevated sialylation or fucosylation at the cell surface might have a similar effect by inhibiting the binding of pro-apoptotic ligands to their cell surface receptors. Along the same line, an increase in sialylation or fucosylation might trigger the interaction between anti-apoptotic factors and their cell surface receptors. Moreover, we have confirmed that L1 reduces ES cell proliferation, and that this effect can be reversed by shRNA knockdown of enzymes that mediate the sialylation and fucosylation on the ESC surface as well. Notably, in fucosylation-deficient mice, a loss of Notch-dependent signal transduction on myeloid progenitor cells was observed, indicating that Notch-dependent control of myelopoiesis is regulated by fucosylation [25]. In addition, it was shown that fucosylation of the epidermal growth factor receptor modulated receptor activity as well as epidermal growth factor-mediated cellular growth [26]. Similarly, in L1-overexpressing stem cells, fucosylation of growth factor receptors could be increased, reducing growth factor signaling and thereby cell proliferation. However, the specific mechanisms by which the pattern of cell surface sialylation and fucosylation induced in ESCs by L1 regulates cell survival and cell proliferation remain to be determined.

All three enzymes upregulated by L1, FUT9, ST6Gal1 and ST3Gal4, had similar effects on L1-dependent regulation of cell survival and proliferation. This observation suggests that both cellular processes require glycosylation of cell surface proteins catalyzed by each of the three enzymes. None of these modifications appears to be dispensable either for regulation of cell growth or for supporting cell survival, underscoring the importance of cell surface glycosylation patterns for L1's biological action.

The cell recognition molecule L1 activates a number of signal transduction pathways, including PLCγ-, PI3 K-, Erk- and PKA-dependent pathways [1]. In order to clarify which of these pathways is responsible for the observed dysregulation of ST6Gal1, ST3Gal4 and FUT9 expression, we applied several inhibitors of different intracellular signaling mediators. We have found that both an inhibitor of PLCγ and PLCγ shRNA reduce FUT9, ST6Gal1, and ST3Gal4 mRNA in L1-transfected ESCs. This suggests that L1 may regulate both sialylation and fucosylation in relation to cell function via a common PLCγ-dependent pathway. Moreover, an inhibition of extracellular signal-regulated kinase (ERK) might contribute to the induction of glycosyltransferases by L1, as an ERK inhibitor caused an increase in FUT9, ST6Gal1, and ST3Gal4 mRNA expression.

The central dogma of molecular biology has been revised to include glycosylation or the attachment of glycans or carbohydrates to proteins or to lipids, which provides for the needed functional diversity to generate extensive phenotypes from a limited genotype [7]. Highly ordered, regulated, and conserved pathways of glycan diversification have evolved to play specific biological roles, many of which are essential and have persisted throughout mammalian speciation [27]. Recently, we have shown that cell surface sialylation and fucosylation in neurons are regulated via PLCγ by L1, modulating neurite outgrowth, cell survival and migration [28]. Together with the present study, we have further demonstrated that L1 is also involved in induction of a specific glycosylation pattern at ESC surfaces, which in turn regulates ESC survival and proliferation.

Conflict of interest statement

The authors have declared that no competing financial interests exist.

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