Biochemical and Biophysical Research Communications 440 (2013) 405–412

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Cell recognition molecule L1 promotes embryonic stem cell differentiation through the regulation of cell surface glycosylation

Ying Li [a,b,1], Xiaohua Huang [a,c,1], Yue An [b], Feng Ren [a], Zara Zhuyun Yang [d,e], Hongmei Zhu [d,e], Lei Zhou [d,e], Xiaowen He [f], Melitta Schachner [f], Zhicheng Xiao [d,e,*], Keli Ma [a,*], Yali Li [a,g,*]

[a] Department of Biochemistry and Molecular Biology, Dalian Medical University, Dalian 116044, China
[b] Department of Clinical Laboratory, Second Affiliated Hospital of Dalian Medical University, Dalian 116023, China
[c] Department of Clinical Biochemistry, College of Laboratory Medicine, Dalian Medical University, Dalian 116044, China
[d] The Key Laboratory of Stem Cell and Regenerative Medicine, Institute of Molecular and Clinical Medicine, Kunming Medical University, Kunming 650228, China
[e] Department of Anatomy and Developmental Biology, Monash University, Clayton 3800, Australia
[f] Keck Center for Collaborative Neuroscience and Department of Cell Biology and Neuroscience, Rutgers University, New Brunswick, NJ, United States
[g] Department of Anatomy, National University of Singapore, Singapore 119078, Singapore

ARTICLE INFO

Article history:
Received 12 September 2013
Available online 23 September 2013

Keywords:
L1
Cell differentiation
ESC (embryonic stem cell)
Glycosylation
PLCγ

ABSTRACT

Cell recognition molecule L1 (CD171) plays an important role in neuronal survival, migration, differentiation, neurite outgrowth, myelination, synaptic plasticity and regeneration after injury. Our previous study has demonstrated that overexpressing L1 enhances cell survival and proliferation of mouse embryonic stem cells (ESCs) through promoting the expression of FUT9 and ST3Gal4, which upregulates cell surface sialylation and fucosylation. In the present study, we examined whether sialylation and fucosylation are involved in ESC differentiation through L1 signaling. RNA interference analysis showed that L1 enhanced differentiation of ESCs into neurons through the upregulation of FUT9 and ST3Gal4. Furthermore, blocking the phospholipase Cγ (PLCγ) signaling pathway with either a specific PLCγ inhibitor or knockdown PLCγ reduced the expression levels of both FUT9 and ST3Gal4 mRNAs and inhibited L1-mediated neuronal differentiation. These results demonstrate that L1 promotes neuronal differentiation from ESCs through the L1-mediated enhancement of FUT9 and ST3Gal4 expression.

1. Introduction

Glycosylation is a crucial post- and/or co-translational modification for most eukaryotic proteins. Evidence indicates that glycan structures play important roles in many biological functions since the glycome contains a tremendous amount of biological information [17,7,23]. Defects in glycosylation pathways have frequently been associated with psychomotor/mental retardation or other neuropathological symptoms, as observed in most congenital glycosylation diseases [14]. Most cell recognition molecules (CRMs) are glycoproteins, and the composition of glycans in these proteins varies with cell type and developmental stage. In contrast, CRMs influence the glycosylation patterns in cells. CRMs, including L1, play an important role in neural functions in the central and peripheral nervous systems of vertebrates [4,24,15]. Mutations in L1 lead to various human genetic disorders with prominent nervous system defects [11,19]. In a previous study, we showed that L1 enhanced neuronal survival and proliferation via the upregulated expression of FUT9 and ST3Gal4, leads to increased cell surface sialylation and fucosylation in mouse ESCs [20,21]. Specific terminal glycan modifications, including fucosylation and sialylation, confer unique functional properties to regulate oligosaccharides during ontogeny and cellular differentiation [5,9]. Fucosylated glycans play important roles in a variety of biological and pathological processes, including tissue development, angiogenesis, fertilization, selectin-mediated leukocyte-endothelial adhesion, inflammation, the host immune response and tumor metastasis [1,25]. Sialic acids are the most abundant terminal monosaccharides [33,31]. These molecules are expressed as terminal sugars with a shared nine-carbon backbone, which have been divided into several classes of cell surface and secreted glycan-carrying molecules [27]. In addition to providing a negative charge and hydrophilicity to vertebrate cell surfaces and masking sub-terminal galactose residues from receptor recognition, sialic acids function as receptors for pathogens and toxins [32]. Sialylation also plays a crucial role in a variety of cellular functions such as cell adhesion and regulates the biological stability of glycoproteins

* Corresponding authors. Addresses: Department of Anatomy and Developmental Biology, Monash University, Clayton 3800, Australia (Z. Xiao). Department of Biochemistry and Molecular Biology, Dalian Medical University, Dalian 116044, China (Y. Li).
E-mail addresses: zhicheng.xiao@monash.edu (Z. Xiao), makeli666@aliyun.com (K. Ma), yalilipaper@gmail.com (Y. Li).
[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.09.082

[10,34]. Previously, we showed that mouse ESCs overexpressing L1 promotes neurite outgrowth, cell survival, and cell migration. ESC proliferation is inhibited in these cells via knockdown fucosyltransferase 9 (FUT9), galactoside 2,6 sialyltransferase (ST6Gal1), and galactoside 2,3 sialyltransferase (ST3Gal4), which are key enzymes for sialylation and fucosylation. FUT9 mediates the synthesis of Lewis X (LeX) in the coagulating gland [35]. Sialyl-Lewis X (sLeX), a major selectin ligand, is expressed on the surfaces of T and B cells in a differentiation or activation stage-specific manner. ST3Gal4 is involved in the regulation of sLeX expression during pre-B cell differentiation [12]. An important neural cell adhesion molecule, CD24, carries both α2,3-linked sialic acid and Lewis X on glycans, and binding to L1 is required for the modulation of CD24-induced neurite outgrowth [22]. It has been reported that ST3Gal4 and FUT9 mediate the synthesis of GlcNAC. Thus, the functional expression of FUT9 and ST3Gal4 might be closely associated with cell differentiation.

In the present study, we examined whether L1 regulates ESC differentiation through the upregulated expression of FUT9 and ST3Gal4. We have shown that L1 indeed enhances ESC differentiation into neurons. Down-regulating the expression of FUT9 and ST3Gal4 in ESCs by RNA interference inhibits this differentiation. Furthermore, blocking the phospholipase Cγ (PLCγ) signaling pathway using either a specific PLCγ inhibitor or knockdown PLCγ decreases the expression of FUT9 and ST3Gal4 and inhibits L1-enhanced neuronal differentiation.

2. Experimental procedures

2.1. Antibodies, lectins and inhibitors

Polyclonal goat anti-mouse, rat and human FUT9 antibodies and polyclonal goat anti-mouse, rat and human ST3Gal4 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal mouse anti-β-actin antibody was purchased from Sigma–Aldrich (St. Louis, MO, USA). Rabbit polyclonal anti-mouse, rat and human PLCγ antibody was purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). L3, L4 and L5 antibodies were produced as previously described [6,16,29,30]. Biotinylated lectins and Texas Red avidin D were purchased from Vector Laboratories, Inc. (Burlingame, CA, USA). Rat anti-mouse IgM microbeads and LS+ positive selection columns were purchased from Miltenyi Biotec Inc. (Bergisch Gladbach, Germany). R-Phycoerythin (R-PE)-conjugated mouse anti-rat monoclonal antibody, PerCP-CY5.5-conjugated rat anti-mouse IgM monoclonal antibody and R-PE-conjugated rat anti-mouse IgM monoclonal antibody were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Leukemia inhibitory factor (LIF) was purchased from Chemicon International (Temecula, CA, USA). The phospholipase Cγ (PLCγ) inhibitor (U73122) and extracellular signal-regulated protein kinase (Erk) inhibitor (ERK Inhibitor II, FR180204) were purchased from Calbiochem (San Diego, CA, USA). The phosphoinositide-3 kinase (PI3 K) inhibitor (LY294002) and protein kinase A inhibitor (KT5720) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell culture

Murine embryonic stem cells (ESCs) stably transfected with L1 (L1-ESCs) and their non-transfected counterparts have been previously described [2]. These cells were maintained in ES-DMEM supplemented with 15% ES-FBS (Invitrogen), 1X non-essential amino acids, 20 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 0.5 U/mL penicillin and 0.5 U/mL streptomycin. Undifferentiated ES cells were cultured on mitomycin C-treated murine embryonic fibroblasts (MEFs) in the presence of 1000 U/mL leukemia inhibitory factor (LIF; Chemicon). The ES cells were passaged every 2 or 3 days using 0.05% trypsin/0.04% EDTA for 2–3 min at 37 °C. The maintenance of undifferentiated ES cells (stage 1), embryoid body (EB) formation (stage 2), selection of nestin-positive cells (stage 3), and expansion of nestin-positive neural precursor cells (stage 4) have been previously described [18,26]. To induce differentiation, basic FGF (FGF-2) (stage 5) was omitted.

2.3. Flow cytometry analysis

Cell surface carbohydrate expression was assessed through indirect immunofluorescence detection using flow cytometry on a FACSCalibur (Becton–Dickinson, San Jose, USA) equipped with an argon laser, with a 488-nm emission wavelength. CellQuest Pro software (Becton–Dickinson) was used for cell acquisition and analysis. The embryonic stem cells were prepared and cultured as described above. The cells were digested with 0.05% trypsin/0.04% EDTA and washed twice with PBS (Gibco, Long Island, USA). A single cell suspension was prepared in PBS containing 10% FBS at a concentration of 10^7 cells/mL for indirect antibody labeling. The antibody labeling was conducted in 4 ml sterile tubes (Falcon, Becton–Dickson). Briefly, 50 μl of the cell suspension (5×10^5 cells) was aliquoted into 4-ml sterile centrifuge tubes (Falcon) and incubated with primary antibodies (1 μg) against the 14 carbohydrates and lectins in PBS containing 10% FBS for 30 min at 4 °C in the darkness. The cells were washed 3 times with cold PBS and subsequently incubated with a secondary antibody conjugated to either R-phycoerythin or Peridinin chlorophyll protein (R-PE or Per-CP, 1 μg) in 50 μl of PBS containing 10% FBS for 30 min at 4 °C in darkness. The cells were washed three times with chilled PBS, fixed in PBS containing 1% formaldehyde (Sigma) and maintained at 4 °C in the darkness before flow cytometric analysis. Approximately 10^4 cells from each tube were acquired for analysis. Unstained cells and cells stained with secondary antibody alone were used as controls for auto-fluorescence.

2.4. RT-PCR analysis

Total RNA was isolated from the cells using Trizol (Invitrogen), and reverse transcription and RT-PCR were conducted with Taq polymerase using standard protocols (Boehringer-Mannheim, Indianapolis, IN, USA). The cycling parameters were implemented as follows: denaturation at 94 °C for 30 s, annealing at 58–61 °C for 1 min, depending on the primer, and elongation at 72 °C for 1 min (according to the description of primers). The number of cycles varied between 25 and 35, depending on the mRNA abundance. The identity of the PCR products was confirmed through sequencing. The relative quantitative analysis was normalized to the endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The following primer sequences (forward and reverse) were used: GAPDH (forward: TACTTATGCCGATGTCGTTGT, reverse: CCAGCCTCGTCCCGTAGA); FUT9 (forward: ATCCAAGTGCCTTATGGCTTCT, reverse: TGCTCAGGGTTCCAGTTACTCA); ST3Gal4 (forward: TAAAGAGCCTCGAGTGTCGTCG, reverse: CCGACTCAGGATAGAAGAGACGTAT).

2.5. Western blot analysis

The cells were harvested and lysed in RIPA buffer (1% Triton X-100, 150 mM NaCl, 25 mM Tris, pH 7.5, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM pyrophosphate, and 50 mM NaF). Samples containing equal amounts of protein were resolved using SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore), followed by incubation with primary antibodies (anti-FucT-IX antibody, anti-St3Gal-IV antibody, anti-PLCγ antibody) and

secondary antibodies and chemiluminescent detection using the ECL kit (Amersham Biosciences, Pittsburgh, PA, USA).

2.6. Immunocytochemical staining

Immunocytochemical staining was performed using standard protocols. The cells were fixed in 4% paraformaldehyde for 30 min at room temperature, washed once with phosphate buffered saline (PBS), pH 7.3, and washed twice with antibody diluents (0.2% BSA and 0.1% Triton X-100 in PBS). Subsequently, the cells were incubated with 5% BSA for 30 min and primary antibodies overnight at 4 °C, followed by washing and incubation with TRITC-labeled secondary antibodies (Santa Cruz Biotechnology) for 1 h at room temperature. Cell type-specific markers, monoclonal mouse anti β-tubulin III (Tuj1) antibody (1:400; Santa Cruz Biotechnology) and polyclonal rabbit anti-GAFP antibody (1:400; Dako, Carpinteria, CA), and anti-MAG antibody (myelin-associated-glycoprotein, 1:400; Santa Cruz Biotechnology) were used. The coverslips were counterstained for 10 min at room temperature using DAPI (50 μg/mL; Sigma–Aldrich).

2.7. Statistical analysis

To determine the total cell numbers, the cells were counterstained with DAPI, and the ratio of cell type-specific, marker-positive DAPI$^+$ cells was calculated. At least 200 DAPI$^+$ cells were counted on every coverslip. The data were analyzed using GraphPad Prism 5 software. All data were expressed as mean ± 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 neuronal differentiation of ESCs

To determine whether L1 affects ESC differentiation, the neuronal differentiation of ESCs and L1-ESCs cells was performed. Cell type-specific markers, nestin for undifferentiated neural precursor cells, β-tubulin for neurons, GFAP for astrocytes and MAG for oligodendrocytes, were used for immunocytochemical staining. At fifth days after the cells were seeded onto PLL substrate in the presence of growth factors, most of the cells were nestin-positive (96 ± 1.2%) (1:400; Santa Cruz Biotechnology) (Fig. 1A). 5 days later, when L1-ESCs were maintained without bFGF, the number of β-tubulin-positive neurons was increased by 28.5 ± 9.5% compared with non-transfected wild-type stem cell-derived neurons (Fig. 1B). The percentage of GFAP-positive astrocytes was reduced by 10.7 ± 3.6% (Fig. 1C). The overall percentage of MAG-positive L1-ESCs (2.7 ± 0.7%) was not significantly changed compared with wild-type ESCs (Fig. 1D). These data demonstrate that L1 promotes neuronal differentiation of ESCs.

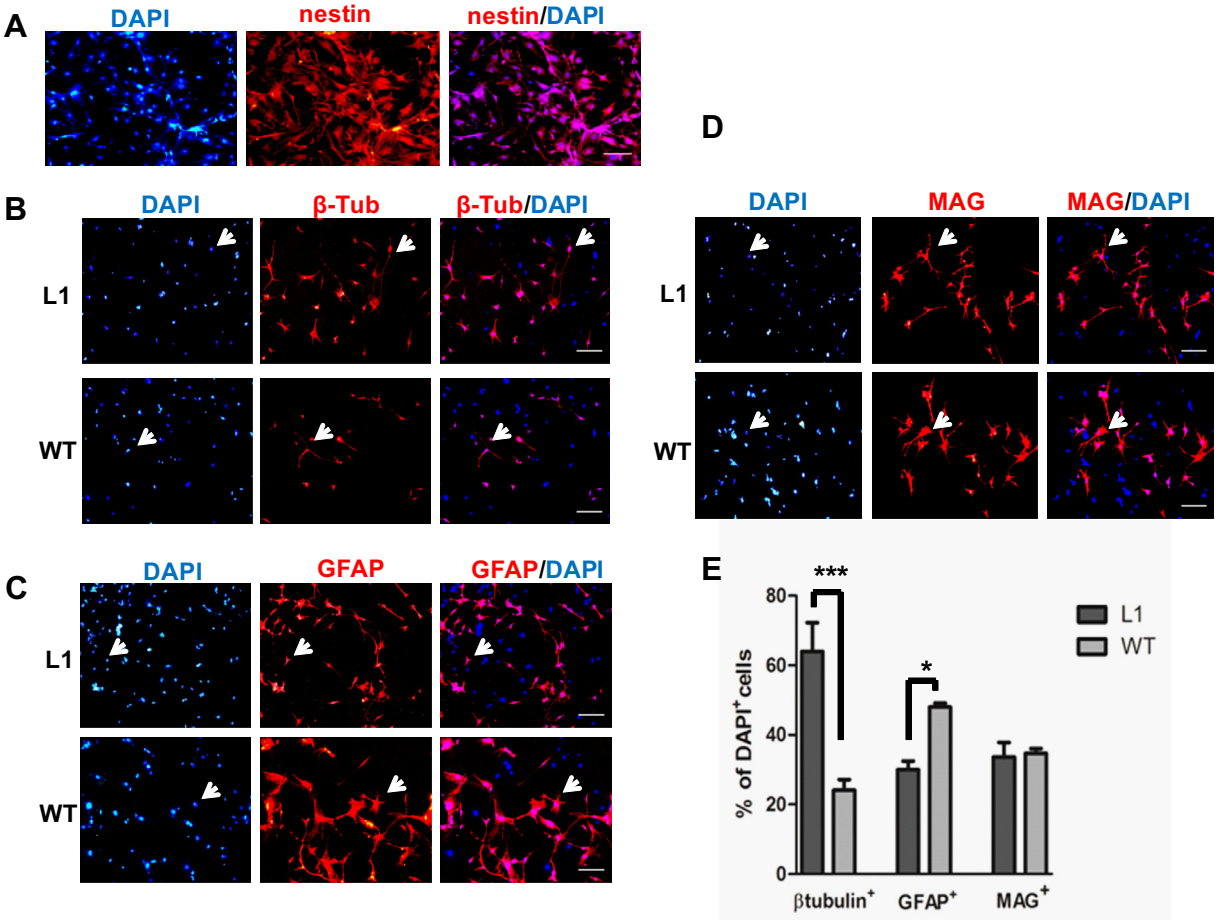

Fig. 1. L1 promotes embryonic stem cell (ESC) differentiation into neurons. After 4 days expansion of neural precursor cells, most of the precursor cells remained nestin-positive (A). After inducing differentiation through growth factor withdrawal, the percentages of β-tubulin-positive cells (B), GFAP-positive cells (C) and MAG-positive cells (D) were determined in L1-ESCs (L1) and wild type parental ESCs (WT). Number of differentiated cells was quantified (E). Scale bar, 100 μm. Data are presented as the mean values ± SEM. $^*p < 0.05$ ($n = 3$); $^{***}p < 0.001$ ($n = 3$).

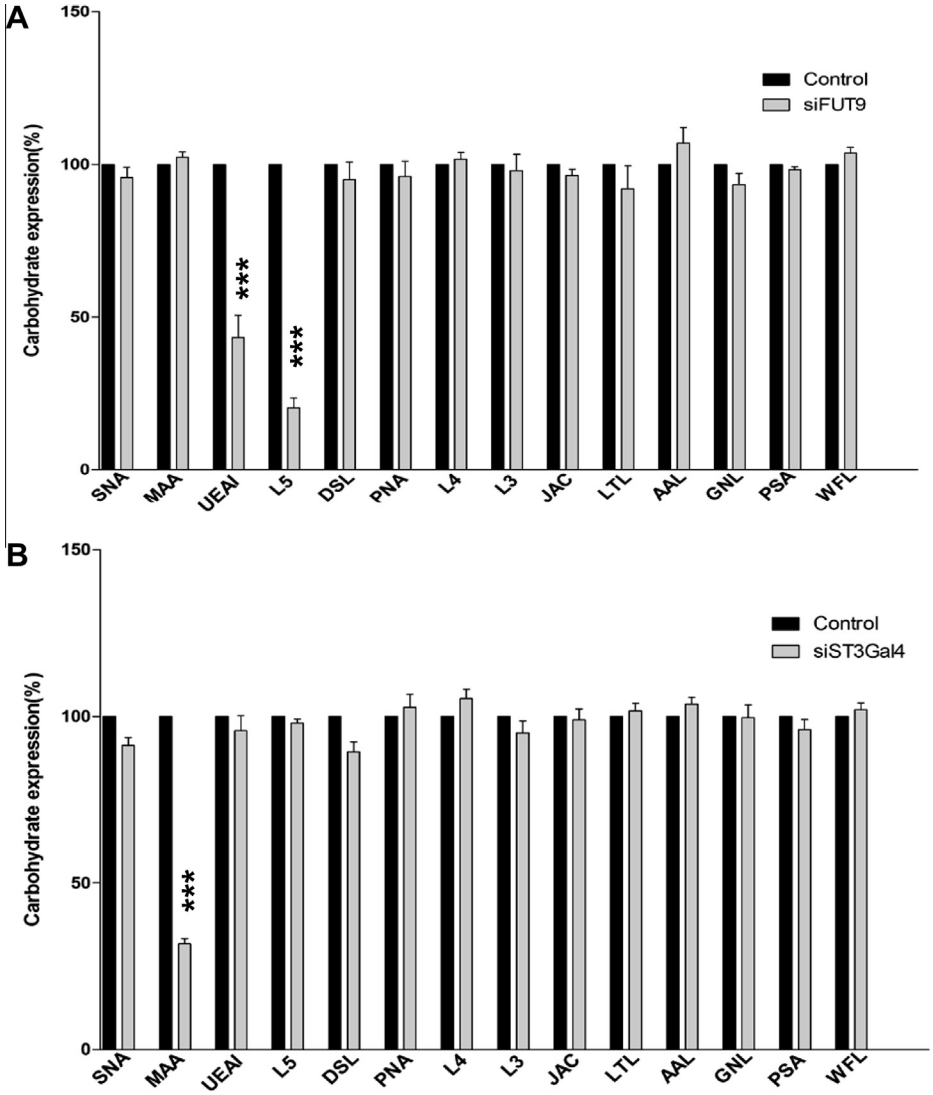

Fig. 2. Glycosylation patterns on the cell surface of L1-ESCs and L1-ESCs after the knockdown of FUT9 and ST3Gal4 expression. L1-ESCs were assayed through flow cytometry after knockdown of FUT9 and ST3Gal4 using a panel of carbohydrate surface markers, including lectins and antibodies against carbohydrates. (A) The quantitative results showed that the expression of carbohydrates recognized with UEAI and the L5 antibody were significantly reduced in L1-ESCs transfected with FUT9 shRNA compared to control cells (L1-ESCs). (B) The quantitative results showed that the expression of carbohydrates recognized with MAA was significantly reduced in L1-ESCs transfected with ST3Gal4 shRNA compared with control cells (L1-ESCs). Data are presented as the mean values ± SEM. $^{***}p < 0.001$ ($n = 3$).

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

In a previous study, we have shown that L1 modulates the expression of specific carbohydrates on the cell surface of ESCs [20]. In the present study, carbohydrate expression on the surface of L1-ESCs and L1-ESCs transfected with FUT9 and ST3Gal4 shRNA was analyzed through flow cytometry, and the patterns of cell surface glycosylation were compared. This analysis showed that both UEAI and L5 were significantly down-regulated by FUT9 shRNA, and MAA was significantly down-regulated by ST3Gal4 shRNA in L1-ESCs (Fig. 2). These results demonstrate that L1 modulates the expression of UEAI, L5 and MAA on the surface of ESCs via the regulation of the expression of FUT9 and ST3Gal4.

3.3. Down-regulating the expression of FUT9 and ST3Gal4 in ESCs blocks L1-induced neuronal differentiation

To examine whether changes in fucosylation and sialylation contribute to the L1-mediated neuronal differentiation of ESCs, we examined the differentiation of L1-ESCs into neurons, astrocytes and oligodendrocytes using cell type-specific markers after knocking down FUT9 and ST3Gal4 through RNA interference. Western blots showed that the expression of either FUT9 or ST3Gal4 in L1-ESCs was significantly reduced through RNA interference compared with that in the control ESCs (Fig. 3A). Ten days after ESC induction into the neural lineage, the cells were cultured for 5 days in the presence of bFGF and subsequently cultured for another 5 days after the bFGF was removed. The percentage of β-tubulin-positive neurons was 31.3 ± 8.2% and 26.7 ± 7.0% lower after knockdown with FUT9 shRNA or ST3Gal4 shRNA, respectively, as compared with the control cells (Fig. 3B). The percentage of GFAP-positive astrocytes increased by 17.3 ± 4.5% and 22.3 ± 5.9% after knockdown with FUT9 shRNA or ST3Gal4 shRNA, respectively, as compared with the control cells (Fig. 3B). The percentage of MAG-positive oligodendrocytes was not influenced after transfection with these shRNAs (Fig. 3B). To confirm shRNA target specificity, we performed a rescue experiment after transiently transfecting the cells with a vector encoding an altered mRNA resistant to shRNA silencing. The effects of RNAi on FUT9 or

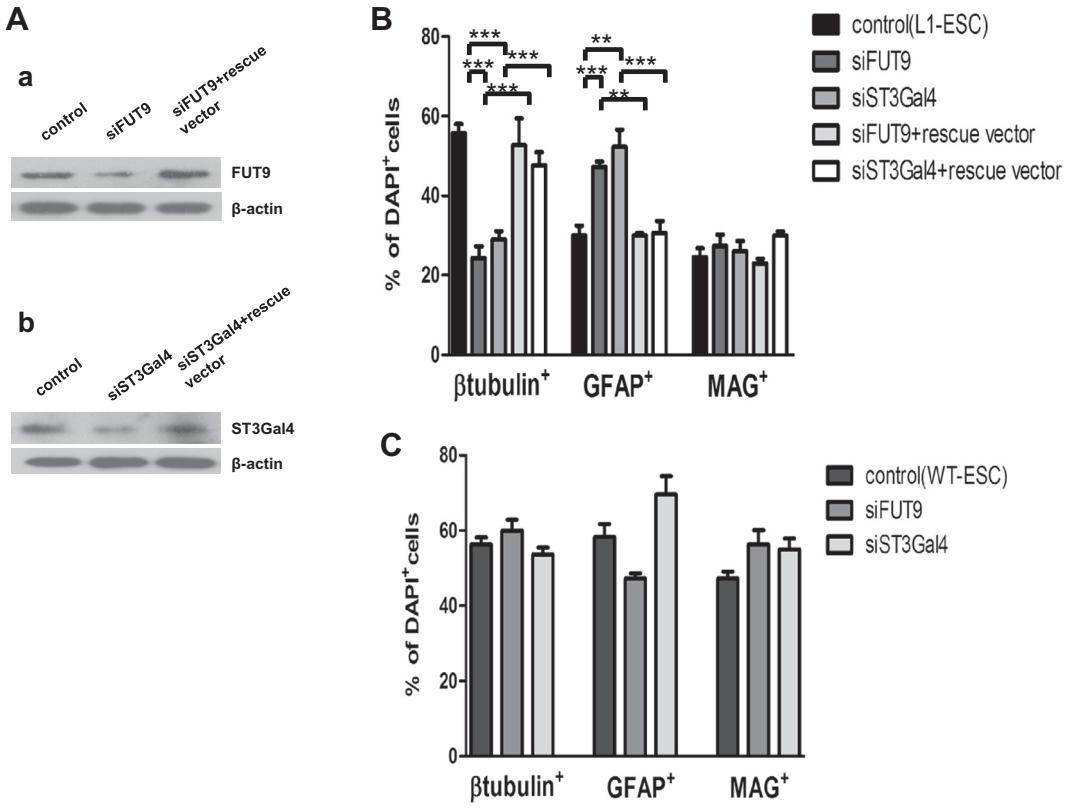

Fig. 3. Expression of FUT9 and ST3Gal4 is reduced after transfection with FUT9 and ST3Gal4 shRNA plasmids and knockdown of FUT9 and ST3Gal4 reduces L1-mediated neuronal differentiation. (A) Undifferentiated L1-ESCs were transfected with empty plasmid (control), FUT9 or ST3Gal4 shRNA or their rescue vector. The cells were seeded at 0 h, transfected with shRNA at 24 h. The cells were collected and analyzed at 96 h. (B) Undifferentiated L1-ESCs and WT-ESCs were transfected with empty plasmid (control) or FUT9 and ST3Gal4 shRNA and seeded on coverslips. The cells were assayed through immunocytochemical staining for β-tubulin-positive, GFAP-positive and MAG-positive cells. Number of differentiated cells was quantified (B and C). Data are presented as the mean values ± SEM. $^{**}p < 0.01$ ($n = 3$); $^{***}p < 0.001$ ($n = 3$).

ST3Gal4 expression were reversed in this rescue experiment, showing that FUT9 or ST3Gal4 expression was restored (Fig. 3A) in the knockdown cells. The percentage of β-tubulin-positive neurons was 28.3 ± 6.7-fold and 18.7 ± 4.4-fold higher in cells transfected with rescue vector compared with the cells transfected with FUT9 or ST3Gal4 shRNA, respectively (Fig. 3B). The percentage of GFAP-positive astrocytes was reduced by 17.3 ± 4.1% and 21.3 ± 5.1% after transfection with the rescue vector compared with cells transfected with FUT9 or ST3Gal4 shRNA, respectively (Fig. 3B). The percentage of MAG-positive oligodendrocytes was not influenced after transfection with the rescue vectors (Fig. 3B). The effects of knocking down FUT9 and ST3Gal4 in parental ES cells (WT-ESC) were investigated to explain the mechanism underlying L1-mediated neuronal differentiation of ESCs associated with changes in fucosylation and sialylation. No significant changes were detected in β-tubulin-positive neurons or GFAP-positive astrocytes after transfection with FUT9 or ST3Gal4 shRNA, respectively, compared with control cells (Fig. 3C). MAG-positive oligodendrocytes were also not influenced after transfection with these shRNAs (Fig. 3C). Together, these results demonstrate that fucosylation and sialylation contribute to the L1-induced neuronal differentiation of ESCs.

3.4. The up-regulated expression of FUT9 and ST3Gal4 in L1-ESCs depends on the activation of phospholipase Cγ signal pathways

To further elucidate the mechanism by which L1 up-regulates fucosylation and sialylation in the neuronal differentiation of ESCs, we blocked L1-activated intracellular signal transduction pathways using specific inhibitors, and determined the mRNA level of FUT9 and ST3Gal4. Then the effects of FUT9 and ST3Gal4 on the neuronal differentiation of ESCs were observed. The treatment of L1-ESCs with the phospholipase Cγ (PLCγ) inhibitor U73122 reduced the mRNA expression of FUT9 and ST3Gal4 (Fig. 4A.a). In contrast, treatment with the phosphoinositide-3 kinase (PI3 K) (LY294002), a protein kinase A (KT5720) and extracellular signal-regulated protein kinase (Erk) (FR180204) inhibitors did not affect FUT9 and ST3Gal4 mRNA expression in L1-ESCs (Fig. 4A.a). Further, the generation of β-tubulin III-positive neurons from L1-ESCs treated with the PLCγ inhibitor U73122 was reduced by 14.0 ± 4.4% compared with control cells (Fig. 4A.b). The generation of GFAP-positive astrocytes from L1-ESCs treated with PLCγ inhibitor U73122 was enhanced by 11.7 ± 3.6% compared with control ESCs (Fig. 4A.b). However, the generation of β-tubulin-positive neurons and GFAP-positive astrocytes from L1-ESCs treated with phosphoinositide-3 kinase (PI3 K) (LY294002), protein kinase A (KT5720) and extracellular signal-regulated protein kinase (Erk) (FR180204) inhibitors was not affected. Moreover, the percentage of MAG-positive oligodendrocytes was not influenced after treatment with these inhibitors (Fig. 4A.b). Furthermore, we used shRNA directed against PLCγ to repeat aforementioned experiments. PLCγ knockdown reduced FUT9 and ST3Gal4 expression in mRNA and protein level (Fig. 4B). The generation of β-tubulin-positive neurons from L1-ESCs treated with PLCγ shRNA was reduced by 34.3 ± 6.9% compared with control cells (Fig. 4B.d). The generation of GFAP-positive astrocytes from L1-ESCs treated with the PLCγ shRNA was enhanced 19.1 ± 3.8-fold in L1-ESCs compared with that in control ESCs (Fig. 4B.d). The percentage of MAG-positive oligodendrocytes was not influenced after treatment with PLCγ shRNA (Fig. 4B.d). Altogether, these data demonstrate that

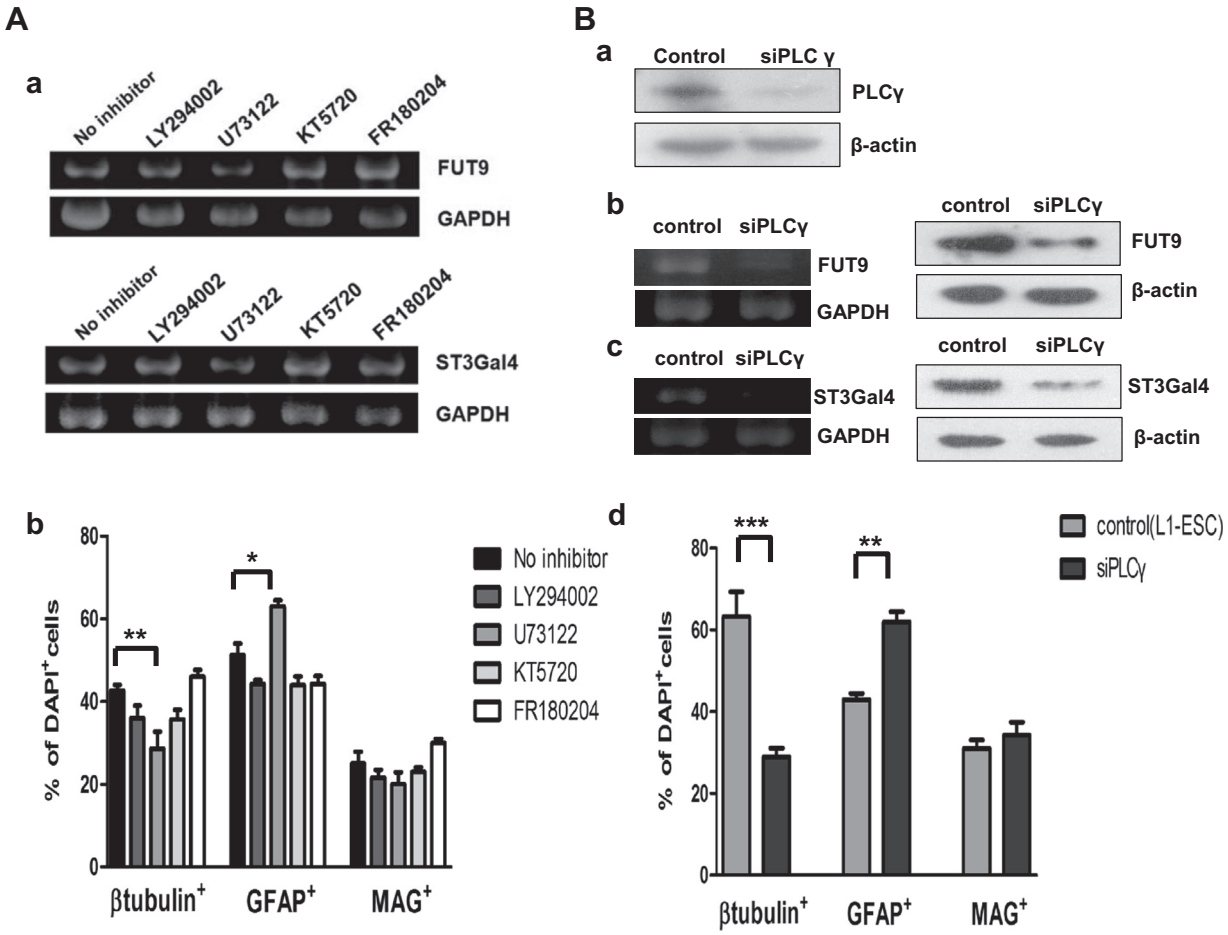

Fig. 4. L1 affects FUT9 and ST3Gal4 expression via phospholipase Cγ intracellular signaling mediators, and the L1-mediated neuronal differentiation of ESCs depends on phospholipase Cγ. (A) During neuronal differentiation, L1-ESCs were treated with the inhibitors for PI3 K (LY294002, 16.5 μM), PLCγ (U73122, 10.5 μM), PKA (KT5720, 280 nM) or Erk (FR180204, 50 μM) for 24 h, respectively. The levels of FUT9 and ST3Gal4 mRNA level were subsequently analyzed through RT-PCR (a). Undifferentiated L1-ESCs were seeded on coverslips and assayed for differentiation after growth factor withdrawal in the presence of inhibitors (same as above). Twenty-four hours after the addition of the inhibitors, immunocytochemical analysis was performed for β-tubulin-positive, GFAP-positive and MAG-positive cells. Number of differentiated cells was quantified (b). (B) During neuronal differentiation, L1-ESCs were transfected with empty vector and PLCγ shRNA, respectively. After 48 h, the cells were treated with RIPA buffer, and total protein was obtained. Western blot analysis was used to detect the expression of PLCγ (a). Both mRNA and protein expression of ST3Gal4 and FUT9 were detected through RT-PCR and Western blot after L1-ESCs were transfected with PLCγ shRNA (b, c). Undifferentiated L1-ESCs were transfected with empty plasmid (control) or PLCγ shRNA and seeded on coverslips. The cells were assayed through immunocytochemical analysis for β-tubulin-positive, GFAP-positive and MAG-positive cells. Number of differentiated cells was quantified (d). Data are presented as the mean values ± SEM. $^{*}p < 0.05$ ($n = 3$); $^{**}p < 0.01$ ($n = 3$); $^{***}p < 0.001$ ($n = 3$).

L1 regulates fucosylation and sialylation via a PLCγ-dependent pathway in the promotion of the neuronal differentiation of ESCs.

4. Discussion

Glycans have increasingly been recognized as important players in cell–cell interactions. In the nervous system, diverse functions, which depend on cell recognition, are mediated through distinct carbohydrate structures. For instance, the neural cell adhesion molecule (NCAM), which is expressed in all nervous system cell types, carries functionally important glycan structures and promotes neuron-neuron and neuron-glia adhesion via homophilic and heterophilic interactions with other cell adhesion and extracellular matrix molecules [8]. In a previous study, we demonstrated that the expression of both fucose and sialic acid on the cell surface is increased in L1-ES cells, in L1 enhanced neuronal survival; and promoted the proliferation of ESCs. These findings suggested that changes in the specific pattern of glycosylation might be an important event for the regulation of the neuronal differentiation of ESCs. Consistent with this notion, using a murine ESC line constitutively expressing L1 at all stages of neural differentiation, we have confirmed that L1 enhanced neuronal differentiation and inhibited astrocytic differentiation. This effect can be blocked through the shRNA knockdown of FUT9 and ST3Gal4, which mediate fucosylation and sialylation on the surface of ESCs. Therefore, we hypothesized that L1-mediated fucosylation and sialylation are also involved in the L1-dependent regulation of the neuronal differentiation of ESCs.

Several intracellular signal pathways, including PLCγ-, PI3 K-, Erk- and PKA dependent pathways, are activated by L1 [36,3]. To clarify which of these pathways is responsible for L1-mediated ESC differentiation through the regulation of cell surface glycosylation, we applied several inhibitors of different intracellular signaling mediators to block the signal transduction pathways. We observed that U73122, a PLCγ inhibitor, reduced the expression of FUT9 and ST3Gal4 and inhibited the L1-enhanced neuronal differentiation of ESCs, indicating that PLCγ activation is the upstream event to regulate the expression of FUT9 and ST3Gal4. Indeed, we have found that L1 mediates the expression of FUT9 and ST3Gal4 and subsequently increases cell fucosylation and sialylation through a common PLCγ-dependent pathway.

However, the molecular mechanisms by which fucosylation and sialylation affect the neuronal differentiation of ESCs remain

elusive. Cell recognition molecules on the cell surface are not only donors, but also acceptors, of carbohydrates. These carbohydrates mediate interactions between recognition molecules in cis or trans, thereby modulating the functions as cell surface receptors and signal transducers [14]. L1 functions via homophilic or heterophilic interactions between L1 and other molecules such as CD24, integrins and CRMs. These carbohydrate-carrying molecules interact with recognition molecules through their glycan structures. Several molecular mechanisms might be involved in the L1-mediated neuronal differentiation of ESCs through the regulation of fucosylation and sialylation. We have shown that L1 regulates sialylation and fucosylation at the cell surface through L1–L1 trans-interactions [20]. L1 at the cell surface is not only a donor, but also an acceptor of certain carbohydrates, thereby forming complexes between apposing partner cell surfaces through trans-interactions [24]. Second, L1 has a binding motif for α2,3-sialyl residues in a single homologous sequence [13,22]. Galactoside 2, 3 sialyltransferase (ST3Gal4) is involved in the synthesis of α2,3-sialyl glycans. It has been reported that expression of Lewis X is controlled through the expression of Fuc-TIX (the FUT9 gene product) [28]. CD24, an important neural cell adhesion molecule, interacts with L1 via its α2,3-sialyl and Lewis X glycans. Both α2,3-linked sialic acid and Lewis X on CD24 glycans are required for the regulation of CD24-induced neurite outgrowth [22]. Third, sialic acid is the most abundant terminal monosaccharide on the eukaryotic cell surface, and this molecule participates in a variety of cellular functions.

In summary, these results suggest that L1 upregulates the expression of FUT9 and ST3Gal4 via the activation of the PLCγ signal pathway to increase fucosylation and sialylation. This cellular event might further enhance the homophilic or heterophilic interactions between L1 and other CRMs in cis or trans to promote the ESC differentiation into neurons.

Authors Contributions

YL and KLM performed majority of the experiments, XHH performed some experiments under the guide of KLM. YA, FR, ZYY, HMZ, LZ and XWH provide technical support and help. YL wrote the manuscript, KLM, YLL, ZCX and M.S corrected the manuscript. YLL, ZCX and M.S participated actively in discussion of the project and editorial work of the manuscript.

Acknowledgments

The authors would like to thank Dr. Gabriele Loers for comments on the manuscript. This work was supported by grants to Y.L. Li from National Natural Science Foundation of China (31070728) and Liaoning Province education bureau (L2010103), and to Z.C. Xiao from the Talent Program, Yunnan Province, China, and The Professorial Fellowship, Monash University, Australia. M.S. was supported through funding from the New Jersey Commission for Spinal Cord Research and the Li Kashing Foundation.

References

[1] D.J. Becker, J.B. Lowe, Fucose: biosynthesis and biological function in mammals, Glycobiology 13 (2003) 41R–53R.

[2] J. Chen, C. Bernreuther, M. Dihne, M. Schachner, Cell adhesion molecule l1-transfected embryonic stem cells with enhanced survival support regrowth of corticospinal tract axons in mice after spinal cord injury, J. Neurotrauma 22 (2005) 896–906.

[3] J. Chen, J. Wu, I. Apostolova, M. Skup, A. Irintchev, S. Kügler, M. Schachner, Adeno-associated virus-mediated L1 expression promotes functional recovery after spinal cord injury, Brain 130 (Pt 4) (2007) 954–969.

[4] M. Dihné, C. Bernreuther, M. Sibbe, W. Paulus, M. Schachner, A new role for the cell adhesion molecule L1 in neural precursor cell proliferation, differentiation, and transmitter-specific subtype generation, J. Neurosci. 23 (16) (2003) 6638–6650.

[5] V.E. Dube, The structural relationship of blood group-related oligosaccharides in human carcinoma to biological function: a perspective, Cancer Metastasis Rev. 6 (4) (1987) 541–557.

[6] T. Fahrig, B. Schmitz, D. Weber, A. Kucherer-Ehret, A. Faissner, M. Schachner, Two monoclonal antibodies recognizing carbohydrate epitopes on neural adhesion molecules interfere with cell interactions, Eur. J. Neurosci. 2 (1990) 153–161.

[7] H.H. Freeze, Genetic defects in the human glycome, Nat. Rev. Genet. 7 (2006) 537–551.

[8] R.S. Haltiwanger, J.B. Lowe, Role of glycosylation in development, Annu. Rev. Biochem. 73 (2004) 491–537.

[9] A.K. Horst, C. Wagener, Bitter sweetness of complexity, Top. Curr. Chem. 288 (2009) 1–15.

[10] T. Kageshita, S. Hirai, T. Kimura, N. Hanai, S. Ohta, T. Ono, Association between sialyl Lewis(a) expression and tumor progression in melanoma, Cancer Res. 55 (1995) 1748–1751.

[11] S. Kenwrick, A. Watkins, E. De Angelis, Neural cell recognition molecule L1: relating biological complexity to human disease mutations, Hum. Mol. Genet. 9 (2000) 879–886.

[12] J. Kikuchi, H. Shinohara, C. Nonomura, H. Ando, S. Takaku, H. Nojiri, M. Nakamura, Not core 2 beta 1,6-N-acetylglucosaminyltransferase-2 or-3 but-1 regulates sialyl- Lewis X expre -ssion in human precursor B cells, Glycobiology 15 (3) (2005) 271–280.

[13] G. Kochlamazashvili, O. Senkov, S. Grebenyuk, C. Robinson, M.F. Xiao, K. Stummeyer, R. Gerardy -Schahn R, Engel AK, Feig L, Semyanov A, Suppiramaniam V, Schachner M, Dityatev A,, A.K. Engel, L. Feig, A. Semyanov, V. Suppiramaniam, M. Schachner, A. Dityatev, Neural cell adhesion molecule-associated polysialic acid regulates synaptic plasticity and learning by restraining the signaling through GluN2B-containing NMDA receptors, J. Neurosci. 30 (11) (2010) 4171–4183.

[14] R. Kleene, M. Schachner, Glycans and neural cell interactions, Nat. Rev. Neurosci. 5 (2004) 195–208.

[15] R. Kleene, H. Yang, M. Kutsche, M. Schachner, The neural recognition molecule L1 is a sialic acid-binding lectin for CD24, which induces promotion and inhibition of neurite outgrowth, J. Biol. Chem. 276 (2001) 21656–21663.

[16] A. Kucherer, A. Faissner, M. Schachner, The novel carbohydrate epitope L3 is shared by some neural cell adhesion molecules, J. Cell Biol. 104 (1987) 1597–1602.

[17] K.S. Lau, E.A. Partridge, A. Grigorian, C.I. Silvescu, V.N. Reinhold, M. Demetriou, J.W. Dennis, Complex N-glycan number and degree of branching cooperate to regulate cell proliferation and differentiation, Cell 29 (2007) 123–134.

[18] V.M. Lee, R.S. Hartley, J.Q. Trojanowski, Neurobiology of human neurons (NT2N) grafted into mouse spinal cord: implications for improving therapy of spinal cord injury, Prog. Brain Res. 128 (2000) 299–307.

[19] V. Lemmon, K.L. Farr, C. Lagenaur, L1-mediated axon outgrowth occurs via a homophilic binding mechanism, Neuron 2 (1989) 1597–1603.

[20] Y.L. Li, G.Z. Wu, G.S. Dawe, L. Zeng, S.S. Cui, G. Loers, T. Tilling, L. Sun, M. Schachner, Z.C. Xiao, Cell surface sialylation and fucosylationare regulated by L1 via phospholipase Cgamma and cooperate to modulate neurite outgrowth, cell survival and migration, PLoS ONE 3 (12) (2008) e3841.

[21] Y.L. Li, G.Z. Wu, L. Zeng, G.S. Dawe, L. Sun, G. Loers, T. Tilling, S.S. Cui, M. Schachner, Z.C. Xiao, 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, FEBS Lett. 583 (4) (2009) 703–710.

[22] A. Lieberoth, F. Splittstoesser, N. Katagihallimath, I. Jakovcevski, G. Loers, B. Ranscht, D. Karagogeos, M. Schachner, R. Kleene, Lewis(x) and alpha2,3-sialyl glycans and their receptors TAG-1, contactin, and L1 mediate CD24-dependent neurite outgrowth, J. Neurosci. 29 (20) (2009) 6677–6690.

[23] J.B. Lowe, J.D. Marth, A genetic approach to mammalian glycan function, Annu. Rev. Biochem. 72 (2003) 643–691.

[24] P.F. Maness, M. Schachner, Neural recognition molecules of the immunoglobulin superfamily: signaling transducers of axon guidance and neuronal migration, Nat. Neurosci. 10 (2007) 19–26.

[25] Y. Ma, P. Katiyar, L.P. Jones, S. Fan, Y. Zhang, P.A. Furth, E.M. Rosen, The breast cancer susceptibility gene BRCA1 regulates progesterone receptor signaling in mammary epithelial cells, Mol. Endocrinol. 20 (2006) 14–34.

[26] S. Okabe, K. Forsberg-Nilsson, A.C. Spiro, M. Segal, R.D. McKay, Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro, Mech. Dev. 59 (1) (1996) 89–102.

[27] M. Schwarzkopf, K.P. Knobeloch, E. Rohde, S. Hinderlich, N. Wiechens, L. Lucka, I. Horak, W. Reutter, R. Horstkorte, Sialylation is essential for early development in mice, Proc. Natl. Acad. Sci. USA 99 (8) (2002) 5267–5270.

[28] Y. Shimoda, Y. Tajima, T. Osanai, A. Katsume, M. Kohara, T. Kudo, H. Narimatsu, N. Takashima, Y. Ishii, S. Nakamura, N. Osumi, Y. Sanai, Pax6 controls the expression of Lewis x epitope in the embryonic forebrain by regulating alpha 1,3-fucosyltransferase IX expression, J. Biol. Chem. 277 (2002) 2033–2039.

[29] A. Streit, A. Faissner, B. Gehrig, M. Schachner, Isolation and biochemical characterization of a neural proteoglycan expressing the L5 carbohydrate epitope, J. Neurochem. 55 (1990) 1494–1506.

[30] A. Streit, C.T. Yuen, R. Wendy Loveless, A.M. Lawson, J. Finne, B. Schmitz, T. Feizi, C.D. Stern, The Lex carbohydrate sequence is recognized by antibody to L5, a functional antigen in early neural development, J. Neurochem. 66 (1996) 834–844.

[31] C. Traving, R. Schauer, Structure, function and metabolism of sialic acids, Cell Mol. Life Sci. 54 (1998) 1330–1349.
[32] A. Varki, Glycan-based interactions involving vertebrate sialic-acidrecognizing proteins, Nature 446 (2007) 1023–1029.
[33] A. Varki, Biological roles of oligosaccharides: all of the theories are correct, Glycobiology 3 (2) (1993) 97–130.
[34] A. Varki, Sialic acids as ligands in recognition phenomena, FASEB J. 11 (1997) 248–255.
[35] C. Wang, C. Huang, Y. Gu, Y. Zhou, Z. Zhu, Y. Zhang, Biosynthesis and distribution of Lewis X- and Lewis Y-containing glycoproteins in the murine male reproductive system, Glycobiology 21 (2) (2011) 225–234.
[36] J. Wu, J.R. Wrathall, M. Schachner, Phosphatidylinositol 3-kinase/protein kinase Cdelta activation induces close homolog of adhesion molecule L1 (CHL1) expression in cultured astrocytes, Glia 58 (3) (2010) 315–328.