Neurochemical Research (2019) 44:839–848
https://doi.org/10.1007/s11064-019-02716-5

ORIGINAL PAPER

SSeCKS promoted lipopolysaccharide-sensitized astrocytes migration via increasing β-1,4-galactosyltransferase-I activity

Hua Wei[1,2] · Leiting Xu[2] · Chunmiao Li[3] · Lianliang Liu[2] · Derry Minyao Ng[2] · Maria Haleem[2] · Lingli Jiang[1] · Ning Sun[1] · Qingzhi Ling[1] · Shaohua Ma[1] · Lingli Zhang[1] · Qinwen Wang[2] · Tao Tao[1,2]

Received: 22 January 2018 / Accepted: 2 January 2019 / Published online: 31 January 2019

Abstract

Astrocytes migration is essential in the formation of the glial scar during the injury response process of the central nervous system (CNS) especially during inflammation. Integrin β1 is part of the extracellular matrix receptors in the CNS and it has been reported that integrin β-deficient astrocytes randomly migrate into wounds. Previous studies have found that β-1,4 Galactosyltransferase-I (β-1,4-GalT-I) enhanced the β-1,4-galactosylation of integrin β1. Src-suppressed C kinase substrate (SSeCKS) is an inflammatory response protein which functionally interacts with β-1,4 Galactosyltransferase-I (β-1,4-GalT-I). In this study we aim to investigate the role of SSeCKS and β-1,4-GalT-I in the migration of astrocytes during lipopolysaccharide (LPS)-induced inflammation. Coimmunoprecipitation and immunofluorescence assays have demonstrated that SSeCKS and β-1,4-GalT-I were significantly enhanced in LPS-treated astrocytes and their interactions may occur in the Trans-Golgi Network. Lectin blot showed that the knockdown of β-1,4-GalT-I could inhibit the β-1,4-galactosylation of glycoproteins including integrin β1 with and without LPS, and that SSeCKS knockdown inhibits the β-1,4-galactosylation of glycoproteins including integrin β1 only in LPS-induced astrocytes. Additionally, wound healing assays indicated that β-1,4-GalT-I knockdown could inhibit astrocytes migration with and without LPS but SSeCKS inhibited cell migration only when LPS was present. Therefore our findings suggest that SSeCKS affects astrocytes migration by regulating the β-1,4-galactosylation of glycoproteins including integrin β1, via β-1,4-GalT-I expression in LPS-sensitized astrocytes.

Keywords Astrocytes · Migration · SSeCKS · β-1,4-Galactosyltransferase-I · Lipopolysaccharide

Introduction

Astrocytes are the most abundant cell type in the CNS and are a central element during development and in disease processes [1, 2]. During development, they function as a glial scaffold for neuroblast migration and are involved in the establishment of the blood–brain barrier [3–5]. In a multitude of pathological processes, astrocytes take part in the pathogenesis of disease and are involved in the injury response process. Astrocytes respond to CNS injury by migrating and proliferating around the affected region to form a glia scar, thus inhibiting axon regeneration in the reactive gliosis process [2]. Furthermore, the inflammatory reaction could enhance astrocyte migration and exacerbate the glia scar formation. Consequently, a better understanding of the factors that influences astrocyte behavior could be beneficial for the design of novel strategies that aim to regulate glial scar formation and subsequently promote CNS recovery.

Integrins are transmembrane receptors, which link the ECM to the intracellular actin cytoskeleton. They form heterodimers consisting of an α-subunit and a β-subunit and can bind different ligands in the ECM and perform distinct functions depending on their subunit composition. Integrin β1 subunit is part of the cell surface receptors in the CNS [6, 7] and astrocytes express several members of the β1 class, which are functional ECM receptors for

✉ Qinwen Wang
wangqinwen@nbu.edu.cn

✉ Tao Tao
taotao@nbu.edu.cn

[1] Ningbo College of Health Sciences, Ningbo 315211, Zhejiang, People's Republic of China

[2] Ningbo University, Ningbo 315211, Zhejiang, People's Republic of China

[3] Medical School of Nantong University, Nantong 226001, Jiangsu, People's Republic of China

laminin (α1β1 and α6β1), collagen (α1β1) and fibronectin (α5β1) [8, 9]. It is reported that β1-deficient astrocytes randomly migrate into the wound [10]. Previous experiments have revealed that aberrant *N*-Glycosylation of integrin β1 causes reduced integrin α5β1 clustering and influences cell migration [11]. However, it is unclear how *N*-glycosylation of integrin β1 is mediated within the astrocytes.

The trans-Golgi network is a highly dynamic series of interconnected tubules and vesicles at the trans face of the Golgi stack. The trans-Golgi network processes and sorts glycoproteins at the interface of the biosynthetic and endosomal pathways [12]. The generation and maintenance of the apical and basolateral membranes rely on the sorting events that occur in the TGN. β-1,4-GalT-I is the first mammalian glycosyltransferase with a resolved crystal structure and has been viewed as a biosynthetic enzyme in the Golgi apparatus, where it transfers galactose from uridine diphosphate-galactose (UDP-Gal) to the terminal *N*-acetylglucosamine (GlcNAc) on oligosaccharide chains of membrane-bound and secretory glycoconjugates [13]. β-1,4-GalT-I also exhibits cell surface expression, and it functions as an adhesion molecule in diverse biological processes, that range from gamete binding to neurite outgrowth [14]. It is known that the glycosylation of proteins is one of the most important post-translational modifications and β-1,4-GalT-I is considered to be the major galactosyltransferase responsible for β4 galactosylation on *N*-glycan [15–17]. Previous studies showed that β-1,4-GalT-I has a regulatory role in cytokine production during inflammatory reactions in the CNS [18]. It remains unknown whether β-1,4-GalT-I is involved in astrocytes migration during inflammation.

SSeCKS is identified as a protein kinase C (PKC) substrate/PKC-binding protein. The major 280 and 290 kDa SSeCKS isoforms are predicted to localize to the cortical cytoskeleton and plasma membrane sites [19]. Previous studies showed that SSeCKS is a major LPS response protein, which is markedly up-regulated in several organs and might participate in the alteration of the cytoskeletal architecture in endothelial cells during inflammation [19, 20]. Previous data indicated that LPS not only induced astrocytes activation but also up-regulated the expression of SSeCKS in astrocytes [21]. Wassler et al. reported that there was a functional interaction between the cytoplasmic domain of β-1, 4-GalT-I and the SSeCKS scaffolding protein [22]. Additionally, it had been reported that SSeCKS and β-1,4-GalT-I could form a positive direction regulation and influence the secretion of TNF-α in LPS-stimulated primary astrocytes [23].

In this study, we constructed the SSeCKS and GalT-I siRNA vectors, the non-specific vector, and the SSeCKS expression vector. We then transfected these vectors into the LPS treated rat's primary astrocytes inflammation model in vitro, to investigate the biological functions of SSeCKS, β-1,4-GalT-I and integrin β1 in LPS-stimulated rat primary astrocytes migration.

Materials and Methods

Cell Culture and Cell Treatment

Primary astrocytes were prepared from the cerebral cortex of newborn Sprague–Dawley rats, which were supplied by the university laboratory animal services centre in Ningbo University. After careful resection of the meninges, the neopallium was isolated and the tissues were cut into 1 mm cubes. They were then treated by 0.125% trypsin at 37 °C for 15 min and subsequently passed through nylon meshes of 70-mm pore size. Cells were seeded onto 75 cm^2 Falcon culture flasks at a seeding density of 2×10^7 cells/flask. Cells were incubated in 1:1 DMEM/F12 medium supplemented with 4-(2-hydroxyethyl)-1-piper-azineethanesulfonic acid (HEPES), sodium bicarbonate, antibiotics, and 10% heat-inactivated fetal bovine serum and were cultured at 37 °C, with 5% CO_2 and 95% air for 7–10 days until the cells were confluent. To obtain a more homogeneous layer of astrocytes, the flasks were placed in a horizontal shaker and shaken for 18 h at 180 rpm. The shaker temperature was monitored at 37 °C. After 8 h, the supernatant, which contained loose astrocytes and oligodendrocytes, was discarded. The purity of the astrocytes was found to be 95% as verified by immunocytochemical staining, with glial fibrillary acidic protein (GFAP) monoclonal antibody (Sigma, SAB2702474) [24]. To investigate the function of SSeCKS and β-1,4-GalT-I in LPS-induced astrocyte, cells were allowed to reach 80% confluence. Non-treated cells were included as controls in all experiments.

Vector Construction and Transfection

The SSeCKS expression vector was a gift from Pro. Gelman (School of Medicine Mount Sinai) and was subcloned to the EGFP vector. The rat SSeCKS siRNA and β-1,4-GalT-I siRNA expression vector was constructed, which targeted the nucleotide residues 5′-AAGGAGATG TCCATGTCCAAG-3′ and 5′-AATTGCACACACAAA GGAGAC-3′. For transient transfection, the SSeCKS and GalT-I expression vector, their siRNA vector, and the non-specific vector were carried out using lipofectamine 2000 (Invitrogen) and plus reagent in OptiMEM (Invitrogen) as suggested by the manufacturer. Transfected cells were used for the subsequent experiments 48 h after transfection.

RNA Isolation and Reverse Transcriptase PCR (RT-PCR) Analysis

The total RNA of the primary astrocytes was extracted using a Trizol extraction kit (Invitrogen) according to the manufacturer's protocol, and reverse transcribed using the ThermoScript RT-PCR system (Thermo Fisher). Primer sequences were listed as follows: SSeCKS: forward, 5′-AAGAATGGCCAGCTGTCTAC-3′; reverse, 5′-GCTTTGGAACTGTCTGTCACT-3′; β-1,4-GalT-I: forward, 5′-TACAACTGCTTTGTGTTCAGTGATG-3′; reverse, 5′-GCAGGCTAAACCCGAACTTG-3′. The GAPDH was used as an internal control. PCR amplification was carried out with an initial denaturing step at 94 °C for 5 min, then 20 cycles at 94 °C for 45 s, at 58 °C for 45 s, and at 72 °C for 45 s, and a further extension at 72 °C for 10 min. The signal intensities of RT-PCR products were quantified with a computing laser densitometer.

Immunofluorescent Staining

Cultures grown on coverslips were fixed with 4% paraformaldehyde for 20 min at 4 °C followed by phosphate-buffered saline (PBS) rinses. Nonspecific binding sites were blocked for 2 h at room temperature with 5% normal horse serum (Sigma) or normal goat serum (Sigma) diluted in 0.1% Triton X-100-PBS. Cells were then incubated overnight at 4 °C with primary antibodies: polyclonal sheep anti SSeCKS (1:100, Sigma, S1562); polyclonal goat anti-β-1,4-GalT-I (1:100, Santa Cruz, sc-515551); monoclonal mouse anti TGN46 (1:100, Abcam, AB2809). Primary antibodies were diluted in blocking buffer. Secondary antibodies conjugated with fluorescein isothiocyanate (FITC) (1:100 dilution in PBS) or Cy3 (1:800 dilution in PBS), and Hoechst (H33342) (Sigma) was used to stain the nucleus for half an hour at 37 °C. In negative controls, non-immune serum was used instead of the primary antibody. Immunopositive cells were observed using Leica fluorescence microscope (Germany).

Lectin Blotting

Primary astrocytes were washed with ice-cold PBS and lysed (0.5% Triton, 50 mM NaCl, 10 mM NaF, 30 mM tetrasodium pyrophosphate, 10% glycerol, 1 mM EDTA, 20 mM Tris, 1 mM Pefabloc, 1% Trasylol, 1 mM sodium orthovanadate, pH 7.4). Extracts were clarified by centrifugation, and protein concentration was determined by the BCA protein assay system (Pierce Company, USA). Proteins were separated with SDS–PAGE (10% gradient gels) and then transferred onto a PVDF membrane. Blots were treated with 25 mM H_2SO_4 at 80 °C for 60 min to remove sialic acid residues and then lectin blotting analysis was conducted using Ricinus communis agglutinin (RCA)-I. The blots were washed and developed with the electrochemiluminescence (ECL) detection system using X-ray film (Fuji Photo Film).

Lectin-Fluorescent Staining with RCA-I

Cells were digested with sialidase for 5 h at 37 °C. After washing in PBS, cells were blocked with 1% BSA 2 h at room temperature and then incubated with monoclonal primary antibody for integrin β1 (1:100,Sigma, SAB4300655) overnight at 4 °C. After washing in PBS three times, the second antibodies (FITC-Donkey-anti-mouse, 1:100, Abcam ab7057) and tetramethyl rhodamine isothiocyanate (TRITC)-labeled RCA-I were added in a dark room and incubated for 2 h at 4 °C. The fluorescence was subsequent detected by Leica fluorescence microscope.

Immunoprecipitation

Primary astrocytes were lysed with ice-cold lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 5 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF) and centrifuged for 30 min at 4 °C. Aliquots of the supernatant (0.2 ml) were incubated with 20 μl of protein G-agarose (Roche) at 4 °C for 3 h followed by centrifugation at 10,000×*g* for 10 min, 0.1 ml aliquots of supernatant were incubated with the monoclonal antibody to SSeCKS, β-1,4-GalT-I and integrin β1 overnight at 4 °C. Thereafter 30 μg protein G-agarose beads were added and the incubation was continued with gentle rotation for an additional 2 h. The beads were washed five times with PBS and then incubated with loading buffer for western blot. The immunoprecipitated integrin β1 subunit was subjected to lectin blotting.

Immunoblot Analysis

After appropriate stimulation, cell lysates were obtained by scratching the cell in a lysate buffer. Proteins were loaded into wells of a 10% acryl/bisacrylamide gel, and after separation, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane. After saturation in Tris-Buffered Saline Tween-20 (TBST) containing 5% milk, the primary antibody and secondary horseradish peroxidase-conjugated antibody that were diluted in TBST, were sequentially added to and incubated with the membranes overnight and 2 h respectively. Results were obtained using ECL.

In Vitro Wound Repair Assay

In vitro scratch wound healing assays were performed using a modification of published procedures [25]. Primary astrocytes (100,000/well) were cultured in six-well plates for 7 days prior to wounding. The cell monolayers

were mechanically wounded by scraping with a 100 µl pipet tip, and the closing of the scratch wound was monitored by Nomarksi contrast light microscopy. The migration distances of the cells were documented with a Leica camera and software immediately and 24 h after wounding.

Statistical Analysis

All the outcomes measurements were made by an investigator that was blinded to the treatment group. At least three assessments were performed for each experiment and all data were expressed as mean ± SE. The density of each band or area in the figures was detected using the Gel Imaging System (GelDoc XR, Bio-Rad) and one-way or two-way ANOVA was used to compare the differences among groups. When used the 2-way ANOVA to analysis the differences between LPS with substrate/enzyme or time with siRNA treatment, we have compared the differences between the LPS treatment and un-treatment in different siRNA transfected, the specific siRNA transfected with non-specific siRNA transfected in different times point. All statistical analyses were conducted using STATA 14.0 software package (Stata Corp., College Station, TX, USA), and all significance levels were set at $p < 0.05$.

Results

The interaction Between SSeCKS and β-1,4-GalT-I was Enhanced in LPS-Treated Astrocytes

Preliminary observations showed that SSeCKS could positively regulate β-1,4-GalT-I expression in LPS-treated astrocytes [23]. In this study, the coimmunoprecipitation experiment results indicated that SSeCKS and β-1,4-GalT-I bind together and their interaction was significantly enhanced in LPS induced primary astrocytes compared to the normal group ($p = 0.023$, LPS treated vs un-treated in SSeCKS/GalT1, $p = 0.018$, LPS treated vs un-treated in GalT1/SSeCKS) (Fig. 1A, B). Immunofluorescent staining using anti-SSeCKS or anti-β-1,4-GalT-I antibody and TGN46 (a marker of the trans-Golgi network) demonstrated that the

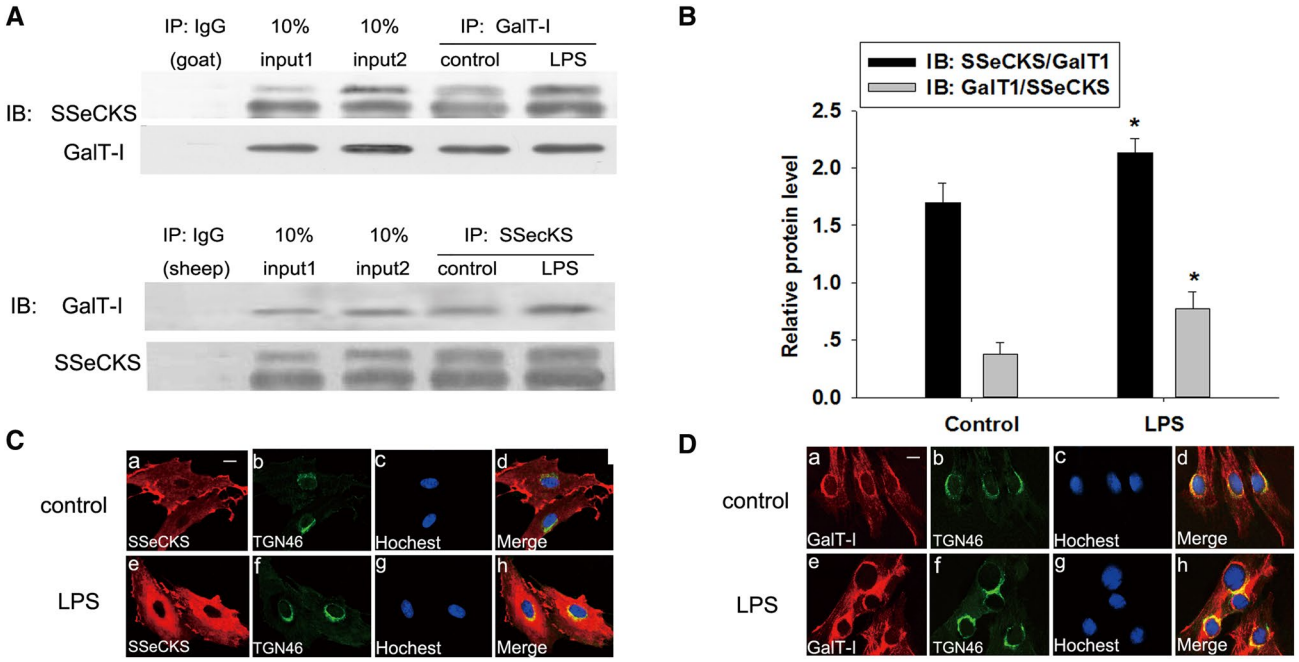

Fig. 1 SSeCKS and β-1,4-GalT-I co-immunoprecipitated in primary astrocytes and co-localized respectively with TGN46. Primary astrocytes were cultured in the medium with and without 1 µg/mL of LPS for 6 h. **A** SSeCKS co-purifies with β-1,4-GalT-I in primary astrocytes. The lysates were immunoprecipitated with antibodies against β-1,4-GalT-I. β-1,4-GalT-I co-purifies with SSeCKS in primary astrocytes. The lysates were immunoprecipitated with antibodies against SSeCKS. Input 1 and Input 2 represents 10% of the total proteins obtained from untreated cells or LPS-treated astrocytes that were used for immunoprecipitation. **B** Quantitative density analysis of the interactions between SSeCKS and β-1,4-GalT-I. Bands were scanned and the intensities were expressed as mean ± SEM of three independent experiments each run in triplicates. Two-way ANOVA was used to compare the differences, and then pairwise comparisons when interactions are significant. Asterisk indicated that the coimmunoprecipitation of both SSeCKS with β-1,4-GalT-I and β-1,4-GalT-I with SSeCKS increased significantly in LPS treated astrocytes. *$p < 0.05$. **C** SSeCKS co-localized with TGN46 in the control group (a–d) and LPS-stimulated group (e–h). **D** β-1,4-GalT-I co-localized with TGN46 in the control group (a–d) and LPS-stimulated group (e–h). The cells were fixed with paraformaldehyde and stained with antibodies to SSeCKS (green), β-1,4-GalT-I (green), TGN46 (red) and Hoechst (blue). Three repetitive assessments were performed for each experiment. Scale bar 20 µm

concentration of both SSeCKS (Fig. 1C, a–h) and β-1,4-GalT-I (Fig. 1D, a–h) were significantly increased in TGN in LPS-induced astrocytes compared with the control groups. It indicates that LPS could be promoting the transport of SSeCKS and β-1,4-GalT-I to the trans-Golgi network.

SSeCKS Promoted β-1,4-Galactosyltransferase-I Mediated Proteins β-1,4-Galactosylation in LPS-Induced Astrocytes Inflammation

Immunofluorescent results showed that SSeCKS and β-1,4-GalT-I are co-located in the perinuclear region, and their concentrations are enhanced in LPS-stimulated astrocytes (Fig. 2A). To study the possible roles of SSeCKS and β-1,4-GalT-I in TGN, we successfully transfected the EGFP-SSeCKS expression vector into astrocytes (Fig. 2C) and found that overexpression of SSeCKS could significantly up-regulate β-1,4-GalT-I expression in LPS-stimulated astrocytes (Fig. 2A,m–p) compared to any other control group (Fig. 2A,a–h). We investigated the relationship among SSeCKS, β-1,4-GalT-I and galactosylation of glycoproteins in LPS-induced astrocytes via knockdown of SSeCKS or β-1,4-GalT-I. Since the RCA lectin blot is considered to be only useful for the identification of β-1,4-linked galactose residues attached to N-linked oligosaccharides [13], therefore in our experiment, the same amount of proteins was analyzed by Coomassie blue staining and RCA-I binding to detect the galactosylation of glycoproteins. The SSeCKS siRNA and β-1,4-GalT-I siRNA transfection significantly decreased the amount of SSeCKS and β-1,4-GalT-I in

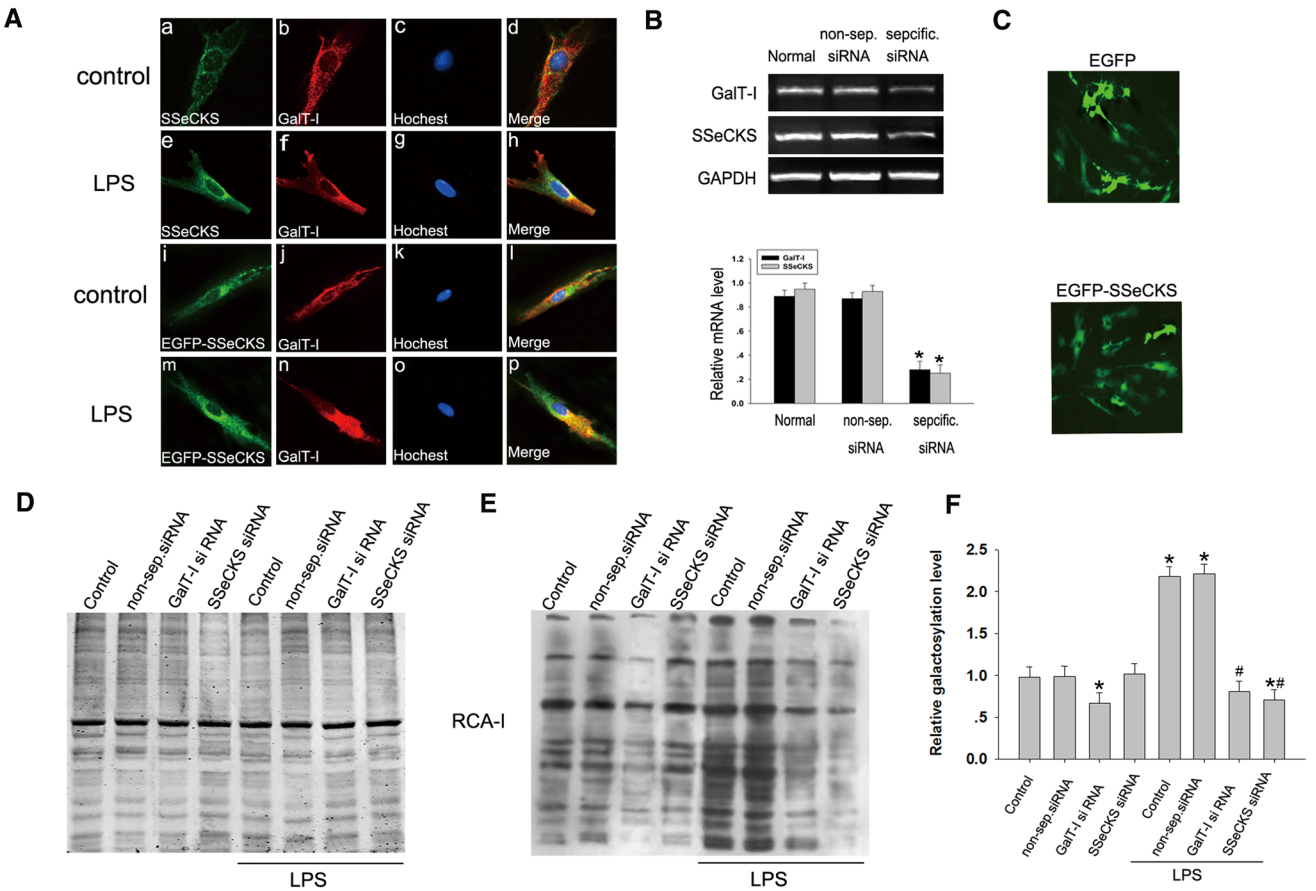

Fig. 2 β-1,4-Galactosylation of Glycoproteins were regulated through the interaction between SSeCKS and β-1,4-GalT-I in LPS-stimulated astrocytes. **A** The co-localization of SSeCKS and β-1,4-GalT-I in the rat astrocytes by double immunofluorescence. Transfected with the SSeCKS overexpression vector and then treated with and without LPS for 3 h (i–p), the WT-control group (a–d), the WT-LPS group (e–h). Scale bar 20 μm. **B** Astrocytes were transfected with non-specific siRNA, SSeCKS siRNA or β-1,4-GalT-I siRNA, and the efficiency of the transfection was assessed by RT-PCR. **C** EGFP and EGFP–SSeCKS expression under a fluorescence microscope. **D, E** The normal and LPS-treated astrocytes were transfected with a non-specific siRNA, SSeCKS siRNA or β-1,4-GalT-I siRNA, and protein extraction of each group was separated by SDS-PAGE and analyzed by Coomassie Blue staining (**D**) and RCA lectin (**E**). **F** Quantitative analysis of RCA lectin results in astrocytes was conducted three times. The densities were detected using the Gel Imaging System (GelDoc XR, Bio-Rad), which captured the entire gel lane and two-way ANOVA was used to compare the differences, and then pairwise comparisons when interactions are significant ($F = 4.231$). $^{*}p < 0.05$ compared with normal astrocytes, $^{\#}p < 0.05$ compared with LPS-treated astrocytes

astrocytes ($p = 0.012$ GalT-1 siRNA transfected with normal, $p = 0.024$ SSeCKS siRNA transfected with normal) (Fig. 2B) and β-1,4-GalT-I knockdown could inhibit β-1,4-Galactosylation of glycoproteins both in the control and the LPS-stimulated groups, and that SSeCKS knockdown significantly inhibited β-1,4-Galactosylation of glycoproteins only in LPS-stimulated astrocytes, but not in the normal group ($p = 0.033$ GalT-1 siRNA transfected with control, $p = 0.022$ LPS treated with untreated, $p = 0.023$ LPS treated non-specific siRNA transfected with control, $p = 0.043$ LPS treated SSeCKS siRNA transfected with control, $p = 0.044$ LPS treated SSeCKS siRNA transfected with un-treated SSeCKS siRNA transfected, $p = 0.038$ LPS treated GalT-1 siRNA transfected with un-treated GalT-1 siRNA transfected) (Fig. 2D, E, F).

Galactosylation of Integrin β1 was Affected by the Interaction Between SSeCKS and β-1,4-Galactosyltransferase-I in LPS-Treated Astrocytes

It is reported that the integrin β1, especially the glycosylated integrin β1, is important for the regulation of astrocyte behavior in CNS. Immunofluorescent staining showed that LPS increased integrin β1 co-localization with RCA-I in astrocytes and promoted its transport from the cell surface into the cell (Fig. 3A), which indicates that LPS could promote integrin β1 galactosylation and may be involved in the signal transmission from the ECM. To further investigate whether SSeCKS and β-1,4-GalT-I catalysis are associated with galactosylation of integrin β1 in LPS-treated astrocytes, we transfected SSeCKS and β-1,4-GalT-I siRNA into astrocytes. Immunoprecipitation followed by RCA-I staining results showed that knockdown of β-1,4-GalT-I inhibited the galactosylation of integrin β1 in astrocytes (Fig. 3B). Knockdown of SSeCKS could also attenuate β-1,4-galactosylation

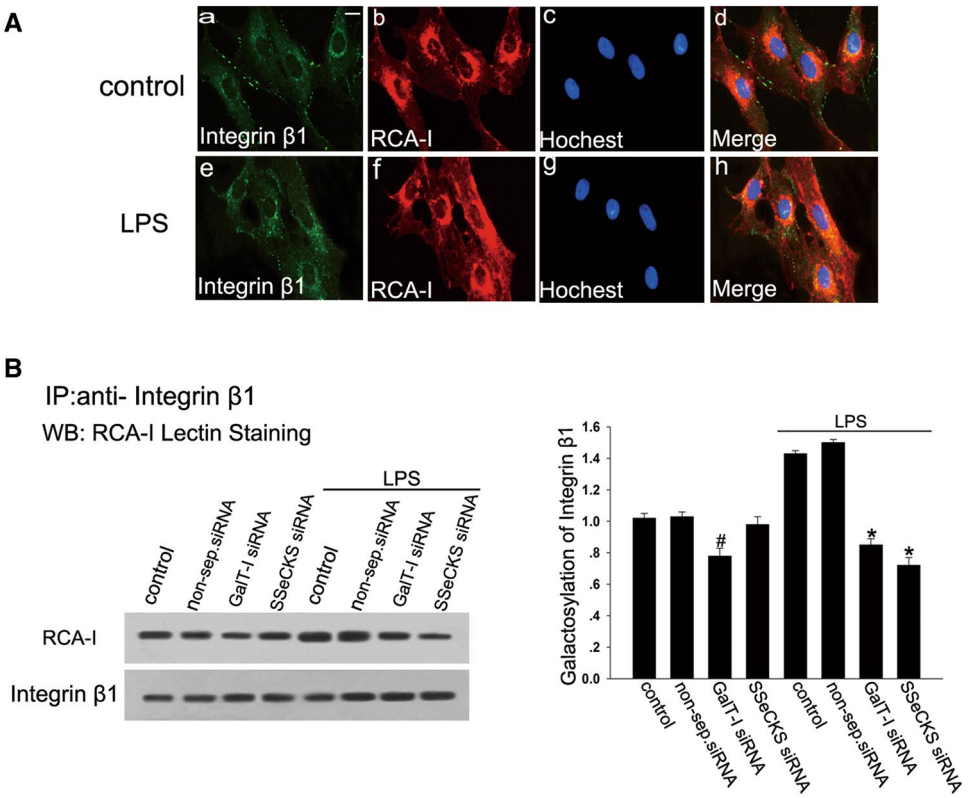

Fig. 3 Galactosylation of integrin β1 on primary astrocytes. **A** Double fluorescence staining was performed using RCA-I (red), and monoclonal antibody against integrin β1 antibodies (green). Pseudocolor in yellow indicates co-localization of RCA-I and integrin β1, which suggests that integrin β1 contains galactose residues. Astrocytes treated with and without 1 μg/mL of LPS, the control group (a–d), the LPS group (e–h). Three repetitive assessments were performed for each experiment. **B** Astrocytes were transfected with a non-specific siRNA, SSeCKS siRNA or β-1,4-GalT-I siRNA, and the integrin β1 was detected by immunoprecipitation followed by RCA-I lectin staining with and without LPS. Quantitative analysis of RCA lectin results in astrocytes was conducted three times for each experiment. Two-way ANOVA was used to compare the differences, and then pairwise comparisons when interactions are significant ($F = 5.342$). $^{\#}p < 0.05$ comparing with LPS untreated control astrocytes, $^{*}p < 0.05$ compared with LPS-treated astrocytes. Scale bars 20 μm

of integrin β1 in LPS-treated astrocytes, but not in the normal group (p = 0.043 GalT-1 siRNA transfected with control, p = 0.044 LPS treated GalT-1 siRNA transfected with un-treated GalT-1 siRNA transfected, p = 0.039 LPS treated SSeCKS siRNA transfected with un-treated SSeCKS siRNA transfected) (Fig. 3B).

SSeCKS Affects Cell Migration Through Functionally Regulating β-1,4-GalT-I in LPS-Treated Astrocytes

As the galactosylation of integrin β1 is affected by SSeCKS and β-1,4-GalT-I, we suspected that regulation of SSeCKS and β-1,4-GalT-I may lead to an alteration in the astrocyte's behavior. Using the wound repair assay with SSeCKS or β-1,4-GalT-I siRNA transfection, we found that cell migration was significantly inhibited in β-1,4-GalT-I—knockdown astrocytes with and without LPS (p = 0.023 24 h group with 0 h group, p = 0.022 24 h group with 0 h group during LPS treated, p = 0.033 GalT-I siRNA transfected 24 h group with GalT-I siRNA transfected 0 h, p = 0.034 GalT-I siRNA transfected 24 h group with GalT-I siRNA transfected 0 h during LPS treated) (Fig. 4). But the migration of SSeCKS deficient astrocytes was inhibited only when it was LPS-stimulated, but not in its normal status (p = 0.029 SSeCKS siRNA transfected 24 h group with SSeCKS siRNA transfected 0 h, p = 0.028 SSeCKS siRNA transfected 24 h group with SSeCKS siRNA transfected 0 h during LPS treated) (Fig. 4).

Based on previous studies and our current results, it is clear that SSeCKS could influence the migration of LPS-induced astrocytes, possibly through the regulation of β-1,4-GalT-I catalysis of β-1,4-galactosylation of glycoproteins which includes integrin β1.

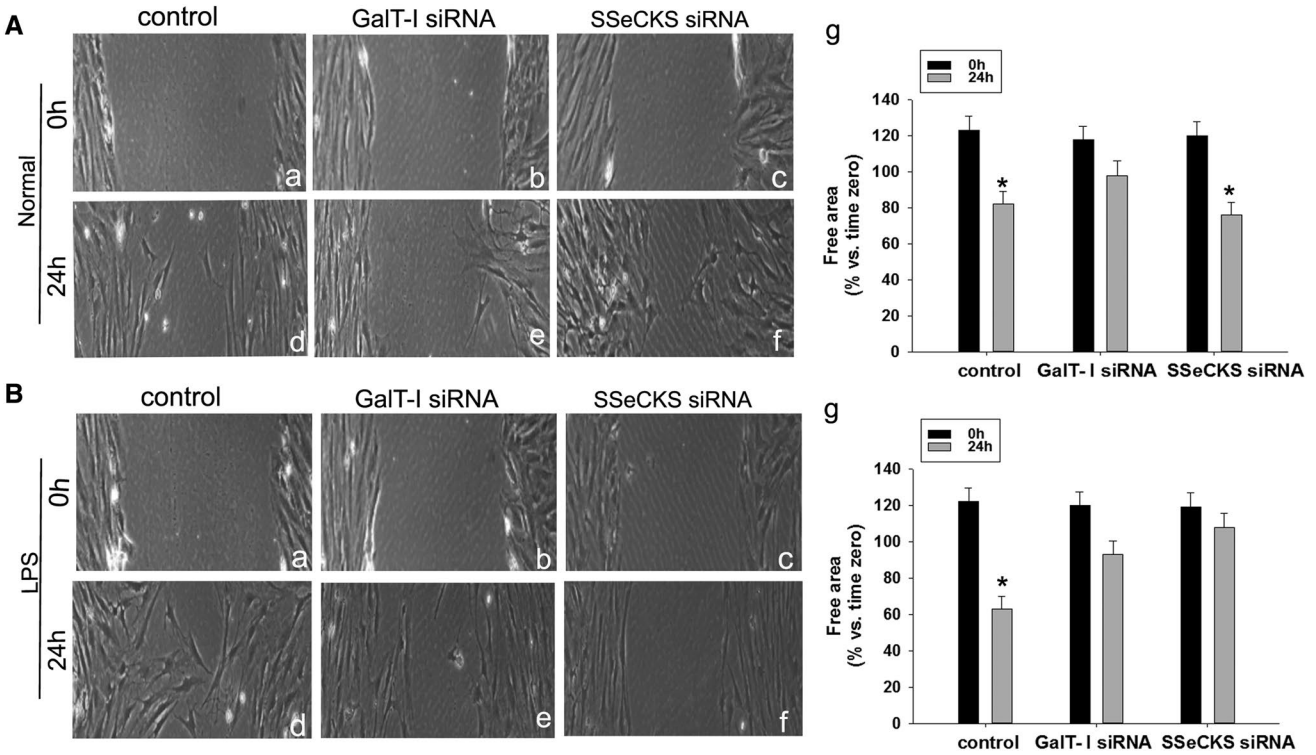

Fig. 4 Cell migration was influenced by knockdown of SSeCKS and β-1,4-GalT-I in LPS-stimulated astrocytes. Primary astrocytes monolayer was scratched with a pipette tip and the wound closure was monitored 24 h after scratching and was expressed as the percentage of the original wound margin distance (recorded at time point 0). Wound closure of the different groups in control astrocytes (**A**) and LPS-treated astrocytes (**B**). Control cells without LPS (**A**-a, f) and with LPS (**B**-a, f); Cells transfected with β-1,4-GalT-I siRNA without LPS (**A**-b, g) and with LPS (**B**-b, g); Cells transfected with SSeCKS siRNA without LPS (**A**-c, h) and with LPS (**B**-c, h). Scale bars 100 μm. **A**-g Quantitative analysis of wound closure in control astrocytes. **B**-g Quantitative analysis of wound closure in LPS-treated astrocytes. Cells of different groups were examined by phase contrast microscopy and wound areas were measured at 0 h and 24 h after scratching. Bars represent mean ± SE of the three to five independent experiments each run in triplicates. Three-way ANOVA was used to compare the differences, and then pairwise comparisons when interactions are significant (F = 5.221, F = 6.132). *indicated p < 0.05 vs control (0 h)

Discussion

Astrocytes migration is essential for the glial scar formation in response to CNS injury. This study provides insights into the possible mechanisms of the interaction among SSeCKS, β-1,4-GalT-I and integrin β1 in LPS-sensitized astrocytes migration. Our results demonstrated that the positive interactions between SSeCKS and β-1,4-GalT-I affected astrocytes behavior possibly through the regulation of β-1,4-Galactosylation of integrin β1 under LPS treatment (Fig. 5).

The yeast two-hybrid system has identified SSeCKS as a cytoplasmic partner for the β-1,4-GalT-I cytoplasmic domain, and SSeCKS also functions as a cytosolic effector associated with β-1,4-GalT-I, which elicits intracellular signal cascades [22]. In this study, the result of overexpression vector transfection experiment showed that an overexpression of SSeCKS could significantly up-regulate the β-1,4-GalT-I expression in LPS-stimulated astrocytes. Moreover, the co-immunoprecipitation test showed that SSeCKS can bind with β-1,4-GalT-I in both the normal and LPS treated astrocyte, and the binding was enhanced when LPS added.

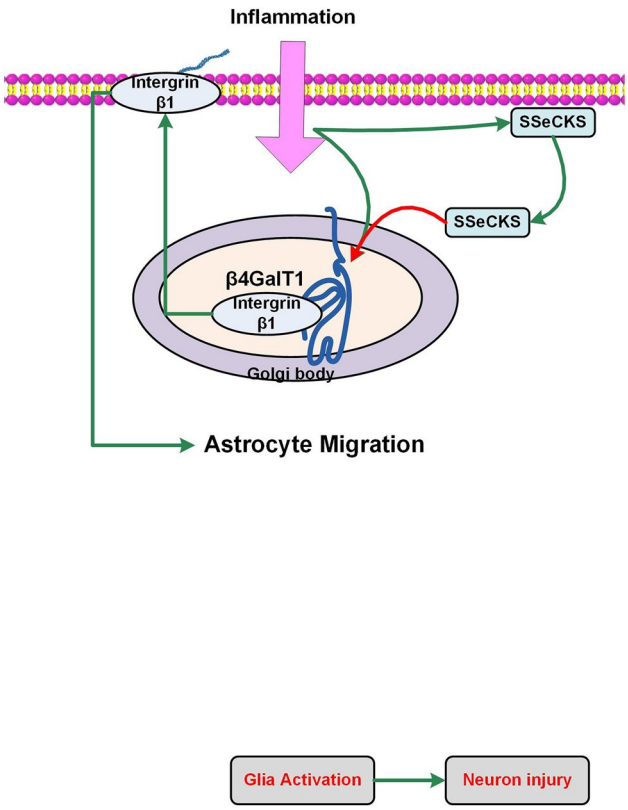

Fig. 5 The proposed model showing the interaction between SSeCKS andβ-1,4-GalT-I in LPS-induced astrocytes migration. In LPS induced astrocyte migration, SSeCKS can bind with β-1,4-GalT-I, which promotes β-1,4-GalT-I mediated β-1,4-Galactosylation of integrin β1

Our results indicated that the interaction between SSeCKS and β-1,4-GalT-I may be induced by LPS and play an essential role in astrocyte activation. PKC activation could also release SSeCKS from the cell surface to the perinuclear region [19]. Our study showed that both SSeCKS and β-1,4-GalT-I could translocate from the cell surface to the perinuclear region and into the TGN in LPS-induced astrocytes. As a scaffolding protein, SSeCKS is involved in the regulation of signaling molecules (e.g. PKC, Rho family members) to β-1,4-GalT-I, in order to influence β-1,4-GalT-I's ability to bind to the actin cytoskeleton [26, 27]. β-1,4-GalT-I is not only a type II membrane glycoprotein, but it also resides in the TGN where it is responsible for the galactosylation of complex glycoconjugates terminating in *N*-acetylglucosamine [28]. Our results demonstrated that the knockdown of SSeCKS could inhibit the β-1,4-Galactosylation of glycoproteins in LPS-stimulated astrocytes. Based on the above data, it is possible that SSeCKS regulates the transport of β-1,4-GalT-I into the TGN, then affects the β-1,4-Galactosylation of glycoproteins in LPS-induced astrocytes.

It is reported that the rate of cell migration on basal lamina matrices was directly dependent upon surface GalTase expression [29]. Moreover, a previous study also found that SSeCKS could regulate the various signaling and cytoskeletal functions associated with β-1,4-GalT-I, and SSeCKS and β-1,4-GalT-I in the cell surface have a coordinating function to cell spread [22]. To better understand the relationship among SSeCKS, β-1,4-GalT-I, and ECM-mediated signaling in LPS-induced astrocytes, we focused our study on the role of the integrin β1 subunit that is part of a family of cell surface ECM receptor molecules in the CNS, which is known to regulate the behavior of many cell types [30, 31]. It is suggested that the conditional deletion of integrin-β1 containing heterodimers during brain development led to defects in glial differentiation and cell migration [32]. Tomoya Isaji et al. revealed that the *N*-glycosylation on the I-like domain of the ββ1 subunit is essential to both heterodimer formation and biological functions, such as cell spreading [33]. Guo et al. also reported that an increase in β-1–6-GlcNAc sugar chains on the integrin β1 subunit stimulated cell migration [11]. Therefore, these studies strongly support the notion that the glycosylation of integrin β1 plays a vital role in cell migration and cell spreading. Our data interestingly showed that SSeCKS affects the galactosylation of integrin β1 and the migration of astrocytes through functionally regulating β-1,4-galactosyltransferase-I catalysis in LPS-induced astrocytes inflammation.

In conclusion, our study clearly found that the interaction between SSeCKS and β-1,4-GalT-I in the TGN influences the galactosylation of glycoproteins including integrin β1, which may affect LPS-induced astrocytes migration. This study provides further insights into the complex mechanisms associated with astrocyte activity. The interactions of the

complex SSeCKS and β-1,4-GalT-I with the β1-integrin subunit is important for astrocytes to acquire ECM-mediated signaling, which may greatly facilitate further studies of the interaction between SSeCKS and β-1,4-GalT-I and the functional regulation of astrocytes.

Acknowledgements This work was supported by the National Natural Science Foundation of China (Grant No. 31600402), China Postdoctoral Science Fund (Grant No. 2017M621895), Zhejiang Province Postdoctoral Research Fund (Grant No. ZX2016000849), Ningbo Postdoctoral Research Fund (Grant No. ZX2017000053), Natural Science Foundation of Ningbo (Grant No. 2017A610216, 2016A610205), Natural Science Foundation of Zhejiang Province (Grant No. Y16H070001), Research Project of Zhejiang Provincial Department of Education (Y201738586), Agricultural Project of Public Welfare Technology Research in Zhejiang Provincial Science and Technology Department (Grant No. ZX2014C32047).

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

References

1. Kniss DA, Burry RW (1988) Serum and fibroblast growth factor stimulate quiescent astrocytes to re-enter the cell cycle. Brain Res 439(1–2):281–288. doi:0006-8993(88)91485-0 [pii]
2. Ridet JL, Malhotra SK, Privat A, Gage FH (1997) Reactive astrocytes: cellular and molecular cues to biological function. Trends Neurosci 20(12):570–577. doi:S0166-2236(97)01139-9 [pii]
3. Kuchler-Bopp S, Delaunoy JP, Artault JC, Zaepfel M, Dietrich JB (1999) Astrocytes induce several blood-brain barrier properties in non-neural endothelial cells. Neuroreport 10(6):1347–1353
4. Rakic P (1971) Neuron-glia relationship during granule cell migration in developing cerebellar cortex. A Golgi and electronmicroscopic study in *Macacus Rhesus*. J Comp Neurol 141(3):283–312. https://doi.org/10.1002/cne.901410303
5. Tao-Cheng JH, Nagy Z, Brightman MW (1987) Tight junctions of brain endothelium in vitro are enhanced by astroglia. J Neurosci 7(10):3293–3299
6. Brakebusch C, Fassler R (2005) beta 1 integrin function in vivo: adhesion, migration and more. Cancer Metastasis Rev 24(3):403–411. https://doi.org/10.1007/s10555-005-5132-5
7. Takada Y, Ye X, Simon S (2007) The integrins. Genome Biol 8 (5):215. https://doi.org/10.1186/gb-2007-8-5-215. doi: gb-2007-8-5-215 [pii]
8. Vogelezang M, Forster UB, Han J, Ginsberg MH, ffrench-Constant C (2007) Neurite outgrowth on a fibronectin isoform expressed during peripheral nerve regeneration is mediated by the interaction of paxillin with alpha4beta1 integrins. BMC Neurosci 8:44. https://doi.org/10.1186/1471-2202-8-44. doi: 1471-2202-8-44
9. Morgan MR, Humphries MJ, Bass MD (2007) Synergistic control of cell adhesion by integrins and syndecans. Nat Rev Mol Cell Biol 8(12):957–969 https://doi.org/10.1038/nrm2289. doi: nrm2289 [pii]
10. Peng H, Shah W, Holland P, Carbonetto S (2008) Integrins and dystroglycan regulate astrocyte wound healing: the integrin beta1 subunit is necessary for process extension and orienting the microtubular network. Dev Neurobiol 68(5):559–574. https://doi.org/10.1002/dneu.20593
11. Guo HB, Lee I, Kamar M, Akiyama SK, Pierce M (2002) Aberrant N-glycosylation of beta1 integrin causes reduced alpha5beta1 integrin clustering and stimulates cell migration. Cancer Res 62(23):6837–6845
12. Kienzle C, von Blume J (2014) Secretory cargo sorting at the trans-Golgi network. Trends in Cell Biol 24(10):584–593. https://doi.org/10.1016/j.tcb.2014.04.007
13. Weingarten S, Thiem J (2004) Formation of LacNAc mimetics employing novel donor substrates for enzymatic beta 1→4 galactosylation. Org Biomol Chem 2(7):961–962. https://doi.org/10.1039/b400916a
14. Owatworakit A, Townsend B, Louveau T, Jenner H, Rejzek M, Hughes RK, Saalbach G, Qi X, Bakht S, Roy AD, Mugford ST, Goss RJ, Field RA, Osbourn A (2013) Glycosyltransferases from oat (Avena) implicated in the acylation of avenacins. J Biol Chem 288(6):3696–3704. https://doi.org/10.1074/jbc.M112.42615. 5M112.426155 [pii]
15. Asano M, Furukawa K, Kido M, Matsumoto S, Umesaki Y, Kochibe N, Iwakura Y (1997) Growth retardation and early death of beta-1,4-galactosyltransferase knockout mice with augmented proliferation and abnormal differentiation of epithelial cells. EMBO J 16(8):1850–1857. https://doi.org/10.1093/emboj/16.8.1850
16. Chui D, Oh-Eda M, Liao YF, Panneerselvam K, Lal A, Marek KW, Freeze HH, Moremen KW, Fukuda MN, Marth JD (1997) Alpha-mannosidase-II deficiency results in dyserythropoiesis and unveils an alternate pathway in oligosaccharide biosynthesis. Cell 90(1):157–167. doi:S0092-8674(00)80322-0 [pii]
17. Yoshida A, Kobayashi K, Manya H, Taniguchi K, Kano H, Mizuno M, Inazu T, Mitsuhashi H, Takahashi S, Takeuchi M, Herrmann R, Straub V, Talim B, Voit T, Topaloglu H, Toda T, Endo T (2001) Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1. Dev Cell 1(5):717–724. doi:S1534-5807(01)00070-3 [pii]
18. Yan M, Xia C, Niu S, Shao X, Cheng C, Zhao J, Shen A (2007) The role of TNF-alpha and its receptors in the production of beta-1,4 galactosyltransferase I and V mRNAs by rat primary astrocytes. J Mol Neurosci 33(2):155–162. doi:JMN:33:2:155 [pii]
19. Lin X, Tombler E, Nelson PJ, Ross M, Gelman IH (1996) A novel src- and ras-suppressed protein kinase C substrate associated with cytoskeletal architecture. J Biol Chem 271(45):28430–28438
20. Gelman IH (2012) Suppression of tumor and metastasis progression through the scaffolding functions of SSeCKS/Gravin/AKAP12. Cancer Metastasis Rev 31(3–4):493–500. https://doi.org/10.1007/s10555-012-9360-1
21. Sun LL, Cheng C, Liu HO, Shen CC, Xiao F, Qin J, Yang JL, Shen AG (2007) Src suppressed C kinase substrate regulates the lipopolysaccharide-induced TNF-alpha biosynthesis in rat astrocytes. J Mol Neurosci 32(1):16–24. doi:JMN:32:1:16 [pii]
22. Wassler MJ, Foote CI, Gelman IH, Shur BD (2001) Functional interaction between the SSeCKS scaffolding protein and the cytoplasmic domain of beta1,4-galactosyltransferase. J Cell Sci 114(Pt 12):2291–2300
23. Shao B, Li C, Yang H, Shen A, Wu X, Yuan Q, Kang L, Liu Z, Zhang G, Lu X, Cheng C (2011) The relationship between Src-suppressed C kinase substrate and beta-1,4 galactosyltransferase-I in the process of lipopolysaccharide-induced TNF-alpha secretion in rat primary astrocytes. Cell Mol Neurobiol 31(7):1047–1056. https://doi.org/10.1007/s10571-011-9704-3
24. Stadlin A, Tsang D, MacDonall JS, Mahadik SP, Karpiak SE (1992) An in vitro study on increased neuronal and astrocytic vulnerability to neurotoxic injury after in utero cocaine exposure: the reversal effects of GM1 treatment. Prog Brain Res 94:339–350
25. Zou XH, Foong WC, Cao T, Bay BH, Ouyang HW, Yip GW (2004) Chondroitin sulfate in palatal wound healing. J Dent Res 83(11):880–885. doi:83/11/880 [pii]
26. Barry ST, Critchley DR (1994) The RhoA-dependent assembly of focal adhesions in Swiss 3T3 cells is associated with increased

tyrosine phosphorylation and the recruitment of both pp125FAK and protein kinase C-delta to focal adhesions. J Cell Sci 107(Pt 7):2033–2045
27. Burnworth B, Pippin J, Karna P, Akakura S, Krofft R, Zhang G, Hudkins K, Alpers CE, Smith K, Shankland SJ, Gelman IH, Nelson PJ (2012) SSeCKS sequesters cyclin D1 in glomerular parietal epithelial cells and influences proliferative injury in the glomerulus. Lab Invest 92(4):499–510. https://doi.org/10.1038/labinvest.2011.199. labinvest2011199 [pii]
28. Amado M, Almeida R, Schwientek T, Clausen H (1999) Identification and characterization of large galactosyltransferase gene families: galactosyltransferases for all functions. Biochim Biophys Acta 1473(1):35–53. doi:S0304-4165(99)00168-3 [pii]
29. Appeddu PA, Shur BD (1994) Molecular analysis of cell surface beta-1,4-galactosyltransferase function during cell migration. Proc Natl Acad Sci USA 91(6):2095–2099
30. Adams JC, Watt FM (1993) Regulation of development and differentiation by the extracellular matrix. Development 117(4):1183–1198
31. Hynes RO (1992) Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69(1):11–25. doi:0092-8674(92)90115-S [pii]
32. King SJ, Worth DC, Scales TM, Monypenny J, Jones GE, Parsons M (2011) beta1 integrins regulate fibroblast chemotaxis through control of N-WASP stability. EMBO J 30(9):1705–1718. https://doi.org/10.1038/emboj.2011.82. emboj201182 [pii]
33. Isaji T, Sato Y, Fukuda T, Gu J (2009) N-glycosylation of the I-like domain of beta1 integrin is essential for beta1 integrin expression and biological function: identification of the minimal *N*-glycosylation requirement for alpha5beta1. J Biol Chem 284(18):12207–12216. https://doi.org/10.1074/jbc.M807920200 M807920200 [pii]