Glycobiology vol. 21 no. 8 pp. 1010–1018, 2011
doi:10.1093/glycob/cwr020
Advance Access publication on March 2, 2011

Polysialylation promotes neural cell adhesion molecule-mediated cell migration in a fibroblast growth factor receptor-dependent manner, but independent of adhesion capability

Jing Li[2,†], Gong Dai[2,†], Ya-Bin Cheng[2], Xin Qi[2], and Mei-Yu Geng[1,2,3]

[2]Department of Pharmacology and Glycobiology, School of Medicine and Pharmacy, Ocean University of China, 5 Yushan Road, Qingdao 266003, PR China and [3]Division of Anti-tumor Pharmacology, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, PR China

Received on August 27, 2010; revised on January 9, 2011; accepted on February 7, 2011

Polysialic acid (PSA), a carbohydrate polymer mainly present in the neural cell adhesion molecule (NCAM), promotes neural plasticity; however, its mode of action in tumor malignancy remains largely unknown. In this study, we investigated the influence of polysialylation on cell migration. PSA consistently promoted cell migration on different extracellular matrices (ECMs) but differentially affected cell adhesion. All of these actions were reversed by endo-*N*-acetylneuraminidase treatment, and PSA-driven migration was inhibited by the specific fibroblast growth factor receptor (FGFR) inhibitor Su5402. Consistent with this latter observation, PSA-stimulated migration on different ECMs was paralleled by activation of the FGFR and its downstream signaling components, PLC-γ, focal adhesion kinase and extracellular signal-regulated kinase 1/2. In contrast, the pattern of $p59^{fyn}$ activation correlated with differential adhesion to different ECMs. Collectively, these results indicate that PSA-conjugated NCAM potentiates signal transduction by the FGFR pathway and thereby enhances cell migration independent of adhesion capability, providing additional insights into the role of PSA in cancer development.

Keywords: adhesion / FGFR / migration / NCAM / PSA

Introduction

The neural cell adhesion molecule (NCAM) is an abundant integral membrane glycoprotein that can promote neuron cell-to-cell adhesion through a homophilic binding mechanism (Rutishauser et al. 1983). NCAM occurs as three major isoforms: two transmembrane isoforms, NCAM-180 and NCAM-140, with apparent molecular weights of 180 and 140 kDa, respectively, and a glycosylphosphatidyl-inositol-anchored isoform, NCAM-120, with an apparent molecular weight of 120 kDa (Kiselyov et al. 2005). The most prominent posttranslational modification of NCAM is the attachment of polysialic acid (PSA), a homopolymer of α-2,8-linked sialic acid residues, that is added to specific *N*-glycan attachment sites in the fifth immunoglobulin-like domain of NCAM (Mühlenhoff et al. 1998). Two enzymes, the polysialyltransferases (PSTs) ST8SiaII (STX) and ST8SiaIV, are known to be involved in the polysialylation of NCAM. PSA modifications are abundant during embryonic development and are downregulated in the course of maturation and differentiation (Crossin and Krushel 2000). In the developing nervous system, polysialylated NCAM (PSA-NCAM) has been shown to promote plasticity of cell–cell interactions during cell migration and neurite outgrowth (Rutishauser and Landmesser 1996; Durbec and Cremer 2001).

In addition to the nervous tissue, PSA-NCAM is expressed in malignant tumors, including neuroblastomas, small-cell lung cancers, nonsmall-cell lung cancer and Wilms' tumor, where its expression is closely related to metastatic potential (Jimbo et al. 2001). In human pituitary tumors, expression of PSA-NCAM is highly correlated with tumor invasion and confirms the clinical diagnosis of aggressiveness (Trouillas et al. 2003). The frequency of upregulated polysialylation activity is highest in diffuse astrocytomas, which spread extensively, and appears to be associated with disease recurrence (Suzuki et al. 2005). Given that PSA attenuates NCAM-mediated adhesion of neural cells, it has been assumed that PSA in tumor cells facilitates the detachment and metastatic migration of tumor cells from the primary sites. However, the underlying mechanisms have not yet been fully elucidated.

The aim of this study was to elucidate the role of PSA in cell metastasis and to investigate the machinery involved in

[1]To whom correspondence should be addressed: Tel: +86-21-50806072; Fax: +86-21-50806072; e-mail: mygeng@mail.shcnc.ac.cn
[†]Both authors contributed equally to this work.

detail. We found that PSA-NCAM promoted cell migration via a fibroblast growth factor receptor (FGFR)-dependent mechanism mediated by focal adhesion kinase (FAK) and extracellular signal-regulated kinase 1/2 (ERK1/2), and did so independent of its effects on cell adhesion.

Results

High levels of PSA trigger alteration in cell behavior

PSA-negative, NCAM-positive murine NIH-3T3 cells (Scheidegger et al. 1995) were transfected with a cDNA encoding the polysialyltransferase ST8SiaII (pcDNA3.1-STX-v5); cells transfected with an empty vector (pcDNA3.1-v5) were used as a mock transfection control. Indirect immunofluorescence analyses demonstrated that STX was stably expressed in NIH-3T3-STX cells (Figure 1A). Direct flow cytometry analyses using a PSA antibody mimic, a catalytically inactive endosialidase fused to green fluorescent protein which is known to bind but not degrade PSA (Jokilammi et al. 2004), revealed that PSA was generated in NIH-3T3-STX cells but not in NIH-3T3-mock cells (Figure 1B). Western blotting further demonstrated that STX catalyzed PSA modification of all three NCAM isoforms, as obvious tailing bands, the character of electrophoresis band for molecules of glycosylation modification, were observed with NCAM antibody. In contrast, endo-N, which specifically hydrolyzes PSA, decreased the tailing of the bands (Figure 1C). To determine whether cell–matrix interactions were affected by stable expression of PSA, we investigated the adhesion, migration and invasion properties of transfected cells. As shown in Figure 2, NIH-3T3-STX cells exhibited a significant decrease in adhesion to Matrigel and an increase in migratory and invasive capacity when compared with NIH-3T3-mock cells. Treatment with endo-N significantly reversed the increase in invasive capacity.

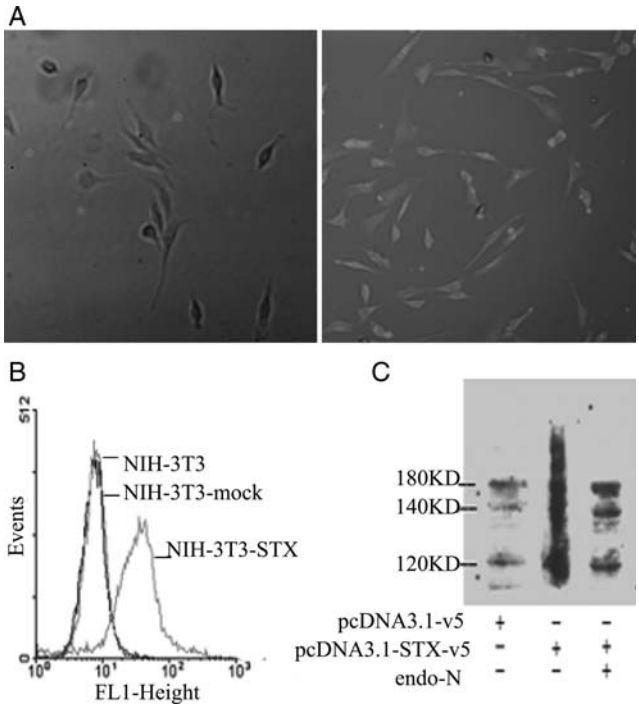

Fig. 1. Detection of NCAM and PSA in NIH-3T3 cells and NIH-3T3 cells transfected with STX cDNA. (**A**) Indirect immunofluorescence analysis of NIH-3T3-STX cells using an anti-V5 antibody. (**B**) Cells were stained with an antibody mimic specific for PSA and subjected to a flow cytometric analysis. (**C**) The cells were subjected to western blotting analysis before (−) and after (+) endo-N treatment. NCAM were detected with the anti-NCAM antibody.

High levels of PSA differentially modulate adhesion to different extracellular matrix substrates

Cell migration is presumed to depend on adhesion to the surrounding extracellular matrix (ECM; Palecek et al. 1997). The ECM is a complex structural entity surrounding and supporting cells, which is composed of three major classes of biomolecules: structural proteins (e.g., collagen), specialized proteins (e.g., fibronectin, laminin) and proteoglycans. To investigate the mechanism underlying the promotion of migration by PSA, we evaluated cellular adhesion to the ECM components, specifically heparin, fibronectin (FN), vitronectin (VN), laminin (LN), collagen IV and Matrigel using a spot cell-adhesion assay; bovine serum albumin (BSA) was used as a control substrate. We found that NIH-3T3-mock cells were highly adherent to FN, moderately adherent to VN, LN and collagen IV, and poorly adherent to heparin. In NIH-3T3-STX cells, adhesion to heparin was increased and adhesion to FN was decreased when compared with NIH-3T3-mock cells, whereas adhesion to VN, LN and collagen IV was unchanged. For further confirmation, we introduced endo-N. We found that endo-N treatment significantly abrogated PSA-mediated alteration in adherence properties (Figure 3). Collectively, these observations indicate a distinct role for PSA in cellular adhesion to different ECM components.

PSA promotes cell migration on different ECM substrates

The turnover of adhesions (assembly and disassembly) is believed to be critical for effective migration of most cells (Horwitz and Webb 2003). Because PSA differentially affected cellular adhesion to different ECM substrates, we presumed that it might also differentially affect migration on different ECMs. To examine this, we measured the motility of NIH-3T3-STX and NIH-3T3-mock cells on FN, VN and heparin. In striking contrast to the differential effects of PSA on adhesion to these three substrates, the effects of PSA on migration on these substrates were similar. Thus, NIH-3T3-STX cells exhibited a similarly marked enhancement of migration on each of these three ECMs compared with NIH-3T3-mock cells. As shown in Figure 4, the distances migrated by NIH-3T3-STX group cells were much greater at all time points tested (6, 12 and 24 h) than those in NIH-3T3-mock control groups. Conversely, the endo–N treatment inhibited the migration of NIH-3T3-STX cells. Together, these results suggest that the cell migration-promoting effects of PSA might not strictly depend on the degree of adhesion to the ECM.

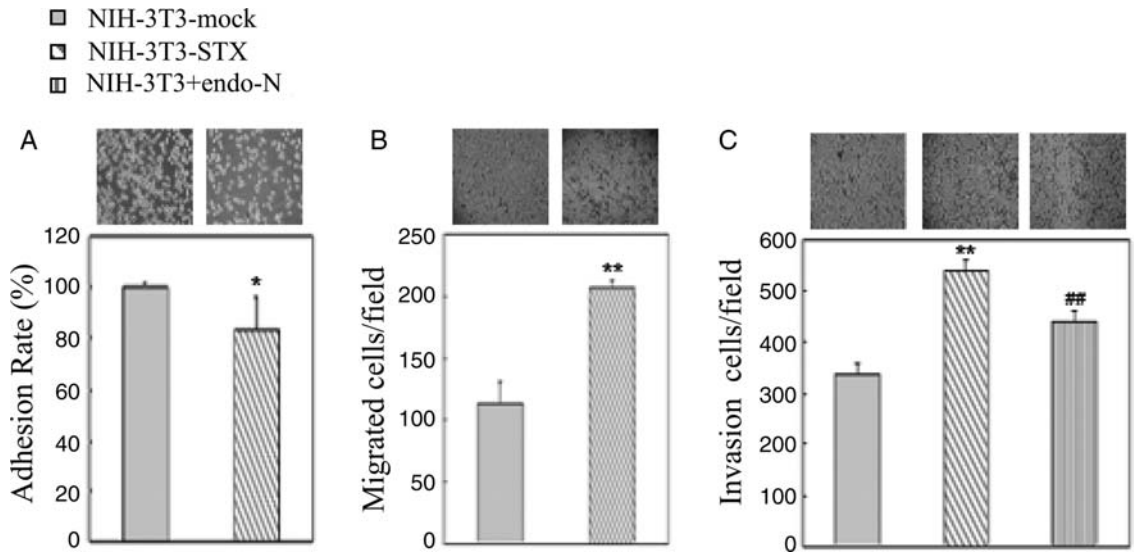

Fig. 2. Cell adhesion, migration and invasion behavior of NIH-3T3-STX and NIH-3T3-mock cells. Each sample was assayed in triplicate, and experiments were repeated at least twice with similar results. Student's *t*-tests were performed to determine the statistical significance (*$P < 0.05$, **$P < 0.01$ vs. NIH-3T3-mock cells; $^{\#}P < 0.01$ vs. NIH-3T3-STX cells; $n = 3$). (**A**) Spot cell-adhesion assay comparing the adhesion rate of NIH-3T3-STX cells to Matrigel relative to that of NIH-3T3-mock cells (defined as 100%). (**B**) Transwell cell migration model evaluating the ability of cells to migrate to the lower side of the filter. (**C**) Transwell model assessing the ability of cells to invade through a Matrigel-coated membrane (**$P < 0.01$ vs. NIH-3T3-mock cells; $^{\#\#}P < 0.01$ vs. NIH-3T3-STX cells; $n = 3$).

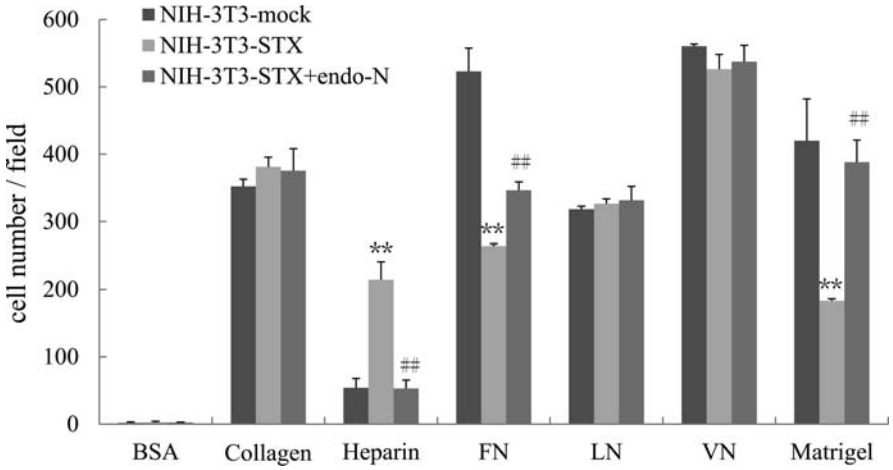

Fig. 3. Adhesion of NIH-3T3 cells to spots of heparin, FN, VN, LN, collagen IV, Matrigel or BSA solution was determined using the spot-adhesion assay (**$P < 0.01$ vs. NIH-3T3-mock cells; $^{\#\#}P < 0.01$ vs. NIH-3T3-STX cells; $n = 3$).

PSA modulates the pattern of FGFR, FAK, ERK1/2 and p59^{fyn} phosphorylation on different ECM substrates

NCAM has been shown to induce neurite outgrowth in an FGFR-dependent manner, a process accompanied by the activation of various signaling cascades, including the phospholipase C (PLC)-γ pathway and the mitogen-activated protein kinase (p42/44 MAPK) pathway (Kolkova et al. 2000). NCAM also associates with the signal-transducing molecules, FAK and the *src*-related tyrosine kinase p59^{fyn} (Beggs et al. 1997). To investigate the mechanisms involved in PSA-induced cell migration, we examined the effects of PSA on FGFR, FAK, ERK1/2 and p59^{fyn} phosphorylation in stably transfected NIH-3T3 cells grown on FN-, VN- or heparin-coated plates, respectively. Compared with NIH-3T3-mock cells, FGFR, FAK and ERK1/2 phosphorylation levels were all significantly enhanced in NIH-3T3-STX cells grown on each of the ECM substrates. In contrast, the pattern of p59^{fyn} phosphorylation in NIH-3T3-STX cells echoed PSA-triggered differences in cell adhesion: phospho-p59^{fyn} levels were upregulated in cells plated on heparin, downregulated in cells plated on FN and unchanged in cells plated on VN (Figure 5). Collectively, these results suggest that the cell migration-promoting effect of PSA may be mainly driven by activation of FGFR, FAK and ERK1/2, whereas

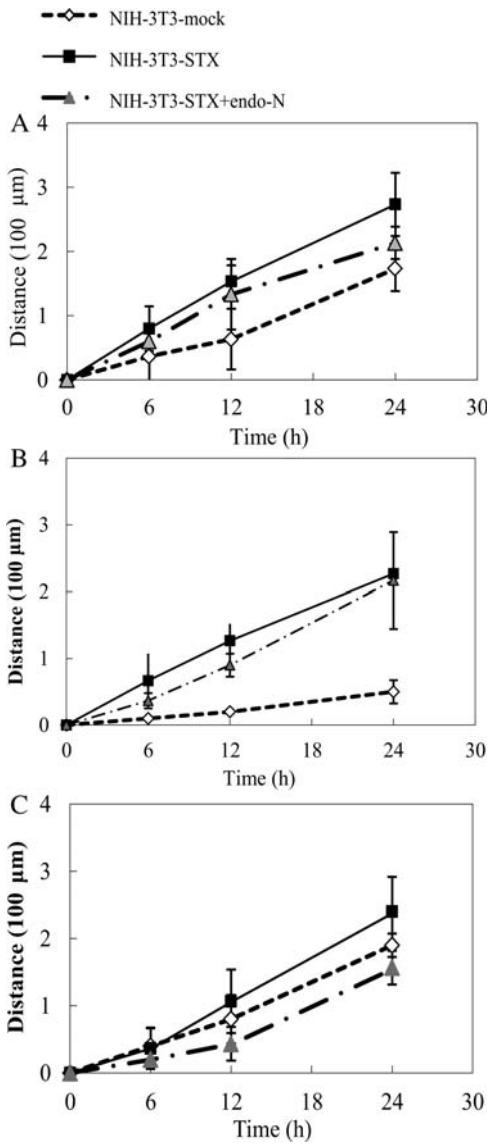

Fig. 4. PSA promotes migration of NIH-3T3 cells. The distances migrated by NIH-3T3-STX and NIH-3T3-mock groups were assessed after 6, 12 and 24 h in wound-healing assays, and compared with those of endo-N-treated cells. Each sample was assayed in triplicate and experiments were repeated at least twice with similar results. NIH-3T3 cells were plated on dishes coated with heparin (**A**), FN (**B**) or VN (**C**).

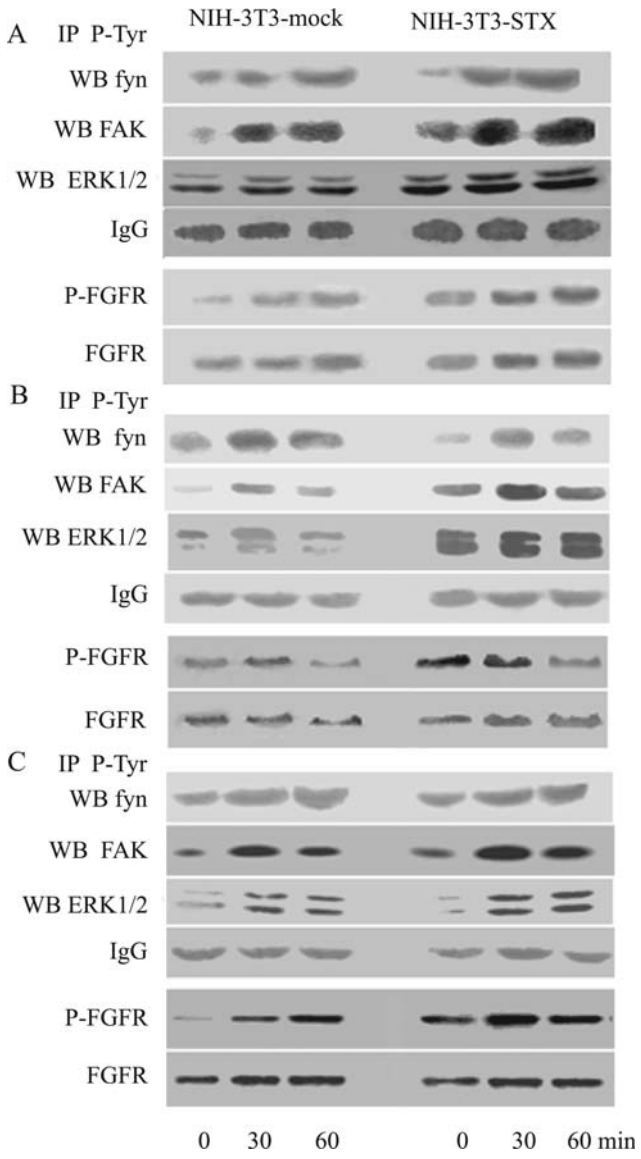

Fig. 5. Phosphorylation of FGFR, FAK, $p59^{fyn}$ and ERK1/2 in NIH-3T3-STX and NIH-3T3-mock cells plated on dishes coated with heparin (**A**), FN (**B**) or VN (**C**). Lysates were analyzed by western blotting using the indicated antibodies.

PSA-mediated cell adhesion is possibly related to activation of $p59^{fyn}$.

PSA-mediated cell migration is dependent on FGFR signaling

To confirm the role of FGFR signaling in PSA-triggered migration, we introduced Su5402, a specific FGFR inhibitor, into the experimental setting. Treatment with Su5402 (10 µM) caused a marked inhibition of NIH-3T3-STX cell migration, decreasing migration by 68, 53 and 57% at 6, 12 and 24 h, respectively. By comparison, migration-inhibition ratios for NIH-3T3-mock cells at 6, 12, 24 h were 27, 27 and 24%, respectively, again substantiating the involvement of the FGFR in PSA-mediated cell migration (Figure 6).

NCAM is involved in PSA-mediated cell migration

Polysialylation is positively correlated with tumor progression. In neural cells, PSA is primarily attached to *N*-glycans of NCAM (Martersteck et al. 1996). To establish that PSA-driven biological events are executed by PSA-NCAM, we used an African green monkey kidney cell line, COS-7 cells that are negative for NCAM and STX (PSA) (Scheidegger et al. 1995), to generate three cell lines, one overexpressing STX (COS-7-STX cells), one overexpressing NCAM (COS-7-Nv cells) and one concurrently

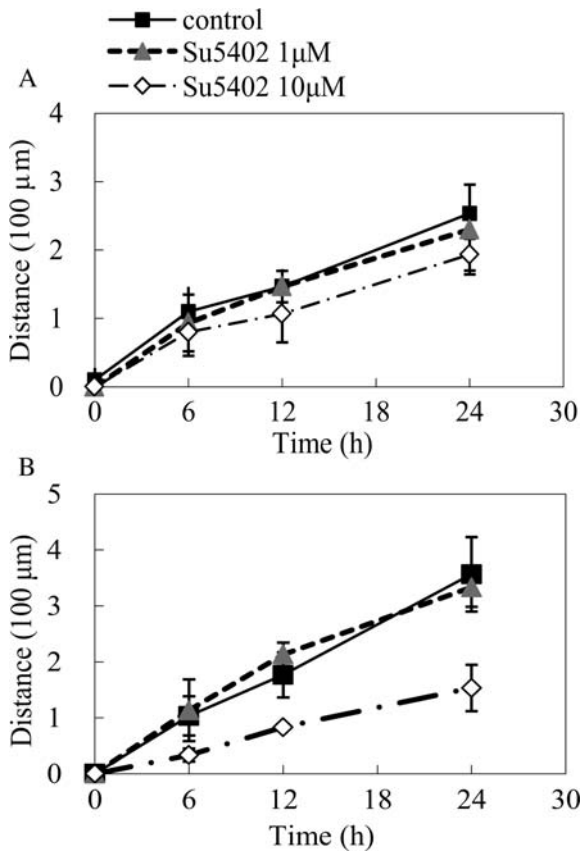

Fig. 6. Effects of the FGFR inhibitor Su5402 on the migration of NIH-3T3-mock cells (**A**) and NIH-3T3-STX cells (**B**) on dishes coated with FN.

overexpressing both STX and NCAM (COS-7-NS cells). Using an antimouse embryonic NCAM monoclonal antibody that recognizes the PSA on NCAM, we found that COS-7-NS cells highly expressed PSA-NCAM, and there was a electrophoresis band by western blotting analysis using the antimouse embryonic NCAM monoclonal antibody for COS-7-Nv cells (the antibody is likely to be directed at least in part toward the NCAM peptide). Similarly, there was a dim electrophoresis band for COS-7-STX cells due to the presence of a few autopolysialylated PSTs on the surface of overexpressing cells (Figure 7A). Flow cytometric analyses further indicated that PSA levels were much higher in COS-7-NS cells than in COS-7-STX and COS-7-Nv cells (Figure 7B), as detected by a PSA antibody mimic. These results indicate that PSA was mainly attached to NCAM.

Further functional analysis showed that adhesion of COS-7-NS cells to FN was significantly decreased compared with that of COS-7-Nv cells, which was consistent with the results obtained in NIH-3T3-STX cells. Interestingly, COS-7-Nv cells showed a remarkable increase in cell adhesion compared with COS-7-STX cells. Although COS-7-NS cells showed a kind of increase in migratory capacity compared with COS-7-STX cells (Figure 7C), however cell migration was increased to a greater degree in COS-7-NS cells possessing PSA and NCAM, indicating that NCAM is involved in PSA-mediated cell migration. In addition, the levels of phosphorylated FGFR, PLCγ, FAK and ERK1/2 were all significantly increased in COS-7-NS cells compared with COS-7-Nv cells and were lowest in COS-7-STX cells (Figure 7D). These results, which parallel those obtained in stably transfected NIH-3T3 cells, strongly suggest that NCAM is involved in PSA-mediated cell migration and further that the FGFR signaling pathway involves in PSA-promoted NCAM-mediated cell migration.

Discussion

It has been increasingly recognized recently that PSA is a positive modulator of tumor malignancy (Tanaka et al. 2000, 2001; Livingston et al. 2005; Suzuki et al. 2005; Fernández-Briera et al. 2010), but the underlying regulatory mechanisms have remained poorly understood. In this study, we provide the first demonstration that PSA-modified NCAM acts through an FGFR-dependent mechanism to play a critical role in cell migration.

It is generally accepted that adhesion of cells to the substratum is a prerequisite for cell migration; however, there are limits to this generalization. For instance, some studies have shown that there is no correlation between the migratory potential of cells and the strength of cell–substratum adhesion and have even suggested that some substrate molecules may act primarily to stimulate cell motility (Calof and Lander 1991). In this study, we found that NIH-3T3-STX cells, which express high levels of PSA, displayed similar migration-promoting capacities on different substrates, despite substantial differences in adhesion to heparin (high), VN, LN and collagen IV (moderate), and FN (poor). Moreover, COS-7 cells transiently transfected with NCAM (COS-7-Nv cells) showed increased adhesion to FN compared with STX-transfected COS-7 (COS-7-STX) cells. Upregulation of PSA in conjunction with NCAM expression (COS-7-NS cells) reversed the increased adhesion when compared with NCAM expression alone, but nonetheless promoted migration. These observations suggest that PSA-driven migration is independent of its ability to promote adhesion, supporting the idea that migration capacity is sometimes unrelated to the degree of adhesion.

There is now an emerging consensus that interactions of NCAM with the FGFR are important not only for neuronal function, but also for contact-dependent survival of some cell types (Peluso 2000; Erez et al. 2004). It has been shown that NCAM stimulation leads to phosphorylation of the FGFR with subsequent neurite outgrowth (through activation of the Ras-MAPK pathway), a process in which differentiating neurons extend axons and dendrites (Kolkova et al. 2000). The initial response of a cancer cell to a migration-promoting agent is to polarize and to extend protrusions in the direction of migration. These protrusions, which can take the form of large, broad lamellipodia or spike-like filopodia, are usually driven by actin polymerization (Kirfel et al. 2004). Indeed, Cavallaro et al. (2001) reported that $NCAM^{+/+}$ tumor cells, but not $NCAM^{-/-}$ tumor cells, extend long, thin protrusions that morphologically resemble neurites and showed that the neurite-like protrusions of tumor cells contain NCAM, neurofilament, N-cadherin and actin, which are all known markers

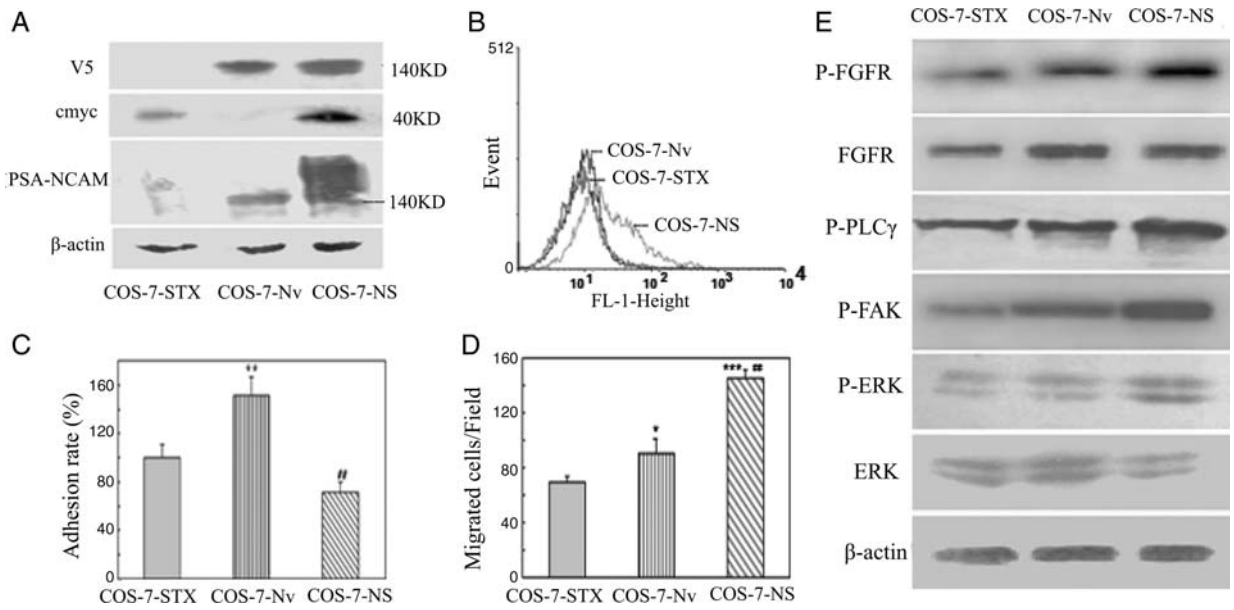

Fig. 7. NCAM is involved in PSA-mediated cell migration. COS-7 cells were transiently transfected with pcDNA3.1-cmyc-STX (COS-7-STX), pcDNA3.1-v5-NCAM (COS-7-Nv) or both pcDNA3.1-v5-NCAM and pcDNA3.1-cmyc-STX (COS-7-NS). (**A**) Lysates were probed by western blotting using anti-V5 antibody, anti-cmyc antibody and an antimouse embryonic NCAM monoclonal antibody. (**B**) COS-7 cells were stained with an antibody mimic specific for PSA and subjected to flow cytometric analyses. (**C**) Adhesion of COS-7-NS cells to FN compared with that of COS-7-Nv cells and COS-7-STX) (**$P < 0.01$ vs. COS-7-STX cells, $^{\#\#}P < 0.01$ vs. COS-7-Nv cells; $n = 3$). (**D**) Migratory ability of COS-7-Nv cells and COS-7-NS cells compared with COS-7-STX cells (*$P < 0.05$ vs. COS-7-STX cells, ***$P < 0.001$ vs. COS-7-STX cells, $^{\#\#}P < 0.01$ vs. COS-7-Nv cells; $n = 3$). (**E**) FGFR, PLCγ, FAK and ERK phosphorylation was assessed by western blotting using the indicated antibodies.

of neurite outgrowth. This suggests a mechanism similar to that in nerve cells, where in cancer cells NCAM appears to be able to act as a surrogate ligand for FGFRs and signal via these receptors independent of FGFRs, and this process is further facilitated by PSA. Indeed, PSA overexpression in NIH-3T3 cells increased phosphorylation of the FGFR in different ECM contexts (FN, VN and heparin). The fact that the FGFR inhibitor Su5402 greatly abrogated PSA-driven migration further favors such a story.

NCAM-activated FGFR pathway is important for neurite outgrowth through activation of $p59^{fyn}$ and its downstream molecule FAK (Beggs et al. 1997; Schmid et al. 1999). In our experiments, FAK phosphorylation was enhanced in NIH-3T3-STX cells compared with NIH-3T3-mock cells on all ECMs. Given FAK has been reported (Nurcombe et al. 2000) to be upregulated directly by FGFR1 or FGFR2, hence the increase in FAK phosphorylation was contributed to PSA-triggered FGFR activation. In contrast, phosphorylation of $p59^{fyn}$ was increased in cells plated on heparin, decreased in cells plated on FN and unchanged in cells plated on VN, a pattern that paralleled the differential adhesion of cells to these ECM components. This suggests that phosphorylation of $p59^{fyn}$ is involved in regulating adhesion activity and does not contribute to cell migration.

FAK-mediated phosphorylation can lead to activation of the Ras-ERK signaling cascade and promote cell migration and invasion (Schlaepfer and Mitra 2004). In the current study, we found that upregulation of polysialylation in NIH-3T3 cells increased phosphorylation of ERK1/2 in different ECMs (FN, VN or heparin). Taking the PSA-promoting cell migration on various ECM into consideration, our findings are in consistent with previous reports that the ERK pathway inhibitors PD98059 and U0126 inhibit the migration of diverse cell types in response to cell–matrix proteins, including FN, VN and collagen (Huang et al. 2004; Kim and Choi 2010). We conclude that increase of PSA in NIH-3T3 cells enhances NCAM-mediated phosphorylation of ERK1/2 and thus promotes cell migration.

The 2,8-polysialylation of glycoproteins is a relatively universal phenomenon (Martersteck et al. 1996). It is thus necessary to clarify whether PSA promotes migration involving in NCAM. Using three different PSA- and NCAM-expressing CHO cells, we found the critical roles of PSA on NCAM in promoting cell migration by subsequent activation of PLCγ, FAK and ERK1/2 in COS-7-Ns compared with COS-7-Nv cells. This notion is in striking contrast to the findings by Angata et al. (2007), in that they found that although PSA modifications on NCAM affect molecular interactions involved in neural development and synaptic plasticity, yet PSA rather than NCAM inhibits the onset of PDGF-induced differentiation of neurosphere cells to glial cell lineages. In this paper, we demonstrate in a different way that the PSA facilitates NCAM-mediated cell mobility. The discrepancy might be attributable to different molecules involved that are downstream to PSA-NCAM pathways. Of course, detailed mechanisms underlying this notion need further elucidation.

Klint and Claesson-Welsh (1999) reported that activation of FGFRs by tyrosine phosphorylation leads to signal transduction through multiple pathways, such as MAPK pathway, Src/

FAK-associated pathways and the PLC-γ/PKC pathway. From all of the above results, we believe that PSA facilitates activation of the FGFR signal transduction pathway, triggering subsequent activation of PLCγ, FAK and ERK1/2, and promoting cell migration. The association of PSA with NCAM, which facilitates the ligand-independent activation of the FGFR, appears to provide to cells an important platform for creating the signaling diversity that leads to cell migration on all ECM components despite differences in their adhesive capacities. In conclusion, we have identified potentiation of the NCAM/FGFR signaling pathway as a new mechanism involved in the regulation of PSA-induced migration in cancer metastasis, providing valuable insights into the molecular aspects of PSA in cell migration.

Materials and methods

Materials

All culture media were from Life Technologies, Inc (Carlsbad, CA). Fetal bovine serum (FBS) was from Hyclone Laboratories (Logan, UT). Erk1/2 kinase antibody, phospho-Erk1/2 kinase (Thr202/Tyr204) antibody, human Fyn (Ser25) polyclonal antibodies, phospho-FGF receptor (Tyr653/654) antibody, PLCγ and phospho-PLCγ (Tyr783) antibody, phospho-tyrosine mouse mAb (Sepharose Bead Conjugate) were all purchased from Cell Signaling Technology, Inc (Danvers, MA). Antifocal adhesion kinase (PP125FAK) and antiphospho-FAK (pTyr397) developed in rabbit were purchased from Sigma (St. Louis, MO). Antibody against FGFR-1 was from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). SU5402 were from Merck. Purified rat antimouse embryonic NCAM monoclonal antibody and purified rat antimouse CD56 were all purchased from BD Pharmingen™, Inc (San Diego, CA). Anti-V5 antibody and anti-cmyc-antibody were from Invitrogen (Carlsbad, CA).

Cells and cell culture

NIH-3T3 cells were obtained from the American Type Culture Collection (Rockville, MD). COS-7 cells were kindly provided by the Shanghai Institute of Biochemistry and Cell Biology (IBCB, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% (w/v) FBS (Gibco), 100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 2 mM glutamine. All cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Stable and transient transfections

NIH-3T3 cells stably expressing the *ST8SIA2* gene encoding human sialyltransferase X (STX), designated NIH-3T3-STX, were prepared by transfecting with pcDNA3.1-STX-v5 (a kind gift from Kristin Geiger) followed by selection with G418. NIH-3T3 cells transfected with pcDNA3.1 (negative control), termed NIH-3T3-mock cells, were also prepared in the same manner.

PSA-NCAM was transiently expressed in COS-7 cells (COS-7-NS) by transfecting with pcDNA3.1-v5-NCAM and pcDNA3.1-cmyc-STX. As negative controls, COS-7 cells were transfected with pcDNA3.1-v5-NCAM (COS-7-Nv) or pcDNA3.1-cmyc-STX (COS-7-STX) expression plasmids alone. All transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Cell adhesion assay

The spot cell-adhesion assay has been described previously (Murray and Jensen 1992; Storms, Anvekar, Adams et al. 1996; Storms, Kim, Tran, Cole 1996). Briefly, 0.25-mL aliquots of nitrocellulose solution (5 cm^2 nitrocellulose in 6 mL of methanol) were applied to 3-cm tissue culture plates and allowed to air dry for 30 min. Then, 2 mL spots of substrate solutions (0.5 mg per spot) were applied to nitrocellulose and then incubated for 1 h. Each plate had about 10 spots, and the spots were labeled on the reverse side of the plates for identification. The nitrocellulose was blocked for 1 h with BSA solution [10 mg mL^{-1} in phosphate-buffered saline (PBS)], then the plates were washed twice with PBS.

Transfected HeLa cells treated with or without 600 ng mL^{-1} purified endo–N prepared as described (El Maarouf and Rutishauser 2003) for 4 h were then incubated in 0.1 mM EDTA for 5 min, after which cells were distributed to appropriate experimental dishes (2 mL per dish) and incubated in a tissue culture incubator for 1 h. Experimental dishes were washed twice and the cells remaining in contact with each substrate spot were counted using an inverted phase-contrast microscope. Each experiment was repeated at least three times.

For transiently transfected COS-7 cells, 96-well microtiter plates were first coated with 0.1 mL of 10 μg mL^{-1} FN, incubated at 4°C overnight, and then blocked with 1% BSA by incubating at 37°C for 1 h. Cells were added to each coated well and incubated for 30 min at 37°C. The wells were then washed gently with PBS to remove unattached cells. Adherent cells were fixed with 5% glutaraldehyde and stained with 0.1% crystal violet. Bound dye was released with 100 μL 10% (v/v) acetic acid and monitored by measuring absorbance at 595 nm.

Transwell cell migration assays

Migration of cells was evaluated using a transwell Boyden chamber (Costar, Bedford, MA). Cells were seeded into the upper compartment of each well (5×10^4 cells per well) in serum-free DMEM. The lower compartment contained 600 μL of culture media supplemented with 10% FBS. After a 6-h incubation at 37°C, cells were fixed and stained with 0.1% crystal violet. Nonmigrating cells on the upper surface of the filter were removed, and stained cells that had migrated to the lower side were photographed and counted under a phase-contrast microscope (Olympus, Japan). Data are the average of five randomly chosen fields.

Wound healing assay

Cells were seeded onto ECM-coated 96-well plates at 1×10^4 cells per well. After reaching confluence, the cell monolayers were scratched with a pipette tip to obtain a "wounded" monolayer culture. The media and dislodged cells were aspirated and replaced by fresh media with or without

600 ng mL^{-1} endo-N and/or 10 μM FGFR inhibitor Su5402 (Merck). After incubation at 37°C, cell migration was photographed under a phase-contrast microscope. The distance migrated from the scratch line by cells at each time point was then measured.

Cell invasion assays

A 20-μL aliquot of a 20% (v/v) Matrigel solution was applied to filters (8-μm pore size) and incubated at 37°C for 45 min. Cells were resuspended in DMEM supplemented with 0.1% FBS and added to the upper compartment of the invasion chamber; the lower compartment contained conditioned medium from NIH-3T3 cells. After incubation for 20 h at 37°C, invasion was determined by visualizing and counting the number of cells on the lower surfaces of the filter as described for the migration assay.

Immunoprecipitation and western blotting analyses

To analyze cellular kinase activation, immunoprecipitation or western blotting were performed alternatively. Transfected HeLa cells or COS-7 cells, grown to 80% confluence, were serum starved for 16–18 h. The cells were then digested and seeded onto ECM-coated 6-well plates at 2×10^6 cells per well for 30 or 60 min. The cells were rapidly washed with ice-cold PBS and lysed with ice-cold radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 50 mM Tris, pH 8.0, 20 mg mL^{-1} aprotinin, 2 mg mL^{-1} leupeptin, 1 mg mL^{-1} pepstatin, 2 mM phenylmethylsulfonyl fluoride, 1 mM sodiumorthovanadate) for 20 min at 4°C. Total cell lysates were sonicated for 5 s and clarified by centrifugation at 10,000 × *g* for 10 min. Protein concentrations were determined using Bradford assay. For the immunoprecipitation studies, identical amounts of protein from each sample were incubated for 2 h at 4°C with phospho-tyrosine mouse antibodies. The antibody–antigen complexes were immunoprecipitated by incubation for 2 h to overnight at 4°C with 20 mL of the protein A–agarose. Nonspecific proteins were removed by washing the agarose beads three times with the RIPA buffer and once with PBS. Bound proteins were solubilized in 40 mL of lysis buffer (62.5 mM Tris–HCl, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% bromophenol) and further analyzed by immunoblotting. Samples were separated by SDS–polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. The membranes were blocked with 5% BSA in Tris-buffered saline plus Tween-20 (TBS-T) and probed with primary antibody overnight at 4°C. Immunoreactive bands were visualized by using horseradish peroxidase-conjugated secondary antibody and the enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL).

Flow cytometry analysis

Cells were grown to subconfluence and detached with 2 mM EDTA in PBS. Cells (1×10^6) were washed and resuspended in 100 μL of fluorescence activated cell sorter (FACS) buffer (PBS containing 1% BSA and 0.01% sodium azide). Isolated cells were incubated for 30 min in the dark with a PSA antibody mimic, followed by three washes with FACS buffer. Cells were then analyzed using flow cytometry (Vantage, BD Biosciences).

Statistical analysis

Experiments were run in triplicate, unless otherwise stated. Date were analyzed by unpaired Student's *t*-test for comparison between two means. Differences were considered statistically significant at $P < 0.05$. *P*-values are indicated in the legend of each figure.

Funding

This work was supported by Program for Changjiang Scholars and Innovative Research Team in University (IRT0944), the Natural Science Foundation of China for Distinguished Young Scholars (30725046) and the State Plan for High-Tech Research and Development of China (2007AA09Z405).

Acknowledgements

We thank Dr. Kristin Geiger for the pcDNA3.1-stx-v5 plasmid. We would also like to thank Prof. Christopher Proud from University of Southampton for his proofreading and revision of the manuscript.

Conflict of interest

None declared.

Abbreviations

BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; ERK1/2, extracellular signal-regulated kinase 1/2; FAK, focal adhesion kinase; FBS, fetal bovine serum; FGFR, fibroblast growth factor receptor; FN, fibronectin; LN, laminin; MAPK, mitogen-activated protein kinase; NCAM, neural cell adhesion molecule; PBS, phosphate-buffered saline; PLCγ, phospholipase C-gamma; PSA, polysialic acid; PSA-NCAM, polysialylated NCAM; PST, polysialyltransferase; RIPA, radioimmunoprecipitation assay; SDS, sodium dodecyl sulfate; STX, sialyltransferase X; VN, vitronectin.

References

Angata K, Huckaby V, Ranscht B, Terskikh A, Marth JD, Fukuda M. 2007. Polysialic acid-directed migration and differentiation of neural precursors are essential for mouse brain development. *Mol Cell Biol*. 27 (19):6659–6668.

Beggs HE, Baragona SC, Hemperly JJ, Maness PF. 1997. NCAM140 interacts with the focal adhesion kinase p125(fak) and the SRC-related tyrosine kinase p59(fyn). *J Biol Chem*. 272(13):8310–8319.

Calof AL, Lander AD. 1991. Relationship between neuronal migration and cell–substratum adhesion: Laminin and merosin promote olfactory neuronal migration but are anti-adhesive. *J Cell Biol*. 115(3):779–794.

Cavallaro U, Niedermeyer J, Fuxa M, Christofori G. 2001. N-CAM modulates tumour-cell adhesion to matrix by inducing FGF-receptor signalling. *Nat Cell Biol*. 3(7):650–657.

Crossin KL, Krushel LA. 2000. Cellular signaling by neural cell adhesion molecules of the immunoglobulin superfamily. *Dev Dyn*. 218(2):260–279.

Durbec P, Cremer H. 2001. Revisiting the function of PSA-NCAM in the nervous system. *Mol Neurobiol*. 24(1–3):53–64.
El Maarouf A, Rutishauser U. 2003. Removal of polysialic acid induces aberrant pathways, synaptic vesicle distribution, and terminal arborization of retinotectal axons. *J Comp Neurol*. 460(2):203–211.
Erez N, Zamir E, Gour BJ, Blaschuk OW, Geiger B. 2004. Induction of apoptosis in cultured endothelial cells by a cadherin antagonist peptide: Involvement of fibroblast growth factor receptor-mediated signalling. *Exp Cell Res*. 294(2):366–378.
Fernández-Briera A, García-Parceiro I, Cuevas E, Gil-Martín E. 2010. Effect of human colorectal carcinogenesis on the neural cell adhesion molecule expression and polysialylation. *Oncology*. 78(3–4):196–204.
Horwitz R, Webb D. 2003. Cell migration. *Curr Biol*. 13(19):756–759.
Huang C, Jacobson K, Schaller MD. 2004. MAP kinases and cell migration. *J Cell Sci*. 117(20):4619–4628.
Jimbo T, Nakayama J, Akahane K, Fukuda M. 2001. Effect of polysialic acid on the tumor xenografts implanted into nude mice. *Int J Cancer*. 94 (2):192–199.
Jokilammi A, Ollikka P, Korja M, Jakobsson E, Loimaranta V, Haataja S, Hirvonen H, Finne J. 2004. Construction of antibody mimics from a non-catalytic enzyme-detection of polysialic acid. *J Immunol Methods*. 295(1–2):149–160.
Kim EK, Choi EJ. 2010. Pathological roles of MAPK signaling pathways in human diseases. *Biochim Biophys Acta*. 1802(4):396–405.
Kirfel G, Rigort A, Borm B, Herzog V. 2004. Cell migration: Mechanisms of rear detachment and the formation of migration tracks. *Eur J Cell Biol*. 83 (11–12):717–724.
Kiselyov VV, Soroka V, Berezin V. 2005. Structural biology of NCAM homophilic binding and activation of FGFR. *J Neurochem*. 94 (5):1169–1179.
Klint P, Claesson-Welsh L. 1999. Signal transduction by fibroblast growth factor receptors. *Front Biosci*. 4:165–177.
Kolkova K, Novitskaya V, Pedersen N, Berezin V, Bock E. 2000. Neural cell adhesion molecule-stimulated neurite outgrowth depends on activation of protein kinase C and the Ras–mitogen-activated protein kinase pathway. *J Neurosci*. 20(6):2238–2246.
Livingston PO, Hood C, Krug LM, Warren N, Kris MG, Brezicka T, Ragupathi G. 2005. Selection of GM2, fucosyl GM1, globo H and polysialic acid as targets on small cell lung cancers for antibody mediated immunotherapy. *Cancer Immunol Immunother*. 54(10):1018–1025.
Martersteck CM, Kedersha NL, Drapp DA, Tsui TG, Colley KJ. 1996. Unique alpha2,8-polysialylated glycoproteins in breast cancer and leukemia cells. *Glycobiology*. 6(3):289–301.
Mühlenhoff M, Eckhardt M, Gerardy-Schahn R. 1998. Polysialic acid: Three-dimensional structure, biosynthesis and function. *Curr Opin Struct Biol*. 8(5):558–564.
Murray BA, Jensen JJ. 1992. Evidence for heterophilic adhesion of embryonic retinal cells and neuroblastoma cells to substratum-adsorbed NCAM. *J Cell Biol*. 117(6):1311–1320.
Nurcombe V, Smart CE, Chipperfield H, Cool SM, Boilly B, Hondermarck H. 2000. The proliferative and migratory activities of breast cancer cells can be differentially regulated by heparan sulfates. *J Biol Chem*. 275 (39):30009–30018.
Palecek SP, Loftus JC, Ginsberg MH, Lauffenburger DA, Horwitz AF. 1997. Integrin–ligand binding properties govern cell migration speed through cell–substratum adhesiveness. *Nature*. 385(6616):537–540.
Peluso JJ. 2000. N-cadherin-mediated cell contact regulates ovarian surface epithelial cell survival. *Biol Signals Recept*. 9(2):115–121.
Rutishauser U, Grumet M, Edelman GM. 1983. Neural cell adhesion molecule mediates initial interactions between spinal cord neurons and muscle cells in culture. *J Cell Biol*. 97(1):145–152.
Rutishauser U, Landmesser L. 1996. Polysialic acid in the vertebrate nervous system: A promoter of plasticity in cell–cell interactions. *Trends Neurosci*. 19(10):422–427.
Scheidegger EP, Sternberg LR, Roth J, Lowe JB. 1995. A human STX cDNA confers polysialic acid expression in mammalian cells. *J Biol Chem*. 270 (39):22685–22688.
Schlaepfer DD, Mitra SK. 2004. Multiple connections link FAK to cell motility and invasion. *Curr Opin Genet Dev*. 14(1):92–101.
Schmid RS, Graff RD, Schaller MD, Chen S, Schachner M, Hemperly JJ, Maness PF. 1999. NCAM stimulates the Ras-MAPK pathway and CREB phosphorylation in neuronal cells. *J Neurobiol*. 38(4):542–558.
Storms SD, Anvekar VM, Adams LD, Murray BA. 1996 Heterophilic NCAM-mediated cell adhesion to proteoglycans from chick embryonic brain membranes. *Exp Cell Res*. 223(2):385–394.
Storms SD, Kim AC, Tran BH, Cole GJ, Murray BA. 1996. NCAM-mediated adhesion of transfected cells to agrin. *Cell Adhes Commun*. 3(6):497–509.
Suzuki M, Suzuki M, Nakayama J, Suzuki A, Angata K, Chen S, Sakai K, Hagihara K, Yamaguchi Y, Fukuda M. 2005. Polysialic acid facilitates tumor invasion by glioma cells. *Glycobiology*. 15(9):887–894.
Tanaka F, Otake Y, Nakagawa T, Kawano Y, Miyahara R, Li M, Yanagihara K, Inui K, Oyanagi H, Yamada T. 2001. Prognostic significance of polysialic acid expression in resected non-small cell lung cancer. *Cancer Res*. 61 (4):1666–1670.
Tanaka F, Otake Y, Nakagawa T, Kawano Y, Miyahara R, Li M, Yanagihara K, Nakayama J, Fujimoto I, Ikenaka K. 2000. Expression of polysialic acid and STX, a human polysialyltransferase, is correlated with tumor progression in non-small cell lung cancer. *Cancer Res*. 60(11):3072–3080.
Trouillas J, Daniel L, Guigard MP, Tong S, Gouvernet J, Jouanneau E, Jan M, Perrin G, Fischer G, Tabarin A. 2003. Polysialylated neural cell adhesion molecules expressed in human pituitary tumors and related to extrasellar invasion. *J Neurosurg*. 98(5):1084–1093.