Enhanced expression of polysialic acid correlates with malignant phenotype in breast cancer cell lines and clinical tissue samples

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Abstract. Polysialic acid (PSA) is highly expressed during embryonic development, but barely expressed during postnatal development, and may be 're-expressed' in cancer tissues. In this study, motility and migration assays were performed to compare the changes in cell behavior between non-malignant and maligant cells. Next, the expression levels of PSA were evaluated in 4 human and mouse normal breast or breast cancer (BC) cell lines using 1,2-diamino-4,5-methylenedioxybenzene-labeling HPLC technology, as well as in human clinical BC tissue samples. PSA expression was significantly higher in malignant cells (where it appeared to facilitate cell migration and motility) than in non-malignant cells. Enhanced PSA expression levels were also observed during epithelial-mesenchymal transition (EMT), a leading cause of cancer cell metastasis, which was induced in the NMuMG and MCF10A cells by treatment with transforming growth factor-β1 (TGF-β1). An increased PSA expression also correlated with the disease stage in the patients with BC ($P<0.0001$). Using RT-qPCR, we found that polysialyltransferase ST8SiaIV (PST) and polysialyltransferase ST8SiaII (STX), which are responsible for PSA synthesis, were differently expressed in the tested BC samples. However, PST, but not STX, was re-expressed in 14 out of 20 clinical BC samples. The findings of the present study indicate that the pathophysiology of BC involves the aberrant regulation of PSA expression and PST gene expression.

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Introduction

Sialic acids, which are mainly attached to the terminal of N-glycans, are abundantly present in a number of organisms, particularly during embryonic development. Sialic acids have been found to mediate various cellular processes in mammals. Polysialic acid (PSA) is a negatively charged homopolymer composed of α-(2,8)-linked sialic acid residues, which is involved in brain development and certain psychiatric disorders, such as schizophrenia (1-3). PSA is barely expressed during postnatal development, but may be 're-expressed' in a number of types of tumor (such as lung cancer, pancreatic cancer, neuroblastoma and glioma), where it modulates cell adhesion, migration and invasion (4-7). The biosynthesis of PSA is catalyzed by two Golgi-resident polysialyltransferases, ST8SiaIV (PST) and ST8SiaII (STX) (8). It has been demonstrated that the mRNA levels of *PST* and *STX* are closely associated with the development of pancreatic cancer (9) and non-small cell lung cancer (10). In patients with breast cancer (BC), serum levels of PSA and sialyltransferases have been shown to be positively associated with the presence of malignant tumors and negatively with responses to anticancer treatment (11). However, to the best of our knowledge, few studies to date have examined the expression of PSA and polysialyltransferases in tissues from patients with BC.

Epithelial-mesenchymal transition (EMT) plays a role in tissue repair and pathological processes, notably in tissue fibrosis and facilitates tumor metastasis (12). During EMT, epithelial cancer cells acquire a mesenchymal phenotype and express mesenchymal markers, such as vimentin (13,14). EMT is therefore a potential target for the development of novel immunotherapeutic approaches. Sialic acids, the ligands for the sialic-acid-binding immunoglobulin-like lectins (Siglec) family of cell adhesion molecules, appear to be involved in regulating the immune response (15). Exploiting the enzymatic permissiveness of sialic acids has been successfully used for the immunotargeting of cancer cells (16). Thus, PSA may be a useful immunotherapeutic target for cancer cells undergoing EMT. The cell lines, NMuMG and MCF10A, derived respectively from mouse mammary glands and non-malignant human breast epithelial tissues, are commonly used for studies

on EMT induced by treatment with transforming growth factor-β1 (TGF-β1) (17). The elucidation of changes in PSA and polysialyltransferase levels during EMT in these two cell lines will help to guide future studies on BC.

The major goals of the present study were to i) use a fluorescence-labeling method followed by HPLC to quantify the PSA expression levels in normal and malignant breast epithelial cells, in cells undergoing TGF-β1-induced EMT, and in 24 clinical BC specimens; ii) evaluate the effects of PSA on cell motility and migration; iii) examine the correlation between PSA expression with that of the related polysialyltransferase, PST, in clinical BC samples.

Materials and methods

Antibodies and reagents. The following antibodies were used: mouse anti-E-cadherin IgG2a (1:50,000; #610181); mouse anti-β-catenin IgG1 (1:10,000; #610153) (BD Biosciences, San Jose, CA, USA); mouse anti-N-cadherin IgG1 (1:1,000; sc-59987) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); mouse anti-vimentin IgG1 (1:1,000; V5255); mouse anti-PSA-neural cell adhesion molecule (NCAM) IgM 5A5 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA); anti-β-tubulin I IgG1 (1:1,000,000; T7816) (Sigma, St. Louis, MO, USA); and horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (1:5,000; A0216) (Beyotime Institute of Biotechnology, Haimen, China).

The reagents used in this study were as follows: TGF-β1 (BD Biosciences), PNGase F (New England BioLabs, Inc., Ipswich, MA, USA), urea, DL-dithiothreitol, iodoacetamide, tetrachloroaurate ($AuCl_4$), Sepharose 4B, methanol, tunicamycin and 1-butanol (Sigma).

Cell lines and cell culture. Mouse mammary epithelial cells (NMuMG), mouse mammary carcinoma cells (4T1), normal human breast cells (MCF10A) and human mammary carcinoma cells (MDA-MB-231; MB-231) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) or RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (both from HyClone, Logan, UT, USA), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco, Carlsbad, CA, USA) in a humidified 5% CO_2 atmosphere at 37˚C. The NMuMG cells were cultured in DMEM containing 10 μg/ml insulin (Sigma). The MCF10A and MB-231 cells were cultured in DMEM containing 1% sodium pyruvate (Solarbio, Beijing, China). The 4T1 cells were cultured in RPMI-1640.

Ethics approval. All procedures performed in experiments involving human participants were carried out in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Patients and tissue samples. BC tissues were obtained from 20 patients with TNM stage I, II and III BC, and 4 normal breast tissue samples were obtained from surgical or autopsy specimens performed at the First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China). All the tissues were snap-frozen in liquid nitrogen and stored at -70˚C until use. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki. The present study was approved by the Research Ethics Committee of Jiangnan University (Wuxi, China).

Western blot analysis. The cells were cultured in 6-well plates, washed with phosphate-buffered saline (PBS), harvested, homogenized in T-PER lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing 10 U/ml aprotinin, and centrifuged at 14,000 x g at 4˚C for 15 min. The supernatants were mixed with loading buffer (which contained 1% β-mercaptoethanol) and heated at 100˚C for 10 min. Proteins were loaded on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto a PVDF membrane. The membrane was blocked with 5% non-fat milk, incubated with primary antibody overnight at 4˚C, probed with appropriate HRP-conjugated secondary antibody, visualized using the Pro-Light HRP kit (Tiangen Biotech Co., Ltd., Beijing, China), and photographed using a Molecular Imager Chemi DOC™ XRS$^+$ system (Bio-Rad, Richmond, CA, USA).

Separation of glycans. Total proteins from the tissues were isolated using an E.Z.N.A. DNA/RNA/Protein Isolation kit (Omega Bio-Tek, Doraville, CA, USA) according to the manufacturer's instructions. Glycans on proteins were released by PNGase F and desalted as previously described (18,19). The glycan sample was lyophilized and dissolved in methanol/H_2O (1:1, v/v) for further analysis.

Fluorescence labeling of free sialic acids for 1,2-diamino-4,5-methylenedioxybenzene (DMB) and HPLC analysis. Free sialic acids were labeled with DMB using a Sialic Acid Fluorescence Labeling kit (Takara Bio Inc., Otsu, Japan), according to the instructions provided by the manufacturer. Briefly, the cell lysate was hydrolyzed with 2 M acetic acid and labeled with DMB, as described in a previous study (20). A 10-μl aliquot of each sample was loaded on a Zorbax SB-C18 column (4.6x250 mm) (Agilent Technologies, Inc., Santa Clara, CA, USA) and eluted with methanol/acetonitrile/water (8:7:85, v/v/v) on a Waters e2695 HPLC system with a fluorescence detector (excitation, 373 nm; emission, 448 nm). Free sialic acids were quantified based on peak areas obtained from a defined standard.

Reverse transcription-quantitative (real-time) polymerase chain reaction (RT-qPCR). Total RNA from the cells was isolated using an RNApure Tissue kit (CWBIO, Beijing, China) and RNA from human tissues was isolated using an E.Z.N.A. DNA/RNA/Protein Isolation kit (Omega Bio-Tek) according to the manufacturer's instructions. First-strand cDNA was synthesized using a ReverTra Ace-α® kit (Toyobo, Osaka, Japan). Primers were designed using DNAman software as follows: mouse *STX* (167 bp) sense, 5'-CTTGGATGCGGAGAAGGAT and antisense, 5'-GGCACAAGTCTGGAAATGCT; mouse *PST* (126 bp) sense, 5'-GCGAACTGCCTATCCATCAC and antisense, 5'-TCACAGAATCTGGTGGCAAG; human *STX* (141 bp) sense, 5'-TCAGAACCAGAACCCAGTCA and antisense, 5'-CGACAG TCAGTTTCAAAGCC; human *PST* (106 bp) sense, 5'-ACTGA AAGTGCGAACTGCCT and antisense, 5'-GAGAAGAC

CTGTGCTGGGTC; mouse *γ-tubulin* (107 bp) sense, 5'-ATCTA CCTGTCGGAGCATGG and antisense, 5'-GCCTCCCGA TCTATGATGTC; and human *β-actin* (232 bp) sense, 5'-CTTCC TGGGCATGGAGTC and antisense, 5'-GCCGATCCACA CGGAGTA. Semi-quantitative PCR was performed as follows: 94˚C, 4 min; 94˚C, 45 sec, 60˚C, 45 sec, 72˚C, 15 sec for 30 cycles; 72˚C, 5 min; quantitative PCR (qPCR) was performed using Ultra SYBR Mixture (Cat. no. CW0957; CWBIO) on a CFX96 Real-Time PCR detection system (Bio-Rad). The transcriptional levels of target genes were quantified using the $2^{-\Delta\Delta Ct}$ method, as previously described (21) and expressed as the means ± SD from triplicate experiments.

Cell motility assay. Phagokinetic gold sol assay was performed as previously described (22,23). Briefly, 1.8 ml of a 14.5 mM $AuCl_4$ solution and 6 ml of a 36.5 mM Na_2CO_3 solution were added to 11 ml distilled H_2O, heated to a boil, and then 1.8 ml of 0.1% formaldehyde was added. Colloidal gold solution (gold sol) was added to 24-well plates, and the plates were blocked with filter-sterilized 1% BSA, as previously described (22). Cells ($2x10^3$) in complete culture medium were seeded onto the gold sol-coated wells and incubated for 18 h. Images were captured using an inverted microscope (model NA0.3OWD72; Sunny Optical Technology, Ningbo, China). The track areas of 50 cells were measured using the ToupView imaging system, as previously described (24) and expressed as square pixels.

Gene silencing with small interfering RNA (siRNA). Duplexes of 21 nucleotides of human and mouse STX siRNA target sequences (hSTXi or mSTXi) and negative control siRNA (NC), without homology to other known human and mouse genes, were designed and synthesized by Invitrogen (Carlsbad, CA, USA) as follows: hSTXi, 5'-GCCUGGAGAUAUUAUUCA UTT (sense); and mSTXi, 5'-CCUGAAGCCUGGAGACAU UTT (sense). siRNA (30 pmol) was transfected using Lipofectamine 2000 reagent (3 µl) (Invitrogen) and the 4T1 or MB-231 cells were examined 24 h following transfection. The suppression of the expression of *STX* was verified by semi-quantitative and quantitative RT-PCR.

Transwell migration assay. The cells were cultured in 6-well plates and treated with or without TGF-β1 (5 ng/ml) for 48 h. Tunicamycin (4 µg/ml) was added together with TGF-β1 into 6-well plates, and the NMuMG or MCF10A cells were cultured for an additional 48 h, as previously described (25,26). The cells ($5x10^4$) were plated in an upper Transwell insert (12/24-well Transwell; 8 µm polycarbonate membrane; Costar, Corning, NY, USA) in DMEM or RPMI-1640 medium containing 0.1 ml of 0.2% BSA (RuiTaibio, Beijing, China). A total of 0.6 ml of DMEM or RPMI-1640 medium supplemented with 10% FBS, serving as a chemoattractant, was deposited in the lower chamber. Following incubation for 16 h at 37˚C in a 5% CO_2 atmosphere, the cells were washed with PBS and fixed with cold 4% buffered paraformaldehyde. The cells in the upper Transwell filter were removed with a cotton wool tip and stained with crystal violet. The Transwells were rinsed with deionized water and air-dried. The filters were photographed, and the cells in 5 random optical fields were counted to determine migration, as previously described (27,28).

Data analysis. Data were statistically analyzed using the Prism 5 software programs as previously described (29). Differences between the means were assessed by paired or unpaired Student's t-test, and P-values <0.05 were considered to indicate statistically significant differences.

Results

PSA level is higher in malignant cancer cells than in normal cells. As EMT is one of the main mechanisms involved in the development of BC metastasis (30), the expression of EMT-related markers in malignant 4T1 and MB-231 cells, in comparison with non-malignant NMuMG and MCF10A cells was determined by western blot analysis (Fig. 1A). The decreased expression of E-cadherin and β-catenin, as well as the increased expression of vimentin, were observed in the 4T1 and MB-231 cells. Tumor progression towards metastasis is often depicted as a multistage process in which malignant cells spread from their original site to colonize distant organs through acquired mobility and migration ability (31,32). In this experiment, increased motility (Fig. 1B) and migration (Fig. 1C) were observed in malignant 4T1 and MB-231 cells compared with normal NMuMG and MCF 10A cells.

In view of the findings that PSA is re-expressed in many types of tumor, we thus compared PSAs on N-glycans in these 4 cell lines. PSAs display unique modifications of N-glycans, particularly of NCAM (33). As shown in Fig. 2A, PSAs on N-glycans were hydrolyzed under mild acidic conditions, and free sialic acids (reflecting PSA levels) were detected using a fluorescence-labeling method followed by HPLC. In comparison with the NMuMG or MCF10A cells, the PSA level was slightly higher in the 4T1 cells and significantly higher in the MB-231 cells (Fig. 2B). These findings suggest that a high PSA expression is associated with the enhanced invasiveness and metastasis of BC cells.

PSA expression level is increased in NMuMG and MCF10A cells undergoing EMT. The EMT process has been shown to be associated with cancer cell motility and metastasis. The non-malignant NMuMG and MCF10A cells were treated with TGF-β1 to induce EMT, as previously described (34). The treated cells displayed a spindle-like, elongated morphology (Fig. 3A), a reduced β-catenin expression, an increased vimentin expression, and exhibited the typical 'cadherin switch' from E-cadherin to N-cadherin (Fig. 3B). The TGF-β1-treated NMuMG cells displayed an enhanced motility and migration, whereas the TGF-β1-treated MCF10A cells exhibited only enhanced migration (Fig. 3C and D). The levels of free sialic acid released from N-glycan as a function of dose and time were examined in the NMuMG cells following treatment with TGF-β1. The NMuMG cells treated with 2 and 5 ng/ml TGF-β1 exhibited free sialic acid levels approximately 2.5- and 4-fold higher, respectively, than those of the untreated cells (Fig. 4A). In order to examine the effects of high levels of PSA on cell behavior, 5 ng/ml TGF-β1 was used in the following experiment. The PSA levels were higher in the TGF-β1-treated NMuMG cells at various time points (Fig. 4B). Similar results were obtained for the TGF-β1-treated MCF10A cells (Fig. 4C). Moreover, a high expression of polysialylated NCAM (PSA-NCAM) was observed in the NMuMG and MCF10A cells undergoing EMT (Fig. 4D).

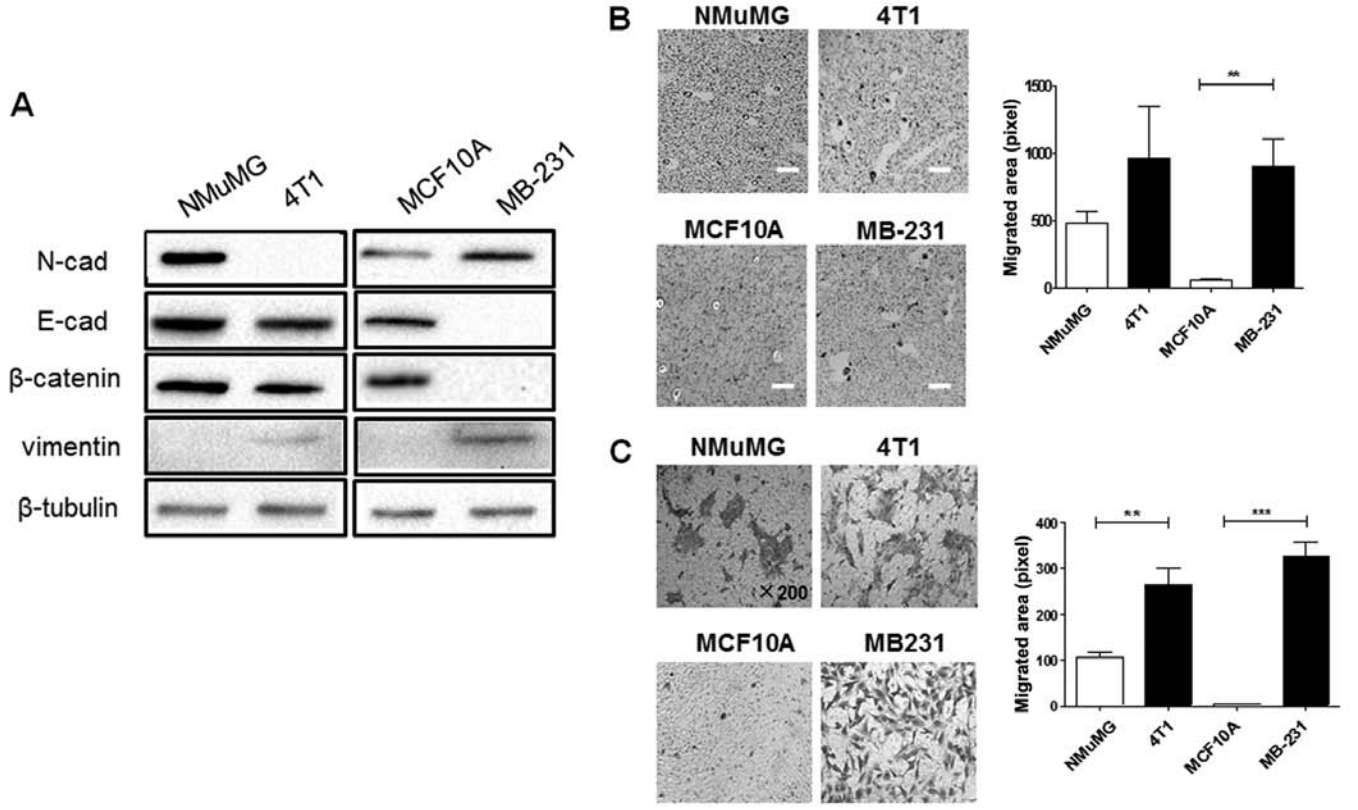

Figure 1. Epithelial-mesenchymal transition (EMT)-related markers, motility and migration of 4 mouse and human cell lines. (A) Western blot analysis of protein markers. Protein aliquots (equal weight) were immunoblotted with antibodies vs. the indicated components (β-tubulin, loading control). E-cad, E-cadherin; N-cad, N-cadherin. (B) Motility assays. The cells ($2x10^3$) were added to 24-well plates coated with gold sol particles. Images of the track areas of 50 cells were taken after 12-18 h of incubation. Cleared areas on gold sol were measured as square pixels using the ToupView Image program and are shown as the means ± SD (right). Two independent experiments yielded similar results. Values are the means ± SD. Scale bar, 10 μm. **P=0.001 to 0.005. (C) Migration assays. The cells were seeded in a chamber containing culture medium with 10% FBS in the bottom layer of the Transwell membrane. The cells that migrated to the bottom part of the Transwell membrane were fixed, stained with 0.2% crystal violet, and counted. Phase-contrast images were captured. The number of migrated cells was quantified (right). The values shown are the means ± SD from 3 independent experiments. Magnification, x200. **P=0.001 to 0.005; ***P<0.001.

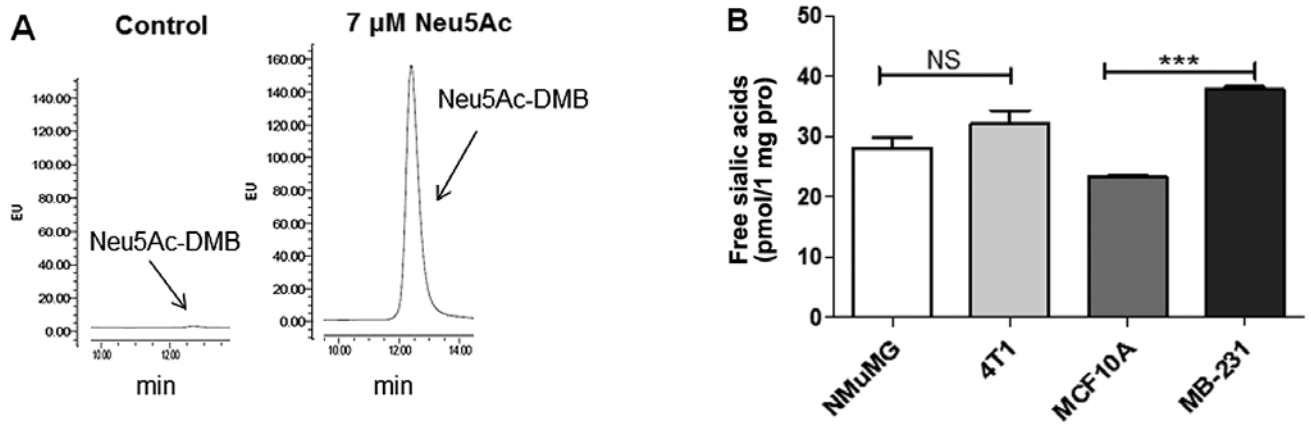

Figure 2. Polysialic acid (PSA) expression in normal breast and breast cancer (BC) cell lines. (A) Sialic acids were separated by HPLC. DMB-labeled Neu5Ac was detected based on a standard curve as described in the Materials and methods. Left, reagent with water (control). Right, 7 μM Neu5Ac. (B) PSA expression. Cells were harvested after 48 h of culture, and glycans were released as described in the Materials and methods. Glycans were subjected to hydrolysis and DMB labeling, and Neu5Ac-DMB was separated by reversed-phase HPLC. The values shown are the means ± SD from 3 independent experiments. NS, not significant; ***P<0.001.

PSA facilitates cell migration in malignant breast cells and in normal breast cells undergoing EMT. In view of the correlation of high PSA levels with the facilitation of cell migration and cell motility during EMT, we examined the possibility that the downregulation of PSA reverses EMT in malignant cells. Semi-quantitative RT-PCR was used to evaluate the mRNA levels of *PST* and *STX*, two major polysialyltransferases responsible for PSA synthesis. The 4T1 and MB-231 cells exhibited high

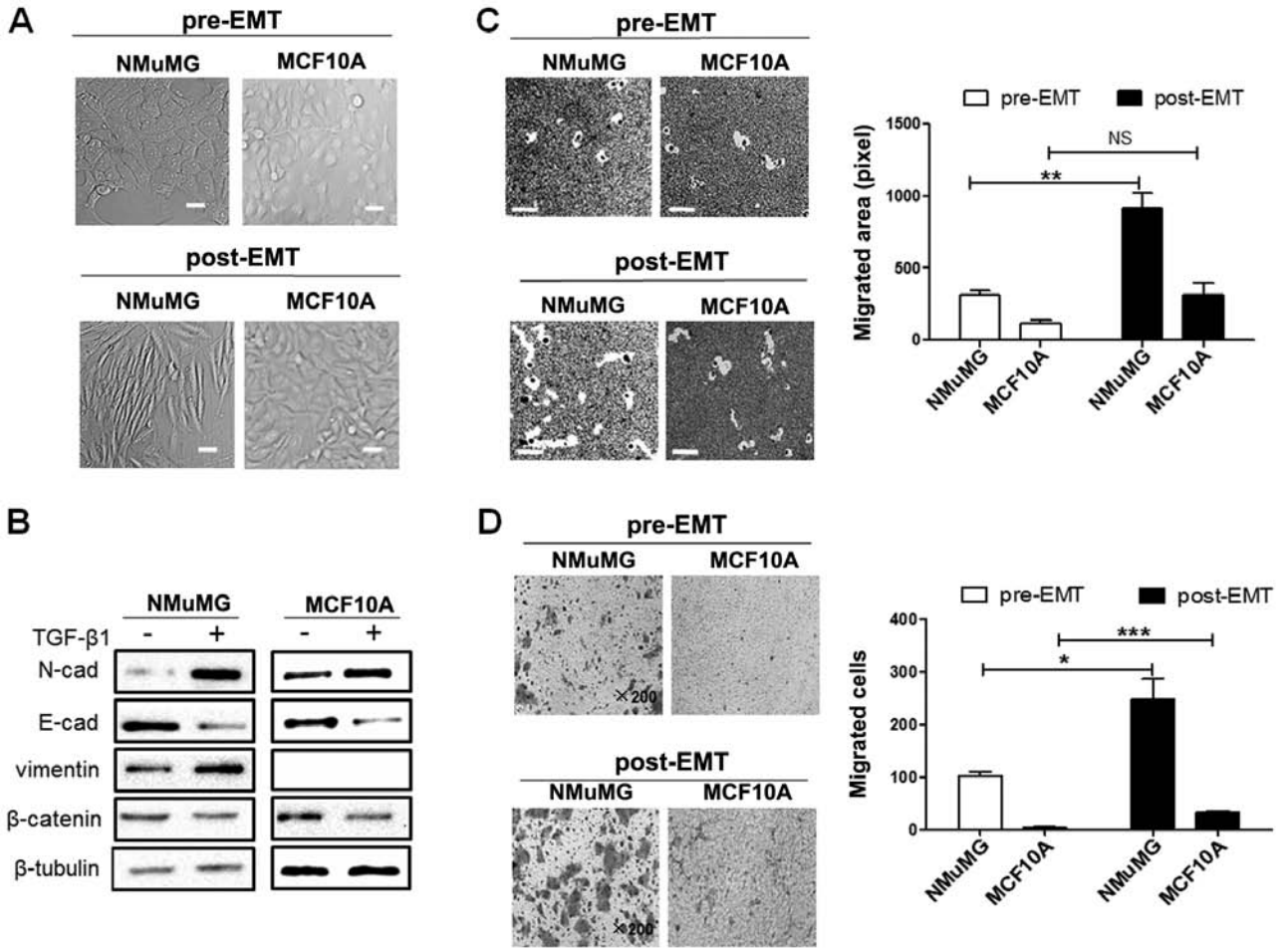

Figure 3. Changes in cell motility and migration during transforming growth factor-β1 (TGF-β1)-induced epithelial-mesenchymal transition (EMT) of NMuMG and MCF10A cells. (A) Morphological changes induced by TGF-β1 treatment. NMuMG and MCF10A cells were treated (+) or not (-) with TGF-β1 (5 ng/ml) for 48 h, and phase-contrast images were captured. Scale bar, 20 μm. (B) Detection of EMT-related markers. NMuMG and MCF10A cells were treated (+) or not (-) with TGF-β1 (5 ng/ml) for 48 h. β-tubulin, loading control. (C) Motility assays. Gold sol assay was performed as described in Fig. 1B. Cleared areas on gold sol were measured as square pixels using the ToupView Image program and are shown as the means ± SD (right panels). Two independent experiments yielded similar results. Scale bar, 10 μm. The values shown are the means ± SD. NS, not significant; **P=0.001 to 0.005. (D) Migration assays. NMuMG and MCF10A cells before and after treatment with TGF-β1 were plated in an upper Transwell insert containing 0.5% BSA and 1 ng/ml TGF-β1. The migration assay was performed as described in Fig. 1C. The number of migrated cells was quantified. The values of the means ± SD were analyzed based on 3 independent experiments. Magnification, x200. *P<0.05; ***P<0.001.

levels of *STX*, but undetectable levels of *PST* (data not shown). The silencing of PSA by the suppression of *STX* resulting in the decreased expression of PSA-NCAM (Fig. 5A and B), or by treatment with tunicamycin (which blocks N-glycan synthesis), significantly decreased the migration of both malignant cell lines (4T1 and MB-231) (Fig. 5C). The downregulation of PSA resulted in the decreased motility of the malignant BC cells (MB-231), but not of the 4T1 cells (Fig. 5C).

To further investigate the role of PSA during EMT, we used tunicamycin to inhibit N-glycan synthesis in normal cells undergoing EMT. The typical 'cadherin switch' from E-cadherin to N-cadherin and the enhancement of cell migration in the TGF-β1-treated MCF10A and NMuMG cells were reversed by the presence of tunicamycin (Fig. 6).

PSA expression level is related to the BC stage. The patient characteristics and PSA levels in the normal tissues (n=4) and malignant tissues (n=20) are listed in Tables I and II. The PSA levels were lower (pmol level) in the normal tissues and higher in the malignant tumor tissues (Fig. 7A). The difference in the PSA levels between the normal tissues and advanced-stage BC tissues was significant (P<0.0001). The PSA level was consistently higher in the tumor tissues compared to the normal controls. In comparison with the controls, the PSA level was highest in the samples obtained from patients with TNM stage III BC (n=6, P<0.0001), followed by stage II (n=8, P<0.0001) and stage I (n=6, P=0.0085), suggesting that an increased PSA expression correlates with disease progression (Fig. 7B).

PST mRNA level may serve as an indicator for human BC. The aberrant expression of polysialyltransferases is often observed in malignant tumors and has thus been considered as a novel target for detection or treatment of metastatic cancer (35). As an enhanced level of PSA was observed in our clinical BC samples, we wished to examine the expression of polysialyltransferases between normal tissues and malignant tissues. We used semi-quantitative and quantitative RT-PCR to

Table I. Sialic acid, STX and PST levels in normal and malignant breast tissue samples.

	Diagnosis	Age (years)	LN(P)	LN	Stage	D	Sia	STX (fd)	PST (fd)
1	N	36	-	-	-	-	4.18±0.11	0.89±0.05	0.87±0.04
2	N	49	-	-	-	-	5.23±0.03	0.58±0.09	1.00±0.03
3	N	35	-	-	-	-	1.70±0.03	1.25±0.09	0.70±0.50
4	N	54	-	-	-	-	0.20±0.005	1.0±0.00	1.00±0.00
5	IDC	54	28	33	T2N3M0 IIIc	U	20.34±0.24	0.76±0.10	2.07±0.42
6	IDC	50	22	24	T1N3M0 IIIc	2	38.39±0.54	0.12±0.01	19.04±2.93
7	IDC	32	16	16	T2N3M0 IIIc	2.1	21.33±0.39	0.20±0.05	8.75±0.50
8	IDC	60	3	14	T4N1M0 IIIb	6	20.89±0.31	12.16±1.00	0.85±0.04
9	IDC	65	2	25	T4N1M0 IIIb	6.5	8.24±0.11	1.26±0.18	3.66±0.35
10	IC	73	U	U	T2N2M0 IIIa	3.4	13.04±0.02	2.03±0.09	3.43±0.27
11	SMC	57	1	28	T2N1M0 IIb	4	18.31±0.08	1.45±0.16	9.15±0.32
12	IDC	35	2	18	T2N1M0 IIb	2.3	11.47±0.23	2.07±0.49	5.97±0.33
13	ILC	42	3	22	T2N1M0 IIb	2.5	10.88±0.005	0.64±0.07	13.91±0.25
14	IDLC	57	0	23	T2N0M0 IIa	2.5	13.27±0.02	0.57±0.16	17.46±1.45
15	MC	51	0	19	T2N0M0 IIa	2.3	15.73±0.29	0.46±0.07	5.05±0.65
16	IDC-NS	33	1	25	T1N1M0 IIa	2	3.93±0.05	1.22±0.07	0.78±0.06
17	IDLC	72	1	24	T1N1M0 IIa	2	12.85±0.20	1.40±0.15	0.83±0.05
18	IDC	54	0	20	T2N0M0 IIa	2.8	7.67±0.05	0.19±0.10	5.26±0.64
19	IDC	39	0	12	T1N0M0 I	1.6	6.20±0.08	1.15±0.08	4.40±0.68
20	IDC	54	0	U	T1N0M0 I	1.2	18.47±0.19	0.65±0.09	7.86±0.35
21	IDC	32	0	U	T1N0M0 I	U	9.37±0.09	1.27±0.08	24.94±0.92
22	IDC	65	0	U	T1N0M0 I	1.0	5.79±0.05	0.46±0.06	0.72±0.07
23	IDC	35	0	U	T1N0M0 I	1.5	5.00±0.08	2.70±0.62	0.21±0.02
24	IDC	39	0	U	T1N0M0 I	U	4.95±0.03	2.73±0.31	0.56±0.08

LN(P), lymph node positivity; D, diameter (cm); fd, fold change; N, normal; IDC, infiltrating ductal carcinoma; IC, infiltrating carcinoma; SMC, squamous metaplastic carcinoma; ILC, infiltrating lobular carcinoma; IDLC, infiltrating ductal-lobular carcinoma; MC, mucinous carcinoma; IDC-NS, infiltrating ductal carcinoma with neural secretion; U, unknown. Sia, sialic acid level (pmol/1mg pro), as determined by HPLC. *STX* and *PST* levels were determined by quantitative RT-qPCR and normalized relative to sample 4 (fold change) (Materials and methods. Values are the means ± SEM).

examine *STX* and *PST* expression at the mRNA level in BC tissues (Table I). *STX* was widely and highly expressed in all tissues examined (n=24) with no significant differences when compared with the normal controls (Fig. 7C and D). However, *PST* expression was significantly increased in the malignant tumor tissues compared to the normal tissues (Fig. 7C and E). *PST* was expressed distinctively in the tumor samples obtained from patients with stage I (n=6, 50%), II (n=8, 75%), and III (n=6, 83%) BC (Table II and Fig. 7E). The PST enzyme is clearly expressed in high-stage BC tissues, but not in normal tissues. The difference in the *PST* level between the normal tissues (n=4) and high-stage/lymph node-positive tissues (n=6, stage III) from patients with BC was significant at r^2=0.8, indicating that PST expression is increased in BC tissues and correlates with cancer progression.

Discussion

Highly sialylated glycans on the surface of cancer cells often correlate with tumor invasiveness and metastasis (33). α2,6-sialylation on the cell surface has been shown to affect the adhesion of MDA-MB-435 breast carcinoma cells (36). Another study revealed that negatively charged PSA led to the increased motility of pancreatic carcinoma cells and subsequently reduced cell adhesion (9). In this study, to assess PSA levels on N-glycans, we applied a DMB-labeling method followed by HPLC, to quantify PSA expression at the pmol level. Our results revealed an increased motility of breast carcinoma 4T1 and MB-231 cells in comparison with the non-malignant NMuMG and MCF10A cells. PSA levels on N-glycans were higher in malignant mouse and human cell lines.

Altered PSA levels were observed in the NMuMG and MCF10A cells undergoing TGF-β1-induced EMT. The EMT process in our models involved increased motility and migration. PSA expression in the cells undergoing EMT increased in a TGF-β1 dose- and time-dependent manner. We further investigated the role of PSA in malignant and normal breast cells undergoing EMT by downregulating PSA biosynthesis. The partial removal of PSA from malignant BC cells using siRNA decreased migration and motility. The treatment of normal breast cells with tunicamycin, an inhibitor of N-glycan synthesis, indicated a similar role for PSA during EMT. The

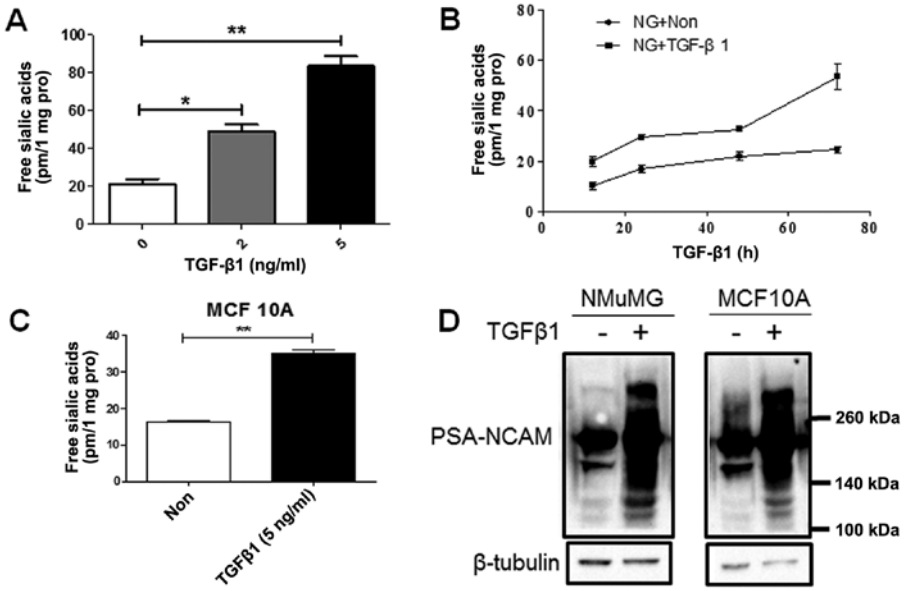

Figure 4. Changes in polysialic acid (PSA) expression during transforming growth factor-β1 (TGF-β1)-induced epithelial-mesenchymal transition (EMT) of NMuMG and MCF10A cells. (A) Dose-dependent sialic acid expression following treatment of NMuMG cells with 2 ng/ml or 5 ng/ml TGF-β1. Sialic acid expression was assessed by HPLC as described in the Materials and methods. The values shown are the means ± SD from 3 independent experiments. *P<0.05; **P=0.001 to 0.005. (B) Time-course effect of TGF-β1 treatment on sialic acid expression. The cells were incubated with 5 ng/ml TGF-β1 for 12, 24, 48 or 72 h, and sialic acid expression was measured. The values shown are the means ± SD from three independent experiments. Non, untreated cells. (C) Detection of sialic acid expression in MCF10A cells undergoing EMT. Sialic acid expression was assessed by HPLC as described above. The values shown are the means ± SD from 3 independent experiments. **P=0.001 to 0.005. (D) Western blot analysis of PSA-neural cell adhesion molecule (NCAM) expression, using anti-PSA-NCAM antibody (5A5) and β-tubulin as a loading control.

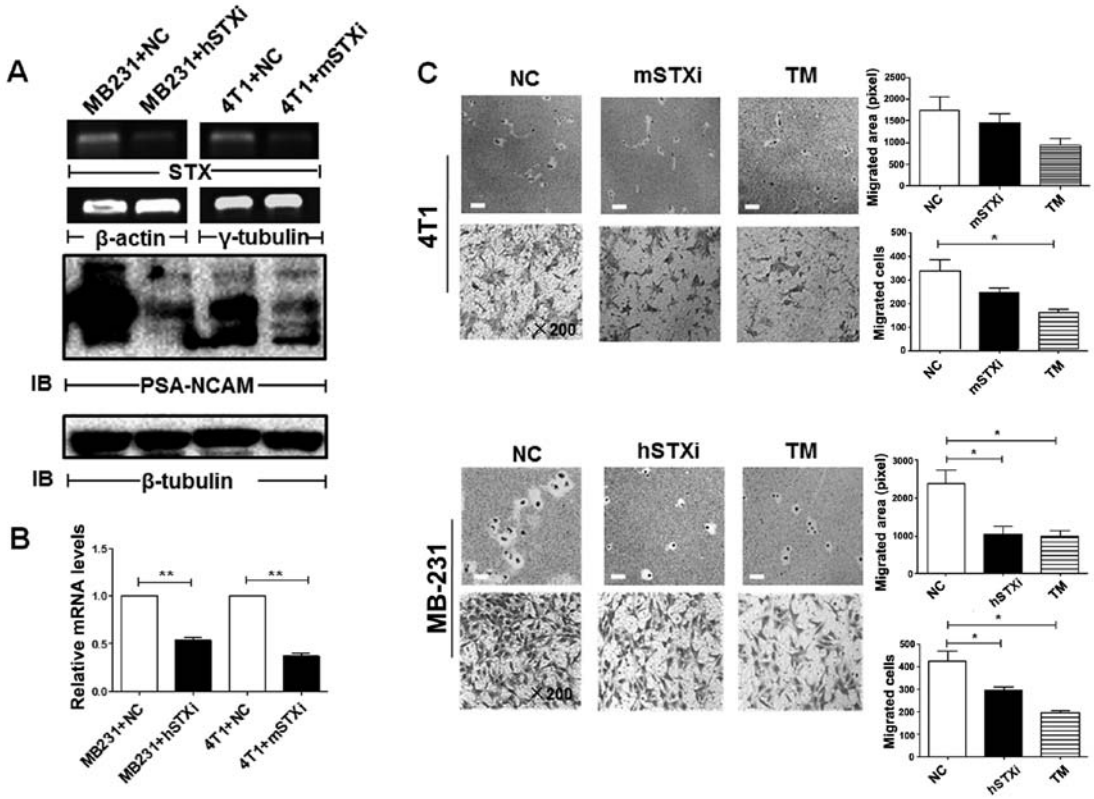

Figure 5. Cell motility and migration by suppression of polysialic acid (PSA) expression in malignant cells. (A and B) Downregulation of *STX* by siRNA. Cultured 4T1 and MCF10A cells were transfected or not with siRNA-targeting mouse STX (mSTXi) or human STX (hSTXi), or with negative control RNA (NC). Total RNA and proteins were isolated as described in the Materials and methods. (A) Semi-quantitative RT-PCR and (B) quantitative RT-PCR were performed for *STX*. Immunoblotting (IB) was performed for PSA-neural cell adhesion molecule (NCAM) using anti-PSA-NCAM antibody (5A5). β-actin, γ-tubulin (at mRNA level) and β-tubulin (at protein level) were used as controls, respectively. **P=0.001 to 0.005. (C) Motility and migration assays. 4T1 and MCF10A cells were transfected respectively with mSTX siRNA and hSTX siRNA or tunicamycin (TM, 4 μg/ml) and subjected to motility and migration assays as described in the Materials and methods. Cleared areas on gold sol and migrated cells were measured as square pixels or numbers, respectively, using the ToupView Image program and are shown as the means ± SD (right panels). Two independent experiments gave similar results. Scale bar, 20 μm. Magnification, x200. *P<0.05.

Table II. Association of disease characteristics with polysialyltransferase (*STX* or *PST*) gene expression in malignant BC patients.

	TNM stage			
Characteristics	I	II	III	Total
No. of patients	6	8	6	20
Site				
Left	2	2	3	7
Right	4	6	3	13
Histology				
Invasive/infiltrating ductal	6	2	5	13
Invasive/infiltrating lobular	0	1	0	1
Invasive/infiltrating ductal-lobular	0	2	0	2
Others	0	3	1	4
Lymph node				
Absent	6	3	0	9
Involvement				
Present	0	5	6	11
Unknown	0	0	0	0
STX-positive	6/6 (100%)	8/8 (100%)	6/6 (100%)	20/20 (100%)
PST-positive	3/6 (50%)	6/8 (75%)	5/6 (83%)	14/20 (70%)

For STX, all samples were set as *STX*-positive; for PST, fd >1, *PST*-positive; fd, fold change. STX, polysialyltransferase ST8SiaII; PST, polysialyltransferase ST8SiaIV.

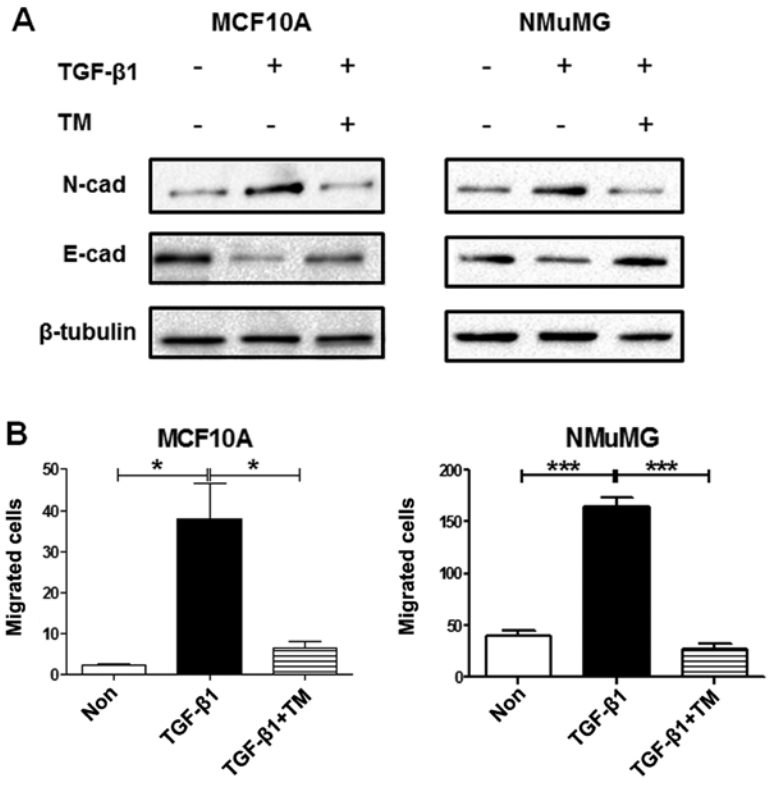

Figure 6. Analysis of the expression of epithelial-mesenchymal transition (EMT) markers, and cell migration, by suppressing polysialic acid (PSA) expression in normal cells undergoing EMT. (A) Detection of EMT markers. Normal breast cells were treated or not with transforming growth factor-β1 (TGF-β1) (5 ng/ml) and tunicamycin (TM) (4 μg/ml). Total proteins were isolated and subjected to western blotting as described in the Materials and methods. β-tubulin was used as a loading control. E-cad, E-cadherin; N-cad, N-cadherin. (B) Migration assays. Normal breast cells were treated or not with TGF-β1 (5 ng/ml) and TM (4 μg/ml). Migration assays were performed as described in the Materials and methods. Migrated cells were quantified, and the results are shown as the means ± SD. Two independent experiments gave similar results. ***P<0.001; *P<0.05. Non, untreated cells.

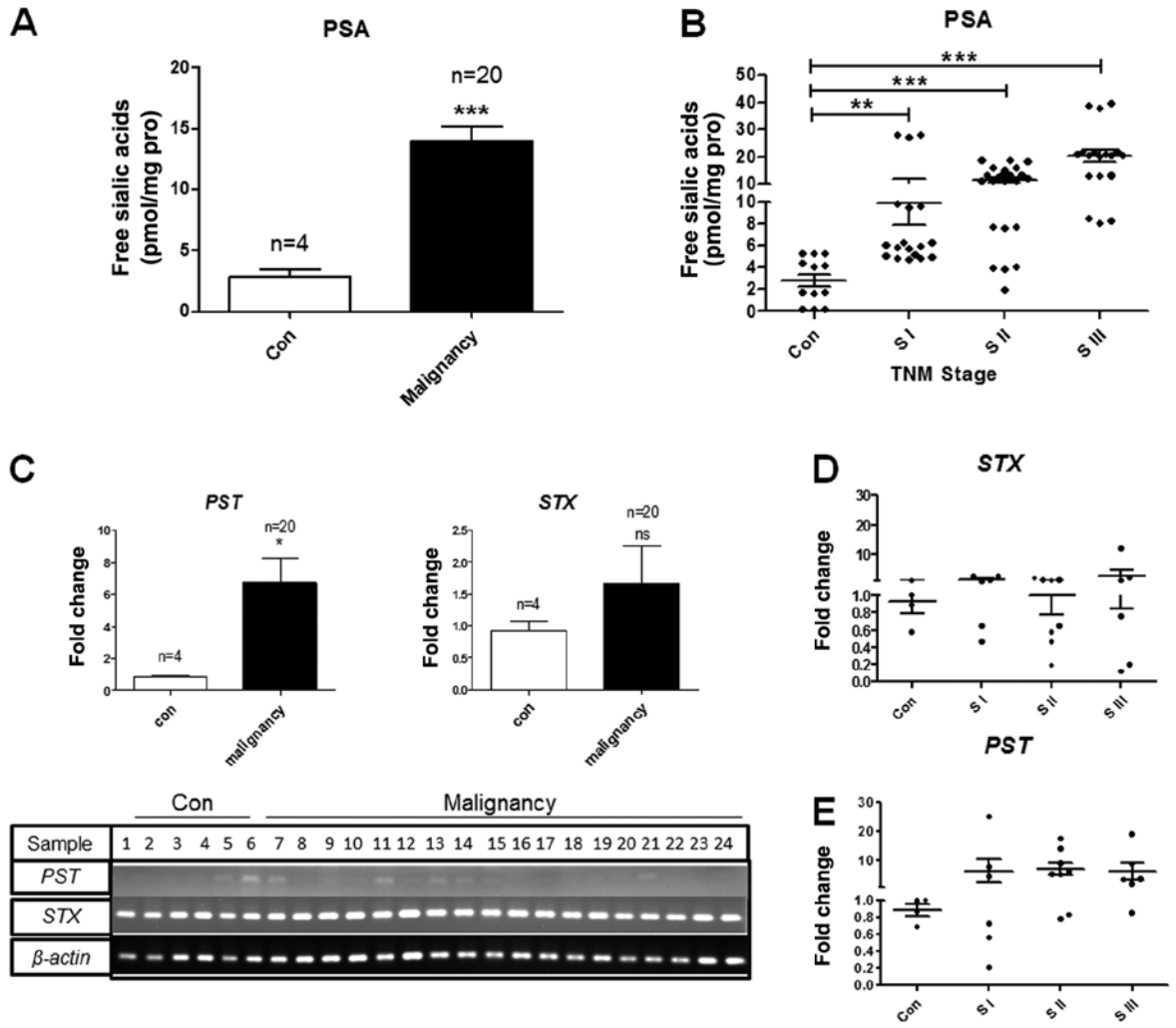

Figure 7. Polysialic acid (PSA) and polysialyltransferase expression in human breast cancer (BC) tissues. (A and B) PSA expression in patients with BC. Glycans were separated and analyzed by HPLC as described in Fig. 2A and B. PSA concentration of each sample was calculated based on a standard sialic acid (Neu5Ac) curve. (A) PSA levels in normal and malignant BC tissues. The values shown are the means ± SD from 3 independent experiments. (B) Correlation of PSA level with BC stages. PSA level was assessed in the 24 samples and classified as normal tissue (control; Con, n=4), TNM stage I (S I, n=6), II (S II, n=8), or III (S III, n=6). Each sample was visualized 3 times (24x3), and data were analyzed by an unpaired t-test. **P=0.001 to 0.005; ***P<0.001. (C) mRNA levels of polysialyltransferase ST8SiaII (*STX*) and polysialyltransferase ST8SiaIV (*PST*) in BC tissues. Expression of *PST* and *STX* genes in clinical BC samples assessed by semi-quantitative and quantitative RT-PCR. mRNA levels of *PST* and *STX* in malignant tissues (malignancy, n=20) were compared with those in normal tissues (con, n=4) by quantitative RT-PCR. The gels showed the mRNA level of *PST* or *STX* in 24 samples (normal, n=4; malignant, n=20). The values shown are the means ± SD from triplicate experiments. β-actin, control. *P<0.05; NS, not significant. (D and E) mRNA level of (D) *STX* and (E) *PST* assessed by RT-qPCR. The values shown are the means ± SD from triplicate experiments. *STX* and *PST* levels were assessed in the 24 samples and classified as normal tissue (control; Con, n=4), TNM stage I (S I, n=6), II (S II, n=8), or III (S III, n=6).

suppression of PSA did not cause significant alterations in the typical EMT markers (aside from reduced cell migration) in these cell models. Our findings suggest that PSA has a greater effect on cell migration than cell motility in malignant cells.

A previous study demonstrated an increased expression of PSA and sialyltransferases in the sera of patients with BC (11). In this study, we directly examined the expression of PSA and polysialyltransferase genes in human BC tissues, and evaluated the correlation between the PSA expression level and disease stage in 24 tissue samples from patients with BC and normal controls. PSA expression was higher in the clinical BC specimens than in the normal tissues obtained from patients with BC. Taken together, our findings indicate that PSA expression in BC tissues is related to the TNM stage.

The polysialyltransferases STX and PST differentially and dependently contribute to the re-expression of PSA based on the histological origin of the tumor (37) and are regarded as anti-metastatic therapeutic targets. It has been demonstrated that STX is more important than PST, due to its dominant expression in cells and tissues (35). In the present study, we observed a marked increase in *PST* mRNA expression. The *PST* gene exhibited a higher expression in the advanced-stage cases than in normal tissues, whereas the *STX* gene was widely and highly expressed in all clinical cases. Polysialyltransferase expression appears to be the basis for the aberrant expression of sialylated structures on N-glycans from BC tissue samples. The *PST* gene plays a crucial role in BC progression and is a potential target for molecular therapy of BC.

In conclusion, our data demonstrate that PSA is highly expressed in malignant BC cells and in normal mouse mammary and human breast cell lines undergoing EMT. The effects of PSA on cell migration were more pronounced than those on

cell motility in malignant BC cells. PSA expression correlates with the TNM stage in human BC samples and depends on PST activity, whereas STX is expressed consistently in both normal and BC tissues. The detailed functions of these two polysialyltransferases remain unclear, however. A previous *in vitro* study found that PST forms more highly polysialylated N-glycans than does STX (38). Our finding of a higher PST expression in advanced-stage BC may reflect the presence of more highly polysialylated N-glycans in these patients. It remains to be clarified whether the increased PSA expression in BC results from synthesis by PST alone or by PST in cooperation with STX.

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