*Original Research*

# Differential effects of Pax3 on expression of polysialyltransferases STX and PST in TGF-β-treated normal murine mammary gland cells

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## Abstract

Glycosylation of certain proteins at the mammalian cell surface is an essential event in carcinogenesis. Sialylation, one type of glycosylation, can act on multiple cell-behaviors, such as migration, growth, and malignant invasion. Two polysialyltransferases, ST8Sia II (STX) and ST8Sia IV (PST), are responsible for synthesis of polysialic acid on neural cell adhesion molecule. We showed previously that STX and PST are oppositely expressed in normal murine mammary gland cells undergoing transforming growth factor-β-induced epithelial-mesenchymal transition. The molecular basis for regulation of STX and PST remained unclear. In the present study, we observed that transcription factor Pax3 upregulates STX expression, downregulates PST expression, and modulates upregulated expression of PSA, which attaches primarily to neural cell adhesion molecule to form PSA-NCAM. Overexpression of Pax3 in normal murine mammary gland cells transformed the expression of epithelial-mesenchymal transition markers E-cadherin and N-cadherin, and significantly promoted cell migration, but had no effect on cell proliferation.

**Keywords:** Pax3, polysialyltransferase, STX, PST, epithelial-mesenchymal transition, PSA-NCAM

***Experimental Biology and Medicine* 2017; 242: 177–183. DOI: 10.1177/1535370216669838**

## Introduction

Glycans, important compositions of animal cell membranes, play pivotal roles in cell–cell interactions, cell–extracellular matrix interactions, and signal transduction. Glycans occur in various forms, and their changes are involved in pivotal pathophysiological events during tumor progression.[1] Polysialic acid (PSA) exists in a linear homopolymer of α2,8-linked form and reduces the interaction of neural cell adhesion molecule (NCAM) based on its large negative charge,[2] and thus plays crucial roles in neural cell migration, neurite outgrowth,[3] and synaptogenesis.[4] PSA expression shows temporal specificity, presenting as high expression during embryogenesis, and a barely detectable level during postnatal development.

PSA synthesis is usually catalyzed synergistically by two polysialyltransferases, STX, and PST,[5] which are highly homologous (59% amino acid sequence similarity).[6] Analysis of genomic structure and promoter activity of mouse STX and PST genes[7–9] revealed their proximal promoter regions and putative binding sites for transcriptions factors. Microarray analysis showed that *STX* is one of the target genes of transcription factor Pax3.[10] It was also reported that the transcription factor, AP-2delta, regulates STX expression in chick retina.[11] Activation of cAMP-CREB cascade increases STX expression, but has no effect on PST expression.[12]. In postnatal mouse visual cortex, expression of STX and PST at the mRNA level is positively regulated by PKC-mediated signaling.[13]

We previously demonstrated significantly higher PSA expression in breast cancer (BC) tissue samples and malignant breast cells in comparison with normal breast tissue samples and nonmalignant cells.[14] We found that *STX* and *PST* were differentially expressed in epithelial-mesenchymal transition (EMT) of normal murine mammary gland (NMuMG) cells induced by transforming growth factor-β (TGF-β).[15]

In the current study, we analyzed the promoter region of *PST* and found a potential binding motif of Pax3, which can also modulate *STX* expression.[10] Our findings demonstrate dual roles of Pax3 in regulating opposite expression of *STX* and *PST* in NMuMG cells. Overexpression of Pax3 had a significant promoting effect on cell migration, but no effect on cell proliferation.

## Materials and methods

### Cells and cell culture

NMuMG cells and mouse mammary tumor 4T1 cells were cultured as described previously.[14] For induction of EMT,

NMuMG cells (~30% confluence) were suffered with 2 ng/ml TGF-β1 (R&D Systems) for 48 h.

### Antibodies and reagents

Mouse anti-E-cadherin IgG2a mAb and anti-NCAM IgG mAb were obtained from BD Biosciences, goat anti-Pax3 IgG polyclonal antibody (C-20) X and mouse anti-N-cadherin IgG1 mAb were purchased from Santa Cruz Biotechnology, mouse anti-β-tubulin IgG1, anti-vimentin IgG1 mAb, and anti-FN polyclonal antibody mAb were obtained from Sigma-Aldrich, and mouse anti-PSA-NCAM IgM mAb was acquired from Developmental Studies Hybridoma Bank. Secondary antibodies with HRP-conjugated were obtained from Beyotime.

### Quantitative real-time PCR analysis

Total RNA was isolated with an RNApure Tissue kit (CoWin Biotech; Beijing, China). An RNA sample (A260/A280>1.8) was reversed transcribed to cDNA using ReverTra Ace-α- ® (Toyobo; Osaka, Japan). Specific primers used for various genes were as follows: forward 5′-GCTCGTGGTCTTCCTCATCT and reverse 5′-GCGGTG AAGAGCCATTTATT for *STX*; forward 5′-GCGAACTGC CTATCCATCAC and reverse 5′-CATGAGGAGACCTG TGCTAGG for *PST*; forward 5′-GGC GGATCTAGAAA GGAAGG and reverse 5′-CGGAGCCTTCATCTGACTG for *Pax3*; forward 5′-CAGTGCGGCAACCAGATAG and reverse 5′-GGAGTGGCACTGTCAACCTC for *β-tubulin*. Quantitative real-time PCR (qRT-PCR) was performed following the procedure described previously,[16] and the data were analyzed by $2^{-\Delta\Delta Ct}$ method.[17]

### Plasmid construction

The Pax3 coding sequence was amplified with the forward primer 5′-CTAGCTAGCATGACCACGCTGGCCG and reverse primer 5′-GGAATT CCGGGCTCCAAGTGGAC AGTT, then inserted into the vector pcDNA3.1(+) (Invitrogen; Carlsbad, CA, USA) at the *Xho*I and *Eco*RI sites. The 5′-flanking promoter region (−443/−162) of *PST* was cloned using forward primer 5′-GGGGTACCCTCACAACGACTCTCCGAGC and reverse primer 5′-CGGAATTCAGCTCTCCCGGTTCTCCAG, and constructed into plasmid TOPFlash at the *Kpn*I and *Eco*RI sites. All plasmids were confirmed by DNA sequencing.

### Transfection and RNA interference

NMuMG cells were stably transfected with the constructed plasmid pcDNA3.1-Pax3 and control vector using Lipofectamine 2000 (Invitrogen), selected with G418 (700 μg/ml), confirmed by Western blot analysis, and designated as NMuMG/Pax3 and NMuMG/Mock.

Three short interfering RNAs (siRNAs) for *Pax3* and negative control dicer substrate duplex were synthesized by RiboBio Co. (Guangzhou, China). The sequences of validated siRNA for *Pax3* were: sense 5′-GCCACAAGAUA GUGGAGAUdTdT, antisense 5′-AUCUCCACUAUCUUG UGGCdTdT.

### Western blot analysis

Cells were harvested and lysed in the pre-cooled radioimmunoprecipitation assay (RIPA) buffer containing with 1% protease inhibitor cocktail (Biotool, Houston, TX, USA). Samples (30 μg protein) were separated by SDS-PAGE, transferred onto PVDF membrane, incubated with primary antibody as indicated and blotted with HRP-conjugated secondary antibody. Protein bands were analyzed by ChemiDoc XRS+ system (Bio-Rad).

### Luciferase assay

NMuMG cells were transiently co-transfected with specific expression vectors for 48 h, then the cells were washed twice with PBS, and lysed in 100 μl lysis buffer. Luciferase reporter assay system (Promega; Madison, WI, USA) was applied to detect the fluorescence. Luminescence intensity was acquired by Synergy H4 Multi-Mode Microplate Reader (Bio Tek; Winooski, VT, USA). Relative luciferase activities were normalized relative to control. Results were expressed as mean ± SD from triplicate experiments.

### Electrophoretic mobility shift assays

Nuclear extracts were separated using nuclear and cytoplasmic extraction kit (CoWin Biotech), and protein concentrations were measured using BCA Protein Quantitation Kit (CoWin Biotech). Electrophoretic mobility shift assays (EMSAs) were performed and analyzed as described previously.[18] In this assay, single-stranded probes covering two putative binding sites were synthesized, annealed to double-stranded oligonucleotide probes, labeled with DIG, and designated as P-1: 5′-CCACC TCCAATGCACAAGG TGTCACATTTG. Mutation probes were designated as P-1-m: 5′-CCACCTCCAATGCA ACCTTTGTCACATTTGAAAAG.

### Immunofluorescence staining

Cell immunofluorescence staining was performed as described previously.[19] In brief, cells were fixed with 4% fresh paraformaldehyde on glass coverslips and blocked with 3% BSA, incubated at 4°C with primary antibody for 16 h, and then stained with secondary antibody. DAPI (Invitrogen; Paisley, UK) was applied to mark the cell nuclei. Laser confocal fluorescence microscopy (model Eclipse Ti-U; Nikon; Tokyo, Japan) was used to detect stained cells.

### Wound healing assay

NMuMG/Pax3 and mock cells were seeded into 6-well plates at $5 \times 10^5$ cells per well. Pipette tips were used to scratch on the confluent monolayer to form linear wounds. Cells were washed with PBS and the images were captured at 0 h. After 24 h culture in medium without FBS, cells were washed with PBS and linear wounds were captured again.

### Proliferation (MTT) assay

Cells ($2 \times 10^3$/well) were plated on 96-well plates and after culture for different hours, 4 μL MTT solution (Cers, Yantai, China) was added to form formazan, then the reaction was stopped by addition of 100 μL DMSO, and absorbance at 595 nm was assessed immediately as previously described.[16]

### Statistical analysis

Data were determined by one-way analysis of variance using the software program GraphPad Prism 5. Differences between means with $P < 0.05$ were considered statistically significant.

## Results

### Pax3 expression was upregulated during EMT

We demonstrated previously that two polysialyltransferases, STX and PST, show significant differential expression during TGF-β-induced EMT process in NMuMG cells, in contrast with the expression in non-treated cells[15] (Figure 1(a)). This finding was confirmed by RT-PCR (Figure 1(b)). Bremer *et al.* showed that transcription factor Pax3, one member of a paired box (PAX), regulates expression of STX by binding to its promoter region.[10] We observed significantly increased Pax3 expression in TGF-β-induced EMT in NMuMG cells (Figure 1(b) and (c)). Metastatic 4T1 cells, in comparison with untreated NMuMG cells, showed higher expression of STX and Pax3 and lower expression of PST (Figure 1(d)), similarly to NMuMG cells undergoing EMT.

### Pax3 was responsible for both upregulation of STX and downregulation of PST in EMT

Bremer *et al.*[20] showed that Pax3 caused specific increase of STX. To clarify whether alteration of STX expression was modulated by Pax3 in NMuMG cells undergoing TGF-β-induced EMT, we established stable Pax3-overexpressing cell line NMuMG/Pax3 (Figure 2(a)). Pax3 overexpression significantly increased STX expression, but reduced PST expression (Figure 2(b)). In 4T1 cells, transient silencing of Pax3 enhanced PST expression, but had no effect on STX expression (Figure 2(a) and (c)). Pax3 was previously shown to positively regulate STX expression through binding to a CAAGG sequence complementary to its paired domain sequence GTTCC.[21,22] However, the effect of Pax3 on PST expression has not been studied. Our analysis of the PST gene promoter region revealed a CAAGG motif between positions −443 and −162 upstream of the transcriptional start point (TSS) (Figure 2(d)). We cloned this promoter region sequence into a luciferase reporter. Luciferase activity was reduced significantly in both TGF-β-treated and Pax3-overexpressing cells co-transfected with luciferase and *Renilla* expression vectors (Figure 2(e)). These findings suggest that Pax3 interacts with the PST promoter.

The binding capacity of Pax3 with PST promoter was evaluated using EMSAs. Treatment with probe P-1 resulted in appearance of a shifted band. Then, 50-fold unlabeled probes were added in competition assay to remove the non-specific binding, resulting in significant reduction of retarded bands. A supershifted band following addition of anti-Pax3 polyclonal antibody indicated specific binding by probe P-1 (Figure 2(f)). The shifted band was still present

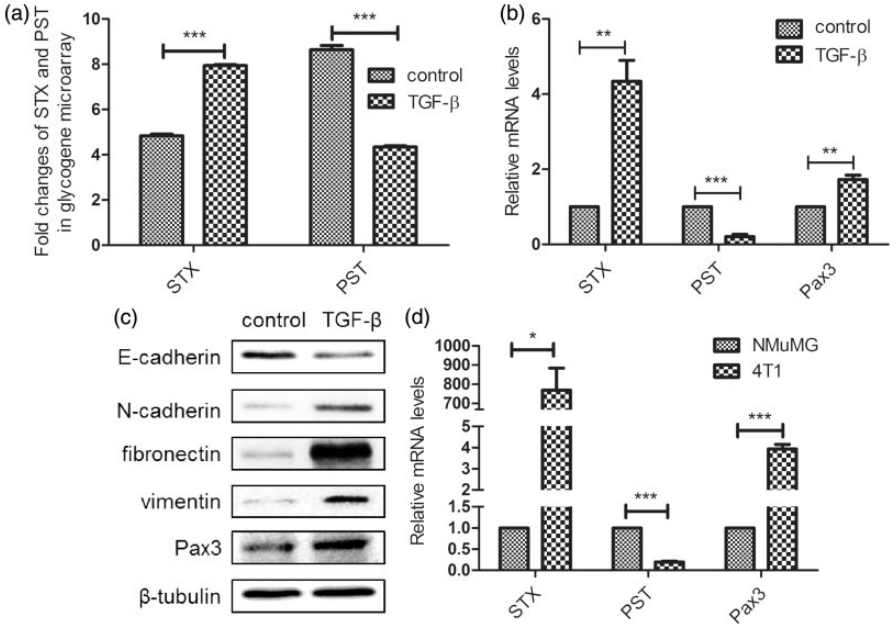


**Figure 1** Pax3 is upregulated in NMuMG cells undergoing TGF-β-induced EMT. (a) Fold changes of *STX* and *PST* in control vs. treated cells were assessed by glycogene chip analysis (GlycoV4 GeneChip). (b) Expression of *STX*, *PST*, and *Pax3* in control vs. treated cells was analyzed by qRT-PCR. (c) Western blot analysis of expression of EMT markers and Pax3 in cells undergoing EMT. Cells were cultured in 6-well plates, treated (or not) with TGF-β for 48 h, harvested, and lysed in RIPA buffer. Lysates (30 μg protein/well) were subjected to SDS-PAGE and analysis of E-cadherin, N-cadherin, fibronectin, vimentin, and Pax3 expression. (d) Expression of *STX*, *PST*, and *Pax3* in NMuMG and 4T1 cells was analyzed by qRT-PCR, with β-tubulin as internal control. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; NS: not significant

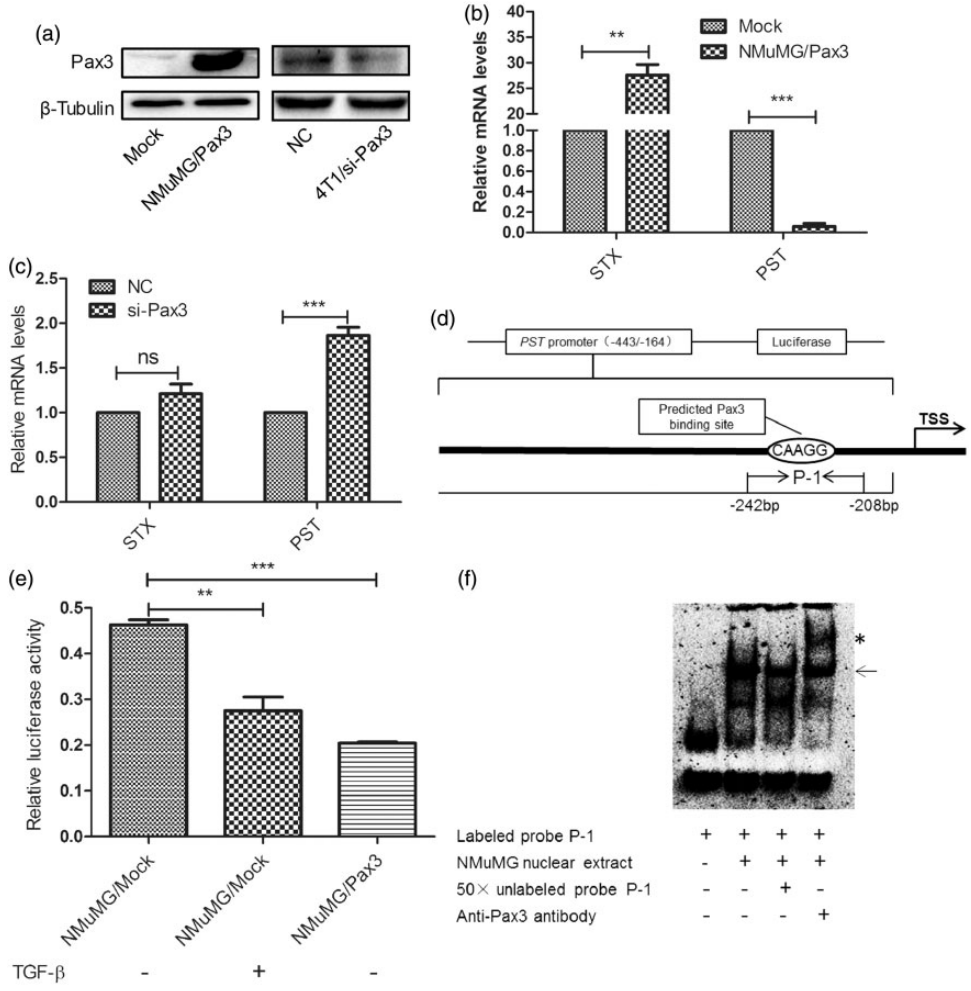


**Figure 2** Pax3 causes upregulation of STX and downregulation of PST. (a) Western blot analysis of Pax3 expression. Mock: pcDNA3.1(+). NC: scrambled sequence control. (b) and (c) Changes in *STX* and *PST* expression levels in NMuMG/Pax3 and 4T1/si-Pax3 cells, respectively, were analyzed by qRT-PCR, with β-tubulin as internal control. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method and presented as fold change. $^{**}P < 0.01$; $^{***}P < 0.001$; NS: not significant. (d) Schematic summary of *PST* promoter sequence analysis, showing two predicted Pax3 binding sites. TSS: translation start site. (e) Luciferase activity analysis of *PST* promoter (−443/−162) for untreated NMuMG/pcDNA3.1(+) cells (lane 1), TGF-β-treated NMuMG/pcDNA3.1(+) cells (lane 2), and NMuMG/Pax3 cells (lane 3). Firefly luciferase activity was normalized to *Renilla* luciferase activity, and presented as mean ± SD from three independent experiments. (f) EMSA was performed using DIG-labeled probe P-1 and nuclear extracts from NMuMG cells. Arrow: DNA-protein complexes. Competitive assay was performed with 50-fold unlabeled probe P-1 (lane 3). Supershift analysis was performed by adding anti-Pax3 antibody (lane 4; *)

following addition of labeled mutation probe P-1-m (data not shown).

Results of luciferase assays and EMSAs, taken together, indicate that Pax3 binds to the PST promoter and thereby suppresses PST expression.

### Effects of Pax3 on expression of PSA-NCAM and NCAM

Previously observation showed an enhanced PSA expression levels during TGF-β-induced EMT in both NMuMG and MCF10A cells.[14] STX and PST are both involved in addition of PSA to NCAM, the major PSA substrate. We were interested in the effect of Pax3 on expression of PSA-NCAM. In comparison with control cells, PSA-NCAM expression was enhanced in NMuMG/Pax3 cells and in NMuMG cells undergoing EMT (Figure 3(a) and (b)). NCAM was moderately increased in NMuMG/Pax3 cells, and greatly increased in NMuMG cells undergoing EMT. These findings, taken together, suggest that Pax3 enhances NCAM expression and promotes NCAM sialylation.

### Effects of Pax3 on migration and proliferation of NMuMG cells

It has been revealed that Pax3 can contribute to regulation of migratory events in embryogenesis, particularly in malignant melanoma and neuroblastoma. We examined the impacts of Pax3 on migration and proliferation in NMuMG cells. Pax3 overexpression obviously receded expression of epithelial cell marker E-cadherin, meanwhile, enhanced expression of mesenchymal cell marker N-cadherin (Figure 4(a) and (b)), and significantly increased cell migration evaluated by wound healing assay (Figure 4(c) and (d)), but had no effect on cell proliferation (Figure 4(e)).

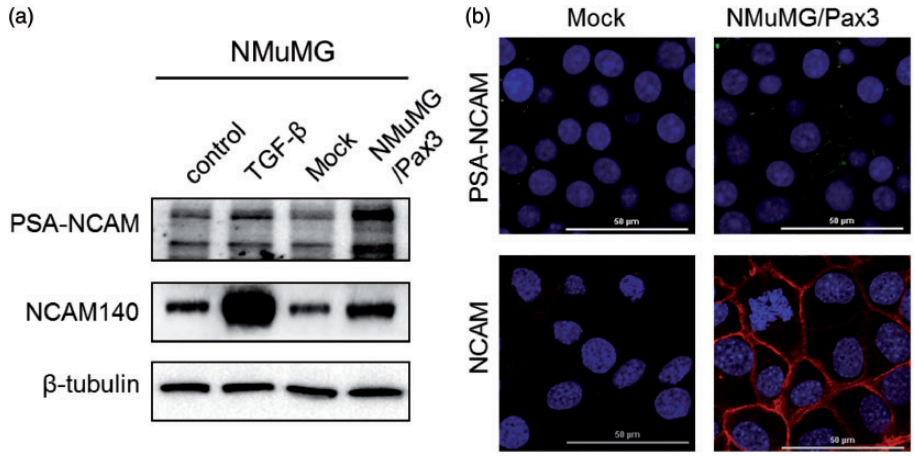


**Figure 3** Effects of Pax3 on PSA-NCAM and NCAM expression. (a) Western blot analysis of PSA-NCAM and NCAM in NMuMG cells. (b) Immunofluorescence staining of PSA-NCAM and NCAM in NMuMG/Mock and NMuMG/Pax3 cells. Nuclei were visualized by DAPI staining. Bars: 50 μm. (A color version of this figure is available in the online journal.)

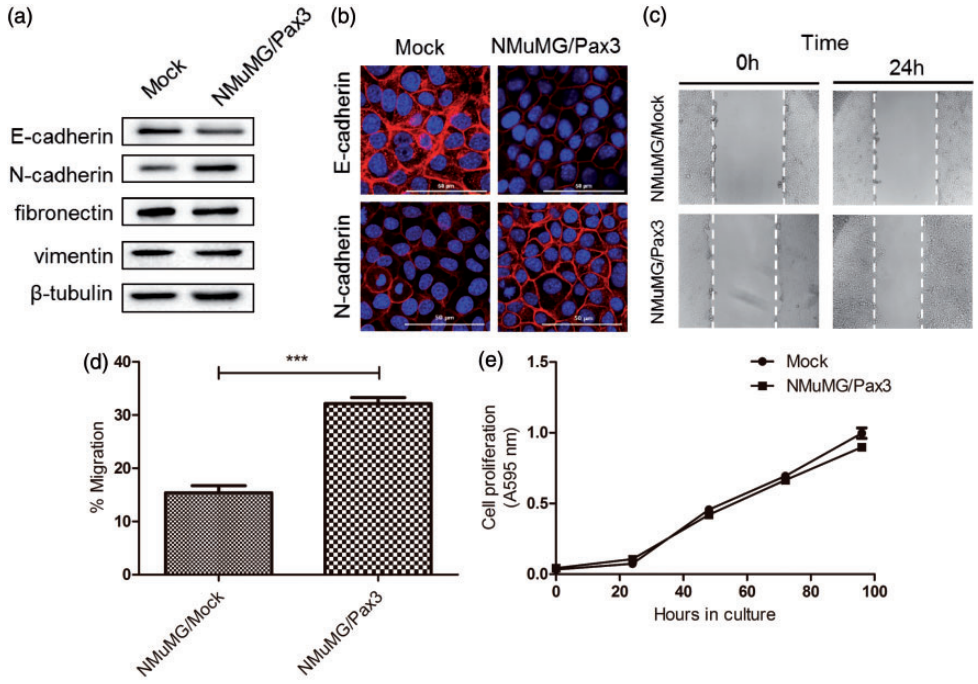


**Figure 4** Effects of Pax3 on migration and proliferation of NMuMG cells. (a) Western blot analysis of EMT markers. Cells were cultured in 6-well plates, harvested, and lysed in RIPA buffer. Lysates (30 μg protein per lane) were subjected to SDS-PAGE and analysis of E-cadherin, N-cadherin, fibronectin, and vimentin expression. (b) Immunofluorescence staining of E-cadherin and N-cadherin in NMuMG/Mock and NMuMG/Pax3 cells, and visualization of nuclei by DAPI staining. Bars: 50 μm. (c) Representative phase-contrast images showing increased migration of NMuMG/Pax3 cells, with NMuMG/Mock cells as control. After cells reached 100% confluence, scratches were made, regions were photographed (0 h), and the same regions were photographed at 24 h to assess the degree of wound healing. (d) Results of wound healing assay were analyzed using the Scion Image software program, and expressed as mean ± SD percentage area covered by moving cells at 24 h. All experiments were performed in triplicate. $^{***}P < 0.001$. (e) Proliferation (MTT) assays. NMuMG/Mock and NMuMG/Pax3 cells were cultured, and MTT assays were performed as described in Materials and Methods. Data are presented as mean ± SD from three independent experiments. (A color version of this figure is available in the online journal.)

## Discussion

Synthesis of heavily sialylated glycans is an event very frequently associated with malignant transformation. The change of terminal sialic acid structures, in particular, can be used as a tumor biomarker.[23] NCAM is the major substrate of PSA, and PSA-NCAM plays crucial function in neuronal differentiation and maturation.[24] There is steadily increasing evidence that aberrantly re-expressed PSA-NCAM modulates cell adhesion, migration, and invasion in many kinds of tumor,[25–27] and shows a strong relationship with severe clinical prognosis.[28] The polysialyltransferases STX and PST catalyze synthesis of PSA on NCAM, either individually or synergistically. Results of the present study show that PST expression is significantly reduced but

PSA-NCAM expression is increased in NMuMG cells undergoing TGF-β-induced EMT, implying that change of STX played a dominant role over PST, and upregulation of STX was primarily accounted for the increase of PSA-NCAM in this model.

Pax3 could be re-detected in some kinds of tumors, including melanoma, neuroblastoma, and rhabdomyosarcoma, and promotes tumor progression via multiple signaling pathways.[29–31] Knockdown of Pax3 in neuroblastoma and melanoma cells strongly inhibited proliferation and migration.[26] In the present study, Pax3 overexpression strikingly enhanced cell migration (Figure 4(c) and (d)), but had no effect on cell proliferation (Figure 4(e)). In a study by Bremer and his co-wokers,[22] Pax3 bound a CAAGG motif on the sense strand of STX and promoted STX expression, in agreement with the promoting effect of Pax3 on STX expression in the present study (Figure 2(b)). The following observations indicate that PST is a downstream target gene of Pax3: (i) the promoter region of *PST* includes a possible Pax3 binding site; (ii) PST expression was strikingly reduced in Pax3-overexpressing NMuMG cells (Figure 2(b)), but increased when endogenous Pax3 was inhibited (Figure 2(c)); (iii) luciferase assays and EMSAs showed that Pax3 can interact with the *PST* promoter region and thereby decrease its expression (Figure 2(e) and (f)). Pax3 evidently plays dual roles (activation or suppression of gene expression) that are dependent on the cellular ''context". Dual roles of transcription factors have been reported previously.[32] For example, to remodel a tumor microenvironment, Snail is acetylated and then transactivates its target gene; however, Snail also can act as a repressor of E-cadherin and induces EMT.[33] Pax3 can function as a repressor by binding to a target gene promoter through its paired domain directly, or through interaction with other protein.[34,35] The present findings suggest a negative regulatory role of Pax3 in PST expression. Studies are in progress to elucidate the molecular mechanism whereby Pax3 interacts with the PST promoter.

The mRNA levels of STX and PST expressed differently in both tissue- and time-specific patterns. STX is the major polysialyltransferase during embryogenesis, and plays dominated role in PSA synthesis in cancer cells,[28] whereas PST is mainly expressed in mouse or human adult brain.[36,37] In our current study, mRNA level of STX was obviously higher than that of PST in NMuMG cells undergoing TGF-β-induced EMT, as a result of Pax3 upregulation.

In conclusion, our findings demonstrate that the transcription factor Pax3 enhances expression of PSA on NCAM in NMuMG cells by upregulating STX expression and downregulating PST expression.

**Authors' contributions:** FG and XL designed the experiments; DG and JG carried out the experiments and analyzed the data; DG, FG and XL wrote the paper.

#### ACKNOWLEDGMENTS

This study was funded by the National Natural Science Foundation of China (No. 81672537), the Natural Science Foundation of Jiangsu Province, China (No.BK20161132, 20160173) and the Fundamental Research Funds for the Central Universities (No. JUSRP51619B, JUSRP116032). We thank Dr. S. Anderson for the English revision of the manuscript.

#### DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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*(Received May 11, 2016, Accepted August 21, 2016)*