Overexpression of Murine Pax3 Increases NCAM Polysialylation in a Human Medulloblastoma Cell Line*

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C. S. K. Mayanil‡, David George‡, Barbara Mania-Farnell §§, Christopher L. Bremer ‡, David G. McLeod ‡, and Eric G. Bremer §§

From the ‡Pediatric Brain Tumor Research Program, Department of Neurosurgery, Children's Memorial Institute of Education and Research, Chicago, Illinois 60614, the §Department of Biology, Purdue University Calumet, Hammond, Indiana 46323, and the ¶Robert H. Lurie Comprehensive Cancer Center, Northwestern University Medical School, Chicago, Illinois 60614

Polysialic acid (PSA) is a developmentally regulated carbohydrate found primarily on neural cell adhesion molecules (NCAM) in embryonic tissues. The majority of NCAM in adult tissues lacks this unique carbohydrate, but polysialylated NCAM (PSA-NCAM) is present in adult brain regions where neural regeneration persists and in some pediatric brain tumors such as medulloblastoma, which show greater propensity for leptomeningeal spread. Pax3, a developmentally regulated paired homeodomain transcription factor, is thought to be involved in the regulation of neural cell adhesion molecules. Overexpression of murine Pax3 into a human medulloblastoma cell line (DAOY) resulted in an increase in NCAM polysialylation and a 2–4-fold increase in α2,8-polysialyltransferase type II mRNA levels. No difference was observed in α2,8-polysialyltransferase type IV message. The addition of PSA to NCAM changed the adhesive behavior of these Pax3 transfectants. Transfectants expressing high PSA-NCAM show much less NCAM-dependent aggregation than those with less PSA-NCAM. In addition, Pax3 transfectants having high PSA-NCAM show heterophilic adhesion involving polysialic acid to heparan sulfate proteoglycan and agrin. These observations suggest that a developmentally regulated transcription factor, Pax3, could affect NCAM polysialylation and subsequently cell-cell and cell-substratum interaction.

Polysialic acid (PSA) is a large negatively charged linear homopolymer of α2,8-sialic acid residues mainly associated with neural cell adhesion molecule (NCAM). The kinetics of the homophilic adhesion mediated by NCAM correlate inversely with the degree of NCAM glycosylation and specifically with differences in the amount of α2,8-linked polysialic acid (1–5). Heterophilic binding of NCAM to extracellular matrix proteoglycans, on the other hand, appears to increase when PSA is present on NCAM (6). This suggests that PSA on NCAM promote cell migration. Migration of neural crest cells is critical for the developing embryo, failure of which causes neural tube defects. Studies done on brain tumors indicate that PSA may be a critical factor in facilitating neuroinvasive tumor metastasis in the brain (2). Whereas adult brain typically lacks this unique carbohydrate, medulloblastoma, neuroblastoma, and alveolar rhabdomyosarcoma are characterized by highly polysialylated NCAM (7–10).

Pax3 is a developmental transcription factor that may play a role in regulating cell adhesion molecules (11) and in oncogenesis (12, 13). Neale and Trasler (14) detected abnormal sialylation of NCAM in Splotch mice, which exhibit a neural tube defect. These authors suggested that Pax3 might be involved in modifying NCAM post-translationally, thus affecting cell adhesion. Gain-of-function mutations in Pax3 have been shown to cause cancer in human cell lines (15). A chromosomal translocation of Pax3, the human homolog of mouse Pax3, is implicated in the generation of pediatric solid tumor alveolar rhabdomyosarcoma (15–17). A chimeric transcription factor Pax3-FKHR, produced by this t(2;13)(q35;q14) chromosomal translocation in alveolar rhabdomyosarcoma binds to the NCAM promoter through its Pax3 homeodomain recognition helix. Although polysialylated NCAM isoform is expressed in rhabdomyosarcoma (10), the levels of NCAM are unaffected in alveolar rhabdomyosarcoma (18). Therefore, it seems plausible to suggest that Pax3/FKHR may not regulate the NCAM gene but could regulate NCAM polysialylation.

Although it is suggested that Pax3 may be involved in the post-translational modification of NCAM, the mechanism for this modification has not been identified. One possibility is that Pax3 may influence PSA levels on NCAM by altering the levels or activity of polysialyltransferase enzymes responsible for the synthesis of PSA chains. The primary objective of this study was to evaluate the role of Pax3 in NCAM polysialylation. In order to examine the effects of Pax3 on the expression of NCAM and PSA-NCAM, we transfected mouse Pax3 into a human medulloblastoma cell line, DAOY. The Pax3 transfectants showed increased NCAM polysialylation, as well as an up-regulation of α2,8-polysialyltransferase (ST8SiaII/STX) message levels and activity. Additionally, cell adhesion was altered in the transfectants. Transfectants expressing high levels of PSA-NCAM showed lower Ca2+-independent aggregation than those with lower levels of PSA-NCAM. These transfectants also showed heterophilic adhesion, involving PSA to heparan sulfate proteoglycans (HSPG). Taken together, these observations
suggest that Pax3 may affect NCAM polysialylation and as a result cell-cell and cell-substratum interaction.

**EXPERIMENTAL PROCEDURES**

Transfection of Pax3 cDNA into DAOY Cells—For the production of stable transfectants of mouse Pax3 in the human medulloblastoma derived cell line, DAOY, a 2.3-kb cDNA was inserted into the pCDNA expression vector (Invitrogen) at the EcoRI sites. The 2.3-kb Pax3 cDNA was prepared from pH3.2, which was kindly provided by Dr. Peter Gruss, Max Planck Institute, Göttingen, Germany (19). pH3.2 was digested with EcoRI (Amersham Pharmacia Biotech) to remove the 2.3-kb Pax3 cDNA, this was then ligated into pCDNA3 at the EcoRI site. Restriction digests confirmed orientation of the cDNA insert. The pCDNA3/Pax3 construct, or pCDNA3 alone as the vector control, was then transfected into the cells using a cationic liposome system, DOTAP (Roche Molecular Biochemicals). Transfectants were selected by anti-biotic resistance in cell medium containing 900 μg/ml Genetin G418 (Life Technologies Inc.). After 4 weeks in culture in the presence of G418, surviving colonies were tested for the presence of Pax3 mRNA.

Selection of Transfectants—Colonies were cloned by limiting dilution, cells were grown in 96-well plates with a cell density of 1 cell/well. The cells were grown in DMEM supplemented with 10% fetal bovine serum (Life Technologies Inc.), penicillin (50 units/ml), streptomycin (50 μg/ml), and L-glutamine (2 mM) supplemented with Geneticin G418 (900 mg/ml). When cells were near confluence, mRNA was isolated using the Poly(A) Tract series 9600 system (Promega), following the instructions in the Promega manual. To synthesize cDNA, 5 μl of mRNA from each well was added to each well in 96-well PCR reaction plates. The following were then added to the wells in a total volume of 50 μl for each PCR reaction (Perkin Elmer/Applied Biosystems, Inc.): 5 μl of 10× TaqMan buffer A (composed of 500 mM KCl, 100 mM Tris-HCl, 0.5 mM EDTA, 600 mM NaCl, 20 mM NaOH, 5 mM MgCl2, 1.5 μl of each dNTP (10 mM), 0.5 μl of forward and reverse primers (10 μM), 1 μl of AmpliTaq Gold (5000 units/ml), 0.25 μl of AmpliTaq Gold supplied at 5 units/ml and 0.25 μl of Murine Moloney leukemia virus. RT-PCR cycle parameters were 48 °C for 30 min, 95 °C for 15 min, followed by 40 cycles at 95 °C for 15 s and 55 °C for 1 min. The primers and the probes used in the study were designed using Primer Express software (Perkin Elmer/Applied Biosystems Inc.) and synthesized by Life Technologies Inc., and are shown in Table 1. Clones with a threshold cycle (Ct) value of 30 or less were selected for Pax3. A definition of Ct value is described in the section below, “Real-time Quantitative RT-PCR.”

Real-time Quantitative RT-PCR—The TaqMan probe consists of a specific oligonucleotide with both a 5′-reporter dye and a 3′-quencher dye. The fluorescent reporter dye such as 6-carboxyfluorescein, is covalently linked to the 5′ end of the oligonucleotide. The reporter is quenched by the 9-carboxyfluorescein-9-carboxylmethylamine (9-CFMA) which is a sequence specific oligonucleotide with both a 5′ end modification and a 3′ dCMP-C-Ptable-Neu5Ac (1.11 × 106 dpn), 1 mM diethiothreitol, 50 mM MOPS buffer, pH 6.1. This buffer and pH maximized the ratio of polysialyltransferase to monosialyltransferase activities. After incubation for 3 h at 33 °C, the reaction was terminated by the addition of EDTA to a final concentration of 50 mM. Each sample was then centrifuged for 30 min at 150,000 × g at 4 °C, to remove excess substrate. The pellets were resuspended in 100 μl of 20 mM Tris-HCl, pH 7.4, and divided into two equal aliquots. Endo-N-acetyleneuraminidase (EndoN) was a generous gift from Dr. Fredrick Troy (Department of Biological Chemistry, University of California School of Medicine, Davis, CA). EndoN, a bacteriophage-derived endosialidase that specifically cleaves α2,8-linked polysialic acid chains, was added to one of the two split samples, and both samples were incubated at 37 °C for 1 h (23). Each sample was subsequently spotted on Whatman 3MM paper. Descending paper chromatography was carried out overnight with ethanol and 1 N ammonium acetate, pH 7.5, in a ratio of 7:3. The papers were then allowed to dry. A 12 × 1-inch portion encompassing each origin was cut out from the paper, placed in vials containing scintillation fluid and quantitated in a Beckman scintillation counter. The difference in the counts between controls and EndoN-treated samples were taken as a measure of the amount of sialic acid that had been enzymatically incorporated into α2,8-linked polysialic acid chains.

**Cell Aggregation Assays—**To evaluate aggregation in a Ca2+-independent manner, transfectants and parental DAOY cells were plated 24 h prior to the assay, so that the cells would be in their exponential growth phase when the assay was performed. Half of the dishes were then treated with EndoN (0.5 unit/ml) for 1 h while the other non-treated dishes were used as a control. For both conditions, the cells were washed with Hepes-buffered saline (HBS), containing 100 mM NaCl, 20 mM Hepes, pH 7.2, and then incubated with HBS containing 3 mM EDTA for 10 min at 37 °C. Cells were collected by centrifugation at 500 rpm (800 × g) for 5 min at room temperature. The cells were then washed in HBS/EDTA and collected as above, after which they were resuspended in RPMI/10% fetal calf serum (2.5 × 105 cells/ml). The viability of cells was ascertained by trypan blue dye exclusion. 200-μl aliquots from EndoN-treated and untreated cells were incubated in plastic tubes at 37 °C for 40 min, followed by four cycles of gentle up-down rotation to ensure sampling from a homogeneous population (24). Single cells, obtained by passing cells 10 times through a 25-gauge needle (25), were counted with a hemocytometer before and after allowing cells to aggregate at 37 °C. Single cells were defined as cells that did not share a common border with neighboring cells (26). Particles that measured less than 4.5 μm in diameter were excluded from evaluation.

Western Blotting—Pax3 transfectants, selected by the above method, were seeded into 75-cm² flasks containing DMEM/10% fetal bovine serum and Genetin (200 μg/ml). When cells were near confluence, monolayers were washed twice with cold PBS, and solubilized by the addition of 200 μl of lysis buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 100 mM NaF, 1 mM MgCl2, 1.5 mM EGTA, and 1% Triton X-100). A separate aliquot of 25 μg of protein as determined by the method of Bradford (21) was used for this purpose, with 0.05 units of type X neureaminidase (Sigma) and incubated at 37 °C for 1 h. Western blots were performed according to Towbin et al. (22). 25 μg of protein was applied to a 10% polyacrylamide gel and subjected to electrophoresis and protein electrotransferred to an Immobilon P membrane. Incubating the membrane in 5% nonfat dry milk for 1 h at room temperature blocked nonspecific sites. The membrane was then incubated with respective antibodies at a dilution of 1:500: anti-NCAM, SBS (Developmental Study Hybridoma Bank), and anti-PSA (Sigma), and anti-MyoD (Santa Cruz Biotechnol-
nonspecific binding, the BSA solution was heat-inactivated at 56 °C for 1 h and filtered through a 0.2-μm Amicon filter. After blocking, the plates were washed twice with PBS.

Cells were then prepared in 0.1 mm EDTA for 5 min and gently removed from the culture dishes. Cells were then washed twice in PBS, centrifuged and resuspended in DMEM, counted, and adjusted to a final concentration of 2 × 10⁶ cells/ml in DMEM containing 0.1% BSA. Nearly confluent Pax3 transfectants as well as the vector and wild type control DAOY cells were treated with EndoN for 4 h. EndoN-treated and untreated cells (2 × 10⁶ cells/ml) were added to appropriate experimental dishes and incubated in a tissue culture incubator for 1 h. The experimental dishes were washed twice with DMEM, and the cells remaining in contact with the substrate were counted using an inverted phase-contrast microscope (magnification, ×200). Three 1-mm² fields of view were counted for each spot. Each experiment was repeated at least three times.

RESULTS

Expression of Pax3 Message and Functional Pax3 Protein in Pax3 Transfectants—Pax3 message expression was confirmed in selected clones by real-time quantitative RT-PCR using the probe and primers for murine Pax3 (Table I). Amplification plots generated by the real-time quantitative RT-PCR analysis showed the presence of Pax3 in the transfected clones (Fig. 1A). Seven highly positive clones were identified in subsequent experiments. These were designated B9, E7, E11, E12, F8, G8, and H6. The threshold cycle values (Cₜ values) of different Pax3 transfectants were normalized against β actin in real-time quantitative RT-PCR. All the transfectants showed lower Cₜ values ranging from −3.77 to −6.44 (β actin Cₜ – Pax3 Cₜ) cycles greater than the β actin (Table II). This corresponds to Pax3 expression levels of 150–625 copies of Pax3 per 10,000 copies of β actin. DAOY or vector controls showed no detectable murine Pax3 message. These observations indicated that mouse Pax3 message was expressed in transfected human DAOY cells. To investigate whether the Pax3 transfectants had functional Pax3 transcription factor, immunoblots were run with antibody against MyoD, a known downstream target of Pax3 (27). Immunoblots were performed using the seven transfectants against MyoD, a known downstream target of Pax3 (27). Immunoblots were performed using the seven

| Gene         | Forward primer | Reverse primer | TaqMan probe |
|--------------|----------------|----------------|--------------|
| Mouse Pax3   | 5′-TCT TAG AGA CGC AAC | CAT GGG-3′ | 6 FAM-ATG GCA TGC GGC CTI |
| Human STX    | 5′-AGC CCT ATT AGA TTT | GCT ATG TAA GCT GTT-3′ | GGC TCA TTT-TAMRA |
| Human PST    | 5′-GCC CGG TTT TGG GGA | GAT AGT-3′ | 6 FAM-ATG GCA TGC GGT CTC |
| Human NCAM   | 5′-GCC TGG GTA CCT GAC | GAG AT-3′ | CTC GCA-TAMRA |
| Human β-actin| 5′-CAG CGG AAC CGG TCA | TGG CCA ATG G-3′ | 6 FAM-ATG GCA TGC GGT CTC |

FIG. 1. The expression of Pax3, β-actin, STX, and PST in Pax3-transfected clone F8 and parental DAOY cells. The expression of murine Pax3 or human β-actin, STX, and PST were analyzed by RT-PCR reaction using the 7700 Sequence Detection System (Perkin Elmer) as described under "Experimental Procedures." The plots show the fluorescence intensity (ΔRₙ) at each PCR cycle. ΔRₙ is normalized reporter signal corrected for initial reporter signal. To calculate the ΔRₙ, the initial reporter signal is subtracted from the normalized reporter signal at each PCR cycle (20). A cycle threshold (Cₜ) is defined as the fractional cycle number at which the reporter fluorescence passes a fixed threshold value above baseline. Samples with higher message expression will have a lower Cₜ value. The cycle threshold was set at 0.06 ΔRₙ for this experiment. The amplification plots for messages originating from the parental DAOY cells are shown in black lines, whereas the plots originating from clone F8 are shown with gray lines. Panel A, amplification plots for human β-actin and murine Pax3. The cycle at which human β-actin and murine Pax3 reporter fluorescence crosses the threshold value in clone F8 is approximately 18 and 26, respectively. For DAOY cells, the β-actin crosses the threshold cycle at approximately 18. Pax3 never crosses the threshold in DAOY cells. Panel B, amplification plots for human STX. The cycle at which human STX reporter fluorescence crosses the threshold value in F8 clone and DAOY cells is approximately 27 and 29, respectively. Panel C, amplification plots for human PST. The cycle at which human PST reporter fluorescence crosses the threshold value in F8 clone and DAOY cells is approximately 26.
Pax3 Transfectants Show an Increase in PSA on NCAM—To examine NCAM polysialylation in Pax3 transfectants, Western blots were performed with anti-NCAM and anti-PSA antibodies. The blots with anti-NCAM antibody (5B8) showed an increased high molecular mass “smearing” ranging from 140 kDa to 200 kDa. This observation was suggestive of increased polysialylation. Similar blots were probed with anti-PSA antibody. In the transfectants two immunoreactive bands were observed. In the pcDNA3-transfected and wild type DAOY controls, the anti-PSA immunoreactivity was observed, suggesting the presence of PSA in these cells. However, the upper immunoreactive band was not present in the controls (Fig. 2).

An increase in high molecular weight immunoreactive NCAM, as shown in Fig. 2, could be due to increased NCAM levels or additional forms of NCAM. To test this possibility anti-NCAM antibody, 5B8 (directed against the protein) was used on the immunoblots. Once again, higher molecular weight immunoreactivity was observed. After neuraminidase treatment, the antibody recognized a band at ~140 kDa only. The transfectants did not show any change in the form of NCAM expression. Overall NCAM levels did not appear to be significantly altered in any of the clones (Fig. 3). These results suggest that Pax3 did not alter NCAM protein type or levels in the transfectants. Most likely, the increased high molecular weight immunoreactivity of anti-NCAM was due to increased levels of PSA on NCAM in the transfectants.

Pax3 Transfectants Show an Increase in ST8SiaII/STX mRNA—The increase in NCAM polysialylation observed by Western blot could be due to an increase in the levels of polysialyltransferase message. To examine if the increase in NCAM polysialylation was due to an increase in polysialyltransferase message or an increase in NCAM message, we performed real-time quantitative RT PCR using probes and primers for human NCAM mRNA—

| Clone         | β actin C_T | β actin STX C_T | β actin PST C_T | β actin-NCAM C_T | β actin Pax3 C_T |
|---------------|-------------|-----------------|-----------------|------------------|-----------------|
| DAOY         | 22.00       | 22.00           | 22.00           | 22.00            | 22.00           |
| pcDNA3       | 22.25       | 22.25           | 22.25           | 22.25            | 22.25           |
| B9           | 22.10       | 22.10           | 22.10           | 22.10            | 22.10           |
| E7           | 22.25       | 22.25           | 22.25           | 22.25            | 22.25           |
| E11          | 22.18       | 22.18           | 22.18           | 22.18            | 22.18           |
| F8           | 22.45       | 22.45           | 22.45           | 22.45            | 22.45           |
| H6           | 22.05       | 22.05           | 22.05           | 22.05            | 22.05           |

* C_T value is defined as the PCR cycle at which fluorescence increase above a predetermined baseline. RT-PCR was performed, and the base line was determined as described under “Experimental Procedures.”

All data were normalized against β actin expression. The numbers represent the cycle difference between β actin C_T and the gene of interest C_T (β actin C_T — gene C_T).

Significantly different from wild type DAOY or pcDNA3-transfected DAOY cells by Mann-Whitney U test, p < 0.0500.

In order to determine if approximately 4-fold increase in ST8SiaII/STX message level could account for the difference in NCAM polysialylation, total polysialyltransferase activity in polysialyltransferase enzyme rich membrane preparations were measured from the clones, the wild type and vector-transfected controls. Clones B9, E7, and H6 showed a significant increase in the polysialyltransferase activity as compared with wild type and pcDNA3-transfected control cells. Student’s t test showed the p values of <0.018, <0.005, and <0.0009, respectively, when B9, E7, and H6 were compared with vector pCDNA3-transfected DAOY cells (Fig. 4). These data showed that the increase in the level of ST8SiaII/STX message resulted in measurable increase in total polysialyltransferase activity for some of the clones. Since the polysialyltransferase activity assay cannot discriminate between ST8SiaII/STX and ST8SiaIV/PST activity, the lack of measurable increase with other clones may be due to the ST8SiaIV/PST activity. The clones with measurable increase in the polysialyltransferase activity also appeared to be the clones showing the greatest increase in PSA immunoreactivity (Fig. 2).

Pax3 Transfectants Show Decrease in Cell-Cell Aggregation—Polysialic acid on NCAM is known to alter cell-cell interaction. In order to determine whether the increased polysialylation in Pax3-transfected clones altered this cellular behavior, Ca²⁺-independent aggregation assays were performed. Fig. 5 shows the percentage of cell-cell aggregation in the Pax3 transfectants, parental DAOY, and vector control cells, with and without EndoN treatment. EndoN cleaves off α2–8-linked polysialic acid from NCAM; cell aggregation in the absence of α2–8-linked polysialic acid is an index of the aggregation due to NCAM alone. The results from the aggregation assays show that the parental and vector-transfected controls showed about 40–60% aggregation as compared with the Pax3-transfected clones, which showed 10–40% aggregation. Clones B9, E7, and H6, which had the highest levels of PSA on NCAM (Western blots; see Fig. 2), as well as the highest polysialyltransferase activity had the lowest aggregation. However, when the cell surface PSA was removed by EndoN treatment, cells aggregation returned to ~80% in all cells. Therefore, these observations suggest that the change in aggregation was due to increased PSA in Pax3 transfectants.

Pax3 Transfectants Show Increased Heterophilic Adhesion to Heparan Sulfate Proteoglycans—Polysialic acid was previously known to be a negative regulator of cell-cell adhesion (28).
Recently, Storms and Rutishauser (6) have shown that PSA may act as a positive regulator of cell-extracellular matrix interaction. In this study, spot cell adhesion assays were used to measure the interaction of Pax3 transfectants with the two extracellular matrix proteoglycans used by Storms and Rutishauser (6), agrin and HSPG (Fig. 6). Laminin was used as a control substrate, where binding should be independent of PSA content on NCAM (6). Clones B9 and H6 showed highest interaction with agrin and HSPG. This increased adhesion was eliminated by EndoN treatment, suggesting a requirement for PSA. In general the Pax3 clones were less adhesive to laminin substratum, but this adhesiveness was not affected by EndoN treatment. The increased adhesion was eliminated by EndoN treatment, suggesting a requirement for PSA. In general the Pax3 clones were less adhesive to laminin substratum, but this adhesiveness was not affected by EndoN treatment.

**DISCUSSION**

Several studies have indicated that Pax3 may be involved in regulating NCAM expression (11) and post-translational modification (14). Our in vitro data on Pax3 transfection in DAOY cells showed that Pax3 overexpression resulted in an increase in PSA-NCAM as observed on Western blots. Using real-time quantitative RT-PCR, the message levels of NCAM, ST8SiaII/STX, and ST8SiaIV/PST were determined in Pax3 transfectants. These data indicated an approximately 4-fold increase in the level of ST8SiaII/STX message. There was no change in ST8SiaIV/PST expression. All of the NCAM expressed in these cells appeared to be the 140-kDa isoform. In the clones that showed the greatest increase in PSA-NCAM, there was a measurable increase in total polysialyltransferase activity. Thus, the increase in NCAM polysialylation may be explained by a Pax3-dependent increase in ST8SiaII/STX message levels, resulting in increased polysialyltransferase activity. These changes in the message and activity of polysialyltransferase also appeared to be significant enough to alter NCAM-mediated cell-cell and cell-substratum adhesion.

Previously, Chalepakis et al. (11) have shown that Pax3 could regulate NCAM expression. Our data also indicate an increase in NCAM message expression in the transfectants. Ginsberg et al. (18) reported that a highly sialylated NCAM isoform was expressed in rhabdomyosarcoma (10), but the levels of NCAM itself were not changed. Rhabdomyosarcoma contains the fusion protein PAX3/FKHR transcription factor. This
The increased expression of ST8SiaII/STX suggests that it may be a downstream target of Pax3. Other molecules known to be regulated by Pax3 are NCAM (11), MyoD (27), c-Met (29), and other genes, Bennett et al. (37) reported that ST8SiaIV/PST preferred the sixth N-glycosylation site (Asn-478), which is closer to the transmembrane domain, over the fifth site (Asn-449). These observations suggested that ST8SiaIV/PST preferred the sixth site, whereas ST8SiaII/STX added polysialic acid on the fifth as well as on the sixth site. The size of the polysialic acid chain synthesized by ST8SiaII/STX was smaller than that synthesized by ST8SiaIV/PST, even though the total amount of polysialic acid synthesized by ST8SiaII/STX was comparable to that synthesized by ST8SiaIV/PST (35). Preferential increase in the message of ST8SiaII/STX in Pax3 transfectants could mean increased polysialylation on the fifth as well as sixth glycosylation site. This may explain the appearance of the upper and lower bands in the Pax3 transfectants. The upper band may represent PSA chains at both fifth and sixth glycosylation sites, whereas in the lower band only one of these sites is used. Interestingly, the presence of both upper and lower bands correlates with increased adhesion to agrin and HSPG substrates (clones B9 and H6).

The significance of these findings is that Pax3 may regulate ST8SiaII/STX, which confers polysialic acid on NCAM. Mouse Pax3 is expressed with a distinct spatiotemporal pattern beginning between day embryonic day 8 (E8) and embryonic day 9.5 (E9.5). Splotch phenotype results from the failure or partial failure of Pax3 to execute a specific genetic program (36). NCAM expression starts around E11 in mouse embryos, and reaches its peak around E18, whereas ST8SiaII/STX expression starts around E14 and reaches its peak around E20 (37, 38). ST8SiaIV/PST expression is fairly constant in the developing embryo. Using multivariate statistical analysis on the expression of Pax3, NCAM, and other genes, Bennett et al. (37) suggested that these genes do interact during those gestation times and that this is crucial for the migration of neural crest.
cells. Our present study provides experimental data that are consistent with their analysis.

Not only are Pax3, NCAM, and ST8SiaII/STX involved in embryonic development, they also appear to be involved in oncogenesis and may be predictors of clinical outcomes of pediatric brain tumors. Overexpression or aberrant regulation of the Pax3 gene can transform fibroblasts into tumor cells in nude mice (12, 13), suggesting that this gene may act as a proto-oncogene. Changes in the amount of polysialic acid on NCAM are related to invasive and metastatic growth potential of human tumors (37, 39, 40) and linked to the modulation of adhesive properties of NCAM. Highly polysialylated NCAM is a cell surface marker for medulloblastoma (41–43), neuroblastoma, and rhabdomyosarcoma (42–44). This polysialylation may give the tumor the propensity for leptomeningeal spread to other areas of the brain and adversely affect tumor outcomes, because the polysialylated NCAM on tumor cell has less cell-cell adhesion and more cell-extracellular matrix proteoglycan adhesion.

In sum, the present findings demonstrate that Pax3 overexpression in DAOY cells resulted in an increase in NCAM polysialylation. Increased polysialylation was due to an increase in the levels of ST8SiaII/STX message and subsequently in the activity of polysialyltransferase enzyme. Increased sialylation of NCAM caused a decrease in cell-cell aggregation and an increase in heterophilic binding to extracellular matrix proteoglycans. It is significant that Pax3 regulates the function of NCAM by regulating a developmentally regulated enzyme.
ST8SiaII/STX, that confers polysialic acid on NCAM. Improper expression or a mutation in Pax3 could adversely affect NCAM expression and polysialylation. This may partially explain the defects in Pax3 homozygous embryos. It may also affect tumor outcomes in medulloblastoma (41–43).

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REFERENCES
1. Troy, F. A. (1992) Glycobiology 2, 5–23
2. Troy, F. A. (1995) Static Acid: Molecular, Regulatory and Abnormal Biology, pp. 95–144, Plenum, New York
3. Doherty, P., Fruns, M., Seaton, P., Dickson, G., Barton, C. H., Sears, T. A., and Walsh, P. S. (1990) Nature 343, 464–466
4. Hoffmann, S. B., and Edelman, G. M. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 5762–5766
5. Rutishauser, U., Watanabe, M., Silver, J., Troy, F. A., and Vimr, E. R. (1985) J. Cell Biol. 101, 1842–1849
6. Stroms, S. D., and Rutishauser, U. (1998) J. Biol. Chem. 273, 27124–27129
7. Kojima, N., Tachida, Y., and Tsuji, S. (1997) J. Biochem. (Tokyo) 122, 1265–1273
8. Gluer, S., Schelp, C., von Schweinitz, D., and Gerardy-Schahn, R. (1998) Pediatr. Res. 43, 145–147
9. Wikstrand, C. J., Friedman, H. S., and Bigner, D. D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 310–314
10. Soler, A. P., Johnson, K. R., Wheelock, M. J., and Krudsen, K. A. (1993) Exp. Cell Res. 208, 84–93
11. Chalapakis, G., Jones, F. S., Edelman, G. M., and Gruss, P. (1994) J. Biol. Chem. 269, 12745–12749
12. Maalbecker, C. C., and Gruss, P. (1993) EMBO J. 12, 2361–2367
13. Stuart, E. T., and Gruss, P. (1995) Hum. Mol. Genet. 4, 1717–1720
14. Neale, S. A., and Trasher, D. G. (1994) Teratog. Teratol. Mutat. Res. 285, 118–124
15. Galili, N., Davis, R. J., Federicks, W. J., Mahapatra, S., Rauscher, F. J III., Emanuel, B. S., Rovera, G., and Barr, F. G. (1993) Nat. Genet. 5, 230–235
16. Barr, F. G., Galili, N., Holick, J., Biegel, J. A., Rovera, G., and Emanuel, B. S. (1995) Proc. Natl. Acad. Sci. U. S. A. 93, 5455–5459
17. Ginsberg, J. P., Davis, R. J., Bennicelli, J. L., Nauta, L. E., and Barr, F. G. (1998) Cancer Res. 58, 3542–3546
18. Higuchi, R., Fockler, C., Dollinger, G., and Watson, R. (1993) BioTechnology 11, 1026–1030
19. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
20. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
21. Sevigny, M. B., Ye, J., Kitazume-Kawaguchi, S., and Troy II, F. A. (1998) Glycobiology 8, 857–867
22. Ye, J., Kitajima, K., Inoue, Y., Inoue, S., and Troy, F. A. (1994) Methods Enzymol. 230, 460–484
23. Kai, J., Wu, X.-F., Gariepy, J., Rutishauser, U., and Shi, C.-H. (1992) J. Cell Biol. 118, 937–949
24. Scheidegger, P. E., Lackie, P. M., Papay, J., and Roth, J. (1994) Lab. Invest. 70, 95–106
25. Maroto, M., Reshef, R., Musterberg, A. E., Koester, S., Goulding, M., and Lassar, A. B. (1997) Cell 89, 139–148
26. Walsh, P. S., and Doherty, P. (1997) Annu. Rev. Cell Dev. Biol. 13, 425–456
27. Epstein, J. A., Shapiro, D. N., Cheng, J., Lam, P. Y. P., and Mass, R. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4213–4218
28. Hill, A. L., Phelan, S. A., and Loken, M. R. (1998) Dev. Genes Evol. 208, 128–134
29. Cai, J., Phelan, S. A., Hill, A. L., and Loken, M. R. (1998) Diabetes 47, 1803–1805
30. Kioussi, C., Gross, M. K., and Gruss, P. (1995) Neuron 15, 553–562
31. Yoshida, Y., Kurosawa, N., Kanematsu, T., Kojima, N., and Tsuji, S. (1996) J. Biol. Chem. 271, 30167–30173
32. Phelan, S. A., and Loken, M. R. (1998) J. Biol. Chem. 273, 19153–19159
33. Angata, K., Suzuki, M., and Fukada, M. (1998) J. Biol. Chem. 273, 28524–28532
34. Gruss, P., and Wallher, C. (1992) Cell 69, 719–722
35. Livingstone, B. D., Jacob, J. L., Glick, M. C., and Troy, F. A. (1988) J. Biol. Chem. 263, 9443–9448
36. Tsuji, S. (1996) J. Biochem. (Tokyo) 120, 1–13
37. Bennett, G. D., An, J., Craig, J. C., Gefrides, L. A., Calvin, J. A., and Finnell, R. H. (1998) Teratog. Teratol. Mutat. Res. 120, 57–70
38. Roth, J., Zuber, C., Wagner, P., Taatsjes, D. J., Weisgerber, C., Heitz, P. U., Goridis, C., and Bitter-Suermann, D. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2999–3003
39. Rougon, G., Durbec, P., and Figarella-Branger, D. (1992) Cancer Res. 52, 3542–3546
40. Rougon, G., Durbec, P., and Figarella-Branger, D. (1992) Cancer Res. 52, 137–144