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Antigens and cancer pathways targeted by de-N-acetyl polysialic acid monoclonal antibodies

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ANTIGENS AND CANCER PATHWAYS TARGETED BY DE-N-ACETYL POLYSIALIC ACID MONOCLONAL ANTIBODIES

by

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ANTIGENS AND CANCER PATHWAYS TARGETED BY DE-N-ACETYL POLYSIALIC ACID MONOCLONAL ANTIBODIES

ADARSHA SHIVAKUMAR

ABSTRACT

Polysialic acid (PSA) is a developmentally regulated glycan made of repeating sialic acid monomers with α2-8 linkages. PSA has very limited expression in adults, and modifies only a few cell-surface proteins. However, PSA is overexpressed in several human cancers and is associated with metastasis and poor prognosis. We have described a derivative of PSA containing a mixture of de-N-acetyl and N-acetyl neuraminic acid residues (dPSA) found intracellularly in many normal human tissues but expressed at much higher levels on the cell surface of many human cancer cell lines. The proteins modified with dPSA and dPSA function in normal and abnormal human biology are unknown. The purpose of this study was to identify protein(s) modified with PSA and possible dPSA-dependent functions in cancer cell lines that express dPSA antigens. Using co-immunoprecipitation with the anti-dPSA monoclonal antibody SEAM 2 and mass spectroscopy, we identified membrane-associated nucleolin that is either directly modified or associated with dPSA. In addition, knocking down expression of the polysialyltransferase ST8SiaII (STX) in SK-MEL-28 human melanoma cells nearly eliminated dPSA and nucleolin from membranes but had no effect on the levels of nuclear nucleolin, and resulted in aberrant cell morphology, cell adhesion, and motility. The data suggest that cell-surface nucleolin depends on modification with dPSA, and that dPSA-modified nucleolin has an important role in cell adhesion and migration.
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LIST OF ABBREVIATIONS

dPSA .......................................................... derivative of PSA containing mix of de-N-acetyl and N-acetyl neuraminic acid residues
mAb .............................................................. Monoclonal Antibody
NCAM .............................................................. Neural Cell Adhesion Molecule
NCL ................................................................. Nucleolin
PSA ................................................................. Polysialic Acid
PSA-NCAM ......................................................... Polysialylated Neural Cell Adhesion Molecule
RNAi .............................................................. RNA Interference
INTRODUCTION

Sialic acids are N- or O-substituted derivatives of the 9-carbon monosaccharide neuraminic acid, and are common terminal carbohydrates on eukaryotic cell surface glycoproteins and glycolipids. Two common mammalian sialic acids are N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). Polysialic acid (PSA) is a glycan made of repeating sialic acid monomers with α2-8 linkages. All N-linked glycans have a common core pentasaccharide, Man$_3$GlcNAc$_2$, as shown in Figure 1. Singular sialic acid modifications are common terminal additions to N-linked glycans as seen in Figure 2. PSA is made up of 50 to over 370 sialic acid residues (1, 2), making them one of the larger glycans added to proteins. Despite its size, PSA is synthesized by just two polysialyltransferases that act on N-linked and O-linked glycans (3, 4). These polysialyltransferases, ST8SiaIV (PST) and ST8SiaII (STX), are expressed in different tissues and amounts postnatally, but have been shown to work cooperatively to polysialate proteins (5). Normally, localization of where glycan synthesis occurs is difficult because several enzymes are required for their biosynthesis. However, identifying the sites of PSA synthesis is less complex since it involves only two specific polysialyltransferases. Polysialylation was found to be typically added to N-glycans on proteins in the trans most cisternae of the rough endoplasmic reticulum and the trans Golgi network (6).
Figure 1: Core structure for all N-linked glycans, a common core pentasaccharide, Man$_3$GlcNAc$_2$ (7).

Figure 2: Structure of a complex glycan that contains terminal sialic acid residues, a bisecting GlcNAc on the pentasaccharide core, and fucosylation on the core GlcNAc (7).

Polysialylation appears not just to occur in normal human cells, but also in certain human tumors: Increased PSA expression has been observed in astrocytoma (8), small cell and non-small cell lung carcinoma (9), neuroblastoma (10), and rhabdomyosarcomas (11). The antibodies used to detect the PSA expression in these cell lines were all created through different means and thus likely have differing epitopes.

Most glycosyltransferases can modify a huge number of proteins, while polysialyltransferases appear to be modify a select few proteins including autopolysialylation of the ST8SiaIV and ST8SiaII enzymes (12), synaptic cell adhesion
molecule 1 (13), the alpha subunit of the voltage sensitive sodium channel (14), the integrin alpha 5 subunit (15), the scavenger receptor CD36 (16), fetuin (17), and neuropilin-2 (18). However, the structural features that determine whether and where a particular protein is polysialylated are not yet clear.

The most studied polysialylated protein is the neural cell adhesion molecule (NCAM) (19), which is prominent in the developing nervous system. NCAM has six N-glycosylation sites, but Foley et al. discovered that polysialylation occurs on two N-glycans located on the fifth immunoglobulin domain (Ig5) of NCAM (20), as shown in Figure 3. They also demonstrated a degree of flexibility in polysialylation sites through forcing limited polysialylation at some of the other N-glycosylation sites.

Figure 3: A schematic representation of the structure of polysialylated NCAM. There are six N-glycosylation sites indicated by the black arrowheads. Of those, only the two on the Ig5 domain can be polysialylated, and an example of a potential polysialylation is shown on the right (21).
While the actual impact of polysialylation on protein function at large is still being studied, the role of polysialylation in NCAM function has been closely investigated (22). When two cell membranes expressed polysialylated-NCAM, the distance between them increased, leading Yang et al. to postulate that the steric hindrance of PSA weakens contacts between neighboring cells (23, 24). However, the effect of PSA on NCAM is not solely inhibitory, as PSA promotes the binding of NCAM to heparan sulfate proteoglycans (25, 26, 27). Overall, the impact of PSA on protein binding ability is variable, and needs to be examined on a case-by-case basis.

Our laboratory discovered a novel derivative of PSA, which contains a mixture of de-N-acetyl and N-acetyl neuraminic acid residues (dPSA) using monoclonal antibodies (mAbs) SEAM 2 and SEAM 3 (28). The mAbs were produced by immunizing mice with a PSA derivative-protein conjugate vaccine where the PSA derivative contained about 16% de-N-acetyl and 84% N-propionyl neuraminic acid residues to enhance immunogenicity (28, 29). By using the murine anti-dPSA mAb SEAM 3 to detect dPSA expression via immunohistochemistry, dPSA was found to be present in the cells of many human tissues (30). However, dPSA is expressed at much higher levels in many human cancers compared to cells in the corresponding normal tissues (31). Given the limited expression of PSA in normal adult humans, this result was intriguing. The presence of dPSA antigens was correlated with increased mRNA expression of the polysialyltransferases PST and STX, therefore this could be the mechanism underlying the increase in dPSA observed in these cancer tissues (30). Further, we showed that dPSA is derived from PSA produced by STX by knocking down expression of that specific
polysialyltransferase (31). While polysaccharide deacetylases are a large family of enzymes (32) widely distributed across many species and known to have an important role in organismal development, the proteins modified with dPSA, the cell types producing dPSA-modified proteins, and the function of dPSA in normal and abnormal human biology are unknown. The purpose of this study was to identify protein(s) modified with PSA and their possible dPSA-dependent functions. This was accomplished by suppressing PSA expression with RNA interference (RNAi) and/or blocking dPSA with anti-dPSA monoclonal antibodies in dPSA-expressing cancer cell lines.
METHODS

Materials

Cell culture medium along with all supplements were purchased from UCSF Tissue Culture Facility (San Francisco, CA) except fetal bovine serum (Gemini Bioproducts, West Sacramento, CA). C23 (MS-3) monoclonal mouse antibody was acquired from Santa Cruz Biotechnology, CA. SEAM 2 monoclonal antibody was generated in Children’s Hospital Oakland Research Institute, as detailed/described below. 14C7, a murine IgG3 mAb specific for Neisseria surface protein A, which is an irrelevant antigen in human cells, was produced by Dr. Gregory Moe in Children’s Hospital Oakland Research Institute (33). All anti-mouse secondary antibodies conjugated with Alexa Fluor fluorochromes were obtained from Invitrogen.

mAb SEAM 2 generation and specificity

We used the monoclonal antibody SEAM 2, which along with SEAM 3, was produced by immunizing a CD1 mouse with an N-Pr MBPS tetanus toxoid conjugate vaccine (29). The specificity and functional activity of SEAM 2 and SEAM 3 have been studied extensively (31, 34–36). In a previous study, we showed that the anti-NPr MBPS mAb SEAM 3 recognizes de-N-acetyl PSA-containing derivatives (34). SEAM 2 is known to recognize a relatively long chain PSA epitope containing more than 10 residues where ∼50% of the residues are neuraminic acid (35). Of note, SEAM 2 is not reactive with PSA-NCAM, even though NCAM is known to have two PSA modifications. This discrepancy can be explained by the fact that SEAM 2 recognizes de-N-acetyl PSA-.
containing derivatives, which are different epitopes than the ones recognized by the antibodies that have been previously targeting PSA. SEAM 2 used in this study was purified by Protein A affinity chromatography from ammonium sulfate precipitated cell culture medium provided by QED, Inc. (San Diego, CA). Sodium sulfate was added to the solution to a final concentration of 0.5 M and incubated at ambient temperature overnight. This step is required to dissociate dPSA antigens that become associated with SEAM 2 during cell culture. This solution was then loaded onto a 5 ml HiTrap Fast Flow Protein A column (GE Healthcare Bioscience) in buffer (20mM histidine, 0.02% tween 20, pH = 6.5 adjusted with NaOH). SEAM 2 was then eluted from the Protein A column with elution buffer (0.1 M histidine, 0.02% tween 20, pH = 2.7 adjusted with acetic acid and HCl). Fractions containing the mAb were immediately neutralized by adding 1M NaHCO₃ to adjust the pH to ~7.5. The fractions were combined, dialyzed (Spectra Pore, 10kDa, ThermoFisher) in 0.1M NaHCO₃, pH 8, and concentrated (Centraprep UltraCel-30, EMD Millipore) to ~2 mg/ml as determined by absorbance at 280nm (Nanodrop, ThermoFisher).

**Cell Culture**

SK-MEL-28 human melanoma cells were purchased from ATCC (HTB-72). Cells were grown routinely in T75 flasks containing RPMI 1640 medium containing 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 2 mM glutamine, penicillin/streptomycin and 10% fetal bovine serum (FBS) at 37°C in 5% CO₂. Confluent cells were sub-cultured (1:3 to 1:8) by treating with 0.25% (w/v) Trypsin/0.53 mM
EDTA solution, and washing in media before re-seeding into new growth medium. SK-MEL-28 cells were only used up to passage 10 from the original ATCC stock of cells. Jurkat cell clone E6-1, a human acute lymphoblastic leukemia T cell line was purchased from ATCC (TIB-152). Jurkat cells were grown in T75 flasks containing the same media as SK-MEL-28 cells in 5% CO₂ at 37°C and subcultured every 3 days with a split ratio of about 1:5. Kelly human neuroblastoma cells were a gift from Dr. Julie Saba, CHORI. Kelly cells were grown in T75 flasks containing the same media as SK-MEL-28 cells in 5% CO₂ at 37°C and subcultured every 3 days with a split ratio of about 1:5. Polysialyltransferase knock-down cell lines in SK-MEL-28 human melanoma cells were produced by Joshua Lee, Lindsay Stierer and Gregory Moe by vector-based interfering RNA (37). The PST1C3 cell line was an STX polysialyltransferase knockdown that did not affect expression of PST, while another cell line which had a non-functional scrambled RNA plasmid was a negative control. Both cell lines were grown in the same media as SK-MEL-28 and Kelly cells in 5% CO₂ at 37°C and subcultured every 3 days with a split ratio of about 1:5. Blasticidin was added to the media and cells in T75 flasks at a concentration of 10μg/mL to select the plasmid-containing cells.

**Protein Extraction from Cells**

Approximately 1×10⁵ cells from each cell culture were extracted using the ProteoExtract® Subcellular Proteome Extraction Kit (EMD Millipore). In brief, the extraction procedure uses four extraction buffers sequentially, along a protease inhibitor cocktail to prevent protein degradation during the extraction and Benzonase nuclease to
degrade contaminating nucleic acids. The first extraction buffer yields cytosolic proteins, the second extraction buffer yields membrane proteins, the third extraction buffer yields nuclear proteins, and the fourth and final extraction buffer yields cytoskeletal components. The listed instructions for extraction were followed (38), and the cell extracts were separated into four fractions: F1 corresponding to the cytosolic fraction, F2 corresponding to the cell membrane fraction, F3 corresponding to the nucleic protein fraction, and F4 corresponding to the cytoskeletal fraction.

**Immunodot Blotting**

A hybridized nitrocellulose membrane (Immobilon-NC, EMD Millipore) was cut to fit the dot blot apparatus (Topac Inc. Instrumentation). The membrane was then pre-wetted in PBS buffer for 2 minutes. The membrane was not allowed to dry out before it was mounted in the apparatus. 2 µ of each extracted fraction was then spotted on the top row of the membrane. Twofold serial dilutions were done for each fraction down each column, up to a 1:16 dilution for each fraction in the appropriate extraction buffer. The membrane was then dried for 30 minutes using a vacuum attached to the dot blot apparatus. After the fraction samples were spotted onto the membrane, the membrane was incubated in a 5% milk solution at 4°C overnight. The membranes were then rinsed with PBS three times for five minutes, and then incubated for ninety minutes at room temperature with either a 1:200 solution of SEAM 2 in PBS/milk or a 1:200 solution of the negative control antibody 14C7 in PBS/milk. The membranes were rinsed again with PBS three times for five minutes. Next, the membranes were incubated for ninety
minutes at room temperature in a 1:5000 solution of the appropriate IgG secondary antibody in PBS/milk. The membranes were rinsed again with PBS three times for five minutes and then placed on a Li-Cor Odyssey Infrared Scanner for imaging at 800 nanometers.

**Co-immunoprecipitation**

As F1, F2, and F3 were the only fractions to react in the immuno-dot blot, they were the only fractions that were further purified through co-immunoprecipitation. Each fraction was incubated with Dynabeads M-270 epoxy magnetic beads (Thermofisher) covalently linked to SEAM 2 or the irrelevant murine IgG3 mAb 14C7. The beads were separated on the side of the tube using a magnet, washed with the respective extraction buffer alone, then buffer containing PSA (50 µg/ml) to remove nonspecific binding antigens. Finally, buffer containing N-propionyl polysialic acid with 36% de-N-acetyl polysialic acid (50 µg/ml, provided by Dr. Gregory Moe) was used to elute the specific binding antigens from each fraction.

**SDS-PAGE and Coomassie staining**

The eluted proteins from each cell fraction were resolved on 4%-12% gradient SDS-PAGE gels, (NuPAGE, Invitrogen) after adding Sample Buffer (Invitrogen) containing reducing agent and heated to 70°C for 10 minutes before loading on the gel. The gel was run for 35 minutes at 200V, and then stained with Simply Blue Coomassie stain (Invitrogen).
**LC/MS/MS protein identification**

The excised gel slices were extracted overnight in AmBic (50 mM ammonium bicarbonate) containing 50% acetonitrile. The disulfide bonds were reduced and modified with iodoacetic acid per UCSF In-Gel Digestion Protocol (39) prior to trypsin digest (Promega). LC/MS/MS protein identification of tryptic peptides was performed by the UC Davis Proteomics Core facility.

**Laser scanning confocal microscopy**

SK-MEL-28, PST1C3, and scrambled RNA negative control cells (∼10⁵ cell/ml) were cultured on multi-well glass coverslips that had been coated with 1:10 human placental Type IV collagen (Sigma Aldrich) in 30% ethanol. After an overnight incubation, cells were gently washed with PBS and fixed with 4% formaldehyde in PBS. After one hour, the cells were washed with PBS, and half the coverslips had 0.5 ml of ice-cold 0.25% Triton X-100 added. The plates were incubated on ice for 10 minute. The Triton and the paraformaldehyde were removed and the plates were then blocked in blocking buffer (0.5ml solution of 2% goat serum in PBS/0.25% Tween) for 1 hour. Next, 200µl of each of the following combinations of primary antibodies was added to the pairs (triton treated and not treated) of wells: 1) negative control 14C7 and 2) SEAM 2 and C23, for one hour at room temperature. The C23 was in 1:50 in goat serum/DPBS buffer, while the other primaries were all 1:100 in goat serum/DPBS buffer. The cells were then gently washed with sterile PBS before goat anti-mouse isotype-specific secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 594 (ThermoFisher) were added (1:200 dilution) in
blocking buffer to each well for at least one hour at room temperature in the dark.

Subsequently, the cells were washed with PBS/Tween, then PBS. Finally, 200µl of .5% DAPI in PBS was added to each well for ten minutes. The cells were then washed with PBS one more time, and then mounted using a hardening mounting medium (Electron Microscopy Sciences) and left to dry overnight. Confocal images were obtained using a Zeiss LSM710 laser scanning confocal microscope (CHORI) and analyzed using ImageJ Software (40) and JACoP (41).
RESULTS

We had established in previous studies that human melanoma SK-MEL-28 cells are relatively high expressers of dPSA antigens that are reactive with anti-dPSA mAbs SEAM 2 and SEAM 3 (31, 37). SEAM 2 recognizes a dPSA epitope containing at least 10 residues, 30%-50% of which are de-N-acetyl polysialic acid (Figure 4). Furthermore, SK-MEL-28 cells are adherent with a relatively large cytoplasm making them advantageous with respect to identifying dPSA antigens in subcellular structures.

SK-MEL-28 cells were processed by differential detergent solubility into fractions enriched in cytosolic, membrane, nuclear, and cytoskeletal proteins (fractions F1, F2, F3, F4, respectively). The presence of dPSA antigens in each fraction was determined by immunodot blot with detection using anti-

![Figure 4](image)

**Figure 4**: The SEAM 2 and SEAM 3 mAbs both bind to the de-N-acetyl PSA containing derivatives, but SEAM 2 binds to derivatives with a longer epitope, as shown in the figure.

![Figure 5](image)

**Figure 5**: Immunodot blot of SK-MEL-28 cell fractions. SEAM 2 marks dPSA antigens mainly in Fraction 2 and to a lesser extent in Fraction 3. Very minor staining of F1 and no staining of Fraction 4 were observed. There was no staining with an irrelevant IgG3 mAb on the left.
dPSA mAb SEAM 2. The membrane fraction (F2) was the most reactive with SEAM 2 (Figure 5).

To identify molecules modified with SEAM 2-reactive dPSA antigens, fractions F1-F3 were combined with SEAM 2 or an irrelevant murine IgG3 mAb (14C7) covalently linked to magnetic beads. Fraction 4 was not subjected to co-immunoprecipitation since this fraction was not reactive with SEAM 2 in the immunodot blot and contains sodium dodecyl sulfate, which inactivated SEAM 2 binding activity. The beads were washed with PSA in the respective extraction buffer to remove antigens binding non-specifically. SEAM 2-specific binding antigens were then eluted with N-propionyl polysialic acid containing 36% de-N-acetyl polysialic acid. SEAM 2 has about 200 times greater avidity for this antigen than dPSA containing N-acetyl neuraminic acid residues (G. Moe, unpublished). The eluted proteins were resolved on SDS-PAGE gels. As shown in Figure Z, only the sample from the SEAM 2-F2 co-immuno-precipitation contained proteins staining with Coomassie, which appear largely as a “smear” rather than distinct bands. Typically, proteins modified with PSA run in SDS-PAGE gels over a relatively wide range of apparent mass because of the
heterogeneity of polysialylation. Variable amounts of PSA de-N-acetylation likely produces additional heterogeneity. Four relatively dark staining regions of the gel were excised from the gel and processed to generate tryptic peptides for LC/MS/MS mass fingerprinting (labeled P1-P4 in Figure 6). As controls, gel slices were taken from the same relative positions in the sample from the F2-irrelevant IgG3 mAb co-immunoprecipitation.

**Identification of SEAM 2 antigen**

The tryptic peptide LC/MS/MS analysis of the bands (figure not shown) excised from the SEAM2-F2 immuno-precipitate identified nucleolin (14 exclusive peptides, 16 unique spectra, 167/710 amino acids identified ranging from residues 139-624) as the predominant protein in slices marked P1 (~145 kDa) and P2 (~115 kDa). In addition, there were trace amounts of several ribosome-associated proteins in both fractions. Slices marked P3 (~71 kDa) contained ribosome-associated proteins and alpha-tubulin and P4 (~48 kDa) contained ribosome-associated proteins and actin. There were no proteins identified in the control slices. The calculated molecular mass of nucleolin is 77 kDa, but due to multiple covalent modifications, the presence of highly acidic regions near the N-terminal region, and self-cleaving activity nucleolin can have several apparent masses ranging from ~100 kDa to ~50 kDa (42, 43). The experiment was repeated and included an additional control of co-immunoprecipitation with the anti-PSA mAb SEAM 12 (data not shown). Again, nucleolin was the predominant protein in gel slices corresponding to P1 and P2 (39% sequence coverage), with smaller amounts of nucleolin also present in the SEAM 12 co-immunoprecipitate and much less in the irrelevant control.
The same co-immunoprecipitation procedure of subcellular enriched cell fractions was repeated for Kelly neuroblastoma and Jurkat T lymphocyte cells. SEAM 2 was found to co-immuno-precipitate nucleolin from F2 of Kelly cells (19 exclusive unique peptides, 22 exclusive unique spectra, 190/710 amino acids) and F1 in Jurkat cells (15 exclusive unique peptides, 25 exclusive unique spectra, 308/710 amino acids) that was not present in corresponding gel slices of the irrelevant negative control mAb (figure not shown).

**Morphological differences between stable, chromosomally expressed RNAi knockdown of polysialyltransferase ST8SiaII (STX) and a scrambled negative control mutant of SK-MEL-28 cells.**

SK-MEL-28 cells express the two polysialyltransferases ST8SiaII (STX) and ST8SiaIV (PST). STX is highly expressed during normal fetal development but is abnormally overexpressed in many human cancers PST is expressed at low levels in a

![Figure 7: Morphological changes resulting from RNAi knockdown of polysialyltransferase ST8SiaII (STX). The negative control “scrambled” SK-MEL-28 cell line has normal morphology characterized by an extended structure with lamellipodia extending from leading and lagging ends of cell movement. The RNAi STX knockdown cell line lacks the extended structure and contains large numbers of lamellipodia.](image-url)
A variety of human tissues (5). Previously, it had been shown that transient STX siRNA knockdown in SK-MEL-28 cells decreased production of PSA and dPSA, demonstrating that dPSA is derived from PSA. Subsequently, mutant SK-MEL-28 cell lines were generated by chromosomal integration of plasmids expressing siRNA targeting PST and STX (37). As shown in Figure 7 above, RNAi knockdown of STX alone resulted in an altered cell morphology compared to a negative control cell line with a chromosomally integrated plasmid expressing a scrambled RNA having wild-type cell morphology. The cells with STX RNAi knockdown are characterized as being smaller with a large number of lamellipodia arrayed in a circular pattern around a dense cytoplasm centered on the nucleus.

**dPSA and nucleolin are co-localized in SK-MEL-28 cells and inhibiting production of PSA decreases dPSA-nucleolin on the cell surface**

Since anti-dPSA mAb SEAM 2 immuno-co-precipitated nucleolin, we asked the question of whether nucleolin is co-localized with dPSA in SK-MEL-28 cells. To address this question, we used laser scanning confocal fluorescence microscopy with anti-nucleolin mAb C23 to mark nucleolin and SEAM 2 to mark dPSA. As a control, the cells were stained with an irrelevant murine IgG3 mAb and the secondary antibodies. As shown in Figure 8 below, there was no staining with the irrelevant mAb or secondary
antibodies alone. In wild-type SK-MEL-28 cells, dPSA (red fluorescence) was present on the surface of cells and was concentrated in vesicle-like structures at the leading and lagging ends of the cell and at points of possible lamellipodia development. Anti-

Figure 8. Fluorescence micrograph of SK-MEL-28 cells containing a chromosomally integrated plasmid expressing negative control “scrambled” RNA stained with an irrelevant murine IgG3 mAb and goat anti-mouse IgG2a conjugated Alexa Fluor 488 (green fluorescence) and goat anti-mouse IgG3 conjugated Alexa Fluor 594 (red fluorescence) secondary antibodies. Nuclear DNA was stained with DAPI (blue fluorescence).

nucleolin staining (green fluorescence) was identical with respect to location and intensity of staining (merge). Next, we looked at staining patterns inside cells by treating fixed cells with the detergent Triton-X100 to permeabilize them and allow entry of primary and secondary antibodies. dPSA inside cells exhibited a web-like pattern typical of the most trans endoplasmic reticulum (ER) and golgi. Both are known to be sites where polysialylation occurs. Also, dPSA staining was concentrated in leading and lagging protrusions and in vesicle-like structures of possible lamellipodia development. Again, anti-nucleolin staining matched that of anti-dPSA inside cells with respect to location and intensity of staining. The exception was the intense anti-nucleolin staining around the nuclear membrane, which was completely devoid of anti-dPSA staining. The negative control SK-MEL-28 mutant expressing the scrambled RNA was similar to the wild-type strain with respect to pattern of staining, co-localization of nucleolin with dPSA, and differences in dPSA staining with nuclear membrane nucleolin. Finally, we
looked at the effect of reduced production of dPSA in the STX RNAi knockdown cell line. As shown in Figure 9, the STX knockdown has the characteristic circular morphology with multiple lamellipodia shown in Figure 7, above. Cell surface dPSA and nucleolin were greatly diminished in the STX RNAi mutant. Similarly, the cytoplasmic distribution of dPSA and nucleolin were reduced but STX knockdown had no effect on anti-nucleolin staining of the nuclear membrane.
Figure 9: Fluorescence micrographs of wild-type SK-MEL-28, negative control mutant expressing a scrambled siRNA, and STX knockdown mutants. dPSA is marked with anti-dPSA mAb SEAM 2 (red fluorescence), nucleolin with anti-nucleolin mAb C23 (green fluorescence, and DNA with DAPI (blue fluorescence). Cells were permeabilized as indicated to allow detection of intracellular dPSA and nucleolin. The figure shows that dPSA is co-localized with nucleolin except for nucleolin in the nuclear membrane, that the presence of nucleolin in the cytoplasm and cell surface depends of the production dPSA, and that dPSA-nucleolin is concentrated in the cell structures at the leading and lagging lamellipodia.
DISCUSSION

Co-immunoprecipitation of the membrane fraction from SK-MEL-28 and Kelly neuroblastoma cells with anti-dPSA mAb SEAM 2 identified nucleolin as either directly modified or associated with de-N-acetyl polysialic acid (dPSA). Furthermore, laser scanning confocal microscopy of SK-MEL-28 cells showed that SEAM 2-reactive dPSA was co-localized with cell surface and intracellular membrane-associated nucleolin but not nuclear nucleolin. The results suggest that dPSA-modified nucleolin is a distinct form of the protein. Nucleolin is an evolutionarily conserved protein, with homologs found in yeast (44) and plants (45). The nucleolin gene contains 14 exons with 13 introns and spans 11kbp (710 AA, 76,614 Da), and there are no known splice variants. The base structure of nucleolin consists of three multifunctional domains: an N-terminal region containing several acidic stretches, a central region containing two to four RNA-binding domains, and a C-terminal region rich in glycine/arginine. Nucleolin is found throughout the cell, and has been detected in the cytoplasm, nucleus, nucleoli, and the cell membrane. In non-cancerous cells, nucleolin is predominantly located intracellularly, except in the cases of epithelial cells, where it is found outside on the cilia (46). Similarly, anti-dPSA reactivity with SEAM 2 is present in cilia but not on the cell surface of normal epithelial cells (G. Moe, unpublished). However, in cancer cells, nucleolin has been shown to be expressed consistently and abundantly cell surface (47, 48). The reason for this difference in nucleolin expression patterns is due to post-translational modifications, which modify the functionality and localization of nucleolin.
Nucleolin’s post-translational modifications include phosphorylation (49), methylation (50), ADP-ribosylation (51), small ubiquitin-like modification (SUMO) (52), and glycosylation-including sialylation although polysialylation of nucleolin was not known to occur (53). Nucleolin is a highly phosphorylated protein, and the N-terminal region is phosphorylated by major protein kinases such as casein kinase 2 (CK2) (49) and cyclin-dependent kinase 1 (CDK1) (54). This phosphorylation has been shown to be necessary for nucleolin to translocate to the cell surface (55). Nucleolin has approximately one-third of the arginine residues being methylated, which impacts its ability to interact with DNA and RNA (56). ADP-ribosylation of nucleolin has been reported, but the residues targeted and the impact on nucleolin’s functionality is unknown.

Carpentier et al. identified two N-glycosylation sites on the extranuclear nucleolin isoform at positions N317 and N492, which are on the edge of the RNA-binding cleft of the RNA-binding domains RBD1 and RBD3 (53). They also identified one other potential N-glycosylation sites along the RNA-binding domains. N317 is also near a highly acidic segment similar to those identified as being essential for polysialyltransferase docking (57). Hoja-Lukowicz, Kedracka-Krok, Duda, and Litynska showed that cell surface but not cytoplasmic nucleolin was modified with α2-3- and α2-6-linked sialic acids (58). Although specific structural determinants for polysialylation are not known, polysialyltransferases require substrate glycans containing at least one sialic acid residue to initiate polymerization (57). SK-MEL-28 cells express both PST and STX polysialyltransferases. However, knocking down expression of just STX
resulted in almost complete loss of SEAM 2 reactivity and membrane-associated nucleolin, which is consistent with cell surface/membrane-associated nucleolin being directly modified with dPSA derived from STX polysialylation. STX expression in normal adult cells is almost completely suppressed (5). In contrast, STX expression is high during fetal development and in many human cancers such as small cell lung cancer, neuroblastoma, and adenocarcinomas of the breast, ovary, pancreas, and stomach (59). Similarly, these cancers are also high expressors of dPSA, as measured by reactivity with anti-dPSA mAbs and cell surface nucleolin (30). Polysialylation of nucleolin may not have been recognized previously because lectins and anti-PSA antibodies do react with dPSA and neuraminidases that degrade PSA have no effect on depolymerizing dPSA (T. Bandarhi and G. Moe, unpublished).

The function of cell surface nucleolin and dPSA is not known. However, the phenotypic effect of knocking down STX expression and the corresponding effect on dPSA-modified/associated nucleolin provides some clues. Cell surface dPSA-modified nucleolin was concentrated in vesicle-like structures in small protrusions at the leading and lagging ends of SK-MEL-28 cells expressing the polysialyltransferase STX but not in cells with STX expression knocked down. The latter cells also exhibited an aberrant morphology characterized by multiple narrow lamellipodia and a defect in cell motility (37). The movement of cells on surfaces is a complex process involving more than 300 proteins (60). The extension of lamellipodia and their adherence to the substrate is a critical component of cell motility and metastasis in normal cancer cells. Disrupting complexes made of cell surface nucleolin and other surface proteins through
pseudopeptide binding has shown a variety of inhibitory effects on various cancer cell
types, including inhibition of cell adhesion and migration (61). Nucleolin has also been
discovered to interact with and affect several cytoskeletal elements. Thus, changes in
nucleolin structure or expression could plausibly impact cellular motility. Hovanessian et
al. found that cell surface nucleolin is able to cluster in specific patterns through
interactions with actin filaments of the cytoskeleton, and that cell surface nucleolin can
mediate the intracellular import of various ligands (62). Gaume et al. discovered that
nucleolin expression plays a role in microtubule stabilization. However, they were unable
to determine if nucleolin directly associated with microtubules or if it indirectly affected
microtubule stability through interactions with actin or through regulation of microtubule
associated proteins (63). As actin filaments and microtubules are critical cytoskeletal
elements of cell movement and lamellipodia formation, the polysialylation of nucleolin
could affect cellular motility and thus tumor progression and metastasis. The discovery
that cell surface nucleolin interacts with integrins to enable cell migration in human
glioblastomas and melanomas supports this idea, as cell surface nucleolin is often
glycosylated (64, 65). Cell surface nucleolin has also been shown to facilitate and
stimulate macropinocytosis in cancer cells (66), a specific type of endocytosis dependent
on the actin skeleton and implicated with cell motility (67).

Separately, polysialylation, especially by STX, and sialic de-N-acetylation have
also been shown to affect cell motility. Cytidine monophosphate-N-acetyl neuraminic
acid (CMP-NANA) is the substrate for polysialyltransferase PSA polymerization. Excess
CMP inhibits CMP-NANA formation without also being cytotoxic. Al-Saireleh et. al.
showed that CMP inhibited cell surface PSA production and significantly reduced the ability of several neuroblastoma cell lines to migrate in a CMP concentration-dependent manner (68). Further, they showed the effect was specifically the result of inhibiting STX. Since the cell lines tested are known to express dPSA antigens (31) and dPSA is derived from PSA, it is possible that the effect of inhibiting STX also affected dPSA. In addition, Liu et. al. provided direct evidence that sialic de-N-acetylation occurs specifically in metastatic melanoma and is important for cell migration and invasion (69). They showed de-N-acetyl ganglioside GM3 promoted cell migration and invasion through urokinase plasminogen activator receptor signaling that activated matrix metallophroteinase-2. Currently, no sialic acid de-N-acetylas have been identified, so it is uncertain whether there are specific sialic acid de-N-acetylas for sialylated molecules that contain only a couple of residues, such as the gangliosides, and longer polymers such as PSA. However, our studies (30, 31, and this study) and previous studies of de-N-acetyl gangliosides in melanoma (69, 70) clearly establish that sialic acid de-N-acetylation is prevalent in melanoma and has a role in cell migration.

Nucleolin is also known to be involved in a large variety of intracellular and extracellular processes in normal and cancer cells. Intracellularly, it is involved in ribosome biogenesis, cytokinesis, nucleogenesis, cell proliferation and growth, chromatin remodeling, DNA repair, regulation of mRNA translation, and receptor signaling (71). The N-terminal domain of NCL also interacts with histones and DNA. Specifically, the phosphorylated N-terminal domain nucleolin has been shown to bind to histone H1 and induce chromatin decondensation (72). The C-terminal domain of nucleolin has been
shown to interact with and bring together ribosomal RNA and proteins (73, 74).
Nucleolin is also shown to be critical for the functionality of RNA Polymerase I (75).
Nucleolin also binds to or interacts with many proteins that have been shown to play a role in tumor proliferation and DNA repair/damage. Wise et al. discovered that nucleolin binds to the critical cancer cell survival factor Fas through glycine/arginine rich domains in the N-terminal, and that B-cell lymphomas with surface nucleolin-Fas complexes are resistant to Fas-mediated apoptosis (76). Farin et al. reported that nucleolin interacts with the oncogenic protein Ras in a unique manner. There are many Ras variants, and each can exist in a GTP and GDP bound state that have different 3D configurations and thus bind to different molecules. However, nucleolin binds to not only all Ras isoforms, but also both the GDP and GTP-bound Ras forms (77). Nucleolin also binds to the human telomerase reverse transcriptase subunit through its fourth RNA-binding domain and the carboxyl-terminal RGG domain in the C-terminal end (78). By doing this, nucleolin appears to be involved in assembly or maturation steps of telomerase (79). Other proteins known to bind to and be regulated by nucleolin include Fragile X Mental Retardation Protein, hepatocyte growth factor, Tumor Necrosis Factor alpha inducing protein, and Proliferating Cell Nuclear Antigen (80-83).

In this study, we have identified cell surface and intracellular membrane-associated nucleolin as a SEAM 2 antigen likely directly modified with dPSA. We have also shown that STX has a critical role in defining SK-MEL-28 cell morphology and enabling cell motility. Since cell adhesion and motility are important in cancer cell metastasis, particularly in melanoma, and anti-dPSA mAbs could have cytotoxic effects
on SK-MEL-28 cells. Therefore, this study has the potential to open the way to new approaches to preventing and treating cancers that produce cell surface dPSA-modified nucleolin.
## LIST OF JOURNAL ABBREVIATIONS

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