A cancer-unique glycan: de-N-acetyl polysialic acid (dPSA) linked to cell surface nucleolin depends on re-expression of the fetal polysialyltransferase ST8SIA2 gene

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Research Article

Keywords: glycans, de-N-acetyl polysialic acid, nucleolin, ST8SIA2, immune shielding, cancer

DOI: https://doi.org/10.21203/rs.3.rs-246416/v1

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Abstract

Background: Polysialic acid (PSA) modifies a few cell surface proteins in humans mainly during fetal development and some blood cells in adults. Two genes in humans, ST8SIA2 and ST8SIA4, code for polysialyltransferases that synthesize PSA. The product of ST8SIA2, STX, is highly expressed during fetal development and in many cancers but not in adult normal human cells. The product of ST8SIA4, PST1, is expressed in fetal and some adult tissues and also in many cancers. We identified a derivative of PSA containing de-N-acetyl neuraminic acid residues (dPSA), which is expressed on the cell surface of human cancer cell lines and tumors but not normal cells.

Methods: dPSA-modified proteins in several human cancer cell lines and normal blood cells were identified using co-immunoprecipitation with anti-dPSA antibodies and mass spectroscopy. RNAi and CRISPR were used to knockdown and knockout, respectively, the polysialyltransferase genes in two different cell lines to determine effect on production of cell surface dPSA measured by flow cytometry and fluorescence microscopy.

Results: We found that dPSA is linked to nucleolin, a nuclear protein reported to be on the cell surface of many cancers but not normal cells. Knocking down expression of ST8SIA2 with RNAi or knocking out each gene individually and in combination using CRISPR showed that cell surface dPSA depended on expression of ST8SIA2 and not ST8SIA4.

Conclusions: The presence of dPSA specifically in a broad range of human cancers offers novel possibilities for targeting the dPSA antigen and synthetic pathway for detection, treatment, and prevention of cancer.

Background

Human polysialic acid (PSA or polySia), is a developmentally regulated homopolymer of a2-8-linked 5-N-acetyl neuraminic acid residues that can be greater than 100 residues in length. Humans have two genes, ST8SIA2 and ST8SIA4, that code for enzymes that synthesize PSA (polysialyltransferases STX and PST1, respectively). While both genes are highly expressed in humans during fetal development [1], PST1 is expressed mainly in lymphoid tissues and lymphocytes [2], while STX appears not to be present at significant levels in any adult normal tissues based on Northern blot [1] and publicly available RNA-seq and protein databases (as summarized, for example, by GeneCards: the human gene database [3]). Of the five proteins confirmed to be polysialylated in humans [4-8], neural cell adhesion molecule (NCAM) is the most abundant, particularly during fetal development, and is the most thoroughly investigated [9]. A number of human cancers are reported to express PSA-NCAM abnormally [10-14] where its role in mediating interactions among cells and between cells and the extracellular matrix is associated with metastasis and poor clinical prognosis [10, 14]. Although both polysialyltransferases STX and PST1 appear to synthesize the same polysaccharide, there may be differences in substrate specificity [15, 16] or functional activity. STX was reported to produce shorter polymers compared to PST1 and, in some
circumstances, the two enzymes may work synergistically [17]. Recently, mutations in the promoter region of ST8SIA2 gene were associated with schizophrenia [18]. However, to date, there are no data suggesting that the two polysialyltransferases have a preference for modifying a specific protein, and functional distinctions between the two are unclear.

Previously, we reported the discovery of a de-N-acetylated form of PSA (dPSA) and of anti-dPSA antibodies that were reactive with dPSA antigens [19, 20] on placent al trophoblasts, the surface of cancer cells, and inside some normal cells within the perinuclear space [21, 22]. Also, human microbial pathogens that produce PSA, including *Neisseria meningitidis* serogroup B [19] and *Leishmania major* [23], display cell surface dPSA under conditions corresponding to encountering a human host [23, 24]. Although de-N-acetyl sialic acid-containing derivatives of gangliosides have been described previously in human melanoma cell lines [25, 26] and de-N-acetylation of polysaccharides occurs in many species [27], proteins modified with dPSA and the function of dPSA in human cell biology, particularly cancer, are unknown.

In this study, we identified nucleolin as the protein modified with dPSA. We investigated the role of STX/ST8SIA2 and PST1/ST8SIA4 related to the production of dPSA and found that STX/ST8SIA2 was responsible for production of dPSA on the surface of cancer cells, and we characterized the effect of interfering with ST8SIA2 and ST8SIA4 gene expression on cell surface dPSA and cancer cell morphology. Importantly, we show that cell surface dPSA is unique to cancer cells and widely expressed among different cancers. The unique presence of dPSA on the surface of human trophoblasts, cancer cells, and microbial pathogens raises the possibility of a role for dPSA in immune shielding.

**Methods**

**Antibody reagents**

Anti-nucleolin monoclonal mouse antibody MS-3 was acquired from Santa Cruz Biotechnology (Santa Cruz, CA), anti-a-tubulin was from Invitrogen and anti-b-tubulin Type III was from Sigma-Aldrich (St. Louis, MO). Irrelevant murine and human subclass control antibodies were obtained from Southern Biotech (Birmingham, AL) and BioXCell (Lebanon, NH). Anti-dPSA monoclonal antibodies (mAbs) SEAM 2 and SEAM 3 and anti-PSA mAb SEAM 12 were produced as described previously [28]. Irrelevant mAb, 14C7 (murine IgG3), was produced as described previously [29]. All anti-mouse secondary antibodies conjugated with Alexa Fluor fluorochromes were obtained from Thermo Fisher Scientific (Waltham, MA). SEAM 2, SEAM 3, SEAM 12 and 14C7 used in this study were purified by Protein A affinity chromatography. Antibody concentrations were determined by absorbance at 280nm.

**Cell culture**

SK-MEL-28 human melanoma and panels of gastric, ovarian, and pancreatic cancer cell lines (HTB-72, TCP-1008, TCP-1021, and TCP-1026, respectively) were obtained from American Type Culture Collection (Manassas, VA) and cultured in medium recommended by ATCC. CHP-134 cells were obtained from...
MilliporeSigma (Burlington, MA). Kelly cells were a gift from J. Saba at UCSF Benioff Children's Hospital Oakland. Kelly, SK-MEL-28, RNAi mutant SK-MEL-28, CHP-134, and CRISPR knockout cells were grown routinely in flasks containing RPMI 1640 medium, 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% (weight/volume) trypsin/0.53 mM EDTA or Accutase® solution (Thermo Fisher Scientific) and washing in media before re-seeding into new growth medium. CHP-134 and CHP-134 CRISPR knockout clone cells were suspended by pipetting. Medium for the SK-MEL-28 mutant cell lines also contained 10 µg/mL blasticidin. Adherent cells used for immunoprecipitation, fluorescence activated cell sorting (FACS) binding, and fluorescence labeling experiments were treated with Accutase® and washed with medium before use. Human normal peripheral blood mononuclear cells (PBMCs) were purchased from AllCells (Alameda, CA).

Protein extraction from cells

Cells from each cell culture were extracted using the ProteoExtract® Subcellular Proteome Extraction Kit (MilliporeSigma). In brief, the differential detergent extraction procedure used four extraction buffers sequentially, along with a protease inhibitor cocktail to prevent protein degradation during the extraction and Benzonase® nuclease (Sigma-Aldrich) to degrade contaminating nucleic acids. The manufacturer's instructions for extraction were followed, and the cell extracts were separated into four fractions: F1 (cytosolic fraction), F2 (cell membrane fraction), F3 (nucleic protein fraction), and F4 (cytoskeletal fraction).

Co-immunoprecipitation

Dynabeads M-270 epoxy magnetic beads (Thermo Fisher Scientific) covalently linked to SEAM 2 or the irrelevant murine IgG3 mAb 14C7 were prepared following the manufacturer's protocol. As F1, F2, and F3 were the only fractions to react in an immuno-dot blot (see, for example, Additional file 1, Supplementary Fig. S1), they were the only fractions that were further purified through co-immunoprecipitation. Each fraction was incubated separately with SEAM 2- or 14C7-linked magnetic beads. The beads were separated using a magnet, washed with the respective extraction buffer alone, and then with buffer containing PSA (50 µg/mL; colominic acid from Sigma-Aldrich) to remove nonspecific binding antigens. Finally, buffer containing 50 µg/mL N-propionyl polysialic acid [22] with 36% de-N-acetyl polysialic acid (N-Pr dPSA) was used to elute the antigens binding specifically to SEAM 2 from each fraction.

LC/MS/MS protein identification

The eluted proteins from each cell fraction were resolved on 4–12% gradient SDS-PAGE gels (NuPAGE, Thermo Fisher Scientific). The gels were stained with SimplyBlue™ Coomassie stain (Thermo Fisher Scientific). The excised gel slices were extracted overnight in 50 mM ammonium bicarbonate containing 50% acetonitrile. The disulfide bonds were reduced and modified with iodoacetamide per University of California San Francisco In-Gel Digestion Protocol [30] prior to trypsin digest (Promega Corp., Madison,
LC/MS/MS protein identification of tryptic peptides was performed by the University of California Davis Proteomics Core facility.

**Laser scanning confocal microscopy**

SK-MEL-28, ST8SIA2 knockdown mutant, and scrambled RNA negative control cells (∼10^5 cells/mL) were cultured on glass coverslips coated with human placental Type IV collagen (Sigma-Aldrich). After an overnight incubation, cells were fixed with 4% formaldehyde for 1 h in phosphate buffered saline (PBS). For staining internal antigens, coverslips were treated with ice-cold 0.25% Triton X-100 in PBS for 10 min. The coverslips were blocked in blocking buffer (2% goat serum in PBS/0.25% Tween). Primary antibodies (5µg/mL) in blocking buffer were added to the coverslips and incubated at ambient temperature. After PBS/Tween washes, goat anti-mouse isotype-specific secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 594 (Thermo Fisher Scientific) were added (1:200 dilution) in blocking buffer at room temperature in the dark. Subsequently, the cells were washed with PBS/Tween, then PBS. Finally, DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) in PBS for 10 min before mounting with mounting medium (Electron Microscopy Sciences, Hatfield, PA). Confocal images were obtained using a Zeiss LSM710 laser scanning confocal microscope and analyzed using ImageJ Software [31] and JACoP [32].

**Creating knockdown cell lines targeting ST8SIA2 and ST8SIA4 by RNA interference**

Knockdown cell lines were produced in SK-MEL-28 human melanoma cells by vector-based interfering RNA using a BLOCK-iT™ Pol II miRNA RNA vector system (Thermo Fisher Scientific). SK-MEL-28 cells were transfected with one of four vector constructs, two targeting each gene, called pcDNA 6.2-GW/+ EmGFP – PolyST, where PolyST represents ST8SIA2-1, ST8SIA2-2, ST8SIA4-1, and ST8SIA4-2. An additional scrambled control construct which does not target any known vertebrate gene was made, for a total of five vector constructs.

For each construct, multiple clonal cells lines were established. Following the Thermo Fisher Scientific TurboFect Transfection Reagent general protocol (up to step 7), approximately 5 × 10^4 cells were seeded in each 24-well plates with growth medium 24 h prior to transfection. Cells were trypsinized and replated after transfection into 6-well plates (continuing with step 3 of the BLOCK-iT™ Pol II miR RNAi Expression Vector Kits - Generating Stable Cell Line protocol), then selected for survival in medium with 5 µg/mL blasticidin. Blasticidin-resistant cell lines were cloned by limiting dilution and a subset of 4 scrambled control constructs, 7 ST8SIA2-1 constructs, 2 ST8SIA2-2 constructs, 4 ST8SIA4-1 constructs, and 3 ST8SIA4-2 constructs were screened by fluorescence microscopy. Cells with EmGFP fluorescence were identified as cells containing the integrated plasmid.

Integration of the vector sequence was confirmed by isolating the genomic DNA (5 Prime PerfectPure DNA Cultured Cell Kit, Thermo Fisher Scientific) from 20 cell lines, amplifying the DNA target sequence with conventional PCR (MJ Research, Inc., South San Francisco, CA) using a T7 primer and running the products on a 0.6% agarose gel containing 0.01% ethidium bromide. After cutting out the PCR products
from the gel (Qiagen QiaQuick Gel Extraction kit, Thermo Fisher Scientific), the PCR product was sequenced (Sequetech, Mountain View, CA).

**Quantifying expression of polysialyltransferase mRNA using real-time qPCR**

RNA from the cells was isolated (Fermentas GeneJet RNA purification kit, Thermo Fisher Scientific), and cDNA was produced (Fermentas Maxima First Strand cDNA Synthesis Kit) for use in a Taqman Gene Expression real-time qPCR assay (Thermo Fisher Scientific). The relative mRNA copy numbers of ST8SIA2 or ST8SIA4 was determined using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control for each of the clonal cell lines (in triplicate samples). Cell lines with decreased mRNA copy number compared to a control cell line with integrated plasmid and cultured in the presence of blasticidin were propagated for further study.

**CRISPR knockout of ST8SIA2 and ST8SIA4 genes in CHP-134 cells**

CHP-134 cell lines with ST8SIA2 and ST8SIA4 genes knocked out individually and in combination were constructed by WuXi AppTec Co. Ltd. (Shanghai, China) The sequences ataaccagagctctctctg and actatgtgcttgacaggcgc were targeted for knocking out ST8SIA2 and ST8SIA4, respectively. After confirming biallelic gene knockouts by reverse transcribing mRNA to cDNA and cloning and sequencing the cDNA, the cell lines were further subcloned a second time as described above for SK-MEL-28 RNAi mutants to ensure clonality.

**Antibody binding to cells**

FACS binding experiments were performed with CHP-134 wild-type and CRISPR knockout clone cells as described previously [22]. Cells were suspended in fresh RPMI 10% FBS medium. The cell count was adjusted to $10^6$ live cells/mL. Test Abs and controls were added (10µg/mL) to cells in tubes and incubated at ambient temperature for 45 min while mixing by mechanically rotating the tubes. The cells were washed with RPMI 10% FBS, then resuspended in the same medium containing secondary goat anti-mouse IgG AlexFluor 647 (Thermo Fisher Scientific). After 30 min incubation with mixing, the cells were washed once with Dulbecco's phosphate buffered saline (DPBS) without Mg$^{2+}$ or Ca$^{2+}$ salts and suspended in DPBS containing 0.5% (volume/volume) formaldehyde for 10 min at room temperature. Binding was analyzed using a LSR Fortessa Flow Cytometer (BD Biosciences, San Jose, CA). FlowJo (TreeStar, Woodburn, OR) was used for data analysis.

**Results**

**Anti-dPSA co-immunoprecipitates dPSA and nucleolin from human cancer cells**

To determine the identity of antigens potentially modified with dPSA, we prepared cytoplasmic, cell membrane, nuclear, and cytoskeletal fractions (F1–F4, respectively) from human SK-MEL-28 melanoma cells. The fractions were combined with anti-dPSA mAb SEAM 2 or an irrelevant control murine IgG3 mAb
(14C7) covalently linked to magnetic beads. The cytoskeletal fraction was not subjected to co-immunoprecipitation since this fraction, which contains SDS, was not reactive with SEAM 2 in an immunodot blot (Additional file 1, Supplementary Fig. S1). The proteins bound to the beads were specifically eluted with N-Pr PSA containing 36% de-N-acetyl sialic acid in extraction buffer after a wash with buffer containing PSA. SEAM 2 has more than 100 times greater avidity for the N-Pr PSA antigen than dPSA containing N-acetyl sialic acid residues based on an inhibition ELISA [19]. The eluted proteins were resolved on SDS-PAGE gels. As shown in Fig. 1, only the sample from the SEAM 2 co-immunoprecipitated membrane fraction (Fig. 1B, lane F2) contained stained proteins. The proteins 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 variable PSA length. Variable amounts of PSA de-N-acetylation may result in additional heterogeneity. Four relatively dark-staining regions of the gel spanning the range of eluted dPSA antigens were excised from the gel and processed to generate tryptic peptides for LC/MS/MS mass fingerprinting (indicated by arrows in Fig. 1B, lane F2). As controls, gel slices were taken from the same relative positions in the sample from the membrane fraction control irrelevant IgG3 mAb co-immunoprecipitation (Fig. 1A, lane F2).

The tryptic peptide LC/MS/MS analysis of the bands excised from the SEAM 2-F2 immuno-precipitate identified nucleolin (14 exclusive peptides, 16 exclusive unique spectra, 167/710 amino acids identified in the segment including 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. One function of nucleolin is to promote assembly of ribosomes in nucleoli. Slices marked P3 (~71 kDa) contained ribosome-associated proteins and a-tubulin in addition to nucleolin, and P4 (~48 kDa) contained ribosome-associated proteins and actin. There were no cell-derived proteins identified in the control slices. The calculated molecular mass of nucleolin is 77 kDa, but nucleolin can have several apparent masses ranging from ~50 kDa to ~100 kDa because of multiple covalent modifications including sialylation, the presence of highly acidic regions near the N-terminal region, and self-cleaving activity [33, 34]. The experiment was repeated and included an additional control of co-immunoprecipitation with the anti-PSA mAb SEAM 12 (Additional file 1, Supplementary Fig. S2). Again, nucleolin was the predominant protein co-immunoprecipitated by SEAM 2 from the membrane fraction in gel slices corresponding to lane F2 bands P1 and P2 in Fig. 1B (38 exclusive unique peptides, 53 exclusive unique spectra, 274/710 amino acids identified in the segment including residues 73–629), with smaller amounts of nucleolin also present in the SEAM 12 co-immuno-precipitate, and trace amounts in the irrelevant control. To determine whether dPSA was unique to SK-MEL-28 cells or common to all dPSA containing cells, the same co-immunoprecipitation procedure of subcellular-enriched cell fraction F2 was repeated for Kelly neuroblastoma, SNU-1 gastric cancer cells, and normal PBMCs (Additional file 1, Supplementary Figs. S3 and S4, respectively). All displayed anti-dPSA reactivity inside and outside cells by FACS and fluorescence microscopy except for PBMCs, which had anti-dPSA reactivity with both SEAM 2 and SEAM 3 inside cells (i.e., detergent-treated) but not on the surface of cells not treated with detergent to permeabilize them (Additional file 2, Supplementary Fig. S1. The cells tested represent a range of STX and PST1 expression based on RNA-seq data from publicly available databases.
Kelly having high STX and low PST1; PBMCs having no STX and high PST1; and SNU-1 having low STX and PST1. Nucleolin was co-immunoprecipitated by SEAM 2 from the membrane fraction (F2) of Kelly (19 exclusive unique peptides, 22 exclusive unique spectra, 190/710 amino acids identified in the segment including residues 73–649), SNU-1 (20 exclusive unique peptides, 32 exclusive unique spectra, 314/710 amino acids identified in the segment including residues 73–648), and PBMCs (10 exclusive unique peptides, 14 exclusive unique spectra, 214/710 amino acids identified in the segment including residues 80–648) that was not present in corresponding gel slices of the irrelevant negative control (Additional file 1, Supplementary Figs. S2-S4). Importantly, there were no peptides observed in any of the anti-dPSA immunoprecipitation experiments identifying proteins known to be modified with PSA [36]. Therefore, none of the known PSA-modified proteins appear to be modified with dPSA.

Anti-dPSA is co-localized with nucleolin on the surface of SK-MEL-28 melanoma cells

Nucleolin is a nuclear protein, but it is also found on cilia of airway epithelial cells, where it serves as a receptor for various pathogens [37] and has been reported to be present on the cell surface of cancer cells [38]. Since anti-dPSA mAb SEAM 2 co-immunoprecipitated nucleolin, we asked whether nucleolin is co-localized with dPSA in SK-MEL-28 cells. Anti-nucleolin mAb MS-3 and anti-dPSA mAb SEAM 2 were used to detect each antigen combined with subclass-specific fluorescently labeled secondary antibodies by laser scanning confocal fluorescence microscopy. As a control, the cells were treated with an irrelevant murine IgG3 mAb and the secondary antibodies. As shown in Fig. 2A, there was no staining with irrelevant mAbs or secondary antibodies alone. In wild-type SK-MEL-28 cells, dPSA (Fig. 2B, red fluorescence) was present on the surface of cells and was concentrated in lamellipodia, podosomes, and filopodia (examples indicated by arrows in Fig. 2B, merge image). Anti-nucleolin staining (Fig. 2B, green fluorescence) was identical with respect to location and intensity of staining to anti-dPSA staining (Fig. 2B, merge).

dPSA-nucleolin is present on the surface of primary and metastatic pancreatic, gastric, and ovarian cancer cell lines

To determine whether dPSA co-localization with nucleolin was unique to SK-MEL-28 cells or common to cancer cells, we performed similar confocal fluorescence microscopy experiments labeling dPSA with SEAM 2 and nucleolin with anti-nucleolin mAb MS-3 on sets of seven pancreatic, five gastric, four ovarian primary and metastatic cell lines (Additional file 2, Supplementary Figs. S6-S8, respectively). The panels of cancer cell lines are representative of different genetic alterations of signaling mechanisms for each cancer [39]. Control antibodies included irrelevant murine IgG1 and IgG3 mAbs and Alexa Fluor-conjugated secondary antibodies (Additional file 2, Supplementary Figs. S5-S8). All the cancer cell lines were positive for both dPSA and cell surface nucleolin, which were co-localized as determined by analysis of Z-stack confocal micrographs using JACoP [32], except for the ovarian teratocarcinoma cell line PA-1 (Additional file 2, Supplementary Fig. S8). dPSA staining was much stronger and consistent for all cell lines that were positive for dPSA, whereas anti-nucleolin staining was less consistent with anti-nucleolin being less reactive for cell lines having high polysialyltransferase expression. There was no surface anti-
dPSA or nucleolin staining on human normal PBMCs (Additional file 2, Supplementary Fig. S5), which include subsets of cells with high levels of PST1 and PSA-NCAM expression.

Membrane-associated dPSA-nucleolin is distinct from nuclear nucleolin

Next, we looked at staining patterns inside cells by treating fixed SK-MEL-28 cells with the detergent Triton X-100 to permeabilize them and allow entry of primary and secondary antibodies. dPSA inside cells exhibited a web-like pattern typical of smooth endoplasmic reticulum and trans golgi network (Fig. 2B, +Triton X-100). Again, anti-nucleolin staining matched that of anti-dPSA inside cells with respect to location and intensity of staining. The exception was the strong anti-nucleolin staining around the nuclear membrane, which was not reactive with anti-dPSA mAbs (Fig. 2B, +Triton X-100).

Effect on dPSA and cell surface nucleolin of knocking down expression of ST8SIA2 with siRNA in human SK-MEL-28 cells

SK-MEL-28 cells express both ST8SIA2 and ST8SIA4 (Fig. 3A). Previously, we showed that transient ST8SIA4 RNAi knockdown in SK-MEL-28 cells decreased production of PSA and dPSA, demonstrating that dPSA was derived from PSA [22]. In this study, we constructed mutant SK-MEL-28 cell lines by chromosomal integration of plasmids expressing siRNA targeting ST8SIA2. The relative expression of both genes in total mRNA was determined by quantitative real-time PCR and normalized using GAPDH mRNA as in internal control (Fig. 3A). There was a significant reduction in the amount of ST8SIA2 mRNA in the ST8SIA2 knockdown compared to the wild-type and the scrambled control constructs (Fig. 3B). There was no effect in the ST8SIA2 knockdown mutant on expression of ST8SIA4 mRNA (Fig. 3B), which was ~3-fold higher than the wild-type level of ST8SIA2 mRNA (Fig. 3A).

RNAi knockdown of ST8SIA2 resulted in altered cell morphology (Fig. 3D) compared to the negative control cell line expressing a scrambled RNA (Fig. 3C). The cells with ST8SIA2 RNAi knockdown had a distinct rounded appearance with relatively short lamellipodia circularly arrayed around the cell (Fig. 3D).

Cell surface dPSA and nucleolin were decreased in the ST8SIA2 RNAi knockdown mutant compared to the control SK-MEL-28 mutant expressing a scrambled RNA (Fig. 3E). Similarly, the cytoplasmic amounts of dPSA and nucleolin in Triton X-100-treated cells (Fig. 3F) were reduced in the ST8SIA2 knockdown mutant. siRNA had no effect on anti-nucleolin staining of the nuclear membrane (Fig. 3F) showing dPSA-modified and nuclear nucleolin were distinct forms of the protein.

CRISPR knockout of ST8SIA2 but not ST8SIA4 eliminated cell surface and intracellular dPSA in human CHP-134 neuroblastoma cells

To determine whether cell surface dPSA depends on the activity of one or both polysialyltransferases, we used CRISPR to knock out each gene individually and in combination in human neuroblastoma CHP-134 cells. CHP-134 cells were chosen since both genes are relatively highly expressed [22] and we wanted to determine whether the effects of interfering with expression of the polysialyltransferases in SK-MEL-28 cells could be replicated in another cancer cell line by a different method. Cell lines transfected with
plasmids coding for Cas9 and RNAs targeting the respective polysialyltransferase gene(s) were cloned, and the targeted gene knockouts were confirmed. When ST8SIA2 alone or in combination with ST8SIA4 was knocked out, cell surface dPSA, as measured by FACS and fluorescence microscopy with SEAM 3, was eliminated (Fig. 4A). Interestingly, knocking out ST8SIA2 also eliminated cell surface PSA (Fig. 4B) suggesting that, in CHP-134 cells, polysialyltransferases may work synergistically as suggested by Angata et al. [17]. PSA in CHP-134 cells mainly modifies NCAM [40]. Knocking out ST8SIA4 had no effect on cell surface dPSA but reduced cell surface PSA (Fig. 4A and B, respectively). Therefore, cell surface dPSA in CHP-134 cells depended entirely on expression of ST8SIA2.

Unlike SK-MEL-28 cells, there were no apparent differences in the morphology of CHP-134 mutants. However, nuclei in cell lines with ST8SIA2 and ST8SIA2+ST8SIA4 knocked out were larger (on average 1.45 ±0.24 times greater based on length times width measurements of 20 nuclei each) compared to wild-type or the ST8SIA4 knockout cell lines (Fig. 4C). The differences in the size of the nuclei depended on cell attachment to a surface, as there were no differences in the size of the nuclei of cells in suspension.

Intracellular antigens reactive with both SEAM 2 and SEAM 3 were reduced but still detected in detergent-treated cells after knocking out ST8SIA2, ST8SIA4 or both genes in CHP-134 cells and in normal PBMCs that do not express ST8SIA2 suggesting that the internal staining described previously [21] may result from cross-reactivity with an antigen that does not depend on either STX or PST1. For example, an intracellular sialylated glycan produced by another sialyltransferase that is subsequently de-N-acetylated [26, 41, 42]. However, the protein identified by immunoprecipitation and mass spectroscopy in both cancer cells and normal PBMCs was nucleolin. Taken together, the data suggest nucleolin modified with de-N-acetyl sialic acid may be an anchor glycan that, when polysialylated by STX, results in transfer of dPSA-nucleolin to the cell surface.

**ST8SIA2 is abnormally expressed in many cancers**

Based on recent data from publicly available RNA-seq and proteomics databases, ST8SIA2 expression in cells of adult normal tissues is either very low or non-existent (see, for example, GeneCards: the human gene database [3]). However, based on RNA-seq data from The Cancer Genome Atlas (TCGA) Program [35] for 37 cancers, most express some level of ST8SIA2 that does not occur in corresponding cells of human normal tissues (Fig. 5). The ST8SIA2 gene expression data are consistent with the reported prevalence of dPSA-linked cell surface nucleolin in human cancers [43]. Based on combined gene expression and proteomics data, the polysialyltransferase does not appear to be produced at measurable levels in any adult normal tissue. However, as shown in Fig. 5, higher expression is observed in most cancers where it is associated with invasiveness, metastasis, and poor prognosis for many cancers [44].

**Discussion**

Altered glycosylation patterns of cell surface proteins occur in nearly all types of cancer. Excessive sialylation of glycoproteins and glycolipids is central to the aberrant regulation of cell adhesion in
metastatic cancer, which in turn can result from re-expression and/or overexpression of genes normally expressed during development but not in cells of adult normal tissues. In this study, we gathered further evidence that dPSA is a novel tumor-associated carbohydrate antigen. We have shown that dPSA is linked to nucleolin, which is normally a nuclear protein but is reported to be on the cell surface of many cancers [43]. We have also shown that the presence of dPSA-nucleolin on the surface of cancer cells is dependent on re-expression of a fetal polysialyltransferase gene, ST8SIA2, which may indicate a novel cancer pathway to further investigate for additional points of therapeutic intervention.

Protein polysialylation is highly restricted. To date, only five proteins in humans are reported to be modified by polysialylation [21]: NCAM [5], E-selectin ligand-1 [7], the scavenger receptor CD36 [8], neuropilin-2 [4], and STX itself [6]. In this study, none of the previously known polysialylated proteins were co-immunoprecipitated from any of the cancer cell lines tested or human normal PBMCs with anti-dPSA antibodies suggesting that dPSA modification is exclusive to nucleolin. The large size and electrostatic repulsion of polyanionic PSA is thought to block homophilic interactions between adhesion molecules such as NCAM [9] during nervous system development. However, partial de-N-acetylation, which is common to many polysaccharides [27], alters the charge of PSA as de-N-acetylation produces zwitterionic polysaccharides (ZPS) with different chemical and biological properties. For example, microbial ZPS are frequently involved in immune shielding such as formation of biofilms [45], modulating T cell function [46], and have increased immunogenicity compared to N-acetylated polysaccharides [47].

Nucleolin is an RNA binding protein found throughout the cell where it has been detected in the cytoplasm, nucleus, nucleoli, cell membrane and on cilia [48]. The reason for the difference in nucleolin subcellular localization is thought to be the result of post-translational modifications, which include sialylation—although polysialylation of nucleolin was not known to occur previously [49]. However, in cancerous cells but not normal cells, nucleolin is reported to be expressed consistently and abundantly on the cell surface [43]. The functional purpose of cell surface nucleolin in cancer is unknown but, based on the data presented here, may be to directly intracellular dPSA to the cell surface.

Cell surface dPSA depended on ST8SIA2 gene expression only. Knocking down or knocking out ST8SIA2 in two different cell lines also reduced or eliminated cell surface nucleolin, respectively. In addition to the cancer cell lines, further evidence for the link between STX and cell surface dPSA-nucleolin was shown with human normal PBMCs, which do not express ST8SIA2 and do not have cell surface dPSA or nucleolin (Additional file 1, Supplementary Fig. S1). Although cumulative data from RNA-seq suggest a low level of ST8SIA2 gene expression in human heart and brain, the presence of STX protein has not been confirmed and we did not find cell surface dPSA in either tissue during a immunohistochemical study of 18 major human organs [21]. Overall, the data presented here show that there are distinct functions of each polysialyltransferase, at least with respect to the subcellular distribution of dPSA and its presence on nucleolin.

Conclusions
Expression of genes coding for polysialyltransferases, in particular, are restricted in adult human normal tissues but commonly expressed in most, if not all, cancers. This study suggests that ST8SIA2 expression and cell surface dPSA-nucleolin are limited to cancer cells and the developing embryo [1, 21]. Since anti-dPSA mAbs have direct cytotoxic effects on multiple cancer cell lines [22], approaches that target ST8SIA2 and dPSA to prevent and treat cancers without adverse effect on normal cells may be possible. The presence of dPSA on human trophoblasts [21], microbial pathogens [23, 24, 47], and cancer cells [21, 22] raises the possibility that the function of dPSA may be related to an unrecognized mechanism of immune shielding, since all of the above have in common a need to avoid being targeted by immunological mechanisms of protection.

List Of Abbreviations

CRISPR, clustered regularly interspaced short palindromic repeats; DPBS, Dulbecco's phosphate buffered saline; dPSA, de-N-acetyl neuraminic acid-containing poly alpha2-8 N-acetyl neuraminic acid; N-Pr dPSA, N-propionyl polysialic acid with 36% de-N-acetyl polysialic acid; FACS, fluorescence activated cell sorting; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mAb, monoclonal antibody; NCAM, neural cell adhesion molecule; PBMCs, peripheral blood mononuclear cells; PSA, poly alpha2-8 N-acetyl neuraminic acid; RNAi, RNA interference; RNA-seq, mRNA sequencing; siRNA, small interfering RNA; ZPS, zwitterionic polysaccharide.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials: The datasets supporting the conclusions of this article are included within the article and its additional files.

Competing interests: G.R.M. is an inventor on several patent applications and issued patents related to therapies and diagnostic applications targeting dPSA. Rights to these inventions have been assigned to UCSF Benioff Children's Hospital Oakland. G.R.M. is also a founder and stockholder of, and consultant to, Saccharo, Inc., a company focused on developing the therapeutic and diagnostic potential of dPSA.

Funding: This work was supported by grants from Saccharo, Inc., Children's Hospital Oakland Research Institute, ImpactAssets, and the Family of Jennifer Leigh Wells.

Authors' contributions: G.R.M and L.M.S. conceived, planned, and performed experiments and wrote the manuscript. A.D.B., J.A.L., and A.S. performed experiments and wrote portions of the manuscript.

Acknowledgements: We thank Eileen Y. Ivasauskas of AccuWrit, Inc. for technical writing and editing the manuscript.
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