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Intracellular patterns of sialophorin expression define a new molecular classification of breast cancer and represent new targets for therapy

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Background: Sialophorin is a transmembrane sialoglycoprotein. Normally, the molecule is only produced by white blood cells where it regulates functions such as intercellular adhesion, intracellular signalling, apoptosis, migration and proliferation.

Methods: Normal breast tissue and primary breast tumours were analysed by immunohistochemistry for sialophorin expression. The sialophorin-positive breast cancer cell line MCF7 was engineered to stably express either non-targeted or sialophorin-targeted small interfering RNA (siRNA). Assays were then performed *in vitro* to assess apoptosis, intracellular adhesion, transendothelial migration and cytotoxicity. An orthotopic mouse model assayed ability to produce tumours *in vivo*.

Results: Normal breast epithelial cells exhibit expression of the N-terminal domain of sialophorin in the cytoplasm but not the nucleus. The majority of these normal cells are also negative for expression of the C-terminal domain. In contrast, malignant breast epithelial cells exhibit N-terminal expression both in the cytoplasm and nucleus and the majority express the C-terminus in the nucleus. Using differential patterns of intracellular expression of the N and C termini of sialophorin, we define six subtypes of breast cancer that are independent of histological and receptor status classification. Targeting sialophorin with siRNA resulted in the MCF7 breast cancer cell line exhibiting increased homotypic adhesion, decreased transendothelial migration, increased susceptibility to apoptosis, increased vulnerability to lysis by natural killer cells and decreased ability to produce tumours in mice.

Conclusion: Our results indicate that intracellular patterns of sialophorin expression define a new molecular classification of breast cancer and that sialophorin represents a novel therapeutic target.

Despite recent improvements in mortality rate, breast cancer remains the second leading cause of cancer-related deaths in American woman (Siegel *et al*, 2011). With an estimated 23 480 new cases and 39 520 deaths in 2011, breast cancer is responsible for approximately 15% of all female cancer deaths (Siegel *et al*, 2011). Advances in treatment will likely stem from the identification and targeting of the underlying molecular defects.

Over a decade ago, an analysis of nine cases of breast cancer found that seven expressed the glycoprotein sialophorin that is normally only produced by leukocytes (Santamaría *et al*, 1996; Ostberg *et al*, 1998; Rosenstein *et al*, 1999). We have now analysed 125 primary breast tumours and determined that in 124 of the cases at least 10% of the malignant tissue is sialophorin positive. In addition, we found that differential patterns of intracellular

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sialophorin expression define six categories of breast cancer that are independent of histological type and expression of epidermal growth factor receptor 2 and the receptors for oestrogen and progesterone (Perou *et al,* 2000; Bertucci *et al*, 2005; Van Laere *et al*, 2006).

A driver rather than a passenger role for sialophorin in disease progression is suggested by extrapolating its normal function in leukocytes. When expressed by non-activated leukocytes, sialophorin acts predominantly as a barrier, preventing cell–cell interactions and allowing blood cells to remain in the circulation (Brown *et al*, 1981; Ardman *et al,* 1992; Manjunath *et al,* 1993; Dragone *et al*, 1995; Soler *et al,* 1997; Fukuoka *et al,* 2000). However, when leukocytes are activated, glycosylation changes, proteolytic cleavage events, transcriptional repression and membrane redistribution lead to sialophorin facilitating adhesion and migration as well as protecting against apoptosis (Piller *et al*, 1988; Remold-O'Donnell and Rosen, 1990; Campanero *et al,* 1991; Rosenstein *et al*, 1991; Bazil and Strominger, 1993; Tomlinson-Jones *et al*, 1994; Sánchez-Mateos *et al*, 1995; Stöckl *et al*, 1996; Soler *et al*, 1997; Serrador *et al*, 1998; Sabri *et al*, 2000; Seveau *et al,* 2000; Shelley *et al*, 2001; van den Berg *et al*, 2001; Da Silva *et al*, 2002; Matsumoto *et al*, 2005; Hernandez *et al*, 2006; Mambole *et al*, 2008; Seo and Ziltener, 2009). If these activated functions of sialophorin are projected to breast cancer cells, then sialophorin could drive pathogenesis by facilitating metastasis and increasing cell survival. If the anti-adhesion function of sialophorin is projected, it could facilitate an escape from immunosurveillance by preventing interaction with immune effectors such as natural killer (NK) or cytotoxic T cells (Burnet, 1970). In addition, the anti-adhesion function could help in turning a primary tumour into a loose cellular mass that sheds potentially metastatic neoplastic cells into the circulation.

In order to test the hypothesis that sialophorin drives the pathogenesis of breast cancer, we stably expressed either non-targeted or sialophorin-targeted small interfering RNA (siRNA) in the sialophorin-positive breast cancer cell line MCF7 (Fernandez-Rodriguez *et al,* 2002). Compared with non-targeted MCF7, sialophorin-targeted MCF7 exhibited increased homotypic adhesion, increased susceptibility to apoptosis and increased vulnerability to lysis by NK cells. Finally, assays performed using an orthotopic mouse model of breast cancer demonstrate that sialophorin targeting reduces primary tumour growth by approximately 76%. Together, these *in vitro* and *in vivo* results indicate that sialophorin contributes to breast cancer pathogenesis and, therefore, represents a putative therapeutic target.

MATERIALS AND METHODS

Patient material. A retrospective search of the files of the Gundersen Foundation BioBank and the Department of Pathology at Gundersen Medical Center identified 125 cases of breast cancer diagnosed between 1976 and 2011. Paraffin-embedded formalin-fixed tissue representing each of these cases was sectioned, stained with haematoxylin and eosin and the histological diagnosis verified. All file searches and subsequent experimental procedures were approved by the Human Subjects Committee of Gundersen Clinic, Ltd. of La Crosse, WI, USA. Slides of normal breast tissue were purchased from US Biomax, Inc., Rockville, MD, USA (catalogue number HuFPT129).

Generation of the polyclonal antibody SSGZ. Covalab SAS (Villeurbanne, France) synthesised a peptide of 26 amino acids with the sequence NH2-PLVASEDGAVDAPAPDEPEGGD-GAAP-COOH. This corresponds to the terminal residues of the intracellular domain of sialophorin. The same company then used glutaraldehyde cross-linking to conjugate the N-terminus of the peptide to keyhole limpet haemocyanin. Next, 0.5 ml containing 100 μg of the conjugated peptide was mixed with 0.5 ml of Complete Freund Adjuvant and this was then injected intradermally into a New Zealand White rabbit. After 21 days and then again after 42 days, intradermal injection was repeated but with Incomplete Freund Adjuvant. After 63 days and then again at 91 days, subcutaneous injection was performed with Incomplete Freund Adjuvant. After 116 days, 55 ml of serum was drawn and IgG affinity purified from 5 ml. The purified antibody was named SSGZ and stored at a concentration of 87.3 μg ml^{-1} in 50% glycerol at $-20\,^{\circ}$C.

Immunohistochemistry. Formalin-fixed paraffin-embedded blocks containing human breast tissue were serially sectioned at 4 μm and dried overnight on Colorfrost Plus microscope slides (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Next, sample slides were deparaffinised by a 60-min incubation at 60 °C followed by four changes of xylene, three changes of 100% ethanol, two changes of 95% ethanol and storage in tap water. One slide from each block was stained with haematoxylin and eosin Y. The remaining slides were subjected to a 20-min incubation at 90–100 °C in the presence of Epitope Retrieval Solution, pH 9 (Dako North America, Inc., Carpinteria, CA, USA). Next, the slides were rocked for 5 min at room temperature with tissue covered by the peroxidase blocking reagent of the EnVision+ System-HRP (DAB) (Dako North America, Inc.). A rocking incubation was then performed at room temperature for 30 min with Surfact-Amps X-100 (Thermo Fisher Scientific, Inc.). One slide from each block was rocked for 45 min at room temperature with either an IgG non-immune rabbit or mouse antibody diluted as recommended by the manufacturer (Epitomics, Inc., Burlingame, CA, USA). One slide from each block was identically incubated with either a 1 : 2400 dilution of the rabbit polyclonal antibody SSGZ or a 1 : 100 dilution of the mouse monoclonal antibody L10 that specifically recognise the C and N termini of sialophorin, respectively. Serial rocking incubations were next performed at room temperature for 30 min with labelled polymer-HRP anti-rabbit or anti-mouse, twice for 5 min with Wash Buffer and 5 min with DAB+ chromogen (Dako North America, Inc.). Counterstaining was accomplished by dipping the slides in haematoxylin, rinsing with tap water, dipping in 1% glacial acetic acid, rinsing again in tap water and then dipping in 1% ammonium hydroxide. Rinsing in 100% ethanol then xylene dehydrated the tissue that was finally protected by glass coverslips mounted with Permount (Thermo Fisher Scientific, Inc.). A pathologist (JJA) certified by the American Board of Pathology and a histotechnician (SEC) certified by the American Society for Clinical Pathology independently scored either at 0 or 1+ the staining of L10 and SSGZ in the cytoplasm or nucleus. A score of 0 indicated that the proportion of the tumour exhibiting staining in a given intracellular structure was $<10\%$. A score of 1+ indicated that over 10% of the tumour was stained. Tumours were classified into six groups defined by their pattern of L10 and SSGZ staining in the nucleus and cytoplasm. LCN/SN: tumours exhibiting a L10 staining score of 1+ in both the nucleus and cytoplasm together with a SSGZ staining score of 1+ in the nucleus and 0 in the cytoplasm. LC/SN: tumours exhibiting a L10 staining score of 0 in the nucleus but 1+ in the cytoplasm together with a SSGZ staining score of 1+ in the nucleus and 0 in the cytoplasm. SN: tumours exhibiting a L10 staining score of 0 in both the nucleus and cytoplasm together with a SSGZ staining score of 1+ in the nucleus but 0 in the cytoplasm. LCN: tumours exhibiting a L10 staining score of 1+ in both the nucleus and cytoplasm together with a SSGZ staining score of 0 in both the nucleus and cytoplasm. LC: tumours exhibiting a L10 staining score of 0 in the nucleus and 1+ in the cytoplasm together with a SSGZ staining score of 0 both in the nucleus and cytoplasm. NR: tumours exhibiting a L10 staining score of 0 in both the nucleus

and cytoplasm together with a SSGZ staining score of 0 both in the nucleus and cytoplasm.

Cell culture. All human breast cancer cell lines were obtained from the American Type Culture Collection and grown according to their specifications (Manassas, VA, USA). The growth medium for MCF7 cells stably expressing siRNA was supplemented with 0.6 μg ml^{-1} of puromycin. The human T-lymphocytic cell line Jurkat was kindly provided by Thomas Wileman (University of East Anglia, Norwich, England). The growth medium for this cell line was composed of RPMI1640, 10% v/v fetal bovine serum (FBS), 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin sulphate. Human microvascular endothelial cells (HMEC-1) were a kind gift of Dr Sean P Colgan (University of Colorado, Aurora, CO, USA; Ades *et al,* 1992). The growth medium for this cell line was composed of medium 131 containing microvascular growth supplement (MVGS; Life Technologies Corp., Carlsbad, CA, USA). In addition, surfaces on which HMEC-1 were grown were coated with attachment factor (AF; Life Technologies, Corp.). The human NK cell line YT2C2-PR was kindly provided by Dr Edgardo E Carosella (Hôpital Saint-Louis, Paris, France; Yoneda *et al*, 1992). The growth medium for this cell line was the same as that for MCF7 except that 20% v/v FBS was used. All cultures were housed in a tissue culture incubator that maintained a humidified atmosphere of 5% carbon dioxide at 37 °C.

Lentivirus expressing siRNA. Four types of SMARTvector shRNA lentivirus particles were purchased from Thermo Scientific Dharmacon (Lafayette, CO, USA). The first lentivirus constitutively expressed siRNA that did not target any known human gene. The three additional lentiviruses expressed siRNA targeting sialophorin mRNA at the 3′ non-coding sequences 5′-GGCAGTT GGTATTTCCCGA-3′ (Sialo-targeted-A) and 5′-AGAGCTGAGG ATTTGGCGA-3′ (Sialo-targeted-B) and the coding sequence 5′-GTACACCACTTCAATAACA-3′ (Sialo-targeted-C). Virus titres ranged from 1.16 to 4.66 $\times$ 10^8 transducing units per ml.

Generation of cell line pools stably expressing siRNA. The three sialophorin-targeted lentiviruses were used together or individually to infect MCF7 cells. The lentivirus expressing non-targeted control siRNA was used independently. All infections were performed overnight at 37 °C in the presence of 3 μg ml^{-1} polybrene at a multiplicity of 10 infectious viral particles to each MCF7 cell. As each siRNA gene contained within the lentiviruses was linked to a puromycin resistance gene, resistance to this antibiotic was used to select for stable shRNA expression. Preliminary experiments determined that the minimum concentration of puromycin required to kill the MCF7 cell line in 3 days is 0.6 μg ml^{-1}. Therefore, following lentiviral infection, cells were treated for 3 days with 0.6 μg ml^{-1} and then for 1 week with 1.0 μg ml^{-1} puromycin. After selection, cells were maintained in media containing 0.6 μg ml^{-1} puromycin. Western blot analysis demonstrated that MCF7 cells selected after simultaneous infection with three sialophorin-targeted lentiviruses exhibited a significant reduction in sialophorin protein expression compared with MCF7 selected after non-targeted infection (Supplementary Figure 1).

Homotypic adhesion assays. Homotypic adhesion assays were performed using confluent monolayers of MCF7 expressing either non-targeted or sialophorin-targeted siRNA. Monolayers were established in 12-well tissue culture plates after seeding at approximately 50% confluence. Once confluent monolayers were established, separate cultures of non-targeted MCF7 and sialophorin-targeted MCF7 were trypinised, washed in Hank's balanced salt solution (HBSS) and incubated for 30 min in a tissue culture incubator with HBSS containing 5 μM 2′7′-bis(carboxyethyl)-5(6)-carboxyfluorescein pentaacetoxymethyl ester (BCECF-AM; Calbiochem, EMD Chemicals, Inc., Gibbstown, NJ, USA). The cells labelled with BCECF-AM were centrifuged at 1000 r.p.m. for 3 min at 25 °C and then washed three times in HBSS warmed to 37 °C. Next, labelled non-targeted MCF7 cells were added to the monolayers of unlabelled non-targeted MCF7 at a multiplicity of 0.1. In the same way, labelled sialophorin-targeted MCF7 were added to monolayers of unlabelled sialophorin-targeted MCF7. Labelled cells were settled by centrifugation at 1000 r.p.m. for 3 min at 25 °C then placed in a tissue culture incubator for 5 min. Following this incubation period, cells were gently washed twice in HBSS warmed to 37 °C and fluorescence intensity measured at excitation and emission wavelengths of 485 and 530 nm, respectively. Measurements were made using a CytoFluor 4000 Series Fluorescence Multi-well Plate Reader (PerSeptive Biosystems, Inc., Framingham, MA, USA).

Migration and invasion assays. Transendothelial migration assays were performed using the BD Falcon HTS FluoroBlok 24-Multiwell Insert system (BD Biosciences, Bedford, MA, USA). The wells of the top insert plate were treated with AF and a monolayer of HMEC-1 cells was established. The monolayer was then activated for 8 h with 100 ng ml^{-1} of phorbol 12-myristate 13-acetate (Sigma-Aldrich, St Louis, MO, USA). After activation, monolayers were washed twice with HMEC-1 medium free of MVGS. Suspensions of non-targeted and sialophorin-targeted MCF7 cells were labelled for 1 h with MCF7 culture media containing 10 μg ml^{-1} DilC$_{12}$(3) fluorescent dye (BD Biosciences). These cells were next centrifuged, resuspended in HMEC-1 medium free of MVGS and 5 $\times$ 10^4 added to each well of the top insert plate that contained an activated monolayer of HMEC-1. The wells of the bottom assay plate contained HMEC-1 medium complete with MVGS that bathed the lower surface of the wells of the top insert plate. Consequently, the lack of MVGS in the top wells and its presence in the bottom wells established a chemoattractant gradient. The completed culture system was then placed in a tissue culture incubator and fluorescence intensity of the under surface of the top wells measured over time at excitation and emission wavelengths of 549 and 565 nm, respectively, using a Synergy 2 Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). Invasion assays were performed using the BD BioCoat Tumour Invasion System (BD Biosciences). The design concept of this system is the same as that used for the assay of transendothelial migration except that the monolayer of HMEC-1 is replaced with a uniform layer of BD Matrigel Basement Membrane Matrix (BD Biosciences). Suspensions of non-targeted and sialophorin-targeted MCF7 were washed twice in serum-free RPMI1640 and 2.5 $\times$ 10^4 added to each well of the top insert plate that contained the matrix. As a control, MCF7 cells were also added to wells of top insert plates that contained no matrix. In all experiments, the wells of the bottom assay plates contained RPMI1640 complete with 10% FBS to set up a chemoattractant gradient. The assembled culture system was incubated for 5 days, the medium was then removed from the wells of the top insert plate and this transferred to a second bottom assay plate. Here each well contained 500 μl of HBSS supplemented with 4 μg ml^{-1} calcein AM (BD Biosciences). After 1 h in a tissue culture incubator, fluorescence intensity was measured at excitation and emission wavelengths of 494 and 517 nm, respectively, using a Synergy 2 Multi-Mode Microplate Reader (BioTek Instruments, Inc.). Percentage invasion was calculated by multiplying by 100 the result of dividing the fluorescence intensity of matrix-coated wells with the intensity of equivalent non-coated wells.

Assay of apoptosis. Wells in 12-well flat-bottomed tissue culture plates were seeded with 5 $\times$ 10^4 non-targeted or sialohorin-targeted MCF7. After 24 h, the culture media was removed and replaced with identical media except for the addition of 10, 20 or 40 ng ml^{-1} of tumour necrosis factor α (TNFα; Immunochemistry Technologies, LLC, Bloomington, MN, USA). Cells were cultured in a tissue culture incubator for 5 days then activated caspases detected

using a FAM-FLICA *in vitro* Poly Caspases Assay Kit (Immunochemistry Technologies, LLC). This assay is based on the observation that the tri-peptide valine–alanine–aspartic acid (VAD) binds to the active site of every known member of the caspase family. In the assay kit, VAD is coupled to a fluoromethyl ketone (FMK) and the green fluorescent dye carboxyfluorescein (FAM). Fluoromethyl ketone causes VAD to be irreversibly linked to activated caspases and FAM allows this binding to be detected. As the FAM–VAD–FMK FLICA reagent becomes covalently coupled to the active caspase enzymes, it is retained within the cell, whereas any unbound FLICA reagent diffuses out of the cell and is washed away. The remaining green fluorescent signal is a direct measure of the amount of caspase activity present in the cell at the time the reagent was added. At least three independent fields were imaged for each cell line both in white-light phase contrast and fluorescence at an excitation wavelength of 490 nm and an emission wavelength of 515 nm. Images were acquired using a PowerShot G9 camera (Canon U.S.A., Inc., Lake Success, NY, USA). White-light and fluorescence images were acquired with exposures of one-fifteenth and one-half of a second, respectively, with an aperture of F6.3 and a speed of ISO 1600. The total number of cells in a given field were counted from the phase contrast image. The numbers of cells undergoing apoptosis were counted in the same field from the fluorescent image. The percentage of cells undergoing apoptosis in a given field was then calculated.

Assay of NK cell cytotoxicity. Wells in 12-well flat-bottomed tissue culture plates were seeded with 1×10^4 of either non-targeted MCF7 or sialophorin-targeted MCF7. Two days later, the NK cell line YT2C2-PR was added at a multiplicity of 1, 5, 10, 15 or 20. Plates were then centrifuged for 1 min at 1000 r.p.m. to effect uniform settling of YT2C2-PR. After 16 h, supernatants were gently mixed and aspirated to remove non-adherent YT2C2-PR and MCF7 cells. Next, 0.7 ml of 0.25% Rose Bengal dye was added to each well and after 3 min the excess removed by two washes in phosphate-buffered saline (PBS). Dye was released from intact, adherent cells by the addition of 0.8 ml of 50% ethanol and optical density (OD) measured at 570 nm. Percentage cytotoxicity was calculated using the formula $100 \times (A - [B - C])/D$ where A is OD of MCF7 cells after mixing to remove non-adherent cells, B is OD of experimental wells, C is OD of adherent YT2C2-PR cells and D is OD of MCF7 cells without mixing to remove those that are non-adherent (Chong and Parish, 1985; Heo *et al,* 1990; Gondolf *et al,* 1996; Adrián-Cabestré *et al*, 1999).

Mouse husbandry. Female mice that were 3 to 4 weeks old and of the strain Hsd:Athymic Nude-*Foxn1*^{nu} were purchased from Harlan Laboratories, Inc. (Indianapolis, IN, USA). Mice were housed in sterilised Super Mouse 750 Micro-Isolator ventilated cages on a RAIR Isosytem rack (Lab Products, Inc., Seaford, DE, USA). This housing system was kept in a barrier room accredited by AAALAC-I that was designed for the husbandry and surgery of immunodeficient animals. Mice were monitored daily and sterile water and Teklad Global 18% Protein Rodent Diet (Harlan Laboratories, Inc.) were provided *ad libitum*. Mice were provided with sterile Teklad Sani-Chips for bedding and iso-BLOX nesting material (Harlan Laboratories, Inc.) as well as LifeSpan Rodent Enrichment (Lab Products, Inc.). Social interaction was ensured by housing mice in groups of 2–3 per sterile cage.

Orthotopic mouse model. Mice were anaesthetised by initial inhalation of 2.5% then subsequent inhalation of 2% isoflurane delivered by facemask using a SurgiVet Universal CDS 9000 Small Animal Anesthesia Machine (Smiths Medical, Dublin, OH, USA). During maintenance anaesthesia mice were taped ventral-side up to a Homeothermic Blanket System set at 37 °C (DC Temperature Control System, FHC, Inc., Bowdoin, ME, USA). Ophthalmic ointment was applied to the eyes to prevent corneal drying and trauma. Buprenorphine at a concentration of 0.1 mg kg^{-1} was injected subcutaneously and the lower abdominal area was wiped liberally with betadine. Surgery was performed to remove the uterus and ovaries and then the peritoneum, muscle wall and skin were serially sutured. Next, mice were turned 45°, betadine applied between the shoulder blades and at this site surgery was performed to place under the skin a pellet formulated to release 0.72 mg of 17β-estradiol over a period of 60 days (Innovative Research of America, Sarasota, FL, USA). After closing the skin with sutures, mammary fat pad no.4 was swabbed with 70% ethanol and here 8×10^6 MCF7 cells in a volume of 200 μl were injected using a 27-gauge needle with the bevel facing up. Injected cells were in suspension in sterile PBS and originated from cultures at 60% confluence that had been trypsinised and subsequently washed twice in their normal growth media. Injections were performed at a 45° angle and aspiration monitored the absence of blood vessel entry. After removing the needle, the injection site was held firmly with sterile gauze for approximately 30 s. Buprenorphine at a concentration of 0.1 mg kg^{-1} was injected subcutaneously for a second time and then mice were returned to their cages to recover from anaesthesia. Buprenorphine was injected again 8 h later and twice a day for the next 5 days. Sixty days after injection, all mice were killed by cervical dislocation following anaesthesia effected by intraperitoneal injection of a mixture of 160 mg kg^{-1} ketamine plus 10 mg kg^{-1} xylazine. At this point, tumours were dissected free of the skin and body tissue and weighed. All animal use protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Wisconsin-La Crosse, WI, USA.

RESULTS

Cytoplasmic and nuclear expression of sialophorin in breast cancer. A rabbit was immunised and boosted with a synthetic peptide of 26 amino acids corresponding to residues 375–400 of the primary translation product of sialophorin mRNA (Pallant *et al*, 1989; Shelley *et al*, 1989). These residues are located at the C-terminus of the intracellular domain of sialophorin. The IgG population of antibodies was purified from the serum of the immunised rabbit and used in western blot analysis of crude protein extracts prepared from Jurkat T-lymphocytic cells. The rabbit polyclonal antibody detected a single major protein with an electrophoretic mobility corresponding to that of the major protein detected by the monoclonal anti-sialophorin antibody L10 (Supplementary Figure 2; Remold-O'Donnell *et al*, 1984). The L10 antibody binds the N-terminus of sialophorin (Remold-O'Donnell and Parent, 1995). The SSGZ and L10 antibodies were then used to analyse formalin-fixed paraffin-embedded normal breast tissue and 125 cases of breast cancer (Figure 1A; Table 1). The breast cancers interrogated comprised 98 invasive carcinomas of no special type (IC-NST), 7 invasive carcinomas of no special type that had lobular features (IC-NST-L), 10 invasive lobular carcinomas and 10 ductal carcinomas *in situ*. This analysis determined that the cytoplasm of most normal breast epithelial cells is L10-positive and SSGZ-negative while the nucleus of most of these cells is negative for both L10 and SSGZ. In contrast, in 119 of the 125 breast cancer cases the nucleus of the majority of malignant epithelial cells was SSZG positive and 61 of the 125 cases expressed L10 not only in the cytoplasm but also in the nucleus. Taken together, the intracellular patterns of L10 and SSGZ reactivity classified breast cancer into three common groups accounting for 95% of cases and three rare groups accounting for 5% of cases (Figure 1B). None of these six groups segregated with either the histological classification of the carcinoma (Table 1) or its receptor status (Table 2).

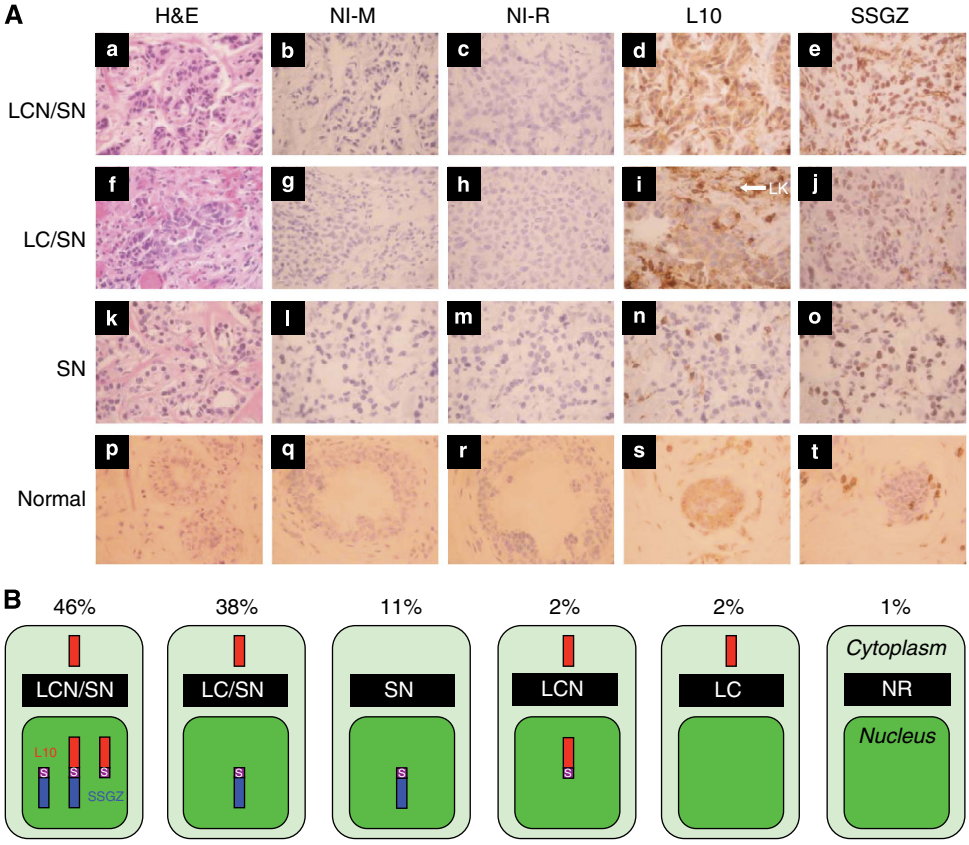

Figure 1. Intracellular patterns of sialophorin expression define a new molecular classification of breast cancer. (**A**) Examples of the three most common of six classes of breast cancer defined by L10 and SSGZ antibody staining. (a–o) Sections of 5 μm cut from formalin-fixed, paraffin-embedded tissue obtained from different patients diagnosed with IC-NST. (p–t) sections of normal human breast tissue purchased from US Biomax, Inc. Sections were stained with haematoxylin and eosin (H&E), a non-immune mouse IgG antibody (NI-M), a non-immune rabbit IgG antibody (NI-R), the mouse monoclonal antibody L10 or the rabbit polyclonal antibody SSGZ. Antibody binding was visualised using the EnVision+ System-HRP (Dako North America, Inc.). Counter staining with haematoxylin visualised nuclei. Brown precipitates indicate L10 and SSGZ binding to sialophorin. The depicted cases of IC-NST exemplify the patterns of staining designated LCN/SN, LC/SN and SN. The origin of this nomenclature is described in the immunohistochemistry section of Materials and Methods section. Arrowed is an example of membrane staining of infiltrating leukocytes (LK). (**B**) Schematic representation of the proteolytic events implied by the intracellular patterns of L10 and SSGZ staining that define six classes of breast cancer. These classes are designated LCN/SN, LC/SN, SN, LCN, LC and NR (Table 1). The L10 antibody binds the N-terminus of sialophorin (red box) and the SSGZ antibody binds the C-terminus (blue box). A nuclear localisation signal (S) spans amino acids 282–296 (purple box). The percentage of cases in each class defined by L10 and SSGZ staining is marked above the corresponding schematic.

Expression of siRNA targeting sialophorin increases homotypic adhesion of MCF7 breast cancer cells. Nuclear expression of the C-terminal domain of sialophorin protects leukocytes against apoptosis, controls gene expression and likely drives proliferation (Andersson *et al*, 2004; Seo and Ziltener, 2009). In addition, domains within the 123 amino acids at the C-terminal end of the molecule directly bind protein kinases (Rosenstein *et al*, 1999). Immunohistochemical analysis using the antibody SSGZ demonstrates that 95% of breast tumours exhibit nuclear expression of the C-terminal domain of sialophorin in the majority of their malignant epithelial cells (Table 1). Therefore, this C-terminus expression could facilitate disease pathogenesis directly by increasing cell survival and proliferation and indirectly by controlling intercellular adhesion through 'inside-out' signalling (Abram and Lowell, 2009).

In order to determine if sialophorin functionally contributes to the pathogenesis of breast cancer, we targeted its expression in the breast cancer cell line MCF7 (Fernandez-Rodriguez *et al*, 2002). A pool of MCF7 was generated that stably expressed a mixture of three different sialophorin-targeted siRNAs. In addition, one control pool was generated that stably expressed a siRNA that fails to target any known human gene. Homotypic adhesion assays were performed on the MCF7 pool expressing sialophorin-targeted siRNA and on the MCF7 pool expressing control non-targeted siRNA. Comparison of these two pools demonstrated that targeting sialophorin increases homotypic adhesion by 2.5-fold (Figure 2). This result indicates that sialophorin inhibits the ability of breast cancer cells to bind to each other. As sialophorin is not expressed at the surface of breast cancer cells, its ability to inhibit homotypic adhesion appears to be mediated by an indirect mechanism.

Targeting sialophorin decreases MCF7 transendothelial migration but not extracellular matrix invasion. Break-up of primary tumours caused by an inhibition of homotypic adhesion represents an initial step in the invasion of surrounding tissue and metastasis. Therefore, as sialophorin targeting increased homotypic adhesion of MCF7, we tested the hypothesis that such targeting inhibited the ability of MCF7 to migrate through an endothelial monolayer. Using a transendothelial migration assay, sialophorin targeting was determined to reduce 9-day migration by 47% (Figure 3A). Therefore, sialophorin expression by MCF7 mediates not only homotypic repulsion but also dynamic transendothelial migration. In contrast to migration through endothelial cells, invasion by MCF7 into a basement membrane extracellular matrix was

Table 1. Breast cancers defined by sialophorin expression distributed between groups defined by histology

Sialophorin Group	Total Cases	IC-NST	IC-NST-L	ILC	DCIS
LCN/SN	58	40	4	9	5
LC/SN	47	40	2	0	5
SN	14	13	0	1	0
LCN	3	3	0	0	0
LC	2	1	1	0	0
NR	1	1	0	0	0
Total cases	125	98	7	10	10

The degree to which individual tumours stained in the cytoplasm or nucleus with the anti-sialophorin antibodies L10 and SSGZ was scored 0 or 1+ by two independent observers as described in Materials and Methods section. The cases interrogated comprised invasive carcinomas of no special type (IC-NST), invasive carcinomas of no special type that had lobular features (IC-NST-L), invasive lobular carcinomas (ILC) and ductal carcinomas *in situ* (DCIS). The cases were diagnosed between 1976 and 2011 at the Gundersen Medical Center. The stage of cancer ranged from 0 to IV. Tumours were categorised into six groups defined by their pattern of L10 and SSGZ staining in the nucleus and cytoplasm. 'L10 scored in the Cytoplasm and Nucleus both as 1+ together with SSGZ scored 1+ in the Nucleus but 0 in the cytoplasm' (LCN/SN). 'L10 scored 1+ in the Cytoplasm but 0 in the nucleus together with SSGZ scored 1+ in the Nucleus but 0 in the cytoplasm' (LC/SN). 'SSGZ scored 1+ in the Nucleus but 0 in the cytoplasm together with L10 scored 0 both in the nucleus and cytoplasm' (SN). 'L10 scored 1+ both in the Cytoplasm and Nucleus together with SSGZ scored 0 both in the cytoplasm and nucleus' (LCN). 'L10 scored 1+ in the Cytoplasm but 0 in the nucleus together with SSGZ scored 0 both in the nucleus and cytoplasm' (LC). 'L10 and SSGZ both scored Non-Reactive in both the nucleus and cytoplasm' (NR).

Table 2. Breast cancers defined by sialophorin expression distributed between groups defined by receptor status

Sialophorin Group	Total Cases	ER	−	−	−	+	+	+	−	+
		PR	−	−	+	−	+	−	+	+
		HR	−	+	−	−	−	+	+	+
LCN/SN	19		3	4	2	2	3	3	0	2
LC/SN	20		3	3	3	2	3	3	0	3
SN	4		0	0	0	2	0	0	1	1
LCN	0		0	0	0	0	0	0	0	0
LC	0		0	0	0	0	0	0	0	0
NR	1		0	0	0	0	0	0	0	1
Total cases	44		6	7	5	6	6	6	1	7

Abbreviations: ER = oestrogen receptor; HR = epidermal growth factor receptor 2; PR = progesterone receptor; Breast cancer cases were diagnosed at the Gundersen Medical Center between 2006 and 2011. The stage of cancer ranged between 0 and IV. Cases were evaluated for expression (+) or lack of expression (−) of the ER, the PR and HR.

unaffected by sialophorin targeting (Figure 3B). Therefore, transendothelial migration mediated by sialophorin would appear to involve integral components of endothelial cells rather than components of the extracellular matrix.

Targeting sialophorin increases the sensitivity of MCF7 cells to apoptosis induced by TNFα. Exogenous overexpression of recombinant sialophorin has been shown to protect the human colorectal cancer cell line SW480 from apoptosis (Kadaja-Saarepuu *et al*, 2008). This observation suggests that endogenous expression of sialophorin in MCF7 may also protect against apoptosis. In order to test this possibility, we attempted to induce apoptosis of non-targeted and sialophorin-targeted MCF7 by a 5-day treatment

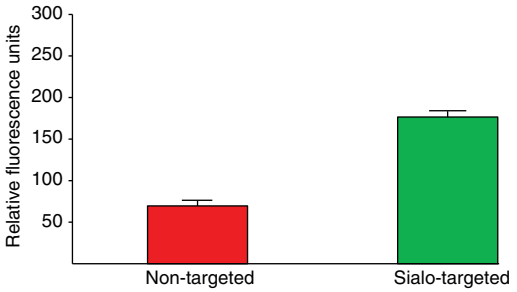

Figure 2. Sialophorin targeting increases homotypic adhesion of MCF7. Adhesion of BCECF-AM-labelled non-targeted and sialophorin (Sialo)-targeted MCF7 to respective confluent monolayers of unlabelled non-targeted and sialophorin-targeted MCF7 ($n = 5$). Error bars represent means ± s.e.m. The difference between adhesion of sialophorin-targeted MCF7 and non-targeted MCF7 was calculated by a one-tailed Student's *t*-test to have a significance level of $P < 0.001$.

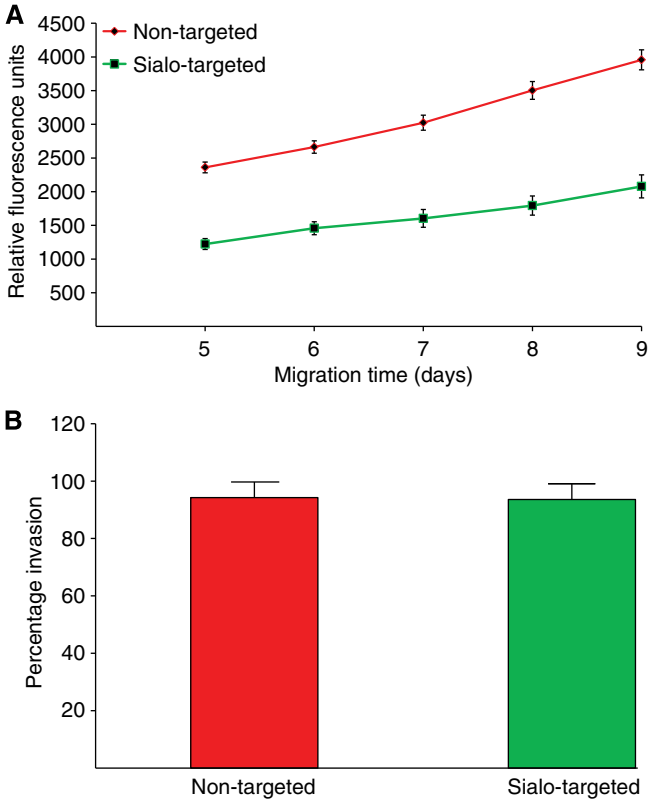

Figure 3. Sialophorin targeting inhibits MCF7 transendothelial migration but not extracellular matrix invasion. (**A**) Transendothelial migration of $DiIC_{12}(3)$-labelled non-targeted and sialophorin (Sialo)-targeted MCF7 through monolayers of HMEC-1 activated with phorbol 12-myristate 13-acetate (PMA; $n = 9$). Error bars represent means ± s.e.m. The difference between migration of sialophorin-targeted MCF7 and non-targeted MCF7 was calculated by a one-tailed Student's *t*-test to have a significance level of $P < 0.001$ for all time points. (**B**) Invasion of non-targeted and sialophorin (Sialo)-targeted MCF7 into BD Matrigel Basement Membrane Matrix (BD Biosciences; $n = 6$). After invasion, MCF7 cells were labelled with calcein AM. Error bars represent means ± s.e.m. No significant difference in the invasive ability of non-targeted and sialophorin-targeted MCF7 was detected.

with 10, 20 or 40 ng ml^{-1} of TNFα (Figure 4). Under these conditions, non-targeted MCF7 exhibited negligible early phase apoptosis, as assessed by expression of activated caspases.

However, in contrast, activated caspases were detected in 29%, 33% and 38% of sialophorin-targeted MCF7 treated with 10, 20 or 40 ng ml^{-1} TNFα, respectively. Consequently, sialophorin appears to protect MCF7 breast cancer cells from at least one anti-tumoural response of the immune system.

Targeting sialophorin increases the susceptibility of MCF7 breast cancer cells to lysis by NK cells. Our analysis indicates that sialophorin expressed by MCF7 protects against apoptosis induced by TNFα (Figure 4). The production of TNFα is a mechanism by which NK cells effect their cytotoxic function (Caron *et al*, 1999). Therefore, our studies suggest that sialophorin expression could contribute to breast cancer pathogenesis by protecting malignant cells from NK attack. We tested this hypothesis by using non-targeted and sialophorin-targeted MCF7 cells in NK cytotoxicity assays. Over a range of NK cell multiplicity, MCF7 cells simultaneously expressing three sialophorin-targeted siRNAs exhibited higher susceptibility to lysis compared with non-targeted cells (Figure 5). The most striking effect occurred at a ratio of NK to MCF7 of 20 : 1. Here 70% of sialophorin-targeted MCF7 underwent lysis compared with only 20% of the non-targeted control. In addition to MCF7 cells simultaneously expressing three sialophorin-targeted siRNAs, MCF7 pools were generated that expressed each of these siRNAs individually. When these pools were tested in the cytotoxicity assay one failed to exhibit any significant difference compared with the pool expressing non-targeted siRNA. The other two pools exhibited 36% and 40% lysis when NK cells were present at a ratio of 20 : 1. Therefore, these data indicate that sialophorin targeting increases breast cancer susceptibility to NK lysis in a dose-dependent additive manner.

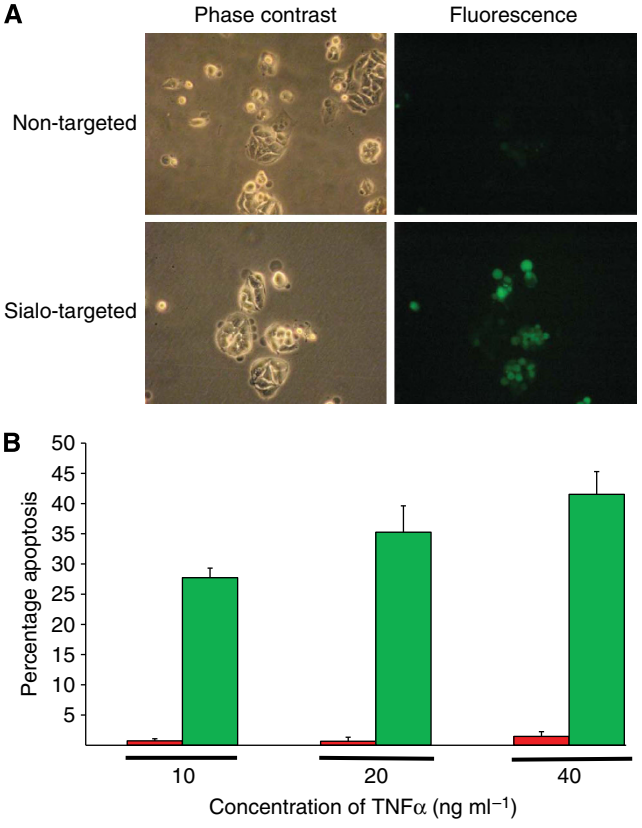

Figure 4. Sialophorin targeting increases the susceptibility of MCF7 to apoptosis induced by TNFα. (**A**) Non-targeted and sialophorin (Sialo)-targeted MCF7 were treated for 5 days with 40 ng ml^{-1} of TNFα and then assayed for activated caspases using a FAM-FLICA *in vitro* Poly Caspases Assay Kit (Immunochemistry Technologies, LLC). Depicted are representative microscope fields imaged either by white-light phase contrast or green fluorescence. (**B**) Non-targeted (red histograms) and sialophorin-targeted MCF7 (green histograms) were treated for 5 days with 10, 20 or 40 ng ml^{-1} of TNFα and then assayed for activated caspases. In a given microscope field, the total number of cells was determined from the white-light image and the number of cells undergoing apoptosis was determined from the fluorescent image. The percentage of apoptotic cells was calculated from the sum of at least three independent fields encompassing a minimum total of 100 cells. Error bars represent means ± s.e.m. of three independent experiments.

Targeting sialophorin limits primary tumour growth in an orthotopic mouse model of breast cancer. Our studies *in vitro* demonstrate that sialophorin targeting increases the homotypic adhesion of breast cancer cells, reduces transendothelial migration and increases susceptibility to apoptosis and NK cytotoxicity. Taken together, these findings provide the basis for the hypothesis that sialophorin targeting could inhibit breast cancer pathogenesis *in vivo*. This hypothesis was tested using an orthotopic mouse model (Figure 6). When non-targeted MCF7 cells were injected into the mammary fat pads of eight oophorohysterectomised female mice all produced primary tumours. The average weight of the tumours at 60 days was 166 mg. In contrast, MCF7 cells stably expressing three sialophorin-targeted siRNAs produced tumours with an average weight of 39 mg at 60 days. Furthermore, 4 of the 10 mice that were injected never produced a detectable tumour. These results show that sialophorin targeting limits *in vivo* the growth of primary breast tumours.

DISCUSSION

Human sialophorin is also known as CD43, large sialoglycoprotein, gpL115 and leukosialin (Ostberg *et al*, 1998; Rosenstein *et al*, 1999). Normally, the molecule is only expressed on the surface of leukocytes and platelets. Mature sialophorin consists of a heavily glycosylated rod-like extracellular domain of 235 amino acids, a 23 residue transmembrane region and a 123-amino-acid C-terminal

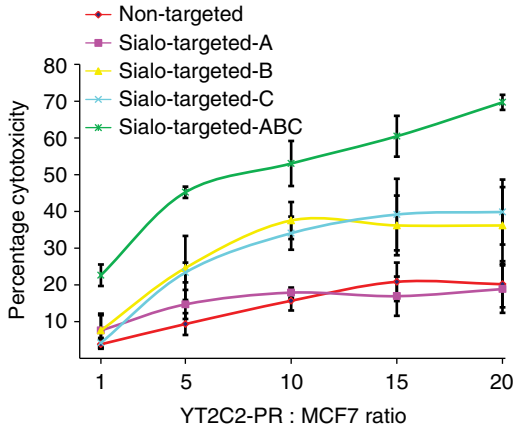

Figure 5. Sialophorin targeting increases the vulnerability of MCF7 to NK-mediated lysis. The NK cell line YT2C2-PR was added at a multiplicity of 1, 5, 10, 15 or 20 to MCF7 expressing non-targeted siRNA, or three sialophorin (Sialo)-targeted siRNAs either simultaneously (ABC) or independently (A, B and C). Following a 16-h incubation, supernatants were gently mixed and aspirated to remove non-adherent YT2C2-PR and MCF7 cells. The remaining intact, adherent cells were labelled with 0.25% Rose Bengal dye, washed and then the dye released by addition of 50% ethanol. Optical density was measured at 570 nm and percentage cytotoxicity calculated. Error bars represent means ± s.e.m. of three independent experiments performed in triplicate.

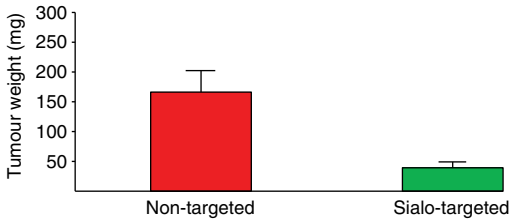

Figure 6. Sialophorin targeting reduces orthotopic tumour growth *in vivo*. MCF7 expressing non-targeted siRNA or three sialophorin (Sialo)-targeted siRNAs were injected into the fourth mammary fat pad of oophorohysterectomised female mice of the strain Hsd:Athymic Nude-*Foxn1*^{nu} ($n=8$ and 10, respectively). After 60 days, mice were killed and visible tumours were removed and weighed. Error bars represent means ± s.e.m. The difference in the weight of tumours produced by non-targeted MCF7 compared with cells expressing three sialophorin-targeted siRNAs had a significance level of $P=0.004$ as calculated by a one-tailed Student's *t*-test.

intracellular region (Pallant *et al*, 1989; Shelley *et al*, 1989; Cyster *et al*, 1991).

Sialophorin can perform diametrically opposite functions. First, depending upon how it is engaged at the cell surface, sialophorin can either induce or protect against leukocyte apoptosis (Brown *et al*, 1996; He and Bevan, 1999; Todeschini *et al*, 2002; Hernandez *et al*, 2006; Kim *et al*, 2006). Second, depending upon the status of leukocyte activation, sialophorin can act either as an anti-adhesion barrier molecule or a pro-adhesion receptor (Brown *et al*, 1981; Rosenstein *et al*, 1991; Ardman *et al,* 1992; Manjunath *et al,* 1993; Dragone *et al*, 1995; Stöckl *et al*, 1996; Soler *et al,* 1997; Fukuoka *et al,* 2000, van den Berg *et al*, 2001; Matsumoto *et al*, 2005; Hernandez *et al*, 2006).

Aberrant expression of sialophorin has been consistently described in colon, salivary gland and lung cancers (Sikut *et al*, 1997, 1999; Woo *et al*, 2006; Seethala *et al*, 2008; Fu *et al*, 2013). In addition, one study of nine breast cancers in 1996 suggested this malignancy might also be characterised by sialophorin expression (Santamaría *et al*, 1996). Our analysis of 125 primary breast tumours representing four histologic subtypes demonstrates that sialophorin expression is indeed a robust breast cancer biomarker. To our knowledge, no study of gene expression profiles has identified sialophorin as being aberrantly expressed in breast cancer. This is consistent with our finding that the majority of normal breast epithelial cells express the N-terminal domain of sialophorin in the cytoplasm and a minority express the C-terminal domain in the nucleus (Figure 1A). Such considerations highlight the limitation of DNA microarrays in determining the molecular mechanisms that underlie disease and normal physiologic processes.

The antibody L10 recognises the N-terminus of sialophorin that in leukocytes is expressed extracellularly (Remold-O'Donnell *et al*, 1984; Remold-O'Donnell and Parent, 1995). In normal breast epithelial cells, the L10 epitope is expressed predominantly in the cytoplasm rather than the nucleus while in breast cancer both nuclear and cytoplasmic expression is often observed. Localised expression at the cell surface either in normal or malignant breast epithelial cells was not seen. Cytoplasmic as opposed to membrane expression of the N-terminal domain of sialophorin has been observed in colon, lung, and salivary gland cancers (Sikut *et al*, 1997, 1999; Woo *et al*, 2006; Seethala *et al*, 2008; Fu *et al*, 2013). Therefore, the pathways that insert sialophorin into the plasma membrane of leukocytes appear to be absent in endothelial and epithelial cells. Nuclear expression of the L10 epitope has been previously reported in lung cancer but not in any other cell type (Fu *et al*, 2013).

The antibody SSGZ recognises the C-terminus of sialophorin. In resting leukocytes, this domain is contiguous with the rest of the molecule and is expressed in the cytoplasm adjacent to the plasma membrane. However, during leukocyte activation, the C-terminus is cleaved away from the rest of sialophorin and translocates to the nucleus where it protects against apoptosis and may drive proliferation (Andersson *et al*, 2004, 2005; Seo and Ziltener, 2009). Our analysis demonstrates that in breast cancer the C-terminus of sialophorin consistently exhibits nuclear rather than cytoplasmic localisation. This suggests that breast cancer is characterised by functional proteolytic pathways analogous to those of activated leukocytes and protection against apoptosis mediated by nuclear expression of sialophorin is constitutive.

The pattern of SSGZ staining linked to the pattern of L10 staining defines six distinct categories of breast cancer (Figure 1B). These categories do not coincide with the cell-of-origin classification of luminal A, luminal B, basal-like, ErbB2-overexpressing and normal-like (Perou *et al,* 2000; Bertucci *et al*, 2005; Van Laere *et al*, 2006). For example, cases of ErbB2-overexpressing breast cancer were present both in the LCN/SN and NR categories defined by sialophorin expression. In addition, both an ErbB2-overexpressing and a basal-like inflammatory tumour were present in the sialophorin LCN/SN category and both luminal A and luminal B tumours were present in the sialophorin category designated SN. The categories of breast cancer defined by sialophorin expression imply they are characterised by distinct proteolyltic cleavage and degradation events (Figure 1B). It is tempting to speculate that these proteolytic pathways may represent new therapeutic targets and new ways to predict treatment outcome.

Western blot analysis demonstrates that the breast cancer cell lines MCF7, MDA-MB-453, MDA-MB-468, MDA-MD-231 and BT-474 all expresses both the N-terminal and C-terminal domains of sialophorin in the nucleus but in the cytoplasm they are either sialophorin negative or express only the C-terminus (Supplementary Figure 3). These patterns of intracellular expression would represent the classifications LN/SN and LN/SCN, respectively. Our analysis of 125 breast tumours has not identified an example of either of these classes (Table 1). Consequently, it appears that LN/SN and LN/SCN are classes common to breast cancer cell lines but rare for breast tumour tissue. This discrepancy may indicate that cell line generation involves the selection of clones with a specific set of molecular processes ideally suited for proliferation *in vitro*.

Acknowledging the limitation of cell lines to mimic the sialophorin classes seen *in vivo*, the functional significance of sialophorin expression in breast cancer was investigated by targeting its expression in the breast cancer cell line MCF7. Such targeting increased homotyptic adhesion supporting the hypothesis that sialophorin facilitates the breakup of primary tumours such that they shed potentially metastatic cells. However, such inhibition of homotypic adhesion must be by an indirect mechanism as sialophorin is expressed inside breast cancer cells not on the outside (Figure 1A). Such 'inside-out' signalling is a well-described means by which the β2-integrin family controls intercellular adhesion (Abram and Lowell, 2009). In the case of sialophorin, analogous signalling could be effected by nuclear expression of the C-terminus changing patterns of gene transcription and/or kinase activity (Rosenstein *et al*, 1999; Andersson *et al,* 2004).

In addition to mediating homotypic repulsion, our targeting studies demonstrate that sialophorin also protects breast cancer cells from apoptosis induced by TNFα. In leukocytes, it has also been shown that sialophorin inhibits apoptosis (He and Bevan, 1999; Todeschini *et al*, 2002; Seo and Ziltener, 2009). However, in this context, sialophorin protects against apoptosis mediated by Fas, growth factor withdrawal and Treg suppression but not against apoptosis induced by TNFα (He and Bevan, 1999; Seo and Ziltener, 2009). Therefore, sialophorin appears to protect against different apoptosis pathways in breast cancer cells compared with

haematopoietic cells. Protection against apoptosis induced by TNFα would be particularly beneficial to cancer cells as this is one of the principal ways by which effectors of the immune system such as NK cells kill tumours (Caron *et al*, 1999). Consistent with this reasoning, we found that sialophorin targeting increased the susceptibility of MCF7 to NK cytotoxicity.

Sialophorin has been shown to affect polarisation and locomotion (Serrador *et al*, 1998; Seveau *et al*, 2000). These findings indicate that sialophorin expressed by breast cancer cells might also mediate migration. Comparison of sialophorin-targeted and non-targeted MCF7 demonstrated that sialophorin targeting reduces transendothelial migration over 9 days by 47%. However, in contrast, sialophorin targeting failed to reduce the invasion of MCF7 into a basement membrane matrix. This extracellular matrix preparation consisted of growth factors, laminin, collagen IV, heparan sulphate, proteoglycans and entactin/nidogen. Therefore, these components appear not to be sufficient for sialophorin-mediated migration.

In summary, our *in vitro* results indicated that sialophorin could contribute to breast cancer pathogenesis *in vivo* in a variety of ways. These range from protecting against apoptosis and attack by NK cells to actively driving metastasis through mechanisms of anti-adhesion and migration. In order to test the role of sialophorin in breast cancer pathogenesis *in vivo*, we used an orthotopic mouse model. Here we established that sialophorin targeting reduced the growth of MCF7 tumours in mammary fat pads by an average of 76%. The intrinsic proliferation rate of MCF7 is unaffected by sialophorin targeting (data not shown). Therefore, the reduction in primary tumour growth *in vivo* suggests increased vulnerability to the murine immune system. Mice of the strain used in our studies produce B-lymphocytes, NK and myeloid cells. Therefore, *in vivo* sialophorin targeting probably makes MCF7 more susceptible to attack by one or more of these immune effectors.

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CONFLICT OF INTEREST

CSS, JJA, QF, SEC and the Gundersen Medical Foundation have intellectual property rights to the polyclonal antibody SSGZ. The remaining authors declare no conflict of interst.

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