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14. ABSTRACT

The overarching goal of this research is to define the role of a cancer-associated glycosyltransferase, ST6Gal-I, in regulating the ovarian tumor cell phenotype. Results obtained as a consequence of DoD pilot funding have established that ST6Gal-I is overexpressed in the great majority of ovarian serous adenocarcinomas and associated metastases. ST6Gal-I adds an α2-6 linked sialic acid to selected membrane receptors including the β1 integrin, as well as the Fas and TNFR1 death receptors. In this final report, we show that ST6Gal-I-mediated receptor sialylation protects ovarian cancer cells against cell death within ascites fluid, suggesting that ST6Gal-I fosters cell survival during tumor cell transit through the peritoneal cavity. Additionally, ST6Gal-I appears to contribute to metastatic targeting of omentum and resistance to cisplatin chemotherapy. These collective findings provide new molecular insight into the important function of the glycosylation machinery in regulating tumor cell behavior. An aberrant profile of cell surface glycans was one of the earliest-identified hallmarks of a tumor cell, however tumor glycobiology has lagged behind most other areas of cancer cell biology. A seminal advance of this study is the determination that a distinct glycosyltransferase, acting on a specific subset of cell surface receptors, plays a critical role in the resistance of ovarian cancer cells to multiple death-inducing stimuli.

15. SUBJECT TERMS
Chemoresistance, death receptors, apoptosis, glycosylation, invasion, metastasis, galectin

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INTRODUCTION

Ovarian cancer is the most lethal gynecologic malignancy, and patient survival rates have not improved appreciably over the last 3 decades. Most patients are initially responsive to treatment, however the majority develop tumor recurrence and succumb to chemoresistant disease. There is a compelling need to uncover novel molecular pathways that can be targeted clinically in order to attack this disease from new directions.

Our group has discovered a unique glycosylation-dependent mechanism that controls ovarian cancer cell invasiveness and survival within the peritoneal milieu. This mechanism involves the addition of an α2-6-linked sialic acid (a negatively charged sugar) to selected cell receptors by the ST6Gal-I sialyltransferase, leading to alterations in receptor function that drive a malignant cell phenotype. For decades, upregulation of ST6Gal-I mRNA has been reported in multiple cancers (reviewed in[1,2]), including ovarian cancer[3], however studies of ST6Gal-I protein expression and function are lacking. Immunohistochemical studies supported by this pilot project confirmed for the first time that ST6Gal-I protein is overexpressed in ovarian cancer, and our recent mechanistic studies have identified specific ST6Gal-I-dependent signaling pathways that control tumor cell behavior. One of the major gaps in our understanding of ST6Gal-I's function is the dearth of studies aimed at defining the substrates for ST6Gal-I, and characterizing the effects of variant sialylation on the activity of these specific targets. Our group identified the β1 integrin as an ST6Gal-I substrate, and showed that elevated integrin sialylation promotes cell migration and invasion through collagen-rich extracellular matrices[4-7]. In addition, integrin sialylation blocks apoptosis induced by the mammalian lectin, galectin-3[8], which our studies show is expressed in human ovarian tumor tissues and in ascitic fluid from patients with metastasis disease. Finally, we have recently identified the Fas and TNFR1 death receptors as ST6Gal-I substrates[9,10], and shown that sialylation of these receptors strongly inhibits apoptotic signaling. Collectively our studies suggest that elevated ST6Gal-I expression provides a selective advantage for tumor cells through multiple molecular pathways. The *central hypotheses* of our study are that increased receptor sialylation (secondary to ST6Gal-I upregulation) contributes to the invasive and apoptosis-resistant phenotype of ovarian cells and (2) ovarian progression can be inhibited by therapeutic targeting of ST6Gal-I expression/activity. The following aims were proposed to address these hypotheses:

Specific Aim 1: *Role of β1 integrin sialylation in regulating ovarian tumor cell association with the omentum*

Specific Aim 2: *Determine whether ovarian tumor tissues upregulate gal-3, and whether integrin sialylation protects tumor cells from gal-3-mediated apoptosis*

Specific Aim 3: *Resistance to apoptosis conferred by ST6Gal-I-mediated sialylation of death receptors*

PROGRESS REPORT (body)

SPECIFIC AIM 1: Role of the β1 integrin receptor in regulating ovarian tumor cell association with the omentum.

Objectives/Tasks of Aim 1:

To characterize molecular events regulating ovarian tumor cell interactions with human and murine omental tissues cultured *ex vivo* (Tasks 1-4), and *in vivo* tumor cell recruitment and invasion of omentum (not part of original Aim).

Progress:

1) The ovarian cancer cell line, OV4, is one of the few lines that does not express ST6Gal-I (unpublished data). To understand the role of ST6Gal-I in the cancer cell phenotype, ST6Gal-I was overexpressed in OV4 cells (Fig 1). Control empty vector OV4 cells (EV), or ST6Gal-I over-expressing OV4 cells (ST6-OE) were injected intraperitoneally into nude mice. At one week following injection, animals were sacrificed, and the omental tissues dissected. Tumor cells within the omentum were stained immunohistochemically (IHC) for human cytokeratin 18 (CK-18). Staining was quantified using imaging software. These studies revealed greater invasion into the omentum by tumor cells that overexpress ST6Gal-I (Fig 1).

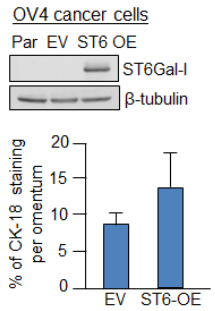

FIG 1: Tumor cell/ omental interaction

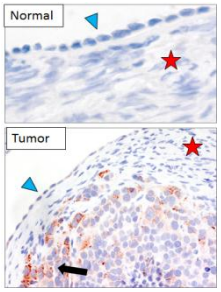

FIG 2: ST6Gal-I in normal vs tumor ovarian tissues
No ST6Gal-I is detected in epithelia (blue arrowhead) or stroma (red star) of normal ovary, or normal-appearing adjacent cells in tumor tissues. In contrast, tumor cells stain positively for ST6Gal-I (black arrow).

2) Due to the prior lack of an effective antibody for IHC, ST6Gal-I protein expression has never before been evaluated in ovarian cancer. Accordingly, IHC staining for ST6Gal-I was conducted in primary and metastatic ovarian tumors. We quantified staining in 33 papillary serous ovarian carcinomas, and 32 had positive staining for ST6Gal-I, whereas the enzyme was undetectable in normal ovarian epithelium or stroma (Fig 2). We also performed side-by-side IHC staining (Fig 3A), as well as Western blot analyses (Fig 3B), of 10 primary and 10 metastatic tumors, with the latter samples primarily composed of metastases within the omentum. Most of the metastatic samples had greater ST6Gal-I staining than primary tumors (Fig 3C), consistent with the concept that tumor cells with high ST6Gal-I expression may have a greater propensity for metastatic targeting of the omentum.

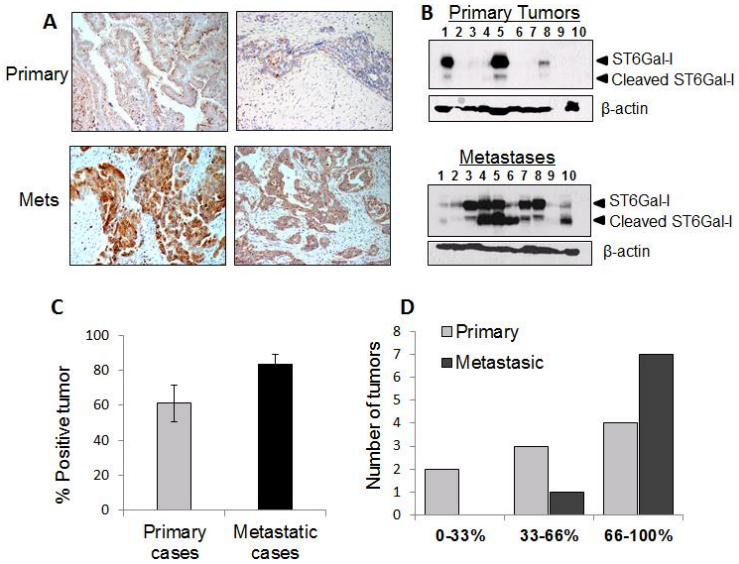

FIG 3: ST6Gal-I expression in primary vs metastatic tumors
A) Representative IHC staining of ST6Gal-I in patient primary vs metastatic tumors
B) Immunoblot of 10 primary and 10 metastatic tumors.
C) The average percent of each tissue section with positive IHC ST6Gal-I staining is shown for 10 primary and 10 metastatic tumors (same patients as panel B).
D) IHC results from the same samples stained in panel C were re-plotted to show the number of tumors that had 0-33, 33-66, or 66-100 percent of the area of the tissue section stained positively for ST6Gal-I.

Challenges:

One challenge we encountered is that the *ex vivo* omental cultures were not very robust. We were not able to sustain the cultures for long enough intervals to probe the role of integrins in ovarian cancer cell invasion into the omentum.

SPECIFIC AIM 2: Determine whether ovarian tumor tissues upregulate gal-3, and whether integrin sialylation protects tumor cells from gal-3-mediated apoptosis.

Objectives/Tasks of Aim 2:

Aim 2 centered on the hypothesis that apoptosis induced by the mammalian lectin, galectin-3 (gal-3), was blocked by sialylation of the β1 integrin. This hypothesis was founded on our prior findings showing that gal-3 secreted by tumor cells binds to the β1 integrin and induces apoptosis, but only if the integrin lacks sialic acid. We showed in cell culture models that forced expression of ST6Gal-I causes integrins to acquire sialic acid, and this sugar structure on the integrin blocks interaction with gal-3. We proposed to examine gal-3 expression in tumor tissues and ascites fluid from ovarian cancer patients (Task 1), and determine whether a gal-3/integrin signaling axis was responsible for regulating tumor cell apoptosis in both cell culture models (Tasks 2-3) and ascites fluid (Task 4).

Progress:

1) We have analyzed five matched patient ascites and tumor tissues by IHC and/or immunoblot and all had significant expression of galectin 3 (representative samples in Fig 4). This is the first demonstration (to our knowledge) that gal-3 levels are high in ascites and tumors from ovarian cancer patients.

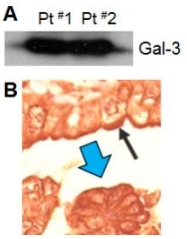

FIG 4: Gal-3 expression in ascites (A) and tumor tissue (B)

2) Our prior studies reporting that integrin sialylation blocks gal-3-mediated apoptosis were conducted with colon carcinoma cells[8]. In the current study we find that, like colon cancer cells, ovarian cancer cell cultures that express sialylated integrins are protected against apoptosis induced by recombinant gal-3 (not shown). To understand the functional importance of the gal-3/integrin pathway within the tumor microenvironment, we incubated ovarian cancer OV4 cells with or without ST6Gal-I expression (EV or ST6-OE cells) with increasing amounts of the soluble fraction of patient ascites fluid (all patient cells were removed prior to these assays). We hypothesized that overexpression of ST6Gal-I would protect tumor cells from apoptosis induced by immune-related molecules including gal-3. Indeed, as shown in Fig 5, cells with forced expression of ST6Gal-I are protected from ascites-induced apoptosis. This is a highly significant finding as it suggests ST6Gal-I plays a critical role in promoting the survival of ovarian cancer cells during transcoelomic metastatic. However, our studies to date do not strongly implicate gal-3/integrin signaling in this process. The addition of galactose to the ascites fluid, to block gal-3 binding and function, did not protect against ascites-mediated apoptosis (not shown). Notably, these studies are preliminary, and it will be important to try other inhibitors of this pathway (anti-gal-3 blocking antibodies or gal-3 depletion). However, as described below, we have identified other ST6Gal-I-dependent mechanisms that appear responsible for conferring resistance to ascites-mediated apoptosis.

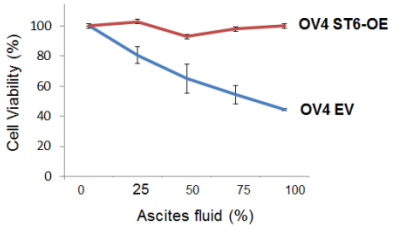

FIG 5: ST6Gal-I confers resistance to ascites-mediated cell death.
EV or ST6-OE cells were incubated for 24 hours with increasing amounts of soluble ascites (mixed with serum-free media).

Challenges:

We did not encounter any technical challenges with this Aim. Given that initial experiments did not strongly implicate gal-3/integrin signaling in apoptosis resistance within ascites, we shifted our attention to Aim 3.

Specific Aim 3: Resistance to apoptosis conferred by ST6Gal-I-mediated sialylation of death receptors.

Objectives/Tasks of Aim 3.

This Aim was focused on understanding the role of sialylation in regulating death receptor-mediated apoptosis. We hypothesized that sialylation of the Fas and TNFR1 receptors would alter some aspect of receptor structure, which in turn would block apoptotic signaling (Tasks 1-2). We proposed to examine the effects of sialylation on receptor oligomerization, internalization and formation of the Death Inducing Signaling Complex (DISC). We also proposed to determine whether sialylation of Fas was responsible for the protection of tumor cells against cisplatin-mediated cell death (Task 3).

Progress:

We have by far made the most progress on Aim 3 and research related to Aim 3 has taken our work in exciting new directions. Importantly, data generated as a consequence of DoD pilot funding were central to an NIH R01 proposal, which was just funded this spring. This R01 is directly relevant to the DoD award as it focuses on the role of ST6Gal-I in ovarian cancer resistance to cisplatin-mediated cell death, as well as death receptor signaling by ovarian cancer cells within the peritoneal cavity.

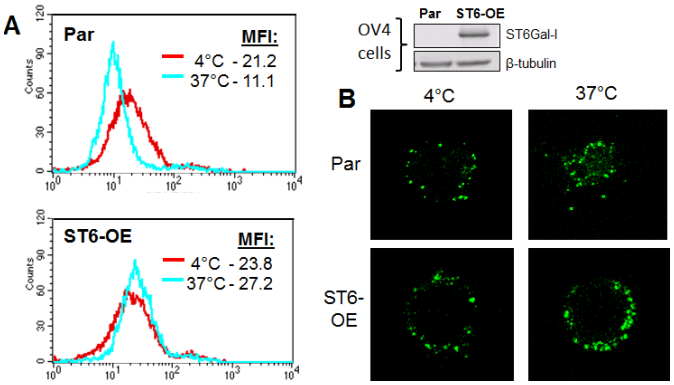

FIG 6: Internalization of TNFR1 is blocked in ST6Gal-I expressing cells

Cells were treated with TNFα-FITC at 4°C to allow binding to TNFR1. Temperature was then switched to 37°C for 10 min to induce TNFR1 activation and internalization.

1) The TNFR1 and Fas (but not other death receptors) have an *N*-glycosylation site in the proximity of the "90s" loop, a conserved domain within death receptors critical for receptor aggregation. We hypothesized that the addition of the bulky, negatively charged sialic acid at this site would interfere with receptor oligomerization. Following activation of TNFR1 and Fas by ligand, receptor aggregation and internalization are essential steps in the formation of the Death Inducing Signaling Complex (DISC), leading to caspase-mediated cell apoptosis[11]. During the time interval of this pilot project, we demonstrated that Fas and TNFR1 are substrates for ST6Gal-I, and that the addition of sialic acid to Fas or TNF1 inhibits apoptosis[9,10]. We further determined that a sialylation-dependent block in receptor internalization is the mechanism responsible for impaired apoptosis. The internalization defect has been published for the Fas receptor[10], and unpublished results show that sialylation of TNFR1 operates through a similar mechanism. OV4 cells (+/- ST6Gal-I) were incubated with TNFα at 4°C, which allows TNFα to bind (but not activate) TNFR1, and then cells were switched to 37°C for 10 min to induce TNFR1 activation and internalization. Flow cytometry experiments (Fig 6A), show that surface TNFR1 is equivalent in parental (Par) and ST6-OE cells treated with TNFα at 4ºC, however

surface TNFR1 is reduced in Par, but not ST6-OE, cells following temperature shift, reflecting internalization (leftward peak shift in Par cells). Similar results were observed by immunocytochemistry (Fig 6B): TNFα treatment at 37ºC causes TNFR1 internalization in cells lacking ST6Gal-I, but not in ST6Gal-I-OE cells. These data are also supported by immunoblotting experiments. Within 10 min of TNFα treatment at 37°C, full-length, surface TNFR1 (upper band, see inset, Fig 7) is lost in Par and EV, but not ST6-OE, cells. A similar decrease in TNFR1 molecular mass was shown by others to result from TNFα-induced translocation of TNFR1 to endosomes, and subsequent processing to a lower MW form[12] (note that substantial TNFR1 is retained intracellularly in resting cells). *Together, flow cytometric, immunocytochemistry and immunoblotting results point to a sialylation-dependent block in TNFR1 internalization following activation by TNFα.*

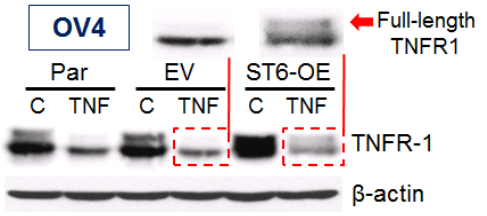

FIG 7: Full-length TNFR1 is retained in ST6Gal-I expressing cells following TNFα treatment
OV4 cells were treated with TNFα for 10 min at 37°C. Cells were then lysed and immunoblotted for TNFR1. Full-length TNFR1 is lost in Par and EV cells, but not in ST6 cells, following TNFα treatment.

3) Having determined that ST6Gal-I-mediated sialylation controls TNFR1 signaling, we evaluated whether this pathway was important for ovarian cancer cell survival within the peritoneal cavity of ovarian cancer patients. Ascites fluid from patients is known to contain significant quantities of TNFα[13,14]. Accordingly, we hypothesized that ovarian cancer cells with upregulated ST6Gal-I would be resistant to apoptosis induced by TNFα within the ascites. To test this hypothesis, OV4 cells with or without ST6Gal-I expression were preincubated with a function-blocking antibody against TNFR1. Cells were then incubated with ascites fluid. It was found that 26% of the amount of ascites-induced apoptosis of OV4 EV cells could be reversed by the TNFR1 antibody. In contrast, the TNFR1 blocking antibody had no effect on ST6Gal-I expressing OV4 cells. These results suggest that ascites-induced apoptosis of ovarian cancer cells with no endogenous ST6Gal-I expression is mediated at least in part by TNFR1 receptors lacking α2-6 sialic acids. These data point to a new glycosylation-dependent molecular mechanism that controls ovarian cancer cell survival within the peritoneal cavity. In future experiments we will perform similar experiments using function-blocking antibodies against both TNFR1 and Fas, since these receptors may cooperate in regulating tumor cell survival within ascites.

4) Studies described above showed that forced ST6Gal-I upregulation in an ovarian cancer cell line can protect against ascites induced apoptosis, however a central question was whether ovarian cancer cells within ascites fluid do in fact express ST6Gal-I. While we have shown that most ovarian cancer cell lines and ovarian cancer tissues overexpress ST6Gal-I, it was important to assess ST6Gal-I expression levels in ovarian cancer cells within the peritoneal cavity. To this end, we obtained ascites fluid and collected the patient cells by centrifugation. Cell pellets were embedded in paraffin, sectioned and stained by IHC for ST6Gal-I. Positive staining for ST6Gal-I was noted in the tumor spheroids (Fig 8A). We next evaluated whether cells with high ST6Gal-I expression exhibited a survival advantage. To address this hypothesis, patient cells within ascites were centrifuged as before, and then cells with high or low ST6Gal-I expression were collected by FACs sorting using the SNA lectin (Fig 8B). SNA is a lectin that specifically recognizes cell surface α2-6-linked sialic acids, which are the product of ST6Gal-I enzymatic activity. Cells that were SNA positive expressed ST6Gal-I, whereas no ST6Gal-I was detected in SNA negative cells. These sorted cells were then placed into culture, and only the SNA positive cells were able to survive in culture, suggesting that ST6Gal-I confers some type of survival benefit. Furthermore, the surviving SNA-positive cells grew as nonadherent spheroid

cultures rather than monolayers, consistent with a cancer stem cell-like phenotype (Fig 8C). Stem-like ovarian tumor spheroids are thought to be directly responsible for metastatic targeting of the peritoneal wall, including omental tissues[15-17].

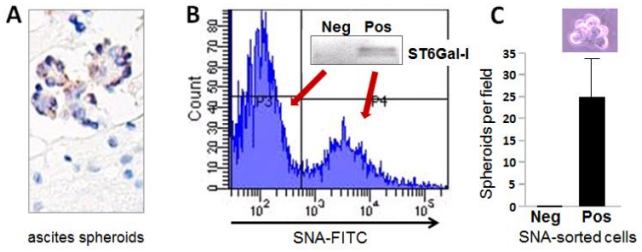

FIG 8: Ascites cells with ST6Gal-I grow as spheroids
(A) Enriched ST6Gal-I expression in spheroids from paraffin-embedded cells pellets.
(B) Cells were sorted for ST6Gal-I by SNA FACs, and placed in culture.
(C) SNA positive cells grew as spheroids; negative cells did not survive.

4) A final hypothesis of Aim 3 was that sialylation of the Fas receptor would be responsible for the role of ST6Gal-I in cisplatin resistance. In a manuscript published last year[18], we showed that: (i) forced expression of ST6Gal-I protects ovarian cancer cells against cisplatin-induced cell death; (ii) forced ST6Gal-I knockdown sensitizes cells to cisplatin and (iii) cell populations selected for stable resistance to cisplatin exhibit upregulation of endogenous ST6Gal-I. To determine the role of sialylated Fas in this process, we pre-incubated cells with Fas function-blocking antibodies, and then evaluated apoptosis. Function-blocking Fas antibodies partially reversed apoptosis of the Pa-1 ovarian cancer cell line, but had little effect on apoptosis of OV4 cells (not shown). We conclude that there are multiple mechanisms by which ST6Gal-I contributes to resistance to cisplatin.

Challenges:

No major technical challenges were encountered, however the mechanisms responsible for cisplatin-induced cell death appeared more complex than initially anticipated. The Fas receptor seemed to play only a minor role in this process. Future studies will be directed at determining whether ST6Gal-I targets other receptors responsible for chemotherapy resistance, such as drug transporters critical for either drug uptake or efflux.

KEY RESEARCH ACCOMPLISHMENTS

- First demonstration that ST6Gal-I protein is upregulated in established ovarian cancer cell lines, primary ovarian cancer cells and spheroids within ascites, and ovarian cancer tissues. 32/33 serous papillary adenocarcinomas were positive for ST6Gal-I, whereas no detectable ST6Gal-I was apparent in normal ovarian epithelium.
- Higher expression of ST6Gal-I in metastases, particularly omental metastases, than in primary tumors.
- First identification of galectin-3 expression in ovarian cancer tumors and ascites.
- First determination that ST6Gal-I blocks ascites-induced apoptosis, implicating variant sialylation in tumor cell survival within the peritoneal cavity.
- Established a new mechanism for ovarian cancer survival within peritoneal cavity involving ST6Gal-I-mediated sialylation of TNFR1 receptor.
- First determination that Fas and TNFR1 receptors are substrates for ST6Gal-I, and that ST6Gal-I-mediated sialylation of these receptors inhibits apoptosis through blocking receptor internalization. These findings provide a major conceptual advance by establishing an important role for glycosylation in death receptor function.
- First report that ST6Gal-I expression confers resistance to cisplatin-mediated apoptosis, highlighting a new role for glycosylation in chemoresistance.

REPORTABLE OUTCOMES (05/15/11-05/14/14)

- Pilot funding from the DoD was instrumental for generating data for an NIH R01 proposal that was recently awarded to the PI. The PI had no other funds to advance this project. The new NIH R01 represents a direct extension of the work pursued in the DoD pilot project. The relevant NIH R01 is described below:

  R01 GM111093 (PI: Bellis) 05/15/14-03/31/17
  NIH/NIGMS $190,000 (adc), $570,000 (tdc)
  Glycosylation-dependent mechanisms regulating ovarian tumor cell survival.

- The work performed as part of the DoD award served as the basis for oral presentations (competitively selected from abstracts) for several national meetings: (1) American Society for Matrix Biology meeting San Diego, CA, 2012; (2) Experimental Biology meeting, Boston, MA, 2013; (3) Society for Glycobiology, St. Petersburg, FL, 2013
- Best poster award (Schultz et al.) NIH Glycosciences Day meeting, Bethesda, MD, May 2013
- Invited talks by the PI (Bellis) – (1) *Sialoglyco* meeting, Taipei, Taiwan, Sept 2012; (2) American Chemical Society meeting, Baton Rouge, LA, Nov 2012; (3) *19th World Congress on Advances in Oncology* conference, Athens, Greece, scheduled for Oct, 2014; (4) seminar speaker: University of Oklahoma Health Sciences Center (Mar 2013), Emory University (Oct 2013), and Roswell Park Cancer Institute (Dec 2013).
- 4 publications related to this work:
  a) Swindall et al. (2011) *J Biol Chem* 286: 22982 - this manuscript was selected as an Editor's Choice manuscript by *Science Signaling.*
  b) Schultz et al (2012) *Cancer Metastasis Rev* 31: 501.
  c) Swindall et al (2013) – *Cancer Res* 73: 2368 - this manuscript was selected as a featured manuscript by *Cancer Stem Cell News*
  d) Schultz et al (2013) *J Ovarian Res* 6: 25 – this manuscript was featured on the Betty Allen Ovarian Cancer Foundation website.
- Two manuscripts are currently in preparation that are directly related to the DoD proposal. One of these manuscripts will focus on the role of TNFR1 sialylation as a survival mechanism for ovarian cancer cells; the second will focus on ST6Gal-I's contribution to a stem cell-like, chemoresistant ovarian cancer cell phenotype.
- The Bellis laboratory was featured in a 2012 Metastasis Research Society newsletter.
- Development of unique reagents and methods including: multiple ovarian cancer cell lines with forced overexpression or knockdown of ST6Gal-I; a method for separating ST6Gal-I expressing tumor cells from solid tumors or ascites; and optimized method for IHC of ST6Gal-I in human tumors.

CONCLUSION

An alteration in the profile of cell surface glycans was one of the earliest identified hallmarks of a tumor cell, however we still know very little regarding the functional contribution of tumor-associated glycoconjugates. The studies described herein provide critical new insights into the role of the ST6Gal-I sialyltransferase in controlling tumor phenotype. In particular, we have shown that sialylation of a subset receptors has a strong inhibitory effect on multiple apoptotic

pathways, including death receptor signaling, and galectin and cisplatin-induced cell death. These findings are important because they establish a strong foundation for pursuing ST6Gal-I as a potential biomarker for ovarian cancer progression, and new molecular target for therapeutic intervention. The identification of new mechanistic pathways involved in ovarian cancer is essential, given the low survival rates of ovarian cancer patients and limited long-term efficacy of current treatments.

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APPENDICES

Manuscript #1

Swindall, AF & Bellis, SL. Sialylation of the Fas death receptor by ST6Gal-I provides protection against Fas-mediated apoptosis in colon carcinoma cells. *J Biol Chem*, **286**, 22982-22990 (2011)

Manuscript #2:

Schultz, M.J., Swindall, A.F. & Bellis, S.L. Regulation of the metastatic cell phenotype by sialylated glycans. *Cancer Metastasis Rev* **31**, 501-18 (2012)

Manuscript #3:

Swindall, A.F. Londoño-Joshi AI, Schultz MJ, Fineberg N, Buchsbaum DJ, Bellis SL. ST6Gal-I protein expression is upregulated in human epithelial tumors and correlates with stem cell markers in normal tissues and colon cancer cell lines. *Cancer Res* **73**, 2368-2378 (2013)

Manuscript #4:

Schultz, M.J., Swindall, AF, Wright, JW, Sztul, ES, Landen, CN, Bellis, SL. ST6Gal-I sialyltransferase confers cisplatin resistance in ovarian tumor cells. *J Ovarian Res* **6**, 25. (2013)

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Sialylation of the Fas Death Receptor by ST6Gal-I Provides Protection against Fas-mediated Apoptosis in Colon Carcinoma Cells*

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The glycosyltransferase, ST6Gal-I, adds sialic acid in an α2–6 linkage to the *N*-glycans of membrane and secreted glycoproteins. Up-regulation of ST6Gal-I occurs in many cancers, including colon carcinoma, and correlates with metastasis and poor prognosis. However, mechanisms by which ST6Gal-I facilitates tumor progression remain poorly understood due to limited knowledge of enzyme substrates. Herein we identify the death receptor, Fas (CD95), as an ST6Gal-I substrate, and show that α2–6 sialylation of Fas confers protection against Fas-mediated apoptosis. Intriguingly, differences in ST6Gal-I activity do not affect the function of DR4 or DR5 death receptors upon treatment with TRAIL, implicating a selective effect of ST6Gal-I on the Fas receptor. Using ST6Gal-I knockdown and forced overexpression colon carcinoma cell models, we find that α2–6 sialylation of Fas prevents apoptosis stimulated by FasL as well as the Fas-activating antibody, CH11, as evidenced by decreased activation of caspases 8 and 3. We also show that α2–6 sialylation of Fas does not alter the binding of CH11, but rather inhibits the capacity of Fas to induce apoptosis by blocking the association of FADD with Fas cytoplasmic tails, an event that initiates death-inducing signaling complex formation. Furthermore, α2–6 sialylation of Fas inhibits Fas internalization, which is required for apoptotic signaling. Although dysregulated Fas activity is a well known mechanism through which tumors evade apoptosis, the current study is the first to link Fas insensitivity to the actions of a specific sialyltransferase. This finding establishes a new paradigm by which death receptor function is impaired for the self-protection of tumors against apoptosis.

The ability to evade apoptosis is one of the defining characteristics of a malignant tumor cell (1). Escape from cell death can be accomplished through alterations in various cellular components, including dysregulation of oncogenes and tumor suppressors, and mutations in apoptotic and anti-apoptotic signaling machinery. The TNF family of death receptors (TNFRs),[3] including TNFR1, DR4, DR5, and Fas (CD95), represents one category of signaling molecules that is commonly disrupted in human tumors and has been strongly implicated in tumor cell survival (2, 3).

The Fas death receptor, like other TNFRs, is a homotrimeric transmembrane receptor that activates multiple intracellular signaling cascades, one of which directs apoptosis. Upon association with activating ligands, Fas undergoes higher order clustering, which facilitates the binding of cytosolic proteins to the Fas cytoplasmic tails. The first protein recruited to the Fas tails is FADD, which binds to Fas through a region known as the death domain. Several other proteins, including procaspase 8 and procaspase 10, are then recruited to the Fas/FADD complex, and together these proteins form the death-inducing signaling complex (DISC). This complex is internalized through clathrin-mediated endocytosis and allows for further DISC formation required for apoptotic signaling (4). This enhanced formation of the DISC leads to the autolytic cleavage and activation of procaspase 8, which goes on to cleave the effector caspase, caspase 3, ultimately resulting in apoptotic endpoints such as membrane blebbing and DNA fragmentation (2).

Diminished Fas expression and activity are well established as mechanisms responsible for the apoptotic resistance of tumor cells. A myriad of studies have reported alterations in the expression of pro- and anti-apoptotic components involved in the Fas pathway such as c-FLIP, BAX, and BCL-2 (5–7). Additionally, in many tumor types there is up-regulation of the endogenous ligand for the Fas receptor, FasL, which is thought to provide a mechanism of self-conservation for tumor cells through FasL-directed killing of tumor-invading immune cells (8). However, despite the extensive research focused on changes in the expression level of Fas and associated signaling molecules, very few studies have investigated molecular mechanisms that alter Fas function independent of variant protein expression, for example, post-translational modifications such as glycosylation.

The Fas receptor is modified with both *O*- and *N*-linked glycans (9), although the functional significance of these glycoconjugates in Fas signaling has received minimal attention. It has been known for decades that tumor cells express an altered profile of cell surface oligosaccharides, and in fact there is a specific subset of glycosylating enzymes exhibiting aberrant activity in human cancers. The ST6Gal-I sialyltransferase is one of the enzymes up-regulated in multiple types of cancer (10–15), and high ST6Gal-I levels are associated with increased metastatic potential (12, 15–17). ST6Gal-I is responsible for the addition of the negatively charged sugar, sialic acid, in an α2–6

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[3] The abbreviations used are: TNFR, TNF family of death receptors; DISC, death-inducing signaling complex; IPG, immobilized pH gradient; MAA, Maackia amurensis agglutinin; PECAM, Platelet Endothelial Cell Adhesion Molecule; SNA, Sambucus Nigra agglutinin.

linkage to the *N*-linked glycans of cell surface or secreted glycoproteins. In the current study we have identified Fas as an ST6Gal-I substrate and further determined that α2–6 sialylation of Fas inhibits Fas-mediated cell death. These collective results elucidate a novel mechanism by which tumor cells evade apoptosis.

EXPERIMENTAL PROCEDURES

Cell Culture—HD3 colon epithelial cells expressing oncogenic *ras* were developed as previously reported (18). These HD3 cells were then transduced with lentivirus (purchased from Sigma) containing either shRNA sequence against *ST6Gal1* (HD3.sh) or an empty vector (HD3.ev). A pooled population of clones stably expressing shRNA was generated by puromycin selection. Down-regulation of ST6Gal-I expression was confirmed by Western blot (19). SW48 cells were purchased from ATCC. These cells were transduced with either lentivirus containing rat liver ST6Gal-I cDNA (SW.ST6) or lentivirus containing an empty vector (SW.ev). The original ST6Gal-I plasmid was obtained from Dr. Karen Colley (University of Illinois, Chicago), and lentiviral vectors developed from this plasmid were constructed by Dr. John Wakefield (Open-Biosystems, Inc.). SW.ST6 cells represent a pooled population of stably transduced clones, isolated by puromycin selection. Confirmation of expression and functionality of ST6Gal-I has been published previously (20). HD3 cells were maintained in low glucose (1 g/liter) DMEM with 7% FBS and 1% antibiotic/antifungal containing penicillin G, streptomycin sulfate, and amphotericin B (Invitrogen). SW48 cells were maintained in 1X-L15 medium with 10% FBS and 1% antibiotic/antifungal.

Western Blotting—To induce apoptosis, cells were treated with either 4 ng/ml membrane-bound FasL (Millipore), 0.5 mg/ml CH11 (Millipore), or 2 mg/ml TRAIL (Biomol International) for 18 h (see Figs. 1–3 below) or 6 h (see Fig. 5*C*). Cells were then lysed in 50 mM Tris-HCl (pH 7.4) with 1% Triton X-100 and protease inhibitors (Roche Applied Bioscience). Proteins were resolved by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked in 5% dried nonfat milk in Tris-buffered saline containing 0.01% Tween 20 blocking buffer at room temperature for 1 h. The membranes were then subjected to primary antibody against cleaved caspase 3 (Cell Signaling), cleaved caspase 8 (Cell Signaling), Fas (Santa Cruz Biotechnology), FADD (Cell Signaling), DR4 (Santa Cruz Biotechnology), DR5 (ProSci Inc.), and β-tubulin (Abcam) overnight at 4 °C. Membranes were washed and then subjected to HRP-conjugated secondary antibodies diluted in blocking buffer for 1 h at room temperature. Proteins were visualized by using Immobilon (Millipore).

Immunofluorescent Staining—Cells were grown on chamber slides and treated with the Fas-activating antibody, CH11 (Millipore) at 0.5 mg/ml or TRAIL (Biomol International) at 2 mg/ml for 18 h. The reaction was then stopped with ice-cold PBS. The protocol for the Caspases 3 and 7 FLICA Apoptosis Detection Kit was followed as recommended by the manufacturer (Immunochemistry Technologies). The slides were imaged by band-pass filter with excitation at 550 nm and emission at 580 nm for the red fluorescence of cleaved caspases 3 and 7, and UV-filter with excitation at 365 nm and emission at 480 nm for Hoescht staining of the nuclei. For quantification, the percentage of apoptosis was calculated as the number of FLICA-positive cells compared with the total number of cells in three independent fields of view. Statistical analysis was performed by repeated-measure analysis of variance, with $p < 0.05$ considered statistically significant.

Lectin Affinity Analysis—Cell lysate protein (1000 μg) was incubated overnight with 50 μl of immobilized SNA-1 or MAA lectin (EY Laboratories) with rotation at 4 °C. The α2–6 sialylated proteins complexed with SNA or α2–3 sialylated proteins complexed with MAA lectin were collected by brief centrifugation and washed. Sialylated proteins were released from the complexes by boiling in SDS-PAGE sample buffer. The proteins were then resolved by SDS-PAGE and immunoblotted to detect Fas.

Two-dimensional Gel Electrophoresis—HD3 whole cell lysates were prepared by lysing in lysis buffer with 50 mM Tris-HCl (pH 7.4) with 1% Triton X-100 and protease inhibitors (Roche Applied Bioscience). Lysates were then diluted in isoelectric focusing rehydration buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 30 mM DTT, 0.5% ampholytes, and trace bromphenol blue. Samples were used to rehydrate 11-cm, 3–10 linear gradient IPG strips for 16 h in Drystrip Reswelling tray.

For the first dimension, IPG strips were focused using Amersham Biosciences IPGphor II isoelectric focusing unit at 50 μA per strip at 20 °C. For the second dimension, strips were equilibrated in DTT equilibration buffer containing 6 M urea, 20% glycerol, 50 mM Tris-HCl, pH 8.8, 2% SDS, 65 mM DTT, and trace bromphenol blue for 30 min and in IA equilibration buffer containing 6 M urea, 20% glycerol, 50 mM Tris-HCl, pH 8.8, 2% SDS, 2.5% iodoacetamide, and trace bromphenol blue for 15 min. Strips were then electrophoresed on 12.5% criterion gels (Bio-Rad) at a 100-V constant in the Bio-Rad Criterion gel box. Proteins were then transferred to PVDF membranes and immunoblotted for Fas (Santa Cruz Biotechnology).

Flow Cytometry—Cells were harvested non-enzymatically from tissue culture dishes using Cell Stripper solution (Cellgro). Cells were then resuspended into ice-cold PBS containing 0.2% heat-denatured BSA. For the antibody binding assay: cells were treated with CH11 (Millipore) at 20 μg/ml or IgM control for 1 h at 4 °C. Cells were then incubated with FITC-tagged secondary antibodies. For the CH11 binding curve, the same experiment was performed using a range of CH11 concentrations, including 1, 10, 20, or 40 μg/ml. For Fas expression: cells were treated with FITC-conjugated anti-human CD95 (BC Pharmingen) for 1 h at 4 °C. After labeling, cells were washed in PBS/BSA and then analyzed with a FACSCalibur (BD Biosciences) at the University of Alabama at Birmingham Arthritis and Musculoskeletal Center Analytic and Preparative Core Facility.

DISC Immunoprecipitation—DISC components were immunoprecipitated by treating 8.0×10^6 cells with 1.0 μg/ml CH11 (Millipore) in media at 4 °C (control) or 37 °C for 30 min. Treatment was stopped by the addition of ice-cold PBS. Cells were centrifuged to remove unbound antibody and washed with ice-cold PBS. Cells were then subjected to lysis buffer containing 50 mM Tris-HCl (pH 7.4) with 1% Triton X-100 and protease inhibitors (Roche Applied Bioscience) on ice for 15

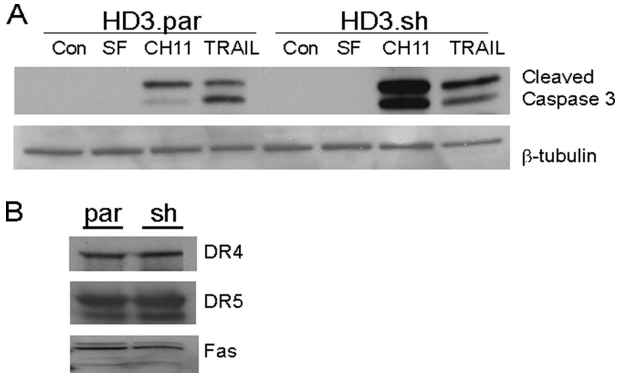

FIGURE 1. **Apoptosis mediated by Fas, but not DR4/DR5, is altered by changes in ST6Gal-I expression.** *A*, after treatment with CH11 (Fas-activating antibody) or TRAIL (activating ligand for DR4/DR5), cells were lysed and lysates were subsequently Western blotted for cleaved caspase 3. The levels of cleaved caspase 3 are much higher in the *HD3.sh CH11* treatment *lane* as compared with the *HD3.par lane*. There is little difference between the TRAIL-treated HD3.sh and HD3.par samples. *B*, there is no change in total expression of DR4, DR5, or Fas (*Con*, serum-containing control media; *SF*, serum-free media).

min. Samples were centrifuged, and supernatant was collected and rotated overnight with 40 μl of prewashed anti-IgM-conjugated agarose beads (Sigma). Precipitated proteins were released from the complexes by boiling in SDS-PAGE sample buffer. Proteins were resolved by SDS-PAGE and immunoblotted for the Fas-binding protein, FADD (Cell Signaling Technology). The PVDF membrane was then stripped with Restore Western Blot Stripping Buffer (Thermo Scientific) and reprobed with Fas (Santa Cruz Biotechnology) as previously described.

Internalization Assay—Cells were lifted from the tissue culture plastic with trypsin, and trypsin activity was stopped with trypsin inhibitor. Cells were resuspended in media with 1.0 μg/ml CH11 (Millipore) at 4 °C (control) or were warmed to 37 °C for 30 min to activate internalization. Cells were washed and resuspended in 3.7% formaldehyde in PBS and fixed for 15 min at room temperature. Cells were then washed and resuspended in media with 1.25 μg/ml anti-mouse IgM Alexa 488 (Invitrogen) at 4 °C for 1 h. Stained cells were then washed and resuspended in Vectashield (Vector Laboratories, Burlingame, CA) and plated on a glass microscope slide. Slides were imaged with the Zeiss LSM 710 laser confocal scanning microscope.

RESULTS

α2–6 Sialylation Confers Protection against Fas-mediated Apoptosis, but Does Not Affect DR4/DR5 Signaling—To evaluate the role of ST6Gal-I-mediated sialylation in regulating tumor cell behavior, we previously developed a cell model system with variant levels of ST6Gal-I expression. HD3 colon carcinoma cells express high levels of endogenous ST6Gal-I as a secondary consequence of oncogenic *ras* activity (18). This cell line was subsequently stably transduced with shRNA to force down-regulation of ST6Gal-I (19). In the current study, the parental HD3 cell line expressing high endogenous ST6Gal-I (HD3.par) and the shRNA knockdown of ST6Gal-I (HD3.sh) were treated with either the ligand for DR4 and DR5, TRAIL, or the Fas-activating antibody, CH11. Levels of apoptosis were then evaluated by immunoblotting for the apoptotic marker, cleaved caspase 3. As shown in Fig. 1*A*, when treated with CH11, HD3.sh cells exhibited a markedly greater degree of cleaved caspase 3 than HD3.par cells, suggesting that low levels of ST6Gal-I-mediated sialylation were associated with enhanced Fas-mediated apoptosis. In contrast, equally high levels of activated caspase 3 were observed in cells treated with TRAIL. Thus, variant levels of ST6Gal-I sialylation appeared to modulate apoptosis induced by Fas receptors, but not DR4 or DR5. To ensure that ST6Gal-I knockdown did not alter the expression of death receptors, Western blots of Fas, DR4, and DR5 were performed, which showed equivalent levels in the HD3.par and HD3.sh cells (Fig. 1*B*).

To further investigate the selective protection by ST6Gal-I against Fas-mediated apoptosis, we again treated the cells with TRAIL or CH11 and monitored apoptosis through immunofluorescence staining with the FLICA reagent, which binds to activated caspases 3 and 7 (cells were counterstained with Hoechst to reveal cell nuclei). HD3.par cells treated with CH11 displayed a small apparent increase in FLICA staining relative to untreated HD3.par cells, however extensive FLICA staining was observed in the CH11-treated HD3.sh cells (Fig. 2*A*). In addition, CH11-treated HD3.sh cells exhibited condensed nuclei, and a disruption in the integrity of cell colonies, both indicative of apoptotic activity (Fig. 2*B*). In contrast to the effects of CH11, treatment with TRAIL induced very strong caspase activation in both the HD3.par and HD3.sh cells (Fig. 2*C*). Analysis of the percentage of apoptosis can be seen in Fig. 2*D*. These data suggest that sialylation by ST6Gal-I protects HD3 cells from apoptosis induced by the Fas receptor, but not DR4/DR5, and importantly, that ST6Gal-I knockdown can sensitize cells to Fas-mediated cell death.

α2–6 Sialylation Confers Protection against Ligand-induced Fas-mediated Apoptosis in Two Cell Models—Although the CH11 antibody is commonly used to stimulate Fas-mediated apoptosis, it was important to examine the effects of ST6Gal-I on apoptosis induced by the biologic ligand for the Fas receptor, FasL. HD3.par, HD3.sh, and additionally, an empty vector control cell line (HD3.ev) were treated with either CH11 or FasL and immunoblotted for cleaved caspase 3. As shown in Fig. 3*A*, HD3.sh cells displayed higher levels of cleaved caspase 3 in response to both FasL and CH11 as compared with HD3.par and HD3.ev cells, confirming that diminished α2–6 sialylation renders cells more susceptible to two independent Fas activators.

Having shown that ST6Gal-I knockdown enhances Fas-mediated apoptosis, we next evaluated whether overexpression of the enzyme in cells with low endogenous ST6Gal-I would inhibit Fas-induced apoptosis. To this end we monitored cell death in the SW48 colon epithelial cell model, which lacks any detectable expression of ST6Gal-I (21). Previously we generated an SW48 cell line that stably expresses ST6Gal-I and verified protein expression and enzyme activity (20). SW48 parental cells (SW.par), which have no ST6Gal-I, SW48 cells with forced expression of ST6Gal-I (SW.ST6), and an SW48 cell line transduced with an empty vector lentivirus (SW.ev) were treated with CH11 or FasL and surveyed for apoptosis (Fig. 3*B*). These experiments revealed substantially lower levels of activated caspase 3 in SW.ST6 cells compared with SW.par and

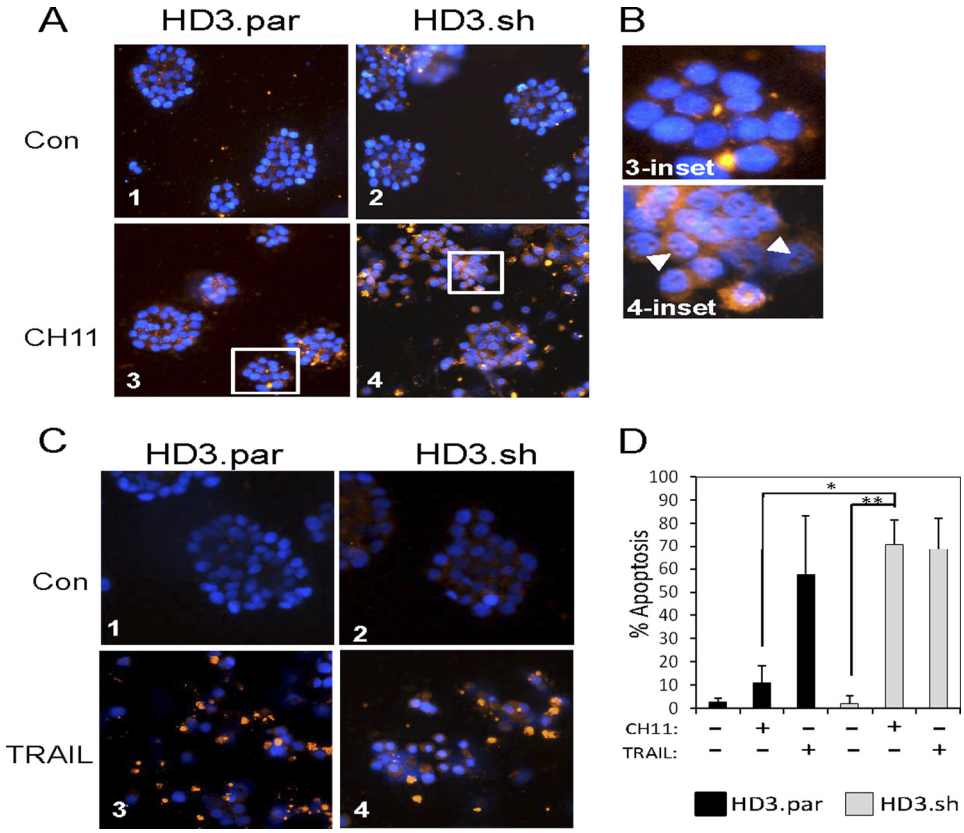

FIGURE 2. **FLICA staining of cleaved caspases 3 and 7 demonstrates that ST6Gal-I protects cells from Fas- but not DR4/DR5-mediated apoptosis.** *A*, HD3.par and HD3.sh cells were stained with FLICA in the absence of CH11 treatment (*panels 1* and *2*), which revealed negligible activation of caspases 3 and 7 in both cell lines. Upon treatment with CH11, there is more activated caspase 3 and 7 staining in HD3.sh (*panel 4*) as compared with HD3.par (*panel 3*). There are also changes in colony and individual cell morphology, indicating apoptosis. Additionally, as can be seen in *B*, there is nuclear condensation verifying apoptosis (*white arrows*) in the HD3.sh after treatment with CH11. *C*, to induce DR4/DR5 signaling, cells were treated with TRAIL. Minimal FLICA staining was observed in the absence of treatment (*panels 1* and *2*), whereas extensive and comparable amounts of cleaved caspases 3 and 7 were observed in HD3.par (*panel 3*) and HD3.sh (*panel 4*) cells, indicating that ST6Gal-I activity does not protect against DR4/5-directed apoptosis (*orange*, FLICA staining of cleaved caspases 3 and 7; *blue*, Hoechst staining for nuclei). *D*, percentage of apoptosis was quantified by counting FLICA-positive *versus* Hoescht-stained cells from multiple microscopic fields. *, $p < 0.01$; **, $p < 0.001$.

SW.ev cells. Hence, the combined results in Fig. 3 establish that ST6Gal-I activity confers protection against apoptosis induced by both the Fas-activating antibody, CH11, and also the endogenous ligand, FasL, in two distinct colon carcinoma cell models.

Fas Is a Target for ST6Gal-I α2–6 Sialylation—We next sought to determine if the Fas receptor is a direct target for α2–6 sialylation by ST6Gal-I, given that this receptor is known to have two possible *N*-linked glycosylation sites (22, 23). To address this, cell lysates were incubated with agarose-conjugated SNA, a lectin that binds specifically to α2–6-linked sialic acids. Samples were centrifuged to selectively precipitate α2–6 sialylated proteins, and α2–6 sialylated proteins were then immunoblotted for the Fas receptor. As depicted in Fig. 4*A*, the band representing Fas in the SNA precipitates from HD3.par cells is denser than that noted in precipitates from HD3.sh cells, indicating more sialylated Fas in the presence of high ST6Gal-I expression. However, no differences were observed in the amount of Fas present in whole cell lysates (representing total Fas protein), indicating that variant ST6Gal-I expression alters Fas sialylation but not Fas protein expression. Also, MAA lectin precipitation (specific for α2–3 sialic acids) revealed that, despite a decrease in α2–6 sialylation, there was no change in α2–3 sialylation of the Fas receptor (Fig. 4*A*). SNA precipitation analyses performed with SW48 cells yielded similar results; Fas was found to be heavily sialylated in SW48 cells with forced expression of ST6Gal-I (SW.ST6 cells), whereas the Fas receptor expressed by parental and empty-vector SW48 cells lacked α2–6 sialylation, as can be seen in Fig. 4*B*. (MAA precipitation of SW48 cell lysate was not performed as these cells have no α2–3 sialyltransferases (21).) As with HD3 cells, variant expression of ST6Gal-I did not alter total Fas protein levels.

To further confirm Fas as an ST6Gal-I substrate, whole cell lysates from HD3.ev and HD3.sh cells were resolved using two-dimensional electrophoresis to separate proteins by both molecular weight and isoelectric point, and then immunoblotting was performed to detect Fas isoforms (Fig. 4*C*). Several Fas isoforms were revealed by this assay; however, a higher molecular weight, more negatively charged band was missing from the HD3.sh lysates. These results are consistent with the loss of a sialylated Fas isoform in cells with ST6Gal-I knockdown. We also used flow cytometry to show that variant Fas sialylation did not change the levels of cell surface Fas expression (Fig. 4*D*).

α2–6 Sialylation of Fas Inhibits DISC Formation—One possible explanation for the decrease in apoptotic signaling from sialylated Fas receptors was that sialic acids might sterically block the binding of Fas activators to the ligand-binding domain. We therefore examined the binding of CH11 antibody to the Fas receptor using flow cytometry. As shown in Fig. 5*A*,

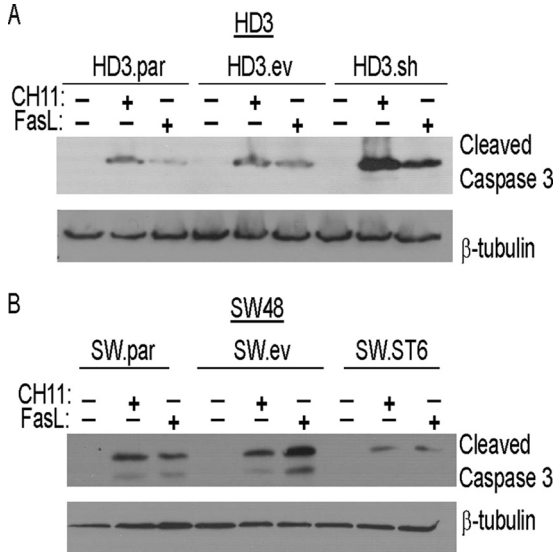

FIGURE 3. **ST6Gal-I protects two distinct cell lines against CH11- and FasL-induced Fas-mediated apoptosis.** *A*, knockdown of ST6Gal-I increases Fas-mediated apoptosis. HD3 cells were treated with either CH11 or FasL. Cell lysates were resolved by SDS-PAGE and immunoblotted for cleaved caspase 3. The levels of cleaved caspase 3 are higher in the HD3.sh cell lysate lanes upon treatment with CH11 and FasL. *B*, forced expression of ST6Gal-I protects cells against Fas-mediated apoptosis. SW48 cells were treated with either CH11 or FasL. The lysates were resolved by SDS-PAGE and immunoblotted for cleaved caspase 3. Levels of cleaved caspase 3 are higher in the *SW.par* and *SW.ev lanes* upon Fas activation, indicating that the presence of ST6Gal-I protects cells from Fas-mediated apoptosis in both cell lines (HD3 and SW48).

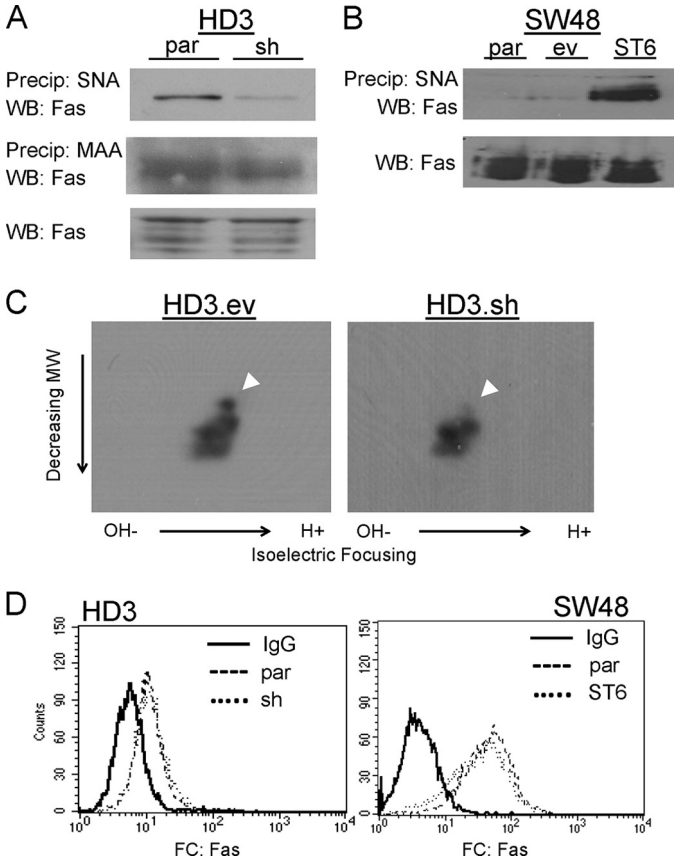

FIGURE 4. **Fas is a substrate for ST6Gal-I sialylation.** *A*, knockdown of ST6Gal-I reduces the amount of α2–6, but not α2–3, sialylation on Fas. HD3 cell lysates were incubated with either SNA lectin (specific for α2–6 sialic acids) or MAA lectin (specific for α2–3 sialic acids) each conjugated to agarose beads. Sialylated proteins were precipitated by centrifugation, and, after extensive washing, precipitates were resolved by SDS-PAGE, and immunoblotted for Fas. As shown, α2–6 sialylated Fas is present at a much higher level in the HD3.par as compared with the HD3.sh cells, whereas levels of α2–3 sialylation were very similar in the two cell lines. Whole cell lysates (*i.e.* not subjected to lectin precipitation) were also immunoblotted for Fas to reveal total levels of Fas protein. *B*, forced expression of ST6Gal-I induces α2–6 sialylation of Fas. SW48 cells were subjected to SNA precipitation as described in *A*. Whole cell lysates were also immunoblotted to detect total Fas levels (MAA precipitation was not performed with SW48 cells due to the known lack of endogenous sialyltransferases in this cell line.) In *A* and *B*, the levels of α2–6 sialylated Fas present are correlated with ST6Gal-I expression, suggesting Fas to be a target for ST6Gal-I sialylation. *C*, loss of a higher molecular weight, more acidic Fas isoform in cells with ST6Gal-I knockdown. Whole cell lysates were subjected to resolution by two-dimensional SDS-PAGE. Proteins were transferred to PVDF membranes and immunoblotted for Fas. The more acidic, higher molecular weight isoform of Fas is not present upon the knockdown of ST6Gal-I (HD3.sh). *D*, surface expression of Fas is not altered by α2–6 sialylation. Cells were stained with FITC-conjugated anti-Human Fas or FITC-conjugated isotype IgG control and characterized by flow cytometry. Surface expression of Fas is not altered as a consequence of differential ST6Gal-I-mediated sialylation.

there was substantial, and equivalent, binding of CH11 to Fas expressed by the HD3.par and HD3.sh cell lines. To further investigate Fas-activator binding, we examined CH11 binding at a range of concentrations from 1 to 40 μg/ml. HD3.par and HD3.sh cell lines showed comparable mean fluorescent intensity at every examined concentration (Fig. 5*B*). Thus, the strong inhibitory effect of α2–6 sialylation on Fas-dependent apoptosis cannot be attributed to diminished binding of the Fas-activating antibody. These data point to a role for sialylation in modulating some aspect of Fas receptor activation rather than ligand binding.

To examine the effect of ST6Gal-I sialylation on Fas signaling, HD3 cells were treated with CH11, and whole cell lysates were immunoblotted for cleaved caspase 8. Caspase 8, an initiator caspase, was evaluated because of its early recruitment to the DISC after Fas activation. As shown in Fig. 5*C*, the amount of cleaved caspase 8 was dramatically increased in CH11-treated HD3.sh cells as compared with the HD3.par cells, suggesting that Fas sialylation alters signaling at some step upstream of caspase 8 activation.

FADD is the initial protein recruited to the DISC that binds directly to the cytoplasmic tail of the Fas receptor after activation. Therefore we examined the amount of FADD associated with the Fas receptor tails by using co-immunoprecipitation experiments. HD3 cells were treated with CH11 at 37 °C to activate DISC formation, and then the Fas receptor and the associated DISC complex were immunoprecipitated. As a control, cells were incubated with CH11 antibody at 4 °C, a treatment that does not induce DISC formation. Immunoprecipitates were then blotted for associated FADD (Fig. 5*D*). These experiments showed that a basal amount of FADD was bound to the Fas cytoplasmic tails in control cells, and no apparent increase in Fas/FADD association was observed in the HD3.par cells upon activation by CH11. In marked contrast, CH11 treatment of HD3.sh cells at 37 °C induced a substantial increase in FADD binding to the Fas cytoplasmic tails, indicative of DISC formation. These results suggest that sialylation of Fas somehow alters the accessibility of Fas cytoplasmic domains for binding to FADD and consequently regulates the first step in DISC formation.

α2–6 Sialylation Inhibits Fas Receptor Internalization—The internalization of Fas after receptor activation is necessary for

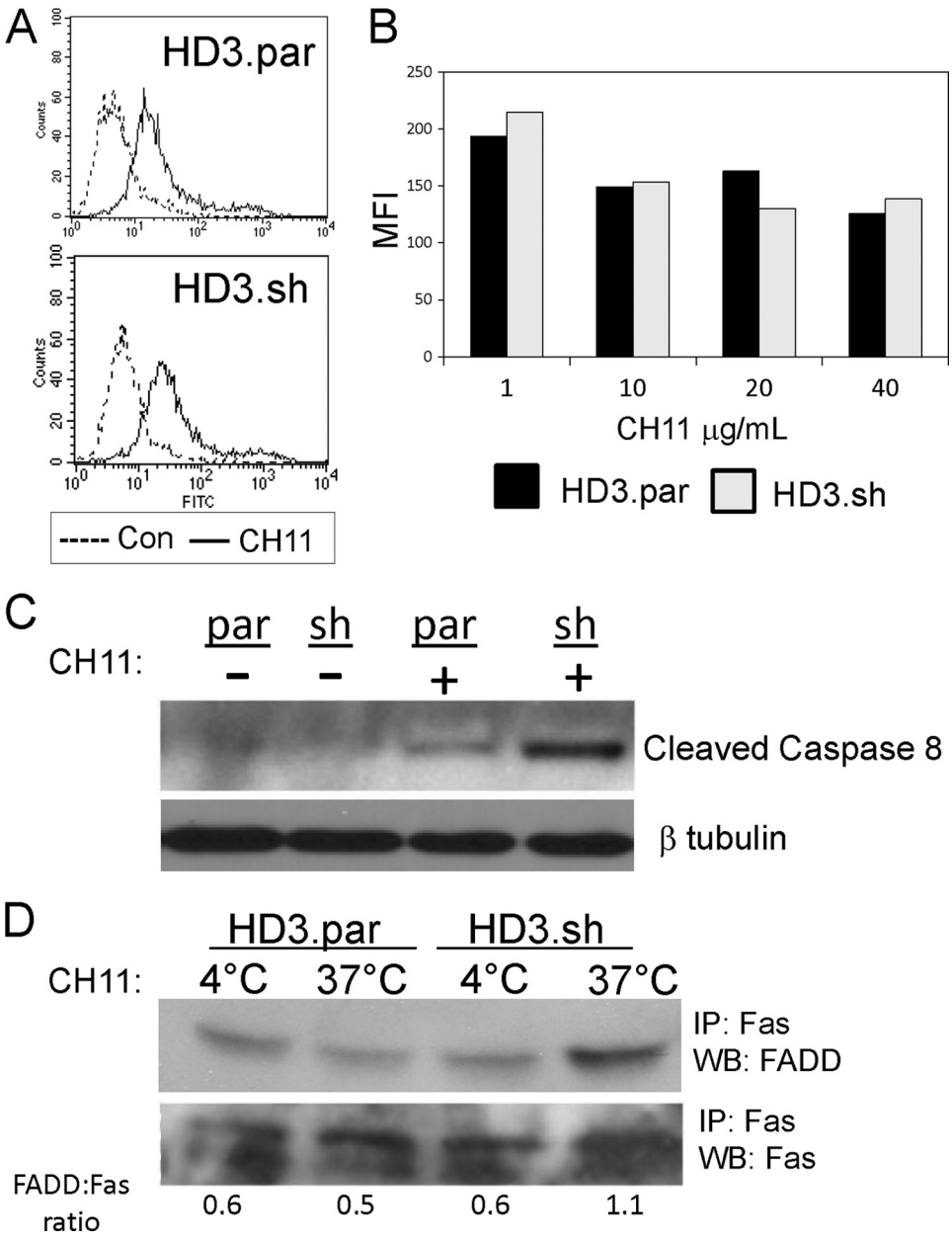

FIGURE 5. **α2–6 sialylation of Fas inhibits DISC formation.** *A*, Fas-activating antibody binds equally well to sialylated and unsialylated Fas receptor. HD3.par and HD3.sh cells were treated with CH11, and antibody binding was measured by flow cytometry (cells were treated at 4 °C to prevent Fas activation). The binding levels for both cell lines are very similar. *B*, CH11 binding curve. Flow cytometric analyses of cells treated with CH11 at 1, 10, 20, or 40 μg/ml revealed comparable mean fluorescent intensities (MFI) at all concentrations. Values for MFI were normalized to the IgM control. *C*, caspase 8 cleavage is affected by variant Fas sialylation. Cells were treated with CH11, and lysates were resolved by SDS-PAGE and immunoblotted for cleaved caspase 8. The level of cleaved caspase 8 is higher in the HD3.sh as compared with HD3.par upon treatment with CH11, indicating enhanced cleavage of caspase 8 when ST6Gal-I is down-regulated. *D*, DISC formation enhanced by knockdown of ST6Gal-I. DISC components were isolated by treating cells at 4 °C (control) or 37 °C for 30 min with CH11 and precipitating through anti-IgM-conjugated agarose beads. Proteins were resolved by SDS-PAGE, and immunoblotted for the Fas-binding protein, FADD. As shown, there is more FADD in HD3.sh after Fas activation with CH11 at 37 °C, indicating more DISC formation in ST6Gal-I deficient cells. The PVDF membrane was stripped and reprobed for the presence of Fas to verify equal immunoprecipitation. Densitometry was used to quantify the FADD and Fas bands, and the FADD:Fas ratio was calculated. Values shown represent the averages from two independent experiments.

Fas-mediated apoptotic signaling (4). Therefore, to further characterize the effects of receptor sialylation, we examined Fas internalization after treatment with CH11. We treated cells with CH11 at either 4 °C (as a control) or 37 °C (to allow for signaling and internalization), fixed the cells, and then used anti-mouse IgM Alexa-fluor 488 (Invitrogen) to visualize the Fas receptor remaining on the cell surface of non-permeabilized cells. As shown in Fig. 6, no major differences were noted in surface Fas levels upon CH11 treatment at 4 °C; however, upon receptor activation at 37 °C, substantially more Fas was internalized in HD3.sh cells. These data suggest that α2–6 sialylation of Fas inhibits receptor internalization, thus limiting Fas-dependent apoptosis.

DISCUSSION

There are multiple sialyltransferases that add sialic acid in an α2–3 linkage to *N*-glycans, however ST6Gal-I is the predominant enzyme that elaborates the α2–6 linkage of sialic acid to

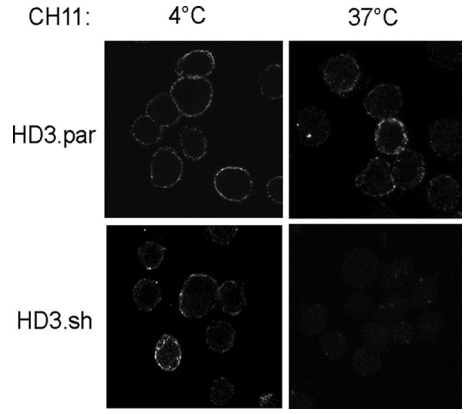

FIGURE 6. **α2–6 sialylation of Fas decreases Fas internalization.** Cells were suspended and treated at 4 °C (control) or 37 °C for 30 min with 1 μg/ml CH11. Cells were then fixed, and secondary anti-mouse IgM Alexa-fluor 488 (Invitrogen) was used to visualize the remaining Fas receptors on the surface of non-permeabilized cells. As can be seen in the HD3.sh cells (*bottom panels*), there is extensive internalization of the Fas receptor at 37 °C, which is not seen in the HD3.par cells (*top panels*).

N-glycosylated proteins (24, 25). Another α2–6 sialyltransferase, ST6Gal-II, has been identified, however this enzyme is localized primarily to the brain and preferentially sialylates oligosaccharides rather than glycoproteins (26, 27). There are also several ST6GalNAc enzymes that add α2–6 sialic acid to the GalNAc residue of *O*-linked glycans or gangliosides (reviewed in Ref. 28). The ST6Gal-I enzyme is overexpressed in at least 13 different types of cancers, including colon, breast, esophageal, oral, ovarian, cervical, leukemias, and brain tumors (10–15, 29–31), and high ST6Gal-I levels are associated with metastasis and poor patient prognosis (15–17). The functional contribution of ST6Gal-I to tumor progression has not been widely investigated, however increased α2–6 sialylation has been linked to enhanced tumor cell migration and invasion (12, 18–20, 32). We and others have reported that ST6Gal-I expression is increased by oncogenic *ras* (reviewed in Ref. 33), which is found in over 30% of human cancers, and Piller's group showed this up-regulation takes place through the ralGEF pathway (34). Studies of ST6Gal-I in colon carcinoma have revealed that 90% of colon tumors screened had up-regulated ST6Gal-I expression (11) and 70% of colorectal cancers were positive for the α2–6 sialic acid modification added by ST6Gal-I (35). Animal studies also support a role for ST6Gal-I up-regulation in tumor progression. Human (16) and murine (36) cancer cells with high levels of α2–6 sialylation were more metastatic to liver following splenic injection in nude mice and enzymatic removal of sialylation from tumor cells prior to injection inhibited metastasis. Furthermore, Varki's group reported that ST6Gal-I-null mice bred to a spontaneous breast cancer model displayed tumors that were more differentiated than tumors from wild-type mice, suggesting that ST6Gal-I activity contributes to the poorly differentiated phenotype of more advanced cancers (37).

Despite these compelling results, there is still a limited understanding of the mechanism by which ST6Gal-I-directed sialylation regulates tumor cell behavior, because of the sparse knowledge of ST6Gal-I substrates, as well as the lack of information regarding the effects of α2–6 sialylation on the function of specific proteins. The current investigation provides a significant advance toward defining the role of ST6Gal-I in tumor progression by showing that the Fas receptor is α2–6 sialylated by ST6Gal-I. Furthermore, we show that α2–6 sialylation of Fas inhibits Fas signaling through both 1) blocking the binding of FADD to the Fas cytoplasmic tail, which is the first step in DISC formation, and 2) inhibiting internalization of the stimulated Fas receptor, which is necessary for Fas apoptotic signaling (4). Sialylation can alter receptor function through several mechanisms, including conformational alteration (38), clustering (39), and differential internalization rate, depending on the specific receptor. Consistent with our work, the CD45 and PECAM receptors have been shown to be targets for α2–6 sialylation by ST6Gal-I, and this sialylation affects internalization in both cases (39–41).

The role of α2–6 sialylation in tumor progression has most often been associated with effects on tumor cell migration and invasion, however it is emerging that ST6Gal-I may be a major regulator of tumor cell survival. This sialylation-dependent survival benefit is likely mediated through multiple molecular pathways. Studies by our group and others have shown that ST6Gal-I-directed α2–6 sialylation of selected receptors serves as a key negative regulator of galectin-induced apoptosis (39, 42, 43). Additionally, the diminished internalization of PECAM due to α2–6 sialylation allows anti-apoptotic signaling from PECAM for a longer time interval (40). It has also been determined that ST6Gal-I levels are up-regulated after radiation treatment in mice (44), and these higher levels confer protection against radiation-induced apoptosis. Our novel finding that α2–6 sialylation inhibits Fas signaling adds to the growing body of literature suggesting that ST6Gal-I modifies a select group of substrates to regulate a multiplicity of apoptotic signaling cascades, thus providing a strong selective advantage for tumor cells.

Changes in expression of the Fas receptor and associated signaling molecules have long been regarded as a mechanism by which tumors evade apoptosis. Several types of cancers are known to down-regulate the Fas receptor as a protective measure, including colon (45), testicular (46), and hepatoma (47). In addition, increased FasL expression on the tumor cell surface has been reported in several cancers (8) and is thought to play a role in tumor evasion from the immune system. Expression levels of downstream effectors in the Fas-induced apoptotic cascade are also altered in tumor tissue, providing additional mechanisms for protection against Fas-mediated apoptosis. Alterations include up-regulation of the anti-apoptotic Bcl and c-FLIP and down-regulation of pro-apoptotic Bax (5–7). Interestingly, Fas is highly expressed on the surface of many tumor types that are not susceptible to Fas-mediated apoptosis (48–51). Results presented herein implicate α2–6 sialylation as a newly identified mechanism by which tumor cells disable Fas signaling. A role for sialylation in regulating the Fas receptor was previously suggested by studies in lymphocytes (52, 53). Keppler *et al.* reported that highly sialylated subclones of the BJA-B B lymphoma cell line were less susceptible to Fas-mediated apoptosis, although α2–3 and α2–6 sialic acids were not distinguished in this study (52). Consistent with these results, Peter *et al.* determined that enzymatic removal of all cell sialic

acids (*i.e.* both α2–3 and α2–6 sialylation) increased the vulnerability of B and T cells to Fas-mediated apoptosis (53). Although these prior studies clearly linked sialylation to Fas activity, they provided limited information regarding the mechanism given that there are more than 20 different sialyltransferases. In the current study we show that Fas activity is regulated through α2–6 sialylation by a distinct sialyltransferase, ST6Gal-I, and of equal importance, this type of sialic acid modification has physiologic relevance given the known up-regulation of ST6Gal-I in cancer. Indeed we previously reported that another ST6Gal-I substrate, the β1 integrin, expresses elevated α2–6 sialylation in 100% of human colon tumors (20). It is also noteworthy that the total levels of sialylation on the two cell model systems evaluated in this study are quite different; HD3 cells express α2–3 sialyltransferases (19), whereas SW48 cells express no endogenous sialyltransferases (21). Thus, ST6Gal-I-mediated sialylation of Fas blocks Fas signaling in cells with both extensive α2–3 cell surface sialylation, including α2–3 sialylation of the Fas receptor (HD3), and cells with no surface sialylation other than that directed by exogenously expressed ST6Gal-I (SW48). These results imply a unique functionality imparted by the α2–6 sialic acid modification added by ST6Gal-I. Further highlighting the specificity of this novel molecular pathway, ST6Gal-I activity appears to have no effect on apoptotic signaling by the DR4 or DR5 death receptors. Intriguingly, work by Ashkenazi's group has shown *O*-glycosylation of the DR5 receptor regulates sensitivity to the DR5 ligand, TRAIL, but does not affect signaling by Fas (54). Moreover, the sites for *O*-glycosylation on DR5 are not conserved in the Fas receptor, and correspondingly, the DR5 receptor does not contain consensus sequences for *N*-glycosylation. A fundamental concept highlighted by these observations is that there is specificity in the effects of certain glycan structures on the function of distinct death receptors.

In conclusion, the current study is the first to demonstrate that a specific sialic acid modification, elaborated by an enzyme known to be up-regulated in cancer, inactivates signaling through the Fas receptor. These results have several important translational implications, including the potential for α2–6 sialylation to serve as a biomarker for Fas insensitivity. In addition, the finding that forced down-regulation of Fas α2–6 sialylation sensitizes tumor cells to apoptosis, even in cells expressing the powerful *ras* oncogene, suggests that ST6Gal-I may be a promising therapeutic target. Finally, ST6Gal-I-dependent regulation of Fas suggests a new paradigm in death receptor signaling, and it more broadly highlights an emerging role for ST6Gal-I as a critical mediator of multiple cell survival pathways.

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Regulation of the metastatic cell phenotype by sialylated glycans

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Abstract Tumor cells exhibit striking changes in cell surface glycosylation as a consequence of dysregulated glycosyltransferases and glycosidases. In particular, an increase in the expression of certain sialylated glycans is a prominent feature of many transformed cells. Altered sialylation has long been associated with metastatic cell behaviors including invasion and enhanced cell survival; however, there is limited information regarding the molecular details of how distinct sialylated structures or sialylated carrier proteins regulate cell signaling to control responses such as adhesion/migration or resistance to specific apoptotic pathways. The goal of this review is to highlight selected examples of sialylated glycans for which there is some knowledge of molecular mechanisms linking aberrant sialylation to critical processes involved in metastasis.

Keywords Sialylation · Metastatic cell phenotype · Sialylated glycoproteins · Invasion

1 Introduction

It has been known for decades that glycoconjugates play an important role in cancer development and progression. An alteration in the profile of cell surface glycans was one of the earliest-identified hallmarks of a tumor cell, and many of the anti-tumor antibodies produced by patients are specific for carbohydrate antigens [1–4]. Cancer-associated glycoconjugates in serum and tissue have been used as important biomarkers for disease progression [5–7]. Notably, the changes in glycan structure following tumorigenic transformation are not random. There is a specific subset of oligosaccharides that becomes enriched on the tumor cell surface, implicating a functional contribution to the tumor phenotype, and many of the glycosyltransferases that synthesize these oligosaccharides are upregulated in response to oncogenes such as Ras [3, 8].

Despite these long-standing observations, our understanding of the molecular mechanisms linking altered glycosylation to tumor cell behavior has lagged behind most other areas of cancer research. This is unfortunate in that this dearth of knowledge has left largely unexplored an important category of potential biomarkers or targets for drug discovery and vaccine development. So why has the field of cancer glycobiology progressed so slowly? While many factors are likely involved, one of the challenges encountered is that, unlike the template-driven synthesis of oligonucleotides and proteins, the synthesis of glycans elaborating cell surface molecules is complex and not readily predictable. Technologies for defining glycoconjugate structure are still evolving, and there are limited methods that can be used to determine the position of an oligosaccharide within the three-dimensional structure of a glycan carrier. For example, X-ray crystallography is difficult to perform with glycosylated proteins, therefore the glycans are typically enzymatically removed prior to crystallization. As a consequence, current literature describing conformational analyses of proteins, or identification of protein–protein interaction domains, often excludes information regarding how glycans might alter protein conformational features or peptide-binding interfaces. It is generally assumed that glycans extend into the extracellular space with high mobility (and many do), however there is evidence that at least some glycans are relatively fixed within the larger

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glycoprotein tertiary structure [9, 10], and glycans can also form direct bonds with primary amino acid sequence [11, 12]. Another important factor not always appreciated is that some monosaccharides, such as sialic acid, are negatively charged at physiologic pH, thus the addition of such a sugar (comparable to a phosphate group) has potential to alter protein conformation and/or oligomerization. As well, sialylation is emerging as a major regulator of cell surface retention of various receptors.

In addition to modulating the conformation, clustering and/or surface retention of an individual glycoprotein, glycoconjugates are ligands for numerous glycan-binding proteins such as lectins [13, 14]. While studies of protein–protein interactions have predominated in cancer research, there is increasing recognition that associations between tumor glycans and lectins are of great importance in regulating many aspects of tumor cell behavior. Lectins exist as intracellular, cell surface, or secreted molecules, depending upon the species, and many secreted lectins are incorporated into the extracellular matrix. Hence, glycan-lectin binding partners represent another fundamental class of molecular regulators of cell–cell and cell–matrix interactions. Elucidating the biochemical details of these interactions has proven challenging, however mounting evidence points to a high degree of specificity, comparable to the role of distinct amino acid motifs that drive protein–protein interactions. Indeed, glycans have been referred to as the "third alphabet of molecular biology" (the other two being proteins and nucleic acids) [15]. This capacity to control cell–cell and cell–matrix interactions, in combination with the known effects of glycans on the structure/function of individual glycoproteins, underlies the presumed role of glycans in most metastatic cell behaviors including migration/invasion through matrix, dissemination through the vasculature or lymphatics, evasion from immune surveillance, and resistance to apoptosis.

Given the multiplicity of carbohydrate modifications associated with human cancer, this review will have a restricted focus on one type of glycan modification, protein sialylation. Experimental results gleaned from patient tissue samples, animal cancer models, and cell culture studies, suggest that altered sialylation is a major contributor to the metastatic cell phenotype [16, 17]. The term "sialic acid" refers to a group of approximately 50 different chemical derivatives of neuraminic acid, with the most common variant represented by *N*-acetylneuraminic acid (Neu5Ac, Fig. 1a) [18]. Sialic acids are added onto the termini of either *N*- or *O*-linked glycans of glycoproteins and can also be added onto glycolipids (Fig. 1b). The overall level of cell surface sialylation is regulated by numerous enzymes including: (1) enzymes that control synthesis and availability of the activated sialic acid substrate, CMP-sialic acid, (2) the sialyltransferase family, which adds sialic acid during glycoprotein biosynthesis, and (3) the sialidase (also called neuraminidase) family, which cleaves sialic acid during glycoprotein degradation. These enzymes typically reside within subcellular compartments, with most sialyltransferases localized to the Golgi, and many of the sialidases localized to lysosomes or endosomes. Aberrant activity of both sialyltransferases and sialidases has been observed in cancer; however, the literature overwhelmingly suggests that sialylation levels are higher on tumor cells [3, 19]. Elevated sialylation is thought to be a relatively static tumor cell characteristic, given that sialic acid is added during glycoprotein biosynthesis, but recent studies indicate that some enzymes involved in sialylation can be expressed on the cell surface, or secreted as active soluble enzymes into the extracellular milieu [20–23]. While not a focus of this review, this opens up the fascinating possibility that the sialylation of certain glycoproteins may be dynamically regulated at the cell surface, providing a unique mechanism for transient control of individual glycoprotein structure or glycan/lectin interactions. One of the major barriers in our understanding of tumor cell sialylation is that much of the prior research was directed at correlating total surface sialylation levels with cell responses, with limited regard for the specific type of sialic acid chemical structure or linkage, or the specific glycoprotein carrier of the sialic acid. This lack of mechanistic knowledge has hindered investigative pursuit

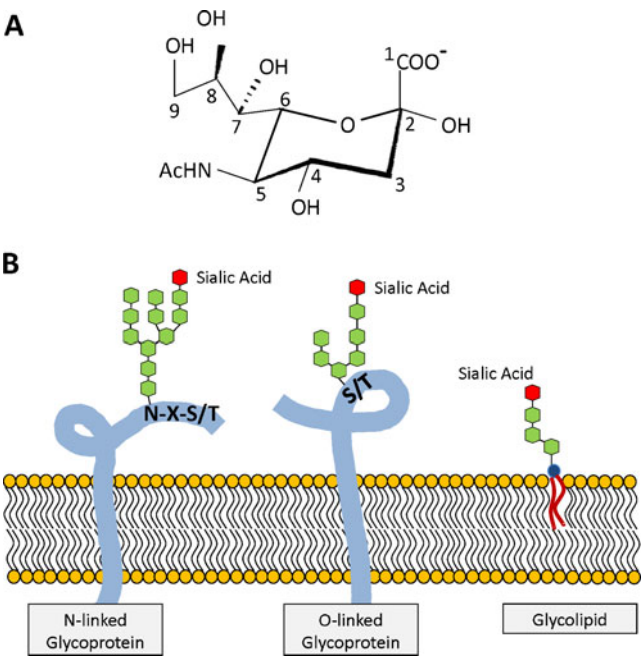

Fig. 1 Types of glycosylation. **a** Structure of the most common sialic acid, Neu5Ac. The negative charge is from the carboxylic acid group on carbon 1. **b** *N*-linked glycans (*left*) are attached to asparagine (N) residues on selected proteins containing the N-X-S/T consensus sequence, while *O*-linked glycans (*center*) are linked to serine (S) or threonine (T) residues. Glycolipids (*right*) are lipids which carry glycan structures

of glycans as clinical targets. The goal of this report is to highlight some of the more prevalent tumor-associated changes in specific types of sialylation, and discuss potential molecular mechanisms by which these modifications influence metastatic progression.

2 Regulation of tumor cell surface sialylation

An upregulation in the expression of selected sialyltransferases is a common event in tumorigenic transformation [16]. Sialyltransferases comprise a family of at least 20 different enzymes that differ in tissue distribution as well as the type of sialic acid linkage elaborated [16, 24]. Some sialyltransferases add sialic acid in an α2-3 linkage to galactose (Gal); whereas others add sialic acid in an α2-6 linkage to either Gal (e.g., the ST6Gal-I and ST6Gal-II sialyltransferases) or *N*-acetylgalactosamine (GalNAc, added by multiple ST6GalNAc sialyltransferases) (Fig. 2). The third type of sialic acid linkage is directed by the polysialyltransferase family, which adds an α2-8 linked sialic acid onto another sialic acid. Cancer-associated

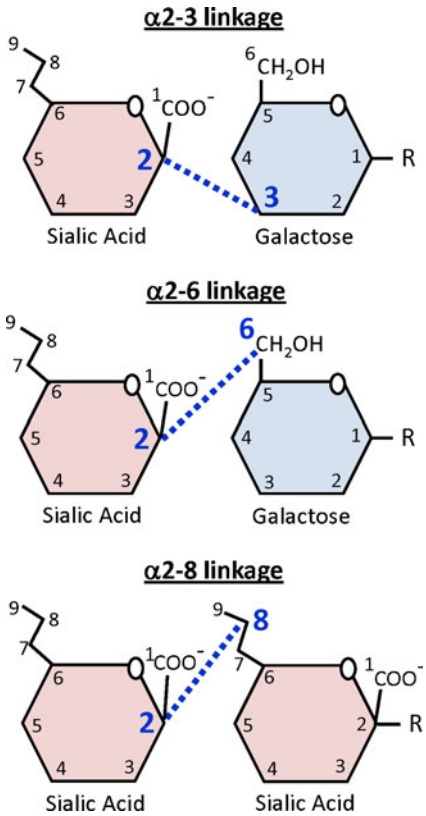

Fig. 2 Sialic acid linkages. Sialic acids are added to the termini of glycans in an α2-3, α2-6, or α2-8 linkage. In the *top two panels*, sialic acid linkage to galactose is depicted, however other sugars, such as GalNAc, can be modified with sialic acid, depending upon the type of linkage. Note that the structures shown in the figure have been simplified (e.g., hydroxyl and acetyl groups are not represented)

dysregulation has been observed for selected members of all three of these sialyltransferase categories. In conjunction with aberrant sialyltransferase expression, certain sialidases are also disrupted in human cancer, although far less is known about the tumorigenic role of this enzyme family. One example is the Neu1 sialidase, which is downregulated in cancer cells, leading to higher levels of cell surface sialylation (due to diminished sialic acid cleavage) [25].

An increase in α2-6-linked sialylation is frequently observed in tumor cells, and is usually attributed to an upregulation in either the ST6Gal-I sialyltransferase [16, 17, 26–28], which primarily sialylates *N*-linked glycans, or members of the ST6GalNAc family, which sialylate either *O*-linked glycans or glycolipids [24, 29]. The selective enrichment of α2-6 sialic acids on tumor cells is significant in that α2-6 sialylation can elicit very distinct biologic outcomes as compared with α2-3 sialylation. One striking example is the effect of α2-6 sialylation on galectin-dependent cell behaviors. Galectins are lectins that bind galactose-containing oligosaccharides [30–32]. Depending upon the galectin species, galectins can be expressed either intracellularly or extracellularly; in the latter case, some galectins are found associated with the extracellular matrix [33–35]. Extensive evidence suggests that α2-6 sialylation of galactose serves as a generic inhibitor of galectin binding [36, 37], unlike α2-3 sialic acids, which have variable effects on binding depending upon the individual galectin (Fig. 3). Accordingly, α2-6 sialylation serves as a key negative regulator of many critical galectin functions. One important activity of cell surface α2-6 sialylation is to block the binding of pro-apoptotic galectins, thereby promoting tumor cell survival [38].

Galectins are not the only glycan-binding proteins influenced by the type of sialylation present on cognate glycoconjugate ligands. Sialic acid linkage, as well as chemical structure (e.g, acetylation), are major determinants in ligand recognition by sialic acid-binding immunoglobulin superfamily lectins (siglecs) [39–41]. Siglecs are mainly expressed by immune cells, and the potential function of siglecs in tumor biology has received minimal attention. One envisions that changes in tumor cell sialylation could affect the activity of siglec-expressing immune cells, and consequently modulate the anti-tumor immune response.

Beyond their pivotal role in regulating interactions with glycan-binding proteins, sialic acids can have direct effects on specific glycoproteins that carry the sialic acid, which is not surprising given the large size and negative charge of this sugar moiety. The effect of sialylation on the structure/function of a given glycoprotein will depend upon the localization of the sialic acid within the larger glycoprotein tertiary structure, which is difficult to determine due to technical challenges. However, it is becoming clear that sialylation can affect glycoprotein activity through many

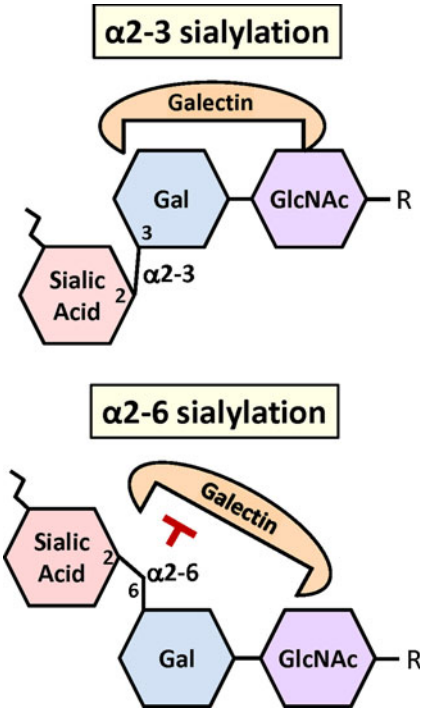

Fig. 3 α2-6 sialylation blocks galectin binding. Galectins require a free hydroxyl group on the 6 carbon of galactose for binding [37], therefore α2-6 sialylation at this site inhibits galectin binding. In contrast, α2-3-linked sialic acids have variable effects on binding, depending upon the specific galectin species

different mechanisms. As examples, sialylation alters: (1) conformation of the β1 integrin [42, 43]; (2) clustering of CD45 [44], EGFR [45], and PECAM [46]; and (3) cell surface retention of PECAM [46] and the Fas death receptor [47]. There is also evidence that sialylation modulates heterotypic associations between two distinct cell surface glycoproteins, as reported for the noncognate interaction between CD8 and MHC class I proteins [48–50]. In addition, many membrane receptors are anchored on the cell surface through a galectin-dependent mechanism that is sensitive to α2-6 sialylation. Extracellular galectins form a multimeric lattice-type structure that binds galactose-containing receptor glycans, and stabilizes glycoprotein surface localization [51–54]. Receptor α2-6 sialylation causes release from the galectin lattice, leading to receptor internalization [20]. Conversely, α2-6 sialylation can facilitate the surface retention of other types of receptors, albeit through pathways that are not generally well-defined. Taken together, the literature indicates that sialylation holds potential to influence tumor cell behavior at many different levels including: regulation of individual glycoprotein conformation, clustering or surface retention, modulation of *cis* or *trans* interactions between two distinct surface receptors, and formation of ligands for glycan-binding proteins that correspondingly control cell–cell and cell–matrix interactions. In the remaining sections of this review, several specific examples of tumor-associated sialoglycans are discussed. These were selected because of the greater knowledge of molecular mechanisms linking these modifications to tumor cell behavior as compared with many of the other prevalent changes in tumor glycosylation.

3 Role of integrin sialylation in tumor cell migration through extracellular matrix

Numerous studies suggest that increased cell surface sialylation contributes to metastasis by stimulating tumor cell movement through the extracellular matrix (ECM). *In vitro* assays performed with many different cancer cell lines indicate a strong positive correlation between migration/invasion and high levels of surface sialylation [55–58]. Likewise, subclones of cell lines selected for enhanced invasiveness often display elevated surface sialylation, including clonal variants from lung [45], colon [59], melanoma [60], and T lymphoma [61] cells. The functional importance of hypersialylation is supported by animal models of metastasis [62, 63]. Intrasplenic injections of two differing populations of 51B colon cells; a heavily α2-6 sialylated population and a poorly sialylated population, resulted in hepatic tumors formed almost exclusively by the highly sialylated cells, indicating selective metastasis [63]. Many other studies have shown that metastasis in murine models can be blocked by pharmacologic inhibitors of sialyltransferase activity [64–66] or sialic acid incorporation [62] or alternately, by pre-treatment of tumor cells with sialidases [63]. Interestingly, certain types of sialic acid linkages may regulate metastatic targeting of selected organs; a breast cancer cell line selected for targeting to bone had higher levels of α2-6-linked sialic acid [67], whereas upregulation of ST6GalNAcV, which adds α2-6 sialic acid to gangliosides, directs breast cancer metastasis to brain [68].

Though the circumstantial evidence linking sialylation to metastasis is extensive, data regarding the specific molecular events driving invasive tumor cell behavior are lacking. The prior experimental use of sialylation inhibitors and sialidases, most of which have low specificity, generally produced widespread ablation of cell surface sialylation, affecting a multitude of glycoproteins and glycolipids. Compounding this issue, such generic approaches are not typically representative of the physiologic changes that occur during metastatic progression, which involve alterations in the expression of specific enzymes. More recent studies applying RNAi technology, or forced overexpression models that better recapitulate the tumor phenotype, are beginning to reveal a more defined view of the role of sialylation in metastasis. Several sialylation-related enzymes have been targeted using this strategy, and such studies are yielding

new insight into how distinct types of sialylation regulate specific receptors to promote tumorigenic cell responses.

Some of the more compelling results implicating a specific sialoprotein in tumor cell migration and invasion have been provided by studies of the integrin family of cell adhesion receptors [69]. Integrin activity is involved in many aspects of tumor metastasis including tumor cell detachment from basement membrane, migration through the stromal matrix, anchorage-independent cell survival in the vasculature, adhesion to endothelium during extravasation, and establishment of metastatic foci in novel ECMs. Glycosylation of integrins has long been known to be required for integrin function [69, 70], and integrins are regulated by several different types of glycan structures [71–73]. Among these, α2-6 sialylation of *N*-glycans is an important modulator of a specific subset of integrins. The β1 integrin subunit (but not β3 or β5 [74]) has been identified as a substrate for the ST6Gal-I sialyltransferase in multiple established cancer cell lines [56, 57, 75, 76]. Furthermore, β1 integrins in human colon cancer tissues display elevated α2-6 sialylation [56], which corresponds to the well-documented overexpression of ST6Gal-I in many different cancers, including colon carcinoma [16, 36]. ST6Gal-I is upregulated in cancer as a consequence of signaling by oncogenic Ras [74, 77–79].

The addition of α2-6-linked sialic acid to the β1 integrin subunit alters the binding activity of several β1-containing heterodimers including receptors for fibronectin (α5β1 [43, 80, 81]), VCAM-1 (α4β1 [42]), laminin (α3β1 [82]), and collagen (α1β1 [74] and α2β1 [75]). Regulation of integrin function by α2-6 sialylation has been confirmed by studies using engineered cell lines, as well as ligand binding assays performed with purified integrin receptors that have been manipulated to express varying levels of sialylation. It has also been shown that, of the ten *N*-linked glycans on the β1 integrin subunit [80], the three *N*-glycans within the β1 I-like domain, a region involved in ligand binding, are essential for heterodimerization of the α and β subunits, and also for ligand-induced cell spreading [83]. These recent results confirm studies performed approximately 20 years ago showing that *N*-glycosylation was indispensible for β1 integrin function [84, 85].

Mechanistic studies of sialylation-related β1 integrin activity are few in number, however experiments using activation-state reporter antibodies suggest that sialylation alters β1 integrin conformation [42, 43], a finding supported by molecular modeling approaches [86]. α2-6 sialylation of collagen-selective integrins increases adhesion to collagen I, enhances coupling of talin to the integrin cytosolic tail, and stimulates cell migration (Fig. 4) [56, 57, 75]. *In vivo* support for sialylation-dependent effects on integrin signaling has been provided by Varki's group, who used the polyomavirus middle T antigen model of spontaneous breast cancer to study ST6Gal-I [87]. Results from this study showed that mammary tumors from ST6Gal-I null mice exhibited a selective alteration in genes associated with focal adhesion signaling, as well as diminished activation of Focal Adhesion Kinase (FAK), a known downstream target of integrin signaling. Tumors from mice lacking ST6Gal-I were also more differentiated, suggesting that the overexpression of ST6Gal-I I that occurs in human carcinoma may contribute to a poorly differentiated tumor phenotype.

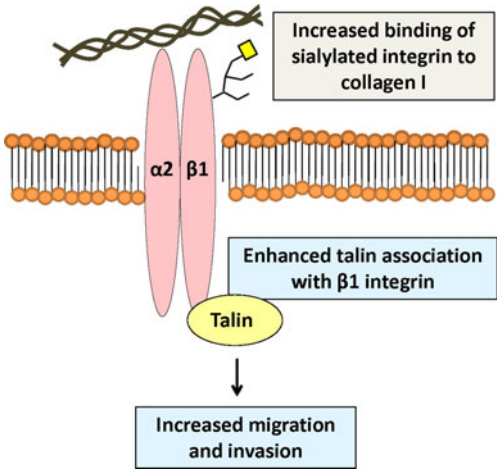

Fig. 4 Regulation of integrins by sialylation. α2-6 sialylation of *N*-linked glycans on the β1 integrin enhances cell adhesion to collagen I and stimulates migration and invasion

ST6Gal-I-directed α2-6 sialylation of the β1 integrin stimulates tumor cell migration and invasion through reconstituted ECM (e.g., Matrigel) [56, 57, 75]. Cells that are null for the β1 integrin do not exhibit differential invasion upon forced ST6Gal-I expression [75], supporting the hypothesis that the effect of upregulated ST6Gal-I is mediated specifically by the β1 integrin. The interaction between integrins and collagen I is thought to be important in metastasis. Microarray studies performed on diverse tumor types identified collagen I as part of a 17-gene signature associated with increased metastasis [88]. The deposition of collagen I in the metastatic microenvironment induces dormant tumor cells to form proliferative metastatic lesions, and this transition is dependent upon β1-integrin signaling [89]. In addition, collagen reorganization at the tumor-stromal interface facilitates local invasion [90] and collagen I fibers provide tracks along which tumor cells migrate during transit to blood vessels [91]. The α2β1 collagen-selective integrin has been suggested as a principal player in metastatic progression; comparative analyses of primary colorectal cancers with corresponding liver and lung metastases suggest that the α2 integrin subunit contributes to liver targeting [92].

Recently a second integrin family member, α6β4 (a laminin-binding receptor), has been reported to be affected by sialylation [25]. In this instance, elevated sialylation of the β4 integrin subunit resulted from decreased expression

of a sialidase rather than increased expression of a sialyltransferase. Downregulation of the Neu1 sialidase, which localizes in part to the plasma membrane [25, 93, 94], was associated with enhanced cell invasiveness and metastatic potential in rat and murine cancer cells [95, 96]. To elucidate the effects of Neu 1 on integrin activity, Neu1 expression was forced in human colon cancer cell lines [25], which led to an accompanying gain in sialylation on the *O*-linked glycans of the β4 subunit. While the effect of sialylation on α6β4 structure has yet to be determined, sialylation clearly influenced α6β4 signaling because the β4 subunit displayed reduced phosphorylation, and α6β4-induced FAK activation was diminished. Importantly, forced Neu1 expression inhibited experimental metastasis to liver [25]. These studies of α6β4, along with β1, point to specific integrin sialoforms (and other glycoforms not discussed herein) as critical mediators of tumor cell migratory and invasive behavior.

4 Sialyl Tn antigen and tumor invasion

The sialyl Thomsen-nouvelle antigen (sialyl Tn) and its unsialylated form, Tn, are well-known tumor-associated carbohydrate antigens, and are highly correlated with cancer invasion and metastasis [97–99]. Sialyl Tn is formed by the sialylation of the Tn antigen: GalNAc linked to serine or threonine (Fig. 5). GalNAc is the first sugar added during *O*-linked glycan synthesis, and this basic unit can be extended to form multiple glycan structures. The sialylation of GalNAc prevents further sugar additions, and effectively truncates the *O*-linked glycan extension [100, 101]. Sialyl Tn is detected in a wide range of cancers including gastric, colorectal, pancreatic, endometrial, breast, and ovarian [102–108], yet sialyl Tn expression is low or absent in normal epithelial cells [109–112]. Sialyl Tn expression is associated with metastatic disease, recurrence, and reduced survival rates in breast cancer [98, 113], and a negative

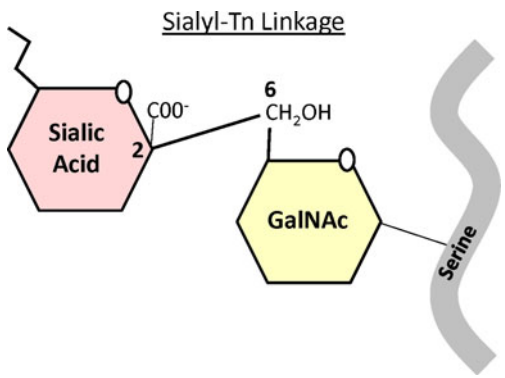

Fig. 5 Sialyl Thomsen-nouvelle antigen. The sialyl Tn antigen is formed by α2-6 sialylation of GalNAc bound to serine or threonine

association between sialyl Tn and survival has been confirmed by many other studies [98, 114–120]; although this is not a universal finding [121, 122]. Approximately 30 % of breast cancers are sialyl Tn positive [123, 124] and over 80 % of all carcinomas express either Tn or sialyl Tn structures [125]. Given that antibodies against sialyl Tn antigen are cancer specific [97, 109], serum sialyl Tn levels are used as a prognostic indicator for cancer aggressiveness and metastatic potential [126]. The relationship between sialyl Tn and cancer progression has been demonstrated experimentally by the forced expression of sialyl Tn structures in cancer cells. Forced sialyl Tn in gastric cancer lines resulted in increased metastasis and decreased survival in nude mice after intraperitoneal injection of tumor cells [127]. This enhanced metastatic capability of sialyl Tn-expressing cells was abrogated by pretreatment with anti-sialyl Tn antibodies. In related studies, various cancer cell lines with forced sialyl Tn expression gained metastatic characteristics such as altered adherence to matrix molecules and increased motility and invasiveness [128–130].

The generation of sialyl Tn is primarily associated with a single sialyltransferase, ST6GalNAc-I, which adds sialic acid in an α2-6 linkage to the Tn antigen [131–133]. Forced expression of ST6GalNAc-I results in sialyl Tn expression in breast and gastric cancer cell lines [127, 129]. Other ST6GalNAc family members may be able to synthesize sialyl Tn; however, while ST6GalNAc-II can create the sialyl Tn structure *in vitro* on peptide-GalNAc substrates [133, 134], to date no other ST6GalNAc has been shown to generate sialyl Tn expression *in vivo*. ST6GalNAc family members have been linked to carcinogenesis: ST6GalNAc-I is upregulated in intestinal metaplasia [132]; ST6GalNAc-II is elevated in colon cancer and is prognostic for patient survival [135], and ST6GalNAc-V is one of four genes upregulated in breast cancer cells with increased metastatic potential to the brain [68]. As an alternate mechanism to ST6GalNAc overexpression, accumulation of sialyl Tn can result from dysregulation of other glycosyltransferases that regulate formation or availability of the Tn substrate. *Cosmc* is a molecular chaperone protein necessary for the activity of T-synthase, an enzyme that adds galactose to GalNAc and therefore competes with ST6GalNAc-I for GalNAc-modified *O*-linked glycan precursors [136, 137]. Cummings' group reported that *Cosmc* disruptions in colon and melanoma cell lines contribute to sialyl Tn expression (due to downregulated T-synthase activity), and further documented two cervical cancer cases with mutations at the *Cosmc* locus, and elevated sialyl Tn expression [125].

Even though there is a strong association between sialyl Tn expression and cancer progression, the specific effects of sialyl Tn on tumor cell behavior remain obscure. Not many studies have been aimed at identifying the carriers of sialyl Tn, or determining the corresponding influence of sialyl Tn

on carrier function. There is also a lack of knowledge regarding physiologically relevant glycan binding proteins that might interact with sialyl Tn in the tumor milieu. However, a few glycoproteins have been determined as carriers of sialyl Tn. CD44 and the mucin, Muc 1, are elaborated with sialyl Tn in breast and gastric cancer cells that have been forced to express ST6GalNAc-I [127, 129]. Mucins are large, densely *O*-glycosylated proteins that act in cell adhesion and signaling [138]. They are upregulated in a variety of cancers and characteristically display truncated *O*-linked glycans as compared with mucins in normal tissue [101, 138, 139]. A general increase in sialylation influences Muc1's role in cell–cell adhesion [140, 141] and elevated levels of Muc 1 with sialyl Tn have been observed in multiple carcinomas [128, 142]. The other sialyl Tn carrier, CD44, is another well-known adhesion protein; this molecule displays a binding specificity for hyaluronan. Certain CD44 splice variants, such as CD44v6, have been associated with breast, lung, colon, and pancreatic carcinomas, among others [143–145]. Two other glycoproteins, β1 integrin [146] and osteopontin [112], are reportedly modified with sialyl Tn in murine cancer cells, although this has not been confirmed in human cells. The β1 integrin was shown to be the primary carrier of sialyl Tn antigen in the non-mucin expressing TS/A murine breast cancer cell line after ST6GalNAc-I forced expression [146]. In this investigation, ST6GalNAc-I expressing cells had reduced mobility and proliferation, suggesting a possible inhibitory effect on the metastatic process. However, in another study which identified Muc1 and CD44 as sialyl Tn carriers in human breast cancer cells, β1 integrin was not found to carry the sialyl Tn antigen [129]. These conflicting reports may be due to differences in mucin expression and competing substrates for ST6GalNAc in the Golgi. Recently, osteopontin was found to carry the sialyl Tn epitope in murine breast cancer cell lines [112]. Osteopontin levels in serum are elevated in a number of human cancers, and increased expression is associated with a negative prognosis [145]. Interestingly, osteopontin serves as a ligand for both CD44 and β1 integrins [145], and it is worth noting that all four proteins identified as sialyl Tn carriers (Muc1, osteopontin, CD44, and β1) are involved in cell adhesion and migration. It is tempting to speculate that the expression of sialyl Tn may subvert the normal function of these proteins to promote an invasive tumor phenotype (Fig. 6).

Sialyl Tn expression may also play an immunologic role in tumor progression. Natural killer cells pre-treated with Muc1 bearing the sialyl Tn antigen, but not Muc1 without sialyl Tn, exhibited diminished capacity for cell-mediated cytotoxicity against K562 leukemia cells [147]. Although mechanistic information is lacking, sialyl Tn structures are capable of binding to siglecs, which are important mediators in immune recognition [148]. The sialyl Tn antigen is the

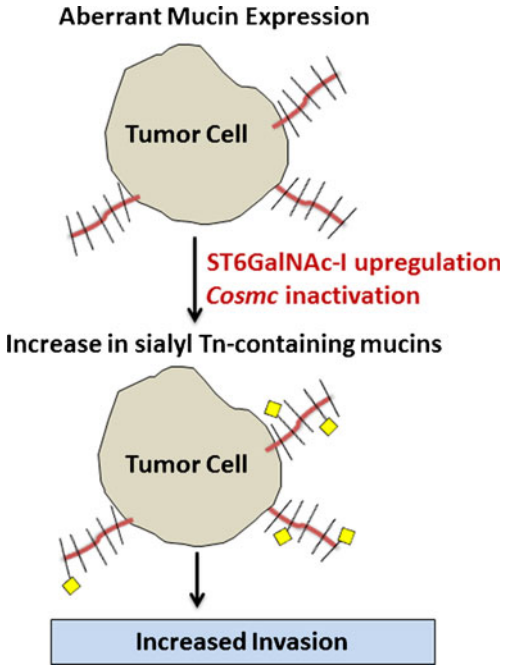

Fig. 6 Sialyl Tn antigen expression correlates to increased invasion. Upregulation of the sialyltransferase, ST6GalNAc-I, or inactivation of the chaperone, *Cosmc*, contributes to an increase in sialyl Tn expression on *O*-linked glycans. Elevated levels of sialyl Tn antigen expressed on tumor cells is correlated to increased invasion; however, the mechanism remains unclear

preferred substrate for siglec 6 [149] while some additional siglecs may bind sialyl Tn along with other sialoglycans [148]. It is possible that altered sialyl Tn expression on tumor cells may shift the immune response in ways that promote tumor development through interactions with different siglecs. As well, sialyl Tn-antibody complexes formed from soluble sialyl Tn and antisera stimulated VEGF release from macrophages and granulocytes, leading to increased tumor angiogenesis and invasion [150, 151]. Enhanced blood vessel formation was similarly observed in SCID mice with subcutaneously injected, sialyl Tn expressing breast cancer cells following the introduction of anti-sialyl Tn antibodies into the systemic circulation. It is still unclear whether this effect was separate from the general immune response to tumor antigens or specific for sialyl Tn.

Theratope® is a cancer vaccine against sialyl Tn conjugated to keyhole limpet hemocyanin, and was initially designed for use in metastatic breast cancer. In a phase II clinical study, patients receiving the vaccine showed a sialyl Tn-specific humoral response and improved overall survival [123]; however, a phase III study concluded that the vaccine did not increase survival in patients with metastatic disease [152]. Independent studies of Theratope in murine models reported detection of sialyl Tn-specific antibodies, and significantly delayed tumor growth [112]. A potential pitfall of

the phase III clinical trial is that the patient population was not evaluated for sialyl Tn expression prior to enrollment, possibly masking any benefit from the vaccine due to heterogeneous sialyl Tn expression between patients. While additional investigation is needed to clarify the discrepant results concerning sialyl Tn, the pursuit of tumor-associated carbohydrate antigens as candidates for vaccine development remains an active area of investigation.

5 Sialyl Lewis structures in tumor dissemination

After entering the systemic circulation, tumor cells must be able to survive within, and then exit, the vasculature in order to metastasize to distant organs. The upregulation of sialyl Lewis (sLe) structures on the tumor cell surface serves as a key mechanism for directing tumor cell adhesion to the endothelium by providing ligands for endothelial selectins [153] (Fig. 7). sLe/selectin interactions also promote the formation of aggregates comprised of tumor cells, platelets and leukocytes, which shields tumor cells from immune attack [154]. sLe glycans are normally present on leukocytes and are critical in leukocyte adhesion and extravasation during an inflammatory response. Conversely, sLe expression is typically low in non-cancerous epithelial cells [19, 155]. sLe structures are tetrasaccharides composed of a GlcNAc-Gal backbone with an α2-3-linked sialic acid attached to Gal, and fucose linked to GlcNAc (Fig. 8). sLe is primarily found on glycolipids or *O*-glycans of glycoproteins [19], and the

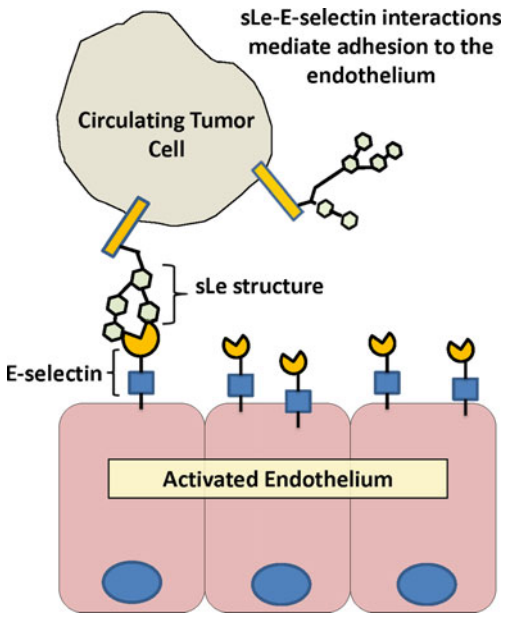

Fig. 7 Sialyl Lewis structures promote tumor dissemination. Sialyl Lewis structures on tumor cell glycoproteins interact with selectins expressed by activated endothelial cells, thereby facilitating tumor cell extravasation

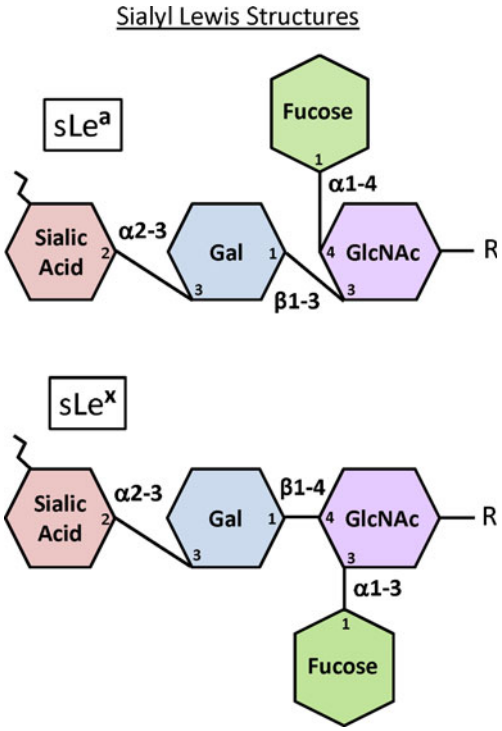

Fig. 8 Sialyl Lewis antigens. Sialyl Lewis (*sLe*) antigens are tetrasaccharide structures composed of a GlcNAc-Gal backbone with fucose linked to GlcNAc and sialic acid α2-3 linked to Gal. sLe^x and sLe^a are different isomers, both of which bind to endothelial selectins

expression of sLe is elevated in many different types of cancer [156–158]. sLe occurs in two isomers, sLe^a and sLe^x (Fig. 8), and cancer cells arising from different organs tend to adhere to endothelium more strongly through one isomer over the other. Colon and pancreatic cancer cells adhere to E-selectin via sLe^a while lung and liver cancer cells adhere through sLe^x [159]. Expression of sLe is increased in patients with metastatic disease and is negatively correlated with patient survival [160–169] although this has been refuted [170]. This association with metastasis is the basis for clinical monitoring of sLe^a structures in cancers of the digestive tract. Screening of serum sLe^a (CA19-9) is part of the standard treatment regimen for colorectal cancer [171–173] and higher preoperative serum CA19-9 levels predict colon cancer recurrence [174]. The CA19-9 antibody is specific for sLe^a and does not recognize the unsialylated Le^a structure [175]. In a cohort of 94 advanced colorectal cancer patients, greater sLe^a expression was positively correlated to hepatic metastasis [176], although this study failed to find a significant association in a similarly sized gastric cancer cohort. A retroactive examination of more than 300 colorectal cancers and their associated metastases found significantly higher sLe^a expression in metastases compared with the primary tumor [171], and a similar finding was reported for breast cancer [162]. In addition to sLe^a,

sLe^x may serve as a prognostic indicator; sLe^x expression predicts outcome in prostate cancer cases after orchiectomy [177] and is also being investigated for use in breast cancer monitoring [178, 179].

A clear clinical correlation continues to prompt investigation of sLe antigens, however the regulation of these structures is far from understood. *O*-linked glycan synthesis requires the coordinated activity of multiple glycosyltransferases, and many of these are altered in carcinogenesis [19, 100]. Glycans are often truncated in cancer cells, due, in part, to incomplete synthesis, contributing to the expression of sLe [100, 173]. Evidence now suggests that the disruption or activation of a single glycosyltransferase may be sufficient to upregulate sLe structures. For instance, epigenetic silencing of ST6GalNAc-VI, as well as experimental reduction of this gene's expression, leads to the accumulation of sLe [173, 180]. ST6GalNAc-VI adds an α2-6-linked sialic acid to GlcNAc, creating the di-sLe (a) structure, which predominates in normal epithelial cells. On the other hand, forced expression of a β1-4 GalNAc transferase reduces sLe expression, and restores a more normal carbohydrate profile [181]. Cells with forced β1-4 GalNAc transferase expression have reduced adhesion to human umbilical cord endothelial cells, and decreased metastasis *in vivo*. Additional factors within the tumor microenvironment likely play a part in sLe expression. Hypoxic conditions stimulate sLe upregulation through HIF-1α signaling [182], while the hormone receptor status of certain cancers appears to influence sLe-E-selectin interactions [183].

Fucosyltransferase activity may also contribute to tumor-associated sLe expression [184–187], although the relative importance of this pathway is debated [173]. Dimitroff's group reported that expression of the Fut-3, Fut-6, or Fut-7 fucosyltransferases in prostate cancer cells was sufficient to stimulate sLe^x production and promote prostate cancer metastasis to bone and liver [185]. Several carriers of sLe structures were identified in this study including CD44, carcinoembryonic antigen, podocalyxin-like protein, and melanoma cell adhesion molecule [185]. Mucins, including Muc1, are also modified with sLe [188, 189]. Antisense strategies directed at Fut-3 reduce sLe expression as well as the number of hepatic metastases observed in mice [190, 191]. sLe structures mediate adhesion to the endothelium through their interactions with selectins on activated endothelial cells [192–195]. The physiologic relevance of tumor cell sLe structures in cancer progression has been confirmed by studies in which sLe/selectin interactions were perturbed. Experimental interventions that blocked sialylation of Lewis structures were effective in inhibiting tumor cell adhesion to both E-selectin-coated plates and endothelial monolayers [196, 197]. Pretreatment of mice with E-selectin peptide agonists decreased the number of metastases in a lung metastasis model [198], and forced liver-specific expression of E-selectin redirected melanoma cell metastasis from the lung to the liver [199]. Finally, E-selectin-deficient SCID mice developed fewer lung metastases in a xenograft colon cancer model [200]. This study also observed a higher number of circulating tumor cells in E-selectin-deficient mice, suggesting metastasis was inhibited at the endothelial binding step of the metastatic cascade.

Despite the established value of sLe as a cancer-associated biomarker, therapeutics targeting these structures have been relatively slow to develop. Cancer vaccines against sLe^a have yielded mixed results [201], even though experimental results support a role for sLe overexpression in stimulating natural killer cell responses [202]. Work by Esko's group demonstrated the utility of disaccharide decoy molecules in reducing overall sialylation and the expression of sLe antigens. Treatment of cells with decoy disaccharides inhibited: sLe^x expression; adhesion to selectin-coated plates; and metastasis to the lung in murine models [203–205], suggesting a possible therapeutic benefit. Additionally, several antibodies against sLe have been shown to be cancer specific and cytotoxic *in vivo*. For example, two monoclonal antibodies developed against sLe^a have demonstrated substantial antitumor effects in an *in vivo* colon cancer model [206]. Although further research is needed, these collective studies highlight the potential for targeting sLe structures in clinical treatment.

6 Tumor cell α2-6 sialylation confers resistance to cell death

Much of the literature regarding tumor cell sialylation has centered on its role in cell adhesion, migration and invasion, but some studies also implicate sialylation in regulating cell death pathways. In particular, α2-6-linked sialic acids may confer an apoptosis-resistant phenotype by modulating the activity of selected receptors and signaling mechanisms. One of the better-characterized functions for α2-6-sialylation is an inhibitory effect on galectin-dependent apoptosis [36]. Many galectins, including gal-1, gal-3, and gal-9, bind to cell surface galactosides and induce cell death [207]. Each galectin exhibits specificity for certain galactosyl structures, and there is evidence that galectins may selectively bind to distinct glycoproteins. The mechanisms underlying galectin selectivity are still under investigation, although some of the documented binding partners for galectins include integrins [38, 208, 209], EGFR [210], CD45 [211], and TRPV5 [20]. Galectins are secreted by immune (and other) cells, therefore α2-6 sialylation on the tumor cell surface may protect tumor cells from the actions of infiltrating immune cells. However, the relationship between galectins and tumor cell α2-6 sialylation is complex.

Many tumor cells overexpress galectins [32], which raises the paradox of why a cancer cell would upregulate an apoptosis-inducing molecule. In fact, galectins have many different tumor-promoting activities; for example, intracellular forms of galectins have anti-apoptotic functions that are independent of cell glycosylation status, and some galectins amplify signaling by the ras oncogene [32, 212]. Thus, tumor cells that coordinately upregulate galectins and α2-6 sialyltransferases would benefit from the pro-tumorigenic activities of intracellular galectins (that are carbohydrate-independent), while simultaneously acquiring resistance to the pro-apoptotic features of secreted galectins (that are carbohydrate-dependent and blocked by α2-6 sialylation).

In tandem with inhibiting galectin-mediated apoptosis, α2-6 sialylation enhances tumor cell survival by regulating the function of individual cell surface receptors. Lee *et al.* reported that treatment of colon tumor cells with ionizing radiation induced increased α2-6 sialylation of the β1 integrin as a secondary consequence of ST6Gal-I upregulation [76, 213]. In this system, α2-6 sialylation of the β1 integrin promoted cell adhesion to fibronectin and contributed to cell survival through the activation of paxillin and AKT [76]. Recently, two members of the TNFR death receptor family, Fas and TNFR1, were also identified as ST6Gal-I substrates, and it was shown that α2-6 sialylation blocked apoptotic signaling by these receptors [47, 214]. Reduced Fas-mediated apoptosis is a well-established factor in tumor cell survival, and Fas expression is downregulated in many different tumor types [215–217]. However, in addition to decreased expression, Fas signaling cascades are disrupted in tumor cells, with Fas activation triggering pro-survival, rather than apoptotic, pathways [218]. These non-apoptotic functions of Fas contribute to tumor-promoting phenotypes [219–222]. Complete knockout of Fas in tumor cell xenografts prevented tumor growth, supporting the hypothesis that Fas is essential for some aspect of tumor cell survival or proliferation [223]. This newer concept is in agreement with many reports that some cancer cells express high levels of Fas, but are yet resistant to Fas-induced apoptosis [224–227].

Several studies have suggested that Fas apoptotic activity is inhibited by sialylation [228–230], although most of these did not define which type of sialic acid linkage is functionally important. In more recent work, forced overexpression or knockdown of ST6Gal-I caused altered α2-6 sialylation of Fas (without affecting α2-3 sialylation) [47]. Elevated α2-6 sialylation of Fas by ST6Gal-I prevented apoptosis stimulated by both Fas-activating antibodies and FasL (the native ligand for Fas). Fas α2-6 sialylation did not interfere with binding of the agonist, but rather inhibited formation of the death-inducing signaling complex, and also restrained Fas receptor internalization (Fig. 9). Intriguingly, there is

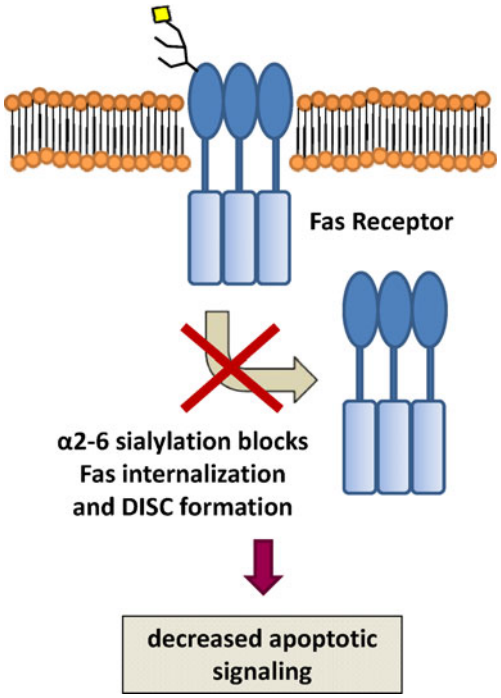

Fig. 9 Inhibition of Fas-mediated apoptosis by α2-6 sialylation. α2-6 sialylation of the Fas receptor blocks apoptosis by preventing receptor internalization and formation of the death-inducing signaling complex (*DISC*)

evidence that plasma membrane-localized Fas receptors may send a pro-survival signal, whereas receptor internalization is important for induction of apoptosis [231]. Hence, the α2-6 sialylation-dependent retention of Fas at the cell surface could serve as a switching mechanism responsible for diverting signaling away from apoptosis and toward survival. It isn't currently known why α2-6 sialylation prevents Fas internalization, but it can be speculated, based on information from other sialylated receptors, that Fas sialylation could regulate: (1) Fas receptor homotrimerization or higher order clustering, (2) tertiary conformation of the receptor, and/or (3) localization of the receptor to lipid raft microdomains.

Similar to Fas, ST6Gal-I-mediated α2-6 sialylation of the TNFR1 death receptor inhibits apoptosis directed by the TNFR1 ligand, TNFα, although at present TNFR1 sialylation has only been evaluated in macrophages [214]. TNFR1 is expressed in epithelial cells, however neither the glycan composition, nor function, of TNFR1 glycans have been characterized in epithelial tumor cells. Nonetheless, the finding that ST6Gal-I-mediated sialylation blocks apoptotic signaling through three major pathways (galectins, TNFR1 and Fas), suggests that upregulated ST6Gal-I may facilitate tumor cell escape from immune surveillance. The ligands for TNFR1 and Fas (TNFα and FasL, respectively) are primarily expressed by immune cells, and immune cells are also a rich source of galectins. Furthermore, as noted

previously, changes in sialylation likely affect tumor cell interactions with siglec-expressing immune cells. These findings underscore the need for elucidating the molecular mechanisms by which distinct tumor-associated sialoglycans or sialoproteins influence the host immune response. The paucity of studies on this topic is noteworthy, particularly given that it has long been believed that surface sialylation shields cancer cells from immune attack. Sialic acids mask antigenic sites on cells, thus weakening immunoreactivity, and sialic acids also protect cells against complement-mediated cell lysis [232]. In addition, loss of sialylation from the cell surface serves as an "eat-me" signal for phagocytes [233] suggesting that high sialylation levels on cancer cells may inhibit phagocytotic targeting by immune cells. These combined observations offer provocative clues that alterations in the profile of tumor cell sialoglycans may be a driving factor in immune escape.

7 Summary

A role for tumor cell sialylation in cancer progression has been presumed for many years, however mechanistic studies of this cell surface modification have been limited when compared with other areas of cancer cell biology. Particularly lacking are studies of: (1) signaling mechanisms that alter transcription or translation of sialylation-related enzymes, (2) sialyltransferase specificity for selected glycoprotein targets, (3) sialylation-dependent changes in glycoprotein structure (e.g., conformation and clustering), and (4) the effects of variant sialylation on the actions of glycan binding proteins. A better understanding of these molecular events is necessary for defining causal relationships between elevated sialylation and metastatic cell behaviors such as invasiveness, hematogenous dissemination, and apoptosis-resistance. The goal of this review was not to comprehensively overview the many reported changes in tumor sialoglycans but rather focus on a select number of examples for which there is substantive information regarding molecular mechanism. The elucidation of sialylation-dependent pathways that control distinct tumor cell responses holds promise for identifying important new diagnostic or prognostic markers, as well as targets for vaccine and drug development.

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Cancer Research

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Tumor and Stem Cell Biology

Cancer Research

ST6Gal-I Protein Expression Is Upregulated in Human Epithelial Tumors and Correlates with Stem Cell Markers in Normal Tissues and Colon Cancer Cell Lines

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Abstract

The ST6Gal-I sialyltransferase adds an α2-6–linked sialic acid to the *N*-glycans of certain receptors. ST6Gal-I mRNA has been reported to be upregulated in human cancer, but a prior lack of antibodies has limited immunochemical analysis of the ST6Gal-I protein. Here, we show upregulated ST6Gal-I protein in several epithelial cancers, including many colon carcinomas. In normal colon, ST6Gal-I localized selectively to the base of crypts, where stem/progenitor cells are found, and the tissue staining patterns were similar to the established stem cell marker ALDH1. Similarly, ST6Gal-I expression was restricted to basal epidermal layers in skin, another stem/progenitor cell compartment. ST6Gal-I was highly expressed in induced pluripotent stem (iPS) cells, with no detectable expression in the fibroblasts from which iPS cells were derived. On the basis of these observations, we investigated further an association of ST6Gal-I with cancer stem cells (CSC). Selection of irinotecan resistance in colon carcinoma cells led to a greater proportion of CSCs compared with parental cells, as measured by the CSC markers CD133 and ALDH1 activity (Aldefluor). These chemoresistant cells exhibited a corresponding upregulation of ST6Gal-I expression. Conversely, short hairpin RNA (shRNA)-mediated attenuation of ST6Gal-I in colon carcinoma cells with elevated endogenous expression decreased the number of CD133/ALDH1-positive cells present in the cell population. Collectively, our results suggest that ST6Gal-I promotes tumorigenesis and may serve as a regulator of the stem cell phenotype in both normal and cancer cell populations. *Cancer Res; 73(7); 2368–78. ©2012 AACR.*

Introduction

Differences in the glycan profile of cancer cells as compared with normal cells are well-documented. These changes are driven by various enzymes responsible for the addition and removal of sugars, such as glycosyltransferases and glycosidases. There is a selected subset of enzymes altered in cancer, suggesting a functional role for distinct glycans in the tumor phenotype. The ST6Gal-I sialyltransferase is an example of a glycosyltransferase commonly upregulated in cancer. This Golgi enzyme adds the negatively charged sugar, sialic acid, in an α2-6 linkage to the termini of *N*-glycans. ST6Gal-I is overexpressed in many types of cancer including colon, breast, and ovarian, and upregulation correlates with increased metastatic potential and poor prognosis (reviewed in refs. 1–3). ST6Gal-I is increased in cancer as a consequence of signaling by the ras oncogene (1–3).

The mechanistic role of ST6Gal-I in tumor progression remains poorly understood. *In vitro* studies suggest that ST6Gal-I promotes cell migration and invasion (4, 5), and this enhanced migratory response is due, at least in part, to ST6Gal-I-mediated sialylation of the β1-integrin receptor (6–8). Animal models also implicate ST6Gal-I in tumor invasiveness. Bresalier and colleagues determined that metastatic murine cell lines were more highly sialylated than less metastatic parental lines, and neuraminidase treatment of the metastatic lines drastically decreased the amount of liver metastases after splenic injection (9). Also, Harvey and colleagues reported decreased metastasis to liver following splenic injections after blocking the transfer of sialic acid from its carrier, CMP sialic acid (10).

In conjunction with cell migration, ST6Gal-I may regulate another important aspect of tumorigenicity, the ability to evade cell death. Work from our group revealed that the Fas death receptor is a substrate for ST6Gal-I, and that α2-6 sialylation of Fas reduces apoptotic signaling by hindering internalization of Fas after ligand-induced activation (11). We similarly reported that ST6Gal-I–mediated sialylation of the TNFR1 death receptor blocks TNFα-induced apoptosis (12). Baum's group showed that sialylation of CD45 by ST6Gal-I prevents CD45 internalization, thereby protecting T cells from

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apoptosis (13), and ST6Gal-I sialylation enhances PECAM surface retention, promoting survival of endothelial cells (14). These studies highlight the capacity of ST6Gal-I to modulate the function of specific receptors, particularly through regulation of cell surface retention. However, additional evidence has established ST6Gal-I as a key negative regulator of galectin-dependent apoptosis. Galectins are galactose-binding lectins that have many functions, including induction of cell death. The addition of α2-6 sialylation to galactosides prevents galectin binding and apoptotic activity (15). For example, our studies have shown that galectin-3 binds directly to the β1-integrin and stimulates apoptosis, but only when the β1-integrin lacks α2-6 sialylation (16). Finally, sialylation of EGF receptor (EGFR) by ST6Gal-I confers resistance to the EGFR-targeted chemotherapy reagent, gefitinib (17). These diverse findings suggest that ST6Gal-I acts as a critical regulator of tumor cell survival by inhibiting a multiplicity of cell death pathways.

While studies of specific receptors and signaling pathways have provided insight into the function of ST6Gal-I within a cellular context, a major gap in our knowledge is that ST6Gal-I expression in normal and tumor tissues has not been well-characterized. Because of a lack of effective anti-ST6Gal-I antibodies, prior investigations relied on measurements of ST6Gal-I mRNA levels, or tissue reactivity toward SNA, a lectin specific for α2-6–linked sialic acid. However, there are limitations associated with both of these approaches. The mRNA pool isolated from tumor tissue homogenates may include mRNA from noncancerous cells such as immune or stromal cells, and SNA reactivity is not completely restricted to ST6Gal-I–mediated α2-6 sialylation, as SNA can also recognize α2-6 sialic acids added to *O*-glycans by the ST6GalNAc family. To address this issue, immunohistochemical and immunoblot analyses of ST6Gal-I protein were conducted in the current study using a newly validated antibody. These studies revealed a dramatic upregulation of ST6Gal-I in tumor specimens compared with pair-matched uninvolved tissues. Surprisingly, the expression of ST6Gal-I in normal epithelium appeared to localize to the stem and/or progenitor cell compartment, and moreover, high ST6Gal-I levels corresponded with the expression of the cancer stem cell (CSC) markers, CD133 and ALDH1. While many questions remain regarding ST6Gal-I function in cancer, these data suggest that ST6Gal-I activity may be involved in maintaining some aspect of stem-like cell behavior.

Materials and Methods

Cell culture

HD3 colon carcinoma cells (18) were maintained in Dulbecco's Modified Eagles Medium (DMEM) low glucose (1 g/L) with 7% FBS and 1% antibiotic/antifungal solution containing streptomycin sulfate, penicillin G, and amphotericin B (Invitrogen). The stable ST6Gal-I knockdown cell line was established as described (8). In brief, HD3 cells were transduced with lentivirus (Sigma) expressing either short hairpin RNA (shRNA) against ST6Gal-I or an empty vector, and a pooled population of clones stably expressing shRNA was isolated by puromycin selection.

SW948 colon carcinoma cells were purchased from American Type Culture Collection. Cells were maintained in DMEM: Liebovitz' L-15 media in a 3:1 ratio with 10% denatured FBS and 2 mmol/L glutamine. To establish a chemoresistant subline, SW948 cells were treated with an initial dose of CPT-11 (irinotecan hydrochloride, Pharmacia & Upjohn Co.) at 4 μg/mL, which is 2-fold the determined IC_{50} dose. Most cells were killed by day 10. Surviving cells were grown in drug-free media for 3 days, and then CPT-11 (4 μg/mL) was added back to the media for 5 days. After a 3-day recovery period in drug-free media, cells were capable of growth in CPT-11 containing media (4 μg/mL). Dosage was then increased stepwise for a period of 185 total days reaching a maximum of 20 μg/mL. Resistant cells were cultured in DMEM:L15 media containing 20 μg/mL CPT-11 and periodically screened for drug resistance. Cells maintained CPT-11 resistance even after growth in drug-free media out to 122 days.

Sample preparation and ST6Gal-I immunoblots

Colon tumor blot. Commercially available membrane containing 3 human colon tumor samples, 1 normal colon, and 1 placental sample was purchased from Biochain Institute (Newark, CA).

Tumor and pair-matched uninvolved colon specimens. Human tissues obtained from the Tissue Procurement Facility at University of Alabama at Birmingham (UAB; Birmingham, AL) were snap-frozen in liquid nitrogen and stored at −80°C. Samples were homogenized using a polytron device in 50 mmol/L Tris-HCl buffer (pH 7.4) with 1% Triton X-100 and protease inhibitors (Roche Applied Bioscience). Samples were centrifuged and supernatants used for immunoblotting.

Induced pluripotent stem cells, individual transcription factor–transduced, and human foreskin fibroblast cell lysates. Frozen lysates from cells including control human foreskin fibroblasts (HFF), induced pluripotent stem (iPS) cells derived from HFFs, or HFFs transduced with one of the following transcription factors, c-Myc, Klf4, Oct4, or Sox2, were obtained from Systems Biotechnologies.

Colon carcinoma cell lines. HD3 and SW948 cells were lysed in 50 mmol/L Tris-HCl buffer containing 1% Triton X-100 and protease inhibitors. Lysates were centrifuged and supernatants collected for immunoblotting.

Samples were separated by SDS-PAGE and transferred to ployvinylidene fluoride (PVDF) membranes. Membranes were blocked in 5% dried non-fat milk (NFM) in TBS containing 0.01% Tween-20 (TBST) at room temperature for 1 hour. The membranes were incubated overnight at 4°C with primary anti-ST6Gal-I antibody (catalog # AF5924, R&D Systems), used at a concentration of 1 μg/mL and diluted into TBST containing 5% NFM. Membranes were washed in TBST and incubated with horseradish peroxidase-conjugated secondary antibody (in 5% NFM/TBST) for 1 hour at room temperature. Blots were developed with Immobilon (Millipore). To control for protein loading, membranes were reprobed for glyceraldeyhde-3-phosphate dehydrogenase (GAPDH) or β-actin (Cell Signaling Technologies). Densitometry was conducted using ImageJ software.

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SNA precipitation and Fas immunoblots

Tissues were homogenized as described above, and 500 μg of homogenate protein was incubated overnight at 4°C with 50 μL SNA-1 conjugated to agarose (EY Laboratories) with rotation. α2-6–sialylated proteins complexed with SNA were collected by centrifugation and washed. Sialylated proteins were released from complexes by boiling in SDS-PAGE sample buffer and immunoblotted for Fas (Santa Cruz Biotechnology, Inc.). To evaluate total Fas protein, Fas immunoblots were conducted using aliquots of the initial tissue homogenates (not subjected to SNA precipitation).

Immunohistochemistry

Slides with paraffin-embedded pair-matched tumor and uninvolved colon tissue were obtained from Biochain Institute. Slides were rehydrated using xylene and a gradient of EtOH solutions including 100%, 95%, 80%, and 70% EtOH in DiH_2O for 5 minutes each. Frozen multitissue arrays were purchased from Biochain Institute. Antigen retrieval was conducted by boiling slides in citrate buffer (Vector Labs) for 30 minutes. Slides were allowed to cool at room temperature for 60 minutes. Slides were blocked for 60 minutes in 10% normalized horse serum diluted in PBS. The following antibodies were then applied overnight at 4°C: 5 μg/mL ST6Gal-I (R&D Systems) or 2.5 μg/mL ALDH1 (BD Pharmingen), each diluted into blocking buffer. Slides were washed in PBS and secondary antibody was applied for 30 minutes at room temperature (Immpress, Vector Labs). Slides were developed with Immpact NovaRed (Vector Labs) and counterstained with hematoxylin (Vector Labs). Slides were dehydrated through 70%, 85%, 95%, and 100% EtOH, and xylene and fixed with Permount (Vector Labs). Images were captured with ISCapture software.

Validation of ST6Gal-I antibody

Specificity of the ST6Gal-I antibody (R&D Systems #AF5924) was validated using 2 established cell lines (Supplementary Fig. S1). SW48 colon cancer cells and OV4 ovarian cancer cells have no endogenous ST6Gal-I, and ST6Gal-I expression was forced in these lines as reported (refs. 6, 7; of note, SW48 and SW948 are distinct cell lines.) Immunoblotting (Supplementary Fig. S1A) was conducted as described above, and immunofluorescent staining (Supplementary Fig. S1B) was conducted using 1 μg/mL anti-ST6Gal-I antibody, followed by Alexa-conjugated secondary antibody (Life Technologies). In addition, immunohistochemical staining was conducted on formaldehyde-fixed OV4 cell cultures (Supplementary Fig. S1C). To control for the effects of paraffin embedding and antigen retrieval, OV4 cells were detached and centrifuged, and the cell pellets were paraffin-embedded and sectioned. Antigen retrieval and immunohistochemical staining were conducted on cell pellet sections (Supplementary Fig. S1D) using the protocol described previously for tissue sections. Validation of tissue staining is shown in Supplementary Fig. S2. Frozen slides containing colon metastasis to liver were subjected to antigen retrieval and immunostaining. Samples were incubated with either primary or an isotype control antibody (Supplementary Fig. S2A). As a final control, paraffin-embedded uninvolved colon and colon tumor specimens were exposed to secondary antibody alone (no primary; Supplementary Fig. S2B).

Flow cytometry

Cells were detached from tissue culture flasks by brief trypsinization. A total of 1×10^6 cells were analyzed for ALDH1 activity using the Aldefluor assay as recommended by the manufacturer (StemCell Technologies). Samples from each cell line with inhibited Aldefluor staining were used as the gating control. CD133/1-PE antibody (AC133) was used according to the manufacturer's protocol (Miltenyi Biotec). Results were gated for nonspecific activity by isotype control (IgG1, Miltenyi Biotec). In addition, for experiments measuring SNA reactivity, TRITC-conjugated SNA-1 (EY Laboratories) was used according to manufacturer instructions. Cells were analyzed by flow cytometry with a FACSCalibur (Becton-Dickinson) at the UAB Rheumatic Diseases Core Center Analytic and Preparative Cytometry Facility. Statistical analysis of the flow cytometry results was accomplished using a z test for 2 proportions. $P < 0.05$ is considered significant.

Results

ST6Gal-I upregulation in human colon tumors

To address the lack of information about ST6Gal-I protein expression in human tissues, we screened several new commercial antibodies and identified one that reliably detects ST6Gal-I protein (Supplementary Figs. S1 and S2). Using this antibody, we evaluated ST6Gal-I levels in human colon cancer tissues using a commercial membrane blot containing three independent cases of human colon carcinoma, along with normal colon and normal placental specimens. As shown in Fig. 1A, higher ST6Gal-I expression was observed in the colon tumors than in normal colon and placenta. The upper band on the blots represents full-length ST6Gal-I, whereas the size of the lower band is consistent with the cleaved, secreted form of ST6Gal-I (19, 20).

We next examined ST6Gal-I expression in tumor and pair-matched uninvolved colon specimens obtained from the Tissue Procurement Shared Facility at UAB. Tissues were homogenized and immunoblotted for ST6Gal-I. Four of the 5 patient samples exhibited upregulated ST6Gal-I in the tumor compared with the cognate uninvolved specimens (Fig. 1B). Patient demographics can be found in Supplementary Table S1.

Elevated α2-6 sialylation of the Fas receptor in human colon carcinoma samples

To assess the functional consequence of ST6Gal-I upregulation in tumors, we measured levels of α2-6 sialylation on the Fas receptor. Using patient samples for which sufficient tissue homogenate was available, tumor and pair-matched uninvolved colon tissue homogenates were incubated with agarose-conjugated SNA-1 lectin. The α2-6–sialylated proteins bound by SNA agarose were isolated by centrifugation, resolved by SDS-PAGE, and immunoblotted for Fas (Fig. 1C, top). To measure total Fas expression, samples of the original tissue homogenates (not subjected to SNA precipitation) were immunoblotted for Fas (Fig. 1C, bottom). We found that total Fas expression was decreased in the tumors, consistent with

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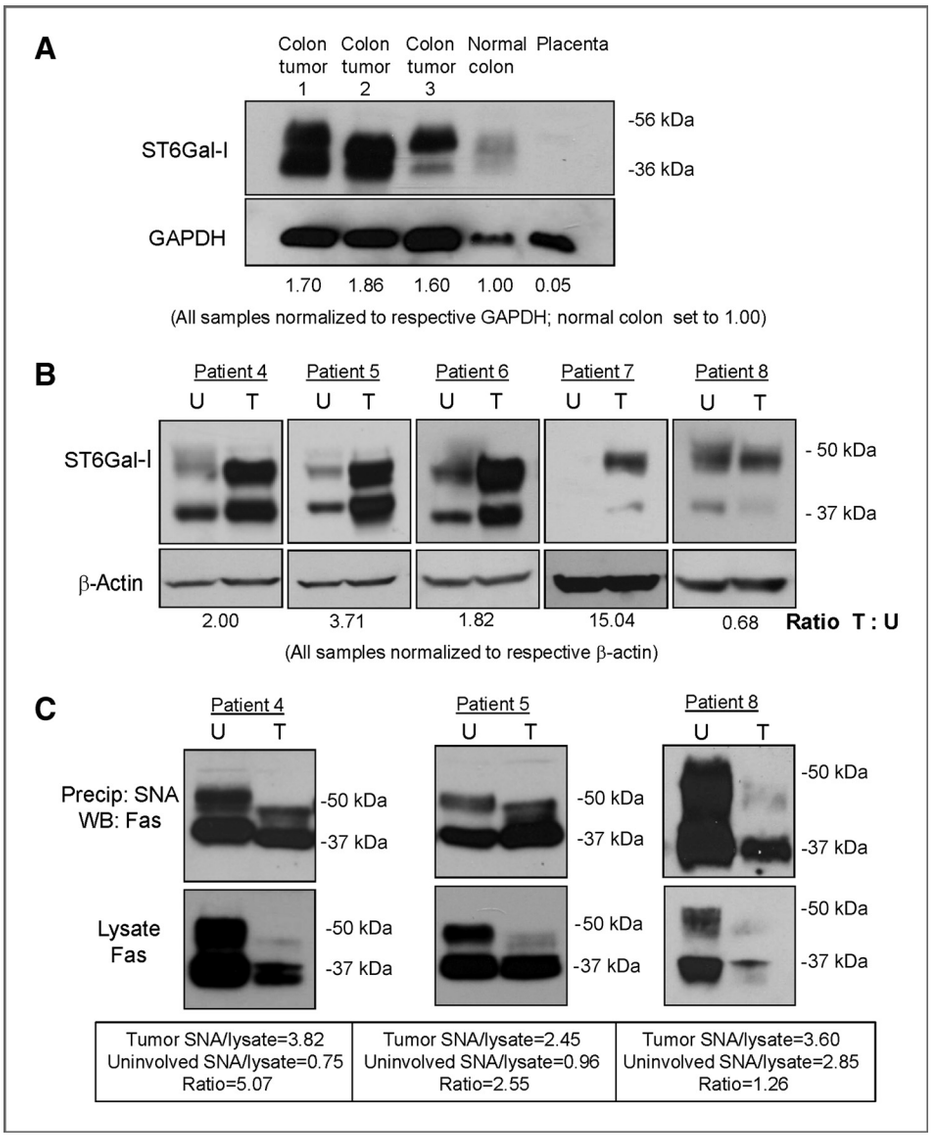

Figure 1. ST6Gal-I is upregulated in human colon tumors and tumor-associated Fas receptors have elevated α2-6 sialylation. A, a commercially purchased membrane for immunoblotting was probed for ST6Gal-I protein expression. Mature ST6Gal-I (top band) is highly expressed in 3 separate colon tumors as compared with normal colon and placenta. The bottom band is consistent with a cleaved, secreted form of ST6Gal-I (19). Densitometry was conducted on the top band. All samples were normalized to GAPDH and then compared with normal colon expression. B, tissue homogenates were prepared from colon tumors and pair-matched uninvolved colon specimens and immunoblotted for ST6Gal-I. ST6Gal-I was upregulated in 4 of 5 of colon tumors as compared to pair-matched uninvolved colon tissues. Densitometry was conducted on the top band. All samples were normalized to β-actin and then tumor samples were compared with pair-matched uninvolved colon. C, α2-6–sialylated proteins were isolated using SNA1-agarose and immunoblotted for Fas. Total Fas levels were assessed by immunoblotting initial tissue homogenates (not subjected to SNA) for Fas. In all 3 patient samples, mature Fas (top band) was downregulated in the tumor tissue relative to the respective uninvolved tissue. In patients with upregulated ST6Gal-I (patients 4 and 5), the proportion of sialylated Fas to total Fas was 5.07 and 2.55, respectively (densitometry was conducted on the top band). In the patient without upregulation of ST6Gal-I (patient 8), the proportion of sialylated Fas to total Fas was much lower at 1.26. U, uninvolved; T, tumor.

other studies suggesting that Fas is downregulated in colon carcinoma as a mechanism for protection against Fas-mediated apoptosis (21). However, despite Fas downregulation, the proportion of α2-6–sialylated Fas in the tumors was distinctly higher than the proportion of α2-6 sialylated Fas in uninvolved colon tissues for those cases that exhibited ST6Gal-I upregulation (patients 4 and 5, Fig. 1C). Conversely, levels of α2-6–sialylated Fas were comparable in tumor and uninvolved tissues from the patient sample that did not exhibit ST6Gal-I upregulation (patient 8). Thus, ST6Gal-I overexpression in tumors acts to hypersialylate Fas despite an overall downregulation in Fas protein. Hypersialylation of Fas, which inhibits Fas receptor internalization and apoptotic signaling (11), may constitute a second line of defense through blocking the activity of Fas receptors remaining on the tumor cell surface. The determination that Fas has enhanced α2-6 sialylation in

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tumors is consistent with our prior studies showing that β1-integrins exhibit elevated α2-6 sialylation in colon tumors (6). These results indicate that upregulation of ST6Gal-I in tumors leads to elevated α2-6 sialylation of functionally important ST6Gal-I targets.

ST6Gal-I upregulation and localization in colon tumors

Although ST6Gal-I upregulation in tumors was observed by immunoblotting, this approach does not address protein localization. Thus, paraffin-embedded tumor and uninvolved colon tissues from seven patients were stained by immunohistochemistry to visualize ST6Gal-I protein. Shown in Fig. 2A are results from a representative patient. In the uninvolved colonic mucosa (en face section), positive ST6Gal-I staining was observed in only a limited number of cells within the crypts. Longitudinal sections of the uninvolved colon tissue (Fig. 2B) revealed that the positively stained cells were localized to the base of the crypts. The staining was focal and located adjacent to the nucleus, typical of Golgi structure. However, the tumor samples showed a dramatic upregulation in ST6Gal-I expression, with punctate-like staining apparent in the majority of the epithelial cancer cells. This type of punctate staining is characteristic of the disrupted Golgi architecture present in cancer cells (22, 23). All 7 of the patients examined by immunohistochemistry exhibited the same type of staining pattern shown in Fig. 2A and B.

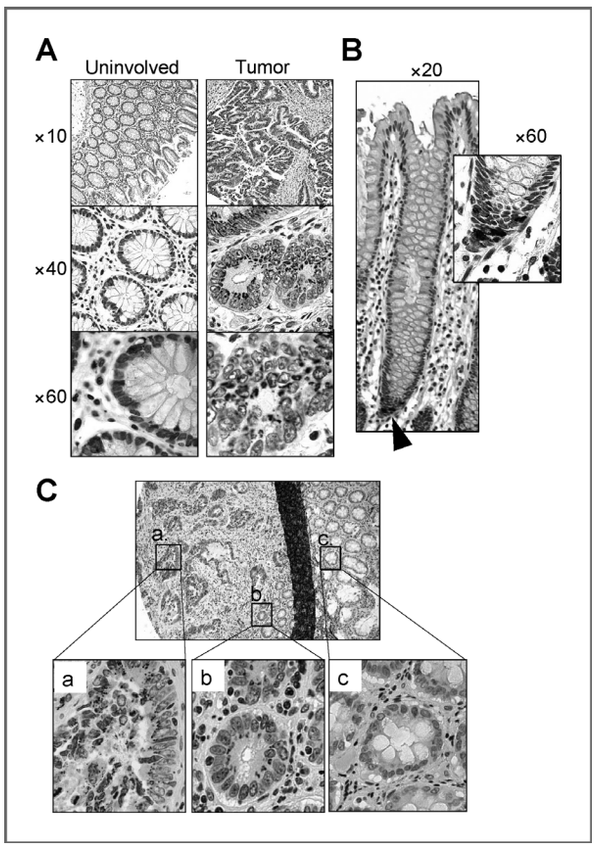

Figure 2. ST6Gal-I upregulation and localization in human colon tumors. A, representative sample of pair-matched tissues stained for ST6Gal-I protein expression. Paraffin-embedded specimens of uninvolved colon tissue and tumor tissues were immunohistologically stained for ST6Gal-I (brown) and counterstained with hematoxylin (blue). ST6Gal-I was highly upregulated in tumor tissue, whereas in uninvolved colon tissue expression was restricted to a very few cells within each crypt structure. B, longitudinal view of a crypt from uninvolved tissue. ST6Gal-I staining was restricted to the base of the crypt (black arrow). Inset shows enlarged view with ST6Gal-I stain in cells at the base of the crypt. C, ST6Gal-I staining in a patient sample showed gradient expression based on proximity to tumor. Upregulated expression of ST6Gal-I in malignant tissue (a), aberrant expression in morphologically normal crypt structures directly adjacent to tumor (b), and low expression in crypts distal to the tumor (c).

Along with increased ST6Gal-I expression, we observed an interesting pattern within several tumor samples; ST6Gal-I levels were elevated in normal appearing crypts immediately adjacent to the tumor. Within the malignant region of the tissue section (Fig. 2C, a), the crypt structure was highly disrupted and ST6Gal-I was upregulated, as in Fig. 2A. However, in the morphologically normal-appearing crypts next to the tumor (Fig. 2C, b), ST6Gal-I staining was increased and distributed in a punctate pattern, similar to staining in cancer cells. In the crypts more distal to the malignant tissue (Fig. 2C, c), ST6Gal-I expression was very low or undetectable, similar to the uninvolved pair-matched specimens. The upregulation of ST6Gal-I in crypts that appear morphologically intact is reminiscent of a "field effect" in which normal-appearing epithelium is in fact the product of expansion of a genetically abnormal clone (24).

ST6Gal-I overexpression in multiple epithelial, but not nonepithelial, tumors

In addition to colon carcinoma, we examined ST6Gal-I protein expression in several other types of tumors. As shown in Fig. 3A, immunohistochemical staining conducted on a multitissue array revealed ST6Gal-I upregulation in ovarian, stomach, pancreatic, and prostate tumors compared with uninvolved tissues. In contrast, ST6Gal-I levels were low or undetectable in malignant and uninvolved tissues from brain and skeletal muscle (Fig. 3B).

ST6Gal-I expression localizes to the stem or progenitor cell compartment in epithelia

The localization of ST6Gal-I within the base of crypts in nonmalignant colon epithelium suggested that ST6Gal-I may be selectively expressed in the stem or progenitor compartment. It is well established that stem and progenitor cells reside in the base of the crypt of normal colon (25). In addition, ST6Gal-I staining was very similar to what has been reported for the ALDH1 stem cell marker in normal colon (26). We therefore stained sections of normal human colon (cancer-free patients) for either ALDH1 or ST6Gal-I. As shown in Fig. 4A and B, both the ST6Gal-I and ALDH1 staining were in the base of the crypt, isolated to only a few cells within each crypt. No detectable staining of ST6Gal-I was observed in the differentiated colonocytes at the apical epithelial surface.

We next examined ST6Gal-I expression in the epidermis, which has clearly defined stem cell compartments (27). One of the compartments for epidermal stem/progenitor cells is the

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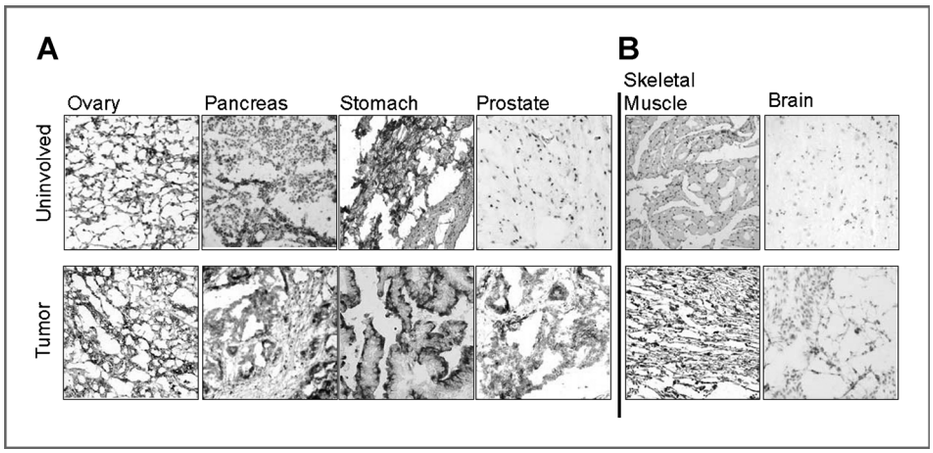

Figure 3. ST6Gal-I is upregulated in several types of epithelial cancers, but not nonepithelial cancers. A, frozen epithelial tumors and pair-matched uninvolved tissues from ovary, pancreas, stomach, and prostate were stained for ST6Gal-I protein expression and counterstained with hematoxylin. ST6Gal-I upregulation was apparent in the tumor samples. B, frozen pair-matched tissues from skeletal muscle and brain exhibited low or undetectable levels of ST6Gal-I.

basal epidermal cell layer, immediately adjacent to the basement membrane. As basal epidermal cells differentiate, they migrate apically and lose the capacity for proliferation. As shown in Fig. 4C, ST6Gal-I expression was restricted to this basal layer, consistent with the concept that ST6Gal-I may be enriched in stem and/or progenitor cells.

ST6Gal-I is highly expressed in human iPS cells

To further explore a link between ST6Gal-I and stem cells, ST6Gal-I levels were evaluated in iPS cells, as well as in the HFF population from which iPS cells were derived. Immunoblots revealed that ST6Gal-I was highly expressed in iPS cells, with no detectable expression in HFFs (Fig. 4D). In addition, ST6Gal-I expression was assessed in HFFs transduced with only 1 of each of the 4 individual transcription factors used in combination to derive iPS cells (c-Myc, Klf4, Oct4, Sox2). As shown in Fig. 4E, ST6Gal-I upregulation was only observed in cells with simultaneous transduction of all 4 transcription factors (iPS cells), suggesting that ST6Gal-I upregulation may require genetic reprogramming. Consistent with these results, Hirabayashi group reported that ST6Gal-I mRNA is elevated in iPS cells relative to somatic cells, and then downregulated upon forced differentiation of iPS cells (28). Intriguingly, somatic cells exhibit both α2-3 and α2-6 sialylation, with α2-3 sialylation predominating, whereas surface sialylation becomes exclusively α2-6–linked following transformation of somatic cells into iPS cells (29). Although the biologic significance of this switch is currently unclear, these findings point to some important and distinct function for α2-6 sialylation in the stem cell phenotype.

ST6Gal-I expression correlates with stem cell enrichment in colon carcinoma cell lines

On the basis of the ST6Gal-I localization in normal and tumor tissues, we hypothesized that ST6Gal-I might be a marker for CSCs. ALDH1 is one of the well-studied markers for both normal and CSCs (26), and furthermore, immunohistochemical analyses revealed a similar staining pattern for ALDH1 and ST6Gal-I (Fig. 4). Hence, we examined whether ST6Gal-I expression was associated with the level of stem cell enrichment in colon cancer cell lines. Our group has generated the human colon carcinoma cell line, HD3, which overexpresses ST6Gal-I secondary to forced oncogenic *ras* expression (18). This line was previously transduced with shRNA to obtain a cell population with stable ST6Gal-I knockdown (8). Parental and ST6Gal-I knockdown cells were analyzed for CSC enrichment by flow cytometry using the ALDH1 activity assay, Aldefluor. As shown in Fig. 5A, in 3 independent experiments cells with high ST6Gal-I expression (HD3.par) exhibited significantly greater CSC enrichment than cells in which ST6Gal-I had been knocked down (HD3.sh). Figure 5B shows a representative dot plot (Run #1, Fig. 5A). In addition, cells were double-labeled with Aldefluor and TRITC-conjugated SNA to detect cell surface α2-6 sialylation to examine the correlation between ST6Gal-I activity and stem cell enrichment (Fig. 5C). Cells with ST6Gal-I knockdown exhibited a decrease in the fluorescent intensity of SNA labeling, indicating reduced α2-6 sialylation, and this was associated with diminished ALDH1 activity (note that there is variation in the level of α2-6 sialylation due to the polyclonal nature of the HD3.sh population). To more stringently assay for stem cell enrichment, cells were double-labeled for ALDH1 and an additional CSC marker, CD133. As shown in Fig. 5D, cells with high endogenous ST6Gal-I expression had significantly greater numbers of cells positive for CD133/ALDH1. This suggests that forced downregulation of ST6Gal-I significantly decreases the number of CSCs within cancer cell populations.

One important characteristic of CSCs is the capacity to survive chemotherapy treatment. To study this cellular behavior, we established a cell line with acquired resistance to the camptothecin analog, irinotecan (CPT-11), a drug used to treat colorectal carcinoma. SW948 colon carcinoma cells were treated serially with CPT-11 to obtain a stable cell line resistant to greater than 10-fold the IC_{50} dosage of parental cells. The parental (SW948.par) and CPT-11-resistant (SW948.CPT) lines were then assayed for ALDH1 activity. As shown in Fig. 6A, 3 independent experiments showed significant enrichment of ALDH1 in the chemoresistant cells. Figure 6B is a representative dot plot (Run #1, Fig 6A). Stem cell enrichment was further evaluated by double-labeling cells with anti-CD133 and Aldefluor, which revealed significantly greater numbers of

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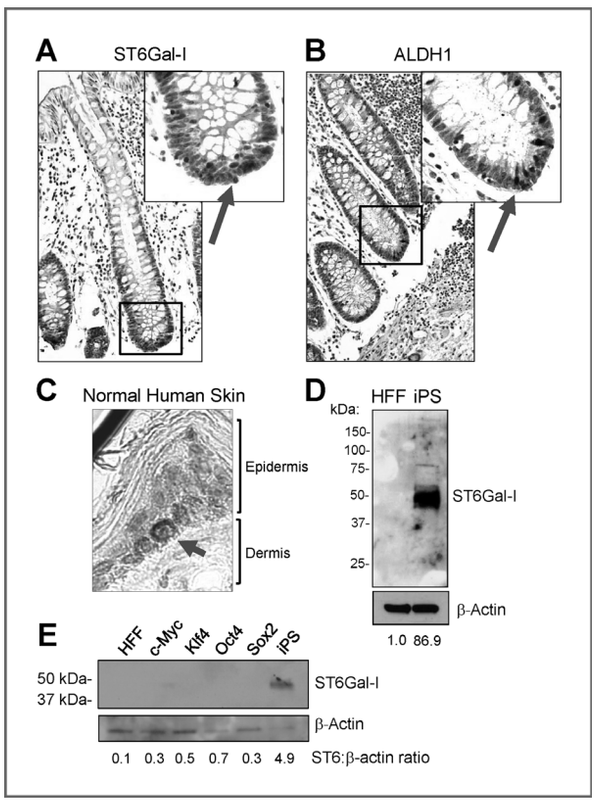

Figure 4. ST6Gal-I expression in stem/progenitor cell populations. A, paraffin-embedded normal colon tissue (from a cancer-free patient) was stained for ST6Gal-I. ST6Gal-I expression was confined to the base of normal colon crypts, with no expression observed in the apical, differentiated epithelium. B, expression of the ALDH1 stem cell marker was localized to the base of the crypts in normal human colon, similar to ST6Gal-I. C, staining for ST6Gal-I in paraffin-embedded normal human skin tissue. ST6Gal-I expression was confined to the basal proliferative compartment of the epidermis in normal skin. D, immunoblot for ST6Gal-I expression in cell lysates obtained from human iPS and the HFF population from which iPS cells were derived. There was no detectable ST6Gal-I expression in HFFs, whereas there was a dramatic upregulation of ST6Gal-I in the iPS cells. E, immunoblot for ST6Gal-I expression in HFFs, iPS cells, or HFFs transduced with only one of the individual Yamanaka factors: c-Myc, Klf4, Oct4, or Sox2. ST6Gal-I upregulation was observed in iPS cells (generated by simultaneous transduction of all 4 factors) but not in HFFs transduced with the single factors alone. Densitometry was completed by normalizing signal to the respective β-actin band and then comparing HFFs with iPS.

CD133+/ALHD1+ cells in the SW948.CPT cells than SW948.par cells (Fig. 6C). We next evaluated ST6Gal-I expression in SW948.par and SW948.CPT cells by immunoblotting. Figure 6D shows an acquired ST6Gal-I expression in the established chemoresistant cells. The chemoresistant cells also exhibit elevated ST6Gal-I activity indicated by increased intensity of SNA-TRITC labeling (Fig. 6E). Taken together, these data show a correlation between CSC enrichment and ST6Gal-I expression in 2 independent cell model systems. Forced ST6Gal-I downregulation decreases CSC number, whereas acquired chemoresistance yields higher CSC numbers with a corresponding increase in ST6Gal-I expression and activity.

Discussion

Studies over the last 2 decades have reported increased ST6Gal-I mRNA in many human cancers (1, 2), and more recent gene expression profiling technologies confirm tumor-associated ST6Gal-I upregulation (30–32). Microarray conducted on colon cancer cells isolated by laser capture microdissection revealed higher ST6Gal-I mRNA in tumors with high versus low risk of recurrence (and cells from both tumor types had higher ST6Gal-I than normal colonocytes; ref. 33). Additional microarray studies indicate that ST6Gal-I is overexpressed in cervical (30), testicular (31), and pancreatic (32) cancers, and ST6Gal-I levels are higher in metastatic versus primary prostate cancer (34). As well, ST6Gal-I is one of the genes downregulated by the metastasis suppressor, BRMS1 (35). However, few investigations have characterized ST6Gal-I protein expression in either cancer or normal tissues due to the prior lack of anti-ST6Gal-I antibodies. In one study, using a privately generated antibody, ST6Gal-I was found to be upregulated in the majority of human colon tumors (36). In the present investigation, we screened multiple new commercial antibodies and identified a reagent with high specificity for ST6Gal-I. Using this antibody, we observed extensive staining for ST6Gal-I in all of the human tumor tissues evaluated by immunohistochemistry and markedly elevated ST6Gal-I expression in 7 of 8 colon tumor samples examined by immunoblotting. Interestingly, the localization of ST6Gal-I in normal tissues was distinctly different from that of tumor tissues. Specifically, ST6Gal-I expression was found within a few cells in the base of the colonic crypts, with no detectable expression in the differentiated epithelial cells. Furthermore, ST6Gal-I expression was high in the basal, proliferative compartment of the epidermis, and high in iPS cells, but undetectable in the somatic cell population from which iPS cells were derived.

Given that ST6Gal-I expression in normal tissues appeared to associate with stem/progenitor cell populations, we evaluated whether ST6Gal-I levels might be elevated in CSCs. CSCs (alternately referred to as "tumor-initiating cells") are posited to represent a subset of cells within the heterogeneous tumor that has a more aggressive and chemoresistant phenotype (37, 38). The level of CSC enrichment within a cancer cell population is identified by a variety of markers, including ALDH1 and CD133, which have been validated in colon carcinoma (26, 39, 40). CSCs are considered to be a driving force behind tumor recurrence due to the self-renewal properties of these cells and resistance to chemotherapeutic drugs. This has been shown in a number of cancer types including breast, ovarian, and colon carcinomas. In this study, we found that high ST6Gal-I expression consistently correlated with ALDH1 and CD133 expression, and forced ST6Gal-I downregulation reduced the percentage of CSCs within a heterogeneous cell population. As well, when SW948 colon cancer cells, which do not usually express ST6Gal-I, were treated serially with increasing concentrations of irinotecan (CPT-11), the stem cell population was selectively protected, evidenced by an increase in ALDH1/CD133-positive cells, and correspondingly, ST6Gal-I expression and activity were markedly increased. Notably, microarray studies comparing gene expression in

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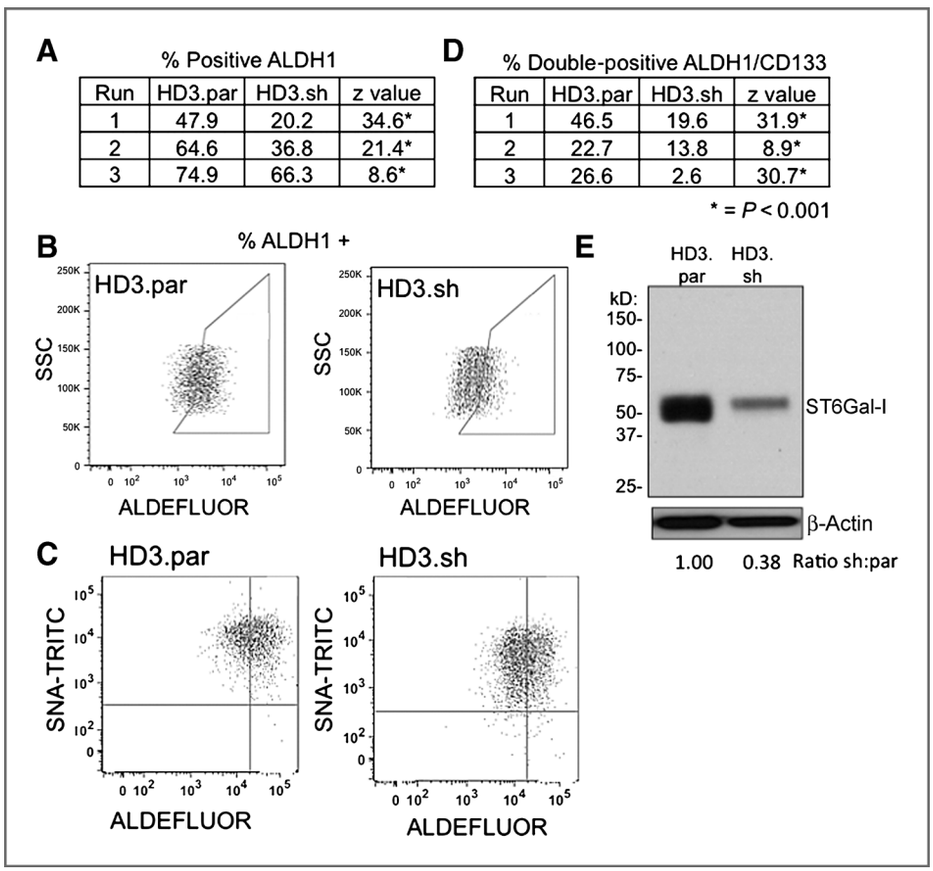

Figure 5. ST6Gal-I expression correlated with CSC enrichment. A, colon carcinoma cells HD3.par and HD3.sh were assayed for ALDH1 activity (Aldefluor) by flow cytometry. Enrichment of ALDH1 staining was significantly higher in HD3.par than in HD3.sh in 3 independent runs. B, representative dot plot (run #1, 5A) showing ALDH1 staining. C, Aldefluor and SNA-TRITC double labeling shows knockdown decreases α2-6 surface sialylation along with stem cell enrichment. D, double labeling for stem cell enrichment of HD3.par and HD3.sh cells with ALDH1 and CD133 by flow cytometry revealed that knockdown of ST6Gal-I leads to significantly decreased enrichment in 3 independent runs. E, immunoblot of HD3.par and HD3.sh cells showed that shRNA transduction reduced ST6Gal-I expression. Densitometry completed by normalizing to respective β-actin and then comparing HD3.sh with HD3.par. *, $P < 0.001$.

$CD133^+$ versus $CD133^-$ colon cancer cells identified ST6Gal-I as one of the 39 genes with the highest selective expression in $CD133^+$ cells, and ST6Gal-I was the only glycosylation-related gene in this pool (41). While further studies are needed, these results suggest that ST6Gal-I may represent a new marker for CSCs.

There are several hypotheses concerning the origin of CSCs. It is widely debated as to whether CSCs are derived from mutated normal stem cells, progenitor cells, or more differentiated cells (that subsequently revert to a less differentiated phenotype). In colon tumorigenesis, it has been suggested that a tumor would more likely arise from a mutated stem or progenitor cell, due to the short half-life of differentiated colonocytes, as well as the clonal nature of crypt development, where the entire crypt is thought to be derived from a single stem cell or stem cell compartment located at the base of the crypt (25, 42). Interestingly, some of the fundamental evidence supporting the clonal crypt hypothesis was obtained from studies of a sialic acid variant, 9-*O*-acetylated sialic acid, which is generated by the enzyme, sialate-*O*-acetyltransferase (OAT). LOH in stem cells of humans heterozygous for the *OAT* gene causes complete repopulation of the crypt by the progeny of the mutant stem cells (43). While the relationship between 9-*O*-acetylated sialic acids and ST6Gal-I activity is unclear, these studies are consistent with the concept that specific types of sialylation may be very important in maintaining some aspect of the stem cell phenotype. This hypothesis is further supported by the recent finding that sialic acids on iPS cells are exclusively α2-6-linked, in contrast to somatic cells, which express a mixture of α2-3 and α2-6 sialylation, with α2-3 predominating (29).

ST6Gal-I–mediated receptor sialylation has been previously correlated with an undifferentiated or immature cell state, particularly in certain immune cell types. We reported that ST6Gal-I expression is decreased as monocytic cells differentiate down the macrophage lineage (19, 44). Others have shown that ST6Gal-I activity is initially important for monocyte-derived dendritic cell generation, but that maturation of dendritic cells is associated with a loss in ST6Gal-I (45). As well, removal of sialic acids via neuraminidase treatment stimulated dendritic cell differentiation, and dendritic cells from ST6Gal-I–null mice have a more mature status than cells from wild-type mice (46). ST6Gal-I is also markedly downregulated upon activation of murine CD4+ and CD8+ T lymphocytes (47). Fewer studies have addressed ST6Gal-I expression in epithelial cell differentiation; however, SNA labeling of epidermis is inversely correlated with cell differentiation status (48). Finally, Varki and colleagues investigated the role of ST6Gal-I in the PyMT spontaneous mammary tumorigenesis model and found that tumors from ST6Gal-I null mice were more differentiated than tumors from wild-type mice (49).

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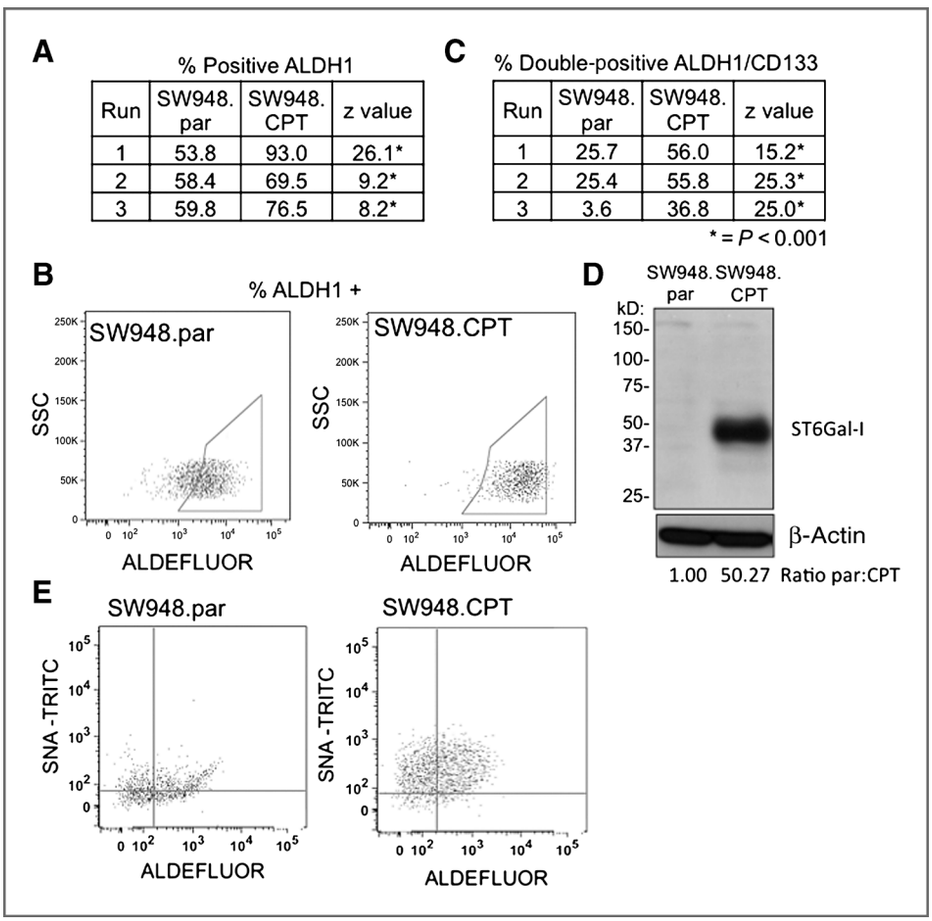

Figure 6. A, ALDH1 activity was assayed by flow cytometry in colon carcinoma cell line SW948. SW948.CPT chemoresistant line had significant enrichment for ALDH1 staining in 3 independent runs as compared with SW948.par. B, representative dot plot of ALDH1 staining (run #1, 6A). C, double labeling of SW948.par and SW948.CPT with ALDH1 and CD133 showed significant increase in stem cell markers in the chemoresistant line (SW948.CPT) in 3 independent runs. D, immunoblot of SW948.par and SW948.CPT shows ST6Gal-I expression was upregulated in the SW948.CPT line. Densitometry completed by normalizing to respective β-actin and then comparing SW948.CPT to SW948.par. E, double labeling with Aldefluor and SNA-TRITC shows chemoresistant line has increased stem cell enrichment as well as increased surface α2-6 sialylation. *, $P < 0.001$.

The functional contribution of ST6Gal-I to an immature or undifferentiated cell phenotype has yet to be elucidated; however, resistance to apoptosis may play a prominent role. Accumulating evidence points to ST6Gal-I as a major inhibitor of cell death pathways initiated by Fas, TNFR1, and galectins (2, 11, 12). Lee group also showed that ST6Gal-I confers radiation resistance in colon cancer cell lines (50). In the aggregate, these studies are consistent with the general concept that ST6Gal-I activity might underlie the survival or self-renewal characteristics of stem/progenitor cells and/or selected cancer cell populations. A corollary hypothesis is that downregulation of ST6Gal-I in differentiated cells may sensitize cells to multiple apoptotic stimuli, thus limiting cell lifespan. Clearly, there is a need for further investigation of ST6Gal-I function; however, the current study provides important new insight into the localization of ST6Gal-I expression in normal and tumor epithelium and also implicates ST6Gal-I as a potential new marker for CSCs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Conception and design: A.F. Swindall, A.I. Londono-Joshi, D.J. Buchsbaum, S.L. Bellis

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.F. Swindall, A.I. Londono-Joshi, M.J. Schultz, D.J. Buchsbaum

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.F. Swindall, A.I. Londono-Joshi, N. Fineberg, D.J. Buchsbaum, S.L. Bellis

Writing, review, and/or revision of the manuscript: A.F. Swindall, A.I. Londono-Joshi, N. Fineberg, D.J. Buchsbaum, S.L. Bellis

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.F. Swindall, D.J. Buchsbaum, S.L. Bellis

Study supervision: D.J. Buchsbaum, S.L. Bellis

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ST6Gal-I sialyltransferase confers cisplatin resistance in ovarian tumor cells

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Abstract

Background: Platinum drugs, including cisplatin, are a frontline therapeutic in ovarian cancer treatment and acquired resistance to these agents is a major contributor to ovarian cancer morbidity and mortality. In this study a novel glycosylation-dependent mechanism for cisplatin resistance is described. Specifically, cisplatin-induced cell death is blocked by the activity of the ST6Gal-I sialyltransferase. ST6Gal-I modifies specific receptors by adding a negatively charged sialic acid sugar which influences diverse receptor functions. Overexpression of ST6Gal-I is a hallmark of ovarian and other cancers and its expression has been correlated to metastasis and poor prognosis.

Methods: Tumor cell viability and apoptotic induction were determined in cell lines with ST6Gal-I overexpression and knockdown. In addition, cell populations with acquired resistance to cisplatin were assayed for endogenous ST6Gal-I expression.

Results: We show that forced expression of ST6Gal-I in OV4 ovarian cancer cells that lack endogenous ST6Gal-I causes reduced activation of caspase 3 and increased cell viability following cisplatin treatment. Conversely, forced ST6Gal-I knockdown in Pa-1 cells with high endogenous ST6Gal-I increases cisplatin-induced caspase activation and cell death. A2780 ovarian cancer cells selected for stable cisplatin resistance display upregulated endogenous ST6Gal-I when compared with parental, cisplatin-sensitive, A2780 cells. Similarly, extended low dose cisplatin treatment of a Pa-1 polyclonal ST6Gal-I shRNA knockdown population led to selection for subclones with elevated ST6Gal-I expression.

Conclusions: Receptor sialylation by ST6Gal-I confers a survival advantage for tumor cells in the presence of cisplatin. These collective findings support a role for ST6Gal-I in chemoresistance and highlight ST6Gal-I as a potential therapeutic target for platinum resistant tumors.

Keywords: Sialic acid, Cisplatin, Ovarian cancer, Apoptosis, Glycosylation

Background

The β-galactoside α2-6-sialyltransferase ST6Gal-I catalyzes the addition of the negatively-charged sugar, sialic acid, to the termini of *N*-linked glycans on selected cell surface or secreted proteins as they transit through the Golgi. ST6Gal-I elaborates an α2-6 linkage of sialic acid to galactose, and this enzyme appears to be the primary sialyltransferase responsible for this modification in most tissues [1,2]. Depending on the specific substrate targeted by ST6Gal-I, α2-6 sialylation can modulate protein conformation, oligomerization and/or receptor internalization (reviewed in [3]). Another important function of α2-6 sialylation is to negatively regulate certain galectin-dependent cell responses [4]. Galectins are lectins that bind galactose-containing glycans, and the addition of α2-6 sialic acid to galactose impedes the ability of most galectins to bind their targets [4]. Given that many glycoprotein receptors are held on the cell surface through an interaction with the extracellular galectin lattice [5-7], ST6Gal-I-mediated sialylation can block glycoprotein binding to the lattice, causing receptor internalization. Conversely, α2-6 sialylation enhances the surface retention of other types of receptor glycoproteins [8], albeit through mechanisms not well-defined. These

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observations suggest that ST6Gal-I may play a role in regulating the complement of receptors on the cell surface, in addition to modulating the function of distinct glycoproteins through effects on receptor conformation and/or clustering.

ST6Gal-I is overexpressed in many different types of cancers including ovarian, breast, and colon carcinoma (reviewed in [3,4]), and ST6Gal-I upregulation is driven by oncogenic ras [9,10]. Elevated expression of ST6Gal-I has been correlated with a negative patient prognosis in breast and colorectal cancers [11,12]. Cell culture studies suggest that ST6Gal-I promotes cell migration and invasion, at least in part through altering the sialylation and function of the β1 integrin [13-15]. More recently ST6Gal-I has also been identified as an inhibitor of several cell death pathways. For example, one important function of extracellular galectins is to induce apoptosis, and this activity is blocked by ST6Gal-I mediated sialylation of galectin substrates [16-18]. Additionally, our group has shown that sialylation of the Fas and TNFR1 death receptors by ST6Gal-I hinders apoptotic signaling in response to their respective ligands, FasL and TNFα [8,19]. Finally, ST6Gal-I activity is associated with resistance to radiation treatment [20].

In view of ST6Gal-I's upregulation in cancer, as well as its emerging role as an inhibitor of cell death pathways, we investigated whether ST6Gal-I activity could influence the sensitivity of tumor cells to cisplatin. Cisplatin is the parent compound of the platinum family of chemotherapeutics commonly used in frontline ovarian cancer treatment. Cisplatin and other platinum derivatives (e.g., oxaliplatin, carboplatin) function by forming inter- and intra-strand crosslinks in DNA, leading to an apoptotic cell death. Resistance to platinum drugs represents a major treatment challenge in ovarian and other cancers. The vast majority of ovarian cancer patients have an initial response to platinum compounds, however up to 75% of patients will relapse, with most exhibiting drug resistant disease [21]. The molecular events underlying resistance are complex, and it is likely that different tumor cells exhibit different mechanisms, or combinations of mechanisms, to escape cisplatin-induced apoptosis. At present, investigations into the mechanisms of tumor cell resistance to platinum agents have focused on drug import or export [22], cytosolic inactivation (e.g. by glutathione and other antioxidants) [23], compensatory DNA repair [24], and defects in apoptotic signaling [25]. The activation of caspases following DNA damage is important for cisplatin-induced cell death, therefore factors impinging on caspase activity can influence drug efficacy. As well, cisplatin may elicit cytotoxicity through mechanisms independent of DNA damage, as cisplatin is known to bind many molecules other than DNA, and can also modulate cytoskeletal structure [26]. In the current study we describe a new mechanism for cisplatin resistance involving α2-6 sialylation of glycoproteins by the ST6Gal-I sialyltransferase.

Methods

Cell lines

The Pa-1 ovarian cancer cell line was purchased commercially through ATCC (Manassas, VA). Pa-1 cells were cultured and grown in Dulbecco's eagle's minimal essential medium (DMEM) with 4.5 g glucose supplemented with 10% fetal bovine serum (FBS)(Hyclone) and 1% antibacterial/antimycotic solution containing penicillin, streptomycin, and amphotericin B (Invitrogen). Pa-1 cells were previously found to express high endogenous levels of ST6Gal-I [13]. To examine the effects of ST6Gal-I expression on cell response to cisplatin treatment a shRNA construct targeting ST6Gal-I as well as an empty vector control were introduced via a lentiviral vector (empty vector and shRNA-expressing lentiviral particles were purchased from Sigma). Pa-1 empty vector (EV) and ST6Gal-I shRNA-mediated knockdown (sh.ST6) lines are stable, polyclonal cell populations initially selected by puromycin at a concentration of 10 μg/ml, and then maintained in 0.5 μg/ml puromycin. The OV4 ovarian cancer cell line was a generous gift from Dr. Timothy Eberlein (Harvard, Cambridge, MA). OV4 cells were cultured and grown in Dulbecco's modified Eagle's MEM/Ham's F-12 50:50 (DMEM/F12) supplemented with 10% FBS and 1% antibiotic/antimycotic solution. OV4 cells lack detectable endogenous ST6Gal-I expression and we previously forced ST6Gal-I expression and an empty vector control by lentiviral transduction (MOI = 3) [13]. Stable, polyclonal populations were isolated through puromycin selection. A2780ip2 and A2780cp20 cell lines were generous gifts from Dr. Anil Sood (MD Anderson Cancer Center). Lines were maintained in RPMI media (Cellgro) supplemented with 10% FBS and 1% antibiotic/antimycotic solution. A2780cp20 cells represent a cisplatin-resistant derivative cell line of A2780ip2 created by repeated cisplatin exposure as previously described [27].

Immunofluorescence imaging

Cells were seeded onto 4-well chamber microscope slides (Beckin Dickinson) and allowed to adhere overnight. Cells were washed and fixed in 4% paraformaldehyde for 10 minutes followed by permeabilization in 5% Triton X-100 (in PBS) for 5 minutes. Cells were then incubated overnight at 4°C or 3 hr at room temperature with the ST6Gal-I antibody (polyclonal, R&D Systems, catalog # AF5924) and 3 hr at room temperature with anti-Golgi Matrix-130 (GM-130) (monoclonal, BD Transduction Laboratories). Following incubation with primary antibody, cells were washed and incubated with anti-goat Alexa-Fluor 594-conjugated or anti-mouse Alexa-Fluor

488 secondary antibody (Molecular Probes) for 30 minutes. Chambers were removed and DAPI-containing mounting solution, Vectamount (Vector Labs), was placed onto each well. Coverslips were added and the slides visualized under a Nikon Eclipe 80i fluorescence microscope fitted with a Photometrics CoolSNAP camera (Roper Scientific). Images were analyzed on NIS elements software. ST6Gal-I co-localization with GM-130 was imaged by confocal microscopy.

Western blot

Prior to lysis cells were grown in puromycin free media for at least one day, passaged normally, and plated onto 6 well tissue culture plates (Fisher) at a density of 7.5×10^5 cells per well. Cells were allowed to adhere overnight and then treated with cisplatin. Cells were lysed on ice in 50 mM Tris-HCl (pH 7.4) containing 1% Triton X-100, and a protease inhibitor cocktail (Roche Applied Bioscience). Cell lysate was kept on ice for 40 minutes vortexing regularly or lysates were sonicated using a sonicator model C-18 (Fisher). Protein concentrations of the lysates were determined using a modified Bradford Assay (Sigma). Proteins were then resolved by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat dry milk in TBS containing 0.1% Tween 20 (TBST). Primary antibodies against ST6Gal-I (R&D Systems) or cleaved caspase-3 (Cell Signaling) were added to the membrane. Membranes were then washed and incubated with horseradish peroxidase-coupled secondary antibody (Amersham) and visualized with Immobilon enhanced chemiluminscence reagent (Millipore). Protein loading was evaluated by immunoblotting for either β-tubulin or β-actin (Cell Signaling).

Cell viability assay

Cells were plated in opaque-sided 96-well plates (Corning) at a density of 10^4 cells per well in 75 μl of media and allowed to adhere overnight. Cisplatin stock solutions were made by dissolving solid cisplatin in distilled water to a concentration of 2.5 mM and stored at 4°C with new solutions made monthly. On the day of each experiment, stock solutions were used to dilute cisplatin into media to obtain the desired concentrations, and then cells were grown in the cisplatin-containing media for 21 hours (Pa-1) or 24 hours (OV4). Cell viability was evaluated by determining ATP content using the CellGlo ATP quantification kit (Promega) following the manufacturer's protocol. Luminescence was measured on a Synergy 2 plate reader (Biotek).

Cell selection with cisplatin

The polyclonal Pa-1 sh.ST6 population contains stable clones with varying levels of ST6Gal-I knockdown. Two cell flasks were grown in parallel; one with DMEM containing 10% FBS and 1% antibiotic/antimycotic solution (control), and the other with this media supplemented with 1 μM cisplatin. After a 3-week interval, greater than 90% of the cells grown in cisplatin-containing media had died, whereas the control population proliferated over this interval. At the end of the 3 week incubation in cisplatin, the remaining viable cells were resuspended in 10% FBS/DMEM lacking cisplatin, and cultures were expanded to gain a sufficient number of cells for lysis and western blot analysis.

Results

Forced overexpression of ST6Gal-I confers tumor cell resistance to cisplatin-induced apoptosis

In order to evaluate the effects of receptor α2-6 sialylation on tumor cell sensitivity to cisplatin, we utilized cell models with engineered ST6Gal-I expression. OV4 ovarian cancer cells have no detectable endogenous ST6Gal-I [13]; we therefore generated a stable cell line with forced ST6Gal-I expression. As shown in Figure 1A, the majority of cells with forced ST6Gal-I expression (ST6) exhibited high levels of immunostaining for ST6Gal-I, whereas none of the parental (Par) or empty vector-transduced (EV) cells were positive for ST6Gal-I. We confirmed forced ST6Gal-I expression by immunoblotting (Figure 1B). The pattern of staining was consistent with Golgi localization, evidenced by ST6Gal-I co-localization with the known Golgi protein, GM-130 (Figure 1C). The Golgi is the expected subcellular compartment for ST6Gal-I, demonstrating that ectopically expressed ST6Gal-I is correctly localized.

Par, EV and ST6 cells were treated with increasing doses of cisplatin and evaluated for cell viability by measuring ATP content. The ST6 cells maintained cell viability at higher cisplatin doses as compared with Par or EV cells (Figure 2A), indicating that ST6Gal-I-mediated sialylation protects against cell death. We also evaluated activation of caspase 3, the principal executioner caspase responsible for directing apoptosis. In concordance with cell viability assays, ST6 cells have decreased activation of caspase 3 as compared with Par or EV cells.

Forced ST6Gal-I knockdown sensitizes tumor cells to cisplatin-induced cell death

To further establish a role for ST6Gal-I in cisplatin sensitivity, ST6Gal-I expression was repressed by shRNA in Pa-1 ovarian cancer cells, a cell line with high endogenous levels of ST6Gal-I [13]. Effective knockdown of ST6Gal-I was confirmed by immunostaining and immunoblotting (Figure 3A and B). Golgi localization of endogenous ST6Gal-I is demonstrated by co-staining cells with GM-130 (Figure 3C). Parental (Par), empty

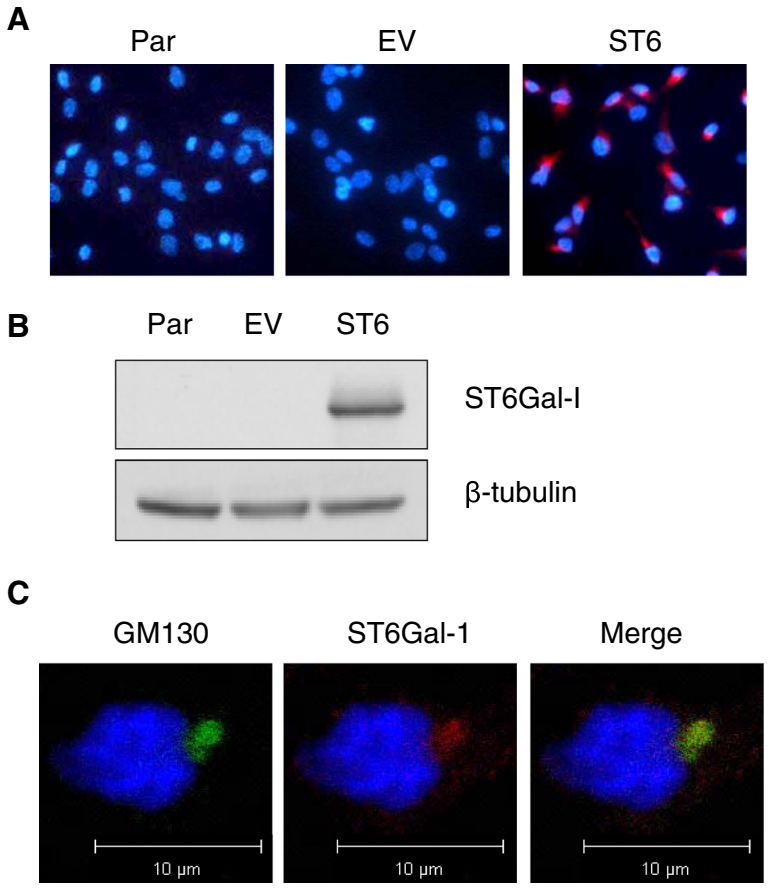

Figure 1 ST6Gal-I expression in OV4 ovarian cancer cells. OV4 cells that have no endogenous ST6Gal-I were stably transduced with either empty vector or ST6Gal-I-expressing lentivirus. ST6Gal-I expression was confirmed by immunocytochemistry (**A**) and immunoblotting (**B**). ST6Gal-I localization to the Golgi is shown by co-localization with the Golgi marker GM-130 in the ST6Gal-I forced expression line (**C**) Par = parental; EV = empty vector; ST6 = cells with forced ST6Gal-I expression.

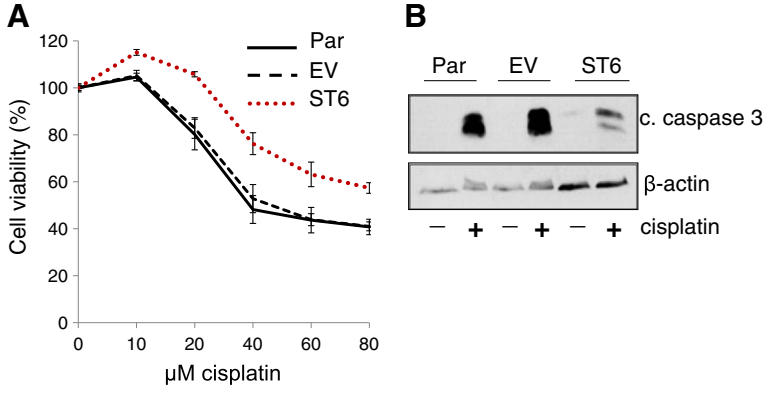

Figure 2 Forced ST6Gal-I expression protects OV4 cells from cisplatin-induced cell death. (**A**) Compared with Par or EV cells, OV4 cells with forced ST6Gal-I expression (ST6) exhibit attenuated cisplatin-induced cell death, as measured by ATP content. Means and S.E.M.s are shown for a representative experiment, with three independent experiments performed, and each experiment performed in triplicate. (**B**) ST6 cells have reduced levels of cleaved caspase-3 (representing caspase activation) following cisplatin treatment.

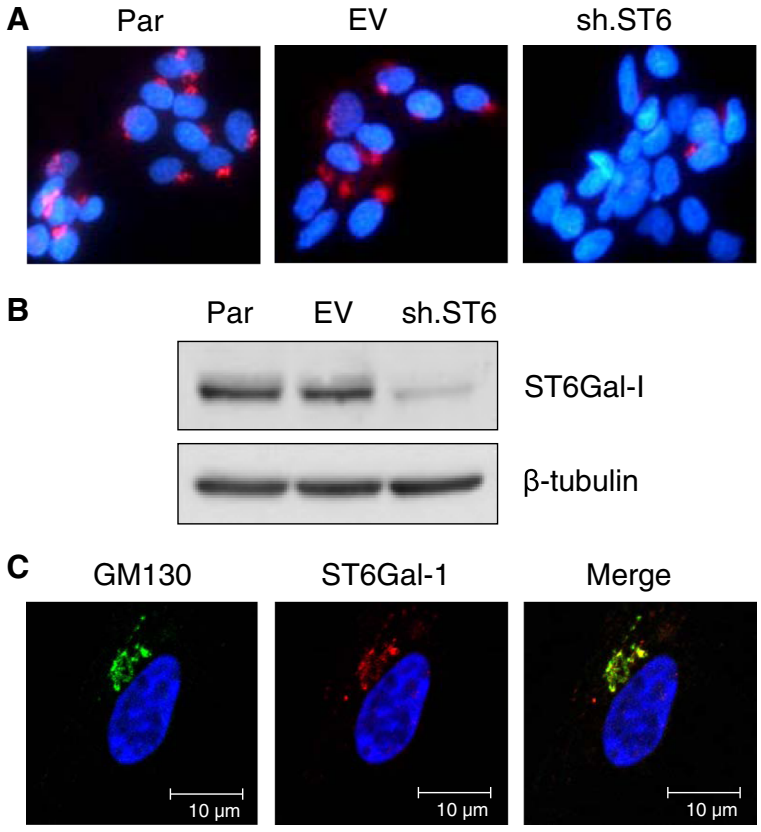

Figure 3 ST6Gal-I knockdown in Pa-1 ovarian cancer cells. Pa-1 cells that have high endogenous ST6Gal-I were stably transduced with either empty vector lentivirus, or virus expressing shRNA for ST6Gal-I. ST6Gal-I knockdown was confirmed by immunocytochemistry (**A**) and immunoblotting (**B**). ST6Gal-I localization to the Golgi was confirmed by co-localization with the Golgi marker GM-130 in the empty vector transduced line (**C**) Par = parental; EV = empty vector; sh.ST6 = cells stably expressing shRNA for ST6Gal-I.

vector (EV) and ST6Gal-I knockdown cells (sh.ST6) were exposed to increasing doses of cisplatin, and monitored for cell viability. As shown in Figure 4A, ST6Gal-I knockdown decreased the viability of Pa-1 cells, indicating enhanced sensitivity to cisplatin. Consistent with these results, sh.ST6 cells displayed greater activation of caspase 3 (Figure 4B).

While results shown in Figures 2 and 4 indicated that ST6Gal-I directly regulates cell response to cisplatin, it is interesting that higher concentrations were needed to

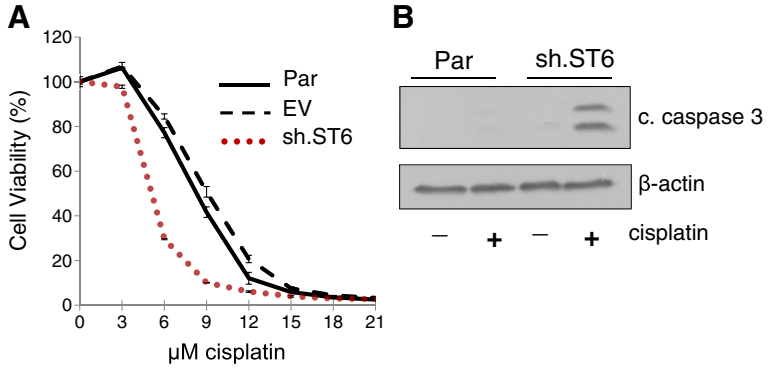

Figure 4 shRNA-mediated ST6Gal-I knockdown sensitizes Pa-1 cells to cisplatin-induced cell death. (**A**) Pa-1 cells with stable ST6Gal-I knockdown (sh.ST6) are sensitized to cisplatin-induced cell death, as measured by ATP content. Means and S.E.M.s are shown for a representative experiment, with three independent experiments performed, and each experiment performed in triplicate. (**B**) sh.ST6 cells have increased levels of cleaved caspase-3 following cisplatin treatment as compared with Par cells.

achieve killing of OV4 cells, suggesting that OV4 cells have an inherent resistance to cisplatin that is independent of ST6Gal-I function. This observation is consistent with the extensive evidence indicating that tumor cells become resistant through many different mechanisms. Another factor to consider is that the efficacy of cell killing depends not only on the amount of cisplatin added extracellularly, but also on the rate of cisplatin uptake as well as intracellular metabolism of cisplatin. The intracellular half-life of cisplatin within OV4 and Pa-1 cells was not measured in the current study, therefore the relationship between intracellular and total cisplatin for the two cell lines is not known. Nonetheless, it is noteworthy that manipulating ST6Gal-I expression in an inherently resistant cell line (OV4) is still effective in regulating cisplatin response, supporting a causal role for ST6Gal-I in cisplatin sensitivity.

Extended cisplatin treatment selects for ST6Gal-I expressing cells

Given that ST6Gal-I is upregulated in many types of cancer, including ovarian carcinoma [28], we hypothesized that cells with high ST6Gal-I expression may have a selective survival advantage. To address this hypothesis, we exposed Pa-1 cells with ST6Gal-I knockdown to prolonged low-dose cisplatin treatment. Notably, the Pa-1 sh.ST6 cell line represents a polyclonal cell population, and some variability in the degree of ST6Gal-I knockdown is observed among individual clones (as seen in Figure 3A). Pa-1 sh.ST6 cells were treated continuously with cisplatin for 3 weeks, during which greater than 90% of the cells were killed. The remaining viable population was then harvested, expanded, and immunoblotted for ST6Gal-I. As shown in Figure 5A, the viable cells exposed to cisplatin (sh.ST6 cis-res) had a higher level of ST6Gal-I, suggesting that ST6Gal-I conferred a survival benefit. To address the possibility that cisplatin may have induced ST6Gal-I expression, sh.ST6 cells were treated for 24 hours with the same dose of cisplatin (sh.ST6 + cis), however no changes in ST6Gal-I were observed (Figure 5B). These data suggest that the enhanced expression of ST6Gal-I in sh.ST6 cells treated with cisplatin for 3 weeks was due to selection for clones with higher ST6Gal-I, rather than induction of gene expression.

If the response of differentially-sialylated tumor cells to cisplatin treatment is indicative of a general protective effect of ST6Gal-I in the presence of cisplatin, then it would be expected that other cell models of cisplatin resistance may exhibit elevated ST6Gal-I expression. Thus, we immunoblotted for ST6Gal-I in the A2780 ovarian carcinoma cell line and its stably cisplatin-resistant derivative, A2780cp20 [27]. As shown in Figure 5C, a marked increase in ST6Gal-I expression was observed in the cisplatin-resistant A2780cp20 cells (Cis-res), providing further evidence of an association between ST6Gal-I upregulation and chemoresistance.

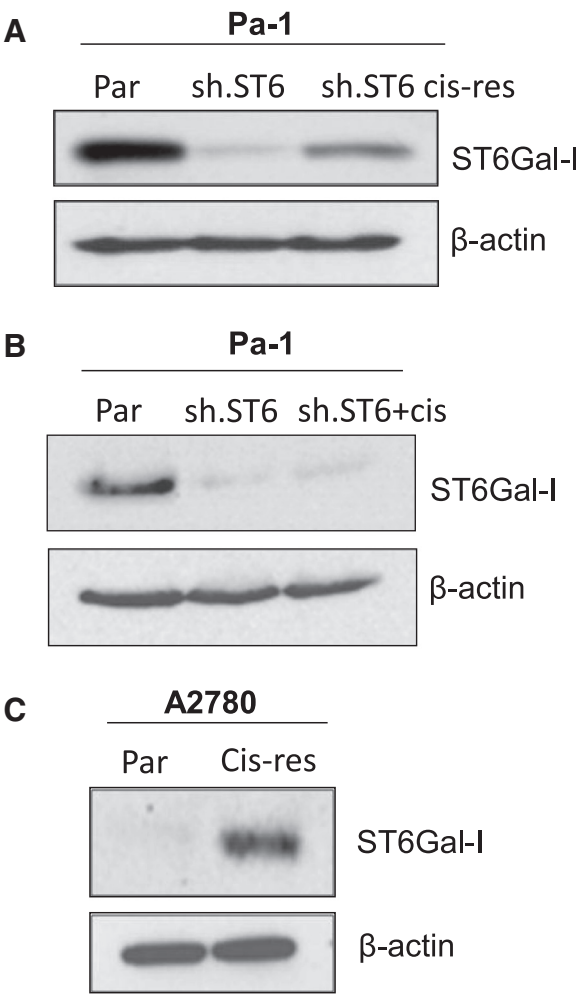

Figure 5 Cells that are resistant to cisplatin have upregulated endogenous ST6Gal-I. (**A**) Pa-1 cells with ST6Gal-I knockdown (sh.ST6) were exposed to cisplatin for 3 weeks, and the remaining viable population (sh.ST6 cis-res) was expanded and immunoblotted for ST6Gal-I. (**B**) Pa-1 cells with ST6Gal-I knockdown do not upregulate ST6Gal-I expression following a 24-hr treatment with cisplatin (sh.ST6 + cis). (**C**) Parental A2780 ovarian cancer cells (Par) and a cisplatin-resistant derivative population (Cis-res) were immunoblotted for endogenous ST6Gal-I.

Discussion

Despite the clear clinical significance of chemotherapy resistance, a single mechanism of resistance has not yet been established for all cases. The multifactorial nature of tumor cell resistance to cisplatin leaves open the possibility of novel mechanisms that remain undiscovered. In this study we show that forced expression of ST6Gal-I confers resistance to cisplatin, whereas ST6Gal-I knockdown conversely sensitizes cells to cisplatin. Furthermore,

cells selected for resistance to cisplatin exhibit an upregulation in endogenous ST6Gal-I protein, suggesting that increased receptor α2-6 sialylation may provide tumor cells with a survival advantage. These findings illuminate a new mechanism for chemoresistance, and underscore the importance of the cellular glycosylation machinery in drug response. An aberrant glycan profile was one of the earliest identified characteristics of a cancer cell, and selective enrichment in α2-6 sialylation (relative to α2-3 sialylation), is a common feature of transformed cells [3]. It is also known that platinum drug-resistant cells have abnormal glycosylation [29,30], and studies spanning more than two decades indicate that cisplatin treatment alters the sialic acid content of tumor cells [31-33]. The link between these glycosylation changes and ST6Gal-I is currently unclear, however the inhibitory effect of ST6Gal-I on cisplatin-induced cell death is likely driven by the activity of variantly-sialylated surface receptors, given that ST6Gal-I modifies glycoproteins bound for the plasma membrane or secretion (and not cytosolic proteins). Interestingly, tumors expressing activating ras mutations [34] or ras overexpression [35] are typically resistant to cisplatin, and ST6Gal-I is one of the targets upregulated by ras signaling [9,10]. Furthermore, we recently reported that high ST6Gal-I expression correlates with expression of the cancer stem cell markers ALDH1 and CD133, suggesting that ST6Gal-I activity may contribute to stem-like cell behaviors including chemoresistance [36].

One predominant surface receptor known to modulate cisplatin sensitivity is the Fas death receptor. Fas is activated by binding to FasL, which in turn causes receptor internalization, formation of the Death Inducing Signaling Complex (DISC), followed by activation of apoptotic caspases. Caspase activation is also a critical downstream event following cisplatin-induced DNA damage, and cisplatin-resistant cells exhibit attenuated activation of caspases 3, 8 and 9 [35]. Cisplatin is reported to cause clustering and activation of the Fas receptor in a ligand independent manner [36], as well as increased Fas expression [37-43]. Additionally, cisplatin stimulates the aggregation of Fas into lipid rafts [44,45], which is correspondingly important for Fas internalization and apoptotic signaling [46]. In mice with subcutaneous tumors formed from syngeneic Lewis lung carcinoma cells, one intraperitoneal dose of cisplatin induced a dramatic increase in Fas expression in the tumors, and also stimulated tumor regression [43]. In this same study, the anti-tumor effects of cisplatin were abrogated in mice deficient in FasL. These results implicate cisplatin-induced Fas upregulation in promoting tumor cell death [43], and further suggest that in order to acquire cisplatin-resistance, tumor cells may evolve mechanisms to disable Fas signaling. Our prior studies demonstrated that Fas is a ST6Gal-I substrate, and that increased α2-6 sialylation of Fas functions to inhibit Fas receptor internalization and DISC formation [8], effectively shutting off Fas apoptotic signaling. Hence, α2-6 sialylated Fas isoforms could play a part in cisplatin resistance.

Another potential mechanism for ST6Gal-I-mediated cisplatin-resistance may involve the differential sialylation of one or more drug transporters. Many cisplatin-resistant cell lines show reduced accumulation of cisplatin [47], pointing to dysfunctions in cell surface transporters that control either drug uptake or efflux. Defective glycosylation of ATP binding cassette (ABC) transporters has been suggested to contribute to cancer development, and possibly, chemoresistance [48]. Liang et al. reported that in epidermoid carcinoma cells selected for resistance to cisplatin, the MRP1 transporter (also known as ABCC1) was aberrantly glycosylated, and this was associated with mislocalization to intracellular compartments and reduced cell surface expression [29]. Similarly, altered N-glycosylation of MRP1 and MRP4 was correlated with cisplatin and oxaliplatin resistance in ovarian cancer cells [49]. In this latter study, *N*-glycosylation defects were linked to reduced levels of two glycosyltransferases: (i) N-acetylglucosamine-1-phosphate transferase, gamma subunit (GNPTG) and (ii) mannosyl (alpha-1,6)-glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase (MGAT5) [49]. *N*-glycans are also known to be crucial for the stability of the ABCG2 transporter in the endoplasmic reticulum [30,50]. These findings indicate the importance of glycosylation in transporter function, and suggest that studies of variant transporter sialylation may be a fruitful area for future research.

Although the mechanisms underlying the effects of ST6Gal-I activity on cisplatin sensitivity are not yet understood, the current study adds to the body of literature implicating this enzyme as a major contributor to tumor cell survival. In addition to conferring cisplatin resistance, ST6Gal-I-mediated receptor sialylation blocks apoptotic signaling by the Fas [8] and TNFR1 [19] death receptors, and also inhibits galectin-induced cell death [16-18]. Taken together, these results suggest that ST6Gal-I may be a promising clinical target, and that inhibition of ST6Gal-I expression or activity could be employed to sensitize tumor cells to platinum drugs, increasing therapeutic efficacy.

Conclusions

We demonstrate that ST6Gal-I expression in ovarian tumor cells confers resistance to cisplatin-mediated cell death and that cell lines selected for resistance to cisplatin strongly express ST6Gal-I. Hence, tumor cell expression of ST6Gal-I possibly contributes to chemotherapy resistance in a clinical setting. This finding points to the potential of targeting ST6Gal-I in ovarian cancer treatment and

identifies ST6Gal-I as a novel contributor to cisplatin resistance.

Competing interests
The authors have no conflicts to disclose.

Authors' contributions
MJS developed the methodology, acquired and interpreted data, and drafted the manuscript. AFS and JWW acquired data. ESS acquired data and aided in study design. CNL conceived and designed the study. SLB conceived and designed the study, developed the methodology, interpreted data, edited the manuscript, and oversaw the study. All authors have read and approved the final manuscript.

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