Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy with a poor prognosis. Gemcitabine, as a single agent or in combination therapy, remains the frontline chemotherapy despite its limited efficacy due to de novo or acquired chemoresistance. There is an acute need to decipher mechanisms underlying chemoresistance and identify new targets to improve patient outcomes. Here, we report a novel role for the ST6Gal-I sialyltransferase in gemcitabine resistance. Utilizing MiaPaCa-2 and BxPC-3 PDAC cells, we found that knockdown (KD) of ST6Gal-I expression, as well as removal of surface α2–6 sialic acids by neuraminidase, enhances gemcitabine-mediated cell death assessed via clonogenic assays and cleaved caspase 3 expression. Additionally, KD of ST6Gal-I potentiates gemcitabine-induced DNA damage as measured by comet assays and quantification of γH2AX foci. ST6Gal-I KD also alters mRNA expression of key gemcitabine metabolic genes, RRM1, RRM2, hENT1, and DCK, leading to an increased gemcitabine sensitivity ratio, an indicator of gemcitabine toxicity. Gemcitabine-resistant MiaPaCa-2 cells display higher ST6Gal-I levels than treatment-naive cells along with a reduced gemcitabine sensitivity ratio, suggesting that chronic chemotherapy selects for clonal variants with more abundant ST6Gal-I. Finally, we examined Suit2 PDAC cells and Suit2 derivatives with enhanced metastatic potential. Intriguingly, three metastatic and chemoresistant subclones, S2-CP9, S2-LM7AA, and S2-013, exhibit up-regulated ST6Gal-I relative to parental Suit2 cells. ST6Gal-I KD in S2-013 cells increases gemcitabine-mediated DNA damage, indicating that suppressing ST6Gal-I activity sensitizes inherently resistant cells to gemcitabine. Together, these findings place ST6Gal-I as a critical player in imparting gemcitabine resistance and as a potential target to restore PDAC chemoresistance.

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death in the United States with a dismal 5-year survival rate of 8% (1, 2). Extensive metastasis, rampant chemoresistance, and lack of early prognostic markers are the major causes of patient mortality. The current standard of care chemotherapy regimen remains gemcitabine alone or in combination with other drugs, particularly nab-paclitaxel (3). There has also been an emergence of additional drug combinations such as FOLFIRINOX; however, most patients are not suitable for this therapy due to its high toxicity (4, 5). Given that inherent or acquired resistance to gemcitabine is one of the chief contributors to PDAC lethality, an understanding of the molecular mechanisms regulating tumor cell response to this drug is of paramount importance.

Gemcitabine, a prodrug, mediates cytotoxicity through inducing robust DNA damage (6, 7). Gemcitabine is taken up by cells and metabolized sequentially to a mono-, di-, and triphosphate form. The triphosphate form (dFdCTP) is a cytidine analog that is incorporated into DNA, causing masked chain termination. Gemcitabine diphosphate (dFdCDP) is an anti-metabolite that inhibits ribonucleotide reductase (RRM1/RRM2), a key enzyme responsible for maintaining the dNTP pool. With dFdCDP hindering the availability of normal dNTPs, the cell is forced to insert dFdCTP into its growing DNA strand, enhancing DNA damage. PDAC resistance to gemcitabine can occur through multiple mechanisms including alterations in gemcitabine metabolism or activation of DNA repair pathways such as the DNA damage response (DDR).

Although much attention has centered on drug metabolism and DDR, there is substantial evidence that cell surface glycoproteins are critical regulators of the cellular response to gemcitabine. For example, surface transporters including hENT1 and hCNT1 control gemcitabine uptake (6, 8, 9), whereas receptors such as EGFR activate pivotal signaling cascades that influence DNA damage and cell survival (10, 11). In the present investigation, we describe a novel gemcitabine resistance...
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mechanism involving altered glycosylation of surface proteins. Specifically, our studies suggest that an increase in surface α2–6 sialic acids, a modification added by the ST6Gal-I sialyltransferase, confers protection against gemcitabine by attenuating DNA damage. ST6Gal-I is the primary enzyme that α2–6 sialylates N-glycosylated proteins destined for the plasma membrane. ST6Gal-I expression is up-regulated in a wide range of cancers (for reviews, see Refs. 12–14), and high expression correlates with poor patient outcomes in ovarian, pancreatic, colon, and breast cancer (15–18). Moreover, ST6Gal-I up-regulation in tumor cells imparts a cancer stem cell (CSC)-like phenotype evidenced by tumor spheroid growth (15); cell invasiveness (19–21); tumor-initiating potential in limiting dilution assays (15); and resistance to cytotoxic stimuli including cisplatin (22), docetaxel (23), radiation (24), and serum starvation (25). ST6Gal-I activity is also necessary for TGF-β-mediated epithelial to mesenchymal transition (26), a cornerstone of malignant disease progression. Consistent with conferring a CSC phenotype, ST6Gal-I activity promotes tumor development and progression in various animal models (15, 16). In one such study, mice engineered with intestine-specific ST6Gal-I overexpression were shown to have greater tumor incidence and burden when subjected to the azoxymethane/dextran sulfate sodium inflammatory model of colon carcinogenesis (15).

Previously, we determined that PDAC cells with high ST6Gal-I expression had reduced gemcitabine-mediated cell death (15); however, the mechanism underlying this protection was not defined. In the current report, ST6Gal-I is shown to attenuate cytotoxicity by blocking gemcitabine-induced DNA damage. Knockdown of ST6Gal-I expression in MiaPaCa-2, BxPC-3, and S2-013 PDAC cell lines potentiates DNA damage by gemcitabine as measured by comet assays and quantification of γH2AX. Removal of surface α2–6 sialic acids by neuraminidase treatment mimics the effect of ST6Gal-I knockdown by sensitizing cells to drug cytotoxicity, supporting a role for ST6Gal-I-mediated sialylation in gemcitabine resistance. ST6Gal-I knockdown also alters the expression of genes involved in gemcitabine metabolism. Finally, cells that acquire stable resistance following chronic gemcitabine treatment have up-regulated endogenous ST6Gal-I. Taken together, these studies implicate ST6Gal-I as a potential therapeutic target to combat gemcitabine resistance in PDAC.

Results

Knockdown of ST6Gal-I enhances cytotoxic response to gemcitabine

ST6Gal-I expression was stably knocked down in MiaPaCa-2 and BxPC-3 PDAC cell lines using lentiviral shRNA transduction. Successful knockdown (KD) was determined by immunoblotting, which showed decreased ST6Gal-I levels in KD cells as compared with control cells transduced with an empty lentiviral vector (EV) (Fig. 1, A and B). Reduced ST6Gal-I activity in KD cells was confirmed by flow cytometric analyses of cells stained with SNA (Fig. 1, C and D), a lectin that binds specifically to surface α2–6 sialic acids. To evaluate the role of ST6Gal-I in chemoresponse, MiaPaCa-2 and BxPC-3 EV and KD cells were exposed to gemcitabine for 72 h, and then cells were plated for clonogenic assays. Replicate wells were seeded with untreated (UT) cells to control for differences in seeding density and to enable calculation of the survival fraction. For both the MiaPaCa-2 and BxPC-3 lines, gemcitabine-treated EV cells had greater colony-forming potential than KD cells as depicted by an increased number of colonies post-treatment (representative images in Fig. 1, E and F). Quantification of the survival fraction confirmed enhanced survival of EV cells (Fig. 1, G and H).

Removal of surface sialylation sensitizes ST6Gal-I–expressing cells to gemcitabine-induced cell death

To more directly link surface sialylation to drug cytotoxicity, gemcitabine-induced cell death was monitored in MiaPaCa-2 cells pretreated with the Arthrobacter ureafaciens neuraminidase. This neuraminidase cleaves both α2–6 and α2–3 sialic acids by neuraminidase treatment mimics the effect of ST6Gal-I knockdown by sensitizing cells to drug cytotoxicity, supporting a role for ST6Gal-I-mediated sialylation in gemcitabine resistance. ST6Gal-I knockdown also alters the expression of genes involved in gemcitabine metabolism. Finally, cells that acquire stable resistance following chronic gemcitabine treatment have up-regulated endogenous ST6Gal-I. Taken together, these studies implicate ST6Gal-I as a potential therapeutic target to combat gemcitabine resistance in PDAC.
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| Mean Fluorescence Intensity (MFI) | EV UT | EV + neura | EV % decrease | KD UT | KD + neura | KD % decrease |
|-----------------------------------|-------|------------|---------------|-------|------------|---------------|
| SNA (α2-6)                        | 304   | 101        | 67%           | 189   | 118        | 38%           |
| MAA (α2-3)                        | 104   | 57         | 45%           | 98    | 55         | 44%           |

Figure 2. Removal of surface sialic acids reverses the protective effect of ST6Gal-I on gemcitabine-induced cell death. A, mean fluorescence intensity (MFI) values for cells stained with either SNA or MAA lectin. Lectin staining was conducted on UT cells or cells incubated with A. ureafaciens neuraminidase (neura) for 30 min. B, EV and KD cells were treated with or without gemcitabine (Gem) for 24 h and monitored for cleavage (activation) of caspase 3. C, cells were pretreated with neuraminidase for 30 min and then incubated with gemcitabine for 24 h. Immunoblots of cleaved caspase 3 show that neuraminidase treatment sensitized EV, but not KD, cells to gemcitabine. D, cells were incubated with anti-TNFR1 function–blocking antibody and gemcitabine for 24 h. The TNFR1-blocking antibody potentiated gemcitabine-induced cell death and eliminated differences in EV and KD apoptotic responses. inhib, inhibitor.

acids; however, it is reported that enriched cleavage of α2–6 sialic acids can be achieved by limiting treatment times to <90 min (27). Accordingly, EV and KD cells were treated with neuraminidase for 30 min, and then levels of α2–6 or α2–3 sialylation were measured by staining cells with SNA or MAA (MAA lectin detects α2–3 sialic acids). Mean fluorescence intensity values presented in Fig. 2A show that for the UT populations KD cells had lower SNA staining than EV cells (189 versus 304), consistent with ST6Gal-I knockdown, whereas MAA staining was comparable (98 versus 104). Following neuraminidase treatment, SNA labeling of EV cells was decreased by 67%, whereas SNA reactivity of KD cells was reduced by only 38%, and the residual levels of α2–6 sialylation after treatment were similar (101 versus 118). Neuraminidase treatment had an equivalent effect on MAA levels for EV and KD cells, reducing staining by 45 and 44%, respectively.

Having established that neuraminidase treatment was effective at reducing surface sialylation, we next evaluated gemcitabine-induced apoptosis. In Fig. 2B, substantially greater activation (cleavage) of caspase 3 was apparent in KD compared with EV cells upon exposure to gemcitabine. Significantly, pre-treatment of EV cells with neuraminidase markedly increased gemcitabine-induced caspase 3 activation, suggesting that removal of surface sialylation reversed the protective effects of ST6Gal-I activity (Fig. 2C). In contrast, neuraminidase treatment had a negligible effect on apoptosis in KD cells. To further link ST6Gal-I activity to receptor sialylation, we assessed the potential role of a known ST6Gal-I substrate, the TNFR1 death receptor (28), in the differential response of EV and KD cells to gemcitabine. Notably, our group has previously determined that ST6Gal-I–mediated sialylation of TNFR1 switches downstream signaling toward survival rather than apoptosis (28, 52). Cells were incubated for 24 h with a TNFR1-blocking antibody in combination with gemcitabine, and then cell lysates were immunoblotted for cleaved caspase 3. As shown in Fig. 2D, inhibition of TNFR1 activity dramatically increased gemcitabine-induced caspase activation and eliminated the differences in apoptosis between EV and KD cells. These data hint that sialylated TNFR1 may play some part in modulating the response to gemcitabine, although further studies will be needed to fully understand the intersection between TNFR1 signaling and gemcitabine-mediated cell death. We also evaluated the possible involvement of another ST6Gal-I substrate, EGFR (29), a receptor known to regulate DNA damage pathways (10, 11). Cells were treated for 24 h with the EGFR tyrosine kinase inhibitor erlotinib in combination with gemcitabine. However, in contrast to the TNFR1-blocking antibody, erlotinib had no effect on gemcitabine-induced caspase 3 activation (data not shown).

ST6Gal-I knockdown alters the mRNA expression of gemcitabine metabolic genes

The expression levels of gemcitabine metabolic genes have been used as a surrogate marker for cell sensitivity to gemcitabine. However, no single gene has emerged as a reliable indicator of gemcitabine efficacy. Conversely, several algorithms have been developed that integrate the expression of multiple gemcitabine metabolic genes into a ratio. As one such example, Nakano et al. (30) devised the “gemcitabine sensitivity ratio” (GSR), which measures the relative mRNA levels of four key metabolic genes, hENT, DCK, RRM1, and RRM2 (see “Experimental procedures” for the formula). High GSR values are associated with greater sensitivity to gemcitabine-mediated cell killing. We adopted this ratio to assess the effect of ST6Gal-I activity on metabolic genes that regulate the gemcitabine response. MiaPaCa-2 and BxPC-3 EV and KD cells were treated with or without gemcitabine for 24 h, and mRNA levels for hENT1, DCK, RRM1, and RRM2 were quantified by qRT-PCR. GSR values were subsequently calculated. As shown in Fig. 3, A and B, a significant increase in GSR was observed in gemcitabine-treated KD cells as compared with gemcitabine-treated EV cells, indicating enhanced gemcitabine sensitivity.

ST6Gal-I knockdown enhances gemcitabine-induced DNA damage

Gemcitabine-mediated cytotoxicity is due to DNA damage potentiated by masked chain termination and replication fork halting. We therefore utilized two DNA damage assays, the alkaline comet assay and quantification of γH2AX foci, to
assess the DNA damage status of MiaPaCa-2 and BxPC-3 cells upon gemcitabine treatment. When subjected to a 24-h gemcitabine treatment, ST6Gal-I KD cells in both cell lines presented enhanced DNA damage as indicated by extended tail length (Fig. 4, A and B) as well as a significant increase in tail moment quantified by comet assay (Fig. 4, C and D).

We further corroborated increased DNA damage in KD cells by quantifying γH2AX foci. γH2AX marks DNA damage sites and aids in the assembly of repair proteins. Formation of γH2AX foci is an early event in DDR, and hence we chose an earlier time point for gemcitabine treatment (6 h) to quantify γH2AX. Gemcitabine-treated cells were stained with an anti-γH2AX antibody, and after quantifying the number of γH2AX foci per cell, the cells were binned into three categories representing 1) a low amount of DNA damage (<5 foci/cell), 2) moderate damage (5–15 foci/cell), or 3) severe damage (>15 foci/cell) (representative images in Fig. 5A). MiaPaCa-2 and BxPC-3 KD populations had a significantly increased percentage of cells with severe gemcitabine-induced DNA damage but a reduced percentage of cells with low damage relative to EV cell lines (Fig. 5, B–E).

**Chronic gemcitabine treatment enriches for ST6Gal-I expression and reduces GSR**

We hypothesized that if ST6Gal-I activity is important for chemoresistance then resistant cells should express elevated levels of endogenous ST6Gal-I. To investigate this hypothesis, we generated MiaPaCa-2 cell lines with stable resistance to gemcitabine. Cells were exposed to continuous gemcitabine treatment, leading to the outgrowth of a resistant population. Cells were termed gemcitabine-resistant when they could replicate in gemcitabine-containing medium without any noticeable cell death. Two independent resistant lines were created by growing cells in either 50 or 100 nM gemcitabine. As shown in Fig. 6A, a dose-dependent increase in ST6Gal-I abundance was observed in the resistant lines as compared with parental MiaPaCa-2 cells. Cells exposed to 50 nM gemcitabine had slightly elevated ST6Gal-I, whereas robust enrichment was noted in the cell line exposed to 100 nM. The resistant lines were then subjected to qRT–PCR to quantify expression of gemcitabine metabolic genes for calculation of the GSR. Although no major difference was observed in the GSR for the 50 nM line (not shown), the 100 nM line had a significantly reduced GSR relative to treatment-naïve parental cells, consistent with decreased sensitivity to gemcitabine (Fig. 6B). We hypothesize that increased levels of ST6Gal-I in stably resistant populations reflect gemcitabine-induced selection for clonal variants with higher ST6Gal-I expression. Supporting this concept, short-term gemcitabine treatment (100 nM for 24–48 h) did not increase ST6Gal-I levels (Fig. 6C), suggesting that gemcitabine does not directly induce ST6Gal-I expression. Furthermore, the up-regulation of ST6Gal-I in gemcitabine-resistant lines is consistent with our prior work pointing to selective expansion of high ST6Gal-I–expressing clones upon exposure to other cytotoxic stressors such as cisplatin, serum deprivation, and anchorage-independent spheroid growth (15, 22, 25).

**ST6Gal-I knockdown is effective in sensitizing highly metastatic, chemoresistant cells to gemcitabine**

We next examined ST6Gal-I expression in the Suit-2 PDAC cell line along with three metastatic Suit2 clonal derivatives. The Suit2 line is one of the few cancer lines with undetectable endogenous ST6Gal-I expression. Interestingly, all three of the metastatic Suit2 variants displayed a pronounced up-regulation in ST6Gal-I (Fig. 7A), consistent with the premise that ST6Gal-I–expressing clones may be selected for during the process of tumor progression. The S2-CP9 line was generated by multiple rounds of *in vivo* selection to obtain a subclone with selective metastasis to the lung (31), whereas the S2-LM7AA subclone was similarly developed for liver-selective metastasis (32). The S2-013 derivative was isolated following soft agar culture (33) and subsequently shown to have greater capacity for lung metastasis (34). In tandem with increased metastatic potential, the S2-LM7AA and S2-013 subclones are significantly more resistant to gemcitabine than parental Suit2 cells (see supplementary results reported in Ref. 32). To evaluate ST6Gal-I’s role in regulating the gemcitabine sensitivity of chemoresistant cells, we knocked down ST6Gal-I expression in the S2-013 subclone. Immunoblot and SNA analyses confirmed reduced ST6Gal-I expression and activity in the KD population (Fig. 7, B and C). The EV and KD lines were then treated with gemcitabine, and reduced cell viability was apparent in KD versus EV cells as measured by increased caspase 3 cleavage and decreased colony-forming potential (Fig. 7, D–F). Additionally, elevated DNA damage was evident in the KD population post-gemcitabine treatment as shown by significantly increased tail moment assessed by comet assay (Fig. 8, A and B) and an increased number of cells with greater than 15 γH2AX foci per cell, indicating severe damage (Fig. 8, C and D). These data suggest that suppressing ST6Gal-I expression in highly metastatic and inherently chemoresistant PDAC cells is effective in restoring response to gemcitabine.

**Discussion**

The literature is replete with studies implicating DDR proteins in gemcitabine resistance, and thus these proteins have been targeted to restore chemoresponsiveness. Both pharmacologic and RNAi strategies have been used to inhibit proteins involved in homologous combination; however, these have yielded inconsistent results (for a review, see Ref. 35). Given the
high mortality of PDAC for which chemoresistance is a major factor (36), there is a pressing need to identify novel molecular vulnerabilities that can be exploited to increase the efficacy of drug therapies.

Studies herein identify ST6Gal-I, a tumor-associated sialyl-transferase, as a new molecular player that dampens DNA damage caused by gemcitabine. ST6Gal-I contributes to malignant cell behavior by modulating the α2–6 sialylation, and thereby function, of select surface receptors including certain growth factor receptors, death receptors, and integrins (19, 28, 29, 37–40). In this manner, ST6Gal-I acts as a master regulator of cell phenotype. Here, we show that suppressing ST6Gal-I expression in multiple PDAC cell lines including metastatic and chemoresistant S2-013 cells augments gemcitabine toxicity by increasing DNA damage. Additionally, we developed cell lines with stable resistance to gemcitabine and determined that the resistant lines have elevated levels of ST6Gal-I relative to the parental, gemcitabine-naïve population. This finding is in accordance with our prior work in which ST6Gal-I expression was measured in gemcitabine-treated mice harboring PDAC patient-derived tumor xenografts (PDXs). Following gemcitabine treatment, the residual PDX tumors displayed an increased number of ST6Gal-I–positive PDAC cells as compared with pair-matched PDX tumors from control (saline-treated) mice (15). Hence, chronic gemcitabine treatment may exert selective pressure that enriches for clonal variants with high endogenous ST6Gal-I.

Significantly, selection for PDAC cells with more abundant ST6Gal-I also appears to occur during metastatic progression. Three metastatic subclones derived from the Suit2 PDAC line (S2-CP9, S2-LM7AA, and S2-013) displayed markedly up-regulated ST6Gal-I relative to parental Suit2 cells. Consistent with this result, metastatic lesions from ovarian cancer patients have higher levels of ST6Gal-I than primary tumors (15). Supporting a causal role for ST6Gal-I in tumor progression, ST6Gal-I knockdown in MiaPaCa-2 cells inhibited the formation of subcutaneous tumors as well as lymph node metastases (15).

Further research will be needed to elucidate the mechanism by which ST6Gal-I protects against DNA damage. One possible pathway may be through sialylation-dependent changes in the activity of cell surface drug transporters; although of note, we did not observe differential α2–6 sialylation of the two principal gemcitabine uptake transporters hCNT1 and hENT1 (data not shown). As a second plausible mechanism, ST6Gal-I may sialylate surface receptors that control intracellular signaling cascades that impinge on apoptosis, DNA damage, or gemcitabine metabolism. For example, ST6Gal-I–mediated sialylation modulates the function of the EGFR and TNFR1 receptors (28, 29, 37), which are major regulators of AKT and NF-κB signaling cascades, respectively. EGFR, AKT, and NF-κB all play well documented roles in PDAC susceptibility to gemcitabine (41–45). Intriguingly, our pilot experiments suggested that blocking the activity of TNFR1, but not EGFR, amplified gemcitabine-induced apoptosis and eliminated the protective effect of ST6Gal-I. In other studies by our group, ST6Gal-I–mediated sialylation of TNFR1 has been shown to potentiate NF-κB activation as well as downstream survival signaling (52). Hyperactivation of NF-κB is strongly implicated in PDAC chemoresistence. Gemcitabine-resistant cells have higher levels of activated NF-κB than -sensitive cells (44), and pharmaco-

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**Figure 4. Gemcitabine-treated ST6Gal-I KD cells have increased DNA damage as measured by comet assay.** Representative images depict increased tail lengths in MiaPaCa-2 KD (A) and BxPC-3 KD (B) cells. MiaPaCa-2 KD (C) and BxPC-3 KD (D) cells treated with gemcitabine (Gem) had a significantly increased tail moment compared with gemcitabine-treated EV cells. Graphs depict results from three independent experiments. Error bars represent S.E. * denotes p < 0.05 as evaluated by Student’s t-test.
logic as well as genetic inhibitors of NF-κB increase the efficacy of gemcitabine-induced tumor cell killing both in vitro and in vivo (44–47). These observations hint at a potential ST6Gal-I/TNFR1/NF-κB axis in gemcitabine resistance; however, future studies will be required to definitively test this hypothesis. Nonetheless, regardless of the specific surface proteins regulated by α2–6 sialylation, ST6Gal-I clearly had some influence on intracellular pathways because manipulating ST6Gal-I expression significantly altered the expression of gemcitabine metabolic genes as evidenced by changes in the GSR. The GSR integrates the expression levels of four primary gemcitabine metabolic genes (hENT1, DCK, RRM1, and RRM2) to provide a predictive measure of gemcitabine response. Cells with ST6Gal-I knockdown had a higher GSR, reflecting greater gemcitabine sensitivity, and furthermore, stably resistant cells had elevated ST6Gal-I expression along with significantly increased GSR.

ST6Gal-I may also modulate gemcitabine response by reprogramming tumor cells into CSCs. CSCs are notoriously resistant to multiple chemotherapeutics in part through up-regulation of drug efflux proteins such as the ATP-binding cassette family of transporters (48). Previously, we determined that ST6Gal-I activity imparts hallmark CSC characteristics including anchorage-independent spheroid growth; tumor-initiating potential; resistance to growth factor withdrawal; and, of relevance to this study, resistance to cisplatin (15, 22, 25).

**Figure 5. Greater DNA damage in KD versus EV cells as indicated by γH2AX foci.** Cells were stained with anti-γH2AX antibody, and the number of foci per cell was counted. Cells were grouped into categories of low DNA damage (<5 foci), moderate DNA damage (5–15 foci), or severe DNA damage (>15 foci). A, representative images of the three categories. Images (B) and quantification (C) of γH2AX foci in MiaPaCa-2 cells are shown. Increased DNA damage in gemcitabine (Gem)-treated KD versus EV cells is demonstrated by a significantly increased number of cells with severe damage and a decreased number of cells with low damage. Images (D) and quantification (E) of γH2AX foci in BxPC-3 show greater DNA damage in gemcitabine-treated KD versus EV cells. Graphs depict results from three independent experiments. Error bars represent S.E. * denotes p < 0.05 as measured by Student’s t test.
with conferring CSC behaviors, ST6Gal-I activity was shown to up-regulate critical stem cell–associated transcription factors such as Sox9 and slug (15). Indeed, Sox9 overexpression protects cells against DNA damage and increases cell survival following exposure to genotoxic stress (49). Sox9 is also one of the central drivers of PDAC initiation (50).

The collective results presented in this report add to the body of evidence pointing to an important function for ST6Gal-I mediated sialylation in tumor cell resistance to chemotherapy. Despite the decades-long knowledge that tumor cells display altered glycosylation and, in particular, elevated sialylation, the overall role of the tumor glycome in regulating cell phenotype and response to cytotoxic drugs remains strikingly underinvestigated. The current study highlights a novel glycosylation-dependent mechanism that confers gemcitabine resistance by preventing DNA damage.

**Experimental procedures**

**Cell culture**

MiaPaCa-2 and BxPC-3 cell lines were obtained from ATCC. Suit-2 cells and the S2-CP9 and S2-013 derivatives were donated by Dr. Michael A. Hollingsworth at the University of Nebraska (Omaha, NE). The S2-LM7AA subclone was developed by Buchsbaum and co-workers (32) at the University of Alabama (Birmingham, AL). Cells were grown in DMEM with 10% FBS and antibiotic/antimitotic supplements (Gibco, catalog number 15240-062). For acute gemcitabine treatments (24–72 h), cells were serum-starved for 4 h prior to treatment with 200 nM gemcitabine (Selleck Chemicals, catalog number S1149) for MiaPaCa-2 and Suit2-013 cells, whereas 400 nM gemcitabine was used for BxPC-3 cells (as they appear to be more inherently resistant). For the generation of stably resistant cell lines, cells were treated with gemcitabine for 2 months (intermittent treatment until cells replicated in gemcitabine without any noticeable cell death). To create stable ST6Gal-I KD lines, cells were transduced with lentiviral vectors expressing shRNA directed against ST6Gal-I (Sigma). Control cells were transduced with an EV. Polyclonal stable lines were isolated following puromycin selection. Successful KD was confirmed by immunoblotting for ST6Gal-I (R&D Systems, goat polyclonal, catalog number AF5924, lot number CDSF0114101) as well as flow cytometric analyses of cells stained with FITC-conjugated SNA lectin (Vector Laboratories), which binds specifically to 2–6–linked sialic acids. Cells were stained for 40 min at 4 °C with a 1:200 dilution of SNA-FITC.

**Neuraminidase treatment**

Neuraminidase from *A. ureafaciens* was utilized for this treatment (Sigma, catalog number 10269611001). Cells were treated with neuraminidase for 30 min, and then loss of surface 2–6 sialylation was confirmed by SNA flow cytometry as discussed earlier. Changes in surface 2–3 sialylation were determined by staining with MAA-FITC lectin, which binds specifically to 2–3 sialic acid. Cells were stained with the respective lectins for 40 min at 4 °C using a 1:200 dilution. To assess the effects of desialylation on drug toxicity, cells were treated for 30 min with neuraminidase and then incubated with gemcitabine for 24 h. The treated cells were lysed in radioimmune precipitation assay buffer (Pierce) supplemented with protease and phosphatase inhibitors (Thermo Fisher). Protein concentration was quantified using a BCA assay kit (Pierce). Equal amounts of protein were loaded for each sample on a denaturing polyacrylamide gel and then transferred to PVDF membrane. Membranes were blotted for cleaved caspase 3 as described below.

**TNFR1 and EGFR blocking**

MiaPaCa-2 cells were incubated with TNFR1-neutralizing antibody (R&D Systems, catalog number MAB225-100) or Erlotinib (Selleck Chemicals, catalog number S1023) along with gemcitabine for 24 h. Post-treatment, protein lysates were collected to assess cell death by immunoblotting for cleaved caspase 3.

**Immunoblotting**

Cells were plated at 50 – 60% confluence for all experiments. Cells were lysed in radioimmune precipitation assay buffer containing protease and phosphatase inhibitors. Following electrophoresis and protein transfer to PVDF membranes, membranes were blocked in 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20. Blots were probed with antibodies against ST6Gal-I (R&D Systems, catalog number AF5924, lot number CDSF0114101) or cleaved caspase 3 (Cell Signaling Technology, catalog number 9661, lot number 43). Protein loading was verified using horseradish peroxidase (HRP)-conjugated anti-actin (Abcam, catalog number ab21058, lot number GR284232). Membranes were incubated with appropriate HRP-coupled secondary antibodies (anti-rabbit and anti-mouse IgG, Cell Signaling Technology; anti-goat IgG, Santa Cruz Biotechnology), and protein was detected by.
ECL (Pierce), Clarity (Bio-Rad), or SuperSignal West Femto substrate (Pierce).

Alkaline comet assay

200,000 cells/well were plated in 6-well plates overnight. Prior to gemcitabine treatment, cells were serum-starved for 4 h. Cells were subjected to gemcitabine treatment for 24 h and harvested using PBS and a cell scraper. The cell suspension was diluted in low melting agar (Trevigen, catalog number 4250-050-02) for a final concentration of 10,000 cells/ml. Agar with cells was plated on precoated comet slides (Trevigen, catalog number 4250-050-03) and incubated at 4 °C to solidify, and then slides were immersed in lysis buffer (Trevigen, catalog number 4250-050-01) overnight to remove cellular and nuclear proteins. Following lysis, cells were placed in alkaline unwinding solution (pH > 13) for 1 h at 4 °C. Post-unwinding, slides were placed in an electrophoresis chamber containing alkaline buffer (pH > 13) for 40 m at 30 V. This voltage was utilized based on the distance between the electrodes, 1 V/cm. After electrophoresis, slides were dehydrated in 70% ethanol and air-dried overnight. Prior to scoring, cells were stained with SYBR green (Invitrogen, catalog number S7563) at a 1:10,000 dilution and air-dried. Tail moment, a quantitation of DNA damage, was calculated using Comet Assay IV software. Three independent experiments were conducted, and at least 100 cells were counted per treatment condition for each individual experiment.

Figure 7. A metastatic, chemoresistant Suit-2 subclone with up-regulated ST6Gal-I can be sensitized to gemcitabine by ST6Gal-I knockdown. A, Suit-2 cells with no detectable ST6Gal-I compared with three Suit2-derived metastatic subclones, S2-CP9, S2-LM7AA, and S2-013. All of the metastatic derivatives have increased ST6Gal-I expression as measured by immunoblotting. B, ST6Gal-I was knocked down in the S2-013 line, and reduced expression was verified by immunoblotting. C, reduction in SNA staining in KD cells, indicative of reduced surface α2–6 sialylation. D, S2-013 KD cells have increased gemcitabine (Gem)-induced cell death relative to EV cells as indicated by increased expression of cleaved caspase 3. S2-013 KD cells have decreased clonogenic potential (E) and significantly reduced survival fraction (F). The graph depicts results from three independent experiments with each experiment performed in triplicate. Error bars represent S.E. * denotes p < 0.05 as measured by Student’s t test.

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Colony formation assay

The colony formation assay was performed as described previously (51). Cells were plated at 50% confluence (~1.0 × 10^6 cells in 10-cm dish) and treated with gemcitabine for 3 days. Post-treatment, cells were trypsinized and then diluted to plate 200 (MiaPaCa-2 and S2-013) or 1,000 (BxPC-3) cells/well in a 6-well plate. Untreated cells (exposed to medium lacking gemcitabine) were plated at 100 (MiaPaCa-2, S2-013) or 200 (BxPC-3) cells/well. Following plating, cells were grown in 10% serum-containing medium until colonies were visible. Colonies of more than 50 cells were counted. To facilitate detection of colonies, the cells were first fixed in 4% paraformaldehyde for 30 min and then stained in 0.5% crystal violet for 30 min. The colonies in the untreated and gemcitabine-treated groups were counted to evaluate plating efficiency and calculate the survival fraction as described by Franken et al. (51). Plating efficiency (PE) and survival fraction (SF) were calculated as follows.

$$\text{PE} = \frac{\text{No. of colonies formed}}{\text{No. of cells plated}} \quad \text{(Eq. 1)}$$

$$\text{SF} = \frac{\text{No. of colonies formed after treatment}}{\text{No. of cells plated} \times \text{PE}} \quad \text{(Eq. 2)}$$

Three independent experiments were performed with three technical replicates included for each independent experiment.

Quantification of γH2AX foci

Cells were plated in 24-well plates on sterile glass coverslips with 30,000 cells/well. Cells were serum-starved for 4 h prior to gemcitabine treatment. Following treatment, cells were fixed with 4% paraformaldehyde and incubated with γH2AX antibody (Millipore, catalog number 05-636, lot number 2806127) at a 1:200 dilution overnight at 4 °C. Following incubation with primary antibody, the slides were incubated with anti-mouse secondary antibody at a 1:400 dilution for 1 h at room temperature and counterstained with Hoechst nuclear stain at a 1:10,000 dilution for 10 s. The coverslips were mounted on slides using Prolong Gold mounting medium (Invitrogen, catalog number P36930). Cells were viewed using NIS elements software at 40× magnification. γH2AX foci were counted, and cells were divided into low, moderate, and severe damage groups based on whether they had <5, between 5 and 15, or >15 foci. 100–150 cells were counted for each treatment from three slides per condition, and data were pooled from three independent biological replicates to calculate significance by Student’s t test.

GSR calculation

For the calculation of GSR, RNA was isolated after 24-h gemcitabine treatment and subjected to qRT-PCR. The four gemcitabine metabolic genes quantified were hENT1, RRM1 and -2 (ribonucleotide reductase gene), and DCK. These four genes...
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