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The role of DNA methylation in ST6Gal1 expression in gliomas

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Abstract

The mechanism of transcriptional silencing of ST6Gal1 in gliomas has not yet been elucidated. Multiple independent promoters govern the expression of the ST6Gal I gene. Here, we investigated whether epigenetic abnormalities involving DNA methylation affect ST6Gal1 expression. Transcript-specific qRT-PCR following exposure of glioma cell lines to 5-aza-2′-deoxycytidine (5-aza-dC), a DNA methyltransferase inhibitor, resulted in the re-expression of the normally quiescent ST6Gal1 mRNA driven exclusively by the P3 promoter sequence. The P3 promoter-specific transcription start site (TSS) was delineated by primer extension and core promoter sequences and associated functional transcription elements identified by deletion analysis utilizing chloramphenicol acetyltransferase reporter constructs. Minimal promoter activity was found to reside within the first 100 bp of the TSS and maximal activity was controlled by functional AP2 binding sites residing between 400 and 500 bp upstream of the initiation site. As altered AP2 binding was not directly associated with AP2 availability, these analyses demonstrate that ST6Gal1 transcription is regulated by DNA methylation within core promoter regions, ultimately by determining critical transcription factor accessibility within these regions. Transcriptional reactivation of ST6Gal1 expression by 5-aza-dC resulted in increased cell surface α2,6 sialoglycoconjugate expression, increased α2,6 sialylation of β1 integrin, and decreased adhesion to fibronectin substrate: functional correlates of decreased invasivity. The effects of global hypomethylation are not glycome-wide. Focused glycotranscriptomic analyses of three invasive glioma cell lines following 5-aza-dC treatment demonstrated the modulation of select glycogene transcripts. Taken together, these results demonstrate that epigenetic modulation of ST6Gal1 expression plays a key role in the glioma phenotype in vitro and that that therapeutic approaches targeting elements of the epigenetic machinery for the treatment of human glioblastoma are warranted.

Key words: DNA methylation, epigenetics, promoter, ST6Gal1, transcription

Introduction

The most fundamental clinical hallmark of malignant gliomas is their ability to invade normal brain tissue and the underlying mechanisms are exceedingly complex and involve multiple interwoven, yet coordinated intra- and extracellular processes (reviewed in Ortensi et al. 2013; Xie et al. 2014; Paw et al. 2015). As such, there are many targetable invasion-related molecules that may have benefit in anti-invasive therapeutic approaches for gliomas. It is well established that virtually all types of human cancers have altered patterns of glycosylation and express aberrant cell-surface glycans. While there is no direct evidence that tumor-associated glycans are oncogenic, a significant number of studies have shown that these carbohydrates

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play key functional roles in tumor progression, invasion, and metastasis (reviewed in Hakomori 2002; Fuster and Esko 2005; Moskal et al. 2009; Stowell et al. 2015).

The effects of modulating glycosyltransferase expression in human glioma cell lines on tumor cell growth, adhesion, and invasivity in both in vitro and in vivo models have also been evaluated and represents a novel approach that has significant therapeutic potential that will also give new insights into the mechanisms of how glycoconjugates influence glioma invasivity (reviewed in Moskal et al. 2009). We have currently analyzed over 200 resected primary human gliomas, all of the available human glioma cell lines, as well as a panel of 24 human glioblastoma xenografts (Giannini et al. 2005), and have never detected measurable ST6 β-galactosamide α2,6-sialyltranferase 1 gene (ST6Gal1) expression. We have found that expressing the normally quiescent ST6Gal1 gene displayed marked inhibition of invasivity in vitro (Yamamoto et al. 1997) and tumorigenesis in vivo (Yamamoto et al. 2001). Our results also showed alterations in integrin sialylation and signal transduction processes (Kroes and Moskal 2013); both associated with regulation of cellular adhesion, migration and glioma cell invasivity. Additionally, a significant increase in sensitivity to apoptotic-inducing chemotherapeutics was observed (Dawson et al. 2004). An adenoviral vector expressing ST6Gal1 was constructed and shown to inhibit tumor formation both in vitro and in vivo (Moskal et al. 2009).

Regulation of ST6Gal1 expression is the result of regulation of a single gene by multiple and distinct promoter regions. At least three major mRNA isoforms are generated in a tissue-specific manner through the use of alternate promoters and a differential assemblage of 5' untranslated sequences (Svensson et al. 1990; Wang et al. 1990). The transcription of these isoforms not only allows for quantitative transcriptional regulation of ST6Gal1 expression, but also potentially at the translational level (Dall'Olio 2000). Several post-translational modifications, including glycosylation, phosphorylation, proteolytic cleavage, and dimerization, affecting enzyme activity have also been described (Weinstein et al. 1987; Ma and Colley 1996; Ma et al. 1997; Ma et al. 1999; Chen and Colley 2000). These data provide the picture of an enzyme encoded by a single gene whose expression is precisely regulated in a tissue and stage-specific manner, owing to the existence of multiple regulatory mechanisms. However, despite the clear, unambiguous importance of ST6Gal1 in the control of glioma cell invasivity, virtually nothing is known about the regulation of ST6Gal1 in the brain.

It has long been well established that DNA methylation is essential for maintaining gene expression states (Maraschio et al. 1988; Trasler et al. 1996; Chen et al. 1998; Hansen et al. 1999; Okano et al. 1999; Xu et al. 1999; Gonzalo et al. 2006). Patterns of methylation are universally disrupted in cancers and play a critical role in tumorigenesis and patient prognosis, with aberrant hypermethylation of CpG islands in promoter regions typically associated with gene silencing (Costello and Plass 2001; Baylin and Bestor 2002; Jones and Baylin 2002; Costello 2003; Feinberg 2005). These studies, however, have primarily focused on a relatively small number of the estimated 30,000 CpG islands in the genome (Antequera and Bird 1993). Concurrent with this hypermethylation at CpG islands, high grade glioblastomas (GBMs) exhibit a net decrease in DNA methylation genome-wide (Feinberg and Vogelstein 1983; Gama-Sosa et al. 1983; Feinberg et al. 1988; Gaudet et al. 2003; Feinberg and Tycko 2004). Genes involved in the regulation of cell migration and invasion are recurring targets for epigenetic silencing, and include genes such as *MGMT* (Esteller et al. 2000), *TIMP3* (Bachman et al. 1999), *EMP3* (Alaminos et al. 2005), cystatin E/M (CST1) (Qiu et al. 2008), and *BEX1*, *BEX2* (Foltz et al. 2006), and *syk* tyrosine kinase (reviewed in Martinez and Schackert 2007). Such silencing is frequently associated with longer survival of newly diagnosed glioblastoma patients and is also an indicator of increased survival in patients treated with radiation plus the alkylating agent temozolomide (Hegi et al. 2005).

Inhibitors of DNA methylation, such as 5-aza-2'-deoxycytidine (5-aza-dC), have been demonstrated to induce the re-expression of epigenetically silenced tumor suppressor genes and restore cell growth control or induce apoptosis (Bender et al. 1998). There have been several published reports describing epigenetic regulation of select glyco-related genes (reviewed in Trinchera et al. 2014). In one of the most noteworthy, Kawamura et al. (2008) demonstrated transcriptional responses of a panel of 12 glycogenes in a panel of gastric and colon carcinoma cell lines and clinical specimens to demethylation by treatment with 5-aza-dC. Their results suggest that epigenetic control of glycogene expression in tumors does not occur in a random manner, but in defined subsets. Taken together with our focused microarray data demonstrating that the majority of glycogenes differentially expressed in GBMs (> 70%) were significantly downregulated (Kroes et al. 2007), epigenetic changes may be among the primary mechanisms of tumor-associated glycogene silencing and resultant modulation in cell surface glycoconjugate expression.

As no information regarding the role of DNA methylation of glycogenes in gliomas currently exists, we focused our initial analyses on the human ST6Gal1 gene as a representative glycogene that has been demonstrated to be critically involved in modulating the invasive phenotype of GBM. Antony et al. (2014) have demonstrated the role of altered methylation in the silencing of ST6Gal1 expression in human bladder tumors. Defining the mechanism by which aberrant DNA methylation specifically alters the expression of ST6Gal1 in gliomas is a critical first step to addressing the overarching hypothesis that aberrant CpG island methylation and gene silencing of a subset of the overall tumor glycome is sufficient to endow glioma cells with altered migratory and invasive properties.

Results

ST6Gal1 promoter predictions

As nothing is known about the methylation status of the ST6Gal1 gene, we analyzed the genomic sequences ± 5 kb from the transcription start site of the three predicted promoters (Figure 1A and Dall'Olio et al. 2004). These sequences were derived from the Ensembl database and analyzed for CpG content (via MethPrimer software). Although there were many CpG-rich regions in each promoter, only five of these met the criteria for bonafide CpG islands (Figure 1B). All of these CpG islands exist within the P3 promoter. There are five islands, two within 650 bp upstream, and two within 900 bp downstream, with the major CpG island spanning the entire first exon (Exon Y) in the Form 3 transcript. However, no data exists as to which transcript is utilized in gliomas.

ST6Gal1 reactivation: transcript specificity

Which of the three alternative promoters are utilized for ST6Gal1 expression in the brain is foundational to our understanding of its regulation. Initially, to choose the optimal treatment regimen, we assessed 5-aza-dC-mediated cytotoxicity by MTT assay and global methylation using a MethylAmp Global DNA methylation kit in all

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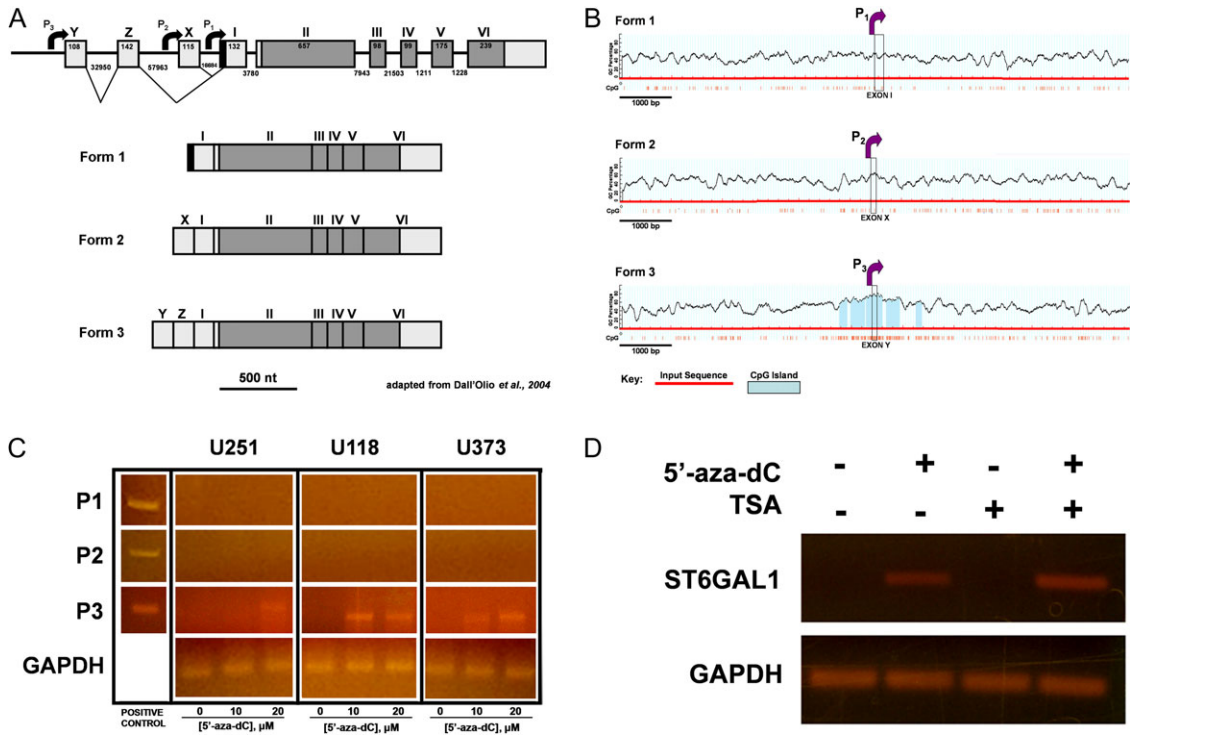

Fig. 1. Predicted CpG islands in the ST6Gal1 promoters. (**A**) Genomic organization of the human ST6Gal1 gene. (**B**) Analysis of CpG content in the human P1, P2, and P3 ST6Gal1 promoters. (**C**) Transcript-specific RT-PCR analysis of ST6Gal1 reactivation in human glioma cell lines. Cells were treated with vehicle or 5-aza-dC (10 µM or 20 µM for 5 days), RNA extracted, cDNA synthesized and subjected to PCR using P1-, P2-, and P3-specific primers (Table I). Human hepG2, Raji, and HT-29 cell lines were used as positive controls for transcript forms 1, 2, and 3, respectively. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serves as a cDNA template control. (**D**) U373 cells were treated with vehicle, 5′-aza-dC (20 mM for 5 days), trichostatin A (TSA, 1 mM for 5 days), or both, as shown. P3 transcript expression was assessed by RT-PCR analysis. This figure is available in black and white in print and in color at *Glycobiology* online.

of the three glioma cell lines used in these studies. We then treated U251, U118, and U373 glioma cells with either vehicle or noncytotoxic doses of 5-aza-dC (10 and 20µM for 5 days) and ST6Gal1 transcript-specific RT-PCR was performed as previously detailed (Dall'Olio et al. 2004). The expression of ST6Gal1 was transcriptionally reactivated by pharmacological manipulation of cytosine methylation by 5-aza-dC and it is clear that this re-expression is driven primarily by the P3 promoter (Figure 1C). Modulation of histone acetylation by trichostatin A (TSA), either alone or in combination with 5-aza-dC, has no effect on this reactivation (Figure 1D). Taken in concert with the predicted methylation data presented above, these data strongly suggest that it is the methylation of the P3 promoter driving the re-expression of ST6Gal1 in the drug treated cells. We subsequently chose to focus our detailed analyses on the U373 cell line.

P3 promoter characterization I: transcription start site determination

For the P3 promoter, the transcription start site (TSS) in U118, U251, and U373 cells was identified via primer extension using two individual [γ-^{32}P]-labeled antisense primers which correspond to the cDNA regions 100 and 200 nt upstream of the intron:exon Y boundary and contiguous to the known exons using total RNA isolated from 5-aza-dC treated U118, U251, and U373 cells (see Figure 3A). The results clearly demonstrate that the TSS of the 5-aza-dC reactivated transcript in each of the three cell lines is at the precise 5' end of Exon Y (Figure 2).

P3 promoter characterization II: methylation analysis

Next, bisulfite conversion of genomic DNA extracted from U373 cells was analyzed using the EZ DNA Methylation Kit™ (Zymo Research), according to the manufacturers' specifications. We chose to use U373 cells as a representative cell line both because it is the most invasive of the glioma cell lines and the large body of literature that exists using this line. As a positive control, assays also included 5'-aza-dC-treated DNA. The P3 promoter (Figure 3A) was covered by 5 total PCR amplicons, each less than 350 bp in length to optimize yield. PCR products were subcloned, a minimum of 10 independent colonies selected and sequenced, and analyzed by Sequencher Software (v4.2, GeneCodes Corp., Ann Arbor, MI). CpG1 and CpG2 were most heavily methylated (100% and 80.8%, respectively), with little to no methylation observed in CpG3 (which contains the entire first transcribed exon), CpG4, or CpG5 (Figure 3B). As expected, treatment with 5-aza-dC totally demethylated both CpG1 and CpG2 (Figure 3C).

P3 promoter characterization: III: promoter deletion analysis

To identify sequences associated with transcriptional reactivation, we transfected U373 cells with the ST6Gal1 promoter-CAT deletion constructs and measured CAT activity at 48 h posttransfection (Figure 4A). Upstream promoter fragments from −100 bp to −400 bp produced promoter activity ~2-fold over the promoter-less pCAT-basic control. However, maximal transcriptional activation (~12-fold) resulted from the addition of heterologous promoter

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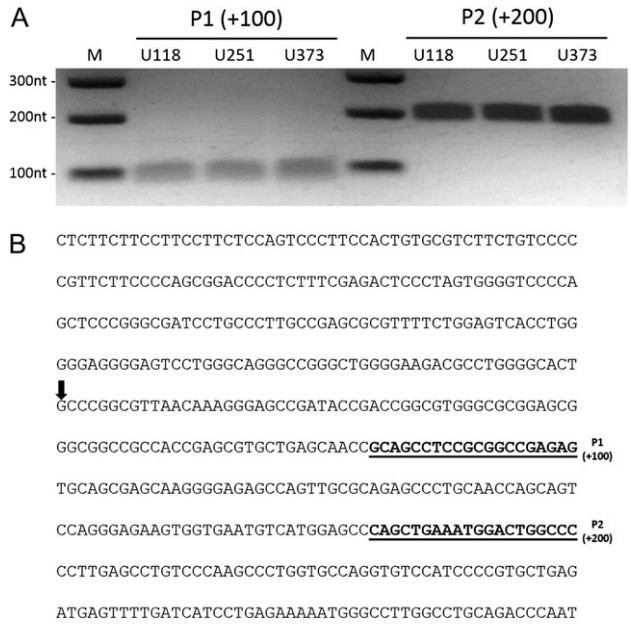

Fig. 2. Analysis of the transcriptional start site (TSS) of the reactivated P3 transcript by primer extension. (**A**) Total RNA from 5-aza-dC-treated U118, U251, and U373 cells was prepared and used for primer extension analysis. Two individual ^{32}P-labeled primers specific for the ST6Gal1 coding sequence and 50 μg template RNA were used and the AMV-RT extended products were separated on a denaturing 8% polyacrylamide/urea gel. (**B**) The sequence of the P3 mRNA transcript (RefSeq NM_173216.2) surrounding the Exon Y and the identified 5′-end of the detected primer extension product is shown by the arrow. The two primers used in the extension analysis are underlined and depicted in bold.

sequences >400 bp upstream of the TSS. From these data, we conclude that sequences within 100 bp upstream of the start of transcriptional initiation are likely associated with minimal promoter activity and required for basal ST6Gal1 expression. Sequences between −400 and −500 bp of the start site, however, are required for maximal promoter activity as well as those required for maximal epigenetic silencing of its expression by DNA methylation in gliomas.

Alterations in in vitro AP2 transcription factor binding (AP2 mutational analyses and ChIP-qPCR)

Guided by the results of the promoter deletion analyses, we surveyed the transcription factor databases specifically for methylation-sensitive transcription factor binding sites. The sole factor present within the region of the 5 CpG islands that met this criteria was AP2, with potential AP2 binding sequences identified at positions −10, −50, −480, −750, −760, −850, and −880. The direct involvement of the AP2 site located at −482 to −465 nt from the TSS (and localized near the 5' end of the ST6Gal1 promoter sequence contained in plasmid p-500) was analyzed by additional promoter deletion analyses (Figure 4B). Mutation and functional inactivation of this single site abolished transcriptional activity to levels observed in the next of the deletion series, p-400; activity that would be expected to result from essential involvement of this AP2 site. Next, the modulation of in vitro AP2 binding activity associated with maximal promoter activity in the presence of 5-aza-dC was assessed by ChIP-qPCR using formaldehyde-crosslinked, immunoprecipitated DNA isolated from control and 5-aza-dC reactivated U118, U251, and U373 cells (Figure 5). In those cells treated with 5-aza-dC, AP2 was clearly and specifically associated with the ST6Gal1 promoter sequences in this region. As the silencing of ST6Gal1 expression is not mediated at the level of AP2 availability in these cell lines (data not shown), the data are consistent with the hypothesis that transcriptional silencing of ST6Gal1 in gliomas is mediated in large part by the accessibility of critical transcription factor binding sites. This also suggests that the presence of methylated AP2 sites at −10 bp and −50 bp may be consistent with the minimal promoter activity observed in the −100 to −400 bp promoter deletion constructs. In addition, these results also suggest that the AP2 sites at −750 to −880 do not contribute to the overall efficiency of transcriptional reactivation by 5-aza-dC.

Functional consequences of reactivation of ST6Gal1 expression

Immunohistochemical analysis using FITC-conjugated SNA lectin, which recognizes terminal Gal- or GalNAc-linked α2,6-linked sialic acid, demonstrated that treatment of U373 cells with 5-aza-dC resulted in the robust *de novo* expression of cell surface α2,6-linked sialoglycoconjugates (Figure 6A). This treatment also resulted in significantly increased α2,6 sialylation of β1 integrin in all 3 glioma lines (Figure 6B) and decreased adhesion to fibronectin (Figure 6C). These data are consistent with data derived from forced, stable (Yamamoto et al. 1997) or transient, adenoviral-mediated (Moskal et al. 2009) overexpression of ST6Gal1 observed initially in U373 cells. Such overexpression has been demonstrated to mediate the inhibition of in vivo tumorigenicity demonstrated in multiple animal models of glioma formation (Yamamoto et al. 2001; Moskal et al. 2009).

Glycome silencing: transcriptomic signature analysis

In order to gain a broader understanding of how cytosine methylation changes directly influences overall glycogene expression, we used our focused glycogene microarray platform, which comprehensively queries mRNA expression of the entire human glycome, (detailed in Kroes et al. 2007) to compare glycogene transcriptome profiles in each of the 3 glioma cell lines treated with 10 μM 5-aza-dC/5 days with vehicle treated cells (Figure 7). The identification of these differentially expressed glycogenes was based on very stringent criteria for these experiments and critical to interpretation of these results. The threshold for significance was set at FDR = 0% and the level of differential expression at > 2-fold, with the threshold for "silencing" as < 2× background fluorescence. These exceptionally stringent criteria set clear and unambiguous thresholds for the level of 5-aza-dC-mediated induction or, alternatively, the level of 5-aza-dC-mediated silencing for those glycogenes already transcriptionally active. As predicted by the transcript-specific RT-PCR analysis, the ST6Gal1 transcript was significantly upregulated in all of the treated cell lines. Modulation of the expression of other glycogenes were observed in at least 2 of the 3 individual cell lines. Interestingly, these data reveal that (a) the effects of hypomethylation do not appear to be glycome-wide, (b) despite the noncytotoxic dose of 5-aza-dC used, there are also glycogenes downregulated by hypomethylation similar to that observed for the fucosyltransferases (FUT) in GI cancer (Kawamura et al. 2008). These results demonstrating a distinct and experimentally manageable subset of the glyco-transcriptome strongly support the hypothesis that a signature of methylated glycogenes associated with a measurable clinical parameter in gliomas can be identified.

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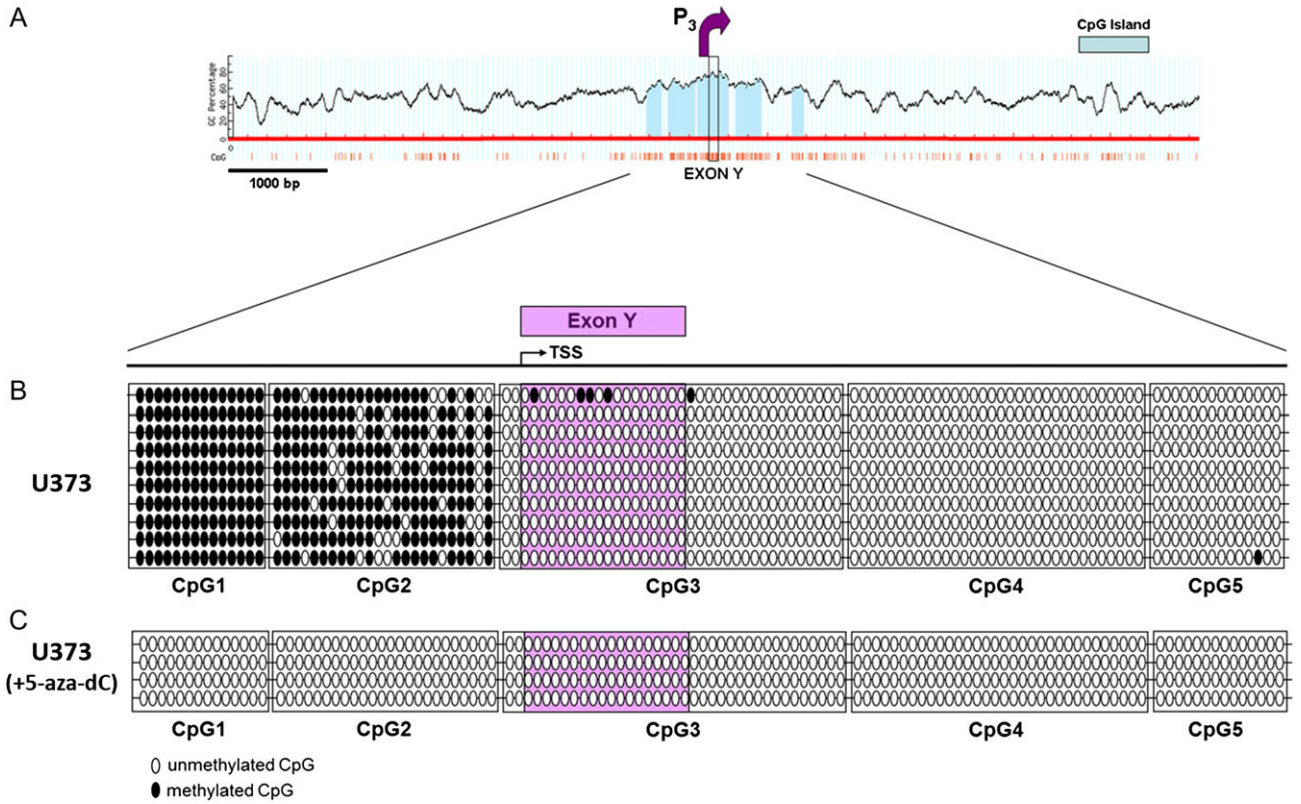

Fig. 3. Analysis of the methylation status of the ST6Gal1 promoter in U373 glioma cells. Schematic representation of the ST6Gal1 gene and bisulfite sequencing of the ST6Gal1 CpG islands. (**A**) Location of the CpG islands in reference to the transcriptional start site of the P3 transcript. (**B**,**C**) Bisulfite sequencing results from the U373 glioma cell line, both without (**B**) and following (**C**) treatment with 5-aza-dC. Each clone is represented by a row, and the CG dinucleotides being interrogated are arranged in columns. White and black circles represent unmethylated and methylated cytosines, respectively. This figure is available in black and white in print and in color at *Glycobiology* online.

Discussion

The present study analyzed the critical role of DNA methylation and in vitro transcription factor:promoter interactions in the expression of the ST6Gal1 gene in glioma cell lines. The role of epigenetic regulation of human glycogene expression is still in its infancy (reviewed in Lauc et al. 2014). Most of what is known has come from studies analyzing the methylation status of gene promoter regions in vitro (Kim and Deng 2008; Kannagi et al. 2010; Yusa et al. 2010), or concomitant with the analysis with histone modifications (Caretti et al. 2012; Kizuka et al. 2014). In our results, only the two CpG islands immediately upstream of the transcriptional start site of the ST6Gal1 gene encoding α2,6 sialyltransferase, the enzyme responsible for the terminal sialylation of N-linked glycoproteins in gliomas, were densely methylated; and this methylation was closely correlated with the transcriptional silencing of the ST6Gal1 gene. These results also showed that all of the CpG islands in the ST6Gal1 gene are not equally methylated. This is consistent with recent evidence that nonpromoter related methylation, including that within the body of the gene (Smith et al. 2007), is related to aberrant DNA methylation, chromatin conformation and gene silencing. We reported earlier (Yamamoto et al. 2001; Moskal et al. 2009) that forced overexpression of ST6Gal1 in gliomas completely inhibited tumorigenicity in in vivo models. This study is the first report demonstrating that the relationship between the glycogene methylation status and transcriptional silencing in gliomas may have clear clinical relevance.

While the use of glioma cell lines may not entirely recapitulate primary tumor biology (Li et al. 2008), some discrete features of glioma molecular biology (including DNA methylation mechanisms) may be adequately conserved in and only amenable to detailed analyses in the established cell lines (Vogel et al. 2005). However, despite these apparent limitations, mechanistic studies may still provide important insight into specific mechanisms of tumor biology and potentially lead to the development of new therapeutic strategies.

The indirect model by which methylation affects gene expression posits that transcription factors interact with unmethylated, accessible promoters but not with methylated, inaccessible promoters (Antequera et al. 1990). According to this model, methylation-related chromatin structure in the promoter of the gene may influence ST6Gal1 expression by allowing or excluding transcription factor access to relevant promoter sequences. However, just as methylated DNA sequences may act by blocking the binding of some transcription factors, they can also modulate expression of surrounding genes by enhancing the binding of proteins containing a methyl-binding domain, referred to as methyl-binding proteins (MBPs). MBPs recruit histone-modifying enzymes, including the histone deacetylases (HDAC) which modify histone proteins, resulting in a compact chromatin structure, also inaccessible to the transcriptional machinery (Klose and Bird 2006; Deaton and Bird 2011). As the reactivation of ST6Gal1 expression was unaffected by treatment with trichostatin A, either alone or together with 5-aza-dC, HDAC-mediated modulation of chromatin structure per se appears to play little role in the epigenetic regulation of ST6Gal1.

While some work regarding the precise transcription initiation site of the P3 transcript has been described in other tissues, the

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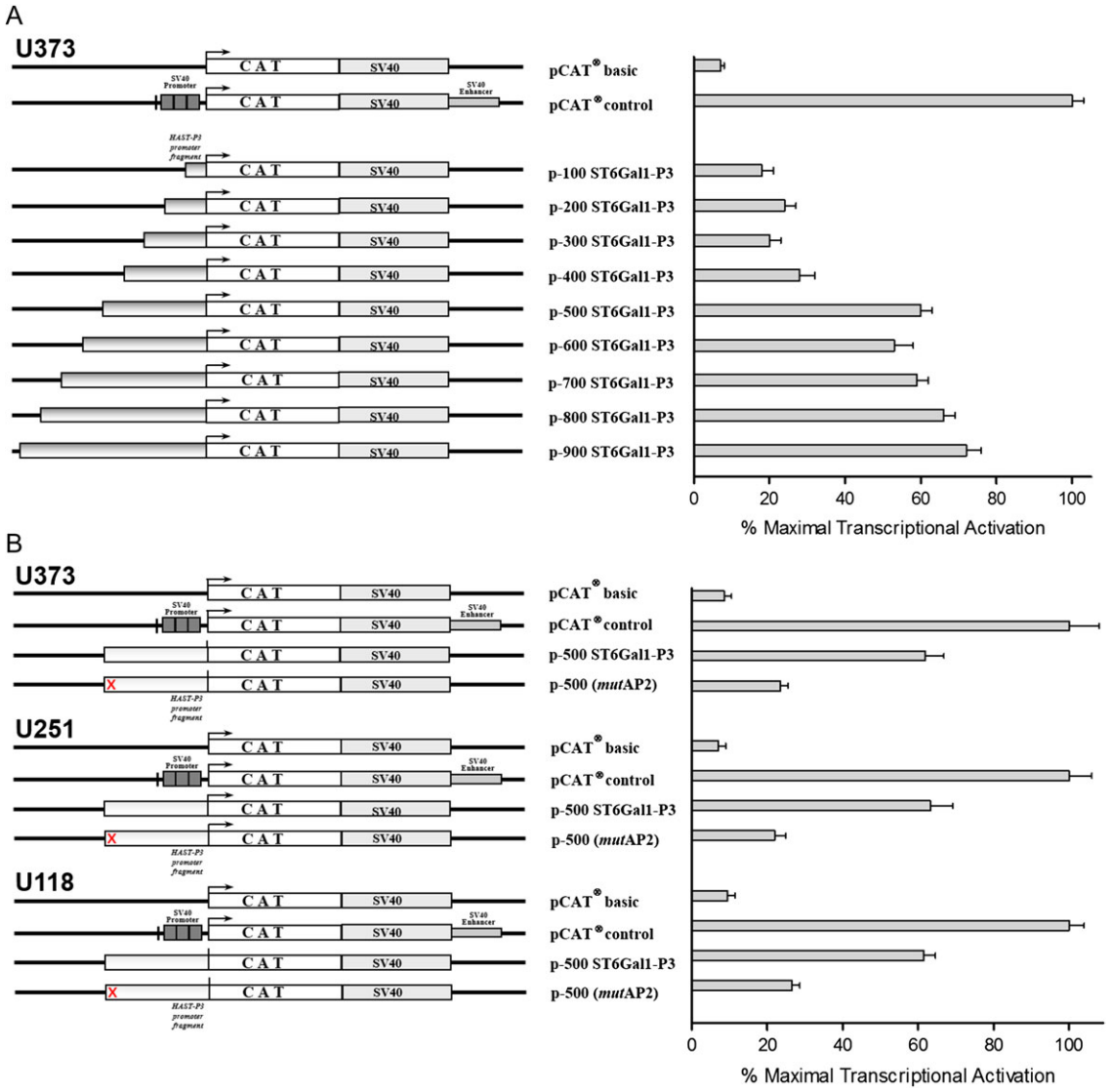

Fig. 4. Transcriptional activity of ST6Gal1-P3 promoter constructs in human glioma cells. (**A**) U373 cells were transiently transfected with individual HAST-P3 deletion mutant constructs using X-fect™ transfection reagent. Whole cell lysate protein was isolated 48 h after transfection by multiple freeze–thaw cycles. 30 μg of cell lysate protein from cells transfected with each of the chimeric CAT constructs (left panel) were incubated at 37°C for 20 h in the presence of 40 μM [^{3}H]chloramphenicol and 240 μM n-butyryl Coenzyme A. The reaction products were extracted with xylene and n-butyrylated chloramphenicol determined by liquid scintillation counting and normalized to pCATcontrol activity (right panel). (**B**) AP2 mutant constructs (left panel). Following transient transfection of U373, U251, and U118 cells with the indicated constructs (right panel), CAT activity relative to pCATcontrol was measured (right panel). This figure is available in black and white in print and in color at *Glycobiology* online.

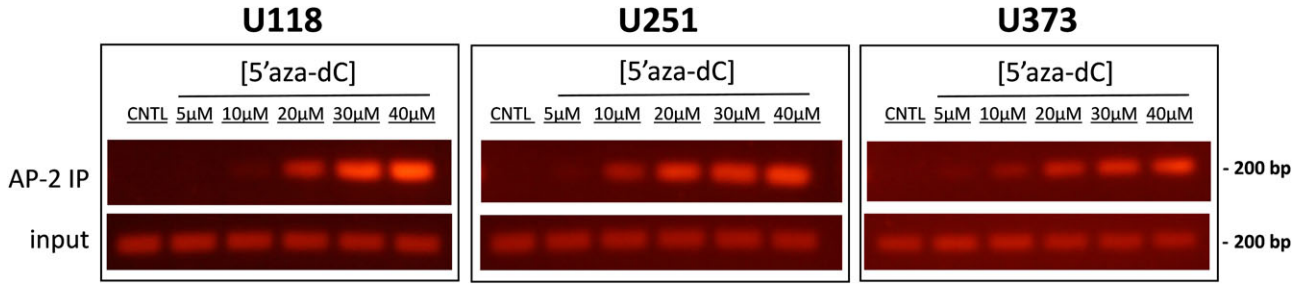

Fig. 5. Dose responsivity of ChIP-qPCR analyses of AP2 activity in control (CNTL) and 5-aza-dC-treated U118, U251, and U373 glioma cells showed that reactivation of ST6Gal1 expression following demethylation by 5-aza-dC treatment concomitantly enhanced AP2 binding to the ST6Gal1 promoter in all 3 cell lines. An anti-AP2α antibody was used for the immunoprecipitation and ST6Gal1 P3-specific primers were used for the qPCR with normal IgG used as a negative control. Input was assessed in sheared chromatin starting material treated with Proteinase K. This figure is available in black and white in print and in color at *Glycobiology* online.

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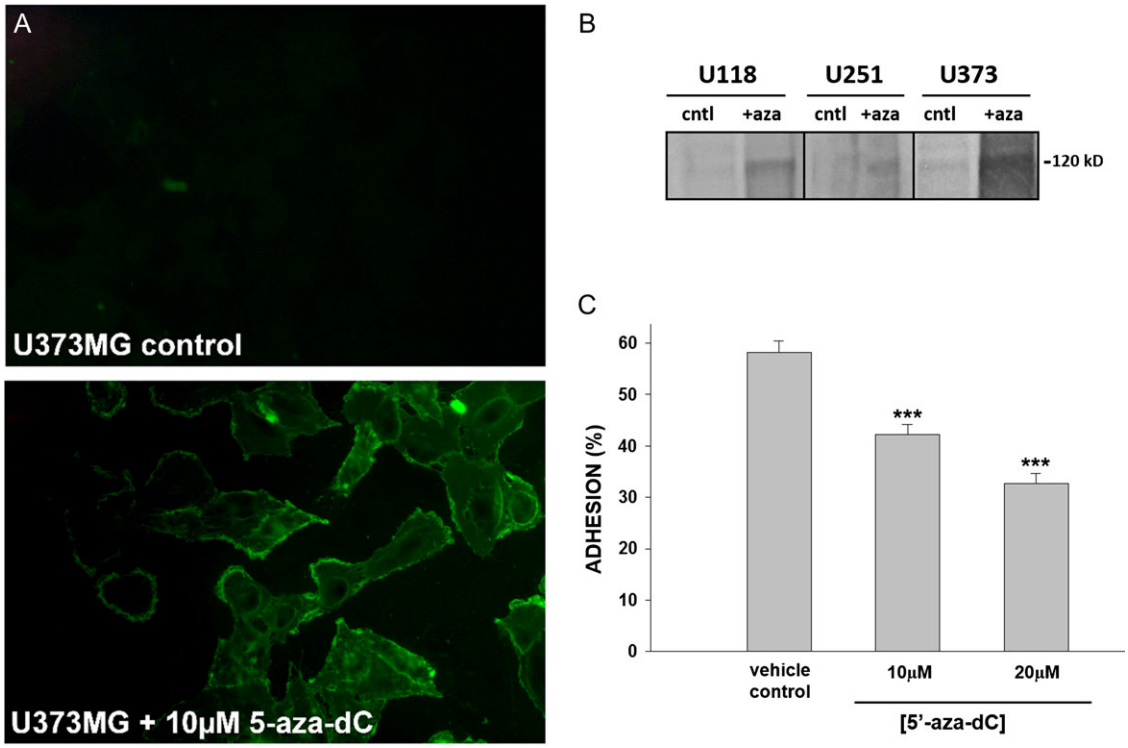

Fig. 6. (**A**) Immunohistochemical analysis of 5-aza-dC-treated cells using SNA lectin. DNA demethylation by 10 μM 5′-aza-dC treatment robustly increases α2,6-linked sialoglycoconjugates on the cell surface of U373MG glioma cells. Immunoreactivity reflects SNA binding to α2,6-linked sialoglycoconjugates. (**B**) β1 integrin sialylation analysis. Control (cntl) or 5-aza-dC treated cells from 3 glioma cell lines (U373, U118, and U251) were homogenized and immunoprecipitated with an anti-β1 integrin antibody immobilized to magnetic beads. Following elution from the beads, samples were electrophoresed through a 7.5% BioRad TGX gel and transferred to a PVDF membrane. Visualization of α2,6-linked protein was evaluated using SNA lectin. (**C**) In vitro adhesion assay. Human fibronectin-coated 96-well plates were used to evaluate the relative adhesion of 5-aza-dC treated U373MG cells. Compared with vehicle-treated parental control, the 5-aza-dC treated cells showed a significant reduction in adhesion to fibronectin substrate. Data are average ± SEM (bars) values of four values taken from a representative experiment (*** $p < 0.001$; two-tailed, unpaired student's *t*-test). This figure is available in black and white in print and in color at *Glycobiology* online.

A

GENE ID	Relative Expression (5′-aza-dC / control) U373	U118	U251
ST6GAL1	9.53	8.38	6.16
UGT2B7	4.20	4.33	2.95
B4GALT1	2.23	3.75	ns
GCNT3	ns	2.44	4.01
LARGE	ns	6.33	4.95
ST3GAL1	ns	2.88	2.01
UTP14C	ns	2.59	2.99
MAN1B1	4.67	ns	3.07

B

GENE ID	Relative Expression (5′-aza-dC / control) U373	U118	U251
B3GNT2	−3.85	−2.97	−5.59
B3GALNT1	−3.00	−2.48	ns
HAS3	−3.97	−5.09	ns
FUCA1	ns	−4.89	−8.30
GLB1L	ns	−3.67	−6.63
GLT25D1	ns	−2.91	−2.44
PIGM	ns	−4.60	−5.76
PLAU	ns	−5.08	−3.01
UGCG	ns	−4.24	−2.43

Fig. 7. Summary of glycogene expression in 5-aza-dC treated cells. Glycogenes re-expressed following treatment with 5-aza-dC are denoted in red and those genes down-regulated depicted in green. Maximally stringent criteria were used for the assessment of differential expression; the cutoff for significance was maximally set at a FDR = 0% with a level of differential expression ≥ 2-fold, with the threshold for "silencing" expressed transcripts at < 2× background fluorescence. This figure is available in black and white in print and in color at *Glycobiology* online.

demonstration of 5-aza-dC reactivated ST6Gal1 expression in gliomas has allowed a unique opportunity to delineate the mechanism and potential differences in transcriptional regulation of this important gene in brain tumors. Delineation of the precise TSS allowed for accurate interpretation of the involvement of cis-acting regulatory elements important in ST6Gal1 expression. Methylationsensitive binding of transcription factors to their cognate sequences represents a relatively small proportion of all defined transcription factors. ST6Gal1

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promoter sequences between 400 and 500 bases upstream of the TSS define the region most sensitive to demethylation by 5-aza-dC. Within this region, AP2 is the only transcription factor that could be responsible for this extreme methylation sensitivity (Comb and Goodman 1990; Hermann and Doerfler 1991). This is not to suggest that AP2 acts alone, but is consistent with the notion that it acts in concert with an ensemble of transcription factors to produce functional P3-driven ST6Gal1 transcripts. Thus, the data support the idea that selective unmasking of a specific AP2 site(s) within the ST6Gal1 promoter would be sufficient to reactivate its expression and lead to the observed phenotypic changes.

DNA methylation changes play prominent roles in the modulation of glioma invasivity, response to chemotherapy, and ultimately patient prognosis. The 5-aza-dC responsive transcriptomic profiles in this study demonstrates that reactivation is not a "glycome-wide" phenomenon and actually impacts a surprisingly small number of transcriptional targets. The decreased expression of specific glycogenes following 5-aza-dC treatment is intriguing. This is consistent with recent evidence that argues against the paradigm that DNA methylation *always* suppresses transcription in which cases where methylation is required for transcriptional activation and therefore positively correlated with gene expression (Rishi et al. 2010). It is also consistent with studies demonstrating that sequence context also plays a critical role in whether 5-aza-dC produces specific or nonspecific (global) demethylation (Baubec and Schubeler 2014). Irrespective of the molecular mechanism, it is clear that our focused glycotranscriptomic approach identifies additional DNA methylation-related targets for further functional analyses.

DNMT inhibitors like 5-aza-dC have proven useful clinically, with antineoplastic activity in patients with leukemia, myelodysplastic syndrome (MDS), and non-small cell lung cancer (NSCLC) (Momparler 2005, 2013). However, they have met with limited clinical success in solid tumors. This limited success appears to be due primarily to pharmacodynamics of drug scheduling (reviewed in Karahoca and Momparler 2013). A large number of novel targets of aberrant epigenetic silencing have been identified in glioblastomas, albeit following combination treatment with both DNMT inhibitors and histone deacetylase (HDAC) inhibitors (reviewed in Kim et al. 2006; Nagarajan and Costello 2009). Despite the fact that this apparent pleiotropism of targets may somewhat confound the issue of causality of effects, there are still a number of agents targeting histone deacetylases in human clinical trials for high grade gliomas and brain metastases (reviewed in Bezecny 2014). Since ST6Gal1 reactivation was totally unaffected by HDAC inhibition, the data from our study seem to provide strong support for potential treatment with DNMT inhibitors alone as well. Additionally, our data suggest that the expression of a very limited subset of glycogenes would be affected by solely hypomethylation-based approaches.

While current treatment with demethylating drugs like 5-aza-dC primarily produce genome-wide demethylation, technological advances in targeted epigenetic intervention bring site-selective approaches closer to fruition. The recent discovery of Ten-eleven Translocation (TET) proteins and their role in epigenetic editing (de Groote et al. 2012) to systematically manipulate epigenetic marks to modulate gene expression, the advent of gene-specific or site-specific demethylation is rapidly approaching. TET enzymes work by converting methylcytosine to 5-hydroxymethylcytosine, a modification that has completely different biological functions. Gene targeting is achieved by fusing a site-specific DNA binding domain to the catalytic domains of the TET proteins, resulting in the re-expression of hypermethylated genes. Importantly, this occurs both in the absence of DNA replication (required for the action of drugs like 5-aza-dC) and without re-expressing unintended genes (Verschure et al. 2006; Chen et al. 2014). While not yet ready for human therapy, the results are nonetheless revitalizing the field, as such "epigenetic editing" results in the reactivation/upregulation of the expression of the target gene of interest within the context of endogenous chromatin. Thus, our observation of the action of defined AP2 binding sites within the ST6Gal1 promoter takes on new life and offers potential hope for "epi-glycomic" approaches for glioma therapeutics.

In summary, the results from these studies yield important new information on DNA methylation-related mechanisms specifically involved in the transcriptional silencing of the ST6Gal1 gene in human glioma cell lines and may contribute to efforts towards the rational development of such effective small molecule treatments and strategies for these tumors. These results also lead to both an increased understanding of the basic mechanisms underlying altered glycoconjugate expression in tumors and the identification of additional targets amenable to the development of novel glycobiology-based therapeutic approaches for their treatment. As a gene therapy approach to modulate glycogene expression in brain tumors has provided a proof of concept that this gene family plays a key role in glioma invasivity and metastasis of nonbrain tumor cells to the brain (Yamamoto et al. 1997; Yamamoto et al. 2000; Yamamoto et al. 2001; Kroes et al. 2007; Moskal et al. 2009; Kroes et al. 2010), the long term scope of the studies described here provides a pathway for the development of small molecule modulators with therapeutic potential for the treatment of malignant brain tumors and metastases to the brain.

Materials and methods

Cell lines, culture conditions, and drug treatment

The human glioma cell lines U373MG, U118MG, and U251 (American Type Culture Collection, Rockville, MD) were maintained using Dulbecco's modified Eagle's medium (DMEM; containing 4.5 g/L glucose) supplemented with 10% heat-inactivated fetal bovine serum (Whittaker BioProducts, Walkersville, MD), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a 10% CO_2 humidified atmosphere. Cells were treated with 5'-aza-dC dissolved in DMSO for 5 days (fresh drug was added every 24 h) and/or trichostatin A dissolved in anhydrous ethanol for 24 h.

MTT assay

Cytotoxicity following 5-aza-dC was determined using an MTT assay (Life Technologies), per manufacturer's recommendations. Briefly, cells were seeded in 96-well plates, allowed to attach overnight, and treated with various concentrations of 5-aza-dC for times up to 5 days at which time, MTT was added to 1.2 mM and incubated at 37°C for 4 h. An equivalent volume of 10%SDS in 10 mM HCl was then added, incubated for an additional 4 h and A_{560} measured spectrophotometrically.

Global methylation assay

Global methylation of control and 5-aza-dC treated genomic DNA was assessed using an anti-5-methylcytosine antibody-based MethylFlash DNA methylation quantification kit (Epigentek, New York, NY), as per manufacturer's specifications and quantitated exactly as described (Novikova et al. 2008).

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Transcript-specific RT-PCR analyses

The expression levels of selected genes were analyzed by gel electrophoresis following reverse transcription of 1 μg of DNAsed, total RNA was primed with oligo(dT) and random hexamers, and was performed exactly as described (Kroes et al. 2006). All primer sets were designed across intron:exon boundaries (Table I), with individual primer concentrations and final amplification conditions optimized for each transcript. Original input RNA amounts were calculated by comparison to standard curves using purified PCR product as a template for the mRNAs of interest and were normalized to amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Experiments were performed in triplicate for each data point.

Determination of the transcriptional start site (TSS) by primer extension

The ST6Gal1 TSS was determined using a modified version of the AMV-RT Primer Extension System (Promega). In brief, 30 μg of total RNA isolated from 3 glioma cell lines (U373, U118, and U251) treated with either vehicle or 5-aza-dC (20 μg/ml for 5 days) was annealed to a ^{32}P-end labeled oligonucleotide at 58°C overnight. Following primer extension with AMV reverse transcriptase at 42°C for 30 min, the reactions were denatured in formamide and electrophoresed through a denaturing 8% acrylamide gel containing 7 M urea, and the primer extension products visualized by autoradiography. The sequences of the primers used are shown in Figure 2.

CpG island analysis, genomic DNA preparation, bisulfite modification, and methylation pattern analysis

Genomic DNA sequences surrounding the putative start sites of each of the 3 unique ST6Gal1 transcripts were obtained from GenBank database search (http://www.ncbi.nlm.nih.gov/nucleotide) and the presence of CpG islands predicted using the MethPrimer online search tool (http://www.urogene.org/methprimer/). Genomic DNA was isolated (Qiagen) from control and 5-aza-dC-treated cells and the methylation status of each of these transcripts was determined by the genomic DNA bisulfite conversion methodology using the EZ DNA Methylation™ Kit (Zymo Research, Irvine, CA) according to manufacturer's specifications. Following bisulfite conversion and desulfonation, *pfu*-PCR was used to amplify five individual 200–350 nt fragments, each comprising one of the CpG island sequences in the ST6Gal1 P3 promoter. Nested bisulfite primers were designed using MethPrimer software either without CpGs in their sequence or one CpG but in the 5' most portion of the primer and synthesized with a wobble nucleotide at that C position. These fragments were then subcloned into pCR®II-TOPO® (blunt) vector (Invitrogen) and a minimum of 10 subclones were sequenced.

ST6Gal1 promoter deletions and AP2 mutant: plasmid construction and transient transfection analysis

The parental pCAT® Reporter vector series (Promega, Madison, WI) were utilized to evaluate the transcriptional activity of the cloned ST6Gal1 promoter fragments in U373 human glioma cells. Varying lengths of the 5' flanking region upstream of Exon Y were *pfu*-PCR amplified using the cloned P3 promoter as template. *SalI* and *XbaI* sites were introduced in the forward and reverse primers, respectively, and used for cloning of these fragments directly upstream of the CAT reporter gene in the *SalI*/*XbaI* site of pCAT®3-Basic vector (Table II). In addition, critical AP2 recognition sequences within the AP2 site (−482 to −465nt from the TSS) localized near the 5' end of the ST6Gal1 promoter sequence contained in plasmid p500 (5'…GGC*CAAGCGGGGA*AGAG…3') was mutated (to 5'…GGC*CAAATATTAA*AGAG…3') by site-directed mutagenesis (QuikChange II XL; Agilent) to render it nonfunctional. All resultant ST6Gal1 -CAT constructs were confirmed by direct sequence analysis. Chloramphenicol acetyltransferase (CAT) activity in cells transiently transfected with these constructs is dependent on insertion of functional promoter sequences upstream from the CAT gene. One day prior to transfection, cells were plated at approximately 3 ×10^6 cells per 100 mm dish. Cells were transiently transfected with 10 μg of each construct using X-fect™ transfection reagent (Clontech), according to the manufacturer's specifications. At 48 h posttransfection, whole cell lysate protein was isolated by

Table I. ST6Gal1 transcript-specific primer sets

PROMOTER PRIMERS		
P1 promoter	Left	5'-CGCCTGGCTTATTTTTAGCTT-3'
	Right	5'-CTGAAGAAGGCAGAGGCTGA-3'
P2 promoter	Left	5'-GTGCAATGGCGTGATCTCT-3'
	Right	5'-ATCATACCCCTCCCTTTTGG-3'
P3 promoter	Left	5'-TCCTGTTGGGCAGTTTTTCT-3'
	Right	5'-TAACAAGCCAAAAGGGGCTA-3'
GAPDH PRIMERS		
	Left	5'-AAGGTCATCCCTGAGCTGAA-3'
	Right	5'-CCCCTCTTCAAGGGGTCTAC-3'
BISULFITE PRIMERS		
P3-CpG island 1	Left	5'-TTTAATGTTAGGAGGTTAATGTTAAA-3'
	Right	5'-CTACCTACTCTCAAAAAAAATAAATC-3'
P3-CpG island 2	Left	5'-TTTTTGAGAGTAGGTAGGATTTTAGTATTA-3'
	Right	5'-ACTAAAAACCCCACTAAAAAATCTC-3'
P3-CpG island 3	Left	5'-TTTTGGAGTTATTTGGGGGA-3'
	Right	5'-CCCTTTAACCCTAACAAAAATACAC-3'
P3-CpG island 4	Left	5'-GGGTTATTTTGGTTTTTTTTTG-3'
	Right	5'-ACCTTTTAAATATCTCTCTC-3'
P3-CpG island 5	Left	5'-AAGGGTTTAAGGGTGTTTTTTAAAA-3'
	Right	5'-AAAATACATTAACAAACCAAAAAAAACTAA-3'

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Table II. ST6Gal1 P3 promoter construct primer sets

P3 PROMOTER FRAGMENT	
−941 to +1060	Left: 5'-TCCTGTTGGGCAGTTTTTCT-3'
	Right: 5'-TAACAAGCCAAAAGGGGCTA-3'
DELETION PRIMERS (+ *SalI* site), FROM TRANSCRIPTIONAL START SITE	
−100	5'-AGTCGACGCTCCCGGGCGATCCTGCCC-3'
−200	5'-AGTCGACCTCTTCTTCCTTCCTTCTCC-3'
−300	5'-AGTCGACGCCGGTGGCGAGAGGTGGAG-3'
−400	5'-AGTCGACGAGCAGGCAGGATTCTAGCA-3'
−500	5'-AGTCGACGCTGGATTGACCCCTCGCGG-3'
−600	5'-AGTCGACTCTTCGGAGAGGTTAAGCGA-3'
−700	5'-AGTCGACTGAGAGCGCCCAGTAGGAGT-3'
−800	5'-AGTCGACCCATCCAGGCTGAGGCTTCA-3'
−900	5'-AGTCGACGGCTATCGGGGATGGTCAGT-3'
COMMON RIGHT PRIMER (+*XbaI* site)	
	5'-ACTCTAGAGTGCCCCAGGCGTCTTCCC-3'

multiple freeze–thaw cycles. CAT enzyme activity in 30 µg of lysate protein was measured following a 20-h incubation at 37°C in the presence of 40 µM [^{3}H]-labeled chloramphenicol and 240 µM n-butyryl Coenzyme A by liquid scintillation counting of xylene-soluble n-butyrylated chloramphenicol product.

Chromatin immunoprecipitation (ChIP)-qPCR analysis

ChIP was conducted on control and 5-aza-dC-treated (5, 10, 20, 30, and 40 µM for 5 days) cells from 3 glioma cell lines (U373, U118, and U251) using the rapid ChIP method, exactly as described (Nelson et al. 2006). In brief, following treatment, cells were cross-linked on the plate with 1.4% formaldehyde at room temperature for 15 min and pelleted by centrifugation. Cells were sonicated, and the lysates were cleared by centrifugation and subsequently incubated with 10 µg of anti-AP2α antibody (ab108311, Abcam) in an ultrasonic bath for 15 min at 4°C. Bound antibody was purified using Protein A agarose and DNA isolated with Chelex 100 resin, exactly as described. Normal IgG was used as a negative control. The abundance of specific DNA sequences in the immunoprecipitates was determined by qPCR performed on equivalent amounts of ChIP-isolated DNA using ST6Gal1 promoter-specific primers spanning the AP2 site (located at −482 to −465 nt): 5'-GCT GGA TTG ACC CCT CGC GG-3' (forward) and 5'-CAC ACC GCG CCC CAA ACC GG-3' (reverse). Input was assessed in an aliquot of the sheared chromatin starting material that was treated with Proteinase K.

Detection of cell surface α2,6-linked sialoglycoproteins

Cell surface α2,6-linked sialoglycoconjugate expression was confirmed by using fluorescein isothiocyanate (FITC)-conjugated Sambucus nigra agglutinin (SNA) (Matreya), exactly as described (Yamamoto et al. 1997). SNA recognizes epitopes containing α2,6-linked sialic acid linked to either Gal or GalNAc. In brief, subconfluent cells, grown on 12-mm glass coverslips, were fixed with 10% buffered formalin for 20 min at 25°C followed by washing once with phosphate-buffered saline (PBS). The fixed cells were incubated for 15 min at room temperature with PBS containing 10 µg/ml FITC-SNA (Vector) and 1% bovine serum albumin. After incubation, excess FITC-SNA was removed by washing the coverslips with PBS three times. The cells were mounted in 70% glycerin and fluorescence microscopy performed using a Nikon model 401 fluorescence microscope.

β1 integrin sialylation analysis

Control or treated cells from 3 glioma cell lines (U373, U118, and U251) were washed with PBS, harvested, and pelleted. Cells were homogenized in RIPA buffer containing 1% protease inhibitor cocktail (Sigma). Three hundred micrograms of protein were immunoprecipitated with an anti-β1 integrin antibody (AbCam) crosslinked to Dynabeads (Life Technologies) and electrophoresed on a 7.5% BioRad TGX gel and transferred to a PVDF membrane. Expression of cell-surface α2,6-linked glycoproteins were evaluated using *Sambucus nigra* (SNA) lectin provided with the DIG Glycan Differentiation Kit (Roche Molecular Biochemical), as per the manufacturer's instructions.

Cell adhesion assay

Cell adhesion to fibronectin was assayed as previously described (Kroes et al. 2010). In brief, flat-bottomed, polystyrene, 24-well plates were incubated overnight at 4°C with 40 µg of human fibronectin (Collaborative Research) in 250 µl of PBS per well. Plates were washed with 500 µl of 1.0% bovine serum albumin in PBS twice to remove unbound extracellular matrix proteins and also to block any remaining reactive surfaces. Nonspecific cellular binding was determined using wells coated only with 1.0% bovine serum albumin. After the plates were washed with PBS, 5×10^4 cells per well in 250 µl of DMEM was plated, and the cells were incubated at 37°C for 30 min for attachment to the fibronectin substrate. After nonadherent cells were washed off, 25 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/ml) was added to the culture and incubated for 3 h, and then 250µl of acidic isopropanol (0.1 M HCI in isopropanol) was added and mixed completely. Optical density (absorbance at 570 nm minus that at 630 nm) was measured to evaluate cells attached to the substrate. The number of cells without the washing procedure was defined as 100%.

Targeted glycotranscriptomic analyses in human glioma cell lines

A custom targeted Human Glycobiology oligonucleotide microarray platform was used to examine the expression of 758 glyco-related transcripts that were compiled from currently available NCBI/EMBL/TIGR human sequence databases and the Consortium for Functional Glycomics-CAZy databases (available at http://www.cazy.org/CAZY/) and represented all of the cloned human glycosyltransferases, glycosylhydrolases, polysaccharide lyases, and carbohydrate esterases. A full description of our targeted transcriptome profiling method has been published (Kroes et al. 2007). Importantly, the quality of this platform has been rigorously evaluated in terms of dynamic range, discrimination power, accuracy, reproducibility, and specificity. The ability to reliably measure even low levels of statistically significant differential gene expression stems from coupling (a) stringently designed and quality controlled chip manufacturing and transcript labeling protocols, and (b) rigorous data analysis algorithms.

In these studies, the relative quantitation of individual transcript abundance in vehicle and 5-aza-dC treated human glioma cell lines was compared. In brief, total RNA extracted and purified from 3 defined glioma cell lines was used as the substrate for RNA amplification and labeling. We employed a universal reference design and comprehensive statistical analysis platforms to facilitate the acquisition of expression profiles. Following hybridization and sequential high-stringency washes, individual Cy3 and Cy5 fluorescence

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hybridization to each spot on the microarray was quantitated by a high-resolution confocal laser scanner. For each cell line, RNA samples from each line were analyzed in triplicate. As each transcript-specific oligonucleotide was spotted in triplicate on the array, there were a total of 27 individual expression measurements per gene in each experimental group. Statistically significant differentially expressed genes were identified using the permutation-based significance analysis of microarrays algorithm (SAM software package, v4.0, Stanford University, Palo Alto, CA). In our analyses, appropriately normalized data were analyzed using two-class, unpaired analysis on a minimum of 5000 permutations and performed by comparing expression data derived from the different 5-aza-dC treated cell lines versus vehicle treated lines. To maximize the confidence of the dataset, we set very stringent criteria for these experiments: the cutoff for significance was maximally set at a FDR = 0% and a level of differential expression $\geq$ 2-fold, with the threshold for "silencing" at $< 2\times$ background fluorescence. These criteria set clear and unambiguous thresholds for the level of induction or, alternatively, silencing for those already transcriptionally active.

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Conflict of interest statement

None declared.

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