EXPERT REVIEWS

The glycobiology of brain tumors: disease relevance and therapeutic potential

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The oligosaccharides that decorate cell surface glycoconjugates play important roles in intercellular recognition and cell–extracellular matrix interactions, and thus the regulation of cellular migration, metastasis and invasivity. Virtually all tumor cells display aberrant cell-surface glycosylation patterns brought about by alterations in their biosynthetic machinery. This holds true for highly invasive, malignant brain tumors as well as tumor cells that metastasize to the brain. The field of glycobiology is well established with essentially all of the biochemical pathways for oligosaccharide metabolism characterized and all of the 'glycogenes' involved in these pathways cloned. Yet there has been a paucity of progress toward the development of therapeutics. However, recent studies aimed at controlled glycosylation of therapeutic antibodies and mucins with anticancer vaccine potential, the emergence of new and highly sensitive tools for the identification of tumor-associated biomarkers and the manipulation of the expression of glycogenes that inhibit brain tumor invasivity have emerged. The opportunity now exists to answer questions as to how glycogenes are regulated at the genomic and transcriptomic level and how altered glycogene expression patterns lead to altered cell surface glycoconjugates. These studies should lead to the development of ways to directly regulate tumor cell glycogene expression, which should have significant therapeutic potential.

Keywords: cell surface glycoconjugate • ganglioside • gene therapy • glioma • glycosyltransferase gene • integrin • invasivity • microarray

Brain tumors & glycosylation

Based on the most recently available published data, more than 200,000 people will be diagnosed with a primary or metastatic brain tumor in the USA this year [1–3]. Brain tumors are presently the second leading cause of cancer death in children under the age of 20 years and they are the third leading cause of cancer death in young adults aged 20–39 years [4,5]. Brain tumors are phenotypically and genotypically diverse, with over 120 different types of brain tumors currently classified [6], with brain metastases, arising primarily from breast and lung cancer, by far the most common type of brain tumor, with an annual incidence greater than four-times that of primary brain tumors [7,8]. Brain tumors in children are clinically different than those in adults and thus are treated differently. Although greater than 60% of children will survive, more often than not they are left with devastating long-term side effects and permanent neurological damage [5]. Glioblastomas represent 23% of all primary brain tumors, rapidly approaching the incidence of meningiomas, which are the most common primary brain tumor and represent 26% of all primary brain tumors [9]. Glioblastoma multiforme (GBM) is nearly uniformly fatal, with median survival between 9 and 12 months from initial diagnosis. GBMs are highly invasive tumors, making complete surgical resection unachievable. Their etiology is unknown. This review focuses on an emerging therapeutic approach for the most devastating of brain tumors: high grade gliomas or GBM.

Glycobiology of brain tumors: basic research

It is estimated that over half of all human proteins are glycosylated. As such, glycosylation represents a more common post-translational modification than phosphorylation. Cell surface carbohydrates contribute to a variety of interactions between the cell and its extracellular environment. The attached oligosaccharides

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have many biological functions, for example, cell–cell communication, signal transduction, protein folding and stability. Glycans cover the entire cell surface as the glycocalyx, and they function both as structural components and as signal transducers. Glycans are essential for many biological processes, including cellular response to oxidative stress, resistance to innate immunity, intracellular protein trafficking, cellular growth and apoptosis, and cell–cell or cell–matrix communication.

It is now clear that virtually all types of human cancers have altered patterns of glycosylation and express aberrant glycosyl moieties on their cell surface glycoconjugates. Moreover, a significant number of studies have shown that these carbohydrates play key functional roles in oncogenic transformation, tumor progression and metastasis (reviewed in [10]).

Research on the glycobiology of brain tumors has been quite active, with studies falling into three categories: descriptive studies identifying brain tumor-associated glycoconjugates, studies examining the expression of specific glycosyltransferase activities in brain tumors and studies aimed at showing a functional relationship between specific glycoconjugates relevant to therapeutic development.

Early descriptive work by Traylor and Hogan, examining a series of human glioblastomas, showed that the concentration of total gangliosides was reduced in the tumors compared with normal brain tissue and that there was an increase in simpler ganglioside structures and a reduction in complex polysialogangliosides [11]. In another series of studies involving the examination of ganglioside expression in human gliomas, Fredman and coworkers also found marked differences in ganglioside structures in the tumors [12] similar to Traylor and Hogan. Later, using monoclonal antibodies directed against oncofetally expressed gangliosides, they found that gliomas both *in vitro* and *in vivo* displayed both 3′-isoLM1 and 3′,6′-isoLD1 lacto series gangliosides [13]. More recently, Jennemann *et al.* characterized glycosphingolipids in human gliomas, reporting a correlation between glycosphingolipid class and glioma tumor grade [14,15]. Shinoura *et al.* evaluated ganglioside profiles in a variety of brain tumors including meningiomas, astrocytomas, neurinomas, ependymomas, metastatic brain tumors, mixed glioma, oligodendroglioma, medulloblastoma, embryonal carcinoma and a cultured glioma cell line [16]. Their results in general agreed with those cited earlier, reinforcing the idea that ganglioside pattern changes occur in virtually all brain tumor types. In further support of this point, Pan *et al.* examined ganglioside patterns in pediatric brain tumors, including primitive neuroectodermal tumors, ependymomas and neuroblastomas [17]. Again, their results showed an increase in GM3 and GD3 gangliosides and reductions in complex gangliosides, such as GT1a, GD1b and GT1b.

In general, recent studies have extended the analyses of brain tumor associated glycoconjugates beyond astrocytomas and gliomas and focused on refining the idea of glycolipids as markers for specific brain tumor types [18,19]. For example, Yates and coworkers have shown that GD1b ganglioside correlated with tumor grade in astrocytomas [20,21]. Hamasaki *et al.* reported GT1b ganglioside as a brain metastasis marker [22]. Mennel and Lell also corroborated earlier reports that simpler ganglioside patterns were associated with malignancy progression, but not the proliferation patterns, of astrocytomas [23]. A series of reports by Ladisch and coworkers [24,25] and Nakamura *et al.* have shown that human neuroblastomas, medulloblastomas and astrocytomas shed gangliosides that can be detected in patient serum and cerebrospinal fluid [26]. Li *et al.* showed that shed ganglioside GD2 was markedly immunosuppressive and, as such, may facilitate tumor formation and progression [27].

One of the most important clinical hallmarks of malignant gliomas is their ability to invade normal brain tissue. In addition, cell-surface glycoproteins have been identified that are associated with the invasive potential of malignant gliomas. The mechanisms underlying the invasive process are exceedingly complex and involve several interwoven, yet coordinated intra- and extracellular processes: specifically, initial decoupling from nascent tumor mass, attachment to the extracellular matrix (ECM), remodeling of the ECM and the migration into normal brain tissue. Each of these processes is profoundly influenced by alterations in both the oligosaccharide content and structure of tumor cell surface glycans.

The initial decoupling of tumor cells is primarily driven by interaction between tumor cell surface adhesion molecules (CAMs) and their surrounding environment, and include proteins such as CD44 [28], neural CAM (NCAM) [29,30], cadherins [31–33] and galectins [34,35].

CD44 is a transmembrane adhesion molecule that functions as the principal cell surface receptor for hyaluronic acid, an important component of the ECM. CD44 is a multifunctional receptor involved in cell–cell and cell–ECM interactions and transmission of signals mediating growth and apoptosis. All forms of CD44 are heavily *N*- and *O*-glycosylated; where changes in glycosylation of CD44 have profound effects on its interaction with hyaluronic acid and provide an important regulatory mechanism of CD44 function.

Neural CAM is also highly glycosylated and is known for its unique expression of polysialic acid sequences on the fifth immunoglobulin-like domain and on the natural killer cell-associated sulfated HNK-1 (CD57) epitope. Polysialic acid plays critical roles in normal neural development by modulating the adhesive properties of NCAM. Increased polysialic acid content has been shown to directly modulate the adhesive properties of NCAM and other CAMs on the surface of childhood neuroblastoma. Additionally, Gratsa *et al.* have shown that a NCAM-deficient clone of an astrocytoma cell line showed significantly more invasivity than a NCAM-positive clone derived from the same parent [36].

E-cadherin is a transmembrane glycoprotein mediating Ca^{2+}-dependent adhesion in gliomas. The disruption of E-cadherin-mediated cell adhesion appears to be a central event in the transition from noninvasive to invasive carcinomas. As the core transmembrane protein of adherens junctions, E-cadherin is required for binding and localization of a number of important cytoplasmic proteins, termed catenins, which connect the cadherin complex to the actin cytoskeleton and several signaling

pathways. E-cadherin is post-translationally modified by both *O*- and *N*-glycosylation. Cytoplasmic *O*-glycosylation of the E-cadherin cytosolic tail occurs in response to endoplasmic reticulum stress and inactivates E-cadherin-mediated intercellular adhesion by preventing its transport to the cell membrane. On the other hand, E-cadherin can be *N*-glycosylated in gliomas, primarily by GnTIII, which is essential for E-cadherin expression, folding and trafficking.

Galectins are specific ligands for β-galactoside-containing molecules and are involved in the modulation of cell adhesion and invasivity, as well as regulation of cell growth and apoptosis of gliomas [37]. The expression of galectin-1 and -3, the two major galectins found in gliomas, directly correlates with tumor grade and patient prognosis. Galectin-1 is also involved in cancer development via anchorage of Ras, which is involved in cellular transformation. The interaction of galectins with integrin glycans modulates cell adhesion and cell migration, primarily via induction of the phosphorylation of FAK, a key regulator of intracellular signaling pathways controlling actin cytoskeleton reorganization and cell migration and invasion. Galectin-3 also regulates cell adhesion via binding to integrin glycans and complexing integrin and the proteoglycan NG2. GM1 and GM3 gangliosides and sulfatides are also major ligands for these galectins. Galectin-8, another glioma-associated galectin, actually possesses two carbohydrate recognition domains and can additionally function to crosslink its ligands. As such, it can organize extracellular CAMs both on the same cell as well as between cells and the ECM, and such a change appears to be very important to tumor invasion.

The next phase of the invasion process involves coordinated expression of molecules mediating the sequential attachment and reattachment of the cell to the surrounding matrix. Prototypical of this most important group of adhesion molecules are the integrins, which are transmembrane proteins forming dimers between 14 different α- and eight different β-subunits [38]. Integrins act as mechanical anchors not only interacting with ECM proteins, but also with CAMs, integrin-associated proteins, including CD44, and growth factor receptors. Adhesion complexes in migrating cells consist of aggregated integrins, cytoskeletal proteins (e.g., vinculin, and p^{125}FAK) and other phosphoproteins. One of the major glioma integrins, the α3β1 integrin, mediates adhesion to the basement membrane, preferentially promoting cell migration and preventing apoptosis. It is, however, expression of the β1 subunit that plays the key role in maintenance of the invasive glioma phenotype, with inhibition of function correlating with decreased motility [39]. These cell surface integrins are all major carriers of N-glycans [40]. *N*-glycosylation of integrins acts as a key regulatory switch for protein activity, playing a critical role in their biological functions. Changes in the N-glycan structures of these integrins directly affect cell–cell and cell–ECM interactions, thereby affecting cell adhesion, migration and tumor malignancy [41]. A growing body of evidence indicates that the presence of the appropriate oligosaccharide can modulate integrin activation. A shift of integrin N-glycans to the highly β1,6 GlcNAc branched type via catalysis by *N*-acetylglucosaminyltransferase (GnT)-V leads to a decrease in cell adhesion, resulting in an increase in both cell motility and tumorigenicity. Conversely, the modification of N-glycans with a bisecting GlcNAc catalyzed by GnT-III inhibits ligand binding ability, subsequently leading to the downregulation of integrin-mediated signaling. In addition, altering terminal sialic acid linkages of N-glycans of α3β1 integrin also plays an important role in cell adhesion, migration, invasivity and tumorigenicity [42,43].

Remodeling of the ECM occurs primarily through the concerted activity of specific ECM-degrading proteases. Gliomas also possess the innate ability to both reconstruct components of the ECM that induce cell migration and decrease expression of matrix components that suppress migration. BEHAB/brevican, a CNS-specific lectican, is upregulated during periods of glial cell motility, such as during prenatal gliogenesis and in human gliomas [44]. BEHAB/brevican upregulation and subsequent proteolytic processing contribute to the infiltration of glioma cells into normal nervous tissue. Fully glycosylated BEHAB/brevican is decorated with a diverse set of oligosaccharides, including *N*- and *O*-linked sugars, and chondroitin sulphate chains. Two novel glioma-specific isoforms of BEHAB/brevican selectively upregulated in human gliomas and absent from normal adult brain have been characterized [45]. The underglycosylated isoform, the major upregulated form of BEHAB/brevican in GBM, plays a significant role in glioma progression.

The ultimate motility of glioma cells is stimulated by individual growth factors, primarily via increased expression of cell surface growth factor receptors, including the EGF receptor (EGFR; reviewed in [46]) and the PDGF receptor. Activation of these receptors leads to alterations in intracellular signaling that ultimately impacts the cells ability to proliferate, invade, escape immune surveillance and alter their responsivity to traditional chemotherapeutic regimens [47,48]. EGFR is amplified in a significant proportion of high-grade gliomas and EGFR activity is regulated by specific addition of N-linked oligosaccharide modifications [49,50]. These modifications, mediated in part by GnT-III, are an essential prerequisite of the conformational change necessary for EGF binding and receptor autophosphorylation that is required for appropriate signaling [51]. Transfection-mediated overexpression of these GnT-III products on EGFRs in glioma cell lines induces significant changes in the proliferative response of the cells to EGF. In addition to these EGFR-driven alterations, there is preliminary evidence that the PDGF receptor is also aberrantly glycosylated and that this leads to altered signaling in gliomas [52].

However, these studies did not investigate the precise role of the glycose moieties of these adhesion molecules in modulating invasivity. For recent reviews see Fish [53], Pilkington [54], Yates [55] and Mikkelsen *et al.* [56].

Glycoconjugate biosynthesis in brain tumors

It is well established that the biosynthetic machinery – the glycosyltransferases, glycosylhydrolases and the genes that regulate their expression – has been significantly altered in all forms

of oncogenic transformation (for recent reviews see [57–60]). Surprisingly, however, there have been very few such studies with brain tumors. Moskal and coworkers reported on the expression of α2,6-sialyltransferase (ST) in a variety of human brain tumors [61]: the altered expression of α2,3-ST mRNA in malignant gliomas [62]; the ability of α2,6-ST gene transfection to inhibit glioma invasivity *in vitro* and *in vivo* [42,43]; the ability of GnT-III and -V to play a role in regulating glioma invasivity in a stable transfectant of a human glioma cell line [63]; and the ability of a variety of glycosyltransferase gene transfection studies to enhance cell death induced by staurosporine, C2-ceramide or etoposide [64]. Xu *et al.* have reported that β1,4 galactosyltransferase-I, -II and -V are overexpressed in human astrocytomas [65] and Hoon *et al.* have shown that ganglioside GM2/GD2 synthetase mRNA can be used as a marker for detecting metastatic neuroblastoma cells in bone marrow [66]. With the cloning and sequencing of essentially all of the human glycosyltransferase and glycosylhydrolase genes having been accomplished, it is probable that more reports on the differential expression of these genes will be forthcoming. Nevertheless, it will be the functional studies showing a direct link between altered glycoconjugate expression and cellular processes that will have the most impact and that are likely to lead to novel therapeutics.

There have been many reports in which some aspect of glycoconjugate biochemistry (e.g., inhibitors of biosynthesis, addition of cell-surface glycosphingolipids and addition of oligosaccharides or glycopeptides) has been manipulated, leading to an inhibition of, for example, tumor growth and metastasis [67]. Of all of these studies, however, only two therapeutic candidates have progressed into clinical trials: swainsonine, an inhibitor of the Golgi-associated α-mannosidase II leading to the inhibition of or alterations in normal N-glycan biosynthesis on many glycoproteins [68], for which dose-limiting toxicity was ultimately an issue [69]; and a GD2-based immunotherapy specifically targeting gliomas [70] that, while showing no toxic side effects, was unable to stimulate antibody formation or effect any tumor regression. Recently, however, Fujimoto *et al.* reported that GM3 ganglioside also has therapeutic potential for patients with gliomas, but no clinical trial data have yet been published [71].

Nevertheless, there are some recent and particularly noteworthy approaches that appear to have therapeutic potential. Ishikawa and coworkers screened phage-display random peptide libraries for peptides that bound to an anti-GD1α monoclonal antibody [72]. A lead peptide was identified with significant adhesion-inhibition properties that was also effective at inhibiting metastasis when injected *in vivo*. Hanessian *et al.* have created functionalized aryl β-D-glycopyranosides called 'glycomers' that they report induce apoptosis in human glioma cell lines [73]. Neurostatin, *O*-acetylated GD1b, has also been found to inhibit the *in vitro* and *in vivo* proliferation of human glioma cell lines [74–76]. A recent review by Rebbaa *et al.* discusses the modulation of growth factor receptors in brain tumors by complex carbohydrates, yet another interesting pathway for the development of therapeutics [77].

Glycogene expression in brain tumors

Moskal and coworkers have attempted to create a program aimed specifically at developing glycobiology-based therapeutics for malignant brain tumors, which is based primarily on an idea elaborated on by Hakomori [78], namely, that glioma invasivity can be disrupted by altering the aberrant cell-surface glycosylation patterns found in these cells. The most direct way to do this would be by directly manipulating glycosyltransferase and/or glycosylhydrolase gene expression in these cells. Our initial strategy was to attempt to identify a glycosyltransferase gene, for example, that was markedly altered in its expression in primary specimens of human brain tumors compared with normal human brain. By starting with the measurement of gene-expression changes, the identification of multiple therapeutic targets would be possible: from the transcriptional regulation of gene expression to post-translational modification of the gene products themselves. Clearly, for each altered gene there are an abundance of approaches to modulating its expression and the function of its product(s).

To date, there are approximately 20 STs that have been cloned including O- and *N*-linked, as well as the entire series of a and b ganglioside STs [79,80]. They comprise a structurally related family of molecules that display substrate specificity, tissue specificity and are all developmentally regulated [81]. Studies by Recchi *et al.* [82], Peyrat *et al.* [83], Julien *et al.* [84] and Marcos *et al.* [85] showed that ST expression in breast cancer cells is markedly altered and that the modulation of their expression can also impact tumor cell behavior *in vivo*. Alterations in the expression of terminal sialic acid residues on glycoconjugates are typically found in cells undergoing or that have undergone oncogenic transformation [86–91]. Increased cell-surface sialylation has been associated with invasivity, metastatic potential, adhesion to endothelial cells and extracellular matrices and resistance to T-cell-mediated cell death [92–94]. While a number of investigators have used cell lines derived from vertebrate brain tumors to study the expression and regulation of various glycosyltransferases [95–99], studies using primary human brain tumor material have been very limited. Shen *et al.* reported that serum ST, using desialylated fetuin as the acceptor, did not significantly differ from controls in glioma patients [100], and Gornati *et al.* found that the ST involved in the biosynthesis of GD3 from GM3 ganglioside was altered in meningiomas [101]. In our initial series of studies, glycosyltransferase and glycosylhydrolase gene expression was evaluated in freshly dissected brain tumor specimens using Northern blot analyses. Glycogenes are abundant – approximately 400 – and are differentially expressed and developmentally regulated. Thus, it seemed probable that a complex pattern of gene expression might be expected in this family of tumors, as well as a different pattern for each tumor. Indeed our data showed that both the qualitative and quantitative expression of the glycogenes that we measured were complex and quite variable from tumor to tumor. However, it was also clear that the α2,6-ST transcript that we measured was virtually absent from all gliomas measured.

This finding led to a second set of studies in which the expression of α2,6-ST mRNA and the cell-surface expression of α2,6-linked sialic acids in a variety of brain tumors was directly

evaluated. These results are shown in Table 1. Epithelial-like tumors, such as meningiomas, chordomas and craniopharyngiomas, often expressed α2,6-ST and α2,6-linked sialic acids. However, glioblastomas, oligodendrogliomas, ependymomas, medulloblastomas and brain metastases were essentially devoid of detectable α2,6-ST mRNA, α2,6-ST immunohistochemical staining and cell surface α2,6-linked sialic acids [61,102].

The lack of expression of the α2,6-ST in malignant gliomas and the fact that the α2,6-ST together with the α2,3-ST (CMP-NeuAc:Galβ1,3(4)GlcNAc α2,3-ST [103]) are the two enzymes responsible for effectively all terminal sialylation of N-linked glycoprotein oligosaccharides led us to the next series of experiments. We examined α2,3-ST mRNA expression in panels of primary human brain tumors, cell lines and fetal astrocytes along with the expression of α2,3-linked cell surface sialic acids [62]. It was concluded from these studies and others demonstrating robust expression of α2,3-ST mRNA, using lectins to examine the expression of α2,3-linked sialic acids [62], that gliomas markedly overexpress terminal sialic acids compared with normal human brain controls and that it is the α2,3-ST as opposed to the α2,6-ST that is the principal enzyme involved in this increase in terminal glycoprotein sialylation.

The next step toward the development of a glycobiology-based brain tumor program was to create a model system that reflected the aforementioned results and was based on two working hypotheses:

- Alterations in glioma cell surface glycosylation should affect tumor cell invasivity
- Altering the expression of glycosyltransferase gene expression in glioma cells should modify cell–surface tumor cell glycosylation patterns

We thus began by creating a stable, α2,6-ST-expressing, human glioma cell line and evaluated it for a number of properties. We chose the cell line U373MG because it is derived from a human glioma, is tumorigenic and does not express α2,6-linked sialic acids [104]. Moreover, adhesion of this cell line to fibronectin or collagen matrices is mediated by the α3β1 integrin receptor since no other β-integrins could be detected [72]. This was important because α3β1 integrin expression is increased in glioblastomas compared with normal brain and probably plays an important role in glioma invasivity [73].

The results from these studies are depicted in Figures 1 & 2 and can be summarized as follows. Stable transfectants were created that now expressed α2,6-ST mRNA, measurable amounts of α2,6-ST enzyme activity and the cell-surface, α2,6-linked-sialic acids. These transfectants showed a significant reduction in adhesivity to the ECM molecules fibronectin and collagen compared with mock transfected controls and the parental glioma cell line. The α3β1 integrin was found to contain α2,6-linked sialic acids and the tyrosine phosphorylation of $p125^{fak}$ was blocked in the transfectants despite increased expression of $p125^{fak}$ mRNA [42]. Integrins interact with the extracellular milieu and act as signal transducers that can mediate glioma cell migration by regulating the function of focal adhesion proteins, such as $p125^{fak}$, through control of their phosphorylation state

Table 1. Histochemical examinations and Northern analyses of human brain tumors.

Case	α2,6-ST	SNA reactivity	α2,6-ST mRNA
Astrocytic tumors			
Astrocytoma (fibrillary)	0/4	0/4	0/1
Anaplastic astrocytoma	0/4	0/4	0/1
Glioblastoma	0/8	0/8	2/4 (0/4)
Piolcytic astrocytoma	0/1	0/1	
Oligodendroglial tumors			
Oligodendroglioma	0/4	0/4	
Anaplastic oligodendroglioma	0/2	0/2	
Ependymal tumors			
Ependymoma	0/5	0/5	
Myxopapillary ependymoma	0/1	0/1	
Embryonal tumors			
Medulloblastoma	0/5	0/5	
Metastatic tumors			
Adenocarcinomas	2/4	2/4	0/2
Squamous cell carcinoma	1/1	1/1	
Clear cell carcinoma	1/1	1/1	0/1
Mixed mesodermal tumor	0/1	0/1	0/1
Adenosquamous carcinoma	1/1	1/1	
Meningiomas			
Meningotherlial type	10/10	10/10	6/6 (5/6)
Transitional type	7/7	7/7	3/4 (1/4)
Fibroblastic meningioma	0/3	0/3	1/2 (0/2)
Anaplastic meningoma	2/2	2/2	0/2
Local extension from regional tumors			
Craniopharyngiomas			
–Adamantinomatous type	0/2	0/2	
–Squamous papillary	2/2	2/2	
–Chordomas	5/5	5/5	

Parenthesis indicates the number of cases that expressed a high level of α2,6-ST mRNA.
SNA: Sambucus nigra agglutinin; ST: Sialyltransferase.
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[42]. Thus, our results suggested that by simply stably expressing the *α2,6-ST* gene we could indeed alter the adhesivity/invasivity of these tumor cells via a well-characterized molecular mechanism [42].

In the next set of studies, the α2,6-ST U373MG stable transfectants were evaluated for their invasive potential using Biocoat Matrigel Invasion Chambers. Clearly, various clones of the α2,6-ST expressing stable transfectants showed marked inhibition of invasivity compared with parental or mock transfectants. These results were robust enough to merit *in vivo* studies, which are shown in Figure 3.

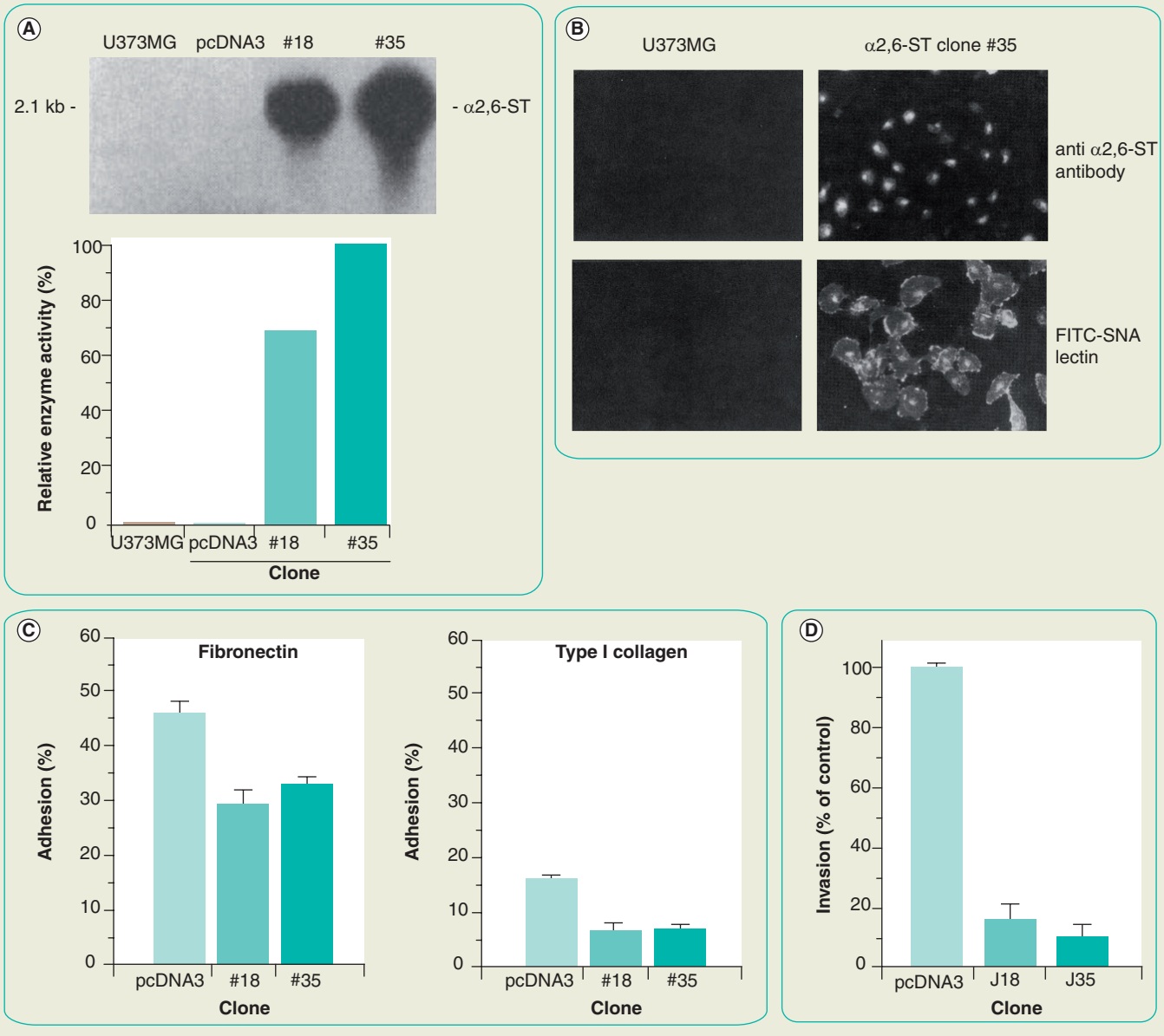

Figure 1. *In vitro* analyses of α2,6-ST stable transfectants. (A) Expression of α2,6-ST mRNA and enzyme activity in U373 MG/α2,6-ST clones. Northern blot analysis of parental glioma U373 MG cells, pcDNA3-transfected cells and two pcDNA3/α2,6-ST-transfected clones (18,and 35; top panel). The relative α2,6-ST enzyme activity expressed by the transfected clones was determined as described previously [138]. The data were normalized to values in the highest expressing clone, 35. No α26-ST enzyme activity was detected in the parental or in the pcDNA3-transfected cells (bottom panel). **(B)** Expression of α2,6-ST protein and α2,6-linked sialoglycoconjugates in stable α2,6ST transfectants. Cells were stained with anti-α2,6-ST antibody or FITC-SNA, as indicated. Fluorescence microscopy of parental U373MG cells and clone 35 are shown. **(C)** *In vitro* adhesion assay of the U373 MG/α2,6-ST transfectants. Human fibronectin- or collagen type I-coated 24-well plates were used to evaluate the relative adhesion of two transfectants (clones 18 and 35). Compared with a pcDNA3 'mock'-transfected control, the transfectants showed statistically significant ($p < 0.05$, two-tailed, unpaired student's t-test) reduction in adhesion to both fibronectin substrate (left panel) and collagen type 1 (right panel). Data are averages ± standard error of the mean (bars) of three values taken from a representative experiment. **(D)** *In vitro* invasion assay of the α-2,6-ST-transfected U-373 MG glioma cells. Biocoat Matrigel Invasion Chambers (Collaborative Research, Bedford, MA, USA) were used to evaluate the relative invasivity of the transfected subclones compared with pcDNA3 mock-transfected controls. Cell invasion was measured by counting the number of cells that pass through the Matrigel and the 8 micron filter pores. In total, 4×10^4 cells/well were plated into the upper compartment and incubated for 24 h, 0.5 ml of U373MG conditioned medium was placed in the lower compartment to facilitate chemoattraction. Cells that migrated through the Matrigel were fixed, stained and the membranes mounted on glass slides and cells counted. Data is expressed as percent of transfected cells migrated versus mock-transfected control cells. Significant differences in *in vitro* invasivity were demonstrated ($p < 0.01$, two-tailed, unpaired Student's t-test). Data are average ± standard error of the mean (bars) values of two separate experiments performed in triplicate. FITC: Fluorescein isothiocyanate; SNA: Sambucus nigra agglutinin; ST: Sialyltransferase.
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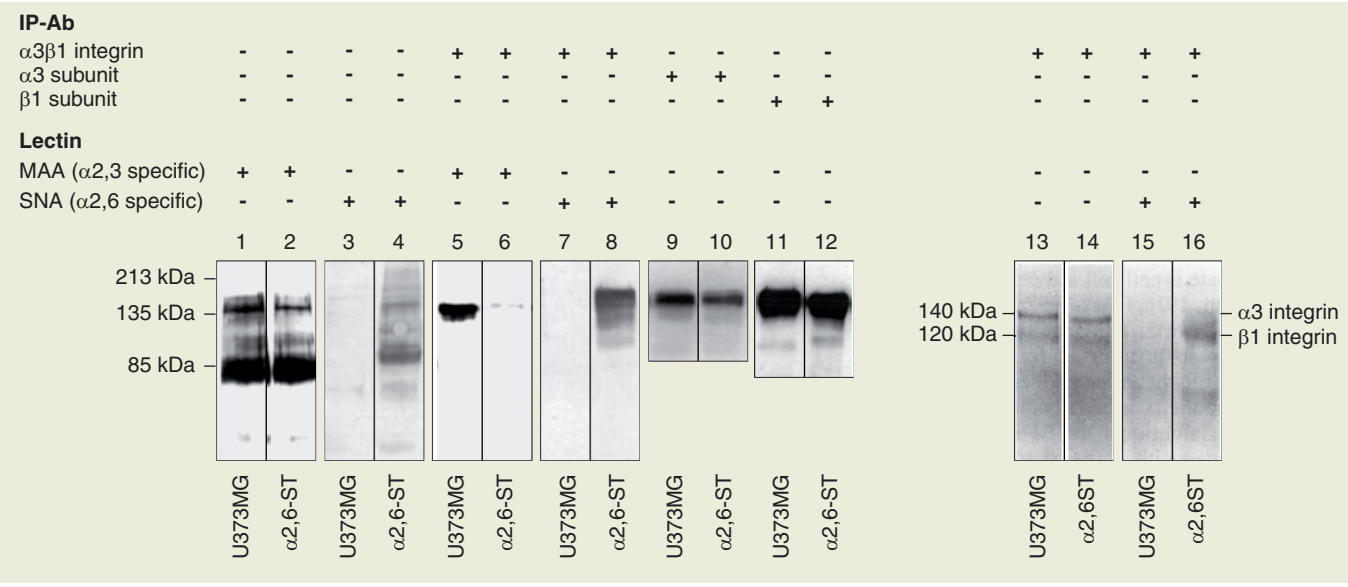

Figure 2. Terminal sialylation of α3β1 integrin in α2,6-ST-transfected U373MG glioma cells. Lanes 1–4: lectin blot analysis of whole cell extracts using the indicated lectins. Robust MAA lectin staining was seen in the both the parental U373MG and α2,6ST stable transfectants, whereas SNA lectin staining was seen only in the α2,6ST transfectants. Lanes 5–8: immunoprecipitation analyses using indicated α3β1 integrin- or α3- or β1-specific antibodies, followed by staining with the indicated lectins. α3β1 integrin subunits were coimmunoprecipitated as a 140-kDa protein, which was intensely stained by MAA lectin in the parental U373MG cells and by SNA lectin in the α2,6ST-transfected cells. Results indicate that the α2,3-linked terminal sialic acids of integrin N-glycans were replaced with α2,6-linked sialic acids by α2,6-ST stable transfection in U373 MG cells. Lanes 9–12: immunoblot analysis of whole cell extracts using α3- or β1-specific antibodies, as indicated. Results indicate that the amount of either α3- or β1-subunit remained unchanged by α2,6ST stable transfection in U373 MG cells. Lanes 13–16: mmunoprecipitation analysis of α3β1 integrin subunits in membrane fractions isolated from [^{35}S]-methionine-labeled cells. Immunoprecipitated proteins were solubilized and were electrophoresed. After electrophoresis, the gel was dried and exposed to x-ray film (lanes 13 and 14). The immunoprecipitated proteins were also transferred to a polyvinylidene fluoride membrane after electrophoresis and stained with SNA lectin to detect α2,6-linked sialic acids (lanes 15 and 16). α2,6-linked sialylation of β1 integrin molecules was detected in the transfectant but not in control cells.
MAA: Maackia amurensis agglutinin; SNA: Sambucus nigra agglutinin.

Using the severe combined immunodeficient (SCID) mouse model (the details of the methods are described in the Figure 3 legend) we found that U373MG clones expressing the α2,6-ST gene showed virtually no brain tumor formation, whereas parental cell or mock transfectant-injected SCID mice typically had quite large tumors by comparison. The right-hand panel shows tumor cross-sectional area as a percent of control versus tumor cell type injected. The left-hand panel shows a typical SCID mouse brain 6 weeks after intracranial injection of either parental cells or transfectants, as indicated. Again, it can be seen that SCID mice injected with the α2,6-ST expressing stable transfectants have no detectable tumor formation [43].

From these results it was felt that our approach had clear therapeutic potential. The α2,6-ST transfectants completely suppressed human glioma invasivity *in vitro* and tumor formation itself *in vivo*. Mechanistically, the data strongly suggested that we had modified the glycosylation of the key integrin in gliomas, α3β1, which, in turn, had altered its signal transducing capabilities and modified cell–extracellular adhesion properties of the tumor cells (Figure 4). In addition, these stable transfectants showed an enhanced sensitivity to apoptotic-inducing drugs (Figure 5); a mechanism of most currently used chemotherapeutics used as the standard of care for brain tumor patients. Thus, a substantial amount of information now existed supporting the idea of trying to develop a therapeutic. However, from a clinical perspective, several key issues remained. First of all, showing that a stable transfectant did not form brain tumors when injected into SCID mice was not the same as delivering a glycosyltransferase gene directly to a pre-existing brain tumor *in vivo*. Second, an *in vivo* delivery system needed to be established.

Glycogene therapeutics: creation & evaluation of an adenovirus delivery system

An adenoviral vector containing the α2,6-ST gene, which characterizes its 'infective potential', was created *in vitro* using the U373MG glioma cell line. Mixing experiments were then performed in which we transiently infected U373MG cells with this adenoviral vector construct and injected the infected cells directly into the brains of SCID mice and evaluated tumor formation. This would provide a simple and cost-effective proof-of-concept for the administration of the *α2,6-ST* gene to tumor cells *in vivo*. The results of these 'preclinical' studies are shown in Figure 6, which is a composite of studies evaluating the efficacy of the adenoviral vector containing the *α2,6-ST* gene. Included are the dose–response relationships of infective potential in terms of plaque forming units per cell, a time course of α2,6-ST mRNA expression and the effects of viral infection on *in vitro* invasivity. Details of these studies are contained in the figure legend. Figure 6 (panel D) shows

the results of mixing adenoviral vector containing the α*2,6-ST* gene with U373MG cells and injecting them directly into the brain of SCID mice. No tumor formation was observed. Under identical conditions, using the same vector without the α*2,6-ST* transgene, significant tumor formation can be seen.

Glycobiology-based microarrays for novel therapeutic target identification

The steady-state expression of the oligosaccharides associated with a specific cell-surface glycoconjugate is the result of the concerted expression of all of the glycosyltransferases involved in its biosynthesis and the glycosidases involved in its degradation [105]. In a system as complex as brain tumorigenesis and invasion it seems as important to determine the global context or pattern of these changes as it does to determine the aberrantly expressed, individual glyco-related gene changes found in such systems. Microarray-based approaches are rapidly becoming one of the cornerstone technologies in rapid throughput gene-expression analyses and have made significant impact defining aberrant gene expression in a multitude of tumor systems [106–108]. In order to begin to grasp the intrinsically complex regulation of genes responsible for the synthesis and degradation of specific carbohydrate structures that are essential for mediating glioma invasivity, it was necessary to utilize custom oligonucleotide microarrays. As genes encoding key glyco-related mRNAs are drastically under-represented on most commercial arrays, we have created a microarray core facility to provide a comprehensive platform of glycoconjugate metabolism-associated oligonucleotides assuring the most up-to-date coverage of these gene families. This technology has not yet been systematically applied to the global analysis of glyco-related gene expression in brain tumors, although small pilot studies have recently been reported in colon tumors [109].

The 359 genes comprising our human glycobiology microarray are compiled from currently available National Center for Biotechnology Information (NCBI)/European Molecular Biology Laboratory (EMBL)/The Institute for Genomic Research (TIGR) human sequence databases and the Consortium for Functional Glycomics-Carbohydrate-Active Enzymes (CAZy) databases. Unique sense 45-mer oligonucleotides corresponding to mRNAs of each gene used as probes are individually synthesized, purified and immobilized via a 5′-amino linker onto aldehyde-coated microarrays. Total RNA is reverse transcribed and used as the substrate for RNA amplification and labeling using the indirect aminoallyl methodology based on the Eberwine protocol [110]. To circumvent the inherent biological heterogeneity of clinical GBM specimens [111], a universal reference design was employed [112], and comprehensive statistical analysis platforms utilized to facilitate acquisition of expression profiles from a necessarily large number

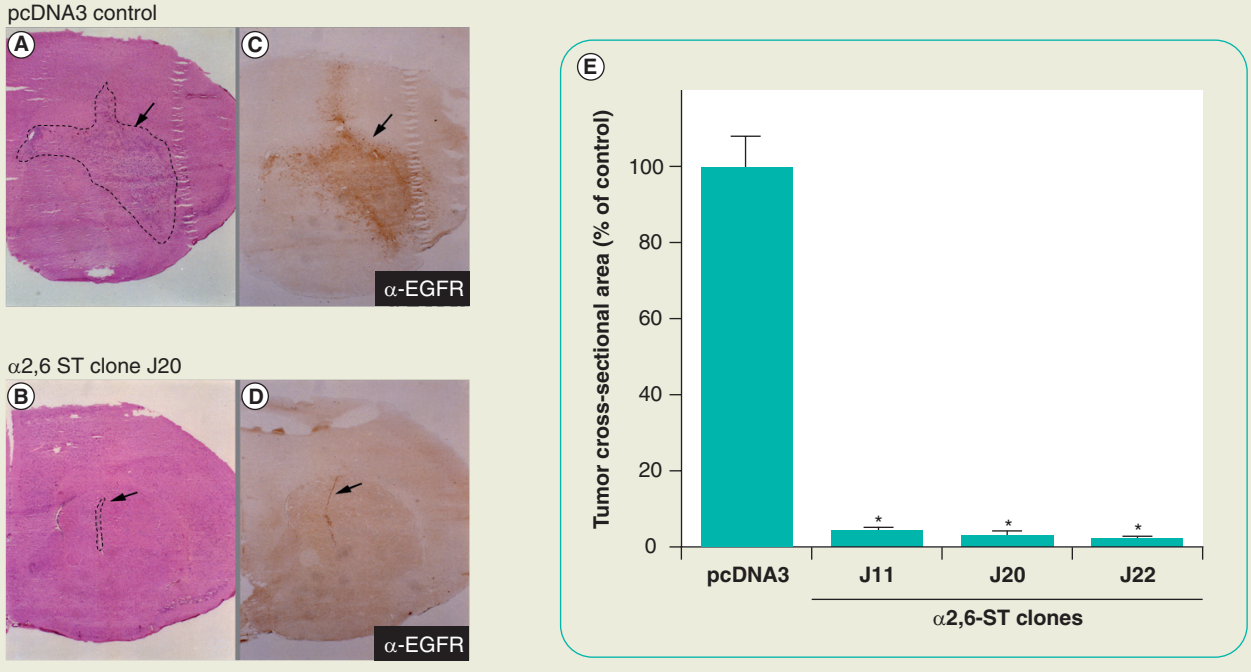

Figure 3. Intracranial tumorigenicity of α2,6ST transfectants. Glioma cells (1.25 x 10^6) were injected into the basal ganglia of severe combined immunodeficient mice at 6 weeks of age with the assistance of a stereotactic frame. The brains were harvested 6 weeks later and sections were stained with H&E **(A)** and **(B)** or antihuman EGFR antibody **(C)** and **(D)** to optimally visualize the resultant tumors. The control pcDNA3 vector-transfected U373MG cells formed large tumors (arrows). The α2,6-ST transfectants formed no tumors, and only the needle-track was stained (arrows). Ten mice/transfectant were used in each group: pcDNA3 vector-transfected U373MG cells as a control, and three α2,6-ST-transfected U373MG clones (J11, J20, and J22). **(E)** The statistical analysis of tumor size in the glioma transfectants. Significant differences in tumor size among the animal groups were determined by χ^2 analysis of the maximum cross-sectional area of each tumor ($^*p < 0.01$).
EGFR: EGF receptor; H&E: Hematoxylin and eosin; ST: Sialyltransferase.

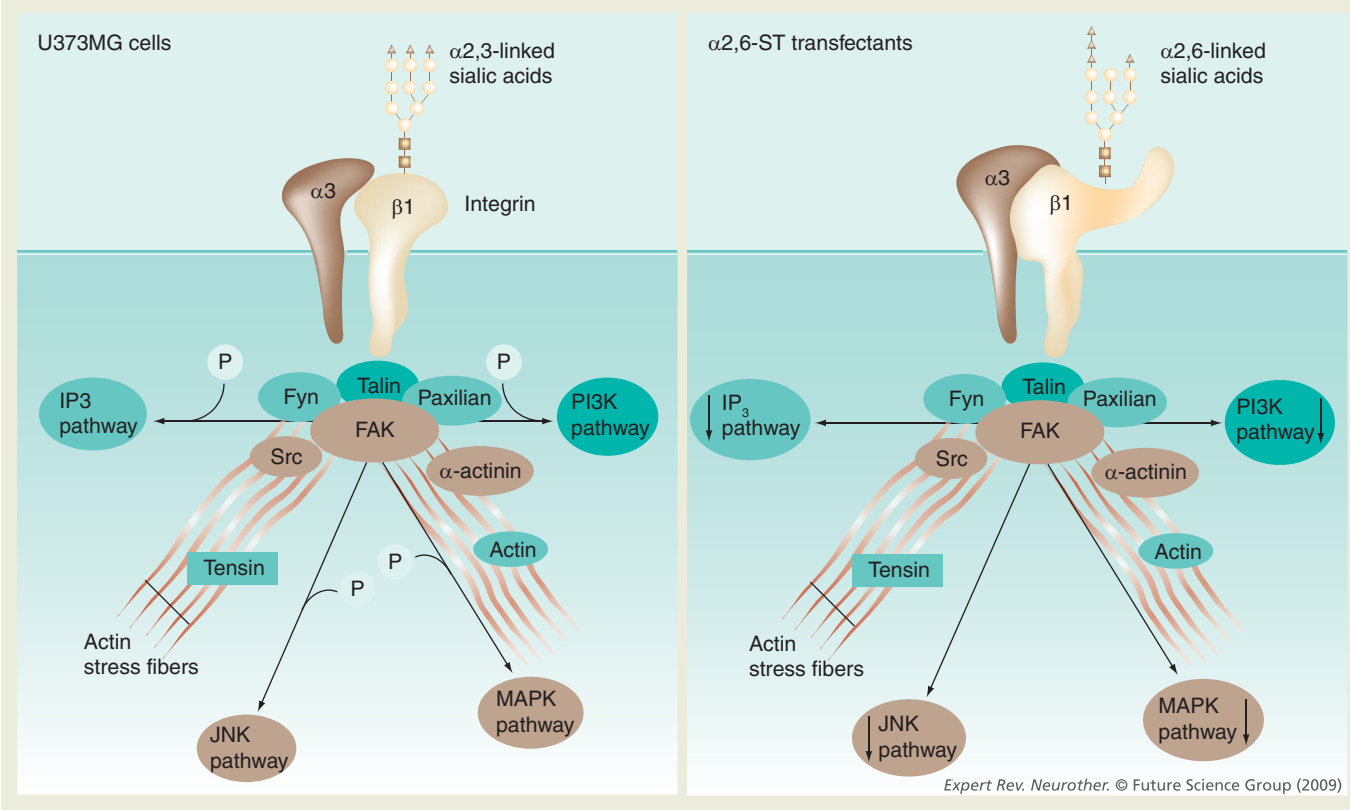

Figure 4. Sialylation-dependent α3β1 integrin signaling. In parental, tumorigenic, U373MG cells, p125^{fak} is present in a phosphorylated form and leads to activation of intracellular signaling cascades mediated by the IP_3, PI3K, JNK and MAPK pathways. Activation of these pathways impacts the increased adhesivity and invasivity in these cells. Expression of α2,6-ST via stable transfection replaces cell surface α2,3-linked sialic acids with α2,6-linked sialic acids specifically on the β1 subunit of the α3β1 integrin receptor, decreases adherence to fibronectin and collagen matrices, decreases adhesion-mediated phosphorylation of p125^{fak} and alters the pattern of focal adhesions, decreases intracellular signaling through the IP_3, PI3K, JNK, and MAPK pathways, and decreases invasivity *in vitro* and tumorigenesis *in vivo*.
ST: Sialyltransferase.

of biological and technical replicates. We have found that our high quality, application-specific, low density microarray platform provides an efficient strategy for such an endeavor.

Initial microarray analyses comparing six grade IV gliomas with six aged-matched normal brain specimens yielded ten significant genes more highly expressed in gliomas compared with normal brain and 24 genes more highly expressed in normal brain compared with gliomas [113]. The expression of two genes, both affecting terminal sialylation, albeit via different mechanisms, in both clinical glioma specimens and glioma cell lines is depicted in Figure 7. There are significant differences in expression of genes associated with glycoconjugate biosynthesis and degradation, many of them novel and all of them potential targets for the development of therapeutics for the treatment of brain tumors. Future studies should include:

- Increasing the number of primary tumors analyzed;
- Evaluating human glioma cell lines by microarray analysis. Cell lines can provide powerful model systems to study the regulation of a given gene or associated gene family;
- Confirming and extending the microarray data with *in situ* hybridization studies and quantitative real time-PCR analyses;
- Continued evaluation of therapeutic candidates using viral vectors in animal models, such as the SCID mouse.

Future directions

Epigenetic control of glycogene expression

DNA methylation is essential for chromosome stability, proper telomere length and maintaining gene-expression states [114–120]. Proper methylation patterns are required for normal development and differentiation, particularly in the brain [121–126]. In primary brain tumors, methylation patterns are severely disrupted. This aberrant hypermethylation of CpG islands in promoter regions is typically associated with gene silencing [121,127–130] and plays a critical role in tumorigenesis and patient prognosis. Many studies on the role of epigenetic silencing in glioma have been carried out using candidate gene approaches and by genome-wide screening methods. Genes regulating cell migration and invasion are frequent targets for epigenetic silencing, and include genes such as *TIMP3*, *EMP3*, cystatin E/M (CST1), *BEX-1* and *-2* and *syk* tyrosine kinase. To date, there has been only one published report describing epigenetic regulation of glyco-related genes in which the authors demonstrated

transcriptional responses of 12 glycogenes in a panel of gastric and colon carcinoma cell lines and clinical specimens to demethylation by treatment with 5′-aza-dC [131]. The expression of the vast majority (ten out of 12) of these was dramatically induced, while the expression of two was actually decreased. Interestingly, these two genes (both fucosyltransferases) transfer terminal fucose to glycoproteins oligosaccharides, and are members of the same functional gene family. Even given the limited subset of glycogenes analyzed, their results suggest that epigenetic control of glycogene expression in tumors does not occur in a random manner, but in defined subsets of glycogenes. Taken in concert with our recent focused microarray data demonstrating that the majority of glycogenes (>70%) differentially expressed in GBMs were significantly downregulated [113], it is tempting to speculate that epigenetic changes and, in particular, DNA hypermethylation, may be the major mechanism of tumor-associated glycogene silencing and subsequent alterations in cell surface glycoconjugate expression.

Improved gene delivery strategies

Preclinical animal studies and human clinical trial data support the use of viral vectors as one of the optimal methods for altering gene expression *in vivo*, as several long-term survivors have been produced. Systemic delivery of viral agents is relatively ineffective, however, as significant sequestration in the liver and the BBB limits the penetration into the tumors. Although intracranial delivery is well tolerated and minimal toxicity has been observed, intratumoral pressure gradients typically limit viral distribution to less than 10 mm from the injection site. In addition, there are still immunogenicity issues to be overcome, as this further limits multiple injections. Recent developments, most notably in convection-enhanced delivery (CED) and conditionally replicative adenoviruses (CRAds), appear to obviate these problems. CED is a regional delivery technique using multiple, strategically placed catheters implanted directly into the tumor and relies on generation of a continuous pressure gradient to significantly enhance intratumoral delivery [132]. CRAds are adenoviruses engineered to preferentially replicate in and kill tumor cells and, in the process, release new viral particles able to enhance the cytotoxic effect by the subsequent infection of neighboring cells (reviewed in [133]). Second-generation CRAds with enhanced tropism to selectively facilitate viral transduction into tumor cells have been developed, have demonstrated enhanced efficacy in preclinical animal models, and are currently in human clinical trials [44,45,134–136]. To date, we envision the use of CED of a CRAd containing one or several glycogenes (depending on the glycogene profile of the patient's tumor) as being an approach with enough therapeutic potential to merit clinical trial development.

Expert commentary & five-year view

The field of glycobiology is now well established in that essentially all of the metabolic pathways for the biosynthesis and degradation of the glycosphingolipids, glycosaminoglycans and glycoproteins have been characterized. Likewise, all of the genes, to all intents and purposes, for their biosynthesis have been cloned and sequenced. Technologies for structure analysis have also matured both in terms of the complexity and detail of structural information obtainable as well as sensitivity of these techniques.

Many specific 'glyco-functions' have also been established. These include, among others, the control of intracellular trafficking, matrix and cell–cell recognition, specific receptor functions, protein–protein binding interactions and allosteric control of cell-surface protein activity [90]. Glycogene expression is also developmentally regulated and cell type-specific [137]. It is also clear that aberrant cell-surface glycosylation patterns accompany cellular transformation and appear on virtually all tumor cells [138].

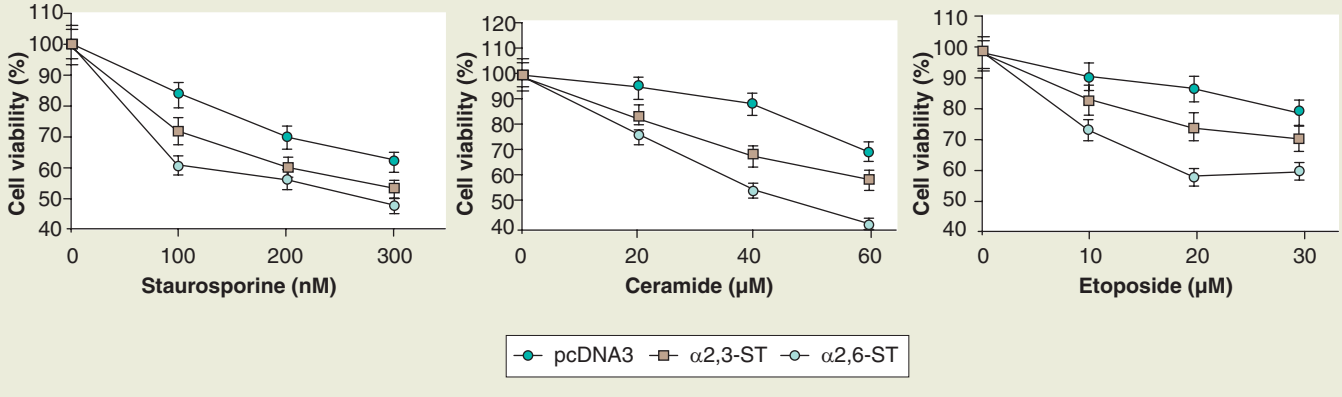

Figure 5. Decreased resistance to proapoptotic drugs following glycosyltransferase transfection. Response of U373MG cells and U373MG following stable transfection of α2,6-ST and α2,3-ST to increasing concentrations of staurosporine, ceramide and etoposide (ETO). Cell death was measured by the MTT method. Results are average ± standard error. Data represent the average ± standard error of two independent experiments with each sample done in triplicate. For each transfectant, differences in chemosensitivity were statistically significant for all drugs at all doses tested (ANOVA, $p < 0.05$).
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ST: Sialyltransferase.
Adapted with permission from [65] ©Wiley-Blackwell.

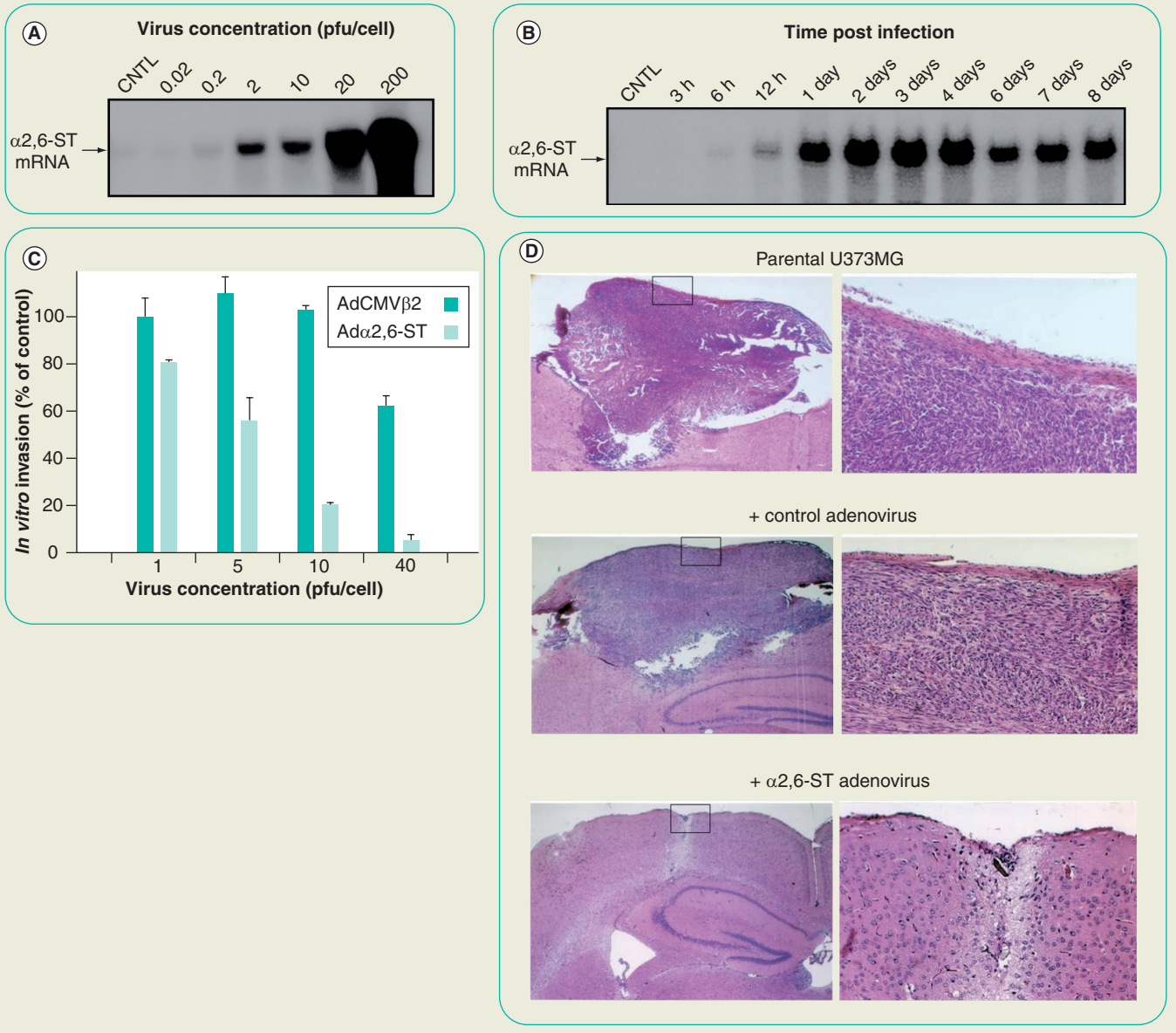

Figure 6. Adenoviral-mediated gene therapy of human gliomas. (A) Dose-dependent expression of α2,6-ST mRNA in U373MG cells. Northern blot analysis probed with radiolabeled α2,6-ST cDNA. Lane 1: U373MG cells with no virus; lanes 2–7: U373MG cells 48 h following infection with, respectively, 0.02, 0.2, 2.0, 10.0, 20.0 and 200 pfu/cell of plaque-purified Adα2,6-ST. **(B)** Time-dependent expression of α2,6-ST mRNA in U373MG cells. Northern blot analysis probed with radiolabeled α2,6-ST cDNA. Lane 1: U373MG cells with no virus; lane 2: U373MG cells 3 h after infection with 10 pfu plaque-purified Adα2,6-ST / cell; lanes 3–1: 6 h, 12 h, 1, 2, 3, 4, 6, 7 and 8 days after infection. **(C)** Inhibition of U373MG glioma cell invasion *in vitro* by Adα2,6-ST. U373MG cells were infected with plaque-purified Adα2,6-ST (grey bars) or a control virus, AdCMVβ2 (black bars), which contains a nonexpressed β-galactosidase marker gene. Cells were infected at 1, 5, 10 and 40 pfu/cell and invasion assay performed following a 4-day incubation period. Data are shown as percentage invasion of nonvirus-infected control U373MG cells. Statistically significant differences ($p < 0.01$, two-tailed, unpaired Student's t-test) between Adα2,6-ST- and control-infected cells were observed at all titers tested. **(D)**. *In vivo* tumorigenicity of Adα2,6-ST-infected U373MG glioma cells. Female severe combined immunodeficiency mice were anesthetized and 1 x 10^8 cells was stereotactically injected over a period of 10 min at a depth of 2.5 mm at a location 1.5 mm anterior to the bregma and 2 mm to the right of the sagittal suture. Ten mice were injected with parental U373MG cells, ten mice were injected with U373MG cells infected with the control adenovirus, AdCMVβ2, and ten mice were injected with U373MG cells infected with the adenovirus Adα2,6-ST. At 28 days postinjection brains were harvested, formalin-fixed, and sectioned with 5 micron slices every 100 microns. The brains were then stained with hematoxylin and eosin and evaluated by light microscopy.
CNTL: Control; pfu: Plaque-forming unit; ST: Sialyltransferase.

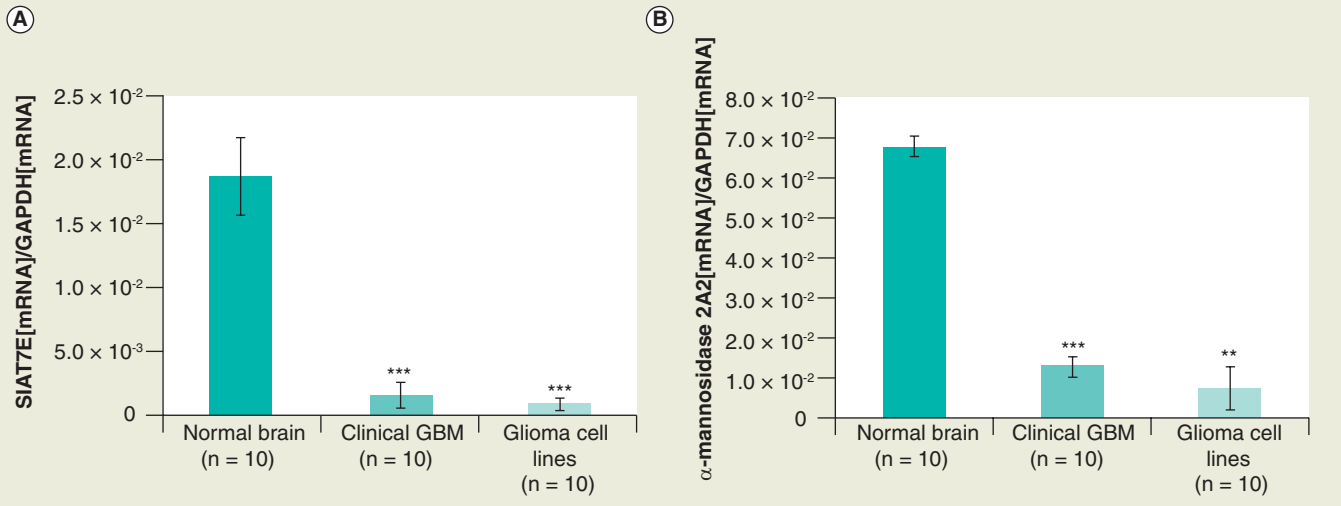

Figure 7. Real time-PCR analysis of selected glyco-targets identified by microarray analysis in clinical glioma specimens, glioma cell lines, compared with normal brain. For each mRNA, transcript abundance, normalized to GAPDH was calculated by direct comparison to standard curves. Data are presented for SIAT7E (ST6GalNAcV) **(A)** and MAN2A2 **(B)**, and represent mean (±SD). Significant differences between GBM and normal brain were observed for both genes.
$^{**}p < 0.01$; $^{***}p < 0.001$; two-tailed, unpaired Student's t-test.
GBM: Glioblastoma; SD: Standard deviation.
Adapted with permission from [114] ©Wiley-Blackwell.

With the exception of the carbohydrate processing inhibitor swainsonine, reaching Phase IB clinical trials [69], there has been very little activity towards developing cancer therapeutics. A recent article by Jefferis reviewed the idea of enhancing antibody efficacy by modifying the oligosaccharides attached to it and thus improving antibody-based therapeutics [139]. Additionally, Tarp and Clausen have suggested that controlled *O*-glycosylation of mucins, particularly MUC1, could significantly impact the design of an effective mucin-based anti-breast cancer vaccine [140].

From the perspective of moving toward a viable glyco-based therapeutic(s) for the treatment of brain tumors, the approach of directly manipulating glycogene expression patterns via gene manipulation holds promise. It seems reasonable to assume that a highly invasive brain tumor cell (e.g., a grade IV glioblastoma) has an optimized pattern of cell-surface oligosaccharides for this behavior. Studies discussed here have shown that altering these patterns by altering glycogene expression, via direct viral delivery to tumor cells, can stop invasivity and tumor formation in *in vivo* animal models. Moreover, enhanced sensitivity to apoptotic agents has been observed, which in and of itself is potentially important but these data also suggest that altered tumor cell surface oligosaccharide expression could enhance radiation sensitivity. These results are very compelling and this approach is the only one nearing clinical trials to date. Until this approach is tested in human clinical trials the verdict will be out as to the true value of this approach. An acceptable delivery system for clinical trials will have to be chosen. The impact of individual glycogene delivery compared with multiple glycogenes to the same tumor will have to be evaluated. Continued glycogene profiling via focused arrays and quantitative real time-PCR and related techniques will be important to carefully fingerprint more and more tumors because of the marked diversity of brain tumors found in children and adults, brain tumors arising from metastases to the brain, possible alterations in oligosaccharide profiles arising from tumor recurrence and the effects of prior chemo and radiation therapies. These are all goals that are within relatively easy reach. In the long run, small molecule, glycogene-expression manipulators that cross the BBB would be ideal. As we learn more about glycogene structure and regulation these kinds of drugs could emerge. In the short term, direct delivery of glycogenes to brain tumor cells appears to have real therapeutic potential and should lead to important information as to how cell-surface expressed oligosaccharides modulate brain tumorigenesis and invasivity.

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Key issues

- Although the annual incidence of malignant gliomas and astrocytomas is small when compared with such cancers as breast, prostate, lung and ovarian, brain tumors are now the second leading cause of cancer death in children under the age of 20 years and the third leading cause of cancer death in young adults between the ages of 20 and 39 years.
- Glycobiology, the study of the biosynthesis, metabolism and function of oligosaccharides attached to lipids and proteins, is now a mature field with virtually all of the metabolic pathways characterized and genes cloned and sequenced. Methods have emerged for highly sensitive molecular identification, as well as fine structure analysis.
- Oligosaccharide functions include adhesion, intercellular communication, conformational control of cell surface receptor functions, including ligand binding and signal transduction, and cell–extracellular matrix interactions.
- To date, brain tumor glycobiology research has primarily been descriptive. Significant alterations in the cell surface expression of oligosaccharides found on glycosphingolipids, such as gangliosides, and adhesion molecules, including cell adhesion molecules, CD44, neural cell adhesion molecule, cadherins and galectins, have been found on all brain tumors, differing to some extent depending on tumor grade, type or invasive potential. All tumor cell-surface oligosaccharide patterns are markedly different compared with their normal brain counterparts.
- Alterations in the genes responsible for oligosaccharide biosynthesis and degradation have also been found in brain tumors. These include sialyltransferases, fucosyltransferases, N-acetylglucosaminyltransferases and mannosidases.
- To date, no therapeutic programs have emerged manipulating oligosaccharide expression; however, an antiganglioside monoclonal antibody-associated peptide has been identified that effectively disrupted metastasis *in vivo*. Neurostatin, *O*-acetylated GD1b, has been found to inhibit glioma proliferation using human cell lines, and direct manipulation of glycosyltransferase gene expression in gliomas has led to inhibition of glioma invasivity and tumor formation *in vitro* and *in vivo* in a variety of model systems.

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