Published in final edited form as:

Exp Cell Res. 2008 October 1; 314(16): 2941–2950. doi:10.1016/j.yexcr.2008.07.021.

Tumor cell migration and invasion are regulated by expression of variant integrin glycoforms

Faheem M Shaikh¹, Eric C Seales², William C Clem³, Kristin M Hennessy¹, Ya Zhuo¹, and Susan L Bellis^{1,3,*}

¹Department of Physiology and Biophysics, University of Alabama at Birmingham, Birmingham, Alabama 35294, USA

²Vivo Biosciences, Birmingham, Alabama, 35205, USA

³Department of Biomedical Engineering, University of Alabama at Birmingham, Birmingham, Alabama 35294, USA

Abstract

The ST6Gal-I glycosyltransferase, which adds α 2-6-linked sialic acids to glycoproteins, is overexpressed in colon adenocarcinoma, and enzyme activity is correlated with tumor cell invasiveness. Previously we reported that forced expression of oncogenic ras in HD3 colonocytes causes upregulation of ST6Gal-I, leading to increased α 2-6 sialylation of β 1 integrins. To determine whether ras-induced sialylation is involved in promoting the tumor cell phenotype, we used shRNA to downregulate ST6Gal-I in ras-expressors, and then monitored integrin-dependent responses. Here we show that forced ST6Gal-I downregulation, leading to diminished α 2-6 sialylation of integrins, inhibits cell adhesion to collagen-I, a β 1 ligand. Correspondingly, collagen binding is reduced by enzymatic removal of cell surface sialic acids from ras-expressors with high ST6Gal-I levels (i.e., no shRNA). Cells with forced ST6Gal-I downregulation also exhibit decreased migration on collagen-I and diminished invasion through Matrigel. Importantly, GD25 cells, which lack β 1 integrins (and ST6Gal-I), do not demonstrate differential invasiveness when forced to express ST6Gal-I, suggesting that the effects of variant sialylation are mediated specifically by β 1 integrins. The observation that cell migration and invasion can be blocked in oncogenic ras-expressing cells by forcing ST6Gal-I downregulation implicates differential sialylation as an important ras effector, and also suggests that ST6Gal-I is a promising therapeutic target.

Keywords

Ras; integrin; sialic acid; metastasis; collagen; colonocytes

Introduction

An altered profile of cell surface oligosaccharides is a well-known hallmark of malignant change. Among the more common cancer-associated carbohydrate modifications are those involving the length and branching of polylactosamine chains and/or the quantity and linkage

*Correspondence to: Susan L. Bellis, Ph.D. Rm. 982A MCLM, 1918 University Blvd. Birmingham, AL 35294 Fax: (205) 975-9028 bellis@physiology.uab.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

of terminal sialic acids [1-7]. Sialic acids are large, negatively charged sugars that can be added in several types of linkages (e.g., α 2-3, α 2-6, or α 2-8) [1,6,8]. The enzyme that adds α 2-6-linked sialic acids, β -galactoside α 2-6 sialyltransferase (ST6Gal-I), is known to be upregulated in many tumor types including colon adenocarcinomas [9-17], and high levels of expression are associated with metastasis and poor patient prognosis [18]. *In vitro* studies suggest that sialylation of membrane glycoproteins by ST6Gal-I contributes to metastasis by enhancing cell motility and invasion through the extracellular matrix. For example, overexpression of ST6Gal-I increases the invasiveness of fibroblasts [19] and breast cancer cells [20], whereas the expression of antisense ST6Gal-I in HT29 colon epithelial cells (having endogenous ST6Gal-I) blocks invasion through Matrigel [21]. Studies of metastasis in animal models corroborate these findings. Harvey et al. showed that pretreatment of colorectal tumor cells with an inhibitor of sialic acid incorporation prior to intrasplenic injection in athymic mice resulted in a marked decrease in the number of animals with hepatic metastasis [22]. Likewise, Bresalier et al. reported that intrasplenic injections of two differing populations of 51B colon cells; a heavily α 2-6 sialylated population and a poorly-sialylated population, resulted in hepatic tumors almost exclusively formed by the highly sialylated cells, indicating selective metastasis [23]. In the latter study it was also shown that metastasis was significantly inhibited if the α 2-6 sialylated cells were pre-treated with a sialidase enzyme to cleave the sialic acids prior to splenic injection.

Despite the extensive literature implicating ST6Gal-I in tumor progression/metastasis, a mechanistic understanding of the role of differential sialylation in regulating tumor cell behavior has been limited by the fact that the spectrum of substrates for ST6Gal-I has not been well-defined. Moreover, few studies have sought to elucidate the effects of α 2-6 sialylation on the function of specific membrane glycoproteins. In prior work [24,25] we identified the β 1 integrin as an ST6Gal-I substrate, and used a cell-free receptor/ligand binding assay to show that purified collagen-binding integrins bind better to collagen I when α 2-6 sialylated. This approach was complemented by studies of integrin-dependent cell behaviors in SW48 colon epithelial cells that either lacked all ST6Gal-I expression, or were alternately engineered to overexpress ST6Gal-I [25]. Specifically, we found that cells with α 2-6 sialylated integrins (due to ST6Gal-I expression) demonstrated significantly greater adhesion and migration on collagen I as compared with unsialylated integrin-expressing cells. As well, the sialylated integrins exhibited a greater degree of association with talin, suggesting that sialylated integrins were in a more activated state.

While comparative studies of cells that either lack, or have forced expression of, ST6Gal-I are useful for dissecting the role of α 2-6 sialylation in modulating integrin function, in an actual tumor cell, endogenous ST6Gal-I would likely be upregulated as a secondary consequence of aberrant signaling mechanisms. Indeed, we and others reported that ST6Gal-I expression is increased in response to signaling by oncogenic ras [24,26,27], and Piller's group showed that the underlying mechanism appears to involve increased ST6Gal-I transcription [27]. In light of these results, one major question that arises is whether, in the context of a cell with oncogenic ras, sialylation-dependent changes in integrin activity would have a significant effect on tumor cell interaction with the matrix. In order to address this question, we manipulated ST6Gal-I expression in oncogenic-ras expressing HD3 colon epithelial cells. We previously reported that stable expression of constitutively-active ras in HD3 cells led to increased expression of ST6Gal-I [24]; in the current study, we reversed expression of ST6Gal-I in these cells using shRNA, and then monitored the effects on integrin-dependent cell behaviors. We now show that downregulation of ST6Gal-I, and accordingly, expression of hyposialylated integrins, in oncogenic ras-expressing cells significantly inhibits cell adhesion and haptotactic migration toward collagen I, and also blocks cell invasion through Matrigel.

Materials & Methods

Cell culture and reagents

HD3 colon epithelial cells were originally obtained from Dr Eileen Friedman (SUNY, Syracuse) and were maintained in low-glucose DMEM containing 7% FBS, penicillin-streptomycin and amphoterecin B. As previously described [24], HD3 cells were stably transfected with an H-ras-expressing plasmid ("V12 ras") containing an activating mutation in the 12th codon (generous gift from Dr Elizabeth Eklund, Northwestern University). These HD3 cells expressing oncogenic ras were then transduced with shRNA to stably silence ST6Gal-I gene expression. Specifically, cells were infected with one of five different lentiviral vectors (MOI=5) containing distinct shRNA sequences. The shRNA-expressing lentiviral vectors were purchased from Sigma (Mission shRNA library), and transduction of cells was accomplished exactly as recommended by the vendor protocol, with the exception that hexadimethrine bromide was not used. Cells with stable integration of the shRNA construct were isolated through puromycin selection, and a pooled population of clones was used for all studies. As shown in Figure 1, all of the five sequences were able to downregulate ST6Gal-I, however sequence #4 (cat. # TRCN0000035432, Sigma) exhibited maximal downregulation, and therefore this population was used for all further studies. GD25 fibroblasts lacking endogenous β 1 integrin expression were a kind gift from Dr Dean Mosher (University of Wisconsin). GD25 cells were maintained in high-glucose DMEM containing 10% FBS, penicillin-streptomycin and amphoterecin B. For some experiments, GD25 cells were transduced with lentivirus containing a V5-tagged ST6Gal-I construct (MOI=3), and a stable population of pooled clones was obtained by puromycin selection. The V5-tagged ST6Gal-I plasmid (gift from Karen Colley, University of Illinois at Chicago) was incorporated into lentivirus as previously described [25], and verification of ST6Gal-I expression was accomplished by immunoblotting for the V5 tag.

Flow Cytometry

Cells were harvested nonenzymatically from tissue culture dishes using Cell Stripper solution (Cellgro). Cells were then resuspended into PBS containing 0.2% heat-denatured BSA as well as unlabeled IgG (Sigma), and incubated on ice for 10 min to block nonspecific binding. Following blocking, cells were resuspended into PBS/BSA solution containing one of the following reagents; a nonspecific IgG-FITC conjugate (Invitrogen), the glycosylation-insensitive anti- β 1 integrin antibody, CBL-481 directly conjugated to FITC (Chemicon), or SNA-FITC (EY Labs), all at the recommended dilutions. For labeling of the β 1 integrin, cells were incubated with FITC-conjugates for 30 min on ice, whereas SNA-FITC labeling was performed at 37°C for 15 min. After labeling, cells were washed in PBS/BSA and then analyzed with FACSCalibur (Becton-Dickinson) by the UAB Arthritis and Musculoskeletal Center Analytic and Preparative Core Facility (APCF).

Western blotting

HD3 cells containing the V12 ras construct, or a combination of V12 ras and shRNA against ST6Gal-I, were solubilized in "lysis buffer" consisting of 50 mM Tris-HCl buffer (pH 7.4) with 1% Triton X-100 and a protease inhibitor cocktail (Roche). Protein concentrations were determined using a Bradford assay (Sigma). Cell lysates (50 μ g) were resolved by reducing SDS-PAGE, and proteins were subsequently transferred to a PVDF membrane. Membranes were blocked for 1-2 h with 5% nonfat dry milk. After blocking, blots were incubated for 2 hours with an antibody specific for either the β 1 integrin (BD Biosciences), ST6Gal-I (a monoclonal generated by the University of Alabama Hybridoma Core Facility) or the V5 epitope (Invitrogen). Blots were then washed several times with Tris-buffered saline containing 0.05% Tween-20 (TBST). Following the washes, blots were incubated for 1 hour with a horseradishperoxidase (HRP)-coupled secondary antibody (Amersham), then washed again

with TBST. Blots were incubated with a luminescent HRP substrate (Amersham) for 1 min and positive signals were detected using the enhanced chemiluminescence method (ECL).

Lectin assays

Cell lysate proteins (1000 µg) were incubated overnight with 50 µl agarose-conjugated SNA-1 lectin with rotation at 4°C (EY Laboratories). α 2-6 sialylated proteins complexed with the SNA lectin were collected by brief centrifugation, and washed three times with lysis buffer, followed by one wash with phosphate-buffered saline (PBS). Sialylated proteins were released from the complexes by boiling in SDS-PAGE sample buffer. The glycoproteins were resolved by SDS-PAGE, then immunoblotted to detect β 1 integrins.

For some experiments, sialylated proteins were detected by blotting with either HRP-conjugated SNA, or biotin-conjugated MAA (EY Labs) followed by incubation with streptavidin-HRP. Briefly, cell lysate glycoproteins or β 1 integrin immunoprecipitates were resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% nonfat dry milk, then incubated for one hour with the lectin at a dilution of 1:200. Lectin-reactive proteins were visualized by enhanced chemiluminescence.

Collagen attachment assays

HD3 cells were detached from culture dishes using Cell Stripper (Cellgro) and then resuspended in serum-free medium. Cells were plated onto tissue culture-treated dishes precoated with 20 µg/mL collagen I (Inamed) and blocked with 2% denatured bovine serum albumin (BSA). Cells were also plated onto dishes coated with 2% denatured BSA alone to control for nonspecific binding. After 90 minutes of incubation at 37°C, nonadherent cells were removed by PBS washing. Adherent cells were fixed for 40 min in 3.7% formaldehyde and then stained for 40 min with 0.1% crystal violet. Stained cells were subsequently solubilized in 10% acetic acid, and absorbance spectroscopy (540 nm) was used to quantify the amount of dye in each sample.

Sialidase treatment

HD3 cells were suspended in serum-free DMEM pH 7.0 at 1×10^6 cells/ml. Cells were then treated with *Vibrio cholerae* sialidase (Roche) for 75 min at 37°C using a concentration range of 1 - 100 mU/ml. Control cells (C) were incubated under the same conditions without sialidase. After washing three times in PBS, the cells were plated at 7.0×10^5 cells/well onto 12-well tissue culture dishes that had been precoated with 30 µg/ml collagen I. After incubating at 37°C for 40 min, nonadherent cells were removed by washing with PBS. Cells were fixed and stained with crystal violet and adhesion was quantified by absorbance spectroscopy (540 nm).

Collagen haptotactic migration assay

Collagen I haptotaxis was evaluated using the QCM Collagen I Quantitative Cell Migration Assay kit (Chemicon International). HD3 cells were detached with nonenzymatic Cell Stripper solution (Cellgro) and then resuspended in serum-free medium. Cells were then seeded 5×10^5 cells per well into the upper wells of Boyden chambers lined with 8.0-µm polyethylene terephthalate (PET) membranes coated on the underside with collagen I. The lower chambers were filled with 300 µL per well of media plus 2% fetal bovine serum (as a chemoattractant). After 24 hours incubation at 37°C, residual cells were carefully and thoroughly wiped from the top surface of the filter and cell migration was quantified by standard crystal violet staining of cells that had migrated to the underside. Stained cells were subsequently solubilized in 10% acetic acid, and absorbance spectroscopy (540 nm) was used to quantify the amount of dye in each sample.

Cell invasion Assay

In vitro invasiveness of the HD3 and GD25 cells was evaluated using the BD BioCoat™ growth factor reduced Matrigel™ Invasion chambers (BD Biosciences). 0.5 ml medium was added to the interior of the inserts and the matrix was allowed to rehydrate at 37°C for 2 hours in a humidified tissue culture incubator. After rehydration the medium was carefully removed without disturbing the layer of matrix on the membrane. HD3 and GD25 cells were detached from tissue culture dishes with Cell Stripper solution and then resuspended in serum free medium. 0.5 ml of cell suspension containing 2.5×10^4 cells was immediately added to the inside of the inserts. 0.75 ml of conditioned media from NIH 3T3 cell cultures was added to the lower chambers to serve as chemoattractant solution. Plates were incubated at 37°C in a humidified 5% CO₂ incubator for 48 hours. After incubation, residual non-invading cells were thoroughly wiped from the top surface of the inserts and the invasive cells at the bottom of the insert were stained and quantified. The highly invasive HT1080 fibrosarcoma cell line was used as a positive control for verification of invasion in this assay (data not shown).

Results

Stable downregulation of ST6Gal-I in cells with oncogenic ras

Previously we engineered HD3 colonocytes to express an oncogenic ras isoform, and found that this caused an increase in the expression and activity of endogenous ST6Gal-I [24]. In order to reverse levels of ST6Gal-I in oncogenic ras-expressing cells, we infected cells with one of five different ST6Gal-I shRNA-expressing lentiviral vectors, and then antibiotic selection was used to obtain stably-transduced cells. Downregulation of ST6Gal-I was verified by Western blotting. As shown in Figure 1A, all of the shRNA constructs were effective in downregulating endogenous ST6Gal-I, with the maximal decrease observed for construct # 4. Accordingly, cells stably-expressing construct #4 were used for all subsequent studies (hereafter referred to as “sh.ST6”).

To assess ST6Gal-I activity, cells were subjected to flow cytometry using SNA, a lectin which specifically binds to α 2-6 linked sialic acids (Figure 1B). These experiments revealed that, on average, cells expressing shRNA for ST6Gal-I had lower amounts of α 2-6 sialic acid on the cell surface than parental cells, although the SNA labeling of sh.ST6 cells was heterogeneous. This heterogeneity likely reflects the fact that sh.ST6 cells are comprised of a pooled population of clones, and would therefore be expected to include both high and low shRNA expressors.

Cells transduced with shRNA for ST6Gal-I have decreased α 2-6 sialylation of β 1 integrins

HD3 cells that express oncogenic ras were shown to have hypersialylated β 1 integrins as a result of ST6Gal-I upregulation [24]. To assess the level of integrin sialylation in cells with downregulated ST6Gal-I, we incubated cell lysates with agarose-conjugated SNA, then precipitated the SNA-reactive proteins, performed SDS-PAGE, and immunoblotted for the β 1 integrin. Whole cell lysates were also Western blotted for the β 1 integrin to quantify total β 1 integrin expression in HD3 cells. As shown in the upper panel of Figure 2A, the ST6Gal-I shRNA construct caused a marked decrease in the level of sialylation on the β 1 integrin, as expected. Blots of total β 1 integrin protein in the cell lysates indicate that similar amounts are expressed (lower panel of Figure 2A). Of note, the upper band in β 1 immunoblots reflects the Golgi-modified mature β 1 integrin species (representing the functional receptor), whereas the lower band depicts a partially-glycosylated precursor isoform which is not α -6 sialylated, given that it is localized to the endoplasmic reticulum [28,29]. Bands were analyzed by densitometry, and ratios of α 2-6 sialylated to total β 1 integrin (mature form) were calculated (Figure 2B). In addition, cells were subjected to flow cytometry using a glycosylation-insensitive antibody to measure total surface levels of β 1 integrin (Figure 2C). As shown, forced downregulation of

ST6Gal-I did not affect surface expression of $\beta 1$, indicating that loss of SNA reactivity was specifically due to a decrease in integrin sialylation.

It is thought that ST6Gal-I can compete with various $\alpha 2$ -3 sialyltransferases for the galactose moieties on the termini of lactosaminyl chains. Thus, we measured the level of $\alpha 2$ -3 sialylation on the $\beta 1$ integrin by evaluating reactivity toward MAA, a lectin that is selective for $\alpha 2$ -3-linked sialic acids (Figure 2D). Interestingly, $\beta 1$ integrins were labeled by MAA, indicating the presence of $\alpha 2$ -3 sialylation, however no differences were apparent in the degree of MAA reactivity between the control and sh.ST6 cells. These results suggest that the loss of $\alpha 2$ -6 sialylation on $\beta 1$ integrins from sh.ST6 cells is not compensated for by a gain in $\alpha 2$ -3 sialylation.

Cells expressing hyposialylated integrins due to downregulated ST6Gal-I adhere poorly on collagen I

Having established a cell model system in which sialylation levels were manipulated within the context of signaling by oncogenic ras, we proceeded to evaluate integrin function. As previously reported [30], the binding of HD3 cells to collagen I is mediated predominantly by $\alpha 2\beta 1$ integrin receptors, therefore we monitored the effects of differential sialylation on integrin function by quantifying cell adhesion to collagen I. As shown in Figure 3A, cells expressing hyposialylated $\beta 1$ integrins (sh.ST6) demonstrated significantly less adhesion to collagen I as compared with control cells.

Enzymatic removal of cell surface sialic acids inhibits cell adhesion to collagen I

In order to further establish that differential sialylation modulates integrin-dependent cell adhesion, oncogenic ras-expressing cells with high endogenous ST6Gal-I (i.e., the cells without shRNA for ST6Gal-I) were treated with sialidase, an enzyme that cleaves sialic acid residues from glycoproteins. The treated cells were then allowed to adhere to collagen I and binding was quantified as before. As shown in Figure 3B, enzymatic removal of cell surface sialic acids by sialidase elicited a dose-dependent reduction in collagen binding.

Cells with hyposialylated $\beta 1$ integrins migrate poorly on collagen-I

Using migration chamber assays (Chemicon), we measured the haptotactic migration of cells toward collagen I. Specifically, cells were resuspended in serum-free medium and then seeded into the upper wells of Boyden chambers coated on the underside with collagen I. The lower chambers contained medium plus 2% FBS as a chemoattractant. After 24 hours, the cells that had migrated through the membrane and attached to the collagen layer were stained with crystal violet, and then solubilized to release the dye into solution. Cell number was quantified by measuring solution absorbance. Figure 4A shows that cells expressing hyposialylated integrins (sh.ST6) migrated poorly toward collagen I as compared to control cells.

Cells with hyposialylated integrins exhibit diminished invasion through Matrigel

To establish that downregulation of ST6Gal-I and consequent $\beta 1$ could modulate the invasive capability of colon tumor cells, we evaluated the invasiveness of our cell lines through Matrigel™ (a reconstituted extracellular matrix), an assay which has been used to assess the metastatic potential of tumor cells [31]. As shown in Figure 4B, we found that the sh.ST6 cells were significantly less invasive through Matrigel as compared to the control cells.

Differential sialylation does not alter the invasiveness of cells lacking expression of $\beta 1$ integrins

Studies from our laboratory suggest that ST6Gal-I may have a restricted substrate specificity [24], however clearly $\beta 1$ integrins are not the only targets for this enzyme. Accordingly, we

questioned whether the diminished invasiveness of cells with downregulated ST6Gal-I was mediated specifically by the $\beta 1$ integrin. To address this hypothesis, we performed invasion assays with GD25 fibroblasts, a cell line that lacks expression of the $\beta 1$ integrin [32]. Briefly, GD25 cells (which express low levels of endogenous ST6Gal-I, not shown) were stably transduced with a V5-tagged ST6Gal-I, and expression of the enzyme was evaluated by immunoblotting for the V5 tag (Figure 5A). To confirm that the exogenous ST6Gal-I enzyme was functional, lysates from parental (Par) or ST6Gal-I-transduced (ST6) GD25 cells were probed for $\alpha 2$ -6 sialylation using the SNA lectin. As shown in Figure 5B, ST6Gal-I expressors displayed an increase in the number and intensity of SNA-reactive bands, indicating upregulated levels of protein sialylation. Parental and ST6Gal-I expressors were then evaluated for invasion through Matrigel as before. As shown in Figure 5C, GD25 cells were able to invade through Matrigel, however in this case, the expression of ST6Gal-I had no effect on the degree of invasiveness. These results are consistent with the hypothesis that the alterations in the invasiveness of HD3 cells are due to changes in sialylation on the $\beta 1$ integrin.

Discussion

Tumor invasion and metastatic spread are complex, multistep processes requiring a dynamic interaction of tumor cells with host environment. Integrins are known to be crucial regulators of tumor cell interactions with the microenvironment. The $\beta 1$ integrin subunit heterodimerizes with at least 12 different α subunits, creating receptors that mediate cell interactions with a wide variety of extracellular matrix ligands such as collagen, laminin, and fibronectin. These interactions, in turn, modulate diverse cell behaviors, including proliferation, survival, adhesion and motility. There is an increasing number of reports in the literature implicating the $\beta 1$ integrin in tumorigenesis, both *in vitro* and *in vivo* [33-35]. The $\beta 1$ integrin has been studied extensively in the biology of solid tumors, and its expression has been shown to correlate with poor prognosis in cancers of the lung [36], pancreas [37], and cutaneous melanoma [38]. The $\beta 1$ integrin is also implicated in the progression and metastasis of human breast cancer [39,40]. Finally, signaling through the $\beta 1$ integrin is thought to mediate resistance to chemotherapy and radiotherapy in lung, breast cancer, and hematologic malignancies [41-46].

Among the several integrin subtypes involved in regulating tumor cell behavior, it is emerging that collagen-binding integrins may play a crucial role. Increased expression of the $\alpha 2\beta 1$ heterodimer has been observed in melanoma and gastric cancer, and this integrin species was suggested to be a predictive marker for metastatic disease [47-49]. As with $\alpha 2\beta 1$, increased expression of collagen I, a ligand for $\alpha 2\beta 1$, is associated with tumor progression. Elevated levels of collagen I mRNA are observed in primary tumors with metastatic potential, and it was recently reported that collagen I is one of 17 upregulated genes that constitute a “metastatic signature” [50-52]. Collagen I is enriched on the surface of endothelial cells within the tumor vasculature, as compared with normal or inflammatory endothelium, suggesting that collagen/integrin interactions may be involved in tumor cell dissemination [53]. Taken together, these findings implicate signaling through the collagen I/ $\alpha 2\beta 1$ axis as an important contributor to metastatic spread. Results described in the current study indicate that, in addition to mechanisms involving increased protein expression, the activity of collagen binding integrins can be enhanced through sialylation by ST6Gal-I, a glycosyltransferase known to be upregulated in human colon carcinoma. Indeed, we previously reported that $\beta 1$ integrins are hypersialylated in human colon tumors as compared with pair-matched normal tissue, suggesting that differential sialylation is a physiologically-relevant integrin regulatory mechanism [25]. Consistent with this latter finding, Varki's group reported elevated activation of the integrin-regulated tyrosine kinase FAK (Focal Adhesion Kinase) in mammary tumors from ST6Gal-I expressing mice as compared with ST6Gal-I-null mice [54], suggesting greater integrin activation. Furthermore, these investigators isolated tumor cells from ST6Gal-I-null

mice, transfected the cells with an inducible ST6Gal-I construct, and found that induction of $\alpha 2$ -6 sialylation led to markedly increased FAK activation. Interestingly, the mammary tumors from ST6Gal-I-null mice were more differentiated than tumors from wild-type mice, which may be related, at least in part, to alterations in integrin activation state.

ST6Gal-I expression [24,25,26] and $\beta 1$ sialylation [24] are upregulated by oncogenic ras, and therefore one important goal of the current study was to determine whether changes in integrin sialylation can significantly affect integrin function, when occurring in the background of robust ras-dependent signaling. Several studies have reported that ras modulates integrin activity, however mechanistically this was previously thought to occur primarily through “inside-out signaling”, a process by which intracellular signaling cascades modulate integrin conformation by acting on integrin cytoplasmic tails. Interestingly, ras can cause either integrin activation or inhibition depending on the cell type, as well as the specific ras isoform [55-57]. In the current study, we stably expressed shRNA for ST6Gal-I in cells that have high levels of endogenous ST6Gal-I due to forced expression of oncogenic ras. We found that downregulation of ST6Gal-I, and accordingly, diminished integrin sialylation, inhibited cell adhesion and migration on collagen I. Similarly, oncogenic ras-expressing cells treated with sialidase enzyme to remove cell surface sialic acids exhibited poor adhesion to collagen I. Thus, it seems that differential sialylation can modulate integrin function independently of inside-out signaling. In addition to effects on adhesion and migration, downregulation of ST6Gal-I caused decreased cell invasion through Matrigel. While we are cognizant of the potential role of multiple cell surface molecules in directing cell invasion, we hypothesize that reduced invasiveness may be dependent upon changes in sialylation that occur specifically on the $\beta 1$ integrin subunit. Notably, we found that GD25 cells, which lack expression of $\beta 1$ integrins, do not exhibit differential invasion when forced to express ST6Gal-I.

There are multiple mechanisms by which sialylation might influence integrin function, however we speculate that addition of sialic acid, a negatively-charged sugar, might stabilize the integrin in a given conformation. Sialic acids are added in the trans-Golgi during integrin synthesis and processing, and presumably remain on the integrin for the lifetime of the molecule. Interestingly, it was reported by Luo et al. that introducing an artificial N-linked glycan into the integrin headpiece can activate both $\beta 1$ and $\beta 3$ integrins by inducing an open conformation of the integrin, which in turn, induces an increase in integrin affinity for ligand [58]. These results established “proof of principle” that changes in glycosylation can regulate integrin conformation.

In the aggregate, results from the current investigation suggest that, in addition to establishing differential sialylation as an important integrin regulatory mechanism, increased sialylation may be one of the pathways by which ras promotes a metastatic cell phenotype. Moreover, our results have translational connotations. Numerous studies have implicated ST6Gal-I in tumor progression; we now show that forced downregulation of ST6Gal-I in cells that express the ras oncogene causes impaired integrin-dependent cell migration and invasion. Constitutively-active ras isoforms are strong inducers of multiple signaling cascades, and therapeutic interventions against ras (for example, farnesyltransferase inhibitors) have yielded only limited success [59-62]. The observation that integrin function can be significantly altered through modulating integrin sialylation suggests that ST6Gal-I is a promising therapeutic target. Blocking $\beta 1$ integrin sialylation, with consequent disruption of tumor cell interaction with the extracellular matrix or tumor endothelium, could potentially reduce rates of metastatic lesions in highly aggressive tumors. This becomes important in malignancies having strong oncogenic ras activation, in which the multiple downstream signaling effectors of ras may not be amenable to therapeutic targeting, or in tumors which are resistant to phenotypic reversal by chemotherapy.

Acknowledgements

This work was supported by NIH grant R01CA84248, as well as a grant from the Mizutani Foundation for Glycoscience (S.L.B.). Dr. Shaikh was supported by an American Heart Association Predoctoral Fellowship.

References

- [1]. Dall'Olio F, Chiricolo M. Sialyltransferases in cancer. *Glycoconj J* 2001;18:841–850. [PubMed: 12820717]
- [2]. Dall'Olio F. The sialyl- α 2,6-lactosaminy-structure: biosynthesis and functional role. *Glycoconj J* 2000;17:669–676. [PubMed: 11425186]
- [3]. Dall'Olio F, Chiricolo M, Ceccarelli C, Minni F, Marrano D, Santini D. Beta-galactoside α 2,6 sialyltransferase in human colon cancer: contribution of multiple transcripts to regulation of enzyme activity and reactivity with *Sambucus nigra* agglutinin. *Int J Cancer* 2000;88:58–65. [PubMed: 10962440]
- [4]. Kim YJ, Varki A. Perspectives on the significance of altered glycosylation of glycoproteins in cancer. *Glycoconj J* 1997;14:569–576. [PubMed: 9298689]
- [5]. Dennis JW, Granovsky M, Warren CE. Protein glycosylation in development and disease. *Bioessays* 1999;21:412–421. [PubMed: 10376012]
- [6]. Varki, A.; Cummings, R.; Esko, J.; Freeze, H.; Hart, G.; Marth, J. *Essentials of Glycobiology*. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 1999.
- [7]. Bellis SL. Variant glycosylation: an underappreciated regulatory mechanism for β 1 integrins. *Biochim Biophys Acta* 2004;1663:52–60. [PubMed: 15157607]
- [8]. Harduin-Lepers A, Vallejo-Ruiz V, Krzewinski-Recchi MA, Samyn-Petit B, Julien S, Delannoy P. The human sialyltransferase family. *Biochimie* 2001;83:727–737. [PubMed: 11530204]
- [9]. Recchi MA, Hebbar M, Hornez L, Harduin-Lepers A, Peyrat JP, Delannoy P. Multiplex reverse transcription polymerase chain reaction assessment of sialyltransferase expression in human breast cancer. *Cancer Res* 1998;58:4066–4070. [PubMed: 9751611]
- [10]. Dall'Olio F, Malagolini N, Serafini-Cessi F. Enhanced CMP-NeuAc:Gal β 1,4GlcNAc-R α 2,6 sialyltransferase activity of human colon cancer xenografts in athymic nude mice and of xenograft-derived cell lines. *Int J Cancer* 1992;50:325–330. [PubMed: 1730528]
- [11]. Dall'Olio F, Malagolini N, di Stefano G, Minni F, Marrano D, Serafini-Cessi F. Increased CMP-NeuAc:Gal β 1,4GlcNAc-R α 2,6 sialyltransferase activity in human colorectal cancer tissues. *Int J Cancer* 1989;44:434–439. [PubMed: 2476402]
- [12]. Gessner P, Riedl S, Quentmaier A, Kemmner W. Enhanced activity of CMP-neuAc:Gal β 1,4GlcNAc: α 2,6-sialyltransferase in metastasizing human colorectal tumor tissue and serum of tumor patients. *Cancer Lett* 1993;75:143–149. [PubMed: 8313349]
- [13]. Skacel PO, Edwards AJ, Harrison CT, Watkins WM. Enzymic control of the expression of the X determinant (CD15) in human myeloid cells during maturation: the regulatory role of 6-sialyltransferase. *Blood* 1991;78:1452–1460. [PubMed: 1679356]
- [14]. Fukushima K, Hara-Kuge S, Seko A, Ikehara Y, Yamashita K. Elevation of α 2 $\rightarrow$ 6 sialyltransferase and α 1 $\rightarrow$ 2 fucosyltransferase activities in human choriocarcinoma. *Cancer Res* 1998;58:4301–4306. [PubMed: 9766657]
- [15]. Wang PH, Li YF, Juang CM, Lee YR, Chao HT, Tsai YC, Yuan CC. Altered mRNA expression of sialyltransferase in squamous cell carcinomas of the cervix. *Gynecol Oncol* 2001;83:121–127. [PubMed: 11585423]
- [16]. Kaneko Y, Yamamoto H, Kersey DS, Colley KJ, Leestma JE, Moskal JR. The expression of Gal β 1,4GlcNAc α 2,6 sialyltransferase and α 2,6-linked sialoglycoconjugates in human brain tumors. *Acta Neuropathol (Berl)* 1996;91:284–292. [PubMed: 8834541]
- [17]. Recchi MA, Harduin-Lepers A, Boilly-Marier Y, Verbert A, Delannoy P. Multiplex RT-PCR method for the analysis of the expression of human sialyltransferases: application to breast cancer cells. *Glycoconj J* 1998;15:19–27. [PubMed: 9530953]

- [18]. Lise M, Belluco C, Perera SP, Patel R, Thomas P, Ganguly A. Clinical correlations of alpha2,6-sialyltransferase expression in colorectal cancer patients. *Hybridoma* 2000;19:281–286. [PubMed: 11001400]
- [19]. Le Marer N, Stehelin D. High alpha-2,6-sialylation of N-acetylglucosamine sequences in ras-transformed rat fibroblasts correlates with high invasive potential. *Glycobiology* 1995;5:219–226. [PubMed: 7780196]
- [20]. Lin S, Kemmner W, Grigull S, Schlag PM. Cell surface alpha 2,6 sialylation affects adhesion of breast carcinoma cells. *Exp Cell Res* 2002;276:101–110. [PubMed: 11978012]
- [21]. Zhu Y, Srivatana U, Ullah A, Gagneja H, Berenson CS, Lance P. Suppression of a sialyltransferase by antisense DNA reduces invasiveness of human colon cancer cells in vitro. *Biochim Biophys Acta* 2001;1536:148–160. [PubMed: 11406350]
- [22]. Harvey BE, Toth CA, Wagner HE, Steele GD Jr. Thomas P. Sialyltransferase activity and hepatic tumor growth in a nude mouse model of colorectal cancer metastases. *Cancer Res* 1992;52:1775–1779. [PubMed: 1312899]
- [23]. Bresalier RS, Rockwell RW, Dahiya R, Duh QY, Kim YS. Cell surface sialoprotein alterations in metastatic murine colon cancer cell lines selected in an animal model for colon cancer metastasis. *Cancer Res* 1990;50:1299–1307. [PubMed: 2297775]
- [24]. Seales EC, Jurado GA, Singhal A, Bellis SL. Ras oncogene directs expression of a differentially sialylated, functionally altered beta1 integrin. *Oncogene* 2003;22:7137–7145. [PubMed: 14562042]
- [25]. Seales EC, Jurado GA, Brunson BA, Wakefield JK, Frost AR, Bellis SL. Hypersialylation of beta1 integrins, observed in colon adenocarcinoma, may contribute to cancer progression by up-regulating cell motility. *Cancer Res* 2005;65:4645–4652. [PubMed: 15930282]
- [26]. Le Marer N, Laudet V, Svensson EC, Cazlaris H, Van Hille B, Lagrou C, Stehelin D, Montreuil J, Verbert A, Delannoy P. The c-Ha-ras oncogene induces increased expression of beta-galactoside alpha-2,6-sialyltransferase in rat fibroblast (FR3T3) cells. *Glycobiology* 1992;2:49–56.
- [27]. Dalziel M, Dall'Olio F, Mungul A, Piller V, Piller F. Ras oncogene induces beta-galactoside alpha2,6-sialyltransferase (ST6Gal I) via a RalGEF-mediated signal to its housekeeping promoter. *Eur J Biochem* 2004;271:3623–3634. [PubMed: 15355339]
- [28]. Heino J, Ignatz RA, Hemler ME, Crouse C, Massague J. Regulation of cell adhesion receptors by transforming growth factor-beta. Concomitant regulation of integrins that share a common beta 1 subunit. *J Biol Chem* 1989;264:380–388. [PubMed: 2491849]
- [29]. Lenter M, Vestweber D. The integrin chains beta 1 and alpha 6 associate with the chaperone calnexin prior to integrin assembly. *J Biol Chem* 1994;269:12263–12268. [PubMed: 8163531]
- [30]. Bellis SL, Newman E, Friedman EA. Steps in integrin beta1-chain glycosylation mediated by TGFbeta1 signaling through Ras. *J Cell Physiol* 1999;181:33–44. [PubMed: 10457351]
- [31]. Melchiori A, Allavena G, Bohm J, Remy W, Schmidt J, Parodi S, Santi L, Albini A. Interferons inhibit chemotaxis of transformed cells and their invasion of a reconstituted basement membrane. *Anticancer Res* 1987;7:475–479. [PubMed: 2443071]
- [32]. Wennerberg K, Lohikangas L, Gullberg D, Pfaff M, Johansson S, Fassler R. Beta 1 integrin-dependent and -independent polymerization of fibronectin. *J Cell Biol* 1996;132:227–238. [PubMed: 8567726]
- [33]. Wang F, Hansen RK, Radisky D, Yoneda T, Barcellos-Hoff MH, Petersen OW, Turley EA, Bissell MJ. Phenotypic reversion or death of cancer cells by altering signaling pathways in three-dimensional contexts. *J Natl Cancer Inst* 2002;94:1494–1503. [PubMed: 12359858]
- [34]. Weaver VM, Petersen OW, Wang F, Larabell CA, Briand P, Damsky C, Bissell MJ. Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. *J Cell Biol* 1997;137:231–245. [PubMed: 9105051]
- [35]. Zutter MM, Santoro SA, Staatz WD, Tsung YL. Re-expression of the alpha 2 beta 1 integrin abrogates the malignant phenotype of breast carcinoma cells. *Proc Natl Acad Sci U S A* 1995;92:7411–7415. [PubMed: 7638207]
- [36]. Oshita F, Kameda Y, Hamanaka N, Saito H, Yamada K, Noda K, Mitsuda A. High expression of integrin beta1 and p53 is a greater poor prognostic factor than clinical stage in small-cell lung cancer. *Am J Clin Oncol* 2004;27:215–219. [PubMed: 15170136]

- [37]. Bottger TC, Maschek H, Lobo M, Gottwohl RG, Brenner W, Junginger T. Prognostic value of immunohistochemical expression of beta-1 integrin in pancreatic carcinoma. *Oncology* 1999;56:308–313. [PubMed: 10343195]
- [38]. Nikkola J, Vihinen P, Vlaykova T, Hahka-Kemppinen M, Heino J, Pyrhonen S. Integrin chains beta1 and alphav as prognostic factors in human metastatic melanoma. *Melanoma Res* 2004;14:29–37. [PubMed: 15091191]
- [39]. Jonjic N, Lucin K, Krstulja M, Iternicka Z, Mustac E. Expression of beta-1 integrins on tumor cells of invasive ductal breast carcinoma. *Pathol Res Pract* 1993;189:979–984. [PubMed: 7508107]
- [40]. Gui GP, Wells CA, Browne PD, Yeomans P, Jordan S, Puddefoot JR, Vinson GP, Carpenter R. Integrin expression in primary breast cancer and its relation to axillary nodal status. *Surgery* 1995;117:102–108. [PubMed: 7809822]
- [41]. Cordes N, Seidler J, Durzok R, Geinitz H, Brakebusch C. beta1-integrin-mediated signaling essentially contributes to cell survival after radiation-induced genotoxic injury. *Oncogene* 2006;25:1378–1390. [PubMed: 16247454]
- [42]. Damiano JS, Hazlehurst LA, Dalton WS. Cell adhesion-mediated drug resistance (CAM-DR) protects the K562 chronic myelogenous leukemia cell line from apoptosis induced by BCR/ABL inhibition, cytotoxic drugs, and gamma-irradiation. *Leukemia* 2001;15:1232–1239. [PubMed: 11480565]
- [43]. Aoudjit F, Vuori K. Integrin signaling inhibits paclitaxel-induced apoptosis in breast cancer cells. *Oncogene* 2001;20:4995–5004. [PubMed: 11526484]
- [44]. Damiano JS, Dalton WS. Integrin-mediated drug resistance in multiple myeloma. *Leuk Lymphoma* 2000;38:71–81. [PubMed: 10811449]
- [45]. Hazlehurst LA, Valkov N, Wisner L, Storey JA, Boulware D, Sullivan DM, Dalton WS. Reduction in drug-induced DNA double-strand breaks associated with beta1 integrin-mediated adhesion correlates with drug resistance in U937 cells. *Blood* 2001;98:1897–1903. [PubMed: 11535527]
- [46]. Hazlehurst LA, Enkemann SA, Beam CA, Argilagos RF, Painter J, Shain KH, Saporta S, Boulware D, Moscinski L, Alsina M, Dalton WS. Genotypic and phenotypic comparisons of de novo and acquired melphalan resistance in an isogenic multiple myeloma cell line model. *Cancer Res* 2003;63:7900–7906. [PubMed: 14633719]
- [47]. Vuoristo M, Vihinen P, Vlaykova T, Nylund C, Heino J, Pyrhonen S. Increased gene expression levels of collagen receptor integrins are associated with decreased survival parameters in patients with advanced melanoma. *Melanoma Res* 2007;17:215–223. [PubMed: 17625451]
- [48]. Matsuoka T, Yashiro M, Nishimura S, Inoue T, Fujihara T, Sawada T, Kato Y, Seki S, Hirakawa-Ys Chung K. Increased expression of alpha2beta1-integrin in the peritoneal dissemination of human gastric carcinoma. *Int J Mol Med* 2000;5:21–25. [PubMed: 10601569]
- [49]. Kawamura T, Endo Y, Yonemura Y, Nojima N, Fujita H, Fujimura T, Obata T, Yamaguchi T, Sasaki T. Significance of integrin alpha2/beta1 in peritoneal dissemination of a human gastric cancer xenograft model. *Int J Oncol* 2001;18:809–815. [PubMed: 11251178]
- [50]. van Kempen LC, Rijntjes J, Mamor-Cornelissen I, Vincent-Naulleau S, Gerritsen MJ, Ruiter DJ, van Dijk MC, Geffrotin C, van Muijen GN. Type I collagen expression contributes to angiogenesis and the development of deeply invasive cutaneous melanoma. *Int J Cancer* 2008;122:1019–1029. [PubMed: 17957794]
- [51]. Fingleton B. Molecular targets in metastasis: lessons from genomic approaches. *Cancer Genomics Proteomics* 2007;4:211–221. [PubMed: 17878524]
- [52]. Ramaswamy S, Ross KN, Lander ES, Golub TR. A molecular signature of metastasis in primary solid tumors. *Nat Genet* 2003;33:49–54. [PubMed: 12469122]
- [53]. St Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, Lal A, Riggins GJ, Lengauer C, Vogelstein B, Kinzler KW. Genes expressed in human tumor endothelium. *Science* 2000;289:1197–1202. [PubMed: 10947988]
- [54]. Hedlund M, Ng E, Varki A, Varki NM. alpha 2-6-Linked sialic acids on N-glycans modulate carcinoma differentiation in vivo. *Cancer Res* 2008;68:388–394. [PubMed: 18199532]
- [55]. Hughes PE, Renshaw MW, Pfaff M, Forsyth J, Keivens VM, Schwartz MA, Ginsberg MH. Suppression of integrin activation: a novel function of a Ras/Raf-initiated MAP kinase pathway. *Cell* 1997;88:521–530. [PubMed: 9038343]

- [56]. Zhang Z, Vuori K, Wang H, Reed JC, Ruoslahti E. Integrin activation by R-ras. *Cell* 1996;85:61–69. [PubMed: 8620538]
- [57]. Kinashi T, Katagiri K, Watanabe S, Vanhaesebroeck B, Downward J, Takatsu K. Distinct mechanisms of alpha 5beta 1 integrin activation by Ha-Ras and R-Ras. *J Biol Chem* 2000;275:22590–22596. [PubMed: 10801791]
- [58]. Luo BH, Springer TA, Takagi J. Stabilizing the open conformation of the integrin headpiece with a glycan wedge increases affinity for ligand. *Proc Natl Acad Sci U S A* 2003;100:2403–2408. [PubMed: 12604783]
- [59]. James G, Goldstein JL, Brown MS. Resistance of K-RasBV12 proteins to farnesyltransferase inhibitors in Rat1 cells. *Proc Natl Acad Sci U S A* 1996;93:4454–4458. [PubMed: 8633088]
- [60]. Prendergast GC, Davide JP, Lebowitz PF, Wechsler-Reya R, Kohl NE. Resistance of a variant ras-transformed cell line to phenotypic reversion by farnesyl transferase inhibitors. *Cancer Res* 1996;56:2626–2632. [PubMed: 8653708]
- [61]. Del Villar K, Urano J, Guo L, Tamanoi F. A mutant form of human protein farnesyltransferase exhibits increased resistance to farnesyltransferase inhibitors. *J Biol Chem* 1999;274:27010–27017. [PubMed: 10480914]
- [62]. Kohl NE, Omer CA, Conner MW, Anthony NJ, Davide JP, deSolms SJ, Giuliani EA, Gomez RP, Graham SL, Hamilton K, Handt LK, Hartman GD, Koblan KS, Kral AM, Miller PJ, Mosser SD, O'Neill TJ, Rands E, Schaber MD, Gibbs JB, Oliff A. Inhibition of farnesyltransferase induces regression of mammary and salivary carcinomas in ras transgenic mice. *Nat Med* 1995;1:792–797. [PubMed: 7585182]

Figure 1. Generation of cell lines with downregulated ST6Gal-I

A) Oncogenic ras-expressing HD3 cells (control) were transduced with lentiviral vectors expressing one of five different anti-ST6Gal-I shRNA sequences. Control cell lysates or shRNA-expressing cell lysates ("sh.ST6", samples 1-5) were Western blotted for ST6Gal-I. All of the shRNA-transduced cells showed downregulation of enzyme levels, with shRNA sequences 2 and 4 giving the greatest suppression of enzyme levels. Cells transduced with sequence #4 (representing a pooled population of clones) were used for all further experiments. **B)** Cells were incubated with FITC-conjugated SNA and evaluated by flow cytometry. The green lines represent labeling by a nonspecific IgG-FITC conjugate (negative control), whereas the red lines reflect SNA labeling. As shown, the SNA labeling of sh.ST6 cells was shifted to the left relative to control cells, indicating a lower level of cell surface α 2-6 sialylation.

Figure 2. Downregulation of ST6Gal-I results in decreased sialylation of β1 integrins

A) Lysates from either control cells or cells expressing shRNA for ST6Gal-I (sh.ST6) were incubated with agarose-conjugated SNA-1, a lectin specific for α2-6 linked sialic acids. Glycoprotein/lectin complexes were precipitated by centrifugation. Glycoproteins were subsequently released from complexes by boiling in SDS-PAGE buffer, resolved by SDS-PAGE, and immunoblotted to detect β1 integrins (upper panel). In the lower panel, whole cell lysates were blotted for the β1 integrin to assess total levels of β1 expression.

B) Bands on immunoblots of SNA precipitates or whole cell homogenates were quantified using densitometry and the ratio of α2-6 sialylated β1 integrin to total β1 integrin was plotted (of note, the “mature” band was used to obtain the value for total β1 integrin expression, since

it is the mature isoform that is a substrate for sialylation). Results represent the means and SEMs of densitometric ratios generated from blots of 4 independent sets of cell lysates.

C) Cells were incubated with a glycosylation-insensitive anti- $\beta 1$ antibody and then subjected to flow cytometry (red lines). A nonspecific IgG was used as an isotype control (green lines). These results show that equivalent amounts of $\beta 1$ integrin are expressed on the cell surface.

D) $\beta 1$ integrins were immunoprecipitated from control and sh.ST6 cell lysates, then blotted with MAA, a lectin specific for $\alpha 2$ -3-linked sialic acids.

Figure 3. Cells expressing hyposialylated integrins adhere poorly to collagen I

A) Control or shRNA-expressing cells were seeded onto dishes coated with collagen I, and adhesion was quantified by a standard colorimetric assay. Cells with downregulated ST6Gal-I expression (sh.ST6), show an approximately 2 fold-reduction in adhesion to collagen I. Results represent means and SEMs of 3 independent experiments performed in triplicate. * = $p < 0.05$

B) Cell surface sialic acids were removed from control HD3 cells (i.e., ras-expressing, but not transduced with shRNA for ST6Gal-I) by incubating with *Vibrio cholerae* sialidase using a concentration range of 1-100 mU/ml. After washing with PBS to remove the sialidase, the cells were seeded onto tissue culture dishes that had been precoated with collagen I. Cell adhesion

was quantified using standard procedures. The binding of untreated (control) cells was set at 100% to minimize baseline variation. Where indicated (*), the adhesion to collagen of sialidase-treated cells was significantly less than that of control cells ($p < 0.05$). Values represent means and SEMs of 3 independent experiments performed in duplicate or triplicate.

Figure 4. Cells with hyposialylated integrins demonstrate reduced migration on collagen I and decreased invasion through Matrigel

A) Control and shRNA-expressing cells were seeded into the upper wells of Boyden chambers lined with 8.0- μ m PET membranes coated on the underside with collagen I. The lower chambers contained medium plus 2% FBS as a chemoattractant. After 24 hours, migration along a collagen I concentration gradient (haptotaxis) was measured using the vendor's protocol. As shown, the haptotactic migration of sh.ST6 cells was significantly less than that of control cells. ($p < 0.05$). Results represent means and SEMs of 3 independent experiments performed in triplicate.

B) HD3 cells were seeded onto the tops of growth factor-reduced Matrigel™ invasion chambers (BD Biosciences) and allowed to invade for 48 hours. NIH3T3-conditioned medium was added to lower chambers as a chemoattractant. Cells invading through the Matrigel layer, and adhering to the underside of the membrane, were stained and quantified by a standard crystal violet staining method. As shown, expression of shRNA for ST6Gal-I significantly inhibits invasion ($p < 0.05$). Results represent means and SEMs of 3 independent experiments performed in triplicate.

Figure 5. Differential α 2-6 sialylation does not alter the invasiveness of β 1 integrin-null GD25 cells

A) GD25 cells were stably transfected with ST6Gal-I enzyme tagged with a V5 epitope for easy detection. Western blots revealed the presence of the V5 tagged-protein in ST6Gal-I transfected cells (ST6), but not in parental cells (Par).

B) Lysates from parental (Par) and ST6Gal-I transfected (ST6) GD25 cells were probed with SNA lectin to identify and compare α 2-6 sialylated proteins. As shown, the ST6Gal-I transfected cells had a significantly higher number and amount of α 2-6 sialylated proteins when compared to the parental cells, indicating that the transfected ST6Gal-I was functional.

C) Cell invasion was monitored for GD25 cells, which are known to lack expression of the β 1 integrin. Briefly, parental GD25 cells (which have low levels of endogenous ST6Gal-I), or GD25 cells forced to express ST6Gal-I, were monitored for invasion through Matrigel as described previously. No significant difference was observed in invasion for the GD25 cells, suggesting that expression of the β 1 integrin is required for the differential effects of sialylation on cell invasion. Results represent means and SEMs of 3 independent experiments performed in triplicate.