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5-Aza-2′-deoxycytidine increases sialyl Lewis X on MUC1 by stimulating β-galactoside:α2,3-sialyltransferase 6 gene

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ABSTRACT

Sialyl Lewis X is a tumor-associated antigen frequently found in the advanced cancers. However, the mechanism for the production of this cancer antigen is not entirely clear. The objective of this study is to examine whether epigenetics is involved in the regulation of the formation of this antigen. We observed an increase of sialyl Lewis X in HCT15 cells, a colon cancer cell line, treated with 5-Aza-2′-deoxycytidine. This treatment enhanced the expression of β-galactoside:α2,3-sialyltransferase 6 gene and sialyl Lewis X on MUC1, and the adherence of these cells to E-selectin under dynamic flow conditions. In addition, 5-Aza-2′-deoxycytidine treatment inhibited methylation of β-galactoside:α2,3-sialyltransferase 6 gene and siRNA knockdown of this gene drastically reduced sialyl Lewis X without affecting MUC1 expression. We conclude that 5-Aza-2′-deoxycytidine treatment increases sialyl Lewis X on MUC1 by stimulating the β-galactoside:α2,3-sialyltransferase 6 gene via inhibition of DNA methylation. Increased sialyl Lewis X by 5-Aza-2′-deoxycytidine raises a concern about the safety of this chemotherapeutic drug. In addition, β-galactoside:α2,3-sialyltransferase 6 gene may be a potential therapeutic target for suppressing tumorigenicity of colon cancer.

1. Introduction

Alterations of mucin glycans have been frequently observed in cancer. Some of these glycans have been identified as tumor-associated antigens and used as markers for diagnosis and prognosis of cancer (Dube and Bertozzi, 2005) or targets for therapy (Taylor-Papadimitriou and Epenetos, 1994). Among these tumor-associated antigens, sialyl Lewis X (sLe^x) receives the most attention because it is associated with poor prognosis. For example, colon carcinoma cells with a high potential for liver metastasis express more abundant amounts of sLe^x than counterparts with low metastatic potential (Amado et al., 1998; Magnani et al., 1982; Matsushita et al., 1990; Nakamori et al., 1993). Increased expression of sLe^x correlated with strong adherence of cancer cells to E-selectin on vascular endothelial cells (Bresalier et al., 1996; Izumi et al., 1995; Saitoh et al., 1992; Sawada et al., 1994). SLe^x is also a key component of the carbohydrate ligands for P and L-selectins (Kim et al., 1999). These results indicate that sLe^x plays an important role in hematogenous cancer metastasis.

SLe^x is a tetrasaccharide located at the non-reducing terminus of a glycan chain. It contains a Galβ1-4GlcNAc backbone with the Gal decorated with α2-3NeuAc and the GlcNAc with α1-3Fuc. The synthesis of sLe^x is initiated by adding α2-3NeuAc to the Gal of *N*-acetyllactosamine as catalyzed by βgal:α2,3-sialyltransferases (ST3Gal) (Carvalho et al., 2010). Then, α1-3Fuc is added to the GlcNAc as catalyzed by α1,3/4-fucosyltransferases (de Vries et al., 1995; Holmes et al., 1986). There are at least four *ST3Gals*, including *ST3Gal3*, *ST3Gal4*, *ST3Gal5*, and *ST3Gal6*, that can produce α2→3 sialyl *N*-acetyllactosamine on Galβ1-4GlcNAc *in vitro* (Carvalho et al., 2010; Kitagawa and Paulson, 1993; Okajima et al., 1999; Sasaki et al., 1993). However, the true target of each of these ST3Gals *in situ* is not known. Similarly, there are at least five α1,3/4-fucosyltransferases which are capable of synthesizing sLe^x (de Vries et al., 1995). The intracellular glycoprotein targets for these enzymes also remain elusive.

In an effort to elucidate the potential epigenetic regulation of glycosyltransferase genes, we treated colon carcinoma HCT15 cells with 5-Aza-2′-deoxycytidine (5-Aza-dC), a DNA methyltransferase inhibitor (Palii et al., 2008), and then monitored subsequent changes in the expression of glycogenes involved in mucin glyco-

Abbreviations: 5-Aza-dC, 5-Aza-2′-deoxycytidine; CHO, Chinese hamster ovary; ST3Gal, β-galactoside:α2,3-sialyltransferase; FUT, fucosyltransferase; Gal, galactose; Fuc, fucose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid; PNGase F, peptide-*N*4-(*N*-acetyl-β-D-glucosaminyl) asparagine amidase F; sLe^x, sialyl Lewis X; ST, sialyltransferase; r.t., room temperature; MAA, Maackia amurensis lectin.

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sylation, particularly the synthesis of sLe^x. We found that 5-Aza-dC treatment induced the production of sLe^x on MUC1 and enhanced the binding of HCT15 cells to E-selectin under dynamic flow conditions through induction of *ST3Gal6* gene by inhibiting methylation of this gene. The results establish that ST3Gal6 is responsible for the synthesis of sLe^x on MUC1 and this contributes to the adhesive property of HCT15 cells. Because expression of sLe^x correlates with cancer metastatic potential, these results raise a concern about the safety of 5-Aza-dC for cancer treatment.

2. Methods

2.1. Cell line and cell culture

Colon carcinoma HCT15 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD). These cells were maintained at 37 °C under a 5% CO_2 and water saturated environment in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml Penicillin and 100 μg/ml Streptomycin.

2.2. 5-Aza-2′-deoxycytidine treatment

A stock solution (1 mg/ml) of 5-Aza-dC (Sigma Chemical Co., St. Louis, MO) was prepared fresh in sterilized PBS within 1 h of the treatment. Final 5-Aza-dC concentrations (0.1–4 μM) were prepared by adding an appropriate amount of the stock solution directly to the culture medium. Drug and PBS (control) treatments of the cells were initiated at 20% confluence and then continued daily for 5 days (d).

2.3. Western blotting

Aliquots of the cell lysates from vehicle and 5-Aza-dC treated cells were boiled (5 min) and then run on 6% SDS-PAGE (7.5 cm × 8.5 cm) under reducing conditions. After electrotransfer, the PVDF membrane (Immobilon-P, 0.2 μ, Millipore, Bedford, MA) was blocked with TBS containing 0.05% Tween 20 and 5% (w/v) nonfat dried milk for 60 min and then exposed to anti-sLe^x (CSLEX1; BD Biosciences), anti-MUC1 (VU-4H5; Life Span Biosciences), anti-MUC4 (8G-7, Santa Cruz Biotechnology, Inc. and 6A134; Abcam, Cambridge, MA), anti-MUC6 (CLH5; Vector Laboratories) or anti-MUC16 (X75; Abcam, Cambridge, MA) antibodies overnight at 4 °C in same buffer at 1:500 for anti-sLe^x, and 1:1000 dilutions for other antibodies. After five washings in same buffer, the membrane was treated for 1 h at room temperature (r.t.) with HRP-conjugated donkey anti-mouse IgM (Jackson Laboratories, West Grove, PA) for anti-sLe^x and donkey anti-mouse IgG (Jackson Laboratories, West Grove, PA) for other antibodies. The membrane was then washed five times (5 min each) with TBS containing 0.05% Tween 20 and once with milli-Q water. Then, the blot was developed with ECL-sensitive film (Amersham Pharmacia Biotech, Uppsala, Sweden). β-Actin probed with mAb (Santa Cruz, Inc.) was used for normalizing the protein loadings.

2.4. Immunoprecipitation

To isolate glycoproteins for characterization of conjugated glycans, cells were lysed in lysis buffer and precleared either in protein L-agarose or in protein G-agarose (Pierce). The precleared cell lysate was incubated with anti-sLe^x (or anti-MUC1) mAb (5 μg antibody per 100 μg protein) for 12 h at 4 °C. The immunocomplexes isolated with protein L-agarose (sLe^x), and protein G-agarose (MUC1) for 1 h at 4 °C, were analyzed for sLe^x, MUC1, or MAA ligand by western blotting. Immunoprecipitates with nonspecific mouse IgM and IgG antibodies were served as negative control. Protein concentration was measured by Coomassie blue dye (Pierce).

2.5. Peptide-N-glycosidase (PNGase) F digestion

The anti-sLe^x immunoprecipitate was boiled (100 °C) for 10 min in 0.5% SDS and 1% 2-mercaptoethanol and then treated with PNGase F (5 U/mg protein in 50 mM phosphate, pH 7.5 containing 1% NP-40) (37 °C, 16 h). The sample was analyzed by anti-sLe^x western blotting.

2.6. Lectin blotting

For lectin blotting, the PVDF membrane prepared above was blocked with TBS containing 3% BSA (60 min), followed by incubation with 2 μg/ml of biotin-conjugated MAA (in TBS containing 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM $CaCl_2$, pH 7.5) for 60 min. The membrane was washed (5×) with TBS containing 0.05% Tween 20 and then incubated with streptavidin-HRP (Pierce) (1:20,000) for 30 min. Finally, the blot was washed (5×) with TBS containing 0.05% Tween 20 and then developed using ECL method as described above.

2.7. Quantitative real-time PCR analysis of glycogene expression

Cells cultured in a T-25 flask were lysed with 1 ml TRI-REAGENT (Molecular Research Center, Inc., Cincinnati, OH) followed by the addition of bromo-3-chloropropane (0.1 ml) after transferred to a 1.5 ml Eppendorf tube. After shaken vigorously for 15 s, the sample was incubated at r.t. for 15 min, and then centrifuged (12,000 rpm) for 15 min at 4 °C. The upper, colorless phase was transferred to a clean Eppendorf tube, which was followed by mixing with 0.5 ml isopropanol, incubated at r.t. for 10 min, and then centrifuged (12,000 rpm) at 4 °C for 8 min. The pelleted RNA was rinsed with 1 ml of cold 75% ethanol in DEPC-treated water and then dissolved in 30–50 μl of DEPC-treated water at r.t. for about 10 min. One half of the total RNA was submitted to the Consortium for Functional Glycomics for GLYCOv4 DNA microarray analysis (see Supplementary materials for detailed protocol) and the other half was utilized for real-time PCR analysis. To prepare cDNA, 2 μg RNA was used in a 20 μl reaction mixture using a Verso reverse transcriptase kit (Thermo scientific) as follows: 5 min at r.t., 60 min at 42 °C, and 2 min at 95 °C. Quantitative real-time PCR was performed in 10 μl reaction volume in a 96-well plate using 2 μl of diluted cDNA with SYBR® *Premix ExTaq*™ (TAKARA BIO INC.) on a Mastercycler Epgradient realplex (Eppendorf AG, Hamburg, Germany). The PCR conditions included 1 cycle at 95 °C for 2 min followed by 45 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 45 s. The data were analyzed using Eppendorf realplex software, version 1.5 (Eppendorf). The amounts of various glycogene transcripts were normalized to the amount of *GAPDH* transcript in same cDNA sample. Relative fold differences in transcript expression were determined using the following comparative CT method: $2^{-[\Delta C_t(5\text{-Aza})-\Delta C_t(\text{control})]} = 2^{-\Delta\Delta C_t}$, where $\Delta C_t = C_t(Target) - C_t(GAPDH)$ as described previously (Tassone et al., 2000). The results were expressed as the amount (%) relative to that (100%) of *GAPDH* and plotted as mean fold changes ± SEM. Primer sequences used for expression analysis of all genes including *GAPDH* are summarized in Table S1 in Supplementary materials.

2.8. Transient transfection of HCT15 cells with ST3Gal6 gene-specific siRNAs

Four different siRNAs targeting *ST3Gal6* RNA along with scrambled siRNA were purchased from Dharmacon (IL, USA). After

treatment with 1 μM 5-Aza-dC for 3 d, HCT15 cells at 50% confluence were transfected for 8 h in a serum and antibiotic-free solution containing 100 nM siRNA and Lipofectamine RNAi MAX reagent (Invitrogen). The medium was then replaced with a fresh RPMI medium containing 10% fetal bovine serum and 1 μM 5-Aza-dC. After cultured for 40 h, the transfected cells were analyzed for *ST3Gal6* mRNA by quantitative PCR and sLe^x by western blotting as described above.

2.9. DNA methylation determination using bisulfite sequencing

DNA methylation was measured by treating 1 μg of genomic DNA with bisulfite using the EZ DNA Methylation-Direct kit (Zymo Research, Orange, CA). This process deaminates unmethylated cytosine residues to generate uracils leaving methylated cytosine residues unchanged (Frommer et al., 1992). Twenty-four ng of bisulfite-modified DNA were used as the template for each PCR reaction. The PCR reactions were performed in a total volume of 25 μl for 35 cycles using Roche Diagnostic Corporation (Indianapolis, IN) FastStart Taq DNA Polymerase (1.0 U), $MgCl_2$ (3.5 mM), dNTP's (0.2 mM), sense primer 5′-GGATGGGAGTGTTTTTTTGGTTGTGG-3′ (0.3 mM), antisense primer 5′-CACCCCCAATTTCCCTCACC-3′ (0.3 mM), with denaturation at 95 °C for 30 s, annealing at 68 °C for 45 s, and extension at 72 °C for 1 min. The primers captured a region of a CpG island covering 319 bases upstream and 283 bases downstream (hg18) of the transcription start site of the *ST3Gal6* gene (Fig. 5). The PCR product was cloned with a TOPO TA cloning Kit (Invitrogen). Colonies were selected and grown in 1.2 ml LB plus Ampicillin for 18 h. A 1:20 dilution of the bacterial cultures was prepared, heated at 95 °C for 12 min and centrifuged (1200 rpm, 3 min). Two μl of the supernatant were used in 25 μl of PCR reaction using M13 sense and antisense primers and 59 °C annealing temperature as described above. PCR products were analyzed on agarose gel and purified using 96-well Millipore MultiScreen HTS PCR purification plates and sequenced by Macrogen sequencing facility (Seoul, Korea).

2.10. Flow cytometric analysis

Quantitative assessment of cell surface sLe^x was performed by flow cytometry using anti-sLe^x antibodies. Both 5-Aza-dC and vehicle treated HCT15 cells were dislodged from culture flasks with a non-enzymatic cell stripper (Mediatec, Inc., USA) (37 °C, 5 min) and washed with PBS (3×) and washing buffer (PBS containing 1% BSA and 0.09% NaN_3) (1×). The cells (0.2×10^6) were treated with 1% BSA in PBS for 30 min on ice to block non-specific sites before exposure to primary antibodies. For primary antibody staining, cells were incubated with anti-sLe^x antibodies (10 μg/ml in washing buffer) on ice for 30 min and then washed with washing buffer (2×). Secondary antibody staining was performed with dylight-488 conjugated goat anti-mouse IgM at 1:200 dilution (Jackson Laboratories, West Grove, PA) for 30 min on ice and excessive antibodies were washed with washing buffer (2 ml). Finally, cells were resuspended in 500 μl of 0.5% paraformaldehyde and analyzed using a FACS Vantage (Becton Dickson San Jose, CA) equipped with 488 nm argon laser and with Cell Quest-pro software. The unstained cells and cells incubated with secondary antibody alone served as negative and antibody controls, respectively.

2.11. Preparation of E-selectin coated cover slips

Recombinant human E-selectin was purified from a CHO cell line transfected with E-selectin cDNA (a gift from Dr. Ajit Varki, UC-San Diego, CA) by immunoaffinity chromatography using anti-E selectin mAb BBII coupled to Sepharose (Borsig et al., 2002). Purified E-selectin dissolved in D-PBS containing 2 mM Ca^{++} and 2 mM Mg^{++} was used to coat the glass coverslips as described by Ross et al. (1998). Twenty μl of the E-selectin solution (1 mg/ml) were placed in the flow path (approximately 160 mm^2) on a glass cover slip (GlycoTech, USA), followed by incubation at 4 °C for 12 h and then rinsing with 1 ml of DPBS to remove unbound E-selectin. Prior to the experiment, slides were incubated with 3% BSA (in DPBS) for 1 h at r.t. to minimize non-specific binding.

2.12. Parallel plate flow chamber assay

Binding of HCT15 cells to E-selectin-coated slides were measured by a parallel-plate flow chamber assay as described previously (Wiese et al., 2009) and evaluated according to Krull et al. (1999). Briefly, cells were dislodged from the flasks with non-enzymatic cell striper and washed (3×) with binding buffer (2 mM HEPES containing 2 mM $CaCl_2$, pH 7.4). A suspension of 2.5×10^5 cells/ml prepared in 4 ml of the binding buffer plus 10 μg/ml DNase I (MP Biomedicals, France) was perfused through the chamber (GlycoTech, USA) at a constant wall shear stress of 1.0 dyne/cm^2 using a Harvard Syringe Pump (Harvard apparatus, USA). The field of observation was chosen randomly and binding was visualized with a phase-contrast video microscope (Olympus 1×70 coupled with camera; Q-Imaging, USA) at 40× magnification and videotaped using Q-pro software. Videotapes were played back at a 10-fold lower speed in Quick time for analysis. The cells at each of 20 different fields were counted for each experiment (Lawrence and Springer, 1991). Binding was expressed as number of adhering cells/high-power field during a 10-min observation period (Sriramarao et al., 1996). To confirm interaction of sLe^x with E-selectin, cells were pre-incubated with MAA lectin (10 μg/ml) for 30 min and then analyzed as described above.

2.13. Statistical analysis

The data were analyzed by the Student's *t*-test using SigmaPlot software (SPSS, Inc.).

3. Results

3.1. 5-Aza-2′-deoxycytidine enhances the expression of sLe^x

The initial objective of the study was to examine if 5-Aza-dC, like sodium butyrate (Radhakrishnan et al., 2007), could increase the production of sLe^x. A preliminary experiment showed that treatment of HCT15 colon cancer cells, which expressed undetectable to low level of sLe^x, with 0.1–4 μM of 5-Aza-dC enhanced the expression of sLe^x in a 250 kDa protein band. One μM of 5-Aza-dC was chosen for subsequent studies because this was the maximal concentration which did not exhibit apparent cytotoxicity (data not shown). Fig. 1A shows that treatment of HCT15 cells with 1 μM 5-Aza-dC for 5 d enhanced sLe^x about 2.3-fold. Because *N*-glycanase treatment did not remove sLe^x from the glycoproteins isolated by immunoprecipitation with sLe^x antibody (Fig. 1B), the sLe^x is likely associated with mucin-type glycans. Removal of *N*-glycans from fetuin with PNGase F showed that the enzyme used for the study was active. Positive MAA lectin staining of same protein band confirmed the presence of terminal $\alpha 2 \rightarrow 3$ sialic acid (Fig. 1B).

3.2. 5-Aza-dC enhances the expression of certain mucin-associated glycogenes

To identify the glycogenes involved in the synthesis of mucin glycan-associated sLe^x, the expression patterns of these genes in control and 5-Aza-dC treated HCT15 cells were analyzed by DNA

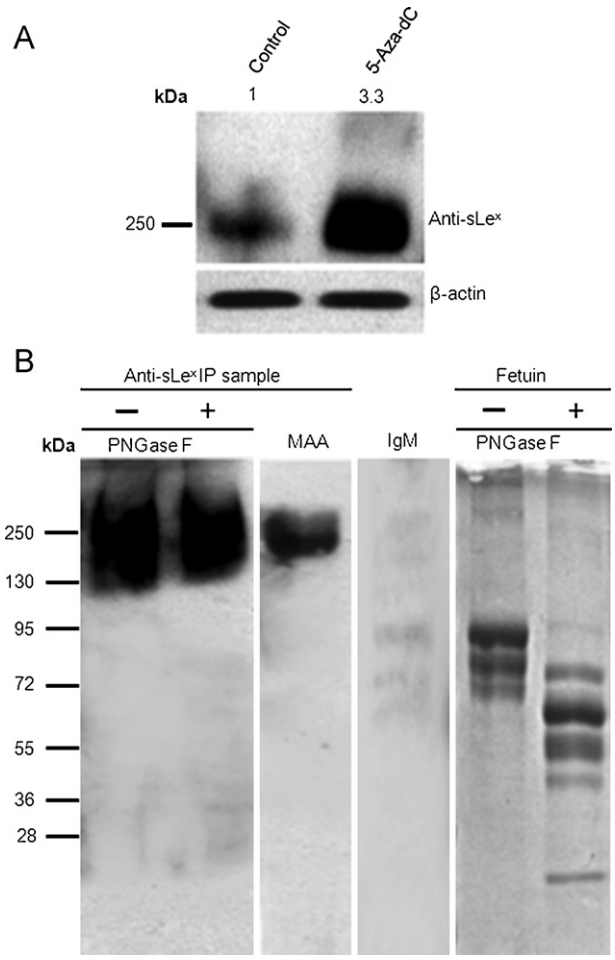

Fig. 1. Effects of 5-Aza-dC treatment on expression of sLe^x in HCT15 colon cancer cells. (A) Cells were treated with 1 μM 5-Aza-dC for 5 d, and total protein (100 μg) was analyzed by CSLEX1 western blotting. A 2.3-fold increase of sLe^x was observed in 5-Aza-dC treated cells as determined by "Image J" software. (B) CSLEX1 Immunoprecipitate was digested with *N*-glycanase, electrophoresed and probed with anti-sLe^x antibodies. Presence of α2 → 3 sialic acid was confirmed by MAA lectin blotting. IgM lane served as a background control. As a positive control, fetuin was digested with *N*-glycanase and stained with Commassie blue R-250. Experiments were repeated (3×) with similar results.

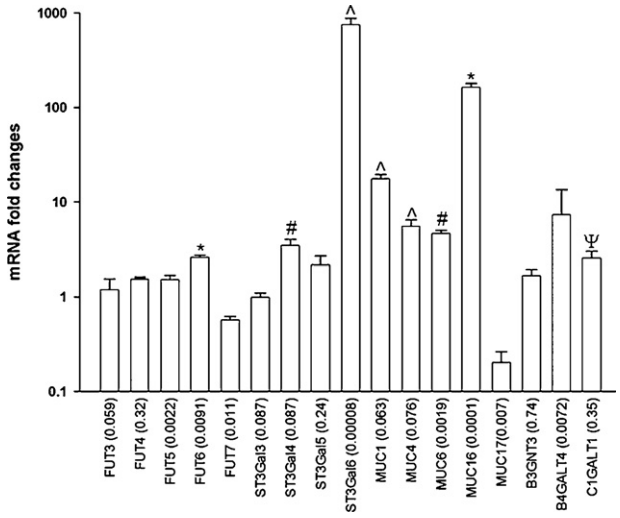

Fig. 2. 5-Aza-dC induces changes in the expression of some MUC genes and key glycogenes involved in the synthesis of sLe^x. Quantitative real time PCR analysis was carried out on HCT15 colon cancer cells with and without 1 μM 5-Aza-dC treatment for 5 d. Relative expression level of each gene was sorted according to ΔC_t (see Section 2) calibrated with *GAPDH* and expressed as fold changes determined by calculating the ratio of the expression level of each gene in 5-Aza-dC treated vs. vehicle-treated cells. Relative amount of each gene vs. that of *GAPDH* (100%) in untreated control cells was given in the parenthesis ($n = 3$). Statistical analysis of the difference in means between treated and control cells for each gene by the Student's *t*-test shows: $^{\Psi}p < .05$; $^{\#}p < .02$; $^{\wedge}p < .01$; and $^{*}p < .001$.

microarray followed by quantitative PCR. The DNA microarray analysis showed enhancement of one mucin gene, *MUC1* (150%), and four glycosyltransferase genes, including *FUT6* (70%), *ST3Gal6* (35%), *B4GALT4* (42%), and *core 1 synthase (C1GALT1)* (60%). The five glycogenes that were found to be suppressed by 5-Aza-dC and the degree of their suppression were: *FUT3* (30%), *FUT4* (11%), *FUT7* (11%), *ST3Gal3* (41%), and *B3GNT3* (23%). The DNA microarray data can be found in Fig. S1 and Table S2 in Supplementary materials and CFG RR#1828. The quantitative PCR analysis of the same RNA samples submitted for microarray analysis confirmed some of the microarray results although the magnitude of the effect varied somewhat (Fig. 2). Relative to that of the control (= 1.0), the expression levels of the five genes, which were found to be enhanced by microarray analysis, as analyzed by the real time PCR were: *MUC1* (17.4), *FUT6* (2.6), *ST3Gal6* (754), *B4GALT4* (10), and *C1GALT1* (1.5). *FUT7* was inhibited 43% while *FUT3*, *FUT4*, *FUT5*, and *B3GNT3* genes were enhanced 18%, 53%, 52%, and 66%, respectively. Some of the glycogenes that were substantially enhanced include *ST3Gal4* (245%), *ST3Gal5* (115%), *MUC4* (451%), and *MUC6* (361%). *MUC16* gene was enhanced 162-fold while *MUC17* gene was inhibited. The *ST3Gal3* gene was not affected.

3.3. *MUC1 is in the 250 kDa protein band that contains sLe^x*

Based on the quantitative PCR result and the size (250 kDa) of the glycoprotein band on SDS-PAGE, we predicted that this band contained MUC1. This prediction was confirmed by a western blot analysis using MUC1 antibodies, which detected MUC1 in the 250 kDa protein band that contains sLe^x and an additional band at the origin (Fig. 3). Employment of two MUC4 antibodies (8G-7 and 6A134) could not detect MUC4 in both bands despite

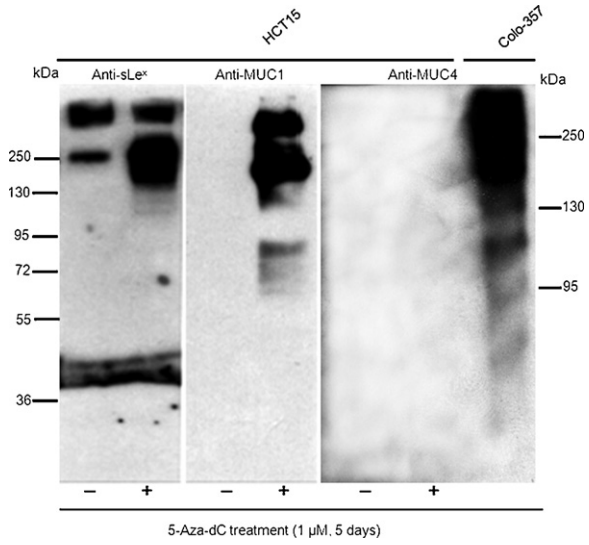

Fig. 3. Anti-sLe^x, MUC1 and MUC4 immunoblots of the lysates of HCT15 cells with and without 5-Aza-dC treatment. Total cellular protein (100 μg) was resolved by SDS-PAGE using 6% gel for sLe^x and MUC1 while 4% gel for MUC4, transferred onto PVDF membrane, and probed with anti-sLe^x (CSLEX1), anti-MUC1 (VU-4H5), or anti-MUC4 (8G-7) antibodies. sLe^x was associated with MUC1 and not MUC4. Cell lysate from Colo-357 cells served as a positive control for anti-MUC4 antibodies. Experiments were repeated at least 3× with similar results. The >250 kDa band was not always detected in all samples analyzed and represent complexes containing MUC1.

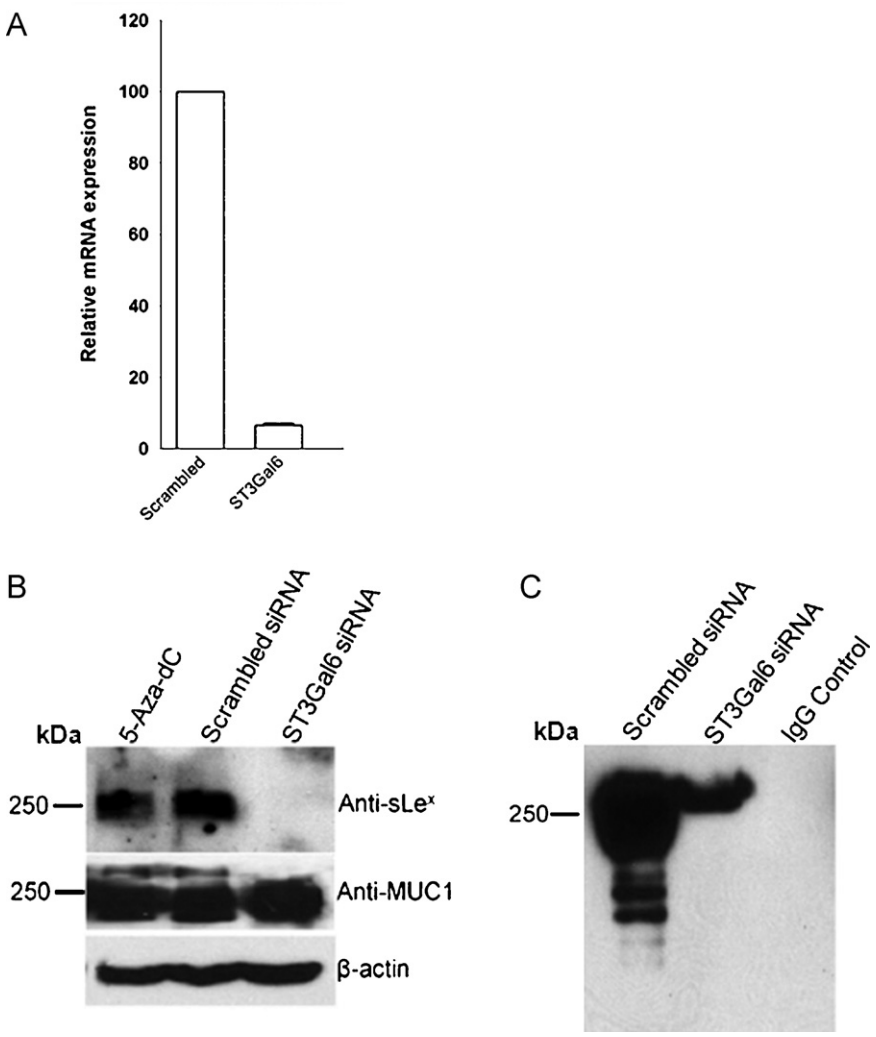

Fig. 4. Knockdown of *ST3Gal6* mRNA (A) and sLe^x (B) in HCT15 cells after treatment with *ST3Gal6*-specific and scrambled siRNAs. Cells were treated with 5-Aza-dC for 5 d and with RNAi for 8 h at d3. At d5, cells were analyzed for *ST3Gal6* and *GAPDH* mRNAs by quantitative PCR, and sLe^x and β-actin by western blotting. (A) Relative expression level of *ST3Gal6* gene was determined according to ΔC_t after normalization with *GAPDH* and expressed as fold changes ($n = 3$). (B) Anti-sLe^x and anti-MUC1 western blots of the lysates of cells treated with 5-Aza-dC, 5-Aza-dC plus scrambled RNAi, and 5-Aza-dC plus *ST3Gal6* RNAi. (C) Anti-sLe^x western blot of anti-MUC1 immunoprecipitates of the lysates from cells treated with 5-Aza-dC plus scrambled siRNA and 5-Aza-dC plus *ST3Gal6* siRNA. IgG lane is the immunoglobulin control.

high expression level of *MUC4* gene in HCT15 cell lysate, suggesting that MUC4 protein backbone was not produced. These two antibodies detected MUC4 in the Colo-357 cell lysate. The result of 8G-7 blot is shown in Fig. 3. The result of 6A134 blot can be found in Supplementary materials (Fig. S2). The >250 kDa band that was positively stained with sLe^x and MUC1 antibodies was not always detected in all samples analyzed and may represent MUC1 aggregate or complexes with other proteins. MUC6 and MUC16 were not detected by western blot analysis (data not shown).

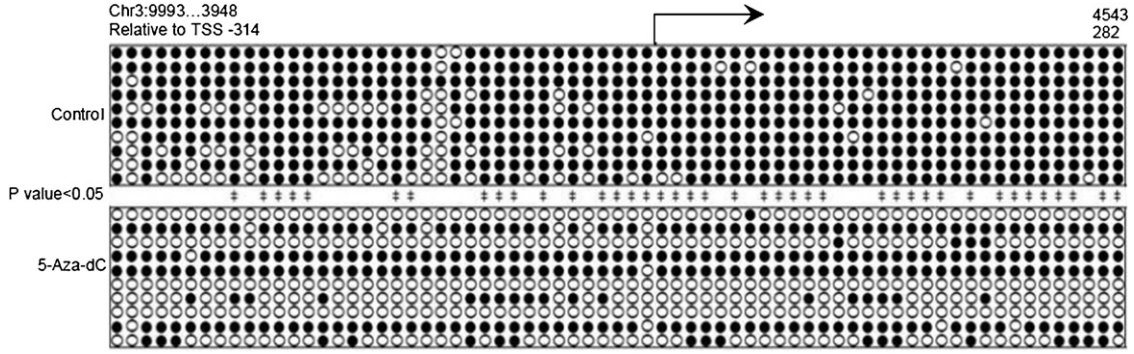

Fig. 5. Methylation patterns of *ST3Gal6* gene around the transcription start site (TSS) in control (top panel) and 5-Aza-dC treated (bottom panel) HCT15 cells. Bisulfite sequencing was performed on genomic DNA isolated from these cells. Significant DNA methylation differences (by the Student's *t*-test) at individual CpGs between control and 5-Aza-dC treated cells at the $p < .05$ level are indicated by ‡.

3.4. ST3Gal6 is involved in the synthesis of sLe^x on MUC1

The next question we asked was whether *ST3Gal6*, the gene stimulated the most by 5-Aza-dC (Fig. 2), was involved in the synthesis of sLe^x and whether sLe^x is on MUC1. sLe^x was monitored after *ST3Gal6* mRNA had been knocked down with RNAi. As shown in Fig. 4A, 95% of the *ST3Gal6* mRNA was knocked down by *ST3Gal6-specific* RNAi, which resulted in essentially a total suppression of sLe^x formation without affecting MUC1 (Fig. 4B). Further, the sLe^x epitope on the MUC1 pulled down with anti-MUC1 antibody was greatly reduced in cells treated with *ST3Gal6*-specific RNAi as compared to that in cells treated with scrambled RNAi (Fig. 4C). The results indicate that *ST3Gal6* is involved in the synthesis of sLe^x on MUC1.

3.5. 5-Aza-dC treatment inhibits methylation of the ST3Gal6 gene

The next question we asked was whether DNA methylation was involved in the suppression of the *ST3Gal6* gene because 5-Aza-dC is a known inhibitor of DNA methyltransferase. Bisulfite sequencing of the region (−314/+282) which covers the upstream and downstream sequences of the transcription start site of the *ST3Gal6* gene showed a significant inhibition of the methylation of this gene after 5-Aza-dC treatment (Fig. 5). These results show that inhibition of the methylation of *ST3Gal6* gene by 5-Aza-dC results in enhancement of sLe^x production on MUC1. We would like to note that the enhancement of *MUC1* gene expression induced by 5-Aza-dC did not involve inhibition of the methylation of *MUC1* gene because 5-Aza-dC treatment did not affect the methylation status of the promoter of *MUC1* gene (unpublished observation).

3.6. 5-Aza-dC treatment increases sLe^x on the surface of HCT15 cells

Flow cytometric analysis showed that 5-Aza-dC treatment of HCT15 cells increased the numbers of cells expressing sLe^x from 5.8% to 34% (Fig. 6). In addition, the mean fluorescence intensity, an arbitrary unit for measuring fluorescence intensity, was increased from 89 in untreated cells to 221 in treated cells, indicating the presence of more sLe^x determinants on the surface of 5-Aza-dC treated cells.

3.7. 5-Aza-dC treatment increases binding of HCT15 cells to immobilized E-selectin under dynamic flow conditions

To determine if the sLe^x generated on HCT15 cells treated with 5-Aza-dC has any biological relevance, E-selectin binding assay under dynamic flow conditions was performed. As shown in Fig. 7, adhesion of the 5-Aza-dC treated cells to immobilized E-selectin at a shear stress of 1.0 dynes/cm^2 was 50% more than that of the control cells. Pre-treatment of the cells with MAA significantly decreased the binding of HCT15 cells to E-selectin. This result confirms that α2-3sialic acid is an essential part of the E-selectin ligand.

4. Discussion

Mucin glycans are frequently altered in tumors (Brockhausen, 1999), but the mechanism for such alterations remains unclear. In this study, we show that 5-Aza-dC, a cancer chemotherapeutic agent, enhances sLe^x production on MUC1 in HCT15 colon cancer cells by stimulating the expression of the *ST3Gal6* gene. We further show that generation of sLe^x increases adhesion of these cells to E-selectin under dynamic flow conditions, suggesting that 5-Aza-dC may enhance the metastatic potential of colon cancer cells.

SLex is a carbohydrate ligand known to help guide, through interactions with selectins, the migration of leukocytes to the site

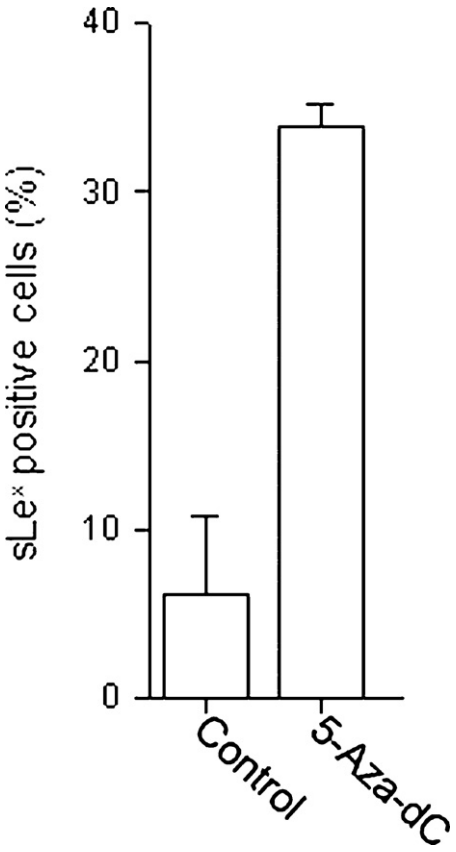

Fig. 6. Flow cytometric analysis of sLe^x expression on HCT15 cells. Treatment of HCT15 cells with 1 μM 5-Aza-dC for 5 d significantly increased the number of cells expressing sLe^x ($p < .05$) as measured by flow cytometry using anti-sLe^x Abs. The data expressed as mean ± SEM were obtained from three independent experiments.

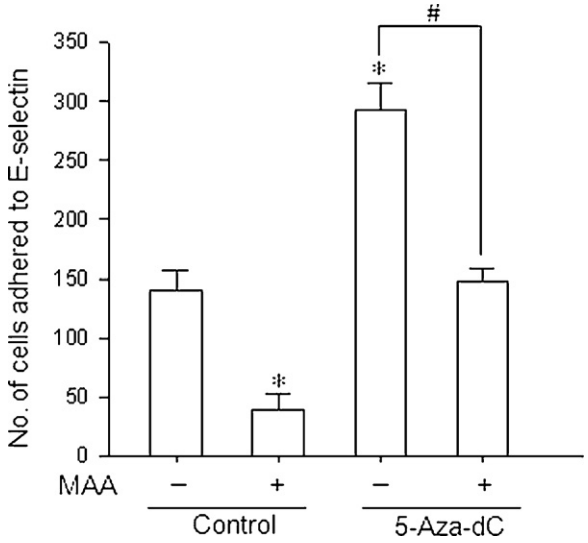

Fig. 7. Binding of HCT15 cells to immobilized E-selectin under dynamic flow conditions. The number of cells that adhered to E-selectin (20 μg) coated on glass slides was measured by parallel plate flow chamber assay. Cells untreated or treated with 5-Aza-dC were perfused through a flow chamber under constant wall shear stress of 1.0 dyne/cm^2. The adhered cells were counted at 40× magnification for 20 different fields and expressed as number of cells adhered/field. To confirm terminal α2 → 3 sialic acid-mediated interaction between sLe^x and E-selectin, cells were pre-treated with MAA lectin (10 μg/ml) and processed similarly (see "Section 2" for details). The data expressed as mean ± SEM were obtained from three independent experiments. $^*p < .05$ for – MAA vs. +MAA in control and 5-Aza-dC – MAA vs. control – MAA; $^\#p < .001$ for – MAA vs. +MAA in 5-Aza-dC treated cells.

of injury and lymphoid tissues to perform innate and acquired immune functions (Varki, 1994). Upon activation by inflammatory cytokines and certain lectins (Ashraf and Khan, 2003; Lowe, 2003), leukocytes were decorated with core 2 mucin glycans capped with sLe^x (Datti and Dennis, 1993; Maemura and Fukuda, 1992). Expression of this glycotope would allow these immune cells to migrate to the target sites to perform their biological functions when needed. This pattern of sLe^x expression in resting and activated leukocytes has also been reported in early and late stage tumors, respectively (Nakamori et al., 1993). This observation was confirmed by forced expression of sLe^x in experimental cell and animal models (Wagers et al., 1997). Acquisition of sLe^x in advanced cancer renders them capable of spreading from primary to distant sites. Our current observation that 1 μM 5-Aza-dC induces the synthesis of sLe^x on the surface of a colon cancer cell line raises a concern about the safety of using this agent for treating cancers. Despite the therapeutic benefit of 5-Aza-dC, this drug may enhance the metastatic potential of the cancer. Therefore, this potential side effect needs to be carefully monitored in patients undergoing chemotherapy with this drug.

SLe^x is found on glycolipids (Huang et al., 2002) and glycoproteins including N-linked (Alon and Rosen, 2007) and O-GalNAc-linked carbohydrates (McEver, 2002). The sLe^x linked to GalNAc has been reported in membrane-tethered mucins, such as MUC1 (Burdick et al., 1997), and secreted mucins, such as MUC2 (Baeckstrom et al., 1995; Hanski et al., 1995). The membrane-associated sLe^x present on cancer cells could promote metastasis (Hollingsworth and Swanson, 2004) as described above. The sLe^x carried by the secreted mucins and membrane-tethered mucins shedded from cell surface could interfere with the immune function by competing with circulating leukocytes for selectins after entering the blood circulation (Dube and Bertozzi, 2005). The sLe^x generated on MUC1 in HCT15 cells as a result of 5-Aza-dC treatment may facilitate metastasis by exhibiting these properties.

It is of interest to note that despite high levels of expression of *MUC4* gene in HCT15 cells, no MUC4 was detected using two different MUC4 antibodies which can detect MUC4 in the lysate of Colo-357 cells. The results suggest that MUC4 protein backbone was not produced from mRNA. It has been known that one of the gene silencing mechanisms of microRNAs is inhibition of mRNA translation (Findlay et al., 2008). This may be one possible explanation that MUC4 was not detected in these cells. Therefore, HCT15 cells could be a useful model for identifying microRNAs that prevent MUC4 protein synthesis from mRNA.

Our results show that the sLe^x induced by 5-Aza-dC is located on mucin-type glycan of MUC1. In these glycans, sLe^x can be built on type 2 *N*-acetyllactosamine generated with B4GALT4. Since *B4GALT4* was not significantly enhanced by 5-Aza-dC, it probably does not play a significant role in the production of sLe^x on MUC1 in 5-Aza-dC treated HCT15 cells. The next biosynthetic step for making sLe^x on mucin-type glycans involves four possible ST3Gals, including ST3Gal3, 4, 5, and 6. It has been reported that ST3Gal 3 and 4 can modify glycoproteins (Carvalho et al., 2010) and ST3Gal5 can modify glycolipids (Kitagawa and Paulson, 1994) while ST3Gal6 can modify both (Carvalho et al., 2010; Kitagawa and Paulson, 1994). Because 5-Aza-dC increased the expression of the *ST3Gal6* gene in HCT15 cells from very low, i.e. <0.0001% of *GAPDH*, to moderate, i.e. 0.06% of *GAPDH*, while the expression levels of three other *ST3Gal* genes were already at sufficiently high levels, i.e. 0.09–0.24% of *GAPDH*, before 5-Aza-dC treatment. This expression level was even higher after 5-Aza-dC treatment, suggesting that ST3Gal6 and not ST3GalT3, 4, or 5 were responsible for the synthesis of sLe^x in HCT15 cells. This prediction was confirmed by suppression of sLe^x on MUC1 after knockdown of the *ST3Gal6* gene using RNAi. Although we do not know the specific FUT(s) involved in the synthesis of sLe^x on MUC1 in HCT15 cells, *FUT7* can be ruled out because its expression level was very low even after 5-Aza-dC treatment. Based on the high expression levels of *FUT3*, *4*, and *5* genes after 5-Aza-dC treatment, they are the likely candidates for catalyzing the formation of sLe^x after NeuAcα2-3Galβ1-4GlcNAc has been generated by ST3Gal6. Involvement of specific FUT(s) in the synthesis of sLe^x on MUC1 in HCT15 cells remains to be established.

In conclusion, our report provides a strong evidence for epigenetic regulation of *ST3Gal6* gene, showing the relationship between methylation status of a sialyltransferase gene and cancer metastatic potential. This finding is consistent with a previous report which showed that inhibition of DNA methylation in colon cancer cells treated with 5-Aza-dC led to the production of tumor-associated antigen Sd^a (Kawamura et al., 2008). However, this study did not examine sLe^x. Present work also provides a valuable insight into the novel concept that each glycosyltransferase acts only on a subset of glycoproteins. In addition, the increased expression of sLe^x upon treatment with 5-Aza-dC observed in this study and with sodium butyrate as demonstrated in our previous work (Radhakrishnan et al., 2007) collectively raises a concern about a potentially serious side effect of using these drugs for cancer therapy. However, it should be pointed out that our observation was made in only one colon cancer cell line. Whether the observation can be reproduced in colon tumors and tumors from other organs remains to be examined.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.biocel.2010.12.015.

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