

Modulation of heparan sulfate biosynthesis by sodium butyrate in recombinant CHO cells

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Received: 22 April 2013 / Accepted: 14 December 2013 / Published online: 29 January 2014
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Abstract Sodium butyrate, a histone deacetylase inhibitor, has been used to improve transgene expression in Chinese hamster ovary (CHO) cells. The current study explores the impact of butyrate treatment on heparan sulfate (HS) biosynthesis and structural composition in a recombinant CHO-S cell line expressing enzymes in the heparin (HP)/(HS) biosynthetic pathway (Dual-10 stably expressing *NDST2* and *HS3st1*). Flow cytometric analysis showed that anti-thrombin binding was increased in Dual-10 cells and basic fibroblast growth factor binding was decreased in response to sodium butyrate treatment. The results were in agreement with the AMAC-LCMS (2-amino-acridine-tagged HS/HP analysis by liquid chromatography mass spectrometry) data that showed that there was an increase in heparan sulfate tri-sulfated disaccharides and a decrease in *N*-sulfated disaccharides in the butyrate-treated cells. However, we could not

detect any changes in the chondroitin sulfate pathway in Dual-10 cells treated with butyrate. The current study is the first to report the effect of butyrate on glycosaminoglycan profiles.

Keywords Chinese hamster ovary cells · Sodium butyrate · Glycomics · Proteoglycans · Bioengineered heparin

Introduction

Sodium butyrate ($\text{CH}_3\text{CH}_2\text{CH}_2\text{CO}_2^-$) is a histone deacetylase inhibitor that modulates gene expression of cellular processes, particularly transgene expression in Chinese hamster ovary (CHO) cells (Chotigeat et al. 1994; Chun et al. 2003; De Leon et al. 2007; Hendrick et al. 2001; Jiang and Sharfstein 2008; Kim and Lee 2000; Mimura et al. 2001; Wang et al. 2002). Butyrate works as a histone deacetylase inhibitor and

Electronic supplementary material The online version of this article (doi:10.1007/s10616-013-9677-9) contains supplementary material, which is available to authorized users.

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may also increase histone acetylation directly in an ATP-citrate-lyase-dependent manner (Donohoe et al. 2012). Acetylation of histones facilitates the relaxation of the chromatin and consequently, transcription factors can bind and alter gene expression of various cellular processes (Riggs et al. 1977). Butyrate selectively increases transcription of genes, and the changes in gene expression due to butyrate treatment in CHO cells have been studied at the transcriptomic and proteomic levels (Yee et al. 2008; De Leon et al. 2007). Butyrate treatment in CHO cells affects cellular processes such as cytoskeleton reorganization, protein transport, nucleosome assembly, nucleotide metabolism, glycosylation, protein folding, oxidative stress responses, lipid metabolism, cholesterol biosynthesis, glycolysis, cell cycle progression, and apoptosis (Yee et al. 2008; De Leon et al. 2007). Butyrate has been used in cell engineering strategies, and butyrate treatment in CHO cells often increases recombinant protein expression (Chun et al. 2003; De Leon et al. 2007; Hendrick et al. 2001; Kim and Lee 2000; Chotigeat et al. 1994; Mimura et al. 2001; Wang et al. 2002; Jiang and Sharfstein 2008). The current work explores the effect of butyrate on glycosaminoglycan (GAG) profiles in recombinant CHO cells in our efforts to produce a bioengineered heparin (HP).

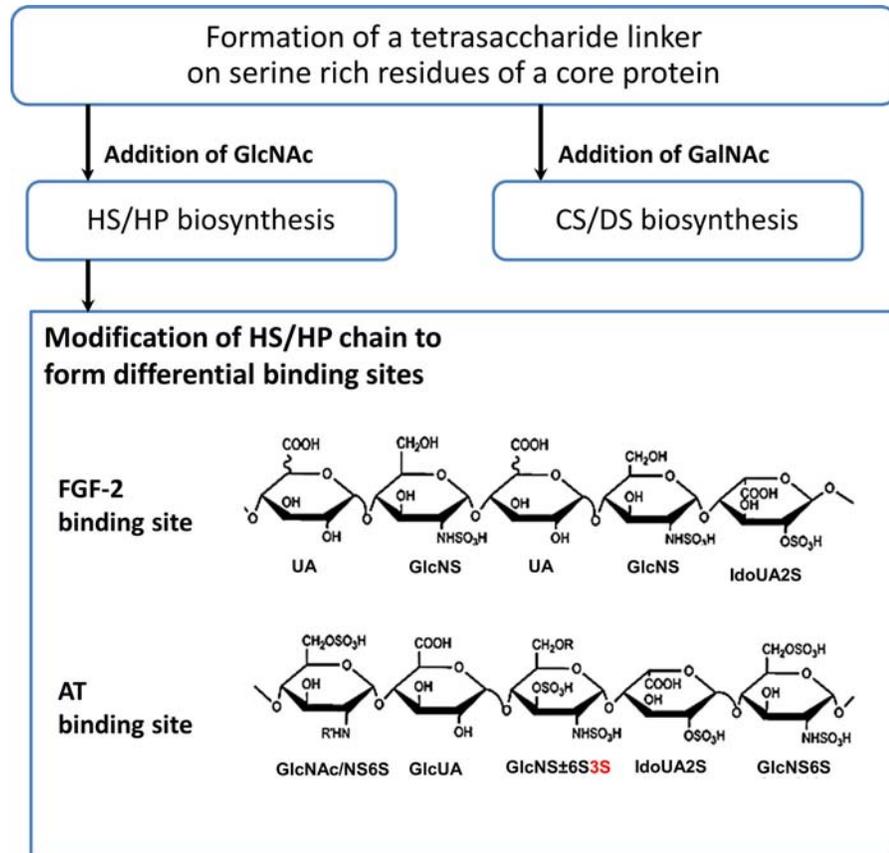
HP is a proteoglycan that shares its biosynthetic pathway with heparan sulfate (HS); HP is specifically produced in mast cells and involved in storage of mast cell proteases (Humphries et al. 1999). HP is widely utilized as a biopharmaceutical anticoagulant drug with over \$7 billion in annual sales. HP is currently derived from mucosal tissues of animals such as pig intestines or cow lungs. Animal-sourced HP is more likely to contain impurities such as viruses and prions. Moreover, as the recent HP contamination crisis suggests, adulterants are much more likely to be present in animal-sourced HP due to the lack of cGMP in the early processing steps (Guerrini et al. 2009; DeAngelis 2012). In contrast, a bioengineered HP would be prepared in a cGMP facility operating under stringent drug-related regulations. Both the possibility of viral and prion contamination, as well as the difficulty in monitoring processing in a non-cGMP facility have led to an interest in the production of biopharmaceutical HP from non-animal sources.

HP and HS are composed of glucosamine and uronic acid residues and share a similar biosynthesis in

the Golgi. Biosynthesis initiates with formation of a tetrasaccharide linker that is common to heparan sulfate (HS), heparin (HP), chondroitin sulfate (CS) and dermatan sulfate (DS) biosynthesis (Fig. 1). Initiation of the tetrasaccharide linker to serine-rich core proteins is followed by chain polymerization and modification (deacetylation, epimerization, and sulfonation) (Carlsson and Kjellen 2012). Chain polymerization and modification involves the action of tissue-specific isoforms of multiple enzymes resulting in a heterogeneous population of HS/HP, having tissue-specific, differential, functional protein binding sites (Sasisekharan et al. 2006). Partial modification yields HS. HS is characterized by: (1) moderate levels of *N*-sulfo-glucosamine (40–60 %); (2) low to moderate levels of sulfonation (0.8–1.8 sulfo groups per hexosamine); (3) a moderate fraction of iduronic acid residues (30–50 %); and (4) minimal antithrombin III (AT) binding (0–0.3 %). Complete modification yields anticoagulant HP characterized by AT binding sites. HP is characterized by: (1) high levels of glucosamine *N*-sulfates (≥ 80 %); (2) moderate to high sulfonation (1.8–2.6 sulfates per hexosamine); (3) a high fraction of iduronic acid residues (≥ 70 %); and (4) AT binding (30 %). Studies related to biosynthesis of anticoagulant HP in mast cells demonstrate: (1) *N*-deacetylase/*N*-sulfotransferase-2 (NDST2) is the predominant NDST isoform in mouse mast cells and critical for HP biosynthesis (Humphries et al. 1999); (2) the presence of *D*-glucuronyl C5-epimerase (GLCE, C5-epimerase) (Feyerabend et al. 2006); (3) 6-*O*-sulfonation is dependent on HS-6-*O*-sulfotransferases isoforms 1 and 2 (HS6st1–2) (Ferdous Anower et al. 2012); and (4) HS-3-*O*-sulfotransferase isoform 1 (HS3st1) is critical for HP production (Shworak et al. 2010).

CHO cells have been extensively used in the biopharmaceutical industry for the production of glycoprotein biologics (Datta et al. 2013). Hence, we hypothesized that they could also be used to produce GAG biopharmaceuticals, specifically HP. CHO cells produce HS and express NDSTs, HS2sts, GLCE, and HS6sts enzymes involved in HS/HP biosynthesis (Zhang et al. 2006; Xu et al. 2011; Kobayashi et al. 1996, 1997; Habuchi et al. 1995, 1998). However, NDST2 and HS3st1, which are critical for the synthesis of anticoagulant HP, are absent in CHO cells (Xu et al. 2011; Zhang et al. 2006; Baik et al. 2012).

Fig. 1 Biosynthesis of GAGs and formation of AT binding site



To create a CHO cell line producing a bioengineered heparin, human NDST2 and murine HS3st1 were sequentially transfected into CHO-S cells. A clone with a high level of NSDT2 expression, as determined by RT-PCR and Western blotting, was transfected with HS3st1 (Baik et al. 2012). From this study, ~40 stable CHO-S dual-transfected clones were isolated. Clones were screened by RT-PCR and Western blotting to ensure that NDST2 expression was maintained and to determine the level of expression of HS3st1. The results of this analysis are shown in Supplementary Figure 1. In that study, two clones (Dual-29 and Dual-3) that express high levels of NDST2 and HS3st1 were selected for characterization of the amount and composition of the GAGs produced, as well as the anticoagulant activity. This analysis showed an increase in the amount of GAGs produced when compared with the wild-type CHO-S cells, particularly for the Dual-29 clone. While the sulfation pattern was altered and an increase in anticoagulant activity was observed, the HS/HP produced still had a much lower

anticoagulant activity and a sulfation pattern distinctly different from pharmaceutical heparin (Baik et al. 2012), suggesting incomplete activity of the transgenes and the importance of balancing exogenous and endogenous genes in metabolic engineering of CHO cells for the production of anticoagulant HP.

Butyrate treatment of murine mastocytoma cells increases sulfonation of HP, including increasing the AT binding ability. Murine mastocytoma cells (an immortalized cell line based on the natural source of heparin) treated with 2.5 mM butyrate produced HS/HP with increased AT binding affinity and an increase in *N*-acetylated glucosamine residues (~35 %, as compared to 10 % in the control) (Jacobsson et al. 1985). While previous studies did not determine the mechanism by which sulfonation was modified, they suggest that butyrate treatment might impact the sulfonation pattern in recombinant CHO cells, with a resultant change in the binding affinity of HS/HP binding proteins such as AT and FGF-2.

Based on the previous observation that butyrate treatment of mastocytoma cells increases sulfonation of HP and other observations that butyrate increases gene expression, particularly for transgenes, we hypothesized that butyrate treatment might increase the sulfonation of the HS/HP produced in our recombinant CHO cells. Thus, the current study evaluates modulation of HS/HP composition in response to butyrate stimulation in a recombinant CHO-S cell line (Dual-10), stably expressing *NDST2* and *HS3st1*, suggesting that butyrate can be an additional tool, coupled with cell engineering strategies for production of bioengineered HP in CHO cells.

Materials and methods

Cell lines and cell culture

CHO-S cells (Life Technologies, Carlsbad, CA, USA) and stable recombinant cell lines (Dual-29 and Dual-10) were routinely cultured in CD-CHO medium (Life Technologies) supplemented with 8 mM Glutamax (Life Technologies) and 2 % HT supplement (mixture of sodium hypoxanthine and thymidine, Invitrogen (Carlsbad, CA, USA) #11067–030). Dual-29 and Dual-10 cells are CHO-S cells that have been sequentially transfected with *NDST2* and *HS3st1* genes; recombinant cell lines were routinely kept under selective pressure of 1 mg/ml G418 and 500 µg/ml Zeocin. The cells were seeded at 2×10^5 cells/ml in 125 ml polycarbonate Erlenmeyer flasks (Corning, Corning, NY, USA) and cultured on orbital shakers agitated at 125 rpm in a humidified 37 °C incubator at 5 % CO₂. All experiments were performed with cells from passage 3 through 10.

Sodium butyrate treatment

100 mM sodium butyrate (Sigma, St. Louis, MO, USA) was prepared in Dulbecco's phosphate-buffered saline (DPBS), filter sterilized, aliquoted, and stored at –20 °C.

On day 0, cell lines were seeded at 2×10^5 cells/ml in 40 ml of medium. On day 3, the cells were counted, centrifuged, and resuspended at a concentration of 1×10^6 cells/ml in 40 ml fresh medium with 2.5 mM sodium butyrate (+NaB) or sterile DPBS (negative control). 5 mM butyrate was also evaluated; however, the cell viability was greatly reduced (<~10 %) as was the cell number (Supplementary Figure 2).

Hence, the subsequent experiments were performed with 2.5 mM butyrate. Fresh medium was used, as GAGs are shed into the conditioned medium. After 48 h of butyrate treatment, cells were counted, and cells and medium were harvested for analyses as described below.

Quantification of AT and fibroblast growth factor-2 (FGF-2) binding by flow cytometry

Flow cytometry was used to compare AT and FGF-2 binding in the cell lines with and without sodium butyrate treatment. AT and FGF-2 were labeled with BODIPY R6G (SE, Invitrogen) as described previously (Baik et al. 2012).

Cells (1×10^6) from each culture condition were washed with cold sterile DPBS containing 10 % fetal bovine serum (FBS) [Sigma-Aldrich (St. Louis, MO, USA)] and incubated with BODIPY R6G-conjugated AT or FGF-2 for 30 min at 4 °C in the dark. The cells were then washed with cold, sterile DPBS containing 10 % FBS. The cells were fixed with freshly prepared 4 % paraformaldehyde and analyzed by flow cytometry. A minimum of 10,000 cells per sample were analyzed on a BD LSRII flow cytometer (BD, San Jose, CA, USA) as described previously (Baik et al. 2012).

For AT and FGF-2 binding assays, the fold induction was calculated by normalizing the mean fluorescence of CHO-S and Dual-10 + NaB cells with respect to untreated Dual-10 cells. Two-way ANOVA was performed on the mean fluorescence, and significant ANOVA was followed by post hoc analysis by post hoc Tukey-HSD test (JMP-IN, SAS, Cary, NC, USA).

Quantification of HS/HP modification enzymes by flow cytometry

To determine whether sodium butyrate treatment affected the expression of HS/HP modification enzymes, expression levels were determined by flow cytometry. Cells were washed once for 10 min with sterile DPBS. Cells were fixed with 4 % paraformaldehyde in sterile DPBS for 15 min at room temperature and further washed for 10 min with cold sterile DPBS. Cells were permeabilized with cold sterile DPBS containing 10 % FBS and permeabilization buffer (Invitrogen) for 10 min and washed with sterile DPBS. Cells were stained with primary antibodies for NDST2, GLCE, HS2st, HS6st1, HS6st2 and HS3st1 overnight at

4 °C in the dark, following the manufacturer's instructions. The primary antibodies used for immunofluorescence are rabbit anti-HS3st1 (ab91065, Abcam), rabbit anti-HS6st1 (ab106095, Abcam, Cambridge, MA, USA), rabbit anti-NDST2 (AP5759B, Abgent, San Diego, CA, USA), rabbit anti-GLCE (H00026035-D01, Abnova, Walnut, CA, USA), rabbit anti-HS6st2 (sc-98287, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-HS2st1 (ab108541, Abcam). After overnight incubation, the cells were washed once with cold sterile DPBS containing 10 % FBS and permeabilization buffer (Invitrogen) for 10 min. Subsequently the cells were stained with secondary antibody (donkey anti-rabbit IgG Alexa Flour 647, Molecular Probes®—Life Technologies) for 30 min at 4 °C in the dark, following the manufacturer's instructions. Following secondary antibody staining, the cells were washed with cold sterile DPBS containing 10 % FBS and analyzed or stored at 4 °C in the dark and analyzed within 24 h.

For flow cytometry analysis, the fold induction was calculated by normalizing the mean fluorescence of CHO-S and Dual-10 + NaB cells with respect to untreated Dual-10 cells. Two-way ANOVA was performed on the mean fluorescence, and significant ANOVA was followed by post hoc analysis by post hoc Tukey-HSD test (JMP-IN, SAS).

Western blot analysis

Western blot analysis was performed by extracting total protein from 10^7 cells with Nonidet-P40 lysis buffer (Boston Bioproducts, Ashland, MA, USA) on ice for 30 min in the presence of a cocktail of protease and phosphatase inhibitors, (Halt™ protease inhibitor cocktail, Thermo Fisher Scientific, Hudson, NH, USA). Protein concentrations were determined using BCA assay (Thermo Fisher Scientific). 40 µg of total protein per sample was loaded and separated on 4–20 % polyacrylamide gels (Thermo Fisher Scientific) at 100 V for 1 h, in Tris–HEPES–SDS running buffer. Proteins were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA), probed with relevant primary antibodies for HS3st1 (rabbit anti-HS3st1, ab91065, Abcam) and NDST2 (rabbit anti-NDST2, AP5759B, Abgent) and detected using the anti-rabbit HRP-conjugated secondary antibody and chemiluminescent detection (Super Signal West Pico ECL substrate, Thermo Fisher Scientific) with exposure

on high performance chemiluminescence film (AmershamHyperfilm ECL, GE Healthcare, Piscataway, NJ, USA).

Isolation of GAGS from cells and cell-conditioned media

Cell samples and cell-conditioned media were used for GAG analysis. The isolation and purification of GAGs from CHO cells has been previously described (Yang et al. 2011). Briefly, the cells and media samples were proteolyzed with actinase E solution (20 mg/ml in HPLC grade water, Kaken Biochemicals, Tokyo, Japan) and the GAG was purified using a strong anion exchange spin column (Vivapure Mini Q H spin column, Viva science, Edgewood, NJ, USA), released with salt and alcohol precipitated. Purification was followed by complete depolymerization of the GAGS using polysaccharides lyases.

Chondroitin lyase ABC (Associates of Cape Cod, Inc., East Falmouth, MA, USA) (5 m-units) and chondroitin lyase ACII (Associates of Cape Cod, Inc.) (2 m-units) in 10 µl of 0.1 % BSA were added to ~5 µg GAG sample in 25 µl of distilled water and incubated at 37 °C for 10 h to depolymerize CS/DS GAGs. The CS and DS disaccharide products were recovered by centrifugal filtration using an YM-30 spin column, and the disaccharides were collected in the flow-through and lyophilized.

A mixture of HP lyase I, II, and III (Iduron Ltd., Manchester, United Kingdom) (10 mU each) in 5 µl of 25 mM Tris, 500 mM NaCl, 300 mM imidazole buffer (pH 7.4) were added and incubated at 37 °C for 10 h to depolymerize HS/HP GAGs. The HP and HS disaccharide products were recovered by centrifugal filtration using an YM-30 spin column (Millipore, Bedford, MA, USA), and the disaccharides were collected in the flow-through and lyophilized.

Disaccharide analysis

Disaccharides were derivatized with 2-aminoacridine (AMAC) (Sigma) as follows: GAG-derived disaccharides (~5 µg) or a mixture of 17 disaccharide standards (Iduron Ltd., Manchester, United Kingdom) (5 µg/ each disaccharide) were added to 10 µl of 0.1 M AMAC in acetic acid (AcOH)/dimethyl sulfoxide (DMSO) (3:17, v/v) and mixed by vortexing for 5 min. Next, 10 µl of 1 M NaBH₃CN was added to the

reaction mixture and incubated at 45 °C for 4 h (Kitagawa et al., 1995). Finally, the AMAC-tagged disaccharide mixtures were diluted to different concentrations (0.5–100 ng) using 50 % (v/v) aqueous DMSO, and LC–MS analysis was performed.

Liquid chromatography mass spectrometry (LC–MS) analyses were performed on an Agilent 1200 LC/MSD instrument (Agilent Technologies, Inc. Wilmington, DE; Santa Clara, CA, USA) equipped with a 6300 ion-trap and a binary pump. The column used was a Poroshell 120 C18 column (2.1 × 150 mm, 2.7 μm, Agilent) at 45 °C. Eluent A was 80 mM ammonium acetate solution and eluent B was methanol. Solution A and 15 % solution B was flowed (150 μl/min) through the column for 5 min followed by a linear gradient of 15–30 % solution B from 5 to 30 min. The column effluent entered the electrospray ionization-MS source for continuous detection by MS. The electrospray interface was set in negative ionization mode with a skimmer potential of –40.0 V, a capillary exit of –40.0 V, and a source temperature of 350 °C, to obtain the maximum abundance of the ions in a full-scan spectrum (150–1,200 Da). Nitrogen (8 l/min, 40 psi) was used as a drying and nebulizing gas (Yang et al. 2012).

Results and discussion

The effects of butyrate on the biosynthesis and the structure of GAGs were analyzed by three approaches: (1) changes in enzymes involved in GAG modification pathway were measured by flow cytometry and Western blot; (2) changes in binding of basic FGF-2 and AT binding were measured by flow cytometry; and (3) the structural compositions of HS and CS were measured by AMAC-LCMS.

Screening of cell lines

CHO-S and recombinant CHO-S cell lines that express *NDST2* and mouse *HS3st1* (Fig. 2a) were treated with sodium butyrate and analyzed for changes in AT binding. AT binding sites are created by complete or nearly complete sulfonation of the HS chain, with the critical modification being sulfonation of the 3' hydroxyl on the glucosamine residue. These cell lines were initially chosen based on their disaccharide analysis

(Fig. 2b). Disaccharide analysis suggests that the dual transfected clones exhibit a highly *N*-sulfated disaccharide pattern that is quite different from biopharmaceutical heparin (right column). The effect of butyrate on AT binding is shown in Fig. 2c ($n = 1$). Treatment with 2.5 mM butyrate had moderate effect on the AT binding in Dual-10 cells and minimal effect on the AT-binding of the wild-type (CHO-S) or the Dual-29 clone. The Dual-29 clone exhibits high levels of both exogenous *NDST2* and *HS3st1* (*NDST2*⁺⁺⁺/*HS3st1*⁺⁺⁺), whereas the Dual-10 clone exhibits lower levels of exogenous *NDST2* and *HS3st1* (*NDST2*⁺⁺/*HS3st1*⁺) expression as shown by Western blot analysis (Baik et al. 2012). As AT binding was only altered in the Dual-10 clone, this cell line was selected for further analysis.

Butyrate interferes with cell growth in Dual-10 cells

Sodium butyrate treatment significantly inhibited growth of Dual-10 cells as shown in Fig. 3. When Dual-10 cells were treated with 2.5 mM sodium butyrate, the mean cell number remained similar to the seeding density when compared to Dual-10 cells treated with vehicle, though no significant cell death was seen. The effect of butyrate on cell growth and apoptosis in CHO cells has previously been observed and strategies to improve cell growth are being considered, including media additives such as silk worm hemolymph, down-regulation of BAK, BAX, Caspase 3 and Caspase 7, and overexpression of bcl-2 (Cost et al. 2010; Kim et al. 2009; Sung et al. 2007; Sung and Lee 2005; Sung et al. 2005; Choi et al. 2005b; Kim and Lee 2002, 2000).

Butyrate significantly increases AT binding sites and significantly reduces FGF-2 binding sites on HS in Dual-10 cells

AT binding sites (Fig. 1) are formed by complete or nearly complete sulfonation of the HS chain. Specifically AT binds to a unique pentasaccharide sequence common in HP and less common in HS. While the biosynthesis of the AT-binding site also requires concerted action of *NDST2*, C-5 epimerase, *HS2st*, and *HS6sts*, it requires the action of *HS3st1* to install a critical, but rare, 3-*O*-sulfo-group on its central

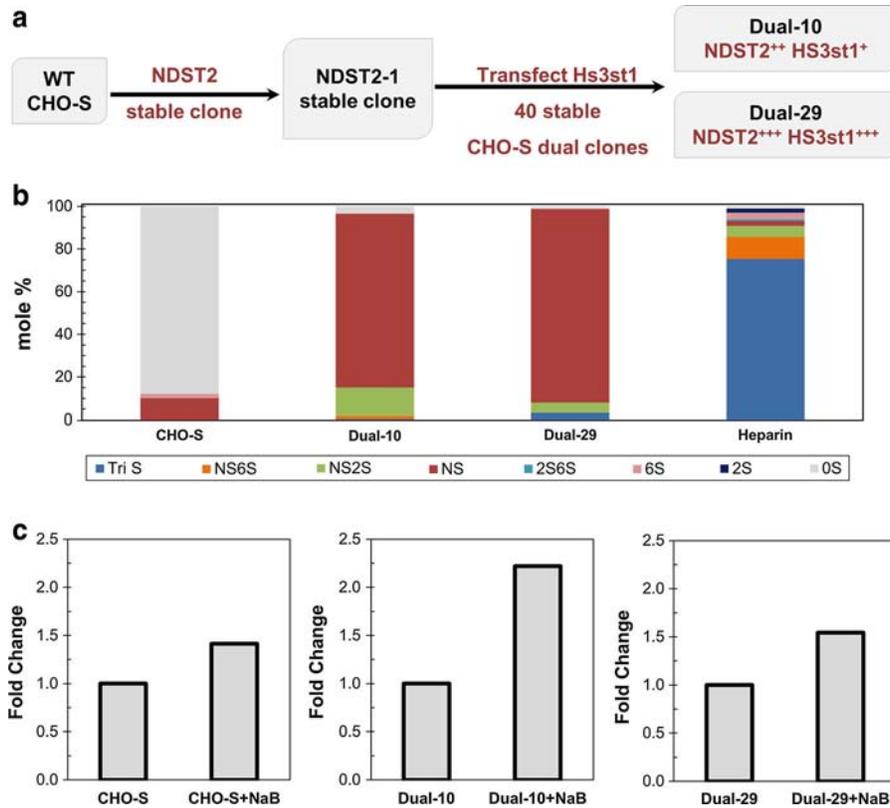


Fig. 2 Screening of cell lines. **a** Schematic diagram of metabolic engineering of Dual-10 and Dual-29 cells from CHO-S cells. **b** Disaccharide composition of GAGs isolated from CHO-S, Dual-10, Dual-29 cells and heparin. Disaccharides consist of glucuronic/iduronic acid (Δ UA) linked (1 \rightarrow 4) to N-acetyl glucosamine (GlcNAc) with various modifications as follows: 0S, Δ UA(1 \rightarrow 4) GlcNAc; NS, Δ UA(1 \rightarrow 4)GlcNS; 6S,

Δ UA(1 \rightarrow 4)GlcNAc6S; 2S, Δ UA2S(1 \rightarrow 4)GlcNAc; NS2S, Δ UA2S(1 \rightarrow 4)GlcNS; NS6S, Δ UA(1 \rightarrow 4)GlcNS6S; 2S6S, Δ UA2S(1 \rightarrow 4)GlcNAc6S; and TriS, Δ UA2S(1 \rightarrow 4)GlcNS6S. **c** Effect of 2.5 mM butyrate treatment (48 h) on AT-binding affinity in CHO-S, Dual-10, and Dual-29 cells evaluated by flow cytometry ($n = 1$)

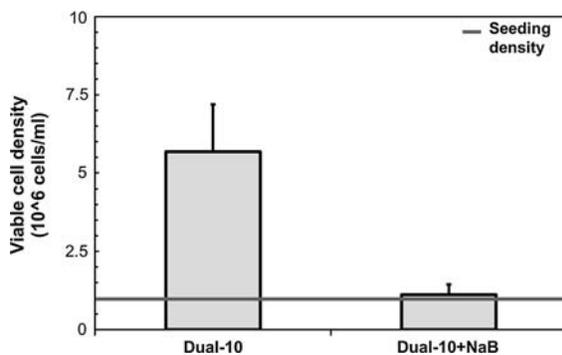
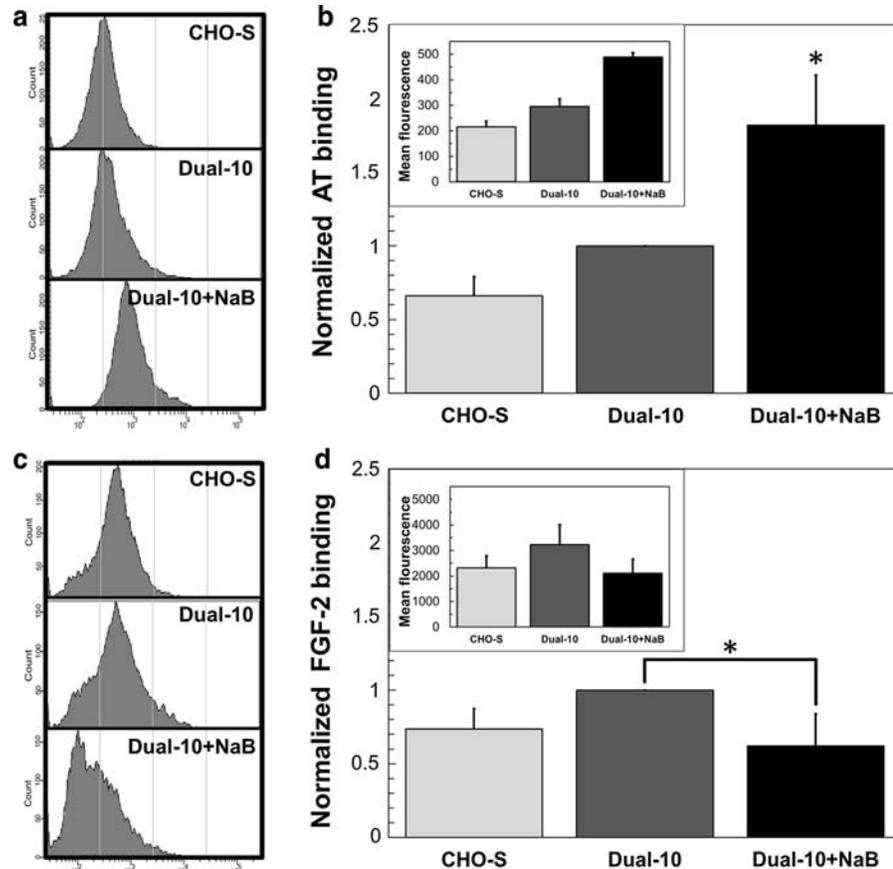


Fig. 3 Effect of butyrate (2.5 mM) treatment (48 h) on cell growth (cell viability \sim 90 %) in Dual-10 cells (mean \pm SD, $n = 5$)

glucosamine residue. The Dual-10 cells are transfected with human NDST2 and mouse HS3st1 under the control of CMV promoters. It has been shown that butyrate affects transgenes regulated by CMV promoters (Choi et al. 2005a). Therefore, butyrate may increase the expression of NDST2 and HS3st1, resulting in an increase in AT binding sites on HS. AT binding was assayed in CHO-S cells, Dual-10 cells, and Dual-10 cells treated with NaB using flow cytometry. Fig. 4a shows the histograms of binding activity for cells fluorescently tagged with AT. Butyrate treatment (2.5 mM for 48 h) increased the AT binding affinity \sim twofold in Dual-10 cells ($n = 4$, $p = 0.0016$).

Fig. 4 Butyrate modulation of AT and FGF-2 binding sites on HS in Dual-10 cells. **a, b** Butyrate (2.5 mM, 48 h) significantly increased AT binding ($n = 4$, mean \pm SD, $p = 0.0016$); **c, d** butyrate significantly decreased FGF-2 binding (**c, d**) in Dual-10 cells to a level similar to FGF-2 binding in CHO-S cells. ($n = 4$, mean \pm SD, $p = 0.0025$)



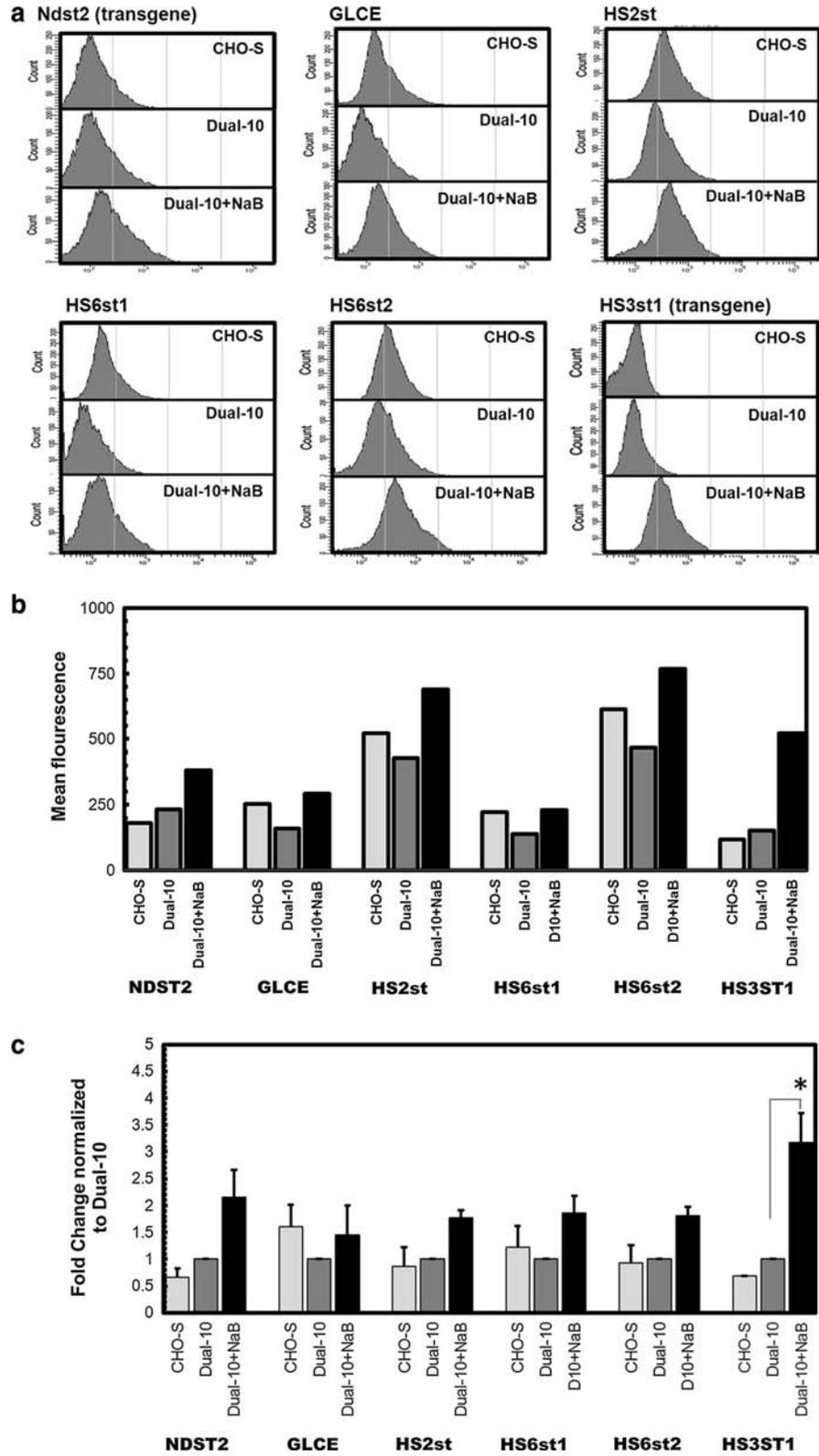
FGF-2 binds preferentially to HP-like structures on HS, corresponding to tri-sulfated disaccharides. FGF-2 is a polypeptide cytokine that is involved in pleiotropic cellular processes, including growth, differentiation and a variety of specialized cell functions. FGF-2 exerts its biological activities in part by tyrosine kinase receptor(s) and HS proteoglycans. HS proteoglycans that are characterized by FGF-2 binding sites, bind and internalize FGF-2 and mediate FGF-2 signal transduction (Zehe et al. 2006; Forsten-Williams et al. 2005; Gleizes et al. 1995; Quarto and Amalric 1994). FGF-2 binding sites on HS (Fig. 1) are a function of specific sulfonation patterns on the HS chain. FGF-2 binding was compared in CHO-S cells, Dual-10 cells, and Dual-10 cells treated with NaB using flow cytometry. Butyrate treatment (2.5 mM for 48 h) significantly reduced FGF-2 binding in Dual-10 cells, reducing it to the same level as CHO-S cells as shown in Fig. 4d ($n = 4$, $p = 0.0025$).

Butyrate effects transgene expressions and endogenous HS2st1, HS6st1 and HS6st2 expression in Dual-10 cells

To determine whether the change in AT and FGF-2 binding was mediated by changes in expression of HS/HP biosynthetic enzymes, expression levels of NDST2, GLCE, HS2st, HS6st1, HS6st2, and HS3st1 were determined by immunofluorescence staining and FACS analysis (Fig. 5a, b). NDST2 and HS3st1 in CHO-S cells exhibited the lowest immunofluorescence. This was expected as previous studies had shown that NDST2 and HS3st1 were absent or at low levels in CHO-S cells (Baik et al. 2012).

Treatment with sodium butyrate did not significantly increase transgene expression of NDST2 in Dual-10 cells ($p = 0.0771$) or endogenous expression of GLCE, HS2st1, HS6st1, or HS6st2 ($p = 0.0373$) in the Dual-10 cells. However, treatment with sodium butyrate significantly increased

Fig. 5 Butyrate (2.5 mM, 48 h) modulates expression of exogenous NDST2 and HS3st1, and endogenous HS2st1, HS6st1, HS6st2 (1-2) in Dual-10 cells. **a** Representative histograms. **b** Mean fluorescence in a representative experiment. **c** Fold change normalized to Dual-10 cell line (mean \pm SD, n = 3)



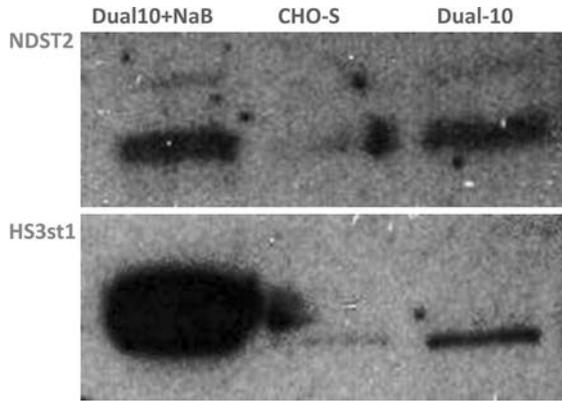


Fig. 6 Western Blot analysis of effects of butyrate on NDST2 (top panel) and HS3st1 (bottom panel) expression in Dual-10 cells treated with 2.5 mM butyrate (48 h), CHO-S and Dual-10 cells

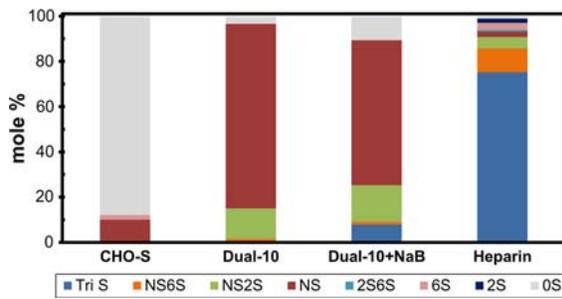


Fig. 7 Effect of butyrate on composition of HS extracted from cells in Dual-10 cells treated with 2.5 mM butyrate (48 h) and compared to HS/HP isolated from CHO-S, Dual-10 and MST10H cells. Regards: Disaccharides consist of glucuronic/iduronic acid (Δ UA) linked (1 \rightarrow 4) to N-acetyl glucosamine (GlcNAc) with various modifications as follows: OS, Δ UA (1 \rightarrow 4) GlcNAc; NS, Δ UA(1 \rightarrow 4)GlcNS; 6S, Δ UA(1 \rightarrow 4)GlcNAc6S; 2S, Δ UA2S(1 \rightarrow 4)GlcNAc; NS2S, Δ UA2S(1 \rightarrow 4)GlcNS; NS6S, Δ UA(1 \rightarrow 4)GlcNS6S; 2S6S, Δ UA2S(1 \rightarrow 4)GlcNAc6S; and TriS, Δ UA2S(1 \rightarrow 4)GlcNS6S

transgene expression of HS3st1 ($p \leq 0.001$, two-way ANOVA, followed by a post hoc Student’s t test) in the Dual-10 cells.

The fold change of biosynthetic enzymes normalized to Dual-10 cells ($n = 3$ at 400–500 V) in flow cytometry experiments are shown in Fig. 5c. The observed fold-change in each enzyme only demonstrates its presence in a form that can be recognized by antibodies. These data do not inform whether these enzymes are correctly folded and biologically active. While all enzymes showed an increase in expression on butyrate treatment over the Dual-10 control, the exogenous HS3st1 and NDST2

Table 1 Effect of butyrate on HS and CS composition of cell pellet and cell-conditioned media from Dual-10 and Dual-10 cells treated with 2.5 mM butyrate (48 h). HS/HP disaccharides are described in the Figure 2 legend. CS and DS disaccharides are comprised of glucuronic/iduronic acid (Δ UA) linked (1 \rightarrow 3) to N-acetyl galactosamine (GalNAc) with various modifications as follows: OS, Δ UA(1 \rightarrow 3)GalNAc; 4S, Δ UA(1 \rightarrow 3)GalNAc4S. Only species observed are listed in the table

| Samples | HS/HP disaccharides | | | | CS/DS disaccharides | | | | Ratio of HS/HP and CS/DS |
|---------------------|---------------------|------------|-------------|-------------|---------------------|-------------|------------|-----|--------------------------|
| | Tri S | | NS2S | | 4S | | OS | | |
| | Tri S | NS6S | NS | OS | 4S | OS | 4S | OS | |
| Dual-10 cells | 0.6 ± 0.31 | 1 ± 0.16 | 13.4 ± 0.35 | 80.6 ± 0.07 | 3.4 ± 0.96 | 94.8 ± 0.25 | 5.2 ± 0.12 | 3:1 | |
| Dual-10 + NaB cells | 7.9 ± 0.25 | 1.2 ± 0.21 | 16.1 ± 0.45 | 64.1 ± 0.08 | 10.8 ± 0.92 | 95.0 ± 0.31 | 5.0 ± 0.08 | 5:1 | |
| Dual-10 media | 1.8 ± 0.23 | 1.1 ± 0.18 | 4.9 ± 0.38 | 88.6 ± 0.12 | 3.6 ± 0.89 | 95.3 ± 0.22 | 4.7 ± 0.15 | 9:1 | |
| Dual-10 + NaB media | 3.3 ± 0.21 | 0.8 ± 0.14 | 11 ± 0.39 | 77.1 ± 0.04 | 7.8 ± 0.86 | 94.2 ± 0.22 | 5.8 ± 0.91 | 3:1 | |

enzymes showed the greatest fold-increase, with only HS3st1 demonstrating a statistically significant increase. Western blot analysis confirmed the changes in protein expression levels of NDST2 and HS3st1 upon sodium butyrate treatment, with NDST2 exhibiting a slight increase in expression upon butyrate treatment and HS3st1 exhibiting a dramatic increase in expression (Fig. 6).

Butyrate effects sulfonation of HS but not CS/DS in Dual-10 cells

The disaccharide composition of HS extracted from cell pellets of CHO-S, Dual-10, and Dual-10 cells treated with 2.5 mM butyrate for 48 h is shown in Fig. 7. The extracted HS was treated with HP lyases to form disaccharides that were analyzed by LC–MS. The major disaccharide isolated from the CHO-S cell pellet was OS (87.9 %), followed by NS (10 %). The major disaccharide isolated from the Dual-10 cell pellet was NS (80.6 %), followed by NS2S (13.4 %). The major disaccharide isolated from the Dual-10 + NaB cells was NS (64.1 %), followed by NS2S (16.1 %), OS (10.8 %), and TriS (7.9 %). The LC–MS disaccharide analysis indicates that under butyrate treatment, there is an increase in NS2S, OS, and TriS disaccharides and a decrease in NS disaccharides. One possible explanation for the decrease in NS on butyrate treatment is that the HS is further sulfated to form other disaccharides, e.g. TriS, NS2S. Alternatively, increased 3-*O*-sulfonation may occur, generating a HP-lyase resistant sequence containing a GlcNS3S residue commonly found in the AT-binding site. As this sequence is HP-lyase resistant, it cannot be detected by disaccharide analysis.

The disaccharide composition of HS present in the cell-conditioned medium of CHO-S, Dual-10 and Dual-10 cells treated with 2.5 mM butyrate for 48 h is shown in Table 1. In general, the shed HS is more highly sulfated (i.e., richer in TriS and poorer in OS) than the cell-associated HS. While butyrate treatment appears to increase the sulfonation level in Dual-10 cells, it also appears that the most highly sulfated chains in Dual-10 cells remain with the cell pellet, while in CHO-S cells, the most highly sulfated chains are shed into the media.

HS biosynthesis begins with initiation of a tetrasaccharide linker on serine rich residues on core proteins (Fig. 1). This tetrasaccharide linker is common for both HS and CS and DS. To determine whether sodium

butyrate treatment altered the sulfonation pattern on CS and DS, CS and DS were extracted from cells and cell-conditioned media. The disaccharide composition of CS/DS extracted from the cell pellet and the cell-conditioned medium of CHO-S, Dual-10 and Dual-10 cells treated with 2.5 mM butyrate (48 h) is shown in Table 1. Interestingly, butyrate treatment had little or no effect on the sulfonation in CS/DS pathway, further demonstrating that the primary effect was to modulate the expression of the transgenes in the HP/HS biosynthetic pathway, rather than altering the expression of endogenous genes.

Conclusion

We demonstrated that butyrate increases HS/HP sulfonation in the recombinant Dual-10 CHO cell lines as shown by structural analysis of cell associated HS using AMAC-LCMS. In contrast, butyrate had little or no effect on the CS/DS sulfonation or on the ratio of HS to CS. Furthermore, butyrate treatment caused a modest increase in the expression of exogenous NDST2 and a significant increase in the expression of exogenous HS3st1 in Dual-10 cells, with a small increase in the expression endogenous of HS2sts and HS6sts. While the resulting cell-associated HS in Dual-10 cells have enhanced the overall incorporation of sulfo groups (Table 1), including increased trisulfated disaccharides, we were unable to identify 3-*O*-sulfo groups by disaccharide analysis. However, the enhanced AT-binding and diminished binding of FGF-2 in Dual-10 cells is consistent with increased 3-*O*-sulfonation. Hence, these butyrate induced changes in sulfonation pattern of HS in recombinant CHO cells suggest that the cell glycome of the recombinant CHO cells may be modulated using butyrate, providing an additional tool for altering glycosylation of proteoglycans produced in bioengineered CHO cells.

Acknowledgments This work was supported in part by a grant from the National Institutes of Health (R01GM090127). Payel Datta was supported in part by a fellowship from Rensselaer Polytechnic Institute.

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