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Epigenetics: methylation-associated repression of heparan sulfate 3-*O*-sulfotransferase gene expression contributes to the invasive phenotype of H-EMC-SS chondrosarcoma cells

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ABSTRACT Heparan sulfate proteoglycans (HSPGs), strategically located at the cell-tissue-organ interface, regulate major biological processes, including cell proliferation, migration, and adhesion. These vital functions are compromised in tumors, due, in part, to alterations in heparan sulfate (HS) expression and structure. How these modifications occur is largely unknown. Here, we investigated whether epigenetic abnormalities involving aberrant DNA methylation affect HS biosynthetic enzymes in cancer cells. Analysis of the methylation status of glycosyltransferase and sulfotransferase genes in H-EMC-SS chondrosarcoma cells showed a typical hypermethylation profile of 3-*OST* sulfotransferase genes. Exposure of chondrosarcoma cells to 5-aza-2'-deoxycytidine (5-Aza-dc), a DNA-methyltransferase inhibitor, up-regulated expression of 3-*OST*1, 3-*OST*2, and 3-*OST*3A mRNAs, indicating that aberrant methylation affects transcription of these genes. Furthermore, HS expression was restored on 5-Aza-dc treatment or reintroduction of 3-*OST* expression, as shown by indirect immunofluorescence microscopy and/or analysis of HS chains by anion-exchange and gel-filtration chromatography. Notably, 5-Aza-dc treatment of EMC cells or expression of 3-*OST*3A cDNA reduced their proliferative and invading properties and augmented adhesion of chondrosarcoma cells. These results provide the first evidence for specific epigenetic regulation of 3-*OST* genes resulting in altered HSPG sulfation and point to a defect of HS-3-*O*-sulfation as a factor in cancer progression.—Bui, C., Ouzzine, M., Talhaoui, I., Sharp, S., Prydz, K., Coughtrie, M. W. H., Fournel-Gigleux, S. Epigenetics: methylation-associated repression of heparan sulfate 3-*O*-sulfotransferase gene expression contributes to the invasive phenotype of H-EMC-SS chondrosarcoma cells. *FASEB J.* 24, 436–450 (2010). www.fasebj.org

Key Words: tumor pathogenesis • proteoglycan synthesis • epigenetic regulation • glycosyltransferase

ACCUMULATING EVIDENCE INDICATES that cell function and phenotype are highly influenced by heparan sul-

fate proteoglycans (HSPGs) present in the extracellular matrix and on the surface of virtually every animal cell type. Located at the cell-tissue-organ interface, HSPGs interact with numerous molecules, including growth factors, cytokines, extracellular matrix components, and coagulation factors, thereby regulating a large number of biological processes (1). These interactions are important not only in physiological processes, such as organogenesis, angiogenesis, blood coagulation, growth factor signaling, and lipid metabolism, but are also implicated in pathological circumstances such as viral/bacterial adherence and cancer (2). There is now compelling evidence that HSPGs regulate several aspects of cancer biology, including tumorigenesis, tumor invasion, and metastasis (3). Tumor HSPGs differ in composition from those in corresponding normal tissue, which might affect the efficiency of growth factor stimulation of cancer cells (4). Similarly, the tumor microenvironment might affect the supporting vasculature, which differs in structure and integrity compared with vessels in normal tissue (5). Thus, it appears that the cellular roles of HSPGs are often misregulated in cancer, as a result of alterations in HSPG structure and expression level.

The synthesis of the glycosaminoglycan (GAG) chains that govern these functions is initiated by the formation of a tetrasaccharide linkage region composed of glucuronic acid-galactose-galactose-xylose (GlcA β 1,3Gal β 1,3Gal β 1,4Xyl β 1-), where xylose is attached to specific serine residues of the core protein of PGs. This linkage tetrasaccharide is built up by the stepwise addition of each sugar residue derived from the corresponding UDP-sugar and catalyzed by *O*-xylosyltransferase I (XT-I) (6), β 1,4-galactosyltransferase 7 (β 4GalT7) (7), β 1,3-galactosyltransferase 6 (β 3GalT6) (8), and β 1,3-glucuronosyltransferase I (GlcAT-I) (9). Following the transfer of α -N-acetylglucosamine (GlcNAc), polymer-

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ization of HS chains proceeds *via* the addition of β 1,4-GlcA and α 1,4-GlcNAc units in alternating sequence to the nonreducing end of the growing polymer. The elongation reaction is carried out by HS-copolymerases encoded by the *EXT1/EXT2* tumor suppressor genes (10). Concomitant with chain elongation, modification of the chain occurs, initiated by *N*-deacetylation/*N*-sulfation of GlcNAc units. Further modification reactions include C5 epimerization of some GlcA into L-iduronic acid (IdoUA), 2-*O*-sulfation of IdoUA, and 6-*O*-sulfation of GlcNAc residues. *O*-sulfation also occurs at position 3 of *N*-sulfated and *N*-unsubstituted GlcNAc units, the latter modifications being the hallmarks for the binding domains of antithrombin and glycoprotein gD of herpes simplex virus type I (HSV-I). These modifications proceed *via* the action of HS-biosynthetic enzymes, including *N*-deacetylase/*N*-sulfotransferases (NDSTs), 2-*O*-sulfotransferases (HS2STs), 3-*O*-sulfotransferases (3-OSTs), and 6-*O*-sulfotransferases (HS6STs) (11).

A number of studies highlight the potential of carbohydrate-based therapeutics in several diseases (12). However, reaching this step requires advanced understanding of the mechanisms involved in the synthesis of these macromolecules. Although the basic biosynthetic scheme for HS has been outlined (13), only a limited number of studies have addressed the mechanisms underlying the assembly process of GAG chains (14–16). We recently showed that modulation of XT-I expression regulates GAG-chain synthesis during cartilage destruction and repair in rheumatoid conditions (17). It is well established that genetic defects in HS biosynthetic enzymes have severe biological consequences. In humans, mutations in the *EXT1* and *EXT2* genes coding for HS-polymerases led to a bone disorder known as hereditary multiple exostoses (HME), characterized by the formation of benign cartilage-capped tumors. This condition is associated with a significantly higher risk of developing malignancies such as chondrosarcomas and osteosarcomas (18). Interestingly, mice deficient in *Ext2* lack HS and also develop exostoses (19). Mutations of the β 4*GalT7* gene coding for a glycosyltransferase involved in the initial step of GAG synthesis are associated with the progeroid form of Ehlers-Danlos syndrome, characterized by aged appearance, developmental delay, dwarfism, and connective tissue abnormalities (20).

Epigenetic phenomena are increasingly implicated in the pathogenesis of various disorders (21), and the role of epigenetic alterations in carcinogenesis is clearly established (22). One main epigenetic change associated with tumorigenesis is aberrant DNA methylation. Malignant cells show major disruptions in their DNA methylation profiles, which manifest as hypermethylation of gene promoters, global hypomethylation, and increased rates of mutation at methylated CpG dinucleotides. Hypermethylation of CpG-enriched regions, also called “CpG islands,” generally located in the promoter and downstream proximal sequence region, has been the most extensively studied area of research of DNA methylation. In cancer cells, hypermethylation of these regions results in the loss of gene expression. Some of these susceptible genes include tumor suppressor

genes, cell cycle regulator genes, DNA repair genes, and genes associated with apoptosis. In addition, a correlation between aberrant methylation of promoter regions and transcriptional activity of various genes coding for enzymes involved in matrix remodeling, such as matrix metalloproteinases and heparanase, has also been described (23). Interestingly, inspection of the 5′ region of the genes coding for the glycosyltransferases and most sulfotransferases involved in HS synthesis and modification reveals the presence of typical CpG islands, raising the possibility that expression of these enzymes might be subject to epigenetic silencing *via* DNA methylation in tumor cells. Supporting this assumption, recent findings highlight the importance of *EXT1* epigenetic inactivation leading to abrogation of HS synthesis in the process of tumor onset and progression (24). Furthermore, aberrant methylation of the *3-OST2* gene has been described in several types of tumors (25).

The critical role of HSPGs in human cancer emphasizes the importance of understanding the regulation of these macromolecules at the molecular genetic level. Here, we have investigated the role of epigenetics in the regulation of HS-biosynthetic enzymes in a human chondrosarcoma cell line, since gene disruption in the HS pathway is associated with bone-cartilage tumors. Specifically, we aimed to determine whether the glycosyltransferase and sulfotransferase genes responsible for HS chain synthesis and modification are affected by an epigenetic mechanism involving DNA methylation. Our results provide strong evidence for aberrant methylation and down-regulation of the *3-OST* gene family in the H-EMC-SS (HEMC) cell line, which is associated with altered HS glycosaminoglycan chains, as demonstrated both at biochemical and cellular levels. Furthermore, we show that treatment of the cells with the DNA-methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-Aza-dc) or reintroduction of 3-OSTs into the chondrosarcoma cell line by cDNA expression modified cell behavior in terms of proliferation, adhesion, and migration. Notably, 5-Aza-dc exposure or 3-OST3A cDNA expression resulted in a decrease in the proliferative and migration properties of chondrosarcoma cells, and augmented their adhesion capacity. These findings underline the significance of HS alterations in the phenotype of invasive HEMC chondrosarcoma cells and identify 3-*O*-sulfation as a contributing factor to this process. To our knowledge, this is the first report showing down-regulation of a specific set of HS-*O*-sulfotransferase enzymes in chondrosarcoma cells involving an epigenetic mechanism. Our observation may be of particular relevance in understanding the process of tumor progression and metastasis and in supporting the development of novel therapies targeting DNA methylation.

MATERIALS AND METHODS

Cell lines, culture conditions, and 5-Aza-dc treatment

The human chondrosarcoma cell line HEMC was obtained from the European Collection of Animal Cell Cultures

(ECACC; Salisbury, UK) and was cultured in F12/Dulbecco's modified Eagle's medium (F12/DMEM) (1:1) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco-Invitrogen, Cergy-Pontoise, France), penicillin (100 U/ml)/streptomycin (100 µg/ml), and 1 mM glutamine. The HL-60 acute promyelocytic leukemia-derived cell line was obtained from ECACC and cultured in RPMI-1640 supplemented with 10% (v/v) FBS, penicillin (100 U/ml)/streptomycin (100 µg/ml), and 1 mM glutamine. HeLa cells were cultured in DMEM supplemented with 10% (v/v) FBS, penicillin (100 U/ml)/streptomycin (100 µg/ml), and 1 mM glutamine. Cells were maintained in a 37°C incubator in a 5% CO₂ humidified atmosphere.

Chondrosarcoma cells were seeded in 25-cm² flasks at 5 × 10⁵ cells, allowed to attach overnight, and treated with 5-Aza-dc (Sigma-Aldrich, Gillingham, UK) dissolved in phosphate-buffered saline (PBS) at 2 and 4 µM final concentration for 7 d. Control cultures contained the vehicle only. During the course of treatment, medium was changed every day; cells were passaged twice and placed in fresh medium containing the same concentration of 5-Aza-dc or vehicle. At the end of the treatment period, cells were washed once with PBS and harvested for genomic DNA or mRNA analysis. Alternatively, cells were subjected to the appropriate medium for metabolic radiolabeling prior to GAG analysis or for other cellular assays (see below).

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

The DNA sequences around the transcription start site (−1000 bp) and the first exon of each glycosyltransferase and sulfotransferase gene were obtained by a GenBank database search (<http://genome-www.stanford.edu/genecards>), and the presence of CpG islands was predicted using the MethPrimer online search tool (<http://www.urogene.org/methprimer/>) (26). The criteria used for the definition of CpG islands included regions of DNA >500 bp with G+C content ≥55%. The following genes contain a typical CpG island in the proximal promoter/first exon sequence: *XT-I*, *β4GalT7*, *β3GalT6*, *GlcAT-I*, *EXT1*, *NDST2*, *NDST3*, *HS2ST1*, *3-OST1*, *3-OST2*, *3-OST3A*, *3-OST3B*, *3-OST6*, *HS6ST1*, *HS6ST2*, and *HS6ST3*. The *NDST1* gene contains a cluster of CpG nucleotides encompassing 366 bp (+302 to +668) located in the 5' untranslated region of the first exon, and the *NDST4* sequence contains no region meeting the defined criteria of a CpG island.

The methylation status of the glycosyltransferase and sulfotransferase genes was determined by the genomic DNA bisulfite conversion method, which induces chemical modification of cytosine residues to uracil in unmethylated DNA, whereas those that are methylated are resistant to the treatment and remain as cytosines (27). Genomic DNA was extracted from untreated or 5-Aza-dc-treated HEMC cells using GenomicPrep DNA isolation kit (GE Healthcare, Amersham, UK). The amount and purity of DNA were determined using a Nanodrop spectrophotometer (Fisher Scientific, Loughborough, UK), and 0.2 µg DNA was used for bisulfite conversion using the EpiTect Bisulfite kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Genomic DNA from human whole blood (Clontech, Mountain View, CA, USA) was used as a source of control DNA. Following bisulfite conversion, two sequential PCR reactions were performed to amplify the modified fragments of interest. The first round of amplification was carried out with 0.1 µg of DNA template using primer sets ("Out sens" and "Out rev"). Nested primers ("In sens" and "In rev") were used in the subsequent PCR reaction. Primer sets were designed, including the use of Primer3 Input 0.4.0 software

(primer3-web/htdocs/input-040.htm) for the prediction of primer hairpins, dimers, and false primers and are listed in Supplemental Table 1. PCR conditions for the first PCR reaction were 94°C for 8 min followed by 20 cycles at 94°C for 30 s, 52°C for 30 s, 72°C for 30 s, ending with a final extension of 10 min at 72°C. PCR conditions for the second step were 94°C for 8 min followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and finally 10 min at 72°C. The resultant PCR products were purified with the QIAquick PCR Purification kit (Qiagen). Initial screening experiments were performed by direct sequencing PCR products from bisulfite converted DNA samples. In a second step, the PCR amplicons were subcloned into pGEM-T-easy TA (Promega, Madison, WI, USA), and at least two recombinant clones were sequenced for each gene of interest. Nonmethylated cytosines were successfully converted by the bisulfite reaction in the PCR amplicons or recombinant clones.

Reverse transcription-PCR analysis of mRNA expression level

Total RNA was isolated from untreated and 5-Aza-dc-treated HEMC cells using the SV RNA Isolation System (Promega). RNA (0.5 µg) was reversed-transcribed into cDNA using oligodT primer and PrimeScript reverse transcriptase (Clontech). The resultant cDNA was used for PCR amplification with Bio-X-ACT DNA polymerase (Bioline, London, UK) following optimization of MgCl₂ concentrations and annealing temperatures. To determine the linear amplification segment for each gene of interest, different amplification steps were initially carried out between 20 and 40 cycles, and the number of cycles for quantification was established to remain in the exponential phase of amplification. The primers and specific PCR conditions used are listed in Supplemental Table 2. The PCR products were electrophoresed on 2.0% (w/v) agarose gels and stained with ethidium bromide, and the expression levels were evaluated following image capture using GeneFlash (Syngene, Cambridge UK) and Scion Image 1.63 (Scion Corp., Frederick, MD, USA) software. The mRNA expression levels of *EXT1*, *NDST1*, *NDST2*, *HS2ST1*, *HS6ST1*, *HS6ST2*, *3-OST1*, *3-OST2*, *3-OST3A*, *3-OST3B*, and *3-OST6* were quantified relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an internal control. For *NDST3*, *NDST4*, and *HS6ST3*, no transcript could be detected in control or 5-Aza-dc-treated HEMC cells when amplification was performed up to 40 cycles. However, these transcripts were successfully amplified from brain or placenta total mRNA (Clontech) used as positive control (not shown).

3-OST1 and 3-OST3A cDNA expression in HEMC cells

3-OST1 and 3-OST3A cDNA were amplified by PCR from IMAGE clones (Geneservice, Cambridge, UK) as templates, using a 5' primer containing a *Hind*III site and a Kozak sequence, and a 3' primer containing the sequence encoding YPYDVPDYA-hemagglutinin (HA) peptide tag and an *Xho*I site. The amplified fragments were inserted into *Hind*III-*Xho*I sites of pCDNA3.1 expression vector (Invitrogen) to generate pCDNA-3OST1 and pCDNA-3OST3A plasmids. Each vector was transiently transfected into HEMC cells, as described previously (28). Briefly, HEMC cells plated into 6-well plates, grown at 80% confluency, were transfected with 2.5 µg of plasmid using ExGen 500 reagent (Euromedex, Souffelweyersheim, France). The empty vector was used for mock-transfection, as a control. Forty-eight hours after transfection, cells were trypsinized and plated for cellular assays, as described below. Simultaneously, expression of recombinant proteins 3-OST1 and 3-OST3A was analyzed by Western

blotting using anti-HA antibodies (Abcam, Cambridge, UK), as described previously (29).

Indirect immunofluorescence staining of chondrosarcoma and HeLa cells

For indirect immunofluorescence staining of cell/matrix HS chains, untreated, 5-Aza-dc-treated cells, or cells transfected with pCDNA-3OST1 and pCDNA-3OST3A were washed with PBS and fixed with 3% paraformaldehyde/PBS for 20 min at room temperature. For HS staining, coverslips were rinsed 3 times in PBS and blocked in 10% (v/v) goat serum/PBS. Cells were then incubated with mouse anti-human HS antibody (10E4 epitope) at 1:50 dilution (Seikagaku Corp., Tokyo, Japan) for 1 h, washed 3 times with PBS, incubated with AlexaFluor 594-conjugated goat anti-mouse antibodies (Molecular Probes/Invitrogen) for 30 min and finally washed with PBS. For 3-OST1 and 3-OST3A-tagged protein staining, cells were permeabilized with a PBS solution containing 0.1% (w/v) Triton-X100 for 4 min and incubated in 10% (v/v) goat serum/PBS. Recombinant cells were incubated with FITC-conjugated mouse anti-HA antibody (Sigma-Aldrich) for 30 min, and washed with PBS. Coverslips were treated with a 5 µg/ml Hoechst/PBS solution for nucleus visualization and were finally mounted with Hydromount or Moviol (National Diagnostics, Hull, UK) containing 2.5% 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma-Aldrich).

Metabolic labeling and ion-exchange and gel-filtration chromatography analysis of cell HSPGs

Subconfluent untreated and 5-Aza-dc-treated HEMC cells were transferred to appropriate labeling media containing 2% FBS with [³⁵S]SO₄ (PerkinElmer Life Sciences, Courtabœuf, France) in Fisher's medium without sulfate (GIBCO-Invitrogen), or [³H]glucosamine (PerkinElmer, Life Sciences, Courtabœuf, France) in RPMI 1640 (Gibco-Invitrogen) without glutamine, or [³⁵S] Easytag Express Protein Labeling Mix (73% L-[³⁵S]methionine and 22% L-[³⁵S]cysteine) in DMEM without L-methionine and L-cysteine for 22 h. Cells were washed twice with PBS, harvested by scraping, and pelleted by centrifugation. Cell pellets were resuspended in 50 mM Tris-HCl buffer (pH 7.5) and 1% (w/v) Triton X-100, and total DNA amounts were quantified using the Quant-iT dsDNA HS Assay Kit (Molecular Probes/Invitrogen). Nonincorporated radioactive label was removed from cell extracts by Sephadex G-50 fine chromatography, as described previously (30), and radio-labeled macromolecules were analyzed by anion-exchange chromatography or gel filtration based on previously described methods (31).

For ion-exchange chromatography, [³⁵S]SO₄ or L-[³⁵S]methionine/L-[³⁵S]cysteine radiolabeled samples corresponding to the same amount of treated or untreated cells (based on DNA quantification) were incubated with chondroitinase ABC from *Proteus vulgaris* (Sigma-Aldrich) (32) and then subjected to DEAE-anion-exchange chromatography (Bio-LogicLP system; Bio-Rad, Hercules, CA, USA), as described previously (33). Briefly, samples were diluted in 1 ml of buffer A (8 M urea, 0.02 M Bis-tris, and 0.05 M NaCl, pH 6.0) and applied onto a 2.5-ml Macro-Prep DEAE support (Bio-Rad). After 20 ml of buffer A, a linear gradient mixing buffer A with buffer B (8 M urea, 0.02 M Bis-tris, and 0.8 M NaCl, pH 6.0) was applied at a rate of 0.5 ml/min. Fractions of 1 ml were collected and analyzed for radioactivity by scintillation counting.

For gel-filtration chromatography, [³⁵S]SO₄ or [³H]glucosamine-radiolabeled macromolecules were treated with proteinase K, as described previously (34). Following heat

inactivation, samples were submitted to chondroitinase ABC digestion, as described above. Samples corresponding to 5-Aza-dc-treated or untreated cells were applied to a column (1×40 cm) of Sepharose CL-6B (Sigma-Aldrich) along with blue dextran and K₂CrO₄ as internal standards, and elution was performed with 0.15 M NaCl in 0.05 M Tris, HCl buffer (pH 8.0), and 0.1% Triton X-100 at a rate of 6 ml/h, as described previously (35). Fractions of 1 ml were collected and analyzed for radioactivity by scintillation counting.

Cell proliferation, adhesion, and migration assays

For proliferation assays, 5-Aza-dc-treated or transfected cells were seeded onto 24-well plates (Costar; Dutscher, Brumath, France) at 25 × 10³ cells/well. Cells were harvested each day during a 5-d period by scraping in lysis buffer [PBS containing 1% (w/v) sodium dodecyl sulfate]. Cell lysates were sonicated in a total volume of 1 ml PBS buffer, and cell proliferation was evaluated by measuring the DNA content of each sample by spectrofluorimetry on a fluorimeter (Qubit; Molecular Probes-Invitrogen, Cergy Pontoise, France) using the Quant-iT dsDNA HS Assay kit.

For adhesion assays, 96-well plates were coated by adsorption with 100 or 200 µg fibronectin (from human foreskin fibroblasts, Sigma-Aldrich) per well for 1 h at 37°C, or left untreated for control experiments. After washing with medium containing 0.1% BSA, wells were saturated by incubation with F12/DMEM containing 0.5% BSA for 1 h at 37°C. Plates were then washed once with medium containing 0.1% BSA and placed on ice before cells were harvested by trypsinization, counted and resuspended in complete F12/DMEM at a density of 5 × 10⁵ cells/ml. Each well was inoculated with 25 × 10³ cells and incubated for 30 min at 37°C, and nonadherent cells were removed by washing. The extent of adhesion was determined after fixing cells with 4% paraformaldehyde for 15 min, staining adherent cells with 1‰ crystal violet for 10 min. Plates were rinsed 3 times in water, and 10% (v/v) acetic acid solution was added to each well to allow staining quantification by absorbance measurement at 550 nm on a spectrophotometer (SAFAS flx-Xenius, Monaco, France).

HEMC cell migration was analyzed using a Boyden chamber chemotaxis assay (36). Following 5-Aza-dc treatment or 3-OST cDNA expression, cells were incubated with 1 µg/ml mitomycin C (Sigma-Aldrich) in complete F12/DMEM for 2 h and seeded at 5 × 10⁴ cells/well on top of 24-well plate inserts (Corning Costar; Sigma-Aldrich) containing a polycarbonate filter (6.5-mm diameter, 8-µm pores). Cells were incubated for 8, 16, or 24 h for 5-Aza-dc treated cells, and for 18 h in the case of transfected cells at 37°C in the upper chamber in F12/DMEM containing 0.1% BSA, whereas the lower chamber contained F12/DMEM supplemented with 5% FBS. At the end of the incubation period, nonmotile cells were removed from the upper side of the filters with cotton-tipped swabs. Migration of cells was quantified by staining with methyl blue, and measuring the optical density at 663 nm of cells that had migrated to the underside of the filters.

Statistical analysis

All data are presented as means ± SE of 3 to 6 observations. For comparison among multiple groups, either 1-way or 2-way ANOVA followed by Bonferroni *post hoc* correction test was used. Statistical analyses were performed using GraphPad Prism 4.0 software (GraphPad, San Diego, CA, USA).

RESULTS

DNA methylation profiling of *N*- and *O*-sulfotransferase genes in HEMC chondrosarcoma cells

To test whether the HS synthesis pathway is regulated *via* epigenetic mechanisms involving DNA methylation in chondrosarcoma cells, we systematically searched the genomic sequences encoding the biosynthetic enzymes for the presence of clustered CpG dinucleotides, representing putative hypermethylation loci.

A region encompassing 1000 bp upstream of the transcription start site and the first exon of *XT-I*, $\beta 4\text{GalT}7$, $\beta 3\text{GalT}6$, *GlcAT-I*, and *EXT1* genes coding for the glycosyltransferases involved in HS chains synthesis was analyzed. We found that each of these genes contains a typical 5' CpG island, indicating that they have the potential for hypermethylation-associated silencing. We determined the methylation status of the CpG islands associated with the *XT-I*, $\beta 4\text{GalT}7$, $\beta 3\text{GalT}6$, and *GlcAT-I* genes by the bisulfite-sequencing method, using primers presented in Supplemental Table 1. These genes encode the glycosyltransferases responsible for the formation of the tetrasaccharide linkage region, which is common to the biosynthetic pathways of both HS and chondroitin sulfate (CS) chains, and is a prerequisite step for GAG-chain elongation. We also examined the sequence of the promoter CpG island of *EXT1*, which encodes a glycosyltransferase specific for HS-chain polymerization, which has been reported to be hypermethylated in leukemia cell lines (24). Our results show that all glycosyltransferase DNA sequences analyzed within the proximal promoter region and first exon were unmethylated or exhibited a low frequency of methylated cytosine residues within their CpG islands in HEMC cells (Fig. 1). The methylation state of these genes in chondrosarcoma cells was similar to that of normal whole blood

Figure 1. Analysis of the methylation status of glycosyltransferase genes in HEMC and HL-60 cells. Genomic DNA was extracted from chondrosarcoma HEMC (HE) or HL-60 (HL) cells, and the methylation profile of the CpG island of the glycosyltransferase genes was analyzed by bisulfite sequencing. Number of methylated CpG sites was determined by counting unconverted cytosine residues. Bars correspond to number of CpG sites in region of interest (Supplemental Table 1). Number of methylated sites is indicated by hatched areas and represents the mean value from sequencing data of 2 independent clones.

DNA (data not shown). For comparison purposes, we also performed bisulfite genomic sequencing of the CpG island regions of the *XT-I*, $\beta 4\text{GalT}7$, $\beta 3\text{GalT}6$, *GlcAT-I*, and *EXT1* genes in HL-60 leukemia cells. The results showed a lack or low number of methylated loci in *XT-I*, $\beta 4\text{GalT}7$, $\beta 3\text{GalT}6$, and *GlcAT-I* glycosyltransferase genes, similar to our observations in HEMC cells. By contrast, in the HL-60 leukemia cell line, the *EXT1*-promoter region analyzed (corresponding to positions -536 to +203 relative to the transcription start site) contained 98% methylated cytosines among 106 CpG sites (Fig. 1). This is indicative of the typical hypermethylation profile of the *EXT1*-promoter region in certain types of leukemia cell lines, in agreement with a previous report (24). Together, these data provide no evidence for methylation differences between HEMC and normal cells in the glycosyltransferase genes involved in the initiation and polymerization of HS chains, whereas the *EXT1* CpG island exhibits a hypermethylation profile in HL-60 cells.

Concomitant with the synthesis of HS chains, substitution of the saccharide residues with sulfate groups occurs *via* the action of several families of *N*- and *O*-sulfotransferases governing key biological functions (11). The first modification enzymes, NDSTs, remove *N*-acetyl groups from selected GlcNAc residues and replace them with sulfate groups. Sulfation of IdoUA and GlcA residues is carried out by 2-*O*-sulfotransferases, followed by 6-*O*-sulfation and 3-*O*-sulfation of glucosamine units. To determine whether sulfation of HS chains might be epigenetically regulated through DNA methylation, we examined the promoter region/first exon of NDST and HS-*O*-sulfotransferase genes in HEMC cells. Although the promoter of the *NDST1* gene contained no canonical CpG island, a cluster of CpG dinucleotides (22 sites) located in the 5' untranslated region of the first exon of this gene (+302 to +668) was identified. Bisulfite conversion analysis showed that this region was fully methylated both in HEMC cells and in blood DNA (Fig. 2A, B). Analysis of the methylation pattern of the CpG island present in the *NDST2* and *NDST3* genes indicated a low methylation frequency in the case of *NDST2* (0–4 of 39 sites), as well as in the *NDST3* sequence (6–7 of 46 sites) (Fig. 2A). Analysis of the *NDST4* gene revealed the presence of a small number of CpG sites (5 in a 453-bp region located in the proximal promoter sequence; Supplemental Table 1).

Interestingly, analysis of HS-*O*-sulfotransferase genes, including *HS2ST*, *HS6ST*, and 3-*OST* families, revealed that a majority of these genes contain a canonical CpG island. In HEMC cells, as in control genomic DNA, the predicted CpG island present in the *HS2ST1* and *HS6ST1* genes exhibited a very low methylation frequency (1–1 of 68 CpG sites and 0–2 of 115 methylated CpG sites, respectively) (Fig. 2A). On the other hand, the *HS6ST2* gene showed a typical hypermethylation profile in HEMC cells (44–45–46 methylated cytosines of 48 CpG sites, 45 ± 1), which was also observed in blood samples (Fig. 2A, C). Analysis of the *HS6ST3* gene showed a highly dense CpG dinucleotide region (-721 to +8) comprising 123 CpG sites. About 30 and

Figure 2. Analysis of the methylation status of *N*- and *O*-sulfotransferase genes in HEMC cells. **A)** DNA methylation profile of sulfotransferase genes in HEMC (HE) and in control blood DNA (BI). Bars correspond to number of CpG sites in region of interest (Supplemental Table 1). Number of methylated sites is indicated by hatched areas and represents the mean value from sequencing data of ≥ 2 independent clones. **B, C)** Schematic representation of the methylation status of *NDST1* and *HS6ST2* genes, respectively, in HEMC cells. Core promoter, exon 1 (black rectangle) and CpG sites (short vertical lines) are shown in the top part of each panel. Predicted transcription start site is marked by a curved arrow; primers are indicated by arrows. Methylated cytosines (solid circles) and unmethylated cytosines (open circles) in the region of interest are represented in the bottom part of each panel. Results are from bisulfite sequencing of 3 different clones.

5.7% of these sites were found to be methylated in HEMC cells and blood DNA, respectively (Fig. 2A).

We finally investigated the methylation state of the largest HS-*O*-sulfotransferase gene family, *3-OST*, which comprises 7 members known to biosynthesize key structures involved in several important physiological processes and possibly in the development and/or progression of tumors (25). The CpG island encompassing 682 bp located in the proximal promoter and 5' region of the first exon (−546 to +136) of the *3-OST1* gene is illustrated in Fig. 3A. Analysis of the methylation status of this fragment showed $\sim 88\%$ methylated sites (51–51–54 methylated cytosines among 59 CpG dinucleotides, 52.0 ± 1.7), revealing a typical hypermethylation profile in HEMC cells (Fig. 3A, C). No methylated site was found when the same region was studied in blood DNA (Fig. 3C). Surprisingly, bisulfite sequencing of the *3-OST2* gene, previously reported to be hypermethylated in a range of human cancers (25), showed only a moderate methylation frequency in HEMC cells, with $\sim 10\%$ methylated sites in a 612 bp-fragment (−520 to +92, 5–8 methylated cytosines among 64 CpG sites) (Fig. 3C). The 5' domain of the *3-OST3A* gene contains a high density of CpG dinucleotides typical of a CpG island, as illustrated in Fig. 3B. Examination of the methylation state of two regions corresponding to positions −338 to +100 and +282 to +726 relative to the transcription start site revealed 35.5% (9–14–26 among 46 sites) and 97% (50–54–54 among 54 CpG sites, 52.7 ± 2.3) methylated cytosines, respectively (Fig. 3B, C). By contrast, bisulfite sequencing of the *3-OST3B* gene showed a lack of methylated loci from position −354 to +218, despite the presence of a typical CpG

cluster (47 CpG sites) (Fig. 3C). Finally, analysis of the methylation profile of the *3-OST6* CpG island revealed that 38% of CpG sites in a 739 bp-fragment (−647 to +92) were methylated (28–37 methylated cytosines among 86 CpG sites, Fig. 3C). Analysis of the methylation profile of the *3-OST* genes performed on blood DNA sample showed $< 2\%$ methylated sites (Fig. 3C). Altogether, the *3-OST* gene family revealed a typical aberrant methylation pattern specific to HEMC cells, with the most striking hypermethylation profile observed in the case of *3-OST1* and *3-OST3A*.

Hypermethylated sulfotransferase gene expression is elevated by 5-Aza-dc treatment in HEMC cells

To study the potential role of CpG methylation on the transcriptional activity of glycosyltransferase and sulfotransferase genes, we analyzed the effect of an inhibitor of DNA-methyltransferase, 5-Aza-dc (37), on mRNA expression. HEMC cells were treated with two concentrations of 5-Aza-dc and mRNA expression levels were compared in treated and untreated cells by semiquantitative RT-PCR. Expression analysis of *EXT1* in the chondrosarcoma cell line showed that HEMC cells expressed a high level of *EXT1* transcript and that mRNA expression was unchanged on 5-Aza-dc treatment (Fig. 4A, C). This result is consistent with the unmethylated status of the CpG island of the *EXT1* proximal promoter/first exon in HEMC cells.

Because aberrant methylation was essentially observed in the case of sulfotransferase genes, further investigation of the consequences of 5-Aza-dc treatment

Figure 3. Analysis of the methylation status of *3-OST* genes in HEMC cells. *A, B*) Schematic representation of the *3-OST1*-promoter (*A*) and *3-OST3A*-promoter region (*B*). CpG island is filled in dotted gray. Short vertical lines below diagram indicate CpG sites; the predicted transcription start site is marked by a curved arrow. Methylation status of the region of interest (Supplemental Table 1) is represented as in Fig. 2. Results are from bisulfite sequencing of 3 different clones. Dotted lines correspond to sequencing data of 3 dif-

ferent clones. *C*) Methylation profile of the different genes of the *3-OST* family. Bars correspond to number of CpG sites in region (Reg) of interest (Supplemental Table 1). Number of methylated sites is indicated by hatched areas and represents the mean value from sequencing data of ≥ 2 independent clones.

on sulfotransferase mRNA expression was carried out. RT-PCR analysis showed that *NDST1* mRNA level was significantly increased (up to 1.7-fold) on exposure of HEMC cells to the highest dose of 5-Aza-dc (Fig. 4A, C), whereas *NDST2* mRNA expression was unchanged, in agreement with the unmethylated state of the promoter CpG island of this gene in chondrosarcoma cells. By contrast, no *NDST3* or *NDST4* transcripts were detected in control or 5-Aza-dc-treated cells, although these transcripts could be amplified from total brain or placenta mRNA, respectively (data not shown). Interestingly, the *NDST3* CpG island was unmethylated in HEMC cells, and the *NDST4* gene exhibited no typical CpG island. Furthermore, the results show that 5-Aza-dc treatment did not modify *HS2ST1* and *HS6ST1* mRNA expression levels in HEMC cells (Fig. 4A, C). This could be expected, since the CpG island of these genes exhibited no apparent hypermethylation pattern in chondrosarcoma cells. By contrast, a significant increase (up to 2-fold) in *HS6ST2* expression level was observed in HEMC cells when treated with the highest dose of demethylating agent. Despite the presence of methylated sites in the *HS6ST3* gene, the corresponding transcript could not be detected in treated or untreated HEMC cells (data not shown). In summary, our results show that *NDST1* and *HS6ST2* mRNA expression only was upregulated in HEMC cells on 5-Aza-dc treatment, suggesting that methylation events are involved in the regulation of these genes. Bisulfite sequencing data indicate that 5-Aza-dc treatment of HEMC cells reduced the number of methylated sites by

30% in *NDST1* gene and by 35% in *HS6ST2* gene, which may contribute, at least in part, to transcription activation (Supplemental Fig. 1). However, since 5-Aza-dc would certainly hypomethylate many other genes coding for transcription factors involved in the regulation of *NDST1* and *HS6ST2*, it is possible that the increase in gene expression may also be due to other mechanisms of promoter activation. Further work is required to establish that point.

Because several members of the *3-OST* gene family exhibited aberrant DNA methylation patterns, it was interesting to analyze the effect of the demethylating agent on expression of these genes. RT-PCR analysis of *3-OST* mRNA transcripts revealed different effects of the demethylating agent among the *3-OST* members in HEMC cells. 5-Aza-dc treatment failed to significantly increase the expression of *3-OST3B* mRNA transcript, concordant with the low methylation state of the CpG island of this gene. By contrast, *3-OST1* and *3-OST2* mRNA expression levels were significantly increased (1.5-fold) at the higher dose of the demethylating agent compared to untreated cells (Fig. 4B, D). The transcriptional activity of the *3-OST3A* gene was also stimulated by treatment with both doses of 5-Aza-dc, which produced ~ 2.7 -fold increase in mRNA expression levels (Fig. 4B, D). Analysis of the methylation status of this gene following 5-Aza-dc treatment of HEMC cells showed $\sim 54\%$ promoter demethylation, suggesting that this effect could account, at least in part, for the increase in *3-OST3A* mRNA expression. Altogether, the results indicate that treatment with

Figure 4. Effect of 5-Aza-dc on mRNA expression of glycosyltransferase and sulfotransferases involved in HSPG synthesis in HEMC cells. Cells were treated with 5-Aza-dc for 7 d, and mRNA expression was analyzed by semiquantitative RT-PCR using specific sets of primers (Supplemental Table 2). GAPDH mRNA was used to normalize for input of total RNA. *A, B*) Typical results of mRNA analysis of EXT1, NDST1, NDST2, HS2ST1, HS6ST1, and HS6ST2 transcripts (*A*) and 3-OST1, 3-OST2, 3-OST3A, 3-OST3B, 3-OST6, and control GAPDH transcripts (*B*) after treatment with 5-Aza-dc (2 and 4 μ M) compared to untreated cells (control). *C, D*) Quantification of RT-PCR products by scanning densitometry. mRNA expression levels in treated cells are expressed relative to untreated cells (control set to 1) and are means $\pm$ SE of 3 to 6 observations. * $P < 0.05$; 1-way ANOVA with Bonferroni's *post hoc* test.

5-Aza-dc restores the expression of 3-OST3A and, to a lesser extent, of 3-OST1 and 3-OST2, supporting the view that DNA methylation is a mechanism affecting the transcription of these genes in HEMC cells.

The HS sulfation pattern is modified by up-regulation of sulfotransferase expression

Unlike mutational inactivation, epigenetic alterations can be reversed by pharmacological means, such as the use of DNA demethylating agents, for example 5-Aza-dc. The next series of experiments was designed to determine whether this compound was able to rescue gene functionality. To provide evidence for possible changes in HS expression on 5-Aza-dc treatment in HEMC cells, we examined the effect of the demethylating agent on GAG-chain structure using biochemical approaches. To assess possible changes in the overall charge density of GAG chains on 5-Aza-dc treatment, [35 S]sulfate-labeled and [35 S]methionine/cysteine-labeled macromolecules isolated from treated and untreated HEMC cells were subjected to anion-exchange chromatography. **Figure 5A** shows the elution pattern of [35 S]sulfate-radiolabeled macromolecules following treatment with chondroitinase ABC. This endoglycosidase degrades galactosaminoglycan chains, leaving intact HSPGs bound to the column. As shown in Fig. 5A, HSPGs from untreated cells emerged as a single peak when the salt gradient was applied (at ~ 0.45 M NaCl). Comparison of the gel chromatography profiles of the chondroitinase digests of [35 S]sulfate-labeled macromolecules extracted from untreated and 5-Aza-dc-

treated HEMC cells showed a shift of the resistant peak corresponding to HSPGs (fractions 45–55), suggesting a charge increase of HSPGs on 5-Aza-dc treatment. By contrast, [35 S]methionine/cysteine-labeled macromolecules from untreated and treated cells exhibited a similar profile on anion-exchange chromatography (Fig. 5B), indicating no major modification in protein synthesis on 5-Aza-dc exposure.

To further investigate changes in GAG synthesis induced by 5-Aza-dc, treated and untreated cells were metabolically labeled with [35 S]sulfate or [3 H]glucosamine, and PGs isolated from cell/matrix extracts were treated with proteinase K to release GAG chains from their core proteins. GAG chains were then subjected to digestion with chondroitinase ABC and analyzed by gel exclusion chromatography. Analysis of the length of HS chains released by proteinase K treatment did not show a marked difference, as indicated by comparison of the chromatography profiles for HS chains from treated and untreated cells (Fig. 5C). However, these HS chains contained more [35 S]sulfate relative to incorporated [3 H]glucosamine, corroborating the idea that the sulfate density of HS chains is increased on 5-Aza-dc treatment, whereas the chain length is not affected (Fig. 5C). Together, these results indicate that treatment with the demethylating agent 5-Aza-dc affects the extent of HS sulfation, rather than HS-chain length in HEMC cells.

To further examine the effect of 5-Aza-dc on HS-GAG chains, we carried out indirect immunofluorescence analysis of the chondrosarcoma cells, using 10E4 anti-HS monoclonal antibody, which is commonly used

Figure 5. Effect of 5-Aza-dc treatment on cellular HSPG negative charge density and chain length in HEMC cells. *A, B*) Anion-exchange chromatography analysis of HSPGs. Untreated and 5-Aza-dc-treated HEMC cells (4 μ M) were radiolabeled with [35 S]SO $_4$ or [35 S]methionine/cysteine for 22 h. After removal of free [35 S] by Sephadex G-50 gel filtration, cellular samples were subjected to specific digestion by chondroitinase ABC and resolved by anion-exchange chromatography. Elution was carried out with a NaCl gradient (0.05–0.8 M). Fractions were collected, and radioactivity was evaluated by scintillation counting. *A*) Elution profile of [35 S]SO $_4$ -radiolabeled macromolecules. *B*) Elution profile of [35 S]methionine/cysteine-radiolabeled macromolecules. *C*) Gel-filtration analysis of HS chains. Untreated and 5-Aza-dc-treated HEMC cells (4 μ M) were radiolabeled with [35 S]SO $_4$ or [3 H]glucosamine for 22 h. After removal of free radioactive label by Sephadex G-50, samples were treated with proteinase K for 2 h prior to digestion by chondroitinase ABC. Samples were subjected to gel-filtration chromatography on Sepharose Cl-6B, and radioactivity associated with the collected fractions was evaluated by scintillation counting. Results are expressed as [35 S]SO $_4$ /[3 H]glucosamine ratio. Moving mobile phase column volume (V_0) and total liquid phase column volume (V_i), corresponding to the volume of elution of blue dextran and K $_2$ CrO $_4$, respectively, are indicated by arrows. Each chromatogram shows a profile that is a representative of 3 separate experiments.

to detect HS chains of PGs (38–40). Prominent staining of the cell membrane was observed in HEMC cells treated with the demethylating agent, whereas only a very low signal could be observed in untreated cells (Fig. 6A, B). Since 10E4 antibodies were initially described to recognize *N*-sulfated glucosamine residues normally present on HS chains (38), the absence of staining in HEMC cells was unexpected (Fig. 6A).

Indeed, HeLa cells used as positive controls exhibited a strong plasma membrane signal (Fig. 6C). However, several studies indicate that the 10E4 epitope associated with HSPGs may be inaccessible in some cell lines (39) and highlight that further characterization of the epitope is needed (38). Recent evidence indicates that removal of *O*-sulfate groups from HS chains by expression of recombinant sulfatase (Sulf) isoforms in hepatocarcinoma (SNU 449) (41) or pancreatic (Panc-I) cell lines (42), abolished 10E4 staining, suggesting that *O*-sulfation might contribute to the antigenicity of HS chains toward 10E4 antibodies. In agreement, we show that transfection of HEMC cells with 3-OST1 cDNA, followed by immunostaining using the 10E4 antibody, gave a strong punctate pattern (Fig. 6E), similar to that observed on 5-Aza-dc treatment, whereas mock transfected cells exhibited low or no staining (Fig. 6D). When recombinant cells were probed with anti-HA antibodies, efficient expression of 3-OST1 was revealed (Fig. 6H), whereas mock-transfected cells showed no staining (Fig. 6G). Similarly, 3-OST3A cDNA expression in HEMC cells was shown using anti-HA antibodies (Fig. 6I) and was associated with a pronounced staining of cell surface HS probed with anti-10E4 antibodies (Fig. 6F). Altogether, these observations indicate that 3-OST1 or 3-OST3A expression produced HS epitopes containing 3-*O*-sulfated glucosamine residues that are recognized by the 10E4 antibody. Furthermore, our data suggest that 5-Aza-dc treatment induces modifications of HS chains that are

similar or identical to those resulting from induction of 3-OST expression.

Effect of restoration of 3-OST expression on proliferation, adhesion, and migration in HEMC cells

The next series of experiments were designed to assess the functional consequences of exposing chondrosarcoma cells to the demethylating agent 5-Aza-dc and of expression of 3-OST isoforms by cDNA transfection on proliferation, adhesion, and migration properties. As shown in Fig. 7A, 5-Aza-dc produced a significant inhibitory effect on HEMC cell proliferation, as rapidly as 2 d after drug exposure, that reached 43% of inhibition after 5 d. No cytotoxicity of 5-Aza-dc at the concentration used (4 μ M) was observed as verified using trypan blue (not shown). Since the antiproliferative effect of the demethylating agent may result from up-regulation of several genes, it was important to determine the specific effect of 3-*O*-sulfation on the proliferation rate of cells transfected with 3-OST cDNAs. Interestingly, expression of the 3-OST3A isoform significantly reduced HEMC cell proliferation up to 20%, whereas 3-OST1 did not exert an antiproliferative effect compared to mock-transfected cells (Fig. 7B). Protein expression of each isoform was verified by immunoblot analysis using anti-HA antibodies (not shown).

Next, the effect of 5-Aza-dc on HEMC cell adhesion onto fibronectin-coated plates or plates coated with BSA only was assayed. No significant difference between 5-Aza-dc-treated and control cells was observed when cells were seeded on plates coated with BSA only or low-dose fibronectin. However, a moderate but significant increase in adhesion was seen when 5-Aza-dc-treated cells were seeded on plates coated with 200 μ g of fibronectin (Fig. 7C). Interestingly, this effect was enhanced when HEMC cells were transfected with the

Figure 6. 5-Aza-dc treatment or expression of recombinant human 3-OST1 or 3-OST3A increases cell surface/matrix HSPG expression in HEMC cells. Cells were treated with 5-Aza-dc (4 μ M) for 7 d or transfected with 3-OST1 or 3-OST3A, and indirect immunofluorescence staining was performed as described in Materials and Methods with 10E4 anti-HSPGs (A–F) or following permeabilization with anti-HA antibodies (G–I). A) Untreated HEMC cells. B) 5-Aza-dc-treated HEMC cells. C) HeLa cells. D, G) Mock-transfected HEMC cells. E, H) pCDNA-3OST1 HEMC transfected cells. F, I) pCDNA-3OST3A-transfected HEMC cells.

3-OST3A expression vector. Indeed, 3-OST3A-expressing cells showed a dose-dependent increase of adhesion when seeded onto fibronectin-coated plates, reaching 65% over control mock-transfected cells (Fig. 7D). However, no significant effect was observed when cells were transfected with the 3-OST1 expression vector.

Finally, we analyzed the effect of 5-Aza-dc on cell motility using an *in vitro* migration assay. Untreated HEMC cells were found to be highly motile, reaching a 45–50% cell migration rate after 24 h (Fig. 7E). However, cells treated with 5-Aza-dc exhibited much less migration potency through the polycarbonate membrane using 5% serum as a chemoattractant over a period of 8, 16, or 24 h (Fig. 7E). The consequences of 3-OST1 and 3-OST3A cDNA expression in HEMC cells

on the migration rate were next examined. The expression of 3-OST1 and 3-OST3A reduced the migration rate by 22 and 15%, respectively, compared to mock-transfected cells (Fig. 7F). These results indicate that overexpression of either 3-OST1 or 3-OST3A cDNA resulted in a less motile phenotype with a larger effect in the case of 3-OST1.

Altogether, our data showed that 5-Aza-dc inhibits the proliferative rate and migratory ability of HEMC cells and produces a moderate increase of cell adhesion on fibronectin-coated plates. 3-OST3A cDNA expression acted in a comparable way to that of the demethylating agent, whereas 3-OST1 only produced an antimigratory effect. These results indicate that reversing the epigenetic loss of 3-OST expression or restoring expres-

Figure 7. Effect of 3-OST expression on HEMC cell proliferation, adhesion, and migration. **A)** Untreated and 4 μ M 5-Aza-dc-treated HEMC cells were seeded onto 24-well plates, and proliferation was evaluated by measurement of DNA content after 1 to 5 d culture time. **B)** HEMC cells were transiently transfected with 3-OST1 or 3-OST3A cDNA for 48 h and analyzed for proliferation as described for **A**. Results are expressed as DNA concentration and are means $\pm$ SE of 3 to 6 observations. $*P < 0.05$, $***P < 0.001$ vs. untreated or mock-transfected cells; 2-way ANOVA with Bonferroni's multiple comparison *post hoc* test. **C)** Untreated and 4 μ M 5-Aza-dc-treated HEMC cells were plated onto wells coated with BSA only or with 100 or 200 μ g of fibronectin for 30 min at 37°C. After washing, adherent cells were quantified after fixation by crystal violet staining and absorbance measurement at a wavelength of 550 nm. Attachment of cells to plates coated with BSA only was assigned a value of 100%. **D)** HEMC cells were transiently transfected by 3-OST1 or 3-OST3A cDNA for 48 h and assayed for adhesion as described for **C**. Attachment of cells to plates coated with BSA only was assigned a value of 100%. Results are expressed as means $\pm$ SE of 3 to 6 observations. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. corresponding control; 1-way ANOVA with Bonferroni's multiple comparison *post hoc* test. **E)** Untreated and 4 μ M 5-Aza-dc-treated HEMC cells were incubated with mitomycin C to block cell proliferation. Cells were plated on Transwell inserts in serum-free medium containing 0.1% BSA and left to migrate through the polycarbonate membrane to the lower chamber, containing medium with 5% FBS. After 8, 16, and 24 h, untreated and 5-Aza-dc-treated cells were washed, fixed, and stained with methyl blue. Quantification was performed by optical density reading at a wavelength of 663 nm. **F)** HEMC cells were transiently transfected with 3-OST1 or 3-OST3A cDNA for 48 h and analyzed for migration potency after 18 h as described for **E**. Results are expressed as percentage of migration and are means $\pm$ SE of 3 to 6 observations. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. corresponding control; 1-way ANOVA with Bonferroni's multiple comparison *post hoc* test.

sion by transient 3-OST3A expression is accompanied by a reduction of the proliferation and migration capacity of HEMC cells, together with an increased adhesion ability that could counteract the invasiveness of chondrosarcoma cells.

DISCUSSION

A growing body of evidence supports an important role for GAGs at key pathophysiological stages of tumorigenesis and progression. HS-GAGs regulate tumor growth, tumor-cell adhesion, invasion, hematogenous metastasis, and angiogenesis (3). These effects are thought to be mediated by multiple interactions with growth factors, cytokines, chemokines, extracellular matrix proteins, and coagulation factors (1). In some cases, these interactions are highly specific in terms of the degree and position of sulfation required to achieve

high-affinity binding. The classic example is antithrombin III, in which a specific pentasaccharide, including a 3-*O*-sulfated glucosamine residue, is essential for high-affinity interactions (43, 44). Specific patterns of sulfation are required for binding members of the fibroblast growth factor (FGF) family and signal transduction mediated by these growth factors (45). *In vivo* studies in transgenic mice clearly indicate that changes in the fine structure of cell surface HS have profound effects on tumor cell growth and metastasis (46). However, although it is well documented that the profile of cellular HS-GAGs is critical for tumor behavior, our understanding of the regulation of the biosynthetic machinery governing the structure of HS chains in cancer cells remains limited. In this study, we show for the first time that a specific expression pattern of HS-3-*O*-sulfotransferases is driven by an epigenetic mechanism involving DNA methylation in a chondrosarcoma cell line, selected as a model of invasive tumors involving connec-

tive tissue. We demonstrate that CpG islands within the proximal region of the *3-OST* genes were hypermethylated, an event associated with down-regulation of the expression of several members of the *3-OST* family in HEMC cells. Notably, exposure of chondrosarcoma cells to a demethylating agent or overexpression of *3-OST3A* cDNA led to the reversion of certain characters associated with invasiveness, namely cell proliferation, attachment to extracellular matrix proteins, and cell migration. Overexpression of *3-OST1* produced a moderate but significant antimigratory effect on HEMC cells. To our knowledge, this study provides the first evidence for the effect of epigenetic regulation of HS-*O*-sulfotransferase gene expression on cancer cell phenotype.

These findings are the result of an extensive DNA methylation analysis of the genes coding for the biosynthetic enzymes involved in HS-chain formation and modification in HEMC cells, where we first observed that the CpG island of the glycosyltransferase genes involved in HS synthesis exhibit a low number of methylated sites in the chondrosarcoma cells. The biosynthetic pathway of HS chains is initiated by the sequential addition of 4 monosaccharides, forming a linker sequence that serves as a primer for further polymerization of the polysaccharide chains *via* the successive and coordinate action of an array of glycosyltransferase enzymes. The formation of the tetrasccharide linker region catalyzed by XT-I, β 4GalT7, β 3GalT6, and GlcAT-I is common to HS- and CS-chain synthesis pathways. The observation that this process is unaffected by inhibition of CpG methylation is not unexpected, since the initiation steps are absolutely required prior to the elongation of both types of GAG chains and thus are predicted to be tightly regulated (47). Indeed, a defect in initiation of GAG synthesis would compromise not only HS but also CS expression and would be expected to be deleterious to cell and tissue functions.

Our results also provide strong evidence that DNA methylation-associated gene silencing alters neither the initiation nor the polymerization of HS chains in HEMC cells. This is based on the following: the *EXT1* gene promoter displayed no hypermethylation profile, no change in *EXT1* mRNA transcript level was observed on 5-Aza-dc treatment, and analysis of cellular HSPGs showed no modification of HS-chain length on cell treatment with a DNA-methyltransferase inhibitor. These data, based on genetic and biochemical analysis, indicate that HS-chain formation catalyzed by *EXT1*, the glycosyltransferase playing a primordial role in HS chain polymerization, is not regulated by an epigenetic mechanism involving DNA methylation in chondrosarcoma cells. The lack of hypermethylation of the *EXT1* gene in HEMC cells is consistent with other studies indicating that neither chondrosarcomas nor osteosarcomas exhibited aberrant methylation at the *EXT1* locus (24, 48). Interestingly, Roperio *et al.* (24) provided evidence for aberrant methylation of the *EXT1* promoter in acute types of leukemia, suggesting that the defect in HS chains resulting from epigenetic silencing of an HS biosynthetic glycosyltransferase is a critical step in the development of this group of malignancies.

However, in chondrosarcoma, our results and other studies indicate that although genetic alteration of the *EXT1* locus leads to cartilaginous and bone malignancies in HME, silencing of the *EXT* gene is not a characteristic feature of these tumor cells. Altogether, our results show that in HEMC cells, regulation of the glycosyltransferases that are responsible for the initiation and polymerization of the saccharide backbone of HS chains does not involve an epigenetic mechanism driven by DNA methylation.

A strong body of evidence has demonstrated that HS sequence patterns generated by sulfotransferases have profound consequences on the cellular functions of proteoglycans (11). This led us to examine the potential effect of epigenetic regulation on properties and functions of these enzymes, which modify the polysaccharide GAG chains, in a model tumor cell line. Interestingly, examination of the methylation status of the *NDST* and *OST* gene families revealed aberrant methylation status of a specific set of genes in chondrosarcoma cells. Indeed, establishment of the methylation pattern of the genes that contain CpG islands showed extensive DNA methylation of the *3-OST1*, *3-OST3A*, and *3-OST6* gene family members in HEMC cells, and a lower number of methylated sites in the proximal promoter region of the *3-OST2* gene. By contrast, low-level or no promoter methylation was observed in the *NDST2*, *NDST3*, *HS2ST1*, and *HS6ST1* genes. The role of DNA methylation as a regulatory mechanism influencing HSPG 3-*O*-sulfation was confirmed by the observation that treatment with 5-Aza-dc, widely used as a DNA-methyltransferase inhibitor, enhanced *3-OST1*, *3-OST2*, and to the largest extent, *3-OST3A* mRNA expression level in HEMC cells. Further analysis of the methylation profile of the *3-OST3A* CpG island following treatment of HEMC cells with 5-Aza-dc indicated that the drug significantly reduced the number of methylated sites, suggesting that the increase in *3-OST3A* gene expression could be attributed, at least in part, to 5-Aza-dc-induced demethylation. However, it is also possible that demethylation of transcription factor genes involved in *3-OST3A* regulation on 5-Aza-dc treatment could contribute to sulfotransferase promoter activation.

The lack of effect of 5-Aza-dc on *3-OST3B*, *HS2ST1*, and *HS6ST1* transcript levels is correlated with the low number or absence of methylated sites at the CpG island of these genes. On the other hand, *NDST1* and *HS6ST2* were found to be hypermethylated in chondrosarcoma cells, and exposure to 5-Aza-dc reduced their methylation content and induced their mRNA levels, indicating that expression of these genes might be regulated at the epigenetic level. However, since *NDST1* and *HS6ST2* exhibited a similar hypermethylation profile in blood cells, no exclusive link between the methylation status of these genes and the pathogenesis of chondrosarcoma could be established at that stage.

Since a methylation event was found to be involved in the regulation of several members of the *3-OST* family in HEMC cells, a key issue was next to determine whether up-regulation of *3-OST* expression had an effect on HSPG structure and function. Following [35 S]sulfate metabolic labeling, analysis of cellular

HSPGs by anion-exchange chromatography provided evidence for an increase in charge on 5-Aza-dc treatment. This effect was likely due to an increase in HS-chain sulfation, based on the following: the degree of HS-GAG chain sulfation was increased, whereas the chain length was unchanged, as shown by gel-filtration chromatography; the synthesis and charge of proteins were not affected by 5-Aza-dc treatment; and a strong increase in HEMC cell membrane/pericellular HS expression on 5-Aza-dc treatment, as well as on 3-OST cDNA transfection, was evidenced by indirect immunofluorescence microscopy. Altogether, our results suggest that reversing the hypermethylation state of HS-biosynthetic genes by 5-Aza-dc treatment in HEMC cells mainly affects HS-chain sulfation, an effect that can be attributed, at least in part, to enhanced 3-OST expression.

Epigenetic changes play predominant roles in the promotion or suppression of tumor growth, invasion, and metastasis. It was thus essential to evaluate the potential biological significance of aberrant methylation of HS-biosynthetic enzymes in the chondrosarcoma cells. To this aim, we determined the effects of 5-Aza-dc treatment, on one hand, and those of 3-OST1 and 3-OST3A cDNA expression, on the other hand, on several parameters linked to the invasive character of HEMC cells. Our results show that not only 5-Aza-dc treatment but also expression of 3-OST3A cDNA was able to produce a significant decrease in chondrosarcoma cell proliferation. In addition, 3-OST3A overexpression promoted cell adhesion to a fibronectin substrate, an important finding since attachment of tumor cells to the extracellular matrix is required for cell motility and invasion. Concordantly, it was recently shown that the fine structure of HS is critical in binding the HepII domain of fibronectin for optimal cell attachment (49). Our results suggest that 3-*O*-sulfation catalyzed by 3-OST3A may also be a determinant for fibronectin binding, although further studies are required to confirm this assumption. Finally, an *in vitro* migration assay clearly showed that either treatment of HEMC cells with the demethylating agent or expression of 3-OST1 and 3-OST3A cDNA resulted in a less motile cell phenotype. Although the mechanisms underlying these observations warrant further investigation, our findings suggest that dysregulation of 3-OST expression, with the consequent effects on HS-3-*O*-sulfation, contributes to several characteristics associated with tumor behavior, at least in HEMC cells. Should this be found to be a more widespread effect, it would have significant implications for our understanding of the molecular basis of tumor cell invasiveness and metastasis. The regulation of 3-OST expression in different types of tumor cells is currently under investigation. The key structure for the 3-*O*-sulfated functional domains of heparin and HSPGs are produced *via* the action of 7 distinct enzymes belonging to the largest HS-*O*-sulfotransferase family. 3-OST1 transfers sulfate groups to the 3-OH position of the *N*-sulfated glucosamine residue that is linked to a GlcA residue at the nonreducing end, to provide a binding site for antithrombin (43). HS modified by 3-OST2, OST3A, OST3B, OST4, and OST6 binds to HSV-1 gD and assists

viral entry (50, 51). Nonetheless, the biological significance of 3-*O*-sulfate other than regulation of blood coagulation and HSV-1 entry remains unknown at the present time, although several recent reports support additional functions, such as in glomerular filtration or FGF-7 [keratinocyte growth factor (KGF)] binding (52). Surprisingly, *Hs3st1*^{-/-} knockout mice did not exhibit a procoagulant phenotype, but showed postnatal lethality and intrauterine growth retardation (53). Reduction of the expression of *Drosophila melanogaster* 3OSTB, a homologue of mammalian 3-OST enzymes, by transgenic RNAi resulted in a phenotype of Notch signaling deficiency (54). Interestingly, Mochizuki *et al.* (55) recently demonstrated that several 3-OST enzymes produce HS-tetrasulfated disaccharides present in most tissues, suggesting a broader, although unknown, physiological role. Recently, Vanpouille *et al.* (56) demonstrated that 3-*O*-sulfated glucosamine residues were responsible for HS interactions with the inflammatory mediator cyclophilin B. An intriguing possibility being raised is that the promigratory activity of this inflammatory mediator might be dependent on interactions with specific binding HS domains present at the cell surface of responsive T lymphocytes. Our investigations of the functional properties triggered by 3-OST expression support the possibility of a novel role for HS-3-*O*-sulfation as an antimigratory/antiinvasiveness modulator, at least in chondrosarcoma cells. In this regard, the importance of 3-*O*-sulfate groups in the specificity of interactions between heparin/HS and FGF-7 and FGF-10 is of special interest (52), taking into account the effect of these growth factors in the regulation of the proliferation of several cell types (57) and cell-stroma interactions (58). Our study reinforces the concept that regulation of sulfotransferases, which in turn determines specific carbohydrate structural modifications of HS chains, has a vital influence in the modulation of glycan function in cancer (59).

CONCLUSIONS

We have demonstrated that aberrant methylation of a specific set of sulfotransferase genes responsible for the modification of HS chains alters GAG-chain sulfation and contributes to alteration of the cell phenotype and invasive properties of HEMC chondrosarcoma cells, an important aspect of cancer growth and metastasis control. Epigenetic abnormalities, such as aberrant methylation of CpG islands, have a major effect on carcinogenesis, and methylation of many genes is found to be associated with poor response to chemotherapy and with poor prognosis (60). However, epigenetic alterations are potentially reversible. The use of DNA demethylating agents has turned out to be effective for hematological malignancies and is being used to treat solid tumors (60). Chondrosarcomas remain challenging malignancies both in terms of therapeutic options and prognosis, since they are resistant to conventional chemotherapy and radiation therapy (61). Our results uncover a new mechanism that might play a role in chondrosarcoma pathogenesis

and lead to the identification of novel therapeutic approaches. FJ

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