MATRIX PATHOBIOLOGY

Heparan Sulfate Proteoglycan Synthesis Is Dysregulated in Human Osteoarthritic Cartilage

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Osteoarthritis (OA) is a common degenerative joint disease, characterized by cartilage loss and subchondral bone remodeling in response to abnormal mechanical load. Heparan sulfate (HS) proteoglycans bind to many proteins that regulate cartilage homeostasis, including growth factors, morphogens, proteases, and their inhibitors, and modulate their localization, retention, and biological activity. Changes in HS expression and structure may thus have important consequences for joint health. We analyzed normal and osteoarthritic human knee cartilage, and found HS biosynthesis was markedly disrupted in OA, with 45% of the 38 genes analyzed differentially regulated in diseased cartilage. The expression of several HS core proteins, biosynthesis, and modification enzymes was increased in OA cartilage, whereas the expression of the HS proteoglycans syndecan 4 and betaglycan was reduced. The structure of HS was also altered, with increased levels of 6-O-sulfation in osteoarthritic samples, which correlated with increased expression of *HS6ST1*, a 6-O-sulfotransferase, and *GLCE*, an epimerase that promotes 6-O-sulfation. siRNA silencing of *HS6ST1* expression in primary OA chondrocytes inhibited extracellular signal-regulated kinase phosphorylation in response to fibroblast growth factor 2, showing that changes in 6-O-sulfation impact a key cartilage signaling pathway. Given the broad range of homeostatic and repair pathways that HS regulates, these changes in proteoglycan expression and HS structure are likely to have significant effects on joint health and progression of OA. (*Am J Pathol* 2019, 189: 632–647; <https://doi.org/10.1016/j.ajpath.2018.11.011>)

Osteoarthritis (OA) is a common degenerative joint disease in which articular cartilage loss and subchondral bone remodeling cause pain and impair movement of affected joints. There are currently no disease-modifying therapies available, and patients are largely treated with analgesia or joint replacement surgery. There is thus a substantial need to develop effective therapeutics that can slow or halt the progression of joint damage.

The primary risk factors for development of OA are aging and injury, which alter the mechanical environment of the joint and stimulate chondrocytes to produce catabolic proteinases that degrade the cartilage extracellular matrix. The pericellular matrix immediately adjacent to chondrocytes is thought to play a central role in transducing mechanical stimuli to biochemical signals within the cells.¹ For example, loading of cartilage causes release of fibroblast

growth factor 2 (FGF2) from the pericellular matrix, and activates downstream extracellular signal-regulated kinase (ERK) signaling pathways in chondrocytes.^{2,3} FGF2 is localized in the pericellular matrix through its interaction with the heparan sulfate (HS) chains of perlecan,² the major HS proteoglycan in cartilage. Pericellular matrix and cell surface HS also binds to many other bioactive proteins, including growth factors [eg, connective tissue growth

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factor and transforming growth factor- β (TGF- β), morphogens [eg, bone morphogenetic protein (BMP)-4 and BMP-7], proteinases (eg, matrix metalloproteinase 13 and adamalysin with thrombospondin motifs 4 and 5), and proteinase inhibitors (eg, tissue inhibitor of metalloproteinases-3), and regulates their retention, localization, and biological activity.^{4,5} HS is thus a critical regulator of chondrocyte homeostasis and cartilage health. Previous studies have shown increased expression of some of the HS core proteins in OA, including perlecan,^{6–9} syndecan 1,^{9–11} and syndecan 4,^{11–13} but nothing is yet known about how the structure of HS itself or its interactions with ligands change in OA.

HS interacts with proteins through electrostatic interactions between its negatively charged sulfate groups and positively charged lysine and arginine residues on the protein ligand. The number and arrangement of sulfate groups along the HS glycosaminoglycan chain determines the affinity of HS for its protein ligands and its ability to modulate their biological activity.^{14–16} This sulfation pattern is generated by the template-independent action of a series of sulfotransferases during HS synthesis in the Golgi apparatus.¹⁷ The exostosin (EXT) family of glycosyltransferases initiate HS synthesis by adding sequential glucuronic acid (GlcA) and N-acetyl glucosamine (GlcNAc) residues to a linker tetrasaccharide attached to the core protein. GlcA can subsequently be epimerized to iduronic acid (IdoA) by glucuronyl C5-epimerase (GLCE). The growing HS chain can then be N-sulfated on GlcNAc residues [by the N-deacetylase/N-sulfotransferases (NDSTs)], and/or O-sulfated on GlcNAc [by the 6-O-sulfotransferases (HS6STs) and 3-O-sulfotransferases (HS3STs)] and GlcA/IdoA [by the 2-O-sulfotransferase (HS2ST1)].¹⁷ HS can also be edited in the extracellular environment by sulfatase 1 (SULF1) and sulfatase 2 (SULF2) that remove 6-O-sulfate groups, or degraded by the heparanases heparanase 1 (HSPE1) and heparanase 2 (HSPE2). These reactions do not always occur to completion, generating a highly variable sulfation pattern that gives HS a structural complexity several orders of magnitude larger than the proteome. Temporal and spatial changes in HS sulfation occur during development^{18–21} and in diseases, such as Alzheimer disease,^{22,23} cancer,²⁴ diabetes,²⁵ and fibrosis,²⁶ and are thought to enable HS to coordinately regulate the retention and activity of multiple bioactive ligands, and thus to powerfully regulate tissue homeostasis.

Herein, we isolated RNA from knee cartilage of normal and osteoarthritic donors, and profiled expression of the 13 core proteins and 25 biosynthesis and modifying enzymes that govern HS structure and sulfation. Notably, disaccharide composition was examined by isolating HS from normal and OA cartilage. HS enzyme expression and glycan structure were found to be markedly dysregulated in OA cartilage, and increased 6-O-sulfation of HS was identified

Table 1 Primer/Probe Sets Used on Custom-Designed TaqMan Low-Density Arrays

Gene name	Primer/probe set
Chain elongation	
<i>EXT1</i>	Hs00609162_m1
<i>EXT2</i>	Hs00181158_m1
<i>EXTL1</i>	Hs00184929_m1
<i>EXTL2</i>	Hs01018237_m1
<i>EXTL3</i>	Hs00918601_m1
N-deacetylase/N-sulfotransferases	
<i>NDST1</i>	Hs00925442_m1
<i>NDST2</i>	Hs00234335_m1
<i>NDST3</i>	Hs01128584_m1
<i>NDST4</i>	Hs00224024_m1
Glucuronyl C5-epimerase	
<i>GLCE</i>	Hs00392011_m1
2-O-sulfotransferase	
<i>HS2ST1</i>	Hs00202138_m1
3-O-sulfotransferases	
<i>HS3ST1</i>	Hs00245421_s1
<i>HS3ST2</i>	Hs00428644_m1
<i>HS3ST3A1</i>	Hs00925624_s1
<i>HS3ST3B1</i>	Hs00797512_s1
<i>HS3ST4</i>	Hs00901124_s1
<i>HS3ST5</i>	Hs00999394_m1
<i>HS3ST6</i>	Hs03007244_m1
6-O-sulfotransferases	
<i>HS6ST1</i>	Hs00757137_m1
<i>HS6ST2</i>	Hs02925656_m1
<i>HS6ST3</i>	Hs00542178_m1
Editing enzymes	
<i>HSPE</i>	Hs00935036_m1
<i>HSPE2</i>	Hs00222435_m1
<i>SULF1</i>	Hs00290918_m1
<i>SULF2</i>	Hs01016476_m1
Core proteins	
<i>AGRN</i>	Hs00394748_m1
<i>GPC1</i>	Hs00892476_m1
<i>GPC2</i>	Hs00415099_m1
<i>GPC3</i>	Hs01018936_m1
<i>GPC4</i>	Hs00155059_m1
<i>GPC5</i>	Hs00270114_m1
<i>GPC6</i>	Hs00170677_m1
<i>HSPG2</i>	Hs00194179_m1
<i>SDC1</i>	Hs00896423_m1
<i>SDC2</i>	Hs00299807_m1
<i>SDC3</i>	Hs01568665_m1
<i>SDC4</i>	Hs00161617_m1
<i>TGFB3</i>	Hs01114253_m1
Normalizing genes	
<i>18S</i>	Hs99999901_s1
<i>RPLP0</i>	Hs99999902_m1
<i>GAPDH</i>	Hs99999905_m1

AGRN, agrin; EXT, exostosin; EXTL, exostosin-like; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPC, glypican; HSPE, heparanase; HSPG, HS proteoglycan 2; SDC, syndecan; RPLP0, ribosomal protein lateral stalk subunit P0; SULF, sulfatase; TGFB3, transforming growth factor- β receptor III.

Figure 1 Changes in heparan sulfate (HS)-related gene expression in human osteoarthritic (OA) cartilage. Expression of HS-associated genes in normal and OA cartilage was analyzed using TaqMan low-density arrays. Fold changes in expression were determined by the $\Delta\Delta C_t$ method against the average of the normal samples. **A:** Fold changes in expression of HS core protein genes. **B:** Fold changes in expression of HS chain elongation and sulfatase genes. **C:** Fold changes in expression of sulfotransferase genes. Error bars represent 95% CIs of the mean. $n = 12$ per group (**A–C**). ** $q < 0.01$, *** $q < 0.001$, and **** $q < 0.0001$ (Benjamini, Krieger, and Yekutieli-corrected multiple t -test). AGRN, agrin; EXT, exostosin; EXLT, exostosin-like; GLCE, glucuronyl C5-epimerase; GPC, glypican; HS3ST, 3-O-sulfotransferase; HS6ST, 6-O-sulfotransferase; HSPG, heparan sulfate proteoglycan; NDST, N-deacetylase/N-sulfotransferase; SDC, syndecan; SULF, sulfatase; TGFBR3, transforming growth factor- β receptor III.

as the primary structural change associated with impaired cartilage homeostasis.

Materials and Methods

Human Cartilage Samples

Human articular cartilage was obtained in full compliance with national and institutional ethical requirements, the UK Human Tissue Act, and the Declaration of Helsinki.

Normal human knee cartilage specimens used for RNA and glycosaminoglycan analyses were obtained from Articular Engineering LLC (Northbrook, IL) from donors (six females of 44 to 68 years of age, six males of 32 to 75 years

of age) with clinically healthy, normal cartilage and no history of OA, following informed donor consent and approval by local ethics committees and the University of Oxford Research Ethics Committee (R45926/RE001). Cartilage was aseptically harvested within 48 hours of death, flash frozen in liquid nitrogen, stored at -80°C , and shipped in dry ice.

Normal human knee cartilage specimens used for immunohistochemistry were obtained from Stanmore Biobank (Royal National Orthopedic Hospital, Stanmore, UK) from donors undergoing amputation for low limb malignancies with no involvement of the cartilage, following informed donor consent and approval by the Royal Veterinary College Ethics and Welfare Committee (institutional approval unique reference number 2012 0048H). Cartilage was aseptically

Figure 2 Expression of heparan sulfate (HS)—associated genes is altered in osteoarthritic (OA) cartilage. **A:** Forest plot showing there is no significant difference between the ages and sexes of normal and OA cartilage donors used for gene expression analysis. Bars show 95% CIs of the mean, **dotted line** shows $Y = 0$. **B:** Expression of HS-associated genes in normal and OA cartilage was analyzed using TaqMan low-density arrays. Fold change in expression was determined by the $\Delta\Delta C_t$ method against the average of the normal samples. The heatmap shows only those HS-associated genes whose expression was significantly different in the two groups (Benjamini, Krieger, and Yekutieli—corrected multiple t -test; $q < 0.05$), with increased expression in OA shown in red, reduced expression shown in blue, and no change indicated in green. Cluster analysis based on the expression levels of significantly regulated genes separated the samples into two clusters, with the first cluster containing all of the OA samples and one of the normal samples, and the second cluster containing the remaining normal samples. $n = 12$ for each group (**A** and **B**). F, female; M, male; AGRN, agrin; EXT, exostosin; EXLT, exostosin-like; GLCE, glucuronyl C5-epimerase; GPC, glypican; HS3ST, 3-O-sulfotransferase; HS6ST, 6-O-sulfotransferase; HSPG, heparan sulfate proteoglycan; NDST, N-deacetylase/N-sulfotransferase; SDC, syndecan; SULF, sulfatase; TGFB3, transforming growth factor- β receptor III.

harvested from all surfaces of the joint within 4 hours of surgery and processed for downstream analyses.

Human OA cartilage samples used for immunohistochemistry, RNA, and glycosaminoglycan analyses were obtained from the Oxford Musculoskeletal Biobank from donors (six females of 58 to 83 years of age, six males of 52 to 84 years of age) undergoing total knee replacement surgery for advanced OA, following informed consent and approval by the Oxford Research Ethics Committee C (09/H0606/11). Cartilage was aseptically harvested from all surfaces of the joint within 4 hours of surgery and processed for downstream analyses.

RNA Isolation

Cartilage (300 mg) from 12 normal and 12 OA donors was ground under liquid nitrogen with a pestle and mortar. The ground cartilage powder was added to 1.5 mL of RLT lysis buffer supplemented with 10 μ L/mL β -mercaptoethanol (Qiagen, Manchester, UK), digested with proteinase K (6 to 10 mAU/100 mg of cartilage, 10 minutes at 55°C; Qiagen, Manchester, UK), and transferred to an RNeasy micro column (Qiagen, Manchester, UK). RNA was extracted according to the manufacturer's protocol with an intermediate DNA digestion step. The eluted RNA was quantified on a Nanodrop spectrophotometer and examined on a Bioanalyser (Agilent,

Stockport, UK). RNA from healthy and OA cartilage had comparable RNA integrity numbers (6 to 7.7) and RNA yields.

RT-PCR and Quantitative PCR

Total RNA (250 ng) was reverse transcribed to cDNA using a reverse transcription kit (ThermoFisher Scientific, Loughborough, UK), and cDNA was amplified on a TaqMan low-density array (Table 1). Fold changes against the control samples were calculated using the $\Delta\Delta C_t$ method. For calculating the ΔC_t , the average of the three normalizing genes (*18S*, *RLPL0*, and *GAPDH*) was used.

Extraction of HS Proteoglycans and Disaccharide Analysis

Approximately 300 mg of frozen cartilage explants was ground in a pestle and mortar under liquid nitrogen, and the powder was placed in 1 mL of Trizol (ThermoFisher Scientific). After phase separation with the addition of chloroform, the aqueous phase was added to equilibrated diethylaminoethyl-Sepharose beads and washed with phosphate-buffered saline (PBS) and 0.25 mol/L NaCl before elution with 2 mol/L NaCl. The eluate was desalted on PD10 columns and freeze dried. The samples were then treated with DNase (Qiagen, Manchester, UK) and RNase,

before incubation with chondroitinase ABC (Sigma Aldrich, Gillingham, UK), neuraminidase, keratanase, and pronase. After the digestions, the samples were once more purified on diethylaminoethyl beads and freeze dried. The rest of the procedure was as described before.²⁷ Briefly, samples were sequentially digested with heparanase I, II, and III (IBEX Pharmaceuticals, Montreal, Canada), purified on C18 and graphite spin columns (ThermoFisher Scientific), and labeled with BIODIPY hydrazine (ThermoFisher Scientific), as previously described.²⁸ Finally, samples underwent ethanol precipitation to remove any copurified impurities. Samples were then analyzed by SAX high-performance liquid chromatography using a Propac PA-1 strong anion-exchange column (4.6 × 250 nm; Dionex, Loughborough, UK). Samples were eluted with a linear gradient of 0 to −1 mol/L sodium chloride and isocratic sodium hydroxide (150 mmol/L) over 30 minutes at 1 mL/minute on a Shimadzu high-performance liquid chromatography system (Shimadzu, Milton Keynes, UK). An inline fluorimeter was used to detect eluted peaks (excitation λ = 488 nm, emission λ = 520 nm). Previously calculated correction factors were applied to quantify the observed disaccharides.²⁷

Immunofluorescence Staining Analysis

On receipt, cartilage explants were embedded in OCT (ThermoFisher Scientific) and frozen in isopentane for storage at −80°C until sectioning. Samples were mounted on a cryostat (CM1860UV; Leica, Milton Keynes, UK) and sections (5 μ m thick) were cut and mounted on Superfrost Plus microscope slides (ThermoFisher Scientific) and stored at −80°C. Frozen sections were air dried for 1 hour at room temperature and fixed with neutral-buffered formalin for 5 minutes at room temperature before their incubation in ice-cold acetone for 10 minutes at −20°C, and incubated at 37°C for 30 minutes with 0.1 U chondroitinase ABC (C3667; Sigma-Aldrich) in 1× chondroitinase buffer (40 mmol/L tris-acetate, pH 8). The digested sections were blocked in 1% goat serum and 5% bovine serum albumin in PBS for 1 hour at room temperature. After 15 minutes of permeabilization with 0.1% Triton X-100 in PBS, sections were washed in PBS and the primary antibodies or isotype negative control antibodies were applied diluted in block buffer overnight at 4°C. The following antibodies were used: rat antiperlecan [HS proteoglycan 2 (HSPG2); 1:1000; Millipore, Watford, UK], rabbit anti-GLCE (1:20; HPA04821; Atlas Antibodies, Bromma, Sweden), rabbit anti-exostosin-like (EXTL2) (1:100; NBP2-16394; Novus Biologicals,

Abingdon, UK), mouse anti-NDST1 (3 μ g/mL; ab55296; Abcam, Cambridge, UK), rabbit anti-glypican 1 (GPC1; 1:250; HPA303571; Sigma-Aldrich), and rabbit anti-HS6ST1 (1:250; ab106195; Abcam). After washing in PBS at room temperature, the appropriate secondary antibodies were added in block buffer, either in combination or in unison: goat anti-rat Alexa Fluor 488 (1:1000; ThermoFisher Scientific), goat anti-rabbit Alexa Fluor 568 (1:500; ThermoFisher Scientific), or goat anti-mouse Alexa Fluor 568 (1:500; ThermoFisher Scientific). The secondary antibodies were washed off with PBS after 3 hours at room temperature. Nuclei were stained with DAPI (1:200 in PBS; ThermoFisher Scientific) for 1 hour before a final wash in PBS and mounting in Prolong Diamond antifade (ThermoFisher Scientific).

Sections were viewed under an Olympus BX51 fluorescent microscope (Olympus, Southend-on-Sea, UK). For each protein, the optimal duration of exposure was determined as that giving a signal in positively stained sections and no detectable staining in negatively stained sections, and avoiding over-exposure and signal saturation. The exposures used were as follows: HSPG2, 200 to 250 milliseconds; EXTL2, 125 milliseconds; GLCE, 200 milliseconds; GPC1, 125 milliseconds; NDST1, 280 to 300 milliseconds; and HS6ST1, 275 to 300 milliseconds. Images were obtained from at least six random regions of the cartilage under a 40× objective lens and then analyzed on ImageJ software version 1.52h (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>), as described previously.^{29,30} For representative images, the intensity of the red and green signal was adjusted for illustrative purposes. For quantification, raw unaltered images were analyzed.

Analysis of ERK Phosphorylation in Chondrocytes

Primary OA chondrocytes were isolated from freshly harvested human OA cartilage by chopping the tissue finely and incubating in Dulbecco's modified Eagle's medium with 10% fetal calf serum (ThermoFisher Scientific) and 1.5 mg/mL type II collagenase (Roche Diagnostics, Burgess Hill, UK) (18 hours, 37°C). The suspension was passed through a cell strainer, pelleted, and washed twice with medium. Isolated chondrocytes were cultured for 7 days until confluent³¹ and stored at −80°C until required. For siRNA transfection, chondrocytes (3 × 10⁵ per well) were transfected with 20 nmol/L of either Silencer Select Negative control number 1 or Silencer Select *HS6ST1* siRNA (s17978; ThermoFisher Scientific, UK) using Lipofectamine 2000 (ThermoFisher Scientific, UK) in serum-free Opti-MEM I (ThermoFisher Scientific).³¹ After 4 hours, the medium was replaced with

Figure 3 Expression of HS proteoglycan 2 (HSPG2), exostosin-like (EXTL2), and glucuronyl C5-epimerase (GLCE) is increased in human osteoarthritic (OA) cartilage. **A:** Representative images showing expression of HSPG2 and EXTL2 in human normal and OA cartilage. **B:** Expression of HSPG2 and EXTL2 was semiquantitatively evaluated in three normal donors (N1, N2, and N3) and three OA donors (OA1, OA2, and OA3), with each dot representing integrated fluorescence intensity per single cell, calculated from at least six random fields of view on three sections per donor. For each donor, the median fluorescence intensity with 95% CIs of the mean are shown. **C:** Representative images showing expression of HSPG2 and GLCE in human normal and OA cartilage. **D:** Expression of HSPG2 and GLCE was semiquantitatively evaluated in three normal donors (N4, N5, and N6) and three OA donors (OA1, OA2, and OA4), as described in **B**. **P < 0.01, ***P < 0.001, and ****P < 0.0001 (Kolmogorov-Smirnov test). Scale bars = 260 μ m (**A** and **C**).

Dulbecco's modified Eagle's medium containing 10% fetal calf serum and cell cultured for a further 72 hours.

To analyze ERK phosphorylation, siRNA-treated chondrocytes were cultured in serum-free Dulbecco's modified Eagle's medium for 24 hours and treated with recombinant human FGF2 (R&D Systems, Minneapolis, MN) (20 ng/mL, 10 minutes). Cells were then washed twice with ice-cold PBS and lysed on ice in 150 μ L radioimmunoprecipitation assay buffer containing protease inhibitors (SIGMAFAST; Sigma-Aldrich) and phosphatase inhibitors (20 mmol/L NaF, 100 mmol/L Na_3VO_4 , 100 mmol/L β -glycerophosphate, and 100 mmol/L sodium pyrophosphate). Lysates were centrifuged ($2500 \times g$, 3 minutes), and 20 μ g of protein was analyzed by immunoblotting using antibodies against phosphorylated ERK1/2 (1:2500; E7028; Sigma-Aldrich) and total ERK (1:5000; 4695S; Cell Signaling Technology, London, UK) in block buffer (5% milk/PBS with Tween 20). After 3×5 -minute washes with PBS with Tween 20, the membranes were incubated with an alkaline phosphatase-conjugated anti-rabbit secondary antibody (1:2000; Promega, UK) and developed using Western Blue stabilized substrate (Promega, Southampton, UK). Immunoblots were analyzed by densitometry using Phoretix 1D software version 11.5 (TotalLab, Newcastle-upon-Tyne, UK), and the levels of phosphorylated ERK were normalized to total ERK for each treatment, and expressed relative to the corresponding untreated sample (defined as 1).

Efficacy of siRNA silencing was confirmed by isolating RNA from radioimmunoprecipitation assay buffer lysates (20 to 50 μ L, diluted to 350 μ L with RLT buffer containing 1% v/v β -mercaptoethanol) using RNeasy micro kits, according to the manufacturer's protocol (Qiagen, Manchester, UK). Isolated RNA was reverse transcribed, and expression of *18S*, *GAPDH*, and *HS6ST1* was quantified as described above.

Statistical Analysis

All statistical tests, except correlations and linear regression models, were performed in GraphPad Prism version 7.0d (GraphPad Software, La Jolla, CA). All other statistical tests were performed in R version 3.4.4 (The R Foundation, Vienna, Austria; <https://www.r-project.org>). All significant changes are annotated in the figures.

Power calculations were based on pilot data from four samples per group. According to the pilot experiments, the sample size required for a two-tailed *t*-test was calculated with an effect size of 1.5 (difference between means of 0.6 and SD of 0.4) and a type I error rate α at 5%, to be 12

samples per group. Therefore, the groups were extended to the appropriate sizes.

Before performing any parametric test, groups were tested for normality using the D'Agostino and Pearson omnibus test.³²

Two-way analysis of variance was performed, and the forest plots (Figure 1) were examined to evaluate whether there was significant variation in the age or sex of the donors.

All tests on the PCR results were performed on the $\Delta\Delta\text{Ct}$ values because they were normally distributed. Multiple two-tailed *t*-tests were performed, comparing the expression of each gene between the two groups. The derived *P* values were corrected for multiplicity using the two-stage step-up method of Benjamini, Krieger, and Yekutieli, with a false-discovery rate cutoff at 5%.³³

For HS disaccharide composition, site of sulfation, and extent of sulfation, two-way analysis of variance was performed, with a Benjamini, Krieger, and Yekutieli multiplicity correction.

Hierarchical clustering was performed in R using the package *gplots* β and *RColorBrewer*. Canberra distance was used as the clustering metric.

For analysis of immunofluorescence images, the background-corrected integrated intensity values per cell were aggregated for the normal and OA groups ($n = 3$ to 6 per group), and the cumulative distributions of signal were analyzed using the Kolmogorov-Smirnov test because the values were not normally distributed.

Results

Expression of HS Proteoglycan Core Proteins Is Disrupted in OA Cartilage

RNA was isolated from normal and osteoarthritic tibial knee cartilage ($n = 12$ per group), and expression of 13 genes encoding HS proteoglycan core proteins was examined. Genes with a significant change in expression are shown in Figure 1.

Expression of agrin (*AGRN*) and perlecan (*HSPG2*) was significantly increased in OA samples (multiple *t*-tests, Benjamini, Krieger, and Yekutieli-corrected $q < 0.05$), whereas expression of betaglycan (TGF- β receptor III, *TGFBR3*) was reduced.

Among the syndecan family, syndecan 2 (*SDC2*) was not differentially regulated between the groups, whereas expression of syndecan 4 (*SDC4*) was reduced in OA, and expression of syndecan 1 (*SDC1*) and syndecan 3 (*SDC3*) was

Figure 4 Expression of perlecan (heparan sulfate proteoglycan 2; HSPG2), 6-O-sulfotransferase 1 (HS6ST1), and N-deacetylase/N-sulfotransferase 1 (NDST1) is increased in human osteoarthritic (OA) cartilage. **A:** Representative images showing expression of HSPG2 and HS6ST1 in human normal and OA cartilage. **B:** Expression of HSPG2 and HS6ST1 was semiquantitatively evaluated in three normal donors (N4, N5, and N6) and three OA donors (OA1, OA2, and OA4), with each dot representing integrated fluorescence intensity per cell, calculated from at least six random fields of view on three sections per donor. For each donor, the median fluorescence intensity with 95% CIs of the mean are shown. **C:** Representative images showing expression of HSPG2 and NDST1 in human normal and OA cartilage. **D:** Expression of HSPG2 and NDST1 was semiquantitatively evaluated in three normal donors (N1, N2, and N3) and three OA donors (OA1, OA2, and OA3), as described in **B**. * $P < 0.05$, *** $P < 0.0001$ (Kolmogorov-Smirnov test). Scale bars = 260 μ m (**A** and **C**).

increased. *SDC1* was the most strongly regulated of the HS core proteins, with mean expression increasing 17-fold in OA.

Glypican core proteins showed fewer changes in expression in OA cartilage, with only *GPC1* showing significantly higher expression in OA samples. Expression of *GPC4*, *GPC5*, and *GPC6* was not significantly regulated, and *GPC2* and *GPC3* were minimally expressed in both normal and OA cartilage.

Expression of HS Biosynthesis and Modifying Enzymes Was Also Disrupted in OA Cartilage

Using the same samples, expression of 25 genes encoding HS biosynthesis and modifying enzymes was analyzed (Figure 1), and 10 of these were found to be expressed at higher levels in OA cartilage.

Expression of *EXT1* and *EXT2*, which extend the growing HS chain, was increased in OA, as was expression of the related *EXTL1* and *EXTL2* genes. *EXTL1* was the most strongly regulated of all of the genes examined, showing a 24-fold increase in expression in OA. Expression of *EXTL3* was not significantly regulated. Mean expression of epimerase (*GLCE*), which epimerizes glucuronic acid to IdoA, was increased twofold in OA cartilage.

Mean expression of the N-sulfotransferase *NDST1* was threefold higher in OA cartilage. *NDST2* was expressed at similar levels in both sample groups, and *NDST3* and *NDST4* were not appreciably expressed in either normal or OA cartilage.

Expression of the 2-O-sulfotransferase *HS2ST1* was not significantly altered in OA samples. Of the seven isoforms of 3-O-sulfotransferase, only *HS3ST1* showed increased expression in OA cartilage, with expression of *HS3ST3A1* and *HS3ST3B1* not differentially regulated and four of the isoforms (*HS3ST2*, *HS3ST3*, *HS3ST5*, and *HS3ST6*) not detectably expressed in cartilage. Among the three 6-O-sulfotransferase isoforms, expression of *HS6ST1* was increased in OA cartilage, whereas *HS6ST3* expression was similar in both sample groups and *HS6ST2* was expressed at low levels.

Expression of both *SULF1* and *SULF2* was increased in OA cartilage, with *HSPE* and *HSPE2* not differentially expressed.

Cluster analysis of the samples, based on the levels of the significantly regulated genes, separated the samples into two clusters (Figure 2), with the first cluster containing all of the OA samples and one of the normal samples, and the second cluster containing the remaining normal samples. This indicates that levels of expression of the 17 significantly regulated genes are largely able to discriminate normal and osteoarthritic cartilage.

Selected Immunohistochemical Analysis Validates Observed Changes in Expression

To establish whether the observed gene regulation resulted in corresponding changes in protein expression, six

proteins (*HSPG2*, *EXTL2*, *GLCE*, *NDST1*, *HS6ST1*, and *GPC1*) were selected to be analyzed by semiquantitative immunofluorescence staining on frozen sections of normal and OA cartilage. *HSPG2* was selected as a marker of pericellular matrix localization, and the other five proteins were selected according to functional antibody availability and to have at least one from each category of HS core proteins and biosynthetic enzymes. The intensity of staining did not vary with cartilage depth for any of the antibodies examined.

As expected, *HSPG2* exhibited a predominantly pericellular localization,³⁴ with approximately 50% increased expression per cell in the four OA samples examined compared with the six normal cartilage samples.

EXTL2 had a predominantly perinuclear localization in both normal and OA cartilage (Figure 3A). In OA cartilage, expression of *EXTL2* was increased 1.6-fold, with a decrease in the number of cells not expressing *EXTL2* and a shift toward more cells expressing moderate amounts of the protein (Figure 3B).

GLCE was similarly localized in the perinuclear region of cells (Figure 3C), with 2.4-fold higher expression in OA and a shift toward more cells expressing modest amount of the enzyme (Figure 3D).

HS6ST1 intensity levels were approximately three times higher in OA (Figure 4B). The protein appeared to be expressed at low levels around the nucleus in normal cartilage and to show some pericellular staining in the OA samples (Figure 4A).

NDST1 was localized in the perinuclear region in normal cells, with more expression toward the periphery of the cell and overlapping with *HSPG2* in the OA samples (Figure 4C). Intensity levels were approximately 3.6-fold higher in the OA samples (Figure 4D).

Staining for *GPC1* was predominantly intracellular (Figure 5A), with 5.5-fold higher intensity in OA samples (Figure 5B).

Increased 6-O-Sulfation of HS in OA Cartilage

The amount and disaccharide composition of HS isolated from normal and OA cartilage was analyzed ($n = 8$ per group) (Figure 6A).

There was no significant difference in the total amount of HS in the normal and OA cartilage samples (5.7 ± 2.3 $\mu\text{g}/100$ mg in the normal samples, 3.02 ± 0.73 $\mu\text{g}/100$ mg of tissue in OA; $P > 0.05$ by t -test) (Figure 6B), or in the ratio of sulfated/total HS disaccharides (36.5% in normal cartilage, 53% in OA cartilage; Mann Whitney test, $P > 0.05$) (Figure 6C).

Normal and OA cartilage did not differ significantly in the abundance of any single HS disaccharide (Figure 6D), or in the percentages of nonsulfated, monosulfated, disulfated, or triple-sulfated disaccharides (data not shown). However, the percentage of 6-O-sulfated disaccharides was increased in OA cartilage (19.8% versus 12.1%; Mann Whitney test, $P = 0.0379$) (Figure 6E).

Figure 5 Expression of perlecan (heparan sulfate proteoglycan 2; HSPG2) and glypican 1 (GPC1) is increased in human osteoarthritic (OA) cartilage. **A:** Representative images showing expression of HSPG2 and GPC1 in human normal and OA cartilage. **B:** Expression of HSPG2 and GPC1 was semiquantitatively evaluated in three normal donors (N1, N2, and N3) and three OA donors (OA1, OA2, and OA3), with each dot representing integrated fluorescence intensity per cell, calculated from at least six random fields of view on three sections per donor. For each donor, the median fluorescence intensity with 95% CIs of the mean are shown. $^{**}P < 0.01$ (Kolmogorov-Smirnov test). Scale bars = 260 μm (A).

6-O-Sulfation of HS Correlates with Expression of *GLCE*, *HS6ST1*, *EXTL2*, and *HS3ST3A1*

Correlation between expression of HS biosynthesis genes and HS disaccharide compositions were quantified.

The percentage of 6-O-sulfated disaccharides correlated with expression of *HS6ST1* ($r = 0.74$, $P = 0.0016$) (Figure 6F), *GLCE* ($r = 0.775$, $P = 0.0007$) (Figure 6G), *EXTL2* ($r = 0.57$, $P = 0.026$), and *HS3ST3A1* ($r = 0.575$, $P = 0.025$). The relationship between 6-O-sulfation and *HS6ST1* expression was linear, with a 4% increase in abundance of 6-O-sulfated disaccharides for every doubling in the amount of *HS6ST1* (adjusted $R^2 = 0.525$, $P = 0.0014$). This relationship was independent of age, OA, and sex. The relationship between 6-O-sulfation and *GLCE* expression was linear, with a doubling in expression of *GLCE* increasing the percentage of 6-O-sulfation by approximately 4%. This relationship was dependent on age and sex, such that for every year increase in age there was a 0.17% increase in percentage 6-

O-sulfation, and that females had approximately 4% more 6-O-sulfation for the same amount of *GLCE* (adjusted $R^2 = 0.42$, $P = 0.028$).

The percentage of 2-O-sulfated disaccharides correlated with expression of *HS3ST3A1* ($r = 0.796$, $P = 0.0004$) and *HS6ST1* ($r = 0.57$, $P = 0.026$). The relationship between 2-O-sulfation and *HS3ST3A1* expression was linear, with a doubling in the levels of *HS3ST3A1* increasing the percentage of 2-O-sulfated disaccharides by 4%. This association was OA and sex dependent, such that females with OA had a 0.62% increase in 2-O-sulfation for the same level of *HS3ST3A1* expression (adjusted $R^2 = 0.67$, $P = 0.0036$).

Correlations were also examined between expression of HS biosynthesis genes and the relative abundance of individual disaccharide residues. This showed that *HS3ST3A1* expression correlated with the percentage of uronic acid (UA; 2-O-sulfate)–GlcNAc ($r = 0.772$, $P < 0.05$), UA(2-O-sulfate)–*N*-sulfoglucosamine ($r = 0.538$, $P < 0.05$), and UA–*N*-sulfoglucosamine(6-O-sulfate) ($r = 0.521$,

$P < 0.05$), whereas *HS6ST1* expression correlated with the percentage of UA(2-O-sulfate)-GlcNAc ($r = 0.53$, $P < 0.05$), UA-GlcNAc(6-O-sulfate) ($r = 0.58$, $P < 0.05$), and UA-GlcNAc ($r = -0.686$, $P = 0.005$).

Finally, *HS3ST2* expression was found to be linearly associated with age, independent of OA and sex, with expression of *HS3ST2* increasing by 6% to 21% every year (adjusted $R^2 = 0.341$, $P = 0.035$).

siRNA Silencing of *HS6ST1* Inhibits FGF2 Signaling

To investigate whether changes in *HS6ST1* expression and 6-O-sulfation have the capacity to alter signaling in OA chondrocytes, chondrocytes were isolated from knee cartilage of five donors with late-stage OA, and FGF2-dependent ERK phosphorylation was examined after siRNA knockdown of *HS6ST1*. siRNA treatment suppressed *HS6ST1* mRNA levels by >90% in all five donors (Figure 6H). Addition of 20 ng/mL FGF2 stimulated rapid phosphorylation of ERK, and this was significantly reduced (by 40%) in *HS6ST1*-silenced cells (Figure 6I).

Discussion

HS proteoglycans control the retention, localization, and biological activity of >400 bioactive molecules, including growth factors (eg, FGF2 and TGF- β), morphogens (eg, BMP-4 and BMP-7), proteinases (eg, matrix metalloproteinase 13 and adamalysin with thrombospondin motifs 4 and 5), and proteinase inhibitors (eg, tissue inhibitor of metalloproteinases-3).^{4,35–37} Many HS ligands have significant impacts on cartilage homeostasis, making HS a critical regulator of joint health. Despite this important role, nothing was previously known about how the structure and sulfation pattern of HS may change during the development of osteoarthritis. This study shows that the expression of HS biosynthesis and modifying enzymes and the resulting HS disaccharide composition differs significantly in normal and osteoarthritic human knee cartilage.

Expression of the HS polymerases *EXT1* and *EXT2*, as well as *EXTL1* and *EXTL2*, was significantly increased in OA cartilage. The roles of these enzymes in homeostasis

of adult cartilage have not been explored, but *EXT1* and *EXT2* are known to be important in musculoskeletal development. *EXT1* regulates Indian hedgehog (Ihh) signaling during endochondral ossification,³⁸ and mutations in both *EXT1* and *EXT2* are associated with development of hereditary multiple exostoses, an inherited pediatric disorder characterized by osteochondroma formation.³⁹ Aberrant activation of chondrocyte hypertrophy has been implicated in development of OA,⁴⁰ and may underlie the elevated expression of *EXT1* and *EXT2* observed. The role of the related *EXTL* genes in HS synthesis is less well understood, but they are thought to regulate early stages of HS chain elongation.⁴¹ Mutations in *EXTL3* have been shown to impair musculoskeletal development,⁴² and although *EXTL3* expression was not regulated in OA, expression of both *EXTL1* and *EXTL2* was strongly up-regulated.

Once polymerized, the HS chain can be modified by epimerization of GlcA to IdoA by a glucuronyl epimerase (GLCE) or by addition of sulfate groups to specific positions in the disaccharide units, by multiple sulfotransferases. The expression of 11 human HS sulfotransferases was measured, and it was found that the expression of *NDST1*, *HS3ST1*, and *HS6ST1* was elevated in OA cartilage. Most notably, a significant increase was observed in the level of 6-O-sulfated disaccharides in OA cartilage, which correlated strongly with expression of both *HS6ST1* and *GLCE*. Epimerization of GlcA to IdoA by GLCE is thought to favor subsequent 6-O-sulfation,⁴³ suggesting that these two enzymes drive the increase in 6-O-sulfation in OA cartilage. Previous studies have shown that levels of 6-O-sulfation are spatially and temporally regulated during development,^{18,19,21} and in diseases such as fibrosis,^{44,45} Alzheimer disease,²² and cancer.^{46,47} In the case of fibrosis, a doubling in 6-O-sulfation is thought to exacerbate disease by amplifying TGF- β ⁴⁴ and FGF2⁴⁵ signaling. We hypothesized that increased 6-O-sulfation in OA cartilage may similarly amplify downstream signaling pathways and alter tissue homeostasis. Consistent with this, using primary OA chondrocytes isolated from five different donors, it was found that siRNA silencing of *HS6ST1* suppressed ERK phosphorylation in response to FGF2. This is in line with previous studies showing that 6-O-sulfation of HS promotes

Figure 6 6-O-sulfation increases in osteoarthritic (OA) cartilage. Heparan sulfate (HS) was isolated from normal and OA cartilage and its composition analyzed by disaccharide analysis. **A:** Forest plot showing there is no significant difference between the ages and sexes of normal and OA cartilage donors used for HS analysis. Bars show 95% CIs of the mean, dotted line shows $Y = 0$. **B:** The total amount of HS in OA cartilage is not statistically different from that in normal cartilage. **C:** The ratio of sulfated/total HS is not statistically different between the two groups of samples. **D:** Abundance of individual disaccharide species is not significantly different in normal and OA cartilage. **E:** 6-O-sulfated HS disaccharides are more abundant in OA cartilage, whereas levels of N- and 2-O-sulfation are not significantly altered. **F:** The level of 6-O-sulfation in donor cartilage correlates with expression of *HS6ST1* (normal cartilage shown in black, OA cartilage in gray, males as squares, female samples as circles). **G:** The level of 6-O-sulfation in donor cartilage also correlates with expression of *GLCE* (normal cartilage shown in black, OA cartilage in gray, males as squares, female samples as circles). **H:** Expression of *HS6ST1* in primary OA chondrocytes is silenced using siRNA. **I:** Silencing of *HS6ST1* in primary OA chondrocytes reduces phosphorylation of extracellular signal-regulated kinase (p-ERK) in response to fibroblast growth factor 2 (FGF2; 20 ng/mL, 10 minutes). $n = 8$ (**A**); $n = 15$ (**F** and **G**); $n = 5$ donors (**H**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (multiple t -tests, Benjamini, Krieger, and Yekutieli corrected). F, female; M, male; 2S, 2-O-sulfate; 6S, 6-O-sulfate; GlcNAc, N-acetyl glucosamine; GlcNS, N-sulfoglucosamine; NT, transfected with nontargeting siRNA; UA, uronic acid.

FGF2 signaling,^{48–51} and confirms that changes in 6-O-sulfation can alter biologically relevant signaling pathways in cartilage. 6-O-Sulfation is known to alter HS affinity for a range of ligands,⁵² with varying effects on their downstream activity. For example, 6-O-sulfation promotes TGF- β ^{53,54} signaling and inhibits Wnt^{55,56} and BMP^{57,58} signaling. An unbiased approach, such as phosphoproteomics, will be required to identify pathways regulated by 6-O-sulfation in normal and OA cartilage.

The importance of 6-O-sulfation is underlined by the fact that it is the only HS modification that is regulated at both the on and the off level, being added during HS biosynthesis in the Golgi apparatus by HS6ST1, HS6ST2, and/or HS6ST3,⁴³ and removed in the extracellular environment by the 6-O-endosulfatases SULF1 and/or SULF2.⁵⁹ This permits remodeling of HS sulfation that is apparently critical to regulation of its functions. Indeed, increased expression of SULF1 and SULF2 was observed in OA cartilage, in line with previous reports examining both human and murine OA cartilage.^{7,9,11,58,60,61} Despite this, net levels of 6-O-sulfation were increased in our OA cartilage samples. As noted in recent studies from HS in muscle,²⁷ this highlights the importance of analyzing HS structure directly and may indicate post-translational regulation of HS6ST and/or SULF activity, or differences in the catalytic efficiency, location, or stability of the enzymes. *Sulf1*^{-/-} and *Sulf2*^{-/-} mice developed accelerated OA, and injection of recombinant SULF1 into the joint reduced cartilage damage,⁶² indicating the enzymes play a chondroprotective role. Elevated 6-O-sulfation may contribute to or perpetuate cartilage damage, potentially through driving anabolic pathways or impairing catabolic repair mechanisms.

No increase was seen in N-sulfation in OA cartilage, despite increased expression of NDST1 at the mRNA and protein level. The abundance of 3-O-sulfated disaccharides was not quantified because of their relative scarcity, but expression of *HS3ST1* was elevated in OA samples. Further investigation may be warranted as knowledge of the functional impact of 3-O-sulfation and the extent of the 3-O-proteome expands.^{63,64}

With regard to the HS core proteins, the previously reported increase in expression of HSPG2,^{6–9} glypican 1,¹¹ syndecan 1,^{9–11} and syndecan 3⁶⁵ was observed in OA cartilage, but not the previously observed increase in syndecan 4^{9,11–13} or decrease in agrin.⁶⁶ This may suggest species- or joint-specific variation in expression of these genes. In addition, the expression of many HS-associated genes was significantly altered by isolation and culture of chondrocytes (data not shown), suggesting that HS biosynthesis is dynamically regulated by the chondrocyte microenvironment and that expression may change during the course of OA development.

A significant reduction was observed in the expression of TGFBR3 in OA cartilage. This HS core protein, alias betaglycan, does not have the ability to signal directly, but

promotes TGF- β signaling by recruiting the growth factor and presenting it to the type II TGF- β receptor. In chondrocytes, TGFBR3 was recently shown to promote activation of latent TGF- β in a connective tissue growth factor- and HS-dependent manner.⁶⁷ Reduction in expression of TGFBR3 in OA is thus likely to impair the chondroprotective effects of TGF- β signaling.

Previous rodent microarray studies indicate that expression of some HS core proteins, biosynthesis, and modifying enzymes is regulated early in the course of OA development.^{7,8,11,68–72} For example, expression of *Ext1*,^{11,68} *Sdc1*,^{11,68,69} *Gpc1*,^{11,69} and *Hspg2*^{11,68} is significantly increased within 2 to 4 weeks of surgical OA induction (Supplemental Table S1),^{70–72} in line with our findings herein. Data on early changes in expression of sulfotransferases are variable, and early changes in *Hs6st1* were not reported. This may suggest that expression of this gene is stimulated once cartilage damage starts to escalate but may also reflect differences between rodent post-traumatic OA and more variable human disease.

Age is a primary risk factor for the development of OA, and it is important to understand the cellular mechanisms that increase risk with age if we are to develop effective therapies to treat the disease. Previous studies have shown that HS sulfation patterns change with age in various tissues, including the aorta,⁷³ myocardium,⁷⁴ muscle stem cell niche,²⁷ and brain,^{75,76} with concomitant changes in growth factor signaling. Although this study was not powered to detect changes in HS disaccharide composition with age, the expression of *HS3ST2* in cartilage was found to increase with age, in contrast to what has previously been reported in skin.⁷⁷ However, this observation is based on a low number of individuals ($n = 11$) because some values were missing for *HS3ST2* expression. Further analysis of age-dependent changes in HS structure may identify changes that impair cartilage repair with age and so increase the risk of OA after injury.

Our analysis identified 6-O-sulfation as the primary HS motif that is altered in end-stage OA cartilage, validating it as a new target for potential therapeutic interventions. To understand the impact this change has on cartilage repair and OA progression, it will be necessary to perform unbiased analyses of the 6-O-sulfate-binding proteome in normal and OA cartilage. Comparative mass spectrometry approaches have proved useful in examining the 3-O-sulfate-binding proteome in cultured cells,⁶⁴ and similar methods could be developed for cartilage analysis. Analysis of other joint tissues (eg, bone, synovium, meniscus) and earlier stages of OA progression can also be expected to shed further light on the role of HS in adult joint homeostasis.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.11.011>.

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