Down-regulated HS6ST2 in osteoarthritis and Kashin–Beck disease inhibits cell viability and influences expression of the genes relevant to aggrecan metabolism of human chondrocytes

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Abstract

Objective. Primary OA and Kashin–Beck disease (KBD) show similar pathological changes in articular cartilage. The objective was to screen differentially expressed genes between OA and normal cartilage, confirm the candidate gene expression among OA, KBD and normal cartilage, and then clarify its role in vitro.

Methods. Differentially expressed genes in OA cartilage were screened by suppression subtractive hybridization (SSH) and verified by real-time quantitative PCR (Q-PCR) analysis. Heparan sulphate 6-O-sulphotransferase 2 (HS6ST2) expression was identified by Q-PCR and immunohistochemistry. After suppressing HS6ST2 by RNA interference in C28/I2 human chondrocyte line, the effects were analysed through determining the cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), the aggrecan contents by toluidine blue staining and the mRNA expression levels of SRY-type high mobility group box 9 (SOX9), AGC1, MMP3, a disintegrin and metalloproteinase domain with thrombospondin motifs 4 (ADAMTS4) and ADAMTS5 by Q-PCR.

Results. HS6ST2 in the reverse subtraction library was identified as a down-regulated gene in OA and KBD at both mRNA and protein levels. The percentage of safranion O staining area was correlated positively with the percentage of HS6ST2-positive chondrocytes in OA and KBD cartilage. After HS6ST2-specific short interfering RNA (siRNA) transfection to C28/I2 cells, the cell viability was inhibited significantly, and the mRNA expression levels of SOX9 and AGC1 were reduced markedly, while MMP3 expression was increased significantly.

Conclusion. HS6ST2 down-regulation was identified in both OA and KBD cartilage. The findings first suggest that HS6ST2 may participate in the pathogenesis of OA and KBD by influencing aggrecan metabolism.

Key words: Osteoarthritis, Kashin–Beck disease, Suppression subtractive hybridization, Heparan sulphate 6-O-sulphotransferase 2.
Introduction

Primary OA is the most prevalent degenerative joint disease in the world and its aetiology remains unclear, though age, obesity, injury and physical activity, and other factors, have been considered as the risk factors for OA. Clinically, OA manifests as arthralgia, joint stiffness and dysfunction based primarily on pathological changes in affected articular cartilage. Kashin–Beck disease (KBD) is a chronic, endemic degenerative osteochondropathy in China and other countries of Asia. Thus far, there are three hypotheses to explain the environmental aetiology of KBD [1–3]: endemic selenium deficiency, serious cereal contamination by mycotoxin-producing fungi and high humic acid levels in drinking water. Nonetheless, none of them has obtained epidemiological and experimental evidence, so it has been indiscriminately considered as a multifactorial disease [4, 5]. Importantly, KBD shares its pathology and semeiology with OA, especially at the advanced stage of the disease [6]. Apoptosis and necrosis of chondrocytes and predominant degradation of extracellular matrix (ECM) in affected articular cartilage, for example, have been observed in both OA and KBD [7, 8]. Therefore, KBD is regarded as a specific type of OA with a rare aetiology [9], although there is a different gene expression pattern in KBD compared with OA [10].

The pathological destruction of OA cartilage is caused by an imbalance between the synthesis and degradation of ECM, but its underlying molecular mechanisms remain unknown [11]. It is plausible that the genes susceptible to the disease are present and the deregulation of gene expression is involved in the pathogenesis of the disease [12]. Thus, gene expression analysis can provide promising information to understand the pathogenesis of OA. In recent years, microarray analysis has been commonly used to study the gene expression profile in OA. Many differentially expressed genes have been identified from disease cartilage, including the anabolic and catabolic matrix genes such as COL1A1, COL2A1, MMPs, a disintegrin and metalloproteinase domain with thrombospondin motifs (ADAMTSs) and oxidative defence genes such as SOD3, GPx3 [13–15]. Compared with the microarray analysis, the suppression subtractive hybridization (SSH) technique offers greater reliability [16] and an opportunity to detect the unknown genes, non-coding RNAs and the genes with low expression [17, 18]. Therefore we used SSH to screen differentially expressed genes in OA cartilage.

In this study we used SSH technology to construct the forward and reverse libraries of differentially expressed genes in OA cartilage compared with normal control. Then we identified heparan sulphate 6-O-sulphotransferase 2 (HS6ST2) as a candidate gene from the reverse subtraction library. After confirming the differential expression of HS6ST2 among OA, KBD and normal cartilage, we further investigated the effects of the HS6ST2 gene on the behaviour of human chondrocyte in vitro. To our knowledge, this is first time HS6ST2 expression decreased in OA and KBD cartilage and its gene knockdown can reduce the cell viability and alter the expression of cartilage-related genes.

Materials and methods

Patients and articular cartilage collection

Primary OA patients were diagnosed according to the ACR classification criteria for OA of the knee [19]. All OA patients came from non-KBD-endemic areas of Shaanxi province, China. The patients with Grade II or III KBD were selected on the basis of the clinical criteria for the diagnosis of KBD in China (diagnostic code GB16395-1996) [6], and they were from the KBD-endemic areas of Linyou and Yongshou counties, Shaanxi province, China. Normal donors, who died in traffic accidents, were from non-KBD areas and had no history of joint diseases, such as genetic bone and cartilage diseases, OA and RA. This study was performed with the approval of the Ethical Committee of the Xi’an Jiaotong University College of Medicine, and each patient or relative of a donor provided informed consent.

Specimens of OA and KBD cartilage were obtained from patients undergoing total knee replacement surgery. Normal cartilage specimens were obtained at autopsy within 8 h of death, and the integrity of the cartilage was evaluated by haematoxylin and eosin (H&E) staining. All the cartilage specimens were obtained from the same anatomic area of medial and lateral femoral condyles and tibial articular surfaces. For RNA extraction, cartilage samples were chopped into 2–5 mm pieces by a razor blade, frozen in liquid nitrogen immediately and then stored at −80°C.

For the SSH experiment, RNA was pooled from three patients with OA (two males and one female; ages 51, 58, and 47 years, respectively) and two normal donors (one male and one female; ages 55 and 39 years, respectively) to avoid individual differences responsible for subtraction results [20]. For the verification experiment, the OA group consisted of eight patients (four males and four females; age range 50–69 years); the KBD group consisted of six patients (three males and three females; age range 39–66 years); and the normal group consisted of four normal donors (three males and one female; age range 39–66 years), two cases of which were also used for the SSH experiment.

RNA isolation and SSH

Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. mRNA was isolated with PolyATtract mRNA Isolation Systems (Promega Corporation, Madison, WI, USA).

The SSH experiment was performed with a Clontech PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer’s protocol. This procedure was performed on a PTC-100 Peltier thermal cycler (BIO-RAD, Hercules, CA, USA). Briefly, double-stranded cDNA (dscDNA) of each group was synthesized from 1.5 μg mRNA by the avian myeloblastosis virus (AMV) reverse transcriptase. The forward-subtracted cDNA library included highly expressed genes in OA; dscDNA from OA cartilage was defined as the tester and from
normal control as the driver. The reverse-subtracted cDNA library included lowly expressed genes in OA; dscDNA from normal control was defined as the tester and from OA cartilage as the driver. The testes and drivers were digested by Rsa I to generate shorter, blunt-ended cDNA fragments. Next, each tester was divided into two equal parts: one was ligated with Adaptor 1, and the other was ligated with Adaptor 2 R, and then hybridized, respectively, to excessive and freshly denatured driver at 68°C for 8 h. During the second hybridization, these two hybridized cDNA and freshly denatured driver cDNA were simultaneously mixed together. The mixture was hybridized at 68°C overnight. Subsequently the reaction mix was incubated at 75°C for 5 min, and then the first and the nested PCR amplifications were performed in order by using adaptor-specific primers. At last, the sequences overexpressed in the tester were amplified. The subtraction efficiency was estimated by comparing the abundance of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) before and after subtraction. GAPDH products in subtracted samples should be detected at ~5–15 cycles later than those in unsubtracted samples. The information of adaptors and primers is depicted in Table 1.

Construction and analysis of the subtracted cDNA libraries

Freshly prepared 15-cycle secondary SSH-PCR products from the forward and reverse subtractions were cloned into pGEM-T-Easy II Vector by using the T/A Cloning Kit (Promega, Madison, USA), and then the recombinants were transformed into DH5α super-competent cells. Individual transformants were cultured on LB/X-gal/IPTG agar plates with ampicillin at 37°C for 16 h, and then white colonies were isolated randomly. Plasmids were isolated with the Plasmid Minipreps Purification system (BioDev-Tech, Beijing, China) and the inserts were sequenced by the company (AuGCT Biotechnology, Beijing, China). The sequences were analysed for similarity to the sequences in GenBank/EMBL/DDJB/PDB and expressed sequence tag (EST) databases by using the basic local alignment search tool (BLAST) program (http://www.ncbi.nlm.nih.gov/BLAST).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Adaptor and primer sequences in SSH</th>
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<tr>
<td>Name</td>
<td>Sequence (5′-3′)</td>
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<tr>
<td>cDNA synthesis primer</td>
<td>TTTTGTACAAGCTT30N1N</td>
</tr>
<tr>
<td>Adaptor 1</td>
<td>CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT</td>
</tr>
<tr>
<td>Adaptor 2 R</td>
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<tr>
<td>Nested PCR primer 2 R</td>
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</tr>
<tr>
<td>GAPDH 5′ primer</td>
<td>ACCACAGTCCATGCCATCAC</td>
</tr>
<tr>
<td>GAPDH 3′ primer</td>
<td>TCCACACCCTGTGCTGTA</td>
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RNA quantification

cDNA was synthesized from 2 μg total RNA using the First-Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, Canada). mRNA quantification was performed by using Q-PCR on an iQ5 system (Bio-RAD, Hercules, CA, USA) with SYBR Premix Ex Taq II (TakaRa, Ohtsu, Shiga, Japan). Gene expression was normalized by GAPDH. The cycle parameters were as follows: denaturing for 15 s at 95°C, annealing for 20 s at a specific temperature, and extending for 20 s at 72°C for a total of 40 cycles. Information on primers and products is depicted in Table 2.

Histopathological examinations

Knee cartilage was fixed in 4% neutral buffered formalin, decalcified in 14% EDTA, paraffin-embedded and then dissected at 5 μm thickness. The sections were stained with H&E or safranin O/fast green.

Immunohistochemistry was performed with the Non-Biotin HRP Detection System (Zhongshan, Beijing, China). The paraffin-embedded sections were deparaffinized, rehydrated and then covered with 3% H2O2 at room temperature for 10 min. After washing in PBS, the slides were pre-incubated with 5% BSA at room temperature for 20 min and then incubated with 1:80 diluted rabbit anti-HS6ST2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. After washing in PBS, the sections were incubated with the secondary antibody of goat anti-rabbit IgG (Zhongshan, Beijing, China) at 37°C for 30 min. Finally, colour reaction was developed by the substrate reagent 3',3'-diaminobenzidine tetrahydrochloride (DAB) (Zhongshan, Beijing, China) and the sections were counterstained with haematoxylin. As a negative control, normal serum of rabbit was used instead of anti-HS6ST2 antibody.

In order to determine the aggrecan content, C28/I2 chondrocytes at 72 h after transfection with HS6ST2-siRNA or NC-siRNA were fixed with 4% paraformaldehyde for 20 min and stained with 1% toluidine blue (Sigma, St Louis, MO, USA) for 5 min.

Images of stained sections were obtained through an optical microscope (Olympus BX51, Tokyo, Japan)
equipped with a digital camera (Olympus PD71, Tokyo, Japan). The percentage of HS6ST2-positive chondrocytes, the safranin O staining area in articular cartilage and the density of toluidine blue staining in cell culture were analysed by Image-Pro Plus 6.0 software. Four different areas were chosen randomly for each section, and mean density and area thresholds were defined.

Cell culture and transfection with HS6ST2 siRNA in chondrocytes

C28/I2 chondrocytes, a human cell line (kindly provided by Dr Mary B. Goldring from the Harvard Institutes of Medicine, Boston, MA, USA), were cultured in DMEM–Ham’s F-12 with a ratio of 1 : 1 (v/v) (DMEM/F12; Hyclone, Logan, UT, USA) containing 10% fetal calf serum (FCS; Hyclone, Logan, UT, USA) as previously described [21].

siRNAs specific to HS6ST2 were designed that relied on the coding sequence of human HS6ST2, transcript variant L. Three siRNA sequences were CCUCUCUGUCAUCGCUGCU GAAtt, named HS6ST2-siRNA1; GCAAAAGAAACAAGGCUCUUt, named HS6ST2-siRNA2; and CAGCUAUUGCACAAA GACCUUUt, named HS6ST2-siRNA3. An oligonucleotide, UUCUCGGAAAAGGUGUCGCUUt has been used as a negative control, named NC-siRNA. The oligonucleotides were designed and synthesized by the company (Genechem, Shanghai, China). A fluorescein-labelled NC-siRNA (GenePharma, Shanghai, China) was used to evaluate transfection efficiency. Transfection of siRNAs to the cells was performed with LipofectamineTM 2000 reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer’s instructions. Briefly, 1 day before transfection, exponentially growing cells were seeded into six-well plates at a density of $1.5 \times 10^5$ cells/well in the medium without antibiotics. When they reached ~70% confluence, cells were transfected with two doses (40 and 60 nM) of siRNA using Lipofectamine 2000. Transfection efficiency was evaluated with fluorescein NC-siRNA by the fluorescence microscopy 6 h after transfection. After 24 and 48 h, total RNA was isolated and the expression levels of the relative transcripts were detected by Q-PCR.

Cell viability assay

Cell viability after transfection of siRNA was determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. One day before transfection, the cells were seeded into 96-well plates (6 $\times 10^3$ cells/well). Cells were transfected with HS6ST2-siRNA or NC-siRNA. At 24 and 48 h after transfection, culture medium was removed and 20 ml MTT solution (5 mg/ml in PBS) dissolved in 200 ml DMEM was added into each well and incubated at 37°C for 4 h. The supernatant was then carefully removed and 150 ml DMSO (Sigma, St Louis, MO, USA) was added to each well. The spectrophotometric absorbance was measured at a wavelength of 490 nm by a microtiter reader (Thermo Electron Corporation, Vantaa, Finland). Each experiment was performed three times and the representative results are shown in Figs 1 and 2.

Statistical analysis

Data were expressed as the mean (S.E.M.). The statistical analysis of differences between experimental groups was performed by Mann–Whitney U test or Student’s t-test. One-way analysis of variance (ANOVA) was used to analyse the differences among three experimental groups. Correlation analysis between the percentage of safranin O staining area and HS6ST2-positive chondrocytes was
Results

Electrophoresis of undigested and Rsa I-digested cDNA in 2% agarose gel showed that the undigested cDNA appeared as a smear from 0.5 to 10 kb and the average cDNA size was smaller (0.1–2 kb) after Rsa I digestion, indicating that tester and driver cDNAs from each experiment were digested successfully (Fig. 3A). The nested PCR products from both the forward and reverse subtracted samples displayed clearly ladder-like bands, in contrast to the smear without clear bands from the unsubtracted samples, suggesting that differentially expressed sequences were enriched (Fig. 3B). GAPDH PCR products appeared first at 30 cycles in the unsubtracted cDNA, but at 40 cycles in the subtracted cDNA, indicating successful subtraction in both the forward and reverse directions (Fig. 3C).

From the forward and reverse subtraction libraries, 215 and 210 positive clones, respectively, were randomly chosen. Then 9 of 210 positive clones from the reverse library were randomly selected to perform sequence analysis and a similarity search with the BLAST program. Three known genes were identified as Homo sapiens heparan sulphate (HS) 6-O-sulphotransferase 2, transcript variant L (HS6ST2), SHC SH2-domain-binding protein 1 (SHCBP1) and Scm-like with four mbt domains 2 (SFMBT2). The Q-PCR results showed that the expression of SFMBT2 and SHCBP1 had no significant difference between the OA and normal groups in spite of a lower trend in the OA group (data not shown). HS6ST2 expression determined by Q-PCR was significantly lower in OA ($P < 0.01$) and KBD cartilage ($P < 0.05$) than in normal controls, but no significant difference was found between the OA and KBD groups ($P > 0.05$) (Fig. 4A). In addition, HS6ST2 protein expression was determined by immunohistochemistry. In normal cartilage, HS6ST2 was expressed mainly in the cytoplasm and cell membrane of chondrocytes that stained brown, and positive chondrocytes were present predominantly in the superficial zone and the upper-middle zones (Fig. 4B). In contrast, much fewer positive chondrocytes could be

**Fig. 1** Effects of HS6ST2 siRNA on cell viability and SOX9 expression in chondrocytes. (A) Interfering efficiency determined by Q-PCR after HS6ST2 siRNA at 40 nM (left panel) and 60 nM (right panel) transfection. Levels of significance were calculated by one-way ANOVA. (B) Cell viability detected by MTT assay with a set of five wells for each experiment. (C) The mRNA expression levels of SOX9. Relative transcript levels were normalized to GAPDH and values are the mean (s.e.m.) of three independent experiments with a set of two wells for each experiment. Levels of significance were calculated by Student’s $t$-test. *$P < 0.05$ and **$P < 0.01$ between HS6ST2-siRNA1 and NC-siRNA groups.
found in OA and KBD cartilage, especially in OA cartilage (Fig. 4C). The statistical result showed that the percentage of positive chondrocytes was decreased significantly in groups of OA ($P < 0.01$) and KBD ($P < 0.05$) compared with normal controls (Fig. 4D).

A percentage of safranin O staining area was used to evaluate the presence of abundant aggrecan among three groups (Fig. 5A). This percentage decreased dramatically in OA and KBD cartilage compared with normal cartilage ($P < 0.001$). However, no significant difference was found between the OA and KBD groups ($P > 0.05$; Fig. 5B). The correlation analysis showed a positive relationship between the percentage of safranin O staining area and HS6ST2-positive chondrocytes in the samples of OA and KBD cartilage ($r = 0.77$, $P < 0.001$) (Fig. 5C).

C28/I2 chondrocytes were transfected with three different HS6ST2 siRNA oligonucleotides. Nearly 90% of cells were transfected at 6 h after transfection, indicating high transfection efficiency. Q-PCR results at 24 h after transfection showed that HS6ST2 expression was not inhibited at 40 nM of HS6ST2-siRNA, while the gene abundance was knockdowned by 70% at 60 nM of HS6ST2-siRNA1 vs NC-siRNA group ($P < 0.01$; Fig. 1A). So, HS6ST2-siRNA1 at a dose of 60 nM as an interfering sequence and NC-siRNA at a dose of 60 nM as a negative control were applied in the following experiments.

Cell viability in the HS6ST2-siRNA1 group was significantly decreased at 48 h after transfection ($P < 0.01$), but no significant difference was found between the HS6ST2-siRNA1 and NC-siRNA groups at 24 h after transfection ($P > 0.05$), indicating that HS6ST2 affected cell viability more severely at a relatively later stage (Fig. 1B). The mRNA expression level of SRY-type high mobility group box 9 (SOX9), the key transcription factor known to be relevant to chondrocyte phenotypic stability, was considerably down-regulated at 24 h after HS6ST2-siRNA1 transfection ($P < 0.05$; Fig. 1C).

The mRNA expression level of AGC1 encoding the core protein of aggrecan was remarkably decreased in the HS6ST2-siRNA1 group compared with the NC-siRNA group at 48 h after transfection ($P < 0.01$) (Fig. 2A). Moreover, the chondrocytes in the HS6ST2-siRNA1 group produced less aggrecan as determined by toluidine blue staining at 72 h after transfection. After image analysis, the mean density of the staining in the
HS6ST2-siRNA1 group was significantly lower than that of the NC-siRNA group (P < 0.05) (Fig. 2B). We also determined the mRNA expression level of MMP3, ADAMTS4 and ADAMTS5, which is relevant to the degradation of aggrecan in cartilage, and found that MMP3 expression was increased significantly at 24 h after HS6ST2-siRNA1 transfection (P < 0.001), but ADAMTS5 expression was decreased significantly at 48 h after transfection (P < 0.01). ADAMTS4 expression in the HS6ST2-siRNA1 and NC-siRNA groups showed no significant difference at two time points after the transfection (P > 0.05) (Fig. 2C).

Discussion
In this study we constructed the forward and reverse libraries of differentially expressed genes in OA cartilage, and identified HS6ST2 in the reverse library as a down-regulated gene associated with the pathological development of OA and KBD. Interfering HS6ST2 gene expression in human chondrocytes inhibited cell viability and altered some phenotypes of chondrocytes.

HS6ST2 mapped to Xq26.1 of the human genome belongs to the HS6STs family and encodes two forms (an original long form, hHS6ST-2, and a short alternative spliced form, hHS6ST-2 S) of HS6ST2. Both forms of HS6ST2 have similar enzymic activity and catalyse the transfer of sulphate from adenosine 3'-phosphate 5'-phosphosulphate (PAPS) to the sixth position of the N-sulphoglucosamine residue in HS, which is attached to the core protein in HS proteoglycans (HSPGs) [22]. HSPGs not only are a major component of ECM, but also reside on the plasmatic membrane of all animal
In addition, HSPGs via the 6-O-sulfation of HS bind to many heparin-binding factors that are important regulators of chondrocytes, such as fibroblast growth factor (FGF), transforming growth factor (TGF), Wnt proteins and bone morphogenetic proteins (BMPs) [24–26]. This binding serves to protect these factors from proteolysis to enhance biological stability, and provides a reservoir of growth and migration factors that can be mobilized in accordance with physiological demand. On the other hand, cell-surface HSPGs act as mandatory co-receptors for heparin-binding growth factors to trigger the signal transduction pathway [27].

To date, few studies have been reported about the distribution of HS6ST2 in cartilage. Our study showed that HS6ST2 is highly expressed in normal knee articular cartilage. Positive chondrocytes were predominantly localized in the superficial and the upper-middle zones, implicating its expression in the zone-specific differences of chondrocyte subsets [28, 29]. Recent studies have demonstrated that HS6ST2 is associated with malignant tumours, such as ovarian tumours and lung cancer [30, 31]. In our study, we found that HS6ST2 expression was significantly down-regulated in OA and KBD cartilage, suggesting that HS6ST2 could be involved in OA and KBD pathogenesis. Our data also showed that the samples of OA and KBD cartilage had a low aggrecan content, and the percentage of safranion O staining area was positively correlated with the percentage of HS6ST2-positive chondrocytes. Low sulfation of glycosaminoglycan (GAG) on chondroitin sulphate and dermatan
sulphate have been observed in the cartilage of the elderly and OA patients [32], but it is still unknown whether a change in heparan sulfation induced by HS6ST2 could be involved in the pathogenesis of OA and KBD. Our study showed that both diseases had similar sulfation disturbances induced by a reduction in HS6ST2. A previous study showed that low heparan sulfation may exist in OA cartilage since HS 6-O-endosulfatase (sulf1, sulf2), enzymes to hydrolyse 6-O-sulphate from HS, are highly expressed in OA cartilage [33]. It has been reported that the increased level of sulf2 or decreased expression of HS6ST2, whose function is opposite to sulf2, may activate the GAG degradation pathway [34, 35]. Future studies require verifying HS sulfation and its relationship with HS6ST2 activity. But it is necessary to know the effects of HS6ST2 on chondrocyte biology.

Therefore we used RNA-interfering technology to knock down HS6ST2 expression in C28/I2 chondrocytes. We found that cell viability was reduced after interfering with HS6ST2 expression. Previous studies showed that FGFs, the most potent mitogens for chondrocytes [36], stimulate the cell proliferation mediated by HSPGs, and 6-O-sulfation of HS is required for receptor dimerization for FGF signalling [37]. In particular, FGF-4 activity is most strongly affected by the 6-O-sulfation of HS [38]. Loss of 6-O-sulfotransferase can reduce the affinity of FGFs to HS, and consequently decrease the effects of FGF signalling. We found that HS6ST2 may affect cell viability through the...
HS6ST2 down-regulation in the cartilage of OA and KBD

FGF signalling pathway. SOX9, a transcription factor of the SRY family, is considered a major transcription factor relevant to chondrocyte phenotypic stability, differentiation [39] and proliferation [40]. It is also essential for regulating the expression of cartilage-specific genes such as aggrecan. After HS6ST2 suppression in our experiments, SOX9 mRNA expression was significantly decreased earlier than the inhibition of cell viability, the decrease of AGC1 expression and the reduction of aggrecan content, suggesting that HS6ST2 affected cell viability and aggrecan expression via regulating SOX9 expression. Our data were consistent with a previous study that SOX9 homozygous mutant cells are unable to express aggrecan [41]. It has been reported recently that SOX9 down-regulation may induce angiogenesis, cartilage resorption and formation of bone marrow and endochondral bone trabeculae [42], which are associated with the progression of OA. In addition, several signalling pathways can regulate SOX9 expression. For example, FGFs can enhance SOX9 expression in chondrocytes mediated by the MEK-MAPK pathway [43], and the Wnt signalling pathway can repress SOX9 transcription [44].

In our experiment, suppressing HS6ST2 increased MMP3 expression. MMP3 may be linked specifically to the loss of proteoglycan [45]. It has been suggested that a common regulation of anabolic and catabolic metabolism of cartilage is based on the correlation between the expression levels of aggrecan and MMP3 [46]. Our data also demonstrated that HS6ST2 may regulate aggrecan degradation through MMP3, for ADAMTS4 expression in the HS6ST2–siRNA1 and NC-siRNA groups was significantly decreased earlier than the inhibition of cell viability, the decrease of AGC1 expression and the reduction aggrecan content, suggesting that HS6ST2 affected cell viability and aggrecan expression via regulating SOX9 expression.

In conclusion, the current study demonstrates that HS6ST2 participates in the pathogenesis of OA and KBD. Knock down of HS6ST2 expression can inhibit cell viability and affect the expression of genes relevant to aggrecan metabolism in human chondrocytes. The findings provide the experimental evidence to elucidate the role of HS6ST2 in the pathogenesis of OA and KBD.

Rheumatology key messages
- HS6ST2 is down-regulated in OA and KBD cartilage.
- HS6ST2 may participate in the pathogenesis of OA and KBD through influencing aggrecan metabolism.

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