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Expression profiles of genes encoding glycosyltransferases synthesizing the glycosaminoglycan linker region during chondrogenic differentiation of ATDC5 cells

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Abstract

Several glycosaminoglycans (GAGs) are covalently linked to specific serine residues in the core proteins of proteoglycans at the GAG–protein linker region, synthesized by five glycosyltransferases, each encoded by *Xylt1*, *Xylt2*, *B4galt7*, *B3galt6*, and *B3gat3*. We quantitatively analyzed the mRNA expression levels of these five genes in the undifferentiated, differentiated, and well-differentiated stages of ATDC5 cell line, which enables the monitoring of chondrogenic differentiation. *Xylt1* and *B4galt7* expressions were markedly increased in the well-differentiated cells, whereas *B3galt6* expression was slightly inhibited. In contrast, *Xylt2* and *B3gat3* expressions remained unaltered across the three stages. These results help us better understand the regulatory mechanisms of the linker region synthesis in chondrocytes. ATDC5 is a useful *in vitro* model for examining these mechanisms.

Key Words: Chondrocyte, Glycosaminoglycan linker region, Glycosyltransferase

Glycosaminoglycans (GAGs), including chondroitin sulfate (CS), dermatan sulfate (DS), and heparan sulfate (HS), are linear polysaccharides that form the side chains of proteoglycans (PGs)[5,16,18,21]. PGs are ubiquitous macromolecules present in the extracellular matrix (ECM) and at the cell surface[11,24]. GAGs are involved in numerous functions, including regulation of the mechanical properties of a tissue, cell proliferation, cell adhesion, growth factor signaling, and immune cell function[5,16,18,21]. CS, DS, and HS chains consist of repeating disaccharide units, namely, glucuronic acid (GlcA)–*N*-acetyl-galactosamine (GalNAc), iduronic acid–GalNAc, and GlcA–*N*-acetyl-glucosamine. These disaccharide chains are covalently linked to specific serine (Ser) residues in the core proteins of PGs through the so-called GAG–protein linker region, the tetrasaccharide GlcA–galactose (Gal)–Gal–xylose (Xyl) (Fig. 1). Biosynthesis of the linker region is initiated by the transfer of a Xyl from uridine diphosphate (UDP)-Xyl to the specific Ser residues by xylosyltransferases (XylTs) encoded by *XYLT1* and *XYLT2* in the endoplasmic reticulum[5,16,18,21]. Subsequently, galactosyltransferase-I (GalT-I),

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Table 1. Primer list used in this study

Gene name	Gene bank number	Primer sequence (5' - 3')	Product Size
Hprt1	NM_013556.2	Fw : CTGGTGAAAAGGACCTCTCGAA Rv : CTGAAGTACTCATTATAGTCAAGGGCAT	110
Acan	NM_001361500.1	Fw : GCAGAAACAACCATGTCCCT Rv : TTGAGAGGCACTCGTCAATG	103
Col2a1	NM_001113515.3	Fw : TGGTGACAAGGGAGAAAAGG Rv : AGTTTCCTGCACTAAGGCCA	128
Col10a1	NM_009925.4	Fw : CATAAAGGGCCCACTTGCTA Rv : ACCAGGAATGCCTTGTTCTC	100
Xylt1	NM_175645.3	Fw : GACCTTTTCCAACAAGCAGC Rv : GACTTGGGCAGGATTGATGT	139
Xylt2	NM_145828.3	Fw : ATCCAACGAGGACGAATGAG Rv : CCAGGCCTTGCTTCTTAATG	114
B4galt7	NM_001311137.1	Fw : TTGAGGAGCTGCTGGTCTTT Rv : GGAAGCCCACATTGATGAGT	135
B3galt6	NM_080445.4	Fw : CGAGGCTGCAACAATCAGTA Rv : ACATAGGAAAGGCGCAACTG	128
B3gat3	NM_024256.2	Fw : AGTTGCAAGCTGATCTCCGT Rv : TCTGCCTTTTGTACCAGCCT	118

Hprt1 was used as a reference gene.
Hprt1, hypoxanthine phosphoribosyltransferase 1; *Acan*, aggrecan; *Col2a1*, collagen, type II, alpha 1; *Col10a1*, collagen, type X, alpha 1; *Xylt1*, xylosyltransferase 1; *Xylt2*, xylosyltransferase 2; *B4galt7*, beta-1,4-galactosyltransferase 7; *B3galt6*, beta-1,3-galactosyltransferase 6; *B3gat3*, beta-1,3-glucuronyltransferase 3.

galactosyltransferase-II (GalT-II), and glucuronosyltransferase-I (GlcAT-I), which are encoded by *B4GALT7*, *B3GALT6*, and *B3GAT3*, respectively, transfer two Gals and a GlcA from UDP-Gal and UDP-GlcA to the Xyl residue. The elongation and sulfation of each GAG disaccharide unit were caused by GAG-specific glycosyltransferases and sulfotransferases, respectively.

GAGs are abundant in the cartilage[10,11]. The cartilage ECM primarily comprises two components: type II collagen and aggrecan. Aggrecan is a large PG containing numerous CS chains. Therefore, CS is the most abundant GAG in the cartilage. Although chondrocytes produce most of the cartilage ECM, including GAGs, the chondrogenic differentiation stage-dependent expression of the glycosyltransferase genes involved in the GAG–protein linker region synthesis has hardly been investigated. Therefore, we sought to investigate these aspects using the mouse prechondrogenic ATDC5 cell line, which is a useful *in vitro* model for monitoring the multiple-step differentiation of chondrocytes[2,19,20,23]. Chondrogenic differentiation of ATDC5 cells was induced by insulin and enhanced further by treatment with bone morphogenetic protein (BMP). We measured the mRNA expression levels of *Xylt1*, *Xylt2*, *B4galt7*, *B3Balt6*, and *B3galt3* in the undifferentiated, differentiated, and well-differentiated stages of this cell line.

The ATDC5 cell line was obtained from RIKEN Cell Bank (Tsukuba, Japan). The cells were maintained in Dulbecco's Modified Eagle Medium/Ham's F12 (1:1) medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5% fetal bovine serum and antibiotics (100 U/ml penicillin-G and 100 mg/ml streptomycin), hereinafter the control medium (CM), at 37°C in a humidified 5% CO_2 atmosphere. To induce chondrogenic differentiation, ATDC5 cells were seeded at confluency and cultured in the CM plus ITS (10 mg/l insulin, 5.5 mg/l transferrin, and 5 μg/ml selenium; Sigma Aldrich), hereinafter the differentiation medium (DM), for 10 or 15 days. The medium was changed every 2–3 days. To strongly induce differentiation, BMP-4 was added to the DM at 100 ng/ml. BMP-4 was purchased from Cosmo Bio Co., Ltd (Tokyo, Japan). Total RNA was isolated from ATDC5 cells using RNAiso Plus (Takara Bio, Otsu, Japan).

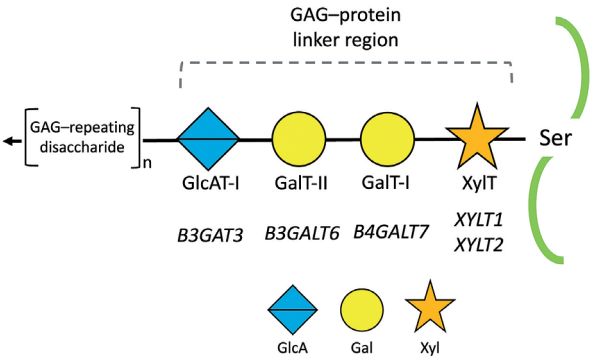

Fig. 1.
Structure of the glycosaminoglycan (GAG)–protein linker region. Several GAG repeating disaccharide units are linked covalently to specific serine (Ser) residues in the core proteins of proteoglycans (PGs) through the GAG¬–protein linker region, the tetrasaccharide glucuronic acid (GlcA)–galactose (Gal)–Gal–xylose (Xyl). The transfer of a Xyl to specific Ser residues on the core proteins of PGs was mediated by xylosyltransferases (XylT) encoded by *XYLT1* and *XYLT2*. Subsequently, galactosyltransferase-I (GalT-I), GalT-II, and glucuronosyltransferase-I (GlcAT-I), which are encoded by *B4GALT7*, *B3GALT6*, and *B3GAT3*, respectively, transfer two Gals and a GlcA to the Xyl residue.

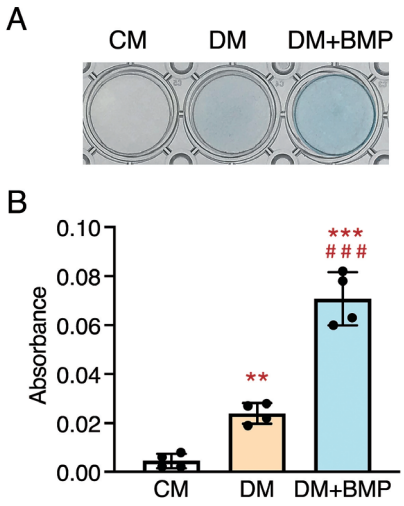

Fig. 2.
Alcian blue staining of ATDC5 cells cultured in the control medium (CM), in the CM plus ITS (differentiation medium: DM), and in the DM plus BMP-4, for 15 days. (A) The stained culture plates. (B) Alcian blue staining was extracted using 6 M guanidine/HCl, and the absorbance of the extracts was measured. Data are expressed as the mean ± SD (n = 4). *P*-value was determined using one-way ANOVA followed by Tukey–Kramer multiple comparison test. ** $P < 0.01$ and *** $P < 0.001$ vs. CM group; ### $P < 0.001$ vs. DM group.

Reverse transcription reactions were performed from 1 µg of total RNA using ReverTra Ace qPCR RT Master Mix (TOYOBO, Tokyo, Japan). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the LightCycler 96 System (Roche, Basel, Switzerland) and THUNDERBIRD SYBR qPCR Mix (TOYOBO). The primer sequences used are described in Table 1. The expression levels of the genes were normalized to those of *Hprt1*, which is a suitable reference gene for qRT-PCR during chondrogenic differentiation of ATDC5[25]. Relative quantification was performed using the ΔΔ CT method, and absolute quantification was performed using the standard curve method. Statistical analysis was performed using Graphpad Prism9 software (GraphPad Software, San Diego, CA, USA). *P* value was determined using one-way analysis of variance and analyzed further using the Tukey–Kramer multiple comparison test. $P < 0.05$ was considered statistically significant. The sulfated GAGs in the ECM produced by ATDC5 cells were visualized using Alcian blue staining. Cells were rinsed with phosphate-buffered saline, fixed with 4% paraformaldehyde (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for 10 minutes, stained with 0.1% Alcian blue 8GX (Merck Millipore, Darmstadt, Germany)/0.1 M HCl at room temperature overnight, and rinsed with distilled water. For quantification, the staining was extracted using 6 M guanidine HCl (FUJIFILM Wako Pure Chemical Corporation) for 2 h at room temperature. The absorbance of the extracts was measured at 630 nm using a V-630BIO UV–Vis spectrophotometer (JASCO Corporation, Tokyo, Japan).

As shown in Fig. 2, the Alcian blue staining intensity of the ATDC5 cells cultured in the DM for 15 days was higher than that in the CM. The addition of BMP-4 to the DM further enhanced the stain intensity. These results suggest that the production of ECM containing sulfated GAGs by ATDC5 cells increased with the addition of ITS to the CM and further increased with the addition of BMP-4 to the DM. Using qRT-PCR analysis, we measured the mRNA expression levels of

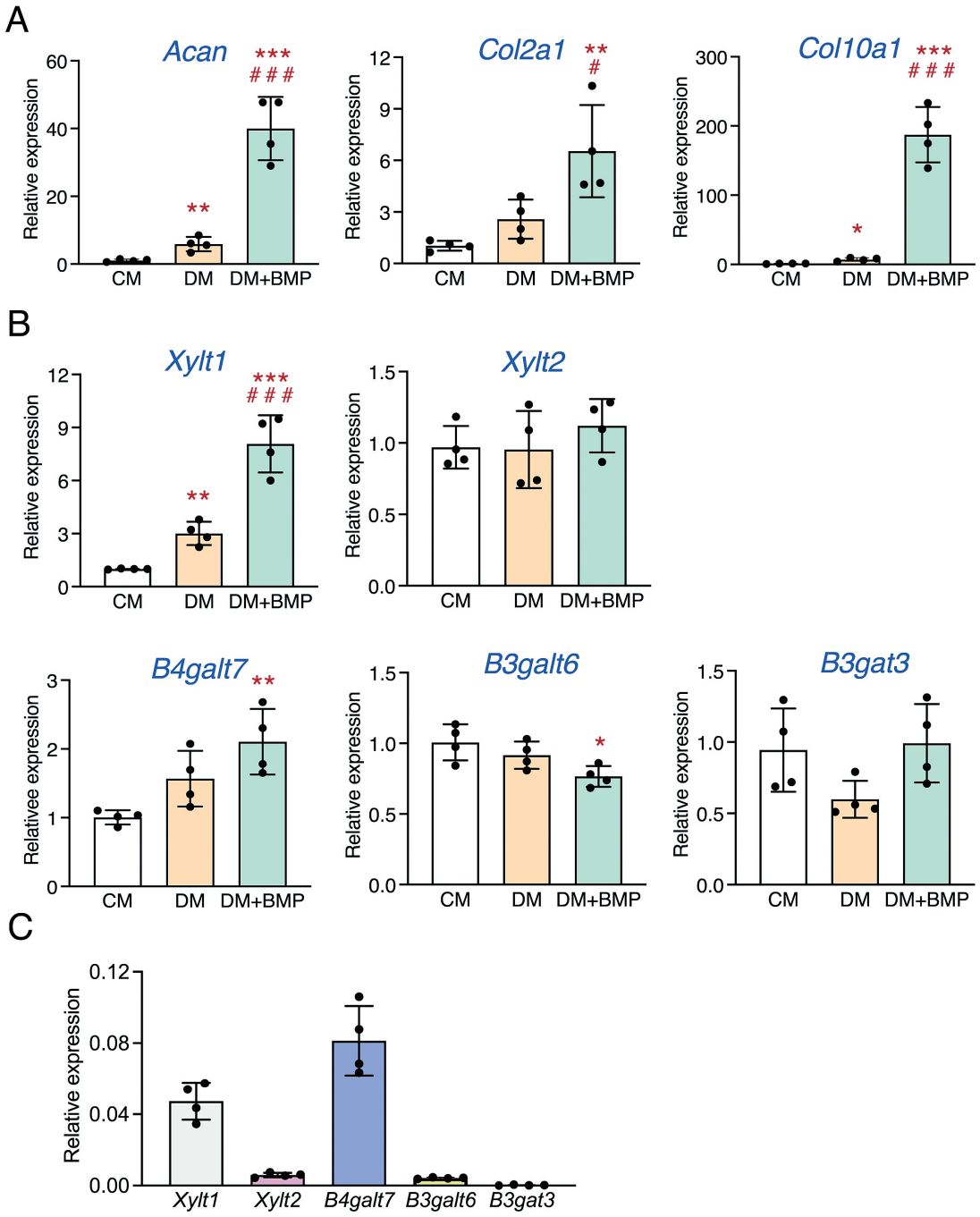

Fig. 3.
Measurement of mRNA expression levels by quantitative real-time PCR. (A) mRNA expression levels of three chondrocyte marker genes, *Acan*, *Col2a1*, and *Col10a1*, in the three differentiation stages of ATDC5 cells. (B) mRNA expression levels of genes encode the glycosyltransferases synthesizing the GAG linker region, *Xylt1*, *Xylt2*, *B4galt7*, *B6galt6*, and *B3gat3*, in the three differentiation stages of ATDC5 cells. (C) Comparison of the expression levels of the five genes in the well-differentiated ATDC5 cells cultured in the differentiation medium (DM) plus BMP-4. Undifferentiated ATDC5 cells were maintained in the control medium (CM). To induce chondrogenic differentiation, ATDC5 cells were cultured in the CM plus ITS (DM) for 10 days. To strongly induce differentiation, cells were cultured in the DM plus BMP-4 at 100 ng/ml. The relative quantification was performed using the ΔΔ CT method with *Hprt1* used as a reference gene (A, B). Absolute quantification was performed using the standard curve method, and the relative expression levels were expressed as the ratio of the absolute level of the gene to that of *Hprt1* (C). Data are expressed as the mean ± SD (n = 4). *P*-value was determined using one-way ANOVA followed by Tukey–Kramer multiple comparison test. * $P < 0.05$, ** $P < 0.01$, and ***$P < 0.001$ vs. CM group; $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ vs. DM group.

three chondrogenic differentiation marker genes, i.e., *Acan*, *Co12a1*, and *Col10a1*[7], in ATDC5 cells cultured in each medium for 10 days (Fig. 3A). Undifferentiated ATDC5 cells differentiate into chondrocytes expressing *Acan* and *Col2a1* and then to mature chondrocytes expressing *Col10A1*. The expression levels of these three genes in the cells in the DM plus BMP-4 were significantly higher than those in the CM or DM alone. The expression levels of *Acan* and *Col10a1* in the DM were significantly higher than those in the CM. The combined results of the two experiments clearly show that the chondrogenic differentiation of ATDC5 cells was induced by the addition of ITS to the CM and increased further with the addition of BMP-4 to the DM, as reported previously[19,23].

We measured the mRNA expression levels of the five genes encoding the glycosyltransferases synthesizing the GAG linker region (Fig. 3B). The expression level of *Xylt1* significantly increased with the degree of chondrogenic differentiation, similar to those of the chondrocyte marker genes. The expression level of *B4galt7* in the cells cultured in the DM plus BMP-4 was significantly higher than that in the CM. Conversely, the expression of *B3galt6* in the cells in the DM plus BMP-4 slightly but significantly decreased from that in the CM. However, the expression levels of *Xylt2* and *B3gat3* remained unaltered across the three differentiation stages of ATDC5. Finally, we compared the mRNA expression levels of the five genes in well-differentiated ATDC5 cells (Fig. 3C). Highest mRNA expression levels in a descending order as follows: *B4galt7*, *Xylt1*, *Xylt2*, *B3galt6*, and *B3gat3*.

In this study, we measured the mRNA expression levels of the five glycosyltransferase genes involved in the GAG linker region synthesis in the three differentiation stages of ATDC5 cells. These profiles strongly suggest that the mRNA expressions of these five genes are differentially regulated during chondrogenic differentiation even though they share a common role in GAG synthesis. For GAG synthesis in chondrocytes, chondrogenic differentiation-dependent increased *Xylt1* and *B4galt7* and sustained *Xylt2*, *B3Galt6*, and *B3galt3* expression levels from undifferentiated to mature chondrocytes might be important. To elucidate the roles of the five genes in chondrogenesis, the knockdown effect of expression of each gene on GAG synthesis and chondrogenic differentiation in ATDC5 cells should be addressed.

Xylt1 was the most significantly upregulated depending on the chondrogenic differentiation stage. Human *XYLT1* mutations cause Desbuquois dysplasia type 2, which is characterized by various skeletal abnormalities, including joint dislocations and laxity, short stature, flat face, and advanced carpal ossification[4,16,18]. *Xylt1* is expressed in the resting, proliferative, and pre-hypertrophic chondrocytes in the growth plate of mouse embryos at E18.5[22]. *Xylt1*-knockout (KO) mice exhibited dwarfism and skeletal abnormalities, along with the shortening of GAG chains[15,22]. In the growth plate of the mutant mice, accelerated chondrocyte maturation and impaired ECM deposition were observed, suggesting that XylT-1 controls chondrocyte maturation and matrix organization. The ATDC5 cell line should be useful for further examination of these speculated XylT-1 functions. *Xylt2* expression remained unchanged across the three differentiation stages of ATDC5. *Xylt2*-KO mice did not exhibit any abnormal skeletal phenotypes. However, the mutant mice exhibited liver abnormalities, such as liver fibrosis and biliary cysts, and renal abnormalities, such as dilated tubules and hydronephrosis[6]. Because the expression level of *Xylt1* in well-differentiated ATDC5 cells was significantly higher than that of *Xylt2*, XylT-2 deficiency may be compensated by XylT-1 in the chondrocytes. *B3gat3* expression was significantly low and remained unchanged across the three differentiation stages. *B3gat3*-KO mice showed embryonic lethality before the 8-cell stage due to failed cytokinesis[8]. Moreover, neither CS nor HS was detected in blastocysts from *B3gat3*-KO embryos. Therefore, GlcAT-I encoded by *B3gat3* is indispensable for embryonic cell division before the 8-cell stage. Conversely, human *B3GAT3* mutations result in B3GAT3-related disorders, which are characterized by developmental delay, multiple dislocations,

bone fragility, and cutis laxa[3,9,16,18]. Because the extremely low expression level of *B3gat3* may be an ATDC5-specific feature and not a chondrocyte-specific one, the functional importance of GlcAT-I encoded by *B3gat3* in mouse chondrocytes remains obscure. To elucidate the function of mouse GlcAT-I, establishing chondrocyte-specific *B3gat3*-KO mice is required. The expression of *B4galt7* was significantly highly expressed and enhanced depending on the chondrogenic differentiation stage. Conversely, the expression level of *B3galt6* in the well-differentiated cells was slightly but significantly lower than in the undifferentiated cells. Although both *B4Galt7*- and *B3galt6*-KO mice have not yet been reported, Human *B4GALT7* and *B3GAL6* are known to be the causative genes for Ehlers–Danlos syndrome (EDS) spondylodysplastic types 1 and 2, respectively[1,13,14,16-18]. EDS types 1 and 2 are characterized by skeletal abnormalities, including short stature, joint laxity, and osteopenia, as well as other abnormalities, such as hypotonic muscles and loose skin. Most skeletal elements are formed through endochondral ossification, in which chondrocytes play essential roles[12]; therefore, GalT-I and GalT-II, encoded by *B4GALT7* and *B3GALT6*, respectively, are essential components of human chondrocyte functions. To elucidate the roles of the two genes in mouse chondrocytes, KO mice deleted for the two genes need to be established.

The expression profiles of the five genes presented in this study would be useful in furthering current understanding of the mechanisms that regulate the GAG–protein linker region synthesis in chondrocyte. Furthermore, this study demonstrated the suitability of the ATDC5 cell line as an *in vitro* model for examining these mechanisms.

Conflict of interest

The authors declare no conflicts of interest associated with this manuscript.

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