Effect of glucosamine on expression of type II collagen, matrix metalloproteinase and sirtuin genes in a human chondrocyte cell line

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Abstract. Glucosamine (GlcN) has been widely used to treat osteoarthritis (OA) in humans. However, the effects of GlcN on genes related to cartilage metabolism are still unknown. In the present study, to elucidate the chondroprotective action of GlcN on OA, we examined the effects of GlcN (0.1-10 mM) on the expression of the sirtuin (SIRT) genes as well as type II collagen and matrix metalloproteinases (MMPs) using a human chondrocyte cell line SW 1353. SW 1353 cells were incubated in the absence or presence of GlcN. RT-PCR analyses revealed that GlcN markedly increased the mRNA expression of type II collagen (*COL2A1*). By contrast, the levels of *MMP-1* and *MMP-9* mRNA were only slightly increased by GlcN. Furthermore, western blot analyses revealed that GlcN significantly increased the protein level of COL2A1. Importantly, GlcN enhanced the mRNA expression and protein level of SIRT1, an upstream-regulating gene of *COL2A1*. Moreover, a SIRT1 inhibitor suppressed GlcN-induced *COL2A1* gene expression. Together these observations suggest that GlcN enhances the mRNA expression and protein level of SIRT1 and its downstream gene *COL2A1* in chondrocytes, thereby possibly exhibiting chondroprotective action on OA.

Introduction

Osteoarthritis (OA) is the most common joint disorder among the elderly presenting with joint pain and deformities. OA is considered a major public health issue causing chronic disability worldwide in the increasing number of aging humans (1). OA is characterized by qualitative and quantitative changes in the architecture and composition of joint structures (2,3). OA is a multifactorial disease of the cartilage, and aging is one of the important risk factor for OA (4). However, the mechanisms, such as inflammation, apoptosis, and degradation of major extracellular matrix (ECM) components (including type II collagen and aggrecan) are commonly involved in the cartilage degradation in OA (5).

Glucosamine (GlcN), a naturally occurring amino monosaccharide, is present in connective and cartilage tissues as a component of glycosaminoglycans (GAGs), and contributes to maintaining the strength, flexibility and elasticity of these tissues. Thus, GlcN has been widely used to treat OA for more than three decades in humans (6-9). In fact, several short-term and long-term clinical trials in OA have shown the significant symptom-modifying effect of GlcN (10-12). We previously revealed that GlcN can induce hyaluronic acid (HA) production by synovial cells and chondrocytes (13). Furthermore, the balance between the synthesis and degradation of ECM components in the cartilage is important for the maintenance of articular metabolism, and the disturbance of this balance leads to the progressive destruction of cartilage in OA (14-17). Thus, GlcN is expected to exert a protective effect on the balance between the synthesis and degradation of ECM components in the cartilage. However, the effects of GlcN on the expression of the genes related to cartilage metabolism are not fully understood.

In the present study, therefore, to further elucidate the chondroprotective action of GlcN, we examined the effect of GlcN on the expression of genes related to cartilage metabolism, such as type II collagen and the matrix metalloproteinases (MMPs).

Materials and methods

Reagents. D-Glucosamine hydrochloride (GlcN) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Penicillin-streptomycin mixed solution, dimethyl sulfoxide (DMSO) and dithiothreitol (DTT) were purchased both from Nacalai Tesque, Inc. (Kyoto, Japan). EX527 [a sirtuin 1 (SIRT1) inhibitor] was purchased from Selleckchem (Houston, TX, USA).

Cells. Human chondrocytes (SW 1353) were purchased from the American Type Culture Collection (HTB-94, ATCC; Manassas, VA, USA). SW 1353 cells were maintained in Leibovitz's L-15 medium (Gibco-Invitrogen Life Technologies, Carlsbad, CA,

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USA) containing 10% fetal bovine serum (FBS; Biological Industries, Cromwell, CT, USA), penicillin and streptomycin at 37˚C under a humidified atmosphere without CO_2.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). SW 1353 cells ($3.0x10^6$ cells/flask) were plated into T-75 cm^2 flasks (Corning Inc., Corning, NY, USA) overnight. The cells were incubated in the absence or presence of GlcN (0.1, 1 and 10 mM) for 24 h, or incubated with GlcN (1 mM) in the absence or presence of EX527 (1 μM) or DMSO (as a solvent) for 6 h (18). After incubation, the cells were washed twice with ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and collected by a cell scraper (Sumitomo Bakelite Co., Ltd., Tokyo, Japan). Then, total RNA was purified using an RNeasy Plus Mini kit and QIAshredder (both from Qiagen, Hilden, Germany) by removing contaminated DNA, according to the manufacturer's instructions, and stored at -80˚C. Reverse transcription was performed using a ReverTra Ace® (Toyobo Co., Ltd., Osaka, Japan), and PCR amplification was performed with GoTaq® Hot Start Green Master Mix (Promega, Madison, WI, USA) in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems, Foster City, CA, USA) for type II collagen (*COL2A1*), *MMP-1*, *MMP-2*, *MMP-9 MMP-13*, *SIRT1-SIRT7* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), according to the manufacturer's instructions. In brief, cDNA was synthesized by reverse transcription using total RNA (500 ng), ReverTra Ace® reverse transcriptase and oligo$(dT)_{20}$. To discriminate mRNA-derived PCR products from genomic DNA-derived products, the intron-spanning PCR primers were used with the annealing temperature and cycle number, as shown in Table I. PCR products were resolved by 1% agarose gel electrophoresis in 1X Tris-acetate-EDTA buffer and stained with ethidium bromide. In our preliminary experiments, we tried to semi-quantitatively detect mRNA by using different cycle numbers of PCR. The results revealed that the amounts of RT-PCR products increased dependently on the cycle number. Thus, we decided to measure the mRNA levels by RT-PCR with the cycle number indicated in Table I. The expression of *GAPDH* was used as a standard. The detected bands were quantified using BioDoc-it Imaging System (UVP LLC, Upland, CA, USA) and quantified using Multi Gauge version 3.0 (Fujifilm, Tokyo, Japan).

Western blot analysis. SW1353 cells ($3.0x10^6$ cells/flask) were plated into T-75 cm^2 flasks overnight. The cells were incubated in the absence or presence of GlcN (0.1, 1 and 10 mM) for 24 h. After incubation, the cells were washed twice with ice-cold PBS and collected by a cell scraper (Sumitomo Bakelite Co., Ltd.). For the detection of COL2A1, the cells were added together with 100 μl of RIPA buffer [50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)] with a protease inhibitor (Complete Protease Inhibitor Cocktail set; Roche Diagnostics, Mannheim, Germany), sonicated (Model UD-201 ultrasonic disruptor, output 20 W, duty 10 for 3 times; Tomy Digital Biology, Co., Ltd., Tokyo, Japan) and placed on ice for 15 min. After centrifugation (8,000 x g, 4˚C, 15 min), the supernatants were used for SDS-polyacrylamide gel electrophoresis (PAGE). For the detection of SIRT1, the cells were added together with 100 μl of lysis buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM $MgCl_2$, 1% NP-40, 10% glycerol, 1 mM DTT) with a protease inhibitor (Roche Diagnostics), sonicated (Model UD-201 ultrasonic disruptor, output 20 W, duty 10 for 5 times) and placed on ice for 15 min. After centrifugation (8000 x g, 4˚C, 15 min), the supernatants were used for SDS-PAGE. Cell lysates (20 μg protein/lane for COL2A1 and 10 μg protein/lane for SIRT1) were subjected to SDS-PAGE and transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore, Billerica, MA, USA). The membranes were blocked overnight with Blocking One (Nacalai Tesque, Inc.) at 4˚C, washed with PBS containing 0.1% Tween-20 (PBST) at room temperature, and then probed with rabbit anti-human COL2A1 antibody (sc-28887; 1,000-fold dilution) or rabbit anti-human SIRT1 antibody (sc-15404; 1,000-fold dilution) (both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (AQ132P; 5,000-fold dilution; Chemicon International, Inc., Temecula, CA, USA). Signals were detected with SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc., Rockford, IL, USA), and quantified using LAS-3000 luminescent image analyzer and Multi Gauge (both from Fujifilm). The expression of GAPDH was analyzed with mouse anti-human GAPDH antibody (MAB374; 30,000-fold dilution; Chemicon International, Inc.) and HRP-conjugated goat anti-mouse IgG/IgM (115-035-044; 5,000-fold dilution; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) as an internal standard.

Statistical analysis. Data are shown as mean ± SD of three separate experiments, and were analyzed for significant difference by a Student's t-test in Excel analysis. Differences were considered statistically significant at $p<0.05$.

Results

Effect of GlcN on the genes related to cartilage metabolism in chondrocytes. First, the effect of GlcN on the expression of genes related to cartilage metabolism, such as *COL2A1* and *MMPs*, was evaluated. As shown in Fig. 1, the expression of *COL2A1* mRNA was significantly (>5-fold) increased by GlcN (0.1, 1 and 10 mM) compared with the expression noted in the control without GlcN ($p<0.05$). Furthermore, the expression of *MMP-1* mRNA was significantly but only 1.2-fold increased by GlcN (10 mM) ($p<0.05$). Moreover, the expression of *MMP-9* mRNA was significantly but only 1.2-fold increased by GlcN (1 and 10 mM). By contrast, the expression of *MMP-2* and *MMP-13* mRNAs was not essentially changed by GlcN, although the expression of *MMP-13* mRNA was slightly increased without statistically significant difference.

Second, the protein level of COL2A1 was evaluated, since the expression of *COL2A1* mRNA was greatly increased by GlcN (Fig. 1). As shown in Fig. 2, the protein level of COL2A1 was significantly (>2-fold) increased by GlcN (1 mM) compared with this level in the control without GlcN.

Effect of GlcN on SIRT gene expression in chondrocytes. The expression of *COL2A1* mRNA was significantly increased

Table I. Gene-specific PCR primers, annealing temperature (˚C) and the cycle number for PCR.

Gene	Forward primer	Reverse primer	Annealing temperature (˚C)	Cycle nos.	Refs.
COL2A1	5'-atgacaatctggctcccaacactgc-3'	5'-gaccggccctatgtccacaccgaat-3'	52	41	(19)
MMP-1	5'-gccagatttgccaagagcaga-3'	5'-cggcaaattcgtaagcagcttc-3'	55	34	(20)
MMP-2	5'-ggccctgtcactcctgagat-3'	5'-ggcatccaggttatcgggga-3'	58	29	(21)
MMP-9	5'-cggagcacggagacgggtat-3'	5'-tgaaggggaagacgcacagc-3'	58	35	(21)
MMP-13	5'-gacttcacgatggcattgctg-3'	5'-gcatcaacctgctgaggatgc-3'	59	39	(22)
SIRT1	5'-gggatggtatttatgctcgc-3'	5'-ctatgatttgtttgatggatagttc-3'	55	34	(23)
SIRT2	5'-agcaaggcacccctctccacc-3'	5'-ggtttctccctctctgttgtc-3'	57	31	(24)
SIRT3	5'-tgagagagtgtcgggcatccctg-3'	5'-tcatcctatttgtctggtccatcaa-3'	55	33	(25)
SIRT4	5'-accctgagaaggtcaaagagttac-3'	5'-ttccccacaatccaagcac-3'	55	33	(26)
SIRT5	5'-ccgagtgtgagacccggctgggca-3'	5'-ttgtaattctcagccacaactcc-3'	54	31	(27)
SIRT6	5'-ccaagttcgacaccaccttt-3'	5'-cggacgtactgcgtcttaca-3'	55	31	(28)
SIRT7	5'-gggagtacgtgcgggtgttcgatg-3'	5'-ggccgccggctaggggggcttggtc-3'	60	33	(27)
GAPDH[a]	5'-accacagtccatgccatcac-3'	5'-tccaccaccctgttgctgta-3'	60	20	

[a]Toyobo Co., Ltd. COL2A1, type II collagen; MMP, matrix metalloproteinase; SIRT, sirtuin.

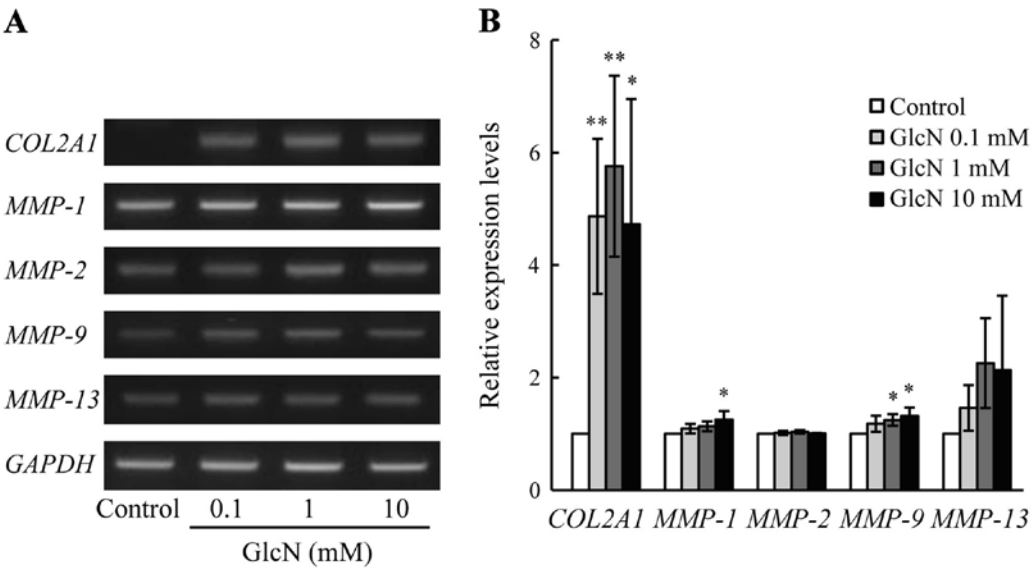

Figure 1. Effect of glucosamine (GlcN) on the mRNA expression of genes related to cartilage metabolism. Chondrocytes (SW 1353) were cultured in the absence (Control) or presence of GlcN (0.1, 1 and 10 mM) for 24 h. After incubation, total RNA was purified, and mRNA expression of the genes related to cartilage metabolism was evaluated by RT-PCR. PCR products were resolved by 1% agarose gel electrophoresis, and stained with ethidium bromide. (A) mRNA levels of genes related to cartilage metabolism. Data represent one of three independent experiments. (B) Quantification of mRNA levels of genes related to cartilage metabolism. The *GAPDH* expression levels were evaluated as an internal standard. The relative expression of mRNAs for type II collagen (*COL2A1)* and matrix metalloproteinases (*MMPs)* corrected for the expression of *GAPDH* is expressed as a ratio to the control cells incubated without GlcN. Data represent the mean ± SD of three independent experiments. Values were compared between the absence (Control) and presence of GlcN. $^{*}p<0.05$, $^{**}p<0.01$.

by GlcN (Fig. 1). Thus, the effect of GlcN on the expression of *SIRT1*, an upstream-regulating gene of *COL2A1* (29), was evaluated. As shown in Fig. 3, the expression of *SIRT1* mRNA was significantly (3- to 4-fold) increased by GlcN (0.1, 1 and 10 mM) compared with that noted in the control without GlcN ($p<0.05$). In humans, the *SIRT* gene family consists of 7 genes, *SIRT1-SIRT7* (30); thus, the effect of GlcN on the expression of *SIRT2-SIRT7* genes was evaluated. As shown in Fig. 3, the expression of *SIRT5* mRNA was significantly but only slightly (~2-fold) increased by GlcN ($p<0.05$). By contrast, the mRNA expression of *SIRT2*, *SIRT3*, *SIRT4*, *SIRT6* and *SIRT7* was not essentially changed by GlcN. Moreover, the protein level of SIRT1 was evaluated, since the expression of *SIRT1* mRNA was found to be greatly increased among the SIRT family genes by GlcN. As shown in Fig. 4, the protein level of SIRT1 was significantly (5- to 6-fold) increased by

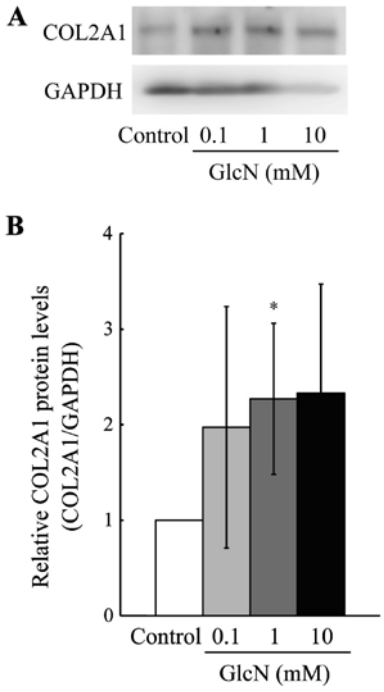

Figure 2. Effect of glucosamine (GlcN) on the protein levels of type II collagen (COL2A1). Chondrocytes (SW 1353) were cultured in the absence (Control) or presence of GlcN (0.1, 1 and 10 mM) for 24 h. After the incubation, total protein was extracted, and the protein levels of COL2A1 were detected by western blot analysis. (A) Protein levels of COL2A1. Data represent one of three independent experiments. (B) Quantification of COL2A1 protein levels. The protein levels of GAPDH were evaluated as an internal standard. The relative protein level of COL2A1 corrected for the level of GAPDH is expressed as a ratio to the control cells incubated without GlcN. Data represent the mean ± SD of three independent experiments. Values were compared between the absence (Control) and presence of GlcN. $^{*}p<0.05$.

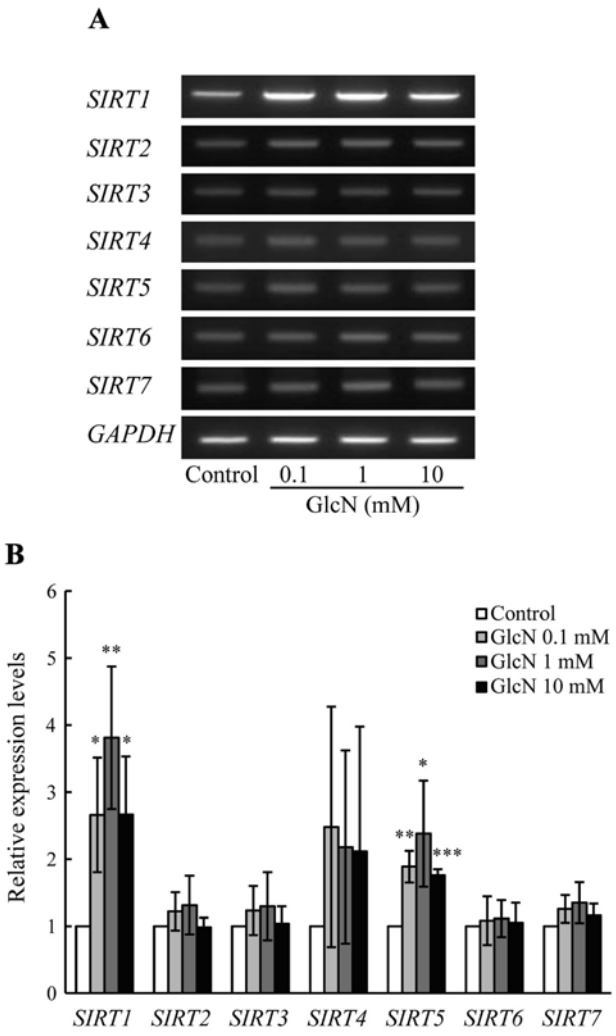

Figure 3. Effect of glucosamine (GlcN) on the mRNA expression of sirtuins (*SIRTs*). Chondrocytes (SW 1353) were cultured in the absence (Control) or presence of GlcN (0.1, 1 and 10 mM) for 24 h. After incubation, total RNA was purified, and the expression of mRNA for *SIRTs* was evaluated by RT-PCR. PCR products were resolved by 1% agarose gel electrophoresis, and stained with ethidium bromide. (A) mRNA levels of *SIRTs*. Data represent one of three independent experiments. (B) Quantification of mRNA levels of *SIRTs*. The *GAPDH* expression levels were evaluated as an internal standard. The relative expression of mRNAs for *SIRT1-7* corrected for the expression of *GAPDH* is expressed as a ratio to control cells incubated without GlcN. Data represent the mean ± SD of three independent experiments. Values were compared between the absence (Control) and presence of GlcN. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

GlcN (1 mM) compared with this level in the control without GlcN ($p<0.01$).

Effect of a SIRT1 inhibitor on COL2A1 and SIRT1 gene expression in chondrocytes. As mentioned above, COL2A1 and SIRT1 expression levels were increased by GlcN. Thus, we aimed to ascertain whether the GlcN-induced *COL2A1* gene expression is mediated by SIRT1 gene expression. For this purpose, the effect of EX527, a specific SIRT1 inhibitor (31), on the expression of *COL2A1* gene expression was evaluated. Importantly, the GlcN-induced *COL2A1* gene expression was significantly suppressed by EX527, although the GlcN-induced *SIRT1* gene expression was not affected by EX527 (Fig. 5). These observations obviously indicate that the GlcN-induced *COL2A1* gene expression was regulated by the *SIRT1* gene expression.

Discussion

GlcN exhibits a symptom-modifying effect on OA (6-8), and has been used to relieve the symptoms of OA in humans (32). Previously, we demonstrated that GlcN can induce HA production by synovial cells and chondrocytes (13). However, the effects of GlcN on genes related to cartilage metabolism are not fully understood.

Collagens and proteoglycans are the major components in the ECM (33). COL2A1 is the most abundant collagen in articular cartilage, and provides cartilage tissue with tensile strength (34,35). In this study, we revealed that GlcN obviously enhanced the mRNA and protein levels of COL2A1 in the chondrocytes (Figs. 1 and 2), although GlcN has been previously reported to increase type II collagen synthesis (36-39). By contrast, GlcN only slightly increased the mRNA expression of the *MMPs* (Fig. 1). These observations suggest that GlcN upregulates the expression of anabolic genes (such as *COL2A1*) rather than catoblic genes (such as *MMPs*), thereby possibly exhibiting chondroprotective actions on degenerative joint diseases such as OA.

SIRT1 is known as an upstream-regulating gene of *COL2A1* in chondrocytes (29) and exerts chondroprotective action in OA (40-42). In this context, it is important to note

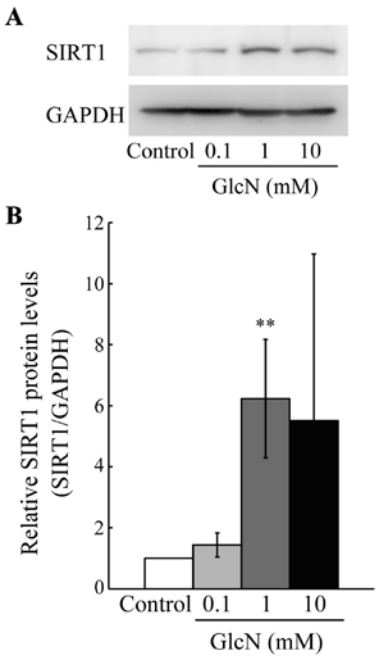

Figure 4. Effect of glucosamine (GlcN) on the protein levels of sirtuin 1 (SIRT1). Chondrocytes (SW 1353) were cultured in the absence (Control) or presence of GlcN (0.1, 1 and 10 mM) for 24 h. After incubation, total protein was extracted, and the protein levels of SIRT1 were detected by western blot analysis. (A) Protein levels of SIRT1. Data represent one of three independent experiments. (B) Quantification of SIRT1 protein levels. The protein levels of GAPDH were evaluated as an internal standard. The relative protein level of SIRT1 corrected for the level of GAPDH is expressed as a ratio to control cells incubated without glucosamine. Data represent the mean ± SD of three independent experiments. Values were compared between the absence (Control) and presence of GlcN. $^{**}p<0.01$.

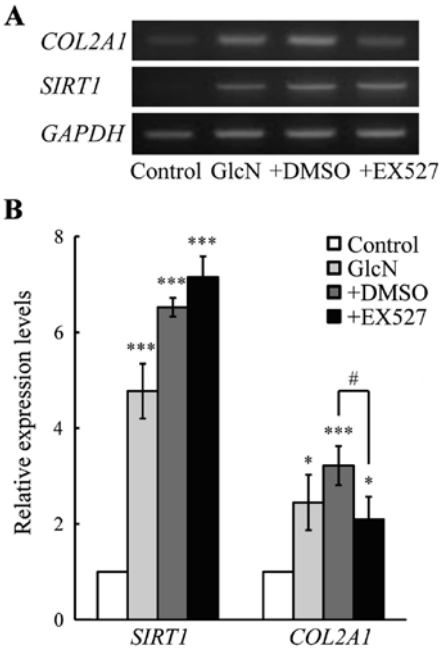

Figure 5. Effects of sirtuin 1 (SIRT1) inhibitor on glucosamine (GlcN)-induced type II collagen (*COL2A1)* mRNA expression. Chondrocytes (SW 1353) were cultured without or with GlcN (1 mM) in the absence or presence of EX527 (+EX527, 1 μM) or DMSO (+DMSO) for 6 h. After incubation, total RNA was purified, and the expression of mRNA for *COL2A1* and *SIRT1* was evaluated by RT-PCR. PCR products were resolved by 1% agarose gel electrophoresis, and stained with ethidium bromide. (A) mRNA levels of *COL2A1* and *SIRT1*. Data represent one of three independent experiments. (B) Quantification of the mRNA levels for *COL2A1* and *SIRT1* genes. The *GAPDH* expression levels were evaluated as an internal standard. The relative expression of mRNAs for *SIRT1* and *COL2A1* corrected for the expression of *GAPDH* is expressed as a ratio to control cells incubated without GlcN, DMSO or EX527. Data represent the mean ± SD of three independent experiments. Values were compared between the absence (Control) and presence of GlcN ($^{*}p<0.05$, $^{***}p<0.001$) or +EX527 and +DMSO ($^{\#}p<0.05$).

that adult heterozygous *Sirt1*-knockout mice and chondrocyte-specific *Sirt1*-conditional knockout mice presented with reduced intensity of Safranin-O staining and increased OA progression compared with wild-type mice (40,41); *Sirt1* point mutation-knockin mice were found to exhibit OA progression (42). Furthermore, SIRT1 promotes cartilage-specific gene expression, such as *COL2A1* (29) in chondrocytes, and protects chondrocytes from senescence (43). Moreover, SIRT1 was found to inhibit the apoptosis of human chondrocytes (44,45), whereas knockdown of *SIRT1* led to osteoarthritic gene expression in human chondrocytes (46). In addition, overexpression of SIRT1 inhibited interleukin (IL)-1β- or tumor necrosis factor (TNF)-α-induced expression of cartilage-degrading enzymes (such as MMPs) by modulating the nuclear factor (NF)-κB pathway (47,48). These observations suggest that SIRT1 exhibits a chondroprotective action on OA by upregulating cartilage-specific gene expression (such as *COL2A1*) but inhibiting cartilage-degrading enzymes (such as MMPs) in chondrocytes. Notably, the present results demonstrated that GlcN markedly enhanced the mRNA and protein levels of SIRT1 as well as COL2A1 in chondrocytes among the SIRT genes examined (Figs. 3 and 4). Importantly, the GlcN-induced *COL2A1* gene expression was significantly suppressed by EX527, a SIRT1-specific inhibitor (Fig. 5). Together these observations suggest the possibility that GlcN exhibits protective actions on chondrocytes by upregulating COL2A1 expression via SIRT1 expression.

On the other hand, it has been noted that the *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) modification of target proteins modulates cellular functions, such as nuclear transport, transcription, translation, cell signaling, apoptosis and cell shape, and the modification is mediated via the addition of *O*-GlcNAc to the hydroxy group in a serine or threonine residue by *O*-GlcNAc transferase (49,50). In this context, it has been demonstrated that several transcription factors are modified by *O*-GlcNAc, and such modification regulates their transcriptional activities of genes (51-55). Moreover, we previously reported that *O*-GlcNAc modification is increased by GlcN in endothelial cells and synovial cells (56-58). Thus, elucidation of the involvement of GlcN-mediated *O*-GlcNAc modification in the transcriptional regulation of SIRT1 in chondrocytes in the future is warranted.

In conclusion, the present study revealed that GlcN upregulates COL2A1 and SIRT1 expression in chondrocytes. Moreover, a SIRT1 inhibitor suppressed the GlcN-induced upregulation of *COL2A1* gene expression. Together these observations suggest that GlcN enhances the mRNA expression and protein level of SIRT1 and its downstream gene *COL2A1* in chondrocytes, thereby possibly exhibiting chondroprotective action. However, the present study has a limitation, as we only evaluated the effects of GlcN on the expression of COL2A1, MMPs and SIRTs using a chondro-

cyte cell line SW 1353. Thus, it remains to be elucidated whether GlcN modulates the expression of these genes in articular chondrocytes.

Acknowledgements

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