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From Cell Biology to Tissue Engineering

Suppression of cartilage degeneration by intra-articular injection of heparan sulfate 6-O endosulfatase in a mouse osteoarthritis model

Shuhei Otsuki, Tomohiko Murakami, Yoshinori Okamoto, Yoshiaki Hoshiyama, Shuhei Oda and Masashi Neo

Department of Orthopedic Surgery, Osaka Medical College, Osaka, Japan

Summary. We previously reported that heparan sulfate 6-O endosulfatases (Sulfs) were expressed in articular cartilage, and that the Sulf-1 knockout mouse developed severe knee osteoarthritis. In this study, we hypothesised that intra-articular injection of Sulf-1 would prevent cartilage degeneration. After confirming that 1 mg/ml Sulf-1 did not induce ATDC5 cell death *in vitro*, gene expression of type II collagen and matrix metalloproteinase (MMP)-13 in the presence of Sulf-1 (1-100 ng/ml) were determined by quantitative real-time polymerase chain reaction. Sulf-1 was also injected intra-articularly into mice following surgical destabilisation of the medial meniscus to produce a model of osteoarthritis, and cartilage degeneration was evaluated by safranin O and MMP-13 staining. We also investigated fibroblast growth factor 2 (FGF2)/extracellular signal-regulated kinase (Erk) cell signalling by western blotting. Exposure to Sulf-1 *in vitro* increased type II collagen expression and decreased MMP-13 expression in a concentration-dependent manner. Sulf-1 injection into the mouse osteoarthritic knee significantly suppressed glycosaminoglycan loss and MMP-13 expression. Erk1/2 signalling pathway activation was significantly reduced by Sulf-1 and FGF2. These findings indicate that Sulf-1 prevents cartilage degeneration by suppressing MMP-13 via an effect on FGF2/Erk1/2 signalling.

Offprint requests to: Shuhei Otsuki M.D., Ph.D., Department of Orthopedic Surgery, Osaka Medical College, 2-7 Daigakumachi, Takatsuki city, Osaka569-8686, Japan. e-mail: ort182@osaka-med.ac.jp

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Introduction

Osteoarthritis (OA) is the most prevalent of all joint diseases and it is characterised by a loss of cartilage (Sandell and Aigner, 2001; Lane et al., 2011; Musumeci et al., 2015). Articular cartilage is maintained by the interaction between chondrocytes and the extracellular matrix, which is composed of collagens, non-collagen proteins, and proteoglycans. Heparan sulfate proteoglycans such as perlecan, syndecan, agrin and decorin mainly act at the cell membrane and are critical for regulating cell signalling pathways (Pacifici et al., 2005; Bishop et al., 2007).

Heparan sulfate 6-O endosulfatases (Sulfs) edit the sulfation pattern of heparan sulfate proteoglycans (Hanson et al., 2004) and were first reported to regulate fibroblast growth factor (FGF) and Wnt protein signalling in quail development (Dhoot et al., 2001). Subsequently, Sulfs were detected in various organs such as nerves (Morimoto-Tomita et al., 2002; Higginson et al., 2012), digestive organs (Lai et al., 2004; Ai et al., 2007), genitals (Viviano et al., 2004) and articular cartilage (Otsuki et al., 2008, 2010). Our previous research revealed that Sulfs were expressed at higher levels in human osteoarthritic knee cartilage than in normal cartilage (Otsuki et al., 2008) and that Sulf-1 knockout (KO) mice showed more severe OA than Sulf-2 KO mice (Otsuki et al., 2010). Taken together, these results indicate that Sulfs are critical for cartilage

homeostasis. However, little is known about the therapeutic effects of exogenous Sulf-1 administration on cartilage degeneration. In this study, we tested the hypothesis that Sulf-1 has the potential to prevent cartilage degeneration.

Materials and methods

Cell viability analysis

Chondrogenesis was induced in the ATDC5 mouse chondrogenic cell line (RIKEN Cell Bank, Ibaraki, Japan), which was supplemented with 10 μg/ml of ITS (insulin, transferrin and selenium) over a period of 3 weeks (Shukunami et al., 1996). These cells (2.0×10^5) were incubated with either phosphate-buffered saline (PBS; control) or with varying concentrations of Sulf-1 (1, 10, 100, or 1000 ng/ml). After 24 h, the cells were harvested and cell viability was determined using the Muse® Annexin V & Dead Cell Assay Kit (Merck Millipore, Billerica, MA) along with the Muse® Cell Analyzer (Merck Millipore), according to the manufacturer's instructions.

Gene expression analysis

ATDC5 cells were seeded at 0.5×10^6 cells/well in a 6-well plate and grown in cell culture medium for 24 h prior to aspiration of the culture medium and washing with PBS. RNA lysis buffer (350 μL) from the RNeasy Mini Kit (Qiagen, Tokyo, Japan) was then added to each well, and total RNA was obtained using the manufacturer's protocol. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine the effect of Sulf-1 exposure (1-100 ng/ml) on type II collagen, matrix metalloproteinase (MMP)-13, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression (Oda et al., 2015). This was performed in a LightCycler (Roche Diagnostics, Mannheim, Germany) using TaqMan Gene Expression Assay probes (Roche Diagnostics) and the protocol provided by the manufacturer.

Animal experiments

All experiments were performed in accordance with the guidelines set by the Osaka Medical College Animal Care Committee (No.27010). We used 8-week-old C57BL/6 male mice in this study.

Surgical destabilisation of the medial meniscus (DMM) and intra-articular injection

Mice were anaesthetised by intraperitoneal injection of 32.4 mg/kg sodium pentobarbital (Somnopentyl®; Kyoritsu, Japan) and their right knees were prepared for aseptic surgery. DMM was performed as previously described (Glasson et al., 2007). This was achieved by resecting the medial meniscotibial ligament, which induced cartilage degeneration, especially in the anteromedial region. In the sham-operated mice, the medial meniscotibial ligaments were identified but not resected. Sixty mice were divided between a DMM group (n=40) and a sham group (control; n=20). The DMM and sham groups were each divided into four sub-groups, based on a different dose of Sulf-1. Intra-articular injection through the patellar tendon was performed using a micro needle (U-100 insulin syringe, 30 G; Becton Dickinson, Fukushima, Japan), as described previously (van der Kraan et al., 1990). The knees were injected with either 10 μl PBS, or with the indicated concentration of recombinant Sulf-1 (Abnova, Taipei, Taiwan), diluted with PBS. Thereafter, injections under anaesthesia were administered weekly on a total of 4 occasions.

Histological analysis

The mice were euthanised 4 or 8 weeks after surgical induction of DMM. Their knees were fixed in 4% paraformaldehyde at room temperature for 24 h and then decalcified in 50% TBD-2 (Fisher Scientific, Pittsburgh, PA) at 4°C for 48 h. The tissues were then dehydrated and embedded in paraffin using standard methods, and 5-μm sagittal sections were prepared. Sections were stained with safranin O and then graded by three independent observers using the Osteoarthritis Research Society International (OARSI) scoring system (Glasson et al., 2010). The average grade assigned to at least 3 consecutive slides was utilised, where "0"=normal; "0.5"=loss of safranin O without structural changes; "1"=small fibrillations without loss of cartilage; "2"=vertical clefts down to the layer immediately below the superficial layer and some loss of surface lamina; and "3" to "6"=vertical clefts/erosion of the calcified cartilage extending to <25%, 25 50%, 50 75%, and >75% of the articular surface, respectively.

Immunohistochemical analysis

The deparaffinised and rehydrated tissue sections were treated with peroxidase for 5 min at room temperature. After washing with PBS, the sections were blocked with 4% skimmed milk for 10 min at room temperature. The rabbit anti-MMP-13 primary antibody was applied (1:100; Abcam, Cambridge, MA) and incubated overnight at 4°C. The sections were washed again with PBS and then incubated with the anti-rabbit IgG secondary antibody (1:100; Vector Laboratories) for 30 min at 4°C before incubating with horseradish peroxidase-streptavidin (1:200; Vector Laboratories Inc., Burlingame, CA). Finally, the sections were washed with PBS and the signals were visualised using the Vectastain ABC Kit (Vector Laboratories). Cells showing severe OA on the anterior femoral cartilage were counted at ×400 magnification using a 100×100-μm grid, and the percentage of MMP-13-positive cells was calculated (Pichler et al., 2013; Di Rosa et al., 2014).

Western blotting and densitometry

Total protein (45 μg) prepared from ATDC5 cells treated as indicated was resolved on polyacrylamide gels (Life Technologies, Carlsbad, CA). The separated proteins were transferred to polyvinylidene difluoride membranes (GE Healthcare, Aliso Viejo, CA), which were blocked using 4% skimmed milk for 1 h at room temperature. The primary antibodies were applied and incubated with the membranes overnight at 4°C. After washing with PBS for 1 h, the appropriate secondary antibodies were applied and incubated for 1 h at room temperature. The primary antibodies employed were rabbit anti-phosphorylated extracellular signal-regulated kinase (Erk) 1/2 (1:1000; Cell Signaling Technology, Danvers, MA), rabbit anti-Erk 1/2 (1:2000; Cell Signaling Technology), rabbit anti-phosphorylated FGF receptor 1 (1:500; Abcam), rabbit anti-FGF receptor 1 (1:500; Cell Signaling Technology), rabbit anti-phosphorylated FGF receptor 3 (1:100, Santa Cruz, Dallas TX), rabbit anti-FGF receptor 3 (1:100, Santa Cruz) and mouse anti-GAPDH (1:5000; Wako Pure Chemical Industries, Osaka, Japan). Immunoreactive bands were visualised using enhanced chemoluminescence (ECL Plus system; GE Healthcare) and the LAS-3000 Mini Chemilumino Analyzer (Fujifilm, Tokyo, Japan). The bands were quantified by densitometry (Multi Gauge; Fujifilm, Tokyo, Japan) and analysed using NIH Image J software (1.49v).

Statistical analysis

The results of each evaluation were analysed using the Mann-Whitney U test. They were considered significant if the p value was <0.05. All statistical analyses were performed using SAS statistical software, version 22 (SAS Institute Inc., Cary, NC). The results were reported as the mean ± standard deviation.

Results

The effects of Sulf-1 on cell viability in vitro

As shown in Fig. 1, exposure to up to 1,000 ng/ml

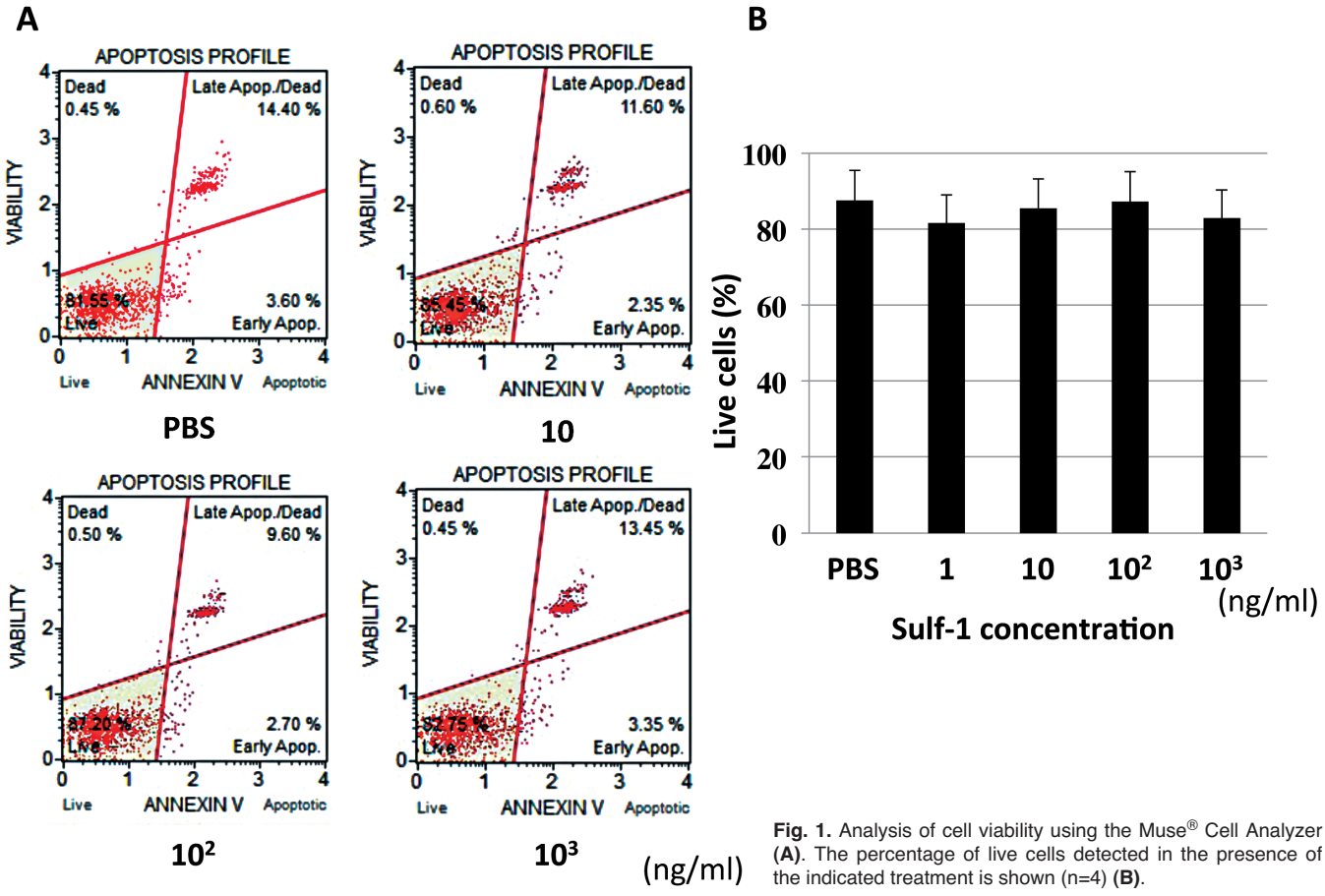

Fig. 1. Analysis of cell viability using the Muse® Cell Analyzer **(A)**. The percentage of live cells detected in the presence of the indicated treatment is shown (n=4) **(B)**.

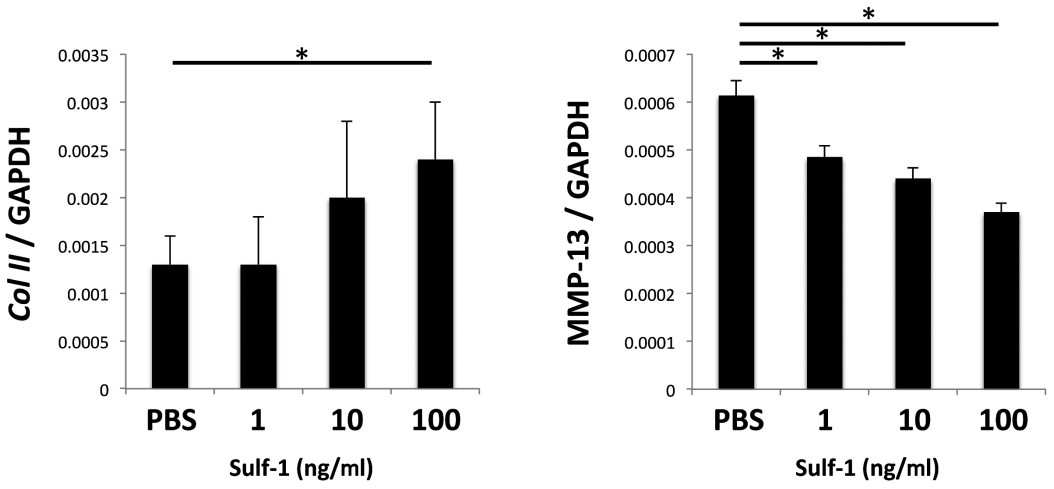

Fig. 2. Type II collagen and MMP-13 mRNA levels were determined using qRT-PCR. Type II collagen mRNA was significantly increased in ATDC5 cells exposed to 100 ng/ml Sulf-1, as compared with control cells, while MMP-13 gene expression decreased in a Sulf-1 concentration-dependent manner; *p<0.05.

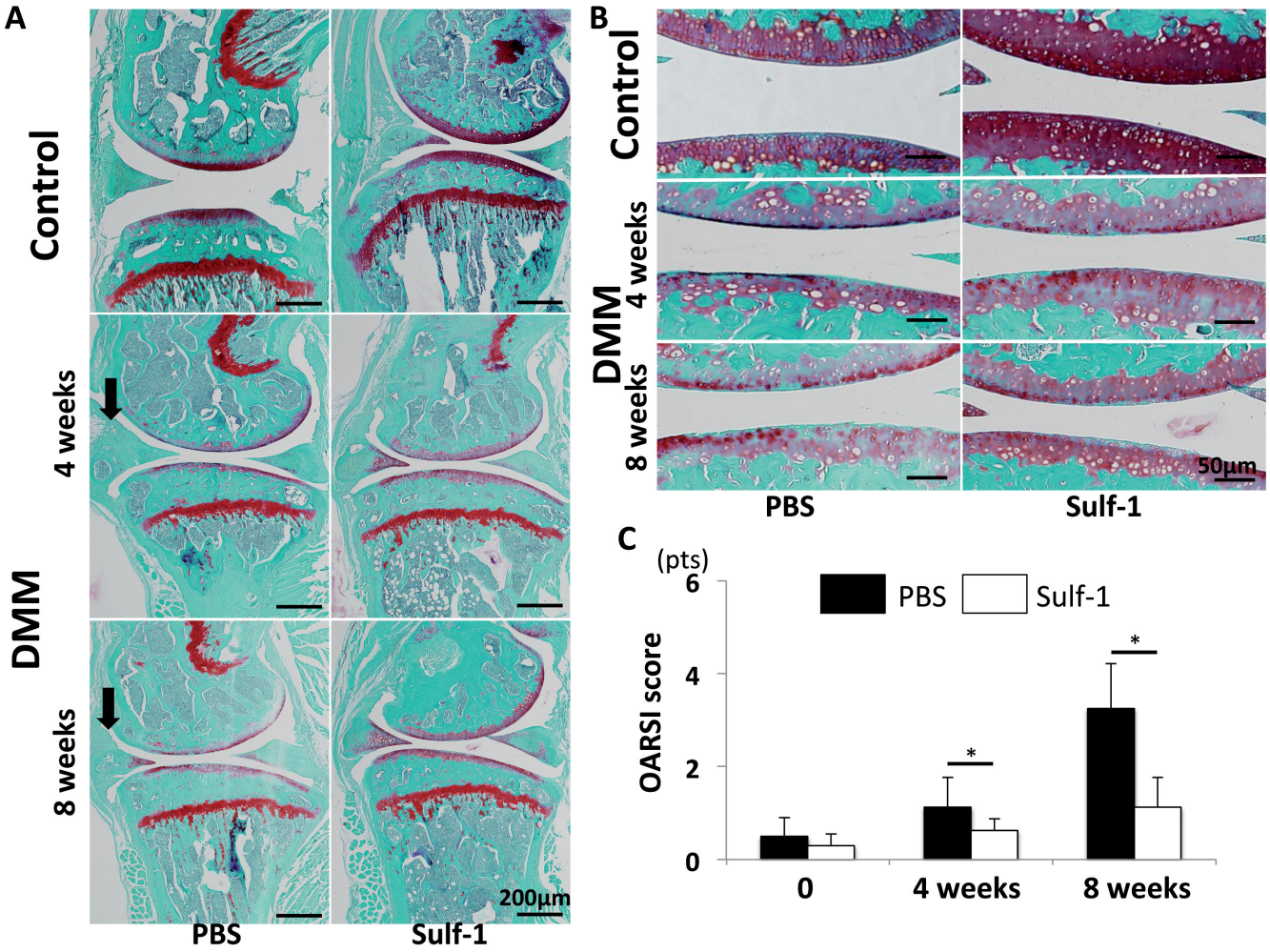

Fig. 3. Safranin O staining of the DMM and control knee joints, injected with the indicated doses of Sulf-1 or PBS. Synovial hyperplasia (black arrows) was identified at 4 and 8 weeks in this mouse model **(A)**. Glycosaminoglycan loss and loss of cartilage thickness progressed in the DMM model at 8 weeks, however these were maintained in mice receiving Sulf-1 injections **(B)**. The OARSI scores (n=5) showed that cartilage degeneration was significantly prevented by Sulf-1 injection (**C**, *p<0.05). Scale bars: A, 200 μm; B, 50 μm.

Sulf-1 did not significantly affect ATDC5 cell viability.

The effects of Sulf-1 on gene expression in vitro

As shown in Fig. 2, the level of type II collagen mRNA was significantly increased in ATDC5 cells exposed to 100 ng/ml Sulf-1, as compared with control cells, while MMP-13 gene expression decreased in a Sulf-1 concentration-dependent manner.

The effects of Sulf-1 on the mouse DMM model of OA

To determine the effect of Sulf-1 on cartilage homeostasis *in vivo*, the progression of OA in the mouse DMM model was evaluated using the OARSI score. Synovial hyperplasia was present at 4 and 8 weeks in this model (Fig. 3A). Glycosaminoglycan loss and loss of cartilage thickness had progressed in the DMM model by 8 weeks, although these features were maintained in Sulf-1-treated mice (Fig. 3B). OARSI scores showed that cartilage degeneration was significantly prevented by Sulf-1 injection (Fig. 3C).

In vivo MMP-13 expression was suppressed by Sulf-1

MMP-13 expression was elevated in both the

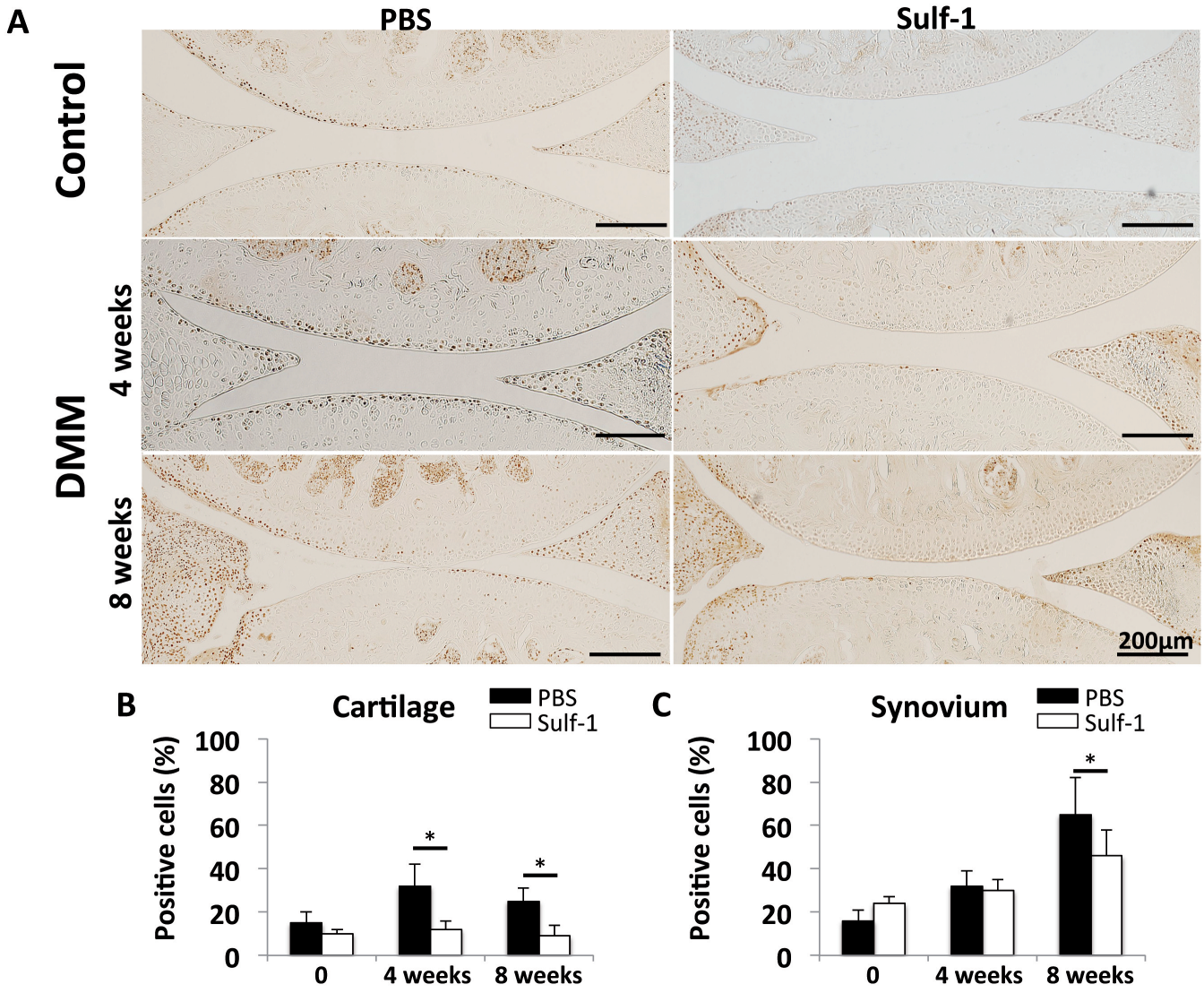

Fig. 4. MMP-13 expression in the mouse knee joint. MMP-13 was overexpressed in both cartilage and synovium at 4 and 8 weeks in the DMM model; this was attenuated by Sulf-1 administration (**A**). MMP-13-positive cells in articular cartilage were significantly reduced by Sulf-1 administration, as compared with PBS injection, at both 4 and 8 weeks in the DMM model (**B**, *p<0.05). Synovial hyperplasia was significantly suppressed around the anteromedial meniscus at 8 weeks in the DMM model animals injected with Sulf-1 (**C**, *p<0.05). Scale bars: A, 200 μm.

cartilage and synovium around the dislocated meniscus in the DMM model at 4 and 8 weeks; this elevation was suppressed by Sulf-1 administration (Fig. 4A). MMP-13 positive cells in the articular cartilage were significantly decreased in mice receiving Sulf-1, as compared to those receiving PBS injections, at both 4 and 8 weeks after DMM (Fig. 4B). Synovial hyperplasia around the anteromedial meniscus was significantly suppressed at 8 weeks in the DMM model mice that were injected with Sulf-1 (Fig. 4C).

The effect of Sulf-1 on FGF signalling in vitro

To further investigate the mechanism underlying the effect of Sulf-1 on MMP-13 suppression, we studied FGF2 and Erk1/2 cell signalling using western blotting. Erk1/2 phosphorylation was significantly decreased after 30-min and 24-h exposure of ATDC5 cells to Sulf-1 and FGF2 (Fig. 5A,C). Interestingly, Sulf-1 did not activate the FGF receptor 1, but FGF receptor 3 was activated 30 min after exposure to FGF2 and Sulf-1 (Fig. 5B,C).

Discussion

The most important finding of this study was that intra-articular injection of Sulf-1 prevented cartilage degeneration by suppressing MMP-13 expression via the FGF2/Erk1/2 cell signalling pathway. The over-expression of Sulfs has previously been reported in osteoarthritic cartilage, especially at cluster formations (Otsuki et al., 2008), which have some self-repairing potential because of the progenitor cells in cluster chondrocytes (Hoshiyama et al., 2015). Our previous results indicated that Sulfs might play a role in chondrocyte homeostasis.

We started the present study by confirming that the presence of high concentrations of Sulf-1 would not induce apoptosis in chondrogenic ATDC5 cells. Moreover, qRT-PCR analyses revealed that Sulf-1 showed some therapeutic potential. Sulfs had been reported to regulate the FGF2-Erk1/2 and BMP7-Smad1/5 cell signalling pathways in Sulf KO mice (Otsuki et al., 2010). Regarding the relationship between MMP-13 and collagen II expression, Settle and colleagues reported that an MMP-13 inhibitor produced chondroprotective effects and decreased levels of the type II collagen biomarker (Settle et al., 2010). Our present results identified increased expression of type II collagen and reduced expression of MMP-13 *in vitro* and *in vivo* in the presence of Sulf-1. This indicated that Sulf-1 might have the potential to maintain cartilage homeostasis. To determine its ability to prevent cartilage

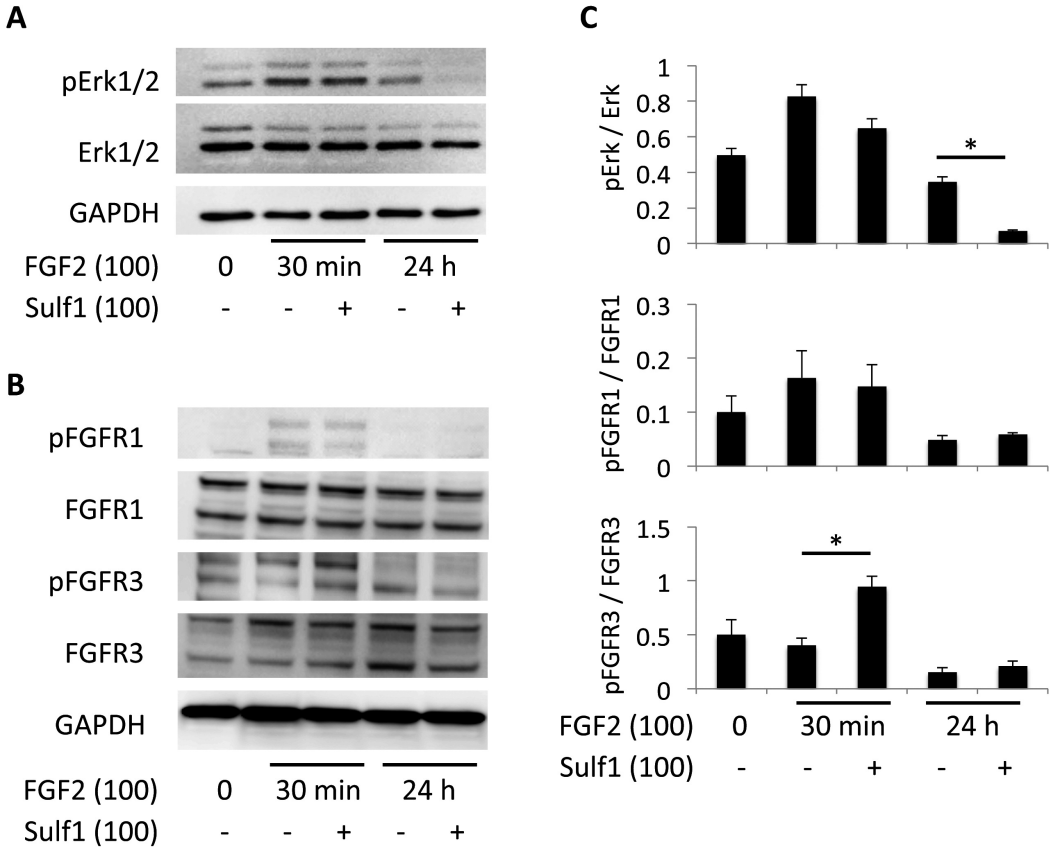

Fig. 5. Western blot analysis of the indicated FGF2/Erk1/2 cell signalling pathway proteins (**A and B**). Protein was harvested 0, 30 min and 24 h after exposure of ATDC5 cells to FGF2 (100 ng/ml) and Sulf-1 (100 ng/ml). Densitometric analysis of the expression of the indicated proteins (**C**, *p<0.05).

degeneration, a mouse DMM model showing mild OA changes was used in this study because we did not anticipate that this would prevent more severe pathology. In the DMM joints, cartilage degeneration progressed gradually within the 8-week study period, as described previously (Glasson et al., 2007). However, Sulf-1 injections suppressed cartilage degeneration and synovial hyperplasia. Interestingly, cartilage degeneration was also prevented at 4 and 8 weeks after DMM, when we had finished administering Sulf-1 injections, indicating that Sulf-1 acted on early OA. Moreover, MMP-13 protein expression, which was considered downstream of Erk1/2 cell signalling, was determined immunohistochemically. MMP-13 expression was reduced in both the cartilage and in the synovial hyperplasia around the anteromedial meniscus in DMM mice treated with Sulf-1, suggesting that Sulf-1 modulated cartilage homeostasis via an effect on synovial inflammation.

To further explore the mechanism underlying MMP-13 suppression, we focused on the FGF2/Erk1/2 cell signalling pathway, which exerts a major influence on MMP-13 expression (Im et al., 2007; Ellman et al., 2013). The level of phosphorylated Erk1/2 was significantly reduced after 24 h in the presence of FGF2 and Sulf-1, suggesting that Sulf-1 suppressed MMP-13 expression via an effect on FGF2/Erk1/2 signalling. Interestingly, the phosphorylation of FGF receptor 1 was not induced, whereas that of FGF receptor 3 was slightly activated by Sulf-1 and FGF2 stimulation. Activation of FGF receptor 3 may therefore protect cartilage from degeneration. Recently, FGF receptor 3 deletion was reported to up-regulate MMP-13 and collagen X, while down-regulating collagen II (Tang et al., 2016); this was consistent with the present results. In the future, the effect of Sulf-1 on FGF18 should also be determined, because this interacts more closely with FGF receptor 3 (Davidson et al., 2005). Although further research is required, the present study indicates that Sulf-1 has the potential to both prevent catabolic effects of FGF signalling and stimulate its anabolic activity (Fig. 6).

This study had some limitations. First, we injected Sulf-1 4 times after the establishment of the DMM model and this approach thus provides an insight into the therapeutic potential of Sulf-1 for early OA. It would also be useful to evaluate Sulf-1 in animals with more advanced OA. Second, we did not study Sulf-2, because Sulf-1 KO mice had more severe OA than Sulf-2 KO mice, and Sulf-1 was considered more critical for cartilage homeostasis than Sulf-2. The effects of Sulf-1 on runt-related transcription factor 2 and collagen X, which serve as markers of OA, should also be determined by future studies (Tetsunaga et al., 2011).

In conclusion, intra-articular injection of Sulf-1 prevented progression of cartilage degeneration by suppressing MMP-13 expression. Although further studies are needed, we believe that Sulf-1 has novel therapeutic potential for preventing cartilage degenera-

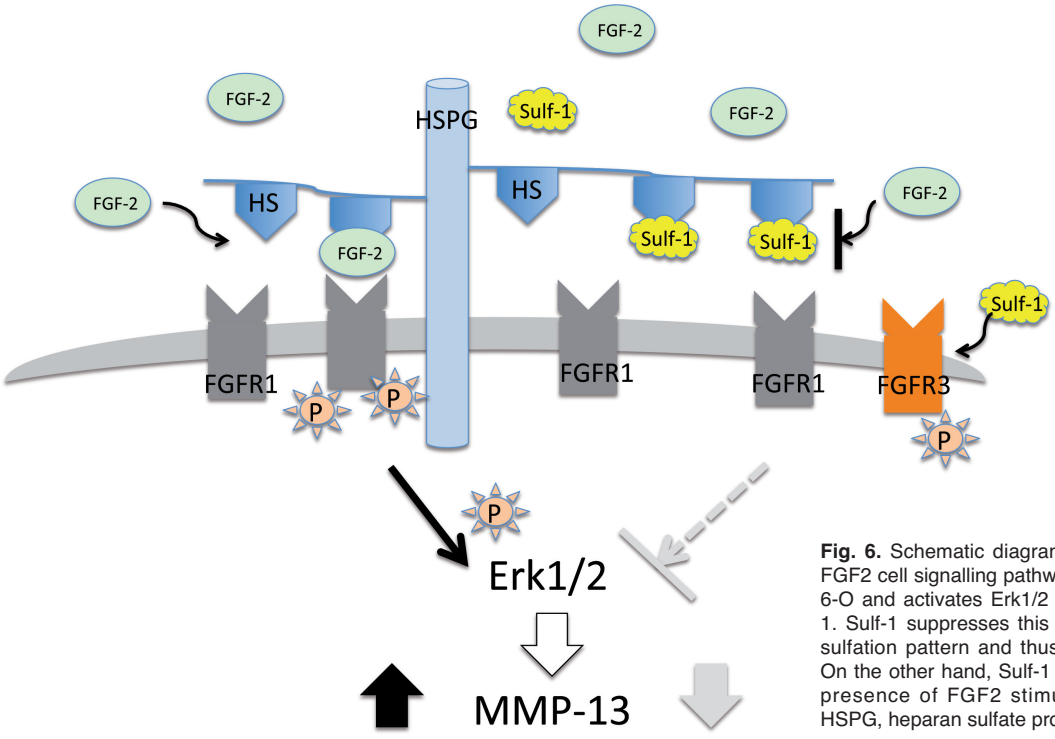

Fig. 6. Schematic diagram of the effect of Sulf-1 on the FGF2 cell signalling pathway. FGF2 binds heparan sulfate 6-O and activates Erk1/2 signalling via the FGF receptor 1. Sulf-1 suppresses this signalling by changing the 6-O sulfation pattern and thus reduces MMP-13 expression. On the other hand, Sulf-1 activates FGF receptor 3 in the presence of FGF2 stimulation. HS, heparan sulfate; HSPG, heparan sulfate proteoglycan.

tion in early OA.

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Authors contributors. Dr. Otsuki had full access to all of the data in the study and takes responsibility for the integrity of these data and the accuracy of the data analyses. Study design: Otsuki, Neo; Acquisition of data: Otsuki, Murakami, Oda, Hoshiyama; Analysis and interpretation of data: Otsuki, Murakami, Neo; Manuscript preparation: Otsuki, Hoshiyama, Oda, Murakami, Okamoto, Neo; Statistical analysis: Otsuki, Murakami, Hoshiyama, Okamoto, Oda

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