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Intraperitoneal injection of the SIRT1 activator SRT1720 attenuates the progression of experimental osteoarthritis in mice

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Objectives
This study aimed to examine the effects of SRT1720, a potent SIRT1 activator, on osteoarthritis (OA) progression using an experimental OA model.

Methods
Osteoarthritis was surgically induced by destabilization of the medial meniscus in eight-week-old C57BL/6 male mice. SRT1720 was administered intraperitoneally twice a week after surgery. Osteoarthritis progression was evaluated histologically using the Osteoarthritis Research Society International (OARSI) score at four, eight, 12 and 16 weeks. The expression of SIRT1, matrix metalloproteinase 13 (MMP-13), a disintegrin and metalloproteinase with thrombospondin motifs-5 (ADAMTS-5), cleaved caspase-3, PARP p85, and acetylated nuclear factor (NF)κB p65 in chondrocytes and the severity of synovitis were examined by immunohistochemistry. Primary mouse epiphyseal chondrocytes were treated with SRT1720 in the presence or absence of interleukin 1 beta (IL-1β), and gene expression changes were examined by real-time polymerase chain reaction (PCR).

Results
The OARSI score was significantly lower in mice treated with SRT1720 than in control mice at eight and 12 weeks associated with the decreased size of osteophytes at four and eight weeks. The delayed OA progression in the mice treated with SRT1720 was also associated with increased SIRT1-positive chondrocytes and decreased MMP-13-, ADAMTS-5-, cleaved caspase-3-, PARP p85-, and acetylated NFκB p65-positive chondrocytes and decreased synovitis at four and eight weeks. SRT1720 treatment partially rescued the decreases in collagen type II alpha 1 (COL2A1) and aggrecan caused by IL-1β, while also reducing the induction of MMP-13 by IL-1β in vitro.

Conclusion
The intraperitoneal injection of SRT1720 attenuated experimental OA progression in mice, indicating that SRT1720 could be a new therapeutic approach for OA.

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Keywords: SIRT1, SRT1720, Osteoarthritis, Synovitis

Article focus
To examine the effects of SRT1720, a potent SIRT1 activator, on osteoarthritis (OA) progression using an experimental OA model.

Key messages
- The intraperitoneal injection of SRT1720 attenuated experimental OA progression in mice.
- The attenuation of OA progression was associated with a decrease in cartilage-degrading enzymes, apoptotic markers and acetylated nuclear factor kappa B (NFκB) p65 in chondrocytes and the severity of synovitis.

Strengths and limitations
- Our study may provide a new therapeutic approach for OA.
- In this study, young mice (eight weeks old) were used. Therefore, our experimental model may not represent OA pathology in humans.
It was not determined whether SRT1720 reached chondrocytes inside the knee joints or whether the action of SRT1720 was through local effects or systemic effects. Although the complete pharmacological effects of SRT1720 are unknown, the results of this study suggest that the systemic administration of SRT1720 could attenuate OA progression.

Introduction
Osteoarthritis (OA) is one of the most common human joint diseases, and it causes joint pain and deformities, resulting in the impairment of daily activities. The main characteristics of OA include the progressive breakdown of articular cartilage, remodelling of subchondral bone, and inflammation of the synovium. Previous attempts at treating established OA, for example by inhibiting cartilage-degrading enzymes, failed to show efficacy or were associated with adverse events in clinical trials. Therefore, an alternative treatment approach needs to be developed.

SIRT1 is a histone deacetylase which regulates gene expression and protein function by deacetylating lysine residues in histone3-5 and non-histone proteins such as p53, forkhead box protein O (FOXO), the RelA/p65 subunit of nuclear factor-κB (NF-κB), and peroxisome proliferator-activated receptor-γ (PPAR-γ). Previous studies have shown that SIRT1 inhibits apoptosis in human chondrocytes and increases the expression of the cartilage extracellular matrix (ECM) genes. In addition, it has been reported that SIRT1 inhibition causes OA-like gene expression changes, namely the downregulation of cartilage ECM genes and the upregulation of matrix-degrading enzymes. Furthermore, several genetic SIRT1-deficient mouse models exhibit accelerated OA progression. These previous reports strongly suggest that SIRT1 plays a protective role in chondrocytes and prevents the development of OA.

We have previously reported that the overexpression of SIRT1 downregulated the interleukin (IL)-1β-induced expression of matrix metalloproteinases (MMPs) while upregulating the expression of cartilage ECM genes in human chondrocytes in vitro. In addition, it has been reported that the intra-articular injection of resveratrol, a natural phytoalexin and an activator of SIRT1, had a protective effect against OA in mouse and rabbit OA models. Therefore, these results suggest that the overexpression or stimulation of SIRT1 might represent a new therapeutic approach for OA.

SRT1720 is a small molecule activator developed by Feige et al. Previous biochemical studies have shown that the half-life of SRT1720 is longer than that of resveratrol in mice (5.0 hours versus 0.1 hour) and that its affinity with SIRT1 is approximately 1000 times as strong as that with resveratrol. Furthermore, it has been reported that the administration of SRT1720 improves metabolic states in mouse ageing models through SIRT1 activation. Therefore, SRT1720 has been suggested to have beneficial effects on ageing-related metabolic diseases. However, the effects of SRT1720 on the development of OA remain unclear.

The purpose of this study was to investigate the effects of SRT1720 on the development of OA in a murine experimental OA model.

Materials and Methods
Experimental osteoarthritis in mice. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at Kobe University Graduate School of Medicine. Male C57BL/6J mice (wild-type; Charles River Laboratories Japan, Inc., Yokohama, Japan) were used in this study. Mice were anaesthetized using an intraperitoneal injection of ketamine (100 mg/kg) and the knee joint was exposed through the medial parapatellar approach. Experimental OA was induced in the knee joint of eight-week-old mice by resecting the medial meniscotibial ligament under a microscope to destabilize the medial meniscus. The joint capsule and skin were closed by 3-0 nylon sutures. Mice were maintained under pathogen-free conditions and were allowed free access to food, water, and activity. The body weight of each mouse was measured before sacrifice.

SRT1720 treatment. SRT1720 was obtained from Selleck Chemicals (Houston, Texas, USA), dissolved in dimethyl sulfoxide (DMSO) at 38 mg/ml and stored at -20°C. The stock solution was diluted in phosphate-buffered saline (PBS) and prepared for injection. Immediately after surgery, the mice were injected intraperitoneally twice weekly with SRT1720 (SRT-treated group; 25 mg/kg, 0.2 ml) or the vehicle (control group; 0.2% DMSO in isotonic saline, 0.2 ml) until the animals were sacrificed. The dosage of SRT1720 was determined based on previous studies.

Histological analysis. The SRT1720-treated mice and control mice were killed at four, eight, 12 and 16 weeks after surgery (n = 5 for each timepoint/group), and the entire knee joints were fixed in 4% paraformaldehyde in 0.1 M PBS overnight at 4°C, decalcified for two weeks with 10% ethylenediaminetetraacetic acid (EDTA) and embedded in paraffin wax. Each specimen was cut into slices of 5 μm along the sagittal plane and stained with safranin O-fast green. Three slices were selected from each medial femoral condyle and medial tibial plateau, and pictures were obtained at a magnification of 40. The histological OA grade for each field was evaluated using the Osteoarthritis Research Society International (OARSI) cartilage OA histopathology grading system (score 0 to 6). Osteoarthritis grading was assessed by a single observer (KN) who was blinded to the study groups. In addition, osteophyte maturity and osteophyte size were scored on coded digital images of the same location of the anteromedial tibia in each mouse as previously described.
Immunohistochemistry. Deparaffinized sections were digested with proteinase (Dako Denmark A/S, Glostrup, Denmark) for ten minutes and treated with 3% hydrogen peroxide (Wako Pure Chemical Industries Ltd, Osaka, Japan) to block endogenous peroxidase activity. After epitope retrieval, sections were treated with a 1:50 dilution of the following primary antibodies at 4°C overnight: SIRT1 (Millipore, Billerica, Massachusetts); MMP-13 (Abcam, Cambridge, Massachusetts); ADAMTS-5 (Santa Cruz Biotechnology Inc., Santa Cruz, California); cleaved caspase-3 (Cell Signaling Technology, Tokyo, Japan); poly(ADP-ribose) polymerase (PARP) p85 (Promega, Madison, Wisconsin); acetylated NF-κB p65 (Sigma-Aldrich, St. Louis, Missouri); and type II collagen (Abcam). All of these antibodies were rabbit polyclonal except the anti-SIRT1 antibody which was mouse monoclonal. Sections were then incubated with peroxidase-labelled anti-rabbit or mouse immunoglobulin (Histofine Simple Stain MAX PO; Nichirei Bioscience, Tokyo, Japan) at room temperature for 30 minutes, and the signal was developed as a brown reaction product using the peroxidase substrate, 3,3’-diaminobenzidine, with methyl green counterstaining or haematoxylin counterstaining. As negative controls, a non-immune mouse or rabbit IgG with a 1:50 dilution, was used instead of the primary antibodies. All of the images were obtained under a microscope (Biozero; KEYENCE Corp., Itasca, Illinois).

Histological analysis of inflammation. The severity of synovitis was graded using a scoring system that was previously described\(^3^0\). The synovitis grade was evaluated by haematoxylin and eosin (H&E) staining at four, eight, 12 and 16 weeks after surgery. IL-1β (Abcam) expression in

Table I. Genes used in this study

| Genes   | Description                                      |
|---------|--------------------------------------------------|
| SIRT1   | Sirtuin 1                                        |
| Col1a1  | Collagen type I, alpha 1                         |
| Col2a1  | Collagen type II, alpha 1                        |
| Adamts-5| A disintegrin and metalloproteinase with thrombospondin motifs 5 |
| Mmp-13  | Matrix metalloproteinase 13                      |
| PARP p85| Poly ADP-ribose polymerase p85                   |
| NF-κB   | Nuclear factor-kappa B                           |

Table II. Changes in the mean body weight (g) of the control mice and mice treated with SRT1720 after surgery

|          | Control  | SRT1720  | p-value |
|----------|----------|----------|---------|
| 4 wks    | 24.9 sd 1.5 | 25.1 sd 1.1 | 0.90    |
| 8 wks    | 26.3 sd 1.7 | 26.4 sd 1.4 | 0.98    |
| 12 wks   | 28.2 sd 1.7 | 26.7 sd 0.9  | 0.08    |
| 16 wks   | 30.1 sd 3.2 | 28.2 sd 1.7  | 0.13    |

Fig. 1a

a) Safranin O staining of the knee joints at four, eight, 12 and 16 weeks (scale bar = 100 µm). Representative images are shown from repeated experiments; b) the OARSI scores for the medial femoral condyle and tibial condyle in the control mice and mice treated with SRT1720. Three sections were selected from the lateral, middle and medial one-third of the condyle from each mouse. *p < 0.05.

(Continued)
the synovial tissue was evaluated by immunohistochemistry at four and eight weeks after surgery.

**Cell culture and real-time PCR.** Primary mouse epiphyseal chondrocytes were obtained from five- to seven-day-old mice as has previously been described. Chondrocytes were seeded on six-well plates ($2 \times 10^5$/well). After reaching 60% to 70% confluency, cells were cultured with 0.1 ng/ml IL-1β (R&D Systems, Minneapolis, Minnesota) for 24 hours and then stimulated with different concentrations of SRT1720 (0 µM control, 0.5 µM) for 48 hours. RNA was isolated using a QIAGEN RNeasy Kit (QIAGEN Inc., Valencia, California) and cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Waltham, Massachusetts). Real-time polymerase chain reaction (PCR) for SIRT1, Col1a1, Col2a1, aggrecan, Adamts-5 and Mmp-13 (all Applied Biosystems) (Table I) was performed in duplicate for each sample to determine relative gene expression using
glyceraldehyde 3-phosphate dehydrogenase (GADPH) (Applied Biosystems) as a housekeeping control and the comparative cycle threshold method.

**Statistical analysis.** All values are expressed as the mean and SD. An unpaired two-tailed Student’s t-test was used to compare differences between groups. One-way analysis of variance (ANOVA) was used to compare multiple groups with the Bonferroni method as a post hoc test; p-values < 0.05 were considered statistically significant.

**Results**

Intraperitoneal injection of SRT1720 slowed the progression of OA in a mouse OA model.

There was no statistically significant difference in the mean body weight between the SRT1720-treated mice and the control mice at any of the timepoints examined (Table II). Although the degeneration of articular cartilage progressed gradually over time in both the SRT group and control group, it tended to progress more in the control mice (Fig. 1a). The semi-quantitative analysis showed that the OARSI scores of the medial femoral and tibial condyles in the control group were significantly higher than those in the SRT group at eight and 12 weeks, but not at four and 16 weeks (Fig. 1b). Immunohistochemical analyses showed that type II collagen was more strongly stained and that significantly more type II collagen-positive chondrocytes were detected in the SRT group compared with the control group (Fig. 1c). Osteophytes developed on the anteromedial aspect of the tibial plateau following surgery in both groups. The generated osteophytes were predominantly cartilaginous at four weeks after surgery and developed to bony tissue eight weeks after surgery (Fig. 1d). In both groups, osteophyte maturity increased with postoperative time (Fig. 1e). There was no statistically significant difference between the groups in osteophyte maturity at each timepoint. However, the cartilaginous osteophytes were significantly larger in the control mice compared with SRT1720-treated mice four and eight weeks after surgery (Fig. 1f). There was no significant difference in osteophyte size between the two groups 12 weeks after surgery.

**SIRT1 expression in cartilage was associated with OA progression.** The effects of the intraperitoneal injection of SRT1720 on SIRT1 expression in cartilage was evaluated by immunohistochemistry (Fig. 2a). SIRT1-positive chondrocytes were distributed from the superficial to the deep zones of the cartilage (Fig. 2a). The time-course analysis showed that there was no significant difference between the two groups at four weeks, whereas significantly more SIRT1-positive chondrocytes were detected in the SRT group compared with the control group at eight and 12 weeks. SIRT1-positive chondrocytes gradually decreased in association with OA progression in both groups, and there was no significant difference between the two groups at 16 weeks (Fig. 2b).

Cartilage-degrading enzymes, apoptotic markers and acetylated NF-κB p65 positive-chondrocytes decreased in mice treated with SRT1720.

The expression of cartilage-degrading enzymes, including MMP-13 and ADAMTS-5, and apoptotic markers, including cleaved caspase-3 and PARP p85 fragment, were then examined. All of these markers significantly decreased in the SRT group compared with the control group at four and eight weeks after surgery, but not at 12
a) Immunohistochemistry analysis of ADAMTS-5 at four, eight and 12 weeks; b) the percentage of ADAMTS-5-positive cells; c) immunohistochemistry analysis of MMP-13 at four, eight and 12 weeks; d) the percentage of MMP-13-positive cells; e) immunohistochemistry analysis of cleaved caspase-3, PARP p85 and acetylated NF-κB at eight weeks, and the percentage of positive cells. Scale bar = 50 μm. The positive cells were counted using a total of five male mice for each timepoint/group. Three sections were selected from each mouse (*p < 0.05).
weeks after surgery (Figs 3a and 3c). The percentage of ADAMTS-5 and MMP-13 positive chondrocytes also significantly decreased in the SRT group compared with the control group at four and eight weeks after surgery but not at 12 weeks (Figs 3b and 3d). In addition, the percentage of cleaved caspase-3- and PARP p85 fragment-positive chondrocytes also significantly decreased in the SRT group compared with the control group at eight weeks (Fig. 3e). The NF-κB pathway mediates inflammatory processes, such as MMPs, and when acetylated NF-κB P65 was examined it was found that acetylated NF-κB P65-positive chondrocytes were significantly decreased in the SRT group compared with the control group (Fig. 3e).

**Synovitis was reduced in mice treated with SRT1720.** Inflammation in the synovium was also examined histologically at four, eight, 12 and 16 weeks. The synovial lining cell layer in the control mice was thicker than that in the mice treated with SRT1720 (Fig. 4a). The synovitis score was significantly lower in the SRT group than in the control group at four and eight weeks (p < 0.05) but not at 12 and 16 weeks (Fig. 4b). In addition, the staining intensity of IL-1β in the synovium of mice treated with SRT1720 was weaker than that in control mice at four and eight weeks (Fig. 4c).

Treatement with SRT1720 partially inhibited IL-1β-induced OA-like gene expression changes in mouse chondrocytes. To further determine the effects of SRT1720 on chondrocytes, gene expression changes in mouse epiphyseal chondrocytes were examined. SIRT1 mRNA expression significantly increased in SRT1720-treated chondrocytes compared with untreated chondrocytes (Fig. 5). Although treatment with SRT1720 did not change Col2a1 and aggrecan expression levels in the absence of IL-1β, it partially rescued the decreases in Col2a1 and aggrecan expression levels caused by IL-1β. In addition, treatment with SRT1720 decreased the IL-1β-induced Mmp-13 expression (Fig. 5).

**Discussion**

The main finding of this study was that the intraperitoneal injection of SRT1720 attenuated experimental OA progression in mice. The attenuation of OA progression was associated with increases in type II collagen- and SIRT1-positive chondrocytes and decreases in MMP-13-, ADAMTS-5- and cleaved caspase-3-positive chondrocytes.

SRT1720 was developed as a potent SIRT1 activator and it has been reported that SIRT1 activation by SRT1720 extends the lifespan and improves metabolic states in various mouse models. Therefore, it has been suggested that it has beneficial effects on ageing-related metabolic diseases.

In this study, the expression levels of SIRT1 were significantly increased by treatment with SRT1720 in vitro. Although SRT1720 was originally reported as an activator of SIRT1 enzymatic activities, our results suggested that SRT1720 can increase the expression level of SIRT1. Supporting our results, it has been reported that the expression levels of SIRT1 were significantly increased following treatment with SRT1720 in rabbit chondrocytes cultured with sodium nitroprusside (SNP). In our in vivo study, significantly more SIRT1-positive chondrocytes were detected in the knee joints of mice injected with SRT1720 at eight and 12 weeks but not at four and 16 weeks. This observation suggested that the intraperitoneal injection of SRT1720 did not increase the expression...
of SIRT1 or only increased it by an undetectable level in chondrocytes in the in vivo model. We have previously examined SIRT1 expression in a mouse OA model and observed that SIRT1 protein first increased in the early phase of OA development and then gradually decreased during the advancement of OA. Furthermore, it has been reported that SIRT1 was clearly expressed in less damaged human articular cartilage, but decreased in severely degenerated cartilage. Therefore, the increased SIRT1 expression in chondrocytes from mice treated with SRT1720 at eight and 12 weeks might be a consequence of delayed OA progression in the SRT group rather than an indication of a direct positive effect on SIRT1 expression in chondrocytes.

In this study, type II collagen was more strongly stained, and significantly more type II collagen positive-chondrocytes were detected in the SRT group compared with the control group eight weeks after surgery. In addition, treatment with SRT1720 partially rescued the decreases in Col2a1 and aggrecan expression levels caused by IL-1β. Previous studies have reported that SIRT1 stimulated type II collagen expression and
aggreca through an interaction with SOX9 in vitro. Therefore, increases in type II collagen and aggrecan expression through SIRT1 might be a mechanism by which treatment with SRT1720 attenuates OA progression.

We also observed fewer apoptotic marker-positive chondrocytes in the mice treated with SRT1720 compared with the control mice. Previous studies have shown anti-apoptotic roles of SIRT in human chondrocytes through the modulation of various pathways. In addition, advanced OA progression associated with increases in apoptotic chondrocytes was observed in systemic SIRT1-knockout mice and cartilage-specific SIRT1-conditional knockout mice. Considering these protective roles of SIRT1 against apoptosis in chondrocytes, it is possible that decreases in apoptosis in chondrocytes through the upregulation of SIRT1 by SRT1720 also contributed to the attenuation of OA progression in the mice treated with SRT1720.

We observed decreased expression of cartilage-degrading enzymes, including MMP-13 and ADAMTS-5, in the mice treated with SRT1720. In addition, the acetylation of the NF-κB p65 subunit was decreased in the SRT1720-treated mice compared with the control mice. A previous study reported that SIRT1 suppressed NF-κB signalling through deacetylation of the NF-κB p65 subunit and that NF-κB p65 positively regulated the expression of MMPs and ADAMTS-5 in chondrocytes. We have reported previously that the overexpression of SIRT1 in chondrocytes decreased the expression of cartilage-degrading enzymes such as MMPs and ADAMTS-5 induced by IL-1β in vitro. Therefore, the attenuation of OA development in the mice treated with SRT1720 may have occurred though decreases in cartilage-degrading enzymes via the modulation of the NF-κB pathway by SIRT1.

Inflammatory changes in the synovium are associated with OA progression in humans and in animal experimental OA models. In the present study, treatment with SRT1720 reduced the severity of synovitis and IL-1β expression in the synovium. Similar to our observations, anti-inflammatory effects of SRT1720 have been observed in various organs. Taken together, the reductions in synovitis, the IL-1β expression level, and inflammation in the synovium caused by SRT1720 may also have contributed to the attenuation of OA progression by SRT1720.

SRT1720 was administered intraperitoneally in our study, and the administration of SRT1720 did not significantly change the body weight during the study period. Jiang et al reported that the oral administration of resveratrol attenuated OA progression in mice fed a high-fat diet without significantly affecting their body weights. In the study, OA progression was attenuated in the mice treated with resveratrol, and this attenuation was associated with decreased serum levels of IL-1β and leptin, a mediator of inflammation. Therefore, the delayed OA progression in the mice treated with SRT1720 may have been due to the decreased serum levels of these systemic inflammatory factors rather than the direct effects of SRT1720 on chondrocytes in the knee joint. Further studies are required to elucidate the mechanisms of the attenuation of OA by the intraperitoneal injection of SRT1720.

Although there was a tendency for the OAASI score to be better in the SRT-treated group than in the control group at all examined timepoints, there was no statistical significant difference between the two groups at four and 16 weeks. The possible reason why we did not see remarkable differences in OA progression between the two groups at four weeks may be due to the speed of OA progression in our OA model, since OA changes in both groups did not noticeably advance and the OAASI score remained low in both groups at four weeks. Therefore, the difference may not be large enough to be detected by the scoring system and it did not reach statistically significant difference.

While most of the previous studies have examined OA changes only until around eight weeks after surgery, we examined the effects of SRT1720 on OA progression for up to 16 weeks. However, we found that OA progression in the SRT group also advanced, and the difference between the two groups became minimal at 16 weeks. Since most of the cartilage had disappeared in the control group at 16 weeks, it is possible that the OA progression in the control group had reached a plateau by this time and the speed of the OA progression had slowed. Therefore, it is possible to suggest that the OA progression in the SRT1720-treated group had by then caught up with the changes seen in the control mice.

Based on the results in this study, the effect of systemic administration of SRT 1720 against OA progression appears to be modest. Although, currently, the precise mechanism of the delayed OA progression by the systemic administration of SRT1720 is unknown, the systemic administration of SRT1720 may mainly modulate inflammatory responses locally or systemically in the early phase of OA similar to other immunomodulatory drugs. As regards future practical use, it is necessary to test the efficacy using intra-articular injection or other drug-delivery systems such as gelatin hydrogels incorporating drug micelles.

There are some limitations to this study. First, young mice (eight weeks old) were used and consequently our experimental model may not reflect OA in humans. Second, only five male mice were used in an in vivo study. Therefore, the statistical analysis may be underpowered and also the results could be different in female mice. Third, it was not determined whether SRT1720 reached the chondrocytes inside the knee joint or indeed whether the action of SRT1720 was through local or systemic effects. Although the complete pharmacological effects
of SRT1720 are unknown, the results of our study suggest that the systemic administration SRT1720 could attenuate OA progression.

In conclusion, we have demonstrated that the intraarticular injection of SRT1720 attenuated experimental OA progression in the mice knee joint. This attenuation of OA progression was associated with decreases in cartilage-degrading enzymes, apoptotic markers and acetylated NF-κB p65 in chondrocytes as well as the severity of synovitis. Hence SRT1720 may represent a new therapeutic approach for OA.

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Author Contributions
K. Nishida: Contributed to the conception of the study and the experimental design. Performed all animal surgeries, SRT1720 and vehicle injections, histological staining and analysis, gene expression analysis and data analysis. Drafted the manuscript, read and approved the final manuscript.

T. Matsushita: Contributed to the conception of the study and the experimental design. Analyzed the data, drafted the manuscript, read and approved the final manuscript.

K. Takayama: Contributed to the conception of the study and the experimental design. Read and approved the final manuscript.

T. Tanaka: Performed all animal surgeries and chondrocytes culture. Read and approved the final manuscript.

N. Miyaji: Performed all animal operations and chondrocytes culture. Read and approved the final manuscript.

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N. Kanzaki: Conception of the study, read and approved the final manuscript.

T. Matsumoto: Conception of the study, read and approved the final manuscript.

R. Kuroda: Experimental design, helped to revise the manuscript. Read and approved the final manuscript.

Conflicts of Interest Statement
None declared

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