SPRY4 acts as an indicator of osteoarthritis severity and regulates chondrocyte hypertrophy and ECM protease expression

Sunghyun Park1,2, Yoshie Arai3, Alvin Bello3, Hansoo Park3, Dohyun Kim1, Kyung-Soon Park2 and Soo-Hong Lee1,*

Osteoarthritis (OA) causes serious changes in the metabolic and signaling pathways of chondrocytes, including the mitogen-activated protein kinase (MAPK) pathway. However, the role of sprouty RTK signaling antagonist 4 (SPRY4), an inhibitor of MAPK, in the human cartilage tissues and chondrocytes remains to be understood. Here, using SPRY4 gene delivery into healthy and degenerated chondrocytes, we elucidated the role of SPRY4 in preventing chondrocyte hypertrophy. In addition to using the human cartilage tissues with the destabilization of the medial meniscus (DMM) model in Sprague-Dawley (SD) rats, the role of SPRY4 in cartilage tissues and chondrocytes was explored through their molecular and histological analyses. In order to determine the effects of SPRY4 on healthy human chondrocyte hypertrophy, small interfering RNA (siRNA) was used to knock down SPRY4. Lentiviral transduction of SPRY4 into degenerated human chondrocytes allowed us to investigate its ability to prevent hypertrophy. SPRY4 expression levels were higher in healthy human cartilage tissue and chondrocytes than in degenerated human cartilage tissues and hypertrophy-induced chondrocytes. The knockdown of SPRY4 in healthy chondrocytes caused an increase in hypertrophy, senescence, reactive oxygen species (ROS) production, and extracellular matrix (ECM) protease expression. However, all these factors decreased upon overexpression of SPRY4 in degenerated chondrocytes via regulation of the MAPK signaling pathway. We conclude that SPRY4 is a crucial indicator of osteoarthritis (OA) severity and could play an important role in preventing OA in the cartilage by inhibiting chondrocyte hypertrophy.

**INTRODUCTION**

The molecular pathogenesis of OA involves numerous molecular, cellular, and niche alterations, including chondrocyte hypertrophy, reactive oxygen species (ROS) generation, chondrocytes senescence, decrease in cell proliferation, protease activation, extracellular matrix (ECM) collapse of cartilage, inflammation, and osteophyte formation1,2. Of these, chondrocyte hypertrophy is a representative pathogenic symptom characterized by volume enlargement and protease activation and typically associated with early stages of OA3,4. Although chondrocyte hypertrophy is essential for endochondral ossification in healthy tissue, it is also implicated in the pathogenesis of OA5. Chondrocyte hypertrophy is tightly regulated by genes such as Indian hedgehog (IHH), collagen type X (COL10), matrix metalloproteinase-13 (MMP13), and runt-related transcription factor-2 (RUNX2)2,5,6. RUNX2, a member of the runt family of transcription factors that plays a crucial role in osteoblast and chondrocyte differentiation, is also tightly associated with OA development and progression6,7. In OA, RUNX2 regulates chondrocyte hypertrophy by inducing ECM mineralization and vascular invasion, thereby inducing chondrocyte apoptosis, osteogenic lineage differentiation, cartilage fibrosis, and ECM protease activation8,10. ECM protease activation is a crucial factor in accelerating OA and is typically responsible for degrading and decomposing the cartilage's fibrous ECM proteins such as collagen and aggrecan8. Many proteases such as MMP13 and disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5) have been associated with OA7,12. Increases in RUNX2 expression and ECM protease activation are critical biological markers of chondrocyte hypertrophy, OA progression, and cartilage collapse.

OA pathogenesis also involves the activation of specific signaling pathways that initiate a protein chain reaction. Among these, the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling pathway is crucially responsible for OA progression13,14. MAPK/ERK activation and phosphorylation induce an increase in RUNX2 expression, MMP13 expression, chondrocyte apoptosis, and cartilage decomposition5,15,16. Therefore, the involvement of the MAPK/ERK pathway needs to be investigated further to better understand chondrocyte hypertrophy and OA.

The SPRY family comprising four known isoforms (SPRY 1, 2, 3, and 4) function as antagonists of MAPK signaling. Although the roles of SPRY 1, 2, and 3 in MAPK signaling have been investigated in other studies17,18, the sprouty RTK signaling antagonist 4 (SPRY4) requires further investigation to understand its role and mechanism in various organs. Previous research has elucidated that SPRY4, which acts as an antagonist of MAPK signaling by inhibiting the formation of active GTP-Ras in the MAPK/ERK pathway, regulates cytokine secretion, cell proliferation, and cell differentiation19–21. Furthermore, we have reported that the suppression of SPRY4 promotes osteogenic differentiation of mesenchymal stem cells by upregulating ERK 1/2 phosphorylation22. We have also reported that SPRY4 knockdown induces cellular hypertrophy in mesenchymal stem cells (MSC). Both, osteogenic differentiation and cellular hypertrophy are essential processes in endochondral ossification23,24. Cellular hypertrophy was induced by SPRY4, an antagonist of MAPK/ERK signaling, in

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1Department Medical Biotechnology, Dongguk University Biomedical Campus, Goyang-si, Gyeonggi-do, Republic of Korea. 2Department of Biomedical Science, CHA University, Seongnam-si, Gyeonggi-do, Republic of Korea. 3School of Integrative Engineering, Chung-ang University, Seoul, Republic of Korea. 4email: soohong@dongguk.edu
S. Park et al.

npj Regenerative Medicine (2021) 56

Difference in Unpaired T-test with Welch’s correction; MMP13: Mann Whitney test)
the GAG content, in the HCs. The trends observed for mRNA expression were similar to those for protein expression. SPRY4 knockdown decreased the expression levels of chondrogenic marker genes (ACAN, SOX9, and COL2) and increased the expression levels of hypertrophic marker genes (RUNX2 and COL10) and ECM protease marker genes (MMP13 and ADAMTS5) compared to those of the control (Fig. 3a). Protein analysis showed that chondrogenic marker protein levels (ACAN, SOX9, and COL2) were decreased, whereas hypertrophic marker protein levels (RUNX2, COL10, and MMP13) and ECM protease marker protein levels (MMP13 and ADAMTS5) were increased in siSPRY4-treated HCs (Fig. 3b). Interestingly, pERK 1/2 protein expression, which is related to RUNX2 and MMP expression, was increased in siSPRY4-treated HCs. In contrast, other MAPK signaling proteins such as p38 and JNK, as well as their phosphorylated forms, showed no significant differences between the control group and the siSPRY4-treated HCs (Supplementary Fig. 4). These results indicate that SPRY4 regulates chondrocyte hypertrophy and OA progression through the ERK-MAPK signaling pathway. Alcian blue staining and safranin-O staining revealed that GAG content decreased in siSPRY4-treated HCs compared to that in the control (Fig. 3c). These results imply that SPRY4 knockdown affects chondrocyte hypertrophy and senescence.

### Effect of SPRY4 overexpression on cellular morphology, hypertrophy, and senescence in DCs

In order to understand the role that SPRY4 plays in regulating hypertrophic changes and senescence in HCs and DCs, we utilized a lentiviral vector to overexpress SPRY4 in DCs. First, the efficiency of the lentiviral delivery system was examined. Lenti-SPRY4 increased SPRY4 mRNA expression level compared to that of the negative control (Supplementary Fig. 5a); this increase was also observed by IF staining (Supplementary Fig. 5b). Using FACS analysis, the transduction efficiency of Lenti-SPRY4 was determined to be 80.23% (Supplementary Fig. 5c). After transfecting the lentiviral vectors into the DCs, we investigated the effect of SPRY4 overexpression on cellular morphology, hypertrophy, and senescence.

SPRY4 overexpression in DCs had a negligible effect on the cell growth rate compared to that of the control (Fig. 4a). However, as confirmed by quantification, SPRY4 overexpression reduced the size of the cell body (Fig. 4b) and the nucleus in the DC compared to that in the control (Fig. 4c). ROS level and senescence, indicated by β-gal staining, of the DCs were also decreased by SPRY4 overexpression (Fig. 4d, e). These results suggest that SPRY4 overexpression might be useful in restoring chondrogenic characteristics to the DCs in terms of morphology, ROS level, and senescence.
DISCUSSION

During OA progression, the MAPK/ERK signaling pathway regulates chondrocyte hypertrophy through the phosphorylation of ERK 1/2, leading to increased expression of RUNX2 and multiple MMPs, which are crucial in regulating chondrocyte hypertrophy and inducing OA. Beier et al. reported that the MAPK signaling cascade also leads to the activation of chondrocyte hypertrophic markers, such as COL10. Furthermore, MAPK/ERK signaling induces senescence-related accumulation of ROS and cell death via intrinsic or extrinsic apoptotic pathways, including mitochondrial cytochrome C release, caspase-8 activation, and autophagic vacuolization. It has been established that SPRY4 is an inhibitor and regulator of the MAPK/ERK signaling pathway. Therefore, we hypothesized that SPRY4 plays a critical role in OA by influencing chondrocyte hypertrophy and senescence.

In order to determine whether SPRY4 is related to OA, we compared its expression levels in healthy and degenerated cartilage tissues. Interestingly, the expression level of SPRY4 was significantly lower in degenerated cartilage tissues compared to that in the healthy cartilage tissues. This finding supported our hypothesis that the expression of SPRY4 in the cartilage is associated with OA progression, characterized by increased chondrocyte hypertrophy and inflammatory conditions. In order to compare the differences between the HCs and DCs further, we measured the expression levels of several chondrogenic and hypertrophic markers. As expected, chondrogenic markers (ACAN, SOX9, and COL2) were upregulated in HCs, whereas hypertrophic markers (RUNX2 and MMP13) were upregulated in the DCs. Notably, contradictory results exist on the use of ACAN and COL2 as chondrogenic markers, which primarily depend on the developmental phase of OA. However, both ACAN and COL2 are still widely accepted as chondrogenic markers and therefore used in this study. The expression of COL10, however, was found to be slightly lower in DCs, presumably because of population variation. Gene expression in samples of human cartilages is known to differ depending on the donor's age, genetic background, and pathologic conditions.

In order to determine whether the induction of hypertrophy would affect the expression levels of SPRY4 in chondrocytes, we treated the HCs with IL-1β. As expected, IL-1β treatment in HCs caused a significant reduction in chondrogenic marker genes; however, it upregulated ECM protease expression levels and increased chondrocyte hypertrophy. Interestingly, SPRY4 expression...
in IL-1β-treated HCs was also reduced. This result supports the idea that SPRY4 expression is a crucial indicator of cartilage health and chondrocyte hypertrophy. Meanwhile, we found that RUNX2 expression was not significantly different in untreated and IL-1β-treated HCs, presumably because of the inhibition of RUNX2 by IL-1β. Ding J et al. reported that treating human mesenchymal stem cells (hMSCs) with IL-1β increased alkaline phosphatase activity and mineralization and inhibited RUNX2. Although chondrocyte hypertrophy induces OA, it is also well established that it increases the secretion of alkaline phosphatase, osteocalcin, and osteopontin similar to endochondral ossification in osteogenesis in hMSCs. Considering the similarities in endochondral ossification in hMSCs and hypertrophic chondrocytes, a similar result might be observed.

Next, we investigated the effect of SPRY4 on HCs and DCs in vitro using SPRY4 knockdown and overexpression studies. We utilized siSPRY4 to knock down SPRY4 and lentiviral SPRY4 to induce its overexpression. Downregulation of SPRY4 induced chondrocyte hypertrophy in HCs and increased ERK 1/2 phosphorylation. In contrast, upregulation of SPRY4 inhibited hypertrophic changes in DCs and decreased ERK 1/2 phosphorylation. Based on the mRNA and protein expression levels of hypertrophic markers and GAG staining analysis, the upregulation of SPRY4 in DCs significantly reduced chondrocyte hypertrophy compared to that in the control. However, chondrogenic marker gene expression levels were not significantly affected SPRY4 upregulation. We believe that because the DCs have already undergone hypertrophy, they primarily express RUNX2, COL10, bone sialoprotein (BSP), and MMP13 while expressing fewer chondrogenic factors, such as ACAN, SOX9, and COL2. Moreover, many studies have reported that MAPK signaling induces chondrocyte hypertrophy, but not chondrocyte regeneration. Taking together, our results suggest that SPRY4 overexpression is critical to OA progression through regulation of the MAPK signaling pathway by inhibiting ERK1/2 phosphorylation. In our previous study, we confirmed that SPRY4 knockdown directly affects the increase in ERK1/2 phosphorylation after u0126 treatment. However, we only investigated the possible role of SPRY4 as a therapeutic for OA. Therefore, our results about the effects of SPRY4 overexpression on MAPK/ERK signaling to require further validation and additional drug targeting to elucidate its complete mechanism.

Furthermore, various studies involving chemicals and scaffolds have focused on MAPK signaling with chondrocytes hypertrophy. For example, Indira et al. reported that MAPK signaling was regulated by u0126 with hyaluronic acid (HA); they reported positive synergistic effects on both chondrocyte hypertrophy and a rat meniscectomy (MSX) OA-induced model. Wang et al. reported that chondrocyte hypertrophy was inhibited and redifferentiated by PD0325901. However, studies applying chemicals and scaffolds may not be able to solve the fundamental problem in OA. Overexpression of SPRY4 might be a good therapeutic candidate because it prevents hypertrophy on a genetic level. We also confirmed that SPRY4 overexpression did not have any negative effects on chondrocyte viability (Supplementary Fig. 6), which indicates its safety for long-term use. Although further studies and additional experiments are required
to validate its effectiveness and efficiency, we believe that SPRY4 will ultimately be a strong candidate for direct OA treatment.

In this study, we compared SPRY4 expression in healthy and degenerated (OA) human cartilage and found that it was higher in healthy cartilage tissue. IL-1β treatment in HCs significantly reduced SPRY4 expression. Furthermore, although the siRNA-mediated downregulation of SPRY4 in HCs reduced characteristics such as GAG content, it increased hypertrophic markers such as SOX9, COL10, and ADAMT5. In contrast, the upregulation of SPRY4 expression in healthy and degenerated cartilage tissues were separated based on a previously described protocol. Brieﬂy, the white-colored, intact, abundant hyaline cartilage tissues were classiﬁed as relatively healthy tissue, whereas the red-colored, collapsed, ﬁbrous cartilage tissues were classiﬁed as relatively degenerated tissue. Cells isolated from the healthy cartilage were denoted as healthy chondrocytes (HCs), while cells from degenerated tissues were denoted as degenerated chondrocytes (DCs). Both tissues were washed three times with phosphate-buffered saline (PBS), and then chopped using a surgical blade until they become ﬁne particles. Chopped tissues were re-washed in PBS and digested with 0.5 mg/ml collagenase (Sigma Aldrich, MO) in DMEM low-glucose media (HyClone, MA) containing 1% antibiotics (P/S) overnight (O/N) at 37 °C in a 5% CO2 incubator. The cells were harvested with 0.5% Trypsin-EDTA (Invitrogen, NY) and passaged at a seeding density of 3 × 10⁴ cells/cm². Whole-cell experiments were performed at passage 3.

**Interleukin 1β (IL-1β) treatment**

Human-derived chondrocytes were used for IL-1β treatment at passage 3. Chondrocytes were seeded at a density of 1 × 10⁵ per well in a 6-well plate (Falcon, NC). After O/N incubation, chondrocytes were incubated for 12 h in 0.5% FBS low-glucose DMEM. Subsequently, 200 ng of IL-1β (R&D, MN) was added to each well and the chondrocytes (n = 3) were incubated for an additional 2 days.

**Cytological and histological staining**

Chondrocytes and cartilage samples were ﬁxed with 4% (w/v) paraformaldehyde (PFA; Biosesang, Seongnam, Korea). In the case of cartilage, samples were decalcified in a decalcification solution (BBC Biochemical, WA) for 1 week and processed as described previously. Briefly, the white-colored, intact, abundant hyaline cartilage tissues were classiﬁed as relatively healthy tissue, whereas the red-colored, collapsed, ﬁbrous cartilage tissues were classiﬁed as relatively degenerated tissue. Cells isolated from the healthy cartilage were denoted as healthy chondrocytes (HCs), while cells from degenerated tissues were denoted as degenerated chondrocytes (DCs). Both tissues were washed three times with phosphate-buffered saline (PBS), and then chopped using a surgical blade until they become ﬁne particles. Chopped tissues were re-washed in PBS and digested with 0.5 mg/ml collagenase (Sigma Aldrich, MO) in DMEM low-glucose media (HyClone, MA) containing 1% antibiotics (P/S) overnight (O/N) at 37 °C in a 5% CO2 incubator. The cells were harvested with 0.5% Trypsin-EDTA (Invitrogen, NY) and passaged at a seeding density of 3 × 10⁴ cells/cm². Whole-cell experiments were performed at passage 3.

**METHODS**

**Human chondrocyte isolation and culture**

Human cartilage was obtained by manual isolation from the knee of donor patients, who provided written informed consent to the CHA Stem Cell Institute with approval from the Institutional Review Board of the CHA University Hospital Ethics Committee (IRB NO. 2014-096). Healthy and degenerated cartilage tissues were separated based on a previously described protocol. Brieﬂy, the white-colored, intact, abundant hyaline cartilage tissues were classiﬁed as relatively healthy tissue, whereas the red-colored, collapsed, ﬁbrous cartilage tissues were classiﬁed as relatively degenerated tissue. Cells isolated from the healthy cartilage were denoted as healthy chondrocytes (HCs), while cells from degenerated tissues were denoted as degenerated chondrocytes (DCs). Both tissues were washed three times with phosphate-buffered saline (PBS), and then chopped using a surgical blade until they become ﬁne particles. Chopped tissues were re-washed in PBS and digested with 0.5 mg/ml collagenase (Sigma Aldrich, MO) in DMEM low-glucose media (HyClone, MA) containing 1% antibiotics (P/S) overnight (O/N) at 37 °C in a 5% CO2 incubator. The cells were harvested with 0.5% Trypsin-EDTA (Invitrogen, NY) and passaged at a seeding density of 3 × 10⁴ cells/cm². Whole-cell experiments were performed at passage 3.

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All IHC analyses were performed using a GBI kit (GBI Labs, WA). For IF, fluorescence signals were detected with a Cytation 3 Cell Imaging Reader (BioTek, VT). For DAPI staining, fixed chondrocytes were treated with 20 μl of 4′,6-diamidino-2-phenylindole (DAPI) solution (Invitrogen, CA) per 1 ml of PBS. After a 10-min incubation, fluorescence was detected with a Cytation 3 Cell Imaging Reader. For reactive oxygen species (ROS) staining, 5 × 10^5 chondrocytes were seeded and cultured on 12-well plates for 1 week. After washing once with PBS, PBS containing 5 μM of Mito SOX Red (M36008, Invitrogen) probe was added and incubated for 15 min at 37 °C in the dark. After washing twice with PBS, red fluorescence was detected using a fluorescence microscope (Olympus, Tokyo, Japan).

Senescence-associated β-galactosidase (β-gal) staining was performed with a staining kit (Cell Signaling Technology, MA). Briefly, chondrocytes were incubated for 7 days after seeding. Subsequently, chondrocytes were fixed and stained according to the manufacturer’s protocol. Blue-stained cells were counted as positive for senescence. Subsequently, stained specimens were mounted (StatLab, TX) and observed using light microscopy. Whole staining results and microscopic images (n = 5) were selected randomly, and size or fluorescence intensity was measured using the ImageJ software.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Chondrocytes were treated with 1 ml of TRIzol reagent (Invitrogen, CA) (n = 3). Total RNA was extracted and then reverse-transcribed using a reverse-transcription kit (Enzymomics, Daejeon, Korea) following the manufacturer’s instructions. The primers for 18s ribosomal RNA (RPS18), SPRY4, Aggreccan (ACAN), SRY-related HMG-box gene 9 (SOX9), Collagen type II (COL2), RUNX2, COL10, MMP13, matrix metalloproteinase 1 (MMP1), matrix metalloproteinase 3 (MMP3), and ADAMTS5 are shown in Supplementary Table 1. qRT-PCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Warrington, UK) with the Power SYBR Green PCR Master Mix 2x (Applied Biosystems, Warrington, UK).

Knockdown of SPRY4 using small interfering RNA (siRNA) in healthy chondrocytes

Chondrocytes were cultured at an initial seeding density of 4 × 10^4 cells. After 24 h, chondrocytes were transfected with 50 nM of siRNA-SPRY4 (siSPRY4, Bioneer, Daejeon, Korea) using Lipofectamine RNAiMAX Reagent (Invitrogen, CA) and Opti-MEM I Reduced Serum Medium (Gibco, MA) following the standard procedure (n = 3). After siRNA treatment, chondrocytes were incubated for 6 h, and then the medium was replaced with fresh low-glucose DMEM. After O/N incubation, chondrocytes were harvested and used in experiments.

Overexpression of SPRY4 by lentiviral transduction in DCs

Lentivirus was generated by co-transfection of 2.5 μg of plenti-SPRY4-mGFP (Origene, MD), 1.25 μg of pMDLg/pRRE (Addgene, MA), 0.625 μg of pRSV-Rev (Addgene, MA), and 0.625 μg of pMD2.G (Addgene, MA) into 293T cells (TakaRa, Kyoto, Japan) (2 × 10^5 cells per 100 mm dish) using the ConvyTM transfection reagent (ACTGene, NJ). After 24 h, the medium was exchanged with a fresh medium. After an additional 24 h incubation at 37 °C, the supernatant containing the lentivirus was collected in a conical tube, and a fresh medium was added to the transected 293T cells. The lentivirus containing supernatant collected in the conical tube was centrifuged at 1000 rpm for 5 min to remove cellular debris. The supernant was filtered using a Millex-HV 0.45 μm filter (Merck Millipore, MA) and then ultra-centrifugated at 22,000 rpm for 2 h. The virus pellet was resuspended with a 10 ml fresh growth medium containing 8 ng/ml hexadimethrine bromide (Sigma Aldrich, MO) and then incubated with chondrocytes for 24 h (n = 3).

Cell viability and proliferation assays

CCK-8 assays (Dojindo, MD) were used to analyze chondrocyte proliferation following the manufacturer’s protocol. Briefly, CCK-8 stock was diluted 1:10 in DMEM low-glucose medium, and 350 μl was added to each well. Treated cells were incubated for 1 h at 37 °C and analyzed with a Cytation 3 Cell Imaging Reader (BioTek, VT) (n = 3).

Western blot analysis

Chondrocytes were washed three times with PBS on ice, lysed with 200 μl RIPA buffer (Sigma Aldrich, MO), and then centrifuged at 13,000 rpm for 20 min. Samples (20 μg) of the total protein extract were then separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose (NC) membrane using a Trans-Blot semi-dry transfer kit (Bio-Rad, CA) and blocked for 1 h with 5% skimmed milk in Tris-buffered saline (TBS) with 0.1% Tween-20 (TBS-T). Blots were incubated O/N with primary antibodies in 5% BSA solution with TBS-T. Membranes were washed 3 times in TBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies (0.1 mg/ml; Jackson Immunoresearch Laboratories) diluted in 5% skimmed milk with TBS-T. Protein bands were analyzed using a Chemi-doc detection system (Bio-Rad, CA).

Primary antibodies for SPRY4 (ab228712, 1:1000 diluted), RUNX2 (ab236639, 1:2000 diluted), ACAN (ab386861, 1:2000 diluted), SOX9 (ab185230, 1:2000 diluted), and ADAMTS5 (ab14037, 1:1000 diluted) were obtained from Abcam (Cambridge, UK). Antibodies for signaling molecules pERK1/2 (4377, 1:1000 diluted), ERK1/2 (9102, 1:2000 diluted), pJNK (4668, 1:1000 diluted), JNK (9258, 1:2000 diluted), pp38 (4631, 1:1000 diluted), and p38 (9212, 1:2000 diluted) were purchased from Cell Signaling Technology (CST) (MA, USA), Antibodies for COL2 (AB8887, 1:2000 diluted) from Calbiochem (Merck Millipore, MA, USA), antibodies for MMP13 (MAB511-100, 1:1000 diluted) from R&D Systems (MN, USA), and COL10 (C7974-100UL, 1:2000 diluted) from Calbiochem Merck (Merck Millipore, MA, USA). The β-actin (G046, 1:1000 diluted) antibodies were obtained from Applied Biological Materials (Richmond BC, Canada). We ensured that all blots derived from the same experiment and were processed in parallel.

Flow cytometry

Flow cytometry was used to measure the efficiency of lentiviral transduction into chondrocytes using green fluorescent protein (GFP) expression. Transduced chondrocytes were trypsinized, washed with PBS, and collected in a 1.5 ml tube. For each sample, a suspension of 5 × 10^6 cells was prepared and used for detection.

In vivo destabilization of the medial meniscus (DMM) model for osteoarthritis induction

Eight-week-old healthy, male Sprague-Dawley (SD) rats (Orient-bio, Seongnam, Korea) were anesthetized with zoletil (50 mg/kg) and rompun (10 mg/kg). OA was induced by dissecting the medial meniscus to initiate degeneration and collapse of the right knee joint of the hind limb for the OA-induced group (n = 4), as described previously26. Rats in the control group (n = 4) were surgically treated the same without dissecting the medial meniscus. Two rats were housed together in clean, pathogen-free cages with dust-free woodchips as substrate bedding, raised at 55–65% humidity at a controlled temperature of 24 ± 3 °C with a 12 h light/dark cycle, and cared for according to the institutional protocol. At eight weeks post-surgery, all experimental animals were sacrificed, and their right knee joints were collected. Whole staining experiments were performed on the medial tibia cartilage layer of the right knee joint. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of CHA University (IACUC180026).

Statistical analysis

All statistics were analyzed using Prism v8.0 (GraphPad software). A two-tailed unpaired t-test with or without Welch’s correction or a Mann Whitney test was used to compare experimental groups following normality (see Supplementary Table 2) and the equal variance test (see Supplementary Table 3). All data were expressed as mean ± standard error of the mean (SEM). The exact P values are presented in the figures and summarized in Supplementary Table 4.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.
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AUTHOR CONTRIBUTIONS

S.P. and Y.A. conceptualized and designed this study. D.K. supported histological analyses. The graphical abstract was prepared by A.B. All authors contributed to the final version of the manuscript. K.-S.P., H.P. and S.-H.L. elaborately reviewed and revised the manuscript. The entire study was conducted under the supervision of S.-H.L.

COMPETING INTERESTS

The authors declare no competing interests.
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Correspondence and requests for materials should be addressed to Soo-Hong Lee.

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