1 **The role of substance P on maintaining ligament homeostasis by inhibiting endochondral**
2 **ossification during osteoarthritis progression**

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25

26 **Abstract**

27 **Purpose:** Osteoarthritis (OA) is characterized by the degeneration of various tissues,
28 including ligaments. However, pathological changes such as chondrogenesis and
29 ossification in ligaments during OA are still unclear. Substance P (SP), a neuropeptide,
30 has various functions including bone metabolism. This study aimed to analyze the
31 expression and function of SP in OA ligaments, and the therapeutic potential of SP
32 agonists in OA mice.

33 **Materials and methods:** Expressions of SP, SOX9, and MMP13 were histologically
34 analyzed in the posterior cruciate ligament (PCL) in humans with OA and Senescence-
35 accelerated mouse-prone 8 (SAMP8) mice as a spontaneous OA model. The effect of
36 SP agonists on chondrogenesis was evaluated using human ligament cells. Finally, SP
37 agonists were administered intraperitoneally to destabilized medial meniscus (DMM)
38 mice, and the PCL was histologically evaluated.

39 **Results:** In PCL of humans and mice, the expression of SP, SOX9, and MMP13 was
40 upregulated as OA progressed, but their expression was downregulated in severe
41 degeneration. SP and SOX9 were co-expressed in chondrocyte-like cells. In ligament
42 cells, SP agonists downregulated SOX9, RUNX2, and COL10A1. On evaluating
43 chondrogenesis in ligament cells, pellet diameter was reduced in those treated with the
44 SP agonists compared to those untreated. Administration of SP agonists ameliorated PCL
45 degeneration in DMM mice. The Osteoarthritis Research Society and ligament scores
46 in mice with SP agonists were significantly lower than those without SP agonists.

Conclusions: SP plays an important role in maintaining ligament homeostasis by inhibiting endochondral ossification during OA progression. Targeting SP has therapeutic potential for preventing ligament degeneration.

Keywords: ligament; degeneration; osteoarthritis; substance P; endochondral ossification

Introduction

Osteoarthritis (OA) is a progressive degenerative joint disorder often encountered in the primary clinical setting. OA is recognized as a whole joint disorder that is characterized by the degeneration of various tissues, including the articular cartilage, subchondral bone, meniscus, joint capsule, and ligament[1]. However, its pathogenesis remains unclear. Although many studies have attempted to elucidate the mechanism of OA, there have been few studies on the degeneration mechanism of the ligaments compared to other tissues, such as the articular cartilage, bone, and meniscus.[2] While ligaments are important structures, degradation of ligaments may accelerate joint degeneration due to joint instability. The histological degeneration of the posterior cruciate ligament (PCL) occurs prior to articular cartilage degeneration of the knee joint[3]. Joint instability due to the anterior cruciate ligament (ACL) and/or PCL insufficiency induces articular cartilage and meniscus injuries, which cause OA progression[4,5]. Since ligament degeneration is one of the triggers of OA pathogenesis, it is necessary to elucidate the mechanism of ligament degeneration in OA and establish treatments to prevent ligament degeneration. In the

69 progression of OA, increasing numbers of chondrocyte-like cells are associated with
70 ligament degeneration, and these cells induce ossification of the ligament through
71 endochondral ossification[6,7]. Therefore, it is important to identify the factors that regulate
72 endochondral ossification in ligaments to prevent ligament degeneration by inhibiting
73 their function.

74 Neuropeptides in OA pathogenesis have attracted attention, because they play important
75 roles in pain and various functions such as bone metabolism, angiogenesis, and
76 inflammation[8,9]. **Sensory and sympathetic nerve fibers distribute in the bone and**
77 **synovium in the joint, but these nerve fibers innervate in the osteochondral junction**
78 **and osteophytes during OA progression with perturbation of joint homeostasis.[10,11]**
79 Neuropeptides are released from these nerves and participate in the deterioration of joint
80 homeostasis in addition to joint pain. Substance P (SP), a neuropeptide, is composed of
81 11 amino acids, is widely distributed in both the central and peripheral nervous systems.
82 SP plays a crucial role in pain, including joint pain in OA, where SP is distributed
83 throughout the subchondral bone[9,11,12]. With regards to bone metabolism, the neurokinin-
84 1 receptor (NK1R) is expressed in osteoblast and osteoclast precursors and stimulates
85 osteoblast and osteoclast differentiation and function[13,14]. In addition, SP has anabolic
86 functions ranging from anti-inflammatory effects to tissue repair through the recruitment
87 of mesenchymal stem cells[15,16,17]. As sensory nerves extend into the joint in OA, it is
88 important to investigate the expression pattern of SP in the ligament to elucidate the
89 mechanism of ligament degeneration, which is promoted by endochondral ossification.
90 The expression of SP in the articular cartilage decreases as cartilage degeneration
91 progresses in OA in humans and mice[18]. Therefore, we hypothesized that the expression
92 of SP in the ligament would decrease as ligament degeneration progressed, and the

93 administration of an SP agonist would prevent ligament degeneration. The purpose of this
94 study was to analyze both the expression pattern of SP in the PCL of humans and mice
95 and the function of SP in ligament cells. Finally, the effect of SP agonist administration
96 on an OA mouse model was evaluated.

97

98 **Materials and methods**

99 ***Human samples***

100 Human ligament tissue from the PCL was obtained from 30 patients (11 men and 19
101 women with a mean age of 73.5 years (range, 66 to 89 years)) who had undergone total
102 knee arthroplasty (TKA) for OA between June 2016 and October 2018. All knee joints
103 were classified as Kellgren-Lawrence grade 3 or 4. Patients with a history of a ligament
104 injury in the knee, fractures around the knee, intra-articular steroid injection in the
105 previous 6 months, infection of the knee joint, or previous knee surgery were excluded.
106 During surgery, PCL tissue was harvested for histological analysis as described
107 previously[19]. This study was approved by the institutional review board and ethics
108 committee of our hospital and conducted in accordance with the Helsinki Declaration.
109 Informed consent was obtained from all the patients.

110

111 ***Histological analysis of human ligament***

112 The harvested PCL tissue was fixed in 4% paraformaldehyde (PFA) and embedded in
113 paraffin. Four-micrometer-thick sections were prepared and stained with safranin- O/fast
114 green. Specimens were histologically graded using a previously established scoring

115 system[20]. Ligaments were scored based on the following categories: (1) inflammation of
116 the ligament substance; (2) mucoid degeneration; (3) chondroid metaplasia; (4) cystic
117 changes; and (5) orientation of collagen fibers. Five fields of view were evaluated from
118 each slide. Histological changes were scored and graded as follows: 0, no changes; 0.5,
119 minimal changes; 1, mild changes; 2, moderate changes; and 3, severe changes. The
120 highest summed ligament degeneration score (total score) was 15 if all five histological
121 categories were scored as severe. The total score was classified into one of three groups:
122 mild (0-5); moderate (6-10); or severe (11-15).

123

124 ***Animals***

125 The study protocols involving animals were approved by the Ethics Committee for
126 Experimental Animals of Hiroshima University and were performed in strict accordance
127 with the committee guidelines. All animals were provided free access to food and water
128 and allowed unrestricted weight-bearing. Senescence-accelerated mouse-prone 8
129 (SAMP8) mice that spontaneously developed OA were used in this study. They were
130 sacrificed at the ages of 4, 18, and 42 weeks (n=9 at each time point), and their knee joints
131 were harvested. They were then fixed in 4% PFA and decalcified for 2 weeks in 20%
132 EDTA. For histological analysis, they were embedded in paraffin and 4-µm-thick sagittal
133 sections, where the entire length of the PCL in the knee joint was observed. Sections were
134 stained with safranin-O and graded histologically using the Osteoarthritis Research
135 Society (OARSI) score and the same ligament degeneration score used for humans[20,21].
136 **For the assessment of the OARSI score, three sections per mouse knee that represent**
137 **the central weight-bearing area of the medial femoral and tibial plateau cartilage**

138 **were analyzed. The average of the quantified parameter of the three sections was**
139 **calculated.**

140 Male 10-week-old C57BL/6 mice were used to evaluate the effect of SP agonist
141 administration on the prevention of ligament degeneration. For the OA model, the medial
142 meniscotibial ligament of the right knee joint was resected (destabilization of the medial
143 meniscus [DMM]) in accordance with the previous literature[22]. Immediately after surgery,
144 the following drugs were administered through intraperitoneal injection: control group
145 (n=7): phosphate-buffered saline (PBS) at a dose of 100 μL/animal; SP group (n=7):
146 NK1R agonist (Septide; Bachem, Bubendorf, Switzerland) dissolved in 100 μL PBS at a
147 dose of 10^{-8} mol/kg[18,22]. **SP agonists or PBS were administered in a single dose when**
148 **the DMM mice model was created.** The animals were sacrificed at 8 weeks and knee
149 joints harvested. Four-micrometer-thick paraffin-embedded sagittal sections were
150 prepared and safranin-O staining and immunohistochemistry were performed. **The**
151 **ligament degeneration was assessed by the ligament score used for humans and**
152 **SAMP8[20]. OA development was evaluated using the OARSI score[21]. Three sections**
153 **per mouse knee that represent the central weight-bearing area of the articular**
154 **surface of the medial tibial plateau were analyzed. The average of the quantified**
155 **parameter of the three sections was calculated.**

156

157 ***Immunohistochemical analysis***

158 For immunohistochemical analysis, each section was immunostained with anti-SP
159 antibody (1:100 dilution, Santa Cruz Biotechnology: sc-58591), anti-SOX 9 (1:800
160 dilution; Abcam, Cambridge, MA), and anti-MMP13 (1:20 dilution; Neo Markers,

161 Fremont, CA) antibodies. **Sections in immunoreactive pH 6.0 (Matsunami Glass,**
162 **Osaka, Japan) were heated in a microwave oven and kept at 85℃ for 1.5 minutes.**
163 **Slides were cooled for 20 minutes at room temperature after antigen unmasking.**
164 **After washing with PBS, sections were treated with 3% H_2O_2 for 10 minutes and**
165 **were blocked with 10% serum for 20 minutes at room temperature. Antibodies were**
166 **applied and incubated overnight at 4℃. After washing with PBS, sections were**
167 **incubated with secondary antibodies for 30 minutes at room temperature and then**
168 **incubated using the peroxidase-based Elite ABC system (Vector Laboratories,**
169 **Burlingame, CA, USA) for 30 minutes. Slides were washed, and sections were**
170 **incubated with 3,3-diaminobenzidine (DAB) substrate.** Five fields from each ligament
171 were randomly selected. **The number of total cells, SOX9-, MMP13-, and SP-positive**
172 **cells was counted and the number of SOX9-, MMP13-, and SP-positive cells were**
173 **divided by the total cell number in each area. Then, the average of these ratios was**
174 **calculated.**
175 For double immunofluorescence staining of SP and SOX9, anti-SP and anti-SOX9
176 antibodies were labeled using the Dojindo Ab-10 Rapid HiLyte Fluor 488 and 568
177 Labeling Kit (Kumamoto, Japan), respectively. 4',6-diamidino-2-phenylindole (DAPI)
178 (Dojindo Laboratories, Kumamoto, Japan) solution was used for nuclear staining.
179
180 ***Cell culture***
181 **Human ligament cells were obtained from the ACL of five patients who had**
182 **undergone TKA.** They were placed in 10-cm diameter Petri dishes under sterile

183 conditions and washed five times with PBS to remove blood cells following the removal
184 of synovial tissue, adipose tissue, and small blood vessels. The ligament was then
185 hollowed out and the inner ligament tissue was used. The tissue was minced into 1 -2 mm
186 pieces. Tissue with 0.25% type 1 collagenase was shaken in a water bath at 37 °C for 60
187 min, and then an equal volume of Dulbecco's modified Eagle's medium (DMEM; Life
188 Technologies, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine
189 serum (FBS; Sigma-Aldrich Corp., St. Louis, MO, USA) and antibiotics (at a final
190 concentration of 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml
191 amphotericin B; Nacalai Tesque, Kyoto, Japan) were added to stop degradation. The
192 tissue was then filtered through a 200-mesh nylon filter and centrifuged at 400 rpm for
193 10 min. The supernatant was discarded, and the cells were suspended in DMEM medium
194 containing 10% FBS and 1% antibiotics. When cell coverage reached 80 -90%, the cells
195 were purified and passaged. The cells from passages 2 and 3 were seeded onto 24-well
196 plates (BD Falcon, Franklin Lakes, NJ, USA), and incubated either with or without 10
197 nM and 100 nM NK1 receptor agonist (Septide), 100 nM NK1 antagonist (Aprepitant;
198 MK-0869, Adooq Bioscience, Irvine, CA, USA), or the same amount of PBS (WAKO)
199 as a control group added to each well twice a week in a humidified 5% CO2/95% air
200 atmosphere at 37 °C. After 24 h, 48 h, and 21 days, RNA was extracted for polymerase
201 chain reaction (PCR) analysis. To induce chondrogenesis, 5×10^5 cells were placed in
202 15-ml polypropylene tubes (BD Falcon) and pelleted by centrifugation at 500 × g for 5
203 min. The pellets were cultured in a chondrogenic medium (StemPro Chondrogenesis
204 Differentiation Kit, Life Technologies) and antibiotics, to which 100 nM of an NK1
205 receptor agonist or the same amount of PBS was added for 3 weeks. Chondrogenic
206 capacity was evaluated by PCR and histological analysis. For histological analysis, the

207 pellets were embedded in paraffin, cut into 6-μm sections, stained with safranin-O/fast
208 green, and their diameters were measured.

209

210 ***Real-time PCR***

211 RNA was isolated using TRIzol (Life Technologies) for real-time PCR analysis, and
212 complementary DNA was synthesized using 1 μg of total RNA using the Superscript
213 VLIO kit (Invitrogen) according to the manufacturer's protocol: MMP13 (Hs
214 00233992_m1), RUNX2 (Hs 00231692_m1), VEGFA (Hs 0090055_m1), SOX9 (Hs
215 00165814_m1), COL2A1(Hs 01064869_m1), COL10A1(Hs 00166657_m1), and
216 GAPDH(Hs99999905_m1) using TaqMan gene expression assays probes (Life
217 Technologies) and real-time PCR assays were performed. The expression level of each
218 gene was evaluated relative to that of GAPDH. The ΔΔCt method was used to analyze
219 real-time PCR data. **The control was set as 1 and its relative expressions were**
220 **compared. The experiment was conducted at least three times with samples of five**
221 **patients, and the average of the data from the real-time PCR was compared.**

222

223 ***Statistical analysis***

224 All results are expressed as a mean ± standard deviation (SD). The Mann–Whitney U
225 test was used to analyze the differences between the two groups. Comparisons among
226 three or four groups were performed using the Tukey–Kramer post hoc test. Statistical
227 significance was set at $P<0.05$.

228

229 **Results**

230 ***Expression pattern of SP in the PCL***

231 Out of 30 human PCL cases, 7 were classified as mild, 12 as moderate, and 11 as severe.
232 In the mild group, **parallel fiber arrangement with fibroblasts in the ligament was**
233 **disrupted and became wavy**. In the moderate group, the parallel fiber arrangement in
234 the ligament was **completely** disrupted with an increased number of chondrocyte-like
235 cells with round nuclei observed. The area stained with safranin O was also increased in
236 this group. In the severe group, the fiber arrangement almost disappeared, with an
237 increase in the number of chondrocyte-like cells. The area stained with safranin O was
238 also increased. In addition, ossified areas were observed in this group (Figure 1A).

239 The ratio of SOX9-positive cells was significantly higher in the moderate group than in
240 the mild and severe groups (P<0.01), and the ratio of MMP-13 positive cells was
241 significantly higher in the mild and moderate groups than in the severe group (P<0.01).
242 The ratio of SP-positive cells was higher in the moderate group than in the mild and severe
243 groups (P<0.01), and the expression pattern of SOX9 was similar to this (Figure 1B).
244 Immunofluorescence analysis showed that SP and SOX9 were co-expressed in
245 chondrocyte-like cells in the ligament (Figure 1C).

246 To investigate longitudinal changes in ligament degeneration, the PCLs of SAMP-8
247 mice were analyzed. As the weeks progressed, the OARSI and ligament scores increased
248 significantly (Figure 2A). Ligaments at 4 weeks displayed a parallel fiber arrangement
249 with spindle-shaped **cell** nuclei. At 18 weeks, the area stained with safranin- O and the
250 number of chondrocyte-like cells in the ligament increased, and the parallel fiber
251 arrangement was decreased. At 42 weeks, the parallel fiber structure had disappeared, and

252 the area stained with safranin-O and the number of chondrocyte-like cells were again
253 increased (Figure 2A). These changes are similar to those observed in human ligament
254 degeneration. In the immunohistochemistry of SP, SP-positive cells were sparse in the
255 PCL at 4 weeks, but their expression was significantly increased at 18 weeks ($P<0.01$).
256 However, the number of SP- expressing cells was decreased in the degenerated PCL at
257 42 weeks ($P<0.01$) (Figure 2B).

258

259 ***In vitro functional analysis of SP in a ligament***

260 To investigate the effects of SP on ligament cells **from human ACL**, the expression
261 of SOX9, Runx2, Col10a1, VEGF, and MMP13 was assessed by real-time PCR. SOX9
262 expression was significantly decreased by SP agonist treatment and significantly
263 increased by SP antagonist treatment at 24 h. At 3 weeks, the SP antagonist had
264 upregulated SOX9 expression. RUNX2 expression at 48 h was decreased by SP agonist
265 treatment in a dose-dependent manner. COL10A1 expression at 3 weeks showed that the
266 SP agonist had suppressed its expression. VEGF expression was significantly
267 downregulated at 48 h post-treatment with the SP agonist, and its expression was
268 upregulated by SP antagonist treatment compared to that by SP agonist treatment at 3
269 weeks. MMP13 expression by SP antagonist treatment increased compared to that by SP
270 agonist treatment at 3 weeks (Figure 3). To analyze the effect of SP on chondrogenesis,
271 pellet cultures of ligament cells with and without SP agonist were used. The diameter of
272 the pellets treated with the SP agonist was significantly smaller than in those not treated
273 ($P<0.05$). The expression of SOX9 and Col2a1 was significantly lower in the pellets
274 treated with the SP antagonist than in those not treated (Figure 4).

275

276 ***Effect of SP agonist administration on ligament degeneration***

277 The PCL in the control group displayed disrupted fiber arrangement with an increased
278 safranin-O- stained area. In contrast, fiber arrangement was mostly maintained in the SP
279 agonist group, and the area stained with safranin-O was hardly observed. The OARSI and
280 ligament scores in the SP agonist group were significantly lower than those in the control
281 group ($P<0.01$, respectively) (Figure 5A). Immunohistochemistry showed that SOX9
282 expression in chondrocyte-like cells was significantly lower in the SP agonist group than
283 in the control group ($P<0.01$).

284

285 **Discussion**

286 This study demonstrated that SP expression in the ligament increased as ligament
287 degeneration progressed, but its expression decreased once degeneration was severe. The
288 results of our study suggest that SP is expressed in chondrocyte-like cells and prevents
289 chondrogenesis of ligament cells during endochondral ossification in the progression of
290 OA. Moreover, the administration of SP receptor **agonists** prevented the progression of
291 ligament degeneration in DMM mice.

292 Ligament degeneration is characterized by disruption of fiber arrangement, increased
293 chondrocyte-like cells, and ossification[24]. Kumagai et al. demonstrated that SCX-positive
294 cells decreased while SOX9-positive cells increased as ligament degeneration
295 progressed[6]. **Since ligaments have stem cells with multi-differentiation potential, it is**
296 **unclear whether the ligament cells themselves transdifferentiate or ligament-**

297 **derived stem cells differentiate into chondrocyte-like cells.**[25] **The regulation of the**
298 **transdifferentiation from ligament cells or chondrogenic differentiation of ligament-**
299 **derived stem cells to the chondrocyte-like cells may be important for preventing**
300 **ligament degeneration.** In this study, we focused on SP because sensory innervation into
301 the joint occurs during the development of OA[10], and SP expression increases in the OA
302 joint[9]. SP is secreted by sensory nerve endings in various tissue, such as the synovium,
303 subchondral bone, and periosteum, and its receptor is expressed in various cells of the
304 musculoskeletal system, enabling responses to stimuli from peripheral nerves[26]. In
305 advanced OA, SP is released from sensory nerve endings in inflamed **cartilage** and the
306 synovium and SP is increased in joint fluid[27,28]. As ligaments are constantly exposed to
307 the synovium and synovial fluid, they are susceptible to SP expression. Moreover, tendon
308 fibroblasts, which have properties similar to ligament cells, endogenously produce SP
309 upon mechanical stress[29]. This evidence supports our observations that the number of SP-
310 positive cells increases with the progression of ligament degeneration. **While increasing**
311 **SP induces pain**[9]**, SP prevents the endochondral ossification in the ligament.**
312 **Ligament degeneration progresses by increasing endochondral ossification through**
313 **decreasing SP expression. Ligament degeneration induces abnormal kinematics of**
314 **the joint, subsequently leading to OA progression.**

315 In our study, SP and SOX9 were co-expressed in chondrocyte-like cells in the
316 degenerated ligaments. SOX9, which is typically a cartilage-specific marker for
317 endochondral ossification processes, is associated with OA development in human
318 ACLs[24]. Levy et al. reported that human cruciate ligaments from OA patients show
319 important chondroid and cartilage metaplasia, which involves a change in the ligament
320 cell phenotype to a more chondrocyte-like round cell morphology[3]. The formation of

321 perivascular cell aggregates and islands of chondrocyte-like cells increases in degenerated
322 ACL, while collagen type II and X have been detected only in the areas with chondroid
323 metaplasia[7]. In the aforementioned study, SOX9 and RUNX2 expressions were also
324 increased in chondrocyte-like cells. Our research revealed that SP inhibits SOX9 and
325 RUNX2 expression in the early phase of degeneration and that persistent SP treatment
326 also downregulates COL10A1 expression in human ligament cells. This indicates that
327 loss of SP expression leads to the progression of ligament degeneration through the
328 enhancement of endochondral ossification. SP also has a negative effect on
329 chondrogenesis. During the progression of the degeneration, SP expression increases to
330 inhibit endochondral ossification. Subsequent decreasing SP expression leads to the
331 expression of VEGF and MMP13, which **accelerate** ligament degeneration. Once OA
332 progresses further despite the inhibitory functions of SP, sensory nerves extending around
333 and/or into ligaments may be damaged. As a result of this, secretion of SP will decrease[30].
334 Maintaining SP expression is important for inhibiting the progression of ligament
335 degeneration.

336 The administration of SP receptor agonists ameliorates ligament degeneration in our
337 study. Previous reports have demonstrated that SP functions as an anabolic factor and has
338 therapeutic potential for a variety of diseases. SP promotes tissue repair via the
339 mobilization of CD29(+) stromal-like cells from the bone marrow to the injured
340 site[31,32,33,34]. SP also plays role in anti-inflammatory responses and tissue repair through
341 the recruitment of mesenchymal stem cells (MSCs)[16,35]. SP treatment ameliorates
342 collagen II-induced arthritis in mice by suppressing the inflammatory response[36]. In an
343 OA animal study, intra-articular injection of SP coupled with self-assembled peptide
344 hydrogels markedly improved cartilage regeneration through the recruitment of MSCs[15].

345 Moreover, SP induces the proliferation of human tenocytes through EGFR signaling[29].

346 Shirakawa et al. demonstrated that intraperitoneal injection of SP agonists could inhibit

347 OA progression in DMM mice[18]. **They focused on the effect of SP on the osteochondral**

348 **unit of the human and DMM mice, and showed the expression of the SP in the**

349 **cartilage and subchondral bone decreased as OA progressed. However, ligaments in**

350 **OA were not evaluated although the administration of the SP agonists successfully**

351 **ameliorated the cartilage degeneration and subchondral bone sclerosis in their study.**

352 **Therefore, we examined the effect of SP agonists on ligament degeneration in the**

353 **same mice models in this study, and the** administration of SP agonists to DMM mice

354 ameliorated ligament degeneration through inhibition of endochondral ossification as

355 well as the anabolic effects that have been described in the previous reports. **Since the**

356 **effect of SP agonists was examined using human-derived ACL cells in *vitro* study, it**

357 **is expected similar effects on the prevention of ligament degeneration in humans as**

358 **in DMM mice. In addition, our study evaluated the expression of SP in the human**

359 **and spontaneous OA mice models and they exhibited the same expression pattern of**

360 **SP. However, it is unclear whether SP agonists directly acted on cells in the PCL to**

361 **prevent ligament degeneration or whether ligament degeneration was suppressed as**

362 **a consequence of decreasing the cartilage degeneration. Since mechanical stress to**

363 **the PCL should be continued due to the instability induced by the meniscotibial**

364 **ligament resection, PCL degeneration might progress even if the cartilage**

365 **degeneration was ameliorated by the SP agonists. In our results of the *in vitro***

366 **experiments, SP agonists suppressed the gene expression regarding endochondral**

367 **ossification. Therefore, SP agonists are effective in preventing the degeneration of**

368 **ligaments as well as cartilage. In the current study, two types of mouse models were**

used. SAMP8 mice are characterized by rapid aging and they have been used as model mice for aging-related diseases such as neurodegenerative disorder, cardiovascular disease, and OA[36,37,38]. Since SAMP8 develops OA spontaneously and progresses to severe OA which exhibited degenerative changes including cartilage, meniscus and ligament[38], it is ideal to analyze the transition of the expression pattern of SP. DMM mice were used to examine the effect of SP agonist on ligament degeneration *in vivo* because severe OA with ligament degeneration develops at 8 weeks[39] and a previous report showed that a single administration of SP agonist could ameliorate OA development in DMM mice[18]. Although it takes a long time to develop a severe OA in the SAMP8 mice, the use of SAMP8 mice may be useful to evaluate the efficacy, dosing regimens, and adverse effect of the administration of SP agonists for spontaneous OA. The administration of SP agonists may develop a novel therapeutic strategy to ameliorate the OA progression through the prevention of cartilage and ligament degeneration.

This study has several limitations. First, the natural course of ligament degeneration cannot be histologically analyzed in human samples. Especially, normal PCL in humans was not analyzed because it is not possible to collect normal PCL from healthy subjects. As an alternative, we evaluated ligament degeneration in aging by using SAMP8 mice as the abnormal patterns of chondrogenesis, ossification, cell hypertrophy, and loss of fiber alignment in the ligaments of mice are similar to that of humans[40]. Second, human ACL-derived cells were used *in vitro* studies although *in vivo* studies focused on the PCL. ACL has less synovium than PCL, making it easier to isolate ligament cells without contamination of synovial cells. Since histological findings of PCL are correlated with those of ACL in OA[3], using ACL-derived cells

may yield the same results as using the PCL-derived cells. **In addition, there was a possibility that chondrocyte-like cells might include in the isolated cells because ACL was harvested from OA patients. It is desirable to use normal ACL without OA to evaluate the effect of SP on pure ligament cells. Third**, while SP plays an important role in pain regulation[11], the adverse effects of SP agonists administration, including pain, have not been evaluated, although **a previous report showed that administration of SP agonists in the knee joint of sham mice had no effect on subchondral bone, cartilage, and synovium[18].** To evaluate the inhibitory effect of SP agonists on endochondral ossification in the ligament during OA progression *in vivo*, a single systemic dose of an SP agonist was administered to DMM mice. Appropriate dosage, the use of multiple administrations, and alternative methods of administration, including intra-articular injection, were not considered in our study and should be investigated in the future. Finally, the mechanisms regulating SP expression have not yet been elucidated. SP expression was upregulated in moderate degeneration but downregulated in severe degeneration. As SP is multifunctional and is expressed in various cells, the mechanisms that regulate SP expression may be complex. Moreover, targeting other neuropeptides, such as calcitonin gene-related peptide and vasoactive intestinal peptide, could ameliorate OA progression *in vivo*[41,42]. However, the relationship between these neuropeptides and SP in ligament degeneration remains unclear. Further studies to explore the adverse effects of SP agonists and their effects on the regulatory mechanisms of SP expression in the ligament during OA progression are necessary.

In conclusion, SP plays an important role in maintaining ligament homeostasis by inhibiting endochondral ossification during OA progression. Targeting SP has therapeutic potential for preventing ligament degeneration.

Data presentation

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Declaration of interests

None

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583

584

585 **Legends**

586 Figure 1.

587 The expression of substance P (SP) in a human posterior cruciate ligament (PCL) from
588 osteoarthritis (OA) patients. (A) **Hematoxylin & Eosin (HE) staining, safranin O**
589 **staining, and** immunohistochemistry of SOX9, MMP13, and SP in the PCL with mild,
590 moderate, and severe degeneration. **HE staining showed fiber arrangements**
591 **disappeared as the ligament degeneration progressed. Low magnification of the**
592 **safranin O staining revealed that the safranin O positive area expanded as the**
593 **ligament degeneration progressed.** Arrows indicate immune-positive cells. ***; ossified**
594 **area.** (B) The rate of SOX9-, MMP13-, and SP- positive cells. (C) Immunohistochemistry
595 of SOX9 and SP, SOX9, and SP were co-expressed in chondrocyte-like cells (arrows).
596 The bar indicates 100μm.

597

598 Figure 2.

599 Histological analyses of posterior cruciate ligament (PCLs) in senescence-accelerated

600 mouse-prone 8 (SAMP8) mice. (A) Osteoarthritis Research Society Score (OARSI) and

601 ligament scores. **These scores increased as the OA progressed. At 4 weeks, parallel**

602 **fiber arrangements were observed and there was no safranin O positive area in the**

603 **PCL. At 18 weeks, fiber arrangement became wavy, and safranin O positive areas**

604 **increased at 42 weeks.** Arrows indicate PCL. (B) Immunohistochemistry of substance P

605 (SP) and the rate of SP-positive cells. **SP positive cells in the PCL were most frequently**

606 **observed at 18 weeks.** Dotted lines indicate PCL. The bar indicates 100µm.

607

608 Figure 3.

609 Real-time PCR of SOX9, RUNX2, COL10A1, VEGF, and MMP13 in human ligament

610 cells.

611

612 Figure 4.

613 Chondrogenesis of human **anterior cruciate** ligament cells. (A) Macroscopic appearance

614 and safranin O staining of pellets with and without the substance P (SP) agonist. **The size**

615 **of the pellet with SP agonist was smaller than that without SP agonist.** (B) Pellet size,

616 and **gene expressions** of SOX9 and COL2A1. **SP agonist reduced the gene expression**

617 **of SOX9 and COL2A1.**

618

619 Figure 5.

Administration of substance P (SP) agonist into the destabilized medial meniscus (DMM) mice. (A) Safranin O staining, Osteoarthritis Research Society Score (OARSI), and ligament scores in control and SP agonist groups. **The upper column is the intercondylar space, and the lower column is the medial compartment.** Arrows indicate posterior cruciate ligament (PCL). The bar indicates 500µm. (B) Immunohistochemistry of Sox9 and SP, and the rate of Sox9 and SP positive cells in control and SP agonist groups. The bar indicates 100µm. *;$p<0.05$, **;$p<0.01$.

Figure 1

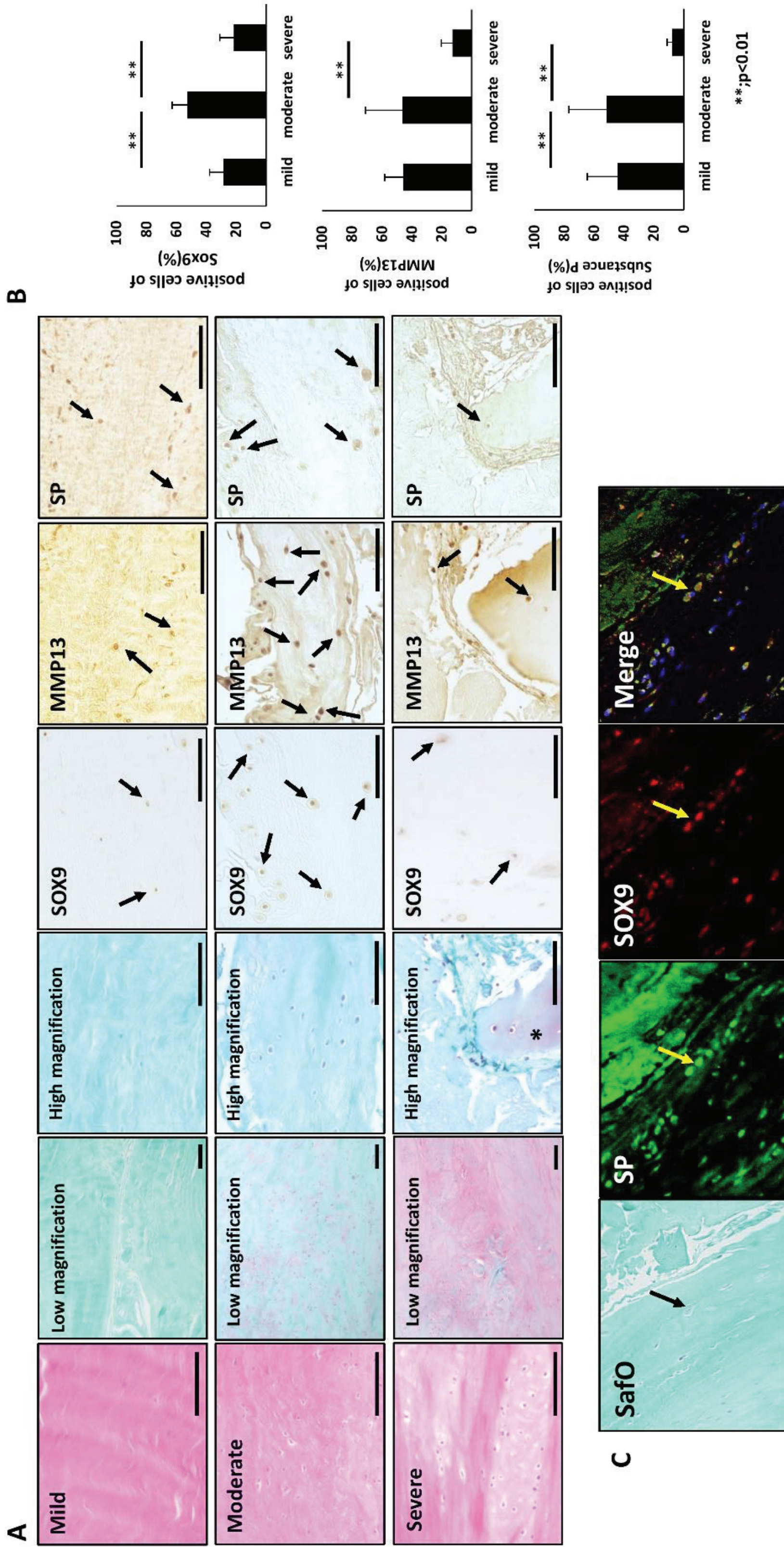
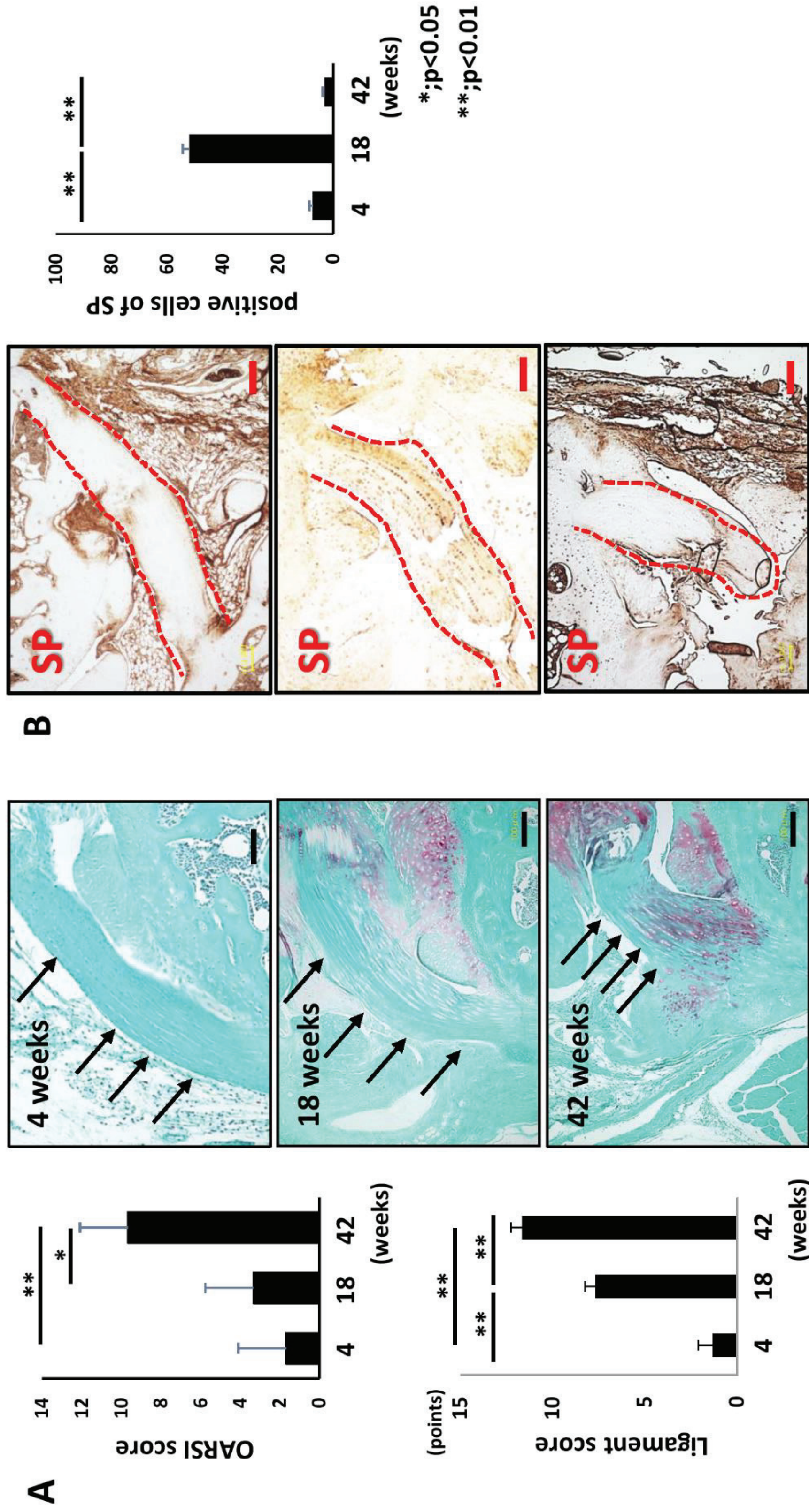


Figure 2



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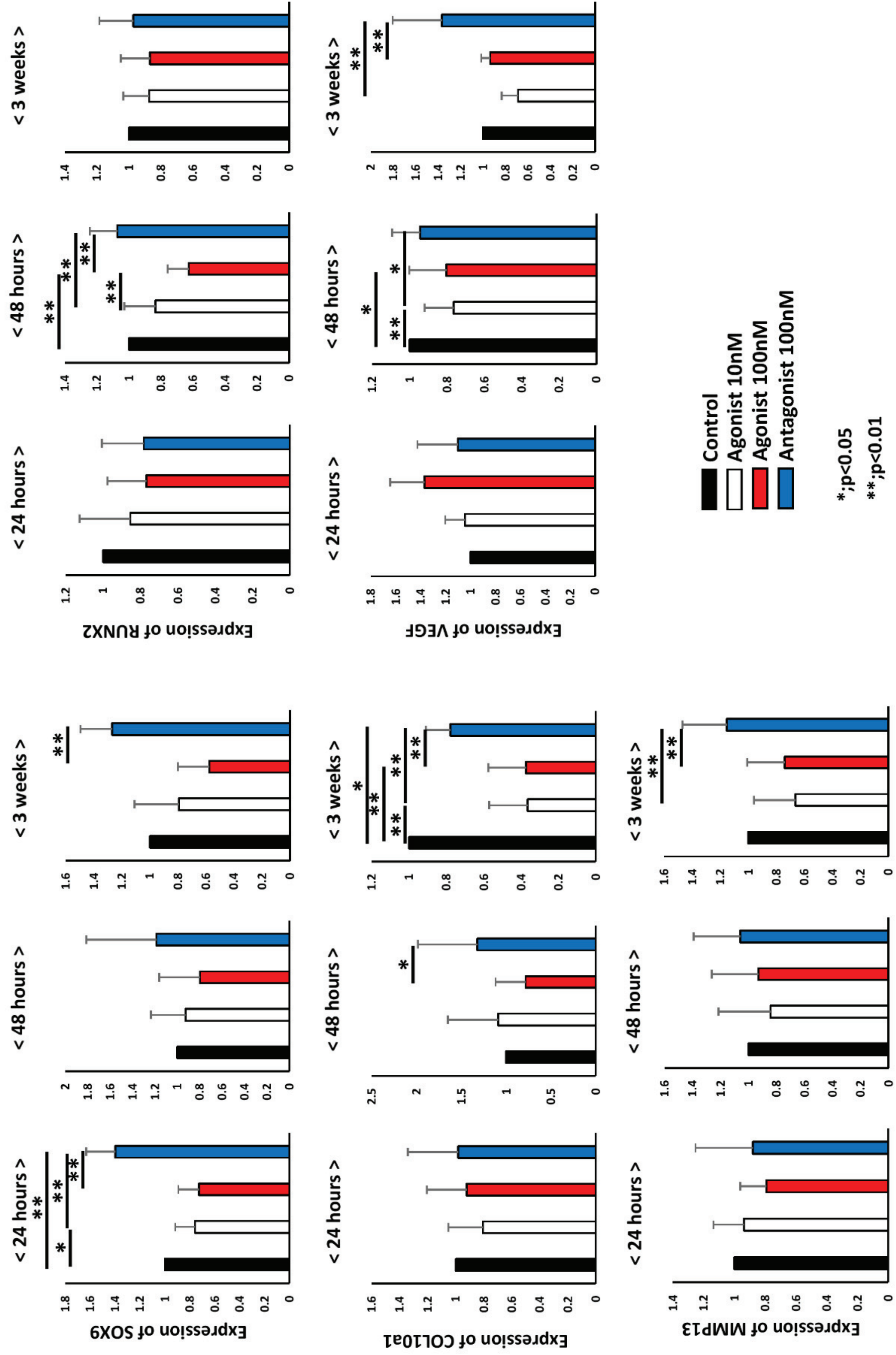

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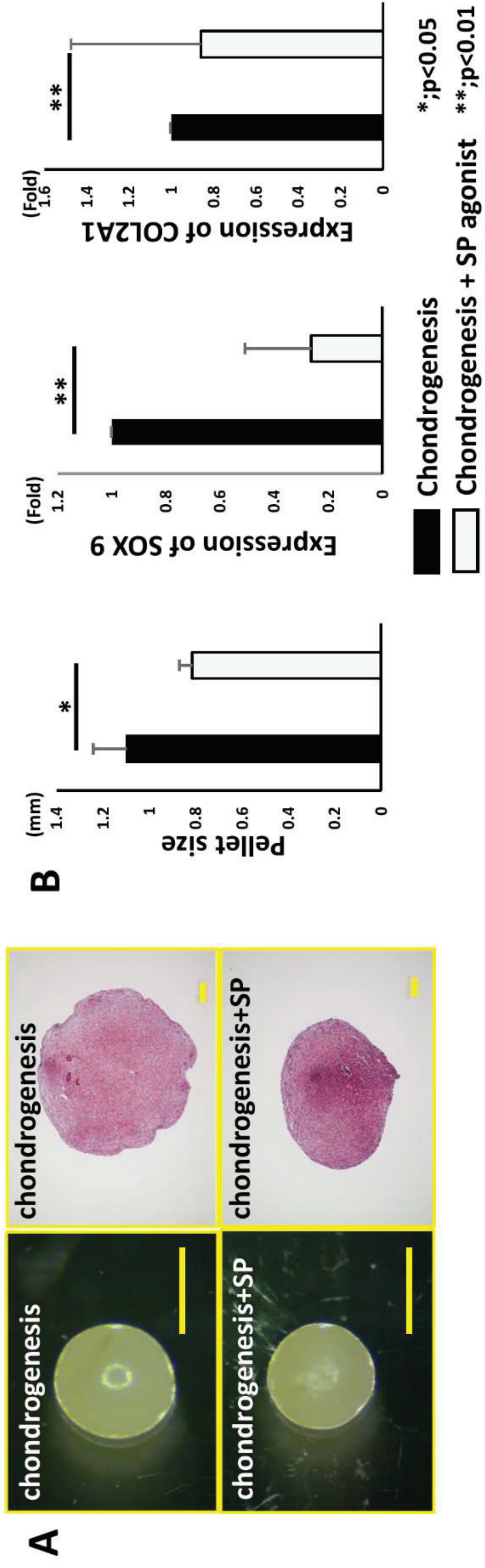
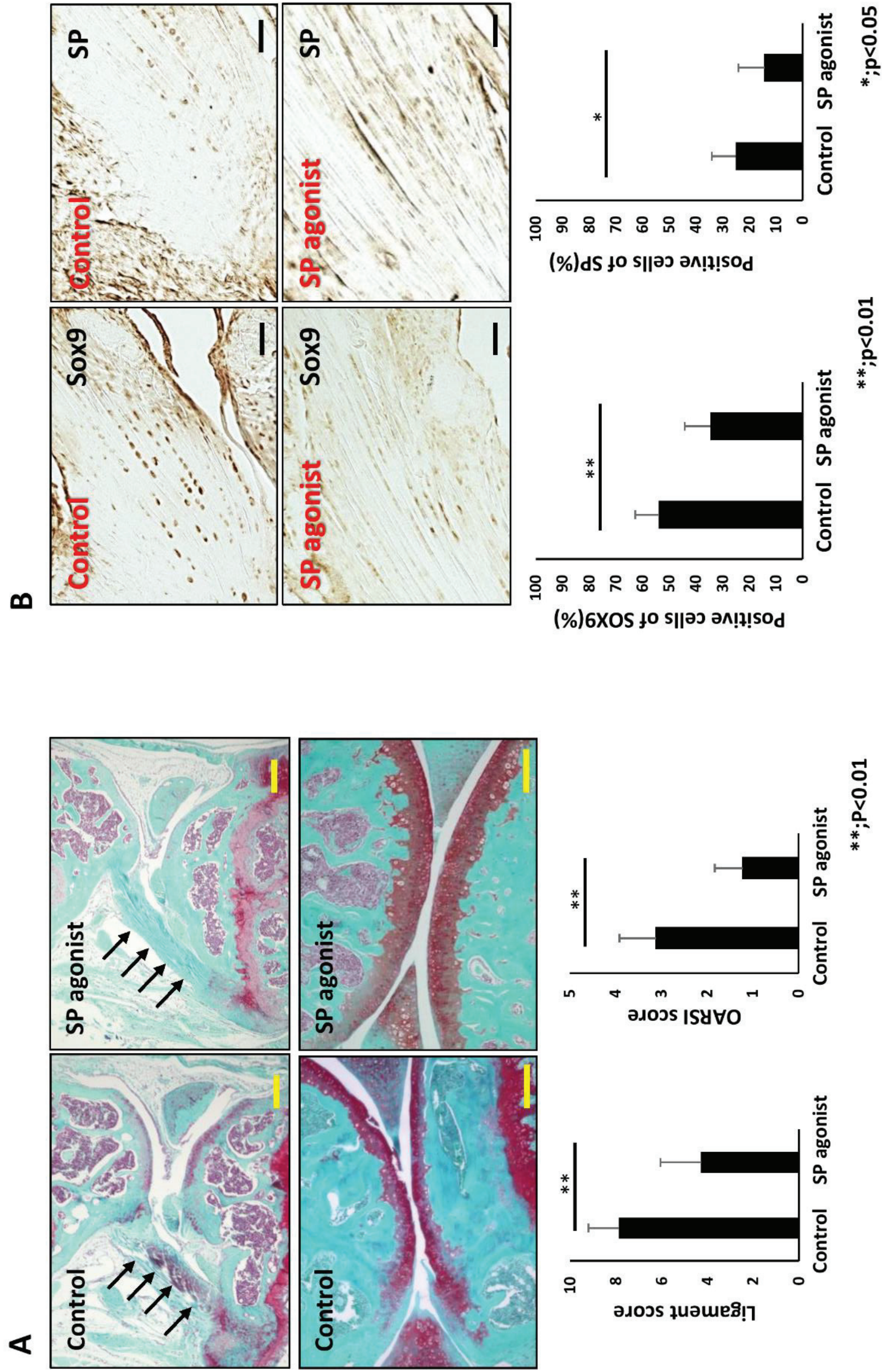


Figure 5



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