

Transplantation of Parathyroid Hormone–Treated Achilles Tendon Promotes Meniscal Regeneration in a Rat Meniscal Defect Model

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Highlights	◇骨粗鬆症治療薬としてすでに広く臨床利用されている副甲状腺ホルモン（PTH）に着目。 ◇ラットの組織と遺伝子レベルで軟骨形成を確認
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<p>Description</p>	<p><概要></p> <p>研究グループは、腱組織に PTH を注入して半月板切除部位へ移植することで、正常な半月板に近い軟骨のような組織が再現できることを明らかにしました。本研究成果は、患者自身の腱を移植する半月板再建手術の治療成績向上につながることで期待されます。</p> <p>膝半月板はスポーツ外傷や加齢的变化によって損傷しますが、自然治癒の見込みは少なく、場合によっては手術が必要です。手術法としては縫合術と切除術があり、切除術では膝の軟骨の損傷が進むため、日本国内では患者自身の腱を移植する半月板再建縫合手術も行われています。しかし、患者自身の腱と半月板は組織が異なるため、治療成績は安定していないのが現状です。</p> <p>本研究グループは、ラットのアキレス腱より採取した腱細胞に PTH を投与した群では、リン酸緩衝生理食塩水（PBS）を腱細胞に注入した群に比べて再建した半月板での被覆率が高く、関節軟骨の保護作用も高いことを動物実験で明らかにしました。また、半月板組織のトルイジンブルー染色では PTH 注入腱で異染性を認め、正常半月板に近い組織像を認めました。そして、ラットの内側半月板を部分切除し、PTH を注入したラットのアキレス腱を半月板欠損部に縫合して軟骨化を評価したところ、4 週間後に軟骨形成に関与する遺伝子の発現上昇を認めました。</p> <p>以上の結果から、腱細胞あるいは腱内の未分化間葉系細胞に対して PTH が作用し、軟骨分化を維持させることにより、半月板様組織が再生する可能性が示唆されました。</p> <p>‘新たな治療法に光 副甲状腺ホルモンと腱組織で正常半月板を再現 ～半月板再建手術の治療成績向上に期待～’. 大阪公立大学. https://www.omu.ac.jp/info/research_news/entry-01712.html (参照 2022-08-09)</p>
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Transplantation of parathyroid hormone-treated Achilles tendon promotes meniscus regeneration in a rat meniscal defect model

Abstract

Background: Autologous tendon grafts are used for meniscal reconstruction of surgically removed knee joint meniscus. However, as meniscal reconstruction cannot prevent the progression of cartilage degeneration, additional procedures that confer meniscus-like histological properties to the transplanted tendon are required for improved outcomes.

Hypothesis: Parathyroid hormone (PTH) (1-34) induces cartilage formation in the rat tendon, and transplantation of PTH-treated tendon promotes meniscal regeneration.

Study Design: Controlled Laboratory Study

Methods: Rat Achilles tendon-derived cells were cultured with/without PTH for 28 d and stained with Alcian blue to determine chondrogenic differentiation. After 14 and 28 d of incubation, gene expression was assessed using quantitative real-time PCR (qRT-PCR). The rat Achilles tendon was injected with PTH and then transplanted onto a medial meniscal defect. Macroscopic and histological assessments of the regenerated meniscus and of cartilage degeneration in the tibial plateau were performed at 4 and 8 weeks post-surgery.

Results: *In vitro*, PTH-treated cells showed better staining with Alcian blue than the control (normal

medium) group. PTH1R, Col2a1, Sox9, and RUNX2 were significantly upregulated in PTH-treated cells ($P < 0.05$). Macroscopically, the *in vivo* results revealed more prominent meniscal coverage and lesser progression of articular cartilage degeneration in the PTH group than in the phosphate-buffered saline-injected group. Histologically, toluidine blue staining revealed metachromasia in the PTH-injected tissue at 4 and 8 weeks. The PTH-treated regenerated meniscus showed positive immunostaining for type II collagen in the area exhibiting metachromasia. Moreover, PTH-treated tendon had an enhanced histological score compared with the untreated group at 4 and 8 weeks ($P < 0.05$).

Conclusion: PTH (1-34) induced cartilage formation in the rat tendon. Transplantation of PTH (1-34)-treated Achilles tendon in a rat meniscal defect model induced meniscal regeneration and preserved knee articular cartilage. Macroscopically, PTH groups showed a greater coverage of the regenerated meniscus. Histologically, the regenerated meniscus had higher cartilaginous matrix content in rats transplanted with PTH-treated tendons. PTH (1-34) stimulated tendon-derived cells to promote chondrogenic differentiation.

Clinical Relevance: Meniscal transplantation using PTH-injected autologous tendon grafts might promote meniscal regeneration and prevent progression of cartilage degeneration by stimulating chondrogenic differentiation of tendon-derived cells.

Key Terms: meniscal reconstruction, parathyroid hormone, meniscus, autologous tendon graft

What is known about the subject: Autologous tendon grafts are one of the materials used for meniscal reconstruction of the surgically removed knee joint meniscus. However, meniscal reconstruction fails to satisfactorily prevent the progression of cartilage degeneration. However, continuous PTH (1-34) administration can enhance chondrocyte proliferation and suppress chondrocyte differentiation.

What this study adds to existing knowledge: We report the novel finding that PTH(1-34) stimulates chondrogenic differentiation in rat tendon-derived cells. Transplantation of the Achilles tendon treated with PTH(1-34) in a rat meniscal defect model promoted meniscus regeneration and prevented articular cartilage degeneration.

Introduction

The meniscus is a semilunar-shaped fibrocartilaginous tissue involved in load bearing and shock absorption functions in the knee joint.⁴⁰ Meniscectomy is a common arthroscopic surgery performed primarily in an avascular area with limited ability for spontaneous healing⁹; this results in articular cartilage degeneration and progression of osteoarthritis¹. Meniscal allograft transplantation has been reported to demonstrate good clinical results in both the short and intermediate postoperative period.^{23,34} However, whether allografts can prevent joint degeneration remains unclear. Use of allograft menisci, which can potentially transmit diseases, may be limited by supply. Alternatively,

synthetic meniscal transplantation has shown varying degrees of success.^{39,42,44}

Autologous tendon grafts are often used for meniscal reconstruction especially in countries where allograft meniscus and artificial meniscus implants are unavailable.^{16,17} This technique has been performed and reported on in the past in a human and animal model and this area may still hold promise because of its safety and biological properties similar to those of the circumferential fibers of meniscus.⁸ In a sheep model, a patellar tendon graft was found to be identical to the native meniscus at 12 months after transplantation. Rönblad et al. presented a pilot clinical study that showed an improved clinical score after a semitendinosus tendon graft.³⁵ However, human clinical studies have not yet shown whether meniscal reconstruction with an autologous tendon prevents progression of cartilage degeneration.¹⁶

Therefore, additional procedures are needed to improve the outcome of tendon transplantation in meniscal defects by conferring the tendon with histological properties similar to those of the meniscus. Recombinant human parathyroid hormone (PTH) (1–34) treatment (teriparatide) is a bone anabolic therapy for osteoporosis. In addition to the bone as a target, the downstream signaling of type I parathyroid hormone receptor (PTH1R) regulates chondrocyte differentiation.^{18,19} A previous study reported that intermittent PTH(1-34) administration promotes chondrocyte differentiation and endochondral ossification, whereas continuous PTH(1-34) administration enhances chondrocyte proliferation and suppresses chondrocyte differentiation.^{11,21} The Achilles tendon is reported to

express PTH receptor and PTH-induced tendons have been shown to possess potential for fibrocartilage formation.^{12,20} These results collectively indicate that PTH(1-34)-induced tendon grafts may have potential to promote cartilaginous differentiation rather than osteogenic differentiation under certain conditions. Therefore, this study aimed 1) to assess whether PTH(1-34) promotes chondrogenic differentiation in tendon-derived cells and 2) to assess whether transplantation of autologous tendon treated with PTH(1-34) induced meniscal regeneration in a rat model.

Materials and Methods

Animals

All experimental animal procedures were approved by and conducted following the regulations of the Osaka City University Graduate School of Medicine Committee on Animal Research (approval no. 11002). Wild-type male Lewis rats (Nihon SLC, Shizuoka, Japan) aged 8 weeks, weighing 250–300 g, were used for *in vitro* and *in vivo* experiments. All animals were maintained under a 24 h light-dark cycle, with food and water available *ad libitum*. Rats were randomly divided into control and PTH-treated groups. There were no excluded cases.

***In vitro* Experiments: Cell Preparation**

Achilles tendons were excised from 10 healthy, 8-week-old, male Lewis rats. Tendons were washed twice with phosphate-buffered saline (PBS) and cut into small pieces, each measuring approximately

1.0 mm³. The tissues were then digested for 1 h at 37 °C with type I collagenase (250 U/mL; Worthington Biochemical Corp, Lakewood, NJ, USA). The released cells were washed in PBS by centrifugation at 300 × g for 5 min and resuspended in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The isolated cells were plated and cultured at 37 °C with 5% CO₂ to form colonies. On day 2, after initial plating, the cells were washed twice with PBS to remove non-adherent cells. On day 7, the cells were trypsinized and labeled as passage 0 (P0). Cells from passages 1 to 3 (P1–P3) were used for the experiments.^{27,36,38} The culture medium was changed every 2 d during the experiments.

In vitro Experiment: PTH Administration

Cells were plated in a six-well plate (1.0×10^5 cells/well) and cultured with PTH(1-34) (Teribone, Asahi-Kasei, Japan) at concentrations of 0 (control), 1, 10, and 100 nM.^{21,43} PTH(1-34) was administered continuously by adding it freshly after each medium change. After 28 d of incubation, the cells were fixed with 10% formalin, washed with PBS, and stained with Alcian blue solution to detect cartilage matrix deposition. Alizarin Red S (Sigma-Aldrich) staining was also performed to visualize calcium deposition. Six random fields from each group were observed at ×200 magnification, and the percentages of the areas positive for Alcian blue or Alizarin red staining were quantified using Image J (National Institutes of Health).

In vitro Experiment: Quantitative Real-time PCR (qRT-PCR) Analysis

Cells were plated on a six-well plate (1.0×10^5 cells/well) and cultured with 0 (control) and 10 nM PTH(1-34). Gene expression was evaluated at 14 and 28 d after incubation, using qRT-PCR analysis. Total RNA from PTH-treated cells was extracted using an RNeasy Mini Kit (Qiagen). Total RNA from tendon-derived cells without PTH administration was also extracted as a control. Furthermore, cDNA was synthesized using a High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific) and stored at -30 °C until PCR analysis. The expression levels of PTH1R, the receptor for PTH; Col2a1, a chondrogenic marker; Sox9, a transcription factor for chondrogenic differentiation; Colla1, a tenogenic and osteogenic markers; RUNX2, a transcription factor for both osteogenic and chondrogenic differentiation; and Scx, a tendon marker, were evaluated using the primer sequences listed in Table 1. Expression levels were normalized to those of β -actin. All reactions were performed using a 7500 Fast Real-time PCR system (Applied Biosystems/Thermo Fisher Scientific, Foster City, CA, USA) and SYBR Green Premix (SYBR Premix Ex Taq; Takara, Shiga, Japan). β -Actin was used as a control for the quantity and quality of cDNA. The threshold cycle (Ct) value, defined as the cycle number at which the fluorescence intensity exceeded the stipulated threshold, and comparative Ct were used to evaluate the relative gene expression levels. Six replicates were assessed for each group.

Table 1

Primers use for quantitative real time PCR

Gene	Forward (5' to 3')	Reverse (5' to 3')
β -actin	CCCGCGAGTACAACCTTCT	CGTCATCCATGGCGAACT
PTH1R	GCTGCTCAAGGAAGTTCTGC	GTCCAGCCCTTGTCTGACTC
Col2a1	CCAGGTCCTGCTGGAAAA	CCTCTTTCTCCGGCCTTT
Sox9	ATCTTCAAGGCGCTGCAA	CGGTGGACCCTGAGATTG
Col1a1	TCCTGGCAAGAACGGAGAT	CAGGAGGTCCACGCTCAC
RUNX2	CCACAGAGCTATTAAAGTGACAGTG	AACAACTAGGTTTAGAGTCATCAAGC
Scx	CCCAAACAGATCTGCACCTT	CCGTCTTTCTGTCACGGTCT

In vivo experiment: *In situ* PTH Injection into Rat Achilles Tendon

Wild-type male Lewis rats, aged 8 weeks, were used for the *in vivo* experiments. Rats were anesthetized using a subcutaneous injection of ketamine (50 mg/mL; Sankyo, Tokyo, Japan) and xylazine (0.2 mg/mL; Bayer HealthCare, Tokyo, Japan) at a ratio of 10:3 and a dose of 1 mL/kg body weight. Both tendon ends were ligated using 5-0 nylon sutures, followed by injection with 0 (control), 9, 22.5, or 45 μ g of PTH(1-34) in 15 μ L of PBS with 0.02% indocyanine green (ICG), using a 29-gauge needle⁹ (Figure 1). ICG was added to visualize the localization of PTH solution within the tendon during injection. After skin closure, the rats were allowed to walk freely in their cages; the animals were then sacrificed at 4 or 8 weeks to evaluate cartilage formation. Treatment was performed on six rats for each concentration for analysis at 4 weeks and on 12 rats for each concentration for analysis at 8 weeks. Six rats from each group at 8 weeks were separately used for micro-CT assessment.

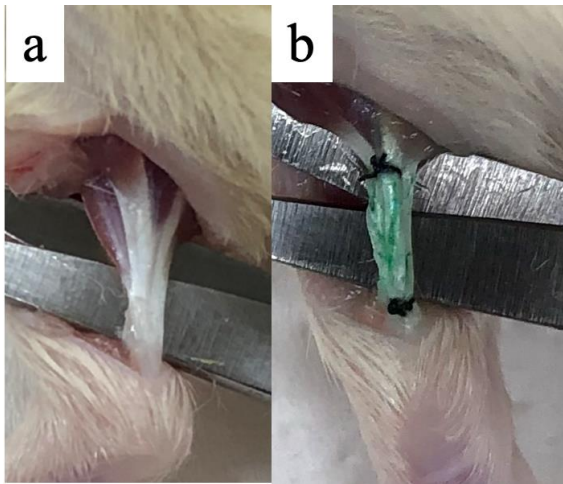


Figure 1. *In situ* PTH injection into rat Achilles tendon. (a) Rat Achilles tendon was exposed. (b) PTH solution containing a green dye was injected using a microsyringe and a 29-gauge needle into the Achilles tendon.

In vivo: Transplantation of PTH-induced Achilles Tendon

The Achilles tendon was dissected from the left lower leg at both ends and ligated using 5-0 nylon sutures, followed by injection with 9 μ g of PTH(1-34). The tendon was then harvested and trimmed to fit the meniscal defect. The right knee joint was exposed using a medial para-patellar approach. The patellar tendon was dislocated laterally, and the anterior insertion of the medial meniscus was cut; the anterior half of the medial meniscus was then withdrawn and excised at the level of the medial collateral ligament. Further, the tendon was placed in the meniscal defect and sutured with the anterior part of the capsule and medial collateral ligament using 6-0 nylon sutures (Figure 2). The rats could walk freely in their cages and were then sacrificed at 4 or 8 weeks (PTH-treated tendon group; $n = 6$

per group for 4 weeks, and n = 12 per group for 8 weeks). The same number of rats were treated with PBS-injected Achilles tendons (control group). Six rats from each group in the 8 weeks model were separately used for micro-CT assessment.

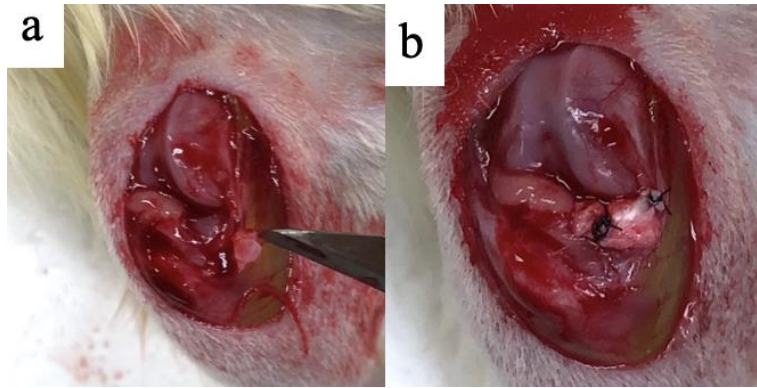


Figure 2. Transplantation of autologous Achilles tendon. (a) A partial meniscectomy was performed for the anterior half of the medial meniscus. (b) The rat Achilles tendon was transplanted into the meniscal defect and sutured with the anterior part of the capsule and medial collateral ligament using 6-0 nylon sutures.

Macroscopic Observation

The proximal tibia with the treated menisci was detached from the femoral condyle at 4 or 8 weeks after transplantation. Macroscopic images of the tibial plateau with the meniscus were captured using a stereomicroscope (SMZ1500, Nikon, Tokyo, Japan). The size of the regenerated meniscus was evaluated using ImageJ software (version 1.46, National Institutes of Health, Bethesda, MD, USA) to determine the ratio of the medial meniscus area, including the regenerated and normal regions, for the

medial tibial plateau area.^{28,30} The meniscus covering ratio of the native knee was also measured using the left knees of the 8 weeks model rats (n = 6). The cartilage of the medial tibial plateau was stained with India ink to identify fibrillation and erosion. The damaged area was quantified using ImageJ software to determine the ratio of the stained area to the entire area of the medial tibial plateau.¹⁵ A high India ink stain ratio indicated severe cartilage degeneration.

Histological Examination

Achilles tendon-regenerated rat meniscal tissue (*in situ* injection model) was fixed in 4% paraformaldehyde for 24 h. The tibial plateau was decalcified in 20% ethylenediaminetetraacetic acid (EDTA) solution for 7 d. Each tissue sample was then embedded in paraffin wax. Specimens were cut radially to a thickness of 5 μ m. The Achilles tendons and regenerated meniscal tissues were stained with hematoxylin and eosin (HE) and toluidine blue. The tibial plateau was stained with safranin O–fast green. Histological sections were visualized using an Olympus BX53 microscope (Olympus, Tokyo, Japan). A modified Pauli's scoring system was used (0–15 points).^{14,31} Degeneration of articular cartilage in the medial tibial plateau was assessed using a modified Mankin score on a scale of 0–13 points²² (Supplemental Table 1,2).

Immunohistochemistry

Specimens were deparaffinized in xylene, rehydrated in graded alcohol, and washed with PBS. Slides were incubated with citrate buffer (S1699, Target Retrieval Solution, 10 \times ; DAKO Japan, Tokyo,

Japan) in PBS for 20 min at room temperature (RT) for optimal antigen retrieval. All subsequent incubations were performed in a humidified chamber. Endogenous peroxidases were quenched using 1.0% hydrogen peroxidase in methanol for 30 min at room temperature. Slides were then rinsed with PBS and incubated with 10% goat serum for 1 h at room temperature. The specimens were then incubated with anti-type II collagen primary antibody (dilution 1:200; mouse IgG fraction, Kyowa Pharma Chemical Co. Ltd., Takaoka, Japan) for 1 h at RT and washed thrice with PBS for 10 min each. The slides were then incubated with a peroxidase-labeled secondary antibody (Histofine Simple Stain, Nichirei Biosciences Inc., Tokyo, Japan) for 1 h at RT. After the specimens were washed thrice with PBS for 10 min each, the immunoreaction was visualized by incubating the sections for 3 min in 3,3'-diaminobenzidine (Histofine Simple DAB solution, Nichirei Biosciences Inc.). An Olympus BX53 microscope (Olympus) was used for microscopic evaluation.

Micro-CT Assessment

Six rats from each group at 8 weeks after Achilles tendon injection or autologous tendon transplantation were subjected to micro-CT assessment for evaluating ossification. The lower leg, including the Achilles tendon or tendon-transplanted knee, was harvested and scanned using a μ CT scanner (LaTheta LCT-100A scanner, Hitachi-Aloka Medical Ltd., Tokyo, Japan). Sequential 240- μ m image slices with a resolution of 120 μ m were used for the calculations.

Statistical Analysis

Student's t-test was used for all comparisons between the PTH and control groups. One-way analysis of variance (ANOVA) with a *post hoc* t-test was used to compare differences between the control and PTH groups. Analysis was performed using R (version 3.5.1, patched, <http://www.r-project.org>, The R Foundation, Vienna, Austria) software, and significance was set at $P < 0.05$. Outcome assessment and data analyses were conducted by an independent researcher.

Results

In vitro: PTH administration

Alcian blue staining of tendon-derived cells at 28 d after incubation is shown in Figure 3. Blue-stained cartilage matrix deposits were observed in PTH-treated cultures. The percentage of the area positive for Alcian blue staining was significantly higher in the 10 nM treatment group than in the control group. As the most abundant cartilage matrix was found at 10 nM, this concentration was used for qRT-PCR (Figure 3).

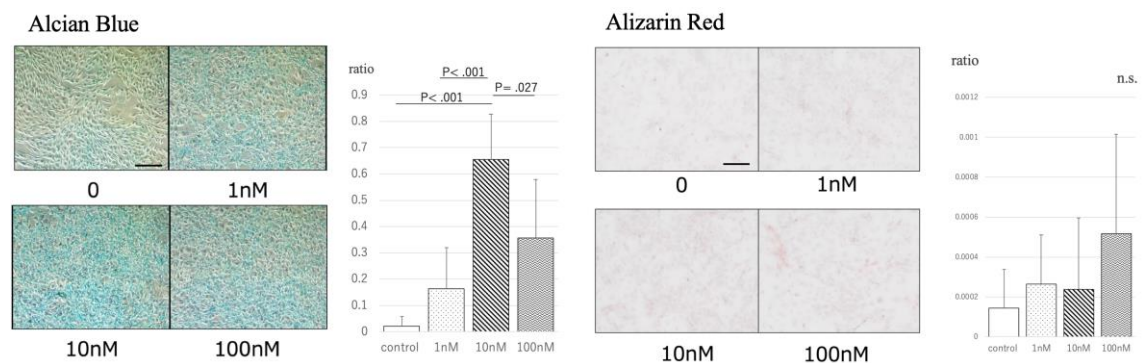


Figure 3. Achilles tendon-derived cells cultured with PTH (0, 1, 10, 100 nM) for 28 d. PTH-treated cells were stained with Alcian blue and Alizarin red S. Scale bar: 300 μ m. The percentage of the area positive for Alcian blue was significantly higher in the 10 nM treatment groups than in the untreated groups. There was no significant difference in the Alizarin red-stained area.

In vitro: qRT-PCR assay

mRNA expression of PTH1R, Col2a1, Sox9, Col1a1, RUNX2, and β -actin was compared between PTH-treated and untreated cells. The total Sox9 and RUNX2 RNA levels were significantly higher in the PTH group than in the control group at 14 d after incubation ($P < 0.05$). Total PTH1R, Col2a1, Sox9, and RUNX2 RNA levels were significantly higher in the PTH group than in the control group at 28 d after incubation ($P < 0.05$). There were no significant differences in Col1a1 expression levels at any time point (Figure 4).

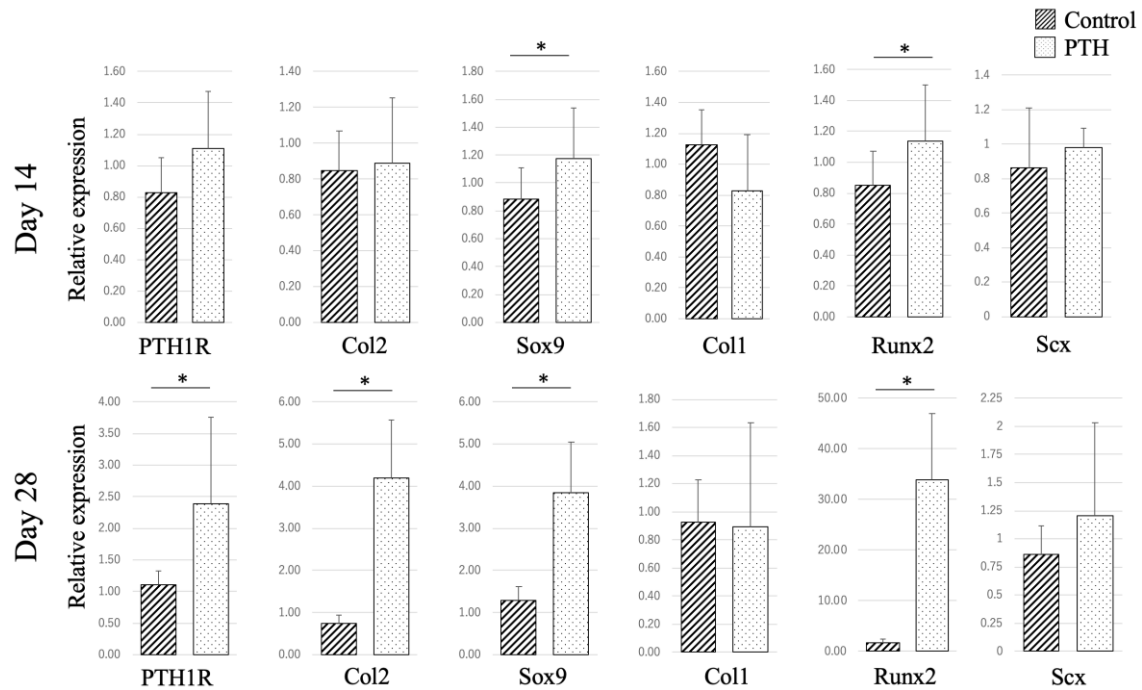


Figure 4. qRT-PCR assay for tendon-derived cells with and without PTH treatment. The total Sox9 and RUNX2 RNA levels were significantly higher in the PTH group than in the control group at 14 d after incubation. The total PTH1R, Col2a1, Sox9, and RUNX2 RNA levels were significantly higher in the PTH group than in the control group at 28 d after incubation (* $P < 0.05$).

In vivo: In situ PTH injection into rat Achilles tendon

HE staining of the PTH(1-34)-injected Achilles tendon showed disordered fibrillar patterns at 4 and 8 weeks post-treatment (Figure 5). Toluidine blue staining revealed metachromasia in the PTH group at 4 and 8 weeks. However, 22.5 and 45 μ g PTH(1-34)-injected tendons showed ossification at 8 weeks after injection (Figure 5). Therefore, 9 μ g of PTH(1-34) was used for transplantation of the autologous Achilles tendon in the rat model.

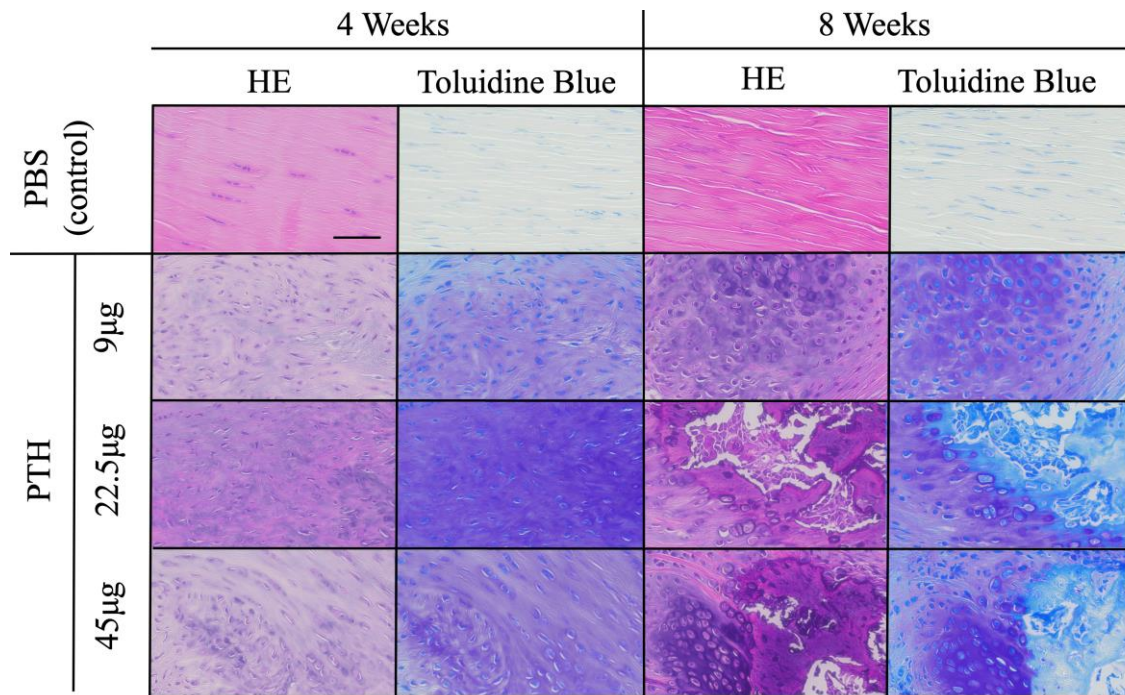


Figure 5. Histological examination of the rat Achilles tendon injected with PBS or PTH(1-34). HE staining of PTH(1-34)-injected Achilles tendon showed disordered fibrillar patterns at 4 and 8 weeks. Toluidine blue staining revealed metachromasia in the PTH group at 4 and 8 weeks. However, tendons injected with 22.5 and 45 µg of PTH(1-34) showed significant levels of ossification at 8 weeks post-injection. Scale bar: 40 µm.

In vivo: Transplantation of PTH-induced Achilles tendon

Based on our macroscopic assessment, the meniscus coverage ratio was significantly higher in the PTH group than in the control group both at 4 and 8 weeks after transplantation (at 4 weeks: 0.57 in control vs. 0.63 in PTH, $P = 0.043$; at 8 weeks: 0.42 vs. 0.60, $P < 0.001$) (Figure 6). The ratio of the native medial meniscus was 0.71. The India ink-stained area was significantly larger in the control

group than in the PTH group at 8 weeks (0.31 vs. 0.12, $P = 0.031$) (Figure 6). These results indicate that the PTH group showed more extensive meniscal coverage and lesser progression of the articular cartilage degeneration than that in the control group.

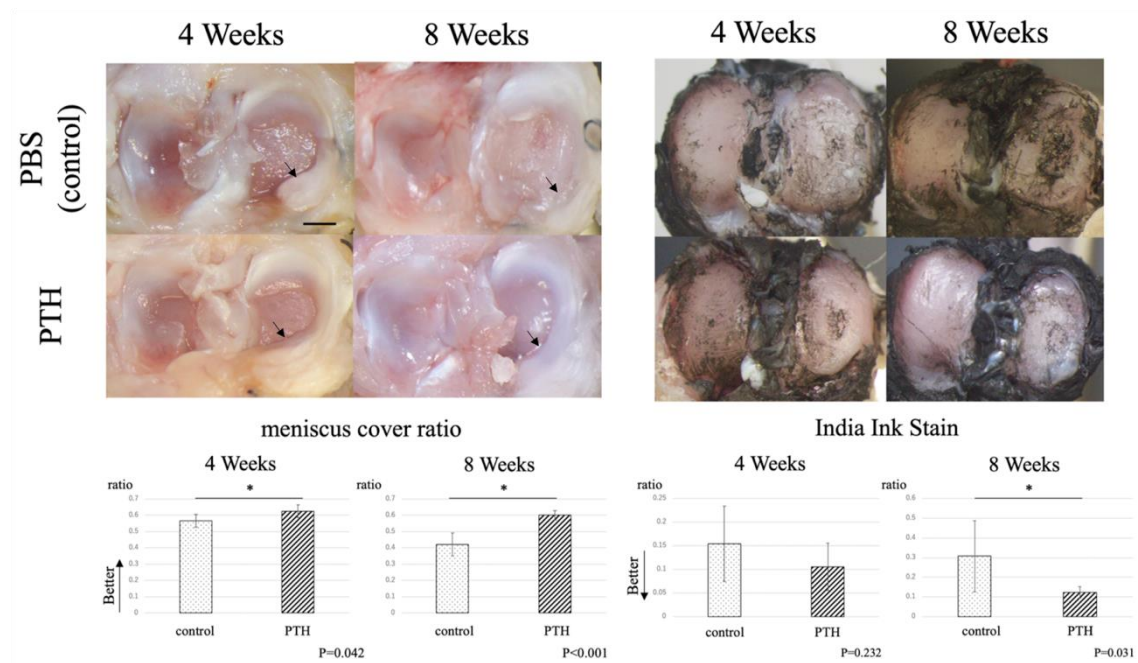


Figure 6. Macroscopic examination of the regenerated meniscus and tibial plateau. Black arrows indicate regenerated tissue. The meniscus coverage ratio, defined as the ratio of medial meniscus area to the medial tibial plateau area, was significantly higher in the PTH group than in the control group, both at 4 and 8 weeks after transplantation. The damaged area of the tibial plateau was quantified by India ink staining to calculate the ratio of the stained area to the entire area of the medial tibial plateau. The India ink-stained area was significantly larger in the control group than in the PTH group at 8 weeks (* $P < 0.05$). Scale bar: 2 mm

Histologically, toluidine blue staining revealed metachromasia in the PTH-injected tissue at 4 weeks and showed stronger staining at 8 weeks (Figure 7). An improved histological score was obtained in the PTH-treated tendon compared with that in the untreated group at 4 and 8 weeks (at 4 weeks: 8.3 in control vs. 11.5 in PTH, $P = 0.024$; at 8 weeks: 7.2 vs. 12.8, $P = 0.002$). Immunostaining for type II collagen was positive in the PTH-treated regenerated meniscus in the region exhibiting metachromasia after toluidine blue staining. Cartilage degeneration of the tibial plateau progressed from 4 weeks after transplantation to 8 weeks in both groups; the modified Mankin score at 8 weeks was significantly better in rats transplanted with PTH-treated tendons (at 4 weeks: 3.5 in control vs. 2.8 in PTH, $P = 0.603$; at 8 weeks: 8.0 vs. 2.7, $P = 0.002$) (Figure 8). These results indicate that transplantation of the PTH-treated Achilles tendon promoted meniscal regeneration and prevented the progression of cartilage degeneration.

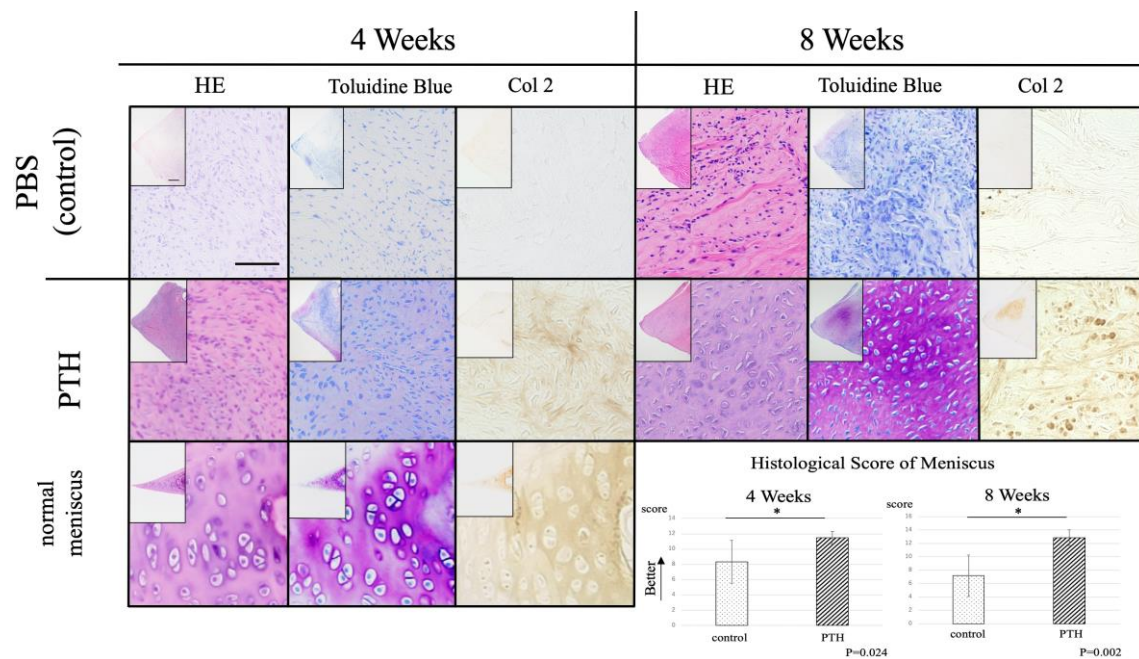


Figure 7. Histologic analysis of regenerated rat meniscus. Toluidine blue staining revealed metachromasia in the PTH group both at 4 and 8 weeks. Immunostaining of type 2 collagen was observed in the PTH group both at 4 and 8 weeks. An improved histological score was observed in the PTH-treated tendons compared with that in the untreated group at 4 and 8 weeks. (* $P < 0.05$) Scale bar: 200 μm for inset images and 40 μm for high magnification images. Col 2; type 2 collagen

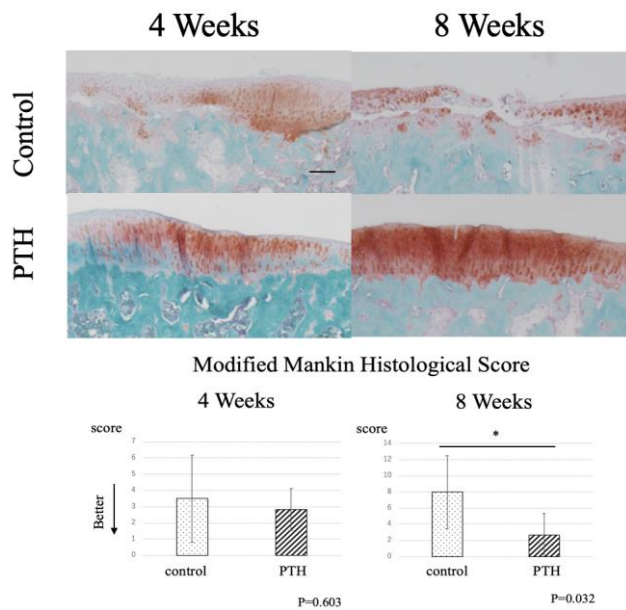


Figure 8. Safranin O staining of the medial tibial plateau. The histological score was significantly better in the PTH-treated group. Scale bar: 200 μ m (* $P < 0.05$).

Micro-CT assessment

No ossification was observed upon micro-CT in either the PTH-injected Achilles tendon or the control group. Similarly, in the transplanted model, no ossification was observed in either group. (Figure 10)

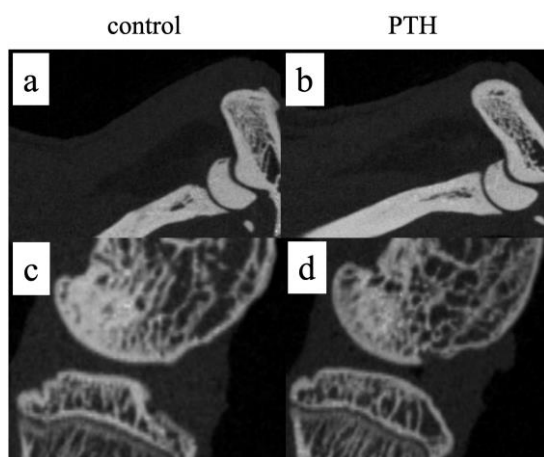


Figure 9. Micro-CT images of rat Achilles tendons (a, b) and knees (c, d) at 8 weeks after surgery. No ossification was observed in both the control (a, c) and PTH (b, d) groups.

Discussion

The most important findings of this study are as follows: PTH (1-34) induces cartilage formation in rat tendons; transplantation of the PTH-injected Achilles tendon into meniscal defects in regenerated tissue covered the tibial plateau more effectively than the untreated tissue; the regenerated meniscus had a cartilaginous matrix, as indicated by toluidine blue staining and type II collagen immunostaining; articular cartilage degeneration was delayed in the PTH group compared with that in the control group; and PTH(1-34) directly acted on tendon-derived cells to stimulate cartilage matrix production.

PTH(1-34) is a bone anabolic agent approved by the US Food and Drug Administration (FDA) for osteoporosis treatment.³² When PTH is administered intermittently, its anabolic effect increases bone mass, density, and strength as well as improves bone fracture prognosis. Parathyroid hormone-related protein (PTHrP), an analog of PTH, binds to the same receptor as PTH.⁵ PTHrP affects chondrocyte proliferation and differentiation and is an essential factor in regulating the pace of chondrocyte differentiation and endochondral ossification.^{11,33} Liu et al. investigated the effects of different PTH administration methods on condylar chondrocytes during osteogenic culture.²¹ They reported that continuous PTH administration promotes condylar chondrocyte proliferation and suppresses differentiation, as demonstrated by upregulated collagen type II mRNA expression. According to the methods reported in previous studies, we used continuous PTH administration for *in vitro* experiments

and found upregulated expression of Col2a1 and Sox9, which are involved in chondrogenesis.

The effects of PTH(1-34) on tendon-derived cells remain unclear, whereas its effects on mesenchymal stem cells (MSCs) have been well investigated.^{7,26,43} Zang et al. reported that a 10 nM concentration of PTH(1-34) promoted chondrogenic differentiation of MSCs with increased expression of Sox9, Col2a1, and PTH1R, whereas chondrogenesis of MSCs was inhibited rather than stimulated with a higher concentration of PTH(1-34).⁴³ Sox9 is a key transcription factor for chondrogenesis and regulates the gene encoding type-II collagen.³ In a previous study, upregulation of Sox9 was observed during meniscal regeneration in a meniscal defect model.² In the present study, we assessed the chondrogenic differentiation of tenocytes; therefore, it is unclear whether the gene expression changes observed by qRT-PCR are identical to those found in meniscal cells. The rat Achilles tendon has abundant multipotent stem cells, called tendon-derived stem cells.^{10,36} In the present study, PTH(1-34) might have stimulated tendon-derived stem cells resulting in upregulated PTH1R expression and activation of the transcription factor, Sox9. This stimulatory action may have promoted the expression of SOX9-sensitive genes, such as COL2A, and chondrogenic differentiation.

If the meniscus is excised and left untreated, only a small amount of synovial tissue fills the meniscal defect area. However, the regenerated tissue is insufficient in size and incapable of preserving the articular cartilage.²⁸ The autologous tendon, transplanted into the meniscal defect, could serve as a scaffold for meniscal regeneration. However, tendons could not independently convert to

fibrocartilage like the native meniscus and need additional procedures to prevent the progression of cartilage degeneration.²⁹

Naka et al. previously reported autogenous semitendinosus tendon transplantation in a rabbit model of meniscal defect using recombinant human bone morphogenetic protein-2 (rhBMP-2).²⁵ They also confirmed cartilaginous tissue formation within the graft. However, ossicles were formed by vascular invasion into rhBMP-2-induced cartilage. Further, Oseki et al. assessed the transplantation of BMP-7-treated Achilles tendon in a rat model and reported ectopic cartilage formation in the tendon.²⁹ Furthermore, ossification was not detectable in their experimental procedure because EDTA was used to decalcify the tissues. In our study, we used PTH to promote chondrogenic differentiation in the early stages and inhibit terminal differentiation. We found 9 µg of PTH(1-34) injection to be appropriate to avoid ossification. This amount corresponds to a single dose recommended by the manufacturer when treating osteoporosis in rats (30 µg/kg), and side effects such as tumorigenesis are not a concern.³⁷ Moreover, as the concentration of 10 nM *in vitro* was equivalent to approximately 41 µg/L, the appropriate dose was consistently maintained throughout the study. When tendons treated with PTH are transplanted into a meniscal defect, the tendon acts as a scaffold in the early stage. PTH addition promotes fibrocartilage differentiation of tendon cells to enhance extracellular cartilage matrix synthesis.

Our study does have some limitations. First, biomechanical examination was not performed. Therefore,

biomechanical properties of the regenerated meniscus could not be confirmed, even though its histologic features were close to those of the native meniscus. However, transplantation of the PTH-induced Achilles tendon prevented the progression of cartilage degeneration, compared with that in the untreated groups. Fischenich et al. reported in their biomechanical study that compressive integrity is reduced by the decreased cartilage matrix content of the meniscus, which reduces the protective effect of articular cartilage.⁶ Histological assessment in this study showed that the regenerated tissue in rats that received PTH-treated tendon transplants had more cartilaginous matrix content than that in control group. These results collectively indicate that the biomechanical properties of the regenerated tissue in PTH groups resulted in stronger integration. Another limitation of this study is the different properties of the meniscus from small animals and humans. The meniscus of small animals has more cells than that from humans.⁴ Further, the distributions of type I and type II collagen are similar but not the same.²⁴ Nevertheless, the results of the present study provide novel evidence of meniscal regeneration. However, these experimental methods must be applied to higher mammals and human trials to confirm that the regenerated meniscus with PTH-treated tendons is clinically relevant.

Conclusion

PTH (1-34) induced cartilage formation in the rat tendon. Transplantation of PTH (1-34)-treated Achilles tendon induced meniscal regeneration and preserved knee articular cartilage in a rat meniscal

defect model. Macroscopically, the PTH groups showed greater coverage of the regenerated meniscus. Histologically, the regenerated meniscus showed more cartilaginous matrix content in rats transplanted with PTH-treated tendons than that in control group. PTH (1-34) stimulated tendon-derived cells showed promotion of chondrogenic differentiation.

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