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TITLE:

In vivo cartilage regeneration induced by a double-network hydrogel: Evaluation of a novel therapeutic strategy for femoral articular cartilage defects in a sheep model

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We have no financial competing interests. We do not hold or are not currently applying for any patents relating to the content of the manuscript.

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Abstract: The purpose of this study was to establish the efficacy of a therapeutic strategy for an articular cartilage defect using a PAMPS/PDMAAm DN gel in a sheep model. Seventeen mature sheep were used in this study. We created a 6.0-mm osteochondral defect in the femoral trochlea of the patellofemoral (PF) joint and the medial condyle of the tibiofemoral (TF) joint. A cylindrical DN gel plug was implanted into the defect of the right knee so that a vacant space of the planned depths of 2.0 mm in Group I, 3.0 mm in Group II, and 4.0 mm in Group III were left. In the left knee, we created a defect with the same depth as the right knee. The regenerated tissues were evaluated with the O'Driscoll score and real time PCR analysis of the cartilage marker genes at 12 weeks. The DN-gel implanted defect of Group II in the PF and TF joints was completely filled with a sufficient volume of the proteoglycan-rich tissue stained with Safranin-O. The score showed that Group II was significantly greater than Groups I and III when treated with DN gel in the PF joint ($p=0.0441$, $p=0.0174$, respectively) and in the TF joint ($p=0.0019$, $p=0.0006$, respectively). This study has clarified the short-term efficacy of the cartilage regeneration strategy using the DN gel in a sheep model.

Key words: Cartilage repair; Double-network hydrogel; Polymer, In vivo; Sheep model

Introduction

Articular cartilage, being an avascular structure, has limited potential for regeneration.^{1,2} Therefore, functional repair of osteochondral defects remains a major challenge in the field of joint surgery. The most prevalent strategy to repair the osteochondral defect is to fill the defect with a tissue-engineered cartilage-like tissue or a cell-seeded scaffold material.³⁻¹⁰ Despite a promising outcome, this strategy has demonstrated inherent disadvantages such as donor site morbidity, two surgeries being needed, and the long non-weight bearing period.¹¹⁻¹⁶ Recently, cell-free approaches have shown their potential in the treatment of osteochondral defect, which offers a one-step procedure. Some investigators have tried to fill an osteochondral defect with acellular polymer or collagen-based scaffolds to induce cartilage regeneration.¹⁷⁻²⁰ In addition to the role as carrier for cell delivery, these materials alone may have potential to induce and promote cartilage regeneration by exploiting the intrinsic regeneration ability.

As a potential biomaterial to develop a cell-free method for cartilage repair, a PAMPS/PDMAAm double-network (DN) gel composed of poly-(2-Acrylamido-2-methylpropanesulfonic acid) (PAMPS) and poly-(N,N'-dimethyl acrylamide) (PDMAAm) may be a promising candidate. The PAMPS/PDMAAm DN gel has excellent biocompatibility and is resistant to biodegradation.^{21, 22} Additionally, we found that hyaline cartilage regeneration occurred in vivo in the defect within 4 weeks after surgery when a PAMPS/PDMAAm DN gel plug was implanted at the bottom of a large osteochondral defect, which was created in the patellofemoral joint of the rabbit knee by intentionally leaving a 1.5 to 3.5-mm deep vacant space defect.²³ This discovery contributes to a novel strategy to repair an osteochondral defect in the field of joint surgery: That is, induction of the cartilage regeneration in a vacant defect using the artificially synthesized hydrogel without any cultured cells or

mammalian-derived scaffolds. It has been confirmed that the regeneration phenomenon occurs not only in the patellofemoral (PF) but also in the tibiofemoral (TF) joints using a rabbit model.²⁴ However, the rabbit model is not practical enough to establish the efficacy of this potential therapeutic strategy. Therefore, the feasibility of this strategy must be evaluated using a large animal.

The purpose of this in vivo study using 17 sheep was to establish the efficacy of a novel therapeutic strategy that induces cartilage regeneration using the PAMPS/PDMAAm DN gel, for osteochondral defects created in the patellofemoral (PF) and the tibiofemoral (TF) joints.

Materials and Methods

Materials

The PAMPS/PDMAAm DN gel was synthesized using the previously reported two-step sequential polymerization method.²⁵ 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) (Toagosei Co. Ltd., Japan) and N,N'-dimethylacrylamide (DMAAm) (KOHJIN Co., Ltd., Tokyo, Japan) were used as purchased. Briefly, PAMPS hydrogel was obtained by radical polymerization using N,N'-methylenebisacrylamide (MBAA) (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) as a cross-linker and 2-oxoglutaric acid (Wako Pure Chemical Industries, Ltd, Osaka, Japan) as an initiator. The monomer concentration was 1 mol/L for PAMPS, 4 mol% for the cross-linker, and 0.1 mol% for the initiator. Aqueous solution containing a monomer, cross-linker, and the initiator was injected into a cell consisting of a pair of glass plates separated by a silicone rubber. The cell was irradiated with a UV lamp (wave length 365 nm) for about 8 hours under argon gas atmosphere. The DN gel was synthesized by the sequential network formation technique (two-step method). The PAMPS hydrogel (1st network) was immersed in an

aqueous solution of 2 mol/L DMAAm, containing 0.1 mol% MBAA, and 0.1 mol% 2-oxoglutaric acid for one day until reaching the equilibrium. The 2nd network (PDMAAm) was subsequently polymerized in the presence of the PAMPS hydrogel by irradiating UV for 8 hours between two plates of glasses under argon gas atmosphere. After polymerization, the PAMPS/PDMAAm DN gel was immersed in 0.9% NaCl solution for 1 week and the water was changed twice daily to remove any un-reacted materials. From the PAMPS/PDMAAm DN gel, we created cylindrical plugs 6-mm in diameter and 10-mm long.

Study design

Animal experiments were carried out in the Institute of Animal Experimentation, under the Rules and Regulation of the University's Animal Care and Use Committee.

A total of 17 skeletally mature female sheep (Suffolk, Sankyo Labo Service Corporation Inc., Tokyo, Japan), 4 to 6 years old and weighing 65 to 85 kg, were used in this study. In the bilateral knees of each animal, we created a cylindrical osteochondral defect having a diameter of 6.0-mm in the femoral trochlea of the PF joint and the medial condyle of the TF joint (Figure 1). Then, 15 of the 17 sheep were randomly divided into 3 groups (Group I, II, and III) of 5 animals each. In each group, the defect was created so that the depth of the final vacant space in the defect with or without DN gel implantation became 2.0 mm in Group I, 3.0 mm in Group II, and 4.0 mm in Group III. We implanted a cylindrical DN gel plug into the defect of the right knee so that a vacant space of the planned depth for each group was left. In the left knee, we created the defect having the same depth as the right knee, and we did not apply any treatment to obtain the non-treated control. All animals were sacrificed at 12 weeks after surgery, using an

overdose of potassium chloride under general anesthesia, and their knee joints were used for the gross and histological evaluations.

Based on the histological findings, because the degree of cartilage regeneration in Group II was the greatest among the groups, the 2 sheep were used for real time PCR analysis to evaluate gene expression in the tissue regenerated in the defect at 12 weeks after surgery.

Surgical procedure

Surgery was performed under anesthesia induced by an intravenous injection of ketamine hydrochloride (3 mg/kg). After intubation, anesthesia was maintained with halothane in pure oxygen via endotracheal intubation. The animal was positioned in dorsal recumbency in a cradle, leaving hindlimbs free. The bilateral hindlimbs were shaved and prepared in the standard sterile fashion. A midline longitudinal skin incision was made over the knee joint. A medial para-patellar arthrotomy was performed to expose the articular surface of the femoral trochlea and the weight-bearing area of the medial femoral condyle. Then, an osteochondral defect having a 6.0-mm diameter was created in the femoral trochlea of the PF joint and the medial femoral condyle of the TF joint using a motorized drill. The right knee joint received the cylindrical DN gel plug implantation, while the left knee was left untreated to serve as a control. The animals were divided into 3 groups depending upon the depth of the final vacant space after the DN gel implantation in the defect or the drilled defect in the non-treated control. The depth of the final vacant space was set at 2.0 mm in Group I, 3.0 mm in Group II, and 4.0 mm in Group III. We selected the three different vacant space depth in this study because the regeneration effect of DN gel implantation was influenced by the vacant space depth.^{23, 30} The incision was closed routinely in layers beginning with the deep fascia.

Postoperatively, the animals were returned to their cages (2 x 2 m) and allowed to put full weight on their limbs without restriction of motion. At day 6 after the surgery, they were allowed to roam freely in a fenced area (approximately 50 m²) and walk around outdoors for 2 hours per day on a licensed farm.

Examination methods

Histological examination of in vivo regenerated tissues

A distal portion of the resected femur was fixed in a 10 % neutral buffered formalin solution for 3 days, decalcified with 50mM EDTA for a period of 3-4 weeks, and then cast in a paraffin block. The femur was sectioned perpendicular to the longitudinal axis, and stained with hematoxylin-eosin and Safranin-O.

We used the scoring system reported by O’Driscoll et al,^{26, 27} because this scale could finely evaluate the histological findings. Histology was evaluated for cellular structure (hyaline articular cartilage, 4 points; incompletely differentiated mesenchyme, 2 points; fibrous tissue or bone, 0 points), Safranin-O staining of the matrix (normal or nearly normal, 3 points; moderate, 2 points; slight, 1 point; none, 0 points), surface regularity (smooth and intact, 3 points; superficial horizontal lamination, 2 points; fissures of 25%-100% of thickness, 1 point; severe disruption including fibrillation, 0 points), structural integrity (normal, 2 points; slight disruption including cysts, 1 point; severe disintegration, 0 points), thickness (100% of normal adjacent cartilage, 2 points; 50%-100% of normal cartilage, 1 point; 0%-50% of normal cartilage, 0 points), bonding to the adjacent cartilage (bonded at both ends of the graft, 2 points; bonded at one end or partially at both ends, 1 point; not bonded, 0 points), hypocellularity (normal cellularity, 3 points; slight hypocellularity, 2 points; moderate hypocellularity, 1 point; severe

hypocellularity, 0 points), chondrocyte clustering (no clusters, 2 points; <25% of the cells, 1 point; 25%-100% of the cells, 0 points), and adjacent cartilage degradation (normal, no clusters, and normal staining, 3 points; normal cell, mild clusters, and moderate staining, 2 points; mild/moderate hypocellularity and slight staining, 1 point; severe hypocellularity, 0 points). The full mark totaled 24 points.

Real time PCR analysis

Total RNA was extracted from the tissues regenerated in the defect, using the RNeasy mini kit (Qiagen Inc., Valencia, CA). RNA quality from each sample was assured by the A260/280 absorbance ratio. The RNA (100ng) was reverse-transcribed into a single strand of cDNA using PrimeScript® RT reagent Kit (TakaraBio, Ohtsu, Japan). The RT reaction was carried out for 15 minutes at 37 degrees Celsius and then for 5 seconds at 85 degrees Celsius. All oligonucleotide primer sets were designed based upon the published mRNA sequence. The sequences of primers used in real time PCR analyses for sheep regenerative tissues were described in Table 1. The real time PCR was performed by using the Thermal Cycler Dice® TP800 (TakaraBio, Ohtsu, Japan) with SYBR® Premix Ex Taq™ (TakaraBio, Ohtsu, Japan). cDNA template (5 ng) was used for real time PCR in a final volume of 25 microliter. cDNA was amplified according to the following conditions: 95 degrees Celsius for 5 seconds and 60 degrees Celsius for 30 seconds at 40 amplification cycles. Fluorescence changes were monitored with SYBR Green after every cycle. A dissociation curve analysis was performed (0.5 degrees Celsius /sec increase from 60 to 95 degrees Celsius with continuous fluorescence readings) at the end of the cycles to ensure that single PCR products were obtained. The amplicon size and reaction specificity were confirmed by 2.5% agarose gel electrophoresis. The results were evaluated using

the Thermal Cycler Dice® Real Time System software program (TakaraBio, Ohtsu, Japan). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used to normalize samples.

Statistical analysis

All data were described as the mean and standard deviation values. A commercially available software program (StatView 5.0, SAS Institute Inc., Cary, NC, USA) was used for statistical calculation. The mean value of the O'Driscoll's score was statistically compared among the groups using a two-way analysis of variance (ANOVA) followed by Fisher's protected least significance difference test for multiple comparisons. The significance level was set at $p=0.05$.

Results

Histological evaluations

In histological analysis, the DN gel implanted defect of Group II in the PF and TF joints was completely filled with a sufficient volume of the proteoglycan-rich tissue stained with Safranin-O (Figure 2). Regarding the defect of Groups I and III, cartilage tissue was not homogenous (Figure 2). High magnification histology showed that fairly large round cells rich in cytoplasm were scattered singly or as an isogenous group in a proteoglycan-rich matrix in the regenerated tissue of the DN gel implanted defect and the regenerated cartilage was integrated with the adjacent cartilage (Figure 3). On the other hand, some untreated defects were filled with the fibrous and bone tissues and the others were not completely filled with a regenerative tissue and rather remained as a defect, independent of the depth or the location.

Quantitative evaluations with the O’Driscoll’s grading scale

Concerning the O’Driscoll score (Figure 4), the ANOVA demonstrated that there was a significant difference not only on the effect of the DN gel implantation ($p < 0.0001$) but also on the effect of the depth of the final vacant space ($p = 0.0006$), while there was no statistical difference on the effect of the defect location. The post-hoc test showed that Group II was significantly greater than Groups I and III when treated with DN gel in the PF joint ($p = 0.0441$, $p = 0.0174$, respectively) and in the TF joint ($p = 0.0019$, $p = 0.0006$, respectively).

Real time PCR analysis

In real time PCR analysis, the type-2 collagen, aggrecan and Sox 9 mRNAs were highly expressed in the regenerated tissue treated with DN gel in both the trochlea and the medial condyle of Group II, although their expression level was relatively low in the untreated control (Figure 5: Two pairs of knees were analyzed).

Discussion

This study demonstrated that cartilage regeneration can be induced in vivo in an osteochondral defect created not only in the PF joint but also in the TF joint of the sheep by implanting the DN gel plug at the bottom of the defect. It is noted that the regeneration effect was affected by the position (depth) of the implanted gel plug in the defect, and that the greatest effect was obtained when a 3-mm deep vacant space was intentionally left in the defect. The quality of regenerated cartilage was confirmed as hyaline-like since the cartilage markers including the type-2 collagen, aggrecan, and Sox9 were highly expressed in the regenerated

tissue treated with DN gel. These findings are consistent with the results obtained in the rabbit model.^{23, 24, 28-31} It is also noted that the implantation of the DN gel is significantly effective in inducing the cartilage regeneration in the TF joint defect, to which a cartilage regeneration therapy is the most frequently required in the clinical field.

In the cartilage repair strategies, hydrogels have been utilized as cell carrier materials or scaffolds, or as artificial cartilage. As presented in this study, the concept of this innovative strategy with the DN gel implantation for cartilage repair is based on recruiting cells onto the DN gel surface in the vacant space. The appropriate size of space is very important for regeneration of a high quality cartilage tissue, but the DN gel itself is the most important key material in this method because the DN gel is an excellent material for inducing cartilage regeneration. Previous in vitro study showed that the DN gel induced chondrogenic differentiation of ATDC5 cells without insulin.³² In addition, previous in vivo studies demonstrated that the cartilage regeneration was induced by the DN gel at 4 weeks after implantation but not induced by the poly(vinyl alcohol) (PVA) gel or the ultra-high molecular weight polyethylene (UHMWPE) in the same experimental condition²³ and that the degree of the cartilage regeneration by the DN gel was significantly greater than that by the PAMPS or the PDMAAm gel,³¹ which is a component single-network gel of the DN gel. A series of our studies indicate that the DN gel has the potential to induce chondrogenesis both in vitro and in vivo.

The regeneration phenomena induced by DN gel in this sheep model is almost the same as presented in a series of studies using a rabbit model; where the effect was due to the position (depth) of the implanted gel plug in the defect. Yasuda et al. demonstrated that cartilage regeneration occurs in the blood clot formed on the DN gel, which contains mesenchymal stem cells and various cytokines.²³ In this DN gel cartilage repair strategy, a sufficient amount of

blood clot is formed in the defect immediately after surgery and an appropriate space is a prerequisite for cartilage regeneration. Therefore, the cartilage regeneration was not induced in Group I because the space was too narrow to accommodate sufficient formation of the blood clot in the vacant space. On the other hand, the cartilage regeneration was not induced in Group III even with a large space. We speculate that the vacant space in Group III was so large that the biomechanical environment was similar to the untreated control given that the biomechanical condition is known to be an important factor when inducing chondrogenesis. Kelly et al. reported that mechanical signals play an important role in differentiation of bone-marrow derived stem cells,³³ and Engler et al. found that matrix elasticity influences the differentiation of mesenchymal stem cells.³⁴

There are some limitations to this study. The first limitation is that we used an animal model in cartilage repair experiments. The second limitation is that we evaluated the effect of the DN gel on in vivo cartilage regeneration only at 12 weeks. The cartilage regeneration induced by the gel may deteriorate for further follow-up. We need to perform further long-term studies to confirm the safety and efficacy of the DN gel on cartilage regeneration. The third limitation is that we did not actually examine the properties of the gel plug implanted in the bone tissue after cartilage repair began. In a previous study,²¹ however, the mechanical properties of this DN gel implanted in the subcutaneous tissue did not deteriorate at 6 weeks after implantation. Therefore, we speculate that the mechanical properties of the hydrogel plug implanted in the bone tissue may not significantly change at 12 weeks after implantation. The fourth limitation is that we did not perform biomechanical evaluations of the regenerated cartilage. We recognize that the biomechanical quality of cartilage is of vital importance for cartilage tissue restoration and durability. The fifth limitation is that the absolute values in the results of the present study with

the sheep cannot directly be ascribed to the human patient, even though large animal models are typically required for regulatory approval of any cartilage repair strategy.^{35, 36}

In conclusion, the present study has added new important information to the cell-free strategy for cartilage regeneration using the DN gel: The short-term efficacy of a cartilage regeneration strategy using the synthetic PAMPS/PDMAAm DN gel in a sheep model. This method appears to generate an excellent hyaline cartilage repair without the use of exogenous cells and without fully filling the osteochondral defect. This novel gel strategy needs to be further validated in long-term studies to elucidate its potential.

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Figure legends

Figure 1. Surgical implantation of a PAMPS/PDMAAm DN gel plug. A: A cylindrical osteochondral defect having a diameter of 6.0 mm was created in the femoral trochlea of the patellofemoral (PF) joint and the medial condyle of the tibiofemoral (TF) joint. Then, a DN gel plug was implanted into the bottom of the defect. B: A schematic cross-section of the osteochondral defect into which the plug was implanted. The depth of the final vacant space in the defect with or without DN gel implantation became 2.0 mm in Group I, 3.0 mm in Group II, and 4.0 mm in Group III.

Figure 2. Histological evaluation by Safranin-O staining at 12 weeks (2x original magnification). Black scale bar = 1 mm.

Figure 3. High magnification histological observations in DN gel implanted specimen of Group II at 12 weeks (a,b: Safranin-O, c,d: HE). White scale bar = 200 μm , and yellow scale bar = 50 μm .

Figure 4. Quantitative evaluations of histology according to the grading scale reported by O'Driscoll et al.²⁷

*Significantly different from the 2- and 4-mm depths of the final vacant space in the DN gel implanted knees ($p=0.0441$ and $p=0.0174$, respectively).

#Significantly different from the 2- and 4-mm depths of the final vacant space in the DN gel implanted knees ($p=0.0019$ and $p=0.0006$, respectively).

Figure 5. Gene expression of type-2 collagen (Col-2), aggrecan, and Sox9 (Two samples, number 1 and 2 were analyzed).

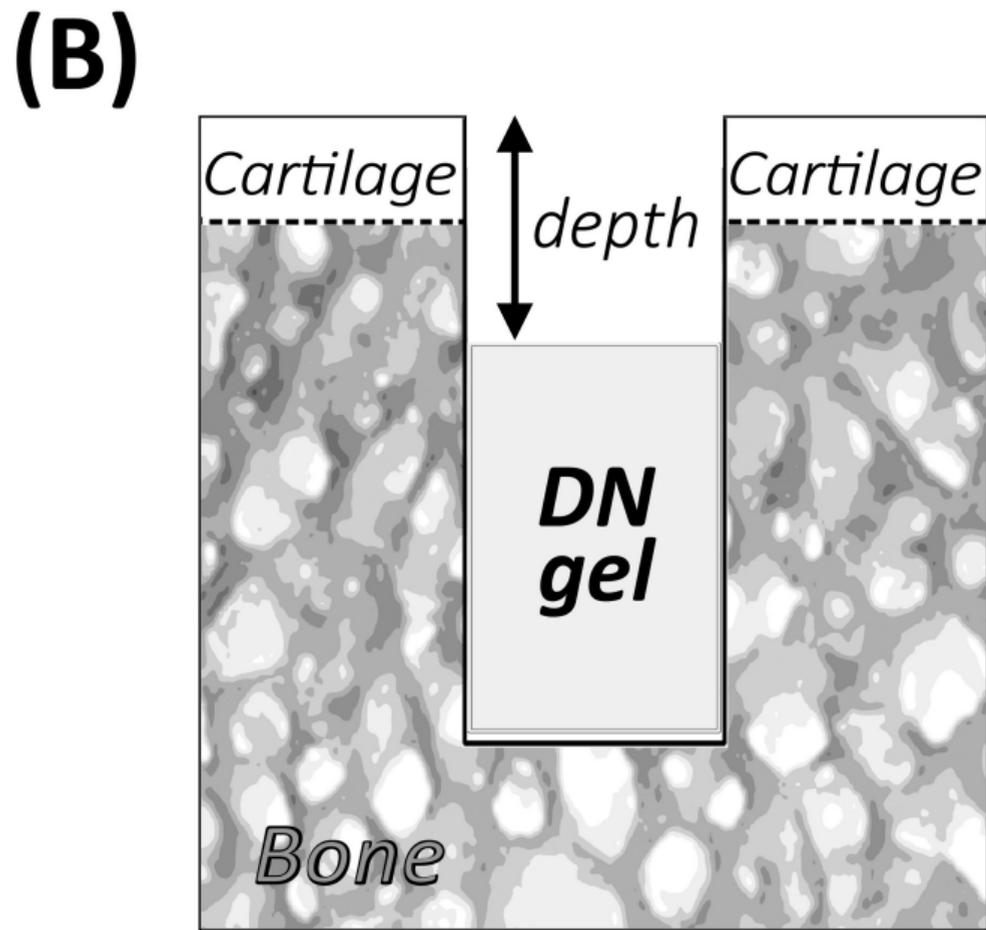
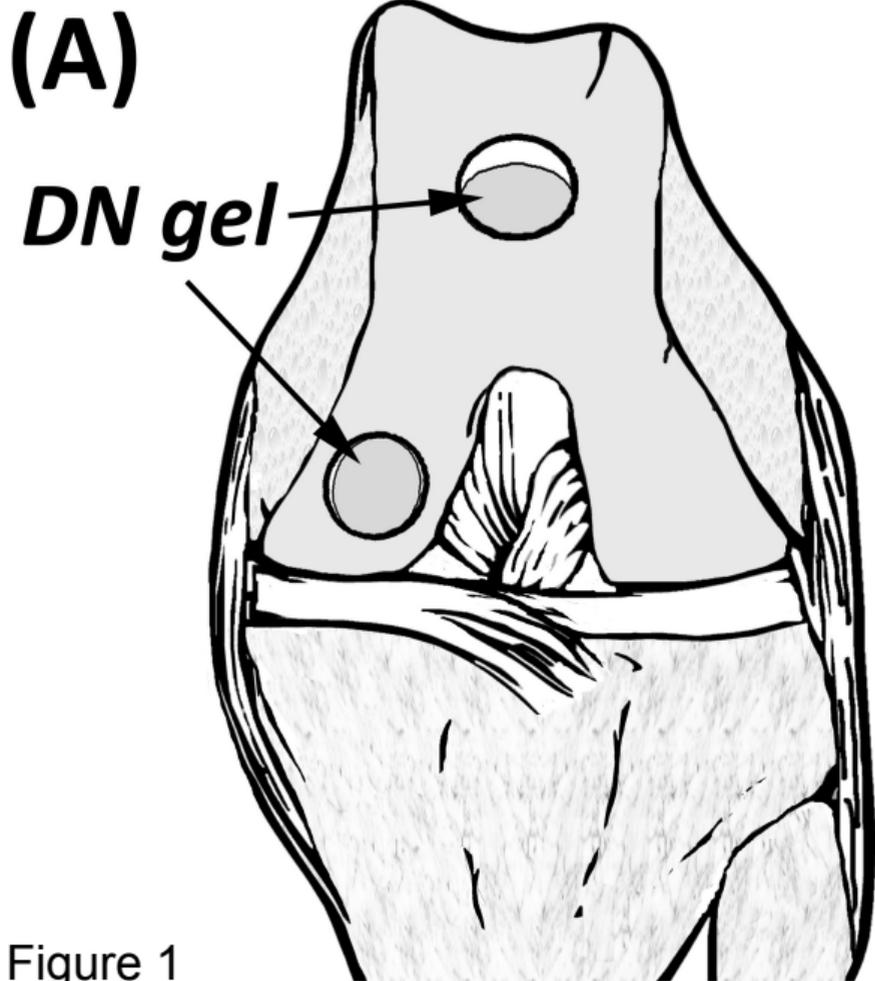


Figure 1

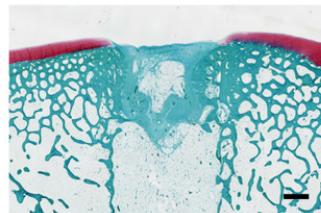
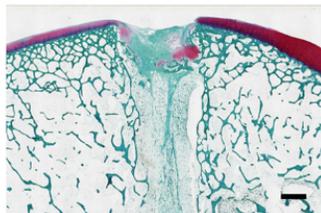
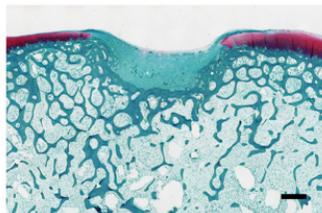
Depth of vacant space (mm)

2

3

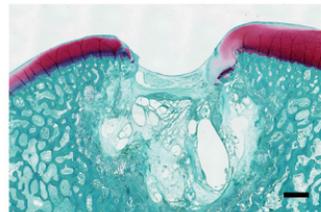
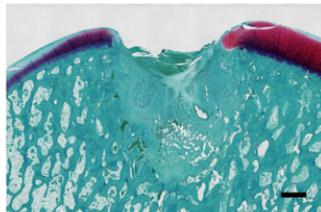
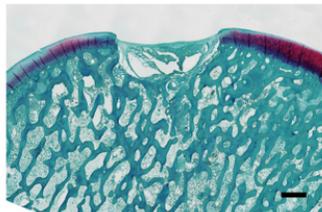
4

PF

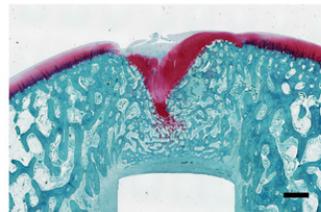
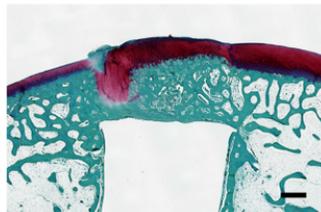
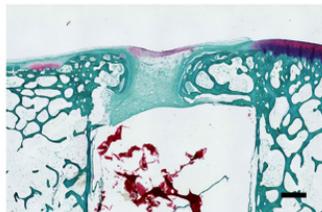


Ctl

TF



PF



DN

TF

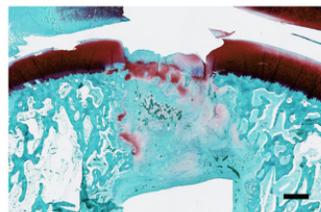
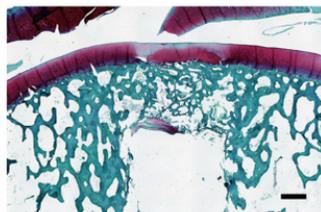
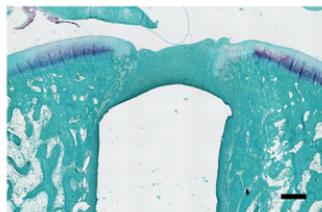


Figure 2

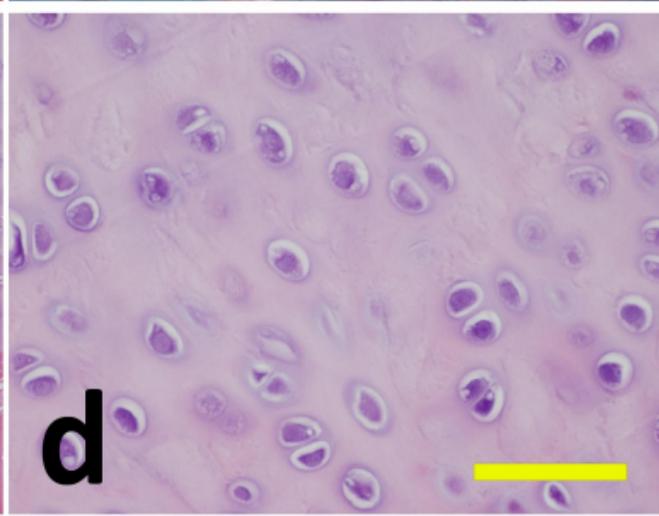
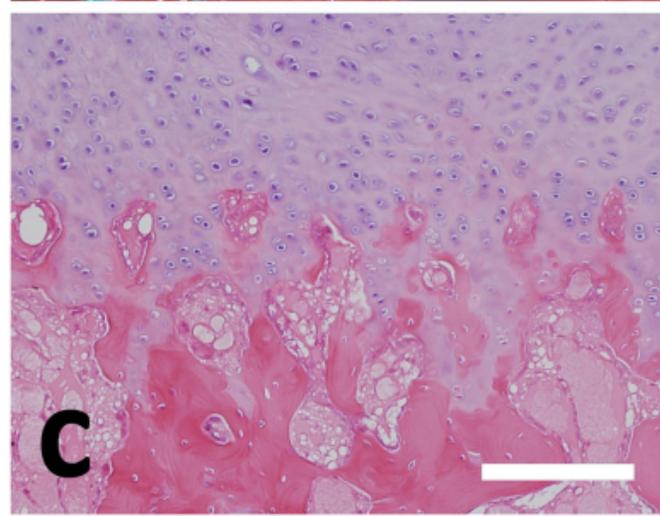
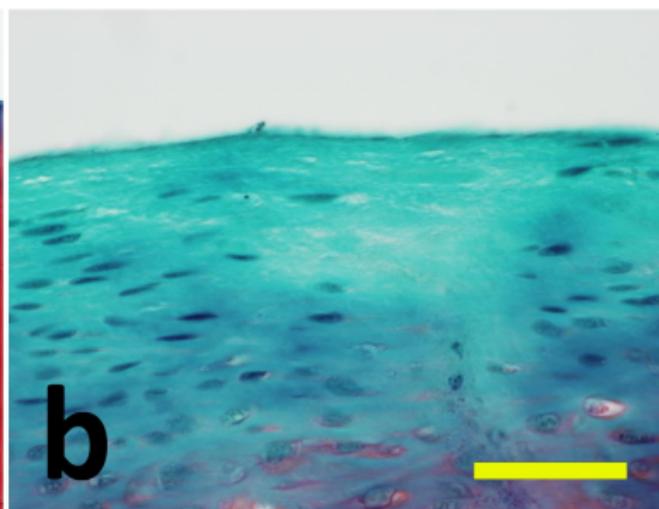
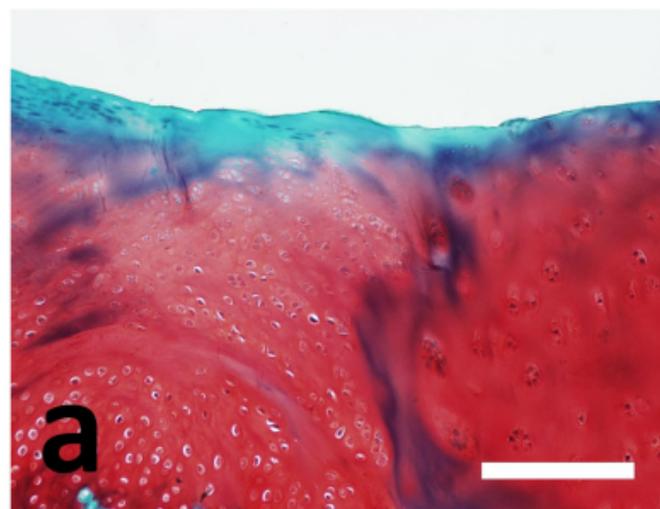
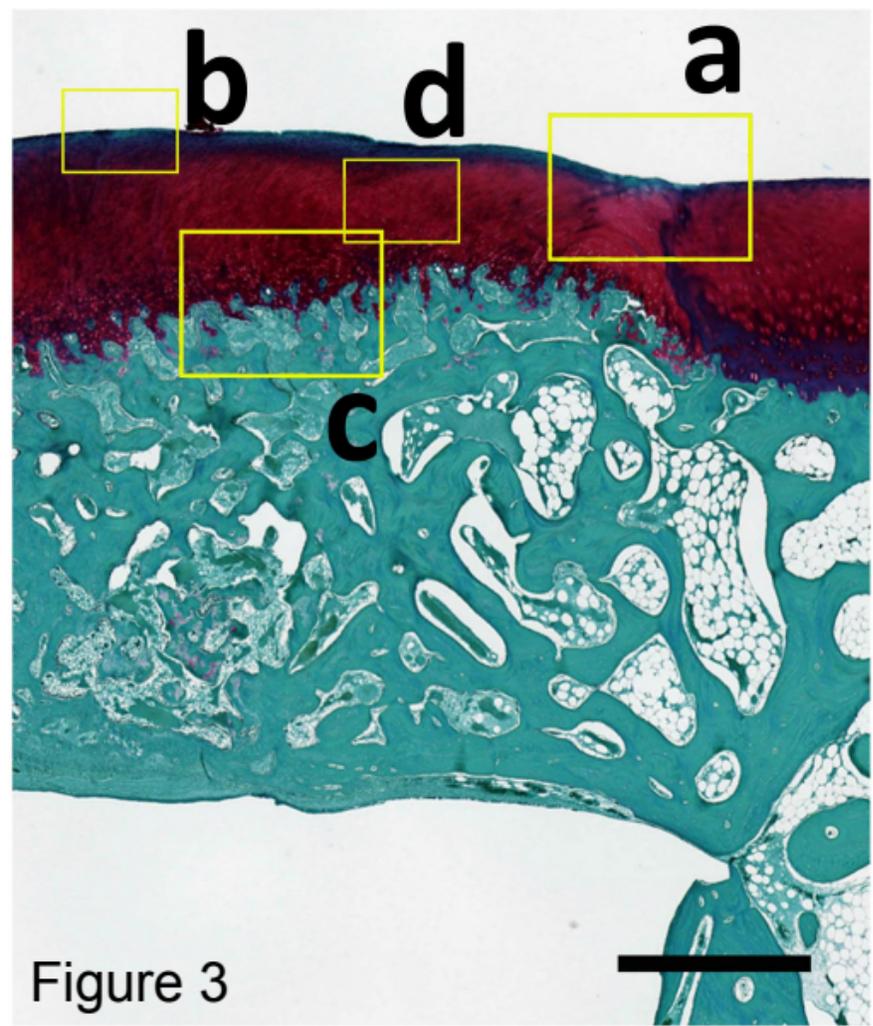


Figure 3

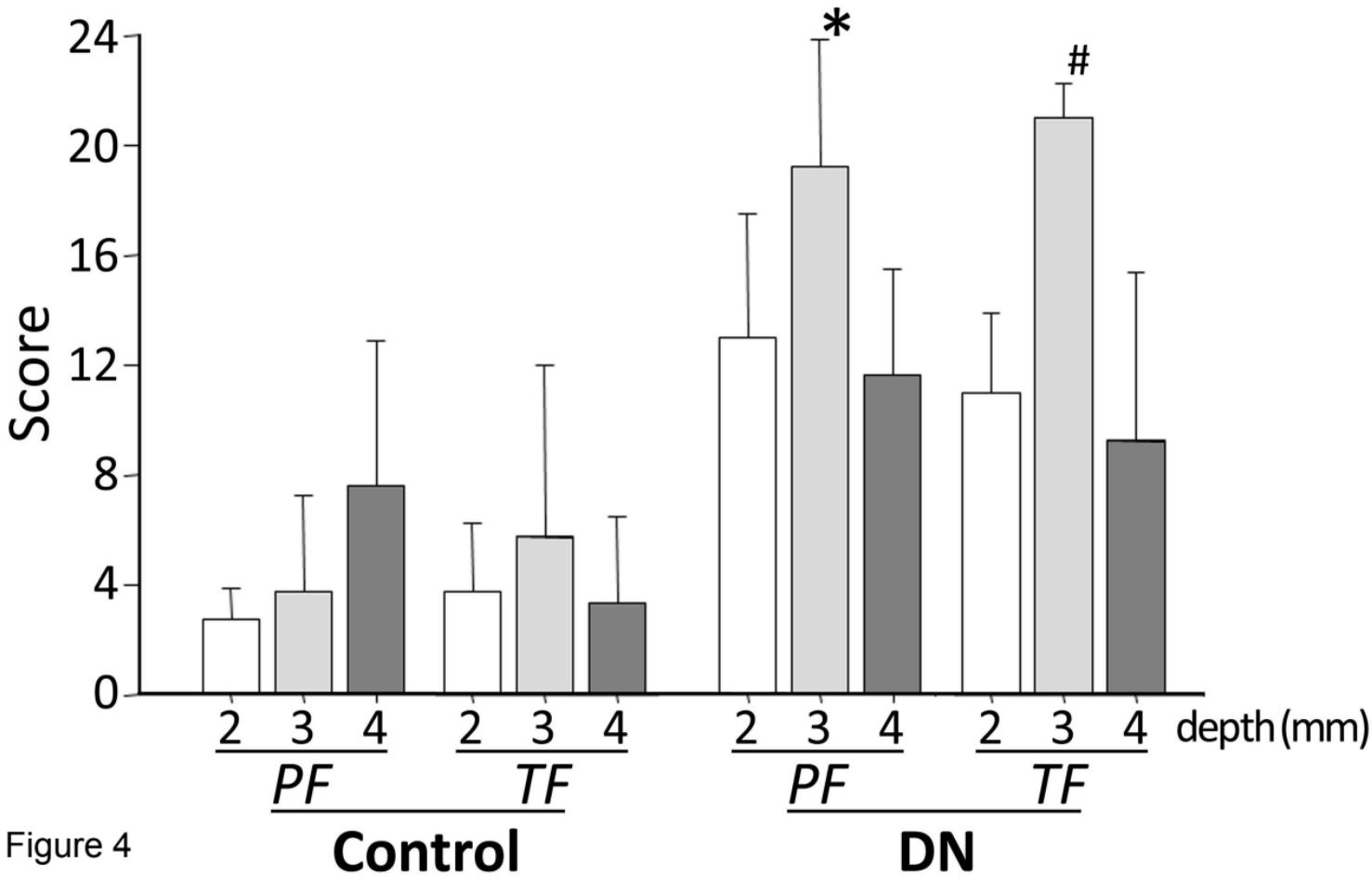


Figure 4

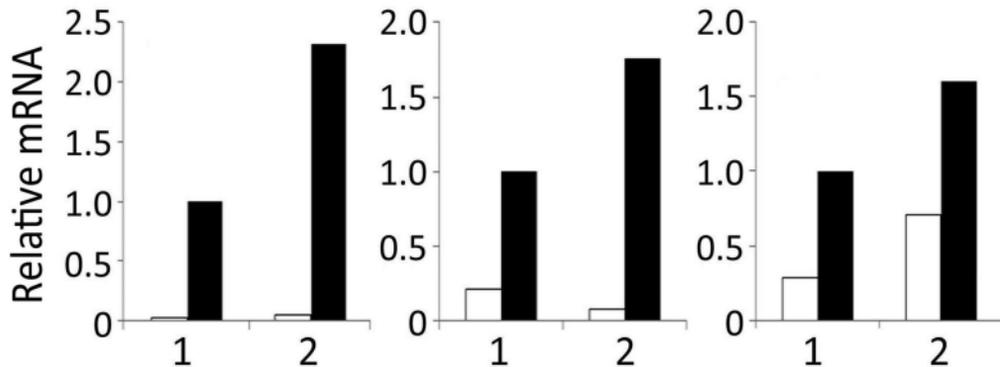
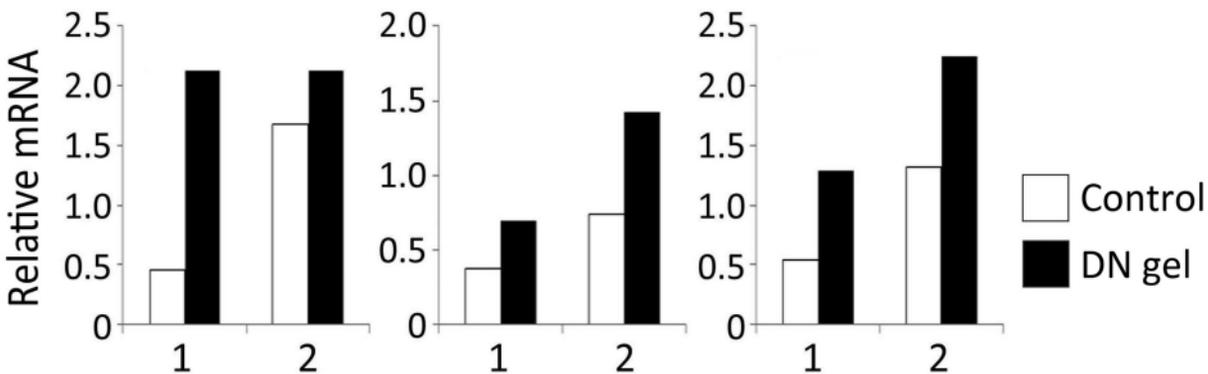
PF*Col-2**Aggrecan**Sox9***TF***Col-2**Aggrecan**Sox9*

Table 1. List of primers used for real time PCR.

		Primers (5'-3')	Accession No.
Type-2 collagen	Forward:	GCAGGAATTCGGTGTGGACATAG	FJ378650
	Reverse:	TCAGGTCAGGTCAGCCATTCAG	
Aggrecan	Forward:	GGCAAGCTCCAGAAGCAAGTG	FJ200438
	Reverse:	ACAGTTGGGAACCCAGATGGAA	
Sox9	Forward:	CACAGATCTTTGGGCTGTCTGAAC	AF012022
	Reverse:	GCTGCTGACACATCCTTGAC	
GAPDH	Forward:	TGGTGAAGCAGGCGTCAGA	AF030943
	Reverse:	CGCTGTTGAAGTCGCAGGAG	