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Joint immobilization inhibits spontaneous hyaline cartilage regeneration induced by a novel double-network gel implantation

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Abstract We have recently discovered that spontaneous hyaline cartilage regeneration can be induced in an osteochondral defect in the rabbit, when we implant a novel double-network (DN) gel plug at the bottom of the defect. To clarify whether joint immobilization inhibits the spontaneous hyaline cartilage regeneration, we conducted this study with 20 rabbits. At 4 or 12 weeks after surgery, the defect in the mobile knees was filled with a sufficient volume of the hyaline cartilage tissue rich in proteoglycan and type-2 collagen, while no cartilage tissues were observed in the defect in the immobilized knees. Type-2 collagen, Aggrecan, and SOX9 mRNAs were expressed only in the mobile knees at each period. This study demonstrated that joint immobilization significantly inhibits the spontaneous hyaline cartilage regeneration induced by the DN gel implantation. This fact suggested that the mechanical environment is one of the significant factors to induce this phenomenon.

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1 Introduction

Articular cartilage defect is a significant health care concern. It has been a commonly established concept that the articular cartilage tissue cannot spontaneously regenerate in vivo [1, 2]. Recently, however, we have found that, when we implant a novel PAMPS/PDMAAm double-network (DN) gel plug [3], which is composed of poly-(2-Acrylamido-2-methylpropanesulfonic acid) (PAMPS) and poly-(*N,N′*-Dimetyl acrylamide) (PDMAAm), at the bottom of a large osteochondral defect created in the rabbit patellofemoral joint so that a 1.5 to 3.5-mm deep vacant space is intentionally left in the defect, spontaneous hyaline cartilage regeneration can be induced in vivo in the vacant space within 4 weeks [4]. This fact has given a major modification to the above-described commonly established concept. However, the mechanism of this phenomenon has not been clarified as of yet. Therefore, we have recently conducted a few ex vivo and in vivo studies to elucidate a part of the mechanism [4–6]. Concerning biological aspects of the mechanism, our ex vivo studies showed that the PAMPS/PDMAAm DN gel surface can enhance differentiation of chondrogenic ATDC5 cells into chondrocytes in the ex vivo condition [4], and that the PAMPS network with a sulphonic acid base plays an important role in the enhancement of chondrogenic differentiation [5]. In addition, our in vivo study indicated that the PAMPS gel, which is negatively charged, plays a significant role in the in vivo spontaneous hyaline cartilage regeneration [6]. However, no studies have been conducted to clarify biomechanical aspects of the mechanism of the spontaneous hyaline cartilage regeneration.

In the actual in vivo treatment of the spontaneous hyaline cartilage regeneration, we have allowed the rabbits to move the knee without any restraints after the DN gel

implantation surgery. It is well known that continuous joint motion is an important factor for the cartilage regeneration [7]. During knee motion, compression forces are repetitively loaded on the joint surface. Previous studies have reported that repetitive compressive loading significantly enhances chondrocyte proliferation as well as aggrecan and collagen synthesis in chondrocytes [8–11]. Recently, it is also reported that repetitive compression loading enhances chondrogenic differentiation [12–14]. Therefore, we have considered that allowing free joint motion may be one of the most significant factors to induce the spontaneous hyaline cartilage regeneration phenomenon using the PAMPS/PDMAAm DN gel. To verify this consideration, we have made a specific hypothesis that joint immobilization may significantly inhibit the spontaneous hyaline cartilage regeneration induced by the PAMPS/PDMAAm DN gel implantation treatment. The effect of joint immobilization on the native articular cartilage has been one of the important foci in the field of biomechanics. However, no studies to clarify the in vivo effect of joint immobilization on cartilage regeneration have been reported as of yet. The purpose of this in vivo study is to test this hypothesis, using a rabbit model.

2 Materials and methods

2.1 Materials

The PAMPS/PDMAAm DN gel is a kind of interpenetrating network hydrogel, but with an asymmetric structure. In this DN gel, the two independently cross-linked polymer networks are physically entangled with each other. The first network, which is rigid and brittle, is composed of densely cross-linked polyelectrolyte, and the second network, which is soft and ductile, consists of loosely or even non-crosslinked neutral polymers. Furthermore, the molar composition of the second network is more than 10 times greater than that of the first network. Although the fracture energy G of the first component is about 1 J/m^2 and the second one is about 10 J/m^2, G of the DN gels becomes anomalously high to about 1000 J/m^2 at its maximum, which is 100–1000 times higher than the primary gels [15]. The PAMPS/PDMAAm DN gel has the elastic modulus of 0.20 MPa and the compressive fracture strength of 3.1 MPa [16, 17].

The PAMPS/PDMAAm DN gel was synthesized using the previously reported two-step sequential polymerization method [3]. Briefly, PAMPS gel was obtained by radical polymerization using *N,N′*-methylenebisacrylamide as a cross-linker and 2-oxoglutaric acid 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 bubbled with nitrogen for 30 min, and then injected into a cell consisting of a pair of glass plates separated by a silicon rubber. The cell was irradiated with an UV lamp (wave length 365 nm) for about 6 h. The DN hydrogel was synthesized by the sequential network formation technique (two-step method). The PAMPS gel (1st network) was immersed in an aqueous solution of 3 M DMAAm, containing 0.1 mol% MBAA, and 0.1 mol% potassium persulfate for 1 day until reaching equilibrium. The 2nd network (PDMAAm) was subsequently polymerized in the presence of the PAMPS gel at 60°C for 6 h between two plates of glasses. After polymerization, the PAMPS-PDMAAm DN gel was immersed in pure water for 1 week and the water was changed 2 times every day to remove any un-reacted materials. From the PAMPS/PDMAAm DN gel, we created cylindrical plugs having a 4.5-mm diameter and a 8-mm length.

2.2 Study design and animal experimentation

A total of 20 mature female rabbits, weighing 3.5 ± 0.3 kg, were used in this study. Animal experimentation was carried out in the Institute of Animal Experimentation, Hokkaido University School of Medicine under the Rules and Regulation of the Animal Care and Use Committee, Hokkaido University School of Medicine.

An operation for each animal was performed under intravenous anesthesia (pentobarbital, 25 mg/kg) under sterile conditions. In 16 out of the 20 rabbits, an osteochondral defect having a 4.3-mm diameter was created in the bilateral femoral grooves of the patellofemoral joint, and a cylindrical DN gel plug was implanted into the bottom of each defect so that a 2-mm deep vacant space remained between the gel and joint surface (Fig. 1). The depth of 2 mm was chosen because this depth was the most effective to induce the spontaneous cartilage regeneration in our previous preliminary study [4]. The actual defect depth was precisely measured in the histological sections after sacrifice. After implantation of the gels, only the right knee was immobilized (defined as "immobilized knee") with a previously validated method [18]: Briefly, the knee was fixed with a 1.6-mm stainless-steel wire extra-articularly passed through the tibia and femur at approximately 150–160° of flexion (Fig. 2). In the immobilized joint, the patella was located slightly distal to the osteochondral defect, as shown in Fig. 2. Therefore, the patella did not seal the defect, and synovial fluid could penetrate into the tissues regenerated in the defect. The quadriceps tendon applied compression forces to the tissues regenerated in the defect. On the other hand, the left knee was left mobile without any additional treatment (defined as "mobile knee"). The incised joint capsule and the skin wound were

closed in layers with 3–0 nylon sutures, and an antiseptic spray dressing was applied. Postoperatively, each animal was allowed unrestricted activity in a cage (310 × 550 × 320 mm). Eight rabbits were sacrificed at 4 and 12 weeks after surgery, respectively. At each period, 5 out of the 8 rabbits were used for quantitative gross observations and histological examinations, and the remaining 3 rabbits were used for real-time PCR analyses.

Additionally, in the remaining 4 out of the 20 rabbits, osteochondral defects were created in the same manner, but the DN gel implantation or the joint immobilization treatment was not applied to obtain the untreated control data. These animals were used to clarify if we really used the osteochondral defect model in which hyaline cartilage regeneration never spontaneously occurred. Two rabbits were sacrificed at 4 and 12 weeks after surgery, respectively. Those rabbits were used for histological and immunohistochemical examinations after gross observations.

2.3 Evaluation methods

2.3.1 Gross observation for regenerated tissues

Immediately after sacrifice, the tissue regenerated in the osteochondral defect was quantitatively evaluated with the grading scale reported by Wayne et al. [19]. Gross appearance of each defect on the femoral condyle was graded for coverage (>75% fill, 4 points; 50–75% fill, 3; 25–50% fill, 2; <25% fill, 1; no fill, 0), tissue color (normal, 4 points; 25% yellow/brown, 3; 50% yellow/brown, 2; 75% yellow/brown, 1; 100% yellow/brown, 0), defect margins (invisible, 4 points; 25% circumference visible, 3; 50% circumference visible, 2; 75% circumference visible, 1; entire circumference visible, 0), and surface (smooth/level with normal, 4 points; smooth but raised, 3; irregular 25–50%, 2; irregular 50–75%, 1; irregular >75%, 0). Thus, the maximum total score was 16 points.

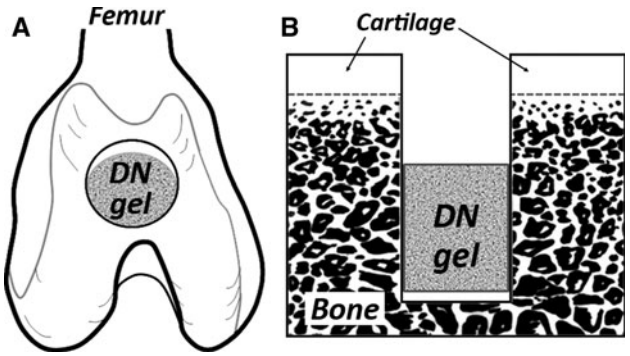

Fig. 1 How to induce cartilage regeneration. **a** We created a cylindrical osteochondral defect having a 4.3-mm diameter in the femoral groove of the patellofemoral joint. Then, we implanted a double network (DN) gel plug into a bottom of the defect. **b** A schematic cross-section of the osteochondral defect into which the plug was implanted. Note that a defect having a few millimeter depth from the cartilage surface remained after surgery

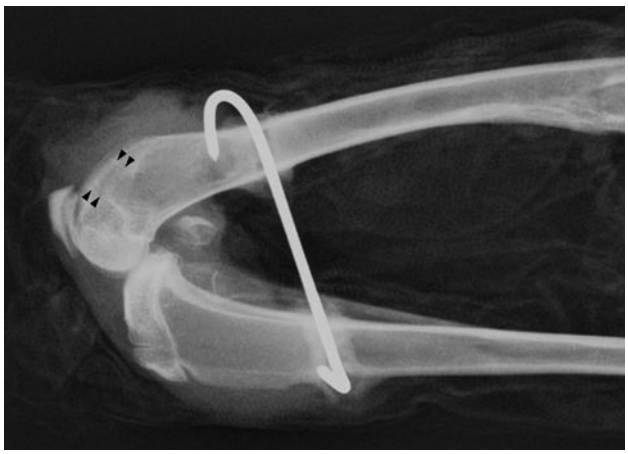

Fig. 2 How to immobilize the knee joint. Using a previously validated method [18], the knee was fixed with a 1.6-mm stainless-steel wire extra-articularly passed through the tibia and femur at approximately 150–160° of flexion. Note that the patella was located slightly distal to the osteochondral defect (shown with black arrows) in the immobilized joint

2.3.2 Histological and immunohistochemical examinations

A distal portion of the resected femur was fixed in a 10% neutral buffered formalin solution for 3 days, decalcified with 50 mM 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. For immunohistochemical evaluations, monoclonal antibody (anti-hCL (II), purified IgG, Fuji Chemical Industries Ltd, Toyama, Japan) was used as primary antibodies. Immunostaining was carried out according to the manufacturer's instructions using the Envision immunostaining system (DAKO Japan, Kyoto, Japan). Finally, the sections were counterstained with hematoxylin.

Histology was evaluated with a scoring system reported by Wayne et al. [19], which was composed of matrix points (hyaline-like cartilage, 4 points; mostly hyaline-like, 3 points; hyaline and fibrocartilage, 2 points; fibrocartilage, 1 point; nonchondrocytic cells, 0 point), cell distribution points (columnar, 3 points; mixed/columnar clusters, 2; clusters, 1; individual cells/disorganized, 0), smoothness points of the surface (smooth/level with normal, 4 points; smooth 3; irregular, 2; clefts, 1; clefts to bone, 0), safranin O stain points (normal, 4; slight reduction, 3; moderate reduction, 2: severe reduction, 1; no stain, 0), safranin O-stained area points (75–100%, 4 points; 50–75%, 3;

25–50%, 2; 0–25%, 1; no stain, 0). Subsequently, the maximum total score was 19 points.

2.3.3 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 (100 ng) was reverse-transcribed into single strand cDNA using PrimeScript® RT reagent Kit (TakaraBio, Ohtsu, Japan). The RT reaction was carried out for 15 min at 37°C and then for 5 s at 85°C. All oligonucleotide primer sets were designed based upon the published mRNA sequence. The expected amplicon lengths ranged from 93 to 189 bp. The sequences of primers used in real time PCR analyses for rabbit regenerative tissues were as follows: type-2 collagen forward GACCATCAATGGCGGCTTC; reverse CACGCTGTTCTTGCAGTGGTAG. Aggrecan forward GC TACGACGCCATCTGCTAC; reverse GTCTGGACCGTG ATGTCCTC. SOX9 forward AACGCCGAGCTCAGCA AGA; reverse TGGTACTTGTAGTCCGGGTGGTC. GAP DH forward CCCTCAATGACCACTTTGTGAA; reverse AGGCCATGTGGACCATGAG. The real time PCR was performed in Thermal Cycler Dice® TP800 (TakaraBio, Ohtsu, Japan) by using SYBR® Premix Ex TaqTM (TakaraBio, Ohtsu, Japan). cDNA template (5 ng) was used for real time PCR in a final volume of 25 microlitter. cDNA was amplified according to the following condition: 95°C for 5 s and 60°C for 30 s at 40 amplification cycles. Fluorescence changes were monitored with SYBR Green after every cycle. A dissociation curve analysis was performed (0.5°C/s increase from 60 to 95°C with continuous fluorescence readings) at the end of 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). Glyceroaldehyde-3-phosphate dehydrogenase (GAPDH) primers were used to normalize samples.

2.4 Statistical analysis

Statistical analysis was performed using commercially available software (StatView 5.0, SAS Institute Inc., Cary, NC, USA). All data were described as the mean and standard deviation values. The paired t test was used to assess the differences in the gross appearance, histology, and total scores between the mobile and the immobile knees to test the above-described hypothesis. The significance limit was set at $P = 0.05$.

3 Results

In gross observation of the joint surface, the defect in the mobile knees was mostly filled with a white opaque tissue

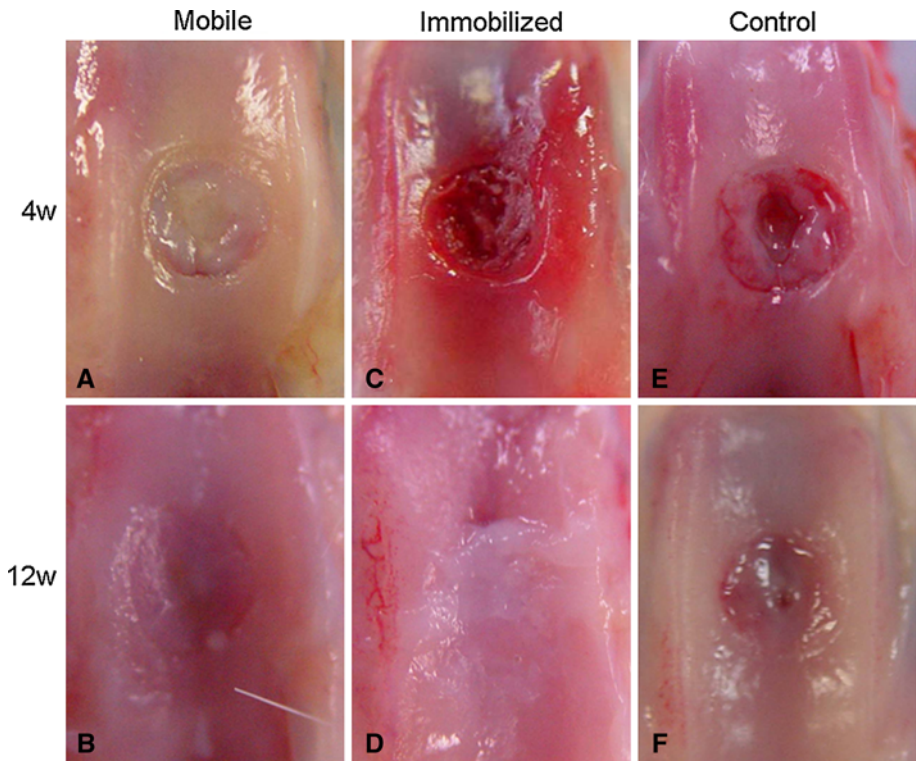

Fig. 3 Gross observations. The defect in the mobile knees was mostly filled with a white opaque tissue at 4 and 12 weeks (**a**, **b**). The defect in the immobilized knees was filled with an irregular surface reddish tissue at 4 weeks (**c**). Fibrous adhesion was observed between the defect site and the opposite cartilage surface at 12 weeks (**d**). In the control knees, the untreated defect was filled with white or reddish, opaque, patchy, stiff tissues (**e**, **f**)

Table 1 Quantitative evaluations of gross appearance and histology at 4 and 12 weeks using the grading scale reported by Wayne et al. [21]

	Gross score	Histology score	Total score
4 weeks			
Mobile	10.2 ± 2.5	8.2 ± 4.8	18.4 ± 7.2
Immobilized	5.6 ± 0.9	2.0 ± 1.2	7.6 ± 2.1
	($P = 0.0093$*)	($P = 0.0327$*)	($P = 0.0201$*)
12 weeks			
Mobile	11.8 ± 2.2	7.6 ± 5.0	19.4 ± 6.8
Immobilized	6.6 ± 2.5	2.8 ± 1.8	9.4 ± 3.5
	($P = 0.0004$*)	($P = 0.0488$*)	($P = 0.0039$*)

Concerning each score (mean ± standard deviation), the immobilized knees (Immobilized) was significantly less than the mobile knees (Mobile)

* P value shows the results of statistical comparison between the mobile and immobilized knees

at 4 and 12 weeks (Fig. 3a, b). The defect in the immobilized knees was insufficiently filled with a reddish stiff tissue at 4 weeks (Fig. 3c), and showed fibrous adhesion between the defect portion and the opposite cartilage surface at 12 weeks (Fig. 3d). The Wayne's gross appearance score of the immobilized knees was significantly lower than that of the mobile knees at 4 and 12 weeks ($P = 0.0093$ and $P = 0.0004$, respectively) (Table 1). The untreated defect in the control knee showed white or reddish, opaque, patchy, stiff tissues, independent of the depth (Fig. 3e, f).

In histological observations, the defect in the mobile knees was filled with a sufficient volume of the proteoglycan-rich tissue stained with Safranin-O at 4 weeks (Fig. 4a). Immunohistochemical staining showed that type-2 collagen was richly expressed in this tissue (Fig. 4b). Beneath this hyaline cartilage tissue, the bone tissue resembling the normal subchondral bone was regenerated (Fig. 4a). In the matrix, fairly large round cells rich in cytoplasm were scattered singly or as an isogenous group at 4 weeks (Fig. 5a). At 12 weeks, the defect in the mobile knees was filled with the same tissue as found at 4 weeks (Figs. 4c, d and 5b), although the amount of the tissue stained with Safranin-O appeared to vary in a few knees. In contrast, the defect in the immobilized knees was insufficiently filled with bone and fibrous tissues at 4 weeks (Figs. 4e and 5c), and the loose fibrous tissue was seen between the regenerated bone tissue and the opposite joint surface at 12 weeks (Fig. 4g). These tissues were not positively stained by Safranin-O or type-2 collagen staining (Fig. 4f, h). In the control knee, the defect was filled with the fibrous and bone tissues at 4 and 12 weeks (Fig. 4i–l).

The Wayne's histology score of the immobilized knees was significantly less than that of the mobile knees at 4 and 12 weeks ($P = 0.0327$ and $P = 0.0488$, respectively) (Table 1). In addition, the Wayne's total score of the immobilized knees was significantly lower than that of the mobile knees at 4 and 12 weeks ($P = 0.0201$ and $P = 0.0039$, respectively) (Table 1). Real time PCR

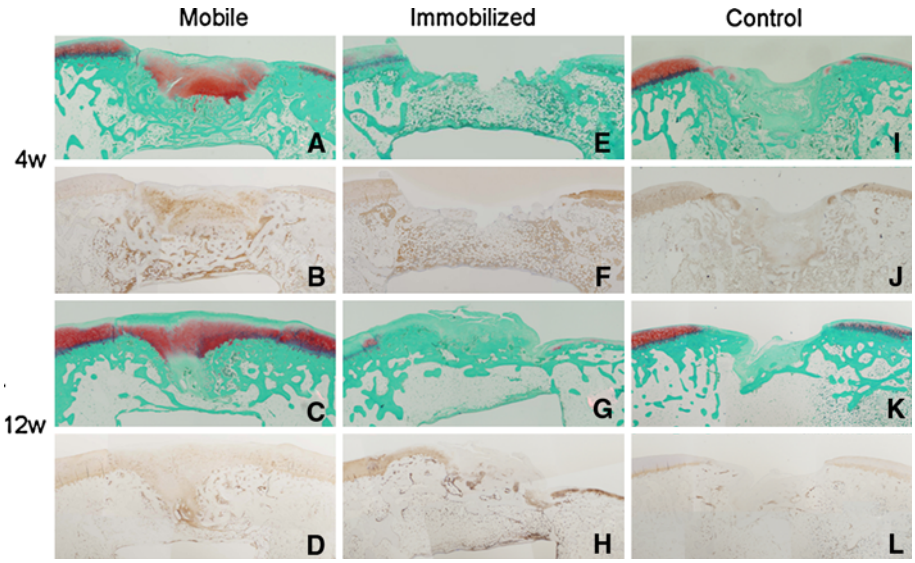

Fig. 4 Histological observations. The defect in the mobile knees was filled with the proteoglycan-rich tissue stained with Safranin-O at 4 weeks (**a**). Type-2 collagen was richly expressed in this tissue (**b**). The proteoglycan-rich tissue was observed at 12 weeks (**c**, **d**). In contrast, all defects in the immobilized knees were insufficiently filled with bone and fibrous tissues at 4 weeks (**e**), and the loose fibrous tissue was formed on the defect surface at 12 weeks (**g**). These tissues were not positively stained by Safranin-O staining or type-2 collagen staining (**f**, **h**). In the control knee, the defect was filled with the fibrous and bone tissues at 4 and 12 weeks (**i–l**)

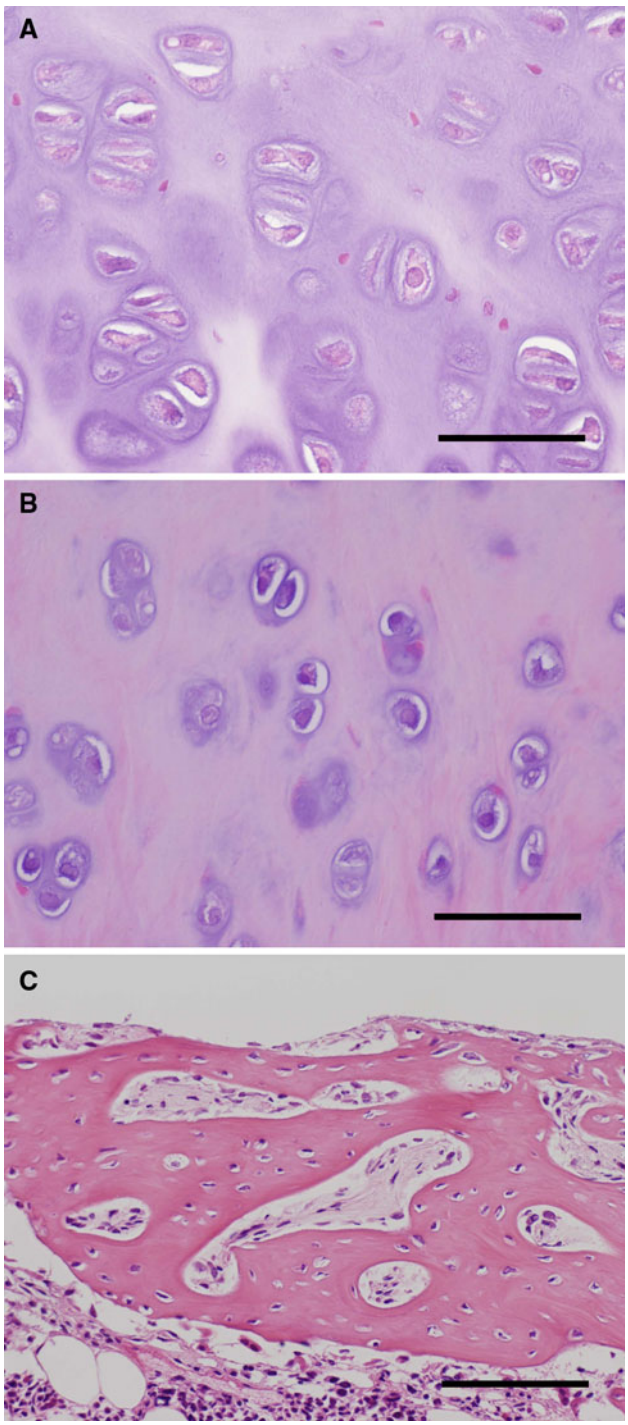

Fig. 5 High magnification histology (hematoxylin-eosin staining) of the tissues regenerated in the defect. Black scale bars show a length of 20.0 micrometers. In the mobile knee, fairly large round cells rich in cytoplasm were scattered singly or as an isogenous group in the matrix at 4 weeks (**a**) and 12 weeks (**b**). In the immobilized knee, bone and fibrous tissues with numerous inflammatory cells were observed at 4 weeks (**c**)

analyses showed great differences (Fig. 6): In the mobile knees, type-2 collagen, Aggrecan, and SOX9 mRNAs were obviously expressed not only at 4 weeks but also at 12 weeks. In the immobilized knees, however, these mRNAs were rarely expressed at each period.

4 Discussion

In the present study, we implanted a PAMPS/PDMAAm DN gel plug at the bottom of a large osteochondral defect so that a 2-mm deep vacant space, which was the most effective depth to induce the cartilage regeneration according to the foregoing study [4], was left in the defect. In the results, it was noted that any cartilage tissues did not regenerate in the immobilized knees at the 4- or 12-week periods. In contrast, in the mobile knees, a sufficient amount of the hyaline cartilage regenerated at 4 and 12 weeks. The quantitative comparisons using the Wayne's scoring system clearly demonstrated that joint immobilization significantly inhibits the spontaneous hyaline cartilage regeneration induced by the PAMPS/PDMAAm DN gel implantation treatment. This fact strongly suggested that joint motion is one of the most significant factors to induce the spontaneous hyaline cartilage regeneration using the PAMPS/PDMAAm DN gel.

Joint immobilization can leave the joint cartilage in an unphysiological mechanical environment where a static compression force is continuously loaded on the cartilage surface. It is well known that functions of chondrocytes in the joint cartilage are extremely affected by mechanical environments. Previous in vivo studies have shown that immobilization reduces the cartilage thickness [20, 21] and changes cartilage metabolism, resulting in a decrease of proteoglycan synthesis [21–24]. Previous ex vivo studies have reported that glucosaminoglycan synthesis in chondrocytes is significantly inhibited in the mechanical condition in which a static compression force is continuously loaded [8, 25, 26]. In the present study, we found that relative expression level of type-2 collagen, Aggrecan, and SOX9 mRNAs in condrocytes in the regenerated tissues was rarely found in the immobilized knees at 4 or 12 weeks. Therefore, we speculate that the unphysiological mechanical condition created by joint immobilization may inhibit chondrogenic differentiation of stem cells that generated in the osteochondral defect or may provide detrimental effects to the metabolism of the chondrocytes differentiated from the stem cells.

Concerning the positive effect of joint motion or repetitive compression loading on chondrocyte function and metabolism, many studies have been recently reported as introduced in the Introduction section of this paper [8–14]. Therefore, we believe that the repetitive compression loading on differentiated chondrocytes is one of the significant factors to induce the in vivo spontaneous hyaline cartilage regeneration phenomenon by means of

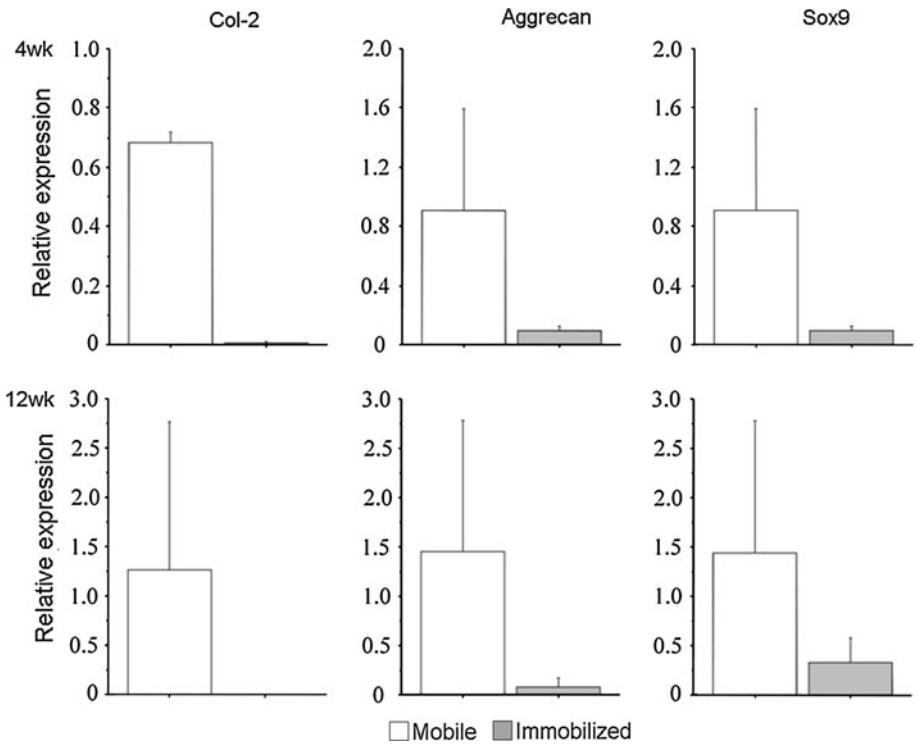

Fig. 6 Real time PCR analysis. Relative expression level of type-2 collagen, Aggrecan, and SOX9 mRNAs in the regenerated tissues was obviously less in the immobilized knees than in the mobile knees not only at 4 weeks but also at 12 weeks

implanting the PAMPS/PDMAAm DN gel. In addition, we have also paid close attention to the effect of the mechanical microenvironment created by the DN gel implantation on chondrogenic deferentiation of the stem cells. For example, in autologous chondrocyte transplantation, quality of the tissue located just beneath the transplanted cells significantly affects quality of the regenerated cartilage [27–29]. It is also known that mechanical microenvironment significantly affects cartilage differentiation of bone-marrow derived stem cells [13, 30]. Recently, Engler et al. [31] reported that elasticity of the material on which cultured cells attach directs stem cell differentiation. For example, elastic materials induce differentiation to the cartilage tissue, and stiff materials induce differentiation to the bone tissue. The PAMPS/PDMAAm DN gel is an elastic, having the elastic modulus of 0.20 MPa [16, 17]. Thus, we speculate that the mechanical environment generated by a combination of the above-discussed physiological joint motion and the elastic DN gel located at the bottom of the defect may be the most significant biomechanical factor in the in vivo spontaneous cartilage regeneration.

There are some limitations in this study. The first limitation is that we did not perform long-term observation of the regenerated cartilage. However, the purpose of the present study was to clarify the effect of joint immobilization on the hyaline cartilage regeneration effect induced by the PAMPS/PDMAAm DN gel implantation treatment, and the results in our short-term observation clearly showed the effect of joint immobilization. Therefore, we can say that we could achieve the study purpose. The second limitation is that we did not examine the properties of the hydrogel plug implanted in the bone tissue after implantation. In our previous study [16], 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 4 weeks after implantation. The third limitation is that we did not perform biomechanical evaluations of the regenerated cartilage. However, we believe that the biological evaluations are the most essential to evaluate the cartilage regeneration, and the most fundamental to demonstrate the effect of joint immobilization on the hyaline cartilage regeneration effect induced by the PAMPS/PDMAAm DN gel implantation treatment. The fourth limitation is that we could not completely clarify the mechanism of the in vivo cartilage tissue induction with the DN gel. Further in vitro and in vivo studies should be made to clarify the mechanisms in the future. However, this limitation did not affect the results or the conclusion observed in the present study.

As for clinical relevance, this study suggested that it is one of the significant factors to allow free joint motion after surgery in order to lead the spontaneous articular cartilage regeneration treatment to success. In addition, the present study first reported that the hyaline cartilage tissue that regenerated in an osteochondral defect at 4 weeks by

means of the above-described treatment with the DN gel can continue to exist even at 12 weeks after surgery. The results have prompted us to develop a potential innovative strategy to repair an osteochondral defect in the field of joint surgery, namely, induction of the spontaneous cartilage regeneration in a vacant defect without any cultured cells or scaffolds. We should note that this strategy is completely different in the concept from the currently prevalent strategies that completely fill the defected space with the tissue-engineered cartilage tissue, cell-seeded scaffold material implantation, or acellular polymer scaffolds with signaling molecules [19, 32–34]. Concerning management of autologous cells and mammalian-derived molecules, various realistic problems including serious disease transmission, donor site morbidity, 2 times of surgeries, a long period until weight bearing, an enormous amount of money to establish a therapeutic system, and etc., have recently been pointed out [4]. We believe that the spontaneous cartilage regeneration strategy has great potential to solve almost all of the above-described current problems. Therefore, the spontaneous regeneration strategy should be studied as a realistic research focus in greater detail in the near future. However, we have not completed to establish the clinical safety of the PAMPS/PDMAAm DN gel as an implant, although any harmful effects due to the PAMPS/PDMAAm DN gel were not detected at 6 weeks in our implantation tests into the muscle and subcutaneous tissue [35]. Further studies are needed to establish the safety of this hydrogel as an implant in the near future.

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Conflict of interest The authors declared that they had no conflicts of interests in their authorship and publication of the contribution.

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