Early tissue formation on whole-area osteochondral defect of rabbit patella by covering with fibroin sponge

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Title Page

Title

Early tissue formation on whole-area osteochondral defect of rabbit patella by covering with fibroin sponge

Running Title

Cartilage repair with fibroin-sponge covering

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ABSTRACT

Large osteochondral defects have been difficult to repair via tissue engineering treatments due to the lack of a sufficient number of source cells for repairing the defect and to the severe mechanical stresses affecting the replacement tissue. In the present study, whole-area osteochondral defects of rabbit patella were covered and wrapped with a fibroin sponge containing chondrocytes, with or without Green Fluorescent Protein (GFP) transgenic marking, on the surface facing the osteochondral defect. Five out of eight osteochondral defects that were covered with the chondrocyte-seeded fibroin sponges showed hyaline cartilage-like repair containing no fibroin fragments at 6 weeks after surgery. The repaired tissue showed a layer formation, which showed intensive safranin-O and toluidine blue staining, and which showed positive type II collagen immunostaining. The average surface coverage of the repaired cartilage was 53%. On average, 48% of the cells in the repaired tissue were derived from GFP transgenic chondrocytes, which had been seeded in the fibroin sponge. The fibroin-sponge covering had the potential to allow the early repair of large osteochondral defects.

Key words: Fibroin sponge; Cell delivery; Cartilage repair; Osteochondral defect; Osteoarthritis
INTRODUCTION

In recent years, cartilage repairing treatments, such as autologous chondrocyte implantation (7,8,38,55), autologous osteochondral grafts (mosaic plasty) (14,30,31) and bone marrow stimulation techniques, which include microfracture (5,47), multiple perforations (42) and abrasion (20), have been evaluated as treatment methods for osteochondral defects. However, various problems, including osteoarthritic changes (29,33,46,47) and avulsion to the laminae of grafted cartilage (14), have raised questions about the comparative superiority of these methods (4,18,19,23,24). In addition, large osteochondral defects have been difficult to repair via tissue engineering treatments due to the lack of a sufficient number of source cells for repairing the defect and to the severe mechanical stresses that affect the replacement tissue (27). The compressive forces that are applied to the patello-femoral joint are approximately 1.2 times the body weight when walking and approximately 5.7 times the body weight when descending stairs (6).

In the present study, we attempted to protect osteochondral defects from severe mechanical stresses with the aid of a "fibroin sponge covering." The fibroin sponge was also used to deliver chondrocytes to the defects. Fibroin sponges with chondrocytes
were used to cover and wrap the whole repair area of a large osteochondral defect,
which spanned the whole articular surface. Our previous report (21,40) demonstrated
that when the pore size of the sponge was small (40-80µm) the majority of
chondrocytes remained within 0-200 µm from the sponge surface, forming
aggregates. In the present study, a pore diameter of 40-80 µm was adopted,
expecting that the cell aggregates would be delivered to the osteochondral defect.
Fibroin purified from silkworm cocoons has previously been used to make
sponge materials that have been used as tissue engineering scaffolds
(21,22,37,40,50,58). Altman et al. showed that pure silk fibroin was a biocompatible
material (2). Minoura et al. showed that fibroin has superior mechanical strength for
biomedical applications (34). Furthermore, Nazarov et al. showed that porous 3-D
fibroin materials have the proper mechanical properties for use in tissue engineering
(37).
The mechanical properties of fibroin sponges can be adjusted (21,50), which can
be advantageous for protecting repairing tissue from severe mechanical stress. In
addition, our previous reports (3,21,36,40,44) suggested that fibroin sponge would be a
functional scaffold for cartilage regeneration, in which chondrocytes tend to form
aggregates and cartilage tissue with a layered structure without losing their phenotypic
character or differentiation ability. It has also been reported to be possible to modify the
structure of fibroin sponge through the transgenic modification of silkworms (16,25,51).

Purpose The purpose of the present study is to examine our hypotheses
hypothesis that the cell aggregates located near the fibroin-sponge surface are would be
delivered to the osteochondral defect and that the sponge would protect the
repairing tissue from severe mechanical stress to create a better repair environment.

We also investigated whether it is necessary to include the inclusion of chondrocytes in the fibroin sponge was
necessary to achieve cartilage repair and whether a significant number of the cells doing
the repair involved in the repair have come originated from the fibroin sponge, are also
observed using green fluorescent protein (GFP) transgenic rabbits.

MATERIALS AND METHODS

Animals and Materials

The experimental animals (Japanese white rabbits and cytomegalovirus
Immediate early enhancer/beta-actin promoter (CAG)/enhanced green fluorescent protein (EGFP) transgenic Japanese white rabbits were provided by Kitayama Labes Co., Ltd. (Nagano, Japan) and Phoenix Bio CO., Ltd. (Hiroshima, Japan). The rabbits had already been used to successfully establish a transgenic rabbit line that ubiquitously expresses an EGFP biomarker gene (49). Twelve-week-old male non-transgenic Japanese white rabbits (body weight: 2.4-2.5 kg) were used as recipients. Four-week-old male non-transgenic Japanese white rabbits (body weight: 450-550 g) and four-week-old male CAG/EGFP transgenic Japanese white rabbits (body weight: 450-550 g) were used to obtain donor cells.

The protocols of the present study were approved by the Kyoto University Committee for Animal Experimentation (G-58-2; 12/02/2009). Animal experiments were carried out in accordance with the National Institute of Health guidelines for the care and use of laboratory animals.

Chemicals were obtained from Nacalai Tesque, Inc. (Kyoto, Japan) unless stated otherwise.

Preparation of Fibroin Sponges
The fibroin sponge was designed and manufactured in the same manner as previously reported (21,50). Briefly, silk fibroin 5% aqueous solutions were prepared from *Bombyx mori* silkworm cocoons (National Institute of Agrobiological Sciences, Ibaraki, Japan) by dissolving them in a 9M lithium bromide solution and then dialyzing the solution against pure water. After the addition of 1% (vol) dimethyl sulfoxide (DMSO) as the sponge making reagent to the fibroin solution, each silk fibroin solution was frozen at -20°C (Programmable Low temp. Precision Bath; EYELA., Japan) for 14 hours and thawed at room temperature to form a sponge structure. After the removal of DMSO by immersing the sponges in pure water, the sponges were immersed in phosphate-buffered saline (PBS) and autoclaved for sterilization. The fibroin sponge formed by this typical process has a thin membrane structure on the pore structure on the surfaces of both sides. The membrane was removed from one side of the sponges in the present study. Thus the sponges had a porous internal structure and a sheet-like structure on one surface (Fig. 1). The diameters of the pores within the fibroin sponges were measured by scanning electron microscopy (SEM) and were found to range from 40-80 µm. The thickness of the sheet-like surface layer was found to be approximately 50 µm.

The sponges were cut into discs measuring 8 mm in diameter and 3 mm in...
thickness. The typical mechanical properties of the fibroin sponges prepared by the above process included a tensile strength of 0.2-0.5 MPa and a compressive modulus of 0.03-0.05 MPa (50).

Cell Culture in Fibroin Sponges

In vitro culturing was performed using the same types of materials as reported previously (3,21,36,40,44). Briefly, as outlined in Figure 2, articular cartilage slices were aseptically harvested from the proximal humerus, distal femur, and proximal tibia of four-week-old male Japanese white rabbits or from four-week-old male CAG/EGFP transgenic Japanese white rabbits (GFP transgenic rabbits). After the removal of all adherent connective tissue, the cartilage tissue was cut into 1 mm$^3$ segments, and chondrocytes were isolated by digesting small segments of the cartilage with 0.25% trypsin-EDTA for 30 min in a temperature-controlled bath at 37$^\circ$C. After the samples were rinsed twice with PBS and centrifuged at 180 x g for 5 min, the cartilage was enzymatically digested with 0.25% collagenase (CLS-2, Worthington Biochemical Co., USA) for six hours at 37$^\circ$C. The solution was then filtered through a cell strainer (BD Falcon, USA) and washed twice with PBS to obtain a single-cell suspension.
The resulting cells were cultured in T-flasks (IWAKI Glass Co., Ltd, Japan) with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotics at 37°C in a humidified atmosphere of 5% CO₂. The medium was changed every 2 days. The first passage cells were used for experiments. There was an approximately 10-fold increase in the number of cells during the one-week period before cell seeding. Aliquots of 90 μl containing 1.5 × 10⁶ cells were seeded onto the top (porous side) of the fibroin sponges in 12-well culture plates (IWAKI Glass Co., Ltd, Japan). The plates were kept in an incubator at 37°C for one hour to allow the cells to infiltrate the sponge. As above, the medium (DMEM, containing 10% FBS, 1% antibiotics and 1% ascorbic acid) was changed every two days. All cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ for two weeks (3,21,36).

Hyaline cartilage-like tissue with a thickness of 100-200 μm, which was measured in a single sagittal plane which passed through the center of the fibroin sponge, was formed on the porous surfaces of the fibroin sponges after two weeks of culturing. These were used as donor tissues. 

Surgical Procedure
Four knees from two 12-week-old male Japanese white rabbits (2.4-2.5 kg) were used. The rabbits were anesthetized by an intravenous injection of thiopental sodium (Mitsubishi Tanabe Pharma Co., Osaka, Japan) (30 mg/kg). Skin incisions were made in the midline of the patella following the medial parapatellar approach to the inside of the knee joint. The patella was then turned over and the articular surface was exposed. The whole area of the articular surface of the patella was shaved at a constant depth of 1.0 mm in the following manner. First, 10 1.0 mm deep holes were made on the articular surface of the patella using a drill (Minimo; MINITOR CO., LTD., Tokyo, Japan) fitted with a rubber cover, saving the top 1.0 mm. Note that during drilling, bleeding with fat was seen from the hole. Following drilling, the whole area of the articular surface was shaved until all holes were worn away, resulting in a continuous 1.0 mm deep osteochondral defect over the whole articular surface of the patella. The average thickness for normal patella articular cartilage in a 12-week-old rabbit is approximately 0.5 mm. Therefore, the 1.0 mm shaving depth of this model was sufficient for creating an osteochondral defect with no cartilage or subchondral plate. The length of the major
axis of the articular surface of the patella is approximately 10 mm, while that of the
minor axis is approximately 7 mm. The operation was completed after rinsing the site
with 0.9% saline solution following the suturing of the articular capsule, subcutaneous
layer and skin.

Antibiotics (Baytril (enrofloxacin); Bayer Yakuhin, Ltd., Osaka, Japan) (5 mg/kg
i.m./day) were administered daily for five days after the operation. The rabbits were
allowed to move freely in their cages, and exhibited no complications, including
infection or weight loss.

Group 2 (System Control with Sponge-only) and Group 3 (Sample with a Sponge Plus
Cells)

Eight right knees from eight 12-week-old male Japanese white rabbits (2.4-
2.5kg) were used for Group 2, and eight left knees from the same rabbits were used for
Group 3 (four knees for Group 3-a and four knees for Group 3-b) in the recipient
animals. Osteochondral defects with a 1.0 mm constant depth were made over the
whole-area of the articular surface of all patellae in the same manner as described for
Group 1.
When implanting the fibroin sponge, the porous surface was placed facing the osteochondral defect in Group 2, and the hyaline cartilage-like tissue (formed of chondrocytes from non-transgenic rabbits in Group 3-a and from GFP-positive chondrocytes in Group 3-b) on the fibroin sponges was placed facing the osteochondral defect in Group 3. In both groups, the sheet-like surfaces were directed toward the joint cavity, and the whole-area of the osteochondral defect was covered and wrapped with the fibroin sponge. The sheet-like surface layer was sutured with 6-0 nylon (ETHICON, Tokyo, Japan) and fixed to the surrounding soft tissue (Fig. 3). None of the animals exhibited any complications, including infection or weight loss.

Histological Examinations

Six weeks after the surgery, the rabbits were euthanatized by an injected overdose of thiopental sodium and the knee joints were harvested in all groups. After macroscopic assessment, the specimens were fixed in 20% neutral-buffered formalin. Tissues were then dehydrated through a series of graded alcohols, following decalcification with 10% EDTA in distilled water for four weeks. Each
specimen was embedded in paraffin and was sliced into sections of approximately 5 µm thick along the long axis of the patella, in the sagittal plane. Histological examinations were performed after staining.

Staining was performed using hematoxylin and eosin, 0.05% toluidine blue (pH = 7.0) and 0.1% safranin-O using the standard methods. For type I collagen immunostaining, an anti-human collagen I mouse monoclonal antibody clone (1:50; F-56, DAIICHI FINE CHEMICAL Co., Ltd., Toyama, Japan) was used as the primary antibody, and a histofine-labeled POM anti-mouse immunoglobulin goat antibody (414321, NICHIREI BIOSCIENCES INC., Tokyo, Japan) was used as the secondary antibody. Finally, staining was performed with DAB (3,3’-diaminobenzidine tetrahydrochloride) (NICHIREI BIOSCIENCES INC., Tokyo, Japan).

For the type II collagen immunostaining, an anti-human collagen II mouse monoclonal antibody clone (1:50; F-57, DAIICHI FINE CHEMICAL Co., Ltd., Toyama, Japan) was used as the primary antibody, and a histofine-labeled POM anti-mouse immunoglobulin goat antibody (414321, NICHIREI BIOSCIENCES INC., Tokyo, Japan) was used as the secondary antibody. Finally, staining was performed with DAB (NICHIREI BIOSCIENCES INC., Tokyo, Japan).

For GFP (Green Fluorescent Protein) immunostaining, an anti-GFP chicken
antibody IgY fraction (1:50; GFP-1020, AVES LABS, INC., Oregon, USA) was used as the primary antibody, and horseradish peroxidase-labeled goat anti-chicken IgY (heavy and light chains) (affinity-purified) (H-1004, AVES LABS, INC., Oregon, USA) was used as the secondary antibody. Finally, staining was performed with DAB (NICHIREI BIOSCIENCES INC., Tokyo, Japan).

The "Surface Coverage" of the Repaired Cartilage

The percentage of the patellar surface area covered by the repaired cartilage observed along the long axis of the patella in a single plane was evaluated as one of the indicators relating to the whole surface coverage.

This value, which was referred to as the "surface coverage", was calculated as follows. First, a specimen slide was prepared by removing the tissue along the long axis of the patella. Second, the length of the articular surface along the long axis of the slide was defined as A, and the total length of the repaired cartilage along the long axis was defined as B. Finally, the value of B/A (×100%) was calculated and defined as the "surface coverage" of the repaired cartilage.

In the present study, the surface coverage was measured as a reference to assess
the tissue repair. This index shows the ratio of the repaired tissue to the total articular surface area, that is, the original osteochondral defect along the long axis of the rabbit patella on the sagittal slice, which has an oval articular surface. This index was calculated in the same way in all of the specimens. The slice passed through the center of the patella's articular surface (the osteochondral defect) along the long axis. Therefore, the slice used for the surface coverage evaluation was the largest slice of the patella articular surface.

**Histological Evaluation of the Repaired Cartilage**

The characteristics of the repaired cartilage as a whole were evaluated using the ICRS scale (28). The repaired cartilage on the specimen slide was divided into segments of 1.0 mm in length along the long axis. Each segment was scored for each of the six features on the ICRS scale. Then the scores for each category from all of the separate segments were averaged and defined as the average scores for the sample for each ICRS category. The average of these scores were then calculated and defined as the "total average score" for a particular category.
RESULTS

Histological Examinations

The results from the histological examinations are shown in Table 1.

Representative macroscopic images of Group 2 and 3 are shown in Figure 4.

Representative histological images of Group 2 and 3 are shown in Figure 5A and 5B. As shown in Table 1, none of the specimens from Group 1 and 2 resulted in any cartilage repair on the whole-area of the osteochondral defect of the patella. On the other hand, cartilage repair was achieved on the osteochondral defect of the patella in five out of the eight specimens from Group 3, which exhibited hyaline cartilage-like tissue. Intensive staining was achieved with safranin-O, and toluidine blue. An immunohistochemical analysis was performed, with the specimens staining negatively and positively for type I and type II collagen, respectively. The repaired tissues contained no fibroin fragments.

Furthermore, the thickness of the repaired cartilage was approximately 200 to 500 µm, whereas the thickness of normal, undamaged articular patellar cartilage in 12-week-old male Japanese white rabbits was approximately 500 µm. However, no obvious bone
regeneration or transition zone between the bone and cartilage were observed. In each group, all of the fibroin-sponges migrated away from the articular surfaces of the patellae and were broken into small pieces within the joint (Fig. 5B). No joint fluid collection, synovial hyperplasia, or signs of inflammation were observed macroscopically. Furthermore, a macroscopic examination failed to reveal any differences in inflammatory response or in the synovial hyperplasia between the GFP-positive chondrocyte-seeded group (Group 3-b) and the GFP-negative chondrocyte-seeded group (Group 3-a).

With regards to the specimens that did not show any cartilage repair, a histological examination at six weeks after surgery revealed that the trochlea, which faced the osteochondral defect of the patella, showed severe osteoarthritis. In contrast, the trochlea maintained a smooth articular surface in the specimens which showed cartilage repair (Fig. 5C).

**The Surface Coverage of the Repaired Cartilage**

In Group 3-a, three of four specimens achieved 100% surface coverage. In contrast, the surface coverage values of the two Group 3-b specimens that exhibited...
cartilage repair were 49.2% and 70.1% (Table 2).

The Percentage of GFP Stained Cells in the Repaired Tissue

Some of the cells in the repaired hyaline cartilage-like tissue obtained in Group 3-b showed intensive immunostaining for GFP, as shown in Figure 6.

The percentage of GFP-positive cells amongst the total number of cells in the repaired hyaline cartilage-like tissue is shown in Figure 7. The percentages of GFP-stained cells in the two repaired tissues were 34% and 61%.

Histological Evaluation of the Repaired Cartilage

The total average scores (based on the ICRS scale) for surface, matrix, cell distribution, cell population viability, subchondral bone, and cartilage mineralization categories were 1.9, 2.6, 2.0, 3.0, 2.2 and 3.0, respectively (Table 3 and Figure 8).

DISCUSSION
In the present study, an osteochondral defect was created over the whole articular surface and no cartilage was left on the articular surface. From the viewpoint of mechanical stress, this condition was more severe for cartilage repair than that in the case in which normal cartilage remained around the defect (cartilage-hole) for cartilage repair from the aspect of mechanical stress compared to the case in which normal cartilage was left around the defect (cartilage-hole). In the case with remaining cartilage left, the repaired tissue was formed in the cartilage-hole that was made on the articular surface, and mechanical stress over the repairing area by from the opposite side (facing side) of the articular surface was reduced. In contrast, in the present study, the severe mechanical stress was not dispersed in the present study and was directly loaded on the repairing area. In Group 1, it was confirmed that an osteochondral defect made over the whole-area of the articular surface of a rabbit patella with a diameter of approximately 10 mm in the long axis and 7 mm in the short axis could not be repaired by itself, possibly due to the lack of a sufficient number of cells and due to their insufficient differentiation (17, 26, 39, 45). Another possible reason is that severe mechanical stress damaged or prevented cartilage repair (27).

In Groups 2 and 3, the possibility of cartilage repair using a fibroin sponge...
Based system in response to a large osteochondral defect was examined. Cartilage repair with layer formation was observed in Group 3, but not in Group 2. In Group 3-b, the repaired cartilage was also observed to contain GFP-positive chondrocytes. These results suggest that some of the cells near the fibroin-sponge surface are delivered to the osteochondral defect and that some of the sponge protects the repairing tissue from severe mechanical stress to create an environment for better repair conditions for repair. It is possible that other cells (GFP-negative chondrocytes), which made a significant contribution to cartilage repair, were chondroprogenitor cells from the bone marrow of the patella (17,45,53,54,56,57). It is considered that there were no other potential origins of the cells, because the whole-area osteochondral defect had been covered and wrapped with the fibroin sponge and was tightly attached to the cartilage tissue on the fibroin sponge during the early stage of cartilage repair. Therefore, in Group 3, it could be considered that some cartilage repair was achieved by both the delivery of cells from the fibroin sponges and by the cells from the bone marrow.

Although some cartilage repair with layer formation was observed by covering the area with cell-a cell-containing fibroin sponge, the repair rate was unstable, possibly because the mechanical conditions were more severe than those in compared to other animal studies. Without any surrounding cartilage, the whole...
osteochondral defect, without surrounding cartilage at all, causes severe several
mechanical conditions and the repaired tissue may be directly loaded by with mechanical stress from the opposite facing side.

The mechanism underlying the cell delivery function has not yet been clarified, however, another advantage of the fibroin sponge is that it is mechanically adjustable (21,50), which allows it to be tailored to protect the process of cartilage repair. The porous structure of the fibroin sponge is also advantageous, not only for holding cells, but also for protecting the repairing tissue with its elasticity and durability (21,50). In order to prepare for the more severe mechanical conditions that are encountered in clinical cases, it is necessary to improve the additional mechanical design of designing for the sponge is required.

With regard to the degradation properties of the fibroin sponge, Wang et al. showed that the in vivo degradation of the silk fibroin scaffolds depended on the original preparation method and their structural characteristics (59). The fibroin materials have a slower rate of biodegradation than typical scaffolds constructed from PLA or collagen (41). It has been reported that an artificial vein constructed by silk thread took approximately one year to biodegrade in rats (10). In the present study, the breaking up of the fibroin sponge might have been mainly due to the mechanical
stresses caused by the motion of knee joint throughout the six-week period. It is possible that the fibroin sponge had already been broken before the repair process was sufficiently achieved in the 3 of the 8 Group 3 specimens which did not show cartilage repair.

In the cases in which cartilage repair was not achieved, the damage on trochlea articular surface was possibly caused by the friction on the opposite uncovered osteochondral defect of the patella.

An inflammatory response was not observed in the macroscopic examinations of the present study. Silk protein had been used for many years for silk thread, and the older types of silk thread that were used as surgical sutures had the potential to cause inflammatory reactions, because the old silk sutures were used without sufficient degumming treatment (to remove the sericin protein). An in vitro evaluation of silk fibroin by Santin et al. showed that highly purified silk fibroin does not lead to the upregulation of the inflammatory response (43). Several reports have also shown highly purified silk fibroin to be less toxic and associated with a low incidence of inflammation (2,32,34,40,41,43,58). In addition, sericin which can cause foreign-body reactions, was removed from the silk protein (43).

Some researchers have reported studies using the allogeneic transplantation of
chondrocytes (11,44). In the present study, the experiments using allogeneic
transplantation in rabbits were successful. It is possible that the donor cartilage tissues
were protected by the surrounding matrix, which is avascular and has low antigenicity,
from immunological attack by the recipient's immune system.

With the eventual goal of the clinical application in humans of this strategy, it
still remains the concerns in the long-term viability and reliability of cartilage
regeneration remain matters of concern. It will be necessary to perform longer
term animal testing in the next study. Furthermore, it will be necessary to examine
the possibility of autogeneic transplantation and the potential of using non-chondrocytic
cells from bone marrow (15,17,52,53,54,56,57) or blood (1,35,48). Gene transfer could
be used to express a functional protein in the fibroin sponge that would allow for the
induction of cell differentiation, which would have a positive effect on cartilage repair
(16,25,51).

The present study investigated the repair of large osteochondral defects.
However, large chondral defects, which do not penetrate the subchondral bone could
also be treated using this system with drilling or abrasion; making the interface down to
the bone marrow space and covering it with a fibroin sponge.

The results of the present study demonstrate that the fibroin sponge-covering
treatment showed early repair of a whole-area osteochondral defect of the patella and showed the potential of the method for repairing large osteochondral or chondral defects of weight-bearing joints.
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Conflicts of Interest:

The present study was funded by Integrated Research Project for Agri-health Translational Project (Ministry of Agriculture, Forestry and Fisheries, Japan).

The authors declare no conflicts of interest in association with the present study.
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Figure 1. The fibroin sponge was formed using fibroin protein that was purified from silkworm cocoons. The sponges in the present study included a porous internal structure and a sheet-like structure on one surface, which facilitated their easy suturing during surgery. Scale bar (SEM image) = 100 µm.

Figure 2. Articular cartilage slices were taken from the proximal humerus, the distal femur, and the proximal tibia of 4-week-old male Japanese white rabbits. Aliquots of 90 µl containing $1.5 \times 10^6$ cells were seeded onto the top (porous side) of the fibroin sponge. Hyaline cartilage-like tissue (100-200 µm thickness) was formed on the porous surfaces of the fibroin sponges after two weeks of culturing. Scale bar = 200 µm.

Figure 3. Cartilage repair using a fibroin sponge-based system was attempted for whole-area osteochondral defects of the rabbit patella. In Group 3, the whole-area osteochondral defect and the hyaline cartilage-like tissue that formed on the fibroin sponge formed a strong bond when covered with the fibroin sponge, and the sheet-like surface layer of the fibroin sponge was sutured with 6-0 nylon and fixed to the
surrounding soft tissue.

Figure 4. Representative macroscopic appearance of the patella articular surface in Groups 2 and 3 are shown. Cartilage repair was achieved in Group 3, with hyaline cartilage-like tissue at six weeks after surgery (arrows). In contrast, cartilage repair was not observed in Group 2 at six weeks after surgery (dotted arrows). Scale bar = 10 mm.

Figure 5. The representative histological appearance of the patella articular surfaces in Groups 2 and 3 are shown. Figure 5B is magnified view of Figure 5A. Figure 5B also shows the histological findings with type I staining. Cartilage repair with hyaline cartilage-like tissue was observed in Group 3 at six weeks after surgery (arrows). No cartilage repair was observed at six weeks after surgery in Group 2 (dotted arrows). Figure 5B also shows broken pieces of the fibroin sponge(*). Figure 5C shows the histology of the trochlea. In the specimen which showed cartilage repair (Group 3), the trochlea maintained a smooth articular surface. On the other hand, in the specimens which did not show any cartilage repair (Group 2), the trochlea, which faced the osteochondral defect of the patella, showed severe osteoarthritis at six weeks after surgery. Scale bar (Figure 5A) = 1.0 mm. Scale bar (Figure 5B) = 250 µm. Scale bar
(Figure 5C) = 250 µm.

Figure 6. Intensive positive staining of the repaired cartilage tissues in Group 3-b was achieved with safranin-O (Figure 6A). Immunostaining for type II collagen was also positive (Figure 6B). Immunostaining for GFP was partially positive (Figure 6C; arrows). Scale bar = 100 µm.

Figure 7. The percentages of GFP-positive cells among the total number of the cells in the repaired hyaline cartilage-like tissues that were obtained in Group 3-b. The percentages of GFP-stained cells contained in the two specimens with repaired tissue were 34% and 61%.

Figure 8. The "total average score" for each feature of the ICRS scale is shown. The scores for the surface, matrix, cell distribution, cell population viability, subchondral bone, and cartilage mineralization categories were 1.9, 2.6, 2.0, 3.0, 2.2 and 3.0, respectively.
Table 1. Histological Examinations

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of specimens which showed cartilage repair / Number of specimens in the group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Control with an</td>
<td>0 / 4</td>
</tr>
<tr>
<td>empty defect)</td>
<td></td>
</tr>
<tr>
<td>Group 2 (System control with</td>
<td>0 / 8</td>
</tr>
<tr>
<td>just a sponge)</td>
<td></td>
</tr>
<tr>
<td>Group 3-a (Sample with a</td>
<td>3 / 4</td>
</tr>
<tr>
<td>sponge plus cells)</td>
<td></td>
</tr>
<tr>
<td>Group 3-b (GFP(+))</td>
<td>2 / 4</td>
</tr>
<tr>
<td>Total</td>
<td>5 / 8</td>
</tr>
</tbody>
</table>

Eiichi Hirakata
Table 2. The “Surface Coverage” of the Repaired Cartilage *1

<table>
<thead>
<tr>
<th>Experimental number</th>
<th>Surface Coverage (%)</th>
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<tbody>
<tr>
<td>No.1</td>
<td>100</td>
</tr>
<tr>
<td>No.2</td>
<td>100</td>
</tr>
<tr>
<td>No.3</td>
<td>100</td>
</tr>
<tr>
<td>No.4</td>
<td>0</td>
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<tr>
<td>Average</td>
<td>75.0</td>
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<tr>
<td>No.5</td>
<td>49.2</td>
</tr>
<tr>
<td>No.6</td>
<td>70.1</td>
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<tr>
<td>No.7</td>
<td>0</td>
</tr>
<tr>
<td>No.8</td>
<td>0</td>
</tr>
<tr>
<td>Average</td>
<td>27.8</td>
</tr>
<tr>
<td>Total average</td>
<td>52.7</td>
</tr>
</tbody>
</table>

*1 The percentage of the patellar surface area covered by the repaired cartilage observed along the long axis of the patella in a single plane; i ) a specimen slide was prepared by removing the tissue along the long axis of the patella, ii ) the length of the articular surface along the long axis of the slide was defined as A, and the total length of the repaired cartilage along the long axis was defined as B, iii ) the value of B/A (×100%) was calculated and defined as the “surface coverage” of the repaired cartilage.
Table 3. Histological Evaluation of the Repaired Cartilage*²

<table>
<thead>
<tr>
<th>Experimental number which showed cartilage repair</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.1</td>
<td>2.4</td>
<td>2.3</td>
<td>1.7</td>
<td>3.0</td>
<td>2.0</td>
<td>3.0</td>
</tr>
<tr>
<td>No.2</td>
<td>2.1</td>
<td>2.9</td>
<td>2.4</td>
<td>3.0</td>
<td>2.4</td>
<td>3.0</td>
</tr>
<tr>
<td>No.3</td>
<td>2.9</td>
<td>2.1</td>
<td>1.9</td>
<td>3.0</td>
<td>2.1</td>
<td>3.0</td>
</tr>
<tr>
<td>No.5</td>
<td>2.0</td>
<td>2.7</td>
<td>1.7</td>
<td>3.0</td>
<td>2.0</td>
<td>3.0</td>
</tr>
<tr>
<td>No.6</td>
<td>0</td>
<td>2.8</td>
<td>2.3</td>
<td>3.0</td>
<td>2.3</td>
<td>3.0</td>
</tr>
<tr>
<td>Total average score</td>
<td>1.9</td>
<td>2.6</td>
<td>2.0</td>
<td>3.0</td>
<td>2.2</td>
<td>3.0</td>
</tr>
</tbody>
</table>

*² i ) The repaired cartilage on the specimen slide was divided into segments of 1.0 mm in length along the long axis, ii ) Each segment was scored for each of the six features on the ICRS scale. Then the scores for each category from all of the separate segments were averaged and defined as the average scores for the sample for each ICRS category, iii ) The average of these scores were then calculated and defined as the "total average score" for a particular category.
The fibroin sponge was formed using fibroin protein that was purified from silkworm cocoons. The sponges in the present study included a porous internal structure and a sheet-like structure on one surface, which facilitated their easy suturing during surgery. Scale bar (SEM image) = 100 µm.

Figure 1.
Articular cartilage slices were taken from the proximal humerus, the distal femur, and the proximal tibia of 4-week-old male Japanese white rabbits. Aliquots of 90 µl containing $1.5 \times 10^6$ cells were seeded onto the top (porous side) of the fibroin sponge. Hyaline cartilage-like tissue (100-200 µm thickness) was formed on the porous surfaces of the fibroin sponges after two weeks of culturing. Scale bar = 200 µm.

107x69mm (300 x 300 DPI)
Cartilage repair using a fibroin sponge-based system was attempted for whole-area osteochondral defects of the rabbit patella. In Group 3, the whole-area osteochondral defect and the hyaline cartilage-like tissue that formed on the fibroin sponge formed a strong bond when covered with the fibroin sponge, and the sheet-like surface layer of the fibroin sponge was sutured with 6-0 nylon and fixed to the surrounding soft tissue.
Representative macroscopic appearance of the patella articular surface in Groups 2 and 3 are shown. Cartilage repair was achieved in Group 3, with hyaline cartilage-like tissue at six weeks after surgery (arrows). In contrast, cartilage repair was not observed in Group 2 at six weeks after surgery (dotted arrows). Scale bar = 10 mm.

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Intensive positive staining of the repaired cartilage tissues in Group 3-b was achieved with safranin-O (Figure 6A). Immunostaining for type II collagen was also positive (Figure 6B). Immunostaining for GFP was partially positive (Figure 6C; arrows). Scale bar = 100 µm.

78x27mm (300 x 300 DPI)
The percentages of GFP-positive cells among the total number of the cells in the repaired hyaline cartilage-like tissues that were obtained in Group 3-b. The percentages of GFP-stained cells contained in the two specimens with repaired tissue were 34% and 61%.

Figure 7.

The percentages of GFP-positive cells among the total number of the cells in the repaired hyaline cartilage-like tissues that were obtained in Group 3-b. The percentages of GFP-stained cells contained in the two specimens with repaired tissue were 34% and 61%.

63x61mm (600 x 600 DPI)
The "total average score" for each feature of the ICRS scale is shown. The scores for the surface, matrix, cell distribution, cell population viability, subchondral bone, and cartilage mineralization categories were 1.9, 2.6, 2.0, 3.0, 2.2 and 3.0, respectively.

61x53mm (600 x 600 DPI)