Cartilage repair in transplanted scaffold-free chondrocyte sheets using a minipig model

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ABSTRACT

Lacking a blood supply and having a low cellular density, articular cartilage has a minimal ability for self-repair. Therefore, wide-ranging cartilage damage rarely resolves spontaneously. Cartilage damage is typically treated by chondrocyte transplantation, mosaicplasty or microfracture. Recent advances in tissue engineering have prompted research on techniques to repair articular cartilage damage using a variety of transplanted cells. We studied the repair and regeneration of cartilage damage using layered chondrocyte sheets prepared on a temperature-responsive culture dish. We previously reported achieving robust tissue repair when covering only the surface layer with layered chondrocyte sheets when researching partial-thickness defects in the articular cartilage of domestic rabbits. The present study was an experiment on the repair and regeneration of articular cartilage in a minipig model of full-thickness defects. Good safranin-O staining and integration with surrounding tissues was achieved in animals transplanted with layered chondrocyte sheets. However, tissue having poor safranin-O staining—not noted in the domestic rabbit experiments—was identified in some of the animals, and the subchondral bone was poorly repaired in these. Thus, although layered chondrocyte sheets facilitate articular cartilage repair, further investigations into appropriate animal models and culture and transplant conditions are required.

1. Introduction

Articular cartilage is hyaline cartilage featuring an extracellular matrix (ECM) consisting of an intricate collagen network and proteoglycans, and is highly resistant to mechanical loads. However, lacking a blood supply and having a low cellular density, articular cartilage has a minimal potential for self-repair. Therefore, wide-ranging cartilage damage rarely resolves spontaneously. Left untreated, cartilage damage in a load-bearing area causes secondary degeneration of the surrounding cartilage and ultimately exerts negative effects on routine activities. Numerous treatments have been developed to repair articular cartilage damage, typical examples being chondrocyte transplantation [1], mosaicplasty [2], and microfracture [3]. These treatments have produced satisfactory outcomes. However, the literature also documents treatment failures [4–8].

Autologous chondrocyte implantation (ACI), first reported by Brittberg et al. [1], has been performed over 20,000 times worldwide. However, two sites for the resection of cartilage and periosteum must be sacrificed to repair a single cartilaginous lesion. This is the largest drawback of ACI. Other problems include periosteal hyperplasia and incompatibility with surrounding tissues [5]. Problems associated with mosaicplasty include a limited number of donors of healthy cartilage and the need for long-term monitoring for damage at the harvest sites [2]. Microfracture induces cartilage regeneration by promoting the migration of mesenchymal cells from the marrow, but results in the production of fibrocartilage, which is mechanically weaker than hyaline cartilage [9].

Previously, we prepared highly adhesive layered chondrocyte sheets without a scaffold and with a short culture time using a temperature-responsive culture dish (Fig. 1a, b). On transplantation, these layered chondrocyte sheets suppressed degeneration in articular cartilage [10–12]. These temperature-responsive culture dishes have already been applied to research in various fields of regenerative medicine, including the regeneration of myocardium [13,14], vascular epithelium [15], cornea]16], hepatocytes [17], and renal cells [18]. This method has also been applied

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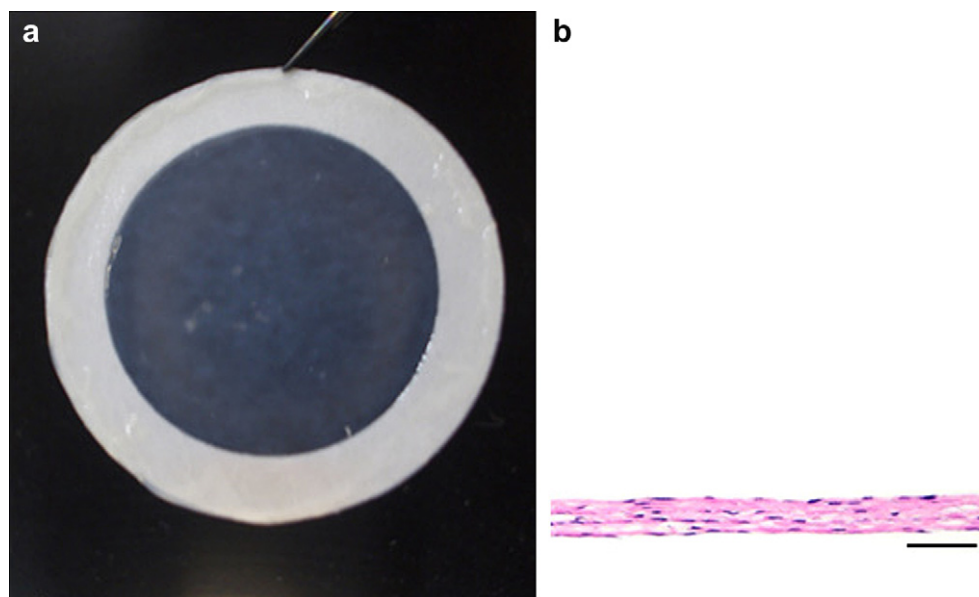E-mail address: sato-m@is.icc.u-tokai.ac.jp (M. Sato).

Fig. 1. (a) Layered chondrocyte sheet. Chondrocytes cultured on a temperature-responsive surface can be released from the dish (4.2 cm^2) by reducing the temperature without the need for proteolytic enzymes. Confluent cultured chondrocytes were harvested as a single contiguous cell sheet retaining cell–cell junctions, and extracellular matrix (ECM) was deposited on the basal side. (b) Chondrocyte sheets can be layered and thereafter adhere to other cell sheets. Culture of five-layered chondrocyte sheets can be continued for up to 1 week (scale bar = 150 μm).

clinically to the myocardium and cornea [16]. The surface of a temperature-responsive culture dish is coated with a polymer (poly(N-isopropylacrylamide)), which becomes hydrophilic or hydrophobic in a reversible manner, depending on the temperature [19]. The polymer has a low critical solution temperature of 32 °C, below which it becomes soluble in water. Based on this characteristic, the temperature-responsive culture dish has a weakly hydrophobic surface, similar to that of commercially available dishes, so it can be used to culture cells in a conventional manner when the temperature is 37 °C or higher. However, the surface of the dish becomes hydrophilic when the temperature falls below the critical solution temperature. Therefore, confluent sheets of cultured cells can be released spontaneously from the hydrophilic dish surface by reducing the temperature to below 32 °C [20]. Using this method, cultured cells can be harvested as a sheet without damaging cell–cell junctions and the ECM because it eliminates the need for conventional enzymatic harvesting with trypsin. Such cell sheets have been reported to have various advantages, including preservation of the normal phenotype and expression of adhesion proteins on the sheet base [21]. Furthermore, these cell sheets can also be superimposed to prepare layered 'tissue' because the ECM is preserved on the base, and such three-dimensional (3D) manufactured tissue has already been used successfully in transplantation [14]. We confirmed previously that such layered chondrocyte sheets are able to maintain a normal chondrocyte phenotype in the knee joints of rabbits. Moreover, they can be attached to injured cartilage, thereby acting as a barrier to prevent the loss of proteoglycan from these sites, while also protecting them from catabolic factors [10].

The objective of this study was to investigate the ability of layered chondrocyte sheets to repair and regenerate tissue in a minipig-based large animal model of full-thickness defects of articular cartilage.

2. Materials and methods

All animal experiments were approved and carried out following the Guidelines of Tokai University on Animal Use.

2.1. Temperature-responsive culture dishes

Specific procedures for the preparation of temperature-responsive culture dishes (provided by CellSeed, Tokyo, Japan) were as described [19]. Briefly, N-isopropylacrylamide (IPAAm) monomer solution was spread onto commercial tissue culture polystyrene dishes. These dishes were then subjected to electron beam irradiation, thus resulting in polymerization and covalent bonding of IPAAm to the dish surface. Poly-IPAAm (PIPAAm)-grafted dishes were rinsed with cold distilled water to remove ungrafted IPAAm. The dishes were then sterilized using ethylene oxide gas [22].

2.2. Chondrocytes from minipigs and proliferation on a temperature-responsive surface

Five minipigs aged 7–8 months and weighing 21.3–21.5 kg were used as the source of chondrocytes. Cartilage samples were collected from the femoral compartment of the knee joint and subjected to enzymatic processing; they were then seeded and cultured according to the method of Sato et al. [23]. Briefly, chondrocytes were digested for 1 h in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Gibco, Grand Island, NY, USA) containing 0.4% Pronase E (Kaken Seiyaku Inc., Tokyo, Japan) and for 4 h further in DMEM/F12 containing 5 mg/ml collagenase type 1/CLS1 (Worthington Inc., Lakewood, NJ, USA) at 37 °C in an atmosphere of 5% CO$_2$ and 95% air. Digested tissue was passed through a Falcon cell strainer (BD Biosciences, Franklin Lakes, NJ, USA) with a pore size of 100 μm. Cells were then seeded at high density (50,000 cells/cm^2) into temperature-responsive dishes (4.2 cm^2; provided by CellSeed, Tokyo, Japan) and were cultured in DMEM/F12 supplemented with 20% fetal bovine serum (FBS; Gibco), 50 μg/ml ascorbic acid (Wakojunyakukougyou Corp., Osaka, Japan) and 1% antibiotic–antimycotic mix (Gibco) at 37 °C in an atmosphere of 5% CO$_2$ and 95% air for a week. Culture dishes were removed from the incubator when the cells reached confluence and were left to stand at 25 °C for 30 min. After the culture medium had been removed, cell sheets were harvested as described by Kaneshiro et al. [10].

2.3. Measurement of chondrocyte proliferative activity

Chondrocytes isolated and cultured as above (3.0 × 10^4 cells) were cultured on 24-well plates and cell proliferation activity was measured using a thiazolyl blue tetrazolium bromide (MTT) assay on days 3, 5 and 7. MTT (Dojindo, Kumamoto, Japan) was added to each well of the 24-well plate and incubated for 2 h at 37 °C in the dark. The resulting crystals were solubilized in dimethyl sulfoxide. Absorbance was read using an enzyme-linked immunosorbent assay (ELISA) plate reader at 590 nm, with absorbance as a function of viable cell number. Data are expressed as the mean ± standard error of the mean.

Table 1
Variables used in the International Cartilage Repair Society (ICRS) grading system and the ICRS remodeling system for evaluating subchondral bone maintenance.

Variable	Comment
Ti:	**Tissue morphology**
	4 = Mostly hyaline cartilage
	3 = Mostly fibrocartilage
	2 = Mostly noncartilage
	1 = Exclusively noncartilage
Matx:	**Matrix staining**
	1 = None
	2 = Slight
	3 = Moderate
	4 = Strong
Stru:	**Structural integrity**
	1 = Severe integration
	2 = Cysts or disruptions
	3 = No organization of chondrocytes
	4 = Beginning of columnar organization of chondrocytes
	5 = Normal, similar to healthy mature cartilage
Clus:	**Chondrocytes clustering in implant**
	1 = 25–100% of cells clustered
	2 = <25% of the cells clustered
	3 = No clusters
Tide:	**Intactness of calcified cartilage layer, formation of tidemark**
	1 = <25% of the calcified cartilage layer intact
	2 = 25–49% of the calcified cartilage layer intact
	3 = 50–75% of the calcified cartilage layer intact
	4 = 76–90% of the calcified cartilage layer intact
	5 = Complete intactness of the calcified cartilage layer intact
Bform:	**Subchondral bone formation**
	1 = No formation
	2 = Slight
	3 = Strong
SurfH:	**Histologic appraisal of surface architecture**
	1 = Severe fibrillation or disruption
	2 = Moderate fibrillation or irregularity
	3 = Slight fibrillation or irregularity
	4 = Normal
FilH:	**Histologic appraisal defect filling**
	1 = <25%
	2 = 26–50%
	3 = 51–75%
	4 = 76–90%
	5 = 91–100%
Latl:	**Lateral integration of implanted material**
	1 = Not bonded
	2 = Bonded at one end/partially both ends
	3 = Bonded at both sides
Basl:	**Basal integration of implanted material**
	1 = <50%
	2 = 50–70%
	3 = 70–90%
	4 = 91–100%
InfH:	**Inflammation**
	1 = No inflammation
	3 = Slight inflammation
	5 = Strong inflammation
Hgtot	**Histologic grading system**
	Some of the histologic variables: tissue morphology (Ti), matrix staining (Matx), structural integrity (Stru), cluster formation (Clus), tidemark opening (Tide), bone formation (Bform), histologic surface architecture (SurfH), histologic degree of defect filling (FilH), lateral integration of defect filling tissue (Latl), basal integration of defect filling tissue (Basl) and histologic signs of inflammation
Remod:	**Subchondral bone remodeling (loose textures of highly cellular tissue composed mostly of fibroblasts)**
	1 = No remodeling
	2 = Discrete cellularity
	3 = Moderate cellularity
	4 = High cellularity

Presentation of the ICRS histological grading system (Hgtot) and remodeling scores (Remod) based on a modified ICRS grading scale [30] developed by O'Driscoll et al. [31] In Hgtot, 11 histologic categories were evaluated and scored. The total score ranged from 11 points (no repair) to 45 points (normal articular cartilage).

2.4. Transplantation of chondrocyte sheets

Before the surgery of implantation, 0.2 mg/kg dormicum (Midazolam 5 mg/1 ml, Astellas Pharma, Tokyo, Japan) and 40 μg/kg medetomidine (Domitor 1 mg/ml, Meiji Seika Pharma Co., Ltd, Tokyo, Japan) were given intramuscularly. Inhalation anesthesia was used during the operation with a combination of isoflurane, dinitrogen monoxide, and oxygen. A chondral defect measuring 6 mm in diameter and 5 mm deep was made in the area of the host animal's medial femoral condyle using a biopsy punch and the damaged cartilage was covered with a three-layered chondrocyte sheet, which was stabilized with a nylon suture until initial fixation was achieved. This was performed in the left knees of 12 minipigs (aged 7–8 months and weighing 21.5–25.0 kg) in the transplantation group. At the same time, the articular cartilage of the medial femoral condyle was holed similarly in the right knees of 12 minipigs in the control group, but not covered with a cell sheet. Cartilage was harvested after 3 weeks, fixed in 4% paraformaldehyde (PFA) for 1 week and decalcified with K-CX decalcifying solution (Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan) for 1 week. Specimens were then embedded in paraffin wax, sectioned and stained with safranin-O for evaluation. Histological scoring of these sections was carried out by two observers, using the International Cartilage Repair Society (ICRS) grading system and the ICRS remodeling system to evaluate subchondral bone maintenance (Table 1).

2.5. Statistical analysis

The Mann–Whitney nonparametric U test was used to analyze histological scores in assessing the efficacy of treatment. $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Chondrocyte sheets

Multilayered chondrocyte sheets were prepared by superimposing sheets and then culturing them together. It was thus possible to continue culturing three-layered sheets. As shown in Fig. 1, the layered articular chondrocyte sheets cultured for 3 weeks maintained their original shape without shrinking when the polyvinylidene difluoride (PVDF) membrane was removed. As a result, it was possible to create round grafts that demonstrated a chondrocytic phenotype.

3.2. Measurement of chondrocyte proliferation activity

Cell proliferation activities examined by MTT assay on days 3, 5 and 7 are illustrated in Fig. 2. Proliferation increased gradually until day 7; the numbers of chondrocytes increased by approximately 2.4-fold by day 5 and by approximately 6-fold by day 7.

3.3. Gross findings in the repaired cartilage

No distinct evidence of infection, articular damage at uninvolved sites, or synovial proliferation was observed in the transplantation or control groups. All defects were filled with white cartilaginous tissue, but defect filling in the control group was insufficient. The surface layer in the transplantation group had been replaced with smooth cartilaginous tissue of a color resembling that of healthy cartilage, but the surface layer in the control group had been replaced with coarse cartilaginous tissue, while the subchondral bone was exposed in some locations (Fig. 3a–d).

3.4. Histological findings of repaired cartilage

Good safranin-O staining and integration with surrounding tissue was noted in the transplantation group, which achieved sufficient cartilaginous repair and regeneration. All animals in the control group exhibited poor safranin-O staining, and tissue repair and regeneration were insufficient (Fig. 4a, b). ICRS scores were compared between the two groups. The mean score in the transplantation group was significantly higher than in the control group
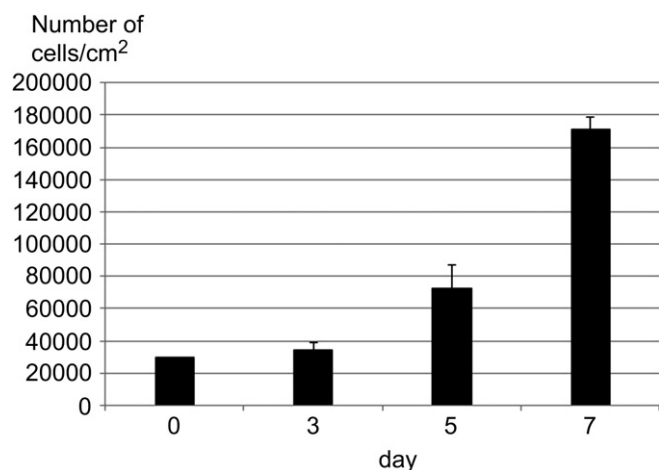
Fig. 2. Cell proliferation activity was examined by MTT assay. Cell proliferation increased steadily until day 7; the numbers of chondrocytes had increased approximately 2.4-fold by day 5 and approximately 6-fold by day 7.

(38.3 points versus 26.3 points, respectively, $p < 0.05$). Mean remodeling scores, an indication of subchondral bone condition, were significantly higher in the transplantation group (3.2 points) than in the control group (2.4 points; Fig. 5a, b). The ICRS scores in the transplantation and control groups are shown in Table 2.

In three of the 12 animals, a more detailed histological examination in the transplantation group revealed that, although tissue filling was present, safranin-O staining was poor and the subchondral bone in these animals was poorly repaired and regenerated (Fig. 4d). A comparison of ICRS scores indicated that subchondral bone repair was satisfactory (remodeling score 4 points) in the animals in the transplantation group that achieved

adequate cartilage repair (histological score 42 points). However, subchondral bone repair was poor (remodeling score 2 points) in animals in the transplantation group with insufficient cartilage repair (histological score 29 points). These findings indicate that the degree of subchondral bone repair reflected that of cartilaginous tissue repair.

4. Discussion

Unless treated, full-thickness defects in knee cartilage cause secondary osteoarthritis and the resulting pain and poor joint function impact substantially on the activities of daily life. Recent advances in tissue engineering have prompted research on techniques to repair articular cartilage damage using a variety of transplanted cells. ACI was the first such technique investigated and is already used clinically in Western countries. However, the technique carries problems such as the potential for leakage of chondrocytes (implanted in suspension) outside the transplantation site, nonuniform distribution of transplanted cells [24] and damage to donor site tissues. Some have claimed that clinical outcomes are not superior to microfracture [25]. More effective treatments must therefore be developed. Ochi et al. [5], hypothesizing that the transplantation of 3D cartilaginous tissues composed of chondrocytes and matrix would yield better outcomes than chondrocyte transplantation, developed and clinically applied the transplantation of cultured chondrocytes embedded in atelocollagen gel. Furthermore, realizing the potential for bone marrow mesenchymal cells to differentiate, Wakitani et al. [26] developed and applied the transplantation of bone marrow mesenchymal cells embedded in collagen gel clinically. However, both types of implant were composites made of periosteum, a scaffold, bone marrow cells, cultured chondrocytes and numerous other elements and were thus unsuitable for creating an optimal environment for articular cartilage regeneration.
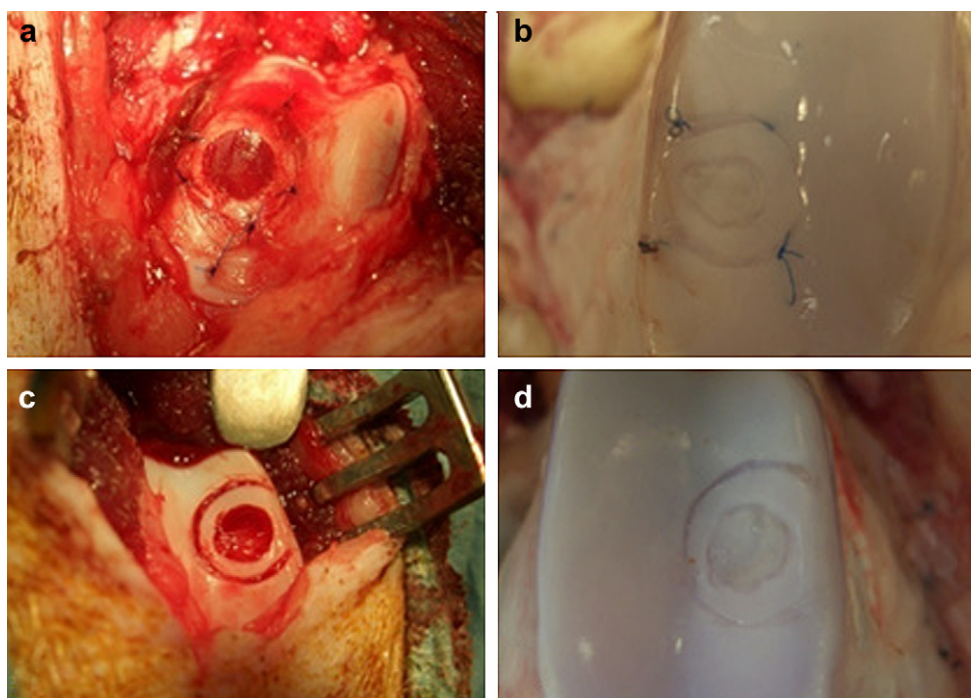

Fig. 3. Gross appearance (a, b, c, d) in the group receiving layered chondrocyte sheets (a, b) and the control group (c, d). Gross appearance is shown at transplantation (a, c) and 3 months later (b, d). The defect in the transplantation group (b) was filled with cartilaginous tissue, but filling in the defect of the control group (d) was insufficient and the subchondral bone was exposed partially. The defect of 6 mm in diameter and 5 mm deep was made, and outside circle of the defect is 8 mm in diameter, and is used for suturing chondrocyte sheet.
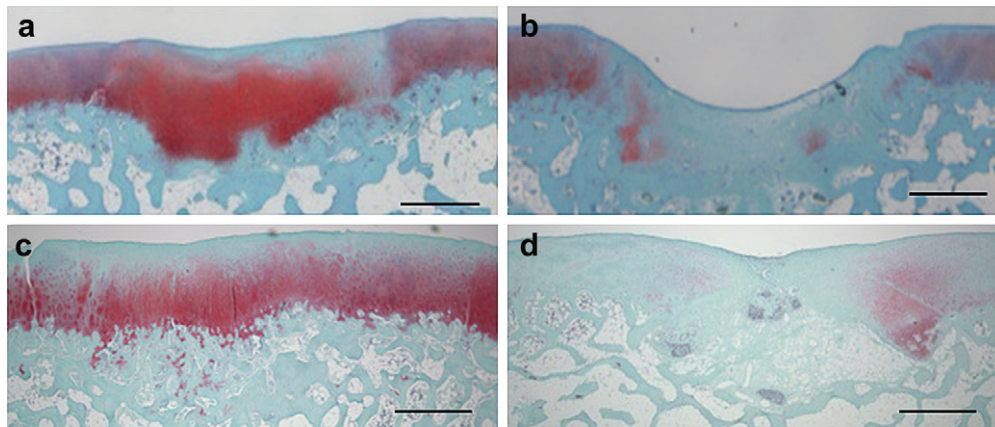
Fig. 4. Histology in the transplantation (a, c, d) and control groups (b). Safranin-O staining was robust and integration with the surrounding tissue was good in the transplantation group (a), but the control group (b) showed poor safranin-O staining and did not achieve satisfactory regeneration or repair. In (c), safranin-O staining was robust and subchondral bone repair and regeneration were good (International Cartilage Repair Society, ICRS histological grading system 42 points; remodeling score 4 points). Although the defect in (d) had been filled with tissue, safranin-O staining was poor and the subchondral bone was insufficiently repaired (ICRS histological grading system 29 points; remodeling score 2 points; scale bar = 2.0 mm).

Convinced that establishing an environment suited to tissue repair is essential for proper articular cartilage regeneration, we began basic research on repair and regeneration using only cells from bone marrow and cultured chondrocytes, without a scaffold or periosteum. Analysis of our layered chondrocyte sheets has shown that the chondrocytes maintain their phenotype, expressing aggrecan, collagen type II (COL2), SOX9 and COL27. The cells also express the adhesion molecules integrin α10 and fibronectin. Immunostaining confirmed the presence of COL2, integrin α10 and fibronectin in the cellular sheets [12,27]. Our findings indicate that these layered chondrocyte sheets demonstrate good adhesiveness and barrier functionality while maintaining a normal phenotype.

We also reported achieving robust tissue repair when covering only the surface layer with a layered chondrocyte sheet in research on partial-thickness defects in the articular cartilage of domestic rabbits [10]. This suggests that liquid factors from the layered sheets, in addition to the basic functionality of the sheets, contribute to repair.

In this study using chondrocytes from minipigs, which are large animals, we found that cultured chondrocytes had a high cell proliferation potential and that—as shown in previous studies—layered chondrocyte sheets created from these chondrocytes also contributed to the repair and regeneration of articular cartilage in this model of full-thickness defects. However, tissue having poor safranin-O staining, which was not noted in our domestic rabbit experiments, was identified in some of the animals (3/12) and the subchondral bone was poorly repaired in these animals. Vasara et al. [28] transplanted chondrocytes into a goat model of full-thickness defects. In animals with poor subchondral bone repair, the transplanted cells showed poor safranin-O staining, and integration with the surrounding tissue was insufficient. Muehleman et al. [29] transplanted chondrocytes into a minipig model of full-thickness defects and compared a group treated with risedronate to an untreated group using histology. Safranin-O staining of the transplanted cells was superior in the treated group and integration with the surrounding tissues was satisfactory,
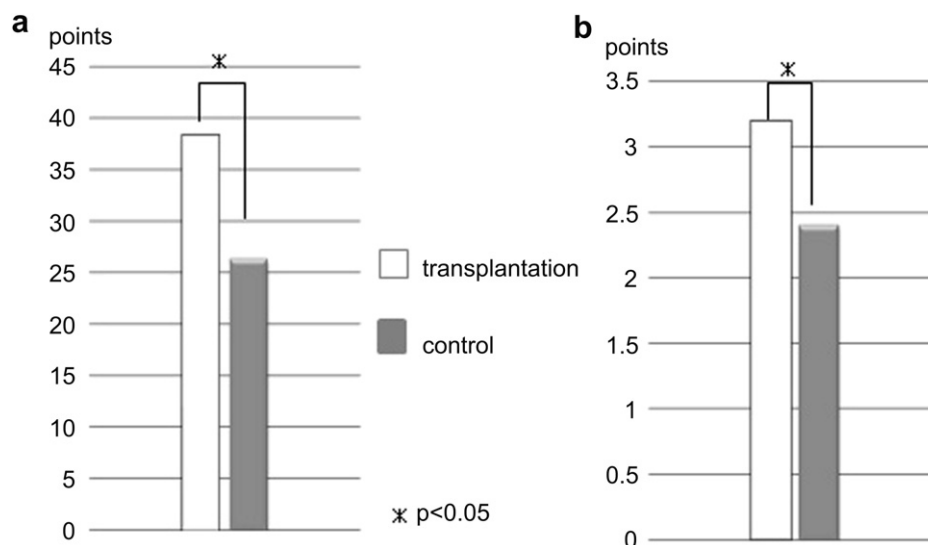

Fig. 5. ICRS histological grading system scores (a) and remodeling scores (b) in the transplantation and control groups. The mean histological score (a) in the transplantation group (38.3 points) was significantly higher than in the control group (26.3 points). Mean remodeling scores (b), an indication of subchondral bone condition, were significantly higher in the transplantation group than in the control group (3.2 points and 2.4 points, respectively).

Table 2
Results of ICRS histological grading system (Hgtot) and remodeling scores (Remod).

Case	Weight (kg)	Age (month)	Transplantation		Control	
			Hgtot	Remod	Hgtot	Remod
			(Points)		(Points)	
1	21.6	7	42	4	28	3
2	23.5	7	40	4	22	2
3	24.2	7	40	4	28	3
4	22.9	7	42	4	30	3
5	24.1	8	41	4	26	2
6	24.0	8	40	3	31	3
7	23.0	8	39	3	24	2
8	25.0	8	39	3	24	2
9	24.0	8	39	3	24	2
10	23.0	8	33	2	33	3
11	23.8	8	29	2	23	2
12	21.5	8	35	2	22	2
Average	23.4	7.7	38.3	3.2	26.2	2.4

These findings indicate that the points awarded in the ICRS histological grading system reflected the remodeling scores.

but greater than expected bone resorption occurred in this large animal model. Similarly, animals with poor subchondral bone repair in the transplantation group in the present study exhibited poor safranin-O staining in the regeneration tissue and insufficient integration with surrounding tissue. Risedronate administration and other measures to suppress bone resorption might thus be necessary in large animals used in future research to determine optimal transplantation conditions.

5. Conclusion

The use of layered chondrocyte sheets facilitated the repair and regeneration of tissue in a minipig model of full-thickness cartilaginous defects in the knee joints. Good safranin-O staining and integration with surrounding tissue was noted in the transplantation group, which achieved sufficient cartilaginous repair and regeneration. Some animals in the group receiving the layered chondrocyte sheets exhibited poor safranin-O staining of the repaired and regenerated tissue in the subchondral bone. Transplantation conditions and other factors must therefore be further investigated.

Acknowledgments

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