Repair Mechanism of Osteochondral Defect Promoted by Bioengineered Chondrocyte Sheet

Ryo Shimizu, MD,¹ Naosuke Kamei, MD, PhD,^{1,2} Nobuo Adachi, MD, PhD,¹ Michio Hamanishi, MD, PhD,¹ Goki Kamei, MD, PhD,¹ Elhussein Elbadry Mahmoud, BVSc,¹ Tomohiro Nakano, CT,³ Takanori Iwata, DDS, PhD,⁴ Masayuki Yamato, PhD,⁴ Teruo Okano, PhD,⁴ and Mitsuo Ochi, MD, PhD¹

Cell sheet engineering has developed as a remarkable method for cell transplantation. In the field of cartilage regeneration, several studies previously reported that cartilage defects could be regenerated by transplantation of a chondrocyte sheet using cell sheet engineering. However, it remains unclear how such a thin cell sheet could repair a deep cartilage defect. We, therefore, focused on the mechanism of cartilage repair using cell sheet engineering in this study. Chondrocyte sheets and synovial cell sheets were fabricated using cell sheet engineering, and these allogenic cell sheets were transplanted to cover an osteochondral defect in a rat model. Macroscopic and histological evaluation was performed at 4 and 12 weeks after transplantation. Analysis of the gene expression of each cell sheet and of the regenerated tissue at 1 week after transplantation was performed. In addition, green fluorescent protein (GFP) transgenic rats were used as donors (transplanted chondrocyte sheets) or recipients (osteochondral defect models) to identify the cell origin of regenerated cartilage.

Cartilage repair was significantly better in the group implanted with a chondrocyte sheet than in that with a synovial cell sheet. The results of gene expression analysis suggest that the possible factor contributing to cartilage repair might be *TGFβ1*. Cell tracking experiments using GFP transgenic rats showed that the regenerated cartilage was largely composed of cells derived from the transplanted chondrocyte sheets.

Introduction

ARTICULAR CARTILAGE IS a hypocellular and avascular tissue with rich extracellular matrix consisting of various types of collagen and proteoglycan. Because of its structural and biological properties, once it is injured, the capacity of cartilage for self-repair is limited. Cartilage injury causes pain and dysfunction of the joint and eventually leads to osteoarthritis. Recently, some treatments for cartilage injury have been developed, such as bone marrow stimulation, osteochondral transplants, and autologous cultured chondrocyte transplantation.^{1,2} Especially in our group, tissue-engineered cartilage-like tissues cultured from autologous chondrocytes in atelocollagen gel have been clinically used for the treatment of articular cartilage defects.³ Meanwhile, cell sheet engineering has been reported as a novel and remarkable method in the field of cell transplantation therapy. In this method, which uses temperature-responsive culture dishes, the cultured cells can be obtained as a cell sheet without the use of proteolytic enzymes.⁴ The cell sheet is released simply by reducing the temperature and has advantages for cell transplantation, because it is scaffold free and

preserves the extracellular matrix and cell–cell junctions. Cell sheet engineering is now applied to several fields of regenerative medicine, and clinical trials are ongoing in the field of regeneration of the myocardium.⁵ Especially in the field of corneal regeneration, an important clinical trial has been successfully completed.⁶ This trial demonstrated the efficacy of cultural oral mucosa epithelial cell sheet transplantation, on 23 eyes of 22 patients, by restoring the eyes' ocular surface.⁷ Chondrocyte sheet transplantation has also been reported to be effective for cartilage repair in an animal model.^{8,9} However, it remains unclear how covering a deep cartilage defect with such a thin cell sheet could markedly promote cartilage repair. In this study, we, therefore, focused on the mechanism of cartilage repair using cell sheet engineering. It was suspected that the chondrocyte sheet might work as a barrier which could prevent proteoglycan loss from damaged cartilage and also protect the injured site from the catabolic factors in synovial fluid.¹⁰ We hypothesized that if the barrier function of a chondrocyte sheet was the main mechanism for cartilage repair, a cell sheet produced from other cell sources besides chondrocytes might also function as a barrier, providing coverage of the defect and contributing to

¹Department of Orthopaedic Surgery, Graduate School of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan.

²Translational Research Medical Center, Hiroshima University Hospital, Hiroshima, Japan.

³Department of Medical Laboratory Science, Faculty of Health Sciences, Junshin Gakuen University, Fukuoka, Japan.

⁴Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan.

its repair. In this study, we, therefore, created a cell sheet using synovial cells as an alternative cell source, and compared the effect of chondrocyte sheets on cartilage repair with that of synovial cell sheets to evaluate the barrier effect of a cell sheet. On the other hand, paracrine growth factors derived from transplanted chondrocyte sheets could be considered one of the mechanisms that promotes cartilage repair. A previous study demonstrated that the expression of beneficial growth factors promoted chondrogenesis *in vitro*.¹¹ However, expression of these factors might be changed under *in vivo* conditions. In this study, expression of factors that are anabolic or catabolic for chondrogenesis in the sheets of chondrocytes and synovial cells were assessed both *in vitro* and *in vivo*. In addition, the cell origin of cartilage tissues repaired after transplantation of chondrocyte cell sheets has not been identified. We hypothesized that the origin of repaired cartilage might be cells from the bone marrow, because covering the cartilage defect could contribute to the accumulation of bone marrow-derived cells, including chondrogenic progenitor cells. To identify the cell origin of repaired tissue, cell tracking of the transplanted cell sheets and cells of the recipient rats was performed using green fluorescent protein (GFP) transgenic rats. The purpose of this study was, therefore, to clarify the mechanisms of articular cartilage repair after transplantation of a bioengineered chondrocyte sheet.

Materials and Methods

All procedures were performed according to an institutionally approved protocol in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals. All of the experimental research protocols were reviewed and approved by the ethics committee of Hiroshima University.

Fabrication of chondrocyte and synovial cell sheets

Articular cartilage and synovial tissue were harvested from the hip and knee joints of 4 week-old Sprague-Dawley (SD) rats. After cutting the cartilage and synovium into pieces, the tissue was digested with trypsin (TrypLE Express; Life Technologies, Carlsbad, CA) for 30 min followed by 0.25% collagenase (Sigma, St. Louis, MO) for 4 h at 37°C. After passing through a cell strainer (70 µm; BD Biosciences, San Jose, CA), the cells were suspended in culture medium and seeded into temperature-responsive culture dishes (UpCell, CellSeed, Tokyo, Japan). Culture medium consisted of Dulbecco's modified Eagle's medium and Ham's F-12 (DMEM/F12; Life Technologies) containing 20% fetal bovine serum (FBS) and 1% antibiotics. The medium for chondrocyte sheets also contained 50 µg/mL L-ascorbic acid (Sigma, Tokyo, Japan). The cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂ for 2 weeks, at the end of which the chondrocyte sheets and synovial cell sheets could be detached simply by reducing the temperature from 37°C to below 32°C. In addition, triple-layered sheets of each cell type could be fabricated using a simple pipetting method¹² followed by culture for a further 1 week.

Transplantation of cell sheets onto the cartilage defect

Thirty-six SD rats, 12 weeks of age, were anesthetized with an intraperitoneal injection of 1 mL/kg sodium pentobarbital.

Using a medial parapatellar approach, the patella groove of the distal femur was exposed. A defect that was 2 mm in diameter and 1 mm in depth was created in the patella groove using a biopsy punch and a metallic drill with a globe-shaped tip of 1 mm diameter. The osteochondral defect that was created affected both articular cartilage and underlying subchondral bone. After lowering the temperature of the culture dishes prepared in advance to a temperature threshold of 32°C, the cultured cell sheets were detached from the dishes using an artificial support membrane (Cell Shifter™ CellSeed, Tokyo, Japan). After cutting the cell sheets and the Cell Shifter to the size of 4 mm square, these allogenic cell sheets were then transplanted to cover the osteochondral defect. The cell sheet was transplanted without suture, because it has a good adhesion property.¹⁰ Animals were divided into the following six groups: 1) sham group (without cartilage defect) (*n* = 12), 2) defect group (without cell sheet) (*n* = 12), 3) monolayer chondrocyte sheet group (*n* = 12), 4) monolayer synovial cell sheet group (*n* = 12), 5) triple-layered chondrocyte sheet group (*n* = 12), 6) and triple-layered synovial cell sheet group (*n* = 12) (Fig. 1). After transplantation, the joint capsule and wound was sutured and all rats were allowed to move freely in their cages.

Histological analysis of the cell sheets

After fabrication of the cell sheets, these were fixed overnight in 4% paraformaldehyde. After cutting to the desired size, pieces were mounted with O.C.T. compound (Sakura Finetek, Tokyo, Japan) and frozen at -20°C. The blocks were then cut into 5 µm sections using a cryostat. After air drying at room temperature, these sections were stained with Hematoxylin and Eosin or Safranin-O/Fast green.

Macroscopic and histological evaluation

At 4 and 12 weeks after creation of the defects and treatment, the rats were sacrificed by an intraperitoneal injection of a lethal dose of sodium pentobarbital. The distal femur was resected and evaluated macroscopically using a macroscopic scoring system that was established by Wayne.¹³ The scale is composed of four categories and assigns a score ranging from 0 (worst) to 16 (best) points (Table 1). After that, the cartilage was fixed in 4% paraformaldehyde for 24 h and decalcified with EDTA for 3 weeks. After embedding in paraffin, the blocks were cut into 5 µm sections in the sagittal plane. The sections were stained with Safranin-O/Fast green and were evaluated histologically using Pineda's semiquantitative scale, which ranges from 0 (best) to 14 (worst) points (Table 2).¹⁴ Furthermore, immunohistochemical staining of type II collagen was performed using the Avidin Biotin Complex (ABC) method with a Vectastain Elite ABC Mouse IgG kit (Vector Laboratories, Burlingame, CA). The primary antibody was a purified mouse monoclonal antibody anti-human type II collagen (Daiichi Fine Chemical, Toyama, Japan).

Analysis of gene expressions in cell sheets in vitro and regenerated tissues in vivo

To elucidate the factors contributing to cartilage repair, the gene expression patterns of chondrocyte sheets and synovial cell sheets *in vitro* and regenerative tissues *in vivo* were analyzed.

FIG. 1. Full-thickness cartilage defects were created in the articular cartilage of rats, and the efficacy of different cell layers in promoting repair when used to cover the defect was assessed, including the schema and gross appearance of each group after transplantation. (1) Sham group: arthrotomy only. (2) Control group: creation of an osteochondral defect without transplantation. (3) Transplantation of a monolayer chondrocyte sheet. (4) Transplantation of a triple-layered chondrocyte sheet. (5) Transplantation of a monolayer synovial cell sheet. (6) Transplantation of a triple-layered synovial cell sheet.

In vitro: Triple-layered chondrocyte sheets and synovial cell sheets were fabricated using temperature-responsive culture dishes. These sheets were homogenized with TRIzol Reagent (Life Technologies), and total RNA was obtained according to the manufacturer's instructions. For cDNA synthesis, reverse transcription of total RNA was performed using reverse transcriptase (SuperScript VILO MasterMix, Life Technologies). A PCR reaction mix containing the primers for the target genes, the cDNA of each sample, and

Fast SYBR[®] Green Master Mix (Life Technologies) was prepared, and real-time PCR was performed using StepOne[®] (Life Technologies). The mRNA expression levels of these samples were normalized to β -actin (*ACTB*) that served as an endogenous control. Data were analyzed using the comparative $\Delta\Delta C_T$ method.

In vivo: The triple-layered chondrocyte sheet and triple-layered synovial cell sheet were prepared as described earlier. One week after transplantation, we harvested the tissue

FIG. 2. (A) Monolayer chondrocyte sheet with Hematoxylin-Eosin staining. (B) Monolayer chondrocyte sheet with Safranin-O/Fast green staining. (C) Triple-layered chondrocyte sheet with Hematoxylin-Eosin staining. (D) Triple-layered chondrocyte sheet with Safranin-O/Fast green staining. (E) Monolayer synovial cell sheet with Hematoxylin-Eosin staining. (F) Monolayer synovial cell sheet with Safranin-O/Fast green staining. (G) Triple-layered synovial cell sheet with Hematoxylin-Eosin staining. (H) Triple-layered synovial cell sheet with Safranin-O/Fast green staining. Each triple-layered sheet was more than thrice the thickness of the monolayer sheet. The thickness of the triple-layered sheet was about 100 μ m. The triple-layered chondrocyte sheets consisted of spherical cells with an abundant matrix that was strongly stained with Safranin-O. (D) Triple-layered synovial cell sheets consisted of abundant fibroblastic cells but with a smaller amount of extracellular matrix (H). Scale bar = 50 μ m.

TABLE 1. GROSS APPEARANCE GRADING SCALE

Coverage	
>75% fill	4
50–75% fill	3
25–50% fill	2
<25% fill	1
No fill	0
Neocartilage color	
Normal	4
25% yellow/brown	3
50% yellow/brown	2
75% yellow/brown	1
100% yellow/brown	0
Defect margins	
Invisible	4
25% circumference visible	3
50% circumference visible	2
75% circumference visible	1
Entire circumference visible	0
Surface	
Smooth/level with normal	4
Smooth but raised	3
Irregular 25–50%	2
Irregular 50–75%	1
Irregular >75%	0

formed in the osteochondral defect of each group. Gene expression in these tissues was analyzed by real-time PCR in the same way as for cell sheets *in vitro*.

The target genes associated with cartilage metabolism were as follows: collagen type I (*COL1*), *COL2*, *COL10*, *Sox9*, bone morphogenetic protein-2 (*BMP2*), fibroblast growth factor-2 (*FGF2*), transforming growth factor- β (*TGF β*), insulin-like growth factor-1 (*IGF1*), cartilage-derived morphogenetic protein-1 (*CDMP1*), fibronectin, aggrecan, matrix

metalloproteinase 13 (*MMP13*), a disintegrin and metalloproteinase with thrombospondin motifs-4 (*ADAMTS4*), *ADAMTS5*, tumor necrosis factor (*TNF*), interleukin-1 β (*IL1b*), and osteocalcin. The primer sequences are shown in Table 3.

Identification of the cell origin of repaired tissue using GFP transgenic rats

GFP transgenic animals have been widely used in cell tracking studies.^{15–17} To identify the cell origin of repaired cartilage, GFP transgenic rats were utilized in this study. We used GFP transgenic rats and SD rats at 4 weeks of age. After chondrocytes were harvested from these rats, the triple-layered chondrocyte sheets were fabricated as described earlier. GFP transgenic rats and SD rats at 12 weeks of age were prepared as the recipients. The cartilage defects of GFP transgenic rats were covered with triple-layered chondrocyte sheets from wild-type SD rats (Group I). In the reverse experiment, the cartilage defects of wild-type SD rats were covered with triple-layered GFP-positive chondrocyte sheets (Group II). These models were also allogenic models. At 4 weeks after transplantation, all rats were sacrificed for histological evaluation. The specimens were prepared in the manner described earlier. To preserve GFP signal for histological analysis, they were embedded in plastic resin (Technovit 8100; Heraeus Kulzer, Wehrheim, Germany). Sections were evaluated under a typical light microscope with Safranin-O/fast green staining for cartilage regeneration and under a fluorescent microscope for GFP fluorescence analysis and DAPI nucleic acid staining.

Statistical analysis

The results of histological scoring are expressed as mean value and standard deviation. Multiple comparisons were performed for the data of each group using the Steel–Dwass method. Gene expression data were analyzed using the Mann–Whitney U test. *p* Values less than 0.05 were considered statistically significant.

TABLE 2. PINEDA'S SEMIQUANTITATIVE SCALE

Filling of defect	
125%	1
100%	0
75%	1
50%	2
25%	3
0%	4
Reconstitution of osteochondral junction	
Yes	0
Almost	1
Not close	2
Matrix staining	
Normal	0
Reduced staining	1
Significantly reduced staining	2
Faint staining	3
No stain	4
Cell morphology	
Normal	0
Mostly hyaline and fibrocartilage	1
Mostly fibrocartilage	2
Some fibrocartilage but mostly nonchondrocytic cells	3
Nonchondrocytic cells only	4

Results

Histology of cell sheets

Comparing the monolayer sheet with the triple-layered sheet, we found that the latter was more than thrice thicker than the former, measuring about 100 μ m. Spherical cells surrounded by lacunae with abundant extracellular matrix were observed in the triple-layered chondrocyte sheet. There was good staining with Safranin-O (Fig. 2C, D). In contrast, fibroblastic cells showed good proliferation in the triple-layered synovial sheet, but the amount of extracellular matrix was relatively poor (Fig. 2G, H).

Macroscopic evaluation

At 4 and 12 weeks after transplantation in each group, some regenerated tissues were found in the cartilage defects (Fig. 3A). There was significant difference between the triple-layered chondrocyte sheet model and the monolayer synovial cell sheet model at 4 weeks after transplantation in the macroscopic score among the five groups excluding the sham group ($p < 0.05$) (Fig. 3B). The categorical values of

TABLE 3. PRIMER SEQUENCES USED FOR REAL TIME POLYMERASE CHAIN REACTION

Target genes	Primer sequence: forward	Primer sequence: reverse
<i>ACTB</i>	5'-GATCATTGCTCCTCCTGAGCG-3'	5'-TGCTGATCCACATCTGCTGGA-3'
<i>COL1</i>	5'-TGCCGTGACCTCAAGATGT-3'	5'-TGGGGTTTGGGCTGATGTA-3'
<i>COL2</i>	5'-CCCAGAACATCACCTACCAC-3'	5'-GGTACTCGATGATGGTGTG-3'
Fibronectin	5'-TCTGGGATCAAAGGGAAACA-3'	5'-ACAGTGCTGCAGATCAGACG-3'
<i>COL10</i>	5'-ATCTCGGAAAGATGCAGGAG-3'	5'-TGTGAACCATGGAGTGATGC-3'
Aggrecan	5'-GATGTCCCCTGCAATTACCA-3'	5'-TCTGTGCAAGTGATTTCGAGG-3'
Osteocalcin	5'-GCATTCTGCCTCTCTGACCT-3'	5'-CTAAACGGTGGTGCCATAGA-3'
<i>TGFβ1</i>	5'-ATCCCTTTTGTGAGCCTGTG-3'	5'-ACTGAAGCGAAAGCCCTGTA-3'
<i>BMP2</i>	5'-TGAACACAGCTGGTCTCAGG-3'	5'-TTAAGACGCTTCCGCTGTTT-3'
<i>BMP7</i>	5'-GTAGCGCGTAGAGCCG-3'	5'-CGAGTCCGTGCAIGG-3'
<i>CDMP1</i>	5'-AGAAGCTGGGGCTATGTCCT-3'	5'-ATCGCACTCGTAGCTCTCGT-3'
<i>FGF2</i>	5'-CGGTACCTGGCTATCAAGGA-3'	5'-CCAGGCGTTCAAAGAAGAAA-3'
<i>IGF1</i>	5'-GCCCTGCCCTTTCTTAACAT-3'	5'-CCACCCAGTTGCTATTGCTT-3'
<i>Sox9</i>	5'-CACTGGGAACAACCCGTCTA-3'	5'-AGGTCTCCTCAGGGTCTGGT-3'
<i>IL1β</i>	5'-CCTCTGACAGGCAACCACTT-3'	5'-CATCCCATACACACGGACAA-3'
<i>TNF</i>	5'-TCACAAGGCTGCTGAAGATG-3'	5'-CAGTCTGGGAAGCTCTGAGG-3'
<i>ADAMTS4</i>	5'-AGGCAGTGATGTGTTGGTGA-3'	5'-GGGTAGAGGGGGCAGTTAAA-3'
<i>ADAMTS5</i>	5'-GGCTATTAGCCACAAAGGA-3'	5'-GCTGTGGCTAATGACGGAAT-3'
<i>MMP13</i>	5'-CCCTTTTCAGACCACCCTTT-3'	5'-CAGGGAAGGGGCTAATGAAC-3'

Wayne's macroscopic scale are shown in Table 4. The results show that the total values of each group improved from 4 weeks to 12 weeks. However, at 12 weeks, there was no significant difference among these groups.

Histological evaluation

In the groups treated with a chondrocyte sheet, the area of Safranin-O staining in the regenerated tissues was larger than that in all other groups except the sham group. Especially in the triple-layered chondrocyte sheet group, larger areas of hyaline-like cartilage and good integration of the surrounding cartilage were observed compared with other groups (Fig. 4A). Assessment using Pineda's semiquantitative scale revealed that cartilage repair was significantly better in the chondrocyte sheet groups than that in the synovial cell sheet groups at 4 weeks ($p < 0.05$). Furthermore, at 12 weeks after transplantation, it revealed that cartilage repair in the triple-layered chondrocyte sheet group was better than in any other group ($p < 0.05$) (Fig. 4B). The immunohistochemical staining also showed better expression of type II collagen in the repaired tissue of the triple-layered chondrocyte sheet group at 12 weeks after transplantation. The categorical values of Pineda's semiquantitative scale are shown in Table 5.

Gene expression of the cell sheets *in vitro* and the repaired tissues *in vivo*

When the chondrocyte sheets and synovial cell sheets were compared *in vitro*, relative expression levels of *COL1*, *FGF2*, *IGF1*, and *ADAMTS4* were significantly higher in the synovial cell sheet group than in the chondrocyte sheet group ($p < 0.05$) (Fig. 5). However, when regenerated tissues were compared between rats treated by chondrocyte sheet transplantation and those treated by synovial cell sheet transplantation *in vivo*, the relative expression level of *TGFβ1* was significantly higher in the chondrocyte sheet group than in the synovial cell sheet group ($p < 0.05$). By contrast, the relative expression levels of *COL1*, *FGF2*, *IGF1*, *ADAMTS4*, and *ADAMTS5* were significantly higher in the synovial cell sheet

group than in the chondrocyte sheet group ($p < 0.05$) (Fig. 6). In short, the expression levels of *TGFβ1* and *ADAMTS5* were different between *in vitro* and *in vivo* conditions.

Cell tracking study using GFP transgenic rats

In both groups, regenerated cartilage-like tissues, containing spherical cells and extracellular matrix with good Safranin-O staining could be observed. Under the fluorescent microscope, GFP-positive cells could be observed in the region of regenerated subchondral bone but almost none were found in the repaired hyaline-like cartilage area in group I (Fig. 7). In contrast, GFP-positive cells were found to correspond closely to the repaired cartilage-like area in group II (Fig. 8). These results suggest that the majority of regenerated hyaline-like cartilage consists of cells derived from the transplanted chondrocyte sheets.

Discussion

This study demonstrates that covering osteochondral defects with chondrocyte sheets but not synovial cell sheets promotes cartilage repair, and that the regenerated hyaline-like cartilage is largely composed of cells derived from transplanted chondrocyte sheets.

Previously, some studies tried to investigate cartilage regeneration in cartilage defects covered with periosteum or artificial membrane. The transplantation of periosteum was reported to be effective for cartilage repair in some studies.^{15,18} Zantop and Petersen demonstrated cartilage regeneration with microfracture when a large chondral defect was covered by resorbable polymer felt and sodium hyaluronan.¹⁹ However, a previous study in our group showed that covering defects with atelocollagen membrane is not effective for cartilage repair but induces osteogenesis.²⁰ Therefore, the effect of the coverage of cartilage defects on cartilage repair remains controversial. In this study, although covering the defects with chondrocyte sheets led to marked cartilage repair, coverage with synovial cell sheets was not so effective. If cartilage defects are mainly repaired as a result of coverage

FIG. 3. (A) Macroscopic findings of the distal femur at 4 and 12 weeks after operation. (1) Sham model: arthrotomy only. (2) Control model: creation of an osteochondral defect without transplantation. (3) Transplantation of a monolayer chondrocyte sheet. (4) Transplantation of a triple-layered chondrocyte sheet. (5) Transplantation of a monolayer synovial cell sheet. (6) Transplantation of a triple-layered synovial cell sheet. (B) Assessment of the five experimental groups using a semiquantitative macroscopic scoring system. There was a significant difference between the triple-layered chondrocyte sheet model and the monolayer synovial cell sheet model at 4 weeks after transplantation.

FIG. 4. (A) Histological findings with Safranin-O/Fast green staining and immunohistochemical staining by type II collagen at 4 and 12 weeks after operation. (1) Sham model: arthrotomy only (2) Control model: creation of an osteochondral defect without transplantation. (3) Transplantation of a monolayer chondrocyte sheet. (4) Transplantation of a triple-layer chondrocyte sheet. (5) Transplantation of a monolayer synovial sheet. (6) Transplantation of a triple-layered synovial sheet. Scale bar=500 μ m (B) Assessment of the five experimental groups using Pineda's semiquantitative scale. At 4 weeks, the cartilage repair was significantly better in the chondrocyte sheet groups than in the synovial sheet groups ($p<0.05$). At 12 weeks, the cartilage repair was significantly better in the triple-layered chondrocyte sheet group than in any of the other groups ($p<0.05$).

TABLE 4. RESULTS OF GROSS APPEARANCE SCALE

	Coverage		Neocartilage color		Defect margins		Surface		Total	
	4 week	12 week	4 week	12 week	4 week	12 week	4 week	12 week	4 week	12 week
Control	3.33±0.52	3.33±0.82	2.17±0.41	2.17±0.75	0.67±0.52	1.50±1.05	1.50±0.84	2.00±1.10	7.67±1.75	9.00±3.92
Monolayer chondrocyte sheet	2.83±0.75	2.83±0.98	1.83±0.98	2.17±0.98	1.17±0.98	1.67±1.51	2.17±0.98	2.33±1.03	8.00±3.03	9.00±4.24
Triple-layered chondrocyte sheet	3.33±0.52	3.33±0.82	2.33±0.52	2.83±0.75	2.17±0.41	2.83±0.75	2.33±0.82	2.17±0.41	10.2±1.94	11.2±2.48
Monolayer synovial cell sheet	2.33±1.03	2.83±0.75	1.83±0.75	1.83±0.75	0.17±0.41	1.33±1.51	1.33±0.52	2.17±0.98	5.67±2.16	8.17±3.87
Triple-layered synovial cell sheet	2.16±1.17	2.66±0.52	2.00±0.63	2.17±0.41	0.33±0.52	1.17±0.75	1.50±0.55	2.33±0.82	6.00±1.79	8.33±1.75

Values are the mean±SD.

TABLE 5. RESULTS OF PINEDA'S HISTOLOGICAL GRADING SCALE

	Filling of defect		Reconstitution of osteochondral junction		Matrix staining		Cell morphology		Total	
	4 week	12 week	4 week	12 week	4 week	12 week	4 week	12 week	4 week	12 week
Control	2.00±1.55	0.67±0.52	1.83±0.41	2.00±0.00	3.00±0.63	3.50±0.55	2.50±0.55	2.83±0.98	9.33±2.42	9.00±1.90
Monolayer chondrocyte sheet	0.33±0.52	1.50±1.38	1.33±0.52	1.50±0.55	1.50±0.84	2.50±1.05	1.50±0.55	2.00±0.63	4.67±1.50	7.50±3.08
Triple-layered chondrocyte sheet	0.17±0.41	0.17±0.41	1.00±0.63	1.00±0.00	0.67±0.82	0.50±0.55	1.00±0.63	1.17±0.75	2.83±2.32	2.13±1.87
Monolayer synovial cell sheet	2.00±1.10	1.83±1.17	2.00±0.00	2.00±0.00	3.00±0.63	3.50±0.55	3.50±0.55	3.00±0.63	10.2±2.23	10.3±2.07
Triple-layered synovial cell sheet	0.67±0.82	0.33±0.52	2.00±0.00	2.00±0.00	3.50±0.84	2.83±0.98	2.83±0.75	2.33±0.52	9.00±1.90	7.50±1.52

Values are the mean±SD.

FIG. 5. Comparison of gene expression between triple-layered chondrocyte sheets and triple-layered synovial sheets *in vitro*, analyzed by real-time PCR. The expression of *COL1*, *FGF2*, *IGF1*, and *ADAMTS4* was significantly higher in the synovial sheet group than in the chondrocyte sheet group ($p < 0.05$).

causing the accumulation of bone marrow cells and protection against the catabolic factors in synovial fluid, articular cartilage should be repaired when covered not only by chondrocyte sheets but also by synovial cell sheets. These findings suggest that simply covering the cartilage defect might not be the main mechanism of cartilage repair.

In this study, gene expression in the chondrocyte or synovial cell sheets *in vitro* and in regenerated tissues *in vivo* was analyzed to investigate production of paracrine factors from cell sheets that could contribute to cartilage repair. Previous studies

also reported gene expression in chondrocyte sheets. Mitani *et al.* investigated the properties of chondrocyte sheets and showed that expression of Sox 9, collagen type 2 and 27, integrin $\alpha 10$, and fibronectin were higher in layered chondrocyte sheets than in a single chondrocyte sheet *in vitro*.²¹ In addition, Hamahashi *et al.* showed that layered chondrocyte sheets produced higher levels of TGF β and PGE2 as humoral factors compared with monolayer chondrocyte sheets and suspected that these factors might contribute to cartilage repair.²² However, these previously reported results might not

FIG. 6. Comparison of gene expression in the regenerative tissue at 1 week after transplantation *in vivo* between the triple-layered chondrocyte sheet and the triple-layered synovial sheet, analyzed by real-time PCR. Expression of *TGFβ1* was significantly higher in the chondrocyte sheet group than in the synovial sheet group ($p < 0.05$). In contrast, the expression of *COL1*, *FGF2*, *IGF1*, *ADAMTS4*, and *ADAMTS5* was significantly higher in the synovial sheet group than in the chondrocyte sheet group ($p < 0.05$).

FIG. 7. In Group I, the cartilage defects created in GFP transgenic rats were treated by covering them with a transplanted triple-layered chondrocyte sheet from control SD rats. (A, D) The repaired tissue, strongly stained by Safranin-O, almost filled the defect. (B, E) Under a fluorescent microscope, the GFP signal was negative in the area corresponding to the repaired tissue, while a positive GFP signal was observed in the region of the repaired subchondral bone and in other tissue of the recipient GFP rat. (C, F) Under a fluorescent microscope with DAPI nucleic acid stain, the *blue* stained cells were observed in the repaired tissue. (A–C) are low-power fields (Scale bar = 500 μ m). (D–F) are high-power fields (Scale bar = 100 μ m).

accurately reflect *in vivo* phenomena, because all these data were collected from cell sheets *in vitro*. In this study, we investigated gene expression in triple-layered chondrocyte and synovial cell sheets not only *in vitro* but also *in vivo*. We found that gene expression patterns of the cell sheets were a little different between *in vitro* and *in vivo* conditions. In particular, the expression of *TGF β 1* in chondrocyte sheets did not differ significantly from that of synovial sheets *in vitro*. In contrast, *in vivo*, *TGF β 1* expression was higher in chondrocyte sheets than in synovial cell sheets. *TGF β 1* is well known as an es-

sential factor for chondrogenesis and has been reported to contribute to chondrogenesis both *in vitro* and *in vivo*.²³ These results indicate that *TGF β 1* expression in chondrocyte sheets could have been activated *in vivo* and might play an important role in cartilage repair. On the other hand, the expression of *FGF2*, *IGF1*, and *ADAMTS* family members was at a higher level in synovial cell sheets than in chondrocyte sheets both *in vitro* and *in vivo*. Since *FGF2* and *IGF1* have been reported to be growth factors which enhance the proliferation or differentiation of chondrocytes and their progenitors, it could be

FIG. 8. In Group II, the cartilage defects created in the SD rats were treated by covering them with a transplanted triple-layered chondrocyte sheet from GFP transgenic rats. (A, D) The repaired tissue, which was strongly stained by Safranin-O, almost filled the defect. (B, E) Under a fluorescent microscope, the positive GFP signal corresponded closely to the region of repaired cartilage. The GFP signal was negative in a few areas of repaired tissue. (C, F) Under a fluorescent microscope with DAPI nucleic acid stain, *blue* stained cells were observed in the repaired tissue, corresponding to a positive GFP signal. (A–C) are low-power fields (Scale bar = 500 μ m). (D–F) are high-power fields (Scale bar = 100 μ m).

expected that high expression of these factors contributes to repair of a cartilage defect.^{24,25} However, *FGF2* may also be disadvantageous for cartilage formation because of its pro-angiogenic and pro-osteogenic potential. Miyakoshi *et al.* demonstrated that intra-articular administration of *FGF2* induced inflammation and osteophyte formation.²⁶ Simultaneously, the expression of both *ADAMTS4* and *ADAMTS5*, which are known as aggrecanases and contribute to cartilage destruction, is at a higher level in synovial cell sheets.²⁷ These differences in the expression of various factors between chondrocyte sheets and synovial cell sheets might be one possible explanation for the difference in the effects of these cell sheets on cartilage repair.

Elucidation of the cell origin of repaired cartilage is one of the important questions that needs to be answered to understand the mechanisms of cartilage regeneration. It is thought that several types of cells around the defect could contribute to the regenerated tissue, because there are many different types of progenitor cells in the synovial joint compartment.²⁸ Recent studies have demonstrated that the superficial layer of articular cartilage contains cells with progenitor characteristics.²⁹ In addition, intra-articular tissues, such as synovium and infrapatellar fat pad, have also been demonstrated to contain mesenchymal stem cells.^{30,31} Bone marrow-derived mesenchymal stem cells could also migrate to the osteochondral defect area. In the case of transplanted chondrocytes as a cell sheet, it was reported that the transplanted cells survived and could be detected for more than 21 months by *in vivo* imaging.³² In this study, cell tracking experiments using GFP transgenic rats revealed that the regenerated hyaline cartilage-like tissues consisted largely of cells derived from the transplanted chondrocyte sheets. We suspect that differences in differentiation capacity between the chondrocyte sheets and synovial cell sheets as a cell source could be responsible for the difference in the effects of these cell sheets on cartilage repair. However, it was reported that synovial tissue contains the synovial mesenchymal stem cells which has chondrogenic differentiation potential and cartilage repair was previously demonstrated by transplantation of synovial mesenchymal stem cells.^{33,34} In this study, if we used mesenchymal stem cells as a cell source, cartilage repair might be promoted.

This study has some limitations. The first limitation is that all experimental models were allotransplantation models. The difference in immune reactions between chondrocytes and synovial cells can induce a difference in repair among those models. According to the GFP rat histology results, transplanted chondrocytes were still alive at 12 weeks after transplantation. Previous studies have demonstrated good repair using cell sheet engineering with animal allotransplantation models. However, there are no clinical data regarding cell sheet allografts. Chondrocyte sheet allografts might be feasible clinically after overcoming allograft-related problems, including immune response and infection. The second limitation is that these defects were made in the rat trochlear groove which is the most conducive model for good repair. However, our control model did not show good repair at 4 and 12 weeks after transplantation. We thought that this model was not conducive to self-repair because of its deep osteochondral defect. The third limitation is the limited evaluation of the repaired tissue. In this study, macroscopic and histological evaluation were performed using the semi-

quantitative scale. We have to take into consideration that these evaluations are likely to be subjective. This study's repaired tissue with its transplantation by the triple-layered chondrocyte sheet did not resemble normal cartilage. Alternative evaluations are necessary, not only histologically but also functionally. Although the results of an ongoing clinical trial using autograft chondrocyte sheet transplantation are yet to be published, when confirmed they will provide more information for cell sheet engineering.

According to our histological analysis of the cell sheets, the thickness of the triple-layered chondrocyte sheet is about 100 μm (Fig. 2). In this study, we have demonstrated that osteochondral defects of 1000 μm in depth are filled with repaired tissue with good staining with Safranin-O and type-2 collagen after being covered with a chondrocyte sheet. In other words, the transplanted chondrocyte sheet is able to fill a defect that is ten times its thickness even without the need for a scaffold. Moreover, even thin chondrocyte sheets could be a cell source for the treatment of full-thickness cartilage defects. However, the repaired tissue far from resembles normal articular cartilage, because of the low level of the subchondral bone along with the fact that osteochondral junction is still not reconstructed. We surmise that these issues would be resolved by improvement in the subchondral bone level, which is a matter of time. Although further investigation is necessary, cell sheet engineering might have a positive impact on clinical application of cartilage regenerative cellular therapy, because chondrocyte sheets can be applied to articular cartilage defects with an area larger than the area of the actual defects, which in the past has been solely treated with conventional thickness tissue-engineered cartilage along with scaffold materials.

Conclusions

Transplantation of chondrocyte sheets but not synovial cell sheets contributes to the repair of full-thickness cartilage defects when used as the cell source. In addition, the high level of expression of *TGF β 1* in chondrocyte sheets *in vivo* compared with that in synovial cell sheets might also participate in the promotion of cartilage repair.

Disclosure Statement

No competing financial interests exist.

References

- Schindler, O.S. Current concepts of articular cartilage repair. *Acta Orthop Belg* **77**, 709, 2011.
- Minas, T. A primer in cartilage repair. *J Bone Joint Surg Br* **94**, 141, 2012.
- Ochi, M., Uchio, Y., Kawasaki, K., Wakitani, S., and Iwasa, J. Transplantation of cartilage-like tissue made by tissue engineering in the treatment of cartilage defects of the knee. *J Bone Joint Surg Br* **84B**, 571, 2002.
- Okano, T., Yamada, N., Sakai, H., and Sakurai, Y. A novel recovery system for cultured cells using plasma-treated polystyrene dishes grafted with poly(N-isopropylacrylamide). *J Biomed Mater Res* **27**, 1243, 1993.
- Sawa, Y., Miyagawa, S., Sakaguchi, T., Fujita, T., Matsuyama, A., Saito, A., *et al.* Tissue engineered myoblast sheets improved cardiac function sufficiently to discontinue LVAS in a patient with DCM: report of a case. *Surg Today* **42**, 181, 2012.

6. Nishida, K., Yamato, M., Hayashida, Y., Watanabe, K., Yamamoto, K., Adachi, E., *et al.* Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. *N Engl J Med* **351**, 1187, 2004.
7. Yamato, M.K.V. Long-term results of cultured autologous oral mucosa epithelial cell-sheet (CAOMECS) graft for the treatment of blindness due to bilateral limbal stem cell deficiency. *J Stem Cell Res Ther* 2014. DOI: 10.4172/2157-7633.1000181.
8. Ebihara, G., Sato, M., Yamato, M., Mitani, G., Kutsuna, T., Nagai, T., *et al.* Cartilage repair in transplanted scaffold-free chondrocyte sheets using a minipig model. *Biomaterials* **33**, 3846, 2012.
9. Ito, S., Sato, M., Yamato, M., Mitani, G., Kutsuna, T., Nagai, T., *et al.* Repair of articular cartilage defect with layered chondrocyte sheets and cultured synovial cells. *Biomaterials* **33**, 5278, 2012.
10. Kaneshiro, N., Sato, M., Ishihara, M., Mitani, G., Sakai, H., and Mochida, J. Bioengineered chondrocyte sheets may be potentially useful for the treatment of partial thickness defects of articular cartilage. *Biochem Biophys Res Commun* **349**, 723, 2006.
11. Fortier, L.A., Barker, J.U., Strauss, E.J., McCarrel, T.M., and Cole, B.J. The role of growth factors in cartilage repair. *Clin Orthop Relat Res* **469**, 2706, 2011.
12. Haraguchi, Y., Shimizu, T., Sasagawa, T., Sekine, H., Sakaguchi, K., Kikuchi, T., *et al.* Fabrication of functional three-dimensional tissues by stacking cell sheets *in vitro*. *Nat Protoc* **7**, 850, 2012.
13. Wayne, J.S., McDowell, C.L., Shields, K.J., and Tuan, R.S. *In vivo* response of polylactic acid-alginate scaffolds and bone marrow-derived cells for cartilage tissue engineering. *Tissue Eng* **11**, 953, 2005.
14. Pineda, S., Pollack, A., Stevenson, S., Goldberg, V., and Caplan, A. A semiquantitative scale for histologic grading of articular cartilage repair. *Acta Anat* **143**, 335, 1992.
15. Shinomiya, R., Ochi, M., Adachi, N., Hachisuka, H., Natsu, K., and Yasunaga, Y. The cellular origin of cartilage-like tissue after periosteal transplantation of full-thickness articular cartilage defects: an experimental study using transgenic rats expressing green fluorescent protein. *Acta Orthop* **76**, 920, 2005.
16. Oshima, Y., Watanabe, N., Matsuda, K., Takai, S., Kawata, M., and Kubo, T. Behavior of transplanted bone marrow-derived GFP mesenchymal cells in osteochondral defect as a simulation of autologous transplantation. *J Histochem Cytochem* **53**, 207, 2005.
17. Miyamoto, A., Deie, M., Yamasaki, T., Nakamae, A., Shinomiya, R., Adachi, N., *et al.* The role of the synovium in repairing cartilage defects. *Knee Surg Sports Traumatol Arthrosc* **15**, 1083, 2007.
18. O'Driscoll, S.W. Articular cartilage regeneration using periosteum. *Clin Orthop Relat Res* **S186**, 1999.
19. Zantop, T., and Petersen, W. Arthroscopic implantation of a matrix to cover large chondral defect during microfracture. *Arthroscopy* **25**, 1354, 2009.
20. Hamanishi, M., Nakasa, T., Kamei, N., Kazusa, H., Kamei, G., and Ochi, M. Treatment of cartilage defects by subchondral drilling combined with covering with atelocollagen membrane induces osteogenesis in a rat model. *J Orthop Sci* **18**, 627, 2013.
21. Mitani, G., Sato, M., Lee, J.I., Kaneshiro, N., Ishihara, M., Ota, N., *et al.* The properties of bioengineered chondrocyte sheets for cartilage regeneration. *BMC Biotechnol* **9**, 17, 2009.
22. Hamahashi, K., Sato, M., Yamato, M., Kokubo, M., Mitani, G., Ito, S., *et al.* Studies of the humoral factors produced by layered chondrocyte sheets. *J Tissue Eng Regen Med* 2012. DOI: 10.1002/term.1610.
23. Madry, H., Rey-Rico, A., Venkatesan, J.K., Johnstone, B., and Cucchiari, M. Transforming growth factor Beta-releasing scaffolds for cartilage tissue engineering. *Tissue Eng Part B Rev* **20**, 106, 2014.
24. Fortier, L.A., Mohammed, H.O., Lust, G., and Nixon, A.J. Insulin-like growth factor-I enhances cell-based repair of articular cartilage. *J Bone Joint Surg Br* **84**, 276, 2002.
25. Stewart, A.A., Byron, C.R., Ponden, H., and Stewart, M.C. Effect of fibroblast growth factor-2 on equine mesenchymal stem cell monolayer expansion and chondrogenesis. *Am J Vet Res* **68**, 941, 2007.
26. Miyakoshi, N., Kobayashi, M., Nozaka, K., Okada, K., Shimada, Y., and Itoi, E. Effects of intraarticular administration of basic fibroblast growth factor with hyaluronic acid on osteochondral defects of the knee in rabbits. *Arch Orthop Trauma Surg* **125**, 683, 2005.
27. Tortorella, M.D., Malfait, A.M., Deccico, C., and Arner, E. The role of ADAM-TS4 (aggrecanase-1) and ADAM-TS5 (aggrecanase-2) in a model of cartilage degradation. *Osteoarthritis Cartilage* **9**, 539, 2001.
28. Yoshimura, H., Muneta, T., Nimura, A., Yokoyama, A., Koga, H., and Sekiya, I. Comparison of rat mesenchymal stem cells derived from bone marrow, synovium, periosteum, adipose tissue, and muscle. *Cell Tissue Res* **327**, 449, 2007.
29. Dowthwaite, G.P., Bishop, J.C., Redman, S.N., Khan, I.M., Rooney, P., Evans, D.J., *et al.* The surface of articular cartilage contains a progenitor cell population. *J Cell Sci* **117**, 889, 2004.
30. Buckley, C.T., Vinardell, T., Thorpe, S.D., Haugh, M.G., Jones, E., McGonagle, D., *et al.* Functional properties of cartilaginous tissues engineered from infrapatellar fat pad-derived mesenchymal stem cells. *J Biomech* **43**, 920, 2010.
31. De Bari, C., Dell'Accio, F., Tylzanowski, P., and Luyten, F.P. Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis Rheum* **44**, 1928, 2001.
32. Takaku, Y., Murai, K., Ukai, T., Ito, S., Kokubo, M., Satoh, M., *et al.* *In vivo* cell tracking by bioluminescence imaging after transplantation of bioengineered cell sheets to the knee joint. *Biomaterials* **35**, 2199, 2014.
33. Shirasawa, S., Sekiya, I., Sakaguchi, Y., Yagishita, K., Ichinose, S., and Muneta, T. *In vitro* chondrogenesis of human synovium-derived mesenchymal stem cells: optimal condition and comparison with bone marrow-derived cells. *J Cell Biochem* **97**, 84, 2006.
34. Suzuki, S., Muneta, T., Tsuji, K., Ichinose, S., Makino, H., Umezawa, A., *et al.* Properties and usefulness of aggregates of synovial mesenchymal stem cells as a source for cartilage regeneration. *Arthritis Res Ther* **14**, R136, 2012.

Address correspondence to:

Ryo Shimizu, MD

Department of Orthopaedic Surgery

Graduate School of Biomedical & Health Sciences

Hiroshima University

1-2-3 Kasumi, Minami-ku

Hiroshima 734-8551

Japan

E-mail: d123437@hiroshima-u.ac.jp

Received: May 27, 2014

Accepted: November 29, 2014

Online Publication Date: December 23, 2014