Articular Cartilage Regeneration Using Cell Sheet Technology

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ABSTRACT

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 in a temperature-responsive culture dish. We previously reported achieving robust tissue repair when covering only the surface layer of partial-thickness defects with layered chondrocyte sheets in domestic rabbits. We also reported good Safranin O staining and integration with surrounding tissue in a minipig model of full-thickness cartilaginous defects in the knee joint. We have continued our studies using human chondrocytes obtained from patients under IRB approval, and have confirmed the safety and efficacy of chondrocyte sheets, and have submitted a report to the Ministry of Health, Labour, and Welfare in Japan. In 2011, the Ministry gave us approval to perform a clinical study of joint repair using cell sheets. We have just started implanting cell sheets in patients at Tokai University Hospital. Anat Rec, 297:36–43, 2014. © 2013 Wiley Periodicals, Inc.

Key words: cell sheet; articular cartilage; tissue engineering; regenerative medicine; temperature responsive culture dish

Articular cartilage is hyaline cartilage characterized by a compact collagen network and an extracellular matrix made up of proteoglycan, and is highly resistant to mechanical loads. However, articular cartilage possesses a limited capacity for complete repair (Paget, 1969). No meaningful repair of the cartilage occurs in partial-thickness lesions that are limited within the cartilage and leads to osteoarthritis (OA). OA, one of the most common joint diseases, is characterized by a slow degradation of cartilage for a long time. As society matures, as for much attention is being focused on OA prevention and countermeasures.

At present, treatments for osteochondral defects have included to date: microfracture (Steadman et al., 2001, 2002; Mithoefer et al., 2006), mosaicplasty (Hangody et al., 1997, 2001; Szerb et al., 2005), cell transplantation (Brittberg et al., 1994; Peterson et al., 2003; Zaslav et al., 2009; Moseley et al., 2010), and implantation of tissue-engineered cartilage with various scaffold materials (Buckwalter and Lohmander, 1994; Freed et al., 1994; Hunziker, 2002; Darling and Athanasiou, 2003; Marcacci et al., 2005) or without scaffold (Mainil-Varlet et al., 2001; Brehm et al., 2006; Park et al., 2006; Nagai et al., 2008a,b) have been developed to overcome this obstacle (Nagai et al.,

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Received 13 September 2013; Accepted 13 September 2013.
DOI 10.1002/ar.22829
Published online 2 December 2013 in Wiley Online Library (wileyonlinelibrary.com).

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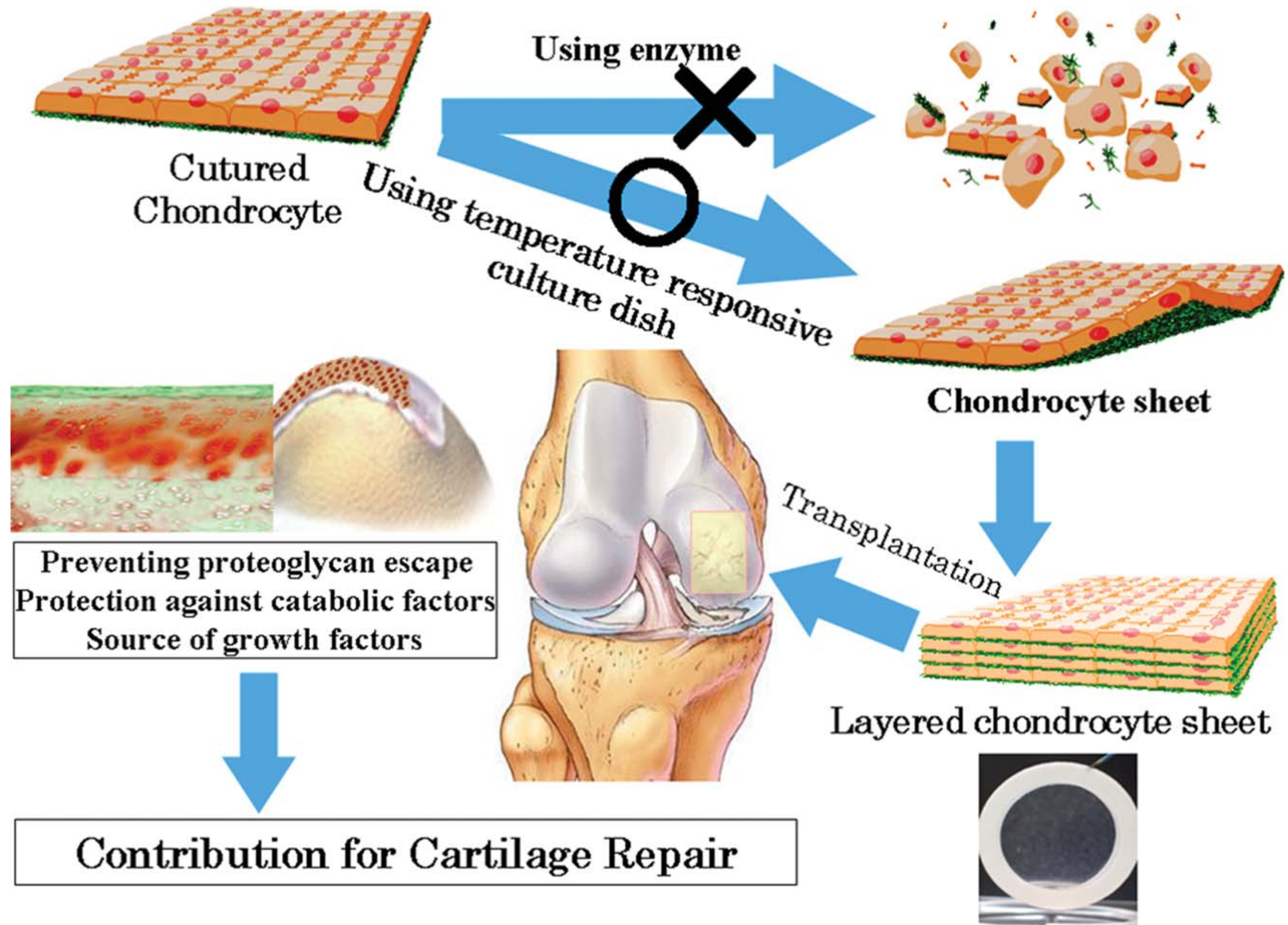

Fig. 1. Regenerative articular cartilage using layered chondrocyte sheets. Our goal is to contribute to the regenerative medicine of articular cartilage using layered chondrocyte sheets. These sheets display excellent adhesiveness and the ability to repair cartilage.

2010). Drilling and microfracture methods which promote to collect reparative cells from bone marrow are the methods that stimulate spontaneous healing (Buckwalter and Lohmander, 1994).

Autologous chondrocyte implantation (ACI), first reported by Brittberg et al. (1994), has been performed for over 20,000 patients worldwide. Normally, an osteochondral defect will induce the production of marrow-derived repair cells (Solchaga et al., 2000). Osteochondral defects are generally thought to be ultimately replaced by subchondral bone after infiltration by blood vessels during endochondral ossification of chondrocytes from multipotent, marrow-derived mesenchymal stem cells (MSCs) (Shapiro et al., 1993; Caplan et al., 1997). Nagai et al. (2008a,b) fabricated tissue-engineered cartilage without a scaffold and reported that “chondrocyte plates” were effective at repairing tissue in animal experiments. Among the established treatments, the preferred mode of treatment is ACI, which requires harvesting healthy articular cartilage cells from the patient and consequently causes donor site morbidity. Despite the promising results brought about by advances in tissue engineering, various limitations of ACI remain unsolved, including adverse events derived from periostium, a lengthy cell expansion period, and the cell delivery system. Improving ACI effectiveness and efficiency as a lifelong therapy for OA requires modifications of the current techniques and identification of other candidate cells for cartilage regeneration.

Cell sheet technology using temperature-responsive culture dishes was first reported by Okano et al., (1993) and is now used widely in corneal, myocardial, hepatic, and other regenerative medicine fields (Kushida et al., 2000; Harimoto et al., 2002; Nishida et al., 2004; Shimizu et al., 2006; Hamahashi et al., in press). Nishida et al. (2004) reported that corneal cell sheets fabricated using these dishes could adhere strongly to the cornea. Kushida et al. (2000) reported that the multilayered, three-dimensional tissue structures can be created without the need for scaffolds because cell sheets have intact extracellular matrix (ECM) and adhesion factors. Shimizu et al. (2006) reported that the maximum thickness of the fabricated rabbit myocardial cell sheet is three layers *in vitro* because thicker sheets receive inadequate nutrition. They also demonstrated that repetitive allografts of cell sheets *in vivo* could not increase the thickness by more than 1 mm in myocardial tissues (Hamahashi et al., 2012).

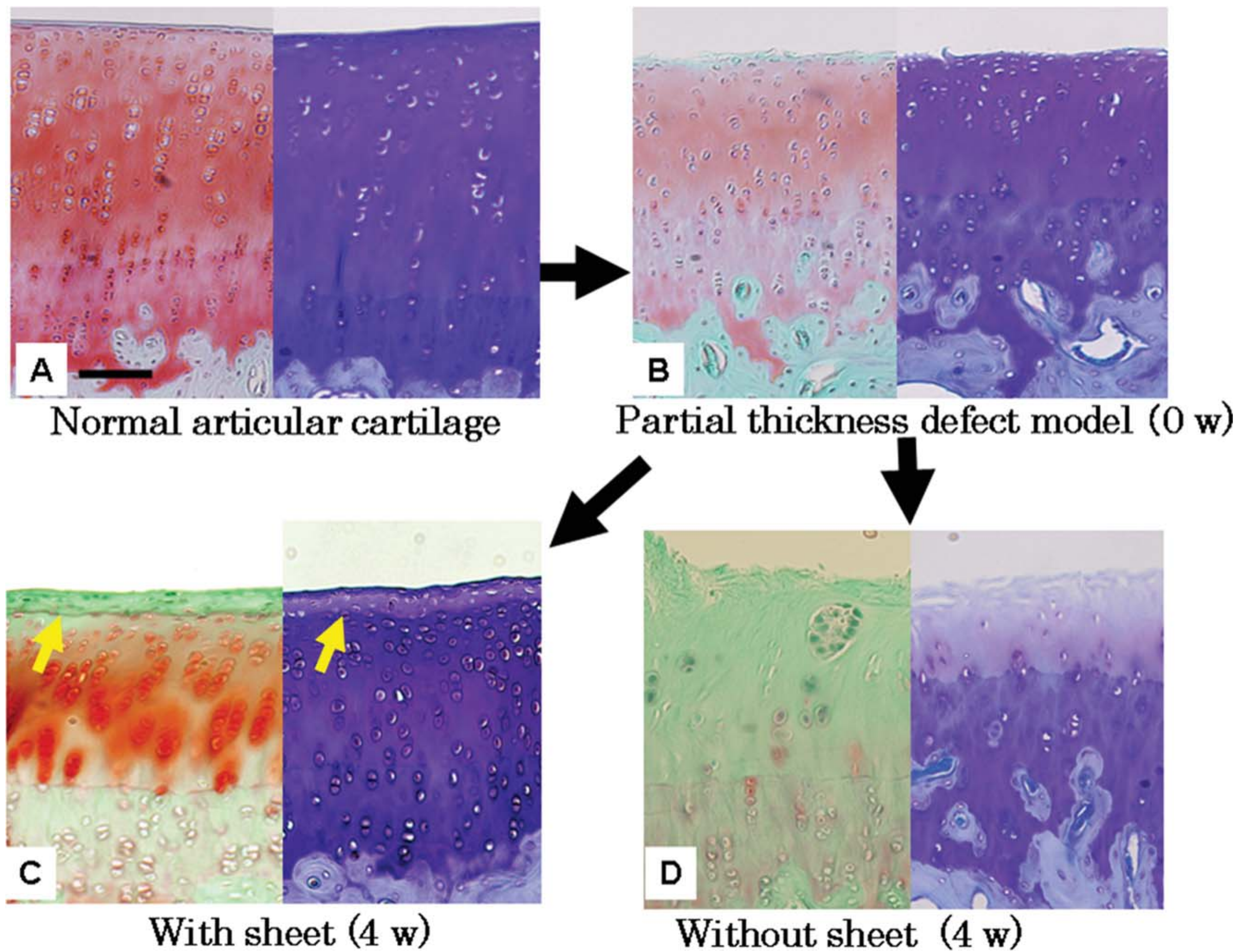

Fig. 2. Partial thickness defect model of rabbit. This is the therapeutic effect on a partial thickness defect. This partial-thickness defect model was produced by removing the surface layer of healthy cartilage. After 4weeks, in the absence of a grafted chondrocyte sheet, proteoglycans in the cartilage matrix flow out, causing cartilage degeneration that leads to OA. However, the matrix was maintained and degeneration failed to proceed in the transplantation group.

Using this innovational technology, we demonstrated chondrocytes sheets with a consistent cartilaginous phenotype and adhesive properties may lead to a new strategy for cartilage regeneration (Mitani et al., 2009) (Fig. 1).

CHONDROCYTE SHEET FOR PARTIAL THICKNESS DEFECT OF ARTICULAR CARTILAGE

The importance of the treatments and prophylaxes for OA is increasing due to the progressively aging society. However, we only have a few conservative therapies at this time, such as non-steroidal anti-inflammatory drugs (NSAIDs) administration and the injection of hyaluronan. Namely, there is still no means to prevent future exacerbations of cartilage degeneration (Kaneshiro et al., 2006). OA contains the partial-thickness defect of cartilage and the osteochondral defect (total thickness defect). Most regenerative therapies are aimed at the small osteochondral defect. Kaneshiro et al. (2006) reported the effects of the layered chondrocyte sheet on a partial-thickness defect in the rabbit. In that study, they allografted the three-layered chondrocyte sheet to repair the defect. This partial-thickness defect model was produced by removing the surface layer from healthy cartilage. The articular cartilage of the medial femoral condyle of Japanese White rabbits weighing about 3 kg was removed to a depth of less than 1 mm using a file to prepare a model of partial thickness cartilage damage. The damaged cartilage was covered with a three-layered chondrocyte sheet, which was stabilized with a nylon suture until the initial fixation was achieved. This was done in four knees of two rabbits as the transplantation group. At the same time, the articular cartilage of the medial femoral condyle was similarly filed, but not covered with a cell sheet, in four knees of the two rabbits (the control group) (Kaneshiro et al., 2006). After four weeks, in the absence of a grafted chondrocyte sheet, the proteoglycans in the cartilage

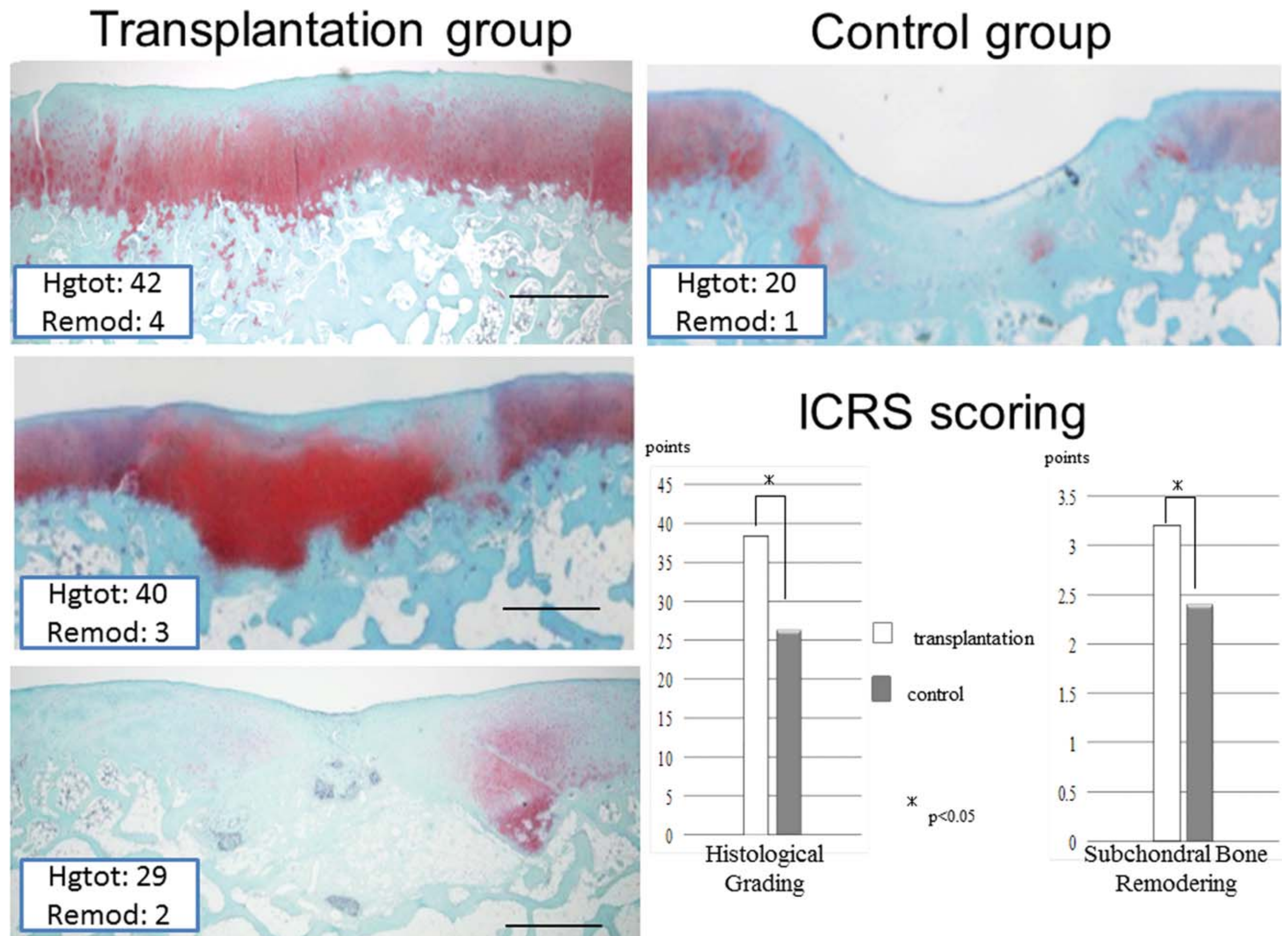

Fig. 3. Total thickness defect of minipig model. In most cases of the transplantation group, the ICRS histological grade was over 35 points, and the remodeling score was over 3 points. The mean histological grade and remodeling score were significantly higher in the transplantation group than in the control group.

matrix flow out, causing cartilage degeneration, which leads to OA. However, the matrix was maintained and degeneration failed to proceed in the transplantation group (Fig. 2). In this study, they confirmed that chondrocytes could be harvested as sheets and thus be made into multilayered "tissue" by culturing in temperature-responsive dishes and then be collected using a temperature recovery system.

Based on the above mentioned facts, they suggest that the use of bioengineered chondrocyte sheets may be potentially useful to treat partial thickness defects of articular cartilage. Because the chondrocyte sheets have good adhesion and barrier function which protect against intra-articular catabolic factors, supplying the growth factors (Kaneshiro et al., 2006).

CHONDROCYTE SHEET FOR TOTAL THICKNESS DEFECT (OSTEOCHONDRAL DEFECT) OF ARTICULAR CARTILAGE

Cartilage repair using synovial cell grafts has been carried out. Hunziker et al. have reported that synovial cells played an important role in the repair of the cartilage defects (Hunziker and Rosenberg, 1996), and Koga et al. (2008) have created osteochondral defects in rabbit knee joints and reported good results from grafts of synovium derived mesenchymal stem cells used in conjunction with periosteum. However, Ando et al. (2007) investigated repair of articular cartilage using chondrocytes and found that the superficial layers of the repaired tissue included fibrous tissue. Further investigation into osteochondral defects using larger animals has been carried out by Ebihara et al. (2012) using the minipig model (Fig. 3) and they have previously reported the efficacy of repairs using layered chondrocyte sheets. In order to solve the problem of fibrous tissue being included in the superficial layer in this experiment, we investigated the effects of treatment with layered chondrocyte sheets and synovial cell transplantation. Forty-eight white Japanese rabbits (female, age: 16–18 weeks, weighing: ~3 kg, with each group $N = 4$, six groups) were used in this study. In order to determine the effects of treatment, the following six groups were produced: (A) synovial cells (1.8×10^6 cells), (B) layered chondrocyte sheets (1.7×10^6 cells), (C) synovial cells (3.0×10^5 cells) + layered chondrocyte sheets, (D) synovial cells

(6.0×10^5 cells) + layered chondrocyte sheets, (E) synovial cells (1.2×10^6 cells) + layered chondrocyte sheets, (F) osteochondral defect. Layered chondrocyte sheets and synovial cells were transplanted, sacrificed 4 and 12 weeks postoperatively. An incapacitance tester (Linton) was used to find trends in the weight distribution ratio of the damaged limbs after surgery. Sections were stained with Safranin-O. Repair sites were evaluated using ICRS grading system. In Groups (A)–(E), the damaged limb weight distribution ratio had improved. The repair tissue stained positively with Safranin-O. Four and twelve weeks after surgery, Groups (A)–(E) exhibited significantly higher scores than Group (F), and Groups (D) and (E) exhibited significantly higher scores than Groups (A) and (B). This suggests the efficacy of combining layered chondrocyte sheets with synovial cells (Ito et al., 2012).

PREPARATION OF HUMAN CHONDROCYTES AND FABRICATION OF CELL SHEET INTO THREE LAYERED SHEETS

Human chondrocytes were obtained from the knee joints of young athletes who underwent anterior cruciate ligament reconstruction. Twenty-nine knees from 29 patients aged 14–49 years (21 males and 8 females) were used as the source of these cells. All subjects provided informed consent. The specimens were stored in basal medium (BM) containing Dulbecco's modified Eagle's medium/F12 (DMEM/F12; GIBCO, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO) and 50 μg/mL ascorbic acid (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and 1% antibiotic–antimycotic mixture (ABAM; 10,000 U/mL penicillin G, 10,000 μg/mL streptomycin sulfate, and 25 μg/mL amphotericin B as Fungizone; GIBCO) until required for the next step. The cartilage samples were cut into small pieces. Thereafter, minced specimens were digested for 1 hr in BM containing 0.4% Pronase E (Kaken Pharmaceutical Inc., Tokyo, Japan), and for a further 4 hr in BM containing 0.016% Collagenase P (Roche Diagnostics GmbH, Mannheim, Germany). The digested cell suspension was passed through a cell strainer (BD Falcon™; BD Bioscience, Bedford, MA) with a pore size of 100 μm, and the isolated cells rinsed twice with chilled Dulbecco's phosphate-buffered saline (PBS; Dainippon Pharmaceutical Co., Osaka, Japan). The chondrocytes were then seeded into 500 cm^2 square dishes (245 mm × 245 mm; Corning Inc., Corning, NY) at a density of 10,000 cells/cm^2 and cultured in BM with 20% FBS (GIBCO) at 37°C in an atmosphere of 5% CO_2 and 95% air (Mitani et al., 2009).

To prepare the single-layer chondrocyte sheets, resuspended chondrocytes were seeded at a density of 10,000 cells/cm^2 in UpCell culture dishes (diameter: 35 mm, provided by CellSeed, Inc.). The seeded chondrocytes were cultured in BM adjusted to 20% FBS (GIBCO) at 37°C in an atmosphere of 5% CO_2 and 95% air. At 100% confluence, the cultured cells were harvested and prepared for gene expression analysis. To release confluent cells as a monolayer chondrocyte sheet from the UpCell temperature-responsive culture dishes, the dishes were removed from the incubator and let stand at about 25°C for 30 min. The culture medium was then removed from the dish, and the cell sheet harvested using polyvinylidene difluoride (PVDF) membrane as a supporting membrane. The lifted chondrocyte sheet edges promptly attached to the overlaid supporting membrane, and the cell sheet and PVDF membrane film were gently detached from the UpCell dish. Each cell sheet prepared as above was transferred onto another confluent chondrocyte sheet to fabricate multilayered sheets. Because the multilayered sheets spontaneously floated in culture medium, a 0.4-μm cell culture insert (Falcon, Becton Dickinson, NJ) was placed on top to prevent floating, and then culture of the sheets was continued for 1 week to obtain firm and perfect integration of the cells in the multilayer chondrocyte sheets (Mitani et al., 2009).

PROPERTIES OF HUMAN CHONDROCYTE SHEET AND HUMERAL FACTORS PRODUCED BY CHONDROCYTE SHEETS

The properties of the human chondrocyte sheets were investigated, including the expression and localization of SOX9, COL1, 2, 27, integrin α10, and fibronectin (Mitani et al., 2009). SOX9 activates COL2A1 in chondrocytes and directly regulates the Type II collagen gene *in vivo* (Wenke et al., 2006). Therefore, SOX9 is the one of the key regulators of chondrogenesis. Moreover, Jenkins et al. (2005) reported that the cartilage collagen gene, COL27A1, contains two enhancer elements that bind SOX9. Integrin α10 is specifically expressed in chondrocytes (Mitani et al., 2009). Chondrocytes, depending on the species and tissue origin, express a characteristic set of integrins, including receptors for collagen Type II (α1β1, α2β1, and α10β1), fibronectin (α5β1, αvβ3, and αvβ5), and laminin (α6β1). Among these receptors, integrin α10β1 is the major integrin mediating chondrocyte–collagen interactions in cartilage (Camper et al., 1998; Bengtsson et al., 2005). Mitani et al. (2009) reported that the significantly higher expressions of SOX9, integrin α10, and COL27 mRNA in the layered chondrocyte sheets revealed characteristics more closely resembling normal chondrocyte phenotype compared with chondrocytes in conventional monolayer culture.

Hamahashi et al. (in press) also investigated the humoral factors produced by layered chondrocyte sheets. Knee articular chondrocytes and synovial cells were harvested during total knee arthroplasty. After coculturing, the samples were divided into three groups: a monolayer, 7-day culture sheet group (Group M); a triple-layered, 7-day culture sheet group (Group L); and a monolayer culture group with a cell count identical to that of Group L (Group C). The secretion of collagen Type 1 (COL1), collagen Type 2 (COL2), matrix metalloproteinase-13 (MMP13), transforming growth factor-β (TGF-β), melanoma inhibitory activity (MIA), and prostaglandin E2 (PGE2) were measured by enzyme-linked immunosorbent assay. Layered chondrocyte sheets produced the most humoral factors. PGE2 expression declined over time in Group C but was significantly higher in Groups M and L. TGF-βexpression was lower in Group C but was significantly higher in Groups M and L ($P < 0.05$). Various humoral factors secreted from Layered chondrocyte sheets contains TGF-β and PGE2, which play an important role in cartilage repair and regeneration (Hamahashi et al., in press).

TGF-β stimulates chondrocytes to produce the extracellular matrices and also compete with the effects of

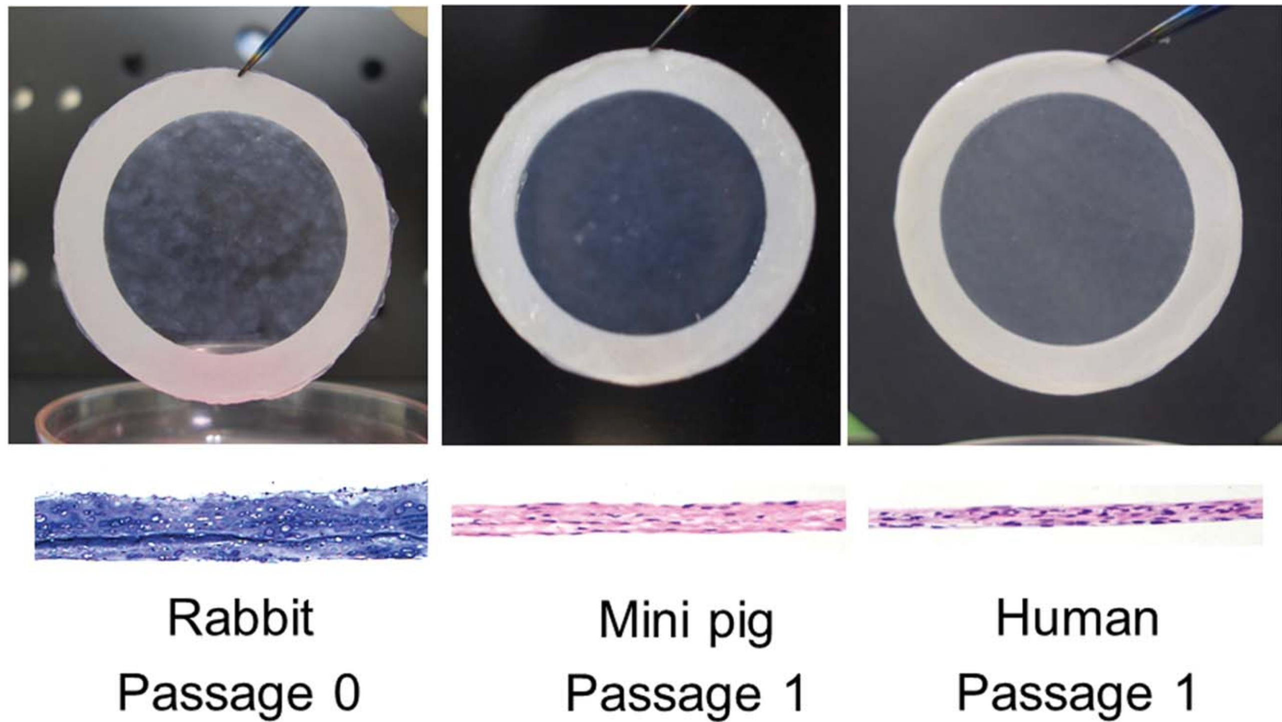

Fig. 4. Layered chondrocyte sheets. Layered chondrocyte sheets, even when composed of the same three layers, have different amounts of matrix accumulation and differing thicknesses depending on the species used. Especially, human chondrocyte sheet was quite thin and difficult to create on a regular schedule.

catabolic factors such as IL-1 (Blaney Davidson et al., 2006). The TGF-β signaling pathway is crucial for both maintaining cartilage homeostasis and preventing its disruption (Roman-Blas et al., 2007). Hamahashi et al. reported that TGF-β secretion levels were high in Group L in P0 and P1 but very low in P2, a pattern similar to that for COL2. PGE2 exerts pleiotropic effects in various tissues through EP1–4 receptors. In this study, they detected the higher secretory capacity for PGE2 in Group L and suggested that the implantation of layered chondrocyte sheets may have a therapeutic effect on partial-thickness cartilage defects. Aoyama et al. (2005) reported that EP2 is the main receptor expressed in articular cartilage, and the PGE2 signal through EP2 stimulates articular chondrocyte growth. And Nishitani et al. (2010) also reported that continuous inhibition of PGE2 accelerates the progression of OA. TGF-β and PGE2 also have an inhibitory effect on T cell proliferation (Di Nicola et al., 2002; Aggarwal and Pittenger, 2005). Hamahashi et al. (in press) also suggested that these inhibitory effects might be beneficial in counteracting immune rejection, such as graft-versus-host disease, suggesting one possible future clinical application.

ONGOING CLINICAL STUDY

Layered chondrocyte sheets, even when composed of the same three layers, accumulate different amounts of matrix and have differing thicknesses, depending on the species used (Fig. 4, cited with permission (a License Agreement of John Wiley and Sons provided by Copyright Clearance Center)). The human chondrocyte sheet is quite thin and difficult to create on a regular schedule. There are individual differences in human cell growth, so we coculture chondrocytes and synovial cells. This may mimic the intra-articular environment and provide an optimal environment for the preparation of chondrocyte sheets for tissue transplantation. This coculture system is particularly useful for reducing the culture period required.

We have confirmed the safety and efficacy of the chondrocyte sheet, and have submitted a report to the Ministry of Health, Labor and Welfare of Japan. In October 2011, the ministry approved a clinical study of joint repair using this cell sheet. We have just started the implantation of the cell sheet into patients at the Tokai University Hospital. We first use arthroscopy to diagnose the chondral defect precisely and to take cartilage and synovium from a nonbearing site. Each sample taken exceeds 1 g. Then we fabricate the layered cell sheet using the coculture method for three weeks at the Cell Processing Center (CPC). On the day of implantation surgery, after the cell sheet has been subjected to many kinds of tests, the layered cell sheets are carried to the operating room from the CPC. After the defect in the cartilage has been refreshed, we transplant the layered cell sheet. We implant the layered cell sheets using an open technique.

In 2008, the Government of Japan established the "Super Special Consortia" for Supporting the Development of Cutting-edge Medical Care. My colleagues and I belong to the "Project for the Realization of Regenerative

Medicine by Cell Sheets." We wish to develop the project as a hub for the rapid clinical application and commercialization of cell sheet technology in many areas of regenerative medicine. Tokai University is responsible for articular cartilage.

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