increased (Fig. 10.6). The layered chondrocyte sheets to be transplanted had low levels of the enzymes that degenerate cartilage and had good adhesion properties that help to both protect and repair the cartilage surface.

## 10.5 Future trends in cartilage repair

The development of a less invasive therapeutic approach for grafting a cell sheet to the injured site may be fundamental in expanding its use for the treatment of patients demonstrating the early stages of osteoarthritis. This section will discuss future trends in cartilage repair.

In the future, it will be important to determine how long such chondrocyte sheets can adhere to and live on the grafted sites, while also clarifying the optimum conditions for the adhesion of other cell sheets, such as synovial sheets, to injured sites. Therefore, use of a combination of both chondrocyte sheets and synovial sheets may also be possible. Although further research is necessary, the use of chondrocyte sheets is useful for the treatment of partial thickness defects of articular cartilage.

Scaffolds may not always be fundamental to the engineering of regenerative tissues. Today, many kinds of scaffold are used, such as atellocollagen, polyglycolide (PGA), poly-lactic-co-glycolic acid (PLGA) and poly-L-lactic acid (PLLA). These compounds are both biocompatible and biodegradable. Although they provide the advantage of initial strength against loading on the engineered tissue, they also introduce the possibility of side effects, such as a foreign-body reaction. Therefore, the long-term effectiveness of these scaffolds is questionable. Scaffold-free tissue engineering using novel technologies could be applied to cartilage repair in the near future.

## 10.6 Regulations regarding regenerative medicine in Japan

Finally, we will discuss some of the problems in the development of laws for regenerative medicine in Japan, which have not been improved as of September 2009. The Japanese medical system is structured to ensure the future safety and validity of both medical products and medical devices during the business development phase, in accordance with the Pharmaceutical Law. However, this Pharmaceutical Law is based on uniform manufacturing and selling practices for the general public and provides for only the two categories of medical products and medical devices. Therefore, if the concepts of the Pharmaceutical Law are applied directly to technology for regenerative medicine, which provides customized processes for autologous cells, it would result in requirements and stipulations that are far removed from the actual treatment situation, thus interfering with the spread of treatments that use the tissue engineering of autologous cells. In 2006, the Ministry of Health, Labour and Welfare presented

a set of guidelines for clinical research using human stem cells, but much like the Pharmaceutical Law, the guidelines fail to differentiate between autologous and allogeneic cells, and the differences between cell provision to the general public and technology provision through customized processes using autologous cells are not specified. The process of applying the achievements of clinical research using autologous cells for practical and general use requires a completely different qualification system from that of the conventional Pharmaceutical Law and the guidelines for stem cells. We strongly feel that new legislation is therefore urgently required in order to quickly realize the full potential of regenerative medicine using autologous cells for patients. Unfortunately, if this process is delayed, we will face a situation in which most of these products and technologies will have to be imported, much like the medical devices and materials currently being used in the clinical field in Japan.

## 10.7 References

[1] T. Okano, N. Yamada, H. Sakai, and Y. Sakurai, A novel recovery system for cultured cells using plasma-treated polystyrene dishes grafted with poly(*N*-isopropylacrylamide), *J Biomed Mater Res* **27** (1993) 1243–1251.

[2] T. Okano, N. Yamada, M. Okuhara, H. Sakai, and Y. Sakurai, Mechanism of cell detachment from temperature-modulated, hydrophilic-hydrophobic polymer surfaces, *Biomaterials* **16** (1995) 297–303.

[3] K. Nishida, M. Yamato, Y. Hayashida, K. Watanabe, N. Maeda, H. Watanabe, K. Yamamoto, S. Nagai, A. Kikuchi, Y. Tano, and T. Okano, Functional bioengineered corneal epithelial sheet grafts from corneal stem cells expanded *ex vivo* on a temperature-responsive cell culture surface, *Transplantation* **77** (2004) 379–385.

[4] K. Nishida, M. Yamato, Y. Hayashida, K. Watanabe, K. Yamamoto, E. Adachi, S. Nagai, A. Kikuchi, N. Maeda, H. Watanabe, T. Okano, and Y. Tano, Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium, *N Engl J Med* **351** (2004) 1187–1196.

[5] S. Sekiya, T. Shimizu, M. Yamato, A. Kikuchi, and T. Okano, Bioengineered cardiac cell sheet grafts have intrinsic angiogenic potential, *Biochem Biophys Res Commun* **341** (2006) 573–582.

[6] T. Shimizu, M. Yamato, Y. Isoi, T. Akutsu, T. Setomaru, K. Abe, A. Kikuchi, M. Umezu, and T. Okano, Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces, *Circ Res* **90** (2002) e40–48.

[7] T. Shimizu, M. Yamato, T. Akutsu, T. Shibata, Y. Isoi, A. Kikuchi, M. Umezu, and T. Okano, Electrically communicating three-dimensional cardiac tissue mimic fabricated by layered cultured cardiomyocyte sheets, *J Biomed Mater Res* **60** (2002) 110–117.

[8] T. Shimizu, M. Yamato, A. Kikuchi, and T. Okano, Two-dimensional manipulation of cardiac myocyte sheets utilizing temperature-responsive culture dishes augments the pulsatile amplitude, *Tissue Eng* **7** (2001) 141–151.

[9] Y. Shiroyanagi, M. Yamato, Y. Yamazaki, H. Toma, and T. Okano, Urothelium regeneration using viable cultured urothelial cell sheets grafted on demucosalized gastric flaps, *BJU Int* **93**(7) (2004) 1069–1075.

[10] D. Murakami, M. Yamato, K. Nishida, T. Ohki, R. Takagi, J. Yang, H. Namiki, and T. Okano, Fabrication of transplantable human oral mucosal epithelial cell sheets using temperature-responsive culture inserts without feeder layer cells, *J Artif Organs* **9**(3) (2006) 185–191.

[11] D. Murakami, M. Yamato, K. Nishida, T. Ohki, R. Takagi, J. Yang, H. Namiki, and T. Okano, The effect of micropores in the surface of temperature-responsive culture inserts on the fabrication of transplantable canine oral mucosal epithelial cell sheets, *Biomaterials* **27**(32) (2006) 5518–5523.

[12] T. Ohki, M. Yamato, D. Murakami, R. Takagi, J. Yang, H. Namiki, T. Okano, and K. Takasaki, Treatment of oesophageal ulcerations using endoscopic transplantation of tissue-engineered autologous oral mucosal epithelial cell sheets in a canine model, *Gut* **55**(12) (2006) 1704–1710.

[13] M. G. Flores, M. Hasegawa, M. Yamato, R. Takagi, T. Okano, and I. Ishikawa, Cementum-periodontal ligament complex regeneration using the cell sheet technique, *J Periodontal Res* **43**(3) (2008) 364–371.

[14] M. Hasegawa, M. Yamato, A. Kikuchi, T. Okano, and I. Ishikawa, Human periodontal ligament cell sheets can regenerate periodontal ligament tissue in an athymic rat model, *Tissue Eng* **11**(3–4) (2005) 469–478.

[15] T. Akizuki, S. Oda, M. Komaki, H. Tsuchioka, N. Kawakatsu, A. Kikuchi, M. Yamato, T. Okano, and I. Ishikawa, Application of periodontal ligament cell sheet for periodontal regeneration: a pilot study in beagle dogs, *J Periodontal Res* **40**(3) (2005) 245–251.

[16] K. Ohashi, T. Yokoyama, M. Yamato, H. Kuge, H. Kanehiro, M. Tsutsumi, T. Amanuma, H. Iwata, J. Yang, T. Okano, and Y. Nakajima, Engineering functional two- and three-dimensional liver systems in vivo using hepatic tissue sheets. *Nat Med* **13**(7) (2007) 880–885.

[17] Y. Tsuda, A. Kikuchi, M. Yamato, A. Nakao, Y. Sakurai, M. Umezu, and T. Okano, The use of patterned dual thermoresponsive surfaces for the collective recovery as co-cultured cell sheets, *Biomaterials* **26**(14) (2005) 1885–1893.

[18] M. Yamato, O. H. Kwon, M. Hirose, A. Kikuchi, and T. Okano, Novel patterned cell coculture utilizing thermally responsive grafted polymer surfaces, *J Biomed Mater Res* **55**(1) (2001) 137–140.

[19] M. Harimoto, M. Yamato, M. Hirose, C. Takahashi, Y. Isoi, A. Kikuchi, and T. Okano, Novel approach for achieving double-layered cell sheets co-culture: overlaying endothelial cell sheets onto monolayer hepatocytes utilizing temperature-responsive culture dishes, *J Biomed Mater Res* **62**(3) (2002) 464–470.

[20] N. Kaneshiro, M. Sato, M. Ishihara, G. Mitani, H. Sakai, and J. Mochida, Bioengineered chondrocyte sheets may be potentially useful for the treatment of partial thickness defects of articular cartilage, *Biochem Biophys Res Commun* **349**(2) (2006) 723–731.

[21] N. Kaneshiro, M. Sato, M. Ishihara, G. Mitani, H. Sakai, T. Kikuchi, and J. Mochida, Cultured articular chondrocytes sheets for partial thickness cartilage defects utilizing temperature-responsive culture dish, *European Cells Mater* **13** (2007) 87–92.

[22] S. W. O'Driscoll, and R. B. Salter, The repair of major osteochondral defects in joint surfaces by neochondrogenesis with autogenous osteoperiosteal grafts stimulated by continuous passive motion. An experimental investigation in the rabbit, *Clin Orthop Relat Res* (1986) 131–140.

[23] Y. Matsusue, T. Yamamuro, and H. Hama, Arthroscopic multiple osteochondral transplantation to the chondral defect in the knee associated with anterior cruciate

ligament disruption, *Arthroscopy* **9** (1993) 318–321.
[24] M. Brittberg, A. Lindahl, A. Nilsson, C. Ohlsson, O. Isaksson, and L. Peterson, Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation, *N Engl J Med* **331** (1994) 889–895.
[25] M. Ochi, Y. Uchio, K. Kawasaki, S. Wakitani, and J. Iwasa, Transplantation of cartilage-like tissue made by tissue engineering in the treatment of cartilage defects of the knee, *J Bone Joint Surg Br* **84** (2002) 571–578.
[26] S. Wakitani, K. Imoto, T. Yamamoto, M. Saito, N. Murata, and M. Yoneda, Human autologous culture expanded bone marrow mesenchymal cell transplantation for repair of cartilage defects in osteoarthritic knees, *Osteoarthritis Cartilage* **10** (2002) 199–206.
[27] E. B. Hunziker, and L. C. Rosenberg, Repair of partial-thickness defects in articular cartilage: cell recruitment from the synovial membrane, *J Bone Joint Surg Am* **78** (1996) 721–733.
[28] M. Yamato, M. Utsumi, A. Kushida, C. Konno, A. Kikuchi, and T. Okano, Thermo-responsive culture dishes allow the intact harvest of multilayered keratinocyte sheets without dispase by reducing temperature, *Tissue Eng* **7** (2001) 473–480.
[29] M. Sato, T. Asazuma, M. Ishihara, T. Kikuchi, K. Masuoka, S. Ichimura, M. Kikuchi, A. Kurita, and K. Fujikawa, An atelocollagen honeycomb-shaped scaffold with a membrane seal (ACHMS-scaffold) for the culture of annulus fibrosus cells from an intervertebral disc, *J Biomed Mater Res A* **64** (2003) 248–256.
[30] A. Fehrenbacher, E. Steck, M. Rickert, W. Roth, and W. Richter, Rapid regulation of collagen but not metalloproteinase 1, 3, 13, 14 and tissue inhibitor of metalloproteinase 1, 2, 3 expression in response to mechanical loading of cartilage explants *in vitro*, *Arch Biochem Biophys* **410** (2003) 39–47.
[31] E. J. Uitterlinden, H. Jahr, J. L. Koevoet, Y. M. Jenniskens, S. M. Bierma-Zeinstra, J. Degroot, J. A. Verhaar, H. Weinans, and G. J. van Osch, Glucosamine decreases expression of anabolic and catabolic genes in human osteoarthritic cartilage explants, *Osteoarthritis Cartilage* **14** (2006) 250–257.
[32] M. Fichter, U. Korner, J. Schomburg, L. Jennings, A. A. Cole, and J. Mollenhauer, Collagen degradation products modulate matrix metalloproteinase expression in cultured articular chondrocytes, *J Orthop Res* **24** (2006) 63–70.
[33] J. Flannelly, M. G. Chambers, J. Dudhia, R. M. Hembry, G. Murphy, R. M. Mason, and M. T. Bayliss, Metalloproteinase and tissue inhibitor of metalloproteinase expression in the murine STR/ort model of osteoarthritis, *Osteoarthritis Cartilage* **10** (2002) 722–733.
[34] A. R. Klatt, G. Klinger, O. Neumuller, B. Eidenmuller, I. Wagner, T. Achenbach, T. Aigner, and E. Bartnik, TAK1 downregulation reduces IL-1beta induced expression of MMP13, MMP1 and TNF-alpha, *Biomed Pharmacother* **60** (2006) 55–61.
[35] H. Ling, and A. D. Recklies, The chitinase 3-like protein human cartilage glycoprotein 39 inhibits cellular responses to the inflammatory cytokines interleukin-1 and tumour necrosis factor-alpha, *Biochem J* **380** (2004) 651–659.
[36] A. M. Malfait, R. Q. Liu, K. Ijiri, S. Komiya, and M. D. Tortorella, Inhibition of ADAM-TS4 and ADAM-TS5 prevents aggrecan degradation in osteoarthritic cartilage, *J Biol Chem* **277** (2002) 22201–22208.
[37] S. S. Glasson, R. Askew, B. Sheppard, B. Carito, T. Blanchet, H. L. Ma, C. R. Flannery, D. Peluso, K. Kanki, Z. Yang, M. K. Majumdar, and E. A. Morris, Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis, *Nature* **434** (2005) 644–648.

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# The properties of bioengineered chondrocyte sheets for cartilage regeneration

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## Abstract

**Background:** Although the clinical results of autologous chondrocyte implantation for articular cartilage defects have recently improved as a result of advanced techniques based on tissue engineering procedures, problems with cell handling and scaffold imperfections remain to be solved. A new cell-sheet technique has been developed, and is potentially able to overcome these obstacles. Chondrocyte sheets applicable to cartilage regeneration can be prepared with this cell-sheet technique using temperature-responsive culture dishes. However, for clinical application, it is necessary to evaluate the characteristics of the cells in these sheets and to identify their similarities to naive cartilage.

**Results:** The expression of SOX 9, collagen type 2, 27, integrin α10, and fibronectin genes in triple-layered chondrocyte sheets was significantly increased in comparison to those in conventional monolayer culture and in a single chondrocyte sheet, implying a nature similar to ordinary cartilage. In addition, immunohistochemistry demonstrated that collagen type II, fibronectin, and integrin α10 were present in the triple-layered chondrocyte sheets.

**Conclusion:** The results of this study indicate that these chondrocyte sheets with a consistent cartilaginous phenotype and adhesive properties may lead to a new strategy for cartilage regeneration.

## Background

Osteoarthritis (OA), the most common articular disorder, is characterized primarily by slow progressive degeneration or destruction of cartilage. However, the exact etiology of OA is not known. The symptoms of osteoarthritis usually appear in middle age and almost everyone has them by age 70. Therefore, adequate treatments for the early stages of degeneration are required.

Cartilage has two important functions, the reduction of friction and the transmission of load. Some of the specific properties of cartilage are a lack of blood vessels, a small number of cell constituents, and a large amount of extracellular matrix (ECM). Once cartilage has been damaged, it is unable to heal itself.[1,2] There are various treatments for damaged cartilage, but few recommended surgical procedures. Drilling, subchondral abrasion[3] and microfracture treatments[4] allow the regeneration of damaged cartilage by activating mesenchymal stem cells derived from the bone marrow; however, previous reports have shown that the regenerated cartilage was fibrocartilage, not hyaline cartilage. The functions and properties of fibrocartilage are inferior to hyaline cartilage, and therefore the outcomes at long-term follow-up after these treatments tend to be poor.[2] Mosaicplasty can be used to transplant hyaline cartilage to the damaged area and reports have shown at long-term follow-up that mosaicplasty is beneficial; however, it has associated donor site morbidity, and only a predetermined defect area can be treated.[5] The clinical results of arthroplasty for severe osteoarthritis have improved with the development of new surgical techniques and the selection of appropriate medical devices. However, many obstacles have yet to be overcome, including limited range of motion and durability, and excessive invasiveness of the surgery. In addition, resulting function is significantly inferior to that of the normal joint. Therefore, the establishment of new protocols for cartilage regeneration using tissue engineering is important. Because of recent progress in tissue engineering, various techniques are available to cure damaged cartilage. Autologous chondrocyte implantation (ACI), first reported by Brittberg *et al.*, [6] has been used clinically. Although clinical results show that this technique can be beneficial, some problems remain, such as limits on the size of lesions that can be treated, periostal hypertrophy, and the lack of appropriate methods to evaluate the regenerated cartilage after ACI. Moreover, although the clinical results of ACI have recently improved as a result of advanced techniques based on tissue engineering procedures, problems relating to cell handling and scaffold imperfections remain. Artificial scaffolds have been adopted to deliver cells into cartilage defect sites, and to reinforce the mechanical stability of three-dimensional tissue engineered chondral grafts. The ideal scaffold is supposed to encourage ECM. Although, some scaffolds have been successfully applied for the cartilage regeneration,[7] there are problems with biocompatibility and cellular viability, including cell attachment, distribution and proliferation.

Recently, a cell-sheet technique[8] has been developed that is potentially able to overcome these problems. Therefore, a new strategy for cartilage regeneration without a scaffold has been studied with cell-sheet technology using temperature-responsive culture dishes (UpCell™ CellSeed Inc., Tokyo, Japan).

We previously reported[9] the implantation of layered chondrocyte sheets, harvested by simply lowering the temperature and with no need for enzyme digestion, in Japanese white rabbits. We also verified the effectiveness of chondrocyte sheets using a swine partial cartilage defect model, which showed reduced degeneration. Interestingly, in layered chondrocyte sheets, it appeared that catabolic factors such as MMP3, MMP13, and ADAMTS5 decrease at the point of layering, while the expression of TIMP1, an inhibitor of MMP3, increases.[9] This indicates that layered chondrocyte sheets have fewer destructive factors than degenerate cartilage and have good adhesion properties, which help to both protect and repair the cartilage surface.[9] However, the precise mechanisms by which such chondrocyte sheets adhere to the damaged cartilage and maintain the cartilage phenotype remain to be elucidated. The purpose of this study was to further investigate the properties of human chondrocyte sheets using scanning electron microscopic evaluation and gene expression and immunohistochemical analyses.

## Results

### *Manipulation of chondrocyte sheets*

Chondrocyte sheets prepared as either cell monolayer sheets or three-layered sheets were obtained by simply reducing the temperature, with no need for an enzymatic digestion step (Fig. 1). The chondrocytes were harvested as a single contiguous cell sheet, retaining the neighboring extracellular structure, which implies that these cell sheets should contain extracellular proteins including cell-cell junction, ECM, and adhesion proteins.

The multilayered sheets could be easily produced by placing one chondrocyte sheet onto other sheets by making use of the supporting PVDF membrane (Fig. 1A–C). By repeating this procedure twice, three-layered cell sheets were obtained (Fig. 1D). When cultured for 1 week, the triple-layered chondrocyte sheets were extendable and were not damaged by mild external force. This extended multilayering process was sufficient to give a single contiguous multilayered structure in which each sheet had adhered firmly and tightly to the other sheets.

With the help of the supporting membrane, the cell-sheet-PVDF film showed good stability and we could easily handle the chondrocyte sheets.

### *Scanning electron microscopy*

SEM analysis revealed that the top and basal aspects of the chondrocyte sheets showed completely different textures. A network of laminated ECM structures was observed on the top aspect of the sheet. These sheets of ECM structures appeared piled up, with several sheet-like configurations and amorphous shapes, and separated edges of the ECM sheet occasionally being observed as dog-ears facing the culture medium side (Fig. 2A).

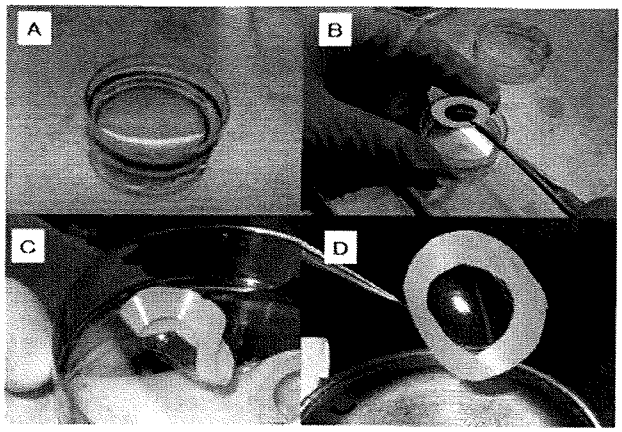


**Figure 1**
**Fabrication of the cell sheets.** Temperature-responsive culture dishes on which chondrocytes had been cultured were removed from the incubator when the cells reached confluence and were let stand at about 25°C for 30 min (A). After the culture medium was removed, a polyvinylidene difluoride (PVDF) membrane was put onto the dish (B), and the sheet was detached gently (C). The chondrocyte sheets could then be easily fabricated into multilayered constructs with the help of the PVDF and without the need for enzyme digestion (D).

The surface of the basal aspect, which had been attached to the bottom of the culture dish, was covered with a smooth ECM pattern, and numerous humps (mound-like elevations) were observed. Compared with the top side of the chondrocyte sheet, the arrangement of the accumulated ECM surface was smoother, with a parallel pattern (Fig. 2B).

***Analysis of gene expression***

The expression of collagen type 1 (COL1) mRNA was observed at significantly lower levels in the layered chondrocyte sheets in comparison to the conventional monolayer cultures and monolayer chondrocyte sheets (Fig. 3A).

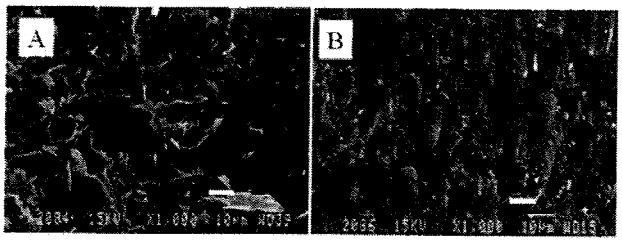


**Figure 2**
**Scanning electron microscopy.** Scanning electron microscopy revealed that the top (A) and the basal aspects (B) of the chondrocyte sheet demonstrated completely different textures. The chondron like texture was only observed on the basal aspect, which had adhesive properties. Scale bar = 10 μm.

In contrast, the expression of collagen type 2 (COL2), SOX9, COL 27, integrin α10 and fibronectin mRNAs were observed at significantly higher levels in the layered chondrocyte sheets in comparison to the conventional monolayer cultures and monolayer chondrocyte sheets (Fig. 3B–F).

***Immunohistochemistry***

Immunohistochemical examination revealed that fibronectin, integrin α10, and COL2 were present in the triple-layered chondrocyte cell sheet (Fig. 4). Interestingly, fibronectin was located in the periphery of the triple-layered chondrocyte sheets. (Fig. 4A, D) and COL2 was observed in the pericellular matrix of the triple-layered chondrocyte sheets (Fig. 4B, E). However, in contrast to these two proteins, integrin α10 was diffusely distributed throughout the triple-layered chondrocyte sheets (Fig. 4C, F). These different immunohistochemical features of target proteins are illustrated in Fig. 5, where the main results are presented together.

## Discussion

Cell-sheet technology using temperature-responsive culture dishes was first reported by Okano *et al.* in 1993.[8] Since their report was published, this technology has been studied with regard to regenerative medicine for the cornea, heart, pancreas, and liver. [10-13] Nishida *et al.* reported that corneal cell sheets cultured in temperature-responsive culture dishes could strongly adhere to the cornea without scaffolding or suturing.[10] Kushida *et al.* reported that fibronectin expression was preserved on the basal side of cell sheets cultured on temperature-responsive culture dishes.[14] As cell sheets can be harvested with the ECM and adhesion factors, it is simple to layer the cell sheets one on top of another, using the natural adhesiveness of the basal side. Therefore, large layered three-dimensional tissues without a scaffold can be constructed in this repeating fashion. Shimizu et al. 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 cannot increase the thickness of the fabrication by more than 1 mm in myocardial tissues in vivo. [12]

To fabricate the multilayered sheets, we extended the culture by 1 week, which was effective enough to consolidate the cell sheets into a single three-dimensional structure. However, the cell sheets tended to float in the culture medium because of their shape. Accordingly, it was necessary to devise strategies to apply a physical force to the sheets to enable them to attach gently to each other and the bottom of dish during culture. Using cell-culture inserts of an appropriate height and the weight of the culture-dish cover, we achieved an appropriately narrow space to facilitate strong adhesion between the cell sheets. Although our experimental study of allografts using lay-

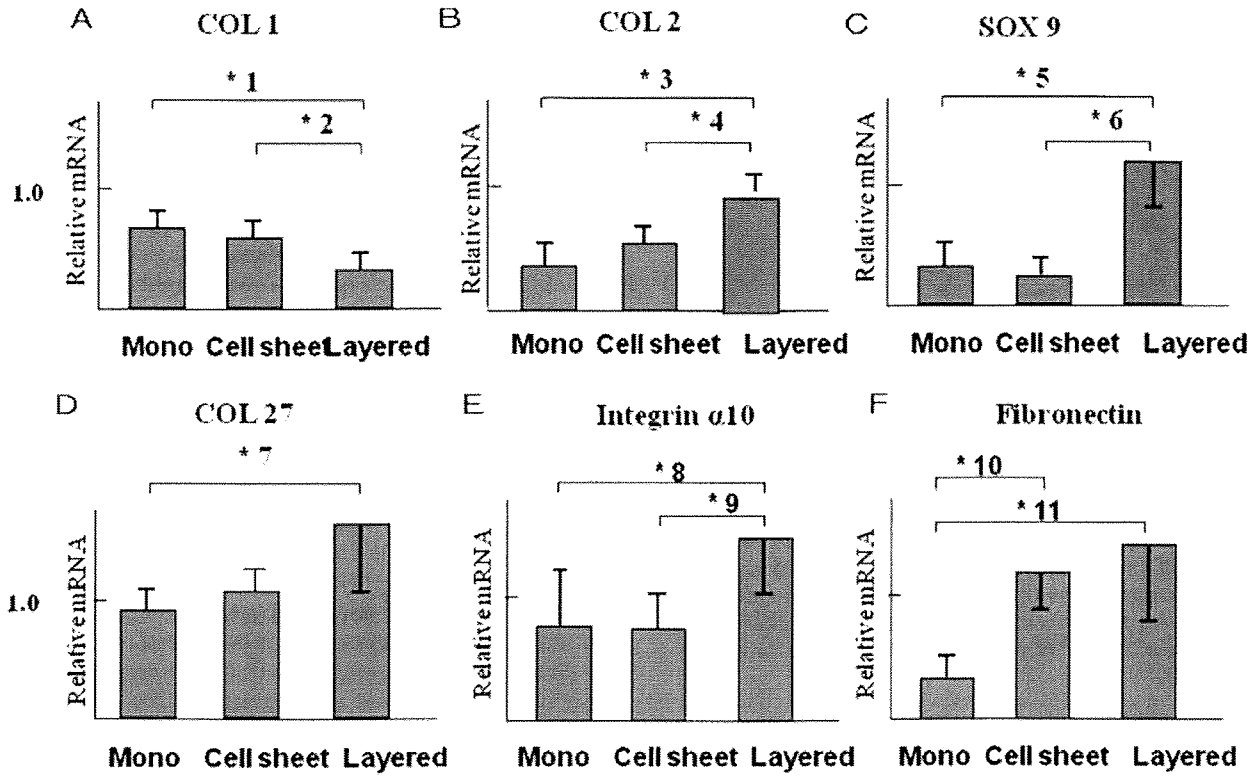


**Figure 3**
**Relative expression of mRNA.** The y-axis shows the mRNA expression relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The results were evaluated using the SmartCycler II software program. GAPDH expression was used to normalize samples. The error bars represent the standard deviation. Type I collagen mRNA expression was present at a low level in the layered chondrocyte cell sheets (A). In contrast, type II collagen mRNA expression was at significantly higher levels in the layered chondrocyte sheets in comparison to the monolayer cultures and monolayer chondrocyte sheets (B). SOX9 and COL27 mRNA expression were observed at significantly higher levels in the layered chondrocyte sheets in comparison to conventional monolayer cultures and monolayer chondrocyte sheets (C, D). Integrin α10 and fibronectin mRNA expression were at significantly higher levels in the layered chondrocyte sheets in comparison to the conventional monolayer cultures and the monolayer chondrocyte sheets (E, F).

ered chondrocyte sheets has been proceeding for only 2 months, good adhesion and an inhibitory effect on cartilage degeneration at injured sites has been confirmed (unpublished data).

The SEM examination of the cell sheets indicated that the top and the adhesive basal aspects were completely different in texture. A network of laminated ECM was observed on the top side of the sheets (Fig. 2A). These sheets of ECM structure resemble the lamination of the normal superficial cartilage zone, the "lamina splendens," as initially proposed by MacConaill and later identified by Clark using SEM;[15] however, the scanning electron micrographs show that the sheets do not have a smoother surface than normal articular cartilage.[15] In this study, it was impossible to observe a distinct collagen fibrous structure, which may exist beneath the lamination. According to scanning electron micrographs of ordinary cartilage,[15] in the superficial zone several layers of collagen fibrils exist immediately beneath the *lamina splendens*, forming a mesh of interwoven fibrils that run parallel to the articular surface, and the chondrocytes in this zone appear to be located beneath the layers of collagen fibrils. Meanwhile, on the basal aspect, numerous mound-like elevations were observed in the surface with a texture similar to an aggregation of chondron-like shapes. This smoother surface more resembled normal cartilage surface than did the top side of the cell sheets (Fig. 2B). The flat and smooth surface of the basal aspect implies abundant accumulation of extracellular proteins therein, and is

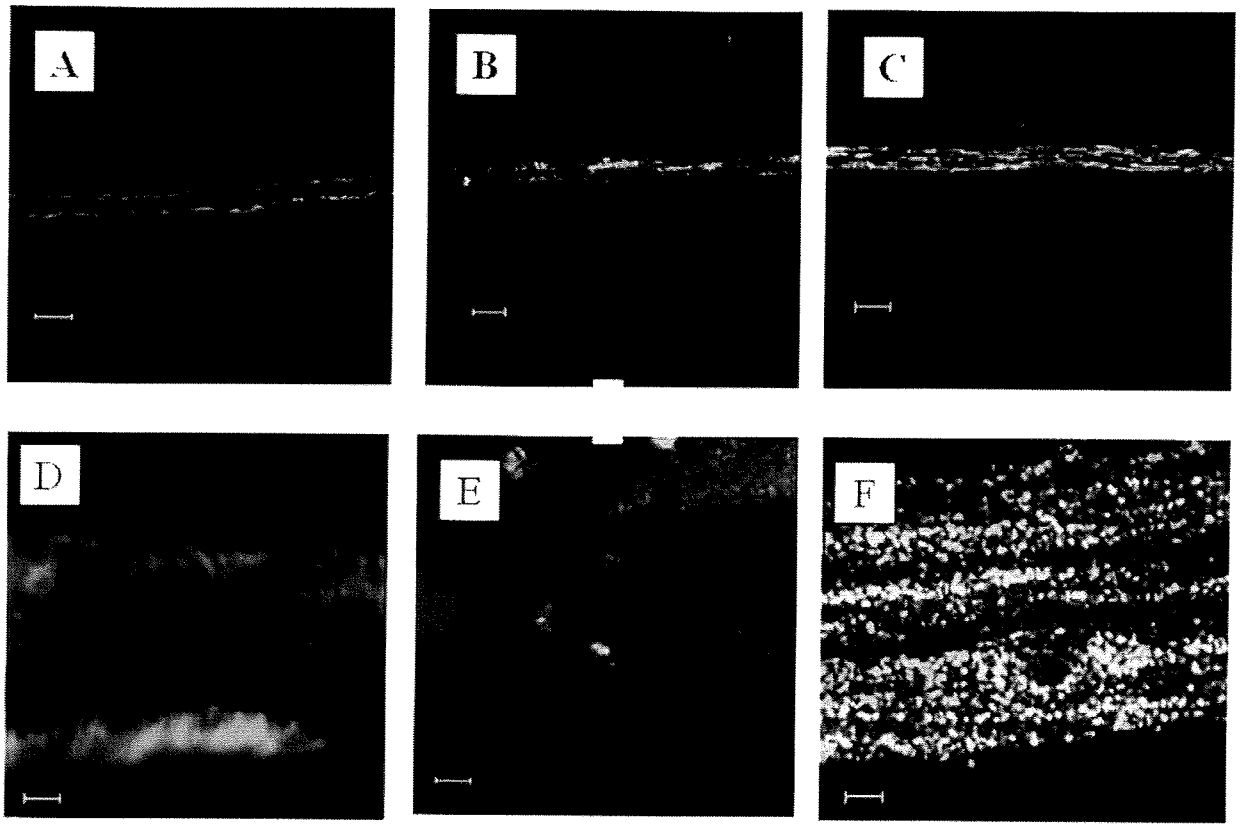


**Figure 4**
**Immunohistochemical examination: fluorescence microscopy of layered chondrocyte sheets.** Fibronectin (shown in green) was detected in the periphery of the triple-layered chondrocyte sheets (A, D). However, collagen type II (shown in green) was located in the pericellular matrix of the triple-layered chondrocyte sheets (B, E) and integrin α10 (shown in green) was scattered diffusely throughout the triple-layered chondrocyte sheets (C, F). The blue color shows counterstained DNA. DAPI excites at about 360 nm and emits at about 460 nm when bound to DNA, producing a blue fluorescence. A, B, C: Scale bar = 20 μm. D, E, F: Scale bar = 2 μm

reflected in their characteristic adhesiveness. The flat bottoms of the culture dish and the effects of gravity during the culture period may also fashion this even surface texture.

The concept of the chondron was first introduces by Benninghoff in 1925, with the chondrocyte and its pericellular capsules together representing the chondron, historically considered the primary structural, functional, and metabolic unit of articular and other hyaline cartilages.[16] During recent decades, many researchers have investigated and established the molecular anatomy, functional properties, and metabolic contribution of the chondron in articular cartilage homeostasis and its failure during the initiation and progression of degenerative osteoarthritis. It is interesting that SEM evaluations of the basal aspect of the cell sheets suggest that chondrocytes with ECM, chondrons, were embedded in opposing sides of the sheet surfaces. Although chondrons were only faintly observed because of the thick ECM, it is clear that our sheets contain the basic structural, functional, and metabolic units of articular cartilage and it is expected that they will maintain their function of reduction of friction and the transmission of load. It is thus suggested that, using our technique, these triple-layered chondrocyte sheets have substantially reconstructed the ordinary superficial zone of articular cartilage. To our knowledge, this is the first report of the morphologic evaluation of the bottom aspect of cultivated chondrocytes, which was made possible because the cell sheets were harvested as a single contiguous shape using a noninvasive method without enzyme digestion, thus keeping their original structure.

In this study, the properties of the chondrocyte sheets were investigated, including the expression and localiza-

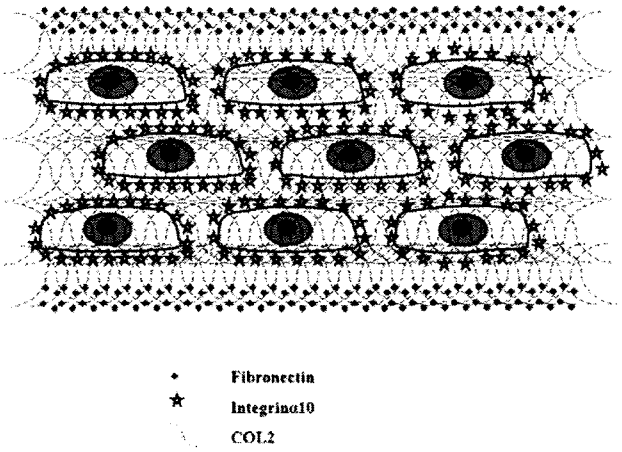


**Figure 5**
**Schematic illustration of the distribution pattern of extracellular adhesion molecules in triple-layered chondrocyte sheets.** Fibronectin was located peripherally in the triple-layered chondrocyte sheet and integrin α10 was observed close to the chondrocytes, in the pericellular matrix, in the layered construct. However, collagen type II was scattered diffusely throughout the layered cell sheets.

tion of SOX9, COL1, 2, 27, integrin α10 and fibronectin. SOX9 has recently been shown to be involved in the control of cell-specific activation of COL2A1 in chondrocytes and to directly regulate the type II collagen gene *in vivo*.[17] Therefore, the high mobility group protein SOX9 is emerging as a key regulator of chondrogenesis. Moreover, Jenkins *et al.* recently reported that the newest cartilage collagen gene, COL27A1, contains two enhancer elements that bind SOX9.[18] Integrin α10 is specifically expressed in chondrocytes. 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, αvβ5), and laminin (α6β1). Among these receptors, integrin α10β1 is the major integrin mediating chondrocyte-collagen interactions in cartilage.[19,20] Compared with chondrocytes in conventional monolayer culture and single chondrocyte sheets, the significantly higher expression of SOX9 and COL27 mRNA in the layered chondrocyte sheets revealed characteristics more closely resembling normal chondrocyte differentiation, which implies the maintenance of a phenotype. In addition, significantly higher expression of fibronectin and integrin α10 mRNA in the layered chondrocyte sheets also demonstrated the adhesiveness of the cell sheets. Furthermore, on immunohistochemical examination, the expression of fibronectin and integrin α10 in the layered chondrocyte cell sheets verified this adhesiveness and illustrated the specific cartilaginous phenotype of the cell sheets. In both this study and in earlier results using temperature-responsive surfaces[14] it was possible to recover monolayer cell sheets together with deposited fibronectin. Fibronectin matrix adhering to the basal side of cell sheets can function as a glue to attach cell sheets onto other surfaces.[14] In fact, cell sheets recovered from temperature-responsive surfaces easily adhere to other surfaces.[21] Interestingly, as illustrated by the immunohistochemical results in this study (Fig. 5), fibronectin was detected peripherally on both sides of the triple-layered chondrocyte sheets, not only on the basal aspect as described previously[14] but also on the top side, while showing intense adhesiveness on the basal side only. It is possible to hypothesize that the culture period prolonged by 1 week with a cell-culture

**Table 1: List of primers used in the real-time PCR.**

| Primer ID | Accesion No. | Sequence | Expect size(bp) |
|---|---|---|---|
| Collagen Type I-F<br>Collagen Type I-R | NM_000088 | AAG GGT GAG ACA GGC GAA CAA<br>TTG CCA GGA GAA CCA GCA AGA | 170 |
| Collagen Type II-F<br>Collagen Type II-R | NM_033150 | GGA CTT TTC TTC CCT CTC T<br>GAC CCG AAG GGT CTT ACA GGA | 113 |
| SOX9-F<br>SOX9-R | NM_000346 | AAC GCC GAG CTC AGC AAG A<br>CCG CGG CTG GTA CTT GTA ATC | 138 |
| Collagen27α1-F<br>Collagen27α1-R | NM_032888 | GGG CCT TAT GGA AAT CCA GGT C<br>GGT CCA GGA TAG CCC TTG TGT C | 176 |
| Integrinα10-F<br>Integrinα10-R | NM_003637 | CTG GGA TAT GTG CCC GTG TG<br>TTG GAG CCA TCC AAG ACA ATG A | 112 |
| Fibronectin1-F<br>Fibronectin1-R | NM_001030524 | GCA CAG GGG AAG AAA AGG AG<br>TTG AGT GGA TGG GAG GAG AG | 189 |

**Table 2: Results of the post hoc test (Scheffe's method).**

| Dependent variable | (I) VI | (J) VI | Difference of averages (I-J) | SEM | P-value | 95% CI | | Figure 3 |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower limit | Upper limit | |
| COL1 | Mono | Cell Sheet | .05250 | .05374 | .635 | -.1043 | .2093 | |
| | | Layered | .39500* | .05374 | .000 | .2382 | .5518 | *1 |
| | Cell Sheet | Mono | -.05250 | .05374 | .635 | -.2093 | .1043 | |
| | | Layered | .34250* | .05374 | .000 | .1857 | .4993 | *2 |
| | Layered | Mono | -.39500* | .05374 | .000 | -.5518 | -.2382 | *1 |
| | | Cell Sheet | -.34250* | .05374 | .000 | -.4993 | -.1857 | *2 |
| COL2 | Mono | Cell Sheet | -.12750 | .05183 | .099 | -.2787 | .0237 | |
| | | Layered | -.52500* | .05183 | .000 | -.6762 | -.3738 | *3 |
| | Cell Sheet | Mono | .12750 | .05183 | .099 | -.0237 | .2787 | |
| | | Layered | -.39750* | .05183 | .000 | -.5487 | -.2463 | *4 |
| | Layered | Mono | .52500* | .05183 | .000 | .3738 | .6762 | *3 |
| | | Cell Sheet | .39750* | .05183 | .000 | .2463 | .5487 | *4 |
| SOX9 | Mono | Cell Sheet | .04500 | .08833 | .880 | -.2127 | .3027 | |
| | | Layered | -.68500* | .08833 | .000 | -.9427 | -.4273 | *5 |
| | Cell Sheet | Mono | -.04500 | .08833 | .880 | -.3027 | .2127 | |
| | | Layered | -.73000* | .08833 | .000 | -.9877 | -.4723 | *6 |
| | Layered | Mono | .68500* | .08833 | .000 | .4273 | .9427 | *5 |
| | | Cell Sheet | .73000* | .08833 | .000 | .4723 | .9877 | *6 |
| COL27 | Mono | Cell Sheet | -.13250 | .17224 | .751 | -.6350 | .3700 | |
| | | Layered | -.43750 | .17224 | .088 | -.9400 | .0650 | *7 |
| | Cell Sheet | Mono | .13250 | .17224 | .751 | -.3700 | .6350 | |
| | | Layered | -.30500 | .17224 | .260 | -.8075 | .1975 | |
| | Layered | Mono | .43750 | .17224 | .088 | -.0650 | .9400 | *7 |
| | | Cell Sheet | .30500 | .17224 | .260 | -.1975 | .8075 | |
| Integrin a10 | Mono | Cell Sheet | .05500 | .10949 | .883 | -.2645 | .3745 | |
| | | Layered | -.36500* | .10949 | .027 | -.6845 | -.0455 | *8 |
| | Cell Sheet | Mono | -.05500 | .10949 | .883 | -.3745 | .2645 | |

**Table 2: Results of the post hoc test (Scheffe's method).** *(Continued)*

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | Layered | -.42000* | .10949 | .013 | -.7395 | -.1005 | *9 |
| | Layered | Mono | .36500* | .10949 | .027 | .0455 | .6845 | *8 |
| | | Cell Sheet | .42000* | .10949 | .013 | .1005 | .7395 | *9 |
| Fibronectin | Mono | Cell Sheet | -.71500* | .16992 | .007 | -1.2108 | -.2192 | *10 |
| | | Layered | -.93000* | .16992 | .001 | -1.4258 | -.4342 | *11 |
| | Cell Sheet | Mono | .71500* | .16992 | .007 | .2192 | 1.2108 | *10 |
| | | Layered | -.21500 | .16992 | .479 | -.7108 | .2808 | |
| | Layered | Mono | .93000* | .16992 | .001 | .4342 | 1.4258 | *11 |
| | | Cell Sheet | .21500 | .16992 | .479 | -.2808 | .7108 | |

insert may affect the localization of fibronectin. In this period, the fibronectin could either be moved from its position in the single-layer cell sheets or be newly secreted in areas of contact with the insert. However, the exact mechanism underlying this phenomenon is unclear in our results and further research into this is required.

It is inevitable that for medical applications and when using vital cells such as in ACI, preparation times, including *in vitro* culture periods, will need to be shortened. Overall, it is important to be able to reduce the chondrocyte culture time before cell-sheet harvesting, with reduced time for cell expansion and ECM production, but without changing the extra week of culture to reinforce the cell sheets as this prolonged step is substantially beneficial to this new strategy to cure OA using bioengineered chondrocyte sheets.

In conventional ACI[6] for cartilage regeneration, transplanted periosteal patches, which are used to enclose the implanted chondrocytes, sometimes cause hypertrophy of the regenerated chondral surface. Some improved methods use alternative scaffolds such as collagen membranes, hyaluronan polymers, and atelocollagen gels.[7,22] In comparison to these conventional ACI techniques, the cell-sheet technology has the specific advantage of generating three-dimensional tissues fabricated by autologous cells without using a scaffold while also showing intense adhesiveness on the basal side. The advantages of such cell sheets are that it is easy to culture and expand, and, most importantly, they have good adhesion and barrier function.[9] This means they can protect against intra-articular catabolic factors and prevent proteoglycan escape from the injured site. Moreover, these cell sheets are considered to contain an advantageous supply of growth factors. Furthermore, such cell sheets could be useful as an alternative to the periosteum itself, which is usually used in ACI. Although cell sheets have good adhesive properties compared with other applications using cell-sheet techniques, such as in the cornea and heart, transplanted chondrocyte sheets will be exposed to a harsh environment resulting from weight bearing and friction. Development of new devices to prevent the sheets peeling from the transplanted site is indispensable to future clinical application.

Although focal gene delivery using a cell sheet has not been addressed in this paper, this may also have clinical potential for treatment of cartilage degeneration. The development of a therapeutic apparatus to deliver the cell sheet to the injured site less invasively may therefore be fundamental to expanding its use in the treatment of patients demonstrating the early stages of osteoarthritis.

The results of this study therefore lead to a new strategy for cartilage regeneration using novel bioengineered chondrocyte sheets produced using a cell-sheet technique.

## Conclusion

These experiments demonstrated that triple-layered chondrocyte sheets contain the phenotypic markers COL2, COL27, SOX9, and the adhesion molecules integrin α10 and fibronectin. Cell-sheet technology therefore provides particular advantages for cartilage regeneration, giving three-dimensional tissue constructed without a scaffold and with good adhesiveness to both itself and to an injured cartilage site.

## Methods

### *Preparation of human chondrocytes*

This study was performed in compliance with the Helsinki Declaration, and was approved by the Institutional Review Board for Clinical Research of Tokai University School of Medicine (ref. 04-056).

Human chondrocytes were obtained from the knee joints of young athletes who underwent anterior cruciate liga-

ment reconstruction at Tokai University Oiso hospital from December 2004 to April 2006. Twenty-nine knees from 29 patients aged 14 to 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, USA) 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 hrs 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, USA) 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 × 245 mm; Corning Inc., Corning, NY, USA) 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 (according to the method of Sato *et al.*).[23]

### *Temperature-responsive culture dishes*

The specific procedures for the preparation of temperature-responsive culture dishes (provided by CellSeed, Inc) have all been previously described.[24] 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 binding of the IPAAm to the dish surface. Poly-IPAAm (PIPAAm)-grafted dishes were rinsed with cold distilled water to remove any ungrafted IPAAm. Finally, the culture dishes were sterilized using ethylene oxide gas.

### *Preparation of conventional monolayer cultures of chondrocytes and single-layer chondrocyte sheets*

To detach the primary passage cells, chondrocytes were digested using 0.05% trypsin:EDTA (GIBCO), and then counted using a hematocytometer. For the conventional monolayer culture, cartilage cells were seeded into culture dishes (diameter: 35 mm, Iwaki Glass Company, LTD., Tokyo, Japan) at a density of 1,000 cells/$cm^2$. 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.

The samples in conventional culture were harvested with a sterile cell scraper. 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.

### *Fabrication of cell sheet into three-layer sheets of chondrocytes*

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, USA) 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.

### *Scanning electron microscopy evaluation*

Triple-layered chondrocyte cell sheets were soaked in 0.1 mol/l phosphate buffer and 2% glutaraldehyde for 2 h. Next, the samples were fixed in 1% osmium solution for 1 h and dehydrated in ascending concentrations of ethanol (50%, 70%, 80%, 90%, 95%, and 100%). The specimens were dried using the critical point drying method, spatter-coated with gold, and affixed to an adhesive interface for observation by SEM (JSM-840; Jeol Ltd., Tokyo, Japan). Both the top and bottom surfaces of the cell sheets were observed.

### *RNA isolation and cDNA synthesis*

Total RNA extraction was carried out using the RNeasy Mini kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. RNA quality from each sample was determined using the A260/280 absorbance ratio and by electrophoresis on 1.2% agarose formaldehyde gel. Total RNA (1.0–2.0 μg) was reverse transcribed into single stand cDNA using MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA). The reverse-transcription reaction was performed in a thermocycler at 42°C for 60 min and then at 95°C for 5 min.

### *Primer design and real-time PCR*

All oligonucleotide primer sets were designed based upon published mRNA sequences. The expected amplicon

lengths ranged from 70 to 200 bp. The oligonucleotide primers used in this study are listed in Table 1. The real-time PCR was performed in a SmartCycler™ (Cepheid, Sunnyvale, CA, USA) using SYBR Green PCR Master Mix (Applied Biosystems). From 2 to 2.5 μl of cDNA template was used for real-time PCR in a final volume of 25 μl. cDNA was amplified according to the following condition: 95°C for 15 s and 60°C for 60 s from 35 to 45 amplification cycles. Fluorescence changes were monitored with SYBR Green after every cycle. A melting curve analysis was performed (a 0.5°C/s increase from 55 to 95°C with continuous fluorescence readings) at the end of cycles to ensure that single PCR products were obtained. The amplicon size and reaction specificity were confirmed by 2.5% agarose gel electrophoresis. All reactions were repeated in six separate PCR runs using RNA isolated from four sets of human samples. The results were evaluated using the SmartCycler™ software program. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used to normalize samples. To monitor crossover contaminations of PCR, RNase-free water (Qiagen Inc) was included in the RNA extraction and was used as a negative control. To ensure the quality of the data, a negative control was always included in each run.

### *Immunohistochemical staining*

Frozen sections (30 × 24 × 5 mm) of triple-layered cell sheets were prepared using OCT compound (Sakura Fine Technical Co., Tokyo, Japan). The sections were then washed in PBS, and were reacted at room temperature for 60 min with three monoclonal antibodies: an anti-fibronectin mouse monoclonal antibody (clone FBN11, diluted 1:500, #MS-1351-P0; Thermo SCIENTIFIC, Lab Vision Co., CA, USA), an anti-human CD11c (integrin α10) mouse monoclonal antibody (clone BU15, diluted 1:200, #SM1834PS; Acris Antibodies GmbH, Herford, Germany), and an anti-human collagen type 2 mouse monoclonal antibody (clone α-4C11, diluted to 5 μg/ml, #F-57; Daiichi Fine Chemical Co., Toyama, Japan). The sections were washed in PBS, and reacted with polyclonal rabbit anti-mouse immunoglobulin/FITC, diluted 1:100 (#F0261; DAKOcytomation, Glostrop, Denmark) as a fluorescent secondary antibody. Mounting with a water-soluble mounting medium (VECTASHIELD® Mounting Medium with DAPI, Vector Laboratories, Inc., Burlingame, CA, USA) was performed to counterstain DNA after washing sections in purified water.

### *Statistical analysis*

The real-time PCR results are expressed as the mean ± standard error of the mean from six determinations and representative results are shown. The statistical software program SPSS (Version 17.0, SPSS, Chicago, IL, USA) was used to perform standard analysis of variance and the Scheffe's post hoc test. Table 2 lists the p-values shown in Figure 3.

## Authors' contributions

GM, MS, NK, MK, and TK performed the research. JIL, MI, HS, and JM analyzed the data. HS provided the temperature-responsive culture dishes for these experiments. NO took charge of the statistical analyses. GM, JIL, MS, and JM wrote the manuscript. All authors have read and approved the final manuscript.

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## References

1. Mankin HJ: **The response of articular cartilage to mechanical injury.** *J Bone Joint Surg Am* 1982, **64(3)**:460-466.
2. Buckwalter JA, Mankin HJ: **Articular cartilage: degeneration and osteoarthritis, repair, regeneration, and transplantation.** *Instr Course Lect* 1998, **47**:487-504.
3. Kim HK, Moran ME, Salter RB: **The potential for regeneration of articular cartilage in defects created by chondral shaving and subchondral abrasion. An experimental investigation in rabbits.** *J Bone Joint Surg Am* 1991, **73(9)**:1301-1315.
4. Steadman JR, Rodkey WG, Singleton SB, Briggs KK: **Microfracture technique for full-thickness chondral defect : technique and clinical results.** *Operative Techniques in Orthopaedics* 1997, **7**:4300-4304.
5. Marcacci M, Kon E, Zaffagnini S, Iacono F, Neri MP, Vascellari A, Visani A, Russo A: **Multiple osteochondral arthroscopic grafting (mosaicplasty) for cartilage defects of the knee: prospective study results at 2-year follow-up.** *Arthroscopy* 2005, **21(4)**:462-470.
6. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L: **Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation.** *N Engl J Med* 1994, **331(14)**:889-895.
7. Masuoka K, Asazuma T, Ishihara M, Sato M, Hattori H, Yoshihara Y, Matsui T, Takase B, Kikuchi M, Nemoto K: **Tissue engineering of articular cartilage using an allograft of cultured chondrocytes in a membrane-sealed atelocollagen honeycomb-shaped scaffold (ACHMS scaffold).** *J Biomed Mater Res B Appl Biomater* 2005, **75(1)**:177-184.
8. Okano T, Yamada N, Sakai H, Sakurai Y: **A novel recovery system for cultured cells using plasma-treated polystyrene dishes grafted with poly(N-isopropylacrylamide).** *J Biomed Mater Res* 1993, **27(10)**:1243-1251.
9. Kaneshiro N, Sato M, Ishihara M, Mitani G, Sakai H, Mochida J: **Bioengineered chondrocyte sheets may be potentially useful for the treatment of partial thickness defects of articular cartilage.** *Biochem Biophys Res Commun* 2006, **349(2)**:723-731.
10. Nishida K, Yamato M, Hayashida Y, Watanabe K, Maeda N, Watanabe H, Yamamoto K, Nagai S, Kikuchi A, Tano Y, *et al.*: **Functional bioengineered corneal epithelial sheet grafts from corneal stem cells expanded ex vivo on a temperature-responsive cell culture surface.** *Transplantation* 2004, **77(3)**:379-385.
11. Shimizu T, Yamato M, Isoi Y, Akutsu T, Setomaru T, Abe K, Kikuchi A, Umezu M, Okano T: **Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces.** *Circ Res* 2002, **90(3)**:e40.
12. Shimizu T, Sekine H, Yang J, Isoi Y, Yamato M, Kikuchi A, Kobayashi E, Okano T: **Polysurgery of cell sheet grafts overcomes diffusion limits to produce thick, vascularized myocardial tissues.** *FASEB J* 2006, **20(6)**:708-10.
13. Harimoto M, Yamato M, Hirose M, Takahashi C, Isoi Y, Kikuchi A, Okano T: **Novel approach for achieving double-layered cell**

**sheets co-culture: overlaying endothelial cell sheets onto monolayer hepatocytes utilizing temperature-responsive culture dishes.** *J Biomed Mater Res* 2002, **62(3)**:464-470.
14. Kushida A, Yamato M, Konno C, Kikuchi A, Sakurai Y, Okano T: **Temperature-responsive culture dishes allow nonenzymatic harvest of differentiated Madin-Darby canine kidney (MDCK) cell sheets.** *J Biomed Mater Res* 2000, **51(2)**:216-223.
15. Hughes LC, Archer CW, ap Gwynn I: **The ultrastructure of mouse articular cartilage: collagen orientation and implications for tissue functionality. A polarised light and scanning electron microscope study and review.** *Eur Cell Mater* 2005, **9**:68-84.
16. Poole CA: **Articular cartilage chondrons: form, function and failure.** *J Anat* 1997, **191(Pt 1)**:1-13.
17. Wenke AK, Rothhammer T, Moser M, Bosserhoff AK: **Regulation of integrin alpha10 expression in chondrocytes by the transcription factors AP-2epsilon and Ets-1.** *Biochem Biophys Res Commun* 2006, **345(1)**:495-501.
18. Jenkins E, Moss JB, Pace JM, Bridgewater LC: **The new collagen gene COL27A1 contains SOX9-responsive enhancer elements.** *Matrix Biol* 2005, **24(3)**:177-184.
19. Camper L, Hellman U, Lundgren-Akerlund E: **Isolation, cloning, and sequence analysis of the integrin subunit alpha10, a beta1-associated collagen binding integrin expressed on chondrocytes.** *J Biol Chem* 1998, **273(32)**:20383-20389.
20. Bengtsson T, Aszodi A, Nicolae C, Hunziker EB, Lundgren-Akerlund E, Fassler R: **Loss of alpha10beta1 integrin expression leads to moderate dysfunction of growth plate chondrocytes.** *J Cell Sci* 2005, **118**:929-936.
21. Kikuchi A, Okuhara M, Karikusa F, Sakurai Y, Okano T: **Two-dimensional manipulation of confluently cultured vascular endothelial cells using temperature-responsive poly(N-isopropylacrylamide)-grafted surfaces.** *J Biomater Sci Polym Ed* 1998, **9(12)**:1331-1348.
22. Ochi M, Uchio Y, Kawasaki K, Wakitani S, 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* 2002, **84(4)**:571-578.
23. Sato M, Asazuma T, Ishihara M, Kikuchi T, Masuoka K, Ichimura S, Kikuchi M, Kurita A, Fujikawa K: **An atelocollagen honeycomb-shaped scaffold with a membrane seal (ACHMS-scaffold) for the culture of annulus fibrosus cells from an intervertebral disc.** *J Biomed Mater Res A* 2003, **64(2)**:248-256.
24. Okano T, Yamada N, Okuhara M, Sakai H, Sakurai Y: **Mechanism of cell detachment from temperature-modulated, hydrophilic-hydrophobic polymer surfaces.** *Biomaterials* 1995, **16(4)**:297-303.

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# Noninvasive Evaluation of Tissue-Engineered Cartilage with Time-Resolved Laser-Induced Fluorescence Spectroscopy

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Regenerative medicine requires noninvasive evaluation. Our objective is to investigate the application of time-resolved laser-induced fluorescence spectroscopy (TR-LIFS) using a nano-second-pulsed laser for evaluation of tissue-engineered cartilage (TEC). To prepare scaffold-free TEC, articular chondrocytes from 4-week-old Japanese white rabbits were harvested, and were inoculated at a high density in a mold. Cells were cultured for 5 weeks by rotating culture (RC) or static culture (SC). The RC group and SC group at each week ($n = 5$), as well as normal articular cartilage and purified collagen type II (as controls), were analyzed by TR-LIFS. The peak wavelength was compared with those of type II collagen immunostaining and type II collagen quantification by enzyme-linked immunosorbent assay and tensile testing. The fluorescence peak wavelength of the TEC analyzed by this method shifted significantly in the RC group at 3 weeks, and in the SC group at 5 weeks ($p < 0.01$). These results correlated with changes in type II collagen (enzyme-linked immunosorbent assay) and changes in Young's modulus on tensile testing. The results were also supported by immunohistologic findings (type II collagen staining). Our findings show that TR-LIFS is useful for evaluating TEC.

## Introduction

NONINVASIVE MEANS ARE ESSENTIAL for evaluating physical properties and tissue characterization of regenerative tissues for implantation. Our research group (Ishihara *et al.*) discovered that stress wave propagation and attenuation by pulsed laser irradiation was influenced by tissue elasticity and, based on this principle, proposed the use of photoacoustic measurement for viscoelastic characterization of biological tissue.[1–5] In this study, we investigated whether pulsed laser irradiation and measurement of excited autofluorescence would enable noninvasive real-time evaluation of viscoelasticity and tissue characterization. Autofluorescence is the basis for photodynamic therapy in malignant lesions, and photosensitizers were originally used. Photosensitizers cause tissue injury and remain in the body or tissue samples for certain periods of time, so they are not suitable for serial monitoring. In addition, they are not suitable for use in regenerative medicine, in which biocompatibility is required. In the late 1970s, background signals that were previously thought to be exogenous fluorescence were found to be due to endogenous autofluorescence.[6] Since that time, the use of autofluorescence for detection and periodic screening of tumors and use in dental lesions, skin lesions, and atherosclerotic plaques have been described in many reports. The use of autofluorescence in regenerative medicine, however, is relatively recent, and few reports have described its application for evaluation of regenerative tissues.[7] Autofluorescent substances include structural proteins (collagen and elastin), amino acids (tryptophan, tyrosine, and phenylalanine), lipids (cholesterol), vitamins (vitamin A, D, and K), and enzyme cofactors (NAD(P)H, FAD).[6,8–9] The structure of these autofluorescent substances is reflected by their fluorescence spectra and lifetime (decay characteristics) in tissue. Thus, fluorescence spectral analysis allows us to predict tissue biochemical composition and metabolic activity.[10,11] Collagen, the major component of cartilage, is now recognized as an important autofluorescent substance. The same is true for enzyme cofactors NAD(P)H in cells.[12] The scaffold-free tissue-engineered cartilage (TEC) used in this study was constructed as follows. Cartilage formation by secretion of molecules forming the extracellular matrix

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(ECM) by chondrocyte differentiation from aggregated mesenchymal cells during the embryonic development stage and dynamic stress is reproduced *in vitro* by a simple system of high-density seeding of cells in a cylindrical mold and rotating flow using a rotary shaker. Then, without specific growth factors, the dedifferentiated monolayer of cultured chondrocytes is redifferentiated in a three-dimensional (3D) culture without a scaffold, and their ability to produce matrix is reacquired. This TEC, based on cell distribution, tissue composition, and quantification of glycosaminoglycans and collagen, has tissue characteristics and physical properties that closely approximate those in normal cartilage.[13–16] Hyaline cartilage has a high moisture content, a sparse cell population, and a collagen and proteoglycan-rich ECM, and thus the autofluorescent substances in cartilage are primarily collagen and NAD(P)H correlating with cell components.[8] In addition, most of the collagen is type II, with trace amounts of types VI, IX, and XI.[17] Therefore, from production to postimplantation of TEC, the monitoring of chondrocyte differentiation and ECM production requires serial measurement of type II collagen and NAD(P)H. Our objective was to investigate the application of time-resolved laser-induced fluorescence spectroscopy (TR-LIFS) for serial monitoring of TEC by comparing the results of TR-LIFS of scaffold-free TEC with the results of conventional evaluation of articular cartilage, including tissue characterization and physical properties. Conventional tissue characterization and evaluation of physical properties of articular cartilage includes biochemical quantification (enzyme-linked immunosorbent assay [ELISA]) of type II collagen and immunostaining. Among the physical properties of articular cartilage, proteoglycans contribute primarily to compression characteristics, whereas type II collagen plays more of a role in tensile characteristics.[18,19] Therefore, we performed tensile testing. Our study showed good correlation between the results and suggests that TR-LIFS is useful for quality noninvasive evaluation of TEC.

## Materials and Methods

### *TR-LIFS setup, spectroscopic measurements, and data analysis*

Figure 1 depicts the experimental setup. The beam was concentrated with a lens attached to a quartz fiber with a core diameter of 400 μm. Fluorescence was induced using the third harmonic wave of a Q switch Nd:YAG laser (wavelength 355 nm, frequency 5 Hz). The pulsed laser was directed onto the sample at a 45° angle of incidence, with a beam diameter of about 5 mm, through a laser transmission optical fiber. Induced fluorescence was collected perpendicularly to this beam. Distance between the probe and sample was 20 mm. The output energy of the optical fiber was adjusted to 50 μJ/pulse,[3] which is substantially lower than the biological damage threshold. Time-resolved fluorescent spectroscopy of the fluorescence induced by pulsed laser was obtained using a photonic multi-channel analyzer with ICCD (wavelength range, 200–860 nm; resolution, 3 nm; gate time, 10 ns). Samples were washed with purified water, and at room temperature, each sample was subjected to measurement at 10 locations.[1–5] We used a sharp cut filter to intercept excitation light (SCF-50S-38L; Sigma–Koki, Tokyo, Japan).

The measured fluorescence was displayed in 3D, and peak wavelength was used as parameters. All calculations were performed using MatLab software (MathWorks Inc.).

### *Cell culture*

Articular chondrocytes were harvested from the shoulders and knees of 4-week-old Japanese white rabbits weighing about 1 kg ($n = 12$). Cartilage was digested in Dulbecco's modified Eagle's medium (DMEM/F12; Gibco–Invitrogen, Carlsbad, CA) containing 0.4% (w/v) actinase E (Kaken Pharmaceutical, Tokyo, Japan) for 1 h, and in DMEM/F12 containing 0.016% (w/v) bacterial collagenase P (Roche

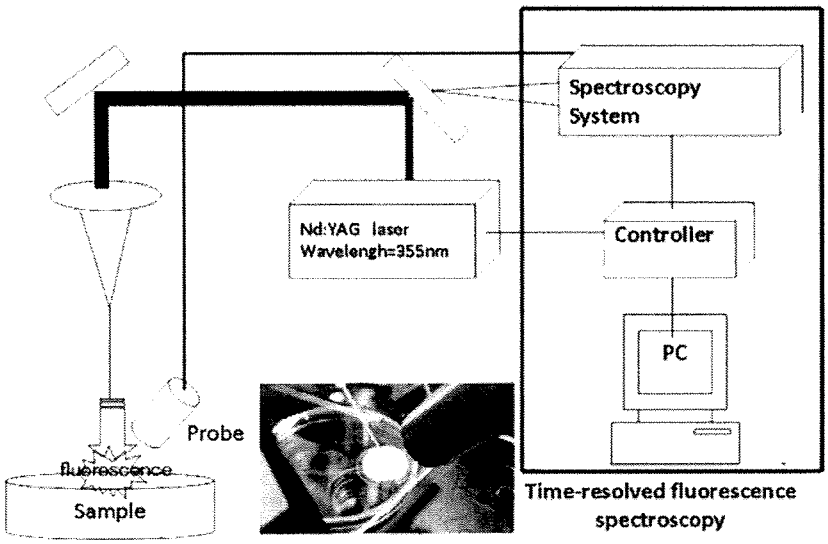



FIG. 1. System for fluorescent measurement. Time-resolved fluorescent spectroscopy was performed using a photonic multi-channel analyzer with a 4-Ch digital signal generator. The fluorescent features of the developed measurement system are as follows: wavelength range, 200–860 nm; wavelength resolution, <3 nm; exposure time, 19 ms; gate time, 10 ns. The parameters of measured fluorescence obtained using MatLab software were peak wavelength at fluorescence maximum, fluorescent spectral bandwidth at half-maximal amplitude (FWHM), and integrated intensity of time-resolved spectrum. Color images available online at www.liebertonline.com/ten.

Diagnostics GmbH, Mannheim, Germany) for 3 h. The digested cartilage was passed through a 70-μm cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ) and was centrifuged at 1500 rpm for 5 min. The pellet was suspended in AU3▶ DMEM/F12, 10% fetal bovine serum (Gibco), 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), 0.25 μg/mL fungizone (Gibco), and 50 μg/mL ascorbic acid (Wako Pure Chemical Industries, Osaka, Japan). Chondrocytes were inoculated on 500-$cm^2$ dishes at $1 \times 104$ cells/$cm^2$ and cultured at 37°C with 5% $CO_2$ and 95% humidity. After 1 week, the primary culture cells (P0) (70% confluence monolayer) were detached using 0.05% trypsin/EDTA (Gibco) at 37°C for 10 min. Cells were then washed with phosphate-buffered saline (PBS), resuspended in medium, and cultured at $1 \times 104$ cells/$cm^2$ for two passages. Medium was replaced every 3 days.[15,16]

### *Cartilage tissue formation*

Second-passage chondrocytes were resuspended at a density of $1.0 \times 10^7$ cells/mL in DMEM/F12, 20% fetal bovine serum (Gibco), 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), 0.25 μg/mL fungizone (Gibco), and 50 μg/mL ascorbic acid (Wako Pure Chemical Industries). Cylindrical glass molds (diameter, 10 mm; height, 10 mm) were placed into a culture insert with a pore size of 0.4 μm (Corning Costar Japan, Tokyo, Japan) to permit oxygen and nutrient diffusion. First, 15 mL of culture medium was added under the culture insert, and 15 mL of culture medium was added onto the culture insert. Next, 0.6 mL of the cell suspension was inoculated onto the mold, and was allowed to stand for 30 min. Gravity-assisted sedimentation of the cell suspension on the insert was confirmed, and about 30 mL of culture medium was then added to the culture insert until the mold was completely filled. This was cultured for 8 h at 37 C under 5% $CO_2$ and 95% humidity. The mold was then removed, and the cell mass (chondrocyte plate) with a form similar to the mold was cultured for 7 days under the same conditions. Constructs, shaped like the mold, were removed using a medicine spoon and moved to a nonadherent six-well culture dish, where they were cultured with 6 mL of medium per plate. Rotating culture (RC) was performed for 5 weeks using an orbital shaker (Taitec; 70 rpm; turn radius, 25 mm). Static culture (SC) and RC were performed at 37°C under 5% $CO_2$ and 95% humidity. Culture medium was replaced every 3 days.[15,16]

### *Fixation and sectioning*

Samples were washed with PBS and fixed in 4% formalin solution. After ethanol dehydration, samples were immersed in isoamyl alcohol, embedded in paraffin, cut into 4-μm sections, and stained with safranin-O and toluidine blue for proteoglycans. Sections were deparaffinized for immunohistochemistry using standard procedures. Sections were then treated with 0.005% proteinase (type XXIV; Sigma–Aldrich, St. Louis, MO) for 30 min at 37°C before incubation with primary antibody. After the slides were washed with PBS, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 15–20 min at room temperature. Next, sections were rinsed with PBS and incubated with normal goat serum (diluted 1:20 in PBS) for 30 min at room temperature. Primary mouse monoclonal antibody directed against human collagen types I and II (Daiichi Fine Chemical, Toyama, Japan) was diluted 1:200 in PBS–1% bovine serum albumin (BSA; Sigma). Slides were incubated ◀AU3 overnight at 4°C, washed 10 times with PBS, and incubated with biotin-conjugated goat anti-mouse secondary antibody (diluted 1:100 in PBS-1% BSA) in a humidified chamber for 1 h at room temperature. Slides were then treated with horseradish peroxidase–labeled streptavidin (streptavidin–horseradish peroxidase) for 1 h. Finally, slides were immersed in a 0.05% solution of diaminobenzidine in Tris-HCl buffer (pH 7.6) containing 0.005% hydrogen peroxide for 2–4 min until the color developed. To improve cell observation, the slides were counterstained with Mayer's hematoxylin. Transverse sections were observed and recorded with an inverted microscope and digital camera (objective lens, ×20).

### *Biochemical analysis*

Type II collagen ELISA. Wet weight and weight after freeze-drying were measured to calculate moisture content. The freeze-dried sample and 0.5 mL of cold distilled water were added to an Eppendorf tube and incubated overnight at 4°C. The sample was centrifuged (10000 rpm, 3 min), and the supernatant was removed. Next, 0.5 mL of 3 M guanidine/0.5 M Tris-HCl (pH 7.5) was added to the sample tube, and this was mixed on a rotator/rocker overnight at 4°C. The sample was homogenized using a small electric homogenizer and centrifuged (10000 rpm, 3 min). The precipitate was washed with cold distilled water, and was then resuspended in 0.8 mL of 0.05 M acetic acid containing 0.5 M NaCl (adjusted to pH 2.9–3.0 with formic acid). Next, 0.1 mL of pepsin (10 mg/mL) dissolved in 0.05 M acetic acid was added and mixed, and this was incubated at 4°C for 48 h. Then, 0.1 mL of 10×TBS (1.0 M Tris-2.0 M NaCl-50 mM CaCl2, pH 7.8–8.0) was added (adjusted to pH 8.0 with 1 N NaOH). In addition, 0.1 mL of pancreatic elastase (1 mg/mL dissolved in 1×TBS, pH 7.8–8.0) was added, followed by mixing on a rotator/rocker overnight at 4°C. The sample was centrifuged (10000 rpm, 5 min), and the supernatant was collected. Type II collagen was assayed by ELISA (Native Type II Collagen ELISA Kit, Catalogue No. 6009; Chondrex) ◀AU3 according to the manufacturer's protocol. ELISA samples were measured by spectrophotometry at 490 nm. The results were compared with the standard curve for type II collagen in the kit.

### *Measurement of thickness and tensile properties*

Sample thickness was measured using a digital micrometer (minimum display, 0.001 mm; error, ± 1 μm; degree of parallelization, 1 μm). Tensile testing was performed with the sample set in a grip and at a pulling speed of 4 mm/min. Young's modulus was calculated from the slope of the linear portion of the load–deflection curve.

### *Statistical analysis*

Samples were divided into the SC and RC groups, with comparisons at each week. All data are shown as means ± SE. ◀AU2 Factorial analysis of variance was used for comparisons. Each significant difference on analysis of variance was analyzed by multiple comparison using Scheffe's test. The level of statistical significance was $p < 0.01$.

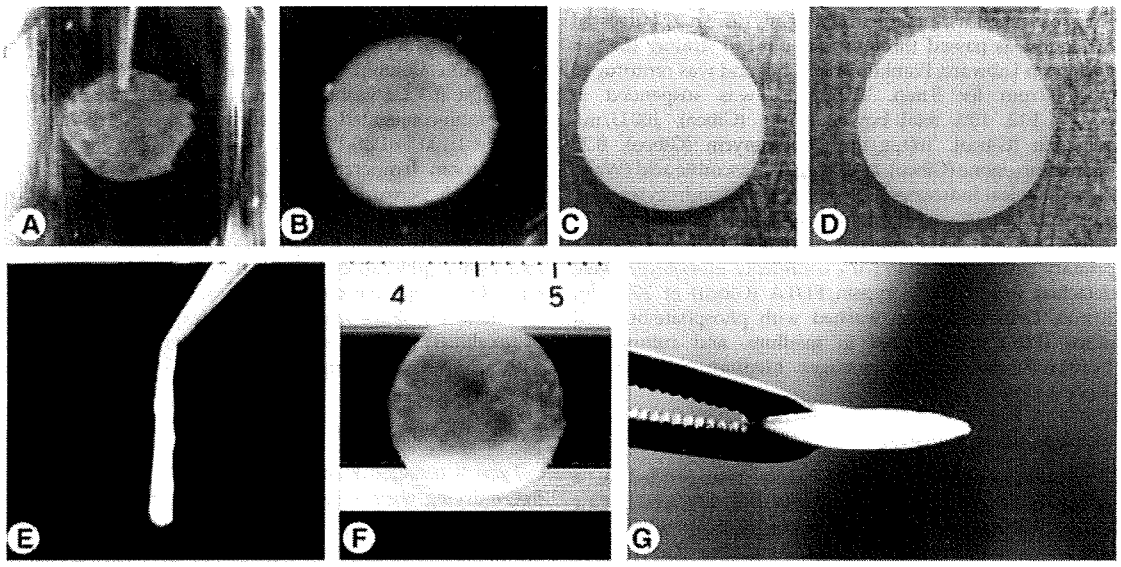



FIG. 2. Macroscopic appearance of the chondrocyte plate, which formed after 7 days of primary static culture (SC) (**A, E**). Subsequently, a chondrocyte plate formed after rotating culture (RC) for 1 week (**B, F**), 2 weeks (**G**), 3 weeks (**C**), or 5 weeks (**D**). Scale bar = 1 mm. Color images available online at www.liebertonline.com/ten.

## Results

### *Macroscopic appearance*

The TEC after 1 week of primary SC retained a form similar to the mold (Fig. 2A). However, after removal from the culture medium, this shape could no longer be maintained (Fig. 2E). With RC and SC, thickness of the chondrocyte plate increased with culture duration (Fig. 2B–D). After 2 weeks of culture, the construct retained its shape even when handled with forceps (Fig. 2F, G).

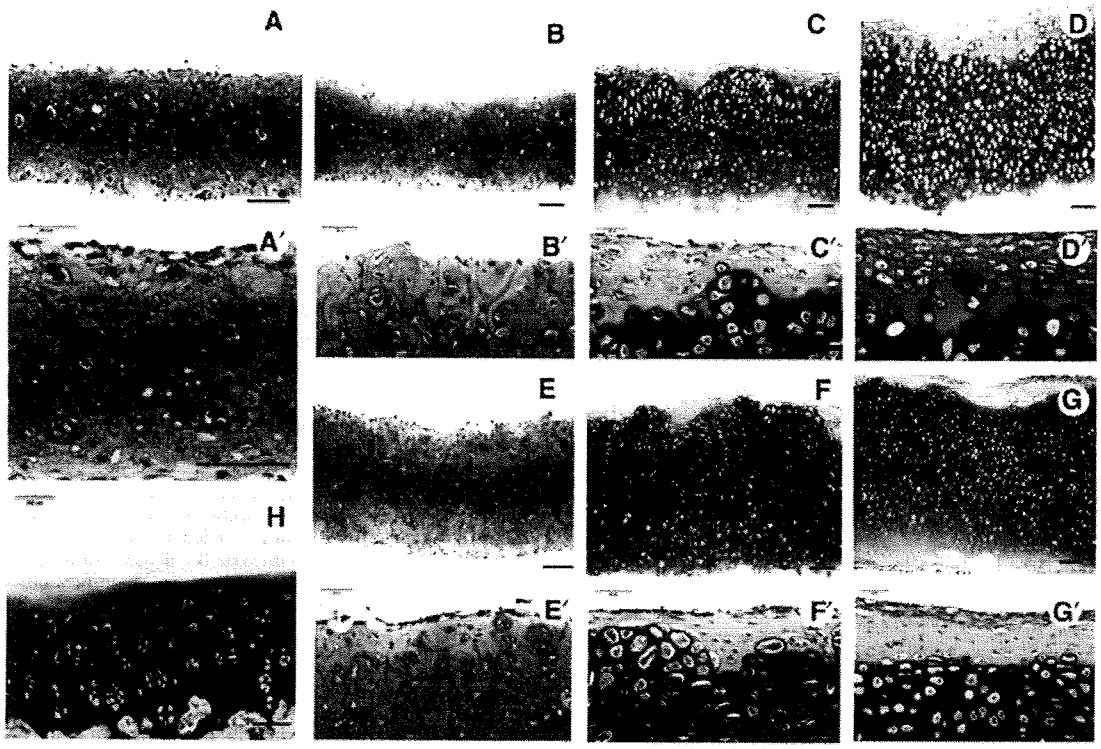
### *Histology and immunohistochemistry*

Figures 3-1H and 3-2H show the normal articular cartilage of knee from 4-week-old Japanese white rabbits. Figure 3-1A shows the toluidine blue staining during the primary 1 week of SC. Cells are uniformly distributed, and the ECM also stains uniformly. In the SCs at 1, 3, and 5 weeks (Fig. 3-1B, B′, C, C′, D, D′); as compared with rotational cultures at 1, 3, and 5 weeks (Fig. 3-1E, E′, F, F′, G, G′); cell distribution was uneven, cell density was lower, and numerous enlarged cells were observed. In addition, staining was uneven near the surface and, overall, tended to be weaker. On the other hand, in the rotational cultures at 1, 3, and 5 weeks (Fig. 3-1E, E′, F, F′, G, G′), cell distribution was uniform, and cell morphology was nearly uniform, with only a few enlarged cells. Further, staining, with the exception of superficial fibroblastic cells, was uniform and tended to be stronger. The superficial fibrous layer, compared to the SCs (Fig. 3-1B, B′, C, C′, D, D′), was thicker and increased in thickness with culture duration (weeks). For the initial 1 week of SC, the SCs at 1, 3, and 5 weeks (Fig. 3-1B, B′, C, C′, D, D′), the rotational cultures at 1, 3, and 5 weeks (Fig. 3-1E, E′, F, F′, G, G′), and the staining characteristics for safranin-O were similar. In the SC group, immunostaining for type II collagen did not clearly differ up to 3 weeks of culture, but at 5 weeks of culture, staining was intense. In the RC group, staining did not clearly differ up to 2 weeks of culture, but staining was intense by 3 weeks of culture and remained so at week 5. In addition, overall staining for type II collagen, as compared to the SC group, tended to be more uniform and intense (Fig. 4). The results were similar to safranin-O and toluidine blue staining.

### *Biochemical analysis*

Type II collagen ELISA. Type II collagen content did not significantly differ between the groups at 2 weeks of culture, but at week 3 and later, type II collagen content was significantly greater in the RC group than in the SC group. In the SC group, there was no significant increase by 3 weeks, but the increase was significant at 5 weeks. In the RC group, there was no significant increase at 2 weeks, but by 3 weeks, there was a significant increase (Fig. 5).

FIG. 3. Distribution of proteoglycans was investigated. Chondrocyte plate formed after 1 week of primary SC was stained with toluidine blue and safranin-O (**1A, A′, 2A, A′**). Chondrocyte plate formed after 1, 3, and 5 weeks of SC was stained with toluidine blue and safranin-O (**1B, B′, C, C′, D, D′, 2B, B′, C, C′, D, D′**). Chondrocyte plate formed after 1, 3, and 5 weeks of RC was stained with toluidine blue and safranin-O (**1E, E′, F, F′, G, G′, 2E, E′ F, F′, G, G′**). Normal articular cartilage (4-week-old Japanese white rabbits) (**1H, 2H**). (**A–H**) Magnifications of objective lens, ×4. (**A′–G′**) Magnifications of objective lens, ×20. Scale bar = 100 μm. Color images available online at www.liebertonline.com/ten.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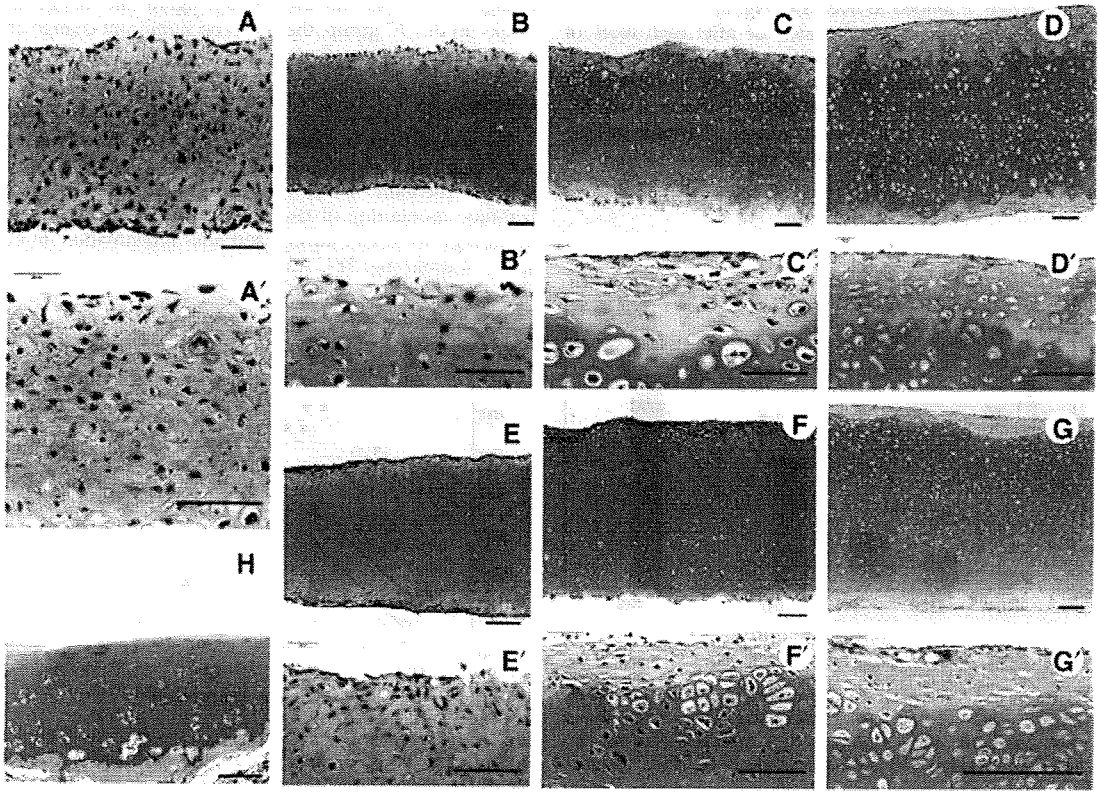
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