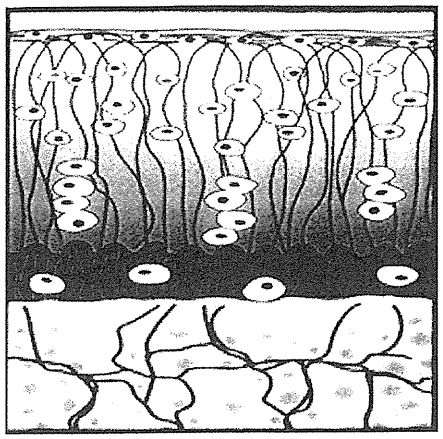
WOODHEAD PUBLISHING IN MATERIALS



# Regenerative medicine and biomaterials for the repair of connective tissues

Edited by Charles Archer and Jim Ralphs

CRC

WP

# 10

# Cell sheet technologies for cartilage repair

M. SATO, Tokai University School of Medicine, Japan

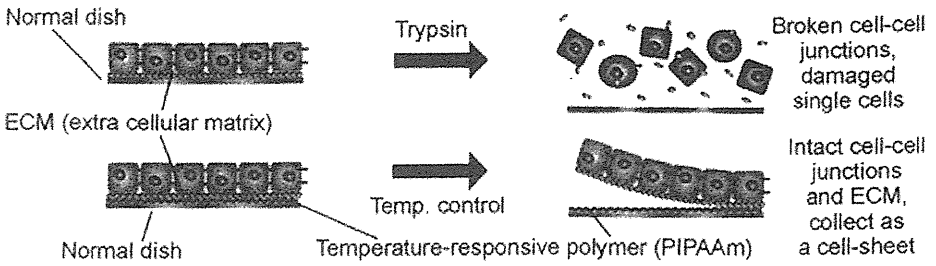
**Abstract:** This chapter outlines the principles of cell sheet technology, its clinical applications, how to repair cartilaginous defects using layered chondrocyte sheets, the properties of layered chondrocyte sheets, future trends in cartilage repair, and regulation of this area in Japan.

**Key words:** cell sheet, temperature-responsive culture dish, articular cartilage, cultured chondrocyte, tissue engineering.

## 10.1 Introduction

This section outlines cell sheet technology, specifically introducing the temperature-responsive culture dish. Cell sheets can be obtained without enzymes. It is also possible to obtain layered constructs. Prof. Okano,[1,2] from Tokyo Women's Medical University, developed this technology involving the building of three-dimensional tissue constructs, consisting of individual units of the cell sheets with tight junctions between the cells and extracellular matrices. A temperature-responsive culture dish is used to obtain the cell sheet. An *N*-isopropylacrylamide monomer solution is spread on commercial tissue culture polystyrene dishes. These dishes are then subjected to electron beam irradiation, thus resulting in polymerization and covalent binding of the isopropylacrylamide to the surface of the dish.

Poly *N*-isopropylacrylamide (PIPAAm) is a unique polymer that exhibits thermally reversible soluble-insoluble changes in an aqueous solution in response to temperature changes across an LCST (lower critical solution temperature) of 32 °C. Polymerized chains of acrylamide hydrate to expand in water below the LCST, while the isopropyl group dehydrates to form compact, insoluble conformations above the LCST. These dishes, therefore, reverse their hydrophobic and hydrophilic properties in response to changing temperature. When the temperature-responsive polymer (PIPAAm) is fixed to a cell-culturing dish using Bionano interface technology, the surface of the plate changes in response to temperature change across an LCST of 32 °C. The surface becomes hydrophobic above 32 °C, which enables cells to attach to the surface and grow. However, when the temperature is reduced to 20 °C, the polymer surface becomes hydrophilic and the hydrated polymer chains allow the cultured cells to




*10.1* Cell detachment mechanism of temperature-responsive surface of the dish. Cultured cells by using a temperature-responsive surface could be released from the dish surface only by reducing the temperature without proteolytic enzyme. *N*-isopropylacrylamide monomer solution was spread onto commercial tissue culture polystyrene dishes. The surface of the temperature-responsive culture dish is grafted with a polymer (poly-*N*-isopropylacrylamide) which becomes either hydrophilic or hydrophobic in a reversible manner, depending on the temperature. Based on this characteristic, the temperature-responsive culture dish has a weakly hydrophobic surface similar to that of commercially available dishes and it can be used to culture cells in a conventional manner when the temperature is 37 °C or higher. However, the surface of the dish becomes hydrophilic when the temperature falls below the critical solution temperature of 32 °C. Therefore, confluent sheets of cultured cells can be spontaneously released from the hydrophilic dish surface by reducing the temperature to below 32 °C.

detach easily (Fig. 10.1, Movie: http://www.cellseed.com/technology-e/003.html). Cells detach because the hydrophobic surface they are attached to disappears below the LCST of 32 °C. Since cell-damaging enzymes are not required, cells can detach while maintaining the cell–cell junction. This enables the cultured cells to be harvested as a single 'sheet'. The cell sheets are highly effective when transplanted into patients due to the tight connections between the cells. The cultured cell sheet can be easily moved and layered upon other cultured cell sheets to generate a 3-dimensional cell culture. If fibronectin is present, the multiple-layer cell sheets are easily constructed by simply laying one cell sheet on top of another. The technology is ultimately applicable to the construction of organs by layering different types of cell sheet.

## 10.2 Overview of present clinical applications

This section will discuss the present clinical applications of cell sheet technology (i.e., the regeneration of the cornea, myocardium, esophageal mucosa, etc.). Prof. Okano and his colleagues are working on building three-dimensional tissue constructs. Cell sheet technology has already been used for regenerative medicine in corneal[3,4] and myocardial tissues.[5–8] In Japan, the lack of cornea and heart donors and the immune system's reaction to a transplanted organ are recognized as serious problems. Prof. Nishida, at Tohoku University, has produced cell sheets from the cultured epithelial stem cells of cornea and oral

mucosal cells. He has transplanted these two types of cell sheet into more than 12 patients without sutures for tissue regeneration and has had excellent clinical results. Prof. Sawa, at Osaka University, made cell sheets from cultured myoblasts of the femur of a patient with dilated cardiomyopathy who underwent surgery for the implantation of a ventricular assist device in February 2006. He transplanted 20 cell sheets into the walls of the left ventricle of the heart in May 2007. The patient recovered well and he no longer needed the ventricular assist device, which was removed in September 2007. This was the first report of successful cell sheet therapy for a patient with dilated cardiomyopathy. The cell sheet therapy can provide regenerative treatment as an alternative for patients who need cardiac transplantation. Prof. Sawa plans to treat six patients, using the same type of cell sheet, in Osaka University Hospital. Other preclinical studies using cell sheets are under way. Clinical studies of cell sheet therapy for urothelial tissue[9] (Tokyo Women's Medical University), oesophageal tissue[10–12] (Tokyo Women's Medical University), periodontal tissue[13–15] (Tokyo Women's Medical University and Tokyo Medical and Dental University), hepatic tissue[16–19] (Tokyo Women's Medical University) and articular cartilaginous tissue[20,21] (Tokai University School of Medicine) are currently taking place.

## 10.3 Challenge for cartilage repair

This section will discuss how to repair cartilaginous defects using layered chondrocyte sheets. Articular cartilage is an avascular tissue that is nourished by synovial fluid. Adult articular cartilage shows poor self-repair after degeneration or injury and it is therefore unlikely to be restored to normal once it has been damaged. The current treatments available for cartilage defects include the application of a periosteal patch to cover the defect[22] and mosaicplasty, in which an osteochondral pillar is grafted from a non-weight-bearing site.[23] However, the use of periosteal patches has limitations owing to problems with ossification and the limited area that can be treated. Although the microfracture technique is widely used, in which drilling is employed to induce bone marrow cells to differentiate into chondrocytes, the cartilage obtained by this technique is fibrocartilage, with different characteristics to those of hyaline cartilage. Since promising results for the transplantation of cultured autologous chondrocytes have been reported,[24] various articular cartilage regeneration techniques have been applied clinically, including the use of scaffolds such as atelocollagen[25] and cell transplantation therapy with bone marrow-derived mesenchymal stem cells.[26] However, current cartilage regeneration techniques are intended for the treatment of full thickness defects and there have been no reports on the clinical application of a technique for partial thickness defects in patients with early osteoarthritis. Defects in articular cartilage are classified as either full or partial thickness defects, according to whether or not they penetrate the marrow spaces of the subchondral bone. Partial thickness defects are analogous to the clefts and

fissures that are seen in the early stages of osteoarthritis in humans. These fibrillated lesions grow larger and deeper during the course of the disease but never repair themselves spontaneously. It has also been suggested that partial thickness defects do not heal because they are walled off from the marrow and thus have no access to the macrophages, endothelial cells and mesenchymal cells that reside therein.[27]

Articular cartilage is composed of scattered chondrocytes embedded in an abundant extracellular matrix (ECM). The matrix is mainly composed of type II collagen and proteoglycans and is responsible for specific joint functions, including smooth movement and shock absorption. When cultured chondrocytes are employed *in vitro*, it is important to harvest the cells without damaging the ECM. However, current methods damage the cultured cells and disrupt the ECM because proteolytic enzymes are used when harvesting the cultured cells. To achieve the repair and regeneration of partial thickness articular cartilage defects, cultured chondrocytes can be harvested without ECM damage by using temperature-responsive culture dishes. Such cell sheets have been reported to have various advantages, including the preservation of the early phenotype and the expression of adhesion proteins on the base.[1] Furthermore, these cell sheets can be layered onto each other to prepare a layered 'tissue' because the ECM is preserved on the base and such three-dimensional manufactured tissues have already been used for transplantation.[5]

In this study, human chondrocyte sheets with the ECM were obtained using the temperature-responsive culture dish method and were then combined in layers. Following this, the 'tissue' was compared with that of a single sheet and the adhesion of the sheets was examined both *in vivo* and *ex vivo*. This demonstrated the first step towards bioengineering cartilaginous tissues for the treatment of partial thickness cartilage defects using cell sheet technology (Fig. 10.2).

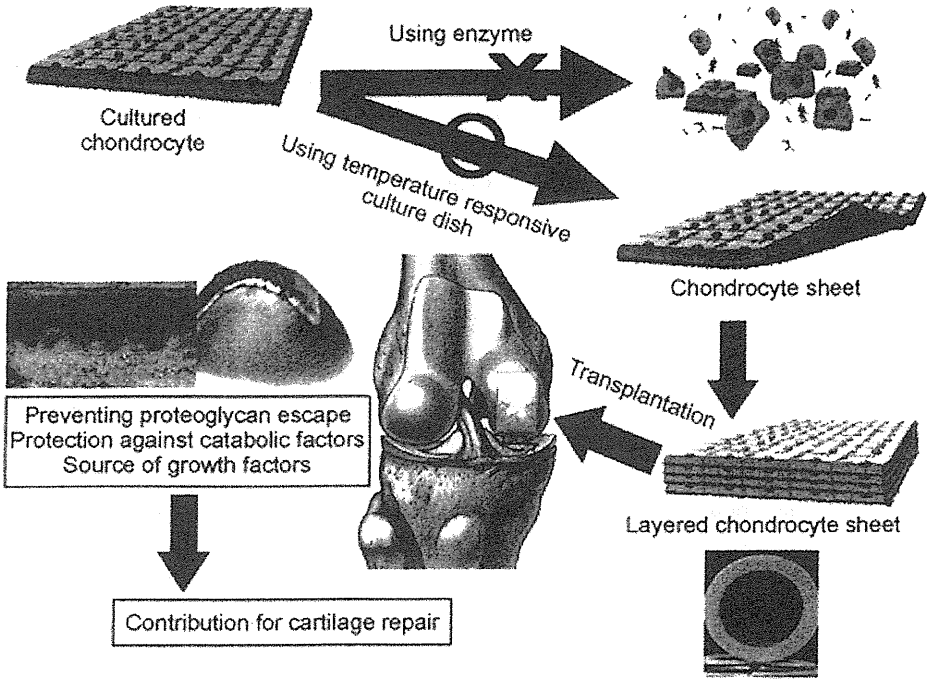
## 10.3.1 Allograft study

*Articular chondrocytes from Japanese white rabbits*

Twelve Japanese white rabbits aged 3–4 weeks and weighing between 800 and 1000 g were used as the source of articular cartilage cells. Cartilage samples were collected from the femoral compartment of the knee joint and were subjected to the same enzymatic treatment process as that used for human articular cartilage cells. Thereafter, the isolated cells were seeded and cultured in temperature-responsive culture dishes.

*Cell proliferations on a temperature-responsive surface*

Chondrocytes were digested for 1 h in Dulbecco's modified Eagle's medium/F12 (D-MEM/F12; GIBCO, NY USA) containing 0.4% Pronase E




*10.2* Cell sheet technology for articular cartilage repair. The advantages of using such cell sheets are that they are easy to culture and they proliferate easily, they have good adhesion, and a barrier function which is also very important because it enables the protection of intra-articular catabolic factors, while also preventing proteoglycans from escaping. Furthermore, cell sheets are useful for various types of cartilageous defect in conjunction with the use of scaffold free tissue-engineered cartilage.

(Kakenseiyaku Inc.) and subsequently for 4 h in DMEM/F12 containing 0.016% Collagenase P (Roche, Mannhein, Germany). The digested tissue was passed through a cell strainer (BD Falcon™) with a pore size of 100 $\mu$m. The cells were then seeded at a high density (10 000 cells/cm$^2$) onto the surfaces of temperature-responsive culture dishes (UpCell™, diameter: 35 mm provided by CellSeed, Tokyo, Japan) and cultured in DMEM/F12 supplemented with 20% Fetal Bovine Serum (FBS; GIBCO, NY) and 50 $\mu$g/ml ascorbic acid (Wakojunyakukougyou Corp. Japan) and 1% Antibiotics-Antimycotic (GIBCO, NY) at 37 °C in an atmosphere of 5% $CO_2$ and 95% air for a week. Human articular cartilage cells were also seeded onto commercially available culture dishes (diameter: 35 mm, Iwaki, Japan) and cultured under the same conditions.

*Harvesting of cell sheets*

Each culture dish was removed from the incubator when the cells reached confluence and was then left to stand at about 25 °C for 30 minutes. After the

culture medium has been removed, the cell sheet was harvested using a polyvinylidene difluoride (PVDF) membrane according to the method reported by Yamato *et al.*[28] In brief, the PVDF membrane was placed on the cell sheet and then the sheet was rolled up with the membrane from one corner. Cultured human chondrocytes were able to be successfully harvested as a single contiguous cell sheet using this method. Then each cell sheet was placed on top of another confluent cell sheet to create multilayered sheets. Since the multilayered sheets floated in the culture medium, a 0.4 $\mu$m cell culture insert (Falcon, USA) was placed on top to prevent this and then the culture of the sheets was continued for 1 week.

Three-layer sheets of cartilage cells from Japanese White rabbits were also prepared. A 0.4 $\mu$m filter was also used to compress these sheets onto the culture dish and sheets were incubated for 3 weeks to prepare the multilayered sheets for transplantation.

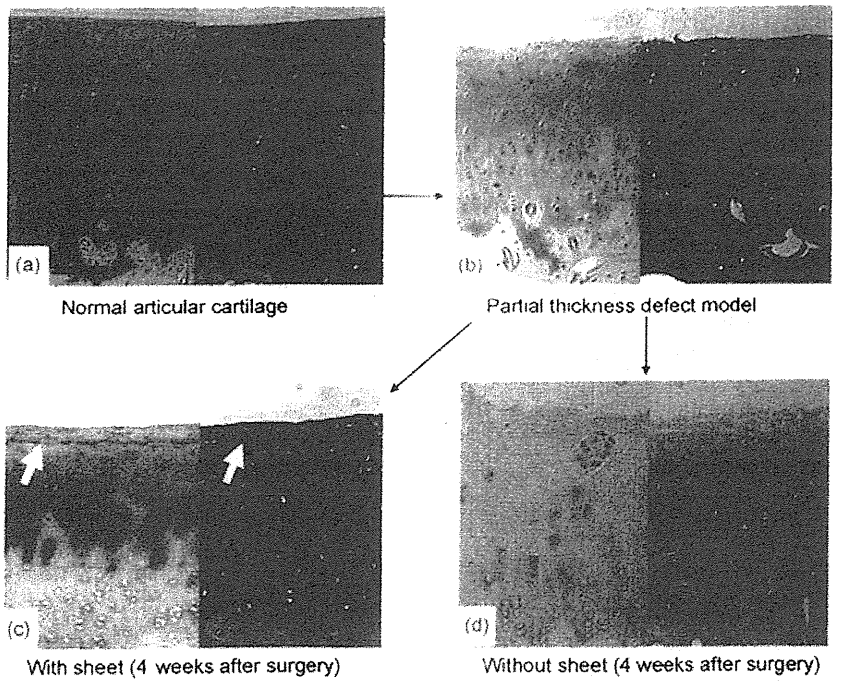
*Transplantation of chondrocyte sheets*

The articular cartilage of the medial femoral condyle of Japanese white rabbits, weighing about 3000 g, 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 two rabbits (the control group). The cartilage was harvested after 4 weeks, fixed in 4% PFA for one week and decalcified with K-CX Decalcifying Solution (Fujisawa Pharmaceutical, Japan) for 1 week. The specimens were then embedded in paraffin, cut into sections and stained with safranin-O and toluidine blue for evaluation.

*Histological findings of the allografted chondrocyte sheet and injured sites*

The three-layered cell sheet remained well attached to tissue sections 4 weeks after transplantation. The area covered by the sheet was better stained than the area not covered with it, as observed in the previously mentioned *ex vivo* experiment. In the partial damaged cartilage model, the area not covered with the multilayered sheets showed progressive cartilage degeneration with fibrillation and poor staining of the matrix at 4 weeks. In contrast, the area covered with the three-layered sheets showed relatively mild degeneration and a well-stained matrix (Figs 10.3 and 10.4). This study confirmed that chondrocytes could be harvested as sheets and thus be made into multilayered 'tissue' by culturing in temperature-responsive dishes and then collecting them using a temperature recovery system.

*10.3* Layered chondrocyte sheets transplantation to the cartilageous defects of the rabbit knee joints. In this rabbit animal model, the allografted cell sheet maintained its cartilageous thickness, but the group without any cell sheets showed an exposure of subchondral bone and severe osteoarthritis.




*10.4* Histological findings after transplantation of layered chondrocyte sheets. A histological analysis of an *in vivo* study (left side: Safranin-O staining, right side: Toluidine blue staining): (a) the normal articular cartilage of the Japanese white rabbit femoral chondrocyte; (b) the partial thickness defect model; (c) the partial thickness defect models which covered the three-layered chondrocyte sheets. These showed a better stainability than those not covered by the chondrocyte sheets (d). The partial thickness defect models themselves showed progressive cartilage degeneration with fibrillation (d). The arrows demonstrate the layered chondrocyte sheets (c) (bar: 100 $\mu$m).

The importance of treatment and prophylaxes for osteoarthritis is increasing due to a progressively ageing society. However, there are only a few conservative therapies available at this time, such as non-steroidal anti-inflammatory drug (NSAID) administration and the injection of hyaluronic acid. There is still no means of preventing the future exacerbation of cartilage degeneration.

Based on the results of this study, the use of bioengineered chondrocyte sheets may be potentially useful in the treatment of partial thickness defects of articular cartilage. The advantages of such cell sheets are that they are easy to culture and grow and, most importantly, they show good adhesion and barrier functions. This means that they can protect against intra-articular catabolic factors while also preventing the escape of proteoglycan from the injured site. They have a promising growth factor supply and furthermore, such cell sheets could be useful as an alternative to periosteum grafting, which is the most commonly used treatment.

Good adhesion and the inhibition of cartilage degeneration at the injured sites were also confirmed even after the experimental study of allografts of layered chondrocyte sheets had been running for 2 months. The sites where the cell sheets showed adhesion were well stained with safranin-O. Therefore, it is suggested that multilayered chondrocyte sheets may serve as a barrier for preventing proteoglycan loss from damaged cartilage, while also protecting the injured site from the catabolic factors in synovial fluid. A strategy has been developed for repairing a full thickness defect of articular cartilage using the layered cell sheets because it is necessary to treat the bleeding from the bone marrow.

## 10.4 Properties of chondrocyte sheets

This section will discuss the properties of chondrocyte sheets. In particular, layered chondrocyte sheets contain few of the destructive factors that cause cartilage to degenerate and they also have good adhesion properties that help to both protect and repair the cartilage surface.

### 10.4.1 Human articular chondrocytes

The cells used for this *in vitro* experiment included human articular chondrocytes obtained from patients who had undergone anterior cruciate ligament reconstruction and had given their informed consent at the Tokai University Oiso Hospital from December 2004 to August 2005. Chondrocytes were obtained while forming the interfoveolar ligament and they were then isolated by enzymatic treatment. Twenty-five knees of 25 patients aged 14 to 49 years (average 23 years, 19 males and 6 females) were used as the source of these cells. The chondrocytes were enzymatically dissociated and were then seeded and cultured according to the method of Sato *et al.*[29]

### 10.4.2 RNA isolation and cDNA synthesis

Total RNA was isolated using the *RNeasy* Mini kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. The RNA quality in each sample was confirmed by the A260/280 absorbance ratio and by electrophoresis on a 1.2% agarose formaldehyde gel. Approximately 1.0–2.0 $\mu$g of total RNA was reverse transcribed into single strand cDNA using Moloney murine leukemia virus (MuLV) reverse transcriptase (Applied Biosystems, Foster City, CA). The reverse transcriptase (RT) reaction was carried out for 60 min at 42 °C and then for 5 min at 95 °C in a thermocycler.

### 10.4.3 Primer design and real-time polymerase chain reaction (PCR)

All oligonucleotide primer sets were designed based upon the published mRNA sequence. The expected amplicon lengths ranged from 70 bp to 200 bp. The oligonucleotide primers used in this study are listed in Table 10.1. Real-time polymerase chain reaction (PCR) was carried out in a SmartCycler II (Cepheid, Sunnyvale, CA) using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). For real-time PCR, 2–2.5 $\mu$l of cDNA template was used in a final volume of 25 $\mu$l. The cDNA was amplified according to the following conditions: 95 °C for 15 s and 60 °C for 60 s for 35 to 45 amplification cycles. Fluorescence changes were monitored with SYBR Green after every cycle. A

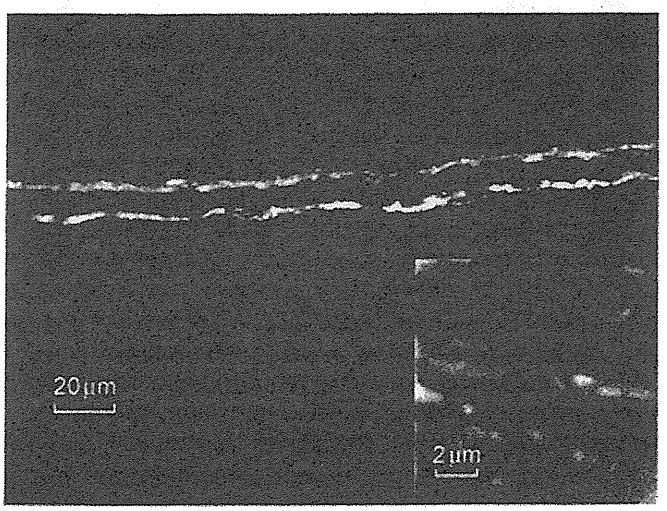
*Table 10.1* List of primers used in real-time PCR

| Primer ID | Accession No. | Sequence | Expected size (bp) |
|---|---|---|---|
| Collagen Type I-F | NM_000088 | AAG GGT GAG ACA GGC GAA CAA | 170 |
| Collagen Type I-R | | TTG CCA GGA GAA CCA GCA AGA | |
| Collagen Type II-F | NM_033150 | GGA CTT TTC TTC CCT CTC T | 113 |
| Collagen Type II-R | | GAC CCG AAG GGT CTT ACA GGA | |
| Aggrecan1-F | NM_001135 | TCG AGG ACA GCG AGG CC | 94 |
| Aggrecan1-R | | TCG AGG GTG TAG GCG TGT AGAGA | |
| Fibronectin1-F | NM_001030524 | GCA CAG GGG AAG AAA AGG AG | 189 |
| Fibronectin1-R | | TTG AGT GGA TGG GAG GAG AG | |
| MMP3-F | NM_002422 | ATT CCA TGG AGC CAG GCT TTC | 138 |
| MMP3-R | | CAT TTG GGT CAA ACT CCA ACT GTG | |
| MMP13-F | NM_002427 | TCA CGA TGG CAT TGC TGA CA | 77 |
| MMP13-R | | AGG GCC CAT CAA ATG GGT AGA | |
| TIMP1-F | NM_003254 | CAG CGT TAT GAG ATC AAG ATG GAC CA | 186 |
| TIMP1-R | | AGT GAT GTG CAA GAG TCC ATC CTG | |
| ADAMTS5-F | NM_007038 | GAG CCA AGG GCA CTG GCT ACT A | 120 |
| ADAMTS5-R | | CGT CAC AGC CAG TTC TCA CACA | |
| GAPDH-F | NM_002046 | GCA CCG TCA AGG CTG AGA AC | 142 |
| GAPGH-R | | ATG GTG GTG AAG ACG CCA GT | |

melting-curve analysis was performed (0.5 °C/s increase from 55 to 95 °C with continuous fluorescence readings) at the end of the 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 II software program. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used to normalize the samples. To monitor crossover contamination of PCR, RNase-free water (Qiagen Inc., Valencia, CA) was included in the RNA extraction and used as a negative control. To ensure the quality of the data, a negative control was always applied in each run.

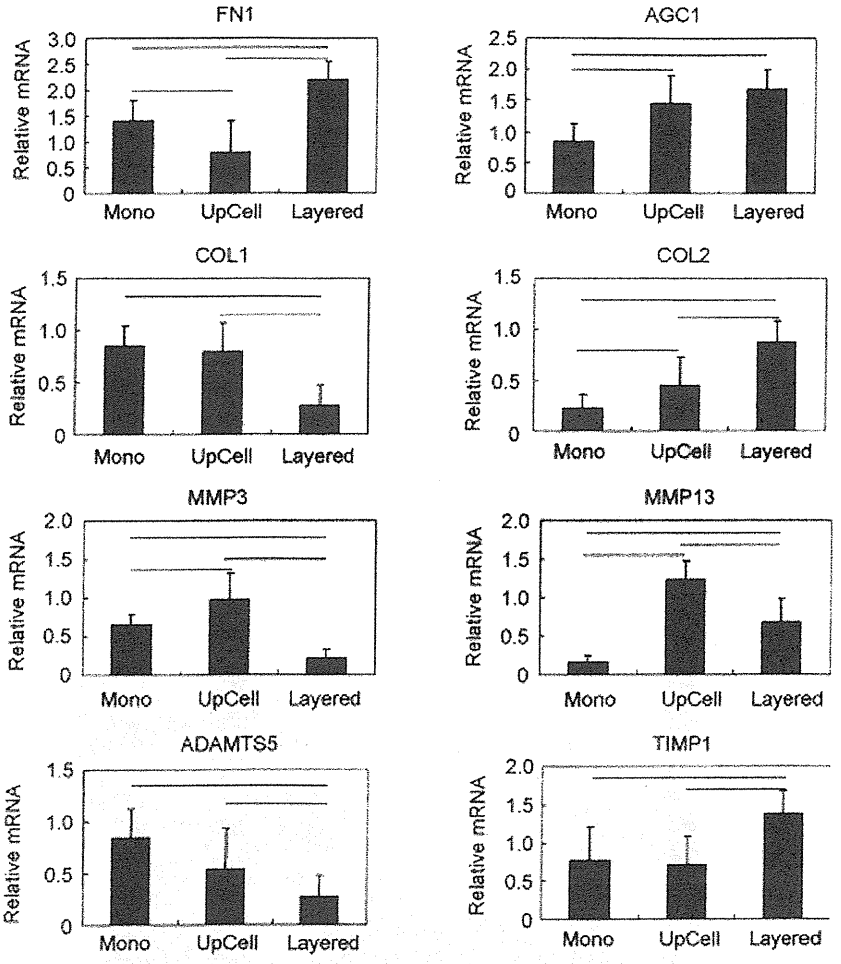
### 10.4.4 Analysis of gene expression

Layered chondrocyte sheets were shown to adhere to porcine cartilage after a one-day organ culture. It is possible that an increase of fibronectin in the multilayered chondrocyte sheets may have been involved (Fig. 10.5). Good adhesion could thus be obtained because harvesting without enzymatic treatment makes it possible to better preserve the activity of both fibronectin and adhesion proteins such as integrin. Matrix–matrix interactions therefore play an important role in the adhesion between chondrocyte sheets and injured cartilage, and specific enzymes may modify the surface of each matrix and permit interaction between integrin proteins and fibronectin. Although this adhesive phenomenon is currently being studied using a cDNA microarray, some of the results were demonstrated here. The expression of fibronectin1, collagen type II, aggrecan 1 and TIMP1 mRNA




*10.5* The expression of fibronectin. The localization of fibronectin was the surface of the layered chondrocyte sheet. The high magnification demonstrated that the expression of fibronectin localized among extracellular matrices and cell-cell junctions.

was observed at significantly high levels, while the expression of collagen type I, MMP3 and ADAMTS5 was at significant levels in the layered chondrocyte sheets in comparison to the monolayer culture. Another interesting aspect of these chondrocyte sheets is the fact that catabolic factors, such as MMP3,[30–33] MMP13[32–35] and ADAMTS5[36,37] were observed to decrease at the time of layering, while the expression of TIMP1 with antagonistic actions against MMP3




*10.6* The relative expressions of mRNA of key genes. The mRNA expression of fibronectin 1 of three-layered chondrocyte sheets was higher than monolayer culture. The mRNA expressions of aggrecan and type II collagen of chondrocyte sheets were higher than that of monolayer culture. The mRNA expression of type I collagen demonstrated a low level in the three-layered chondrocyte sheets. The expressions of MMP3 and ADAMTS5, which promote cartilage degeneration were low in the three-layered chondrocyte sheets while the mRNA expression of TIMP1, which is an antagonistic factor of MMP3, showed a significantly high level in the three-layered chondrocyte sheets. Mono: conventional monolayer culture, UpCell: monolayer culture using temperature-responsive culture dish, Layered: layered chondorocyte sheets (–: $P < 0.05$).

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.

BMC Biotechnology

BioMed Central

Research article

Open Access

# The properties of bioengineered chondrocyte sheets for cartilage regeneration

Genya Mitani[1], Masato Sato*[1], Jeong IK Lee[1], Nagatoshi Kaneshiro[1], Miya Ishihara[1,2], Naoshi Ota[1], Mami Kokubo[1], Hideaki Sakai[3], Tetsutaro Kikuchi[3] and Joji Mochida[1]

Address: [1]Department of Orthopaedic Surgery, Surgical Science, Tokai University School of Medicine, Kanagawa, Japan, [2]Department of Medical Engineering, National Defense Medical Collage, Saitama, Japan and [3]CellSeed Inc., Tokyo, Japan

Email: Genya Mitani - genya@syd.odn.ne.jp; Masato Sato* - sato-m@is.icc.u-tokai.ac.jp; Jeong IK Lee - jeongiklee@hotmail.com; Nagatoshi Kaneshiro - kanesiro@is.icc.u-tokai.ac.jp; Miya Ishihara - kobako@ndmc.ac.jp; Naoshi Ota - naoshi-o@is.icc.u-tokai.ac.jp; Mami Kokubo - mami-k@is.icc.u-tokai.ac.jp; Hideaki Sakai - hsakai@cellseed.com; Tetsutaro Kikuchi - tkikuchi@cellseed.com; Joji Mochida - jomo@is.icc.u-tokai.ac.jp

* Corresponding author

Published: 6 March 2009

*BMC Biotechnology* 2009, **9**:17 doi:10.1186/1472-6750-9-17

Received: 24 September 2008
Accepted: 6 March 2009

This article is available from: http://www.biomedcentral.com/1472-6750/9/17



## 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).

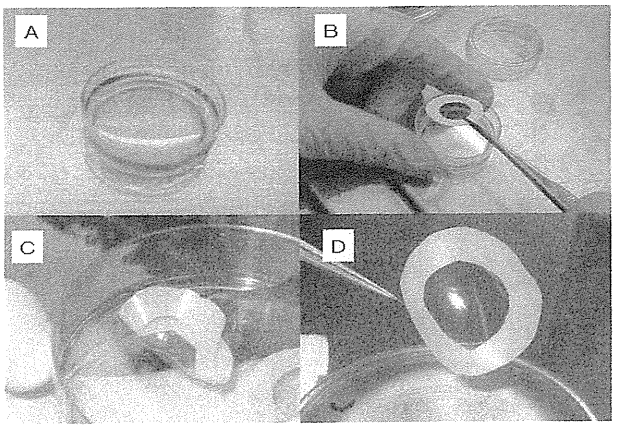



**Figure 1**
**Fabrication of the cell sheets.** Temperature-responsive culture dishes on which chondrocytes had been cultured were removed from the incubator when the cells reached confluence and were let stand at about 25°C for 30 min (A). After the culture medium was removed, a polyvinylidene difluoride (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).

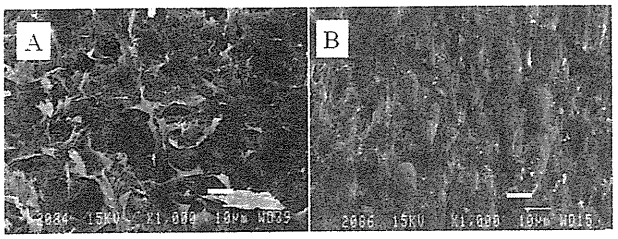



**Figure 2**
**Scanning electron microscopy.** Scanning electron microscopy revealed that the top (A) and the basal aspects (B) of the chondrocyte sheet demonstrated completely different textures. The chondron like texture was only observed on the basal aspect, which had adhesive properties. Scale bar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

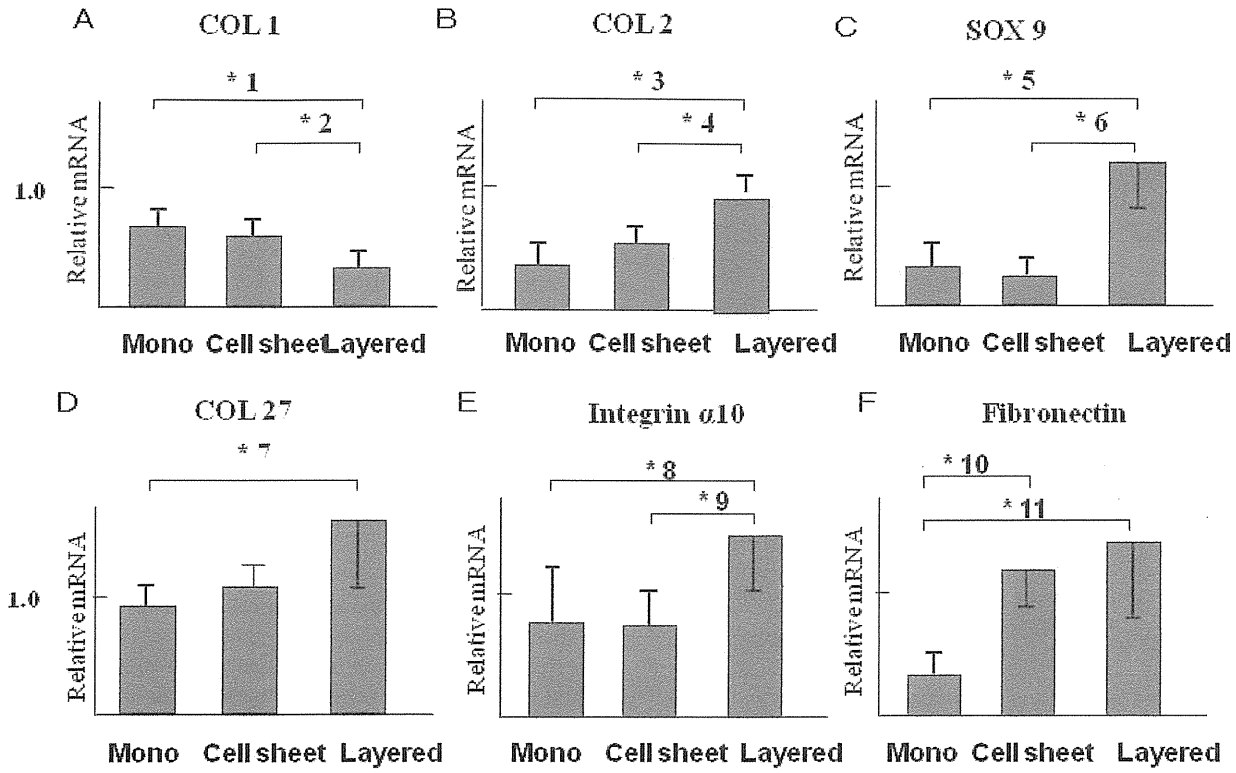



**Figure 3**
**Relative expression of mRNA.** The y-axis shows the mRNA expression relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The results were evaluated using the SmartCycler II software program. GAPDH expression was used to normalize samples. The error bars represent the standard 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