JOURNAL OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE
J Tissue Eng Regen Med (2013)
Published online in Wiley Online Library (wileyonlinelibrary.com) **DOI:** 10.1002/term.1764

RESEARCH ARTICLE

Characterization of chondrocyte sheets prepared using a co-culture method with temperature-responsive culture inserts

Mami Kokubo[1], Masato Sato[1]*, Masayuki Yamato[2], Genya Mitani[1], Toshiharu Kutsuna[1], Goro Ebihara[1], Teruo Okano[2] and Joji Mochida[1]

[1]*Department of Orthopaedic Surgery, Surgical Science, Tokai University School of Medicine, Isehara, Kanagawa, Japan*
[2]*Institute of Advanced Biomedical Engineering and Science, Tokyo, Women's Medical University, Shinjuku-ku, Tokyo, Japan*

Abstract

Conventional culture methods using temperature-responsive culture dishes require 4–5 weeks to prepare layered chondrocyte sheets that can be used in articular cartilage repair and regeneration. This study investigated whether the use of synovial tissue obtained from the same joint as the chondrocyte nutritive supply source could more quickly facilitate the preparation of chondrocyte sheets. After culturing derived synoviocytes and chondrocytes together (i.e. combined culture or co-culture) on temperature-responsive inserts, chondrocyte growth was assessed and a molecular analysis of the chondrocyte sheets was performed. Transplantable tissue could be obtained more quickly using this method (average 10.5 days). Real-time polymerase chain reaction and immunostaining of the three-layer chondrocyte sheets confirmed the significant expression of genes critical to cartilage maintenance, including type II collagen (COL2), aggrecan-1 and tissue metallopeptidase inhibitor 1. However, the expression of COL1, matrix metalloproteinase 3 (MMP3), MMP13 and A-disintegrin and metalloproteinase with thrombospondin motifs 5 was suppressed. The adhesive factor fibronectin-1 (FN1) was observed in all sheet layers, whereas in sheets generated using conventional preparation methods positive FN1 immunostaining was observed only on the surface of the sheets. The results indicate that synoviocyte co-cultures provide an optimal environment for the preparation of chondrocyte sheets for tissue transplantation and are particularly beneficial for shortening the required culture period.

Received 11 September 2012; Revised 2 February 2013; Accepted 13 April 2013

Keywords **chondrocyte; synovial cells; temperature-responsive culture dishes; three-layer chondrocyte sheets; regeneration; co-culture**

1. Introduction

Articular cartilage plays an important role in maintaining joint function but has a low capacity for self-propagation. The main reasons cited for this are the lack of blood vessels in cartilage, relative immobility of chondrocytes inside the abundant extracellular matrix, and age-related loss of proliferative ability in mature chondrocytes. Thus, when cartilage is damaged, the injured cartilage is replaced by fibrous tissue and the surrounding cartilage degenerates, often leading to osteoarthritis (OA). In addition, both aging and joint overuse lead to a wide range of cartilage defects (Convery *et al*., 1972). Currently, various methods of therapy are used to try to repair full-thickness articular cartilage injuries (Skoog *et al*., 1972). However, regardless of the defect type, regeneration of hyaline cartilage is impossible (Hunziker, 2002).

A variety of methods, including autologous chondrocyte implantation (ACI), perichondrium transplantation (Skoog *et al*., 1972), periosteal transplantation (O'Driscoll *et al*., 1986), decalcified bone grafts containing bone morphogenetic protein and mosaicplasty (Yamashita *et al*., 1985) have

*Correspondence to: M. Sato, Department of Orthopaedic Surgery, Surgical Science, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259–1193, Japan. E-mail: sato-m@is.icc.u-tokai.ac.jp

been reported as good short-term options for repairing cartilage injuries (Hangody *et al.*, 2004). First introduced in 1994 (Brittberg *et al.*, 1994), the ACI technique is used commonly in Europe, the USA and Korea for treating osteochondral injuries (Minas and Peterson, 1999). Methods for preparing and transplanting cartilage involve tissue engineering by seeding chondrocytes or mesenchymal stem cells (MSCs) isolated from the unloaded part of the articular cartilage. These methods are used to treat full-thickness osteochondral injuries (Wakitani *et al.*, 2011). The clinical results to date have been favourable, but some authors have claimed that more evidence is needed to support the utility and efficacy of this technique (Nakamura *et al.*, 2009). Hyaline cartilage regeneration is considered difficult (Hunziker, 2002). The most commonly used ACI method currently requires exposure of the joint using an arthrotomy to coat and suture the cell suspension with a periosteal patch. These patches have several potential negative effects, including periosteal thickening, deficiency and intra-articular adhesion (Minas and Peterson, 1999).

Alternative methods of autologous periosteal implantation, including concealment using type I/III porcine collagen (Gomoll *et al.*, 2009), have been used in recent years. Seeding multilayered collagen with chondrocytes (Bartlett *et al.*, 2005) is a common method used for matrix-induced autologous chondrocyte transplantation. Although useful, these techniques could still benefit from improvements such as reducing cytotoxicity and increasing biocompatibility and treatment efficacy.

The current study has observed the treatment of cartilaginous injury using adhesive chondrocyte sheets that contain no artificial components and that have been obtained from temperature-responsive culture dishes (UpCell®; CellSeed Inc., Tokyo, Japan). Developed by Okano and colleagues (1993), UpCells have been used clinically in the regeneration of the cornea (Nishida *et al.*, 2004), myocardium (Shimizu *et al.*, 2002) and oesophageal mucosa (Ohki *et al.*, 2006). Experiments in animals have shown that layered chondrocyte sheets, cultured using UpCells, adhere to the injured articular cartilage and are active in tissue repair (Kaneshiro *et al.*, 2006). Compared with single-layer cell sheets, layered sheets have a stronger chondroprotective effect (Kaneshiro *et al.*, 2007). However, because of the paucity of chondrocytes obtained from the extracellular matrix and their poor proliferative ability, it takes 4–5 weeks to prepare transplantable tissue from the harvested cells. It is established that there are structural and temporal differences in the formation of cell sheets for every animal species (Figure 1). Chondrocyte sheets must be produced more quickly to be useful in a clinical setting.

Various methods have been developed to promote *in vitro* cell growth, including the use of feeder cells or the addition of growth factors to the culture medium (Fujisato *et al.*, 1996; Wakitani *et al.*, 1997; van Osch *et al.*, 1998). However, few agents can be applied clinically and their effects are temporary. *In vivo*, articular cartilage obtains its nutrients mainly from the synovial fluid secreted by the synovial membranes in the same joint (Hodge and McKibbin, 1969). Synovial tissue is thought to help in the repair of damaged cartilage (Hunziker and Rosenberg, 1996) and possesses exceptional capacity for repair, regeneration and growth. A large number of cells can be harvested from synovial tissue and synovium-derived MSCs have excellent musculoskeletal differentiation potential (Fan *et al.*, 2009). We have previously investigated whether layered chondrocyte sheets co-cultured with synovial cells could be transplanted into a porcine full-thickness cartilage defect model, and we found more favourable repair compared with the control group (Ebihara *et al.*, 2012). We also demonstrated the repair

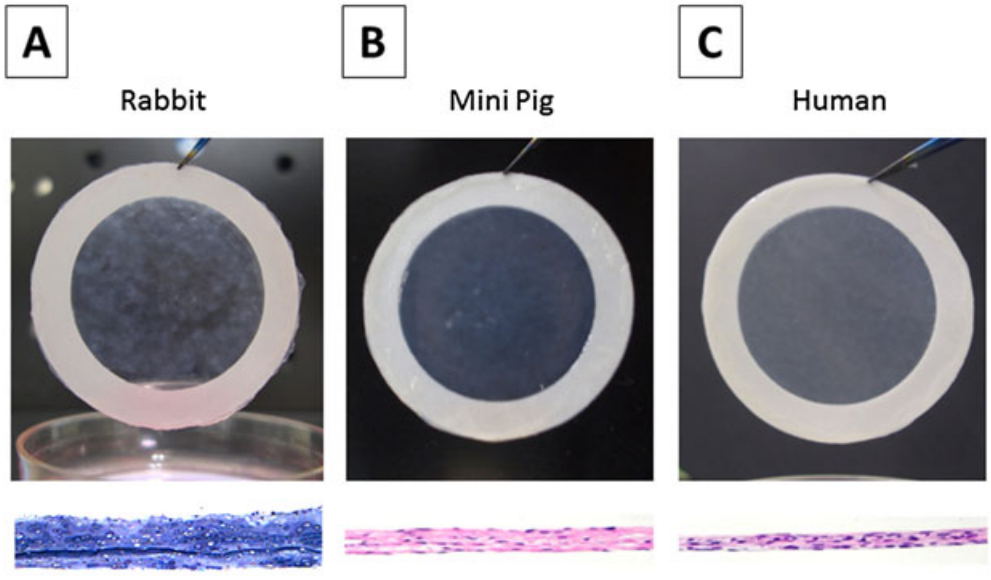

Figure 1. Three-layer chondrocyte sheets for different animal species. (A) A macrophotograph and graft (toluidine blue staining) of rabbit layered chondrocyte sheets. (B) A macrophotograph and graft [haematoxylin and eosin (H&E) staining] of minipig layered chondrocyte sheets. (C) A macrophotograph and graft (H&E staining) of human layered chondrocyte sheets

DOI: 10.1002/term

of articular cartilage defects using layered chondrocyte sheets and cultured synovial cells in a rabbit full-thickness cartilage defect model (Ito *et al*., 2012).

In the current study, simple techniques were applied to try to increase the proliferative activity of chondrocytes. chondrocytes were co-cultured with synoviocytes on inserts to mimic the intra-articular provision of nutrients by synovial fluid. Three-layered chondrocyte sheets were also prepared using the combined culture method. Finally, the molecular properties of single-layered and multilayered chondrocyte sheets were compared to simulate their ability to encourage articular cartilage repair. A preliminary investigation of combined culture using porcine cells was conducted and a greater increase in chondrocyte numbers in co-cultures than in chondrocyte single cultures was observed.

2. Materials and methods

2.1. Patients and cell separation

Cartilage and synovium were obtained from 10 patients (median age: 29 years, age range 20–42 years; six men and four women) who underwent reconstruction surgery of the anterior cruciate ligament at Tokai University Hospital. Cells were separated enzymatically using previously described methods (Sato *et al*., 2003). All patients consented to participate in the study and the research was conducted with the approval of the Tokai University Ethics Committee.

2.2. Isolation and culturing of chondrocytes and synoviocytes

Harvested chondrocytes and synoviocytes were dissected with scissors in Petri dishes. Cells were cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Gibco, Grand Island, NY, USA) containing actinase E (0.4%) (Kaken Seiyaku Inc., Tokyo, Japan) and were incubated with stirring for 1 h at 37°C in 95% air and 5% CO_2. The solution was replaced with DMEM/F12 containing 0.016% collagenase P (Roche, Mannheim, Germany) and then incubated with stirring for 2 h at 37°C and in 95% air 5% CO_2. The digested tissue was passed through a strainer (BD Falcon, Franklin Lakes, NJ, USA) with a pore size of 100 μm.

First-generation chondrocytes were maintained in culture for 4 days in DMEM/F12 supplemented with 20% fetal bovine serum (FBS; Gibco) and 1% antibiotic–antimycotic solution (Gibco). After day 4 of culture, the solution was replaced with medium containing an additional 50 μg/ml of ascorbic acid (Wako Junyaku Kogyo Corp., Osaka, Japan). Synoviocytes were maintained in DMEM/F12 supplemented with 10% FBS and 1% antibiotic–antimycotic solution. All cultures were kept at 37 °C in 95% room air and 5% CO_2. All of the first-generation cultures (P0) and successive cultures (P1 and P2) were seeded at 1×10^4 cells/cm^2; P0 progressed to P1 within 7 days and P1 progressed to P2 within 5 days.

2.3. Generation of chondrocyte sheets using temperature-responsive culture inserts

Temperature-responsive culture inserts (CellSeed, Inc.) were used as the culture dishes for preparing cell sheets. These culture inserts are superficially coated with the temperature-responsive polymer poly(*N*-isopropylacrylamide). This polymer, which reversibly alters its hydration properties at different temperatures, is chemically immobilized in thin films on the cell culture surface, facilitating cell adhesion and growth in normal culture conditions at 37°C. Reducing the temperature of the culture to < 30°C causes the surface to hydrate and swell rapidly, prompting complete detachment of adherent cells without the need for treatment with typical proteolytic enzymes or trypsin. Confluent cell cultures on these surfaces can be harvested conveniently as a single, unsupported contiguous cell sheet, retaining cell-to-cell junctions as well as the extracellular matrix deposited on the basal surface of the sheet. These chondrocyte sheets preserve the extracellular matrix of the cultured cells. We promoted adhesiveness by stacking related cell cultures into three-layered structures, which prevents disruption of the adhesive proteins and membrane receptor ligands.

2.4. Preparation of culture groups

First-generation cell cultures (P0), first successive generation cell cultures (P1) and second successive generation cell cultures (P2) were used in this study. Chondrocytes were initially cultured alone (S group) in a six-well culture dish (BD Falcon) with cell culture inserts (BD Falcon) and a pore size of 0.4 μm. Synoviocytes were seeded into these inserts to form the combined culture group (C group). A 7-day culture group (layered chondrocyte, or CL group) was prepared concurrently in which the combined culture was performed with heat-sensitive inserts that produced three layers by culture day 14. Throughout the study, the same culture medium protocol was used for chondrocyte maintenance.

2.5. Measurement of cell proliferation rate

Cell proliferation ability was measured in 24-well and plate culture dishes. The S group and C group were prepared by seeding 1×10^4 cells/cm^2 into the chondrocyte and synoviocyte cultures. Proliferation was measured using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Dojindo, Kumamoto, Japan) assay on culture days 3, 5, 7, 9, 11 and 14. There were six replicates per experimental condition.

After cultivation for the scheduled period, cell numbers were counted using the MTT assay. Briefly, the culture medium was replaced with 0.1 ml of MTT solution (0.5 mg/ml MTT) in serum-free DMEM without phenol

DOI: 10.1002/term

red (Gibco). The cells were incubated at 37°C for 2 h and the MTT solution was replaced by 0.2 ml of solubilizer solution comprising 80% isopropanol (Sigma Chemical Co., St Louis, MO, USA), 20% dimethyl sulfoxide (Sigma Chemical Co.), and 4% Tween-20 (Sigma Chemical Co.). After the new solution was mixed, absorbance was quantified at 562 nm on a microplate reader (SpectraMax 250; Molecular Devices, Sunnyvale, CA, USA). The cell number was calculated based on the absorbance according to a standard curve of human chondrocytes prepared before the experiment.

2.6. Measurement of gene expression in the cell sheets

The S group, C group and CL group were prepared by seeding chondrocytes at a density of 3×10^4 cells/cm^2; this assay used only cells that had been cultured for 21 days (six cell sheets from six patients per group). Real-time polymerase chain reaction (PCR) to verify the expression of the following genes: those important for maintaining cartilage character, such as *COL2* (type II collagen), *AGC1* (aggrecan-1), *SOX9* (SRY-related HMG Box 9), and *TIMP1* (tissue metallopeptidase inhibitor 1); those with a catabolic function, such as *COL1* (type I collagen), *MMP3* (matrix metalloproteinase 3), *MMP13*, and *ADAMTS5* (A-disintegrin and metalloproteinase with thrombospondin motifs 5); and adhesion factors, such as *FN1* (fibronectin-1) and *ITGα10* (integrin-α10).

2.7. RNA isolation and cDNA synthesis

An SV Total RNA Isolation System (Promega Corp., Madison, WI, USA) was used to extract total RNA according to the manufacturer's instructions. The quality of RNA in each sample was verified using the A260/280 absorbance ratio. Then 1.0–2.0 μg of total RNA was reverse-transcribed into single-strand cDNA using oligo dT primers and MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA). The reverse transfection reaction was performed in a thermocycler set at 42 °C for 60 min and at 95°C for 5 min.

2.8. Primer design and real-time PCR

All oligonucleotide primer sets were designed using 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. Real-time PCR was performed using SYBR® Green PCR Master Mix (Applied Biosystems). cDNA template, 1.0–2.0 μl, was added to bring the final volume of the real-time PCR sample to 25 μl. A total of 35–45 amplification cycles were run during which the samples were heated to 95°C for 15 s and 60°C for 60 s. Changes in fluorescence were monitored with SYBR Green after every cycle. A melting curve analysis was performed (0.5°C/s increase from 55°C to 95°C with continuous fluorescence readings) at the end of all cycles to ensure that single PCR products had been obtained. The results were evaluated using SmartCycler II software (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used to normalize the samples. To monitor crossover contamination during PCR, RNase-free water (Qiagen Inc., Valencia, CA, USA) was included in the RNA extraction and was used as a negative control. To ensure the quality of data, a negative control was included in each run.

2.9. Immunohistochemistry

Immunohistochemical techniques were used to identify COL2 and to localize adhesion factors in the layered chondrocyte sheets.

Table 1. List of primers used in real time PCR

Primer ID	Accession No.	Sequence	Expect size (bp)
ACAN-F	NM_001135.3	CTA TAC CCC AGT GGG CAC AT	108
ACAN-R		GGC ACT TCA GTT GCA GAA GG	
ADAMTS5-F	NM_007038	GAG CCA AGG GCA CTG GCT ACT A	120
ADAMTS5-R		CGT CAC AGC CAG TTC TCA CAC A	
Collagen Type I–F	NM_000089.3	CTG GAG AGG CTG GTA CTG CT	62
Collagen Type I–R		AGC ACC AAG AAG ACC CTG AG	
Collagen Type II–F	NM_001844.4	GTG AAC CTG GTG TCT CTG GTC	94
Collagen Type II–R		TTT CCA GGT TTT CCA GCT TC	
Integrin α10-F	NM_003637	CTG GGA TAT GTG CCC GTG TG	112
Integrin α10-R		TTG GAG CCA TCC AAG ACA ATG A	
Fibronectin1-F	NM_212482.1	GAA CTA TGA TGC CGA CCA GAA	67
Fibronectin1-R		GGT TGT GCA GAT TTC CTC GT	
SOX9-F	NM_000346	AAC GCC GAG CTC AGC AAG A	138
SOX9-R		CCG CGG CTG GTA CTT GTA ATC	
MMP3-F	NM_002422	AAT 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	GCA CCG TCA AGG CTG AGA AC	186
TIMP1-R		ATG GTG GTG AAG ACG CCA GT	
GAPDH-F	NM_002046	GCA CCG TCA AGG CTG AGA AC	142
GAPDH-R		ATG GTG GTG AAG ACG CCA GT	

DOI: 10.1002/term

Frozen sections ($30 \times 24 \times 5$ mm) of three-layered chondrocyte sheets were prepared using Optimal Cutting Temperature compound (Sakura Fine Technical Co., Tokyo, Japan). The sections were washed in phosphate-buffered saline (PBS) and reacted at 4°C overnight with three monoclonal antibodies: anti-fibronectin mouse monoclonal antibody clone (FBN11, diluted 1:500; #MS-1351-P0; Thermo Scientific, Lab Vision Co., Kalamazoo, MI, USA), anti-human CD11c (ITGα10) mouse monoclonal antibody clone (BU15, diluted 1:200; #SM1834PS; Acris Antibodies GmbH, Herford, Germany) and anti-human type II collagen 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 goat anti-mouse immunoglobulin/Alexa Fluor 594 (diluted 1:100, #A-11037; Invitrogen, Glostrup, Denmark) or polyclonal goat anti-mouse immunoglobulin/Alexa Fluor 488 (diluted 1:100, #A-11029; Invitrogen) to provide the fluorescent signal. The sections were washed in PBS and mounted with mounting medium and 4′,6-diamidino-2-phenylindole (DAPI, #H-1500; Vector Laboratories, Inc., Burlingame, CA, USA).

2.10. Statistical analysis

The data are presented as the mean and standard error of the mean (SEM). Analysis of variance (ANOVA) was used to investigate differences between single chondrocyte sheets and layered chondrocyte sheets. In cases where $p < 0.05$, we used the Student–Newman–Keuls test for multiple paired comparisons.

3. Results

3.1. Isolating and culturing chondrocytes and synoviocytes

The average weights of cartilage tissue and synovium tissue at the first collection were 0.45 ± 0.25 g and 2.02 ± 2.95 g, respectively. Subsequently, we isolated an average of $1.20 \pm 0.79 \times 10^6$ cells/g and $2.47 \pm 1.78 \times 10^6$ cells/g, respectively.

3.2. Cell proliferation rates

Regardless of the culture generation, cell proliferation was significantly greater in the C group than in the S group. In P0, the C group contained more than twice the number of cells in the S group on culture day 3, and the cells reached confluence on day 11. Phase-contrast microscopy showed that cell proliferation was faster in the C group than in the S group, resulting in apparently equivalent cell densities in the S group on day 20 and the C group on day 11. In cases where the initial seeding density was 1×10^4 cells/cm^2, the average time to confluence was 12.0 days in P0, 10.2 days in P1 and 9.2 days in P2. Thus, 9.2 days appeared to be the shortest possible period required for the formation of a three-layered chondrocyte sheet (Figure 2). The difference in growth ability was confirmed on day 3 of culture by phase-contrast microscopy. (Figure 3A,F) We confirmed that group C reached confluence on day 7 of culture (Figure 3B,G), whereas group S reached confluence after 20 days of culture (Figure 3J).

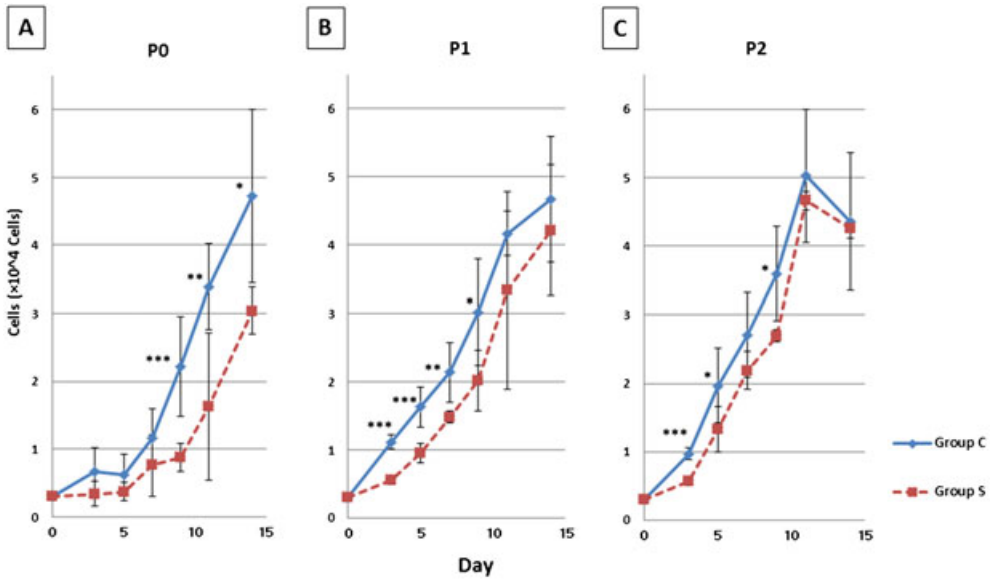

Figure 2. Cell proliferation assessment by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. (A) Proliferation was significantly greater in P0 chondrocytes in group CL than in group S from day 3. The difference in cell count between the groups was significant from day 9. (B) Proliferation was significantly greater in P1 chondrocytes in group CL than in group S from day 3. Proliferation was significantly greater in P2 chondrocytes in group CL than in group S from day 3 to day 9. (C) The cell count decreased on post-culture day 11 as the cells reached confluence on day 11 in both groups. The data are presented as mean ± standard error of the mean (SEM). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

DOI: 10.1002/term

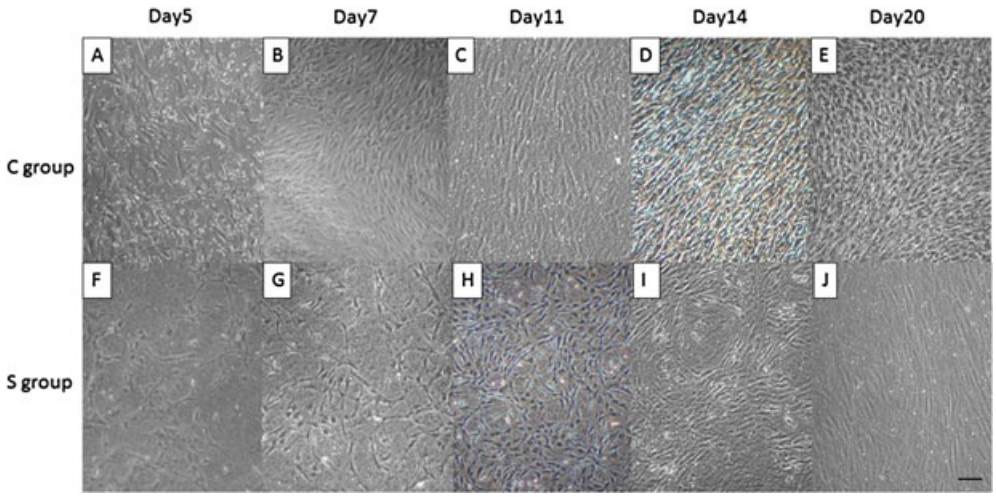

Figure 3. Observation of cell proliferation using phase-contrast microscopy. Phase-contrast micrographs of P1 cartilage in group C (A–E) and group S (F–I) on day 3 (A,F), day 7 (B,G), day 11 (C,H), day 14 (D,I), and day 20 (E,J) (original magnification ×100)

3.3. Real-time PCR results

Genes important for maintaining articular joint characteristics, such as *COL2*, *ITGα10*, *TIMP1* and *SOX9*, were expressed at consistently higher levels in the CL group than in the S group. The difference was also definitive in P0 and P1 (Figure 4A,C, D). The levels of COL2, ITGα10 and TIMP1, particularly in P0 and P1, were higher in the CL group than in the other groups. Significant inhibition of the catabolic genes *MMP3*, *MMP13* and *ADAMTS5* was observed until P1, although gene expression during P2 was similar in all groups (Figure 4E–G). High expression of the COL2 gene and low expression of COL1 gene – hallmarks of articular cartilage – were observed in the layered chondrocyte sheets during every successive generation (Figure 4A,I). However, the treatment groups did not significantly differ in expression of the AGC1 and FN1 genes (Figure 4B,I).

3.4. Immunostaining

The mean thickness of the layered chondrocyte sheets was 24 ± 8.5 μm. The layered chondrocyte sheets adhered strongly to each other and the layers could not be separated even by hand. The COL2 gene was strongly expressed across the whole sheet (Figures 5A and 6A). In the layered chondrocyte sheets produced using the UpCells method, expression of the FN1 gene localized to the culture sheet surface (Mitani *et al.*, 2009); however, the new combined culture method produced sheets with widespread expression of FN1 (Figure 5B). Expression of ITGα10 was not found in the monolayer sheet, but the three layered sheet was strongly expressed across the whole sheet (Figure 5C and 6C).

4. Discussion

The proliferative ability of human chondrocytes varies between individuals and declines with age (Barbero *et al.*, 2004). Other important considerations when producing tissue-engineered cartilage for use in regenerative therapy include the length of time required for cell culture and the simplicity of the culture technique. Although various techniques are available to address these issues, the focus of this study was on developing a method that did not require the use of scaffolds (Nagai *et al.*, 2008a, 2008b). The results indicate that the novel technique described here produces more extracellular matrix than conventional techniques (Kaneshiro *et al.*, 2006, 2007; Mitani *et al.*, 2009) while preserving the characteristics of natural cartilage.

Combined culture methods have been used with inflammatory synoviocytes and normal chondrocytes as *in vitro* models of rheumatoid arthritis and OA – conditions in which the two types of cells interact (Nevo *et al.*, 1993; Kurz and Schunke, 1997; Smolian *et al.*, 2001; Lubke *et al.*, 2005). This is why this study focused on these interactions and the proliferative ability of synoviocytes, although synoviocytes were not harvested from tissues that were chronically inflamed. Cell proliferative activity was significantly higher in the combined chondrocyte–synoviocyte culture (C group) than in the isolated chondrocyte culture (S group), regardless of the generation. Chondrocyte sheets could also be produced in less time using the combined culture technique. It is noted that the methods introduced here do not require the addition of growth factors to the medium and rely only on liquid factors supplied from serum (FBS) and the cultured cells themselves. Thus, the method mimics the intra-articular nutritive conditions to which chondrocytes are exposed naturally *in vivo*.

The strong expression of the FN1 and ITGα10 genes in chondrocyte sheets produced using our new technique was confirmed (Figure 5B,C) compared with the single-layered sheet (Figure 6B,C). The observation that expression of the FN gene was found throughout the sheet rather than being limited to the sheet surface may reflect the use of inserts (Figure 5B). FN, which plays a critical role in the adhesion, proliferation, migration, and differentiation of cells, is a macromolecular (~ 440 kDa) glycoprotein. It is a part of the extracellular matrix and links to integrin,

DOI: 10.1002/term

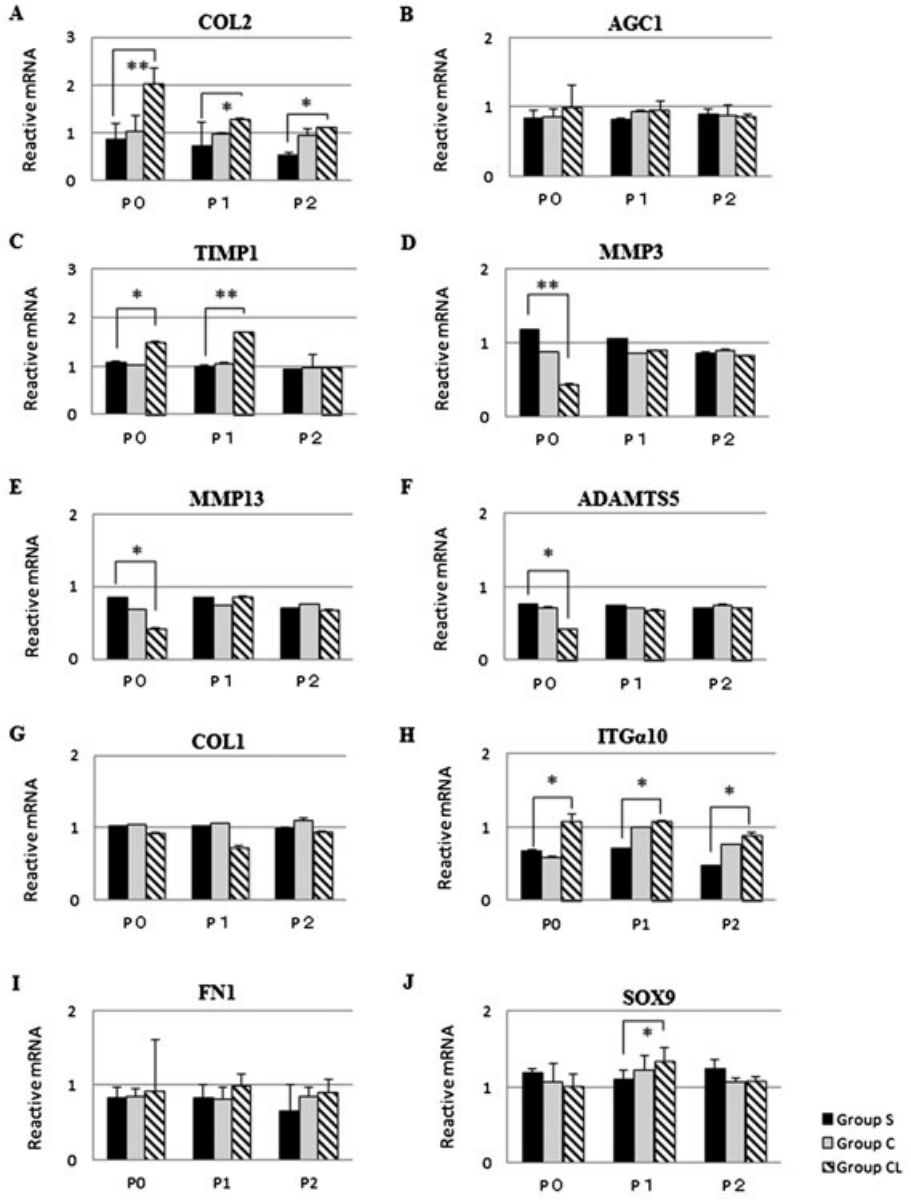

Figure 4. Results of real-time polymerase chain reaction (PCR) analysis comparing mRNA expression between the three culture groups. The type II collagen (COL2) (A), aggrecan-1 (AGC1) (B), tissue metallopeptidase inhibitor 1 (TIMP1) and fibronectin-1 (FN1) (I) genes, which are important for the maintenance of the chondrocyte phenotype, were upregulated significantly more in P0 and P1 cells in the CL group than in the S group. Genes encoding the catabolic factors matrix metalloproteinase 3 (MMP3) (D), MMP13 (E), and A-disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5) (F) were downregulated significantly more in the CL group than in the S group in P0 cells, but not in P1 or P2 cells. Downregulation of the type I collagen (COL1) (G) and integrin-α10 (ITGα10) (H) genes was observed in P0, P1 and P2 cells in all groups, whereas expression of the SOX9 gene (J) was high in P0, P1 and P2 cells in all groups. In all passage numbers, the ITGα10 gene (H) was upregulated significantly more in the CL group than in the S group. In P1 cells, the SRY-related HMG Box 9 (SOX9) gene (J) was upregulated significantly more in the CL group than in the S group. The data are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

a receptor protein on the cell membrane. Integrin is expressed primarily on chondrocytes of the articular cartilage, spine, trachea and cartilage supporting the bronchi (Camper *et al*., 2001). Integrin binds to the extracellular matrix, where it provides signals about the dynamic state of the surrounding matrix to the cell (Boudreau and Jones, 1999; Hering, 1999; Heino, 2000). The genes involved in chondrocyte proliferation and promotion of extracellular matrix formation were found more frequently in the cartilage matrix than were COL2 and AGC1 (Figure 4).

Using animal experiments, Kaneshiro *et al*. (2006) found that layered chondrocyte sheets made using UpCells adhere to damaged parts of articular cartilage and are active in tissue repair. The layered sheets were found to have chondroprotective effects (Kaneshiro *et al*., 2007) and to restrain the catabolic factors MMP3, MMP13 and ADAMTS5 – enzymes that degrade the extracellular matrix (Mitani *et al*., 2009). In this study, it was found that layered chondrocyte sheets fabricated using our co-culture technique expressed genes critical

DOI: 10.1002/term

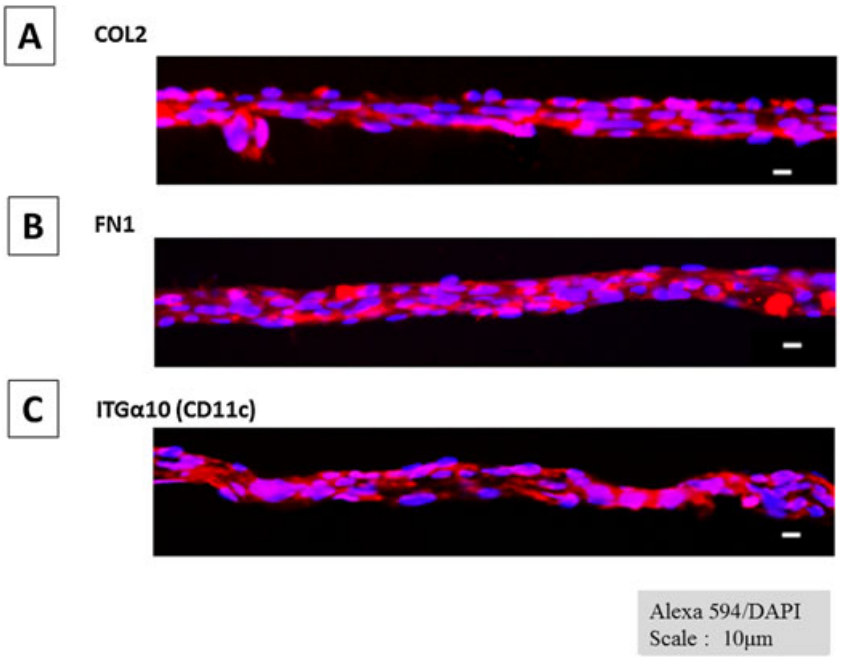

Figure 5. Results of immunostaining in the CL group. Expression of (A) type II collagen (COL2) and (B) fibronectin-1 (FN1) was observed throughout the layered chondrocyte sheet. (C) Integrin-α10 (ITGα10) (shown in red) localized in the pericellular matrix of the triple-layered chondrocyte sheet. Scale bars, 10 μm

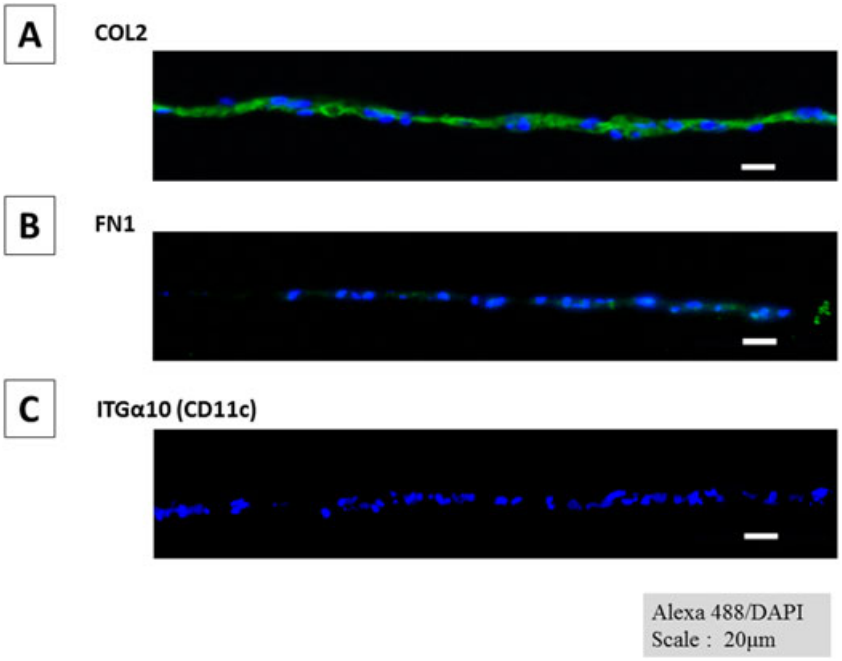

Figure 6. Results of immunostaining in the S group. Expression of (A) type II collagen (COL2) and was observed throughout the layered chondrocyte sheet. (B) fibronectin-1 (FN1) and (C) Integrin-α10 (ITGα10) (shown in green) localized in the pericellular matrix of the triple-layered chondrocyte sheet. Scale bars, 20 μm

to maintaining the characteristics of cartilage. COL2 and AGC1 were found at higher levels in the CL group than in the C group, a difference that was particularly clear during P0 and P1. Thus, layering appears to provide a suitable culture environment for chondrocytes.

In two-dimensional culture conditions, chondrocytes generally tend to lose their cartilage-specific properties and change into fibroblasts. This causes a decrease in proteoglycan production and tends to cause a shift in expression from COL2 to COL1. Yoon *et al*. (2002) reported that, after the fourth generation, there is a complete loss of differentiation in chondrocyte cultures. In contrast to this observation, we did not detect any decreases in COL2 or increases in COL1 in our S group. We found that the CL

DOI: 10.1002/term

group had higher levels of AGC1 and TIMP1 and lower levels of MMP3, MMP13 and ADAMTS5 than did the S and C groups during P0 and P1. However, the expression of these factors was similar in all groups in P2. This suggests that chondrocytes tend toward dedifferentiation in repeated monolayer cultures, thereby decreasing their ability to maintain the chondrocyte phenotype. These results imply that P0 and P1 culture cells may be more suitable for clinical applications.

In summary, a novel technique has been developed for co-culturing chondrocytes and synoviocytes using temperature-responsive culture inserts. Layered chondrocyte sheets fabricated using this technique expressed genes critical to cartilaginous differentiation and the maintenance of cartilaginous characteristics, as well as genes for adhesion factors. Compared with conventional methods, this technique requires much less culture time. However, our method still requires up to 21 days to produce transplantable P0 cell sheets. A further reduction in culture times may be required for clinical applications.

Acknowledgements

This work was supported by a Health Labour Sciences Research Grant from the Japanese Ministry of Health, Labour and Welfare and a Grant-in-aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology. We gratefully acknowledge CellSeed, Inc., for supplying the temperature-responsive culture inserts used in this study. We are grateful to the Education and Research Support Center, Tokai University.

Conflict of interest

The authors have declared that there is no conflict of interest.

References

Barbero A, Grogan S, Schafer D *et al*. 2004; Age related changes in human articular chondrocyte yield, proliferation and post-expansion chondrogenic capacity. *Osteoarthr Cartil* **12**: 476–483.

Bartlett W, Gooding CR, Carrington RW *et al*. 2005; Autologous chondrocyte implantation at the knee using a bilayer collagen membrane with bone graft. A preliminary report. *J Bone Joint Surg Br* **87**: 330–332.

Boudreau NJ, Jones PL. 1999; Extracellular matrix and integrin signalling: the shape of things to come. *Biochem J* **339**: 481–488.

Brittberg M, Lindahl A, Nilsson A *et al*. 1994; Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N Engl J Med* **331**: 889–895.

Camper L, Holmvall K, Wangnerud C *et al*. 2001; Distribution of the collagen-binding integrin α10β1 during mouse development. *Cell Tissue Res* **306**: 107–116.

Convery FR, Akeson WH, Keown GH. 1972; The repair of large osteochondral defects: an experimental study in horses. *Clin Orthop Relat Res* **82**: 253–262.

Ebihara G, Sato M, Yamato M *et al*. 2012; Cartilage repair in transplanted scaffold-free chondrocyte sheets using a minipig model. *Biomaterials* **33**: 3846–3851.

Fan J, Varshney RR, Ren L *et al*. 2009; Synovium-derived mesenchymal stem cells: a new cell source for musculoskeletal regeneration. *Tissue Eng Part B Rev* **15**: 75–86.

Fujisato T, Sajiki T, Liu Q *et al*. 1996; Effect of basic fibroblast growth factor on cartilage regeneration in chondrocyte-seeded collagen sponge scaffold. *Biomaterials* **17**: 155–162.

Gomoll AH, Probst C, Farr J *et al*. 2009; Use of a type I/III bilayer collagen membrane decreases reoperation rates for symptomatic hypertrophy after autologous chondrocyte implantation. *Am J Sports Med* **37**: S20–S23.

Hangody L, Rathonyi GK, Duska Z *et al*. 2004; Autologous osteochondral mosaicplasty. Surgical technique. *J Bone Joint Surg Am* **86A**: 65–72.

Heino J. 2000; The collagen receptor integrins have distinct ligand recognition and signaling functions. *Matrix Biol* **19**: 319–323.

Hering TM. 1999; Regulation of chondrocyte gene expression. *Front Biosci* **4**: D743–D761.

Hodge JA, McKibbin B. 1969; The nutrition of mature and immature cartilage in rabbits. An autoradiographic study. *J Bone Joint Surg Br* **51**: 140–147.

Hunziker EB. 2002; Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. *Osteoarthr Cartil* **10**: 432–463.

Hunziker EB, Rosenberg LC. 1996; Repair of partial-thickness defects in articular cartilage: cell recruitment from the synovial membrane. *J Bone Joint Surg Am* **78**: 721–733.

Ito S, Sato M, Yamato M *et al*. 2012; Repair of articular cartilage defect with layered chondrocyte sheets and cultured synovial cells. *Biomaterials* **33**: 5278–5286.

Kaneshiro N, Sato M, Ishihara M *et al*. 2006; Bioengineered chondrocyte sheets may be potentially useful for the treatment of partial thickness defects of articular cartilage. *Biochem Biophys Res Commun* **349**: 723–731.

Kaneshiro N, Sato M, Ishihara M *et al*. 2007; Cultured articular chondrocytes sheets for partial thickness cartilage defects utilizing temperature-responsive culture dishes. *Eur Cell Mater* **13**: 87–92.

Kurz B, Schunke M. 1997; Articular chondrocytes and synoviocytes in culture: Influence of antioxidants on lipid peroxidation and proliferation. *Ann Anat* **179**: 439–446.

Lubke C, Ringe J, Krenn V *et al*. 2005; Growth characterization of neo porcine cartilage pellets and their use in an interactive culture model. *Osteoarthr Cartil* **13**: 478–487.

Minas T, Peterson L. 1999; Advanced techniques in autologous chondrocyte transplantation. *Clin Sports Med* **18**: 13–44.

Mitani G, Sato M, Lee JI *et al*. 2009; The properties of bioengineered chondrocyte sheets for cartilage regeneration. *BMC Biotechnol* **9**: 17.

Nagai T, Furukawa KS, Sato M *et al*. 2008a; Characteristics of a scaffold-free articular chondrocyte plate grown in rotational culture. *Tissue Eng Part A* **14**: 1183–1193.

Nagai T, Sato M, Furukawa KS *et al*. 2008b; Optimization of allograft implantation using scaffold-free chondrocyte plates. *Tissue Eng Part A* **14**: 1225–1235.

Nakamura N, Miyama T, Engebretsen L *et al*. 2009; Cell-based therapy in articular cartilage lesions of the knee. *Arthroscopy* **25**: 531–552.

Nevo Z, Silver J, Chorev Y *et al*. 1993; Adhesion characteristics of chondrocytes cultured separately and in cocultures with synovial fibroblasts. *Cell Biol Int* **17**: 255–273.

Nishida K, Yamato M, Hayashida Y *et al*. 2004; Functional bioengineered corneal epithelial sheet grafts from corneal stem cells expanded *ex vivo* on a temperature-responsive cell culture surface. *Transplantation* **77**: 379–385.

O'Driscoll SW, Keeley FW, Salter RB. 1986; The chondrogenic potential of free autogenous periosteal grafts for biological resurfacing of major full-thickness defects in joint surfaces under the influence of continuous passive motion. An experimental investigation in the rabbit. *J Bone Joint Surg Am* **68**: 1017–1035.

Ohki T, Yamato M, Murakami D *et al*. 2006; Treatment of oesophageal ulcerations using endoscopic transplantation of tissue-engineered autologous oral mucosal epithelial cell sheets in a canine model. *Gut* **55**: 1704–1710.

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

Sato M, Asazuma T, Ishihara M *et al*. 2003; 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**: 248–256.

Shimizu T, Yamato M, Akutsu T *et al*. 2002; Electrically communicating three-dimensional cardiac tissue mimic fabricated by layered cultured cardiomyocyte sheets. *J Biomed Mater Res* **60**: 110–117.

Skoog T, Ohlsen L, Sohn SA. 1972; Perichondrial potential for cartilaginous regeneration. *Scand J Plast Reconstr Surg* **6**: 123–125.

Smolian H, Thiele S, Kolkenbrock H *et al*. 2001; Establishment of an *in vitro* model for rheumatoid arthritis as test system for therapeutical substances. *ALTEX* **18**: 265–280.

van Osch GJ, van den Berg WB, Hunziker EB *et al*. 1998; Differential effects of IGF-1 and TGFβ-2 on the assembly of proteoglycans in pericellular and territorial matrix by cultured bovine articular chondrocytes. *Osteoarthr Cartil* **6**: 187–195.

Wakitani S, Imoto K, Kimura T *et al*. 1997; Hepatocyte growth factor facilitates cartilage repair. Full thickness articular cartilage defect studied in rabbit knees. *Acta Orthop Scand* **68**: 474–480.

Wakitani S, Okabe T, Horibe S *et al*. 2011; Safety of autologous bone marrow-derived mesenchymal stem cell transplantation for cartilage repair in 41 patients with 45 joints followed for up to 11 years and 5 months. *J Tissue Eng Regen Med* **5**: 146–150.

Yamashita F, Sakakida K, Suzu F *et al*. 1985; The transplantation of an autogeneic osteochondral fragment for osteochondritis dissecans of the knee. *Clin Orthop Relat Res* **201**: 43–50.

Yoon YM, Kim SJ, Oh CD *et al*. 2002; Maintenance of differentiated phenotype of articular chondrocytes by protein kinase C and extracellular signal-regulated protein kinase. *J Biol Chem* **277**: 8412–8420.

DOI: 10.1002/term