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RESEARCH ARTICLE

Characterization of layered chondrocyte sheets created in a co-culture system with synoviocytes in a hypoxic environment

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Abstract

Endeavouring to repair and regenerate articular cartilage using cell sheets, we have previously established a co-culture system of chondrocytes and synoviocytes, and have reported the successful and rapid production of chondrocyte sheets. In the present study, to examine the effects of oxygen concentration on the chondrocyte sheets, we co-cultured human articular chondrocytes and human synoviocytes in 2%, 5% and 21% oxygen, and measured chondrocyte metabolic activity and proliferation activities under each condition for 14 days in culture. Layered chondrocyte sheets were also created under each condition and the proteoglycan (PG) level was compared with the gene expression of type I collagen (*COL1*), *COL2*, *COL27*, tissue metallopeptidase inhibitor 1 (*TIMP1*), fibronectin-1 (*FN1*), SRY-related HMG Box 9 (*SOX9*), aggrecan-1 (*ACAN*), integrin-α10 (*ITGα10*), matrix metalloproteinase 3 (*MMP3*), *MMP13* and a disintegrin and metalloproteinase with thrombospondin motif 5 (*ADAMTS5*). Compared with 5% and 21% oxygen, the 2% condition caused significantly greater cell metabolic activity and proliferation ($p < 0.05$). The 2% condition produced a 10% greater PG level compared with 21% oxygen ($p < 0.05$). All conditions increased the expression of chondrocyte-specific genes, such as *COL2*, and were associated with low expression levels of catabolic factors, such as *MMP3* and *MMP13*. These observations indicated that the specificity of the chondrocyte sheets was maintained under all conditions. The culture times did not differ between the 5% and 21% conditions. Compared with 21% oxygen, layered chondrocyte sheets rich in extracellular matrix were created 2.85 days earlier in 2% oxygen, which is similar to the level found in deep cartilage.

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Keywords **human chondrocyte; hypoxia; layered chondrocyte sheets; temperature-responsive culture dishes; regeneration**

1. Introduction

Cartilage tissues lack blood vessels, nerves and lymphatic vessels (Stockwell, 1978). They are composed of extracellular matrix constituents, such as collagen and proteoglycan (PG), and are characterized by strong type 2 collagen-specific staining. The coefficient of friction of the cartilage surface is extremely low (0.001–0.01), which helps to maintain smooth joint function (Blum and Ovaert, 2013; Charnley, 1960; Forster and Fisher, 1999; Wang *et al.*, 2013).

Osteoarthritis of the knee is a disorder triggered by various factors and leads to a decline in quality of life. At present, a total knee replacement performed at the end stage of the disease is the only method for curative treatment, and symptomatic treatment is provided for most conditions. Autologous chondrocyte implantation (ACI), first reported by Brittberg *et al.* (1994), has been performed in >20 000 patients worldwide for >20 years outside Japan, although it has yet to be applied for knee osteoarthritis (Biant *et al.*, 2014; Brittberg *et al.*, 1994; Minas and Peterson, 1999). The outcomes of ACI are not necessarily positive (Moseley *et al.*, 2010; Niemeyer *et al.*, 2014).

In an endeavour to create an alternative to the present method for ACI, we previously manufactured layered chondrocyte sheets using a temperature-responsive culture dish (UpCell™; CellSeed Inc., Tokyo, Japan). We have demonstrated in animal studies that these sheets can contribute to tissue repair at the sites of articular cartilage damage (Kaneshiro *et al.*, 2006) and full-thickness defects (cartilage damage) (Ebihara *et al.*, 2012; Ito *et al.*, 2012). We have also verified, in characterization studies, that they can function to protect cartilage (Hamahashi *et al.*, 2012; Kaneshiro *et al.*, 2007; Mitani *et al.*, 2009). However, the number of chondrocytes that can be collected from extracellular matrix-rich cartilage tissue is low, and these chondrocytes have poor proliferative ability. Thus, a long time is required from collection to the completion of transplantable tissue production.

Cartilage tissues in the body receive nutrition from synovial fluid secreted by the synovium. The oxygen tension in the environment of articular cartilage is low

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and ranges from 10% at the surface to 1% in the deep layer (Falchuk *et al.*, 1970; Fermor *et al.*, 2007; Malda *et al.*, 2003; Zhou *et al.*, 2004). We hypothesized that a more suitable *in vitro* culture environment for cartilage could be achieved by recreating the *in vivo* environment. We also postulated that, by supplying a suitable culture environment, chondrocytes could be dedifferentiated without using specific media to induce differentiation. Consequently, better quality chondrocyte sheets could be provided for patients.

Previously, we conducted a study that focused on the synovium, which is the source of nutrition for cartilage in vivo. We successfully promoted chondrocyte proliferation by co-culturing these cells with synoviocytes obtained from synovial tissue, and we reported the usefulness of this technique (Kokubo et al., 2013). Since 2011, a clinical study of articular cartilage repair and regeneration, using chondrocyte sheets created using this co-culture system, has been ongoing in the Department of Orthopaedic Surgery, Tokai University School of Medicine; eight patients have received chondrocyte sheet grafts thus far. Biopsy samples taken 1 year later have shown that favourable repairs have been achieved (manuscript in preparation).

In the present study, we aimed to reduce the time required to culture layered chondrocyte sheets, currently about 3 weeks, by reproducing the physiological oxygen environment of cartilage under the experimental culture conditions. Here, we report the results from our investigation of the usefulness of this method.

2. Materials and methods

Cartilage and synovium were obtained from 22 patients, average age 26.8 (range 15–55) years, 18 men and four women, who underwent reconstruction surgery of the anterior cruciate ligament at Tokai University Hospital. All patients consented to participate in the study, and the research was conducted with the approval of the Tokai University Ethics Committee.

2.1. Culture

Harvested chondrocytes and synoviocytes were dissected with scissors in Petri dishes and subsequently digested for 2–4 h at 37°C and in 5% CO_2 in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Gibco, Grand Island, NY, USA) containing 5 mg/ml collagenase type 1 (Worthington, Mannheim, Germany). The digested tissue was passed through a strainer (BD Falcon, Franklin Lakes, NJ, USA) with a pore size of 100 μm (Sato *et al.*, 2003).

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 (AB; Gibco). After 4 days of culture, the solution was replaced with medium containing an additional 50 μg/ml of ascorbic acid (Wako Junyaku Kogyo Corp., Osaka, Japan). Chondrocytes and synoviocytes were maintained in DMEM/F12 supplemented with 20% FBS and 1% AB. All cultures were kept at 37°C in 95% room air and 5% CO_2. Each 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.

Human articular chondrocyte and human synoviocyte samples collected from tissues were co-cultured with the chondrocytes on top of and synoviocytes below the insert, such that the different cell types were kept separate (Figure 1A). The culture conditions were 2%, 5% or 21% oxygen with 5% CO_2 at 37°C.

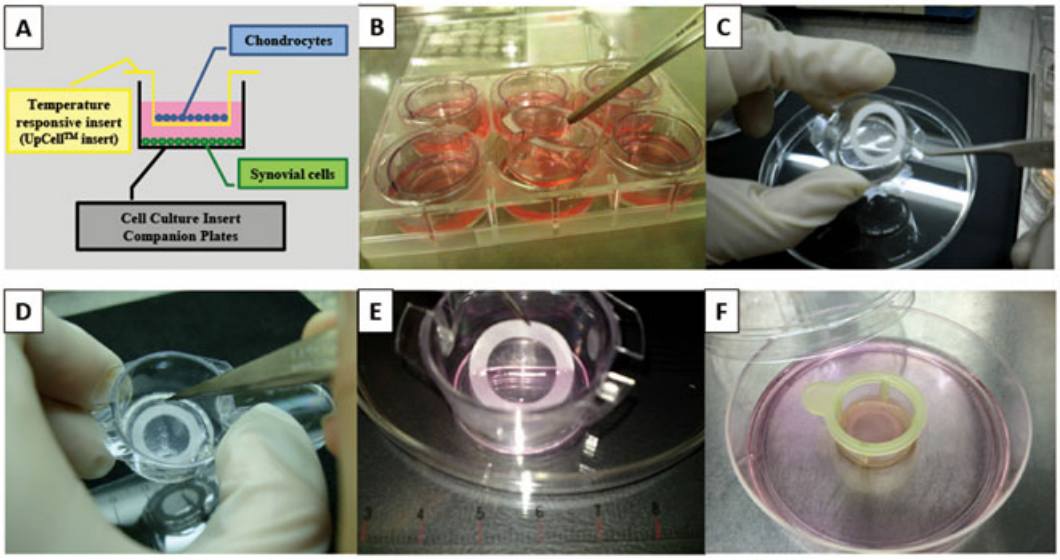

Figure 1. Method of detaching chondrocyte sheets. (A) Chondrocyte sheets are cultured using a temperature-responsive insert (UpCell™). (B, C) The culture plates are left at room temperature for 30 min and the inserts are then removed; after removing the medium, a PVDF membrane is placed in the centre of the insert, using forceps. (D) Using forceps, the cell sheet is rolled up onto the PVDF membrane. (E) A small volume of medium is added and the cell sheet and PVDF membrane are slowly peeled from the temperature-responsive insert. (F) After layering the cell sheet the appropriate number of times, it is transferred to a 100 mm dish; a weight is placed on top of the sheet, which is cultured for 7 days

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The basic culture method was the same as that reported in our previous article, and the only difference was the use of the different hypoxic environments. This culture protocol was approved in 2011 by the Ministry of Health, Labour and Welfare (Japan) and is the standard culture protocol used in clinical studies performed at Tokai University.

2.2. Creation of single-layered chondrocyte sheets

Using temperature-responsive culture inserts (UpCell™ insert; CellSeed Inc., Tokyo, Japan), P0 cells were co-cultured for 14 days at 5×10^4 cells/cm^2 for chondrocytes and 1×10^4 cells/cm^2 for synoviocytes, with 5% or 21% oxygen. Chondrocyte sheets were created using polyvinylidene fluoride (PVDF) membranes as a support. The cells were cultured for a further week under each oxygen condition and were subsequently used as single-layered chondrocyte sheet samples (Figure 1).

2.3. Creation of layered chondrocyte sheets

Using UpCell™ inserts, cells were co-cultured for 14 days in 2%, 5% or 21% oxygen in a CO_2 multi-gas incubator (CDI-325 M; Astec Co. Ltd., Hukuoka, Japan) to create the chondrocyte sheets. Chondrocytes with a three-layered structure were created using a PVDF membrane as a support. These were cultured under each oxygen condition for 1 additional week and were subsequently used as layered chondrocyte sheets (Figure 1).

2.4. 3-(4,5-Di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Using 24-well inserts, the MTT assay was used to compare the proliferative abilities of chondrocytes cultured under each condition over the 14 days. MTT was diluted in serum-free DMEM/F12 without phenol red to a final concentration of 0.5 mg/ml and was used to replace the culture medium. The cells were incubated for 2 h at 37°C, the medium was removed and the precipitated formazan crystals were solubilized in dimethyl sulphoxide. Product formation was calculated by measuring the absorbance at 562 nm on a microplate reader (GE Healthcare Life Science, Pharmacia, Stockholm, Sweden) (Yokoyama *et al.*, 2013).

2.5. 1,9-Dimethyl methylene blue (DMMB) assay

The PG level was measured using a Sulphated Glycosaminoglycan Assay Kit (Seikagaku Biobusiness, Tokyo, Japan); 150 μl protease solution was added and the solution was agitated for 10 s and treated for 2 h at 55°C, after which the solution was boiled for 10 min and restored to room temperature. The pretreated sample fluid was stained for 5 min in DMMB liquid dye; the chromogenic reaction occurred at room temperature and the sulphated PG concentration was calculated by measuring the absorbance at 530 nm. Shark cartilage-derived PG was used as the PG standard and for the calibration curve. DNA content was measured by quantifying the amount of 4′-6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA) fluorescence as follows. As with PG, the pretreated sample fluid was added to a 96-well plate and stained with DAPI (0.4 μg/ml); the chromogenic reaction occurred at room temperature and fluorescence was measured on a luminescence counter (Wallac 1420 ARVO MXLight Luminescence Counter; Perkin-Elmer, Yokohama, Japan). Salmon sperm DNA (Invitrogen, Carlsbad, CA, USA) was used as the DNA standard solution and for the calibration curve (Tanaka *et al.*, 2013).

2.6. Real-time polymerase chain reaction (PCR)

We used real-time PCR to confirm the expression of the following genes: those important for maintaining cartilage characteristics, such as type II collagen (*COL2*), *COL27*, aggrecan-1 (*ACAN*), SRY-related HMG Box 9 (*SOX9*) and tissue metallopeptidase inhibitor 1 (*TIMP1*); those with a catabolic function, such as *COL1*, matrix metalloproteinase 3 (*MMP3*), *MMP13*, and a disintegrin and metalloproteinase with thrombospondin motif 5 (*ADAMTS5*); and adhesion factors, such as fibronectin-1 (*FN1*) and integrin-α10 (ITGα10). All oligonucleotide primer sets were designed using published mRNA sequences. The expected amplicon lengths were in the range 70–200 bp. The oligonucleotide primers used in this study are listed in Table 1.

Real-time PCR was performed in a real-time PCR system (Applied Biosystems, Tokyo, Japan), using SYBR Green PCR Master Mix (Applied Biosystems). We added 1.0–2.0 μl cDNA template to bring the final volume of the real-time PCR sample to 25 μl. We then ran 40–45

Table 1. Oligonucleotide primers used in this study

Primer ID	Accession No.	Sequence	Expected size (bp)
SOX9-F	NM_000346	AAC GCC GAG CTC AGC AAG A	138
SOX9-R		CCG CGG CTG GTA CTT GTA ATC	
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	
Collagen type 27α F	NM_032888	GGG CCT TAT GGA AAT CCA GGT C	176
Collagen type 27α R		GGT CCA GGA TAG CCC TTG TGT C	
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	
ACAN F	NM_001135.3	CTA TAC CCC AGT GGG CAC AT	108
ACAN R		GGC ACT TCA GTT GCA GAA GG	
TIMP1 F	NM_003254	GCA CCG TCA AGG CTG AGA AC	186
TIMP1 R		ATG GTG GTG AAG ACG CCA GT	
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	
ADAMTS5 F	NM_007038	GAG CCA AGG GCA CTG GCT ACT A	120
ADAMTS5 R		CGT CAC AGC CAG TTC TCA CAC A	
Fibronectin 1 F	NM_212482.1	GAA CTA TGA TGC CGA CCA GAA	67
Fibronectin 1R		GGT TGT GCA GAT TTC CTC GT	
Integrin α10 F	NM_003637	CTG GGA TAT GTG CCC GTG TG	112
Integrin α10 R		TTG GAG CCA TCC AAG ACA ATG A	
GAPDH F	NM_002046	GCA CCG TCA AGG CTG AGA AC	142
GAPDH R		ATG GTG GTG AAG ACG CCA GT	

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amplification cycles, during which the samples were heated to 95°C for 15 s and 60°C for 60 s. Fluorescence changes were monitored by measuring SYBR Green fluorescence after every cycle. 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 were 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, 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 (Table 1).

2.7. Histological evaluation of layered chondrocyte sheets

Immunohistochemical techniques were used to identify COL1 and COL2 and to localize PG in the layered chondrocyte sheets. Frozen sections of three-layered chondrocyte sheets were prepared using Optimal Cutting Temperature (OCT) compound (Sakura Fine Technical Co., Tokyo, Japan). The sections were reacted at 4°C overnight with the following monoclonal antibodies: anti-human type II collagen mouse monoclonal antibody clone (a-4C11, diluted to 5 mg/ml; cat. no. F-57, Daiichi Fine Chemical Co., Toyama, Japan) or anti-collagen type I mouse monoclonal antibody clone (cat. no. AM10043SU-N, diluted 1:100; Acris Antibodies, Herford, Germany). The sections were washed in phosphate-buffered saline (PBS) and reacted with polyclonal goat anti-mouse immunoglobulins/biotinylated (diluted 1:600, cat. no. E0433, Dako, Glostrup, Denmark) to provide the fluorescent signal. Immunostaining was performed using a Universal LSAB2 Kit/HRP Rabbit/Mouse (cat. no. K0675, Dako), according to the manufacturer's instructions. The sections were counterstained with Mayer's haematoxylin. Images were scanned on a fluorescence microscope (cat. no. BZ-X700, Keyence Corp., Osaka, Japan).

2.8. Statistical analysis

The results are expressed as mean ± standard error (SE) of the mean. Data were compared between experimental groups using analysis of variance (ANOVA), followed by individual Scheffé *post hoc* comparisons for multiple groups.

3. Results

3.1. Cell metabolic activity and proliferative ability

In the MTT assay, the absorbance was significantly greater from day 7 of culture in cells from all passages grown in 2% oxygen ($p < 0.05$), and these cells reached confluence at an earlier stage. Cell metabolic activity did not differ significantly between chondrocytes cultured in 5% and 21% oxygen (Figure 2). Cell sheets grown in 2% oxygen could be detached 2.85 days earlier compared with cells grown in 21% oxygen (Figure 3). The mean time until chondrocyte sheet attachment did not differ significantly between the 5% and 21% conditions.

3.2. Comparison of matrix production ability of single-layered cell sheets (DMMB assay)

In single-layered chondrocyte sheets, PG levels were non-significantly higher when cultured in 5% oxygen compared with 21% oxygen (Figure 4A). Even after correcting for the amount of DNA by expressing PG content as the PG:DNA ratio, significant differences were not observed (Figure 4B).

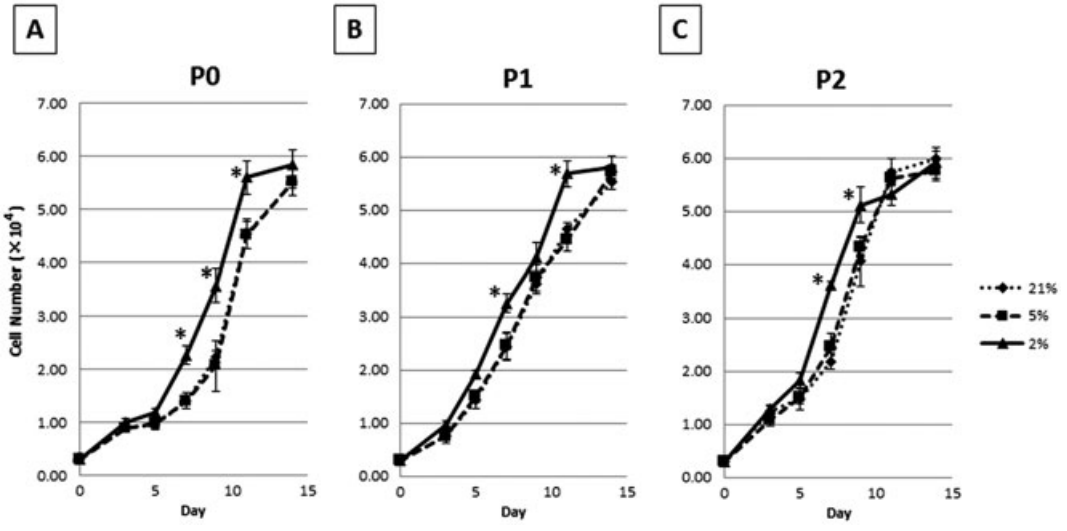

Figure 2. Proliferative ability of chondrocyte from passages P0, P1 and P2 cultured under different oxygen conditions. Chondrocytes from each passage were cultured for 14 days in 2%, 5% or 21% oxygen, and their proliferative ability was measured using the MTT assay: (A) P0 chondrocytes; (B) P1 chondrocytes; and (C) P2 chondrocytes; values in (A–C) are presented as mean ± SE; *$p < 0.05$ vs 21% oxygen

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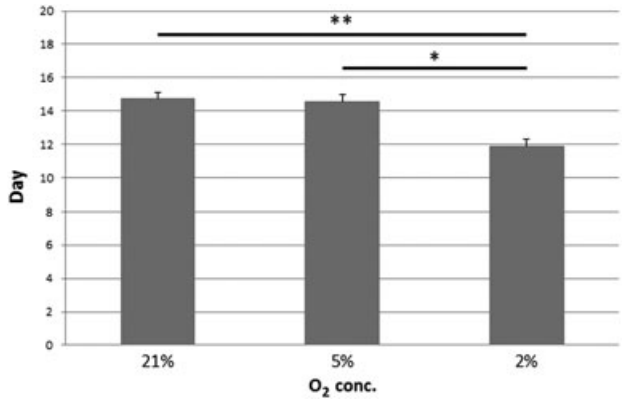

Figure 3. Mean culture period until detachment of chondrocyte sheets created with P0 chondrocytes, showing the mean number of days required until P0 chondrocytes could be detached as cell sheets for each oxygen condition: values are presented as mean ± SE; *$p < 0.05$ vs 5% oxygen; **$p < 0.01$ vs 21% oxygen

3.3. Comparison of matrix production ability of layered cell sheets (DMMB assay)

The total PG content, an indicator of the ability to produce extracellular matrix, was significantly greater in layered P0 chondrocyte sheets cultured in 2% oxygen compared with the other two conditions ($p < 0.05$) (Figure 5A). The total PG content in P0 sheets was non-significantly greater in cells grown in 5% oxygen compared with those grown in 21% oxygen. After correcting for the amount of DNA by expressing PG content as the PG:DNA ratio, the PG level remained significantly greater in cells grown in 2% oxygen compared with the other two oxygen conditions ($p < 0.05$), but did not differ significantly between the 21% and 5% conditions (Figure 5B). In the comparison of layered chondrocyte sheets created with P2 cells, the PG content and PG:DNA ratio were significantly greater in cells grown in 2% oxygen than under the other two oxygen conditions but did not differ significantly between the 21% and 5% oxygen conditions ($p < 0.05$) (Figure 5C, D).

3.4. Gene expression in layered cell sheets (real-time PCR)

The expression of *SOX9*, an important transcription factor for maintaining cartilage-specific characteristics, was significantly greater in the sheets grown in 2% oxygen ($p < 0.05$) (Figure 6A). The expression of *COL2* (Figure 6B) and *COL27* (Figure 6C), which are both cartilage-specific matrix collagens, was significantly greater in the sheets grown in 2% oxygen ($p < 0.05$). *ACAN* expression (Figure 6E), an indicator of the ability to produce extracellular matrix, was significantly greater in the sheets grown in 2% oxygen ($p < 0.05$). The expression of *TIMP1*, a gene involved in inhibiting the degradation of the extracellular matrix, was significantly greater in the sheets grown in 2% oxygen (Figure 6F). By contrast, the expression of the proteases *COL1* (Figure 5D), *MMP3* (Figure 6G) and *MMP13* (Figure 6H) was low in the sheets grown under all conditions and did not differ between conditions. The expression of *ADAMTS5* (Figure 6I), a gene that is closely involved in the progression of cartilage destruction, was also low in sheets grown under all conditions. The expression of the cell adhesion factor *FN1* (Figure 6J) did not differ significantly between conditions. The expression of another adhesion factor, ITGα10 (Figure 6K), which plays an important role in extracellular matrix production in

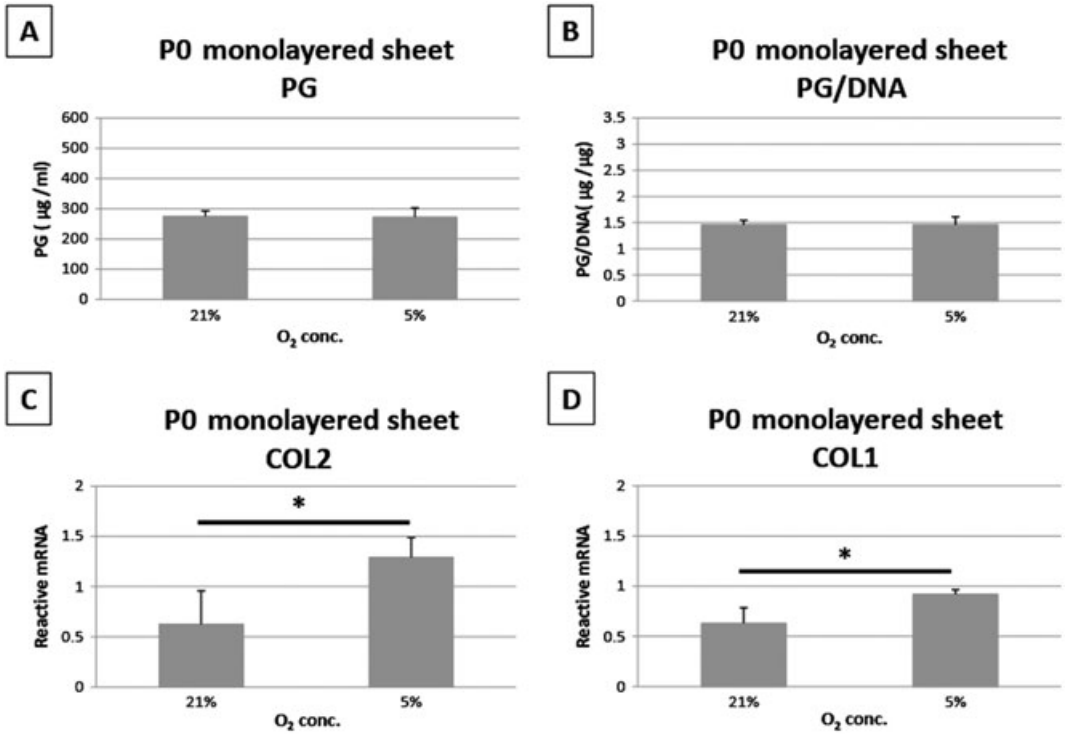

Figure 4. Matrix-producing ability and gene expression of single-layered chondrocyte sheets cultured under each oxygen condition, showing (A) PG, (B) PG/DNA and (C) gene expression of *COL2* and (D) *COL1* in single-layered chondrocyte sheets created with P0 chondrocytes; values in (A–D) are presented as mean ± SE; *$p < 0.05$ vs 21% oxygen

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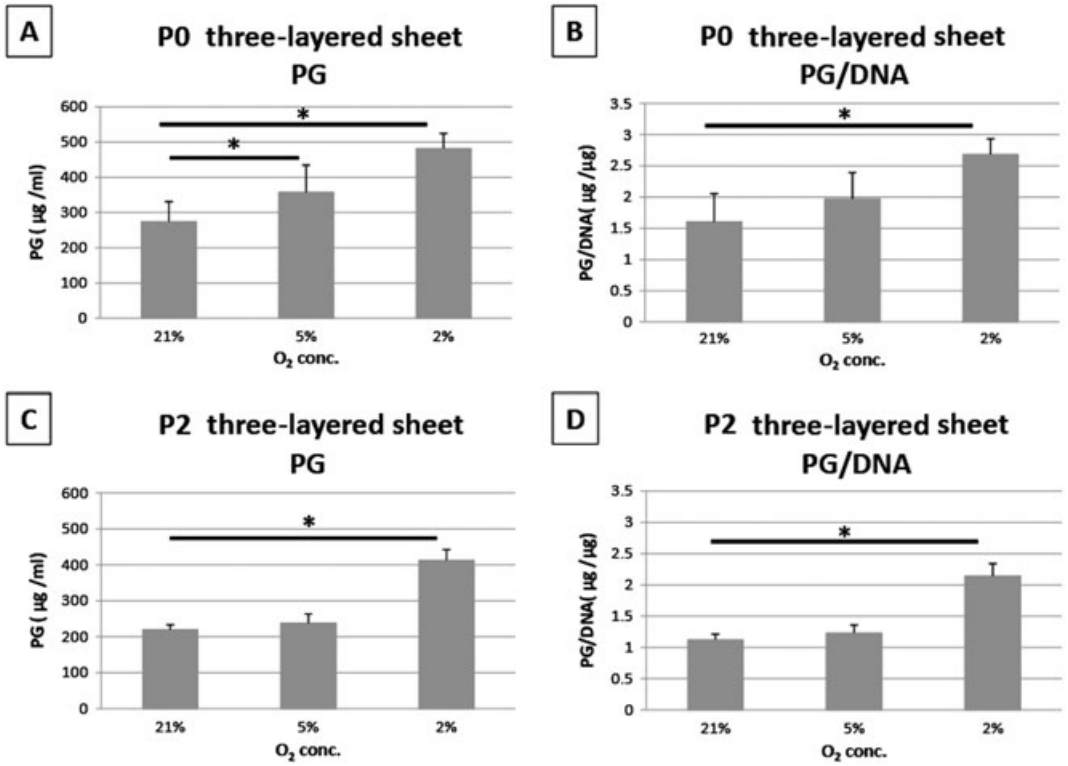

Figure 5. Matrix-producing ability of layered chondrocyte sheets cultured under each oxygen condition, showing (A) PG content and (B) PG/DNA in layered chondrocyte sheets created with P0 chondrocytes, and (C) PG content and (D) PG/DNA in layered chondrocyte sheets created with P2 chondrocytes; values in (A–D) are presented as mean ± SE; $^{*}p < 0.05$ vs 21% oxygen

cartilage, was significantly greater in the sheets grown in 2% oxygen ($p < 0.05$).

3.5. Histological analyses of the layered chondrocyte sheets by staining for safranin O and types I and II collagen

Sections of the layered chondrocyte sheets created using P0 chondrocytes and cultured in 21% oxygen stained positively for safranin O (Figure 7A), type II collagen (Figure 7D) and type I collagen (Figure 7G).

Sections from the layered chondrocyte sheets created using P0 chondrocytes and cultured in 5% oxygen stained positively for safranin O (Figure 7B), type II collagen (Figure 7E) and type I collagen (Figure 7H).

Sections from the layered chondrocyte sheets created using P0 chondrocytes and cultured in 2% oxygen stained positively for safranin O (Figure 7C), type II collagen (Figure 7F) and type I collagen (Figure 7I).

Sheets cultured in 2% oxygen were thicker as compared with the other groups. The nuclei became flat with increasing time in culture (Figure 7A, D, G). However, in 2% oxygen, a round nuclear shape was maintained (Figure 7C, F, I).

4. Discussion

Previously, we co-cultured chondrocytes with synoviocytes obtained from the synovium and achieved a more efficient method for proliferating cells than culturing chondrocytes alone. In that study, we were able to create layered chondrocyte sheets with significantly greater expression of cartilage-specific genes (Kokubo *et al.*, 2013). In the present study, the objectives were to further shorten the culture duration and to generate chondrocyte sheets with greater utility in cartilage repair and regeneration. Thus, we created chondrocyte sheets by co-culturing in a low-oxygen environment.

Significantly greater cell proliferation was observed under the low-oxygen condition (2%), which is similar to the oxygen environment deep within the cartilage, compared with the ambient (21%) and intermediate (5%) oxygen conditions (Figure 2). We succeeded in shortening the culture time required for the creation of transplantable chondrocyte sheets by 2.85 days compared with the previous co-culture method (Figure 3). This reduction in culture time will reduce the delay between tissue collection and transplantation for patients undergoing autologous chondrocyte sheet transplantation.

In the present study, we used two different low-oxygen conditions, 2% and 5% oxygen. In the normal joint, the oxygen tension at the articular cartilage is in the range 10% at the surface to 2% in the deep layer close to the bone. Thus, both low-oxygen tension conditions used in this study were within the physiological range for articular cartilage (Falchuk *et al.*, 1970; Fermor *et al.*, 2007; Malda *et al.*, 2003; Zhou *et al.*, 2004). Low-oxygen conditions of 2–5% have been used in other studies of chondrocyte culture (Murphy and Polak, 2004; Schrobback *et al.*, 2012a, 2012b; Leijten *et al.*, 2014). It is thought that in

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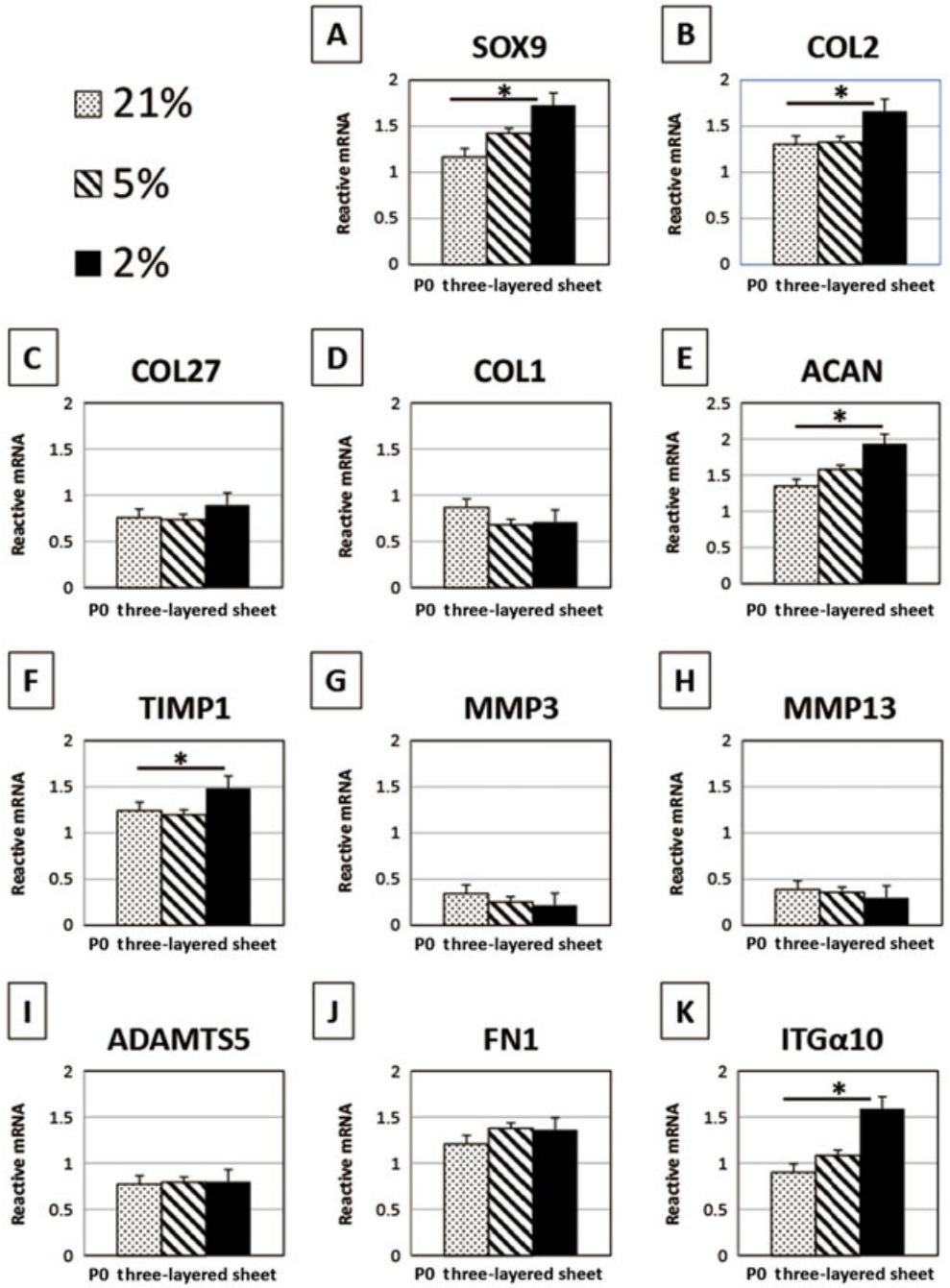

Figure 6. Gene expression in layered chondrocyte sheets cultured under each oxygen condition. The expression of the following genes was quantified in layered chondrocyte sheets created using P0 chondrocytes and cultured in 2%, 5% or 21% oxygen: (A) *SOX9*; (B) *COL2*; (C) *COL27*; (D) *COL1*; (E) *ACAN*; (F) *TIMP1*; (G) *MMP3*; (H) *MMP13*; (I) *ADAMTS5*; (J) *FN1*; and (K) *ITGα10*; *GAPDH* expression was used as an internal control; values in (A–K) are mean ± SE; $*p < 0.05$ vs 21% oxygen

sheets with a high extracellular matrix-producing ability transplanted into a cartilage injury, PG escape is prevented and the cells are protected against catabolic factors, become a source of growth factors, and play the role of an initiator of cartilage repair (Sato *et al.*, 2014). For the single-layered sheets in our previous study, *COL2* and *COL1* expression was significantly greater in cells grown in 5% oxygen compared with 21% oxygen. Conversely, PG levels and mRNA expression of factors essential to cartilage did not differ significantly in layered chondrocyte sheets grown in 5% oxygen compared with 21% oxygen (Figure 4). However, in this study, high expression of *COL2* was found in cells grown in all oxygen concentrations, and layered chondrocyte sheets created under the 5% oxygen condition did not have significantly upregulated gene expression compared with those created under the ambient (21%) oxygen condition (Figure 6B). These findings suggest that it may be possible to manufacture the layered chondrocyte sheets in a hypoxic environment and that these sheets may provide a source of extracellular matrix.

We previously reported that cartilage-specific genes are upregulated when chondrocyte sheets are layered (Hamahashi *et al.*, 2012; Kaneshiro *et al.*, 2006, 2007; Mitani *et al.*, 2009). However, the results of the present study suggest that the 5% oxygen condition is not a

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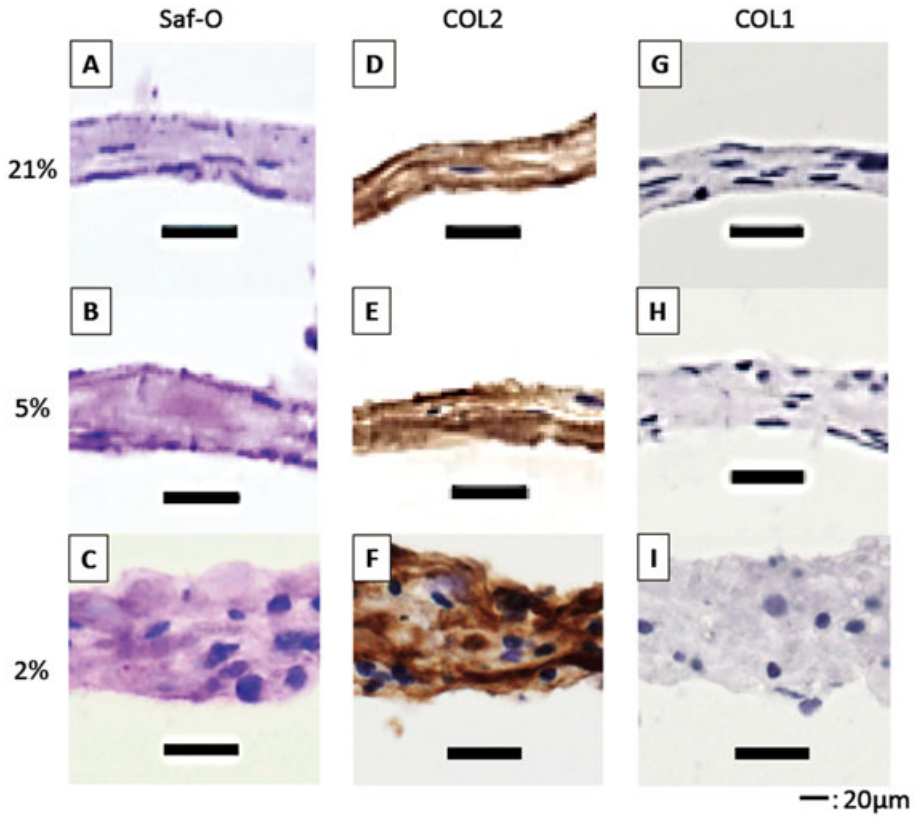

Figure 7. Histological analyses of layered chondrocyte sheets stained for safranin O and types I and II collagen: sections from the layered chondrocyte sheets created using P0 chondrocytes and cultured in 21% (A, D, G), 5% (B, E, H) and 2% (C, F, I) oxygen: staining for (A–C) safranin O, (D–F) type II collagen and (G–I) type I collagen; scale bars =20 μm

suitable culture environment for inducing significant upregulation in these genes compared with the previously used method of layering alone. Additionally, it was postulated that the composition of the collected tissue could explain why the proliferation was not significantly greater in monolayered cells grown in 5% oxygen. Because of the layering, the inside of the cell sheet cultured in 21% oxygen became hypoxic, possibly resembling the 5% oxygen condition compared with 21% oxygen, in unlayered compared with layered cells. These findings suggest that the ability to produce extracellular matrix is facilitated at a low oxygen concentration and that layering is beneficial for creating *COL2*-dominant chondrocyte sheets.

Although the mechanisms underlying the response in the expression of hypoxia-inducible factor 1 (*HIF1*) or *HIF2* under low-oxygen conditions in chondrocytes or cartilage (Fan *et al.*, 2014; Lafont, 2010; Murphy *et al.*, 2009; Srinivas *et al.*, 2009) differ between species, an anabolic response or anticatabolic effect of *HIF* expression is evident in all species (Fan *et al.*, 2014; Murphy and Sambanis, 2001; Murphy *et al.*, 2009; Pfander *et al.*, 2003; Thoms *et al.*, 2013). In human cells, HIF2α binds directly to SOX9, which is especially important for maintaining cartilage-specific characteristics and for consequently inducing cartilage production (Lafont *et al.*, 2007, 2008; Thoms *et al.*, 2013). In the present study, we found that *SOX9* was significantly upregulated in cells grown under the 2% oxygen condition compared with the other two conditions ($p < 0.05$). This presumably led to the elevated expression of *COL2* and *ACAN*, which are extracellular matrix genes that are *SOX9* transcription targets. Moreover, *HIF1*α suppresses *MMP13* expression in human cells (Thoms *et al.*, 2013). We previously demonstrated that catabolic factors such as *MMP3* and *MMP13* are downregulated in layered chondrocyte sheets created by co-culturing with synoviocytes compared with monolayered chondrocyte sheets (Kokubo *et al.*, 2013). In the present study, we combined the co-culture method with a low-oxygen environment. Compared with the culture method used in our clinical study, we obtained results the same class or more. In particular, we found that the ability to produce extracellular matrix was facilitated in 2% oxygen (Figures 5, 7).

We believe that transforming growth factor-β (TGFβ), one of the chondrocytic growth factors, may be involved in chondrocyte activity under the 2% oxygen condition in this study. Hamahashi *et al.* (2012) reported that layering chondrocyte sheets increases the production of humoral factors such as TGFβ and is involved in tissue regeneration at the transplantation site. Sanchez-Elsner *et al.* (2004) reported that *TGFβ* gene expression is increased in a hypoxic environment through SP1 and Smads. As for the significant increase of this 2% oxygen culture group, it is thought with the results that TGFβ was enhanced by making the environment hypoxic. Layered chondrocyte sheets created in a low-oxygen environment can prevent PG 'leaks' at the transplantation site and can activate bone marrow-derived MSCs. A valuable role for these sheets is anticipated because they may help initiate to cartilage repair and regeneration.

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Our data suggest that, although the difference between 2% and 5% oxygen is small, superior tissues can be manufactured by culturing cells under the appropriate oxygen condition. The chondrocytes used in the present study were collected from cartilage tissues that were discarded at the time of surgery. For this reason, the samples were collected from specimens that contained tissues with advanced cartilage degeneration or from tissues primarily comprising a relatively deep portion of the cartilage and lacking surface cartilage tissue. It may be postulated that, in our study, significant experimental results were not attained in cells grown in 5% oxygen, which is within the normal physiological oxygen concentration range of cartilage tissue, because the samples we used contained fewer surface cells. Nonetheless, because the cartilage tissues we used did not have an adequate proportion of surface tissue, we could not compare the effects of oxygen conditions between chondrocytes isolated from the surface vs deep tissues. Future directions include investigating the effects of oxygen tension on cells collected from different tissue sites and details of the associations between these oxygen conditions and HIFs.

5. Conclusion

Under the 2% oxygen condition, we were able to shorten the time needed for the collection of layered cell sheets by about 3 days, promote chondrocyte proliferation, and create chondrocyte sheets that are rich in extracellular matrix. The faster creation of chondrocyte sheets may reduce the length of hospital stay for patients requiring this treatment and may help improve their quality of life.

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Conflicts of interest

M.S. receives research funding from CellSeed Inc. (Tokyo, Japan); T.O. is a founder and member of the board of CellSeed Inc., which has licenses for certain cell sheet-related technologies and patents from Tokyo Women's Medical University; and M.Y. is a shareholder of CellSeed Inc. Tokyo Women's Medical University receives research funding from CellSeed Inc.

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