Regardless of the potential value of these newly developed materials, further research is required to ensure their safe clinical application. Dexon mesh is a bioabsorbable material used widely in surgical treatments. The ability to repair incomplete ACL tears using self-adhesive ACL cell sheets and to fabricate three-dimensional bioengineered ACLs with a combination of cell sheets and materials currently used clinically represents considerable progress in ACL regeneration.

CONCLUSIONS

The elevated expression of TNMD in SCL-, SIF-, and SCF-derived cell sheets indicates that the synovium is a potential cell source for ACL regeneration. The presence of TNMD expression in fabricated cell sheets derived from ACL fibroblasts and synovial cells suggests that these sheets maintain some of the properties of ligaments. Cell sheet technology may be useful for fabricating tissue-engineered ligaments from these cells.

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Development of a novel vitrification method for chondrocyte sheets

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Abstract

Background: There is considerable interest in using cell sheets for the treatment of various lesions as part of regenerative medicine therapy. Cell sheets can be prepared in temperature-responsive culture dishes and applied to injured tissue. For example, cartilage-derived cell sheets are currently under preclinical testing for use in treatment of knee cartilage injuries. The additional use of cryopreservation technology could increase the range and practicality of cell sheet therapies. To date, however, cryopreservation of cell sheets has proved impractical.

Results: Here we have developed a novel and effective method for cryopreserving fragile chondrocyte sheets. We modified the vitrification method previously developed for cryopreservation of mammalian embryos to vitrify a cell sheet through use of a minimum volume of vitrification solution containing 20% dimethyl sulfoxide, 20% ethylene glycol, 0.5 M sucrose, and 10% carboxylated poly-L-lysine. The principal feature of our method is the coating of the cell sheet with a viscous vitrification solution containing permeable and non-permeable cryoprotectants prior to vitrification in liquid nitrogen vapor. This method prevented fracturing of the fragile cell sheet even after vitrification and rewarming. Both the macro- and microstructures of the vitrified cell sheets were maintained without damage or loss of major components. Cell survival in the vitrified sheets was comparable to that in non-vitrified samples.

Conclusions: We have shown here that it is feasible to vitrify chondrocyte cell sheets and that these sheets retain their normal characteristics upon thawing. The availability of a practical cryopreservation method should make a significant contribution to the effectiveness and range of applications of cell sheet therapy.

Keywords: Cell sheet therapy, Chondrocyte sheet, Vitrification, Cryopreservation, Cartilage repair

Background

The use of cell sheets is being actively investigated in the field of regenerative medicine as a potential treatment for various lesions [1,2]. For example, Okano et al. developed a method of preparing various types of cell sheets using temperature-responsive culture dishes [3]; additionally, cell sheets derived from corneal epithelia [4], skin [5], oral mucosal epithelia [6], bladder epithelia [7], myocardial cells [8,9], periodontal ligaments [10], and cartilage [11,12] are currently under investigation in preclinical studies or clinical applications [13-15].

We have been investigating the use of chondrocyte-derived cell sheets for treatment of knee cartilage injuries [11,12,16-18]. Cell sheets can be used as autografts or allografts. In a clinical setting, the preparation of autologous cell sheets involves a defined period of time for culture of the cells. Thus, the timing of transplantation has to be arranged with regard to both the needs of the patient and the condition of the cultured cell sheet. Cryopreservation of cell sheets would simplify the coordination of transplantation timing and would also allow repeated treatments for a single patient. In addition, development of robust cryopreservation methods and distribution protocols would need to be established to facilitate allograft-based cell sheet therapy. There is little doubt that cryopreservation of cell sheets would provide significant benefits to clinical applications of cell sheet therapies.

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Cell sheet therapy involves covering a tissue lesion with a membranous sheet [11,19,20]. Therefore, an indispensable prerequisite of a cryopreservation method is to maintain the integrity of the membranous structure of the cell sheet. However, achieving this has been challenging and although the viability of the cells comprising the sheets can be maintained, damage to the integrity of the sheet often occurs [21]. To date, no practical cryopreservation method has been developed for cell sheets that have been generated in temperature-responsive culture dishes.

Recent developments in vitrification methods have allowed practical cryopreservation of early stage embryos of many mammalian species, including humans [22,23]. Embryos are highly susceptible to damage by various aspects of cryopreservation, including the toxicity of the cryoprotectant (CPA), osmotic shock, and temperature shock [23,24], which makes their successful cryopreservation more difficult compared to somatic cells. Furthermore, embryos of some mammalian species such as pigs [25-28] are especially sensitive to low temperatures. However, the latest vitrification methods have enabled very high post-cryopreservation survival rates for mammalian embryos of a wide range of species, derivations or backgrounds [23,29,30]. The basic principle of the latest high-performance method involves vitrifying embryos with a very small amount of vitrification solution, a process termed the minimum volume cooling (MVC)-vitrification method [29-32]. The MVC-vitrification method is effective at stabilizing the vitreous status of the solution during vitrification and rewarming, and thereby achieves a high rate of survival of embryos.

In light of the success achieved in embryos, we decided to apply the basic principles of the MVC-vitrification method for cryopreservation of chondrocyte sheets. We successfully developed a coating method by which a cell sheet could be vitrified using a minimum amount of solution. This report describes the development of an effective vitrification method that does not impair either the macro- or microstructures of cell sheets, and thereby possesses significant potential for applications related to clinical cell sheet therapy.

Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich Chemical Co. (MO, USA), unless otherwise indicated.

Preparation of rabbit chondrocyte sheets

The study was conducted using commercially available primary cultured cells (Primary Cell, Hokkaido, Japan) derived from the knee cartilage of a Japanese white rabbit. The cells were plated onto temperature-responsive culture dishes (UpCell, diameter: 35 mm; CellSeed, Tokyo, Japan) [3] at a density of 5.0×10^4 to 1.0×10^5 cells/dish and cultured in RPMI-1640 medium (11875; GIBCO, Life Technologies Corporation, CA, USA) supplemented with 10% fetal bovine serum (FBS; 171012, Nichirei Biosciences, Tokyo, Japan) at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium in each dish was replaced with the same medium supplemented with 100 μM L-ascorbic acid (A4544; Wako Pure Chemical Industries, Osaka, Japan) when the cells reached confluence. The cells formed a single thin layer after 2 weeks of plating, at which time the UpCell dishes were placed at 25°C for 30 min to promote detachment of the cell sheet from the bottom surface of the dish [11]. The cell sheet was then removed from the dish using a cell shifter (CellSeed). Three cell sheets were layered together to form a triple-layered sheet, and this sheet was further cultured for 1 week in the UpCell dish.

Vitrification solutions

The vitrification solution developed for cryopreserving mammalian embryos [22,32] was used after slight modifications.

Hepes (20 mM) buffered Tissue Culture Medium-199 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 20% calf serum (12133C; SAFC Biosciences, KS, USA) was used as the basal solution. Dimethyl sulfoxide (DMSO) and ethylene glycol (EG) were used as permeable CPAs. Sucrose was used as a non-permeable CPA for all vitrification experiments. In some experimental groups, carboxylated poly-L-lysine (COOH-PLL) [33] was also added as a supplemental non-permeable CPA.

An equilibration solution (ES) consisting of 10% (v/v) DMSO and 10% (v/v) EG in the basal solution and a vitrification solution (VS) containing 20% (v/v) DMSO, 20% (v/v) EG, and 0.5 M sucrose were prepared. The effect of supplementation of the VS with 10% (w/v) COOH-PLL was examined in some of the experimental groups. A rewarming solution (RS) and a dilution solution (DS) containing 1 M and 0.5 M sucrose, respectively, were prepared and the basal solution was used as the washing solution (WS).

The VS was used at ice temperature (on crushed ice), and pre-warmed RS at 38°C was used to devitrify (rewarm) the vitrified cell sheet. All other solutions were used at room temperature (24-27°C).

Vitrification procedures

Coating method

The development of the coating method for the chondrocyte sheets was guided by the MVC concept [31], which has been shown to be effective for the vitrification of mammalian embryos. The method used here involves vitrification of a cell sheet; this is achieved by treating a cell sheet with ES and VS and then applying a thin coat of VS containing permeable and non-permeable CPAs.

This technique enables vitrification of the cell sheet with the minimum amount of vitrification solution (Figure 1).

First, a triple-layered cell sheet was peeled from the UpCell surface using a cell shifter and forceps, and immersed in 5 ml of ES in a 60 mm dish (Iwaki 3010–060, AGC Techno Glass, Shizuoka, Japan) for 5 min for pre-equilibration. Then, the cell sheet was transferred to the same solution in a fresh dish for 20 min (equilibration). After equilibration, the cell sheet was transferred to VS using forceps for 5 min (VS pre-treatment), and then transferred to fresh VS in another dish for 15 min for dehydration and the permeation of the permeable CPAs. VS containing COOH-PLL and unmodified VS were compared. After VS treatment, the cell sheet was carefully placed on a stainless steel mesh (30 mm) using forceps (Figure 2A). The cell sheet on the mesh was held 1 cm above the surface of liquid nitrogen (LN) in a 1 L Styrofoam bath and was vitrified by a 20 min exposure to the LN vapor (−140°C) (Figure 2B). We observed that vitrification of the cell sheet was completed within the first 5 min (Figure 2C). The use of LN vapor was adopted after preliminary tests demonstrated that the cell sheet fractured when it was directly immersed in LN.

To rewarm a vitrified sheet, the mesh holding the sheet was placed directly onto an electric heating plate (HP-4530; ASONE Corporation, Osaka, Japan) at 38°C for 90 sec (Figure 2D). After the sheet had completely thawed, the mesh holding the sheet was slowly placed into 10 ml of RS in a 60 mm dish and was gently removed from the mesh using forceps (Figure 2E). At this stage, the recovered sheet was checked for cracks. The CPAs were diluted and removed in a stepwise manner. Briefly, the cell sheet was held in RS for 1 min and then transferred into 5 ml of DS using forceps for 3 min. Then, the cell sheet was transferred twice into 5 ml of WS. The cell sheet was gently shaken several times in each solution to help diffusion of the CPAs.

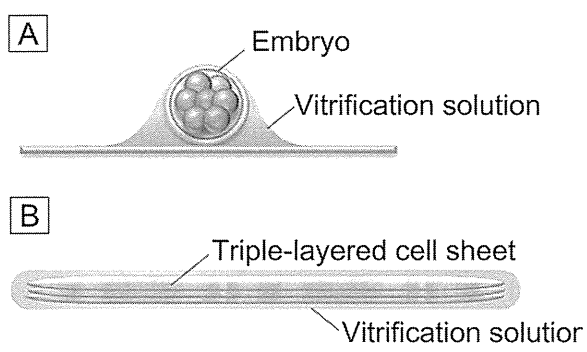

Figure 1 The minimum volume cooling concept applied to vitrification of an embryo and a cell sheet. A: The protocol of embryo vitrification using the minimum volume cooling (MVC) concept; the embryo is vitrified in a minimum volume of vitrification solution on a plastic or metal plate with high thermal conductivity [22,32]. **B**: For cryopreservation of triple-layered rabbit chondrocyte sheets, we developed a coating method in which the cultured cell sheet is thinly coated with a viscous vitrification solution containing permeable and non-permeable cryoprotectants. This enables the cell sheet to be vitrified in a minimum amount of vitrification solution in accordance with the MVC concept.

Envelope method

A cell sheet pre-treated with ES and VS as described above in the coating method, was placed onto a 5 × 10 cm rectangular piece of polyvinylidene chloride kitchen wrap (Kureha Corporation, Tokyo, Japan) using forceps. Then, the wrapping film was folded to enclose the cell sheet (Figure 2F). The wrapped sheet was held 1 cm above the surface of LN and vitrified by exposure to the vapor for 20 min (Figure 2G). Vitrification of the cell sheet was observed within the first 5 min (Figure 2H). As the presence of COOH-PLL in the VS was found to be essential for maintaining the structure of the vitrified sheet in the development of the coating method described above, VS containing COOH-PLL was used for the envelope method.

To rewarm the vitrified sheet, the cell sheet envelope was placed directly onto a heating plate at 38°C for 90 sec (Figure 2I). When the sheet had completely thawed, the wrapping film was slowly opened and the sheet was transferred into RS using forceps (Figure 2J). The recovered sheet was checked for cracks, and the CPAs were diluted, removed, and washed as described above for the coating method.

Dish method

In order to evaluate the effect of VS volume on cell sheet vitrification, a sheet was immersed in 2 ml of VS, a considerably larger volume than used in the coating or envelope methods.

A preliminary study revealed the formation of many cracks and ice crystals when the vitrification/rewarming procedure was performed using 2 ml of VS in a 35 mm plastic dish (Additional file 1: Figure S1A). However, ice crystal and crack formation was significantly reduced when the VS contained 10% COOH-PLL (Additional file 1: Figure S1B). Therefore, the dish method was performed using VS containing COOH-PLL.

A cell sheet was pretreated with ES and VS in the same manner as in the coating and envelope methods, placed in 2 ml of VS in a 35 mm dish (Nunc 150318, Thermo Fisher Scientific, Kanagawa, Japan) for 15 min for further CPA permeation and dehydration (Figure 2K). The dish was held 1 cm above the surface of LN for 20 min (Figure 2L) to induce solidification of the VS containing the sheet (Figure 2M).

To rewarm the vitrified sheet, the dish was allowed to stand for 3 min on a heating plate at 38°C (Figure 2N).

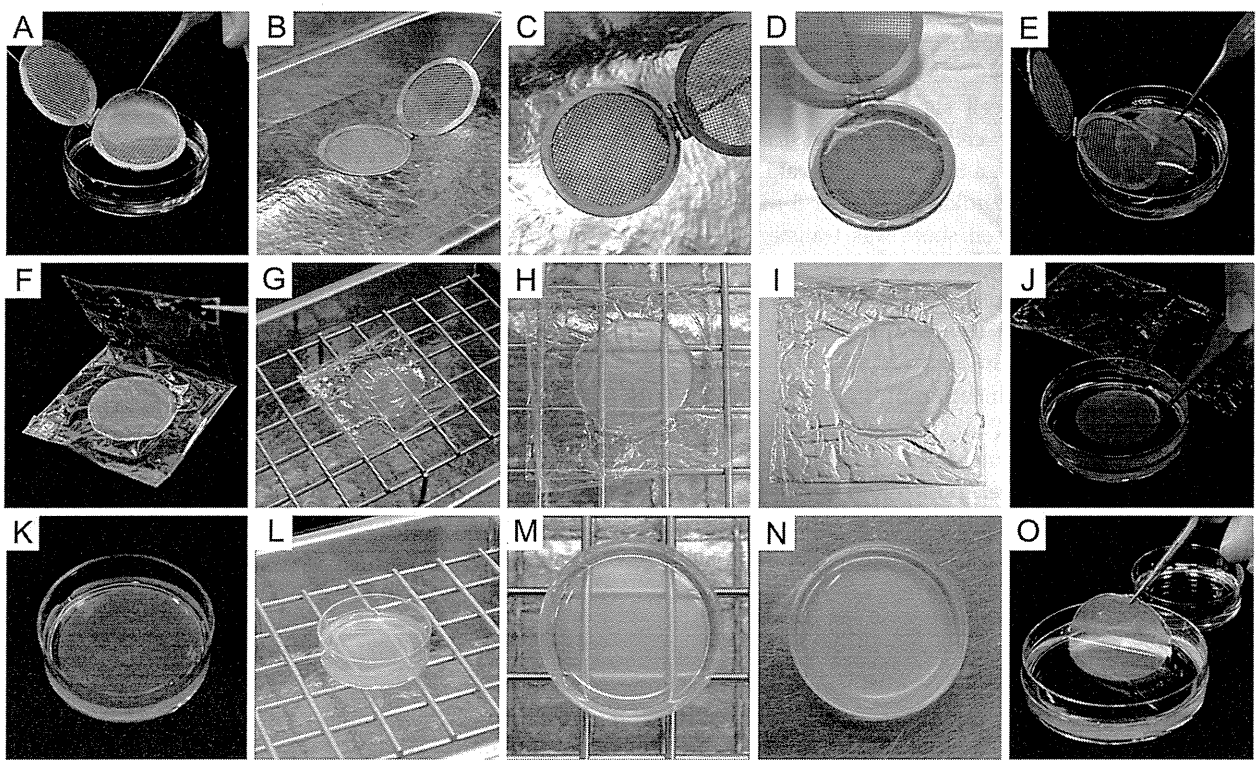

Figure 2 Vitrification and rewarming methods for triple-layered rabbit chondrocyte sheets. (A-E) Coating method. A cell sheet treated with ES and VS is placed on a stainless mesh using forceps **(A)** and exposed to liquid nitrogen (LN) vapor for vitrification **(B)**. **(C)** A cell sheet vitrified on mesh. Note the transparency of the vitrified sheet. For rewarming, the vitrified sheet on a mesh device is placed on a heating plate for thawing **(D)** and then transferred to RS using forceps **(E)**. **(F-J)** Wrapping film method. An ES- and VS-treated cell sheet was wrapped in an envelope of polyvinylidene chloride film **(F)** and exposed to LN vapor for vitrification **(G)**. **(H)** A cell sheet vitrified in wrapping film. Note that the cell sheet is transparent when it solidifies. For rewarming, the sheet vitrified in the wrapping film is placed on a heating plate **(I)**, followed by transfer into RS **(J)**. **(K-O)** Dish method. A cell sheet pretreated with ES and VS is placed in 2 ml VS **(K)** and exposed to LN vapor for vitrification **(L)**. **(M)** A cell sheet solidified with VS in a dish. Note the transparency of the vitrified solution and the lack of any cracks. **(N)** A dish containing a cell sheet on a heating plate for rewarming. The opacity of the solution indicates that ice crystals have formed during the warming process. The thawed cell sheet is then transferred into RS using forceps **(O)**.

After the complete melting of the VS in the dish, the cell sheet was transferred into RS using forceps (Figure 2O) and checked for visible damage (cracks). The CPAs were diluted, removed, and washed in the same manner as in the coating method.

Survival of the cells in the vitrified cell sheet

A vitrified cell sheet recovered after rewarming, rehydration and removal of CPAs was transferred into Dulbecco's phosphate-buffered saline (D-PBS; 10 ml) for washing. The cell sheet was cut into 1–2 mm^2 pieces with ophthalmic scissors, and the pieces were incubated in RPMI-1640 medium containing 2 mg/ml collagenase II (17101; GIBCO) at 37°C for 40–50 min to isolate the cells. The suspension of isolated cells was centrifuged at 1,000 rpm for 5 min, and the precipitated cells were resuspended in RPMI-1640 medium (22400; GIBCO) and their viability was determined after trypan blue staining (viability (%) = live cells/live and dead cells × 100).

Electron microscopy

Triple-layered chondrocyte sheets were soaked in 0.1 mol/l D-PBS containing 2% glutaraldehyde for 2 h, then fixed in 2% osmium tetroxide solution for 1 h, and then dehydrated through an ethanol series (50, 70, 80, 90, 95, and 100%). Next, the ethanol was replaced by 100% *tert*-butyl alcohol, and the samples were dried using freeze dryer (ES-2030; Hitachi High-Technologies Corporation, Tokyo, Japan). The dried specimens were sputter-coated with osmium and affixed to an adhesive interface for observation with a scanning electron microscope (JSM-6700 F; JEOL, Tokyo, Japan). The top surfaces of the cell sheets were observed at magnifications ranging from × 300 to × 2,000.

Histological examination and immunohistochemical staining

Triple-layered cell sheets were harvested after culture or cryopreservation and fixed in 4% paraformaldehyde solution for 1 week. The specimens were embedded in

paraffin and sectioned, and the sections were placed on glass slides. After deparaffinization and rehydration, the sections were stained for proteoglycan with 0.1% Safranin-O or immunostained with type II collagen antibody. For immunohistochemistry, the slides were incubated with a diluted primary anti-human type II collagen antibody (F-57: Daiichi Fine Chemical, Toyama, Japan) for 16 h at 4°C, followed by incubation with the EnVision + Mouse/HRP secondary antibody (K4000: DAKO, Glostrup, Denmark) for 1 h at room temperature. Finally, the sections were stained with diaminobenzidine (K3466: DAKO) and counterstained with hematoxylin. Coverslips were mounted onto the slides and sealed with nail polish. The slides were then examined under a microscope and images were captured (Biozero BZ-8000, KEYENCE, Osaka, Japan).

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 20.0 software (IBM Corporation, NY, USA). The proportional data were subjected to arcsine transformation and evaluated by one-way analysis of variance (ANOVA) followed by multiple comparisons using Tukey's test. The level of significance was set at p values < 0.05.

Results and discussion

Maintenance of cell sheet structure and cell viability after vitrification

Ten cell sheets were vitrified using the coating method in the presence of 10% COOH-PLL (Table 1). After rewarming, all the recovered sheets (100%) showed no visible damage and had same appearance as non-vitrified cell sheets (Figure 3A, B). Eight sheets were vitrified using the coating method in the absence of COOH-PLL (Table 1); only one of the recovered sheets (12.5%) did not have visible damage, all of the remainder exhibited cracks (Figure 3C). Cell viability in the vitrified cell sheets did not differ between protocols with or without COOH-PLL (92.1% vs. 91.9%); the rates of cell viability were comparable to that of the non-vitrified control (94.6%).

These results clearly showed that vitrification by the coating method in the presence of COOH-PLL as a supplemental non-permeable CPA was capable of preserving the membranous structure of the cell sheet with a high survival rate for the constituent cells. To the best of our knowledge, our results represent the first successful vitrification of cell sheets grown in temperature-responsive dishes. In this study, we vitrified cell sheets in LN vapor rather than by direct immersion in the LN. This also had a critical influence on the maintenance of the membranous structure of the cell sheet during vitrification. In preliminary experiments, all of the cell sheets cracked, even in the presence of COOH-PLL, when they were vitrified by direct immersion in LN. Possibly, the direct immersion approach might have had a more drastic impact on membrane integrity than the vapor and, thereby, impaired cell sheet structure.

Since the coating method proved successful for vitrification of cell sheets, we examined whether cell sheets enveloped in a thin film could also be vitrified successfully. Seven cell sheets were placed into film envelopes, vitrified and rewarmed (Table 1). All the sheets (100%) were recovered without visible damage (Figure 3D). However, cell viability (86.8%) was slightly lower compared to that in the coating method ($p < 0.05$).

For use in clinical applications, it would be preferable if vitrified cell sheets could be stored and distributed in hygienic coverings. Our results demonstrated that a cell sheet enveloped in a thin film with a minimum volume of VS could be successfully vitrified. However, wrapping a cell sheet with a film might influence the optimal cooling and rewarming rates during vitrification and rewarming processes. As cooling and warming rates have a crucial influence on the viability of vitrified cells [34,35], it will be important to identify robust film materials that have high thermal conductivity and are protective against invasive pathogens, as well as improving cooling and rewarming methods.

We also examined the influence of the volume of vitrification solution on the morphology and survival of cell sheets. Seven cell sheets were vitrified using the dish method (Table 1) that involves a substantially greater

Table 1 Structural maintenance and cell viability after vitrification of triple-layered rabbit chondrocyte sheets

Vitrification method	Presence of COOH-PLL in VS*	No. of cell sheets recovered without fracture / No. of cell sheets examined (%)	Cell viability (mean ± SEM)
Non-vitrified control		8/8 (100)	94.6 ± 0.5^{a}
Coating	+	10/10 (100)	92.1 ± 0.9^{a}
	-	1/8 (12.5)	91.9 ± 0.7^{a}
Envelope	+	7/7 (100)	86.8 ± 0.7^{b}
Dish	+	7/7 (100)	77.6 ± 3.15^{c}

[a-c]Values with different superscript differ significantly ($p < 0.05$).
*VS: Vitrification Solution.

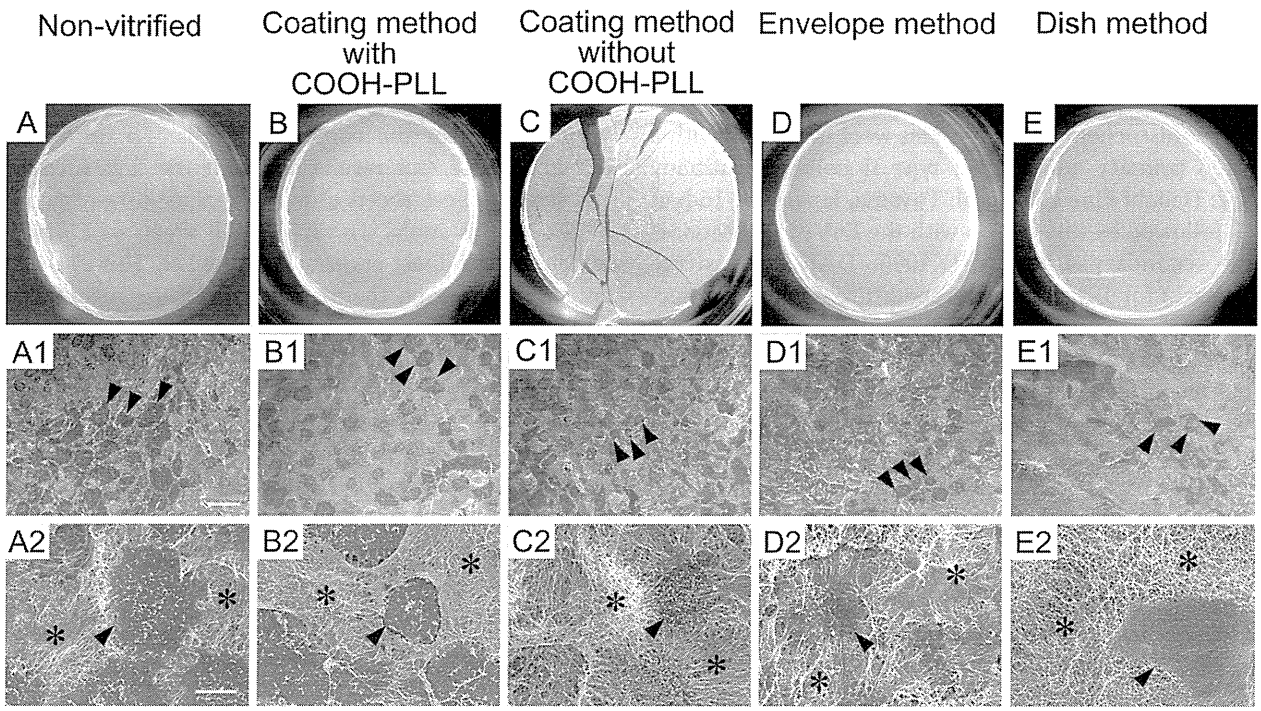

Figure 3 The macro- and microstructures of triple-layered rabbit chondrocyte sheets after vitrification. A-E: Morphological appearance of chondrocyte sheets after vitrification and rewarming. **A1-E2**: Scanning electron microscopic images of the surfaces of cell sheets recovered after vitrification and rewarming. **A1-E1**: ×300, Scale bar = 50 μm; **A2-E2**: ×2,000, Scale bar = 10 μm. The surfaces **(A1-E2)** are irregular, featuring pavement-like cell populations (arrowheads in **A1-E1** indicate representative three cells) and well-developed extracellular matrices with a dense fibrous structure (*). The microstructures of cell sheets vitrified by any of the methods described in this report were similar to those of the non-vitrified control sample.

volume of VS than the coating and envelope methods. VS containing COOH-PLL was used because a preliminary experiment revealed that its presence was essential to ensure crack-free vitrification using the dish method (Additional file 1: Figure S1). All of the 7 sheets (100%) that were vitrified were recovered without any cracks (Figure 3E). However, cell viability after vitrification was 77.6%, which was significantly lower than that observed following vitrification with the coating or envelope methods ($p < 0.05$).

High levels of viability can be achieved by minimizing the volume of the solution used for vitrification of mammalian embryos [31]. In contrast, the vitreous state becomes unstable when larger solution volumes are employed: more cracks tend to occur during the solidification of the solution and more ice crystals form upon rewarming. Our results demonstrated that addition of COOH-PLL as a non-permeable CPA was effective in stabilizing the vitrified state of the solution. However, even when the VS contained COOH-PLL, cell viability was reduced slightly with the dish method. The decrease might be attributed to the slower cooling and warming rates and/or ice crystal formation during rewarming. We observed that the VS appeared opaque for a moment during the rewarming process in the dish method, suggesting the occurrence of ice crystal formation.

Scanning electron microscopic images of the surface of vitrified cell sheets

The microstructures of the cell sheets vitrified in the four experimental groups were compared to those of the non-vitrified sample (Figure 3). Although slight differences were observed among individual sheets, overall, the cell sheets retained their basic structure of pavement-like cells (Figure 3A1-E2) distributed within well-developed extracellular matrices (Figure 3A2-E2). The sheet surfaces were irregular, featuring well-developed extracellular matrices with dense fibrous structures (Figure 3A1-E2).

The microstructures of cell sheets were maintained in the vitrified samples of all the experimental groups under the same conditions as the non-vitrified control groups. The sheets that developed cracks during vitrification with the coating method in the absence of COOH-PLL showed no microstructural abnormalities (Figure 3C1, C2), suggesting that the fracturing of the sheet structure did not affect the microstructure. The sheets vitrified by the envelope method (Figure 3D1, D2) and the dish method (Figure 3E1, E2), where cell viabilities were slightly

decreased, also exhibited no microstructural abnormalities. These results indicate that the microstructure of the vitrified cell sheet, including the extracellular matrix, were well maintained even after vitrification and rewarming under suboptimal conditions.

Histological and immunohistochemical examination of vitrified cell sheets

Cell sheets vitrified with the coating and envelope methods in the presence of COOH-PLL were histochemically and immunohistochemically examined to investigate the distribution of the major components of cartilage, i.e. proteoglycan and type II collagen. In the non-vitrified control (Figure 4A) and the vitrified cell sheet, strong Safranin-O staining was exhibited (coating method: Figure 4C, envelope method: Figure 4E). These results showed that acidic proteoglycan were, in general, densely and evenly distributed throughout the chondrocyte sheet and this distribution pattern was maintained after vitrification in the coating and envelope methods.

The vitrified samples also exhibited large amounts of type II collagen (Figure 4D, F) in a similar manner as in the non-vitrified control (Figure 4B). Overall, these data showed that the extracellular matrix of the vitrified cell sheets had been maintained in both the coating and envelope methods.

Significance of maintaining membranous structure in chondrocyte sheet cryopreservation

In the conventional slow-freezing method, cultured cell sheets are frozen in the presence of a relatively low concentration of a CPA [21]. Thus, extra- and intracellular ice crystal formation is inevitable during freezing, which destroys the cell sheet structure and decreases cell viability [21]. In contrast, with the vitrification method, a solution containing a high concentration of a CPA is rapidly cooled to achieve the transition from the liquid phase to the solid phase (amorphous) without ice crystal formation [36]. Therefore, cell sheets could be sealed in a glassy state that maintained their macro- and microstructures and also allowed high cell viability.

In cell sheet therapy, cytokines and growth factors produced by the cell sheet play an important role in healing damaged tissues [11,19,20]. We found that the formation of a chondrocyte sheet structure enhanced transforming growth factor-β secretion from the cells [37], which implies that maintaining the membranous structure after cryopreservation is a prerequisite for

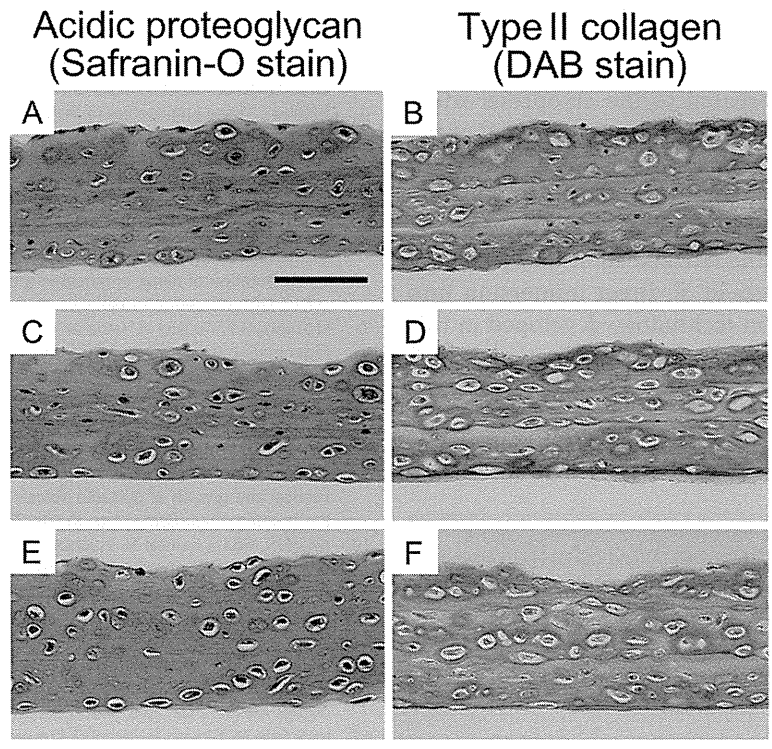

Figure 4 Histological and immunohistochemical examination of triple-layered rabbit chondrocyte sheets. Staining for proteoglycan **(A, C, E)** and type II collagen **(B, D, F)** on cross-sections of cell sheets recovered after vitrification and rewarming. **A, B**: Non-vitrified control cell sheet exhibiting large amounts of proteoglycan and type II collagen in the extracellular matrix. **C, D**: Cell sheet vitrified with the coating method using VS containing COOH-PLL; the sheet exhibits a normal extracellular matrix. **E, F**: Cell sheets vitrified by the envelope method. The extracellular matrix exhibits no difference to samples from control and coating method groups. (Scale bar = 100 μm).

function. Therefore, in the present study, we focused on both the maintenance of sheet structures and of cell viability after vitrification. Thus, our study provides the first demonstration that cryopreservation of cultured chondrocyte sheets with a fragile membranous structure can be achieved using a vitrification method developed on the basis of the MVC concept. Biochemical functions such as cytokine production by the vitrified chondrocyte sheets has yet to be analyzed. Additionally, transplantation experiments using vitrified cell sheets are under consideration.

In our preliminary study, we could successfully vitrify cell sheets with more fragile characteristics including human chondrocyte sheets. It is, therefore, likely that the vitrification method developed in the present study can be applied to different types of cell sheet other than the triple layered rabbit chondrocyte sheets. In application of the vitrification technology to human therapies, toxicity of CPAs to human cells needs to be verified.

Conclusions

In this study, we demonstrate that the vitrification method developed here facilitated the cryopreservation of a chondrocyte sheet while maintaining its macro- and microstructures and allowing a high rate of viability of the constituent cells. The coating method, where the cell sheet was vitrified with a minimum volume of VS in the presence of COOH-PLL, effectively prevented structural damage due to vitrification. Here, we propose three basic principles essential to the cryopreservation of chondrocyte sheets: (i) minimizing the volume of the vitrification solution by using the coating method, (ii) stabilizing the vitreous state via the addition of COOH-PLL as a non-permeable CPA, and (iii) preventing the occurrence of cracks in the vitrified solution by cooling samples in LN vapor instead of direct immersion into LN. The cryopreservation technology developed in this study will play a pivotal role in clinical applications of cell sheet-based therapies.

Additional file

Additional file 1: Figure S1. Protective effect of COOH-PLL against fracture of vitrified solution. COOH-PLL-free (A) and COOH-PLL-containing (B) solutions in the process of rewarming after vitrification in liquid nitrogen vapor. Note the occurrence of many cracks in the COOH-PLL-free solution (A), while the COOH-PLL-containing solution is free of cracks (B). The opacity of the solution in B indicates that ice crystals formed during the warming process.

Abbreviations

COOH-PLL: Carboxylated poly-L-lysine.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HN conceived and designed the experiments and wrote the manuscript. MM performed the experiments and wrote the manuscript together with HN. MW wrote the manuscript together with HN. TK and HM performed the experiments. MS (Michio Sato) scanned electron microscopic images. MY, MK prepared chondrocyte cell sheets. KM and HSH prepared COOH-PLL. MS (Masato Sato) and JM helped to draft the manuscript. All authors read and approved the final manuscript.

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RESEARCH ARTICLE

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

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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. **Copyright © 2013 John Wiley & Sons, Ltd.**

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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

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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

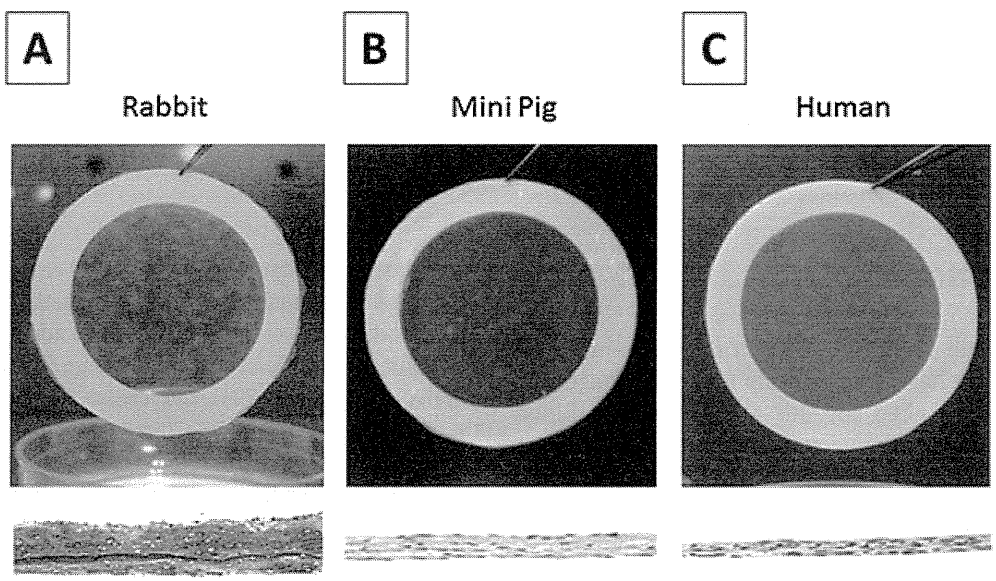

Figure 1. Three-layer chondrocyte sheets for different animal species. (A) A macrophotograph and graft (toluidine blue staining) of rabbit layered chondrocyte sheets. (B) A macrophotograph and graft [haematoxylin and eosin (H&E) staining] of minipig layered chondrocyte sheets. (C) A macrophotograph and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

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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

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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	

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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).

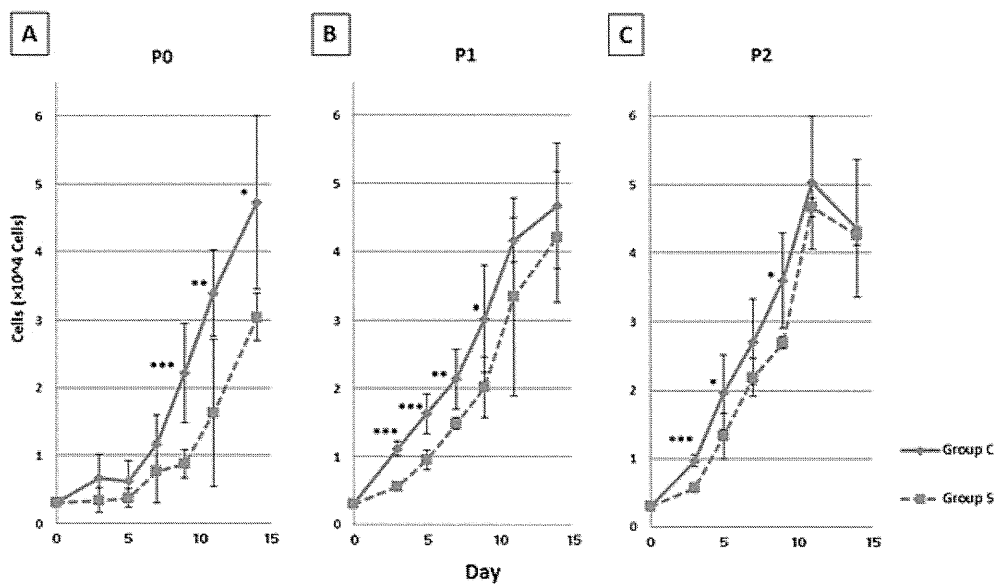

Figure 2. Cell proliferation assessment by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. (A) Proliferation was significantly greater in P0 chondrocytes in group CL than in group S from day 3. The difference in cell count between the groups was significant from day 9. (B) Proliferation 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). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

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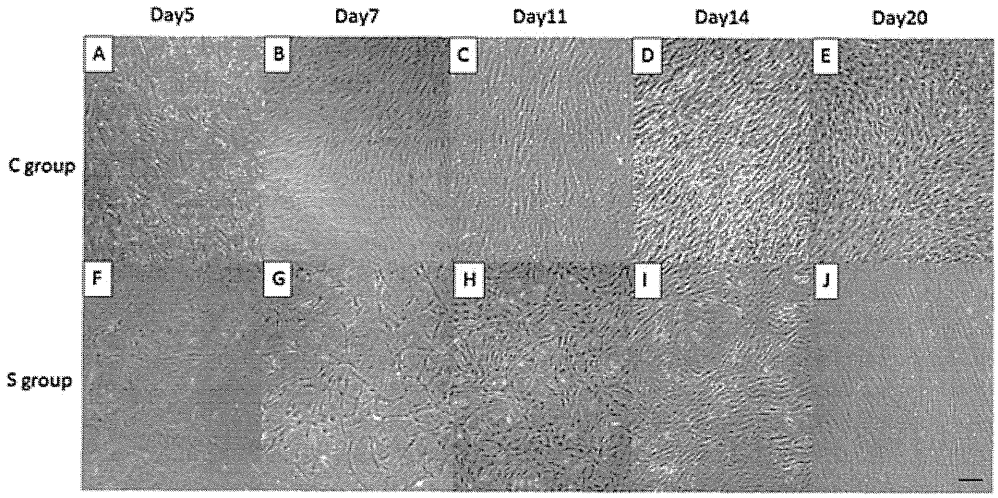

Figure 3. Observation of cell proliferation using phase-contrast microscopy. Phase-contrast micrographs of P1 cartilage in group C (A–E) and group S (F–I) on day 3 (A,F), day 7 (B,G), day 11 (C,H), day 14 (D,I), and day 20 (E,J) (original magnification ×100)

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,

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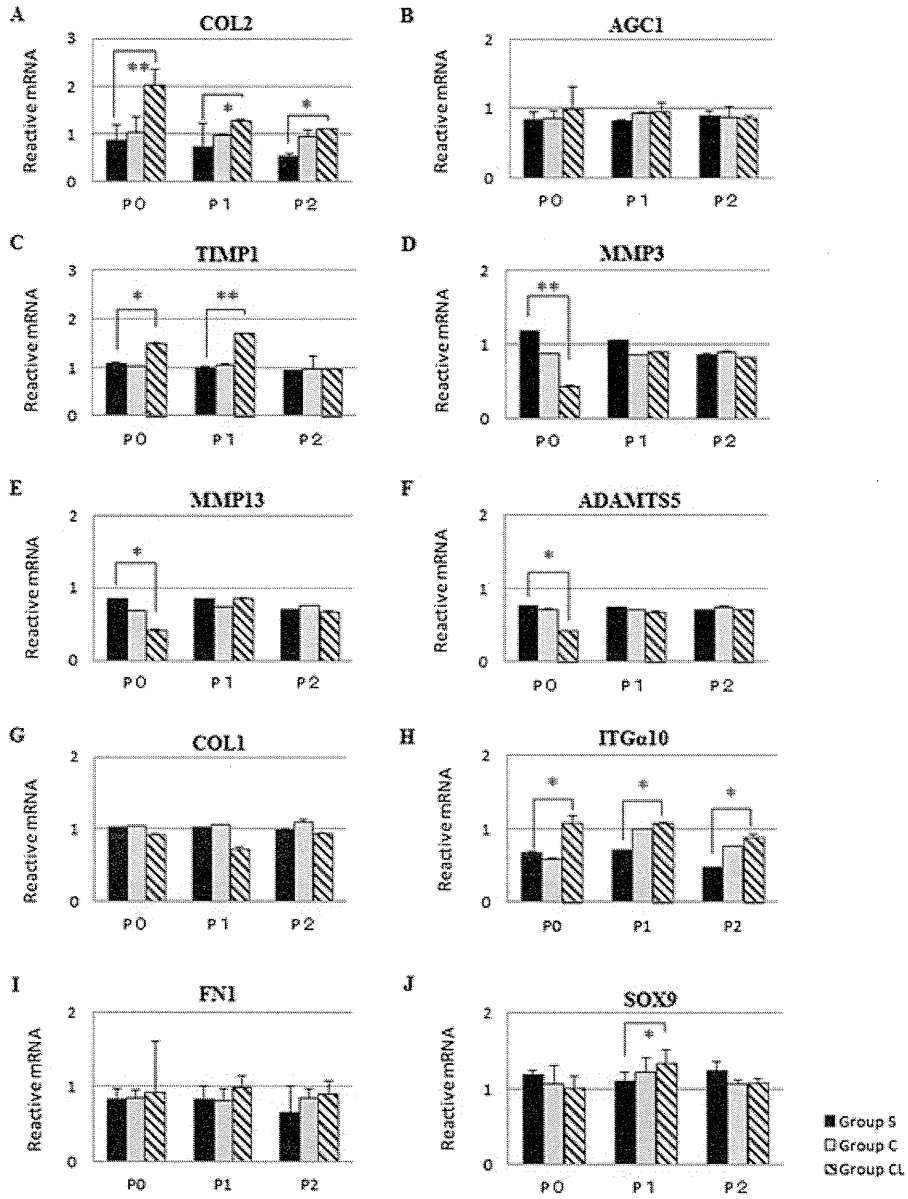

Figure 4. Results of real-time polymerase chain reaction (PCR) analysis comparing mRNA expression between the three culture groups. The type II collagen (COL2) (A), aggrecan-1 (AGC1) (B), tissue metallopeptidase inhibitor 1 (TIMP1) and fibronectin-1 (FN1) (I) genes, which are important for the maintenance 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

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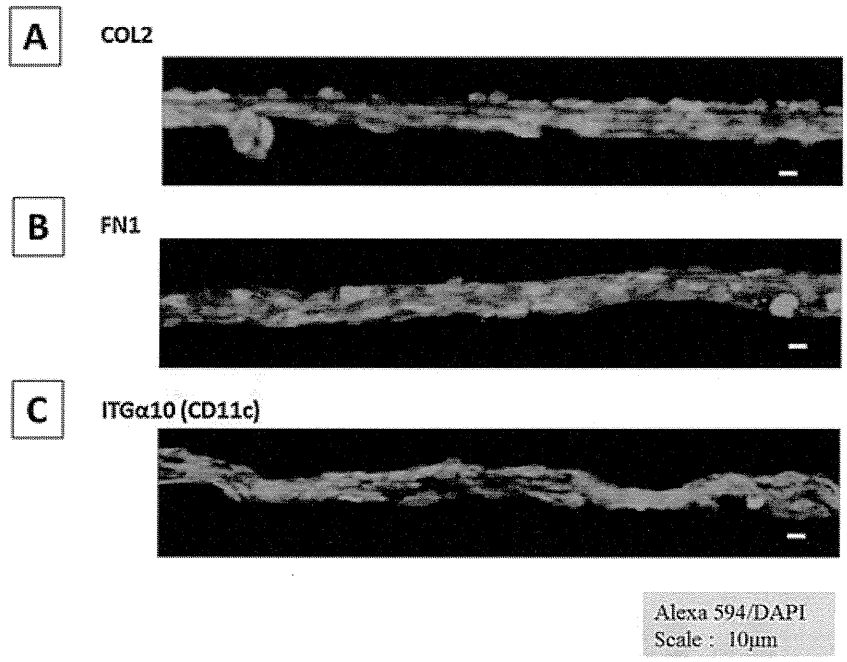

Figure 5. Results of immunostaining in the CL group. Expression of (A) type II collagen (COL2) and (B) fibronectin-1 (FN1) was observed throughout the layered chondrocyte sheet. (C) Integrin-α10 (ITGα10) (shown in red) localized in the pericellular matrix of the triple-layered chondrocyte sheet. Scale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

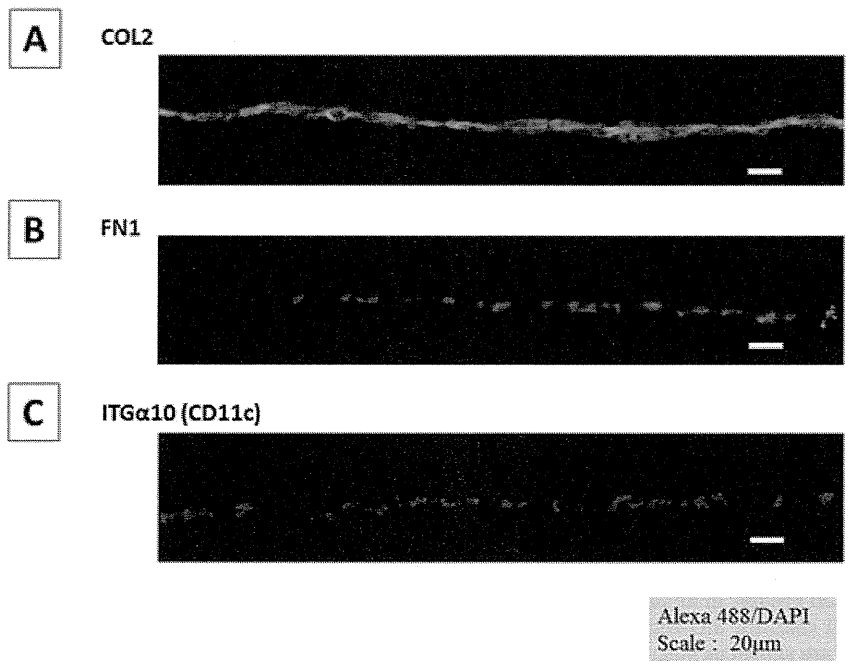

Figure 6. Results of immunostaining in the S group. Expression of (A) type II collagen (COL2) and was observed throughout the layered chondrocyte sheet. (B) fibronectin-1 (FN1) and (C) Integrin-α10 (ITGα10) (shown in green) localized in the pericellular matrix of the triple-layered chondrocyte sheet.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

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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.

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Conflict of interest

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

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