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Original Article

Thickness-wise growth technique for human articular chondrocytes to fabricate three-dimensional cartilage grafts

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ABSTRACT

Introduction: Cutting the cost of manufacturing is important for extending the use of tissue-engineered therapeutic products. The present study aimed to develop a simple method for fabrication of cartilaginous tissues for regenerative therapy, utilizing the phenomenon where human articular chondrocytes grow thickness-wise and spontaneously form three-dimensionally thick tissues.
Methods: Normal human articular chondrocytes (NHACs) were cultured with varying concentrations of transforming growth factor beta 1 (TGF-β1) and/or fibroblast growth factor-2 (FGF-2) to optimize the culture condition for thickness-wise growth of chondrocytes. Next, the tissues grown in the optimal condition were subjected to re-differentiation culture in attached and detached states to assess differentiation capacity by evaluating secreted factors, histological analysis, and a gene expression assay.
Results: NHACs grew thickness-wise efficiently in the presence of 1 ng/mL TGF-β1 and 10 ng/mL FGF-2. After two weeks of culture, NHACs grew with 11-fold higher thickness and 16-fold higher cell number compared to cells which were neither treated with TGF-β1 nor with FGF-2. These thickness-wise-grown chondrocytes could be re-differentiated by a differentiation medium according to the increase in melanoma inhibitory activity (MIA) and positive safranin-O staining. Interestingly, the cartilaginous gene expression was considerably different between the attached and detached conditions even in the same culture medium, indicating the necessity of detachment and shrinkage to achieve further differentiation.
Conclusions: Spontaneous thickness-wise growth might provide a simple tissue-engineering method for manufacturing cartilaginous 3D tissues.

1. Introduction

The main obstacle against extending the use of tissue-engineered therapeutic products includes its cost of manufacturing. While pursuing the simplest method for fabricating a cartilaginous tissue suitable for regeneration of articular cartilage defects, we found that human articular chondrocytes can grow thickness-wise and spontaneously form a three-dimensionally (3D) thick tissue even in a conventional tissue culture dish. Thus, in the present study, we investigated a method for manufacturing tissue-engineered cartilage grafts exploiting this thickness-wise growth technique.

It is widely believed that normal primary cells exhibit “contact inhibition” in tissue culture dishes while malignantly transformed cells break it and grow aggressively. As an exception, stratified epithelial cells, such as keratinocytes, can grow three-dimensionally only in tuned conditions with or without feeder cells [1,2]. Stratification of epithelial cells has been widely investigated in terms of histology, physiology, pathology, and *in vitro* culture techniques [2–8]. In contrast, 3D growth of non-epithelial cells has been less investigated. However, understanding micro environment and behavior of cells in 3D tissue culture is a key factor for elucidating the underlying pathological phenomena, such as wound healing process or fibrosis, and for tissue-engineering of

Abbreviations: FGF-2, Fibroblast growth factor 2; TGF-β1, Transforming growth factor β1; BMP, Bone morphogenetic protein; GDF, Growth and differentiation factor; MIA, Melanoma inhibitory activity; OCT, Optical coherence tomography.

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in vitro models or therapeutic grafts. Few studies have reported that factors such as transforming growth factor beta (TGF-β) or ascorbic acid, which stimulate extracellular matrix secretion, promote the 3D growth of fibroblasts *in vitro*, and these studies indicated the importance of fibronectin for adhesion among cells [9–12]. In addition, Hendriks and colleagues reported that chondrocytes can grow thickness-wise over the confluence in the presence of fibroblast growth factor 2 (FGF-2), TGF-β, platelet-derived growth factor (PDGF), non-essential amino acids (NEAA), and ascorbic acid phosphate [13]. Therefore, we hypothesized that spontaneous thickness-wise growth of chondrocytes might be a simple approach to prepare 3D cartilaginous tissue, where chondrocytes proliferate both two-dimensionally and three-dimensionally in a culture vessel without any extra processing, such as passaging. Although it is known that articular chondrocytes lose their differentiated phenotype upon prolonged passage culture [14–16], they regain their differentiated phenotype partially when cultured with differentiation factors (TGF-βs, BMPs, GDFs, or dexamethasone), especially in 3D cultures including pellet culture. Thus, it was also expected that the thickness-wise-grown tissues could be re-differentiated by adding differentiation factors. To ascertain these hypotheses, in this study, we first optimized the thickness-wise culture condition of propagated human articular chondrocytes, and then, examined the re-differentiation capacity of the resultant construct as a 3D cartilaginous tissue.

2. Methods

2.1. Experimental groups

A total of five culture conditions were compared in this study. The culture condition of each group is summarized in Fig. 1.

2.2. Cell preparation

Adult normal human articular chondrocytes (NHACs) and adult normal human dermal fibroblasts (NHDFs) were purchased from Lonza (Cat.No. CC-2550 and CC-2511, respectively, passage 2 at arrival). NHACs were passaged two more times (passage 4) in CGM-2 medium (Lonza, CC-3216). NHDFs were passaged five more times (passage 7) in FGM-2 medium (Lonza, CC-3132) or DMEM-F12 medium (Thermo Fisher Scientific, Cat.No. 11320-082) supplemented with 10% fetal bovine serum (Japan Bio Serum), 10 ng/mL FGF-2 (Fiblast spray, Kaken Pharmaceutical), and 1% penicillin-streptomycin (Thermo Fisher Scientific, Cat.No. 15140-122). Passaged cells were cryopreserved in CellBanker 1-plus (Takara Bio) at −80 °C until further use.

2.3. Thickness-wise growth culture

The cells were cultured in Cell Culture Inserts for 6-well plates (Corning, Cat.No. 353090) or tissue culture dishes with growth or differentiation media. Growth medium was prepared by adding fetal bovine serum (FBS), 1% penicillin-streptomycin, 0.01% ascorbic acid phosphate magnesium salt n-hydrate (Wako Chemicals, Cat.No. 013-12061), FGF-2 (Fiblast spray, Kaken Pharmaceutical), and TGF-β1 (Miltenyi Biotec, Cat.No. 130-095-066) to DMEM-F12 basal medium (Thermo Fisher Scientific, Cat.No. 11320-082). The concentrations of FBS, TGF-β1, and FGF-2 were determined in the first experiment (10% FBS, 10 ng/mL FGF-2, and 1 ng/mL TGF-β1). Differentiation medium was prepared by adding 1% FBS, 1% penicillin-streptomycin, 1% insulin-transferrin-selenium solution (Thermo Fisher Scientific, Cat.No. 41400-045), 0.01% ascorbic acid phosphate magnesium salt n-hydrate, 10 ng/mL TGF-β1, 10 ng/mL bone morphogenetic protein 2 (BMP-2) (Miltenyi Biotec, Cat.No. 130-094-616), and 10 ng/mL growth and differentiation factor 5 (GDF-5) (BioVision, Cat.No. 4667-50). Thawed NHACs and NHDFs were seeded at 1×10^4 cells/cm^2 in growth medium. For re-differentiation, the medium was changed to differentiation medium at Day 7. In all experiments, cells were cultured in a humidified incubator at 37 °C under 5% CO_2 and the medium was changed 2 or 3 times in a week.

2.4. Pellet culture

A pellet culture of chondrocytes was performed to produce a histological positive control of chondrogenic differentiation induced by a three-dimensional culture and to confirm the differentiation capacity of the chondrocytes used in this study. Thawed NHACs were seeded in a tissue culture dish at 1×10^4 cells/cm^2 and cultured for four days in growth medium. The cells were then harvested by Accutase (Nacalai tesque, 12679-54) and re-suspended in the medium at 1×10^6 cells/mL. Next, 0.5 mL of the suspension was transferred to a 15 mL conical tube (Corning), centrifuged at 230×*g* for 5 min and cultured for four weeks in differentiation medium.

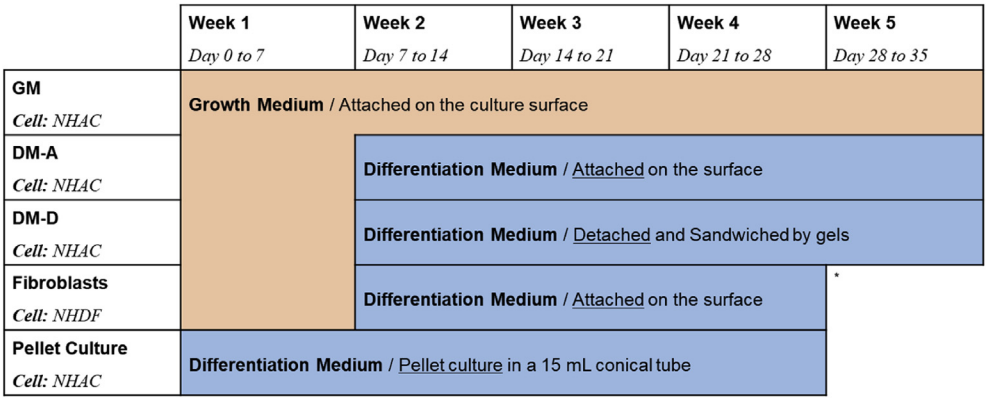

Fig. 1. Diagram of culture conditions. Samples in each group were cultured according to this diagram. * NHDFs were analyzed at day 28 because they detached spontaneously at week 5.

2.5. Detachment and re-culture of tissues

To promote re-differentiation of chondrocytes, a part of NHAC tissues at Day 7 were detached from the culture inserts or dishes mechanically by pipetting. The tissues were then cultured in the differentiation medium sandwiched by 10% agarose gels to prevent folding up. When the tissues were detached, they shrunk in parallel to the culture surface spontaneously.

2.6. Thickness measurement

Three dimensional light scattering images of the tissues were acquired by an optical coherence microscope (OCM) (Panasonic, in development) [17], and the thicknesses were measured from these images using ImageJ software. The resolution of the images was 0.675 μm for the horizontal axis and 0.73 μm for the vertical (stacking) axis. In these images, the surfaces of culture inserts could be detected as peaks of intensity, and surfaces of cultured tissues facing the medium were detected as inflection points of intensity. The images were slightly blurred by a Gaussian filter to reduce noises before the measurement. The average of randomly selected three points was adopted as the representative thickness value for each tissue.

2.7. Cell counting

The number of cells in each tissue was counted after digestion by enzymes. Cultured tissues were rinsed with phosphate buffered saline (PBS) twice, and dispersed by treatment with 5 mg/mL collagenase (Serva, Cat.No. 17454) for 1–3 h in a 37 °C incubator followed by Trypsin–EDTA (Nacalai tesque, Cat.No. 32777-44) treatment for 5 min. The cells were collected and counted using disposable hemocytometers.

2.8. Measurement of secreted factors

To confirm the time-dependent re-differentiation of chondrocytes, melanoma inhibitory activity (MIA) and matrix metalloproteinase 13 (MMP-13) in the supernatants were measured. Culture supernatants were sampled once a week, 24 h after medium change, and stored at −80 °C immediately. Next, an Enzyme-Linked Immuno Sorbent Assay (ELISA) was conducted using commercially available kits for MIA (R&D Systems, Cat.No. DY2050) and total MMP-13 (R&D Systems, Cat.No. DY511), according to the manufacturer's instructions.

2.9. Histological analysis

To examine the extent of chondrocyte proliferation and re-differentiation, histological analyses were conducted. Firstly, the proliferation of chondrocytes in growth medium were demonstrated using a Ki-67 antibody and 4', 6-diamidino-2-phenylindole (DAPI). Secondly, the tissues in all culture groups were embedded in paraffin and sectioned. Then, hematoxylin-eosin (H&E) staining and Safranin-O fast green staining were performed according to the standard procedures. In addition, a supplementary immunohistological analysis for type I collagen and type II collagen was conducted.

2.10. Quantitative polymerase chain reaction (qPCR)

To examine the phenotypes of chondrocytes in 3D tissues, quantitative polymerase chain reaction (qPCR) was performed. Chondrocyte tissues at day 7, 22, and 35 were sampled. Total RNAs were extracted using RNeasy Plus Mini Universal Kit (Qiagen), and cDNAs were synthesized by PrimeScript RT reagent Kit (Takara Bio). Next, Ct values of genes were determined by TaqMan Gene Expression Assay (Applied Biosystems) with a Viia7 apparatus (Applied Biosystems) using 5 ng total RNA per reaction. For ΔCt calculation, the geometric average of expression (arithmetic average of Ct values) of YWHAZ, PUM1, and EIF2B1 genes were used as endogenous control as determined by geNorm method [18] for 32 housekeeping genes in TaqMan Array Human Endogenous Controls Plate (Thermo Fisher Scientific). In addition to the ΔCt analysis for each single gene with endogenous control genes, ΔCt between two opposing genes (COL2A1 to COL1A1, ACAN to VCAN,

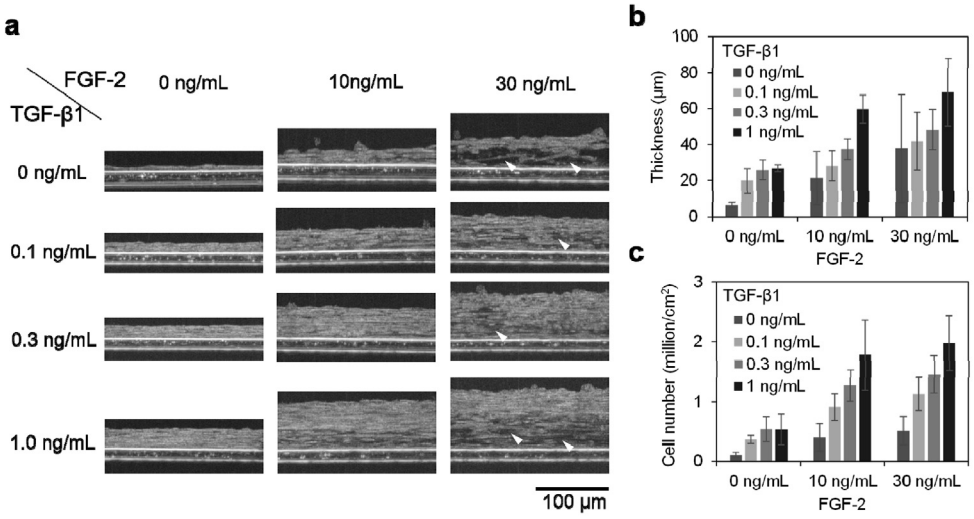

Fig. 2. Thickness-wise growth of chondrocytes. Human articular chondrocytes of three independent strains were cultured in growth medium with varying concentrations of transforming growth factor beta 1 (TGF-β1) and/or fibroblast growth factor-2 (FGF-2) for 2 weeks. **a,** Cross-sectional images of 5 μm thickness re-constructed from stacked horizontal slice images acquired by an optical coherence microscopy (OCT). White arrowheads indicate sparse or void spaces in the tissues. **b,** Thicknesses of the tissues calculated from the OCT images. Bars indicate standard deviations of three strains. Multiple linear regression analysis proved that the thicknesses were positively correlated with concentrations of TGF-β1 ($p = 0.00048$) and FGF-2 ($p = 0.00026$). **c,** Number of cells in each tissue counted after enzymatic digestion. Bars indicate standard deviations of three strains. Multiple linear regression analysis proved that the cell numbers were positively correlated with concentrations of TGF-β1 ($p = 0.0078$) and FGF-2 ($p = 0.011$).

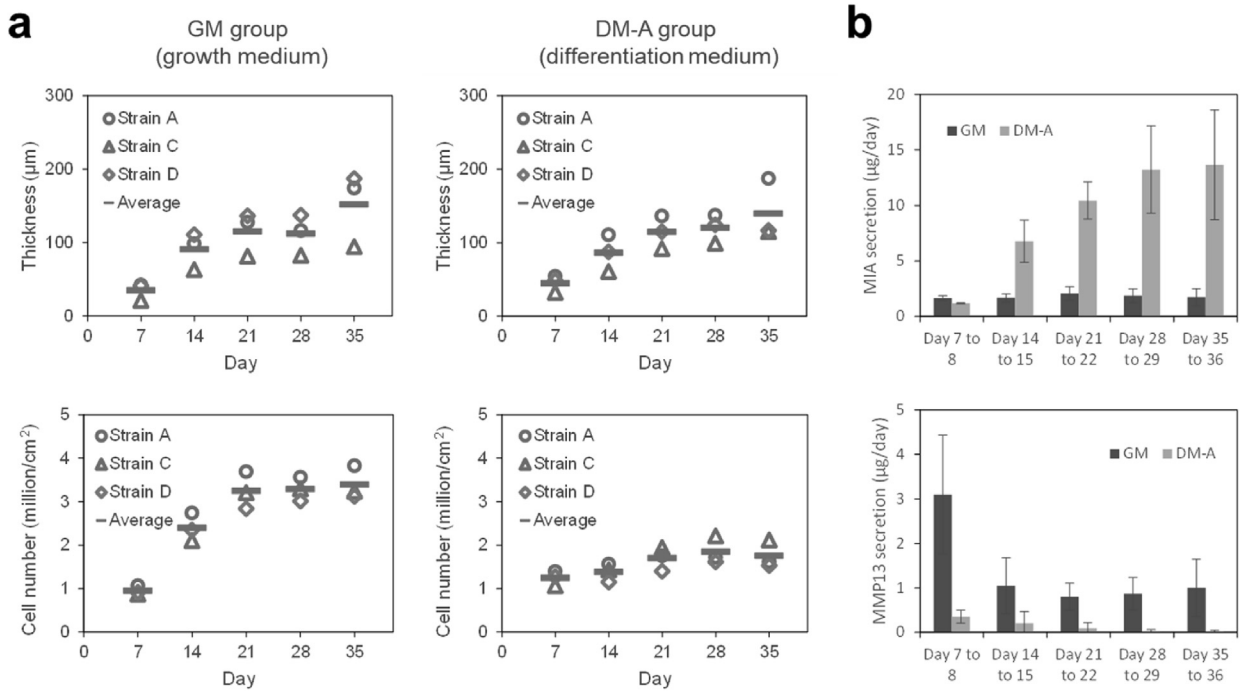

Fig. 3. Time-course of thickness-wise growth of human articular chondrocytes. Three independent strains of human articular chondrocytes were cultured in GM or DM-A culture conditions shown in Fig. 1. **a,** Time-course of thickness and cell number. **b,** MIA and MMP13 secretion in a day.

COL1A2 to COL1A1) were directly calculated from the Ct values as indicators of the state of chondrocytes [19–23]. All the assay targets are listed in Supplementary Material 1. Statistical analysis was conducted using paired t-tests between two groups for each gene and gene combination under the assumption that expressions of a gene in a group converge to a log-normal distribution [24]. The differences where the p-value was less than 0.05 were considered to be significant.

3. Results

3.1. Optimization of culture conditions for thickness-wise growth

Firstly, NHACs were cultured in growth medium (10% FBS) with 0, 0.1, 0.3, or 1 ng/mL TGF-β1 and 0, 10, or 30 ng/mL FGF-2 for 2 weeks, and cell numbers and thicknesses were measured (Fig. 2). Both TGF-β1 and FGF-2 increased cell number and thickness of the tissue concentration-dependently although relatively sparse or void spaces were observed in OCT images in the presence of 30 ng/mL FGF-2. Thus, we determined the optimum concentration of growth factors as 1 ng/mL TGF-β1 and 10 ng/mL FGF-2. Next, the effect of FBS concentration was assessed. The thickness of the tissue slightly increased with FBS concentration. However, tissues cultured with 20% FBS exhibited many void spaces near the culture surfaces (Supplementary Material 2a). Therefore, 10% FBS was adopted for further experiments. In this condition, immunohistological analysis for Ki-67 demonstrated that proliferation of chondrocytes continued at least up to 2 weeks in the tissues (Supplementary Material 2b).

3.2. Time-course of thickness-wise growth

Secondly, how chondrocytes grow thickness-wise in growth medium or differentiation medium was investigated (Fig. 3). In GM group, NHACs were cultured in growth medium (containing 10 ng/mL FGF-2 and 1 ng/mL TGF-β1) for five weeks. In DM-A group, NHACs were cultured in growth medium during the first week, and cultured in differentiation medium in second to fifth weeks (see Fig. 1). Tissues and culture supernatants were sampled weekly, and subjected to the analysis of thickness, cell number, and secreted chondrogenic markers (MIA and MMP-13). In the GM group, both thickness and cell number increased until day 21, and the increase was suppressed after that. MIA secretion was stable over the culture period. MMP-13 secretion was high at day 7–8, but stable after day 14. In DM-A group, the increase of thickness was comparable to that of GM group. However, the increase in cell number was slower than GM group. MIA secretion increased gradually indicating the re-differentiation of chondrocytes, while the secretion of MMP-13 decreased.

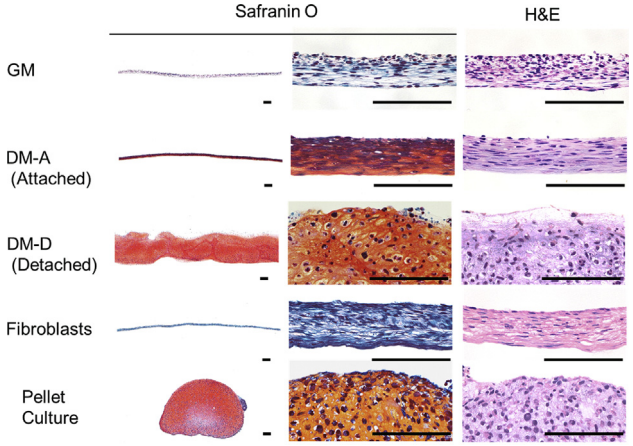

Fig. 4. Histological analysis of cultured tissues. H&E, hematoxylin and eosin staining. Safranin O, Safranin-O and fast green staining. Culture conditions are shown in Fig. 1. Bars indicate 100 μm.

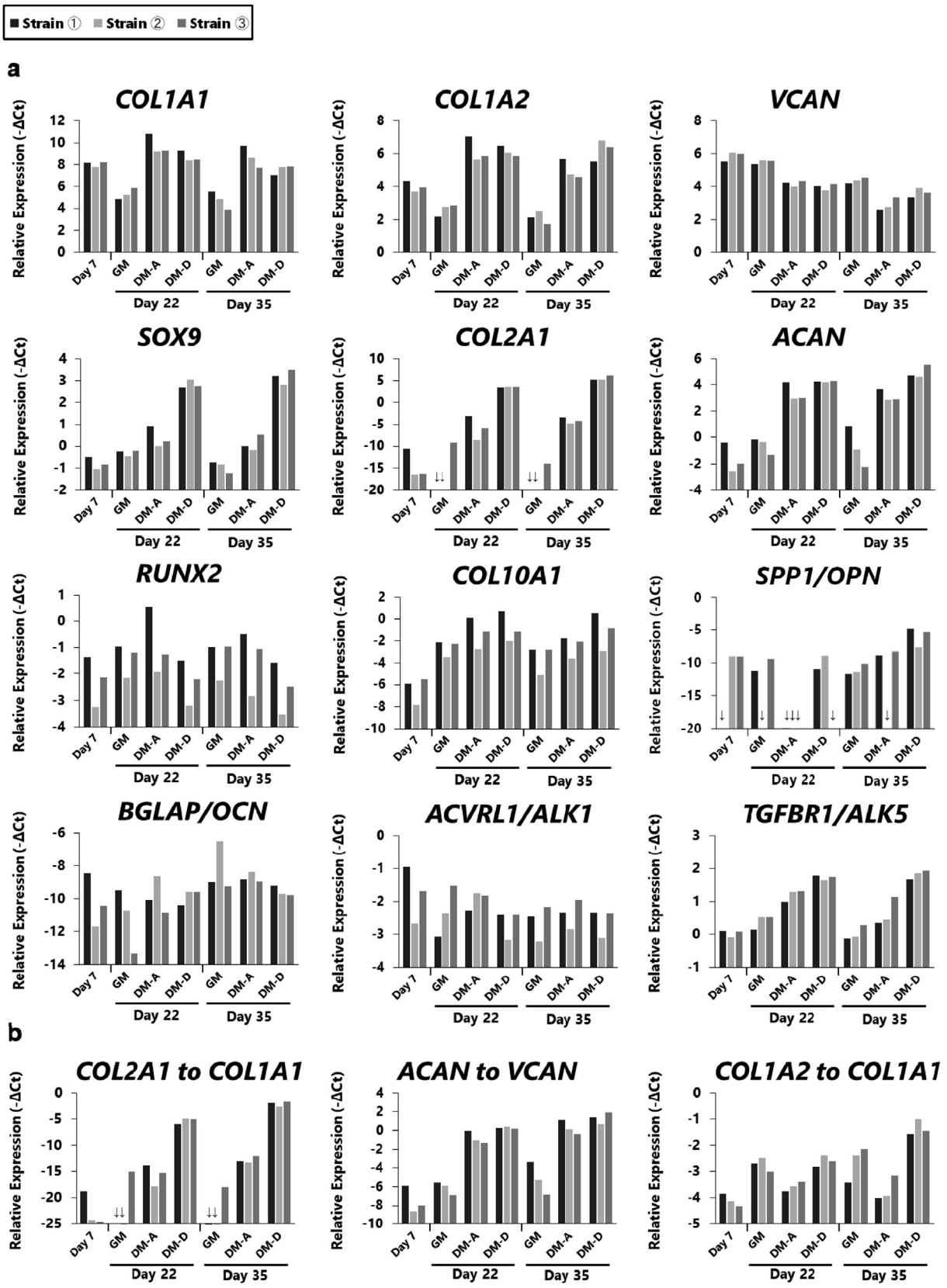

Fig. 5. Expression of cartilage-related genes. Three independent strains of human articular chondrocytes were cultured in three conditions (GM, DM-A, and DM-D). Until day 7, the chondrocytes in all three groups were cultured in the same condition with growth medium. After that, the cells in GM group were cultured in growth medium, the cells in DM-A group were cultured in differentiation medium, and the cells in DM-D group were detached from the culture inserts and cultured in differentiation medium. Total RNAs were extracted at day 7, 22 and 35 for qPCR analysis. **a,** Expression of each gene relative to the endogenous control genes. **b,** Relative expression between two genes. Downward arrows in the graphs indicate instances where the Ct values could not be determined over 50 cycles.

Table 1
Paired t-test of gene expression between GM group and DM-A group.

Gene	Day 22			Day 35		
	ΔΔCt[*1]	P-value	Result[*2]	ΔΔCt[*1]	P-value	Result[*2]
COL1A1	4.4	0.014	GM＜DM-A	4.0	0.00034	GM＜DM-A
ACAN	4.0	0.0039	GM＜DM-A	3.9	0.014	GM＜DM-A
COL10A1	1.3	0.045	GM＜DM-A	1.1	0.021	GM＜DM-A
SOX9	0.67	0.050	GM＜DM-A	1.0	0.051	NS
RUNX2	0.56	0.18	NS	-0.078	0.41	NS
COL1A2	3.6	0.015	GM＜DM-A	2.9	0.0082	GM＜DM-A
COL2A1	11	0.059	NS	14[*3]	0.012[*3]	GM＜DM-A[*3]
VCAN	-1.3	0.0057	GM＞DM-A	-1.5	0.0034	GM＞DM-A
SPP1	-6.3	0.10	NS	-1.3	0.38	NS
BGLAP	1.3	0.15	NS	-0.47	0.29	NS
ACVRL1	0.37	0.20	NS	0.24	0.04	GM＜DM-A
TGFBR1	0.81	0.00042	GM＜DM-A	0.62	0.019	GM＜DM-A
COL2A1 to COL1A1	6.2	0.10	NS	10	0.021	GM＜DM-A
ACAN to VCAN	5.3	0.00077	GM＜DM-A	5.4	0.0053	GM＜DM-A
COL1A2 to COL1A1	-0.85	0.035	GM＞DM-A	-1.1	0.032	GM＞DM-A

*1 Average of three strains.

*2 Judged by $p < 0.05$. NS means not significant.

*3 Calculated under the condition where Ct values of non-detected genes are defined as 50.

3.3. Histological analysis

Paraffin sections of a tissue cultured in each condition of Fig. 1 were subjected to H&E staining and Safranin-O/fast-green staining (Fig. 4). Samples in GM group and fibroblast group were negative for Safranin-O as expected, and samples in DM-A, DM-D, and pellet culture group were vividly positive for Safranin-O proving accumulation of glycosaminoglycan in these tissues. However, chondrocytes in DM-A sample presented flatten morphology while chondrocytes in DM-D and pellet culture samples had rather round shapes. In addition, immunohistological analysis revealed different fluorescence patterns for type II collagen between DM-A and DM-D groups (Supplementary Material 3).

3.4. Quantitative polymerase chain reaction (qPCR) analysis

Finally, the expression of cartilage-related genes in GM, DM-A and DM-D groups were quantified by qPCR (Fig. 5, Table 1, and Table 2) to elucidate the phenotypic differences among these groups. In comparison between GM group and DM-A group, the expression of COL2A1 was higher in DM-A group at day 35, and the expression of ACAN was higher in DM-A group at day 22 and day 35 while the expression of VCAN was higher in GM group at day 22 and day 35, confirming the effects of differentiation medium to promote re-differentiation of chondrocytes. In comparison between DM-A (attached) group and DM-D (detached) group, the expressions of COL2A1 and SOX9 were higher in DM-D group at day 22 and day 35, suggesting the detachment and/or shrinkage had positive effect on differentiation of chondrocytes. The expression of RUNX2 was slightly higher in DM-A group.

4. Discussion

It is known that growth factors, serum, and ascorbic acid induce thickness-wise growth of fibroblasts and chondrocytes [9–13]. Among these factors, we examined the effects of TGF-β1 and FGF-2 because they synergistically promote two-dimensional growth of chondrocytes [25–28]. Accordingly, both TGF-β1 and FGF-2 increased cell number and thickness in a concentration-dependent manner. We also confirmed FBS concentration affected the thickness. Thickness-wise growth occurred even in 1% FBS when supplemented with TGF-β and FGF-2, suggesting that high serum content was not essential for this phenomenon (Supplementary Material 2a).

Secondly, we evaluated the time-course of NHAC thickness-wise growth in terms of thickness and cell numbers. In growth medium, NHACs proliferated up to 3×10^6 cells/cm^2 and the thickness increased up to 120 μm within 3 weeks although the proliferation and the increase of thickness slowed down in week 4 and 5. Immunohistochemistry analysis for Ki-67 demonstrated that chondrocytes can proliferate even at the top of the tissue (Supplementary Material 2b). It indicated that cell-dense tissues and/or secreted extra cellular matrix provided a substrate on which cells adhere and proliferate. Therefore, what is limiting the proliferation

Table 2
Paired t-test of gene expression between DM-A group and DM-D group.

Gene	Day 22			Day 35		
	ΔΔCt[*1]	P-value	Result[*2]	ΔΔCt[*1]	P-value	Result[*2]
COL1A1	-1.0	0.026	DM-A＞DM-D	-1.1	0.14	NS
ACAN	0.85	0.085	NS	1.8	0.028	DM-A＜DM-D
COL10A1	0.42	0.10	NS	1.4	0.045	DM-A＜DM-D
SOX9	2.4	0.011	DM-A＜DM-D	3.0	0.0003	DM-A＜DM-D
RUNX2	-1.4	0.024	DM-A＞DM-D	-1.1	0.020	DM-A＞DM-D
COL1A2	-0.052	0.44	NS	1.2	0.11	NS
COL2A1	9.4	0.014	DM-A＜DM-D	9.6	0.0016	DM-A＜DM-D
VCAN	-0.20	0.0015	DM-A＞DM-D	0.71	0.051	NS
SPP1	6.4	0.11	NS	6.5	0.081	NS
BGLAP	0.0023	0.50	NS	-0.83	0.046	DM-A＞DM-D
ACVRL1	-0.73	0.10	NS	-0.23	0.10	NS
TGFBR1	0.53	0.033	DM-A＜DM-D	1.2	0.013	DM-A＜DM-D
COL2A1 to COL1A1	10	0.0089	DM-A＜DM-D	11	0.00031	DM-A＜DM-D
ACAN to VCAN	1.0	0.062	NS	1.1	0.11	NS
COL1A2 to COL1A1	0.97	0.0089	DM-A＜DM-D	2.4	0.011	DM-A＜DM-D

*1 Average of three strains.

*2 Judged by $p < 0.05$. NS means not significant.

and the increase of thickness is to be investigated. In differentiation medium, the proliferation of NHACs was limited while the thickness increased probably because sizes of the cells enlarged through differentiation.

Then, we examined the differentiation capacity of these thickness-wise-grown tissues. MIA secretion increased after the medium change for differentiation medium in DM-A group. MIA has been reported to be a reliable chondrogenic differentiation marker [29]. Therefore, the medium could promote re-differentiation of chondrocytes. MMP-13 secretion was seen in GM group but declined in DM-A group. MMP-13 is secreted in osteoarthritis cartilages [30–32] and used as a marker for hypertrophic differentiation followed by calcification as well as type X collagen [33,34]. However, primary culture chondrocytes derived from normal cartilage also secrete MMP-13 [32], and the expression is strongly stimulated by FGF-2 [35–37]. Therefore, high secretion of MMP-13 in GM group should come from FGF-2 supplementation in growth medium while low secretion of MMP-13 in DM-A group may reflect their imperfect hypertrophic differentiation. In the histological examination, the tissues in DM-A, DM-D and pellet culture groups were positive for Safranin-O staining indicating accumulation of glycosaminoglycan as expected. However, the chondrocytes in DM-A group presented flattened shapes while the chondrocytes in DM-D and pellet culture groups have spherical shapes. In addition, immunohistochemistry analysis for type II collagen demonstrated different fluorescence patterns between DM-A and DM-D groups (Supplementary Material 3). In DM-A group, the cytosol of chondrocytes was slightly positive and inter-cellular space showed less fluorescent intensity. In the samples of DM-D and pellet culture group, the cytosol of chondrocytes was negative and inter-cellular space had strong intensity. Hence, we performed gene expression analysis for cartilage-related genes to elucidate the difference.

The qPCR analysis revealed a gap in the expression of cartilaginous differentiation marker genes between DM-A group and DM-D group according to the histological observation. For example, the ratio of COL2A1/COL1A1 was higher in DM-D group by $\Delta\Delta Ct = 11$ at day 35 (Table 2). This gap should arise due to the change of pericellular micro environment at the detachment from the substrate, including cytoskeletal shrinkage, thickening of tissue, and gel-sandwiched culture. These results suggested that both the differentiation factors in differentiation medium and the structure of the 3D tissues are necessary for accomplishing the differentiation of chondrocytes as observed from the expressions of chondrogenic markers, such as COL2A1 or SOX9. Interestingly, the direct comparisons of two opposing genes indicated that the soluble differentiation factors (GM vs. DM-A) mainly affected ACAN/VCAN ratio while the structure of the tissue (DM-A vs. DM-D) had great impact on COL2A1/COL1A1 ratio. The expression ratios of COL2A1 to COL1A1 and ACAN to VCAN were reported as quantitative differentiation markers for chondrocytes [19–23] although it was also reported that the rises of both ratios do not necessarily coincide [23]. In addition, COL1A2/COL1A1 ratio (ratio of two types of chains forming type I collagen) decreased from GM to DM-A, and increased from DM-A to DM-D. Basically, type I collagen consists of one α2 chain and two α1 chains [38], and the protein synthesis ratio of COL1A2/COL1A1 is regulated to be 1:2 both at the levels of transcription and translation [39–41]. However, it has been reported that the mRNA ratio of COL1A2/COL1A1 was lower in bones of osteoarthritis patients [19] and higher in developing cartilages [42]. Also, type I collagen α1 chain homotrimer was found in fetal tissues, genetic disorders, fibrotic tissues, carcinomas, and fetal and

cancer cell cultures [43]. At present, we cannot judge weather the result is trivial or profound, the ratio might indicate a kind of normality of cartilaginous tissues.

Articular cartilage regeneration by cultured human chondrocytes was first reported in 1994 by Brittberg et al. [44], and thereafter, several methods to effectively transplant functional chondrocytes at cartilage defect sites have been investigated. The technical challenge of these methods converged on the fact that chondrocytes lost their differentiated state in two-dimensional *in vitro* propagation while the expanded chondrocytes could regain their differentiated phenotype partially by soluble differentiation factors and by three-dimensional cultures [45–48]. In the context of production of cell-based medicines, the simpler method is preferred because a complicated procedure requires trained cell culture technicians and causes risk of quality variance including microorganism contamination. In line with this, our method has an advantage in sweeping away the steps of passaging cells and three-dimensional tissue fabrication from the manufacturing process, which may reduce the total cost of manufacturing. Once chondrocytes are isolated from a donor tissue, all you need is medium changes which can be done by a less-trained person or by an automated medium change apparatus. However, if the re-differentiation of the tissue is required for effective repair of the cartilage defects, the detachment of tissues from culture vessels and the following gel-sandwiched culture is rather elaborated process. That is a drawback of the current method. While searching the simplest method exploiting the forerunners' effort, as a by-product, this study has demonstrated that the structure of tissue affects the phenotype of chondrocytes even in three-dimensional cultures, although previous reports have mentioned the difference only for two-dimensional culture vs. three-dimensional culture. In short, what is essential is not only 'dimension' but also 'structure'.

5. Conclusion

In this study, we demonstrated that propagated human articular chondrocytes grew thickness-wise efficiently in the presence of FGF-2 and TGF-β1, forming three-dimensional thick tissues that could be re-differentiated by a differentiation medium in terms of glycosaminoglycan accumulation. However, further differentiation, in terms of expression of chondrogenic marker genes, required detachment and shrinkage. Collectively, the thickness-wise growth technique might prove to be a simple approach for manufacturing cartilaginous 3D tissue grafts for regenerative therapy, and the detachment from the culture surface is a key factor for chondrogenic re-differentiation of the tissues.

Declaration of Competing Interest

An experimental apparatus was provided from Panasonic Corporation (Osaka, Japan).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2019.12.001.

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