



Original Article

The usefulness of the decellularized matrix from three-dimensional regenerative cartilage as a scaffold material

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ABSTRACT

In cartilage tissue engineering, research on materials for three-dimensional (3D) scaffold has attracted attention. Decellularized matrix can be one of the candidates for the scaffold material. In this study, decellularization of regenerated cartilage was carried out and its effectiveness as a scaffold material was examined. Three-dimensionally-cultured cartilage constructs in the differentiation medium containing IGF-1 produced more cartilage matrix than those in the proliferation medium. Detergent-enzymatic method (DEM) could decellularize 3D-cultured cartilage constructs only by 1 cycle without breaking down the structure of the constructs. In vitro, newly-seeded chondrocytes were infiltrated and engrafted into decellularized constructs in the proliferation medium, and newly formed fibers were observed around the surface where newly-seeded cells were attached. Recellularized constructs could mature similarly as those without decellularization in vivo. The decellularized 3D-cultured matrix from regenerative cartilage is expected to be used as a scaffold material in the future.

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1. Introduction

Cartilage tissue localizes in various parts of the whole body such as the trachea, auricle, and ribs, supporting the tissue structure of the body and maintaining function. In the craniofacial region, congenital diseases such as microtia and cleft lip/palate cause dysplasia and deformity of cartilages. Because these diseases affect not only function but also facial appearance, surgeries are performed to reconstruct the cartilages [1,2]. Currently, microtia is often treated with an auricular frame prepared from costal cartilage, while collection of the costal cartilage is quite invasive [3]. Rib cartilages are also used for rhinoplasty, while complications such as

warping and resorption may occur in addition to the donor site morbidity described above [4]. Although iliac bone is sometimes grafted on the back of the nose for the treatment of nose deformity caused by cleft lip/palate, nose augmented by bone is not elastic, and fracture may occur by minor injuries [5]. A method to prepare 3-dimensional (3D) cartilage without quite invasive procedure is desired to overcome these issues.

Research on regenerative medicine and tissue engineering is progressing recently, and it is expected to be applied to regeneration and reconstruction of cartilage. Autologous chondrocyte implantation (ACI) is an attractive method in the field of articular cartilage regeneration. Brittberg et al. [6] proposed a technique to remove a small number of chondrocytes from a non-weightbearing site of a patient, to proliferate the cells in a monolayer culture and to transplant them locally to their injured site, which has been applied clinically since 1987. Thereafter, based on this technique, various methods for culturing and transplantation of chondrocytes have been researched and studied, including ones which utilize scaffold to retain transplanted cells in 3D environment [7–10]. For

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the reconstruction of the nose cartilage, our group developed an implant-type regenerative cartilage. To fabricate the regenerative cartilage, small piece of autologous auricular cartilage is harvested, and enzymatically-digested to harvest chondrocytes. Chondrocytes are then cultured to obtain enough number of cells to be loaded to a 3D scaffold [11].

Chondrocytes have the property of producing cartilage matrix and maintaining the strength of the tissue, which is lost by dedifferentiation of the cells during the monolayer culture [12–14]. It is known that chondrocytes can be redifferentiated, when cultured in a 3D structure [15–17]. Therefore, many studies to keep chondrocytes in a 3D structure have been progressing using several kinds of scaffolding materials. Typical scaffolding materials are gel-like materials such as collagen [10,15] and fibrin [18], and synthetic polymers such as poly-L-lactic acid (PLLA) and poly-lactic-co-glycolic acid (PLGA) [19,20]. The most of the former is a component derived from animals, which can create a microenvironment that imitates an *in vivo* milieu. Among them, atelocollagen is made from a protein extracted from the dermis of cattle. It has a high biocompatibility because it decomposes and removes the region of the telopeptide, which is greatly involved in antigenicity, while it is inferior in mechanical strength. In contrast, although synthetic polymers have mechanical strength and can maintain the stable 3D structure, these have problems to be improved, such as biodegradability, absorption, maintenance of cell engraftability and proliferation ability.

As a therapeutic strategy of regenerative medicine, the use of decellularized living tissue as a scaffold has attracted attention recently [21]. The extracellular matrix prepared by decellularization can be accepted in an ideal state for the living body because the microenvironment of the tissue is reproduced as it is [22,23]. In addition, it has been reported that the extracellular matrix as a scaffold material supports not only the shape maintenance, but also migration, proliferation and differentiation of endothelial cells and progenitor cells, and contributes to development and homeostasis of the regenerative tissue through signal transduction [[24,25]. Therefore, clinical applications of decellularized matrix are progressing in various organs such as heart [26], liver [27], and lung [28].

Methods of decellularization of cartilage tissue have also been studied. Since cartilage tissue has poor absorbability and response to reagents caused by a high proportion of extracellular matrix and has a high-density structure, decellularization processing is not easy [29]. In addition, there are many problems if this method is to be applied widely, such as limited shape and availability of grafts. However, the concept of decellularization is considered to be very effective in cartilage tissue formed with a dense collagen network because of its low immunogenicity.

In order to apply this method clinically, feasibility is greatly increased by decellularizing the cartilage tissue produced *in vitro* and using it. There are few reports of culturing regenerated cartilage and decellularizing *in vitro*. For example, Nie X et al. demonstrated the efficacy of decellularized regenerative cartilage fabricated using porcine chondrocytes for the treatment of chondral defect, while re-cellularization of the decellularized cartilage matrix is not performed [30]. If cultured regenerated cartilage can be decellularized and the extracellular matrix can be taken out, it can be more moldable, safer, purer and more biocompatible cartilage matrix as compared with that of native cartilage. In this study, we fabricated 3D-cultured cartilage constructs and decellularized it, investigated the function of the decellularized cartilage matrix, and examined its effectiveness as a scaffold material.

2. Materials and methods

2.1. Fabrication of 3D cultured constructs of human auricular chondrocytes

All procedures in the present study were approved by the ethics committee of the University of Tokyo Hospital (ethics permission #622 and #2573). Human auricular cartilage was obtained under informed consent from remnant auricular cartilage of microtia patients who underwent surgery at Nagata Microtia and Reconstructive Plastic Surgery Clinic. Isolation and culture of chondrocytes were conducted as previously described [31]. In brief, cartilage were minced into 1 mm³ pieces and digested with 0.3% collagenase (Wako Chemicals Co, Ltd. Tokyo, Japan) in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12) (Sigma Chemical Co. MO, USA) containing penicillin and streptomycin (Sigma Chemical Co. MO, USA) at 37 °C for 18 h. The digested suspension was centrifuged at 440 g for 5 min, and stored at –80 °C. Auricular chondrocytes were passaged once, and P1 cells were collected with trypsin–EDTA solution (Sigma Chemical Co. MO, USA). P1 human auricular chondrocytes were embedded in 0.4% atelocollagen gel (Kawaken Fine Chemicals Co, Ltd Tokyo, Japan) at 1.0×10^6 cells/100 µL and incubated for gelation at 37 °C in 5% CO₂ for 3 h. Three-dimensionally-shaped constructs were taken out from the mold, and cultured in 10 mL of 3 types of media: (1) Basal medium: DMEM/F12 without any additives (2) Differentiation medium: DMEM/F12 supplemented with insulin-like growth factor-1 (IGF-1) (1 µg/mL) (Astellas Pharma Inc. Tokyo, Japan) (3) Proliferation medium: DMEM/F12 supplemented with 5% human serum, fibroblast growth factor-2 (FGF-2) (100 ng/mL) (Kawaken Fine Chemicals Co, Ltd Tokyo, Japan), and insulin (5 µg/mL) (Novo Nordisk Pharma Ltd. Tokyo, Japan). The medium was exchanged twice a week. Three weeks later, the constructs were collected, and analyzed.

2.2. Decellularization of 3D-cultured constructs

3D-cultured constructs were treated according to the detergent-enzymatic method (DEM) [32,33]. The constructs were rinsed twice in distilled water, then incubated in 4% sodium deoxycholate solution (Wako Chemicals Co, Ltd. Tokyo, Japan) diluted in distilled water and shaken for 3 h at room temperature to induce fracture of cell membranes. After 2 wash steps with distilled water, the constructs were incubated in 1000 units DNase-I (Sigma Chemical Co. MO, USA)/mL and shaken for 3 h at room temperature, to solubilize nuclear contents and degrade DNA. After 2 further wash steps with distilled water, the constructs were stored in PBS containing 1% penicillin-streptomycin at 4 °C. This cycle of decellularization protocol were conducted for 1, 3, and 5 times.

2.3. Recellularization of decellularized constructs

Decellularized constructs were prepared as described above (3D-culture in differentiation medium and DEM 1 cycle). We made 1.0×10^7 cell/mL cell suspension with P1 human auricular chondrocytes (different lots from the cells used by fabricating 3D-cultured constructs) and DMEM/F12. One hundred microliter cells in suspension were injected into each decellularized construct by using a 22-gauge injection needle. After injection, the constructs were incubated in the remaining cell suspension for 30 min at room temperature. Recellularized constructs were cultured at 37 °C in 5% CO₂ in 10 mL of 2 types of media (1) basal medium (2) proliferation medium. The medium was exchanged twice a week. We made the non-cell group by injection of DMEM/F12 without chondrocytes.

After 1 week and 3 weeks, cultured constructs were collected, and analyzed.

2.4. Transplantation of recellularized constructs in nude mice

The dynamics of recellularized constructs were analyzed *in vivo*. Recellularized constructs were prepared as described above (3D-culture in differentiation medium, DEM 1 cycle, recellularizing and incubating in proliferation medium for 3 weeks). Six-weeks-old female Balb/c nu/nu mice (Nisseizai, Tokyo, Japan) were anesthetized by inhalation anesthesia of 2% isoflurane (AbbVie GK, Tokyo, Japan). A 10 mm incision was made on the back of the mouse, and the constructs were transplanted subcutaneously. 3D-cultured constructs and decellularized constructs were transplanted similarly. Each construct was independently transplanted into an individual mouse. Four weeks after transplantation, mice were euthanized, and the constructs were excised from the back. Animal experiment protocols were approved by the Institutional Animal Care and Use Committee of the University of Tokyo (#P14-104).

2.5. Histological staining and cell counting

A harvested sample was cut in half: one half underwent biochemical analysis, and the other was fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 5 μm sections for histological analyses. The sections were stained with toluidine blue (TB) to detect proteoglycans, with hematoxylin and eosin (HE) to observe morphology and with DAPI to identify nuclei. We counted cell numbers in a unit area by DAPI staining at 5 different regions of interests, and average of the number was calculated.

2.6. Biochemical analyses

The sample was minced with scissors and dissolved in 10 mg/mL pepsin and 0.05 M acetic acid at 4 °C for 48 h. Then, 1 mg/mL pancreatic elastase and 10 \times TSB were added to each sample, and they were incubated at 4 °C overnight. After centrifuging at 12,000 g for 5 min, the supernatant was subjected to examinations for glycosaminoglycan (GAG) by Alcian blue binding assay (Wieslab AB, Lund, Sweden) and type I and II collagen by ELISA (Chondrex, Redmond, WA, USA) according to the manufacturer's instructions.

2.7. Measurement of mechanical strength

Mechanical strength was measured in terms of Young's modulus with a Vinustron tactile sensor (Axiom, Fukushima, Japan) as previously described [34]. Young's modulus and compressive strength were measured 5 times for each sample on different positions, and data are expressed as mean \pm standard deviation.

2.8. Scanning electron microscopy (SEM)

The 3D-cultured, decellularized and recellularized constructs were immersed in mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 day at 4 °C. For selected SEM specimens were washed in buffer for several times, post-fixed in 1% osmium tetroxide for 3 h at RT. Such specimens were transferred into t-butylalcohol frozen, then sputter-coated with the platinum-palladium. The morphology of tissue-engineered constructs was examined by SEM (JCM-6000plus, JEOL Ltd., Tokyo, Japan).

2.9. Statistical analysis

Statistical evaluation was performed using JMP Pro software version 13 software (SAS Institute Japan Ltd.). Data were expressed as mean \pm standard deviation. Statistical significance was evaluated using the Tukey–Kramer method. A value of $p < 0.05$ was interpreted to denote statistical significance.

3. Results

3.1. Fabrication of 3D-cultured constructs of human auricular chondrocytes

We initially fabricated 3D-cultured constructs of human auricular chondrocytes to obtain 3D cartilage matrix. Macroscopic findings showed a tendency for the 3D-construct cultured with basal medium to contract, while the size and shape were almost unchanged in that with differentiation medium, and slight expansion and swelling were observed in that with proliferation medium (Fig. 1A). As shown in the histological sections, chondrocytes were uniformly distributed throughout the tissue in all the groups (Fig. 1A). In the higher magnification view, in particular, the cartilage lacunas appeared throughout the tissue in the differentiation medium, indicating that chondrocyte redifferentiation was progressing. By toluidine blue staining, metachromasia indicating the cartilage matrix accumulation was hardly observed in the construct cultured with basal medium, while it could be confirmed in those cultured in other 2 conditions. In particular, metachromasia was more obvious in the construct with differentiation medium, suggesting the progression of matrix maturation. To examine the maturation of constructs quantitatively, amounts of matrix proteins were determined (Fig. 1B). As for GAG contents, the construct cultured with differentiation medium showed significantly higher accumulation compared to those in basal medium. No significant difference was found between those in basal and proliferation media, although the amount tended to be higher in the construct with the differentiation medium. The amount of type II collagen protein, which represents a cartilage-specific matrix, was significantly higher in the construct with the differentiation medium compared with the other 2 groups, whereas the amounts of type I collagen protein was not significantly different in the 3 groups. These results showed that 3D-cultured constructs in the differentiation medium produced more cartilage matrix than the other groups in accordance with the results in the previous report from our group [35]. According to these results, we decided to use the differentiation medium to fabricate 3D-cultured constructs.

3.2. Decellularization of 3D-cultured constructs

To obtain 3D-cartilage matrix without cellular components, we decellularized 3D-constructs by DEM. 3D-cultured constructs were fabricated as noted above by using differentiation medium for 3 weeks. By changing the number of DEM cycles, the appropriate condition for the decellularization of 3D-constructs were determined (Fig. 2A). In the macroscopic findings, opacity was lost gradually with the increase of DEM cycles (Fig. 2B). In HE staining, the nuclei were markedly dropped out from the whole tissue in 1 cycle, and lots of empty lacunas were found. Even if DEM cycles were repeated, the structure of the constructs seemed unchanged, although the size of the construct tended to decrease. In DAPI staining, which showed cell nuclei specifically, it was also confirmed that 3D-cultured constructs were almost totally decellularized only by 1 cycle (Fig. 2B and C). In TB staining, apparent loss of metachromasia was not found even after 5 cycles of DEM (Fig. 2B). The effects of DEM on the

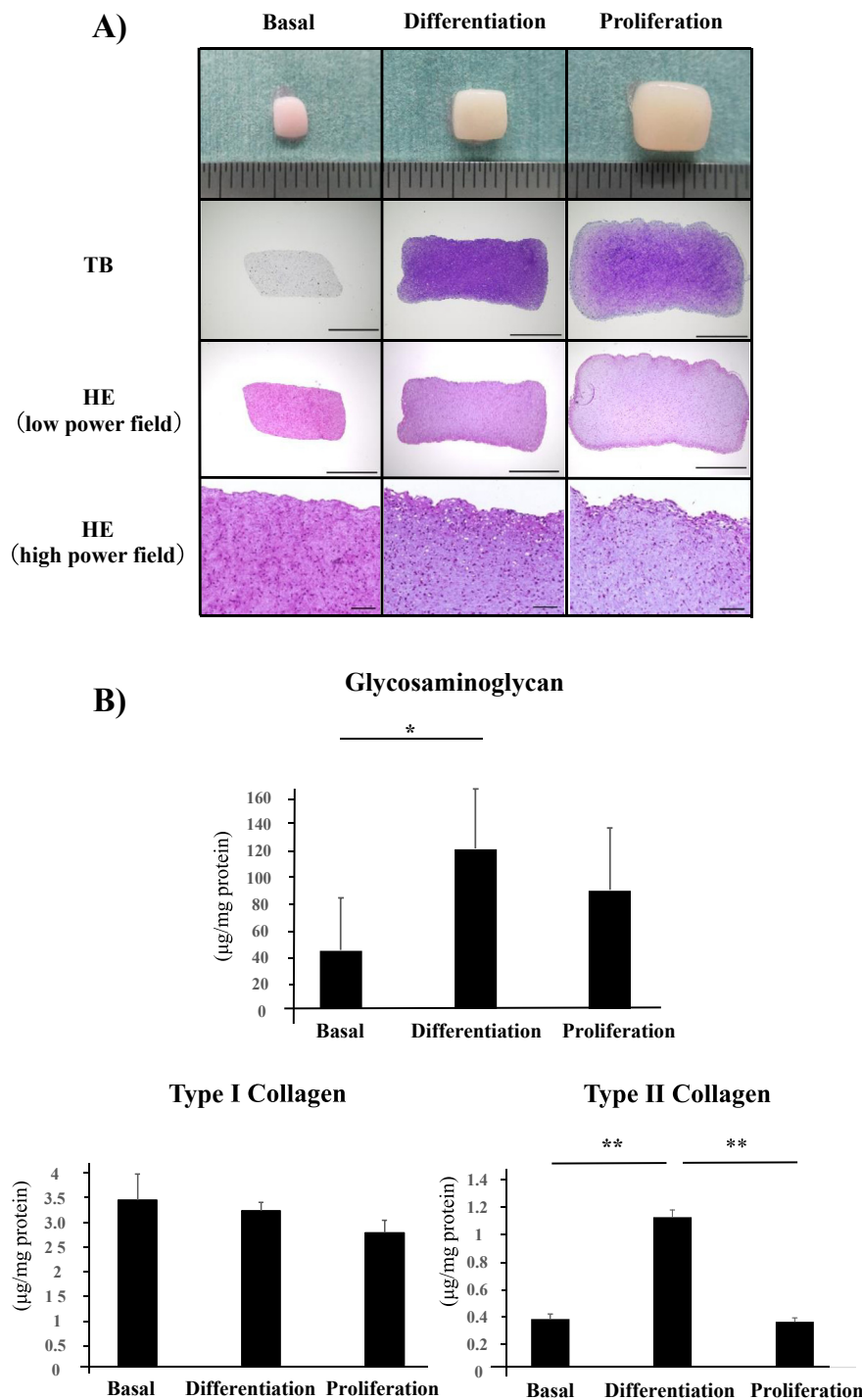


Fig. 1. Morphological, histological and biochemical changes in engineered constructs during 3D culture of human auricular chondrocytes. The constructs were fabricated with P1 human auricular chondrocytes and 0.4% atelocollagen and cultured for 3 weeks in basal, differentiation, or proliferation media. A) Morphological and histological analysis. Upper panels: macroscopic views, TB: toluidine blue staining (scale bars: 1000 µm), HE: hematoxylin and eosin staining, observed in low power fields (scale bars: 1000 µm) and in high power fields (scale bars: 100 µm). B) Biochemical analysis: GAG accumulation was examined by Alcian blue binding assay. Protein synthesis of type I and II collagen were examined by ELISA. Data were expressed as mean (bars) ± standard deviation (error bars) (N = 3) (**p < 0.01, *p < 0.05).

cartilage matrix proteins were also confirmed quantitatively (Fig. 2D). Amounts of total protein, GAG, type I collagen and type II collagen showed no difference among all groups. These results showed that DEM could decellularize 3D-cultured cartilage constructs without breaking down the structure of the constructs, indicating the usefulness of this method for decellularization of 3D-cultured constructs. We decided to use 1 DEM

cycle for decellularization, which was enough to remove almost all the cells from matrix.

3.3. Recellularization of decellularized constructs

To verify the ability of the decellularized constructs for cell engraftment, we tried to recellularize the decellularized constructs.

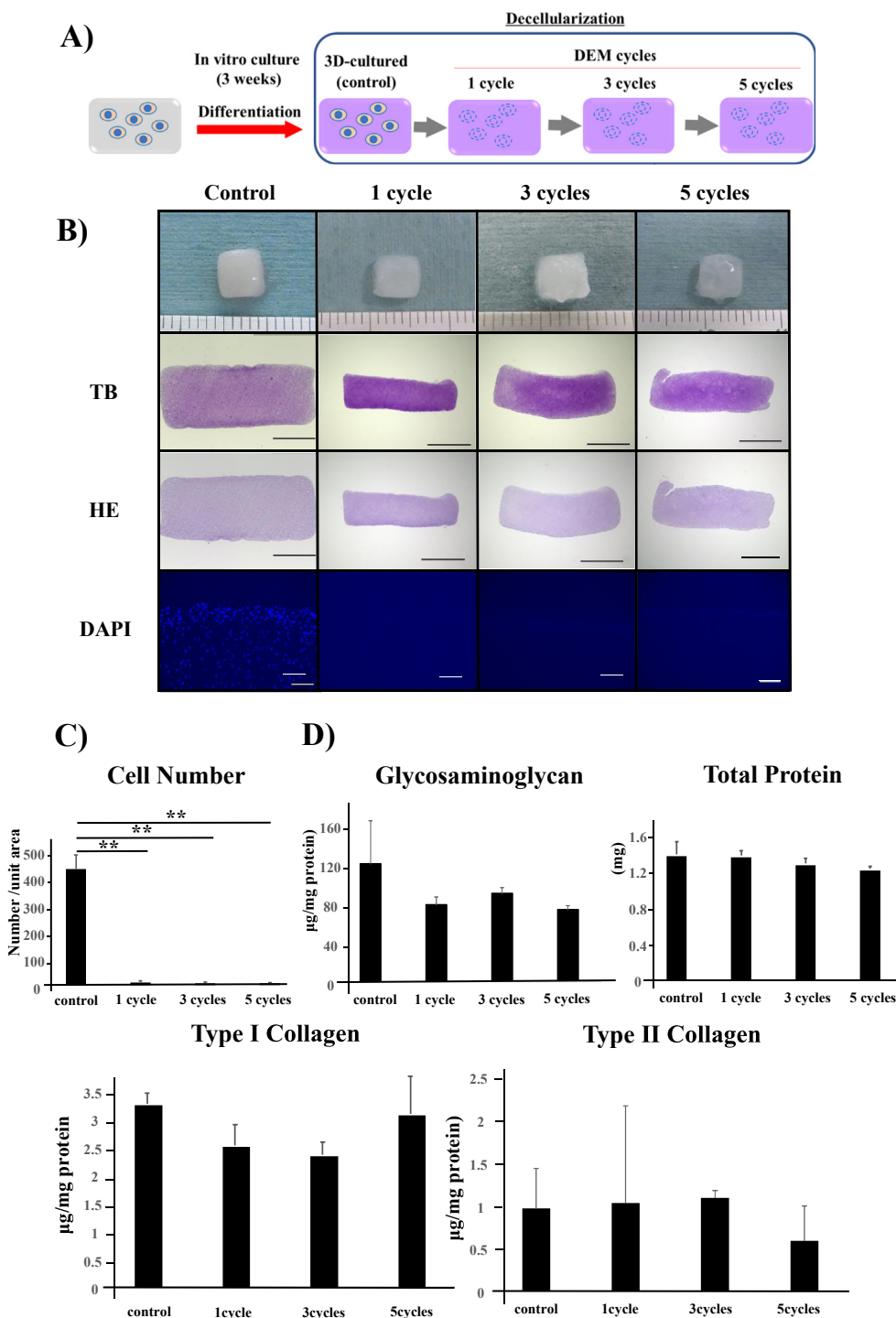


Fig. 2. Morphological, histological, and biochemical changes of 3D cultured constructs of human auricle chondrocytes by decellularization. 3D cultured constructs of human auricle chondrocytes were decellularized by 1, 3, or 5 cycles of DEM. A) A schematic representation of the experiments. B) Morphological and histological analysis. Upper panels: macroscopic views, TB: toluidine blue staining (scale bars: 1000 μm), HE: hematoxylin and eosin staining (scale bars: 1000 μm), DAPI: DAPI staining (scale bars: 100 μm). C) Measurement of cell numbers: Cell numbers were counted for each sample of DAPI staining on 5 different positions. Data were expressed as mean (bars) ± standard deviation (error bars) (**p < 0.01). D) Biochemical analysis: GAG accumulation was examined by Alcian blue binding assay. Protein synthesis of type I and II collagen were examined by ELISA. Data were expressed as mean (bars) ± standard deviation (error bars) (N = 3).

After recellularizing allogenic human auricular chondrocytes, constructs were cultured using basal and proliferation media (Fig. 3A). In a macroscopic view, the shape and the size were not apparently different among control constructs without cells and those with cells in different culture conditions (Fig. 3B). In

histological analysis, cells were rarely found in the basal medium group (Fig. 3D, left). On the other hand, in the proliferation medium group, cells were found around the construct and near the puncture site after 1-week culture (Fig. 3D, upper/right). After 3 weeks, cells were found in the whole area of the construct of the proliferation

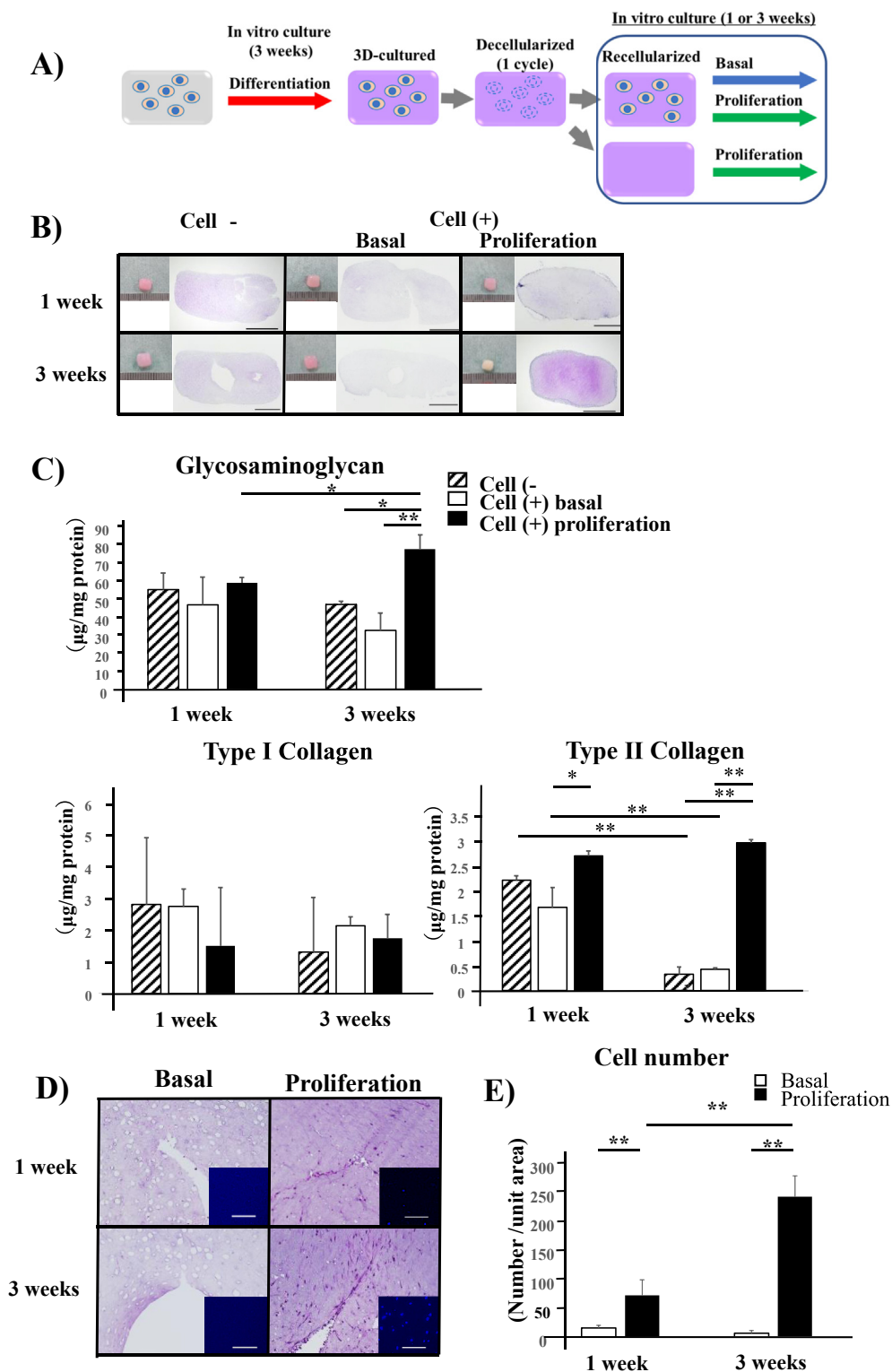


Fig. 3. Recellularization of decellularized constructs and the evaluation of cell engraftment of decellularized-recellularized constructs. Decellularized constructs were recellularized and incubated for 1 or 3 weeks in basal or proliferation media. Non-cellularized constructs were also incubated as controls. A) A schematic representation of the experiments. B) Macroscopic view (left) and histological analysis (right, toluidine blue staining) (scale bars: 1000 μm). C) Biochemical analysis: GAG accumulation was examined by Alcian blue binding assay. Protein synthesis of type I and II collagen were examined by ELISA. Data were expressed as mean (bars) ± standard deviation (error bars) (N = 3) (**p < 0.01, *p < 0.05). D) Histological analysis. Hematoxylin and eosin staining and DAPI staining (lower right) (scale bars: 100 μm). E) Measurement of Cell numbers in the constructs: cell number was counted in a unit area 5 times in different positions for each construct by DAPI staining. Data were expressed as mean (bars) ± standard deviation (error bars) (**p < 0.01).

group (Fig. 3D, lower/right). The cell numbers increased significantly only in the proliferation group (Fig. 3E). These results showed that newly-seeded chondrocytes were infiltrated and engrafted into decellularized constructs by using proliferation medium. Moreover, in TB staining, while metachromasia was vanished in the non-cell group and the basal medium group, it was strongly found in the proliferation group after 3 weeks (Fig. 3B). It suggested that newly-seeded chondrocytes became mature and produced new cartilage matrix. To examine the maturation of cartilage tissue quantitatively, the amounts of matrix proteins were determined (Fig. 3C). After 3 weeks, GAG accumulation rose significantly in the proliferation group, while it tended to decrease gradually in the other groups. Type I collagen accumulation was unchanged in all groups. The protein level of type II collagen was maintained only in the proliferation group, while it decreased in the other 2 groups.

3.4. Analysis of recellularized constructs

To check mechanical strength of the constructs, we measured compressive strength and Young's module (Fig. 4A). The elastic modulus of 3D-cultured construct was significantly higher compared with that of atelocollagen gel. Although the elastic modulus tended to decrease by decellularization, it was still significantly higher compared with that of atelocollagen gel. It suggested that 3D-cultured construct was decellularized without considerably decreasing its mechanical strength. After

recellularizing, the elastic modulus tended to increase. It might be because of new matrix formation by newly-seeded chondrocytes.

To examine the effect of DEM on the microstructures of the matrix, constructs were observed by scanning electron microscopy (Fig. 4B). In 3D-cultured constructs, collagen fibers of several ten to several hundred nm in diameter formed a dense network, in which several cells were found. On the other hand, cells disappeared in decellularized constructs, while collagen structure was kept to some extent. Thick fibers of collagen were more apparent compared to 3D-cultured constructs. In recellularized constructs, a collagen network similar to those of 3D-cultured constructs was observed, and re-inserted cells were also found. In particular, around the surface where newly-seeded cells were attached, networks of thin collagen fibers were observed, which might be newly formed by re-inserted cells.

3.5. Transplantation of recellularized constructs in nude mice

To verify the ability of recellularized constructs to regenerate cartilage in vivo, we transplanted the constructs into the back of nude mice. After 4 weeks, transplanted constructs were harvested and analyzed (Fig. 5A). The volume of decellularized construct was reduced and metachromasia was rarely found in TB staining. On the other hand, metachromasia became more evident in the recellularized construct as same as the 3D-cultured construct (Fig. 5B). Surprisingly, GAG accumulation of the recellularized constructs

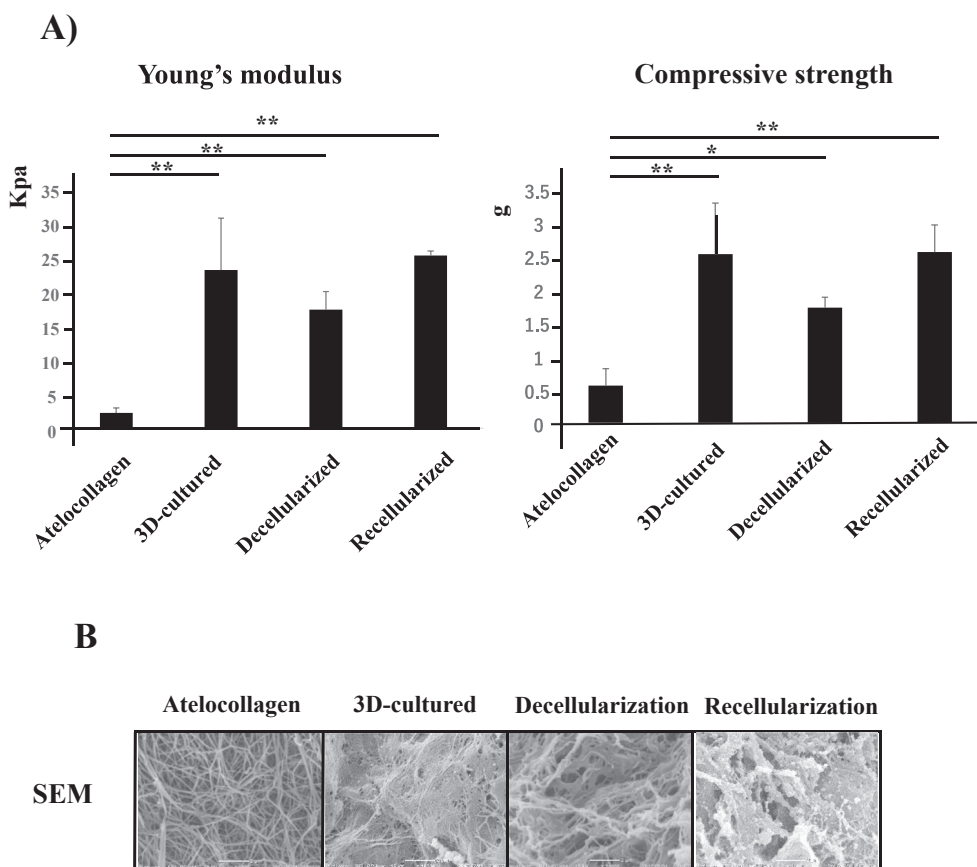


Fig. 4. Mechanical strength and structure of decellularized and recellularized constructs. A) Mechanical strength was measured in terms of Young's modulus with Vinustron tactile sensor (Axiom, Fukushima, Japan). Young's modulus and compressive strength were measured 5 times for each samples on different positions, and data are expressed as mean (bars) ± standard deviation (error bars) (**p < 0.01, *p < 0.05). B) Scanning electron microscopy images of the atelocollagen gel, the 3D-cultured construct, the decellularized construct, and the recellularized construct (scale bars: 2 μm).

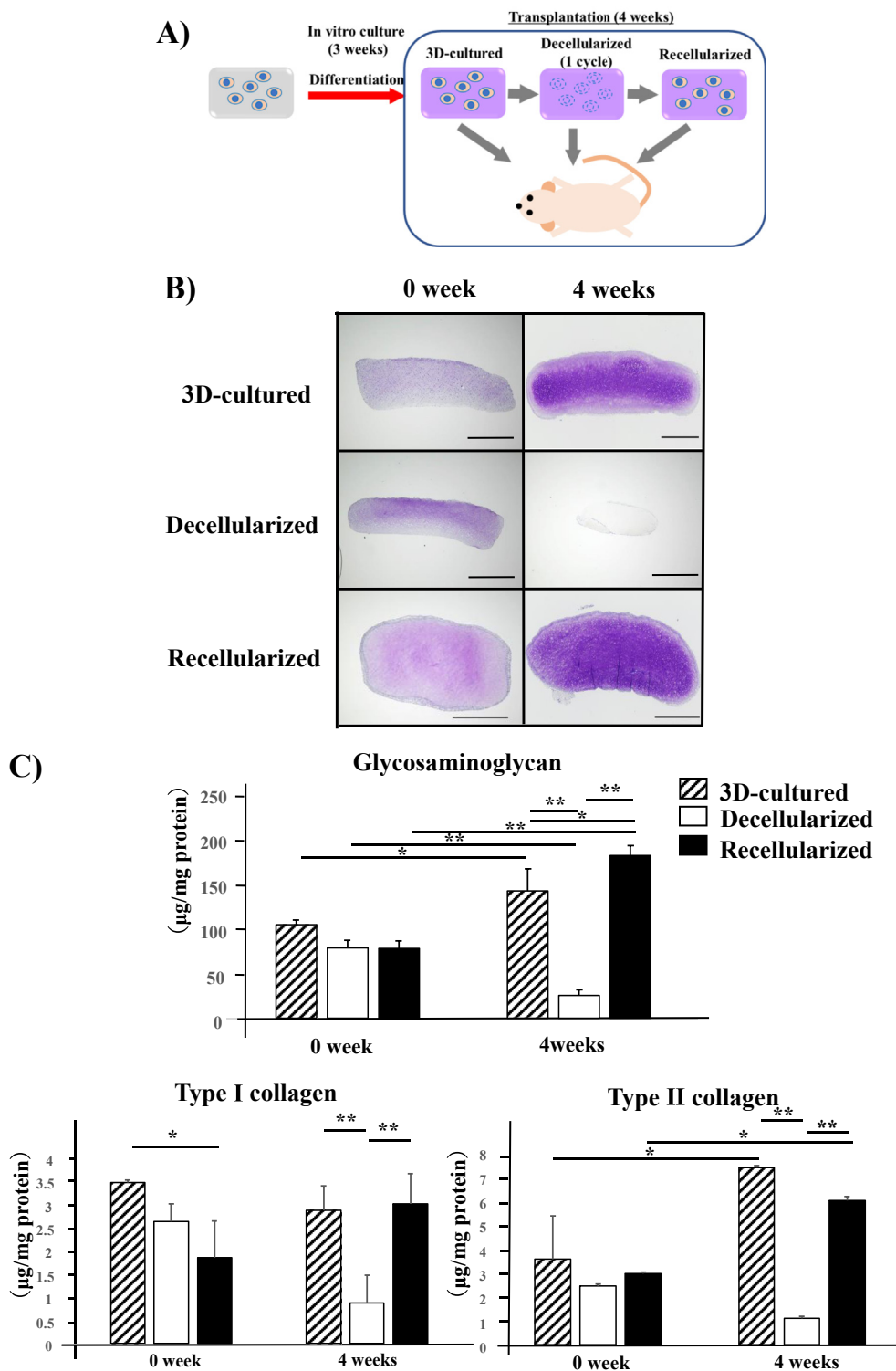


Fig. 5. Transplantation of recellularized constructs in nude mice. Recellularized constructs were transplanted in the back of nude mouse, harvested after 4 weeks, and analyzed together with the constructs at the time of transplantation (0 week). 3D-cultured constructs and decellularized constructs were also transplanted. A) A schematic representation of the experiments. B) Histological analysis (toluidine blue staining, scale bars: 1000 µm). C) Biochemical analysis: GAG accumulation was examined by alcian blue binding assay. Protein synthesis of type I and II collagen were examined by ELISA. Data were expressed as mean (bars) ± standard deviation (error bars) (N = 3) (**p < 0.01, *p < 0.05).

was relatively higher than that of the 3D-cultured constructs (Fig. 5C). While type I collagen accumulation didn't make no difference between transplanted constructs and non-transplanted constructs, type II collagen accumulation increased after 4 weeks.

Type II collagen accumulation was comparable between 3D-cultured construct and recellularized one. Totally, these results showed that recellularized constructs could mature similarly with 3D cultured ones in vivo.

4. Discussion

Cartilage has a very high proportion of extracellular matrix in tissue contents compared with other organs, accounting for over 90% [34]. Therefore, the role of cartilage matrix is considered to be very important. Cartilage matrix does not only give strength to tissue and maintain its shape, but also contributes to cell migration, proliferation and differentiation [24]. In this study, we tried to use the decellularized regenerative cartilage formed by allogenic cells as an ideal scaffold for regenerative cartilage. As a result, the decellularized cartilage showed a comparable ability to form cartilage tissue compared to the original regenerative cartilage, suggesting the possibility for this method to be applied clinically. However, the size and the shape of constructs changed during the culture, the decellularization process, and *in vivo* maturation. The applicability of this method in combination with other scaffolding materials such as PLLA for the retention of the size and shape should be examined in future studies.

In addition, the decellularized regenerative cartilage can be also useful for the basic research of cartilage. Cartilage is a tissue without blood vessels and nerves, and it is considered to be an immunoprivileged tissue [36]. There are some reports which indicate the role of chondrocytes to protect cartilage tissues from immunoreaction. Adkisson et al. [37] showed that neocartilage-derived chondrocytes constantly suppressed the expression of cell surface molecules that induce T cell immune responses without stimulating allogenic T cells. Fujihara et al. [38] reported that macrophage migration inhibitory (MIF) was expressed in regenerated chondrocytes. On the other hand, the role of extracellular matrix in the modulation of immunoreaction has not been studied intensively. It is suggested that cartilage matrix contributes to immune privilege because of its physical characteristics [39]. By using decellularized regenerated cartilage, we can examine the specific roles of cells and the matrix in suppression of the immunoreaction to the cartilage tissue separately.

In this study, we could produce 3D-cultured regenerated cartilage constructs rich in cartilage matrix by using differentiation induction medium with IGF-1. Although it is a differentiation inducer at low concentrations, IGF-1 is known to act as a cell growth factor when its concentration increases [40–42]. We added IGF-1 in a concentration of 1 $\mu\text{g}/\text{mL}$ to induce differentiation of chondrocytes. Various studies have been made on the selection of culture medium for *in vitro* culture. Moretti et al. [43] compared the effects of a differentiation induction medium using TGF- β 1, insulin, ascorbic acid and a cell growth medium using TGF- β 1, FGF-2 and PDGF in 3D culture of chondrocytes. The result showed preculture of 3D constructs in differentiating medium, but not in proliferating medium, supported enhanced *in vivo* development of engineered cartilage. On the other hand, our group has previously compared the effect of pre-culture with differentiation medium and proliferation medium, which were identical to those in the present study, on the cartilage regeneration after transplantation [35]. As a result, the cartilage construct pre-cultured in proliferation medium showed more prominent chondrogenesis *in vivo* which is attributable to the better cell viability, although the differentiation medium induced cartilage maturation *in vitro* better compared to the proliferation medium. Taking these findings into account, in the present study, we chose the differentiation medium for the preparation of scaffold, and used the proliferation medium for the culture after recellularization.

Regarding the method of decellularization, many kinds of methods are examined for various organs [22]. Since cartilage has a dense matrix structure compared to other organs, decellularization is difficult due to poor absorbability [29]. On the other hand, the regenerated cartilage tissue can drop most of the cells from the

tissue at 1 cycle. A possible explanation for the difference in the cycle numbers is that the regenerated cartilage matrix is rough and more absorptive than the cartilage tissue of the living body and that the reaction to deoxycholic acid and DNase-I was carried out efficiently. As problems of decellularization treatment in cartilage tissue, decrease in the amount of GAG and decrease in elastic modulus have been reported [44,45]. On the contrary, our study showed that the decrease in the amount of GAG and elastic modulus by DEM cycle were not substantial. The DEM method is an appropriate method for the decellularization of regenerative cartilage in the sense that decellularization was carried out while maintaining cartilage matrix as much as possible. Although decellularization could be achieved at 1 cycle, there is still a room for consideration such as solution concentration and shaking time to minimize the treatment.

When chondrocytes were newly seeded on the decellularized cartilage matrix and cultured in the proliferation medium, chondrocytes adhered to the surface, gradually invaded into the interior, and proliferated and differentiated, although matrix and chondrocytes were allogenic. There are few studies in which regenerated cartilage is used as a scaffold, chondrocytes are sowed and maturation of the cartilage is observed. Studies have been made to investigate the degree of cell engraftment as a scaffolding material as the size of pores of synthetic fibers are changed [46]. Griffon et al. [47] showed that cell engraftment was actively performed with synthetic polymers with pore sizes of 75–120 μm , becoming less likely to adhere with smaller pores. The SEM images in our study showed that in the decellularized regenerated cartilage, the sizes of the pores were mixed, and a considerably dense collagen network formation was observed. Although it is a denser fiber network compared with synthetic polymers, a favorable course of cell engraftment was obtained.

In our study, decellularized cartilage was stored in PBS and used for reinsertion after washing. For a long-term preservation, it is necessary to consider the risk of infection and hydrolysis. In addition, since the cell suspension was seeded on wet constructs, cells might be lost with the flowing out of the solution. It is also problematic that the number of cells to be seeded on the constructs was not specifically determined. To solve these problems, we are investigating ways to preserve and use decellularized cartilage by freeze-drying. Because the freeze-dried construct will absorb the cell suspension efficiently, loss of cells will be reduced and requirement of cell number must be clarified. The degree of proliferation can be determined if the number of cells seeded can be clarified, which is important for the determination of the effectiveness of the decellularized cartilage as a scaffold material.

If the effectiveness of allogenic decellularized cartilage as a scaffold material is further demonstrated, it is possible to reduce the *in vitro* pre-cultivation period by banking of the decellularizing allogenic regenerative cartilages. As cell sources, not only allogenic chondrocytes but also mesenchymal stem cells or induced pluripotent stem cells (iPS cells) can be applied. In particular, iPS cells can be promising because they can proliferate infinitely and plenty of regenerative cartilage can be fabricated. Although the clinical applications of iPS cells are ongoing in several areas, the risk of tumorigenesis may hinder them. By decellularizing the regenerative cartilage formed by iPS cells, safe application of the cells with great potential can be realized.

In summary, we presented the comparative ability of the decellularized allogenic regenerative cartilage to reproduce cartilage tissues. This will be applicable not only clinically but also for the basic research of cartilage. Once the effectiveness of the allogenic use of the decellularized cartilage can be established, regenerative medicine of cartilage will progress greatly by the

banking of decellularized cartilage and application of iPSC cells as a cell source for the fabrication of them.

5. Conclusion

We succeeded in production of decellularized cartilage matrix from 3D-cultured regenerated cartilage by detergent enzyme method without breaking down the structure.

The decellularized construct was able to infiltrate and engraft allogenic chondrocytes, and the recellularized construct could mature both *in vitro* and *in vivo*. The decellularized 3D-cultured matrix from regenerated chondrocytes is expected to be used as a scaffold material in the future.

Declaration of competing interest

Atsuhiko Hikita and Yukiyo Asawa: Affiliation with an endowed chair from FUJISOFT INCORPORATED.

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