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ORIGINAL ARTICLE

TERM

Disc-Type Hyaline Cartilage Reconstruction Using 3D-Cell Sheet Culture of Human Bone Marrow Stromal Cells and Human Costal Chondrocytes and Maintenance of Its Shape and Phenotype after Transplantation

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In this study, we developed the disc-type bio-cartilage reconstruction strategies for transplantable hyaline cartilage for reconstructive surgery using 3D-cell sheet culture of human bone marrow stromal cells and human costal chondrocytes. We compared chondrogenesis efficiency between different chondrogenic-induction methods such as micromass culture, pellet culture, and 3D-cell sheet culture. Among them, the 3D-cell sheet culture resulted in the best chondrogenesis with the disc-type bio-cartilage (>12 mm diameter in size) *in vitro*, but sometimes spontaneous curling and contraction of 3D-cell sheet culture resulted in the formation of bead-type cartilage, which was prevented by type I collagen coating or by culturing on amniotic membrane. Previously, it was reported that tissue-engineered cartilage reconstructed *in vitro* does not maintain its cartilage phenotype after transplantation but tends to transform to other tissue type such as bone or connective tissue. However, the disc-type bio-cartilage of 3D-cell sheet culture maintained its hyaline cartilage phenotype even after exposure to the osteogenic-induction condition *in vitro* for 3 weeks or after the transplantation for 4 weeks in mouse subcutaneous. Collectively, the disc-type bio-cartilage with 12 mm diameter can be reproducibly reconstructed by the 3D-cell sheet culture, whose hyaline cartilage phenotype and shape can be maintained under the osteogenic-induction condition as well as after the transplantation. This disc-type bio-cartilage can be proposed for the application to reconstructive surgery and repair of disc-type cartilage such as mandibular cartilage and digits.

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Key Words: Costal chondrocyte; Bone marrow stromal cell; Cartilage; Chondrogenesis

INTRODUCTION

Hyaline cartilage is most widespread cartilage type mainly found in articular surface of all long bones trachea, nose, rib tips, and digits. Majority of hyaline cartilage is composed of water-entrapped extracellular matrix (ECM) such as type II collagen fibers and aggrecan which is proteoglycan associated with a variety of glycosaminoglycans (GAGs) such as chondroitin sulfate (CS), keratan sulfate, and hyaluronic acid [1-3]. These ECM constitutes over than 90% weight of cartilage along with entrapped water, provides compressive strength and strain strength to the cartilage tissue, and also defines shapes of each cartilage tissue. Since cartilage is a fully differentiated and avascular tissue, which can hardly be recovered by endogenous tissue repair [4,5], a variety of surgical methods such as osteochondral graft and microfracture [6,7] and therapeutics such as autologous chondrocytes, bone marrow mesenchymal stem cells, and cord blood mesenchymal stem cells have been developed to repair the cartilage defects by sports accidents and age-related defects in cartilages [4,8]. However, these therapeutics cannot meet the need of articular cartilage due to incomplete hyaline cartilage formation, limited mechanical property, and functional deficiency in the long term [9]. Therefore, new tissue engineered bio-cartilage satisfying the mechanical and structural property of naïve hyaline cartilage has to be developed for better outcome after transplantation. Furthermore, demand of small pieces of hyaline cartilage has been increased for plastic surgery such as rhinoplasty.

For tissue engineering of hyaline bio-cartilage, autologous chondrocytes of articular cartilage or costal cartilage, and bone marrow stromal cells (BMSCs) are most commonly used [8,

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10-12]. Among them, articular cartilage may not be available as a donor tissue in the aged osteoarthritis patients but costal cartilage, one of large hyaline cartilage in our body with well reserved proliferating chondrocytes even in the aged, may be served as an alternative donor tissue for hyaline cartilage tissue engineering, whose feasibility was previously reported [11]. Alternatively, BMSCs, easily harvested by bone marrow aspiration and expanded, are possible candidates for tissue engineered hyaline bio-cartilage. However, still differentiation control to hyaline cartilage is difficult but BMSCs tend to form fibrocartilage [13]. Furthermore, BMSCs, with multipotent differentiation capability to osteoblast, adipocytes, stromal cells, and chondrocytes, did not seem to maintain their original differentiated phenotype but tended to be transformed to other type of differentiation after transplantation *in vivo*. Thus, maintenance of tissue property as well as its original shape of tissue engineered bio-cartilage under a variety of different *in vivo* microenvironment is very important task to be solved for successful tissue engineered products.

A variety of chondrogenesis strategies with combinations of growth factors [14-16], hypoxic environment [17-19], pellet culture with high density cell seeding, transwell culture and bioprocessed membraneous scaffold [20-22], have been attempted to reconstruct bio-cartilage similar to naïve hyaline cartilage. High cell density pellet culture in defined serum free media supplemented with transforming growth factor (TGF) beta is used generally for *in vitro* chondrogenesis [14,23]. However, this bead-type bio-cartilage obtained by the pellet culture has several problems such as apoptosis, necrosis, and poor extracellular matrix (ECM) formation in the central part and different structural architecture from naïve cartilage in the body unless merging and integration of bead-type bio-cartilage to the sheet form *in vivo* is not invented. In addition, bio-cartilage which was constructed by *in vitro* chondrogenesis, was lost their cartilage character by occurring calcification after *in vivo* transplantation [24]. Especially, Murdoch et al. [22] reported that transwell culture is able to overcome pellet culture problems. However, transwell culture has a limitation to create a cartilage more than 6 mm diameter due to curling and contraction of bio-cartilage sheet. In this study, we compare chondrogenesis efficiency of a variety of chondrogenesis methods using dedifferentiated human costal chondrocytes (deHCCs) as well as human BMSCs (hBMSCs) and also estimated different coating methods, different oxygen tension, as well as different bio-processed native scaffolds such as amniotic membrane, small intestine submucosa (SIS) for the reconstruction of consistent disc-type bio-cartilage *in vitro*. Then, we also estimated whether this disc-type bio-cartilage reconstructed *in vitro* can maintain its cartilage phenotype under the osteogenic environment and after subcutaneous transplantation *in vivo* or not. The disc-type bio-cartilage larger than 12 mm diameter reconstructed by 3D-cell sheet culture will be applicable for plastic surgery and cartilage repair with similar size requirement.

MATERIALS AND METHODS

Cell harvest and expansion

Human costal cartilage was obtained from 7 donors (20s–70s years) with written agreement of patients to use the remnant of costal cartilages for research purpose (SMC201501097-HE003). The isolations of human costal chondrocytes (hCCs) were performed by mincing cartilage into 1–2 mm^3 pieces, digesting with an enzyme cocktail solution including collagenase D (2 mg/mL), hyaluronidase (1 mg/mL), and DNase (0.75 mg/mL) (all from Roche Diagnostics, Mannheim, Germany) at 37°C and 5% CO_2 overnight, and filtering through 40 μm nylon mesh. The isolated cells were washed twice with mesenchymal stem cell growth medium (MSCGM) (Lonza, Basel, Switzerland) and plated at a density of 5×10^5 cells/100 mm culture dish in MSCGM supplemented with 1 ng/mL basic-FGF (bFGF). In order to reach more than 1×10^5 folds cell expansion and dedifferentiation of hCCs, the hCCs were sub-cultured in MSCGM supplemented with 1 ng/mL bFGF up to passage 6, when all the hCCs were fully dedifferentiated as featured by complete loss of type II collagen expression and regain of type I collagen expression and other MSC-related markers such as CD44 (Supplementary Fig. 1 in the online-only Data Supplement). HBMSCs were obtained from St. Peter's Hospital (5 donors, Seoul, Korea) with written agreement of patients to use the remnant of hBMSCs for research purpose (KPH IRB 2009-003). Mononuclear cell fraction was isolated from the bone marrow aspirate by Ficoll-gradient centrifugation. hBMSCs were selectively cultured by removing one hour attached cells and unattached cells at 1 day culture. hBMSCs colonies were confirmed at 10 days and replated at a density of 2×10^5 cells/100 mm culture dish in MSCGM supplemented with 1 ng/mL bFGF. hBMSC at passage 4–6 were used for this study. In all cases, the medium was changed every 2–3 days.

Comparison of chondrogenic differentiation

Dedifferentiated hCCs at passage 4–8 were used to test chondrogenesis efficiency. For micromass culture, drops of 1×10^5 cells/10 μL were seeded in 100 mm cell culture dish. After 3 hours incubation, 10 mL of culture medium was added. For pellet culture, aliquots of 2.5×10^5 cells were spun down in 15 mL tubes (1 mL of cell culture medium, 1500 RPM, 5 minutes). For 3D-cell sheet culture, aliquots of 1×10^6 cells/200 μL were seeded on 12 mm diameter, 0.4 μm pore size polycarbonate tran-

swell membrane (Merck Millipore, Darmstadt, Germany) and spun in 12 well culture plate (1500 RPM, 5 minutes). Cell culture medium (1 mL) was added to 12 well culture plate. Confluent monolayer culture with 5×10^6 cells/10 mL were seeded in 100 mm cell culture dish as control. After 1 day culture in MSCGM medium supplemented with 1 ng/mL bFGF, all the cultures received chondrogenic induction medium consisting of high glucose DMEM containing 110 μg/mL sodium pyruvate (Gibco Life Technologies, Grand Island, NY, USA), 10 ng/mL TGF-β3 (Peprotech, Rocky Hill, NJ, USA), 100 nM dexamethasone, 1X ITS+3, 0.35 mM proline, and 0.3 mM ascorbic acid (all from Sigma-Aldrich, St. Louis, MO, USA) and the medium was changed every 2–3 days. To estimate ECM coating methods to prevent the curling of the cell sheet, the transwell membrane were coated with 37.5 μg/50 μL type I collagen (Celtrix Laboratories, Palo Alto, CA, USA), 37.5 μg/50 μL CS, or 37.5 μg/50 μL chitosan (Sigma-Aldrich, St. Louis, MO, USA) and dried in biosafety cabinet overnight.

3D-cell sheet culture using bio-membrane

Amniotic membrane was obtained from Bioland Ltd. (Cheonan, Korea) and SIS membrane was obtained from Biomedical Engineering Lab (Chonbuk National University, Jeonju, Korea) [25,26]. SIS membrane was made as one layer on 3M paper, freeze-dried, sterilized in 70% ethanol, and dried before use. Bio-membrane was cut to the proper size for experiment and fixed using CellCrown™ (Scaffdax, Tampere, Finland). To compare chondrogenesis, aliquots of 1×10^6 cells/200 μL were seeded on bio-membrane and spun in 12-well culture plate (1500 RPM, 5 minutes). The rest of procedure was followed as described above.

Osteogenic differentiation on bio-cartilage

In order to test the phenotypic stability of bio-cartilage, the 3D cell sheet culture after 4 weeks chondrogenic induction, was incubated with osteogenic induction medium consisting of high alpha-MEM containing 110 μg/mL sodium pyruvate (Gibco Life Technologies, Grand Island, NY, USA), 20% fetal bovine serum (Gibco Life Technologies, Grand Island, NY, USA), 10 nM dexamethasone, 10 mM β-glycerophosphate, and 0.2 mM ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) and their osteogenic transformation was estimated by Alizarin red-S staining for calcium deposition and their chondrogenic maintenance was estimated by Safranin-O staining.

In vivo transplantation of disc-type bio-cartilage made by 3D-cell sheet culture

In order to estimate optimal culture period for transplantable bio-cartilage, the 3D-cell sheet culture was performed either to 10 days or 28 days in the chondrogenic-induction medium on transwell culture and then was subcutaneously transplanted on back skin of 7–10 weeks old female nude mice (Jung-Ang Lab. Animal, Seoul, Korea). The transplanted bio-cartilage was harvested for histological examination at four weeks after transplantation and fixed with 3.7% formaldehyde (Sigma-Aldrich, St. Louis, MO, USA). All the animal experiments were approved by the institutional animal use committee of Kyung Hee University with the approval number KHU-ASP(SU)-12-07. In order to estimate whether the stacking of the disc-type bio-cartilage can be integrated into one *in vivo*, three disc-type bio-cartilages were stacked and subcutaneously implanted on the back of nude mice.

Blyscan® GAG assay

The GAG content was measured using the Blyscan® (Bicolor, Northern Ireland, UK) 1,9-Dimethylmethylene Blue assay [27]. Aliquot of a 300 μL culture supernatant was analyzed according to the manufacturer's recommendations and calibrated using a standard curve constructed by using 0, 0.5, 1, 2, 3, 4, 5, 7.5, and 10 μg of shark CS (Sigma-Aldrich, St. Louis, MO, USA).

Histology and immunohistochemistry

Bio-cartilages, made by 3D-cell sheet culture or pellet culture, were fixed in 3.7% formaldehyde (Sigma-Aldrich, St. Louis, MO, USA). Bio-cartilages were processed and embedded in paraffin, sectioned (4-μm), and stained with Safranin-O/Fast Green for GAG expression and Alizarin red-S staining for calcium deposition. Histological quality for cartilage induction was evaluated using Bern Score [28]. Sections for immunohistochemistry were treated with pepsin at 37°C for 10 minutes and the goat anti-human type I collagen primary antibody (SouthernBiotech, Birmingham, AL, USA) or mouse anti-human type II collagen primary antibody (Merck Millipore, Darmstadt, Germany), diluted 1:100 in PBS were incubated for 60 minutes. Secondary HRP-conjugated goat anti-mouse antibody or rabbit anti-goat antibody (all from Bio-Rad, Hercules, CA, USA) were applied to catalyze chromogen development in 3,3'-diaminobenzidine (Vector, Burlingame, CA, USA). Sections were counter stained with Nuclear Fast Red (Vector). Images were collected using an Olympus BX41 (Olympus, Tokyo, Japan).

Statistical analysis

Experimental data are presented as mean±standard deviations. Statistical analysis was performed with the software GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). Differences between groups were determined using one-way ANOVA and t-test. p values<0.05 were considered statistically significant.

RESULTS

Comparison of chondrogenesis efficiency between different chondrogenesis methods

Chondrogenesis *in vitro* is induced under high cell density culture such as micromass culture and pellet culture. However, this pellet-like sphere retains different cartilage architecture comparing to naïve cartilage tissue in our body. Furthermore, chondrogenic-induction on the surface of the pellet, more accessible to chondrogenic-induction medium, results in heavy deposition of ECM on the surface, which then inhibits efficient permeation of nutrients and growth factors to the cells in central part of the pellets. If both sides of chondrocyte sheet are exposed to the culture media, more efficient permeation as well as layered cartilage may be provided. For this purpose, mesenchymal stem cells or chondrocyte precursors may be cultured on the polycarbonates surface of Transwell or Millicell, an insert of 12 well plates at very high cell density.

In this study, we compared chondrogenesis efficiency of different chondrogenic-induction methods based on their GAG production (Fig. 1A and B). For confluent monolayer culture, deHCCs were seeded at a density of 5×10^6 cells/100 mm dish, for micromass culture, deHCCs were inoculated at a density of 1×10^5 cells/10 μL on 100 mm cell culture dish, for pellet culture, deHCCs at a density of 2.5×10^5 cells/mL were spun down to 15 mL concave tube. In order to compare chondrogenesis efficiency, all cultures received the chondrogenic-induction media and chondrogenesis efficiency was compared at the actual amount of GAG at 1 week and 4 weeks of the same culture method using Blyscan® GAG assay. Monolayer culture and micromass culture did increase the GAG production only slightly even after 4 weeks culture under the chondrogenic-induction media. However, the total GAG contents of the pellet culture increased approximately 20-fold at 4 weeks comparing to that of the 1 week culture.

Next, we compared the chondrogenesis-efficiency between the pellet culture and the 3D-cell sheet culture in Transwell. For efficient high density cell loading on the polycarbonate surface of Transwell, the deHCCs was loaded at a density of 1×10^6 cells/12 mm Transwell and centrifuged in the 12 well plate, which allow homogenous and compact cell sheet on the bottom of Transwell (Fig. 1C). After 4 weeks induction of chondrogenesis of the 3D deHCC-cell sheet culture in Transwell, the disc-type bio-cartilage was formed in contrast to the bead-type bio-cartilage formation from the pellet culture (Fig. 1D). In the cross sections of the bead-type and disc-type cartilages, Safranin-O staining and immunohistochemical staining were performed (Fig. 1D). Uniformly dense deposition of GAG as well as type II collagen throughout the disc-type bio-cartilage was prominent. In contrast, deposition of GAG and type II collagen was detected only at the outer shell of the bead-type bio-cartilage and chondrogenesis was not efficiently proceed in the central part of the bead-type bio-cartilage. Comparing the Safranin-O positive area of both bio-cartilages (Fig. 1E), the disc-type bio-cartilage showed approximately 74% GAG positive area but the bead-type bio-cartilage showed only 31% GAG positive area. This result suggests that the 3D-cell sheet culture in Transwell or its equivalent may be more efficient in chondrogenesis and provide more homogenous and permeable condition for chondrogenesis.

Type I collagen coating inhibits spontaneous curling and contraction of the chondrocyte-sheet during 3D-cell sheet culture of human BMSC

The 3D-cell sheet culture in Transwell is an efficient culture method for homogenous disc-type bio-cartilage. During the chondrogenesis using hBMSCs, dense deposition of ECM sometimes caused the curling up of the whole sheet, which negatively affect the integrity of the disc-type bio-cartilage and also GAG deposition (Fig. 2A). In order to prevent the curling phenomena of the hBMSC cell sheet and induce firm attachment of the cells to the polycarbonate surface of Transwell, we investigated different ECM coating methods (Fig. 2B). Type I collagen as one of sub-chondral bone ECM, CS as one of cartilaginous ECM, and chitosan as a similar chemical nature to GAG were coated before high density cell seeding. Only type I collagen coating allowed the firm attachment of the whole sheet of hBMSCs during the 4 weeks chondrogenesis in the Transwell. However, cartilage ECM such as CS or chitosan rather enhanced the curling of the whole sheet. The disc-type bio-cartilage on the type I collagen-coated surface maintained its original surface area of 12 mm insert size, which was consistently produced as an integrated uniform disc-type bio-cartilage. The disc-type bio-cartilage was mechanically durable and strong enough to be hold on by the forceps and for the suture (Fig. 2B). Histological analysis by Safranin-O staining and immunohistochemical staining showed no difference in GAG and type I collagen production with different membrane coating materials. Especially, type I collagen coating did not induce type I collagen expression and did not affect chondrogenesis. However, the difference in the type II collagen expression between membrane coating materials was found. The disc-type bio-cartilage in the type I collagen coated one showed uniform expression of type II collagen throughout the bio-cartilage, whereas CS and chitosan coated ones in the curled sphere-like morphology expressed type II collagen outer shell of the bio-cartilage (Fig. 2B). Therefore, thin film coating of type I collagen on the surface of the 3D-cell sheet culture insert of hBMSCs inhibits the cell

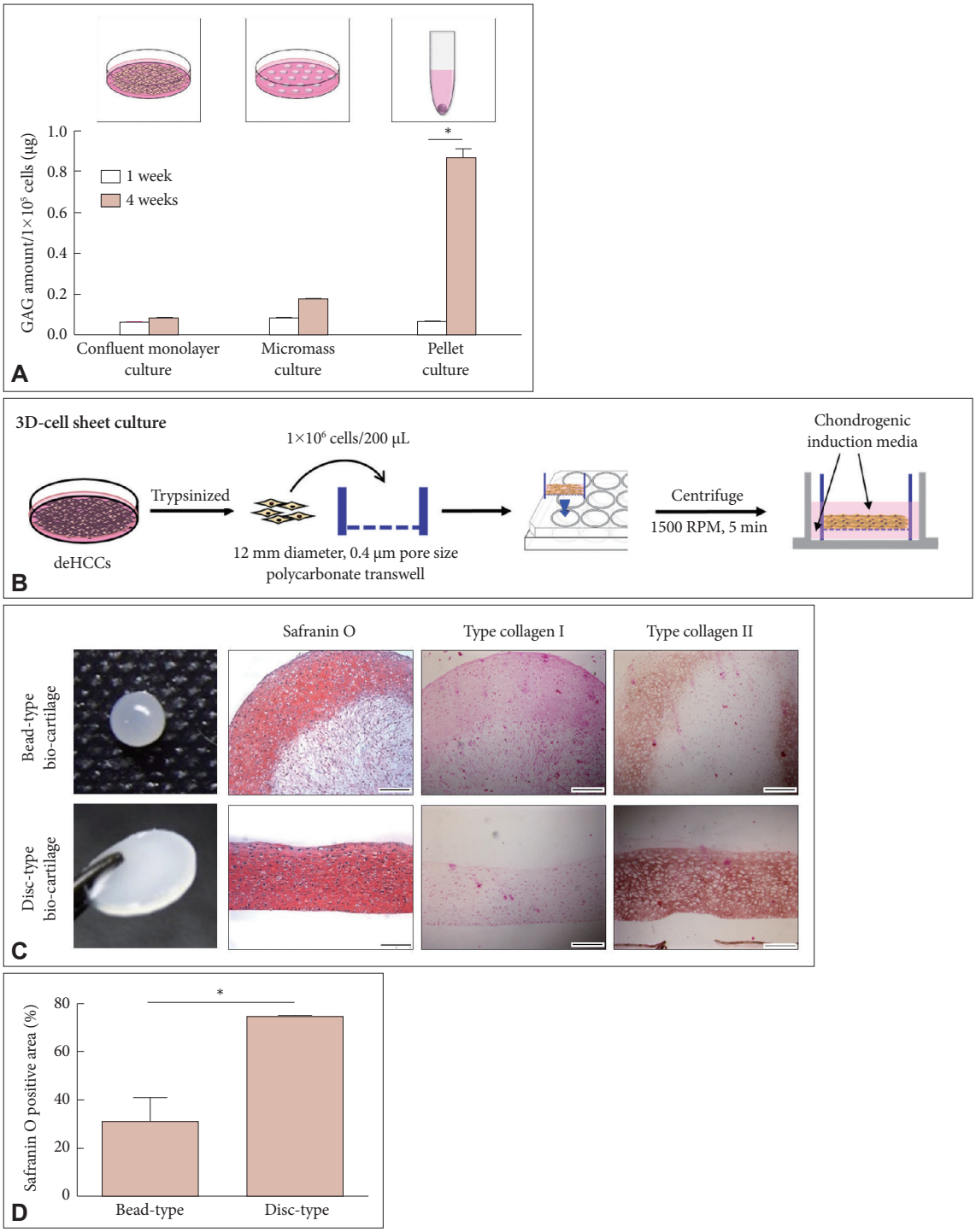

Figure 1. Comparison of chondrogenesis efficiency between different chondrogenic-induction methods. (A) Scheme of chondrogenic induction methods and actual amount of GAG measured by Blyscan® GAG assay at 1 week and at 4 weeks. GAG expression was normalized based on 1×10^5 inoculating cells in all three methods (*p<0.001). (B) Scheme of 3D-cell sheet culture method. (C) Comparison of chondrogenesis efficiency between the pellet culture and the 3D-cell sheet culture. GAG deposition was estimated by Safranin-O staining. Expression of type I and II collagens was estimated by immunohistochemical staining. (D) Analysis of GAG positive area in the total area of cartilage tissue. Scale bar=200 μm. GAG: glycosaminoglycan, deHCC: dedifferentiated human costal chondrocyte.

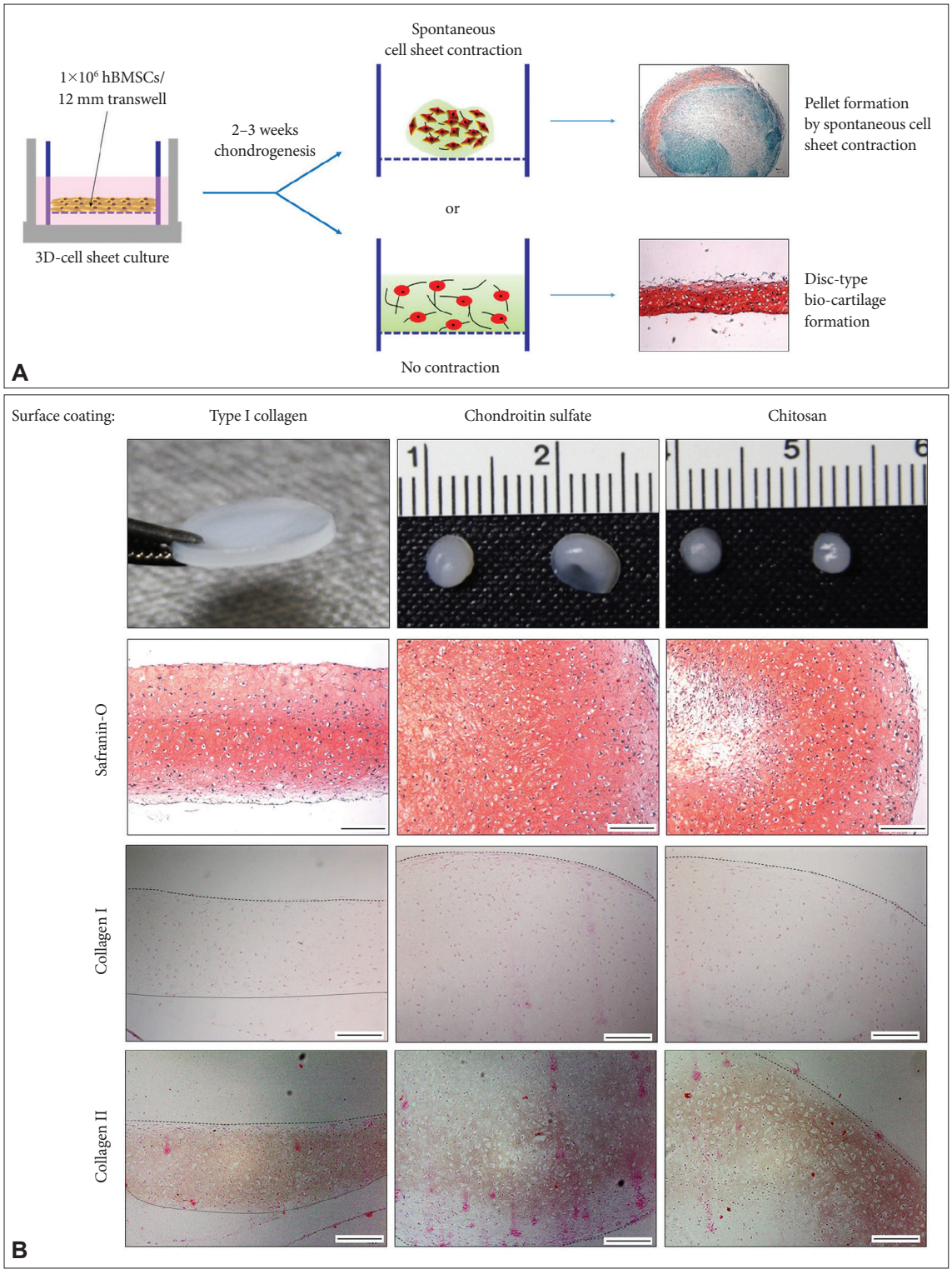

Figure 2. Comparison of ECM coatings to prevent curling of hBMSC-cell sheet during chondrogenesis. (A) Scheme of 3D-cell sheet culture of BMSC and its possible fate without ECM coating. (B) Comparison of bio-cartilage formation after 4 weeks culture under different ECM coating condition. Gross morphologies were shown at upper panel. Safranin-O staining for GAG deposition and immunohistochemical stainings for type I, II collagen were shown. Scale bar=200 μm. ECM: extracellular matrix, GAG: glycosaminoglycan, hBMSC: human bone marrow stromal cell.

contraction and the curling of the whole sheet like a sphere, which makes possible produce uniform disc-type bio-cartilage consistently.

Reconstruction of disc-type bio-cartilage on amniotic membrane and SIS membrane and comparison of their chondrogenesis efficiency

Three-dimensional-cell sheet culture on type I collagen coated transwell enables to make uniform disc-type bio-cartilage. However, Transwell membrane, which is composed of chemically synthesized polycarbonate, is optimal to provide mechanical support for bio-cartilage sheet but type I collagen coating is necessary for reproducible production of disc-type bio-cartilage using hBMSC. Furthermore, the polycarbonate membrane is not transplantable *in vivo*. In order to seek transplantable and collagen abundant bio-membranes which can replace polycarbonate membrane in mechanical property, we evaluated bio-membranes such as amniotic membrane and SIS membrane in their applicability for disc-type bio-cartilage production. Amniotic membrane, which is mainly composed of type I, III, and IV collagens, is currently used as a graft or a dressing in different surgical subspecialties and to reconstruct the ocular surface. SIS membrane, which is mainly composed of type I collagen, is also used for reconstruction of a rotator cuff tendon and other applications. Each bio-membrane was inserted between two rings of the CellCrown™ (Fig. 3A). During 4 weeks chondrogenic induction of hBMSCs, both amniotic membrane and SIS membrane were firmly attached to the membrane as a whole sheet in the CellCrown™. However, histological analysis showed dense GAG depositions by Safranin-O staining and high histological quality by Bern scoring of the disc in amniotic membrane and type I collagen-coated polycarbonate membrane. In contrast, marked retardation of chondrogenesis was obvious in SIS membrane as shown by poor GAG deposition and low Bern score (Fig. 3B and C). In terms of chondrogenesis, amniotic membrane is better than SIS membrane.

The thickness of bio-cartilage was also varied between membrane types. The thickness of the disc-type bio-cartilages on type I collagen-coated polycarbonate membranes was approximately over 600 μm, while those on amniotic membranes were approximately 300 μm and those on SIS membranes were under 200 μm (Fig. 3D). This result suggests that polycarbonate membrane provides better condition for the disc-type bio-cartilage but amniotic membrane may also be recommended for production of a transplantable disc-type bio-cartilage with mechanical support for suture and surgical manipulation.

The disc-type bio-cartilage maintained its hyaline cartilage phenotype even under the osteogenic condition

The disc-type bio-cartilage reconstructed by 3D-cell sheet culture is a typical hyaline cartilage abundant with GAGs and type II collagens. However, after the transplantation, bio-cartilage made by BMSCs is known to decrease GAG and type II collagen expression and be calcified *in vivo* [24]. In order to test whether the disc-type bio-cartilage can be calcified under the osteogenic-induction condition or not, the disc-type bio-cartilage reconstructed by 4 weeks chondrogenesis of hBMSC was transferred to the osteogenic-induction medium and cultured for additional one or 3 weeks (Fig. 4A). Based on Safranin-O staining for GAG deposition and Alizarin red-S staining for calcium deposition, osteogenic environment did not reduce GAG deposition and also did not induce calcium deposition in the disc-type bio-cartilage (Fig. 4B). This result indicates that the disc-type bio-cartilage regenerated from hBMSC 3D-cell sheet culture is a fully differentiated hyaline cartilage, which is not converted to calcified tissue phenotype even under the osteogenesis-favorable environment.

Disc-type bio-cartilage maintained their cartilage phenotype after *in vivo* transplantation

Disc-type bio-cartilage was efficiently reconstructed by 3D-cell sheet culture of hBMSC and deHCC, whose cartilage phenotype was maintained under the osteogenesis environment. In order to test whether *in vitro* reconstructed bio-cartilages can maintain their cartilage phenotype after transplantation *in vivo* or not, the bio-cartilages which was cultured as 3D-cell sheet under the chondrogenic-induction medium for 10 days for immature bio-cartilage and 28 days for mature cartilage, respectively were transplanted subcutaneously on the back skin of nude mice (Fig. 5A). At 4 weeks transplantation, the disc-type bio-cartilages were isolated for histological evaluation (Fig. 5B and C). Subcutaneous transplantation increased ECMs such as GAG, type I, and II collagen contents as well as lacunae formation of the bio-cartilage comparing to the *in vitro* 3D-cell sheet. Original disc-type bio-cartilage shape and phenotype was fairly well maintained in 28 days 3D-cell sheet culture group and further maturation forming ECM-rich lacunae morphology was clearly detected (Fig. 5D). In contrast, in the case of 10 days 3D-cell sheet, deformation and shrinkage of original disc-type cell sheet was accompanied by approximately 70% reduction in size, while further maturation to the lacunae-rich hyaline cartilage was still progressed in *in vivo* environment. However, the bio-cartilage of 28 days 3D-cell sheet culture was approximately 2.5-fold larger in diameter than the 10 days' one. These results indicated that the bio-cartilage reconstructed by *in vitro*

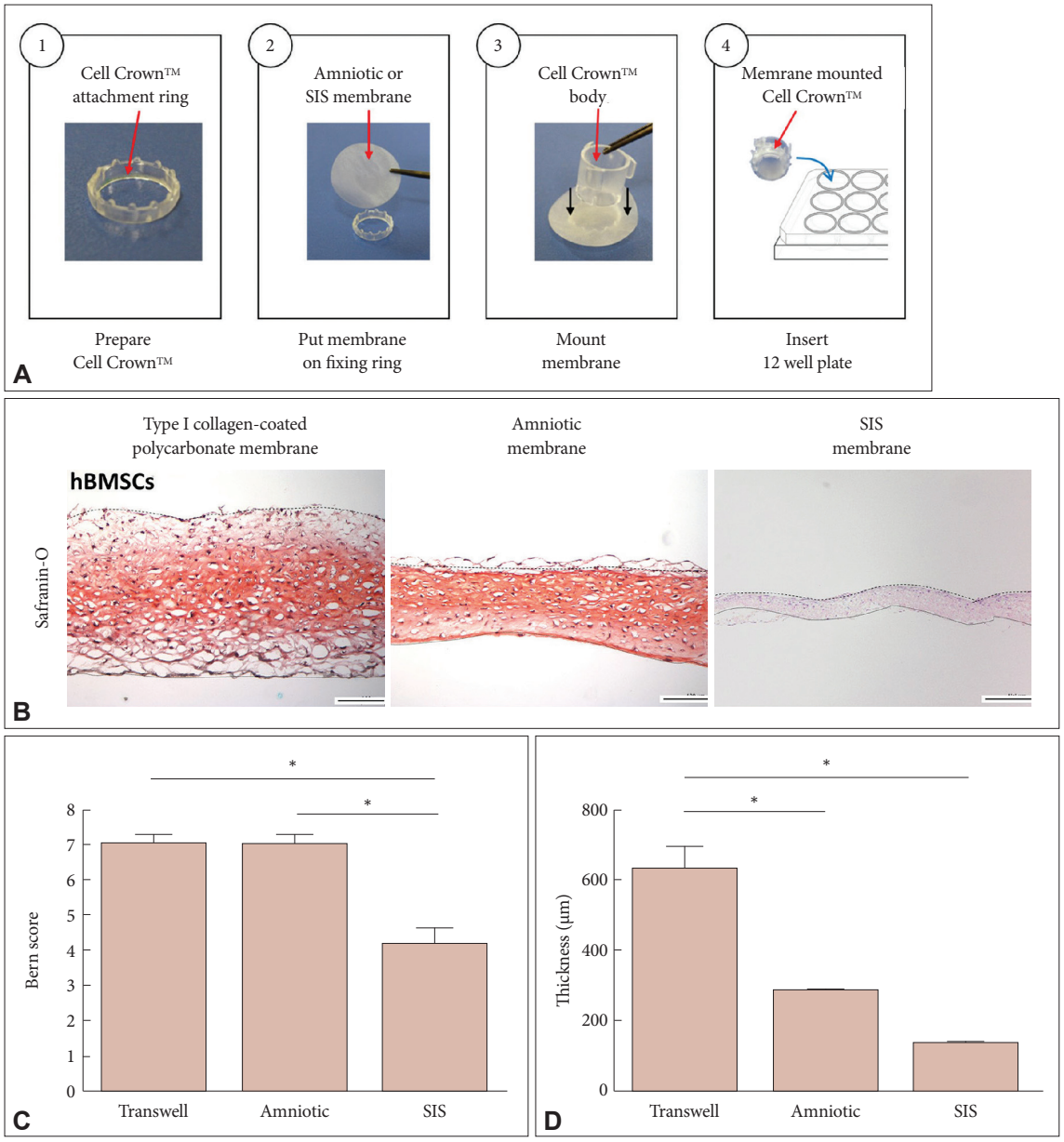

Figure 3. Comparison of bio-cartilage formation cultured on different bio-membranes. (A) Scheme of 3D-cell sheet culture method using bio-membrane with the CellCrown™. (B and C) Histology evaluation of GAG formation by Safranin-O staining (B) and Bern score (C) (*p<0.001). (D) Comparison of bio-cartilage thickness between different membrane types (*p<0.001). Scale bar=200 μm. SIS: small intestine submucosa, hBMSCs: human bone marrow stromal cell, GAG: glycosaminoglycan.

3D cell sheet culture for 28 days maintained its original disc shape and hyaline cartilage phenotype and subcutaneous environment may further facilitate mature lacunae-rich chondrogenesis.

In order to increase thickness of disc-type bio-cartilage, three sheets of both cultures were stacked, transplanted in the subcutaneous tissue of the nude mice, and cured for 4 weeks to induce fusion of three sheets. In the case of 28 days 3D-cell sheet culture as for mature cartilage, its gross appearance suggested the fusion of three discs during the 4 weeks curing *in vivo* by increasing the thickness approximately three folds (Fig. 6B and C) but the histology of cross sections revealed that complete fusion of the disc bio-cartilage was not proceeded (Fig. 6A, yellow arrow). In contrast, the histology of the 10 days 3D-cell sheet, as for the immature cartilage, revealed better fusion of three sheets (Fig. 6A).

DISCUSSION

Many clinical trials for the repair of articular cartilage defects

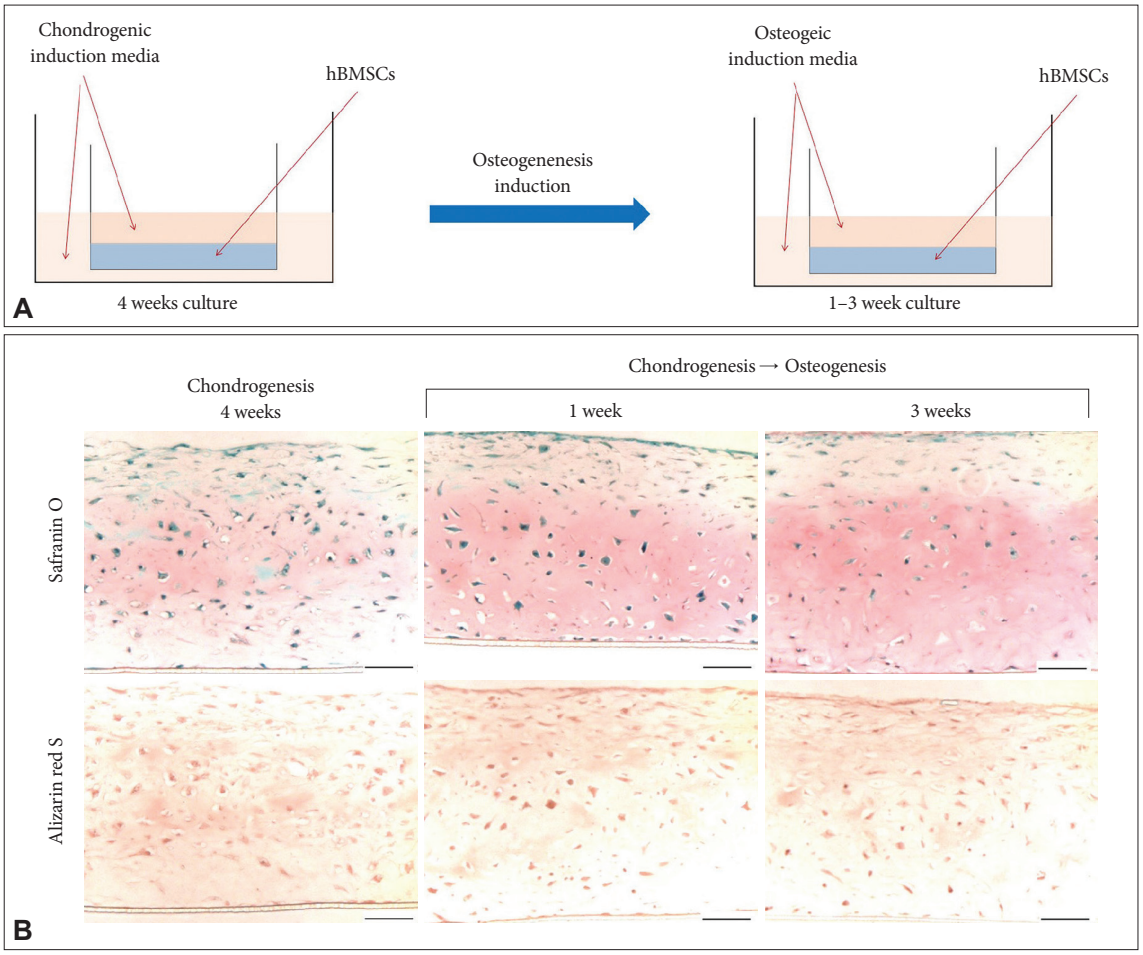

Figure 4. Osteogenic-induction to the disc-type bio-cartilage. (A) Experimental scheme for the osteogenic-induction of disc-type bio-cartilage. (B) Histological evaluation of GAG formation by Safranin-O staining and calcium deposition by Alizarin red-S staining after 1 week and 3 weeks osteogenic-differentiation challenge. Scale bar=100 μm. hBMSCs: human bone marrow stromal cell, GAG: glycosaminoglycan.

have been made using autologous chondrocytes and MSCs from diverse origins but most successful outcome was obtained only in young patients within defects less than 10–20 mm in size [12,29,30]. Also, paracrine effect of MSCs on the control of inflammatory response has been proposed as one of mechanism for the repair [12,31]. Bio-cartilage with transplantable size and mechanical requirement has not been developed so far. In this study, we have developed several strategies to consistently reconstruct the disc-type bio-cartilage in 12 mm diameter and 600 μm thick, whose shape and hyaline cartilage phenotype can be maintained even under the osteogenic-induction condition and after subcutaneous transplantation. Since ample deposition of GAG and type II collagen and lacunae formation were detected in this disc-type bio-cartilage, the disc-type bio-cartilage is structurally and biochemically similar to naïve hyaline cartilage. Furthermore, the disc bio-cartilage is tangible and can be grabbed by forceps, which makes it ready for a variety of surgical procedures. Accordingly, this disc-type bio-cartilage may be clinically applied to repair cartilage defect on the articular surface, reconstructive plastic surgery, mandibular joints, intervertebral disc, digits, and all of which requires cartilage with mechanical strength.

The disc-type cartilage reconstructed by 3D-cell sheet culture of hCCs in this study revealed approximately 12 mm diameter and 600 μm thickness, which is much thinner than human articular cartilage with approximately 3–4 mm thickness [32]. Accordingly, the disc bio-cartilage may be used to repair partial thickness damages or mandibular condyle cartilage with approximately 15 mm in diameter and 1 mm thickness [33]. Our attempt to increase thickness of bio-cartilage by stacking and integrating three disc-type bio-cartilages into one thicker cartilage through the *in vivo* transplantation for 4 weeks was not successful in the case of the 28 day *in vitro* 3D-cell sheet culture but merging of three discs was possible in the case of the 10 days 3D-cell sheet culture even though it was not completely fused. This suggests that immature cartilage may be fused together

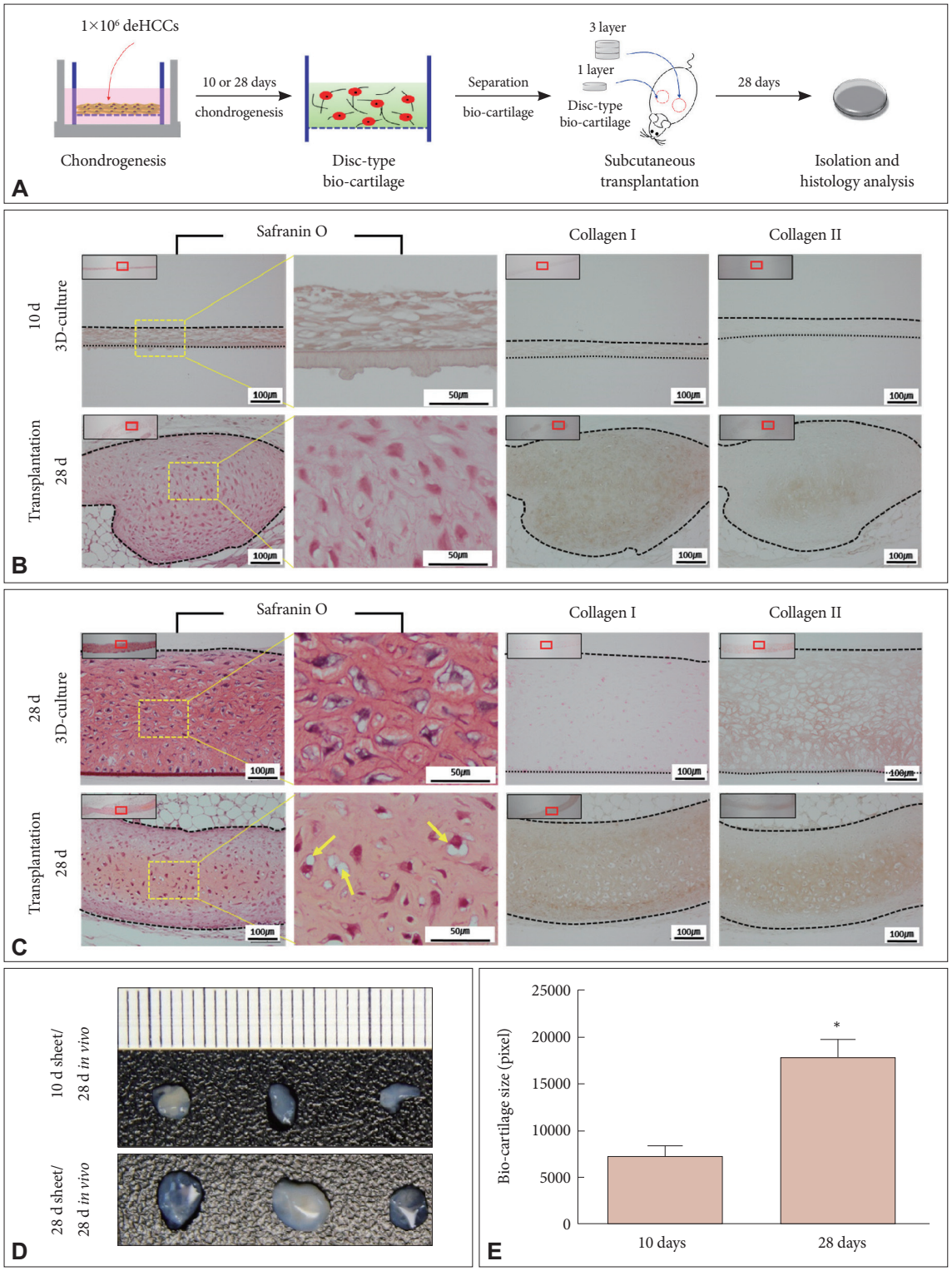

Figure 5. Analysis of disc-type bio-cartilage phenotype change by *in vivo* transplantation. (A) Experimental scheme of disc-type bio-cartilage *in vivo* transplantation for identifying maintenance of their cartilage phenotype and fusion of multiple sheets of bio-cartilage. (B and C) Histological change analysis of (B) 10 day and (C) 28 days chondrogenic differentiation disc-type bio-cartilage after 28 days *in vivo* transplantation. Histological evaluation of GAG formation by Safranin-O staining and type I, II collagen expression by immunohistochemical staining. (D) Gross morphology of disc-type bio-cartilage after 28 days *in vivo* transplantation. (E) Size of disc-type bio-cartilage after 28 days *in vivo* transplantation with 10 days and 28 days chondrogenic differentiation. *p<0.05. deHCCs: dedifferentiated human costal chondrocytes, GAG: glycosaminoglycan.

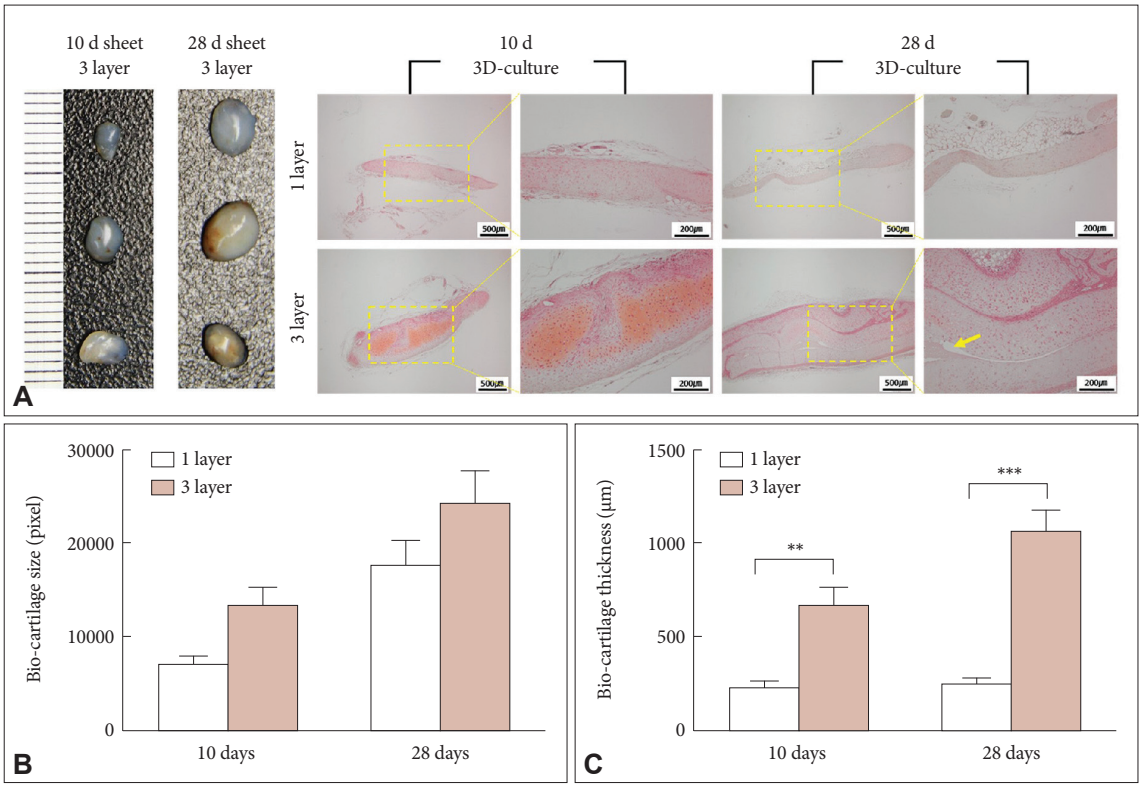

Figure 6. Fusion of multiple sheets of bio-cartilage under the *in vivo* environment. (A) Gross morphology of transplants harvested at 28 days transplantation and Safrannin-O staining of cross sections of transplants. Comparison of the mean area of the disc-type bio-cartilage (B) and thickness (C) between one sheet and three sheets after 28 days *in vivo* transplantation of 10 days or 28 days chondrogenic differentiation. $^{**}p<0.01$, $^{***}p<0.001$.

under the *in vivo* environment. Thus, alternate stacking of mature disc and immature disc and *in vivo* curing by transplantation may be the future study to be attempted.

For successful tissue engineering of transplantable bio-cartilage, more accessible and expandable donor tissue has to be identified. Among them, costal chondrocyte (CC) even after extensive expansion under the MSCGM+bFGF culture condition successfully reproduced the disc-type bio-cartilage by 3D-cell sheet culture. These CCs revealed most MSC phenotype after the dedifferentiation under the MSCGM+bFGF condition. However, CCs still retained the skewed tendency to preferentially differentiate into the hyaline cartilage. In terms of cell expansion and chondrogenic differentiation, CCs is the better donor tissue than ACs in animals [11,34]. Furthermore, costal cartilage is well preserved and existed in active chondrocyte state even at patients 60 years [35,36].

BMSCs derived from bone marrow aspirate can also be differentiated into disc-type bio-cartilage if curling up of the sheet is prevented by type I collagen coating but their tendency to calcification after the transplantation as noted by several previous reports [24] may limit their clinical application. Other MSC-like cells such as adipose-derived stem cells may also be one of easily accessible cell sources, which may also be attempted in the future study.

Supplementary Materials

The online-only Data Supplement is available with this article at http://dx.doi.org/10.1007/s13770-016-9065-6.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

Animal care and operating procedures were approved by the institutional animal use committee of Kyung Hee University, Korea (KHUASP(SU)-12-07). Human samples were collected from donors with written agreement of patients to use the remnant of bone marrow (KPH IRB 2009-003) and costal cartilages (SMC201501097-HE003) for research purpose.

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