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Title

Chondrogenic differentiation of mouse induced pluripotent stem cells (iPSCs) using the three-dimensional culture with ultra-purified alginate gel (UPAL gel)

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Abstract

Since articular cartilages have rarely healed by themselves because of their characteristics of avascularity and low cell density, surgical intervention is ideal for patients with cartilaginous injuries. Because of structural characteristics of the cartilage tissue, a three-dimensional culture of stem cells in biomaterials is a favorable system on cartilage tissue engineering. Induced pluripotent stem cells (iPSCs) are a new cell source in cartilage tissue engineering for its characteristics of self-renewal capability and pluripotency. However, the optimal cultivation condition for chondrogenesis of iPSCs is still unknown. Here we show that a novel chondrogenic differentiation method of iPSCs by using the combination of three-dimensional cultivation in ultra-purified alginate gel (UPAL gel) and multi-step differentiation via mesenchymal stem cell-like cells (iPS-MSCs) could efficiently and specifically differentiate iPSCs into chondrocytes. The iPS-MSCs in UPAL gel culture sequentially enhanced the expression of chondrogenic marker without the up-regulation of that of osteogenic and adipogenic marker and histologically showed homogeneous chondrogenic extracellular matrix formation. Our results suggest that the pluripotency of iPSCs can be controlled when iPSCs are differentiated into iPS-MSCs before embedding in UPAL gel. These results lead to the establishment of an efficient three-dimensional system to engineer artificial cartilage tissue from iPSCs for cartilage regeneration.

Keywords

cartilage repair, iPS cells, mesenchymal stem cells, alginate gel, chondrogenic differentiation

1. Introduction

Articular cartilage, considered as a subtype of hyaline cartilage, is avascular tissue with low cell density. The characteristics of cartilage tissue result in lack of spontaneous healing following cartilage injuries^{1,2}. Therefore, cartilage tissue engineering techniques is ideal for patients with symptomatic cartilaginous lesions³. Although several studies have shown successful clinical outcomes of cartilage tissue engineering by using autologous chondrocytes for cartilaginous lesions^{4,5}, comparative clinical trials have demonstrated no obvious superiority of these procedures over conventional operative techniques⁶. One of the potential reasons for this unexpected results is the limited ability of chondrocytes to proliferate and maintain their phenotype, transforming into fibroblastic cells⁷. A possible strategy for overcoming such drawback is the use of stem cells.

In 2006, Takahashi and Yamanaka first induced pluripotent stem cells (iPSCs) that can be generated by the introduction of several types of reprogramming factors into mature somatic cells^{8,9}. As with embryonic stem cells (ESCs), iPSCs have pluripotency and infinite self-renewal ability, allowing for the provision of a sufficient number of cells of standard quality^{10,11}. In addition, iPSCs can avoid the bioethical issues associated with the destruction of fertilized eggs that ESCs generation entails. Therefore, iPSCs are a highly promising cell source in regenerative medicine.

At the present, several chondrogenic differentiation methods from iPSCs, such as the co-cultivation with chondrocytes¹², the embryoid body-based method¹³, and the differentiation via mesenchymal stem cell-like cells^{14,15} have been established. One of the methods, spontaneous differentiation of iPSCs followed by enrichment of mesenchymal stem cell-like cells by further culturing the iPSC-progeny in MSC medium, was applied because of its simplicity^{16,17}. As chondroprogenitor, mesenchymal stem cell-like cells derived from ESCs/iPSCs were able to be expanded extensively¹⁸; however, their expansion caused loss

of chondrogenic activity.^{16,19}

To avoid loss of chondrogenic potential, a three-dimensional (3D) culture system designed as a mimic of cartilage extracellular matrix (ECM) has received attention^{20,21}. A large number of 3D chondrogenic induction systems using various biomaterials, including alginate^{22,23} have been introduced in the cartilage tissue engineering field. However, very few attempts have been made at using 3D biomaterial systems to control chondrogenesis from iPSCs.

We have recently developed in situ forming gel based on alginate as a scaffold or an injectable delivery vehicle for bone marrow stromal cells (BMSCs)²⁴⁻²⁷. The endotoxin level of this biomaterial was drastically reduced by our own developed ultra-purified technology²⁴. This leads to enhancing the chondrogenesis from BMSCs and promoting reparative process of cartilaginous lesions²⁴⁻²⁸. Based on the previous observations, we hypothesized that a 3D culture using this ultrapurified alginate gel (UPAL gel) could enhance the chondrogenesis from iPSCs. The specific purposes of this study were to clarify the capability of UPAL gel to differentiate mouse iPSCs into chondrocyte-like cells by comparing with that of conventional grade alginate gel and a high density micromass culture, and to determine the optimal differentiation stage of iPSCs to embed them into UPAL gels for better chondrogenesis.

2. Materials and Methods

2.1. Mouse iPSCs culture and maintenance

We used the mouse iPS cell line (ASP0001 iPS-MEFNg-20D-17; RIKEN BRC Cell Bank, Ibaraki, Japan) in which the green fluorescent protein (GFP) was knocked into the endogenous Nanog locus. Undifferentiated mouse iPSCs were grown on a feeder layer of mitotically-inactivated (mitomycin C; Sigma-Aldrich, St. Louis, MO) mouse embryonic

fibroblasts (ORIENTALYEAST, Tokyo, Japan) in the presence of 1000 U/ml of leukemia inhibitory factor (LIF, Millipore, Billerica, MA) contained in iPSC medium.

2.2. Derivation iPS-MSCs (mesenchymal progenitor cells) from mouse iPSCs and subsequent expansion

Mesenchymal progenitor cells were induced from mouse iPSCs as previously described^{29,30}. Colonies of iPSCs were grown for 7–9 days, and then they were treated with accutase (Innovative Cell Technologies, San Diego, CA) and passed through 40 µm cell strainers. The cells were seeded onto gelatin-coated plates at 1×10^5 cells/cm² in MSC induction medium consisting of DMEM-HG (Thermo Fisher Scientific, Tokyo, Japan), 10% defined fetal bovine serum (FBS, Thermo Fisher Scientific), 1% nonessential amino acids, 1% penicillin/streptomycin, and 5 ng/ml human recombinant bFGF (Thermo Fisher Scientific). With subsequent passaging (p5–p7) onto non-coated plates, the mesenchymal stem cell-like population acquired fibroblast-like morphology.

2.3. Preparation of alginate gel

UPAL gel (UPAL® gel, Sea Matrix®, Mochida Pharma. Co. Ltd., Tokyo, Japan) and conventional grade alginate gel (Sodium Alginate 500, 199–09961, Wako Pure Chemical Industries, Ltd., Osaka, Japan) were used in this experiment. The UPAL gel was isolated from brown seaweed (*Lessonia*) and purified by our original clarification procedure^{24,27}. In our original purification process, the contained endotoxin level of UPAL gel was reduced to 5.76 EU/g; whereas that of conventional alginate gel is 169,237EU/g. The gel with a molecular weight of 1700 kDa was used in this experiment.

2.4. Alginate encapsulation and recovery of cells

Cells were suspended in alginate gel (2% solution) at the desired concentration (40 µl alginate beads containing 1×10^6) and dropped from a pipette into CaCl₂ solution. They were solidified completely at 10 min after contact with the CaCl₂ solution. For the recovery of

cells, alginate beads were washed three times with PBS, and then placed in 50 mM EDTA (Thermo Fisher Scientific). 10 minutes later, cells were recovered by centrifugation at 1500 g for 5 min.

2.5. *Chondrogenic differentiation in alginate gel and high density micromass culture*

Chondrogenic differentiation of mouse iPSCs, iPS-MSCs (passage 5–7) and mouse BMSCs (Cyagen Biosciences Inc., Santa Clara, CA) was induced by culturing accutase-dispersed cells in alginate gel²⁴. A 40 µl alginate bead containing 1×10^6 iPSCs, iPS-MSCs or BMSCs were cultured in 1 mL of MSC induction medium. 24 hours after the beads formation, the culture medium was replaced with chondrogenic medium. On Day 2 of alginate bead formation and for the duration of the differentiation assay, cultures were treated with human recombinant BMP-2 (100 ng/ml; R&D Systems, Minneapolis, MN). Medium and growth factor were replaced three times a week.

A high-density micromass culture (1×10^5 cells/10 µl drop) was formed as previously described³¹. Medium and growth factor were replaced in the same way as described above.

2.6. *RNA isolation and quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)*

Total RNA extracted from iPSCs, iPS-MSCs and BMSCs at different time points was harvested by a Trizol reagent (Thermo Fisher Scientific), and analyzed for Oct3/4, Nanog, Klf4, COL2A1, aggrecan, Sox9, Runx2, PPAR γ , ALP, and COL10A1 gene expression. PCR was carried out on triplicate cDNA samples using SYBR Green Master Mix (Finnzymes, Vantaa, Finland). The relative mRNA expression for each targeted gene was expressed as the cycle threshold value of each gene normalized to the cycle threshold value of the glyceraldehyde-3-phosphate dehydrogenase gene as a housekeeping control.

2.7. *Flow cytometry*

The samples of mouse iPS-MSCs (passage 5–7) and mouse BMSCs (Cyagen) were

analyzed by flow cytometry with a BD FACS Canto II flow cytometer. Compensation was set using BD Comp Beads. Cells were tested for MSC positive markers, CD29 PE (eBioscience, San Diego, CA), anti-mouse CD44 FITC (eBioscience), anti-mouse CD73 PE (R&D Systems, Minneapolis, MN), anti-mouse CD90 PE (R&D) and MSC negative markers, anti-mouse CD31 FITC (eBioscience), anti-mouse CD45 FITC (eBioscience), anti-mouse CD86 PE (eBioscience). Data were analyzed using FACS Diva software (BD Biosciences, Franklin Lakes, New Jersey) and Flowjo™ data analysis package (Tree Star, Inc., Ashland, OR).

2.8. *Histological and immunohistochemical analysis*

At 7, 14 and 28 days after cultivation, each alginate bead was washed with PBS and fixed with 10% phosphate-buffered paraformaldehyde for 24 h, embedded within paraffin, cut into a 5 µm-thick section from the center of the bead, and stained with 0.1% Alcian blue. Immunohistochemical stains were performed with anti-type II (Fuji Pharm. Lab., Toyama, Japan) collagen anti-bodies. In high density micromass culture, at 7, 14 and 28 days after cultivation, cells were rinsed with PBS and fixed with 95% methanol (FUJIFILM Wako Pure Chemical Co., Osaka, Japan). They were then stained with 0.1% Alcian blue 8GX (FUJIFILM Wako Pure Chemical Co.) in 0.1 M HCl (Wako) overnight.

2.9. *Cell transplantation into the dorsal flanks of the nude mice*

The animal experiments in this study were approved by the Animal Research and Care Committee of our institution. Mouse iPS-MSCs and mouse BMSCs were suspended in alginate gel at the desired concentration (1×10^6 cells per 40 µl alginate bead). 36 male nude mice (Six-week-old, BALB/cScl-nu/nu, Japan SLC) were used for the transplantation. Before surgery, the mice were given sufficient anesthesia by intraperitoneal administration of pentobarbital sodium (Kyoritsu Seiyaku, Tokyo, Japan) 15 times diluted by physiological saline (10 mL/kg; Otsuka Pharmaceutical, Tokyo, Japan). Each alginate gel bead containing

cells was transplanted subcutaneously into the dorsal flank of nude mice.

2.10. DMMB (dimethylmethylene blue) Assay for quantifying GAG concentration

Alginate gel beads from days 14 and 28 were washed with PBS and crushed. The samples were digested in papain buffer overnight at 60 °C. The digested samples were subjected to biochemical analyses to determine the glycosaminoglycan content. Glycosaminoglycan production was determined by using Blyscan assay kit (Biocolor, arrickfergus, Northern Ireland). Glycosaminoglycan content was determined using a standard curve drawn using standard solutions containing chondroitin 4-sulfate. Alginate gel beads without cells were analyzed in the same manner and the values are used as blank.

2.11. Statistical analysis

All quantitative data are expressed as mean $\pm$ SEM on triplicate samples of 3 independent experiments. Statistical comparisons were performed by ANOVA, with Tukey post-hoc analysis (JMP Pro version 12.0 statistical software, SAS Institute, Cary, NC) when two or more groups were involved. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Generation of chondrogenic progenitor cells (iPS-MSCs) from mouse iPSCs

The typical colony formation of iPSCs and the Nanog expression were confirmed by microscopic observation (Fig.1A a, b). The iPS-MSCs at passage 3 formed cell colonies with a mixture of cubic and spindle cells, which spread out from their original colonies, showing almost no GFP (Fig.1A c, d). Almost all cells acquired a fibroblast-like spindle morphology at the passage 5, matching the typical morphology of MSCs (Fig.1A e).

3.2. Disappearance of pluripotent markers and appearance of mesodermal markers

When mouse iPSCs were induced into iPS-MSCs, qRT-PCR confirmed significant suppression of pluripotent markers (Oct4, Nanog or Klf4). As the culture was repeatedly

201 passaged, the expressions of pluripotent markers further decreased (Fig.1B).

202 To evaluate the cellular property, CD surface antigen marker expressions of the iPS-
203 MSCs was compared with those of BMSCs using FACS analyses. The results showed that
204 the immunophenotype of iPS-MSCs resembled that of BMSCs (Fig.1C). The typical MSC
205 positive markers in iPS-MSCs such as CD29 (95.2%), CD44 (75.1%), CD73 (71.1%) were
206 highly expressed, whereas CD90 was rarely expressed. The iPS-MSCs largely lacked
207 expression of the definitive hematopoietic lineage marker CD45 (3.81%) and the endothelial
208 marker CD31 (0.89%), as well as the antigen-presenting cell surface marker CD86 (2.2%).
209 These results suggested that iPS-MSCs lost the properties of iPSCs and partially acquired
210 those of mesenchymal stem cells.

211 3.3. *Morphological and biochemical characterization of chondrogenic differentiation* 212 *of mouse BMSCs, mouse iPSCs and iPS-MSCs*

213 The mouse BMSCs, iPSCs and iPS-MSCs induced to differentiate into chondrocytes
214 were evaluated by Alcian blue staining and HE staining. Comparing with the conventional
215 grade alginate gel culture, stronger staining of Alcian blue was observed in the UPAL gel
216 3D culture (Fig.2A, B). Although strong staining was also observed in high density
217 micromass culture, the distribution was speckled and heterogeneous (Fig.2C). The
218 quantification of sulfated GAG revealed that the amount of GAG in BMSCs embedded into
219 UPAL gel significantly increased 28 days after the culture; whereas those in iPS-MSCs
220 slightly increased without significance. In addition, BMSCs embedded into conventional
221 alginate gel had decreased the amount of GAG during culturing (Fig.2D).

222 3.4. *Chondrogenic markers in BMSCs, iPSCs and iPS-MSCs derived chondrocytes*

223 The expressions of chondrogenic markers, including SOX9 (the master transcription
224 factor of chondrogenesis), COL2A1 (the matrix formation marker, the key chondrogenic
225 marker), and aggrecan (the matrix formation marker) in iPS-MSCs and BMSCs significantly

increased over time in the UPAL gel 3D culture as well as in the high density micromass 2D culture (Fig.3A). Whereas, the expressions of SOX9 and aggrecan did not increase in conventional grade alginate gel(Fig.3A). The expressions of Runx2, ALP, COL10A1 (the osteogenic marker) and PPAR γ (the adipogenic marker) in BMSCs and iPS-MSCs increased only in the high density micromass culture (Fig.3A). Meanwhile, in UPAL gel 3D culture the expressions of the osteogenic and the adipogenic markers significantly increased only in iPSCs (Fig.3B).

3.5. *In vivo induction of chondrogenic differentiation of mouse BMSCs and iPS-MSCs in UPAL gel and conventional grade alginate gel*

At 7, 14 and 28 days after transplantation the beads were surgically dissected and histologically stained. Mouse BMSCs and iPS-MSCs in UPAL gel were stained with Alcian blue and type II collagen over time (Fig.4A). Although both cells in conventional grade alginate gel were also stained with Alcian blue, they were poorly stained with type II collagen (Fig.4B). There was no significant difference in the GAG amount in either gel (Fig.4C). The chondrogenic gene expression in BMSCs and iPS-MSCs was evaluated by qRT-PCR. Despite the mRNA expressions of COL2A1 and aggrecan significantly increased in UPAL gel, the expression of Sox9 did not continuously elevate in either cell group (Fig.5). Meanwhile, the expressions of the osteogenic and the adipogenic markers did not increase in UPAL gel (Fig.5). On the other hand, the expressions of the several osteogenic and the adipogenic markers significantly increased in conventional grade alginate gel culture (Fig.5).

4. Discussion

The first objective of this study was to clarify the capability of UPAL gel to differentiate mouse iPSCs into chondrocyte-like cells by comparing with that of conventional grade alginate gel and high density micromass culture. The current study suggests that the

UPAL gel more specifically differentiated iPSCs into chondrocyte-like cells, compared to others. The second objective was to determine the optimal differentiation stage of iPSCs to embed them into UPAL gels for better chondrogenesis. Our results suggest that their pluripotency can specifically be controlled into chondrogenic potency when iPSCs are differentiated into mesenchymal stem cell-like cells before embedding them in UPAL gel.

Regarding the transplantation of iPSCs, their pluripotency must be controlled to avoid tumor formation and unexpected differentiation. To control the pluripotency of iPSCs, pericellular environments such as ECM and cell-cell contact are important^{32,33}. Zhang et al reported that stem cells may remain pluripotency with the activation of intercellular adhesion molecules³³. Uto et al reported that mouse iPSCs were successfully differentiated into target cells by collagen hydrogel³⁴. These results indicate that 3D culture using biomaterials is one of the ideal strategies for controlling pluripotency of iPSCs. Alginate is known as a hyaluronic acid (HA)-like biocompatible polymer often used in biomaterial science. In the present study, culturing in UPAL gel increased the expression of chondrogenic markers, but the expression of osteogenic and adipogenic markers remained low during chondrogenic differentiation from iPS-MSCs and BMSCs. In contrast, high density micromass culture increased not only the expression of chondrogenic marker, but also the expression of osteogenic and adipogenic markers, suggesting the difficulty in controlling the pluripotency of stem cells by this culture system. In addition, we have confirmed the effect of the alginate purification by comparing UPAL gel with conventional grade alginate gel. Conventional alginate includes mitogenic and cytotoxic impurities that induce a foreign body reaction or heavy pericapsular fibrosis in a living body. These impurities possibly prevented a chondrogenesis from stem cells and could result in a heterogeneous differentiation. Therefore, we speculated that the purification of alginate enhances the effect of chondrogenesis from iPS-MSCs. In the present study, UPAL gel culture significantly

increased the expression of all chondrogenic markers (SOX9, COL2A1, and aggrecan) without increasing the expression of osteogenic nor adipogenic markers; whereas the conventional grade alginate gel culture didn't increase the expression of aggrecan and slightly increased the expression of osteogenic markers. The results of HE staining seems that conventional alginate does not reduce the number of cells. The most likely cause is that the quality of commercial alginate used in this study is different from that used before²⁴. The conventional alginate used in this study may have fewer contaminants than previously used. We subsequently quantified the amount of glycosaminoglycan by DMMB. Firstly, we found that the values of alginate gel without cells were different between UPAL and commercial alginate (0.752 vs 1.029). It is possibly due to the impurities in the conventional alginate. Hence, we should consider that the background of conventional alginate was higher than that of UPAL. This factor leads observers to underestimate the results of UPAL in DMMB assay. The amount of glycosaminoglycan in BMSCs significantly decreased during the differentiation using conventional alginate, possibly due to its cytotoxicity. These results indicate that the purification of alginate promotes the chondrocyte-specific differentiation from iPS-MSCs and BMSCs. Based on the obtained results, we conclude that the 3D culture system with UPAL gel can specifically induce chondrogenic differentiation from iPS-MSCs.

The UPAL gel could enhance chondrogenic differentiation and cell proliferation of BMSCs, resulting in better reparative tissues in small and large osteochondral defect model^{24,26,27}. Therefore, we hypothesized that UPAL gel could enhance the chondrogenesis from iPSCs. In this study, we first applied UPAL gel to iPSCs and iPS-MSCs *in vitro*. The iPS-MSCs embedded in UPAL gel increased the expression of chondrogenic markers without increasing the expression of osteogenic nor adipogenic markers; whereas the iPSCs embedded in UPAL® increased the expression of all differentiation markers, suggesting that UPAL gel alone could not control the pluripotency of iPSCs sufficiently. The present *in vitro*

study revealed that inducing iPSCs to differentiate into iPS-MSCs before embedding them in UPAL gel could repress their pluripotency and specifically differentiate them into chondrocytes. We subsequently evaluated the chondrogenic capability of the stepwise differentiation method via iPS-MSCs using UPAL gel culture *in vivo*. Although a regenerative cartilage matrix was histologically observed in the iPS-MSCs transplanted into the dorsal flanks of nude mice, the expression of the cartilage transcription factor Sox9 did not increase continuously. Possible causes are: 1. Alginate beads were embedded subcutaneously, so there was a possibility that host cells might contaminate. 2. There was no growth factor or cytokine capable of controlling cartilage differentiation. It suggests that the UPAL gel 3D culture is insufficient to stably differentiate iPS-MSCs into chondrocytes in the subcutaneous transplantation. An addition of chondrogenic cytokines into UPAL gel or a molecular modification of UPAL gel is required for specific chondrogenic differentiation of iPS-MSCs in this subcutaneous transplantation model.

Further studies are necessary for realization of clinical application of iPSCs. First, because the present study was conducted with models using mouse iPSCs, it is necessary to determine whether studies with human iPSCs can yield similar results. Second, the intraarticular environment was not recreated in the subcutaneous transplantation model of nude mice. The experimental systems using subcutaneous implantation can investigate the safety of biomaterials. However, when observing cartilage differentiation, it is necessary to consider the difference in subcutaneous and intraarticular environments (host cell penetration, missing growth factors and cytokines, etc.). For these reasons, the results *in vivo* and *in vitro* are inconsistent. In future studies, it is necessary to establish models of articular defect in which iPS-MSCs embedded in UPAL gel are directly implanted to the lesion and to examine the ability of iPS-MSCs to repair tissue. Such *in vivo* model, mimicking clinical application of this strategy, could provide adequate environment for sufficient chondrogenesis of iPS-

MSCs. Third, it is necessary to compare the alginate gel with other biomaterials such as collagen gel and to clarify the advantages of UPAL gel. Although these considerations should be solved, the present study indicates that UPAL gel culture and multi-staged differentiation through iPS-MSCs are promising and after further study in the future may provide an alternative method to current cartilage restoration techniques.

5. Conclusion

We established a chondrogenic differentiation method of mouse iPS cells by using the combination of three-dimensional culture in UPAL gel and multi-step differentiation via iPS-MSCs. These achievements offer a novel strategy for future clinical application of iPSCs. Our strategy may allow us to repair larger osteochondral defect than conventional technique treated.

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Author Disclosure Statement

We have nothing to disclose related to this study

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FIGURE CAPTIONS

Fig.1 A; Derivation of a mesenchymal progenitor population from mouse iPS cell line: (iPS-MEF-Ng-20D-17). Undifferentiated iPSCs formed typical colonies on a feeder (a) and showed GFP by Nanog (b). iPS-MSCs at passage 3 formed cell colony with a mixture of cubic and spindle cells (c), showing almost no GFP (d). The morphology of the iPS-MSCs at passage 5 resembled spindle shape (e). B; Gene expression analyses of pluripotent markers Oct3/4, Nanog, and Klf4 in the iPSCs and iPS-MSCs (passage 3 & 5) by quantitative RT-PCR. Asterisk (*) denotes significance at $P < 0.05$. C; Expression of surface antigens in

BMSCs and iPS-MSCs by fluorescence activated cell sorting (FACS) analysis. Representative FACS profiles show iPS-MSCs (passages 6) express markers associated with the mesenchymal phenotype (positive for CD29, CD44, CD73 and CD90 as red letter; negative for CD31, CD45, and CD86 as blue letter). Blue histogram indicates antibody stained population; red profile indicates negative isotype stained population.

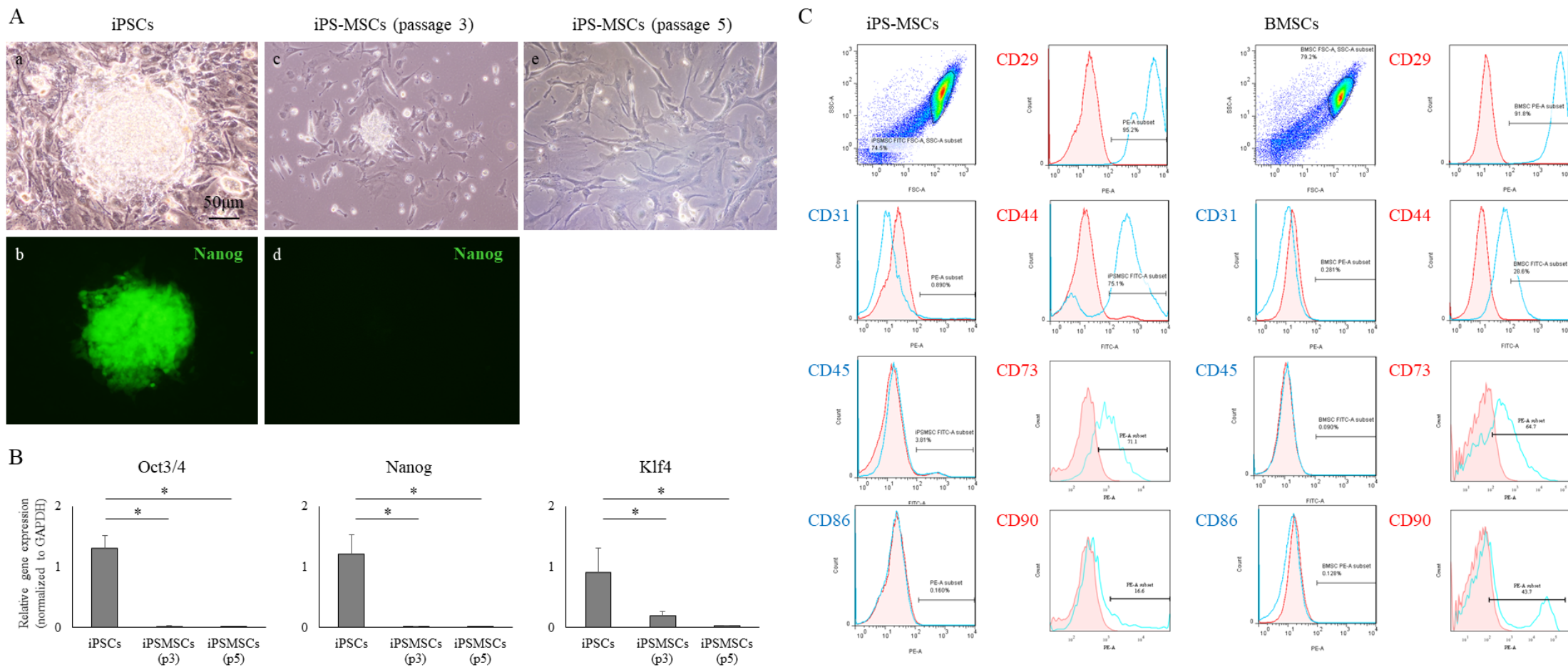
Fig.2 BMSCs, iPSCs, and iPS-MSCs were respectively induced to differentiate into chondrocytes and were stained with Alcian blue and Hematoxylin-Eosin (HE) *in vitro*. Scale bars = 200 μ m. A; UPAL gel 3D culture, B; Commercial gel 3D culture, C; High density micromass culture, D; DMMB (dimethylmethylene blue) Assay for quantifying GAG concentration. Asterisk (*) denotes significance versus parental undifferentiated iPSCs at $P < 0.05$.

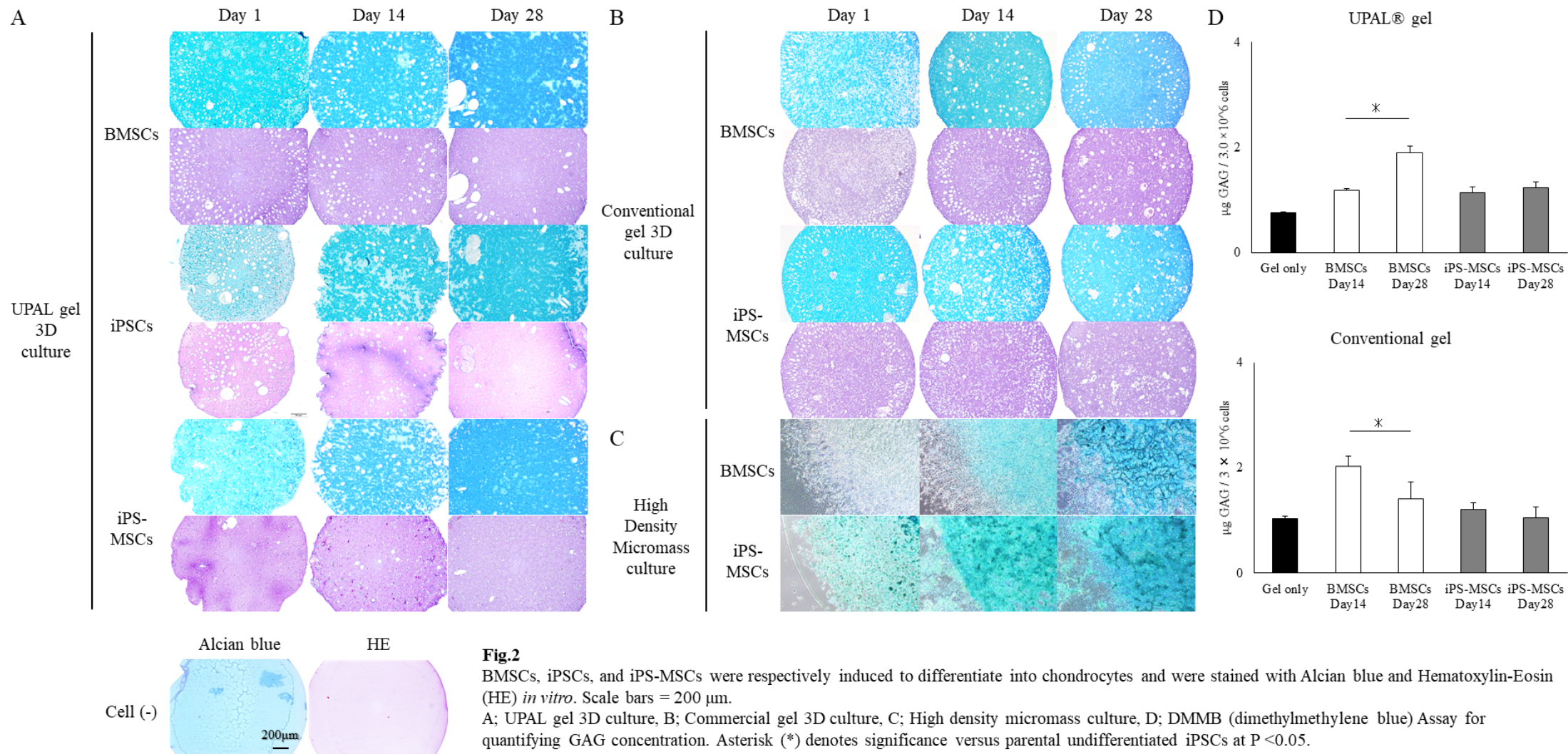
Fig.3 A; Gene expression analyses of BMSCs and iPS-MSCs in each culture system *in vitro* by qRT-PCR. B; Gene expression analyses of iPSCs group and iPS-MSCs group in UPAL gel 3D culture *in vitro* by qRT-PCR. The expressions of chondrogenic markers (SOX9, COL2A1 and aggrecan), osteogenic markers (Runx2, ALP, and COL10A1) and adipogenic marker (PPAR γ) were evaluated.

Fig.4 Alcian blue and type II collagen staining of iPS-MSCs or BMSCs in UPAL gel culture and conventional grade gel culture *in vivo*. The iPS-MSCs or BMSCs in UPAL gel transplanted into the dorsal flank of nude mice were stained with Alcian blue and type II collagen at 7, 14 and 28 days after of the transplantation. Scale bars = 200 μ m. A; UPAL gel 3D culture, B; Conventional grade alginate gel 3D culture, C; DMMB (dimethylmethylene blue) Assay for quantifying GAG concentration

474

475 Fig.5 Gene expression analyses of BMSCs and iPS-MSCs in each culture system *in vivo* by
476 qRT-PCR. The expressions of chondrogenic markers (SOX9, COL2A1 and aggrecan),
477 osteogenic markers (Runx2, ALP, and COL10A1) and adipogenic marker (PPAR γ) were
478 evaluated. Asterisk (*) denotes significance at $P < 0.05$.





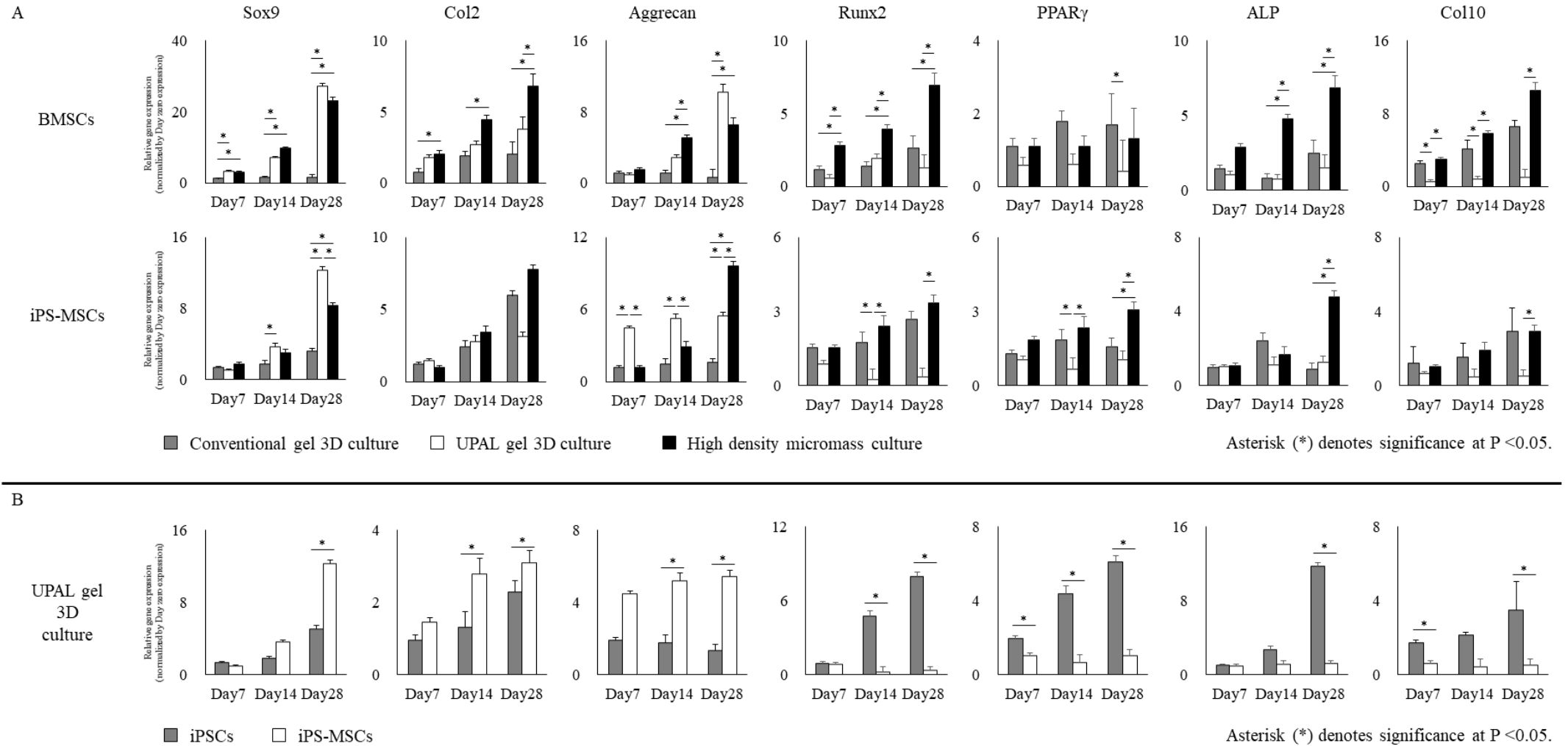


Fig.3

A: Gene expression analyses of BMSCs and iPS-MSCs in each culture system *in vitro* by qRT-PCR.

B: Gene expression analyses of iPSCs group and iPS-MSCs group in UPAL gel 3D culture *in vitro* by qRT-PCR.

The expressions of chondrogenic markers (SOX9, COL2A1 and aggrecan), osteogenic markers (Runx2, ALP, and COL10A1) and adipogenic marker (PPAR γ) were evaluated.

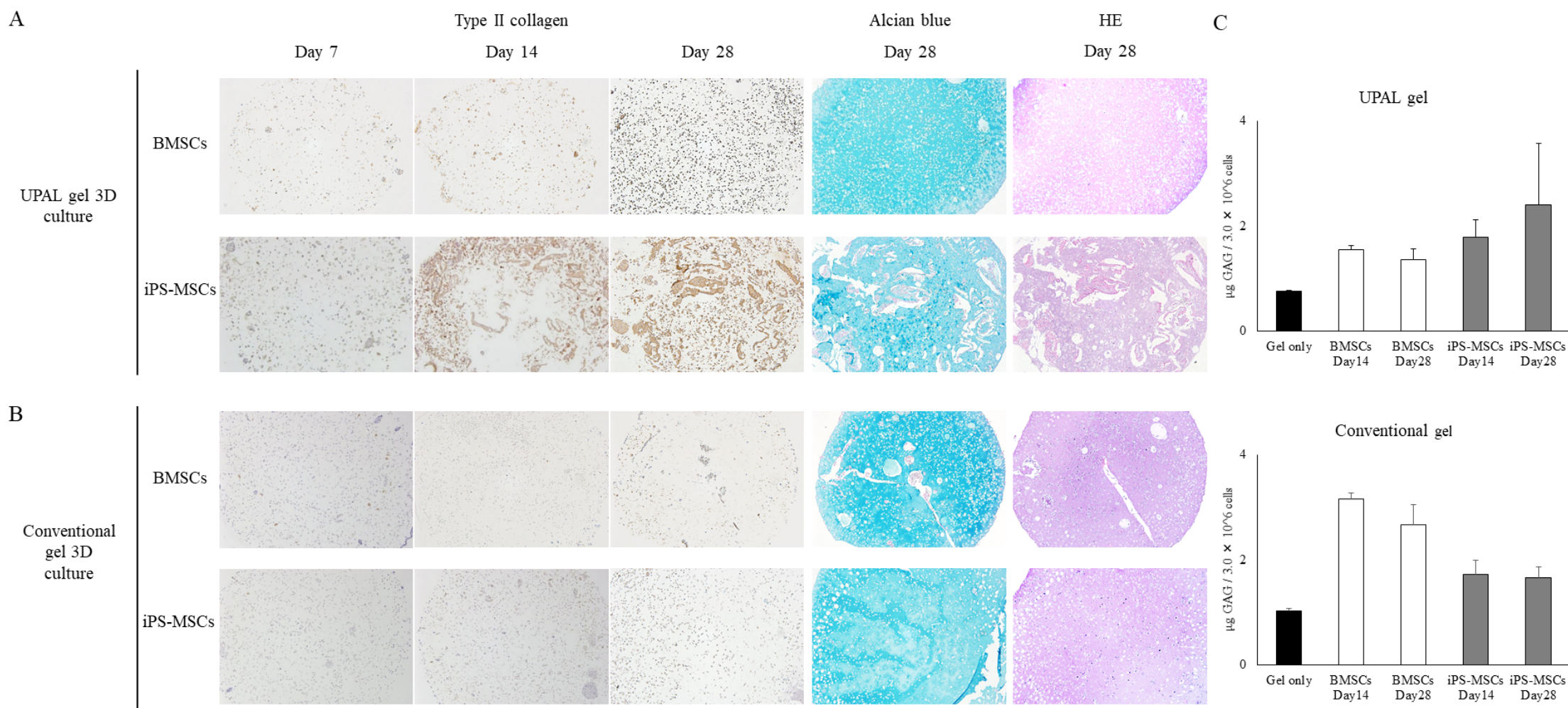


Fig.4
 Alcian blue and type II collagen staining of iPS-MSCs or BMSCs in UPAL gel culture and conventional grade gel culture *in vivo*.
 The iPS-MSCs or BMSCs in UPAL gel transplanted into the dorsal flank of nude mice were stained with Alcian blue and type II collagen at 7, 14 and 28 days after of the transplantation. Scale bars = 200 µm.
A; UPAL gel 3D culture, **B**; Conventional grade alginate gel 3D culture, **C**; DMMB (dimethylmethylene blue) Assay for quantifying GAG concentration

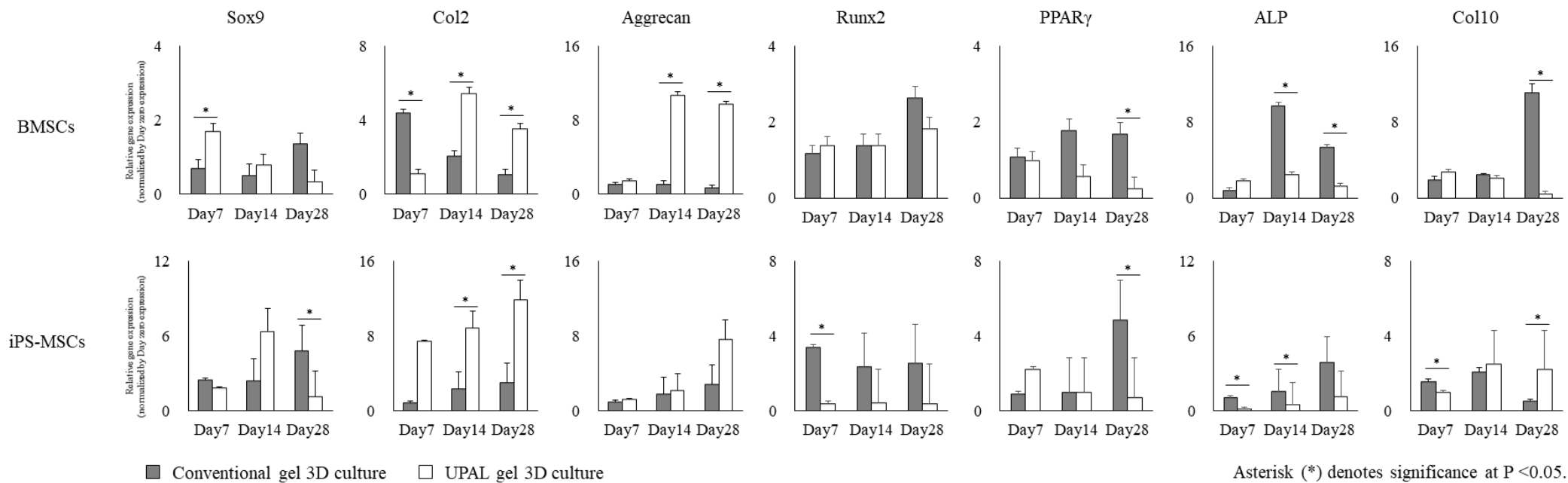


Fig.5

Gene expression analyses of BMSCs and iPS-MSCs in each culture system *in vivo* by qRT-PCR.

The expressions of chondrogenic markers (SOX9, COL2A1 and aggrecan), osteogenic markers (Runx2, ALP, and COL10A1) and adipogenic marker (PPAR γ) were evaluated.