Chondrocyte differentiation of human buccal fat pad-derived dedifferentiated fat cells and adipose stem cells using an atelocollagen sponge

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We evaluated the chondrocyte differentiation potential of dedifferentiated fat cells (DFATs) and adipose stem cells (ASCs) from the human buccal fat pad (BFP). We isolated the cells from two patients who underwent oral and maxillofacial surgery. Chondrocyte differentiation was evaluated based on gene and protein expression analysis in addition to histological analysis of DFATs and ASCs seeded in an atelocollagen sponge and cultured for up to 21 days. The gene expression levels of chondrocyte differentiation markers such as aggrecan, collagen type 2, and SOX9 were higher in DFATs than in ASCs cultured for 14 and 21 days, whereas protein expression levels were higher in DFATs at all time points tested. Additionally, the levels of the embryonic stem cell markers Nanog, SOX2, and OCT4 were higher in DFATs than in ASCs at 72 h. The extracellular matrix of both the cultured ASCs and DFATs was Alcian blue-positive, indicating production of sulfated glycosaminoglycans, and was aggrecan-positive.

The chondrocyte differentiation ability of human DFATs was higher than that of ASCs. Isolation of DFATs from the BFP offers an aesthetic advantage, as the BFP can be obtained from the oral cavity without surface scarring. Therefore, we propose that BFP-derived DFATs are an ideal cell source for cartilage tissue engineering. This study provides evidence that DFATs from the BFP are an ideal cell source for cartilage tissue engineering. (J Osaka Dent Univ 2015；49：185–196)

Key words：Dedifferentiated fat cells；Adipose stem cells；Ceiling culture；Buccal fat pad；Chondrocyte differentiation；Cartilage tissue engineering

INTRODUCTION

Cartilage, a widely dispersed connective tissue found in the maxillofacial region (the nose, ears and various joints) can be affected by various conditions, including congenital morphological anomalies such as cleft lip and palate, joint trauma, or post-surgery trauma after tumor removal. Damage to cartilage tissue has a deleterious effect on facial morphology and can make it difficult for the patient to resume daily activities, which may significantly decrease the patient's quality of life.[1] In such situations, treatment generally involves transplantation of autologous cartilage or implantation of polymer biomaterials such as silicon or hyaluronic acid.[1] However, these materials have certain disadvantages such as a lack of durability, increased risk of infection, and the requirement of invasive approaches to remove tissue from donor sites.[1] Tissue engineering strategies are expected to replace injured articular cartilage tissues with tissue grown *in vitro.* Recently, mature adipocytes, which are the most abundant cell type in adipose tissues, have been considered good candidates for tissue engineering because of their multilineage potential.[2, 3]

These types of cells, which are termed adipose stem cells (ASCs), have been investigated for their capacity to differentiate into three linages : mesoderm, ectoderm, and endoderm.[4] In general, acquisi-

tion of ASCs from adipose tissue is more cost-effective and less invasive than that from other tissues.[5] Moreover, ASCs can be cultured easily and for longer periods than bone marrow stromal cells (BMSCs).[5] Using an *in vitro* dedifferentiation strategy known as ceiling culture,[6, 7] mature adipocytes isolated from fat tissue can be dedifferentiated into fibroblast-like cells that have been termed dedifferentiated fat cells (DFATs). Furthermore, a recent study[8] from our lab demonstrated that the osteoblastic differentiation ability of DFATs is higher than that of human MSCs. Another study from our group evaluated the osteoblastic differentiation abilities of DFATs and ASCs from the buccal fat pad (BFP) and demonstrated that the osteoblastic differentiation potential of DFATs is greater than that of ASCs.[9]

In the present study, we obtained fat cells from the BFP of two male adult patients. Matsumoto *et al.* demonstrated that DFATs can be obtained from donors regardless of their age ;[6] they successfully prepared DFATs from donors between 4 and 81 years of age, although lower proliferative activity was observed in cells from donors over 70 years of age. The multilineage differentiation potential of DFATs was also confirmed in most donors examined except for those of very high age, such as 77 and 81 years. Their observations suggested that DFATs can be used for autologous transplantation in patients of various ages.

The chemical composition and structural characteristics of three-dimensional (3D) scaffolds affect cell behavior, ultimately determining the performance of tissue-engineered constructs.[10] Atelocollagen has been widely applied clinically in various forms such as gels or sponges because it shows good biodegradability, biocompatibility, and absorbability.[11–13] For example, collagen sponges are considered a useful 3D scaffold matrix for several types of tissues. The purpose of this study was to compare the chondrocyte differentiation capacity of BFP-derived DFATs and ASCs cultured in an atelocollagen sponge for tissue engineering.

MATERIALS AND METHODS

Collagen scaffold preparation

A porous collagen sponge was produced as described previously. Briefly, an atelocollagen (KOKEN, Tokyo, Japan) gel was freeze-dried, cross-linked, sterilized with formaldehyde, and γ-ray (10 kGy)-treated to produce a porous collagen sponge with a diameter of 5 mm and thickness of 3 mm (AteloCell atelocollagen sponge MIGHTY ; KOKEN). The pore size was designed to be 30–200 mm, and the pores were inter-connected.

Isolation and culture of DFATs and ASCs

Human BFP was obtained from two male patients who underwent oral and maxillofacial surgery at Osaka Dental University Hospital. The patients were healthy and had no systemic disease. All procedures were approved by the ethics committee of Osaka Dental University (Approval No. 110714). We isolated DFATs using the ceiling culture method as described in our previous report.[9] Briefly, approximately 1 g of BFP was minced into small pieces and then dissociated using a 0.1% (w/v) collagenase solution (collagenase type I ; Wako Pure Chemical, Osaka, Japan) at 37°C for 1 h with gentle agitation. The cell suspension was filtered through 150-μm and 250-μm nylon meshes to allow the cells to pass through while excluding unwanted stromal cells and tissue. Floating mature adipocytes in the top layer were collected and centrifuged at 135 $\times$ g for 3 min. Isolated mature adipocytes were seeded in a 25–cm^2 culture flask (Sumilon ; Sumitomo Bakelite, Tokyo, Japan) that was completely filled with Dulbecco's modified Eagle medium (DMEM ; Nacalai Tesque, Kyoto, Japan) supplemented with 20% (v/v) fetal bovine serum (FBS ; Life Technologies, Carlsbad, CA, USA) and an antibiotic/antimycotic mixed stock solution consisting of 10,000 U/mL penicillin, 10,000 μg/mL streptomycin, and 25 μg/mL amphotericin B (Nacalai Tesque).

The cells were then incubated at 37°C under 5% CO_2. The flask was positioned with the adhesive culture surface facing upward, such that floating adipocytes containing lipid droplets attached to the inner ceiling surface of the flask. This method is referred to as ceiling culture. After 7 d, the medium was removed and the flask was inverted. A small volume of fresh medium (sufficient to barely cover the bottom of the flask) was added. The medium was then changed

twice a week. At confluence, the cells were passaged and used for experiments. We isolated ASCs according to the method of Zuk *et al.*[14] ASCs were obtained by expansion of adherent cells derived from pellets of collagenase-digested BFP by centrifugation. The medium used for DFATs culture was also applied to ASCs culture. The culture medium for both cell types was replaced twice a week. At confluence, the cells were passaged and used for experiments.

Chondrocyte differentiation in the scaffold

A 100-μL cell suspension was seeded in the atelocollagen sponge. ASCs and DFATs from passage 3 were seeded at a density of 5×10^5 cells/scaffold in a 96-well plate, cultured in DMEM, and then mixed with an equal volume of 2% atelocollagen gel on ice. After the cells attached to the scaffolds, the scaffolds were moved to a 24-well plate. ASCs and DFATs within the scaffold were cultured in DMEM for 72 h to investigate embryonic stem cell marker expression. Chondrocyte differentiation was induced by culturing ASCs and DFATs within the scaffold for 1, 2 and 3 weeks in chondrocyte differentiation medium (CDM ; DMEM containing 1% FBS, 50 mM L-ascorbic acid-2-pho-

sphate (Sigma-Aldrich), 40 mg/mL proline (Sigma-Aldrich), 100 mg/mL pyruvate (Sigma-Aldrich), and 10 ng/mL transforming growth factor (TGF)-β3 (R & D Systems, Minneapolis, MN, USA), and $1 \times$ ITS). The medium was changed every 4 days.

RNA analysis

An entire scaffold was crushed in a mixer mill and centrifuged using a QIAshredder (Qiagen, Hilden, Germany). Total RNA was isolated from the cell-seeded matrix by using a total RNA extraction kit (Qiagen). Single-stranded cDNA was synthesized from mRNA using a High-Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA, USA). The real-time polymerase chain reaction (RT-PCR) was conducted using a Universal Probe Library Set, Human (Roche Diagnostics, Mannheim, Germany) and a FastStart Universal Probe Master Mix (Roche Diagnostics) using the two-stage program parameters on a Step One Plus PCR system (Applied Biosystems). The PCR conditions were as follows : 10 min at 95 °C, followed by 45 cycles of 15 s at 95°C and 60 s at 60°C. The primers used for PCR are listed in Table 1.

Table 1 Sequences of the primers used for RT-PCR

Primer	Genebank No.	Probe No.	Forward/Backward	Sequence
ACAN	BC150624	22	F	5'-TCACGTGTAAAAAGGGCACA-'3
			B	5'-CTTCTTCTGCCCGAAGGTC-'3
COL2A1	NM_001844	9	F	5'-GTGAACCTGGTGTCTCTGGTC-'3
			B	5'-TTTCCAGGTTTTCCAGCTTC-'3
SOX9	Z46629	61	F	5'-GTACCCGCACTTGCACAAC-'3
			B	5'-TCTCGCTCTCGTTCAGAAGTC-'3
NANOG	NM_024865	69	F	5'-GAGATGCCTCACACGGAGAC-'3
			B	5'-AGGGCTGTCCTGAATAAGCA-'3
Oct4	NM_002701	60	F	5'-CTTCGCAAGCCCTCATTTC-'3
			B	5'-GAGAAGGCGAAATCCGAAG-'3
SOX2	NM_003106	65	F	5'-GGGGGAATGGACCTTGTATAG-'3
			B	5'-GCAAAGCTCCTACCGTACCA-'3
GAPDH (control)	NM_002046	60	F	5'-AGCCACATCGCTCAGACAC-'3
			B	5'-GCCCAATACGACCAAATCC-'3

Histological analysis

Collagen sponges that had been cultured with ASCs and DFATs in CDM were washed with PBS, fixed with 4% paraformaldehyde in phosphate buffer (Wako Pure Chemical, Osaka, Japan), dehydrated in alcohol, embedded in paraffin, and cut into 5-μm thick sections. Each specimen was stained with hematoxylin-eosin (HE) and Alcian blue at pH 2.5 (Wako) for detection of mucopolysaccharides and glycosaminoglycans.

The presence of aggrecan was detected by immunohistochemical staining using 5-μm sections. Briefly, paraffin sections were deparaffinized followed by hydration in gradient ethanol solutions. Endogenous peroxidases were then inactivated by incubation with 3% hydrogen peroxide for 5 min. Antigen retrieval was performed by chondroitinase ABC processing for 60 min at 37°C. Sections were then incubated at room temperature for 50 min with a monoclonal antibody directed against aggrecan at 1 : 100 (ab 3778 ; Abcam, Boston, MA, USA), followed by incubation with Histofine Simple Stain Rat MAX-PO (MULTI) (Nichirei No. 714191, Nichirei Biosciences, Tokyo, Japan) for 30 min at room temperature. Color development was performed using diaminobenzidine tetrahydrochloride (DAB). Finally, cell nuclei were stained with hematoxylin.

Western blot analysis

Western blot analysis was performed using an atelo-collagen sponge where ASCs and DFATs were cultured for 1, 2 or 3 weeks. Cell lysates were obtained using a cocktail including Bolt LDS sample buffer and Bolt Sample Reducing Agent following the protocol supplied by Life Technologies. PNGase F (Biolabs, Ipswich, MA, USA) was added to the proteins and incubated at 37°C for 1 h. The proteins were separated by SDS-PAGE and transferred onto PVDF membranes using the iBlot Dry Blotting system. The iBind western system was used to perform hands-free blocking, antibody binding, and washes. Signals were detected using SuperSignal West Pico chemiluminescent substrate. An anti-aggrecan (ab 3778 ; Abcam) antibody was used as the primary antibody, whereas a goat anti-mouse IgG (Thermo Fisher Scientific, Waltham, MA, USA) was used as the secondary antibody. A mouse anti-GAPDH antibody at 1 : 100 (MBL International, Woburn, MA, USA) was used as a control for equal loading.

Statistical analysis

All experiments were conducted in quadruplicate. Data are expressed as the mean and standard deviation. Statistical analyses were conducted for DFATs and ASCs cultured in the same medium to compare the pluripotency and chondrocyte differentiation abilities of both cell types. Intergroup comparisons were performed using the Mann-Whitney U-test. Differences were considered significant at $p < 0.05$.
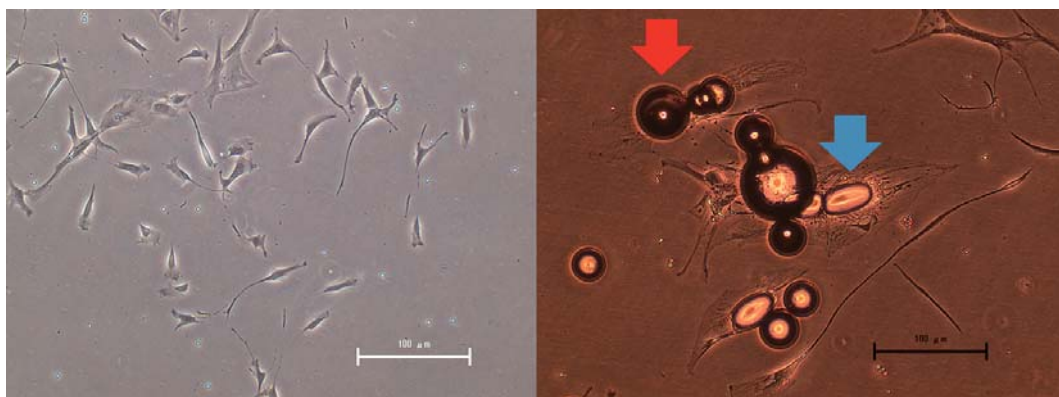

Fig. 1   Morphology of ASCs and DFATs from the buccal fat pad 3 d after dissemination of the stromal-vascular fraction and the beginning of ceiling culture, respectively. The red arrow indicates the reduction of lipid droplets, whereas the blue arrow indicates a cytoplasmic extension (Bar = 100 μm).

RESULTS

Loss of adipocyte morphology after ceiling culture

Both ASCs and DFATs showed fibroblastic and spindle-shaped cell morphology during early passages in adherent cell culture. In particular, DFATs showed characteristic attributes in ceiling culture with a reduction of lipid droplets and cytoplasmic extensions (Fig. 1).
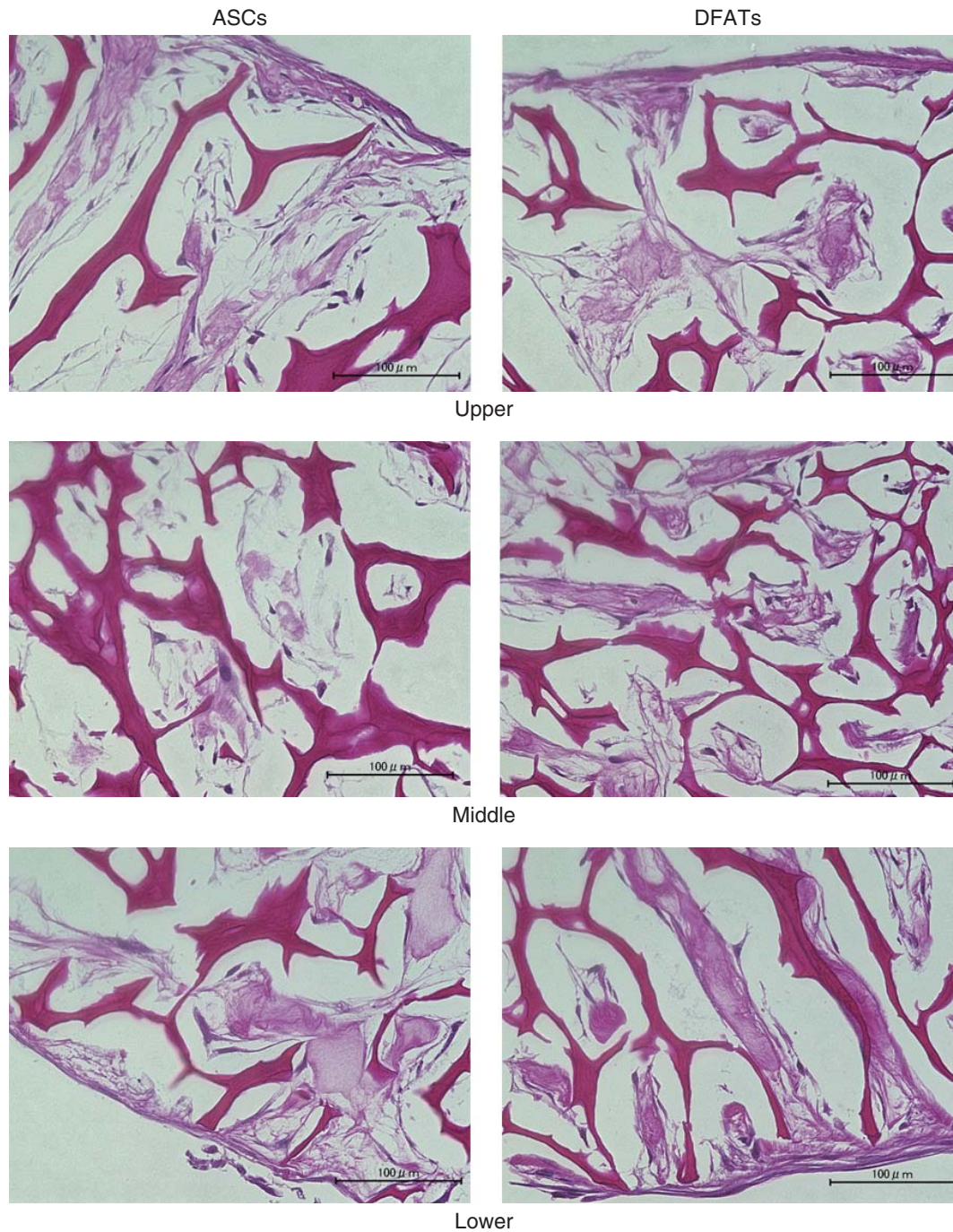
ASCs   DFATs

Upper

Middle

Lower

Fig. 2 Hematoxylin and eosin-stained sections after cell seeding showing ASCs and DFATs in the upper, middle, and the lower layers of construct.

HE staining

Microscopic examination of the HE-stained layers of the scaffold (lower, middle and upper) one week after seeding revealed that the cells in the 3D construct were evenly embedded in the collagen scaffold, with no cell leakage and collagen breakage after cell seeding (Fig. 2).

Chondrocyte differentiation and embryonic stem cell marker expression

Expression of chondrocyte differentiation markers such as aggrecan, collagen type 2, and Sox 9, was higher in DFATs than in ASCs at 14 and 21 d of culture ($p < 0.05$, Fig. 3). The levels of the embryonic stem cell markers Nanog, SOX2, and OCT4 were 6-, 29-, and 1140-fold higher, respectively, in DFATs than in ASCs at 72 h of culture ($p < 0.05$, Fig. 4).

Western blot analysis

Figure 6 shows the western blot results for aggrecan expression in ASCs and DFATs cultured for 1, 2 or 3 weeks. Aggrecan levels were approximately 1.5-, 2.4-, and 1.3-fold higher in DFATs than in ASCs at 1, 2 and 3 weeks, respectively (Fig. 5).

Alcian blue staining

Following 3D culture for 7, 14 and 21 days, Alcian blue staining revealed deposition of a glycosaminoglycan-rich matrix surrounding ASCs and DFATs (Fig. 6). Both ASCs and DFATs showed high amounts of self-produced matrix, and the strength of staining did not differ between ASCs and DFATs.
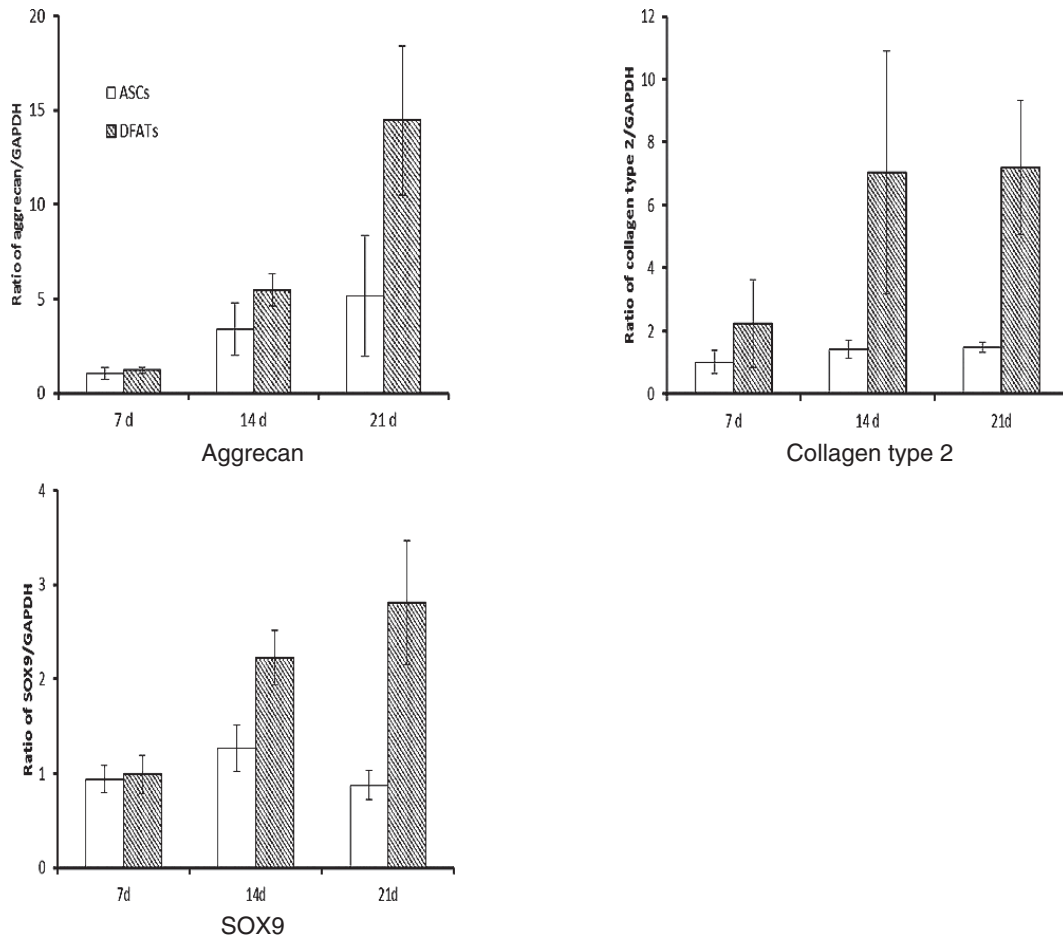

Fig. 3   Chondrocyte differentiation of ASCs and DFATs after 7, 14 and 21 d of culture. Gene expression of aggrecan, collagen type 2, and SOX9 are normalized against that of GAPDH as an internal control (*$p < 0.05$).
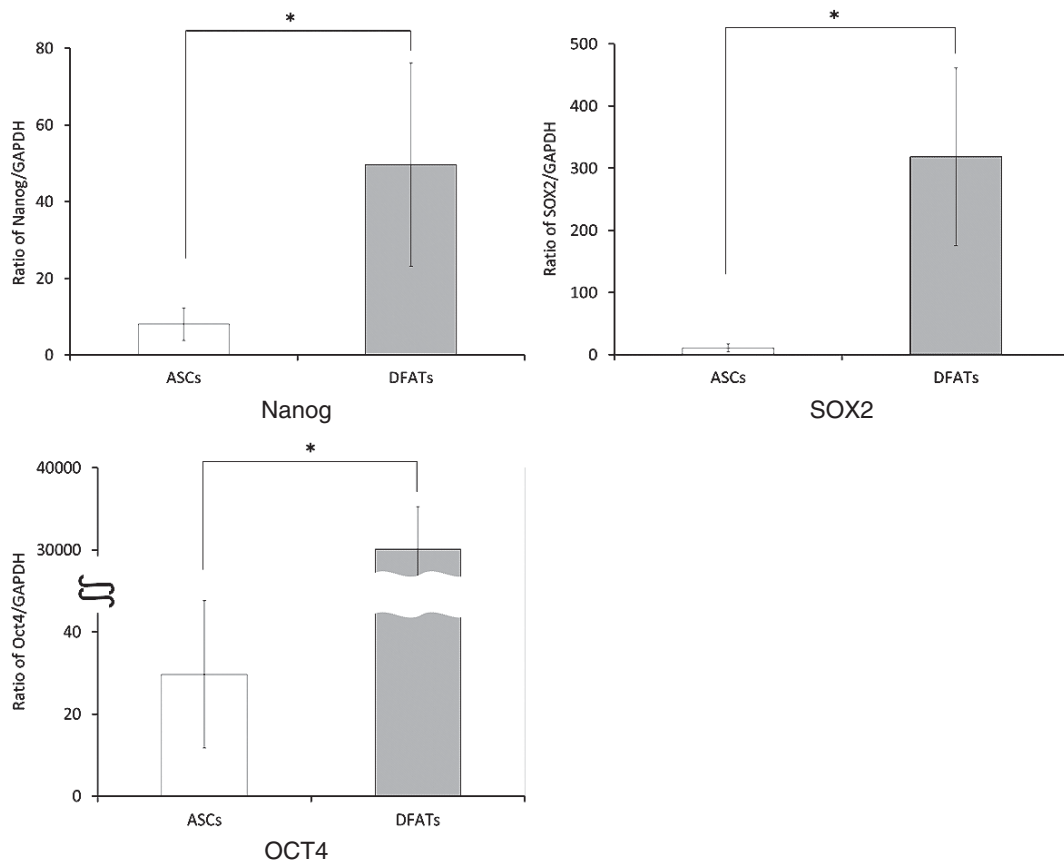
Fig. 4   Gene expression of the embryonic stem cell markers Nanog, Sox2 and Oct4 after 72 h normalized against that of GAPDH as an internal control (*p＜0.05).

Immunohistochemical staining of aggrecan

Figure 7 shows the results of immunohistochemical staining for aggrecan in ASCs and DFATs cultured for 7, 14 or 21 days. Increased aggrecan expression was observed for both ASCs and DFATs, confirming an increase in protein synthesis. Aggrecan antibody positivity was observed in the extracellular matrix for both ASCs and DFATs. However, similar to the results of Alcian blue staining, the strength of the staining did not differ between ASCs and DFATs.

DISCUSSION

Two general types of stem cells are potentially useful for tissue engineering applications : embryonic stem cells (ESCs) and autologous stem cells. Although ESCs have been successfully converted *in vitro* into various tissues or organs because of their pluripotentiality, the practical use of ESCs is limited because of potential problems of ethics, immunorejection, and tumorigenesis. In contrast, autologous stem cells are immunocompatible and have no associated ethical issues. For engineering of mesodermally derived tissues, autologous stem cells have been obtained from bone marrow and adipose tissue. However, differentiated cells frequently contaminate the stem cell populations.[14, 15] Therefore, several passages are usually needed to eliminate such contamination.[14, 16]

In contrast, DFATs contain almost no other cell types even at the first passage. This property of DFATs may lead to higher safety and efficacy for clinical cell therapies.[6] Mature adipocytes, which are the source of DFATs dedifferentiated into fibroblast-like cells by an *in vitro* dedifferentiation strategy known as ceiling culture, are the most abundant cell type in adipose tissue.[6] DFATs also have recently been shown to differentiate into multiple mesodermal linages.[6] The
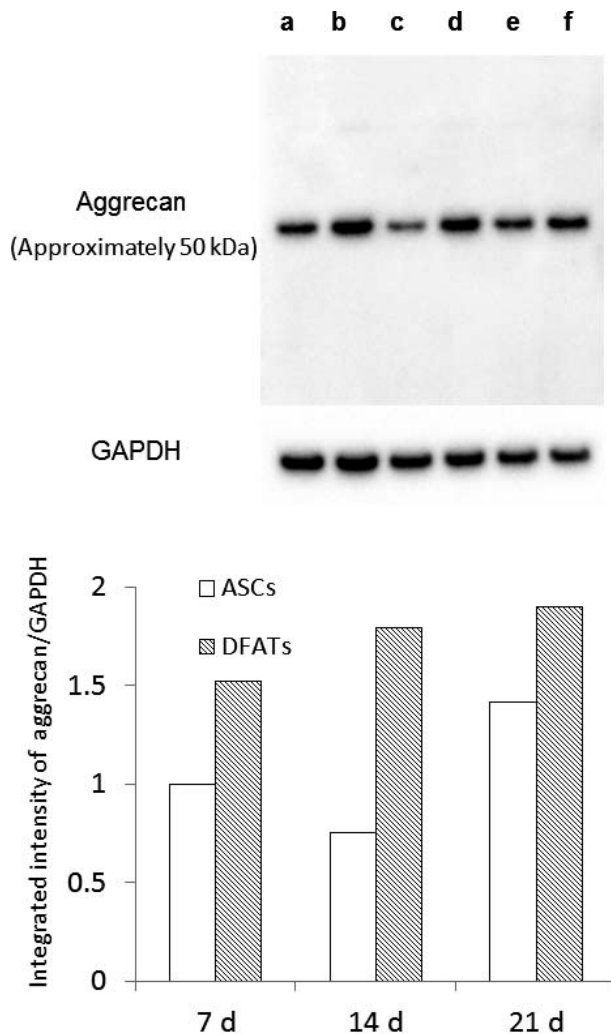
Fig. 5 Expression of aggrecan following chondrocyte differentiation of ASCs and DFATs after 7, 14 and 21 d of culture by western blot analysis. Chondrocyte differentiation of ASCs is shown at 7 d (a), 14 d (c) and 21 d (e), while differentiation of DFATs is shown at 7 d (b), 14 d (d), and 21 d (f). The expression of aggrecan was normalized against that of GAPDH as an internal control.

it shows good biocompatibility and absorbability.[12] However, *in vitro* experiments have revealed that the mechanical strength of the atelocollagen hydrogel is insufficient,[18] even after biological enhancement of matrix production by chondrocytes within the hydrogel. Akamine *et al.* reported that following seeding of human synovial cells onto the same atelocollagen sponge used in this study, neither cell leakage nor tissue destruction of the 3D construct occurred after loading at 40 kPa.[19] In this study, HE staining revealed uniform seeding and growth of DFATs and ASCs cultured within the atelocollagen sponge for one week.

Addition of chondrocyte supplements such as L-ascorbic acid-2-phosphate and transforming growth factor to DFATs cultures can induce chondrogenesis. Real-time RT-PCR analysis showed that the cultured DFATs expressed the chondrocyte markers Sox 9, aggrecan, and collagen type 2, suggesting that DFATs retain the properties of chondrocyte lineage-committed progenitor cells. Matsumoto *et al.* demonstrated that monolayer cultures of DFATs expressed the chondrocyte markers Sox 9 and aggrecan on day 21;[6] however, the expression ratio of these genes at this time point was lower than that on day 14. In contrast, in this study, expression of chondrocyte-specific markers remained high in the atelocollagen sponge-cultured DFATs on day 21. DFATs may present different functional phenotypes depending on the physical environment within a 3D structure. Alcian blue staining was used to visualize the presence of sulfated glycosaminoglycans and collagen within the constructs.[20] Aggrecan is essential for the normal functioning of articular cartilage and intervertebral discs, where it allows the tissues to withstand compressive loading.[21] These histological findings showing production of aggrecan, sulfated glycosaminoglycans, and collagen indicate the formation of a functional extracellular matrix. Quantitative analysis using western blotting showed that aggrecan expression of DFATs cultured in the atelocollagen sponge was higher than that of similarly cultured ASCs. These results suggest that this carrier is a suitable scaffold for DFATs and will be useful as a 3D chondrocyte tissue engineering scaffold.

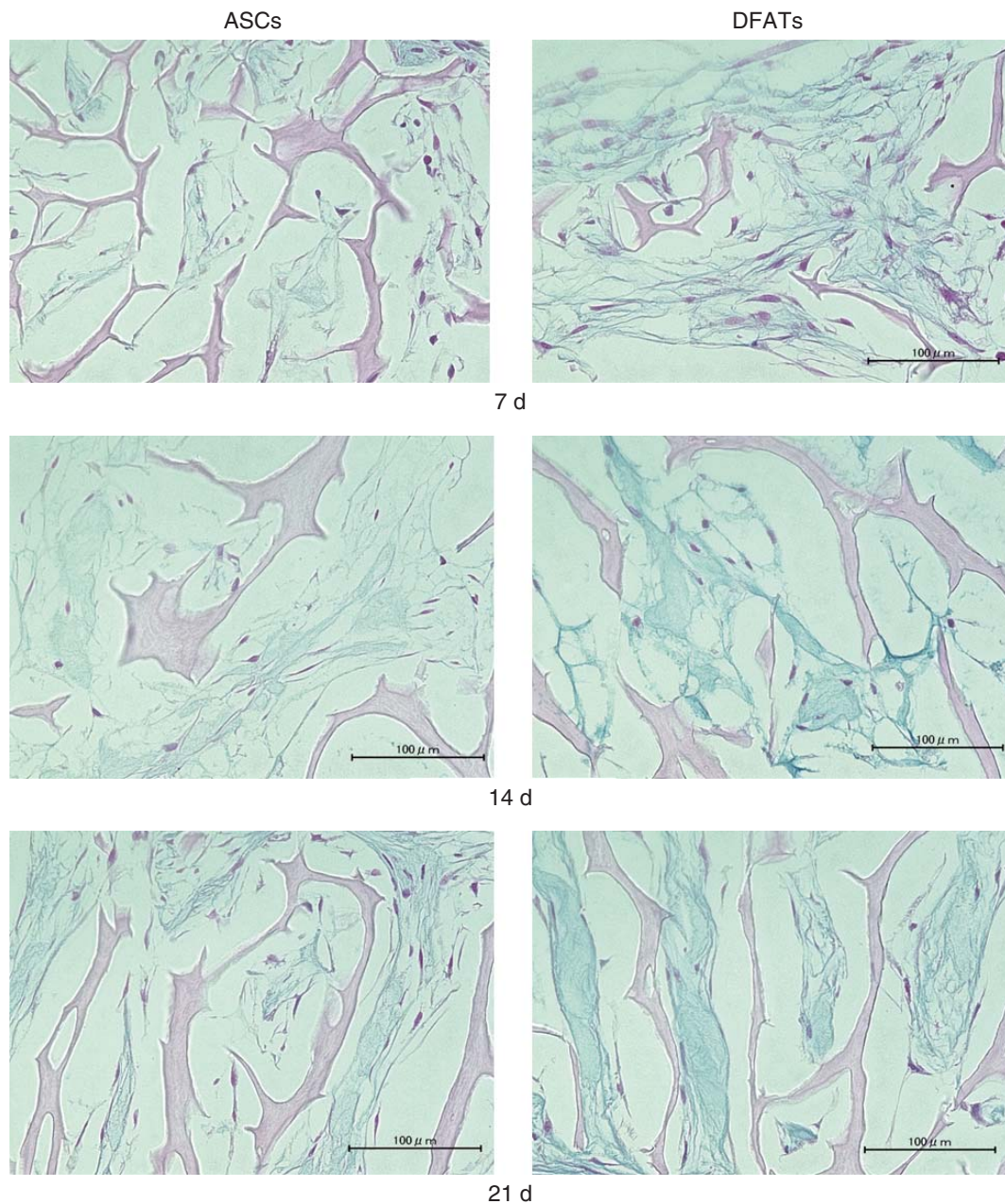ASCs and DFATs are an attractive cell source for

characteristics of the ASCs and DFATs used in this study were demonstrated using FACS analysis, and both cell types were positive for representative stem cell markers at the cell surface (i.e., CD 90 and CD 105), but negative for CD 34, CD 11 b, and CD 45.[9] Matsumoto *et al.* demonstrated that the expression profiles of surface antigens were essentially the same for DFATs and ASCs.[6]

Atelocollagen has been conventionally applied clinically in various forms such as gels or sponges as

ASCs   DFATs

7 d

14 d

21 d

Fig. 6   Alcian blue-stained sections of ASCs and DFATs after culture for 7, 14 and 21 d.

regenerative medicine because of their high proliferation rate and multilineage differentiation capacity. In this study, the chondrogenic differentiation capacity of DFATs was superior to that of ASCs. We speculate that differential expression patterns of embryonic stem cell markers contribute to this property. Embryonic stem cell markers form a regulatory circuit that involves the transcription factors OCT4, SOX2 and Nanog,[22] and is responsible for stem cell self-renewal and differentiation. In the present study, the expression of OCT4, SOX2, and Nanog in the cultured DFATs was dramatically higher than that in the ASCs. Gao *et al.* reported that whereas DFATs expressed some ESC markers and were a candidate source of stem cells such as ASCs, the expression level of embryonic stem cell markers in DFATs was
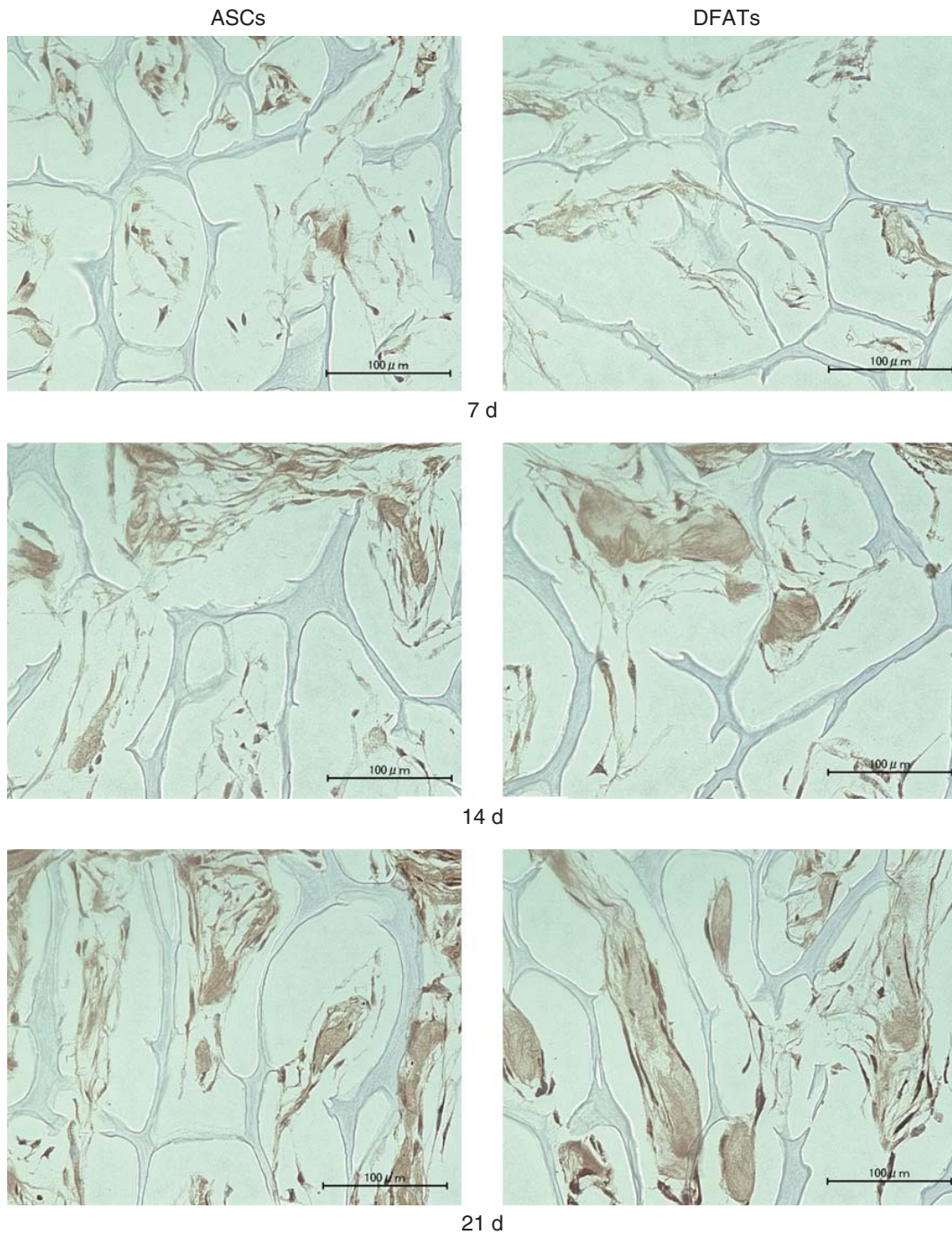
ASCs   DFATs

7 d

14 d

21 d

Fig. 7   Immunohistochemical staining for aggrecan expression of ASCs and DFATs after culture for 7, 14 and 21 d in chondrocyte differentiation medium.

slightly lower than that in ASCs.[22] This discrepancy between studies may have resulted from differences between monolayer and 3D cultures. Biomaterials can provide a 3D culture environment to mimic the physiological microenvironment and guide differentia-

tion of stem cell populations.[23–25] Therefore, we speculate that in 3D culture, spontaneous reprogramming may occur in DFATs, whereas it may not occur in ASCs.

The present study revealed that the chondrocyte

differentiation ability of DFATs is higher than that of ASCs. This study also histologically revealed that under the 3D culture conditions provided by the atelocollagen sponge, DFATs differentiate into chondrocytes as early as day 7 of culture to form cultured cartilage *in vitro.* Therefore, the combined use of DFATs and an atelocollagen sponge may be an attractive option for cartilage tissue engineering. DFATs combined with an atelocollagen sponge may thus be useful for maxillofacial reconstruction. Further *in vivo* studies of cartilage tissue engineering are necessary.

Our previous study was the first to describe DFATs isolated from BFP, which offer the advantage of being easily accessible.[9] Harvesting of BFP cells is a simple procedure that requires a minimal incision with local anesthesia and results in minimal donor site morbidity. In the present study, DFATs isolated from the BFP were uniformly seeded and cultured in an atelocollagen sponge and differentiated *in vitro* to yield cartilage tissue. The shape and pore size of the collagen scaffolds enabled them to be used for high-density cell culture and also to allow diffusion of the newly formed extracellular matrix to provide a better environment for cell-cell and cell-matrix communication, and help regulate chondrocyte differentiation. In addition, the chondrocyte differentiation ability of DFATs from the BFP was greater than that of ASCs. Overall, these studies provide evidence that DFATs from the BFP are an ideal cell source for cartilage tissue engineering.

Conflict of interest statement
The authors declare that they have no conflict of interest.

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