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Original article

Gene expression profiles of early chondrogenic markers in dedifferentiated fat cells stimulated by bone morphogenetic protein 4 under monolayer and spheroid culture conditions *in vitro*

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ABSTRACT

Purpose: Human dedifferentiated fat (hDFAT) cells are thought to be a promising cell source for cartilage regeneration therapy. Nevertheless, the responses of hDFAT cells to bone morphogenetic proteins (BMPs) are still unclear. Here, we elucidated the effects of BMP-4 on the mRNA expression of early chondrogenic markers in hDFAT cells under monolayer or pellet cell culture conditions.

Materials and methods: Monolayer and pellet cell cultures of hDFAT cells were grown with control medium or chondrogenic medium (CM) with or without BMP-2, BMP-4, or BMP-7. Real-time polymerase chain reaction was used to analyze the mRNA expression levels of nine genes: chondrogenic markers, *i.e.*, *SOX9*, *SOX5*, *SOX6*, aggrecan, type 2 collagen, type 10 collagen, and matrix metalloproteinase (MMP) 13; type 1 collagen; and *MMP3*. The BMP signaling inhibitor dorsomorphin was used to verify the mechanisms of BMP-4-induced chondrogenesis.

Results: Recombinant BMP-4 (100 ng/mL) increased the expression of *SOX9*, *SOX6*, and aggrecan mRNAs in monolayer cells compared with that in cells treated with BMP-2 or BMP-7 on day 3. Chondrogenically differentiated hDFAT cells induced by CM containing BMP-4 showed higher expression of eight genes (excluding *SOX5*) in monolayer cultures and nine genes (including *SOX5*) in pellet cultures compared with those in control medium on day 14. Dorsomorphin attenuated the effects of BMP-4.

Conclusion: These results showed that BMP-4 had the potential to modulate the early chondrogenesis of hDFAT cells under both monolayer and pellet cell culture conditions.

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1. Introduction

Injuries and congenital morphological anomalies of cartilage, such as cleft lip and palate, cause deleterious effects on facial morphology and decrease the quality of life of patients [1,2]. Currently, autocartilage, bone grafts, and synthetic materials have been used as conventional reconstructive materials for the treatment of such diseases [1,2]. However, these materials have potential disadvantages, such as lack of durability, absorption, calcification, and inflammation [1]. Regenerative therapy using stem cells has attracted much attention as a prospective method to supply chondrocytes [1], which might be beneficial in the management of the above diseases.

Dedifferentiated fat (DFAT) cells exhibit a fibroblastic cell shape and can be obtained from mature adipocytes using ceiling [3] and gel culture techniques [4]. Similar to bone marrow-derived mesenchymal stem cells (BMSCs) [5] and adipocyte-derived stem cells (ADSCs) [3], DFAT cells can differentiate into multiple lineages, such as osteoblasts [3,6–14], adipocytes [3,14], myocytes [13–16], endothelial cells [17], and chondrocytes [3,18,19], under specific cell culture conditions *in vitro*. Although DFAT cells and ADSCs are isolated from the same fat tissues, DFAT cells differentiate into osteoblasts much earlier than BMSCs and ADSCs [8,20]. Moreover, these cells exhibit higher adipogenic efficiency [21]. Despite these advantages of hDFAT cells for regenerative therapies, application of hDFAT cells in cartilage regeneration has not been extensively investigated, and the chondrogenic differentiation process in these cells has not been fully elucidated.

Chondrogenic differentiation of BMSCs and ADSCs *in vitro* has been widely investigated to obtain chondrocyte by application of a variety of growth factors and synthetic glucocorticoid [22–26]. In particular, combinations of transforming growth factor (TGF)-β3, and other growth factors, such as basic fibroblast growth factor (bFGF) and insulin-like growth factor (IGF), are potent cocktails that can enhance the chondrogenic differentiation of mesenchymal stem cells (MSCs) [22]. Similar to these growth factors, bone morphogenetic proteins (BMPs) are known to stimulate chondrogenesis in MSCs *in vitro* [22,27,28]. BMP-4 enhances cartilage formation and regulates chondrogenic differentiation in adult stem cells [28]. Nevertheless, little information is available regarding the effects of BMPs on the early chondrogenic differentiation of hDFAT cells *in vitro*.

Given the similarity of DFAT cells to BMSCs and ADSCs in terms of multipotency, we hypothesized that the recombinant BMP-4 could also be an effective stimulant for modulating the chondrogenic differentiation of hDFAT cells, which could be applicable in preparing DFAT-derived chondrocytes for stem cell-based cartilage regeneration. To improve our understating of the mechanisms of chondrogenic differentiation in hDFAT cells *in vitro*, we investigated the mRNA expression profiles of monolayer and pellet cell cultures of hDFAT cells undergoing chondrogenic differentiation using chondrogenic medium (CM) containing BMP-4 *in vitro*. BMP-2 and BMP-7 were also used to compare the early chondrogenic capability of BMP-4. Dorsomorphin (DM), a specific BMP signaling inhibitor, was applied to verify the mechanisms underlying the chondrogenic differentiation of hDFAT cells treated with BMP-4.

2. Materials and methods

2.1. Reagents

Recombinant hTGF-β3, bFGF, hBMP-2, and hBMP-7 were purchased from PeproTech (Rocky Hill, NJ, USA). L-proline, L-ascorbic acid 2-phosphate, dexamethasone, and DM were purchased from Sigma (St. Louis, MO, USA). BMP-4 was obtained from HumanZyme (Chicago, IL, USA). ITS-premix was purchased from Corning Inc. (NY, USA).

2.2. Isolation and culture of hDFAT cells

This study conformed to the tenets of the Declaration of Helsinki, and the protocol was approved by the ethics committees of Osaka Dental University and Amagasaki Chuo Hospital (approval numbers: 110760 and 110790). The hDFAT cells used in the present study were prepared using the ceiling culture technique [3,18]. Briefly, fat tissues were obtained from a healthy 63-year-old man who underwent oral and maxillofacial surgery. Isolated mature adipocytes contained in the fat tissue were seeded into a converted culture flask completely filled with growth medium. The floating adipocytes attached to the inner ceiling of the flask. One week later, the flask was inverted to remove the residual mature adipocytes. Fibroblast-like cells attached to the bottom surface were designated DFAT cells. Surface markers of the original cells have been evaluated using fluorescence-assisted cell sorting (FACS): CD90 and CD105 were regarded as positive, while CD34 and CD45 were regarded as negative [18]. These surface markers showed tendencies similar to those of surface markers on DFAT cells reported in previous studies [3,21]. The isolated DFAT cells were maintained in the medium, consisting of Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich) with 10% fetal bovine serum (FBS) and antibiotics in an incubator at 37 °C with 5% CO_2. The cells were used at passages 6–9. Images of cell morphology were obtained using an optical microscope (IX70; Olympus, Tokyo, Japan).

2.3. Chondrogenic differentiation

To compare the effects of BMPs on early chondrogenic differentiation of hDFAT cells, we stimulated the cells by using control (CNTL) medium or using CM without or with different concentrations of BMPs (Fig. 1). For monolayer cell cultures, hDFAT cells were seeded at 1.5×10^4 cells/cm^2 in 24-well plates and were cultured in CNTL medium, consisting of DMEM with 1% FBS, antibiotics, and 5 ng/mL bFGF. From one day after cell seeding, the cells were cultured in CNTL medium or CM with or without 10 or 100 ng/mL BMPs for up to 14 days. CM consisted of CNTL medium with 10 ng/mL TGF-β3, 40 μg/mL L-proline, 1 × ITS premix, 15 μM L-ascorbic acid 2-phosphate, and 100 nM dexamethasone. The media were changed at every 3–4 days. For the pellet culture, hDFAT cells were seeded at 100×10^4 cells/tube in cryotubes (Thermo Fisher Scientific Inc., MA, USA) and were cultured in 1 mL CNTL

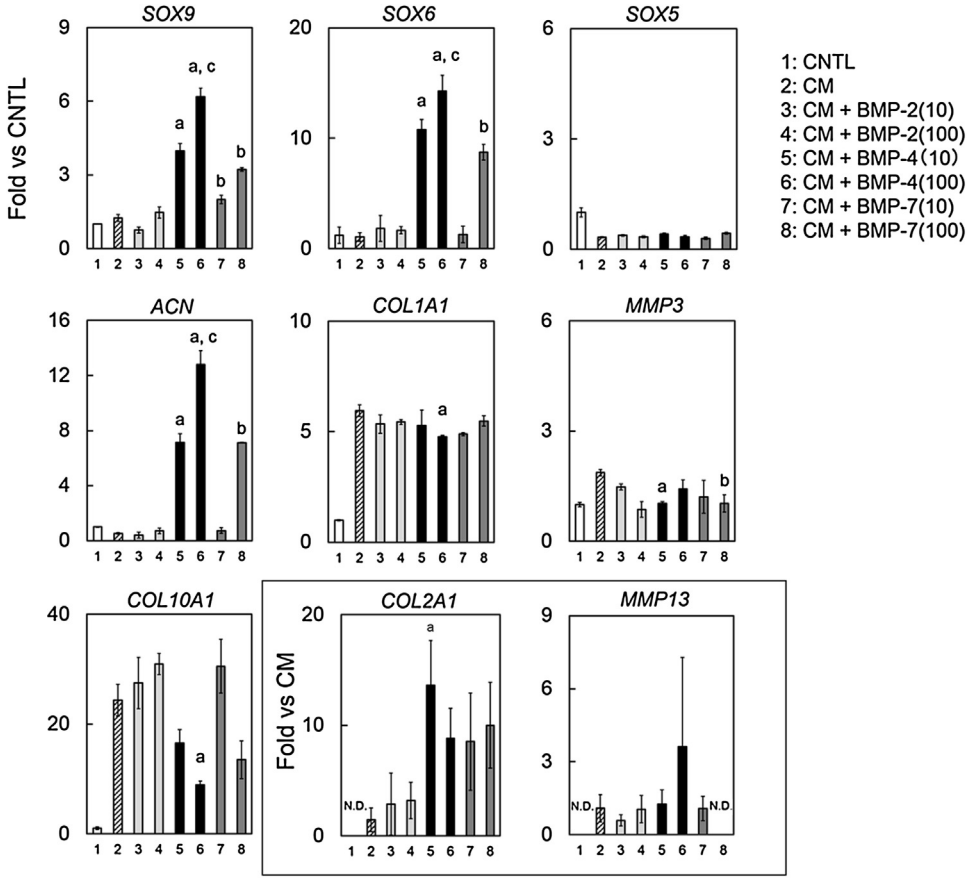

Fig. 1 – mRNA expression profiles of monolayer hDFAT cells undergoing chondrogenic differentiation for 3 days. Cells were treated with or without chondrogenic medium for 3 days. CNTL: control medium; CM: chondrogenic medium; BMP: bone morphogenetic protein; SOX: SRY-box; ACN: aggrecan; COL: collagen; MMP: matrix metalloprotease. Parentheses after BMPs: concentrations of BMPs (ng/mL). Cells were seeded at 1.5×10^4 cells/cm^2 and treated with CNTL medium, CM, or CM+BMPs for 3 days. a: $P<0.05$, CM *vs* CM+BMP-4 (10 or 100 ng/mL); b: $P<0.05$, CM *vs* CM+BMP-7 (10 or 100 ng/mL); c: $P<0.05$, CM+BMP-4 (100 ng/mL) *vs* others (ANOVA with Scheffe test). The bar graphs show means with standard deviations (SDs; n = 3). N.D.: data not available.

medium. From one day after cell seeding, the cells were cultured in CNTL medium or CM with 100 ng/mL BMP-4 for up to 14 days. Half of the medium was exchanged every 3–4 days to avoid removal of the pellet and to add fresh medium.

2.4. *Quantitative real-time polymerase chain reaction (qPCR)*

On day 3 and day 14, total RNA was isolated using an RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA). Reverse transcription was carried out using Transcriptor Universal cDNA Master mix (Roche Diagnostics, Mannheim, Germany). mRNA levels of chondrogenic markers and other genes were evaluated by qPCR using TaqMan Gene Expression Assays (Thermo Fisher Scientific Inc.) and Step One Plus PCR system (Thermo Fisher Scientific Inc.). The assay IDs of TaqMan Gene Expression Assays and the accession numbers for each gene are as follows: SRY-box 9 (*SOX9*), Hs01001343_g1, NM_000346.3; *SOX5*, Hs00753050_s1, NM_001261414.1; *SOX6*, Hs00264525_m1, NM_001145811.1; collagen type 2 alpha 1 (*COL2A1*), Hs00264051_m1, NM_033150.2; collagen type 1 alpha 1 (*COL1A1*), Hs00164004_m1, NM_000088; aggrecan (*ACN*), Hs00202971_m1, NM_013227.3; collagen type X alpha 1 (*COL10A1*), Hs00166657_m1, NM_000493.3; matrix metalloproteinase 3 (*MMP3*), Hs00968305_m1, NM_002422.3; and *MMP13*, Hs00233992_m1, NM_002427.3. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as an internal standard (human GAPDH endogenous control; Thermo Fisher Scientific Inc.). The PCR cycling conditions were as follows: 2 min at 50 °C, 20 s at 95 °C, and 45 cycles of 1 s at 95 °C and 20 s at 60 °C. The signals were normalized to the *GAPDH* signal. mRNA expression levels were calculated using the comparative CT method.

2.5. *Immunostaining*

On day 14, cells in the monolayer culture were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), followed by incubating with primary antibodies (SAB 4500662 for aggrecan and SAB 4500366 for collagen 2; Sigma-Aldrich) produced in rabbits. Secondary antibody staining was performed using a VectaFluor Excel Amplified Fluorescent

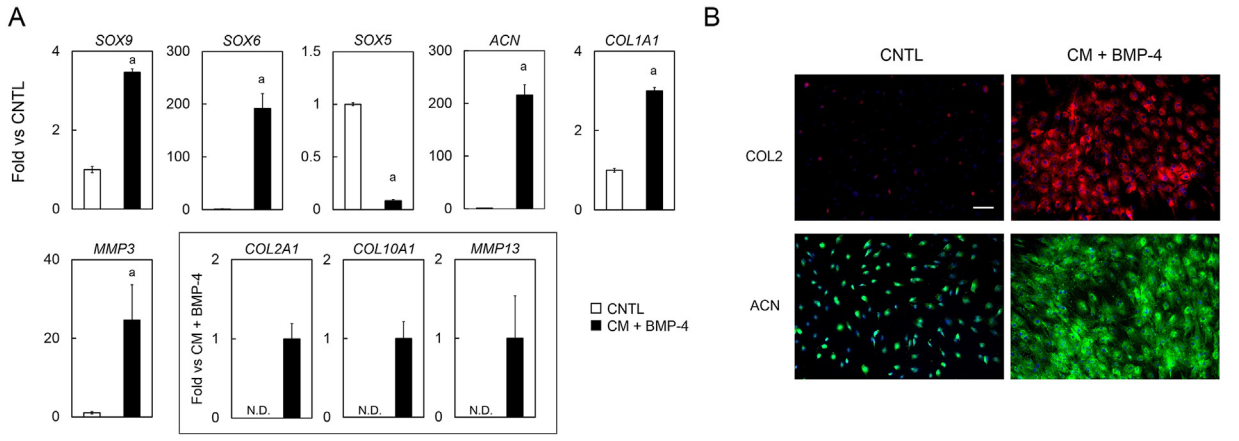

Fig. 2 – mRNA expression profiles (A) and immunostaining (B) of monolayer hDFAT cells undergoing chondrogenic differentiation for 14 days. CNTL: control. CM: chondrogenic medium. (A and B): cells were seeded at 1.5×10^4 cells/cm^2 and treated with CNTL medium or BMP-4-containing CM for 14 days. a: $P < 0.05$, analyzed with Student's t-tests (*vs* CNTL). The bar graphs show the means and SDs (n=3). Bar: 100 μm.

Staining Kit (#DK-1488 for aggrecan [green color] and #DK-1594 for type 2 collagen [red color]; Vector Laboratories Ltd., Burlingame, CA, USA) according to the manufacturer's instructions. Nuclei were stained with Cellstain^R-DAPI solution (Dojindo Laboratories, Kumamoto, Japan). All immunofluorescence images were captured using a ZOE Fluorescent Cell Imager (Bio-Rad, Hercules, CA, USA).

2.6. *Inhibitor assays*

To verify the mechanisms underlying the effects of BMP-4 on the chondrogenic differentiation of hDFAT cells, we used DM, the BMP signaling inhibitor [29]. The chondrogenic differentiation conditions with monolayer cells were applied in inhibitor assays. One day after cell seeding, cells were treated with CM or CM containing 100 ng/mL BMP-4 with or without 1 μM DM for 14 days.

2.7. *Statistical analysis*

Numerical data are represented as means and standard deviations (SDs). Statistical significance was assessed with Student's *t*-tests or one-way analysis of variance (ANOVA) followed by Scheffe methods. Statistical analysis was performed using Microsoft Excel software statistic package. Differences with *P* values of less than 0.05 were considered significant.

3. Results

3.1. *Chondrogenic differentiation under monolayer culture conditions*

qPCR analysis indicated that treatment of cells with BMP-4 and BMP-7 significantly enhanced the expression of *SOX9*, *SOX6*, and *ACN* on day 3 (Fig. 1). BMP-4 (100 ng/mL) in CM induced the highest expression of *SOX9*, *SOX6*, and *ACN*. The *ACN* expression pattern was similar to those of *SOX9* and *SOX6*. Additionally, *SOX5* expression decreased by the chondrogenic differentiation conditions used in this study (*i.e.*, both CM and CM with BMPs). In contrast to BMP-2, BMP-4 and BMP-7 decreased *COL10A1* expression in a concentration-dependent manner.

Based on the results shown in Fig. 1, we next sought to confirm the long-term effects of BMP-4 on chondrogenic differentiation in hDFAT cells under monolayer cell culture conditions for up to 14 days. Fig. 2 shows the gene expression profiles of hDFAT cells cultured in CNTL medium or CM with 100 ng/mL BMP-4 for 14 days. The cells stimulated with CM containing BMP-4 showed increased expression of chondrogenic markers, *i.e.*, *SOX9*, *SOX6*, *ACN*, *COL2A1*, *COL10A1*, and *MMP13*, compared with that in cells cultured with CNTL medium (Fig. 2A). The results of immunofluorescence staining revealed stronger staining of ACN and type 2 collagen in the cells treated with CM containing BMP-4 than in the cells treated with CNTL medium (Fig. 2B). Nevertheless, *SOX5* expression was still lower in cells treated with CM containing BMP-4 than that in cells treated with CNTL medium. Taken together, these results suggested that CM containing BMP-4 induced early chondrogenic differentiation in monolayer hDFAT cells, but inhibited *SOX5* expression.

3.2. *Inhibition of BMP signaling*

Changes in the shapes of cells stimulated with DM at concentrations of up to 1 μM were negligible (Fig. 3A). However, the addition of DM in BMP-4-containing CM significantly inhibited the enhancement of *SOX9* and *ACN* expression and cancelled the inhibition of *COL10A1*, *MMP3*, and *MMP13* expression (Fig. 3B).

3.3. *Chondrogenic differentiation under pellet culture conditions*

A previous study showed that chondrogenesis is elevated in hADSCs under pellet culture conditions [25]. To verify that chondrogenic differentiation induced by BMP-4 still occurred

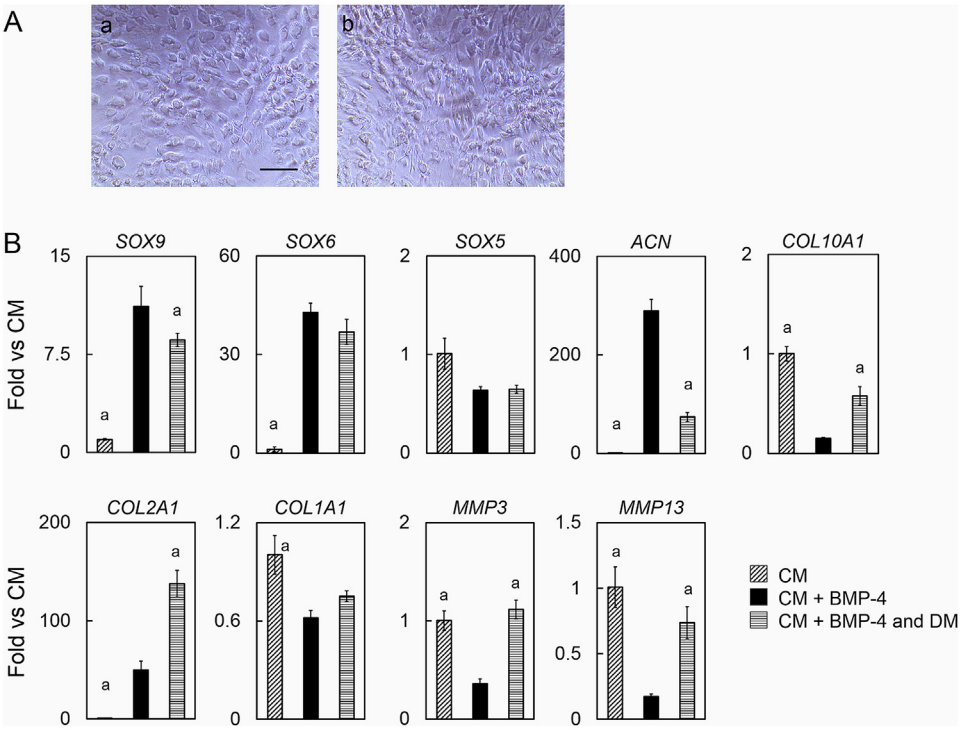

Fig. 3 – Inhibitory effects of BMP signaling on CM+BMP-4-induced chondrogenic differentiation of hDFAT cells in monolayer cell cultures. (A) Phase contrast images of the cells. (B) mRNA expression. The cells were treated with CM or CM containing 100 ng/mL BMP-4 with or without 1 μM dorsomorphin (DM) for 14 days. CM: chondrogenic medium. (A-a): cells treated with CM containing 100 ng/mL BMP-4. (A-b): cells treated with CM containing 100 ng/mL BMP-4 and 1 μM DM. Bar: 50 μm. (B) a: $P<0.05$, analyzed with ANOVA with Scheffe test (*vs* BMP-4-containing CM). The bar graphs show the means and SDs (n=3).

under three-dimensional cell culture conditions, similar to that under monolayer culture conditions, hDFAT cells were treated with CNTL medium or CM containing 100 ng/mL BMP-4 under pellet culture conditions (Fig. 4). CM with BMP-4 robustly enhanced the expression of the chondrogenic markers. In contrast to cells grown under monolayer culture conditions,

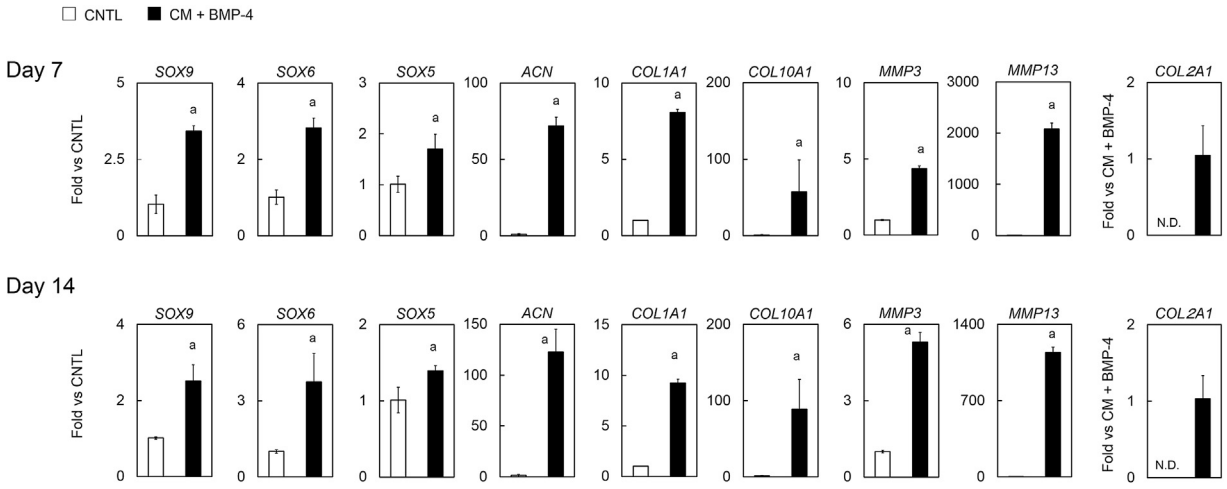

Fig. 4 – mRNA expression profiles of pellet cell cultures of hDFAT cells undergoing chondrogenic differentiation for 14 days. CNTL: control; CM: chondrogenic medium. Cells were seeded at 100×10^4 cells/tube and treated with CNTL medium or CM containing 100 ng/mL BMP-4 for 14 days. a: $P<0.05$, analyzed with Student's *t*-tests (*vs* CNTL). The bar graphs show the means and SDs (n=3).

SOX5 expression in cells treated with CM containing BMP-4 under pellet culture conditions was slightly elevated compared with that in cells grown in CNTL medium (Figs. 2 and 4).

4. Discussion

BMPs exhibit multiple functions in gastrulation, organogenesis, mesoderm induction, embryogenesis, bone formation, and cartilage formation [29]. BMP-2, BMP-4, and BMP-7 have been reported to modulate chondrogenesis [22,26]. However, few studies have evaluated the functions of BMPs in the chondrogenesis of hDFAT cells *in vitro*. In the present study, we verified that BMP-4, when used with CM, modulated the chondrogenic differentiation of hDFAT cells under both monolayer and pellet culture conditions *in vitro*.

Recombinant BMP-4 has been shown to play an important role in promoting early cartilage differentiation in the embryonic limb bud mesenchyme in mice [30] and in MSCs [27]. Moreover, this factor has also been shown to suppress chondrocyte hypertrophy [28]. Consistent with these previous results, we found that administration of BMP-4 in CM significantly enhanced the expression of chondrogenic markers, such as *SOX9*, *SOX6*, and *ACN*, in hDFAT cells much earlier than those induced by BMP-2 and BMP-7 under monolayer conditions (Fig. 1). Conversely, the expression of *COL10A1*, a hypertrophic marker, was strongly inhibited by BMP-4. These results suggest that recombinant hBMP-4 may be a superior inducer of early chondrogenesis but an inhibitor of chondrogenic hypertrophy in hDFAT cells compared with the other two BMPs.

Pellet cell culture is a conventional technique used to differentiate stem cells into chondrocytes for cartilage regeneration therapy [25]. However, in general, pellet cell culture requires a large number of cells [25], which may represent a major bottleneck for the future application of chondrogenically differentiated hDFAT cells for large cartilage defects. Exploring an effective technique with small numbers of cells, such as cell sheet technology (layered sheets of monolayer cells) [31], may represent a prospective approach for cartilage treatment using hDFAT cells. Thus, in this study, we cautiously investigated chondrogenic differentiation of hDFAT cells under the monolayer culture condition in addition to the pellet condition. However, SOX5 expression was completely suppressed in monolayers of hDFAT cells treated with CM with or without BMPs, including BMP-4 (Fig. 1). SOX5 and SOX6 have been reported to function as co-activators of SOX9, the master transcription factor involved in chondrogenesis, in order to enhance the secretion of cartilage extracellular matrix components [32]. Indeed, Ikeda reported that transfection of SOX trio (SOX9, SOX6, and SOX5) genes significantly enhanced the chondrogenesis of hMSC cells compared with that of cells transfected with each SOX alone [33]. Based on these results, further studies are needed to explore costimulants of *SOX5* expression in order to enhance the chondrogenic differentiation of monolayers of hDFAT cells; such studies may be applicable for preparing robust chondrocyte cell sheets [31].

In pellet culture, the expression of *SOX5* was much higher in the cells treated with CM containing BMP4 than that in cells treated with CNTL medium for up to 14 days (Fig. 4). These results may provide insights into the increased expression of *SOX5* in monolayer cultures of cells grown in CM containing BMP-4. Pellet cell culture is generally superior to monolayer culture with regard to cell–cell interactions and hypoxic condition, resulting in enhanced cartilage formation [25,34]. In our previous study, we showed that alterations in cell seeding density do not significantly affect *SOX9* and *COL2A1* expression in hDFAT cells [18]. Additionally, Khan et al. reported that hypoxic conditions increase *SOX5* expression in stem cells isolated from the infrapatellar fat pad [34]. These results suggest that the combination of hypoxic conditions and BMP-4 administration could be applicable for enhancement of *SOX5* expression in hDFAT cells grown in monolayer cultures.

Recombinant BMP-4 is thought to have a potential to enhance the osteogenic differentiation [35]. Type 1 collagen is a well-known early marker of osteoblast differentiation [36]. In the present study, the expression of *COL1A1* was elevated in the cells stimulated by CM with/without BMPs on day 3 (Fig. 1). However, there was negligible difference among the cells stimulated using these media. Weiss et al. have reported that *COL1A1* expression in MSC increased with time during chondrogenic differentiation using TGF-β [22]. In accordance with these results, expression of *COL1A1* in hDFAT cells treated with CM containing BMP-4 could be attributed to chondrogenic differentiation.

Administration of DM in CM attenuated the effects of BMP-4 (Fig. 3B). Yu et al. reported that DM inhibits the BMP-mediated phosphorylation of SMAD1/5/8 [29]. Moreover, BMP-SMAD1/5 signaling is known to crosstalk with TGF-β signaling [37]. These results suggest that the chondrogenic capacity of BMP-4 in hDFAT cells could be related to the synergistic effects of BMP-4 and TGF-β3.

The principal limitation of our study was that one adipose tissue sample from one subject was used to isolate hDFAT cells. Herlofsen et al. have reported that human BMSCs from three different donors showed a similar mRNA expression profile during chondrogenic differentiation [38]. There might be some possibility that the effect of BMP-4 on the chondrogenic differentiation of hDFAT cells was robust; thereby, it was not disturbed by individual and inter-individual variation. However, to date, only limited studies have explored the mechanisms underlying the chondrogenic differentiation of hDFAT cells. Although our data may provide some clue to explore this mechanism, further investigation is imperative to elucidate the response of hDFAT cells stimulated by BMP-4.

5. Conclusion

In the present study, we demonstrated that BMP-4 regulates the early chondrogenic differentiation of hDFAT cells when used with a CM, as with BMSCs and ADSCs. This growth factor significantly induced the early expression of *SOX9*, *SOX6*, and *ACN* on day 3. BMP4-induced chondrogenically differentiated hDFAT cells robustly produced ACN and type 2 collagen protein, even under monolayer cell culture conditions on day 14. In contrast to the monolayer culture, *SOX5* expression was elevated in pellet cell cultures under CM containing BMP-4.

These findings provide insights into the chondrogenic differentiation of hDFAT cells, which may serve as a cell source for chondrocyte generation. However, DFAT cells used in the present study were isolated from one adipose tissue sample from one subject. Additional studies verifying the effect of individual and inter-individual variation must be conducted to develop reliable methods for obtaining chondrocytes from hDFAT cells for applications in cartilage regeneration therapy in patients with craniofacial injuries and abnormalities.

Conflict of interest

All authors declare that there is no conflict of interest.

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REFERENCES

[1] Kobayashi S, Takebe T, Inui M, Iwai S, Kan H, Zheng YW, et al. Reconstruction of human elastic cartilage by a CD44+ CD90+ stem cell in the ear perichondrium. Proc Natl Acad Sci U S A 2011;108:14479-84.
[2] Hoshi K, Fujihara Y, Asawa Y, Nishizawa S, Kanazawa S, Sakamoto T, et al. Recent trends in cartilage regenerative medicine and its application to oral and maxillofacial surgery. Oral Sci Int 2013;10:15-9.
[3] Matsumoto T, Kano K, Kondo D, Fukuda N, Iribe Y, Tanaka N, et al. Mature adipocyte-derived dedifferentiated fat cells exhibit multilineage potential. J Cell Physiol 2008;215:210-22.
[4] Huber B, Kluger PJ. Decelerating mature adipocyte dedifferentiation by media composition. Tissue Eng C Methods 2015;21:1237–45.
[5] Salem HK, Thiemermann C. Mesenchymal stromal cells: current understanding and clinical status. Stem Cells 2010;28:585-96.
[6] Justesen J, Pedersen SB, Stenderup K, Kassem M. Subcutaneous adipocytes can differentiate into bone-forming cells in vitro and in vivo. Tissue Eng 2004;10:381–91.
[7] Kishimoto N, Momota Y, Hashimoto Y, Ando K, Omasa T, Kotani J. Dedifferentiated fat cells differentiate into osteoblasts in titanium fiber mesh. Cytotechnology 2013;65:15-22.
[8] Kishimoto N, Momota Y, Hashimoto Y, Tatsumi S, Ando K, Omasa T, et al. The osteoblastic differentiation ability of human dedifferentiated fat cells is higher than that of adipose stem cells from the buccal fat pad. Clin Oral Investig 2014;18:1893-901.
[9] Kishimoto N, Momota Y, Mori M, Hashimoto Y, Imai K, Omasa T, et al. Bone regenration using dedifferentiated fat cells with pure matrix. J Oral Tissue Eng 2008;6:127–34.
[10] Nakamura T, Shinohara Y, Momozaki S, Yoshimoto T, Noguchi K. Co-stimulation with bone morphogenetic protein-9 and FK506 induces remarkable osteoblastic differentiation in rat dedifferentiated fat cells. Biochem Biophys Res Commun 2013;440:289-94.
[11] Shirakata Y, Nakamura T, Shinohara Y, Taniyama K, Sakoda K, Yoshimoto T, et al. An exploratory study on the efficacy of rat dedifferentiated fat cells (rDFATs) with a poly lactic-co-glycolic acid/hydroxylapatite (PLGA/HA) composite for bone formation in a rat calvarial defect model. J Mater Sci Mater Med 2014;25:899-908.
[12] Oki Y, Watanabe S, Endo T, Kano K. Mature adipocyte-derived dedifferentiated fat cells can trans-differentiate into osteoblasts in vitro and in vivo only by all-trans retinoic acid. Cell Struct Funct 2008;33:211–22.
[13] Sakuma T, Matsumoto T, Kano K, Fukuda N, Obinata D, Yamaguchi K, et al. Mature, adipocyte derived, dedifferentiated fat cells can differentiate into smooth muscle-like cells and contribute to bladder tissue regeneration. J Urol 2009;182:355-65.
[14] Peng X, Song T, Hu X, Zhou Y, Wei H, Peng J, et al. Phenotypic and functional properties of porcine dedifferentiated fat cells during the long-term culture in vitro. Biomed Res Int 2015;2015:673651.
[15] Kazama T, Fujie M, Endo T, Kano K. Mature adipocyte-derived dedifferentiated fat cells can transdifferentiate into skeletal myocytes in vitro. Biochem Biophys Res Commun 2008;377:780-5.
[16] Jumabay M, Matsumoto T, Yokoyama S, Kano K, Kusumi Y, Masuko T, et al. Dedifferentiated fat cells convert to cardiomyocyte phenotype and repair infarcted cardiac tissue in rats. J Mol Cell Cardiol 2009;47:565-75.
[17] Jumabay M, Abdmaulen R, Urs S, Heydarkhan-Hagvall S, Chazenbalk GD, Jordan MC, et al. Endothelial differentiation in multipotent cells derived from mouse and human white mature adipocytes. J Mol Cell Cardiol 2012;53:790-800.
[18] Okita N, Honda Y, Kishimoto N, Liao W, Azumi E, Hashimoto Y, et al. Supplementation of strontium to a chondrogenic medium promotes chondrogenic differentiation of human dedifferentiated fat cells. Tissue Eng A 2015;21:1695-704.
[19] Otaki M, Matsumoto T, Kano K, Tokuhashi Y. Chondrogenic differentiation potential of human dedifferentiated fat (DFAT) cells. J Nihon Univ Med Assoc 2015;74:246–52 [in Japanese].
[20] Sakamoto F, Hashimoto Y, Kishimoto N, Honda Y, Matsumoto N. The utility of human dedifferentiated fat cells in bone tissue engineering in vitro. Cytotechnology 2013;67:75-84.
[21] Watson JE, Patel NA, Carter G, Moor A, Patel R, Ghansah T, et al. Comparison of markers and functional attributes of human adipose-derived stem cells and dedifferentiated adipocyte cells from subcutaneous fat of an obese diabetic donor. Adv Wound Care (New Rochelle) 2014;3:219-28.
[22] Weiss S, Hennig T, Bock R, Steck E, Richter W. Impact of growth factors and PTHrP on early and late chondrogenic differentiation of human mesenchymal stem cells. J Cell Physiol 2010;223:84-93.
[23] Shanmugarajan T, Kim B, Lee H, Im G. Growth factors and signaling pathways in the chondrogenic differentiation of mesenchymal stem cells. Tissue Eng Regen Med 2011;8:292–9.
[24] Shintani N, Hunziker EB. Differential effects of dexamethasone on the chondrogenesis of mesenchymal stromal cells: influence of microenvironment, tissue origin and growth factor. Eur Cell Mater 2011;22:302–19 [discussion 19-20].
[25] Yoon HH, Bhang SH, Shin JY, Shin J, Kim BS. Enhanced cartilage formation via three-dimensional cell engineering of human adipose-derived stem cells. Tissue Eng A 2012;18:1949-56.
[26] Danisovic L, Varga I, Polak S. Growth factors and chondrogenic differentiation of mesenchymal stem cells. Tissue Cell 2012;44:69-73.
[27] Sekiya I, Larson BL, Vuoristo JT, Reger RL, Prockop DJ. Comparison of effect of BMP-2, -4, and -6 on in vitro cartilage formation of human adult stem cells from bone marrow stroma. Cell Tissue Res 2005;320:269-76.

[28] Miljkovic ND, Cooper GM, Marra KG. Chondrogenesis, bone morphogenetic protein-4 and mesenchymal stem cells. Osteoarthritis Cartilage 2008;16:1121–30.
[29] Yu PB, Hong CC, Sachidanandan C, Babitt JL, Deng DY, Hoyng SA, et al. Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. Nat Chem Biol 2008;4:33–41.
[30] Hatakeyama Y, Tuan RS, Shum L. Distinct functions of BMP4 and GDF5 in the regulation of chondrogenesis. J Cell Biochem 2004;91:1204–17.
[31] Mitani G, Sato M, Lee JI, Kaneshiro N, Ishihara M, Ota N, et al. The properties of bioengineered chondrocyte sheets for cartilage regeneration. BMC Biotechnol 2009;9:17.
[32] Giuliani N, Lisignoli G, Magnani M, Racano C, Bolzoni M, Dalla Palma B, et al. New insights into osteogenic and chondrogenic differentiation of human bone marrow mesenchymal stem cells and their potential clinical applications for bone regeneration in pediatric orthopaedics. Stem Cells Int 2013;2013:312501.
[33] Ikeda T, Kamekura S, Mabuchi A, Kou I, Seki S, Takato T, et al. The combination of SOX5, SOX6, and SOX9 (the SOX trio) provides signals sufficient for induction of permanent cartilage. Arthritis Rheum 2004;50:3561–73.
[34] Khan WS, Adesida AB, Hardingham TE. Hypoxic conditions increase hypoxia-inducible transcription factor 2alpha and enhance chondrogenesis in stem cells from the infrapatellar fat pad of osteoarthritis patients. Arthritis Res Ther 2007;9:R55.
[35] Yamaguchi A, Komori T, Suda T. Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfa1. Endocr Rev 2000;21:393–411.
[36] Miron RJ, Zhang YF. Osteoinduction: a review of old concepts with new standards. J Dent Res 2012;91:736–44.
[37] Wharton K, Derynck R. TGFbeta family signaling: novel insights in development and disease. Development 2009;136:3691–7.
[38] Herlofsen SR, Kuchler AM, Melvik JE, Brinchmann JE. Chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells in self-gelling alginate discs reveals novel chondrogenic signature gene clusters. Tissue Eng A 2011;17:1003–13.