

ORIGINAL ARTICLE

Low-Intensity Pulsed Ultrasound Promotes BMP9 Induced Osteoblastic Differentiation in Rat Dedifferentiated Fat Cells

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Dedifferentiated fat cells (DFATs) isolated from mature adipocytes have a multilineage differentiation capacity similar to mesenchymal stem cells and are considered as promising source of cells for tissue engineering. Bone morphogenetic protein 9 (BMP9) and low-intensity pulsed ultrasound (LIPUS) have been reported to stimulate bone formation both *in vitro* and *in vivo*. However, the combined effect of BMP9 and LIPUS on osteoblastic differentiation of DFATs has not been studied. After preparing DFATs from mature adipose tissue from rats, DFATs were treated with different doses of BMP9 and/or LIPUS. The effects on osteoblastic differentiation were assessed by changes in alkaline phosphatase (ALP) activity, mineralization/calcium deposition, and expression of bone related genes; Runx2, osterix, osteopontin. No significant differences for ALP activity, mineralization deposition, as well as expression for bone related genes were observed by LIPUS treatment alone while treatment with BMP9 induced osteoblastic differentiation of DFATs in a dose dependent manner. Further, co-treatment with BMP9 and LIPUS significantly increased osteoblastic differentiation of DFATs compared to those treated with BMP9 alone. In addition, upregulation for BMP9-receptor genes was observed by LIPUS treatment. Indomethacin, an inhibitor of prostaglandin synthesis, significantly inhibited the synergistic effect of BMP9 and LIPUS co-stimulation on osteoblastic differentiation of DFATs. LIPUS promotes BMP9 induced osteoblastic differentiation of DFATs *in vitro* and prostaglandins may be involved in this mechanism.

Keywords: Dedifferentiated fat cells, Bone morphogenetic protein 9, Low-intensity pulsed ultrasound, Prostaglandins

Introduction

Bone tissue engineering is an effective method to treat large bone defects after trauma, peri-implantitis, and

periodontitis. Cells are an important component for tissue engineering and mesenchymal stem cells (MSCs) have been considered suitable for tissue engineering and regeneration because of their high proliferative potential and ability to differentiate into several lineages, including bone forming cells (i.e. osteoprogenitors, osteoblasts). Despite the promising results from preclinical studies, issues such as heterogeneity of cells as well as the need to undergo invasive procedure and *ex vivo* expansion to obtain enough cells related have limited the application of MSCs in clinical practice, fostering the search for alternative cells through a minimally invasive approach. It has been demonstrated that dedifferentiated fat cells (DFATs) isolated from mature adipocytes by the ceiling culture method have multilineage differentiation capacity (1, 2). For bone formation, DFATs have been reported to show higher expression lev-

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el of osteoblastic genes, alkaline phosphatase activity, and calcium deposition than bone marrow derived MSCs *in vitro* (3). Furthermore, DFATs have been successfully prepared from donors aged 4~81 years and can be obtained regardless of the donor's age (1). Therefore, DFATs are a promising cell source for tissue engineering.

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor TGF- β superfamily and regulate bone formation, angiogenesis, neurogenesis, as well as development of multiple organ systems (4). Among several members of BMPs, BMP2 and BMP7 have been characterized for higher osteogenic effects than other BMPs. Indeed, BMP2 is currently used in clinics for treating large bone defect and fracture (5, 6). BMP9, also known as growth differentiation factor 2, was originally identified in fetal mouse liver and recent studies found that BMP9 is more osteoinductive than BMP2 (7, 8). BMP9 has different characteristics to other BMPs including resistance to the inhibitors noggin and BMP3 (9).

Low-intensity pulsed ultrasound (LIPUS) is a FDA-approved non-invasive intervention which has been clinically applied in the treatment of intractable fracture and non-unions by stimulating cell proliferation, angiogenesis, extracellular matrix production and inflammation suppression (10-13). At the cellular level, it has been reported that LIPUS stimulus is converted into biological signaling through integrins on the cell surface and subsequently up-regulates prostaglandin E₂ (PGE₂) and cyclooxygenase 2 (COX2) expression, which promotes osteogenesis in osteoblasts (14, 15).

A previous study showed that osteoblastic differentiation of DFATs was significantly induced by BMP9 and co-stimulation with additional agents such as FK506 (16). Furthermore, combination of BMP9 and LIPUS resulted in higher bone formation compared to the carrier/collagen sponge only-group in rat calvarial bone defects, *in vivo* (17). However, the combined effect of BMP9 and LIPUS on osteoblastic differentiation of DFATs has not been studied, so far. Therefore, the aim of this study was to examine the effects of BMP9 and LIPUS co-stimulation on osteoblastic differentiation of rat DFATs.

Materials and Methods

Reagents

Recombinant human BMP9 was purchased from FUJIFILM Wako (Osaka, Japan) and an inhibitor for prostaglandin synthesis; indomethacin, and Prostaglandin E₂ ELISA Kit were purchased from Cayman Chemical (Ann Arbor, MI).

Isolation and culture of DFATs

All animal experiments were approved by the Ethical Committee of the Animal Research Center of Kagoshima University (Approval No. D19039). Isolation of DFATs from mature adipose tissue was performed by the ceiling culture method as previously described by Jumabay et al. (18), with minor modification (16). In brief, 9~10-week-old male Wister rats were purchased from Charles River Laboratories (Kanagawa, Japan). Mature adipose tissue (2 g) was removed from the inguinal region of the rat and minced followed by digestion using 0.2% collagenase I solution (Invitrogen, Carlsbad, CA) at 37°C for 45 min with gentle shaking. These cells were filtrated through 140 μ m mesh (Sigma-Aldrich, St. Louis, MO) and centrifuged at 135 g for 3 min. The top layer, a suspension of adipocytes, was washed with phosphate-buffered saline (PBS) and centrifuged three times. The cells were cultured in a 25 cm² tissue culture flask filled completely with Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) containing 20% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin G and 100 μ g/ml streptomycin) in this floating condition at 37°C in 5% CO₂. After 1 week, the fibroblast like cells attached to the upper surface of the flasks. The medium was removed, and the flasks were inverted. The adherent cells were cultured in growth culture medium (DMEM supplemented with 10% FBS and antibiotics). DFATs were subcultured and used for experiments at passages 3~7. For osteogenic differentiation, the cells were cultured in osteogenic differentiation medium (ODM) consisting of DMEM supplemented with 10% FBS, antibiotics, 10 mM β -glycerophosphate, 10 μ g/ml ascorbic acid, and 10 μ M all-trans retinoic acid (first 3 days only).

LIPUS application

DFATs were stimulated by a LIPUS exposure device (Teijin Pharma, Tokyo, Japan) consisting of an array of six transducers designed for a 6 well culture plate. Ultrasound transducers were placed under the bottom of each well using a coupling gel (19). The LIPUS signal consisted of a 1.5 MHz, 200 μ s burst sine wave with repetition rate at 1.0 kHz and was delivered at an intensity of 30 mW/cm² spatial and temporal average (SATA). The cells were exposed to LIPUS for 20 min every day. Non-LIPUS-treated cells were handled in the same way using separate culture plates, but the ultrasound generator was not switched on.

ALP activity and Alizarin red S staining

DFATs were seeded at a density of 2×10^4 cells/cm² in 6 well culture plates. Cells were cultured for 6 d for ALP activity assay. After 6 d, cells were washed twice with PBS,

sonicated 10 s on ice and scraped off with lysis buffer (1.5 M Tris-HCl at pH 9.2, 1 mM MgCl₂·6H₂O, and 1% Triton X-100). ALP activity was measured as described previously and measured values were corrected by total protein content (16). For the mineralization assay, DFATs were cultured for 21 d. After 21 d, cells were fixed in 3.7% formaldehyde neutral buffer and stained with alizarin red S. Images of the stained plates were acquired by using a scanner. Further, quantification of the alizarin red S dye was performed by extraction with 10% cetylpyridinium chloride/10 mM sodium phosphate solution followed by measurement with microplate reader at a wavelength of 562 nm (20).

Real-time PCR analysis

For real-time PCR, cells were cultured for 2 d or 6 d. Total RNA was extracted from DFATs using TRIzol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Total RNA was used as the template to synthesize cDNA by the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). Quantitative gene-expression analyses were carried out using real-time PCR by means of the Thunderbird SYBR qPCR mix (Toyobo, Osaka, Japan) and the Real-time PCR System 7300 (Applied Biosystems, Foster City, CA) as previously described (21). The PCR amplifications were carried out under the following conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 15 s, 60°C for 35 s. We used the comparative Ct method to calculate the relative mRNA expression. All quantitation was normalized by the corresponding GAPDH expression. Information for the primer sets is listed in Table 1.

Prostaglandin E₂ assay

The media from DFATs was collected 24 h after treatment with LIPUS and stored at -80°C. The levels of

prostaglandin E₂ (PGE₂) produced by DFATs in the culture media were quantified using a commercially available ELISA kit (Prostaglandin E₂, EIA Monoclonal Kit; Cayman Chemical, Ann Arbor, MI), in accordance with the manufacturer's instructions.

Statistical analysis

All experiments were conducted independently for DFATs from two different donor animals and similar results were obtained. All experiments were repeated at least twice. The statistical significance of differences between treatment groups was analyzed by one-way ANOVA and Bonferroni-Dunn test (IBM SPSS Statistics; IBM SPSS, Chicago, IL). Values of $p < 0.05$ were considered to be statistically significant.

Results

ALP activity of DFATs stimulated with BMP9 and/or LIPUS

We examined the effects of BMP9 and LIPUS alone or in combination on ALP activity in DFATs. LIPUS alone (without BMP9) did not affect ALP activity of DFATs at 6 d. BMP9 (0.1~100 ng/ml) enhanced ALP activity of DFATs in a dose-dependent manner, with 100 ng/ml of BMP9 inducing the highest ALP activity. In the presence of BMP9 at 10 ng/ml or higher, significantly higher ALP activity ($p < 0.05$) was noted in the LIPUS-stimulated group compared to the non-LIPUS-stimulated group (Fig. 1).

Mineralization of DFATs stimulated with BMP9 and/or LIPUS

Effects of BMP9 and LIPUS on mineralization in DFATs were examined at 21 days. In doses less than 10 ng/ml of BMP9, DFATs were not stained by Alizarin red S, but at 100 ng/ml, calcium deposits were detected in both sham

Table 1. The sequences for primers used in present research

Gene	Forward (5'<—> 3')	Reverse (5'<—> 3')	Size (bp)	Accession number
<i>ALK-1</i>	CGTGCTGGTCAAGAGCAACT	GCTTTGCGAGTGCATCACA	69	NM_022441.2
<i>ALK-2</i>	GGAAGTGGCCAGGAGGAT	GGGTCATTGGGAACAACATC	80	NM_024486
<i>BMP receptor II</i>	CCCCGAGGAGATCATTACAA	ACGTGCCACCATTCTTACC	81	NM_080407.1
<i>Endoglin</i>	GCTGCGGCATGAAAGTGA	GGTAAGCCTGATGGCAAATTG	69	NM_001010968.3
<i>Gapdh</i>	CGGCAAGTTCAACGGCACAGTCAAGG	ACGACATACTCAGCACCAGCATCACC	129	NM_017008.4
<i>Opn</i>	GATGAACCAAGCGTGGAAAC	TGAAACTCGTGGCTCTGATG	200	NM_012881.2
<i>Osx</i>	CCCTTCCCCACTCATTTCC	CTGCCACCACTAACCACAA	237	NM_001173467.3
<i>Runx2</i>	ACAACCACAGAACCAACAG	TCTCGGTGGCTGGTAGTGA	105	NM_001278483.1

and LIPUS stimulated groups (Fig. 2A). Quantified result of Alizarin red S stain indicated no significant difference in doses less than 10 ng/ml of BMP9, while at 100 ng/ml, mineralization was significantly enhanced in the LIPUS-stimulated group compared to the sham group ($p < 0.05$, Fig. 2B).

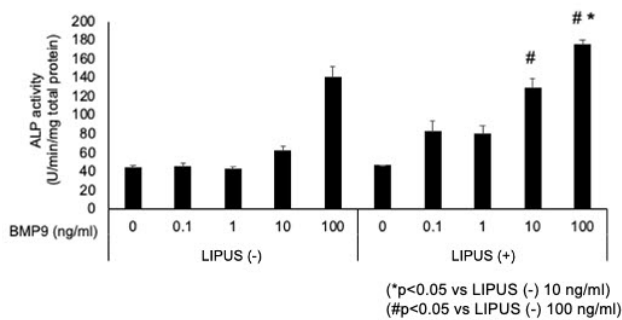


Fig. 1. ALP activity of DFATs stimulated with BMP9 and/or LIPUS. DFATs were seeded at a density of 2×10^4 cells/cm² in CM. After 24 h of incubation, cells were cultured with BMP9 (0~100 ng/ml) for 6 days in ODM. LIPUS treated cells were exposed to LIPUS for 20 minutes every day. Enhanced ALP activity by LIPUS treatment was observed in DFATs with BMP9 concentrations at 10 ng/ml or higher. Data represent means \pm SD ($n = 4$). * $p < 0.05$ vs. LIPUS (-) 100 ng/ml. # $p < 0.05$ vs. LIPUS (-) 10 ng/ml.

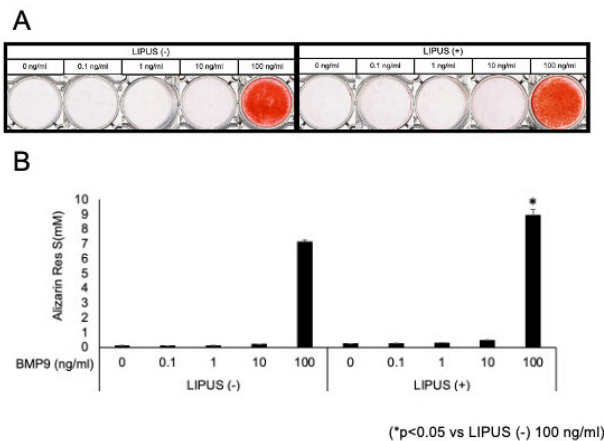


Fig. 2. Mineralization of DFATs treated with BMP9 and/or LIPUS. (A) Alizarin red S staining. (B) Quantification of Alizarin red S staining. The cells were cultured with BMP9 (0~100 ng/ml) for 21 days in ODM. LIPUS treated cells were exposed to LIPUS for 20 minutes every day. Intense Alizarin red S staining was observed in DFATs treated with BMP9 concentrations at 100 ng/ml and higher amount of stain was noted by co-treatment with LIPUS. Values are shown as mean \pm SD ($n = 4$). * $p < 0.05$ vs. LIPUS (-) 100 ng/ml.

mRNA expression of bone-related genes in DFATs stimulated with BMP9 and/or LIPUS

The expression of bone-related genes, including *Runx2*, *osterix* (*Osx*), and *osteopontin* (*Opn*) was investigated by quantitative PCR. *Runx2* and *Osx* were analyzed at 2 d and *Opn* at 6 d. Results for all genes showed a tendency toward an increase in gene expression level in a dose-dependent manner for BMP9. For all genes, including *Runx2*, *Osx*, and *Opn*, a significantly higher level of gene expression was observed in combination of BMP9 at 100 ng/ml and LIPUS compared to BMP9 at 100 ng/ml alone (Fig. 3). Thus, we decided to use 100 ng/ml of BMP9 for the following experiments.

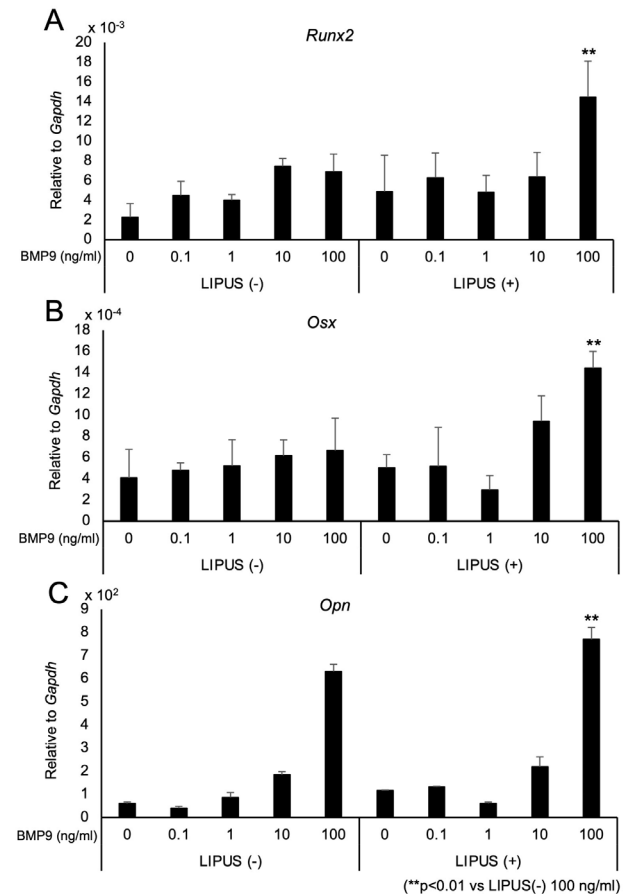


Fig. 3. mRNA expression of bone-related genes in DFATs treated with BMP9 and/or LIPUS. (A~C) The effects of BMP9 and/or LIPUS treatment on the expression of bone-related genes (*Runx2*, *Osx*, *Opn*) were analyzed by quantitative real-time PCR. The cells were cultured with BMP9 (0~100 ng/ml) for 2 days (A, B) and 6 days (C) in ODM. LIPUS treated cells were exposed to LIPUS for 20 minutes every day. Values are shown as mean \pm SD ($n = 3$). ** $p < 0.01$ vs. LIPUS (-) 100 ng/ml.

Expression of BMP9 receptor genes in DFATs stimulated with BMP9 and/or LIPUS

BMP9 binds to activin receptor-like kinase -1 (ALK1), -2 (ALK-2) and BMP receptor II with high affinity. In addition, endoglin is known to act as a co-receptor. Here, we examined the effect of BMP9 and/or LIPUS on expression of BMP9-related-receptor genes in DFATs. Two days after LIPUS treatment, expression of BMP9-related-receptor genes was investigated by real-time PCR. The expression level for *ALK-1*, *ALK-2*, *BMP receptor II* and *Endoglin* was significantly higher in the BMP9 stimulated group than ODM group (Fig. 4). The expression for all BMP9-related-receptor genes elevated by BMP9 was further enhanced by LIPUS stimulation (Fig. 4).

Effects of indomethacin on DFATs stimulated with BMP9 and/or LIPUS

As it has previously been reported that PGE₂ plays an important role during bone remodeling (10), we examined

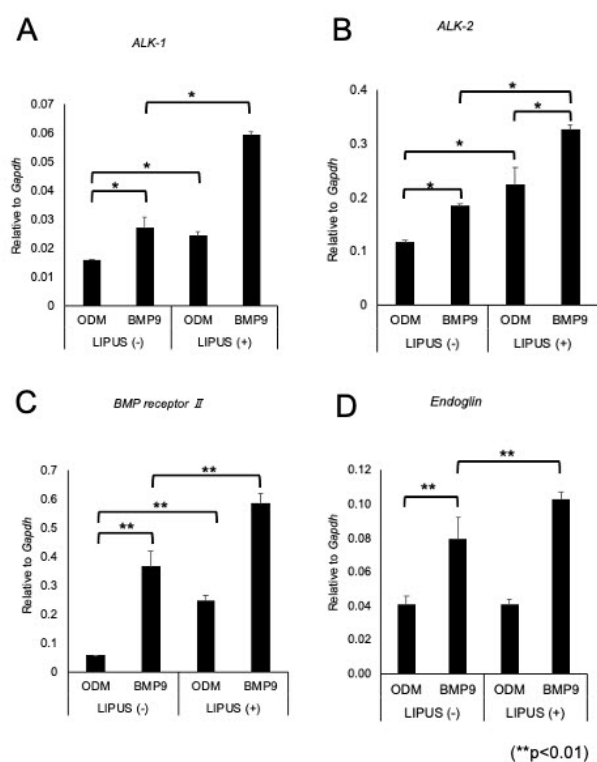


Fig. 4. Expression of BMP9-related receptor genes in DFATs treated with BMP9 and/or LIPUS. (A~D) The expression of (A) *ALK-1*, (B) *ALK-2*, (C) *BMP receptor II* and (D) *Endoglin* in DFATs analyzed by real-time PCR. The cells were cultured with BMP9 (100 ng/ml) for 2 days in ODM. LIPUS treated cells were exposed to LIPUS for 20 minutes every day. Values are shown as mean \pm SD (n=4). *p<0.05, **p<0.01.

the effects of an inhibitor for prostaglandin synthesis, indomethacin, by ALP activity and expression of bone-related genes in DFATs after stimulation with BMP9 and/or LIPUS. For ALP activity, no significant effect of indomethacin was observed in the non-LIPUS-treated group, but in the LIPUS-stimulated groups, addition of indomethacin significantly suppressed ALP activity level induced by BMP9 (Fig. 5A). On the other hand, for the ex-

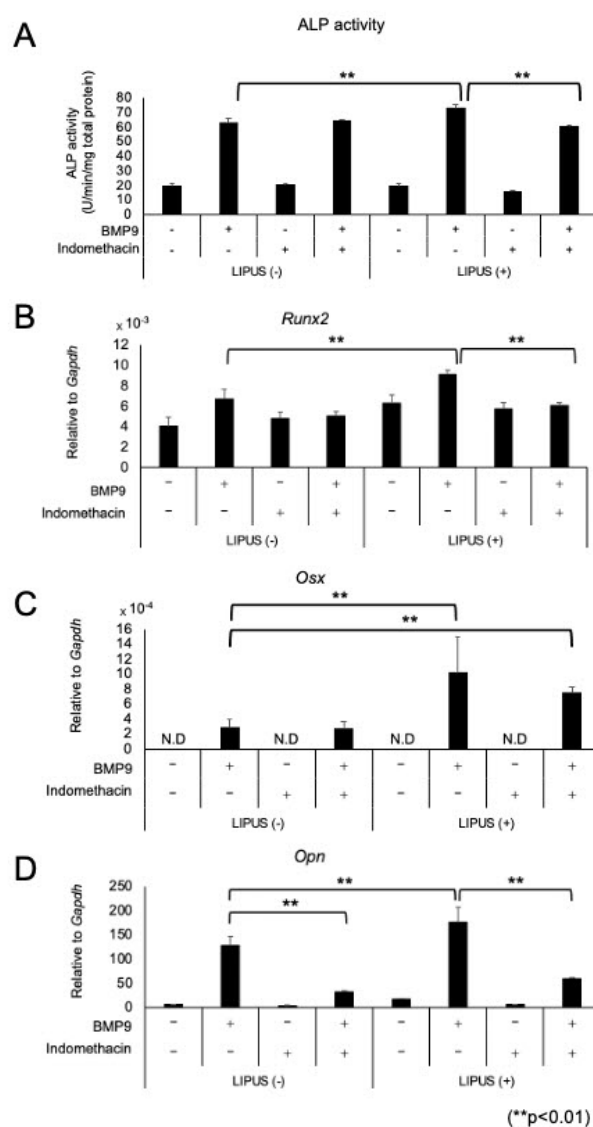


Fig. 5. Effects of indomethacin on ALP activity and mRNA levels of bone-related genes in DFATs treated with BMP9 and/or LIPUS. (A) The effect of indomethacin on ALP activity. (B~D) The effect of indomethacin on expression of bone-related genes; (B) *Runx2*, (C) *Osx*, (D) *Opn*. DFATs were cultured with BMP9 (100 ng/ml) and indomethacin (1 μ M) for 2 days (A~C) and 6 days (D) in ODM. LIPUS treated cells were exposed to LIPUS for 20 minutes every day. Values are shown as mean \pm SD (n=4). **p<0.01.

pression of bone-related genes in the LIPUS-stimulated group, the addition of indomethacin significantly suppressed the expression of *Runx2* and *Opn* (Fig. 5B and 5D) but showed a slight decrease, although not significantly for *Osx* (Fig. 5C).

Release of PGE₂ from DFATs stimulated with BMP9 and/or LIPUS and effect of indomethacin

Next, we analyzed the effect on BMP9 and/or LIPUS treatment on PGE₂ production by DFATs. BMP9 significantly increased PGE₂ production and addition of indomethacin significantly suppressed the release of BMP9-induced PGE₂ (Fig. 6). The addition of LIPUS significantly enhanced the PGE₂ production induced by BMP9 which was suppressed by indomethacin.

Discussion

A previous study showed BMP9 led to differentiation of DFATs into the osteoblastic lineage (16). The present study evaluated the combined effect of BMP9 and LIPUS on osteoblastic differentiation of DFATs *in vitro*. Although LIPUS treatment has been reported to stimulate osteoblastic differentiation of human periodontal ligament stem cells (22), as well as murine stromal cells (23), LIPUS alone did not induce DFATs to differentiate into the osteoblastic lineage. This may be due to different cell types used to study the effect of LIPUS on its inductive effect to differentiate to the osteoblastic lineage. Indeed, a previous report comparing response of bone- and bone mar-

row-derived primary cells to LIPUS by Naruse et al. noted that non-differentiated bone marrow-derived adherent cells obtained from rat femora were insensitive to LIPUS compared to osteoblasts and osteocytes (24). In their study, no significant change in mRNA levels of COX2 (an upstream enzyme for prostaglandin production) as well as other bone proteins were detected in bone marrow-derived adherent cells after LIPUS treatment while higher expression level was observed in osteoblasts and osteocytes. Our results suggest that non-differentiated DFATs may be insensitive to LIPUS similar to that non-differentiated bone marrow-derived adherent cells.

Co-treatment with BMP9 and LIPUS synergistically induced osteoblastic differentiation of DFATs with enhancement of ALP level, the amount of calcium deposition and expression levels for bone related genes, compared to individual stimulation by BMP or LIPUS. Sant'Anna et al. (25) studied the combined effect of BMP2 and LIPUS in rat bone marrow stromal cells and reported changes in the temporal expression patterns of osteogenic genes indicating differences in signal transduction pathways by the stimulus. Lai et al. (26) reported that no significant differences in expression of bone related genes among human MSCs treated with LIPUS or BMP2 alone and as co-treatments. Recently, a study by Han et al. (27) reported co-treatment of rat mesenchymal stem cells with BMP2 and LIPUS led to enhanced ALP activity compared to LIPUS alone or BMP2 alone. *In vivo*, it has also been reported that the co-stimulation with BMP2 and LIPUS enhanced bone formation (28, 29). In addition, we recently reported that that LIPUS promoted BMP9-induced bone formation in a rat calvarial bone defect (17). Our current finding may provide promising support for utilizing DFATs with co-treatment of BMP9 and LIPUS for effective bone tissue engineering. One noteworthy finding in the present study is that the DFATs co-treated with 10 ng/ml of BMP9 and LIPUS showed similar ALP activity to those treated with 100 ng/ml of BMP9 (without LIPUS). Although the results from the mineralization assay did not show a similar effect, combined use of BMP9 and LIPUS may allow for the use of a smaller (1/10) concentration of BMP9.

BMPs are known to transduce signals through a receptor complex consisting of serine/threonine kinases, including two type I receptors and two type II receptors on the cell membrane (30). Especially, BMP9 was reported to have high binding affinity to type I receptors; ALK-1 and ALK-2, type II receptor; BMP receptor II, and co-receptor; endoglin (31, 32). We showed that expression levels for *ALK-1*, *ALK-2*, and *BMP receptor II* gene were upregulated by LIPUS stimulation while BMP9 stimulation upre-

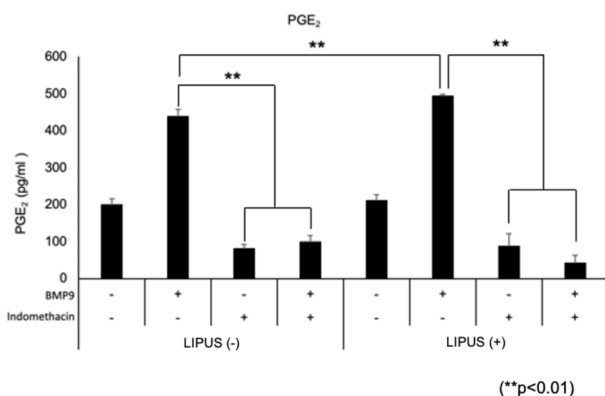


Fig. 6. Release of PGE₂ from DFATs treated with BMP9 and/or LIPUS and effect of indomethacin. DFATs were stimulated with BMP9 and/or LIPUS in ODM. Some samples were treated with indomethacin. The cells were cultured with BMP9 (100 ng/ml) and indomethacin (1 μ M) for 24 h in ODM. LIPUS treated cells were exposed to LIPUS for 20 minutes every day. Values are shown as mean \pm SD (n=4). **p<0.01.

gulated the expression of all BMP9-related receptor genes analyzed. By co-stimulation of DFATs with BMP9 and LIPUS, significantly higher mRNA levels for ALK-1, ALK-2, BMP receptor II and endoglin were observed compared to BMP9 alone (without LIPUS). These results suggest that LIPUS may enhance the responses of DFATs to BMP9 by upregulating the expression of its receptors. Our finding on increased expression of BMP receptor genes by LIPUS stimulation is also in agreement with a previous study which reported elevated expression of BMP receptor genes by LIPUS treatment in osteoblasts (33). Similarly, a more recent study utilizing the rabbit distraction osteogenesis model reported that the application of BMP2 after LIPUS pretreatment led to greater bone volume than the application of BMP2 before LIPUS treatment, *in vivo* (27). Together with our current findings, it is likely that the synergistic effect of BMPs and LIPUS application in osteoblastic differentiation or bone formation may be regulated through enhancing the availability of BMP receptors by LIPUS stimulation. Further investigation is necessary to elucidate how LIPUS upregulates the expression of BMP receptors and whether enhancing the number or availability of BMP receptors in DFATs modulates the function of BMPs on these cells.

Prostaglandins are known as biologically active substances produced from arachidonic acid via cyclooxygenases. In particular, PGE₂ has been known to play an important role in bone metabolism, being involved in both bone resorption and bone formation (34). In this study, indomethacin significantly suppressed elevated ALP activity induced by BMP9 and LIPUS co-stimulation (Fig. 5A) while such an effect was not observed in the non-LIPUS stimulated groups. Similarly, higher expression level for *Runx2* and *Opn* in the BMP9 and LIPUS co-stimulation group was significantly inhibited by indomethacin (Fig. 5B and 5D). These results suggest involvement of prostaglandins in osteoblastic differentiation of DFATs. Since no significant inhibition of osteoblastic differentiation was observed in the sham group, indomethacin may counteract the osteoblastic differentiation promoted by LIPUS stimulation in BMP treated DFATs. From the ELISA results, increased release of PGE₂ was observed after treating DFATs with BMP9 (Fig. 6). LIPUS co-stimulation significantly increased the release of PGE₂ and the addition of indomethacin significantly suppressed the release. These results indicate that the synergistic effect of LIPUS treatment on BMP9 induced osteoblastic differentiation of DFATs is mediated via PGE₂. Indeed, together with COX2, PGE₂ have been strongly suggested as key downstream molecules stimulated by LIPUS stimulation (35). It has been

demonstrated that LIPUS stimulates PGE₂ synthesis from murine long bone osteocyte-like cells (36). In addition, secretion of PGE₂ was significantly upregulated over 24 h after a single 20 min application of LIPUS in murine bone marrow-derived cells (24) and murine osteoblasts (15). Moreover, by treating the DFATs with PGE₂ at different concentration, significantly low ALP level was observed at 0.1 nM (=35.25 pg/ml) PGE₂ treatment, compared to 0 nM, while no difference was observed at 1 nM and significantly higher ALP level was observed after 10 nM (=35.25 ng/ml), 100 nM (=35.25 ng/ml), and 1,000 nM (=352.5 ng/ml) PGE₂ treatment (Supplementary Fig. S1). In our study, the concentration of PGE₂ in the culture medium of DFATs was less than 10 nM (between 50 pg/ml and 500 pg/ml, Fig. 6). For this, significant increase in osteoblastic differentiation of DFATs after BMP9 and LIPUS co-treatment cannot be explained by the concentration of PGE₂. However, involvement of PGE₂ is evident as indicated by the inhibitory effect of indomethacin. Additionally, Takiguchi et al. (37) reported co-treatment of human periodontal ligament cells with bone morphogenetic protein 2 (BMP2) and PGE₂ within concentration between 10⁻¹⁰ M (0.1 nM) and 10⁻⁸ M (10 nM) showed higher osteoblastic differentiation compared to the cells treated with BMP2 alone by ALP activity. Taken together, the level of PGE₂ observed in our study may not be enough to promote osteoblastic differentiation of DFATs only by PGE₂ but may be enough to stimulate BMP-induced osteoblastic differentiation.

Application of DFATs has been shown to be promising for bone repair in several preclinical studies (38-40). Kikuta et al. (38) have reported that autologous transplantation of osteoblastic differentiated DFATs with beta-tricalcium phosphates/collagen sponge promoted bone regeneration in a rabbit tibial defect model. Tateno et al. (39) showed significant bone regeneration by transplanting DFATs combined with a biodegradable type I collagen recombinant peptide scaffold in a rat mandible defect model. In addition, our group also reported poly lactic-co-glycolic acid/hydroxyapatite as an effective carrier for bone formation utilizing DFATs in a rat calvarial defect (40). These reports suggest that DFATs may be a promising cell source for bone tissue engineering. Imafuji et al. (17) recently reported combination of BMP9 and LIPUS resulted in a higher bone formation compared to the carrier/collagen sponge only-group in rat calvarial bone defects. Taken together, in addition to combination of DFATs and BMP9 with an appropriate scaffold, LIPUS stimulation may promote bone formation, suggesting a novel strategy for bone tissue engineering in treating large bone defects.

In conclusion, we have found that LIPUS promotes

BMP9 induced osteoblastic differentiation of DFATs *in vitro*, possibly via PGE₂ and modulation of BMP9-related-receptors are suggested to be involved in this mechanism. Our findings may lead to the use of DFATs in combination with BMP9 and LIPUS for future bone tissue engineering approaches to treat large bone defects.

Acknowledgments

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Potential Conflict of Interest

The authors have no conflicting financial interest.

Supplementary Materials

Supplementary data including one figure can be found with this article online at <https://doi.org/10.15283/ijsc23027>

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