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Co-stimulation with bone morphogenetic protein-9 and FK506 induces remarkable osteoblastic differentiation in rat dedifferentiated fat cells

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ABSTRACT

Dedifferentiated fat (DFAT) cells, which are isolated from mature adipocytes using the ceiling culture method, exhibit similar characteristics to mesenchymal stem cells, and possess adipogenic, osteogenic, chondrogenic, and myogenic potentials. Bone morphogenetic protein (BMP)-2 and -9, members of the transforming growth factor-β superfamily, exhibit the most potent osteogenic activity of this growth factor family. However, the effects of BMP-2 and BMP-9 on the osteogenic differentiation of DFAT remain unknown. Here, we examined the effects of BMP-2 and BMP-9 on osteoblastic differentiation of rat DFAT (rDFAT) cells in the presence or absence of FK506, an immunosuppressive agent. Co-stimulation with BMP-9 and FK506 induced gene expression of *runx2*, *osterix*, and *bone sialoprotein*, and ALP activity compared with BMP-9 alone, BMP-2 alone and BMP-2 + FK506 in rDFAT cells. Furthermore, it caused mineralization of cultures and phosphorylation of smad1/5/8, compared with BMP-9 alone. The ALP activity induced by BMP-9 + FK506 was not influenced by addition of noggin, a BMP antagonist. Our data suggest that the combination of BMP-9 and FK506 potently induces osteoblastic differentiation of rDFAT cells.

1. Introduction

Transplant cells are considered to be a crucial component in the research field of tissue regenerative therapy. Several types of adult mesenchymal stem/stromal cells (MSCs) including bone marrow-derived stromal cells (BMSCs), adipose tissue-derived stem cells (ADSCs), and dental pulp-derived stem cells have been considered to be attractive cell sources in stem cell-based tissue engineering [1]. Recently, dedifferentiated fat (DFAT) cells, which are isolated from mature adipocytes using the ceiling culture method, have become the focus of potential sources of MSCs. It has been reported that DFAT cells have a multilineage differentiation capacity, and that they possess similar properties to BMSCs [2] with a higher purity of stem cell population than ADSCs [3]. Comparative transcriptome analyses have revealed the reduction of genes for lipid metabolism and the increase of genes involved in cell proliferation, altered cell morphology, and regulation of differentiation during the dedifferentiation process of DFAT cells [4]. It has been reported that when DFAT cells are transplanted subcutaneously in mice, osteogenically differentiated DFAT cells can form ectopic osteoid tissue, although non-differentiated DFAT cells cannot [5]. This finding may indicate that the effective stimulation of osteogenic differentiation is critical to the DFAT cells-based bone regeneration.

Bone morphogenetic proteins (BMPs) belong to the major subgroup of the transforming growth factor (TGF)-β superfamily. At least 15 types of BMPs have been identified in humans, and BMPs are well known to function in embryogenesis, hematopoiesis, neurogenesis, and skeletal formation [6]. BMPs exert their biologic effects to bind mainly to type I and II serine/threonine kinase receptors and activate intracellular signaling including smad and/or non-smad pathways. In particular, BMP-2, a member of the BMP-2/-4 subfamily, has been well studied, and possesses potent osteoinductive activity [7]. BMP-2 mainly interacts with activin receptor-like kinase (ALK)-3 (BMPR-IA) and ALK-6 (BMPR IB), type I receptors, and activates smad1/5/8. The phosphorylated smads, which complex with smad4, translocate into the nucleus, activating the transcription of target genes including *runx2* and *osterix*, and regulate transcription of osteogenic genes such as *alkaline phosphatase* (*ALP*) and *bone sialoprotein* (*BSP*) [8–10]. The USA Food and Drug Administration has already approved the use of recombinant human BMP-2 with resorbable collagen sponges to treat tibial bone fractures and for spinal fusion [11]. It is reported that FK506, known as an immunosuppressive drug, modulates smad signaling pathway [12] and promotes the differentiation of osteoblastic cells [13]. Kaihara et al. have demonstrated that FK506 promotes the early stage of BMP-2-induced osteoinduction after short-term administration [14].

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BMP-9 (also known as growth differentiation factor-2 [GDF-2]), which was originally identified from fetal mouse liver [15], has been shown to possess osteogenic activity [16], and to induce osteogenic differentiation and bone formation of MSCs *in vitro* and *in vivo* [17]. Recently, it has been reported that BMP-9 is more osteogenic than BMP-2, and is not antagonized by noggin, which is a BMP antagonist [18,19]. However, it is unknown whether BMP-2 and BMP-9 can induce osteogenic differentiation in rDFAT cells. The aim of the present study was to examine the effects of BMP-2 and BMP-9 in the presence or absence of FK506 on osteoblastic differentiation in rat DFAT (rDFAT) cells.

2. Materials and methods

2.1. Animals

9–10-week-old male Wistar rats were purchased from Kyudo Co. Ltd (Tosu, Japan). All animal experiments were approved by the Ethical Committee of the Animal Research Center of Kagoshima University.

2.2. Reagents

Recombinant human BMP-2, BMP-9, and Noggin were purchased from R&D Systems Inc (Minneapolis, MN, USA), FK506 from Sigma–Aldrich (St. Louis, MO, USA), and anti-phospho-Smad1/5/8 and anti-Smad1 polyclonal antibody from Cell Signaling (Beverly, MA, USA).

2.3. Cell isolation and culture

The preparation of rDFAT cells from adipose tissue was performed according to the method of Jumabay et al. [20], with a minor modification. The adipose tissue (1–2 g) was minced and digested using 0.2% collagenase I solution (Invitrogen, Carlsbad, CA, USA) at 37 °C for 45 min with gentle shaking. After filtration through 140 μm mesh (Sigma–Aldrich) and centrifugation at 135 g for 3 min, the top layer of unilocular adipocytes was collected. The layer containing adipocytes was washed with phosphate-buffered saline (PBS) and centrifuged three times. The cells were placed in a 25 cm^2 culture flask filled completely with Dulbecco's modified Eagle's medium (DMEM, Sigma–Aldrich), supplemented with 20% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin), and cultured in this floating condition at 37 °C in 5% CO_2. After 1 week, cells adhered to the top inner ceiling surface. The medium was removed, and the flasks were inverted. Then the cells were cultured in culture medium (CM: DMEM supplemented with 10% FBS and antibiotics). rDFAT cells were subcultured and used for experiments at passages 4–7. For osteogenic differentiation, the cells were cultured in osteogenic differentiation medium (ODM), which consisted of DMEM supplemented with 10% FBS, antibiotics, 10 mM β-glycerophosphate, 10 μg/ml ascorbic acid, with 10 μM all-trans retinoic acid for the initial 3 days, and without retinoic acid following the first 3 days [5].

2.4. ALP activity assay and mineralization assay

Cells were cultured for 6 days, and then lysed for the purpose of an ALP activity assay. ALP activity was measured as described previously [21], and expressed relative to the protein content of the samples as determined using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). For the mineralization assay, rDFAT cells were cultured for 21 days. The cells were fixed in 3.7% formaldehyde neutral buffer solution and then stained with alizarin red S.

2.5. Reverse-transcriptase PCR and real-time PCR analysis

Total RNA was extracted from cells using Isogen (Nippon Gene, Tokyo, Japan). RT-PCR was conducted as described previously [22]. Total RNA was converted to cDNA using SuperScript III First-strand Synthesis SuperMix (Invitrogen). The PCR amplifications were carried out using a Taq PCR Core Kit (Qiagen, Hilden, Germany) under the following conditions: 94 °C for 3 min, followed by 40 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. The PCR products were separated by electrophoresis in 2% agarose gels, stained with ethidium bromide, and visualized with an ultraviolet light transilluminator. Real-time PCR amplifications were performed using a QuantiTect SYBR Green PCR Kit (Qiagen) according to the manufacturer's instruction. The amplification conditions were 40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. We used the comparative Ct method to calculate the relative mRNA expression. All quantitation was normalized by the corresponding GAPDH expression and presented relative to the control levels. Three measurements were performed for each sample. The primer sequences, GenBank accession numbers, and predicted sizes of the PCR products are shown in Supplementary Tables 1 and 2.

2.6. Western blotting

rDFAT cells were treated with either vehicle, BMP-9 or BMP-9 + FK506, for the indicated times, and the cells were then lysed with RIPA buffer (Nacalai tesque, Kyoto Japan). Extracts containing the same amounts of protein (10 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane using the Trans-blot Turbo Transfer system (Bio-Rad Laboratories). Membranes were blocked in blocking buffer (Block Ace Powder; Dainippon, Osaka, Japan), and then probed with anti-phospho-Smad1/5/8 (1:1000) or anti-Smad1 polyclonal antibody (1:1000), followed by incubation with a horseradish peroxidase-conjugated anti-rabbit IgG antibody (Cell Signaling). The immunoreactive bands were visualized using an ECL plus detection system (GE Healthcare Biosciences, Piscataway, NJ, USA). Band densities were measured with a ChemiDoc XRS Plus system (Bio-Rad Laboratories), and normalized for the optical density of smad1 bands.

2.7. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). The statistical significance of differences between groups was analyzed by one-way analysis of variance (ANOVA) and the Bonferroni–Dunn test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. mRNA expression of BMP receptors in rDFAT cells, and expression levels of bone-related genes in rDFAT cells stimulated with BMP-2, BMP-9, and FK506

We examined the gene expression of BMP receptors in rDFAT cells. RT-PCR analysis revealed the expression of mRNA of BMP receptors, including type I receptors (ALK-1, -2, -3, -6) and type II receptors (BMPRII, ActRIIA, and ActRIIB), in rDFAT cells (Fig. 1A). The level of bone-related gene expression, including *runx2*, *osterix*, and *bone sialoprotein* (*BSP*), was significantly increased in rDFAT

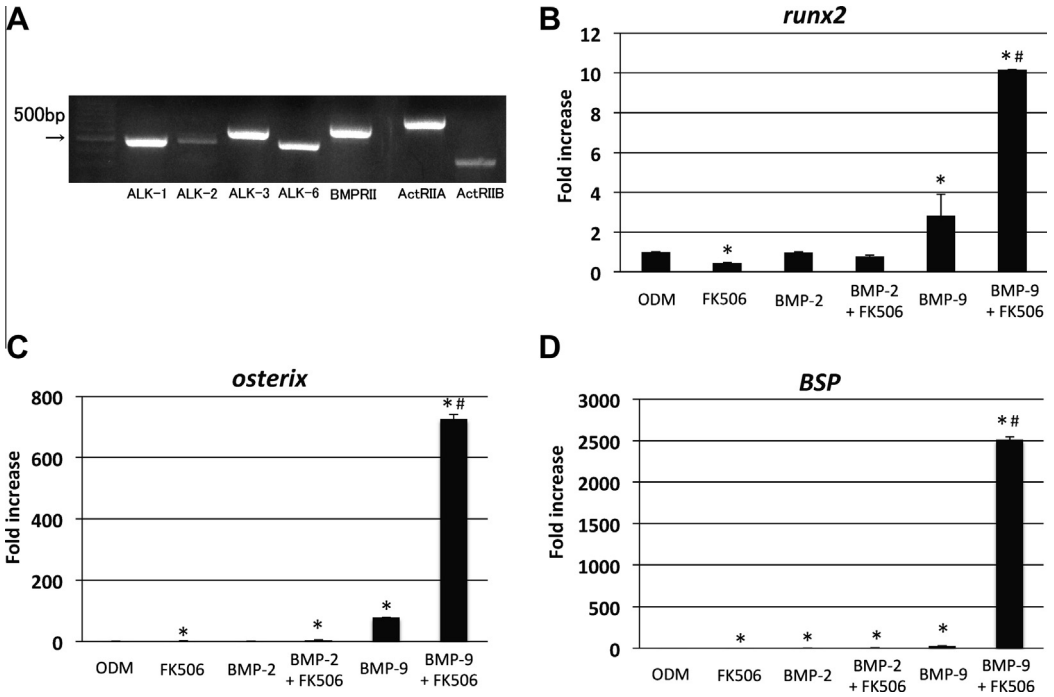

Fig. 1. mRNA expression of BMP receptors and bone-related genes (*runx2*, *osterix*, and *BSP*) in rDFAT cells. (A) mRNA of BMP receptors including type I receptors (ALK-1, -2, -3, -6) and type II receptors (BMPRII, ActRIIA, and ActRIIB). After rDFAT cells reached confluence in CM, total RNA was extracted from the cells, and analyzed by RT-PCR. (B) and (C) The effects of BMP-2 (100 ng/ml), BMP-9 (100 ng/ml) and FK506 (1 μg/ml) on the expression of bone-related genes of rDFAT cells using quantitative PCR. (B)–(D) The cells were seeded at a density of 2×10^4 cells/cm^2 in CM. After 24 h of incubation, the medium was replaced with CM or ODM including the indicated combination of stimulants, and cultured for 48 h (B, C) and 6 days (D). The data shown are mean ± SD ($n = 3$) and are representative of two separate experiments. $^*P < 0.05$, significantly different from rDFAT cells in ODM. $^\#P < 0.05$, significantly different from rDFAT cells with BMP-9 stimulation.

cells stimulated with BMP-9 compared with controls. Furthermore, addition of FK506 synergistically enhanced BMP-9-induced expression of these genes (Fig. 1B,C). In contrast, the effects of BMP-2 alone or co-stimulation with BMP-2 and FK506 on expression of these genes in rDFAT cells were very slight compared with BMP-9 alone or co-stimulation with BMP-9 and FK506.

3.2. ALP activity and mineralization of rDFAT cells induced by co-stimulation with BMP-9 and FK506

Next, we determined whether BMP-9 alone or in synergy with FK506 promoted osteoblastic differentiation of rDFAT cells. Although BMP-9 alone failed to induce ALP activity at 6 days, it significantly enhanced ALP activity in the presence of FK506 (0.01–1 μg/ml) (Fig. 2A). The most effective dose of FK506 was 1 μg/ml (Fig. 2A). However, a high dose (10 μg/ml) of FK506 was not effective (Fig. 2A). Thus, we decided to use 1 μg/ml FK506 for the following experiments. In contrast, BMP-2 alone or in synergy with FK506 failed to enhance ALP activity at 6 days (Fig. 2B).

Fig 2C shows mineralization of rDFAT cells at 21 days. The mineralization was remarkably increased by co-stimulation with BMP-9 and FK506, compared with vehicle, BMP-2 alone and BMP-9 alone.

3.3. Co-stimulation with BMP-9 and FK506 induced phosphorylation of smads

As BMP-9 activates smad1/5/8 in various cell types [23,24], we examined the effects of BMP-9 and BMP-9 + FK506 on the phosphorylation of smad1/5/8 in rDFAT cells. In BMP-9-treated cells, expression of phospho-smad1/5/8 was slightly up-regulated after 30, 60, and 120 min of exposure to the stimuli. However, in cells co-stimulated with BMP-9 and FK506, expression of phospho-smad1/5/8 was potently enhanced (Fig. 3).

3.4. Effects of noggin on rDFAT cells stimulated with BMP-9 + FK506

Noggin is well known to be a BMP antagonist, which binds with varying affinities to BMPs and blocks smad-dependent signaling. The levels of *noggin* mRNA were significantly enhanced in rDFAT cells stimulated with BMP-9. Addition of FK506 significantly up-regulated BMP-9-induced *noggin* mRNA expression (Fig. 4A). Similarly, although BMP-2 alone was unable to induce the expression of *noggin* mRNA, co-stimulation of cells with BMP-2 and FK506 slightly up-regulated expression of the antagonist (Fig. 4A).

Next, we examined the effects of exogenous noggin on ALP activity, induced by co-stimulation with BMP-9 and FK506 in rDFAT cells. The ALP activity was not significantly influenced by exogenous noggin (Fig. 4B).

4. Discussion

It has been demonstrated that DFAT cells can exhibit adipogenic, osteogenic, chondrogenic, and myogenic potentials [25]. In the present study, we showed that a combination of BMP-9 and FK506 induced a significant increase in ALP activity, and promoted mineralization, in rDFAT cells cultured in ODM. In contrast, supplementation of the culture medium with BMP-9 alone, BMP-2 or BMP-2 + FK506 could not induce a significant increase in ALP activity. Stimulation of rDFAT cells with BMP-9 alone mildly enhanced gene expression of *runx2*, *osterix*, and *BSP*, but treatment with the combination of BMP-9 and FK506 markedly up-regulated the expression of the genes. BMP-2 and BMP-2 + FK506 gave no or limited effects on the expression of these genes. From these results,

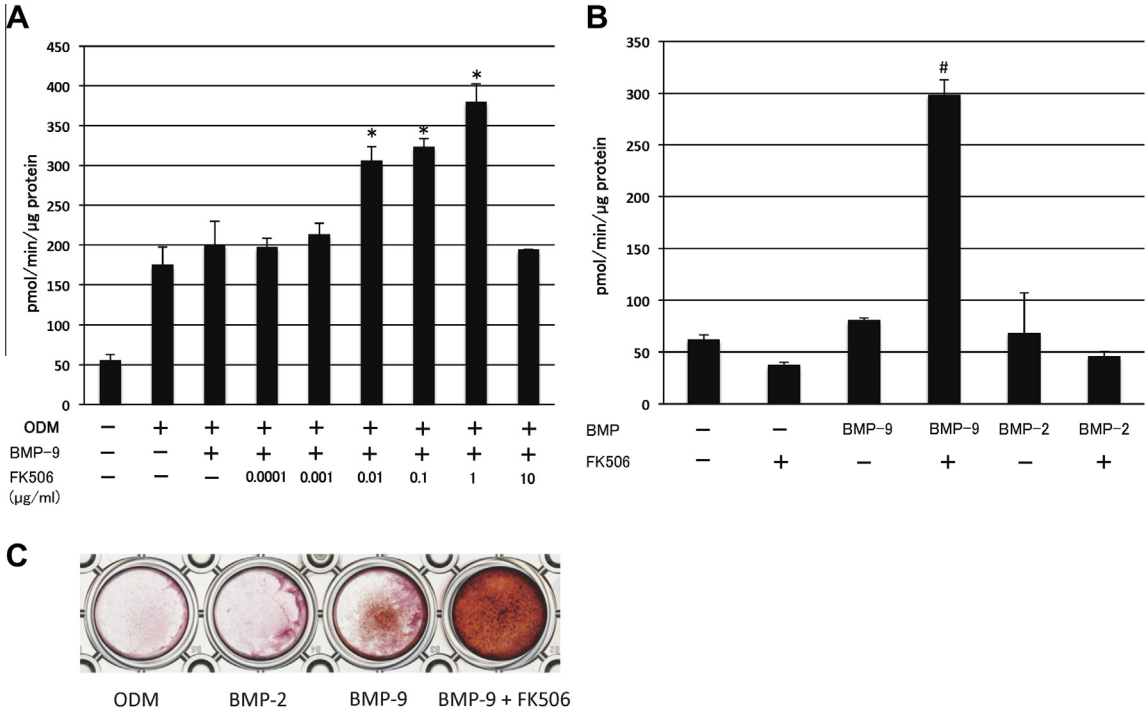

Fig. 2. Co-stimulation with BMP-9 and FK506 induced osteoblastic differentiation of rDFAT cells. (A) Dose-dependent effect of FK506 on ALP activity of rDFAT cells in the absence or presence of BMP-9 (100 ng/ml). (B) The effects of FK506 on ALP activity in BMP-9 or BMP-2-stimulated rDFAT cells. The cells were seeded at a density of 2×10^4 cells/cm^2 in CM. After 24 h of incubation, the cells were stimulated with BMP-9 (100 ng/ml), BMP-2 (100 ng/ml) and FK506 (1 μg/ml) alone or in combination under ODM, and cultured for 6 days. The data shown are mean ± SD ($n = 3$) and are representative of two separate experiments. (C) Alizarin red staining of rDFAT cells cultured in the presence of BMP-2 (100 ng/ml), BMP-9 (100 ng/ml) and BMP-9 + FK506 (1 μg/ml) at 21 days under ODM. $^*P < 0.05$, significantly different from rDFAT cells with BMP-9 stimulation in ODM. $^\#P < 0.05$, significantly different from rDFAT cells with BMP-9 stimulation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

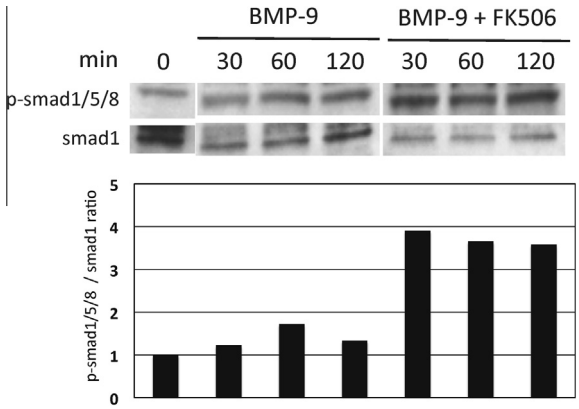

Fig. 3. Western blot analysis of phosphorylated smad1/5/8 in rDFAT cells. The cells were treated with BMP-9 (100 ng/ml) and BMP-9 + FK506 (1 μg/ml) for the indicated times. Proteins were extracted from the cells, and the extracts were analyzed by western blotting for phosphorylated (p) –smad1/5/8 and smad1 (upper panel). Lower panel shows the ratios of band densities of p-smad1/5/8 to smad1.

it is very likely that the combination of BMP-9 and FK506 is a potent inducer of osteoblastic differentiation in rDFAT cells.

BMP dimers bind to a tetrameric receptor complex, composed of pairs of type I and II receptors, both of which are necessary for signaling events via smad and non-smad pathways. Seven type I receptors (ALK-1–7) and three type II receptors have been identified [10]. Recent studies have demonstrated that the type I receptors involved in BMP-9-induced osteoblastic differentiation of MSCs are ALK-1 and ALK-2, and that ALK-1 in particular is highly specific for BMP-9 [26,27]. ALK-1 and ALK-2 activate smad1/5/8 and transduce intracellular signals. In the present study, we showed that ALK-1 and ALK-2 were expressed in rDFAT cells and that smad1/5/8 was phosphorylated following stimulation with BMP-9 in the presence of FK506, which can modulate smad signaling pathway [12]. We demonstrated here that the expression of bone-related genes, ALP activity and mineralization were all remarkably increased by the stimulation of rDFAT cells with BMP-9 in the presence of FK506, compared with BMP-9 alone. It is very likely that these effects of BMP-9 and FK506 on rDFAT cells are regulated at least in part via the smad signaling pathway, since the phosphorylation of smad1/5/8 was induced by the stimulation with BMP-9 + FK506. It has been previously shown that p38 and ERK1/2 mitogen activated protein kinases (MAPKs) are activated by BMP-9, through non-smad pathways of BMP signaling, which act in opposition to regulate BMP-9-induced osteoblastic differentiation in mesenchymal progenitor cells [28]. Thus, there is a possibility that the non-smad pathways are involved in the BMP-9 + FK506-induced osteoblastic differentiation of rDFAT cells. Further investigation is necessary to clarify the mechanism of action of BMP-9 + FK506 in rDFAT cells.

FK506 was seen to act in a cooperative manner with BMP-9 in the osteoblastic differentiation of rDFAT cells, but not with BMP-2, in this study. The different effects observed for BMP-9 and BMP-2, in conjunction with FK506, in rDFAT cells, may in part be due to the influence of BMP antagonists including noggin, which binds with varying affinities to BMPs and inhibits BMP-2, -4, -5, -7, -13, and -14 mediated actions [29]. It has been reported that osteogenic activity mediated by BMP-9 is not antagonized by noggin in W20 cells, a mouse BMSC line [19]. In rDFAT cells, gene expression of *noggin* was induced by the stimulation of BMP-2 or BMP-9 in the presence of FK506, yet a more potent expression of the antagonist was observed with BMP-9 + FK506 than BMP-2 + FK506. However, ALP activity enhanced by BMP-9 + FK506 was not affected by exogenous noggin. Thus, we suggest that BMP-9

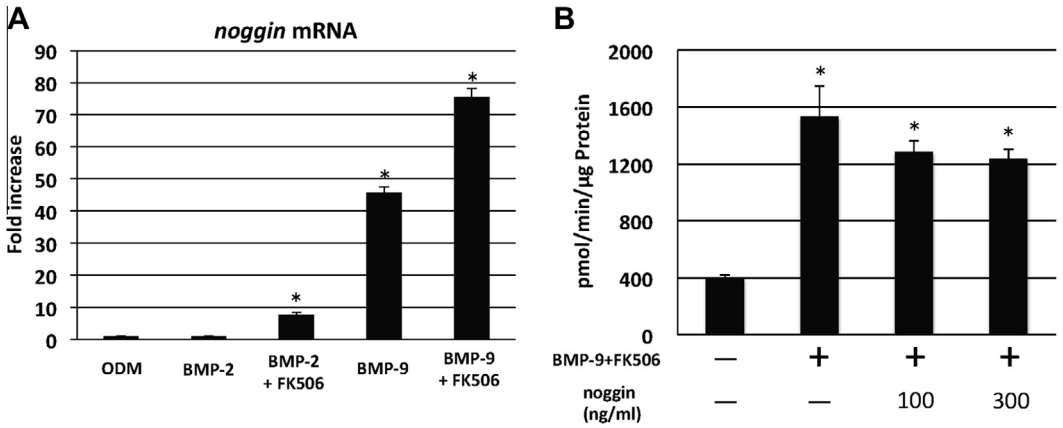

Fig. 4. *Noggin* expression (A) and effects of exogenous noggin on ALP activity (B) in rDFAT cells. rDFAT cells were stimulated with BMP-2 (100 ng/ml), BMP-9 (100 ng/ml) and FK506 (1 μg/ml) alone or in combination under ODM (A), or stimulated with BMP-9 + FK506 with or without noggin (B), and cultured for 6 days. $^{*}P < 0.05$, significantly different from ODM.

+ FK506-induced osteogenic differentiation of rDFAT cells is not inhibited by noggin. On the other hand, in the stimulation with BMP-2 + FK506, endogenous noggin might bind to BMP-2, resulting in the inhibition of BMP-2 action in the culture system of the present study. Diefenderfer et al. have demonstrated that BMP-2 induces mRNA expression of *noggin*, but does not induce ALP activity in human MSCs, suggesting that a defect exists in the system required for the differentiation of the osteoblastic phenotype [30]. Thus, there may be a possibility that rDFAT cells similarly lack the system for osteogenic differentiation in response to BMP-2.

Obinata et al. revealed that transplantation of DFAT cells promoted urethral tissue regeneration and sphincter function in the rat vaginal distension model [31]. Moreover, it has been demonstrated that DFAT cells can spontaneously differentiate into endothelial cells, which is promoted by BMPs [32]. These lines of evidence suggest that DFAT cells could be a promising cell source for transplant across the various fields of regenerative medicine. Very recently, Kikuta et al. have reported that autologous implantation of DFAT cells cultured in ODM for 3 weeks with a scaffold (β-TCP/collagen sponge) contributes to bone regeneration in a rabbit bone defect model [33]. Therefore, implantation of DFAT cells osteo-differentiated by BMP-9 + FK506 with an appropriate scaffold may become effective for bone regeneration.

In conclusion, we suggest that the combination of BMP-9 and FK506 effectively induces osteoblastic differentiation of rDFAT cells. Thus, the associated reduction in both cost and culture time through the use of BMP-9 and FK506 differentiated DFAT cells may make them a viable transplant alternative in bone regenerative therapy. Further studies are required to investigate the effects of BMP-9 + FK506 osteo-differentiated DFAT cells on bone regeneration *in vivo*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.073.

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