

Effect of Prostaglandin E₂ on Human Dental Follicle Cells during Osteogenic Differentiation

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Article History

Received 1 December 2017

Accepted 5 December 2017

Keywords :

human dental follicle cells,
prostaglandin E₂,
osteogenic differentiation,
mineralization

Abstract

Stem/progenitor cells isolated from human dental follicles differentiate into osteogenic cells. To investigate factors associated with osteogenic differentiation/mineralization in human dental follicle cells (hDFCs), we performed gene expression profiling of hDFCs during osteogenic differentiation. Cyclooxygenase (COX)-1 and -2 were up-regulated in hDFCs cultured in osteogenic induction medium (OIM) compared to growth medium (GM). Prostaglandin E₂ (PGE₂), which is the most studied prostanoid derived from arachidonic acid through the actions of COXs, may regulate bone metabolism. All PGE₂ E-type prostanoid receptors (EP), EP1-EP4, were expressed in hDFCs. Real-time PCR showed that the expression of EP2 and EP4 was increased in hDFCs cultured in OIM and GM at days 10 and 17 compared to day 0. We investigated the action of PGE₂ in osteogenic differentiation using hDFCs. PGE₂ decreased gene expression levels of osterix and alkaline phosphatase, which are factors associated with osteogenic differentiation. PGE₂ elicited inhibitory action on matrix mineralization of hDFCs as seen with alizarin red S staining. These findings suggest that PGE₂ may inhibit osteogenic differentiation/mineralization of stem/progenitor cells.

Introduction

Neural crest cells, a specific population of vertebrate cells that originate in the dorsal neural tube, form a variety of tissues including the dorsal root ganglia, peripheral nerves, and craniofacial bone (1-3). The dental follicle, which is an ectomesenchymal tissue derived from the neural crest that surrounds the tooth germ, contains progenitor cells and/or stem cells. Human dental follicle cells (hDFCs) have a capacity to commit to differentiate into multiple cell lineages such as osteoblastic and adipogenic cells (4, 5). hDFCs are a major source of stem cells in adults, as they can be easily got during the extraction of impacted teeth. We previously reported that hDFCs can differentiate into osteogenic cells in osteogenic induction medium (OIM) without dexamethasone (6), which has various biological effects such as anti-inflammatory properties. In addition, we compared the

gene expression profiles between mesenchymal stem cells (MSCs) from human bone marrow (hMSCs) and hDFCs to assess whether hDFCs are a useful cell source for applications in clinical tissue regeneration (7). The expression of MSC markers and growth factor receptors is similar in hDFCs and hMSCs, whereas hDFCs highly express LIM (acronym of Lin-11, Isl-1, Mec-3) homeobox 8, which is associated with development of the palatal mesenchyme and tooth germ (7).

Prostaglandins, which are lipid metabolites derived from arachidonic acid through the actions of cyclooxygenase (COX)-1 and -2, play an important role in bone formation and inflammatory processes. Prostaglandin E₂ (PGE₂) is abundantly expressed in bone and plays important roles in skeletal metabolism (8). PGE₂ increases bone marrow mineralized nodule formation when administered systemically, suggesting that PGE₂ increases the number of committed progenitors (9). In contrast, PGE₂ effectively suppresses MSCs during bone marrow-mediated matrix

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mineralization (10). PGE₂ plays an important role during regulating bone metabolism, although whether its primary mode of action is to promote bone resorption or bone formation remains a topic of debate.

The aim of this study was to investigate the effect of PGE₂ during osteogenic differentiation using hDFCs.

Materials and Methods

Isolation and culture of hDFCs

hDFCs were obtained using according to a previously reported method (5). The use of hDFCs was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo (Recognition number: EC 15-10-036-1). Briefly, normal human impacted third molars were surgically removed and collected from two patients (one female and one male: 14 years of age) who gave informed consent and informed assent. Dental follicle tissues were washed in phosphate-buffered saline (PBS), minced with sterilized scalpels, and digested in a solution of 1 U/ml dispase and 0.1 U/ml collagenase type I (Roche, Basel, Switzerland) for 1 h at 37°C. hDFCs attached to 100 mm culture plates and were grown in growth medium (GM; Mesenchymal stem cell growth medium BulletKit™ consisting MSC basal medium, fetal bovine serum, penicillin/streptomycin, and L-glutamine; Lonza, Basel, Switzerland) in a humidified incubator (CO₂ incubator MCO-175M; Panasonic, Tokyo, Japan) in 5% CO₂ in air at 37°C. hDFCs from the 5 to 6th passage were used for the following experiments.

Osteogenic differentiation

For induction of osteogenic differentiation, hDFCs were seeded at 3.1×10^3 cells/cm² in GM. After 24 h (day 0), media were replaced with OIM (Osteogenic differentiation media BulletKit™ consisting Osteogenic basal medium, L-glutamine, penicillin/streptomycin, ascorbate, and β-glycerophosphate; Lonza) as reported previously (6). Medium was replaced every 3 days (7).

To investigate the effects of PGE₂ on hDFCs, the cells were cultured in OIM containing 10⁻⁵ to 10⁻⁷ M PGE₂ (Cayman, Ann Arbor, MI) or 0.1% dimethyl sulfoxide (DMSO) for the indicated number of days. Media were replaced every 3 days.

Total RNA isolation

Total RNA was isolated using miRNeasy Mini Kits

(QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Microarray analysis

Gene expression profiling was performed using SurePrint G3 Human GE microarray 8 × 60k (Agilent, Santa Clara, CA) according to the manufacturer's protocol. Briefly, cRNA labelled with Gy3 was generated from 100 ng total RNA using the Low Input Quick Amp Labeling kit, one-colour (Agilent) and hybridized to the array using a Gene Expression Hybridization kit. The array was scanned (Agilent DNA Microarray Scanner), and raw data from the microarray were loaded into Gene Spring GX software (Version 11.5; Agilent).

Alizarin red S staining

Cells were washed twice with PBS, fixed with 10% formalin solution for 30 min, and then washed twice with pure water. Cells were placed in 1% alizarin red S (Kanto Chemical, Tokyo, Japan) solution for 10 min, washed three times with pure water, and then air-dried.

Real-time PCR

Complementary DNA (cDNA) was synthesized from total RNA using the GeneAmp RNA PCR Kit (Thermo Fisher Scientific, Waltham, MA). Real-time PCR was performed using the DyNAmo SYBR Green qPCR Kit (Thermo Fisher Scientific). The PCR mixture, containing 20 pmol forward and reverse primers and 2 μl cDNA, was subjected to amplification with a DNA Engine Opticon 1 (Bio-Rad, Hercules, CA), with preheating at 95°C for 15 min, followed by 40 cycles of 94°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec. Primer sequences used for real-time PCR analysis are shown in Table 1. Gene expression levels were calculated using the ΔΔC_T method with normalization to GAPDH (11).

PCR fragments were electrophoresed on 1.5% agarose gels, followed by staining with Midori Green Direct (NIPPON Genetics, Tokyo, Japan) and examination of fragment sizes.

Statistical analysis

Data are shown as mean values ± SD. One-way ANOVA and Two-way ANOVA were used to analyze differences.

Table 1. Primer sequences for real-time PCR

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')	Amplicon size (bp)
COX-1	CACAGTGCCTCCAACCTTATCC	CCAGGACCCATCTTGCCAGAAG	306
COX-2	TTCAAATGAGATTGTGGGAAAATTGCT	AGATCATCTCTGCCTGAGTATCTT	304
EP1	CTGGAACCAGATCCTGGAC	GTGCTTAGAAGTGGCTGAGG	191
EP2	GCTATCATGACCATCACCTT	ACCTAAGAGCTTGGAGGTC	109
EP3	CTTGGGTTTACCTGCTGTT	ACATCAAGGTTGAGGAACAC	123
EP4	CATCTTACTCATTGCCACCT	TTTACTGACTTCTCGCTCCA	112
Runx-2	TGAGAGCCGCTTCTCCAACC	GCGGAAGCATTCTGGAAGGA	266
OSX	GGGCTTTCTCTGGGGTTGCT	AGGCCCTTCGTCTGCACCTG	292
ALP	GATATGTTCTGGGAGA	GAAGGGAACTGTCCATCT	79
BMP-2	CATGTGGACGCTCTTCAAT	GAAGCAGCAACGCTAGAAGA	113
BMP-4	GCTAGGAGCCATTCCGTAGT	CCTAGCAGGACTTGGCATAA	193
TGF-β	TGTGTTATCCCTGCTGTAC	GACTCGCCAGAGTGGTTATC	123
GAPDH	ATCACCATCTTCCAGGAG	ATCGACTGTGGTCATGAG	318

Cyclooxygenase: COX, E-type prostanoid receptor: EP, Runt-related transcription factor 2: Runx-2, Osterix: OSX, Alkaline Phosphatase: ALP, Bone Morphogenetic Protein: BMP, Transforming growth factor-beta: TGF-β, Glyceraldehyde-3-phosphate dehydrogenase: GAPDH.

Table 2. COX DNA Microarray

Gene	raw data		
	Day0	Day17	
	GM	GM	OIM
COX-1	274	651	2925
	1331	821	3463
COX-2	490	160	2521

Growth medium: GM, Osteogenic induction medium: OIM, Cyclooxygenase: COX.

Results

COX gene expression

We performed microarray analysis to determine the genes regulated in hDFCs during osteogenic differentiation (6). As shown in Table 2, the gene expression of COX-1 and COX-2 was increased in hDFCs cultured in OIM at day 17 compared to cells cultured in GM on days 0 and 17.

We examined the gene expression of COX-1 and -2 in hDFCs during osteogenic differentiation using real-time PCR. COX-1 and COX-2 expression was significantly increased in hDFCs cultured in OIM compared to GM on day 17 (Fig. 1). The expression of COXs was not different between cultures grown in OIM and GM on day 3 or day 10.

PGE₂ receptor gene expression

To examine the functional effects of PGE₂ on hDFCs, we

analyzed the expression of PGE₂ receptors, EP1-4, in hDFCs using RT-PCR (Fig. 2). hDFCs before osteogenic differentiation was not induced expressed all EPs (Fig. 2). The expression of EP4 was up-regulated in hDFCs on day 17 compared to day 0 as seen with microarray analysis (Table 3). We confirmed the expression of EPs in hDFCs during osteogenic differentiation using real-time PCR (Fig. 3). The expression of EP4 was significantly increased in hDFCs cultured in OIM on day 10 and 17 compared to day 0 whereas the expression was also significantly increased in hDFCs cultured in GM. The expression of EP4 was slightly increased in OIM than GM on day 10 and 17. The expression of EP2 was significantly increased in hDFCs in a time-dependent manner during osteogenic differentiation. The expression of EP2 was increased in GM culture in a time-dependent manner although the increase level was

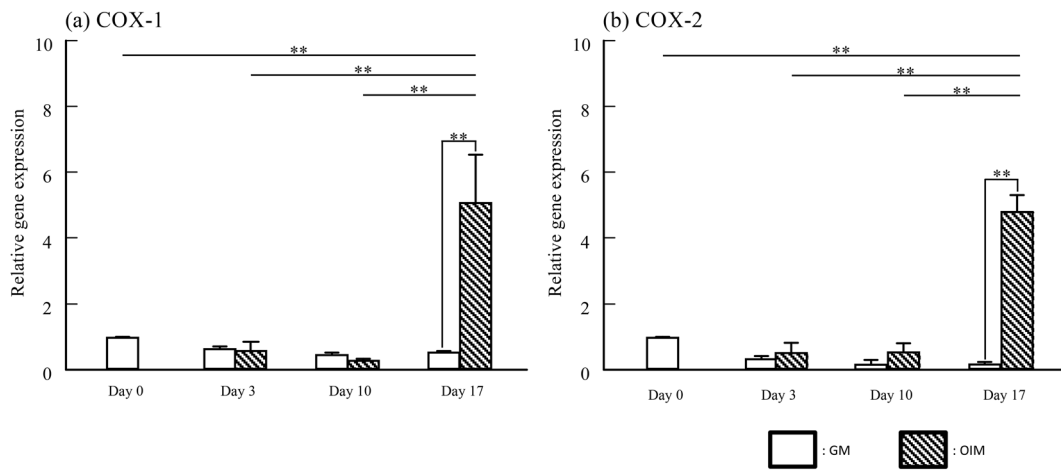


Fig. 1. Gene expression of COXs in hDFCs. hDFCs were cultured in OIM or GM. COX-1 (a), COX-2 (b). Gene expression of COXs was examined with real-time PCR. Values represent the means \pm SD of results from five independent experiments. **P < 0.01 compared to cells grown in GM at the indicated time points.

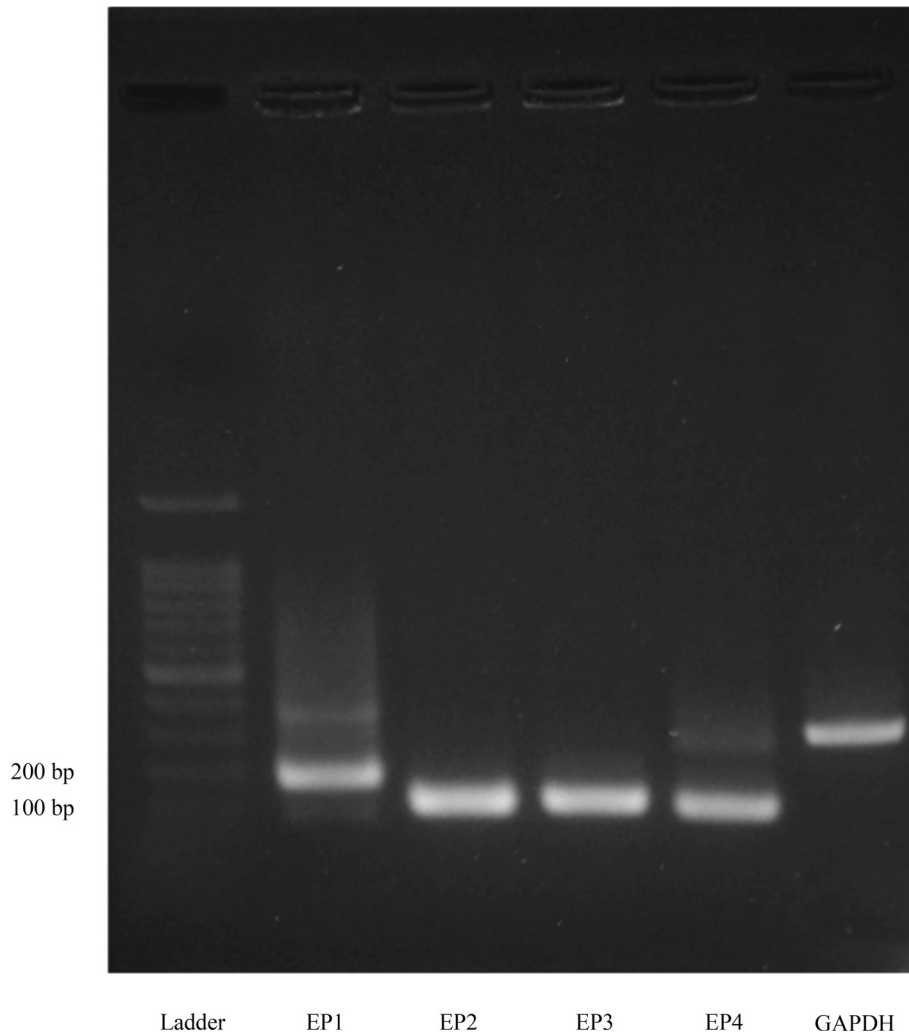


Fig. 2. The expression of PGE₂ receptors in human dental follicle cells. mRNA levels of E-type prostanoid receptors (EP1, EP2, EP3, and EP4), in hDFCs were analyzed using RT-PCR. The PCR products were electrophoresed through an agarose gel.

Table 3. EP DNA Microarray

Gene	raw data		
	Day0		Day17
	GM	GM	OIM
EP1	574	217	87
EP2	901	991	1351
EP3	241	329	106
	159	231	143
	147	280	93
	542	743	318
EP4	502	3816	1861

Growth medium: GM, Osteogenic induction medium: OIM,
E-type prostanoid receptor: EP.

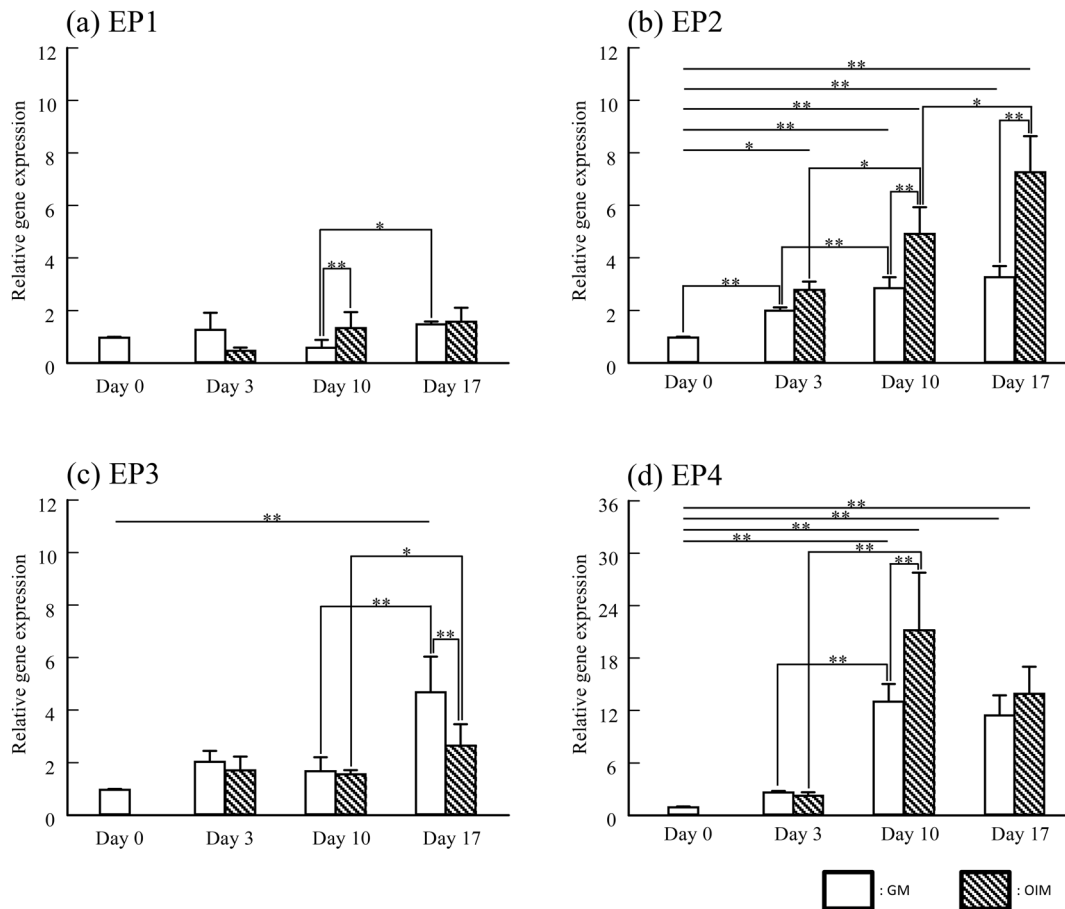


Fig. 3. Time course of EP expression in hDFCs. hDFCs were cultured in OIM or GM. EP1 (a), EP2 (b), EP3 (c), EP4 (d). Gene expression of EPs was examined with real-time PCR. Values represent means \pm SD of the results from three independent experiments. *P < 0.05, **P < 0.01 compared to cells cultured GM at the indicated time points.

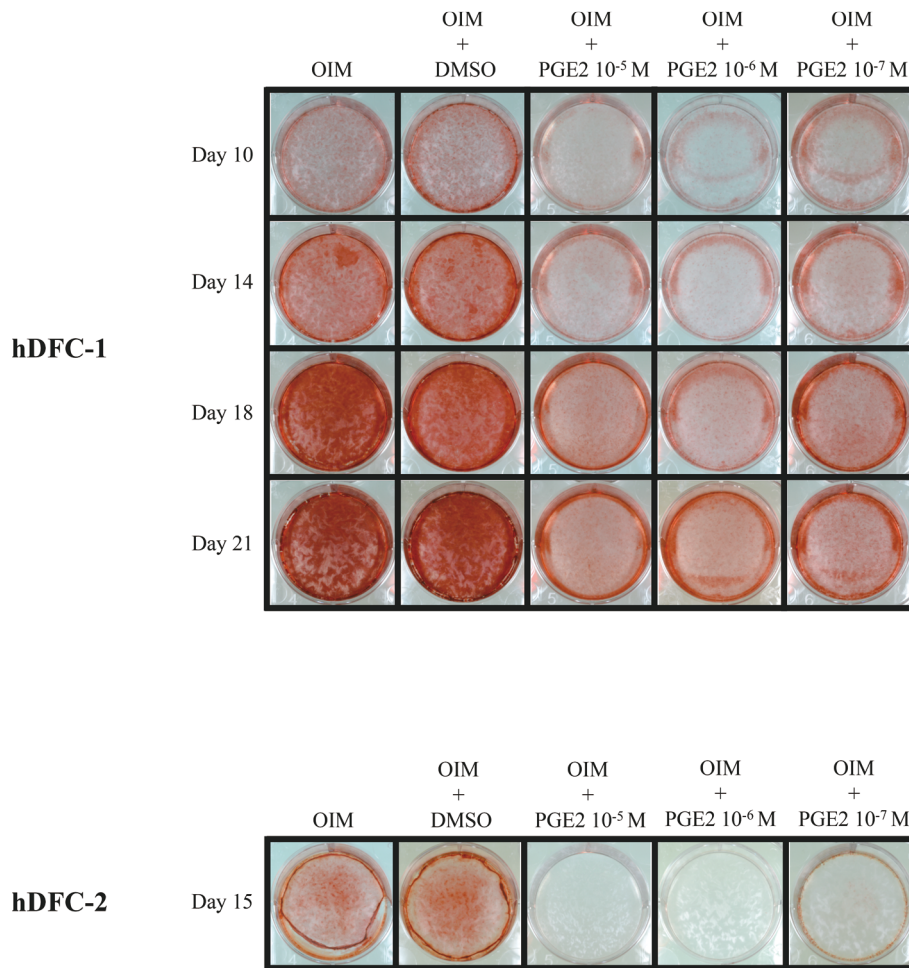


Fig. 4. Effect of PGE_2 on matrix mineralization. hDFCs were cultured in GM or OIM with or without 10^{-5} to 10^{-7} M PGE_2 from day 10 to day 21. Matrix mineralization was examined using alizarin red S staining. hDFC-1: cells from patient 1. hDFC-2: cells from patient 2. DMSO, PGE_2 solvent.

slightly in GM culture compared to OIM culture. In contrast, the expression of EP3 was decreased in hDFCs cultured with OIM compared to with GM on day 17. The expression of EP1 was not different in hDFCs between the cultures in OIM and GM.

Effects of PGE_2

Mineralization was examined by alizarin red S staining in hDFCs (patient 1: hDFC-1) treated with PGE_2 during osteogenic differentiation (Fig. 4). hDFC cells cultures grown in OIM and OIM plus 0.1% DMSO (PGE_2 solvent) stained weakly at day 10, and stained strongly thereafter in a time-dependent manner. From day 10 to 21, hDFCs treated with PGE_2 stained weakly compared to cultures grown in OIM and OIM plus DMSO. At day 10 to 18, hDFCs treated with 10^{-5} and 10^{-6} M PGE_2 stained weakly

compared to cultures treated with 10^{-7} M PGE_2 . The alizarin staining of hDFCs from a different patient (patient 2: hDFC-2) was also weak following treatment with PGE_2 (Fig. 4).

Following addition of PGE_2 to hDFCs cultured in OIM, gene expression related to osteoblast differentiation/mineralization was examined. The expression of osterix (OSX) was significantly decreased in hDFCs following treatment with 10^{-5} and 10^{-6} M PGE_2 (Fig. 5). In addition, the expression of alkaline phosphatase (ALP) was significantly decreased in hDFCs following treatment with 10^{-5} to 10^{-7} M PGE_2 (Fig. 5). The gene expression of runt-related transcription factor 2 (Runx-2), bone morphogenetic protein 4 (BMP-4), and transforming growth factor- β (TGF- β) tended to decrease in hDFCs treated with 10^{-5} and 10^{-6} M PGE_2 compared to non-treated cells grown in OIM (Fig. 5).

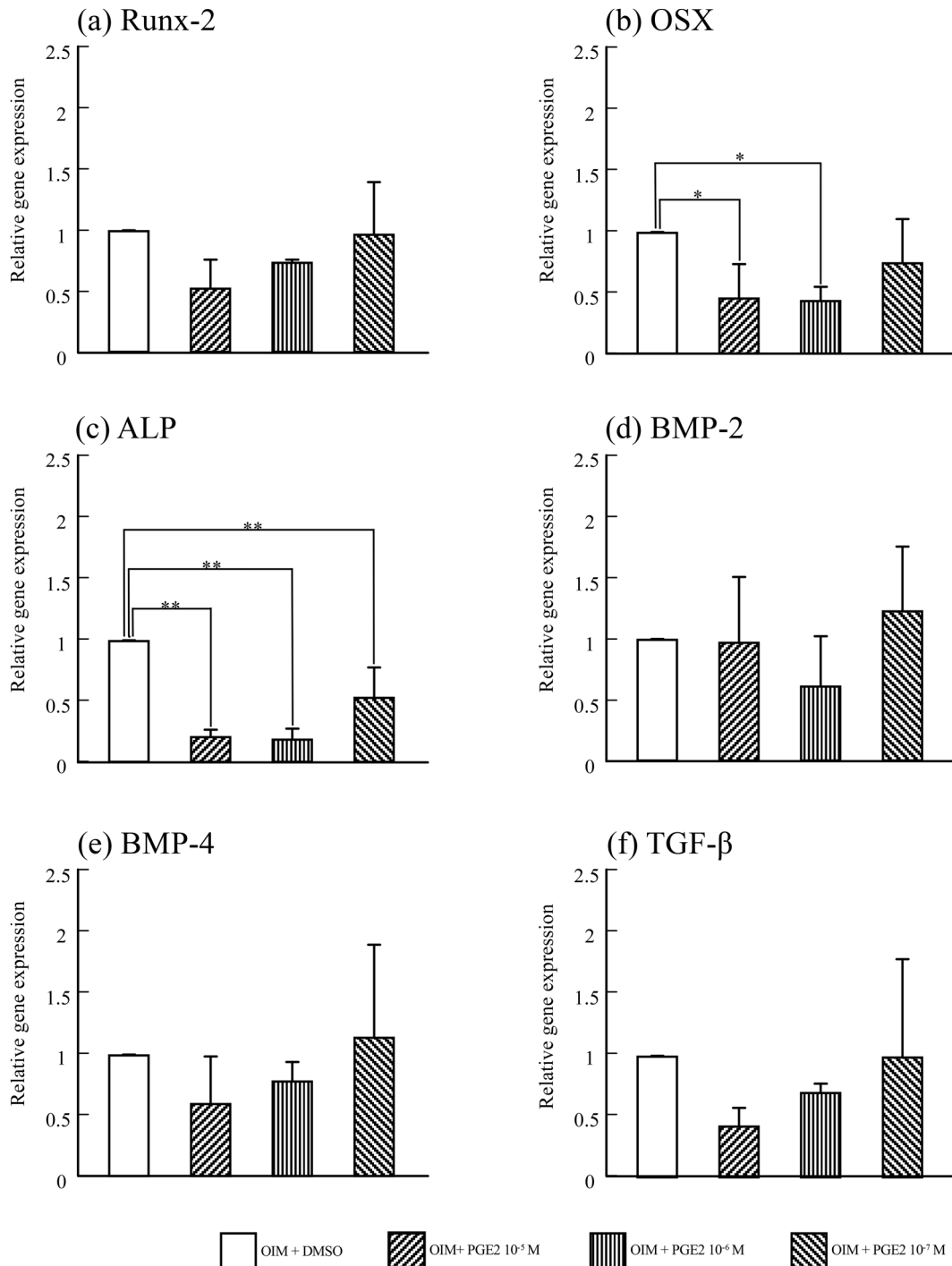


Fig. 5. Effects of PGE₂ on osteogenic differentiation of hDFCs. Cells were cultured in OIM or GM, with 10⁻⁵ to 10⁻⁷ M PGE₂ for 3 days. Gene expression was examined using real-time PCR. Values represent the means ± SD of the results from five independent experiments. *P < 0.05, **P < 0.01 compared to cells cultured in GM at the indicated time points. Runt-related transcription factor 2: Runx-2(a), Osterix: OSX (b), Alkaline Phosphatase: ALP(c), Bone morphogenetic protein: BMP -2(e), -4(d), Transforming growth factor-beta: TGF-β(f).

Discussion

To investigate the genes associated with osteogenic differentiation and/or mineralization, we profiled the changes in gene expression in hDFCs between culture day 0 and day 17, which is the mineralization stage (6). Gene expression analyses showed that COX-1 and -2 are up-regulated in cells cultured in OIM at day 17 compared to day 0 and GM at day 17. We examined the time course of COX expression in hDFCs during osteogenic differentiation using real-time PCR. The expression of COXs was up-regulated in hDFCs cultured in OIM compared to GM at day 17, whereas the expression of COXs was not different in cells cultured in OIM compared to GM at day 3 or day 10. COXs are enzymes that convert arachidonic acid into Prostaglandin G2 and then into Prostaglandin H2 (12). COX-1 is often referred to as “constitutive,” because it is expressed at stable levels in cells, in contrast to COX-2, which is expressed at low levels until a stimulus “induces” expression (12). COX-1 expression is induced in stromal cells and osteoblasts by several stimuli, although the induced level of COX-1 is low compared to that of COX-2 (13). In this study, the expression of COX-1 and -2 was up-regulated in hDFCs during late stages of osteogenic differentiation. Therefore, prostanoids derived from arachidonic acid through the actions of COXs may be associated with mineralization of hDFCs.

PGE₂, which is the most well-studied prostanoid derived from arachidonic acid through the actions of COXs, may play an important role in bone remodeling under physiological and pathological conditions. PGE₂ increases bone formation and bone mass in *in vivo* experiments using rats (14). Local delivery of PGE₂ enhances bone formation at the cortical bone graft junction (15). The pro-osteogenic effect of PGE₂ has been further substantiated by findings from numerous *in vitro* studies using murine- or rat-derived MSCs and osteoblasts (16, 17). The ability of PGE₂ to enhance bone formation has largely been attributed to its stimulatory effects on MSC differentiation (16, 18). In contrast, findings from *in vitro* studies utilizing hMSCs derived from bone marrow and adipose tissue suggest that PGE₂ may also have a negative influence on osteogenesis (10, 19). Our findings showed that continuous treatment with PGE₂ inhibited matrix mineralization by hDFCs during osteoblast differentiation/mineralization. In addition, the markers of early-stage osteoblasts, OSX and ALP, were significantly down-regulated in hDFCs by PGE₂ treatment.

The expression of Runx-2 and BMP-4 was also down-regulated by PGE₂ treatment, although the differences were not significant. It has been reported that PGE₂ down-regulated the expression of Runx-2 in adipose tissue-derived MSCs at culture day 4 (19). PGE₂ inhibited *in vitro* mineralization of human periodontal ligament (PDL) cells, although PGE₂ up-regulated the expression of Runx-2 (20); it was suggested that the inhibitory effect might be caused by the up-regulation of TWIST1, an inhibitor of Runx-2 (20). In a recent study, PGE₂ inhibited matrix mineralization and enhanced adipogenesis in human bone marrow stromal cells (10).

It has also been suggested that the effects of PGE₂ are dependent on the concentration dose. A high dose of PGE₂ (100 ng/ml, 10⁻⁴ M) inhibited the proliferation of PDL cells while a low dose of PGE₂ (0.01 to 1 ng/ml, 10⁻⁸ to 10⁻⁶ M) appeared to play a role in the maintenance of PDL stemness through the enhancement of proliferation and expression of Nanog and Oct4 (21). In addition, the regulation of Runx-2 expression was also dependent on the dose of PGE₂ in the cells with the ability to differentiate into osteoblasts. The expression of Runx-2 was up-regulated in PDL cells treated with 10⁻⁶ M PGE₂ (20) and bone marrow stromal cells treated with 10⁻⁷ to 10⁻⁹ M PGE₂. In our study, hDFCs were treated with 10⁻⁵ to 10⁻⁷ M PGE₂. Examination of the treatment of hDFCs with a low concentration (10⁻⁸ to 10⁻⁹ M) of PGE₂ is needed in future studies.

The various effects of PGE₂ on cell metabolism may be explained in part by the activation of different EP receptors (22). Different efficiencies of PGE₂ may be due to the relative level and distribution of PGE₂ receptors, EP1-EP4. The osteogenic action of PGE₂ in osteoblasts is mediated through EP2 and EP4 (23), based on experimental evidence showing that bone formation is enhanced by a selective EP agonists (24) and also that PGE₂-mediated bone formation is abolished only in EP2 or EP4 knockout mice (25, 26). It also suggested that PGE₂ mediated its effects through EP2 and EP4 in inhibiting matrix mineralization of hMSCs derived from bone marrow (10). The expression of EP2 was increased in hMSCs on day 7 and 14 during osteogenic differentiation. The expression of EP4 was increased in these cells on day 14 whereas decreased on day 7. However, despite identifying dexamethasone as a potent stimulator of EP2 expression, and, to a lesser extent, EP4 expression, the response of hMSCs to PGE₂ was independent of dexamethasone. In addition, they suggested that treatment of hMSCs

with EP4 siRNA proved more effective than EP2 siRNA in rescuing matrix mineralization by hMSCs treated with PGE₂(10). In our study, the expression of EP2 and EP4 was increased in hDFCs toward the late stage of osteoblast differentiation/mineralization, although hDFCs were cultured without dexamethasone. On the other hand, the up-regulation of EP2 and EP4 were earlier than those of COXs in hDFCs. Because the COXs were expressed in hDFCs of early stage for osteoblast differentiation/mineralization, PGE₂ may be produced in hDFCs constitutively. Future investigations should examine the PGE₂ production in hDFCs and the effect of endogenous and exogenous PGE₂ on osteogenic differentiation/mineralization of hDFCs. In addition, comparisons of the effects of EP1-EP4 should be studied on osteoblast differentiation/mineralization of hDFCs using selective agonists and/or antagonists, or receptor siRNAs of these receptors.

In conclusion, we investigated the effect of PGE₂ on osteogenic differentiation and/or mineralization using hDFCs. Our data suggested that PGE₂ inhibits osteoblast differentiation/mineralization of hDFCs. These findings provide important insight into methods to control stem/progenitor cell lineage commitment and may increase understanding of the role of PGE₂ in physiological and pathological conditions.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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