Prostaglandin E_2 inhibits mineralization and enhances matrix metalloproteinase-13 of matured cementoblasts mainly via EP4 pathway.

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RUNNING TITLE: The Role of PGE_2 and EPs in Cementoblasts

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Number of Words: ABSTRUCT (216 words), MANUACRIPT (2,247 words).

Number of table: 1

Number of figures: 3

Key Words: Prostaglandin E_2; PGE receptor subtypes; Cementoblasts; Periodontal tissue.

Abstract

Objective: PGE_2 is an important factor in pathogenesis of periodontal disease because of bone resorting activity and association with attachment loss. PGE_2 and PGE receptor subtypes (EPs) play an important role in modulating bone metabolism via osteoblasts. However, little is known about the effects of PGE_2 on cementoblasts. The aims of this study were to determine the expression of EPs on matured cementoblasts and to examine the effect of PGE_2 and EPs on their cellar function.

Design: Expression of EPs in immortalized mouse cementoblasts (OCCM-30), which were characterized as matured cementoblasts, was determined using RT-PCR. Then effects of PGE_2 and EP agonists on mineralization were examined by studying nodule formation with alizarin red S (ALZ) staining. Alkaline phosphatase (ALP) activity with PGE_2, EP4 agonist was examined by Bessey-Lowry enzymologic method. Effects of PGE_2-EP4 pathway on expression levels of osteocalcin (OCN) and matrix metalloproteinase (MMP)-13 mRNA were examined by real-time RT-PCR.

Results: OCCM-30 expressed EP1, 2, 3 and 4 mRNA. PGE_2 and EP4 agonist caused downregulation of mineralized nodule formation and ALP activity in OCCM-30. OCN mRNA expression was suppressed and MMP-13 mRNA expression was stimulated via PGE_2-EP4 pathway in OCCM-30.

Conclusions: Cementoblasts may downregulate their mineralization ability and upregulate MMP-13

production through PGE_2-EP4 pathway and may contribute to destruction of connective tissue attachment under inflammatory condition.

Introduction

Cementum, a thin mineralized tissue covering the tooth root surface, assists in anchoring teeth to surrounding alveolar bone. It also contributes to the maintenance of structural stability and physiological function of the dentition.[1] Several studies have demonstrated that cementoblasts share many characteristics with osteoblasts, including similar molecular properties and the ability to promote mineralization.[2,3] In the periodontal disease area, osteoblasts function as osteoclastogenesis controlling cell rather than as bone forming cell. It is considered that cementoblasts may be responsible for destruction of connective tissue attachment. However, little has known about the functions of cementoblasts under inflammatory condition. Therefore, it is important to study the mechanisms controlling function of cementoblasts in order to assist in enhancing of our understanding the pathogenesis of periodontal disease.

It is well known that a variety of products such as prostaglandins E_2 (PGE_2), cytokines and chemokines from inflammatory cells and periodontal tissue resident cells may contribute to this destructive process. Especially, PGE_2 is focused as an important factor involved in the pathogenesis of periodontal disease, because of powerful stimulator of bone resorption. In fact, high levels of PGE_2 within the gingival crevicular fluid have been associated with marked attachment loss in patients with periodontitis.[4, 5]

Various biological actions of PGE_2 are mediated by PGE specific G-protein-coupled receptors. PGE receptors (EPs) are divided into 4 subtypes, EP1, EP2, EP3 and EP4.[6, 7, 8]

Recently we reported that cementoblasts at proliferative culture stage were stimulated alkaline phosphatase (ALP) activity with PGE_2 treatment.[9] However, there is no data about expression of EPs mRNA in cementoblasts and detail report about roles of PGE_2 and EPs on cellular function of matured cementoblasts.

In this study, to determine the effect of PGE_2 and EPs on function of cementoblast, we investigated the expression of EPs in cementoblasts and examined the role of PGE_2 on function of matured cementoblasts such as mineralization and ALP activity, osteocalcin (OCN) and matrix metalloproteinase (MMP)-13 mRNA expressions.

Materials and methods

Cell line and cell culture

OCCM-30 cells used for these studies were established by isolating tooth root surface cells from transgenic mice containing a SV40 large T-antigen under control of an OCN promoter and were characterized as highly differentiated cementoblasts.[10, 11] OCCM-30 cells were maintained in DMEM (NISSUI PHARMACEUTICAL CO., LTD., Tokyo, Japan) supplemented with 10 mM HEPES (pH 7.2) (Sigma-Aldrich, Tokyo, Japan), 10% fetal bovine serum (FBS) (Invitrogen Corporation, N.Y. U.S.A.) and 100 U/mL Penicillin-Streptomycin (Invitrogen Corporation, N.Y., U.S.A) at 37 °C in a humidified atmosphere of 5% CO_2.

Reagents

PGE_2 was purchased from Advanced Magnetics Inc. (Massachusetts, USA). ONO-DI-004 (EP1 agonist), ONO-AE1-259-01 (EP2 agonist), ONO-AE-248 (EP3 agonist), ONO-AE1-329 (EP4 agonist) and ONO-AE3-208 (selective EP4 antagonist) were kindly provided from ONO Pharmaceuticals Co. Ltd. (Tokyo, Japan).

I. Gene Expression Experiments

i. Extruction of total mRNA

Expression of EPs: OCCM-30 cells were plated into 60 mm culture dishes (4×10^5 cells/dish) and cultured in α–MEM containing 10% FBS and ascorbic acid (AA)(50 μg/ml). At confluence, total RNA was extracted with TRIzol® Reagent (Invitrogen).

Expression of OCN and MMP-13: OCCM-30 cells were plated in 6 well plates (4×10^5 cells/well) and maintained in α–MEM containing 10% FBS and AA (50 μg/ml). Upon reaching confluence, cells were switched to α–MEM containing 2% FBS and AA (50 μg/ml) with PGE_2 (300 nM) or each EP agonist (1 μM). To determine the effects of PGE_2-EP4 pathway, the cells were pretreated with EP4 antagonist (1 μM) for 2 hours prior to the addition of PGE_2 (300 nM). After treatment for 7 days, total RNA was extracted with TRIzol® Reagent. After digesting contaminating genomic DNA with DEOXYRIBONUCLEASE I (SIGMA-Aldrich), total RNA was purified with Quiaquick® PCR Purification Kit (QIAGEN KK, Tokyo, Japan).

ii. RT-PCR

cDNAs were synthesized from 1 μg of total RNA using Rever Tra Dash (TOYOBO CO. LTD., Osaka, Japan). Aliquots of total cDNA were amplified with KOD-Plus-DNA Polymerase (TOYOBO CO. LTD.). The amplification was performed using a MyCycler^{TM} thermal cycler (BIO-RAD, Tokyo, Japan). PCR followed using primer pairs, annealing temperatures and reaction cycles listed in Table 1. PCR products were reduced on 1.5% agarose gels, electrophoresed at 100 mV and visualized by ethidium bromide.

iii. Quantitative real-time RT-PCR

cDNA was synthesized from 1 μg of total RNA as described above. Real-time RT-PCR was performed in the Light Cycler System (light cycler quick system 350S, Roche Diagnostics GmbH) using LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany) and specific primers for OCN and MMP-13 gene. The primer pairs and annealing temperatures used here listed in Table 1.

Reaction product was quantified (the LightCycler software version 3.5, Roche Diagnostics GmbH) with GAPDH as the reference gene. Each experiment was repeated four times with comparable results.

II. Mineralization

Mineral nodule formation was detected by alizarin red S (ALZ), which stains for calcium.

OCCM-30 cells were plated in 24 well plates (5×10^4 cells/well) and cultured in α-MEM supplemented with 10% FBS and AA (50 μg/ml) for 24 hours. Then medium was changed to α-MEM containing 2% FBS, AA (50 μg/ml), and sodium β−glycerophosphate (10 mM) with PGE_2 (300 nM) or each EP agonist (1 μM). After 3 weeks, the cells were fixed in a 3.5% formaldehyde neutral buffer solution, and then stained with ALZ.

III. Activity of ALPase

OCCM-30 cells were plated into 24 well plates (3×10^3 cells/well) and cultured in α−MEM containing 10% FBS and AA (50 μg/ml). After 4 days, medium was changed to α−MEM containing 2% FBS and AA (50 μg/ml) and cells were treated with PGE_2 (30 nM, 300 nM) or each EP4 agonist (0.1 μM, 1 μM). At 7 days after treatment with regents, the quantitative analysis of ALP activity was performed by Bessey-Lowry enzymologic method (Alkaline-phospha B test; Wako, Osaka, Japan). Each experiment was repeated four times with comparable results.

Statistical analysis

Each experiment was repeated four times with comparable results. Data are expressed as means ± SD for each group. Statistical differences between groups were evaluated by the multivariate

analysis of variance (ANOVA) at 0.05 levels. The fisher's test was used as the post hoc test at the 5 % level of significance.

Results

Expression of EPs

EP1, EP2, EP3 (α, β, γ) and EP4 mRNAs were expressed in OCCM-30 cells (Fig. 1A).

Mineralization

PGE_2 (300 nM) and EP4 agonist (1 μM) markedly suppressed mineral nodule formation by OCCM-30 cells (Fig. 1B). Other EP agonist did not affect mineral nodule formation in OCCM-30 cells.

Effects of PGE_2 and EP agonists on ALP activity

ALP activity was significantly suppressed with 30 nM and 300 nM of PGE_2-stimulation at 7 days in OCCM-30 cells. EP4 agonist (1 μM) also significantly suppressed ALP activity at 7 days (Fig. 2).

Effects of PGE_2 and EP4 agonists on expression of OCN and MMP-13 mRNA

To further clarify the cell phenotype, as a cementum forming cell or a tissue destruction regulating cell, after long term incubation with PGE_2, and EP4 agonist, transcripts for OCN (mineralization

related genes) and MMP-13 (an important inducible MMP involved in degradation of the collagenous matrix of bone and cartilage) were analyzed. PGE_2 (300 nM) and EP4 agonist (1 μM) significantly suppressed OCN mRNA expression and significantly increased mRNA levels of MMP-13 compared to that of control (Fig. 3A). The effects of PGE_2 on the expression levels of OCN mRNA and MMP-13 mRNA were eliminated completely by treatment with EP4 antagonist (Fig. 3B).

Discussion

In the present study we report that EP1, EP2, EP3 (α, β, γ), and EP4 are expressed in the mouse cementoblast cell line, OCCM-30 cells. To our knowledge, this is the first report showing the expression of EPs in cementoblasts at mRNA level. The finding indicates that all PGE_2 –EP pathways exist in cementoblasts.

ALZ staining showed suppressive effects of PGE_2 and EP 4 agonist on mineralization in OCCM-30 cells. Under the same condition, other EP agonists did not show suppressive effect of the mineral nodule formation in OCCM-30 cells. So in further examination, we focused on the effects of EP4 on the function of cementoblasts.

Numerous reports have highlighted the effects of PGE_2 on ALP activity and mineralization in osteoblasts. [12, 13] It is well known that the effects of PGE_2 on ALP activity were mediated by EP2 and EP4 pathway. [14] Stimulation of both EP2 and EP4 causes an upregulation of adenylate

cyclase/cAMP system. [15, 16] In the present study, PGE_2 and EP4 agonist significantly downregulated ALP activity in OCCM-30 cells at 7 days after treatment. There are at least two explanations for this downregulation of ALP activity by PGE_2 and EP4 agonist. One possibility is that these changes are related to the stage of cementoblast differentiation. It is well known that ALP expression and activity are increase with osteoblast maturation and then decreased with osteoid mineralization. [17, 18] Another possibility is that incubation with PGE_2 may alter the cellular function of OCCM-30 from cementum forming cell into tissue destruction modulating cells. Therefore to clarify this point, we examined the effects of application of PGE_2 and EP4 agonist on the expression of OCN (mineralization related genes) and MMP-13 (an important inducible MMP involved in degradation of the collagenous matrix of bone and cartilage [19]) mRNA. PGE_2 and EP4 agonist decreased expression of OCN mRNA, whereas upregulated mRNA level for MMP-13. And EP4 antagonist eliminated the effects of PGE_2 on OCN and MMP-13 mRNA expressions. Parathyroid hormone (PTH) and PTH-related protein downregulated BSP expression through increasing cAMP in cementoblasts and periodontal ligament cells. [20, 21, 22] As described above, PGE_2 stimulates cAMP via EP2 and EP4 pathway. [15, 16] Thinking together, it is suggested that PGE_2 downregulate OCN expression mainly via EP4-cAMP pathway in OCCM-30 cells. MMP-13 is expressed by differentiated phenotypes of the osteoblastic lineage. [23] Inflammatory cytokines such as TNF-α and IL-1β induced MMP-13 expression in chondrocytes and osteoblasts. [24, 25] PGE_2 also stimulated MMP-13 production by osteoblasts via EP4 pathway and contribute to

bone resorption.[26] Osteoblasts obtained from patients with rheumatoid arthritis are known to produce proinflammatory cytokines and PGE_2 and considered to be involved in tissue destruction.[27] Previously, we demonstrated that topical application of LPS to rat periodontal tissues in vivo enhanced the immuno-expression of cyclooxygenase-2 (COX-2; a synthetic enzyme of inducible PGE_2)[28] and proinflammatory cytokines including TNF-α, IL-1β and IL-1α in cementoblasts and osteoblasts in vivo periodontal tissue.[29] Moreover, we demonstrated that IL-1α-stimulated IL-6 production from OCCM-30 was upregulated by COX-2 dependent PGE_2.[30] And it was reported that PGE_2 induced osteoclasts formation mainly via EP4 pathway in osteoblasts.[31, 32] Therefore we consider that exposure to PGE_2 changes cellular function of OCCM-30 from a cementum forming cell type to destruction modulating cell type like osteoblasts.

Recently Camargo et al. showed that PGE_2, EP1 and EP3 activators increased mineralization of OCCM-30.[33] The reason for the discrepancy from our data may be differentiation stage of cementblast with PGE_2 stimulation. In this study, we stimulated cementoblasts at high-cellular condition after confluency, which seemed to be more matured cells than their report.[33] Using MC3T3-E1 cells, Suda *et al.* reported that the effect of PGE_2 is quite different between cells at confluency and those 5 days after confluency.[12] It was also indicated that responsiveness of primary osteoblasts to PGE_2 may change during the culture period.[34] According to their results, PGE_2-addition 3days before confluency stimulated mineralization but addition of PGE_2 2 days after confluency markedly suppressed it. Although PGE_2 may stimulate cell differentiation of

immature cementoblasts via EP1 and EP3, it may inhibit mineralization of highly differentiated cementoblasts via EP4. There is a possibility that this changing in balance of EP expression levels with cellular maturation caused these differences in responsiveness to PGE_2. Further studies are needed to clarify this point.

In conclusion, in mature cementoblasts, OCCM-30, PGE_2-EP4 pathway downregulated mineralization ability and upregulated MMP-13 production. The present study suggests that mature cementoblasts located along tooth root surface may downregulate their mineralization ability and positively contribute to loss of connective tissue attachment through destruction of collagen via MMP production under inflammatory condition.

Acknowledgments

The authors thank Drs. Yumi Mada and Ikuko Ogawa for their advice and assistance. This work was supported by a Grant-in Aid for Scientific Research (C) (number 16591827) from the Ministry of Education, Science, Sports and Culture, Japan.

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TABLE LEGEND

Table 1. Oligonucleotide primer sequences utilized in the RT-PCR.

FIGURE LEGENDS

Fig. 1. Expression of PGE receptor subtypes (EPs) mRNAs in OCCM-30 cells (A). Effects of PGE_2 and EP4 agonist on mineralization in OCCM-30 cells (B). Cells were plated into 60 mm culture dishes (4×10^5 cells/dish) and cultured in α–MEM (10% FBS and 50 μg/ml AA). Total RNA was extracted from confluent cells and expression of mRNA for EPs was examined by RT-PCR analysis. OCCM-30 cells expressed EP1, EP2, EP3α, EP3β, EP3γ, and EP4 mRNAs (A). Mineral nodule formation was detected by alizarin red S (ALZ), which stains for calcium. Cells were placed in a 24 well plate at a density of 5×10^4 cells per well and cultured in α-MEM (10% FBS and 50 μg/ml AA) for 24 hours. Then medium was changed to α-MEM (2% FBS, 50 μg/ml AA, 10mM sodium β–glycerophosphate) with PGE_2 (300 nM) or each EP agonist (1 μM). After 3 weeks, cells were fixed in a 3.5% formaldehyde neutral buffer solution, and then stained with ALZ.

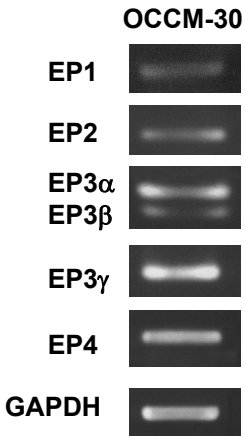
Fig. 2. Effects of PGE_2 and EP4 agonist on ALP activity in OCCM-30 cells. OCCM-30 cells were plated into 24 well plates (3×10^3 cells/well) and cultured in α–MEM (10% FBS and 50 μg/ml AA). After 4 days, medium was changed to α–MEM (2% FBS and 50 μg/ml AA) and cells were treated with PGE_2 (30, 300 nM) or EP4 agonist (0.1, 1 μM). At 7 days after treatment with reagents, the quantitative analysis of ALP activity was performed. PGE_2 and EP4 agonist significantly suppressed ALP activity in OCCM-30 cells. Data are expressed as the mean ± SD of four independent experiments. *; Significantly different from the culture with control ($p<0.05$),

**; ($p<0.01$).

Fig. 3. Effects of EP4 on OCN and MMP-13 mRNA expression in OCCM-30 cells. OCCM-30 cells were plated in 6 well plates (4×10^5 cells/well) and maintained in α–MEM (10% FBS and 50 μg/ml AA). Upon reaching confluence, cells were switched to α–MEM (2% FBS and 50 μg/ml AA) with PGE_2 (300 nM) and EP4 agonist (1 μM). To determine the effects of EP4 antagonists, the cells were pretreated with EP4 antagonist (1 μM) for 2 hours prior to the addition of PGE_2 (300 nM). After treatment for 7 days, total RNA was extracted and used to synthesize cDNA, and quantitative real-time RT-PCR was used to check expression of OCN (mineralization related genes) and MMP-13 (an important inducible MMP involved in degradation of the collagenous matrix of bone and cartilage) mRNA. Results were normalized to GAPDH as a reference gene. Data are expressed as the mean ± SD of four independent experiments. *; Significantly different from the culture with control ($p<0.05$), **; ($p<0.01$).

Fig. 1

(A)


OCCM-30
EP1
EP2
EP3α
EP3β
EP3γ
EP4
GAPDH

(B)

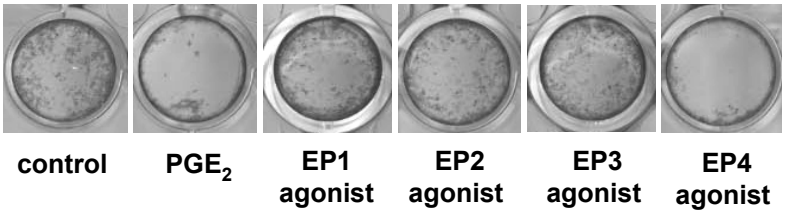

control
PGE2
EP1 agonist
EP2 agonist
EP3 agonist
EP4 agonist

Fig. 2

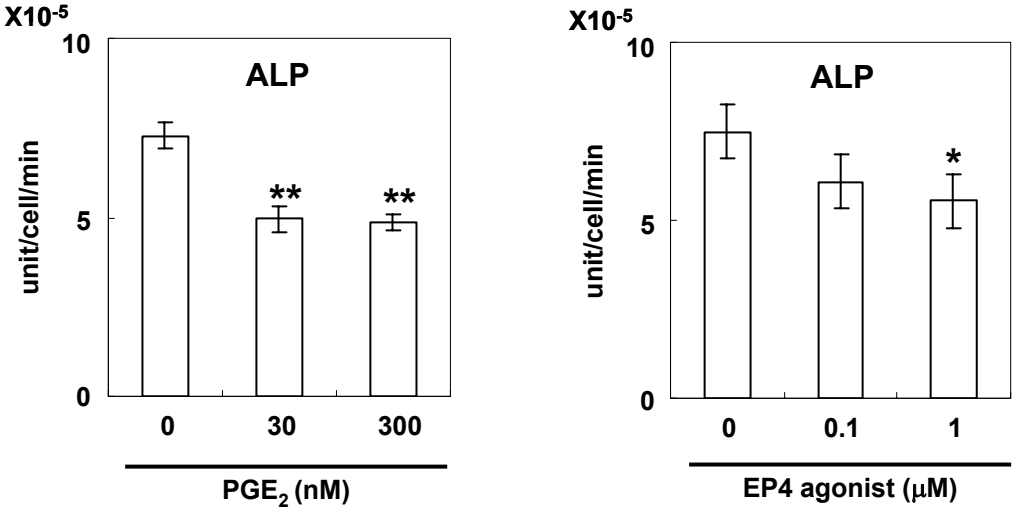
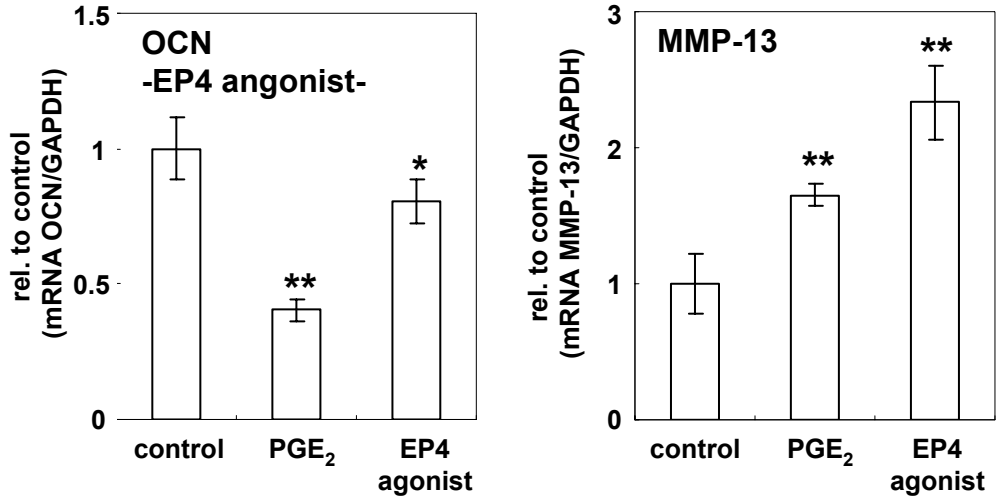

X10-5
10
5
0
ALP
unit/cell/min
**
**
0
30
300
PGE2 (nM)
X10-5
10
5
0
ALP
unit/cell/min
*
0
0.1
1
EP4 agonist (μM)

Fig. 3

(A)


OCN
-EP4 angonist-
rel. to control
(mRNA OCN/GAPDH)
1.5
1
0.5
0
**
*
control
PGE2
EP4
agonist
MMP-13
rel. to control
(mRNA MMP-13/GAPDH)
3
2
1
0
**
**
control
PGE2
EP4
agonist

(B)

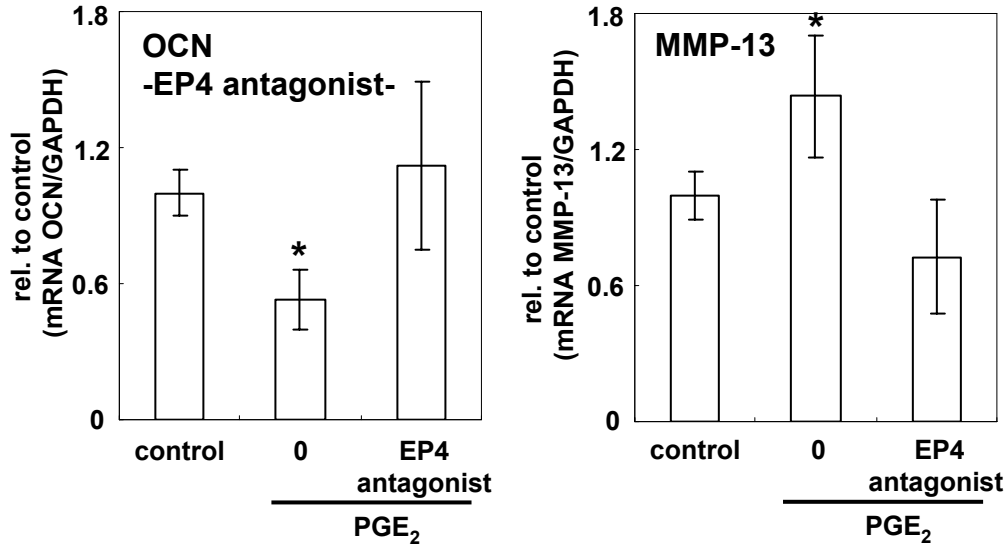

OCN
-EP4 antagonist-
rel. to control
(mRNA OCN/GAPDH)
1.8
1.2
0.6
0
*
control
0
EP4
antagonist
PGE2
MMP-13
rel. to control
(mRNA MMP-13/GAPDH)
1.8
1.2
0.6
0
*
control
0
EP4
antagonist
PGE2

Table 1

		Sequence	Products (bp)	Ta (℃)	Cycles	Accession no.	Reference
RT-PCR							
EP1	Forward	5'-TTAACCTGAGCCTAGCGGATG-3'	670	60	30		Suzawa et al.
	Reverse	5'-CGCTGAGCGTATTGCACACTA-3'					
EP2	Forward	5'-GGTGGTGCTGGCTTCATATT-3'	250	56	35	D50589	
	Reverse	5'-CAGGGAACAGAAGAGCAAGG-3'					
EP3 α	Forward	5'-CCTGGGTTTATCTGCTGCTAAG-3'	293	57	35	D10204	
	Reverse	5'-CTCGGTGTGTTTCCTGGCAAGG-3'					
EP3 β	Forward	5'-CCTGGGTTTATCTGCTGCTAAG-3'	199	57	35	D133321	
	Reverse	5'-CTCGGTGTGTTTAATGGCAAGG-3'					
EP3 γ	Forward	5'-CCTGGGTTTATCTGCTGCTAAG-3'	368	57	35	D17406	
	Reverse	5'-CTCTGGCAAAGACTCAAAATGC-3'					
EP4	Forward	5'-GGTCATCTTACTCATCGCCACCTCTC-	536	61	35		Suzawa et al.
	Reverse	5'-TCCCACTAACCTCATCCACCAACAG-3'					
GAPDH	Forward	5'-CCACTCTTCCACCTTCG-3'	154	60	30	M32599	
	Reverse	5'-GTGGTCCAGGGTTTCTTAC-3'					
Quantitive real-time RT-PCR							
OCN	Forward	5'-TAAGGTAGTGAACAGACTCCG-3	153	58		L24429	
	Reverse	5'-CCGTAGATGCGTTTGTAGG-3'					
MMP-13	Forward	5'-AGGCTGAGCTCTTTTTGACA-3'	133	58		NM_008607	
	Reverse	5'-TCATAACCATTCAGAGCCCA-3'					
GAPDH		see above		65/58			

Ta:annealing temperature