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Relation	

The EP4-ERK-dependent pathway stimulates osteo-adipogenic progenitor proliferation resulting in increased adipogenesis in fetal rat calvaria cell cultures

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1. Introduction

Four specific G protein-coupled Prostaglandin E_2 (PGE_2) receptor subtypes (EP1-4) have been identified and are known to be differentially expressed across tissue types [1]. EP1 couples to Ca^{2+}-dependent protein kinase C (PKC), and EP2 and EP4 share the adenylate cyclase-cAMP-PKA pathway, whereas EP3 antagonizes the EP2/EP4 pathway. These various PGE_2 signaling pathways bring about a large number of physiological and pathophysiological processes [2]. PGE_2 actions in bone are also complicated, but its anabolic effects in rats are obvious, when PGE_2 is administered systemically or locally to the skeleton [3, 4]. Development of selective agonists for each EP receptor subtype agonists, EP1A-4A) [2, 5] and pharmacological [6, 7] and genetic [8] approaches led us conclude that both EP2 and EP4 play a crucial role in PGE_2-mediated bone formation. mitogen-activated protein kinase (MAPK) pathways are considered to be involved in PGE_2 actions in bone [9, 10]. Previously, we found that, of the three principal MAPK pathways, EP2 and EP4 activate the cAMP-p38 MAPK-c-fos/Runx2 pathways, while EP4 also mediates the ERK pathway, possibly via PKC, and c-Jun N-terminal kinase (JNK) in fetal rat calvaria (RC) cells [11].

PGJ_2 appears to be a natural ligand for peroxisome proliferator-activated receptor γ (PPARγ) [12], a master transcription factor of adipogenesis [13], and cyclooxygenase (COX)-2 is necessary for PGJ_2 production and/or adipogenesis in adipose tissue [2, 14, 15]. On the other hand, COX-2 in bone is mostly involved in PGE_2 production, which in turn increases bone morphogenetic protein 2-dependent bone formation [16]. Together with the involvement of EP2 and EP4 in osteogenesis, EP4 mediates the inhibitory effect of PGE_2 on adipogenic differentiation of 3T3-L1 cells with a concomitant decrease in

PPARγ mRNA expression [17]. In aged ovariectomized (OVX) rats, EP4A stimulates bone formation at skeletal sites, while it decreases the number of adipocytes and fatty marrow area [4]. In contrast, when we treated fetal rat calvaria (RC) cells with PGE_2 under osteogenic conditions, we found an increase in adipocyte colonies together with increased bone nodule formation. Osteoblasts and adipocytes share a common mesenchymal progenitor cells, and not only bone marrow stromal cells (a mesenchymal stem cell model) but also RC cells (a committed osteoblast precursor pool) [18], can convert into adipocytes in particular situations, such as ectopic overexpression of PPARγ [12] and treatment with a synthetic PPARγ ligand [18, 19]. We thus explored in detail the role(s) of PGE_2 in adipogenesis in RC cell cultures under osteogenic conditions.

2. Materials and methods

2.1. Reagents

Selective EP agonists (EP1A, ONO-DI-004; EP2A, ONO-AEI-259; EP3A, ONO-AE-248; EP4A, ONO-AE1-437; each product was guaranteed with >90% purity) were gifts from Ono Pharmaceutical Co. (Osaka, Japan). PGE_2, MAPK inhibitors (for p38 MAPK, SB203580; for JNK, dicumarol; for ERK, U0126) and all other chemicals, unless otherwise specified, were purchased from Sigma-Aldrich Co (St Louis, MO). These reagents were dissolved in dimethylsulfoxide (DMSO) at a final concentration of < 0.1%.

2.2. Animals

Animal use and procedures were approved by the Committee of Research Facilities

for Laboratory Animal Science, Hiroshima. Rats were euthanized by cervical dislocation under deep anesthesia.

2.3. Cell cultures

RC cells were routinely obtained from fetal rat calvaria (embryonic day 21) as described [20]. Briefly, calvariae were minced and digested with collagenase (type I) for 10, 20, 30, 50 and 70 min at 37 °C. Cells retrieved from the last four of five digestion fractions were separately grown in αMEM containing 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA) and antibiotics. After 24 h, cells were pooled and grown in multi-well plates in the same medium supplemented additionally with 50 μg/ml of ascorbic acid (osteogenic medium). Cells were treated with or without reagents in regular or serum-deprived conditions (see below), as specified. Medium was changed every 2-3 days, and cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

2.4. 5-bromo-2'-deoxyuridine(BrdU)-labeling index

Cells grown in osteogenic medium for 2 days were adapted to serum-deprived conditions (0.1% FCS) for 24 h and treated with or without EP agonists or PGE_2 for an additional 24 h. BrdU-labeled cells were detected immunohistochemically, as described before [11]. Briefly, cells were labeled with BrdU (10 μM) for 3 h before culture termination, then fixed with 70% ethanol for 30 min, air dried and permeabilized with 2 M HCl for 5 min. Anti-BrdU monoclonal antibody (1:1,000) and horseradish peroxidase-labeled secondary antibody (1:200, Vector Lab, Burlingame, CA) were used with the avidin-biotin complex (ABC) system (Vector Lab).

2.5. MTT assay

Cells were kept under serum-starved conditions (1% FCS) for 24 h and then treated with or without each EP agonist for 48 h, followed by treatment with MTT (3-[4,5-dimethylthiazoyl-2-yl]-2,5-diphenyltetrazolium bromide) for the last 4 h of cultures [11]. Cells were dried, assessed by microscopic examination, and MTT was quantified colorimetrically.

2.6. RNA extraction and real-time RT-PCR

Total RNA was isolated from cells with TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's directions. cDNA was synthesized from ≤2 μg of total RNA using ReverTra Ace (TOYOBO, Osaka, Japan) at 50 °C for 40 min. Primer sets for genes of interest were described elsewhere [11, 21]; ribosomal protein L32 (L32) was used as internal control, 5'-CAT GGC TGC CCT TCG GCC TC-3' and 5'-CAT TCT CTT CGC TGC GTA GCC-3'; PPARγ2, 5'-TGA CAG TGA CTT GGC CAT ATT T-3' and 5'-TTG TCT TGG ATG TCC TCG AT-3'; CCAAT/enhancer binding protein (C/EBP)δ, 5'-AGA CTC CGA ACG ACC GAT AC-3' and 5'-GTG CCC AAG AAA CTG TAG CA-3'; c-fos, 5'-AGA ATC CGA AGG GAA AGG AA-3' and 5'-ATG ATG CCG GAA ACA AGA AG-3' [11, 18, 19]. Real-time RT-PCR was carried out by using the Light Cycler system (Light Cycler™ DNA Master SYBR® Green I; Roche Diagnostics, Indianapolis, IN), according to the manufacturer's instructions.

2.7. Western blotting

Cells under serum-deprived conditions (0.1% FCS) were treated with or without EP4A for 30 min. Cell lysates were obtained and subjected to Western Blotting, as

described [11]. Briefly, cells were lysed in RIPA buffer containing Phosphatase Inhibitor Cocktail (Nacalai Tesque, Kyoto, Japan) and Complete Protease Inhibitor Cocktail (Roche Diagnostics) and, aliquots of the lysates (≤ 5 μg protein/lane) were subjected to SDS-PAGE (15% gels) and electroblotted onto nitrocellulose membranes (Millipore, Bedford, MA). The membranes were probed with antibody against phosphorylated ERK1/2 (p-ERK) (1:1000; Santa Cruz biotechnology), followed by incubation with HRP-conjugated secondary antibody (1:2000, Santa Cruz Biotechnology). Signals were detected by chemiluminescence (Lumi-Light^{PLUS}, Roche Diagnostics). The membranes were reprobed with antibody against non-phosphorylated ERK (1:1000, Santa Cruz Biotechnology).

2.8. Staining for alkaline phosphatase (ALP)/oil red O

Cells were fixed in neutral buffered formalin for 15 min, washed and incubated with AS MX-phosphate/blue LB in 0.1 M Tris-HCl (pH 8.3) to determine osteoid-like nodules. To confirm adipocyte colonies, cells were treated with freshly prepared oil red O for 30 min [19]. In some cases, cells were double stained with ALP and oil red O.

2.9. Statistical analysis

Data from at least three independent experiments are expressed as the mean ± SD. Statistical differences were evaluated by one-way factorial analysis of variance (ANOVA) and post hoc Tukey's test. A P value less than 0.05 was considered to indicate a significant difference.

3. Results

3.1. PGE_2 via EP4 acts on the proliferation of primitive osteoprogenitor cells and a consequent increase in adipocyte colonies

We initially examined the effect of chronic treatment of EP agonists including PGE_2 on adipocyte colony formation during osteoblastogenesis in the RC cell model (14-15 days). Of these ligands, EP4A increased the number of adipocyte colonies (42.5 ± 9.6, $P<0.01$), followed by PGE_2 and EP2A (35.8 ± 8.1, $P<0.01$, and 24.0 ± 2.7, $P<0.05$, respectively), but not by EP1A and EP3A (10.3 ± 3.3 and 10.0 ± 1.6, respectively) (Figures 1A, B). In contrast, EP2A was the most effective compound in altering bone nodule formation (not shown), as we described before [11]. To elucidate target cells for PGE_2 in adipogenesis versus osteoblastogenesis, we pulse-treated RC cells with PGE_2 for 2 days during three typical osteogenic development stages (see below) as defined by osteoblast markers [11] and counted adipocyte colonies at day 14 (d14). PGE_2 increased the number of adipocyte colonies, when treated during proliferation stages (d3-5) but not differentiation (d7-9) and maturation (d10-12) stages (Figure 2A). These findings differed from the effective time windows for the osteogenic effect of PGE_2 (see ref. [11]).

To determine how PGE_2 exerts adipogenic effect in RC cells, we compared the effect of PGE_2, EP2A and EP4A on cell proliferation by using the MTT assay. As expected, when cells were treated during proliferation stages, EP4A, followed by PGE_2, but not EP2A increased MTT activity (Figure 2B). The effect of EP4A was obvious in monolayer cells (Figure 2C) during proliferation stages but not in nodule-forming multilayer cells during differentiation stages (not shown). These results were confirmed by quantifying the BrdU labeling index during proliferation stages (Figure 2D). We also monitored the

formation of adipocyte colonies over time. By d9, adipocyte colonies were not detectable with or without PGE_2. By d12, adipocyte colonies were seen in non-treatment groups (1.0 ± 0.8 and 2.8 ± 1.0, respectively) and PGE_2 enhanced these phenomena (4.8 ± 2.1, $P<0.05$, and 18.8 ± 3.0, $P<0.01$, respectively) (Figure 2E), in parallel with an increase in the number of bone nodules (see ref. [11]). Taken together, these data indicate that an increase in the number of RC cells during proliferation stages is correlated with an increase in number of adipocyte colonies.

3.2. The ERK pathway is involved in EP4-mdeiated adipogenesis

We described previously that the MAPK pathways mediate PGE_2-dependent bone nodule formation in RC cells [11]. To determine whether MAPKs are also crucial for PGE_2-dependent adipogenesis, we evaluated the effect of SB203580, an inhibitor of p38-MAPK, U0126, an inhibitor of ERK1/2 or dicumarol, an inhibitor of JNK. We treated cells with or without PGE_2, in combination with or without MAPK inhibitors, throughout the culture period. In contrast to SB203580 and dicumarol that increased adipocyte colony formation with or without PGE_2, U0126 decreased the number of adipocyte colonies only in the presence of PGE_2 (Figure 3A). Likewise, the adipogenic effect of EP4A was eliminated by cotreatment with U0126 (Figure 3B). We then treated cells at d3 with or without EP4A under serum-deprived conditions (0.1% FBS) and found that EP4 increased ERK phosphporylation within 30 min (Figure 3C).

Whether EP4 affects gene expression levels of the transcription factors necessary for adipogenesis, such as PPARγ2, C/EBPα and C/EBPδ, is of interest. To address this

question, we pretreated cells at d3 with or without U0126, followed by treatment with or without EP4A for 24 h. Using quantitative real-time RT-PCR, we demonstrated that EP4A increased C/EBPδ but not PPARγ2 and C̃EBPα mRNA expression (Figures 4A-C). U0126 alone did not show any effect on these mRNA levels, while it blocked the increased levels of C/EBPδ mRNA induced by EP4A (Figure 4A-C). We also found that EP4A increased mRNA expression of the protooncogene c-fos [22], consistent with our proliferation data; U0126 again attenuated the EP4A effect (Figure 4D). These results suggest that PGE_2 may not be directly involved in adipocyte differentiation. Rather, the prostanoid may increase the proliferation of primitive osteo-adipogenic progenitor cells via the EP4-ERK pathway.

4. Discussion

In addition to our previous report that PGE_2 acts on osteoblastogenesis via both EP2 and EP4 in RC cell cultures, we now provide evidence that PGE_2 also increases adopogenesis in this model, possibly due to its ability to increase the proliferation of primitive osteo-adipogenic progenitor cells principally via the EP4-dependent ERK pathway.

It is worth noting that these results are different from previous studies on 3T3-L1 preadipocytes [17] and aged OVX rats [23], where the EP4 pathway mediates the

anti-adipogenic effect of PGE_2. The ability or not of PGE_2/EP4A to alter the expression of PPARγ may underlie this discrepancy. We acknowledge that the large proportion of bone nodules versus adipocyte colonies may lead to a difficulty in our ability to detect a change in PPARγ mRNA expression. However, PPARγ is constitutively active in the two previous models [13, 24] but not in RC cells; this transcription factor is relatively highly expressed in RC cells during proliferation stages, but its nuclear translocation is not seen without the presence of its ligand [19]. The molecular mechanism(s) of the EP4-dependent downregulation of PPARγ has not been elucidated, but our findings suggest that EP4 is not directly involved in PPARγ transactivation at least in RC cells. Although we have not explored the reason why a small number of adipocyte colonies were seen in RC cell cultures without added PGE_2, it seems likely that PGJ_2, a natural ligand of PPARγ and/or other unknown factors in FBS are involved.

The difference in downstream signaling between EP2A (linked to ERK) and EP4A (linked to p38 MAPK) appears to account for the difference in the adipogenic potency between EP2 and EP4. The difference in target cells between EP2A and EP4A is also notable; EP2A acts mainly on osteogenic cells during differentiation stages, while EP4A acts on less committed primitive osteo-adipogenic progenitor cells with capacity for both osteoblast and adipocyte differentiation [11, 19]. The narrow window of PGE_2 regulation of adipogenesis in RC cells may be the reason why, of four EPs, EP4 is primarily involved in adipogenesisin RC cells. For example, ERK is turned on during proliferation stages, while it is shut-off resulting in low PPARγ phosphorylation during more mature stages in 3T3-L1 [25]. Our data on the positive effect of p38 MAPK and JNK inhibitors on adipogenesis also support the unique activity of EP4A in adipogenesis. Thus, EP4A increases p38 MAPK and JNK phosphorylation during differentiation stages [11], which may downregulate C/EBPδand PPARγ and/or cAMP-response element-binding protein,

as shown in a variety of embryonic and adult adipocyte cell models[26] and mesenchymal stem cells [27].

Even with the activation of PPARγ, a reciprocal relationship between osteogenesis and adipogenesis was not seen in RC cells [18, 19]. Amongst transcription factors directly involved in adipogenesis [28], C/EBPδ is expressed not only in early preadipocytes to induce PPAR expression [29], but also in osteoblastic cells to regulate osteocalcin expression [30]. Our finding of the EP4A-dependent upregulation of C/EBPδ during proliferation stages appears reasonable in this context. c-fos, a member of the intermediate early gene family, also known as a protooncogene, plays an important role in cell proliferation [31, 32] including rat preadipocytes [33]. In 3T3-L1 preadipocytes, a hormonal adipogenic stimulus triggers the sequential activation of C/EBPδ, followed by C/EBP⟨ and PPAR© [34, 35]. Together with evidence that C/EBPδ is associated with Runx2, a master transcription factor for osteoblastogenesis [36], these results suggest that PGE_2 may activate the EP4-c-fos-dependent pathway which promotes the proliferation of osteo-adipogenic progenitor cells and C/EBPδ mRNA expression, resulting in increased adipocyte colonies without a reciprocal decrease in osteoblastogenesis in RC cells.

As summarized in Figure 5, we characterized PGE_2-dependent adipogenic actions in RC cell cultures. Taken together with our previous observation that both EP2 and EP4 act on osteoblastogenesis principally through the MAPK pathways [11], we conclude that EP4 mediates the proliferation of osteo-adipogenic bipotential progenitor cells via the ERK pathway, resulting in adipocyte colony formation concomitant with a massive

increase in bone nodule formation.

5. Acknowledgements

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Figure Legends

Fig. 1. PGE_2 increases the number of adipocyte colonies in RC cell cultures. Cells in 24-well plates were chronically treated with PGE_2, EP2A or EP4A (100 nM each) for 14 days. (A) Representative macrographs of cultures in the presence or absence of PGE_2. The lower right-hand panel is a higher magnification view of the enclosed area in the upper right-hand panel. ALP/oil red O staining. (B) Effect of PGE_2 and EPAs on the number of adipocyte colonies. **P*<0.05 and ***P*<0.01, compared to control (–).

Fig. 2. PGE_2 and EP4A but not EP2A increase proliferation of primitive osteoprogenitor cells. (A) Effect of PGE_2 on the number of adipocyte colonies in three typical development time windows. Cells in 24-well plates were pulse-treated with or without 100 nM PGE_2 for 48 h as indicated and grown up to d14. (B and C) Effect of PGE_2, EP2A and EP4A on cell proliferation. Cells in 96-well plates were treated with PGE_2, EP2A or EP4A (100 nM each) from d3 for 48 h. 0.5% MTT was added for 4 h before culture termination. Panels in (C) show representative micrographs of MTT staining. (D) Effect of PGE_2, EP2A and EP4A on the number of BrdU-labeled cells. Cells in chamber slides under serum-deprived conditions were treated with PGE_2, EP2A or EP4A (100 nM each) for 24 h from d3 to d5 and treated with BrdU for 4 h before culture termination. BrdU-positive cells were detected immunocytochemically. **P*<0.05 and ***P*<0.01, compared to control (–). (E) Chronological changes in adipocyte colony formation. Cells in 24-well plates were treated with or without 100 nM PGE_2 from d3 to d5, and fixed,and stained with oil red O at the days indicated. N.D., not detected.

Fig. 3. The EP4-ERK pathway is involved in adipogenesis. (A) Effect of SB203580,

dicumarol or U0126 on the PGE_2-dependent adipocyte colony formation. Cells were treated with 100 nM PGE_2 in combination with or without 10 μM MAPK inhibitors throughout the culture period. Adipocyte colonies were counted at d14, as described. (B) U0126 inhibits the effect of pulse-treatment with EP4A on adipocyte colony formation. Cells at d3 in 24-well plates were pretreated with or without 10 μM U0126 for 2 h, followed by treatment with or without 100 nM EP4A for an additional 48 h. Adipocyte colonies were counted at d14, as described. (C) Effect of EP4A on ERK phosphorylation. Cells at d3 in 35 mm dishes were treated with or without EP4A for 30 min, and activation of ERK1/2 was determined by Western blot analysis. Left, representative images. Right, quantitative data. **P*<0.05, ***P*<0.01, compared to control (–). $^{\#}P$<0.05, $^{\#\#}P$<0.01, compared to EP4A alone.

Fig. 4. The EP4A-ERK pathway is involved in C/EBPδ and c-fos mRNA expression. Cells at d3 were handled as shown in Figure 3 (B). Total RNA was isolated, and mRNA expression of PPARγ (A), C/EBPα (B), C/EBPδ (C) and c-fos (D) was quantified by real-time RT-PCR. **P*<0.05 and ***P*<0.01, compared to PGE_2 alone. $^{\#\#}P$<0.01, compared to EP4A alone.

Fig. 5. A schematic diagram of deduced PGE_2-MAPK-dependent osteo-adipogenesis in RC cell cultures. PGE_2 appears to act on both primitive and more committed osteoprogenitor cells. Taken together with our previous study [11], the EP4-ERK and EP2-p38 MAPK pathways act mostly on the former and the latter, respectively. EP4 mediates the proliferation of primitive osteprogenitor cells which have the potential to differentiate into both osteocytes and adipocytes. In consequence, EP4 may increase

adipocyte colonies, independent of its massive stimulatory effect on bone nodule formation.

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Figure 1

Figure(s)

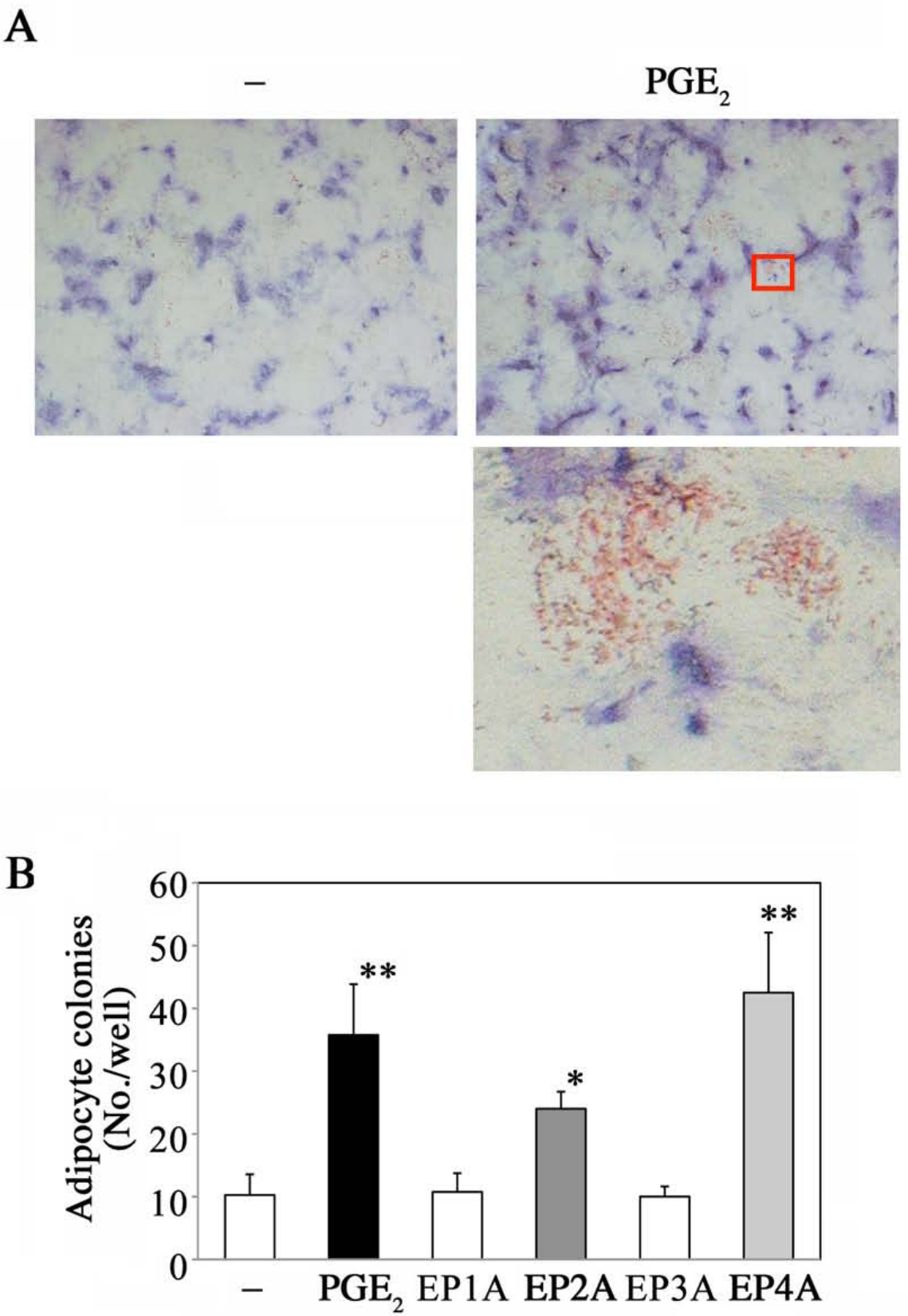


Figure 2

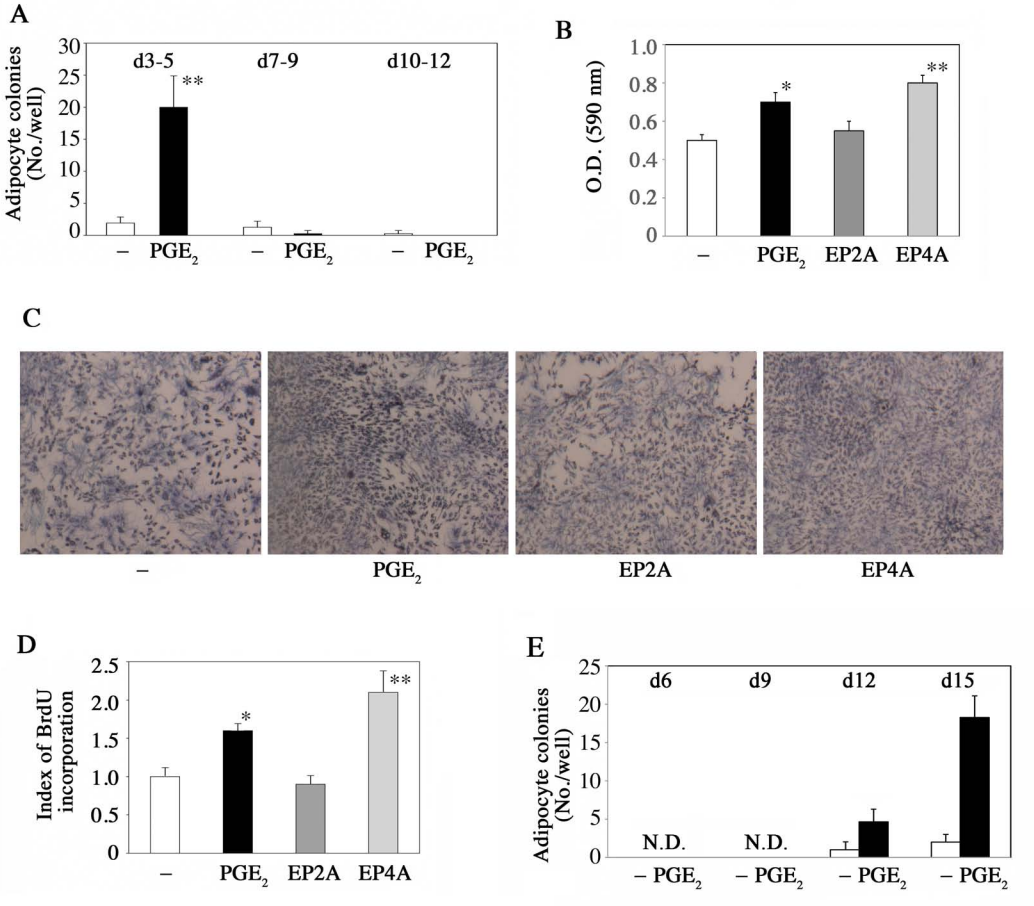

A
Adipocyte colonies (No./well)
30
25
20
15
10
5
0
d3-5
d7-9
d10-12
**
– PGE2 – PGE2 – PGE2
B
O.D. (590 nm)
1.0
0.8
0.6
0.4
0.2
0
*
**
– PGE2 EP2A EP4A
C
– PGE2 EP2A EP4A
D
Index of BrdU incorporation
2.5
2.0
1.5
1.0
0.5
0
*
**
– PGE2 EP2A EP4A
E
Adipocyte colonies (No./well)
25
20
15
10
5
0
d6
d9
d12
d15
N.D.
N.D.
– PGE2 – PGE2 – PGE2 – PGE2

Figure 3

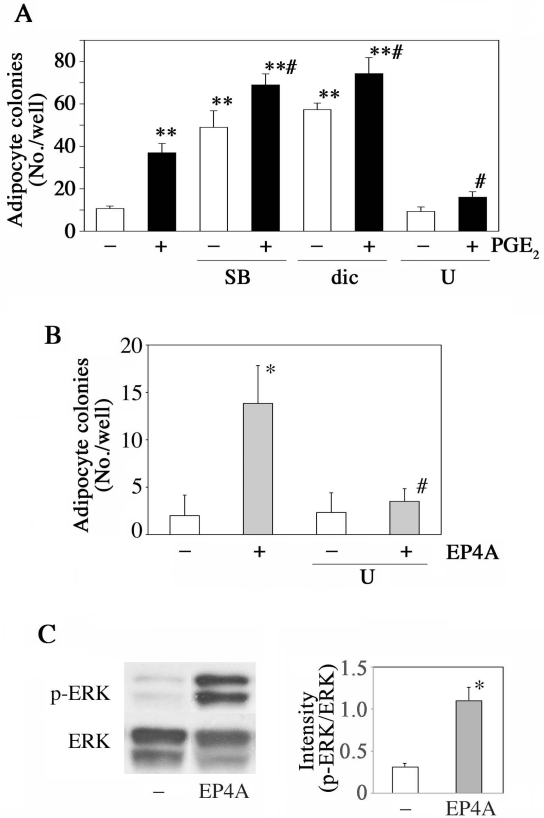

A
Adipocyte colonies (No./well)
80
60
40
20
0
**
**
**#
**
**#
#
– + – + – + – + PGE2
SB
dic
U
B
Adipocyte colonies (No./well)
20
15
10
5
0
*
#
– + – + EP4A
U
C
p-ERK
ERK
– EP4A
Intensity (p-ERK/ERK)
1.5
1.0
0.5
0
*
– EP4A

Figure 4

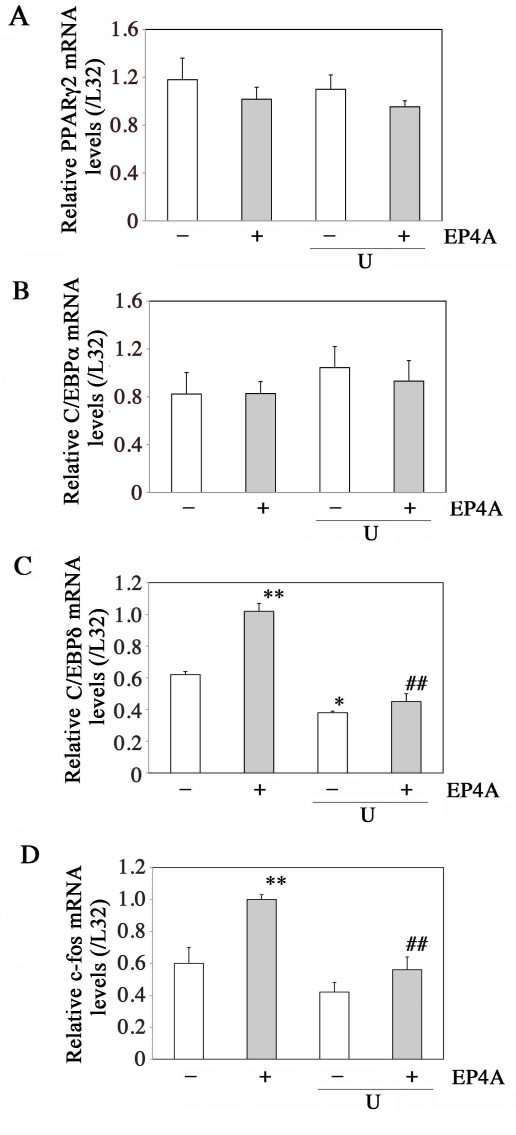
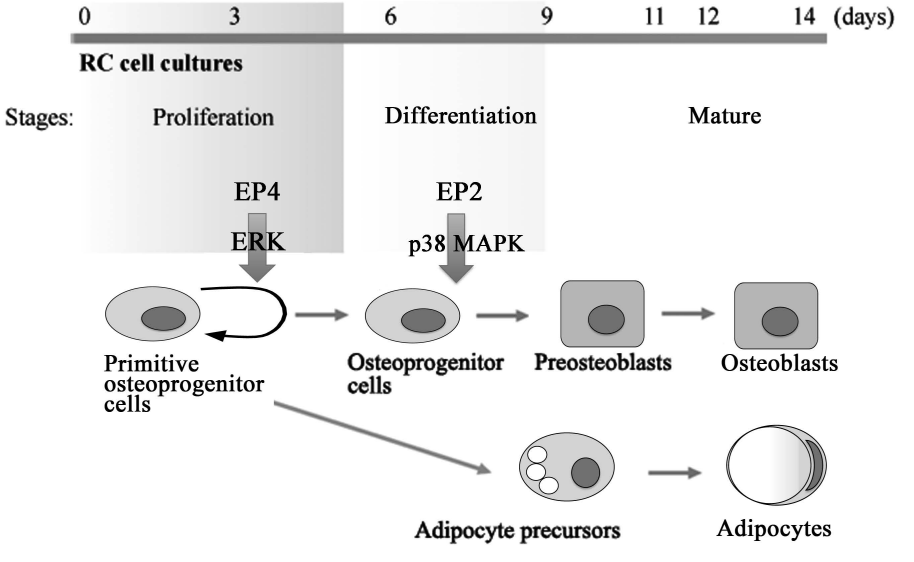

A
Relative PPARγ2 mRNA levels (/L32)
1.6
1.2
0.8
0.4
0
– + – + EP4A
U
B
Relative C/EBPα mRNA levels (/L32)
1.6
1.2
0.8
0.4
0
– + – + EP4A
U
C
Relative C/EBPδ mRNA levels (/L32)
1.2
1.0
0.8
0.6
0.4
0.2
0
**
*
##
– + – + EP4A
U
D
Relative c-fos mRNA levels (/L32)
1.2
1.0
0.8
0.6
0.4
0.2
0
**
##
– + – + EP4A
U

Figure 5


0
3
6
9
11
12
14
(days)
RC cell cultures
Stages:
Proliferation
Differentiation
Mature
EP4
ERK
EP2
p38 MAPK
Primitive osteoprogenitor cells
Osteoprogenitor cells
Preosteoblasts
Osteoblasts
Adipocyte precursors
Adipocytes