

Osteopontin deficiency enhances anabolic action of EP4 agonist at a sub-optimal dose in bone

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Abstract

Osteoporosis is one of the most widespread and destructive bone diseases in our modern world. There is a great need for anabolic agents for bone which could reverse this disease, but few are available for clinical use. Prostaglandin E receptor (EP4) agonist (EP4A) is one of the very few anabolic agents for bone in rat, but its systemic efficacy against bone loss at sub-optimal dose is limited in mice. As osteoblasts are regulated by extracellular matrix proteins, we tested whether deficiency of osteopontin (OPN), a secreted phosphorylated protein, could modulate the effects of EP4A (ONO-AE1-329) treatment at 30 µg/kg body weight, a sub-optimal dose, for 5 days/week for 4 weeks. OPN deficiency enhanced the anabolic effects of EP4A on bone volume.

Histomorphometric analysis indicated that EP4A increased mineral apposition rate as well as bone formation rate in OPN-deficient but not in wild-type mice. Neither OPN deficiency nor EP4A altered osteoclast parameters. Importantly, OPN deficiency enhanced the direct anabolic action of EP4A locally injected onto the parietal bone in inducing new bone formation. Combination of OPN deficiency and EP4A treatment caused an increase in mineralized nodule formation in the cultures of bone marrow cells. Finally, OPN deficiency enhanced anabolic action of EP4A in the mice subjected to ovariectomy. These data indicate that OPN deficiency enhances the actions of EP4A at sub-optimal dose.

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Introduction

The number of patients with osteoporosis is constantly increasing in the world and this disease has become a major health issue. The increased fracture rate in osteoporotic elderly patients due to reduced bone mass is a major issue in terms of quality of life and economy. Several types of drugs have been developed to treat osteoporosis; most of these are inhibitors of bone resorption (Rodan & Martin 2000). Effective treatment of osteoporosis, however, requires not only resorption inhibitors, which so far reduce the fracture risk only by 50%, but also stimulators for bone formation especially in patients who already have lost a significant amount of bone mass (Rosen & Bilezikian 2001). Parathyroid hormone (PTH) has been shown to be an efficacious drug for osteoporosis (Swarthout *et al.* 2002). However, the peptide nature of PTH is a problem with respect to its handling and

administration. Thus, non-peptide anabolic agents for bone would be an ideal option for the treatment of osteoporosis.

Prostaglandins (PGs) are a group of lipid mediators that are derived from arachidonic acid in a variety of tissues under various physiological and pathophysiological conditions (Narumiya *et al.* 1999). PGs produced by the cells in the osteoblastic lineage have been shown to play important roles in both bone formation and bone resorption (Pilbeam *et al.* 2002). Among several PGs, PGE₂ exhibits anabolic effects on bone when administered systemically or locally in rat (Yang *et al.* 1993, Suponitzky & Weinreb 1998). However, PGE₂ administration is associated with side effects that include diarrhea, lethargy, and flushing.

Four subtypes of PGE₂ receptors, EP1, EP2, EP3, and EP4, have been identified. These receptors are encoded by distinct genes and are expressed differentially in the different tissues (Segi *et al.* 1998, Ushikubi *et al.* 1998, Hizaki *et al.* 1999,

Narumiya *et al.* 1999). Previous studies using knockout mice which lack each of the four receptor subtypes revealed that EP4 is the only receptor to mediate the PGE₂-induced anabolic action in bone formation (Yoshida *et al.* 2002, Li *et al.* 2005). There are a few articles describing that EP2 receptor subtype plays a role in bone formation (Li *et al.* 2000, 2003, Akhter *et al.* 2001). It was further demonstrated that administration of an EP4-receptor agonist (EP4A) prevented bone loss in ovariectomized and immobilized rats (Yoshida *et al.* 2002). EP4A was also shown to restore bone mass in established osteopenic aged rats (Ke *et al.* 2006). In addition, the systemic administration of EP4A accelerated bone defect healing in the femoral diaphysis after drill-hole injury in mature rats (Tanaka *et al.* 2004). To our knowledge, there is no previous paper showing that either PGE₂ or EP4A exerts a systemic anabolic effect on bone in mice. Both Perry *et al.* (2000) and Li *et al.* (2002) show that, in sharp contrast to rats, PGE₂ is catabolic in mice for reasons that have not been determined yet. Furthermore, Sasaoka *et al.* (2004) show that administration of another EP4A into mice has no systemic bone anabolic effect. Thus, in mice, understanding the molecular mechanisms regulating systemic actions of EP4 on bone may contribute to improve the use of EP4A.

Osteoblastic activities are regulated not only by hormones and cytokines but also by extracellular matrix proteins. Osteopontin (OPN) is a non-collagenous bone matrix protein, which is expressed in both osteoblasts and osteoclasts in bone in response to cytokines and mechanical signals to modulate osteoblastic functions as well as osteoclastic functions (Noda & Denhardt 2002). OPN inhibits mineral crystal growth both *in vivo* and *in vitro* (Hunter *et al.* 1996, Boskey *et al.* 2002). *In vitro* experiments revealed that OPN can have conflicting activities. OPN promotes cell attachment by its RGD (arginine, glycine, aspartic acid) motif via binding to integrins (Noda & Denhardt 2002). This promotion of attachment provides a positive environment for cell growth (Thalmann *et al.* 1999). Conversely, OPN is a negative regulator of proliferation and differentiation in MC3T3E1 cells (Huang *et al.* 2004). Thus, the physiological role of OPN *in vivo* is still not clear based on these *in vitro* studies, and the elucidation of OPN function especially *in vivo* is still to be established. Furthermore, although PGE₂ regulates OPN expression *in vitro*, it is not known whether there is a functional relationship between the two molecules. We therefore examined whether OPN deficiency modulates effects of EP4A on bone *in vivo* using knockout mice.

Materials and Methods

Animals

OPN-deficient mice were produced as described by Rittling *et al.* (1998). The original chimeric mice were backcrossed with 129S1 mice to generate a 129 mixed (S1, S7) background. WT and OPN^{-/-} mice were maintained as

separate colonies. To examine the effects of OPN deficiency on EP4 agonist (EP4A), 40 female mice (9-week-old) with either OPN-deficient or wild-type genotype were used for systemic treatment and 16 mice (3-week-old) were used for local treatment. For ovariectomy experiments, 15-week-old mice were used. The mice were housed under controlled conditions on a 12 h light/12 h darkness cycle and fed with standard laboratory chow and given tap water. The animal experiments were approved by the committee for animal welfare of Tokyo Medical and Dental University.

Experimental protocol

EP4-receptor agonist (EP4A, ONO-AE1-329; Ono Pharmaceutical Company, Osaka, Japan) was dissolved in saline containing 0.3% ethanol and 0.1% Tween 80. For systemic treatment, the mice were subjected to s.c. injections in the dorsal flank thrice/day with EP4A at 30 µg/kg body weight or vehicle (saline containing 0.3% ethanol and 0.1% Tween 80) for 5 days/week for 4 weeks. This sub-optimal dose was chosen according to the previous study (Yoshida *et al.* 2002). To evaluate dynamic bone formation parameters, the mice were injected subcutaneously with 4 mg/kg calcein, 4 and 2 days before killing. The mice were anesthetized by 150 mg/kg avertin (tribromoethanol) injection and killed 24 h after the last EP4A injection.

Body weight of the animals

To monitor the effects of EP4A on the general body condition of these animals, body weight was measured every day during the experimental periods.

Micro-CT analysis

The femora were used for three-dimensional micro-CT analysis. Distal regions of femora were subjected to high-resolution µCT analysis (µCT20, Scano Medical AG, Bassersdorf, Switzerland; Ishijima *et al.* 2002). Two-dimensional (2D) analysis data were quantified in an area with 0.42 mm² (0.7 × 0.8 mm) in slices 0.2 mm distal to the growth plate of the distal ends of the femora. Some of the femora were also subjected to 2D µCT analysis using another micro-CT apparatus, Musashi (Nittetsu-ELEX, Osaka, Japan). For cancellous bone of vertebrae, 2D µCT slices were made within a midcoronal plane in the trabecular region of the bones to obtain bone volume/tissue volume (BV/TV) values in a 0.42 mm² area (0.7 × 0.8 mm) of the vertebral bodies. The image data were subsequently quantified using Luzex-F automated image analysis system (Nireco, Tokyo, Japan).

Histomorphometry

Histomorphometric studies were conducted as described elsewhere according to the definition described by Parfitt *et al.* (1987).

RT-PCR

Total RNA of the whole humerus was extracted according to acid guanidinium thiocyanate–phenol–chloroform method as described (Ishijima *et al.* 2002). Total RNA (1 µg) was used for first strand cDNA synthesis and synthesized cDNA was then subjected to PCR using the following primers: alkaline phosphatase (ALP), forward, 5'-ATTGCCCTGAAACTCCAAAACC-3' and reverse, 5'-CCTCTGGTGGCATCTCGTTATC-3'; type 1 procollagen, forward, 5'-TTTGTG GACCTCCGGCTC-3' and reverse, 5'-AAGCAGAGCACTCGCCCT-3'; osterix, forward, 5'-CTGGGGAAAGGAGGCACAAAGAAG-3' and reverse, 5'-GGGTTAAGGGGAGCAAAGTCAGAT-3'; and GAPDH, forward, 5'-ACCACAGTCCATGCCATCAC-3' and reverse, 5'-TCCACCACCCTGTTGCTGTA-3'.

Injection of EP4A into calvariae

EP4A (30 µg/kg) or vehicle was directly injected into calvariae of 3-week-old wild-type and OPN-deficient mice thrice/day for 5 days/week for 4 weeks. Animals were killed 2 days after the last injection into calvariae. Calvariae were removed and were subjected to µCT analysis.

Measurement of serum and urinary parameters of bone metabolism

Urine was collected from each mouse during the last 24 h before killing after treatment with EP4A for 4 weeks. Urinary deoxypyridinoline (U-Dpyr) levels were measured using ELISA (Metra Biosystems, Tokyo, Japan). Serum was collected at the final point of killing. ALP in serum was measured (Daiya AUTO T-Alp; Daiya Shiyaku, Tokyo, Japan).

ALP and MTT assay in culture

Calvariae were harvested after systemic EP4A or vehicle treatment (s.c. injection), and minced pieces (about 1–2 mm in diameter) of the calvariae were placed onto plastic dishes in medium and cultured for 3 weeks to allow outgrowth of the cells. Cells derived from the calvarial outgrowth were harvested and replated in 96-well plates (5×10^4 cells/well) and cultured for 8 days. At the end of the cultures, cells were collected for measurement of ALP activity as described previously (Morinobu *et al.* 2005). The proliferation of calvarial cells was examined based on MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay (Usui *et al.* 2002).

Mineralized nodule formation assay

Bone marrow cells from wild-type and OPN-deficient mice after EP4A or vehicle treatment for 4 weeks were flushed out from tibia and a cell suspension was prepared.

These bone marrow cells were plated in 12-well plates ($3.8 \text{ cm}^2/\text{well}$) at a concentration of 5×10^6 cells/well in α -MEM supplemented with 10% FBS, 50 µg/ml ascorbic acid, 10 mM β -glycerophosphate, and antibiotics/antimycotics, with a medium change every 3 days. The culture was continued for 3 weeks in the absence of EP4A or any other drugs in order to examine the effects of EP4A treatment *in vivo* on osteoblastic activity *in vitro*. The cultures were fixed with 95% ethanol for 10 min and were stained for 10 min with a saturated solution of alizarin red (Wako, Tokyo, Japan), washed with water, and dried in air. The area of mineralized nodules and total surface of the culture area were measured by using Luzex-F image analyzer. The data were expressed as mineralized nodule area per total culture surface.

Osteoclastogenesis in bone marrow cultures

Bone marrow cells from wild-type and OPN-deficient mice after treatment with EP4A or vehicle for 4 weeks were flushed out from tibia. The cells were plated in 12-well plates at 5×10^6 cells/well in α -MEM supplemented with 10% FBS, 10 nM 1,25(OH) $_2$ vitamin D $_3$, 100 nM dexamethasone, and antibiotics/antimycotics in the absence of EP4A. Medium was changed every 3 days, and the cells were cultured for 7–9 days. Tartrate-resistant acid phosphatase (TRAP)-positive osteoclast-like multinucleated cells (more than two nuclei) were counted at the end of culture.

Ovariectomy

For ovariectomy, 15-week-old female wild-type and OPN-deficient mice were used. A total of 24 mice per genotype (WT or OPN $^{-/-}$) were randomly assigned in equal numbers into sham operation and ovariectomy groups. After surgery, these mice were injected with EP4A at 30 µg/kg, thrice/day, 5 days/week for 4 weeks before killing. BV/TV levels were analyzed in a 0.42 mm^2 square area located 0.2 mm away from the growth plate of the distal end of femur.

Statistical analysis

The results were presented as mean values \pm S.D. Statistical evaluation was performed based on multiple comparison test using Tukey's method. In ovariectomized data, the statistical analysis consisted of 2×2 factorial analysis of variance (ANOVA) with the factors of operation (sham or ovariectomy) and treatment (control or EP4A). Statistical significance of the main effect of operations and treatments and their interaction was calculated and the *P* values for interaction were presented. In all tests, $P < 0.05$ was considered to be statistically significant.

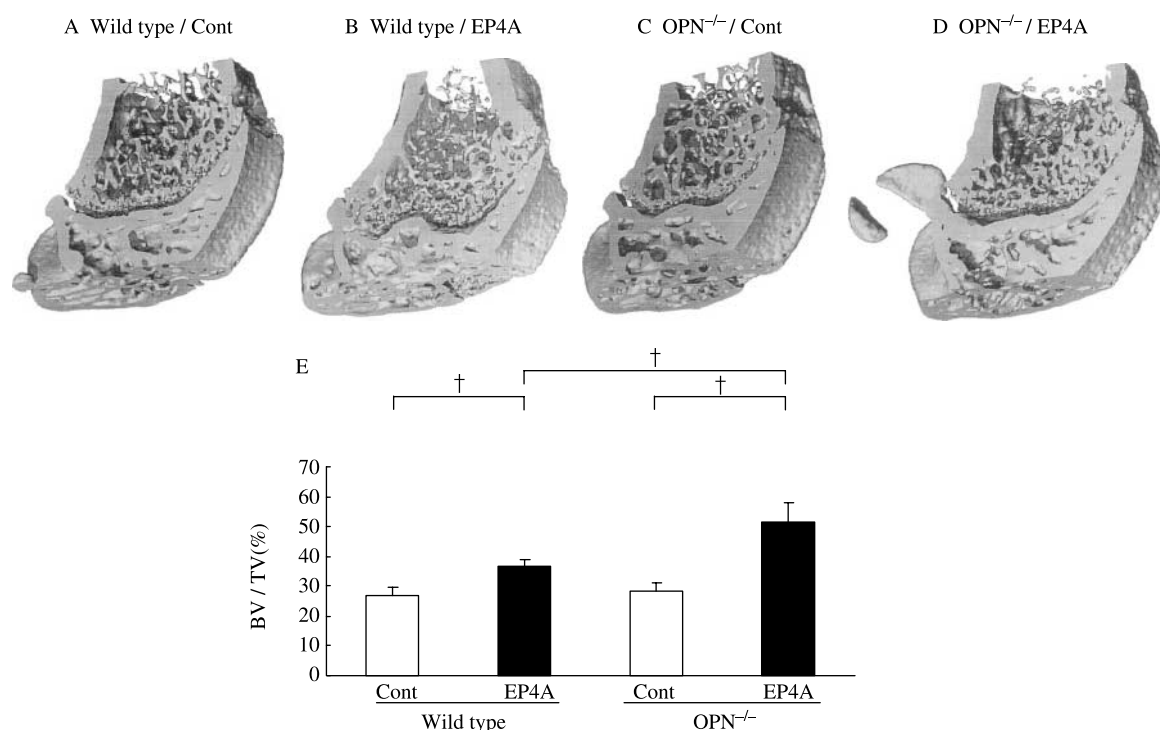


Figure 1 Micro-CT analysis of distal femora. Micro-CT-based reconstruction of distal femora from (A) Wild type/Cont, (B) Wild type/EP4A, (C) OPN^{-/-}/Cont, and (D) OPN^{-/-}/EP4A. (E) Bones analyzed for bone volume per tissue volume (BV/TV). Dagger indicates statistically significant difference ($^{\dagger}P < 0.01$).

Results

OPN deficiency augments anabolic effects of EP4A on bone

In order to evaluate the role of the bone matrix protein OPN on anabolic action of EP4 in bone, we examined the structure of bones in WT and OPN-deficient mice treated either with sub-optimal dose of EP4A or vehicle. Micro-CT evaluation indicated that EP4A treatment moderately enhanced the BV/TV in the cancellous bone of the femur in wild-type mice (Fig. 1B versus A and Fig. 1E). In OPN-deficient mice, the vehicle-treated mice had similar bone volume as vehicle-treated wild-type mice (Fig. 1C versus A and Fig. 1E). OPN deficiency enhanced the effects of EP4A on the bone volume more than those in wild-type mice (Fig. 1D versus B and E).

We further examined whether the enhancement of the anabolic actions of EP4A on cancellous bone by OPN deficiency was limited to the femoral bone or was a general effect on the systemic skeleton. For this purpose, the fourth lumbar vertebrae were subjected to 2D micro-CT analysis. In wild-type mice, EP4A treatment at sub-optimal dose did not significantly alter the bone volume in the lumbar vertebrae (Fig. 2A versus B and Fig. 2E). The bone volume in lumbar vertebrae of OPN-deficient mice was similar to that in wild-type control or EP4A-treated wild-type mice

(Fig. 2C versus A, B and Fig. 2E). In contrast to wild type, the bone volume in the lumbar vertebrae was significantly increased in OPN-deficient mice treated with sub-optimal dose of EP4A (Fig. 2E, column 4 versus column 2). Thus, OPN deficiency revealed the anabolic actions of EP4A at sub-optimal dose, and this effect was observed at least in two distinct locations.

Previous experiments indicated that PGE₂ enhancement of bone formation was associated with a significant loss of body weight and this is an important and serious side effect (Ushikubi *et al.* 1998). In contrast to these PGE₂ effects, neither EP4A treatment at sub-optimal dose nor OPN deficiency altered body weight (Fig. 2F).

To elucidate the target of OPN actions on EP4A effects, we analyzed bone cell activity *in vivo*. The enhancement of the EP4A-induced increase in BV/TV by OPN deficiency could be due to alterations in bone formation or in bone resorption. To address this point, we conducted histomorphometry to evaluate the dynamic activity of bone formation, i.e. bone mineral apposition rate (MAR) and bone formation rate (BFR). In wild type, MAR was not significantly altered by the treatment with EP4A at sub-optimal dose alone (Fig. 3A). BFR was also not enhanced by EP4A treatment at this dose alone in wild-type mice (Fig. 3B). OPN deficiency alone did not alter MAR (Fig. 3A) nor BFR (Fig. 3B). In contrast, both MAR and BFR were increased in OPN-deficient mice

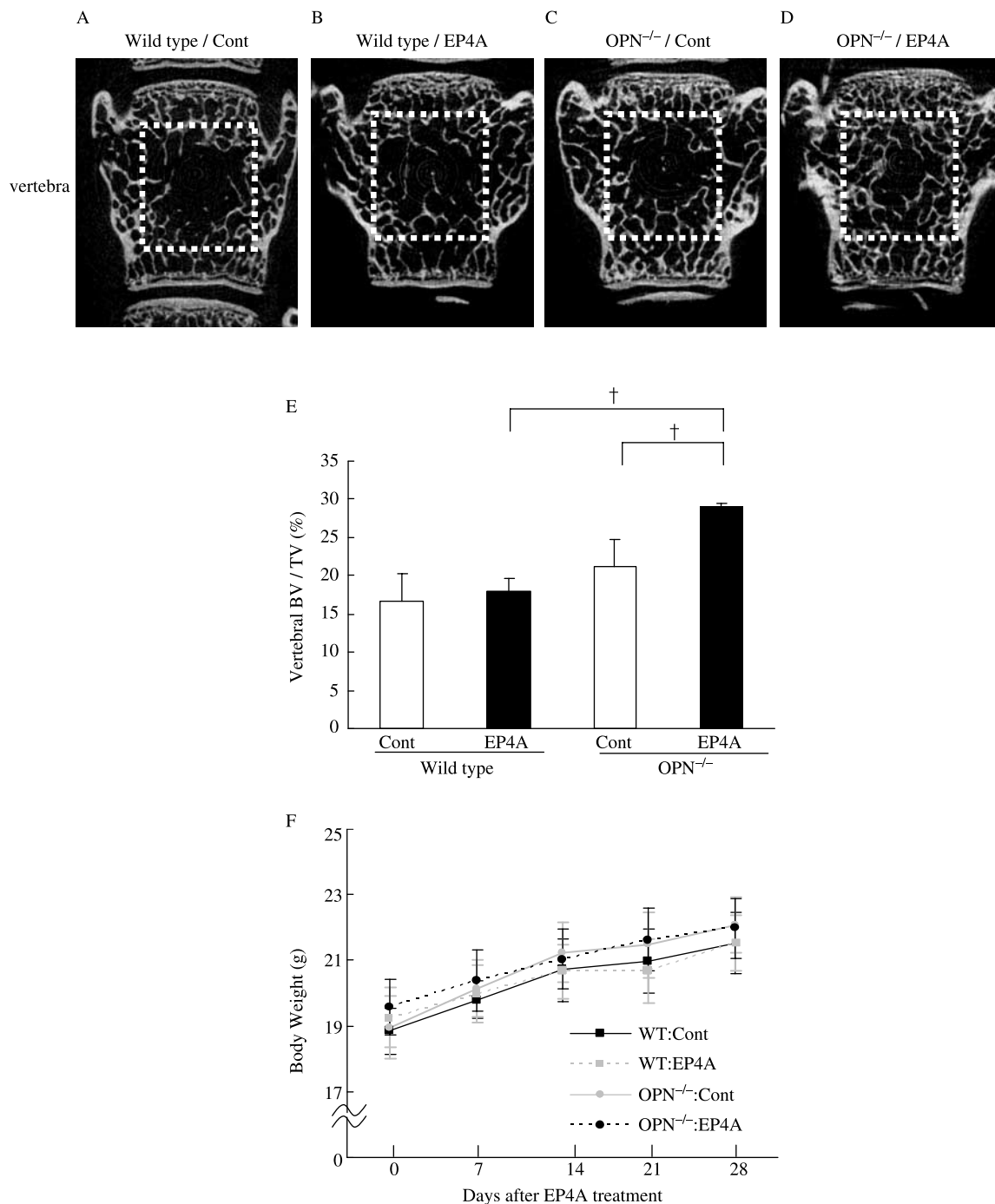


Figure 2 Micro-CT analysis of vertebrae. Micro-CT-based 2D reconstruction of vertebrae of (A) Wild type/Cont, (B) Wild type/EP4A, (C) OPN^{-/-}/Cont, and (D) OPN^{-/-}/EP4A. (E) Coronal sections of vertebrae of Wild type/Cont, Wild type/EP4A, OPN^{-/-}/Cont, and OPN^{-/-}/EP4A analyzed for bone volume per tissue volume (BV/TV). (F) Body weight during treatment with EP4A or vehicle. Dagger indicates statistically significant difference ([†]*P* < 0.01), Cont, control; WT, wild type.

treated with EP4A at sub-optimal dose (Fig. 3A and B). On the other hand, EP4A at this dose did not alter MS/BS regardless of the genotype (Fig. 3C). Thus, the effects of OPN deficiency on EP4A actions on bone volume were at least in

part through its effects on EP4A actions on the bone formation (MAR and BFR) by osteoblasts *in vivo*.

Since bone histomorphometric analysis provides information only on the morphological aspects of bone, we

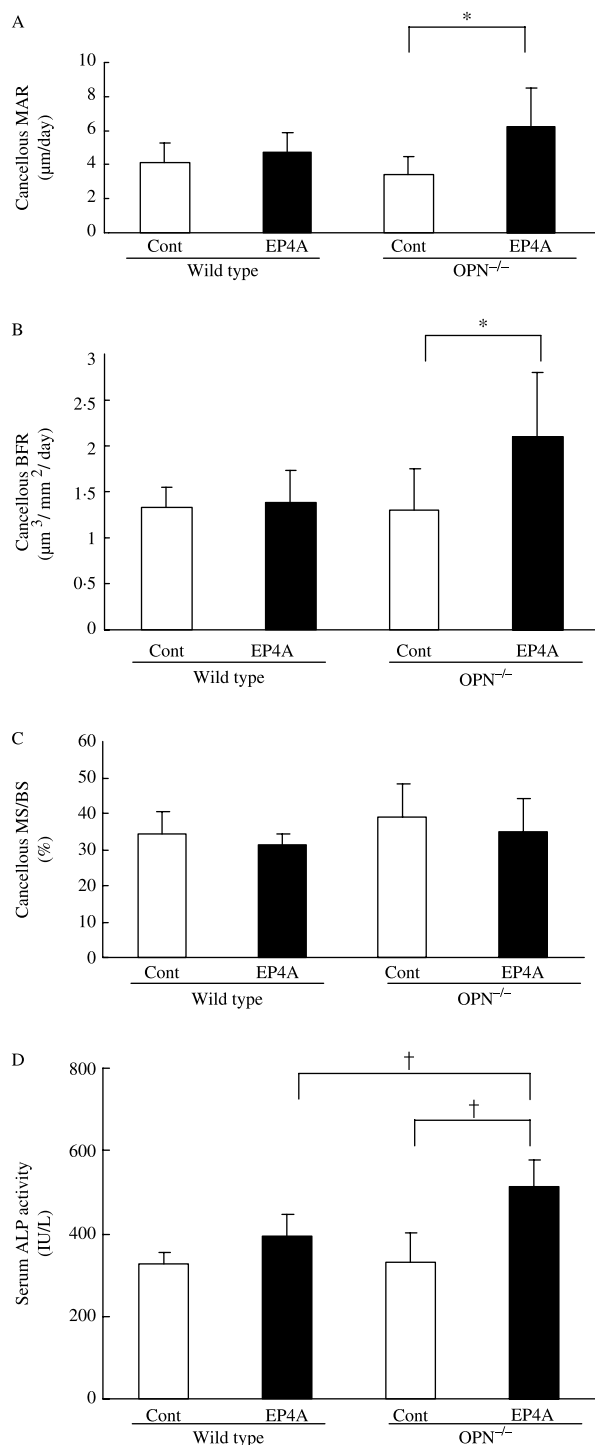


Figure 3 EP4A effects on dynamic histomorphometry on bone formation. (A) MAR, (B) BFR, and (C) MS/BS were measured. (D) EP4A effects on biochemical parameters of bone formation. Serum levels of ALP in Wild type/Cont, Wild type/EP4A, OPN^{-/-}/Cont, and OPN^{-/-}/EP4A. Asterisk and dagger indicate statistically significant difference (* $P < 0.05$, † $P < 0.01$), Cont, control.

addressed the question whether the effect of OPN deficiency on EP4A action could be detected based on biochemical (systemic bone formation) parameters. To examine this point, we measured total ALP levels in the serum of the mice. OPN deficiency enhanced more than threefold the EP4A actions on the levels of serum ALP activity (Fig. 3D).

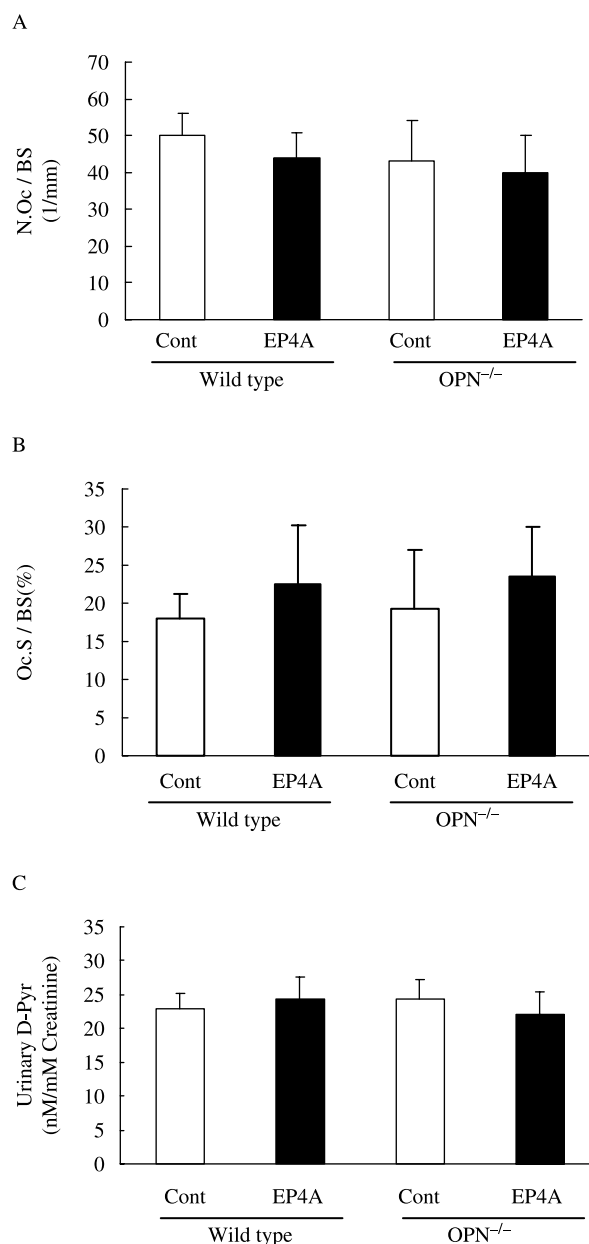


Figure 4 Sections stained for tartrate-resistant acid phosphatase (TRAP) activity. (A) Number of osteoclasts per bone surface (N.Oc/BS) and (B) osteoclast surface per bone surface (Oc.S/BS). (C) Levels of U-Dpyr (see Materials and Methods section), Cont, control.

As bone volume is determined not only by bone formation but also by changes in bone resorption, we investigated the effects of OPN deficiency on EP4A action in terms of osteoclastic bone resorption parameters *in vivo*. Neither EP4A treatment at sub-optimal dose, OPN deficiency, nor a combination of both altered the morphology or number of osteoclasts or the osteoclast surface (Fig. 4A and B). U-Dpyr

levels were examined to evaluate the systemic effects on bone resorption. Neither EP4A treatment, OPN deficiency, nor a combination of both altered U-Dpyr secretion compared with the vehicle-treated control in wild type mice (Fig. 4C). These data suggest that the OPN-deficiency enhancement of the sub-optimal dose EP4A-induced increase in bone volume was due to the effects on bone formation and not on bone resorption.

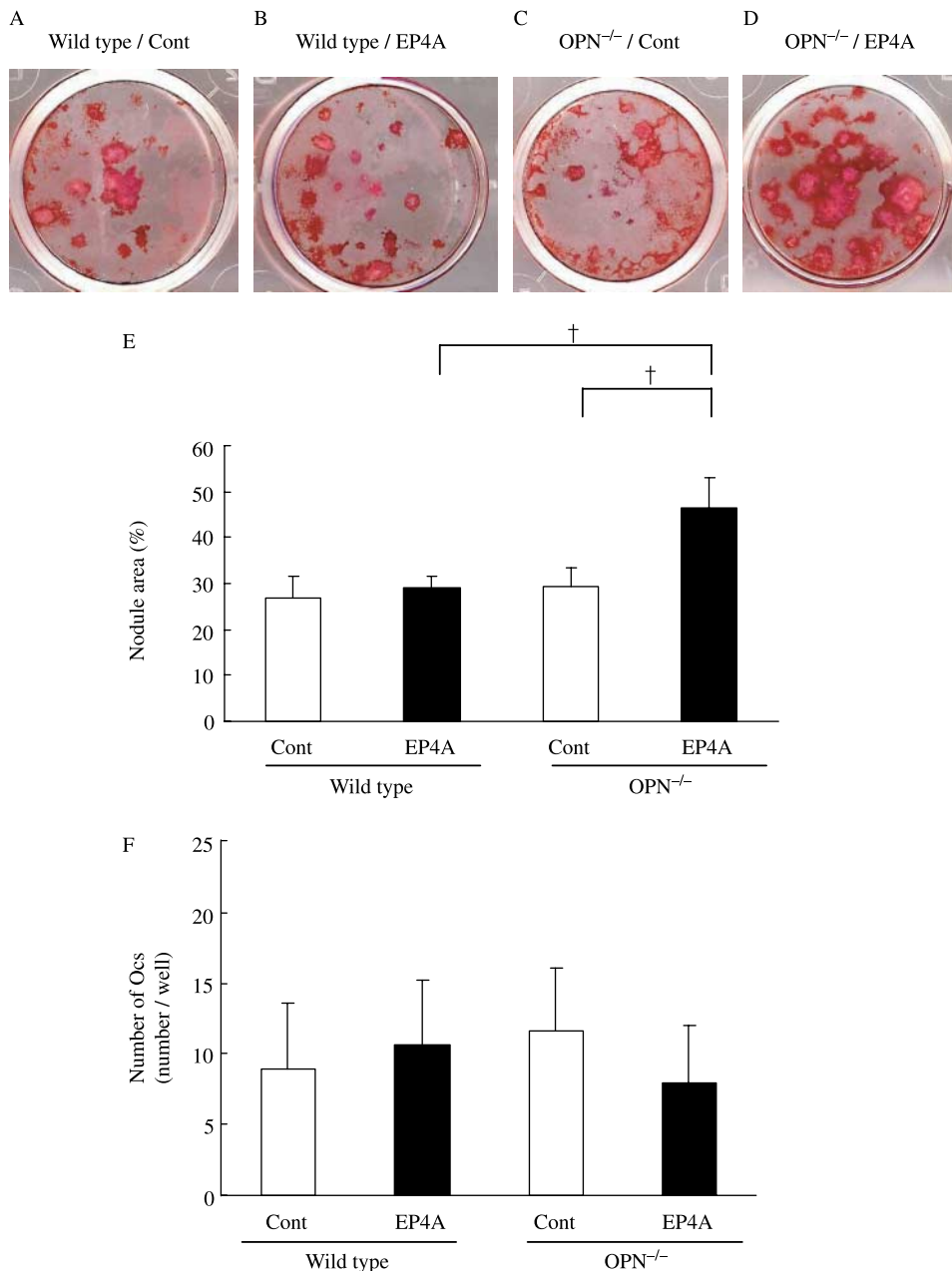


Figure 5 *Ex vivo* analysis of osteoblast formation and osteoclast formation. (A–D) Mineralized nodule formation in the bone marrow cells obtained from wild-type or OPN-deficient (OPN^{-/-}) mice after 4 weeks of vehicle (Cont) or EP4 agonist (EP4A) treatment. (E) Quantification of the mineralized nodules. (F) Analysis of osteoclast development: quantitative analysis of number of osteoclasts. [†]*P* < 0.01.

In order to obtain further insights into the mechanisms of OPN-deficiency enhancement of sub-optimal dose EP4A actions in bone, we evaluated osteogenic activity in bone marrow cell cultures by measuring mineralized nodule formation. Sub-optimal dose EP4A treatment *in vivo* alone did not significantly alter the extent of mineralized nodule formation in bone marrow cells cultured for 3 weeks after the *in vivo* treatment for 4 weeks (Fig. 5B versus A and Fig. 5E). In contrast, OPN deficiency revealed increase in the formation of mineralized nodules *in vitro* of the cells obtained from EP4A-treated mice (Fig. 5E). Thus, treatment of OPN-deficient mice with EP4A at sub-optimal dose for 4 weeks prior to killing causes an increase in the osteogenic activity in the bone marrow cells. Neither EP4A treatment *in vivo*, OPN deficiency, nor the combination of both significantly altered the levels of the development of TRAP-positive multinucleated cells in the cultures of the bone marrow cells (Fig. 5F). This again suggests the action of this bone matrix protein to enhance sub-optimal dose EP4A actions on osteoblasts.

To further examine the OPN-deficiency enhancement on EP4A actions in mineralized nodule formation, we used calvaria-derived cells. As described in Materials and Methods, calvariae were removed from mice treated with EP4A or vehicle for 4 weeks *in vivo* and minced into pieces. These calvarial bone pieces were placed in culture dishes, and about 3 weeks later out-grown cells were harvested from the dishes. These fibroblast-like cells were then replated and cultured for 8 days, and ALP activity was measured. OPN deficiency enhanced the effects of EP4A at sub-optimal dose (administered *in vivo*) on ALP activity in these calvaria-derived cell cultures (Fig. 6A). Neither OPN deficiency, EP4A treatment *in vivo*, nor combination of both affected the proliferation of the cells outgrown from the calvariae based on the MTT assay of cells cultured for 3 days (Fig. 6B). These observations further support the notion that OPN deficiency enhanced the actions of EP4A at sub-optimal dose in the cells of osteoblast lineage.

The molecular basis of the ability of OPN deficiency to enhance of EP4A action on cell differentiation was analyzed using PCR-based evaluation of gene expression in the bones of wild-type and OPN-deficient mice treated with EP4A. EP4A treatment at sub-optimal dose alone *in vivo* did not alter the expression of genes encoding proteins related to osteoblast-related phenotypes including type I collagen, ALP, and osterix (Fig. 7A–D), while the combination of OPN deficiency and EP4A at sub-optimal dose resulted in elevation of the mRNA expression of these genes (Fig. 7A–D). These data suggest that OPN deficiency enhanced sub-optimal dose EP4A actions *in vivo* as reflected in the activation of the expression of genes encoding osteoblast phenotype-related proteins.

To address the question whether OPN deficiency enhances direct action of EP4A on bone formation, we directly injected EP4A at sub-optimal dose into the calvariae of mice. Direct EP4A treatment at sub-optimal dose of OPN-deficient mice resulted in more increase in the calvarial thickness than wild type (Fig. 8D versus B and Fig. 8E). Thus, our prior observations on OPN-deficiency enhancement of the

sub-optimal dose EP4A effects on bone mass were at least in part due to OPN-deficiency effect on the direct EP4A action on bone.

Finally, we addressed the question whether OPN-deficiency enhancement of EP4A actions could be observed under pathological condition. To examine this point, we conducted ovariectomy experiments in wild-type and OPN-deficient mice. In wild-type mice, ovariectomy reduced the bone volume in the femora as shown previously (Fig. 9A). Treatment with EP4A at sub-optimal dose alone modestly increased trabecular bone volume in sham-operated wild-type mice (Fig. 9A), although the total bone loss was partially resumed by the EP4A treatment of ovariectomized wild-type mice (Fig. 9A, $P=0.362$). Thus, EP4A at sub-optimal dose treatment failed to fully prevent bone loss due to ovariectomy compared with sham-operated wild-type mice (Fig. 9A). No interactive effect of ovariectomy on the modest bone mass-increasing effect of EP4A was indicated by two-way ANOVA ($P=0.395$). Same experiments were carried out on OPN-deficient mice (Fig. 9B). OPN deficiency alone did not alter the basal levels of bone mass (Fig. 9; comparing column 1 in A and B), while it suppressed the bone loss due to ovariectomy as reported previously (Yoshitake *et al.* 1999; Fig. 9A). Accordingly, the bone volume in ovariectomized OPN-deficient mice was higher than that in ovariectomized wild-type mice (Fig. 9; comparing column 2 in A and B).

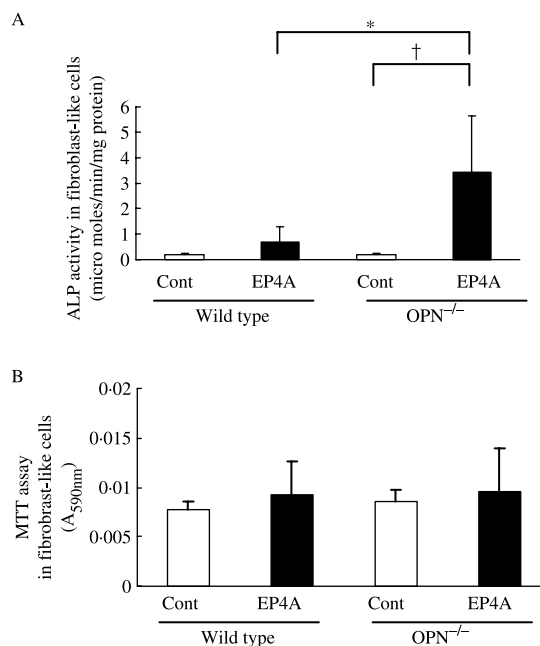


Figure 6 OPN deficiency enhancement of EP4A-induced ALP expression in calvaria-derived cells. (A) Fibroblast-like cells were cultured for 8 days after EP4A treatment for 4 weeks. ALP activities were measured and the values were normalized against protein concentration. (B) Quantitative analysis of proliferation rate (MTT assay) of fibroblast-like cells after culture for 3 days after EP4A treatment for 4 weeks. Asterisk and dagger indicate statistically significant difference (* $P<0.05$, † $P<0.01$), Cont, control.

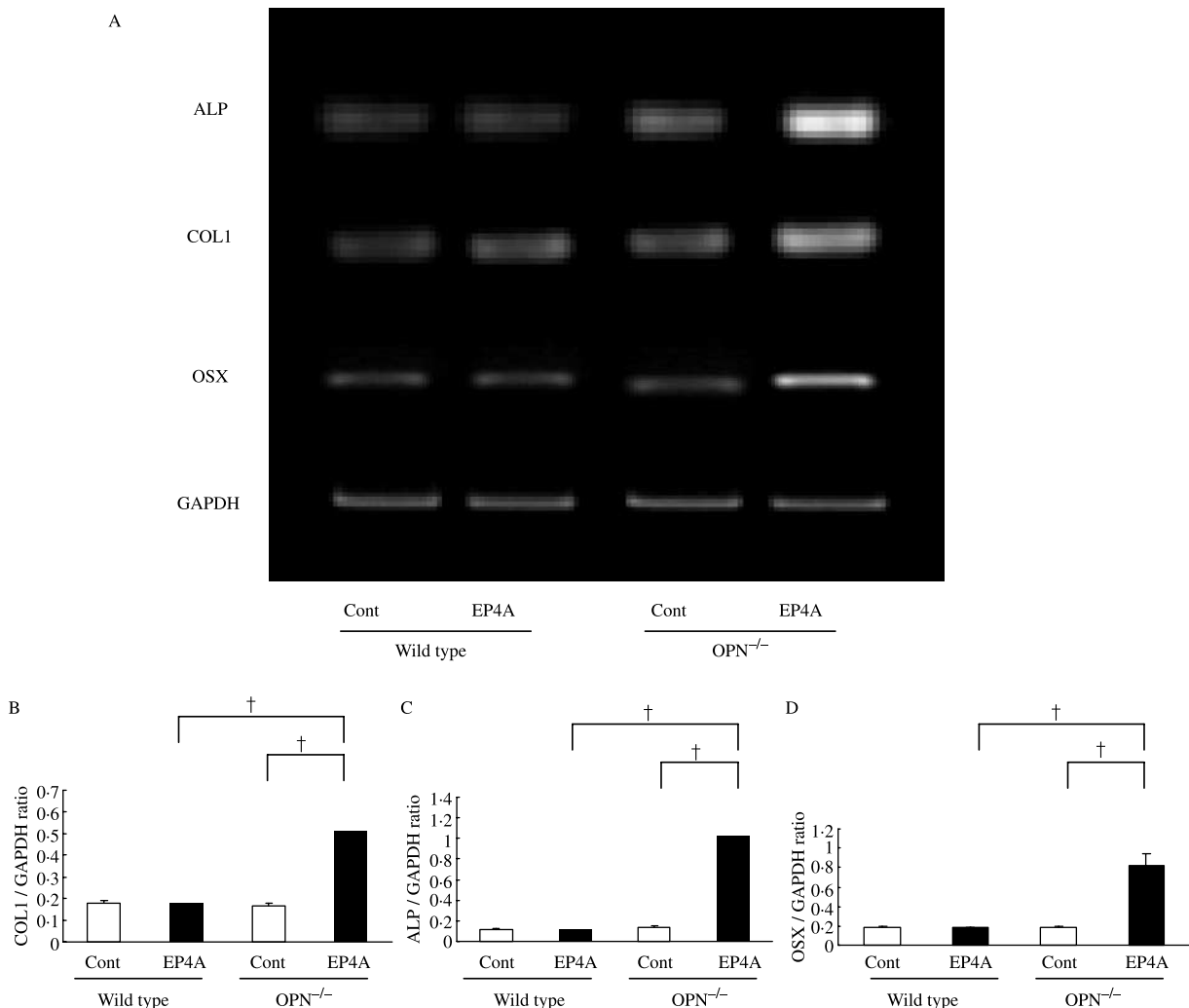


Figure 7 RT-PCR analysis for osterix (OSX), collagen type 1 (COL1), and ALP. (A, B, C, D) Expression levels of COL1 and ALP, and osterix mRNAs in bone enhanced in OPN^{-/-} mice after EP4A treatment. Dagger indicates statistically significant difference ($^{\dagger}P < 0.01$), Cont, control.

In this setting, OPN deficiency enhanced EP4A actions in sham-operated mice, as in the previous observations (Fig. 9; comparing columns 1 and 3 in A and B). In addition, OPN deficiency together with sub-optimal dose of EP4A treatment resulted in almost full suppression of bone loss due to ovariectomy (Fig. 9B). Thus, no interactive effect of ovariectomy was indicated again on the significant bone mass increase by EP4A treatment (two-way ANOVA; $P = 0.995$). Most importantly, there was an increase in bone mass following ovariectomy in mice deficient for OPN expression treated with EP4A (Fig. 9B; $P < 0.01$), suggesting that this combination of EP4A and OPN deficiency can preserve and increase bone mass in the absence of estrogen. Neither the effects of OPN deficiency, EP4A treatment, nor the combination of both affected ovariectomy-induced reduction in uterine weight (Fig. 9C).

Discussion

We observed that OPN deficiency enhanced the otherwise moderate anabolic actions of EP4A at sub-optimal dose in bone. There is no previous paper showing that either PGE₂ or EP4A exerts a systemic anabolic effect on bone in mice. As shown in wild-type mice in our experiments, EP4A treatment at sub-optimal dose only moderately prevented bone loss in ovariectomized mice and did not fully restore the levels of bone mass compared with those in wild-type sham-operated mice. In contrast, OPN deficiency enhanced the EP4A-induced increase in bone mass in the ovariectomized mice and fully prevented bone loss due to estrogen depletion. This suggests that release from the inhibitory action of OPN enhanced the moderate anabolic actions of EP4A at sub-optimal dose in ovariectomized mice.

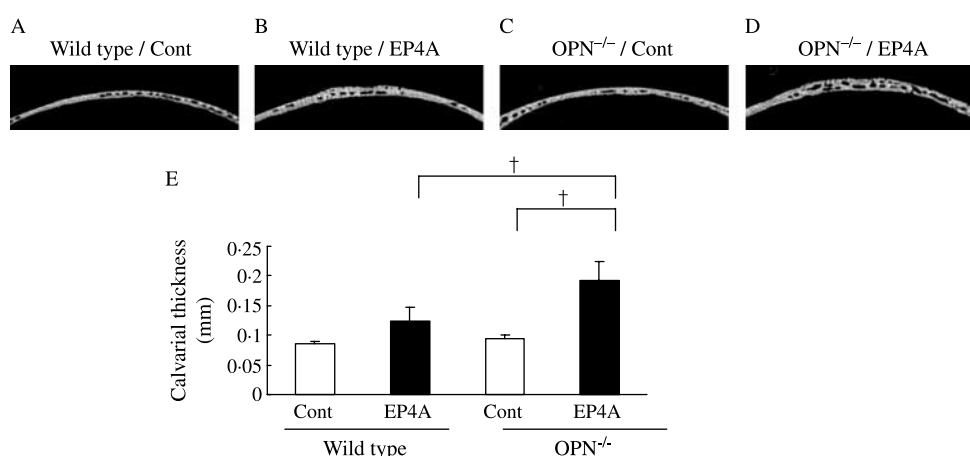


Figure 8 OPN deficiency enhancement of the direct action of EP4A on bone *in vivo*. (A–D) 2D micro-CT determination of calvarial thickness after local treatment with EP4A. (E) Calvarial thickness in calvarial thickness was calculated as EP4A treated/vehicle. Dagger indicates statistically significant difference ($^{\dagger}P < 0.01$), Cont, control.

Our results suggest that differentiation of precursor cells into osteoblasts is enhanced by EP4A together with OPN deficiency, as measured by the accumulation of mineralized nodules in primary osteoblast cultures. These results are consistent with previous work demonstrating that EP4 functions to enhance osteoblast differentiation from precursor cells (Shamir *et al.* 2004). OPN is a major secretory product of osteoblasts, and is a marker of osteoblastic differentiation, with peak expression occurring during mineralization (Lian & Stein 1995). OPN would regulate hydroxyapatite formation, inhibiting nucleation *in vitro* (Pampena *et al.* 2004) as well as calcification in cell cultures (Jono *et al.* 2000). Thus, one possible mechanism for the synergistic effect of OPN deficiency and EP4A at sub-optimal dose is an enhancement of osteoblast differentiation, together with release from inhibition of mineralization by OPN. However, this would not simply explain the observations in our experiments. Alternatively, OPN has been implicated in the regulation of hematopoietic stem cells (Nilsson *et al.* 2005, Stier *et al.* 2005) and may be involved in osteoblastic differentiation from mesenchymal precursors as well. Knockout mice of another bone matrix protein, osteocalcin, were reported to exhibit higher bone mass than wild type (Ducy *et al.* 1996). However, the mechanism of osteocalcin deficiency on bone mass control is not known. Whether our observation on OPN may have any relevance to osteocalcin function is still to be elucidated. Understanding the mechanism of the synergistic effect of OPN deficiency and EP4A is an important next step.

PTH has been considered to be one of the promising approaches for preventing fractures more efficiently than the currently used amino bisphosphonates. In fact, PTH reduces fracture risks by almost 70% according to the large multicenter studies (Swarthout *et al.* 2002). This is more potent than the protection provided by amino bisphosphonates. However, as PTH is a peptide in nature, handling is complex and daily injection is a major problem of this type of treatment.

PGE₂ treatment for the patent ductus arteriosus results in bone formation in infants (Jorgensen *et al.* 1998), and animal studies have revealed the efficacy of PGE₂ as a possible bone anabolic agent (Pilbeam *et al.* 2002). However, the use of PGE₂ is limited by its side effects. Most of the early studies using PGE₂ indicated that although animals certainly increased their bone mass, they suffered from severe side effects, such as diarrhea and body weight loss (Suponitzky & Weinreb 1998). Recent reports indicated that the PGE₂ receptor EP4 is one of the major receptor subtypes in bone. Therefore, specific EP4As could be used to circumvent side effects of PGE₂ (Kabashima *et al.* 2002). The EP4A has been shown to be efficacious in maintaining bone mass in ovariectomized rats (Yoshida *et al.* 2002), in increasing ectopic bone mass induced by bone morphogenetic protein (BMP; Sasaoka *et al.* 2004), and restore bone mass and strength in aged ovariectomized rats (Ke *et al.* 2006). However, such prevention by EP4A at sub-optimal dose had not yet been demonstrated in mice. Furthermore, there are still concerns due to its possible side effects in organs other than bone which express EP4 receptors (Yoshida *et al.* 2002). The combination of EP4A at sub-optimal dose and OPN deficiency resulted in increase (Fig. 9B) in bone mass in ovariectomized mice compared with the sham-operated wild-type mice. If one could develop measures to suppress OPN actions *in vivo* using antibodies, it may be possible to augment the action of EP4A at sub-optimal dose.

EP4A was administered in our experiments through s.c. injection. However, a major strength of this drug is that its structure indicates that it can be effective after oral administration (Yoshida *et al.* 2002). Thus, compared with PTH, which cannot be administered orally due to its peptide nature, EP4A may be superior in terms of possible clinical oral use in the patients. Several forms of PG drugs have already been used in the clinical setting such as those to increase circulation in the case of obstructive arteritis (Makita *et al.*

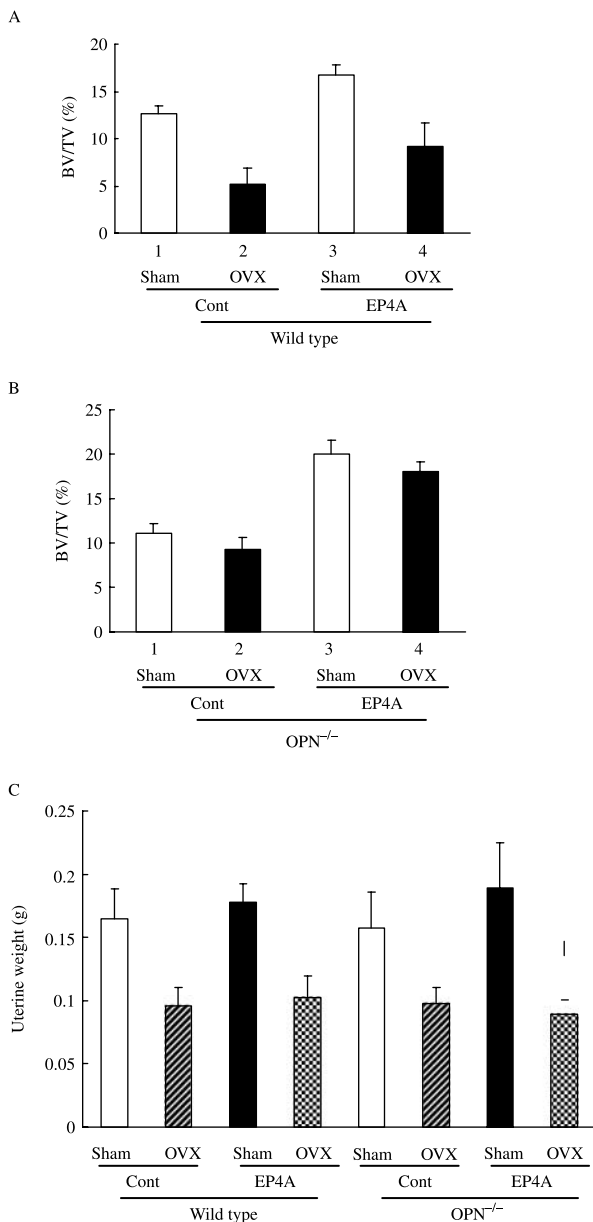


Figure 9 Effects of EP4 signaling induced by OPN deficiency after estrogen depletion. Micro-CT analysis of the femora of mice. Micro-CT-based quantification of the BV/TV after ovariectomy (OVX) in (A) EP4A-treated wild-type mice and (B) OPN-deficient mice. (C) Uterine weight by EP4A after ovariectomy in OPN-deficient mice. Interaction between the effects introduced by operations and treatments was tested by 2×2 factorial ANOVA, Cont, control.

1997) or lumbar canal stenosis (Yone *et al.* 1999). Together, these observations suggest that orally administrated EP4A may be safe and effective in combination with inhibition of OPN action.

In conclusion, anabolic measures to increase bone mass are among the most important therapies for the osteoporosis patients, especially for those with severe bone loss. However,

currently available options such as PTH are not yet regarded as ideal treatment for patients in general, despite its certain efficacy based on evidence. Therefore, efficacious use of non-peptide compounds will be a breakthrough. Our observation on the OPN-deficiency enhancement of anabolic activity of EP4A at sub-optimal dose may provide a clue to develop novel options for the treatment of osteoporosis.

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