

Phospholipase C Signaling via the Parathyroid Hormone (PTH)/PTH-Related Peptide Receptor Is Essential for Normal Bone Responses to PTH

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We have previously shown that differentiation of hypertrophic chondrocytes is delayed in mice expressing a mutated PTH/PTHrP receptor (PTHR) (called DSEL here) that stimulates adenylyl cyclase normally but fails to activate phospholipase C (PLC). To better understand the role of PLC signaling via the PTHR in skeletal and mineral homeostasis, we examined these mice fed a normal or calcium-deficient diet. On a standard diet, DSEL mice displayed a modest decrease in bone mass. Remarkably, when fed a low-calcium diet or infused with PTH, DSEL mice exhibited strikingly curtailed peritrabecular stromal cell responses and attenuated new bone formation when compared with Wt mice. Attenuated *in vitro* colony formation was also observed in bone marrow cells derived from DSEL mice fed a low-calcium diet. Furthermore, PTH stimulated proliferation and increased mRNAs encoding cyclin D1 in primary osteoblasts derived from Wt but not from DSEL mice. Our data indicate that PLC signaling through the PTHR is required for skeletal homeostasis. (**Endocrinology 151: 3502–3513, 2010**)

The PTH/PTHrP receptor (PTHR) is a class II G protein-coupled receptor that, like many other G protein-coupled receptors, activates several signaling pathways, including the G α -linked adenylyl cyclase (AC)-protein kinase A (PKA) signaling pathway and the G $_{q/11}$ -linked phosphatidylinositol-specific phospholipase C (PI-PLC, named PLC here)-protein kinase C (PKC) signaling pathway in studies in cell culture (1, 2). As with other such receptors, however, few studies establish whether the activation of multiple G proteins actually occurs *in vivo* and has importance in the action of such receptors. To explore this possibility, we have generated knock-in mice with a mutated PTHR, named DSEL (3). The DSEL receptors, with four mutated residues in the receptor's second intracellular loop, can activate AC normally but cannot activate PLC (1, 3). In initial studies, we explored the role of signaling via multiple G proteins by the PTHR in the chondrocytes of growing bones. Hypertrophic differentiation of

chondrocytes is modestly delayed in DSEL mice, indicating that the PLC signaling pathway via the PTHR is essential for normal chondrocyte differentiation (3). The PTHR is important not only for chondrocyte differentiation but also for bone formation (4–7) and calcium/phosphate homeostasis. Here we address the roles of signaling by multiple signaling pathways in the role of the PTHR in regulating skeletal and mineral ion homeostasis.

Intermittent PTH administration has been shown to increase bone density, improve skeletal architecture, enhance biomechanical strength, and reduce fracture risk (8–11), whereas continuous infusion of PTH causes pathological changes similar to those seen in clinical hyperparathyroidism, including accumulation of peritrabecular stromal cells, increased bone resorption, and hypercalcemia (12, 13). Despite these striking differences, both intermittent and continuous PTH administration increase both bone formation and

ISSN Print 0013-7227 ISSN Online 1945-7170

Printed in U.S.A.

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doi: 10.1210/en.2009-1494 Received December 24, 2009. Accepted May 6, 2010.

First Published Online May 25, 2010

Abbreviations: AC, Adenylyl cyclase; ALP, alkaline phosphatase; BrdU, bromodeoxyuridine; CFU, colony-forming units; μ CT, microcomputed tomography; CTX, C-terminal telopeptide α 1 chain of type I collagen; FBS, fetal bovine serum; hPTH, human PTH; IP3, inositol triphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phosphatidylinositol-specific phospholipase C; PMA, phorbol 12-myristate 13-acetate; P1NP, N-terminal propeptide of type I procollagen; PTHR, PTH/PTHrP receptor; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; Wt, wild type.

bone resorption; the balance of these two responses and perhaps their mechanisms differ. The mechanistic basis for these varying responses to PTH has been extensively explored but is yet to be fully identified (14–16). Several lines of evidence suggest that intermittent PTH treatment results in increased osteoblast number and activity via increased differentiation and survival of osteoblasts (17–19), whereas continuous PTH treatment leads to increased osteoclast differentiation and activity through effects on cells of the osteoblast lineage (20, 21).

The roles of distinct signaling pathways activated by the PTHR in generating these skeletal responses to PTH are not fully understood. Studies using amino-truncated PTH analogs that cannot stimulate cAMP production suggested that such stimulation was required for the anabolic actions of PTH (22, 23), although subsequent studies suggested that such analogs are defective in PLC activation as well (11). Recently, PTHR PLC signaling stimulated by administration of signal-specific PTH analogs has been shown not to be required for the anabolic effect of PTH on bone (11), but other evidence suggests that the cAMP/PKA signaling pathway alone may not be sufficient to elicit a full anabolic response (24, 25). Of course, in these studies using PTH analogs given to normal rodents, the possible importance of signaling in response to endogenous PTH and PTHrP, expected to activate both cAMP/PKA and PLC, could not be evaluated. Clarification of the roles of distinct signaling pathways downstream from the PTHR may further understanding of the varying actions of PTH on bone and could facilitate the design of suitable agents for the treatment of bone diseases such as osteoporosis.

To better understand the role of PLC signaling via the PTHR in bone modeling and remodeling and also in calcium/phosphate homeostasis, we examined the bone phenotype and serum calcium/phosphate in mutant mice fed either a standard diet or a low-calcium diet designed to provide a model of secondary hyperparathyroidism. Here, we demonstrate that in the basal state, DSEL mice displayed low bone mass perhaps partly due to increased osteoclast activity in the primary spongiosa, whereas the mutant mice, when fed a low-calcium diet or infused with PTH, exhibited strikingly curtailed peritrabecular stromal cell responses and attenuated new bone formation despite elevated serum PTH. Our data indicate that PLC signaling through the PTHR is essential for normal bone turnover.

Materials and Methods

Animals

The DSEL mice used in the present experiment were backcrossed to the C57/B6 background for more than 10 generations. All animals were maintained in facilities operated by the Center

for Comparative Research of the Massachusetts General Hospital, and all animal experimental procedures were approved by the institution's Subcommittee on Research Animal Care.

Sample preparation and histological analysis

Both wild-type (Wt) and DSEL homozygous mice were fed with a standard diet. To induce secondary hyperparathyroidism, 20-d-old mice from both Wt and DSEL homozygous mice were fed with a low-calcium diet (0.02% calcium and 0.4% phosphorus) or a control diet (0.6% calcium and 0.4% phosphorus) for 3 wk (low-calcium diet TD 02279 and control diet TD 97191 were purchased from Harlan Teklad, Madison, WI). For histological analysis, tissues from Wt and DSEL homozygous mice were fixed with 10% formalin and 70% ethanol. In selected cases, hind limbs were decalcified with 20% EDTA, and paraffin blocks were prepared by standard histological procedures. For selected samples, tartrate-resistant acid phosphatase staining was performed using a Sigma Chemical Co. (St. Louis, MO) acid phosphatase detection kit.

Serum biochemistry

Blood was collected by orbital sinus puncture before killing for serum biochemistry. Serum calcium and inorganic phosphorus were assayed with the UV determination kits (Stanbio Laboratory, Boerne, TX). Mouse intact PTH(1-84) was measured in duplicate using an ELISA kit (Immutopics Inc., San Clemente, CA). Serum N-terminal propeptide of type I procollagen (P1NP) and C-terminal telopeptide α 1 chain of type I collagen (CTX) were measured with ELISA kits from Immunodiagnostic Systems (Fountain Hills, AZ).

Microcomputed tomography (μ CT)

μ CT analysis of the distal femur was performed using a desktop microtomographic image system, as described previously (11).

Tibial trabecular bone histomorphometry

For dynamic histomorphometry, 10-wk-old animals were injected ip with fluorochromes calcein (2 mg/ml; Sigma) and demeclocycline (2 mg/ml; Sigma) 3 and 10 d before killing, respectively. Tibiae and vertebrae were collected for histomorphometric analysis. Bones were fixed with 10% formalin overnight and then 70% ethanol and embedded in methyl methacrylate resin. Five-micrometer sections were stained with toluidine blue and von Kossa, and all measurements were performed on the trabecular area of proximal tibia in a region between 0.5 and 2.5 fields (at $\times 200$) distal to the growth plate using a digitizing image analysis system and a morphometric program, Osteomeasure (Osteometrics Inc., Atlanta, GA). Fibrosis volume was measured as peritrabecular stromal cell response and determined by measurement of fibroblastic volume/bone volume in the primary and secondary spongiosa regions. Fibrosis was defined as multiple layers of elongated or fusiform cells surrounded by extracellular matrix that line the trabecular bone surface.

In situ hybridization

In situ hybridizations were performed on paraffin sections using complementary 35 S-labeled riboprobes (cRNAs) transcribed from the plasmids, kindly provided by the Massachusetts General Hospital Endocrine Unit histology core facility (26).

Quantitative real-time PCR

RNA was extracted from bone and cultured primary osteoblastic cells using Trizol (Invitrogen, Carlsbad, CA) and was reverse transcribed into cDNA with SuperscriptRT II (Invitrogen). SYBRGreen Universal Master Mix (Applied Biosystems, Foster City, CA) was used for quantitative, real-time PCR, as described previously (27). The PCR primer sequences used are as follows: cyclin D1 forward 5'-gcaagaggaaggagccagcc-3' and reverse 5'-gggatgcagattctatctct-3' and Gapdh forward 5'-tg-gagtgtgtctctactact-3' and reverse 5'-aagcagttggtggtcaggat-3'. Relative expression was calculated for each gene by the $2^{-\Delta\Delta C_T}$ method with Gapdh for normalization.

Effect of low-calcium diet on colony formation

in vitro

Tibiae were dissected from 4-wk-old Wt and DSEL mice on the low-calcium diet or control diet for 1 wk. Epiphyses were removed and the diaphysis flushed with 15 ml α -MEM using a 20-ml syringe fitted with a 23-gauge needle. Flushed bone marrow cells were plated in 12-well plates at 5×10^5 cells per well and cultured for 14 d with α -MEM supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin at 37 C in a humidified atmosphere of 95% air and 5% carbon dioxide. On d 14, the cells were fixed with 10% neutral buffered formalin in PBS for 10 min. Total number of colonies [colony-forming units (CFU)-F] and colonies positive for alkaline phosphatase (ALP) (CFU-ALP) were determined by staining with methylene blue or with ALP staining buffer (Sigma), respectively. Colonies large enough to be seen with the naked eye were counted, and counts were performed blind on coded plates.

Primary osteoblast cultures

Six-week-old tibiae were dissected from Wt and DSEL mice. After bone marrow was flushed, the dissected bones were minced and cultured in six-well plates with α -MEM containing 10% FBS for 5 d, and then the growing primary osteoblastic cells were plated in slide chambers for proliferation and apoptosis assays or grown in six- and 12-well plates for *in vitro* determination of osteoblast differentiation. Cell proliferation was quantified by bromodeoxyuridine (BrdU) assay with an *in situ* cell proliferation kit (Roche, Indianapolis, IN), and apoptosis was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay with an *in situ* cell death kit (Roche). For cell proliferation experiment, primary osteoblasts were cultured in an eight-well slide chamber with α -MEM containing 10% FBS. Cells at 70% confluence were treated in various ways for 24 h and labeled with BrdU (0.5 mg/ml) for 2 h. To examine apoptosis, primary osteoblasts were plated in an eight-well slide chamber with α -MEM containing 10% FBS. Cells at 90% confluence were cultured with α -MEM containing 0.1% FBS for 24 h and then treated with vehicle or PTH for an additional 24 h. For *in vitro* determination of osteoblast differentiation, primary osteoblasts were cultured in differentiation medium containing 10% FBS, β -glycerophosphate (0.2 mM), and ascorbic acid (50 μ g/ml), and after confluence, cells were refed every 48 h with differentiation medium containing PTH (100 nM) (so-called continuous PTH treatment), or cells were treated with PTH in a 4 h/48 h schedule (so-called intermittent PTH treatment), whereby cells were treated with PTH for 4 h in differentiation medium and then the medium with peptide was aspirated and cells were rinsed with α -MEM twice before being refed with fresh differentiation

medium and incubated for another 44 h before the procedure was repeated. Osteoblastic differentiation was assessed by von Kossa stain, ALP activity, and calcium content measurements, as described previously (28).

cAMP and inositol triphosphate (IP3) measurements

Intracellular cAMP accumulation and IP3 stimulation in primary osteoblastic cells were measured as described previously (28).

In vitro intracellular calcium assay

We used a Fluo-4 direct calcium assay kit from Invitrogen (catalog item F10471) to measure the intracellular calcium response to PTH in primary osteoblastic cells. Primary osteoblastic cells isolated from 6-wk-old tibiae as described above were cultured in a 96-well plates with α -MEM containing 10% FBS. After confluence, primary cells were treated with human PTH (hPTH), ionomycin, phorbol 12-myristate 13-acetate (PMA), or forskolin and incubated with Fluo-4 direct calcium reagent loading solution for 40 min. After incubation, the fluorescence signal was immediately measured using a fluorescence microplate reader at excitation and emission of 509 and 516 nm, respectively.

PTH infusion

PTH was infused in mice at 4 wk of age. Human PTH(1-34) was reconstituted in a solution containing 150 mM NaCl, 1 mM HCl, and 2% heat-inactivated mouse serum and loaded into Alzet osmotic minipumps (model 1002; Durect Corp., Cupertino, CA). The pumps were equilibrated in 0.9% NaCl overnight at 37 C and then implanted into an interscapular sc pocket under Avertin anesthesia. Wt or DSEL mice were infused with vehicle (control) or PTH at a dose of 80 μ g/kg \cdot d for 14 d, with six mice in each group.

Statistics

All values in the paper are expressed as mean \pm SD or SEM. Comparisons between groups were made by unpaired two-tailed Student's *t* test for data from *in vitro* and serum biochemistry assays and by ANOVA (two-way ANOVA) with a *post hoc* Fisher's test for data from μ CT measurements and histomorphometry. *P* values smaller than 0.05 were considered statistically significant.

Results

PTHR signaling in DSEL osteoblastic cells

To verify that the signaling properties of the PTHR in osteoblastic cells in the DSEL mouse resembled those of this mutant receptor in other settings (1, 3), we measured cAMP accumulation as an indication of activation of Gs, and intracellular calcium and IP3 stimulation as indications of activation of Gq/11. As expected, PTH stimulated cAMP production in DSEL-derived primary osteoblastic cells with a dose dependence similar to that seen in Wt primary osteoblastic cells, whereas PTH induced a cal-

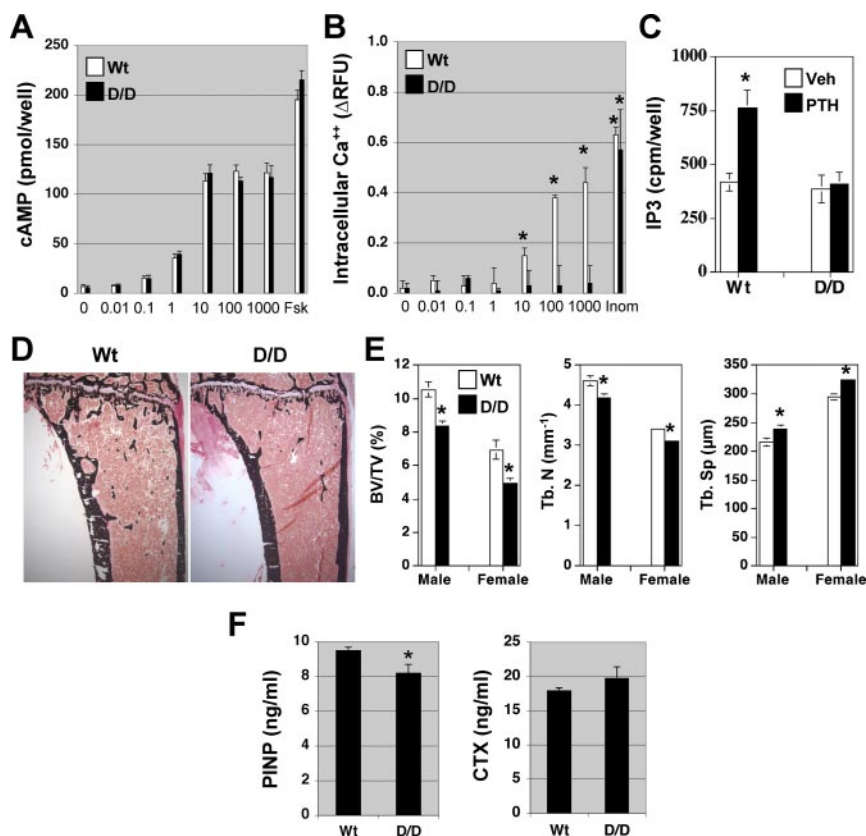


FIG. 1. PTHR signaling and bone mass in DSEL mice. **A**, Dose-dependent stimulation of cAMP by PTH in DSEL-derived primary osteoblastic cells. Primary osteoblastic cells isolated from Wt (*white bars*) and D/D (*black bars*) mice were cultured in 24-well plates, and after confluence, cells were incubated with cAMP assay buffer containing hPTH(1-34) (~0–1000 nM) or forskolin (10 μ M) for 20 min before cAMP measurement. **B**, PTH fails to induce intracellular calcium response in DSEL-derived primary osteoblastic cells. Primary osteoblastic cells (*white bars* for Wt cells and *black bars* for D/D cells) were cultured in 96-well plates, and after confluence, cells were incubated with Fluo-4 direct calcium buffer containing hPTH (~0–1000 nM), ionomycin (Ionom) (10 μ M), PMA (10 nM), or forskolin (Fsk) (10 μ M) for 40 min before fluorescence signal measurement. Data are expressed as relative fluorescence units (RFU). **C**, No PLC activation by PTH in DSEL-derived osteoblastic cells. Primary osteoblastic cells from Wt and D/D mice were cultured in six-well plates. At 80% confluence, cells were labeled with [3 H]myoinositol for 48 h and then stimulated with hPTH (1000 nM) (*black bars*) or vehicle (*white bars*) for 40 min in the presence of 20 mM LiCl. The cellular content of radioactive IP3 was determined. Values are mean \pm SD of triplicate wells. *, $P < 0.05$ vs. vehicle. Each experiment was repeated twice with similar results. **D**, Representative micrographs of plastic sections of tibiae from sex-matched Wt and DSEL homozygous (D/D) male mice at the age of 10 wk, stained with von Kossa. **E**, μ CT analysis of 10-wk-old distal femur from both male and female Wt (*white bars*) and D/D (*black bars*) mice. **F**, Serum P1NP and CTX were measured in both Wt and D/D mice at the age of 10 wk. Error bars in **E** and **F** represent SE ($n = 8$). *, $P < 0.05$ vs. Wt mice. BV/TV, Bone volume/total volume; Tb. N, trabecular number; Tb. Sp, trabecular spacing.

cium response and IP3 stimulation only in Wt-derived osteoblasts but not in DSEL-derived osteoblasts (Fig. 1, A–C). These results indicate that the DSEL mutation of the PTHR blocks PTH signaling through receptor-Gq/11 coupling but not through receptor-Gs coupling in osteoblastic cells.

Bone phenotype in postnatal DSEL mice

To determine the role of the PLC signaling pathway via the PTHR in postnatal bone development, tibiae at age 10 wk

from both Wt and DSEL (D/D) mice were examined. DSEL mice displayed a significant decrease in the amount of trabecular bone with little alteration in the cortical bone (Fig. 1D). Histomorphometry of proximal tibial trabecular bone at 10 wk of age demonstrated a significant decrease (by 25%) in trabecular volume in the secondary spongiosa that was associated with reduced trabecular number and thickness and increased trabecular spacing (Table 1). The decreased trabecular bone volume associated with decreased trabecular number and increased trabecular spacing in the mutant mice was confirmed by μ CT analysis of 10-wk-old distal femurs (Fig. 1E). To determine whether the decreased trabecular bone in the mutant mice was partly caused by reduced osteoblast activity, osteoblast number and dynamic parameters of bone formation were examined. The histomorphometric analysis showed that the number of trabecular osteoblasts, mineral apposition rate, and bone formation rate in the mutant mice were not significantly different from those observed in the Wt control mice (Table 1). No significant difference in osteoclast number was observed by histomorphometric analysis of 10-wk-old proximal tibial trabecular bone in the secondary spongiosa (Table 1). Serum P1NP was modestly decreased in DSEL mice, whereas levels of serum CTX were not significantly different between the two groups (Fig. 1F).

To examine whether PLC signaling via the PTHR is more important for PTH-regulated bone turnover during rapid growth, histomorphometric analysis was also performed in 6-wk-old tibiae (Table 1). Decreased trabecular bone associated with lowered trabecular number and increased trabecular spacing was consistent with that seen in the bones from 10-wk-old mice. Interestingly, unlike the nonsignificant change in trabecular bone cellularity observed in 10-wk-old tibiae, the mutant trabecular bone at age of 6 wk exhibited a significant decrease in osteoblast number per total area and a significant increase in osteoclast number per millimeter of bone surface, suggesting that the PLC signaling pathway via the PTHR may be required for generation of

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TABLE 1. Histomorphometry of proximal tibial metaphyseal trabecular bone

Measurements	10 wk old		6 wk old	
	Wt	D/D	Wt	D/D
Structural parameters				
BV/TV (%)	11.66 ± 1.0	8.82 ± 0.66 ^a	11.34 ± 0.95	8.08 ± 0.80 ^a
Tb.Th (μm)	33.05 ± 1.37	28.91 ± 0.92 ^a	22.92 ± 1.55	21.07 ± 1.19
Tb.Sp (μm)	256.7 ± 19.8	313.9 ± 18.6 ^a	186.8 ± 18.7	238.4 ± 30.5 ^a
Tb.N (mm ⁻¹)	3.65 ± 0.23	3.02 ± 0.17 ^a	5.11 ± 0.61	3.75 ± 0.32 ^a
Static parameters				
Ob/TA (no./mm ²)	191.4 ± 19.5	170.2 ± 8.1	254.1 ± 24.1	204.6 ± 18.5 ^a
N.Ob/BS (no./μm)	27.4 ± 1.3	26.1 ± 1.1	25.33 ± 1.12	25.34 ± 1.14
Oc/TA (no./mm ²)	84.1 ± 10.2	72.5 ± 6.1	27.07 ± 3.48	31.52 ± 5.8
N.Oc/BS (no./mm)	10.6 ± 1.1	11.6 ± 0.41	2.61 ± 0.34	3.71 ± 0.36 ^a
Dynamic parameters				
MAR (μm/yr)	564.8 ± 62.4	526.5 ± 34.6		
BFR/BS (μm ³ /μm ² · yr)	183.2 ± 19.2	193.0 ± 16.5		

Data are presented as group mean ± SEM. BFR, Bone formation rate; BS, bone surface; BV, bone volume; MAR, mineral apposition rate; MS, mineralizing surface; N, number; N.Ob/BS, osteoblast number per bone surface; N.Oc/BS, osteoclast number per bone surface; Ob, osteoblast; Ob.S/BS, osteoblast surface per bone surface; Oc, osteoclast; Oc.S/BS, osteoclast surface per bone surface; Sp, spacing; TA, total area; Tb, trabecular; Th, thickness; TV, total volume.

^a $P < 0.05$ vs. Wt (n = 8 per group).

normal numbers of osteoblasts and osteoclasts in young growing bone.

Effect of low-calcium diet on bone remodeling

We have previously shown that activation of PLC by the PTHR requires much higher concentrations of PTH than activation of AC requires (29). We hypothesized that physiological stresses that lead to high PTH levels might bring out the actions of PTHR-stimulated PLC in bone. Serum PTH can be increased by either continuous infusion of PTH to mimic primary hyperparathyroidism or by feeding mice a low-calcium diet that causes secondary hyperparathyroidism (12, 30). To evaluate the role of PTH-induced PLC signaling in bone turnover, bone responses were examined in a model of secondary hyperparathyroidism caused by a low-calcium diet. Twenty-day-old mice were placed on either a control diet or a low-calcium diet for 3 wk. As expected, blood calcium levels fell and PTH levels rose similarly in both genotypes of mice (Table 2). Interestingly, the blood phos-

phate fell in the Wt mice but increased in the DSEL mice (Table 2). These differing phosphate responses may primarily reflect differences in the renal response to PTH in these mice. The low-calcium diet caused a similar loss of body weight (decreased by approximately 20% compared with control diet) in both Wt and D/D mice. When stained for phosphate through von Kossa staining, both the Wt and DSEL mice exhibited dramatically lowered levels of bone mineral on the low-calcium diet (Fig. 2A). The low-calcium diet caused extensive lowering of cortical bone mass in both Wt and DSEL mice (Fig. 2, B and C), whereas increased trabecular bone volume observed in Wt mice fed a low-calcium diet was attenuated in D/D mice (Fig. 2C). Interestingly, the peritrabecular space, normally occupied by hematopoietic cells, in the Wt mice on the low-calcium diet was filled with the fibroblast-like stromal cells, most striking in the metaphyseal region, whereas such a peritrabecular stromal cell response to the low-calcium diet was not present in the

TABLE 2. Effect of low-calcium diet on serum parameters

Measurements	Male mice		Female mice	
	Wt	D/D	Wt	D/D
Serum intact PTH (pg/ml)				
Control diet	59 ± 12.4	106.2 ± 22.3	83.1 ± 22.1	145.5 ± 29.1
Low-calcium diet	591.5 ± 65.2 ^a	504.7 ± 55.5 ^a	607.1 ± 59.4 ^a	607.6 ± 90.3 ^a
Total serum calcium (mg/dl)				
Control diet	8.39 ± 0.36	8.95 ± 0.41	8.04 ± 0.19	7.3 ± 0.5
Low-calcium diet	7.54 ± 0.2 ^a	6.65 ± 0.48 ^a	7.01 ± 0.33 ^a	5.95 ± 0.43
Serum inorganic phosphorus (mg/dl)				
Control diet	8.13 ± 0.37	9.54 ± 0.62	9.03 ± 0.54	9.44 ± 0.26
Low-calcium diet	6.22 ± 0.24 ^a	13.36 ± 1.3 ^a	7.84 ± 0.51 ^a	12.08 ± 1.01 ^a

Data are presented as group mean ± SEM.

^a $P < 0.01$ vs. control diet (n = 8 per group).

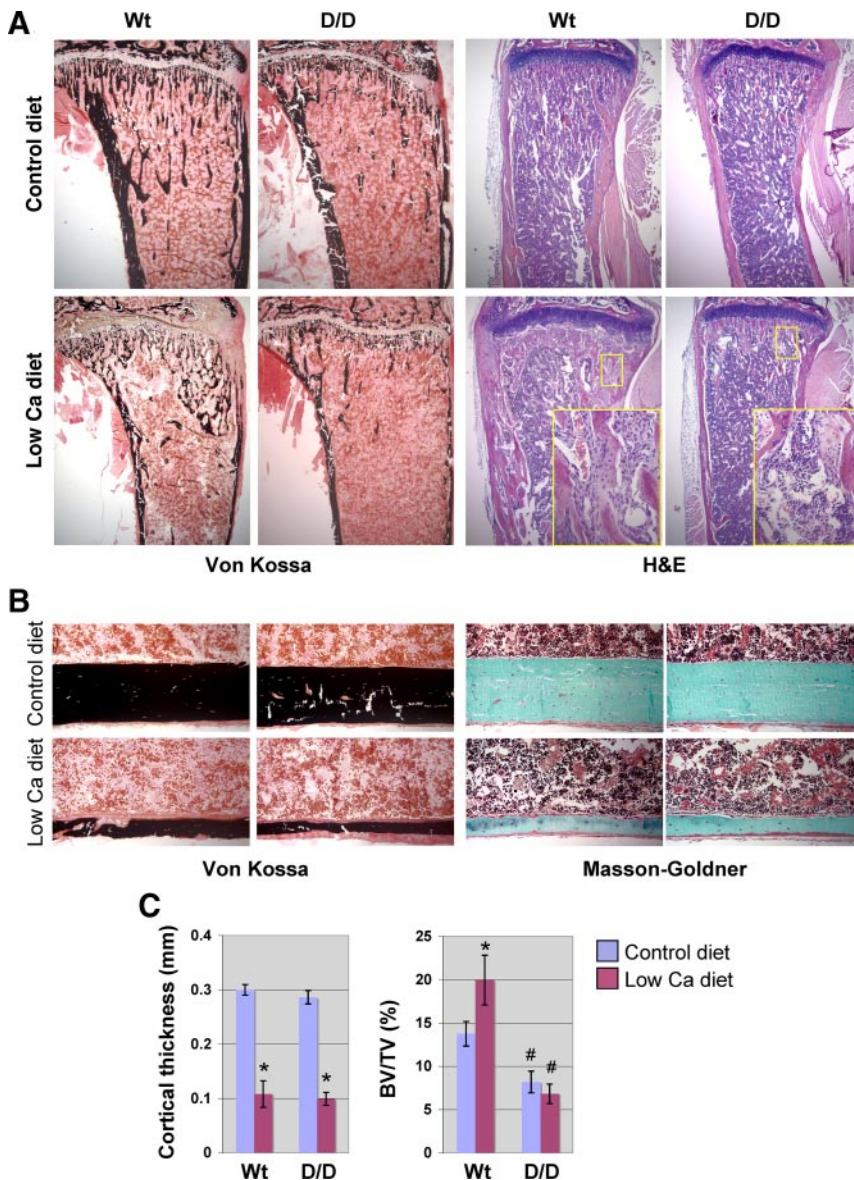


FIG. 2. Effect of calcium-deficient diet on bone turnover. A, Representative micrographs of proximal tibiae; B, diaphyseal cortical bone from both Wt and D/D mice fed a low-calcium diet or a corresponding control diet for 3 wk after weaning, stained with von Kossa for mineral, hematoxylin and eosin (H&E) for histology, and Masson-Goldner trichrome for bone matrix, as indicated. Both groups of mice on the low-calcium diet showed a similar dramatic loss of bone mineral and cortical bone mass, but H&E stain demonstrated a striking difference (indicated by insets) in metaphyseal trabecular bone turnover between Wt and D/D mice on the low-calcium diet. C, Histomorphometric measurements of diaphyseal cortical bone thickness and metaphyseal trabecular bone volume in 6-wk-old Wt and D/D mice fed low-calcium and control diet, as indicated. Error bars represent SE (n = 6); *, P < 0.05 vs. control diet; #, P < 0.05 vs. Wt.

DSEL mice (Figs. 2A and 3A). Substantial amounts of disorganized bone containing a high density of osteocytes and unmineralized matrix, as shown in red by Masson-Goldner trichrome staining (Fig. 3A), were observed only in the Wt but not in the DSEL mice fed the low-calcium diet. Such irregular bones are very similar to woven bone and probably represent newly formed bone; this finding suggests more rapid bone formation in the Wt mice.

Peritrabecular fibroblast-like stromal cells are diminished in DSEL mice

Continuous PTH treatment in rats causes the accumulation of peritrabecular stromal cells, increased bone resorption, and accumulation of poorly mineralized extracellular matrix on bone surfaces (10, 30). Primary hyperparathyroidism is associated with continuously increased PTH levels, and this disease is characterized by accumulation of osteoid, focal bone resorption, increased bone formation, and peritrabecular marrow fibrosis (peritrabecular stromal cell response). To demonstrate the extent of the peritrabecular stromal cell response to the low-calcium diet, we measured the fibrosis volume in the tibial metaphyseal region. No peritrabecular fibrosis is present in the normal bone. Surprisingly, little peritrabecular fibrosis was observed in the mutant mice on the low-calcium diet, whereas the Wt mice on the low-calcium diet displayed extensive peritrabecular fibrosis, most strikingly in the metaphyseal region (Fig. 3B). These peritrabecular stromal cells strongly express osteopontin and collagen a1(I) mRNAs (Fig. 3C), suggesting that they may be preosteoblasts (30).

Diminished stromal cell response and bone formation in PTH-infused DSEL mice

Similar phenomena were also observed in mice with continuous PTH infusion. In Wt mice, continuous PTH infusion caused both peritrabecular stromal cell responses and disorganized bone formation similar to those observed in Wt mice fed a low-calcium diet, whereas such stromal cell responses and disorganized bone formation were dramatically

attenuated in DSEL mice with continuous PTH infusion (Fig. 4A). Consistent with the observed alterations in metaphyseal trabecular bone after PTH infusion, levels of serum P1NP, a marker of bone formation, were significantly increased in Wt but not in DSEL mice receiving continuous PTH administration, whereas levels of serum CTX, a bone resorption marker, were elevated in both groups of mice (Fig. 4B). Continuous PTH infusion caused similarly undetectable serum

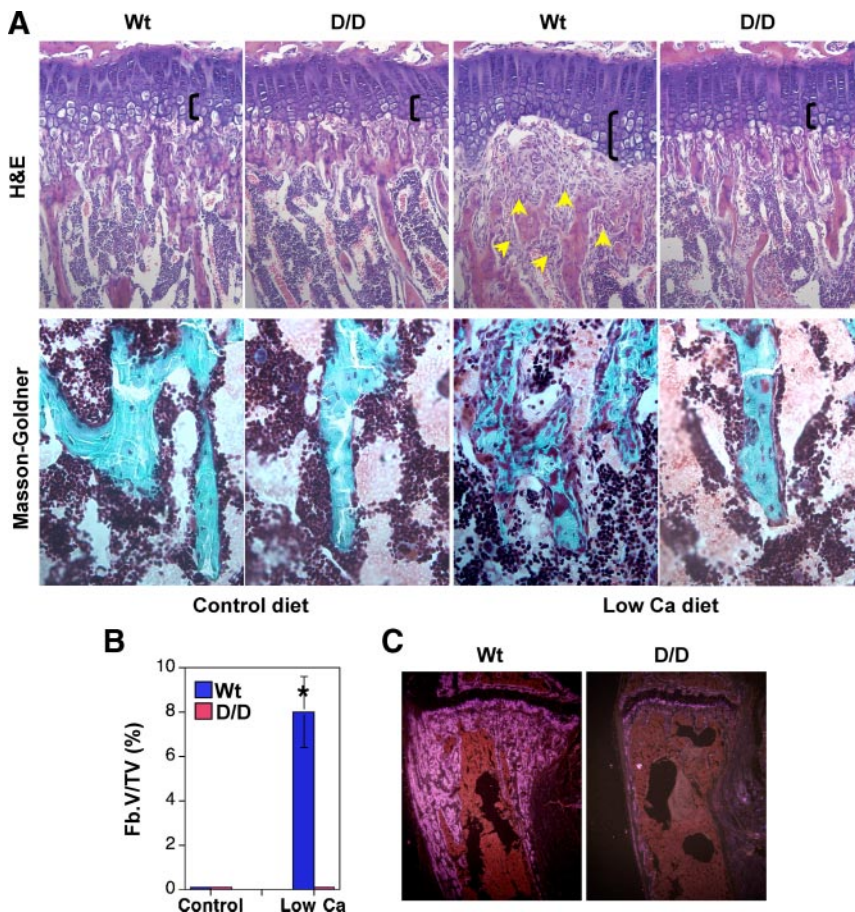


FIG. 3. DSEL mice on the low-calcium diet exhibited strikingly curtailed peritrabecular stromal cell accumulation, new bone formation, and hypertrophic expansion. **A**, Representative micrographs of proximal tibial metaphyseal trabecular bone from mice fed a low-calcium diet or a corresponding control diet for 3 wk after weaning, stained with hematoxylin and eosin (H&E) for histology ($\times 20$ magnification), showing expanded hypertrophic chondrocytes (as indicated by the *black bracket*) and accumulation of peritrabecular fibroblast-like stromal cells (indicated by *arrows*) in Wt but not in D/D mice fed the low-calcium diet, and stained with Masson-Goldner trichrome ($\times 40$ magnification), demonstrating massive disorganized newly formed undermineralized bone, shown in *red*, in Wt but not in D/D mice fed the low-calcium diet. **B**, Measurements of peritrabecular fibrosis. Fb.V/TV, Fibrosis volume/total volume. *, $P < 0.05$ vs. control diet; $n = 6$. **C**, Osteopontin *in situ* in proximal tibiae on the low-calcium diet for 3 wk.

intact PTH and elevated serum calcium in both Wt and D/D mice, whereas serum phosphate was significantly decreased in Wt but not in D/D mice after PTH infusion (Fig. 4B). At the end of PTH infusion, both Wt and D/D mice showed a similar decrease in body weight (by approximately 15% compared with vehicle-infused control mice) and appeared to be sick, but the level of serum creatinine in both Wt and D/D mice with PTH infusion was not significantly increased compared with vehicle-infused control mice (data not shown), suggesting that both mice after PTH infusion were not in severe renal failure.

Attenuated colony formation and proliferation in DSEL-derived bone marrow cells and primary osteoblasts

To examine the effect of the DSEL mutation on osteoblast progenitors, bone marrow cells from mice on the

normal or low-calcium diets for 1 wk were plated in culture for 2 wk. Interestingly, on the low-calcium diet, Wt mice increased the numbers of CFU-F and CFU-ALP, whereas the DSEL mice failed to increase the numbers of both kinds of colonies (Fig. 5A), presumably reflecting a failure of these mice to generate the colony-forming cells *in vivo*. Activation of PKC by PTH signaling has been implicated in the mitogenic action of PTH on osteoblastic cells (31, 32). To determine whether osteoblastic cells from the DSEL mouse exhibited abnormalities in cellular proliferation in response to PTH, we stimulated primary osteoblastic cells derived from both Wt and DSEL mice with PTH. PTH treatment significantly increased the number of BrdU-positive cells in osteoblastic cells derived from Wt but not from DSEL mice. Furthermore, the effect of PTH to increase cell proliferation in the Wt primary osteoblastic cells was not mimicked by treatment with $[G^1, R^{19}]hPTH(1-28)$, a signal-selective peptide with defective activation of PLC/PKC signaling by the PTHR (28, 33). In contrast, basic fibroblast growth factor, a potent mitogenic stimulator in osteoblastic cells (34), had a similar stimulatory effect on cell proliferation in primary osteoblastic cells from both Wt and DSEL mice (Fig. 5B).

PTH fails to induce cyclin D1 mRNA expression in the primary osteoblastic cells derived from DSEL mice

Increased expression of cyclin D1 leads to increased cell proliferation in many tissues, and recently, cyclin D1 has been implicated as a primary target of mitogenic signals in osteoblastic cells (35–39). To determine whether failure of PTH to stimulate proliferation in DSEL-derived primary osteoblasts is in part due to curtailed induction of cyclin D1 by PTH, we next examined cyclin D1 mRNA expression in the primary cultures of osteoblastic cells after PTH treatment. In the primary osteoblasts derived from Wt mice, PTH treatment significantly increased cyclin D1 mRNA levels in a time-dependent manner, whereas such induction of cyclin D1 by PTH was not seen in the DSEL-derived primary osteoblasts (Fig. 5C). Furthermore, PTH-mediated induction of cyclin D1 mRNA was mimicked by treatment with PMA

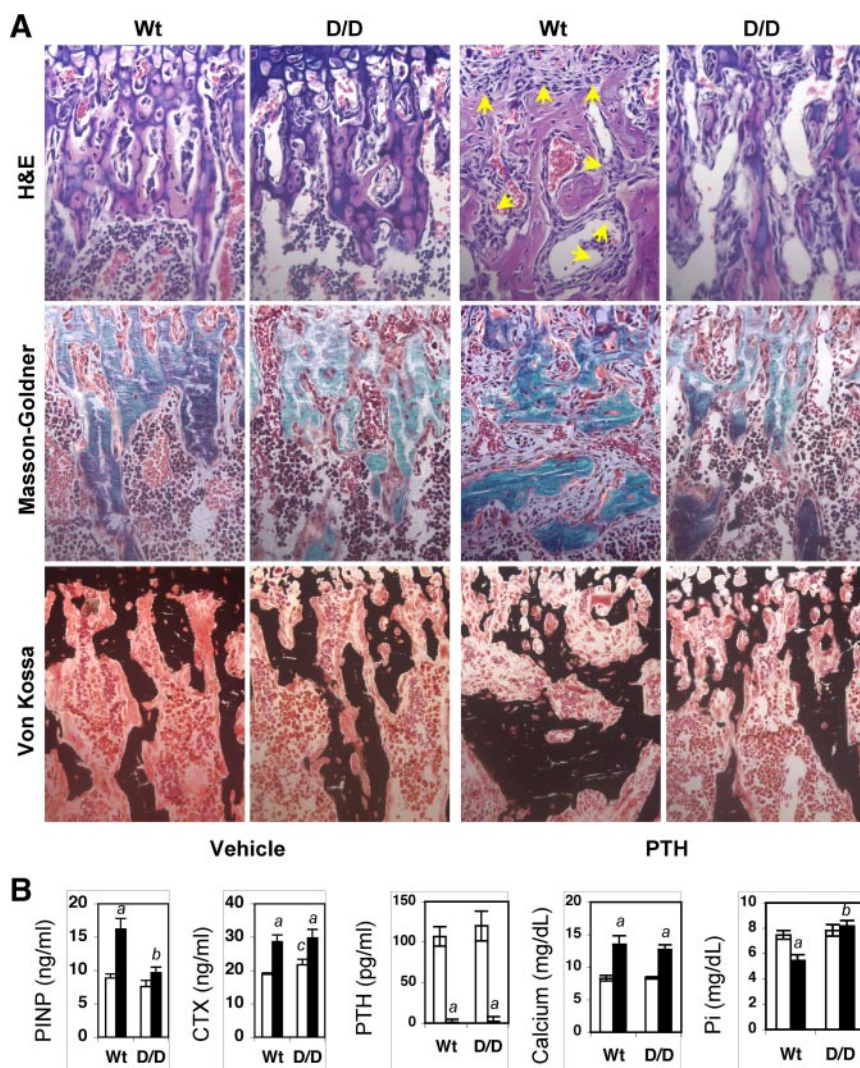


FIG. 4. DSEL mice with PTH infusion exhibited curtailed peritrabecular stromal cell responses and bone formation. **A**, Representative micrographs of proximal tibia from 4-wk-old mice receiving continuous infusion of hPTH (80 $\mu\text{g}/\text{kg} \cdot \text{d}$) or vehicle through sc implantation of Alzet mini-osmotic pumps for 2 wk, stained with hematoxylin and eosin (H&E) for histology ($\times 40$ magnification), showing dramatic accumulation of peritrabecular fibroblast-like stromal cells (indicated by arrows) in Wt but not in D/D mice, and stained with Masson-Goldner trichrome and von Kossa ($\times 40$ magnification) demonstrating massive disorganized bone formation in Wt but not in D/D mice. **B**, Serum P1NP, CTX, intact PTH(1-84) (PTH), total calcium, and phosphorus (Pi) were measured in Wt and D/D mice after infusion with vehicle (white bars) and PTH (black bars). Error bars represent SE ($n = 6$). a, $P < 0.05$ vs. vehicle; b, $P < 0.05$ vs. Wt PTH; c, $P < 0.05$ vs. Wt vehicle.

but not by treatment with either $[G^1, R^{19}]hPTH(1-28)$ or 8-bromo-cAMP (Fig. 5C), indicating that activation of the PLC/PKC pathway by the PTHR is essential for PTH to induce cyclin D1 expression and to stimulate proliferation of osteoblastic cells.

PTH efficiently prevents apoptosis and regulates osteoblast differentiation in DSEL-derived primary osteoblastic cells

An antiapoptotic action of PTH has been proposed to be a mechanism underlying the increased osteoblast number associated with the anabolic response of bone to intermittent

PTH administration (16, 18). To determine whether the antiapoptotic action of PTH might be dampened in bones of DSEL mice, we next examined the effect of PTH on apoptosis of primary osteoblasts derived from both Wt and DSEL tibiae. The proportion of osteoblastic cells undergoing apoptosis, as determined by TUNEL labeling, was significantly decreased by PTH in primary osteoblastic cells from both Wt and DSEL mice (Fig. 6A). This normal antiapoptotic response in DSEL-derived primary osteoblasts suggests that PLC/PKC signaling through the PTHR is not critical for osteoblast survival and that the low bone mass in the DSEL mice probably does not result from altered antiapoptotic actions of PTH in osteoblasts in DSEL mice.

To assess whether PLC signaling through the PTHR is important for PTH-mediated differentiation of osteoblastic cells, primary osteoblasts were isolated from 6-wk-old tibiae of Wt and DSEL mice and treated with PTH continuously or intermittently to determine the effect of PTH on osteoblast differentiation. In cells from both Wt and DSEL mice, continuous treatment with PTH similarly suppressed ALP activity, as determined by staining cells for enzyme activity or by measuring enzymatic activity in cell extracts. PTH also similarly inhibited mineralization, shown by von Kossa stain of cells for phosphate and by measuring calcium content in the cell layer, in both types of primary osteoblastic cells. Furthermore, intermittent PTH administration dramatically increased ALP activity and mineralization in primary osteoblastic cells from both Wt and DSEL mice (Fig. 6B). The similar differentiation responses to PTH in cells from the Wt and DSEL mice suggests that PLC signaling through the PTHR is not required for PTH-mediated osteoblast differentiation at least *in vitro*.

Discussion

Although the PTHR signaling pathways have been extensively studied for decades (40–44), the role of PLC signaling via the PTHR *in vivo* remains incompletely understood, es-

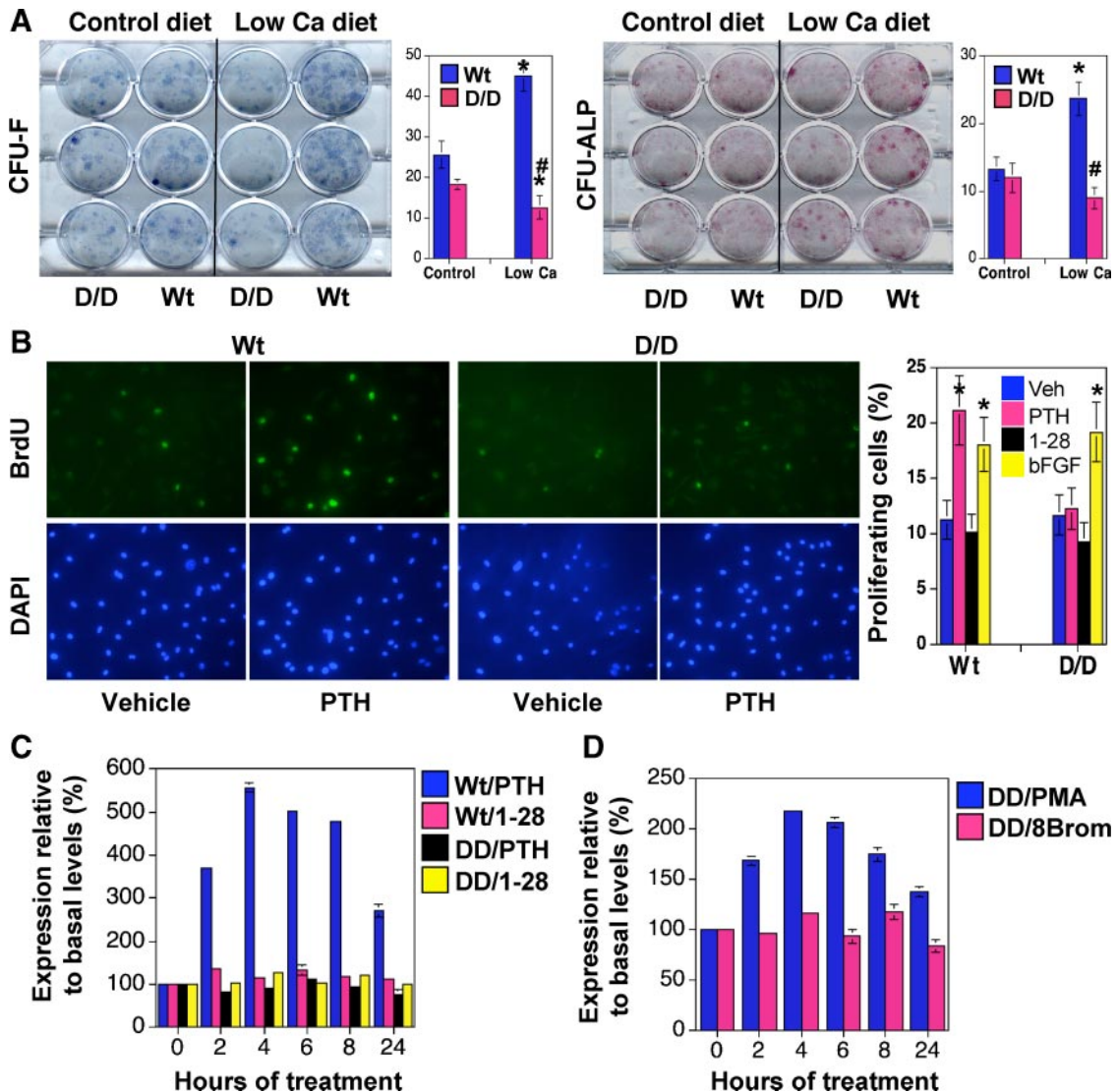


FIG. 5. Decreased colony formation and proliferation in DSEL-derived primary bone and bone marrow cells. **A**, Decreased colony formation in DSEL-derived bone marrow cells. Bone marrow cells from 4-wk-old tibiae on either a low-calcium diet (Ca diet) or a control diet for 1 wk were cultured in 12-well plates for 14 d. The cultured plates were stained with methylene blue for total number of colonies (CFU-F) or with ALP staining solution to count positive colonies for ALP (CFU-ALP). Counts of colonies were performed blind on coded plates. *, $P < 0.05$ vs. Wt control diet; #, $P < 0.05$ vs. Wt low-calcium diet ($n = 4$). **B**, *In vitro* osteoblast proliferation. Primary osteoblasts were treated with vehicle (Veh), 100 nM hPTH(1-34) (PTH), 100 nM $[G^1, R^{19}]hPTH(1-28)$ (1-28), or 10 ng/ml basic fibroblast growth factor (bFGF) for 24 h and labeled with BrdU for 2 h. *In situ* anti-BrdU fluorescein (green) and 4',6-diamidino-2-phenylindole (DAPI) (blue) stain were performed and BrdU-positive cells were counted, as shown in bar graph (two wells per treatment in three independent experiments). *, $P < 0.05$ vs. vehicle. **C** and **D**, Real-time PCR detection of cyclin D1 mRNA expression in primary osteoblastic cells isolated from Wt and D/D mice (C) or from D/D mice (D). Primary osteoblasts were treated with 100 nM hPTH(1-34) (PTH) or $[G^1, R^{19}]hPTH(1-28)$ (1-28) (C) or with 10 nM PMA and 100 μ M 8-bromo-cAMP (8Brom) (D) for 0–24 h, as indicated. Transcript expression of the cultured cells was determined by quantitative RT-PCR with primers specific for cyclin D1. Data are expressed as percent basal levels, and similar results were obtained in three independent experiments.

pecially regarding the regulation of bone modeling and remodeling. To examine the roles of distinct signaling pathways *in vivo*, we have generated a knock-in mutant mouse that expresses the PTHR with the DSEL mutation instead of the Wt receptor (3). The DSEL receptor stimulates AC normally but fails to activate PLC (1, 2). The DSEL mice exhibit a mild delay in hypertrophic differentiation of chondrocytes during embryonic development, indicating that PLC signaling through the PTHR is important for embryonic chondrocyte differentiation (3). In the present study, we demonstrate

that in the basal state, the DSEL mice at 10 wk of age displayed reduced trabecular bone mass in both tibiae and femurs. However, the mechanism underlying the low bone mass in the mutant mice in the basal state is difficult to analyze, because histomorphometric parameters of trabecular bone cellularity and bone formation rate were not significantly altered in the secondary spongiosa of tibiae from 10-wk-old DSEL mice. The decreased bone mass may reflect abnormal modeling early in life. The mutant mice exhibit a modest decrease in osteoblast number and increase in

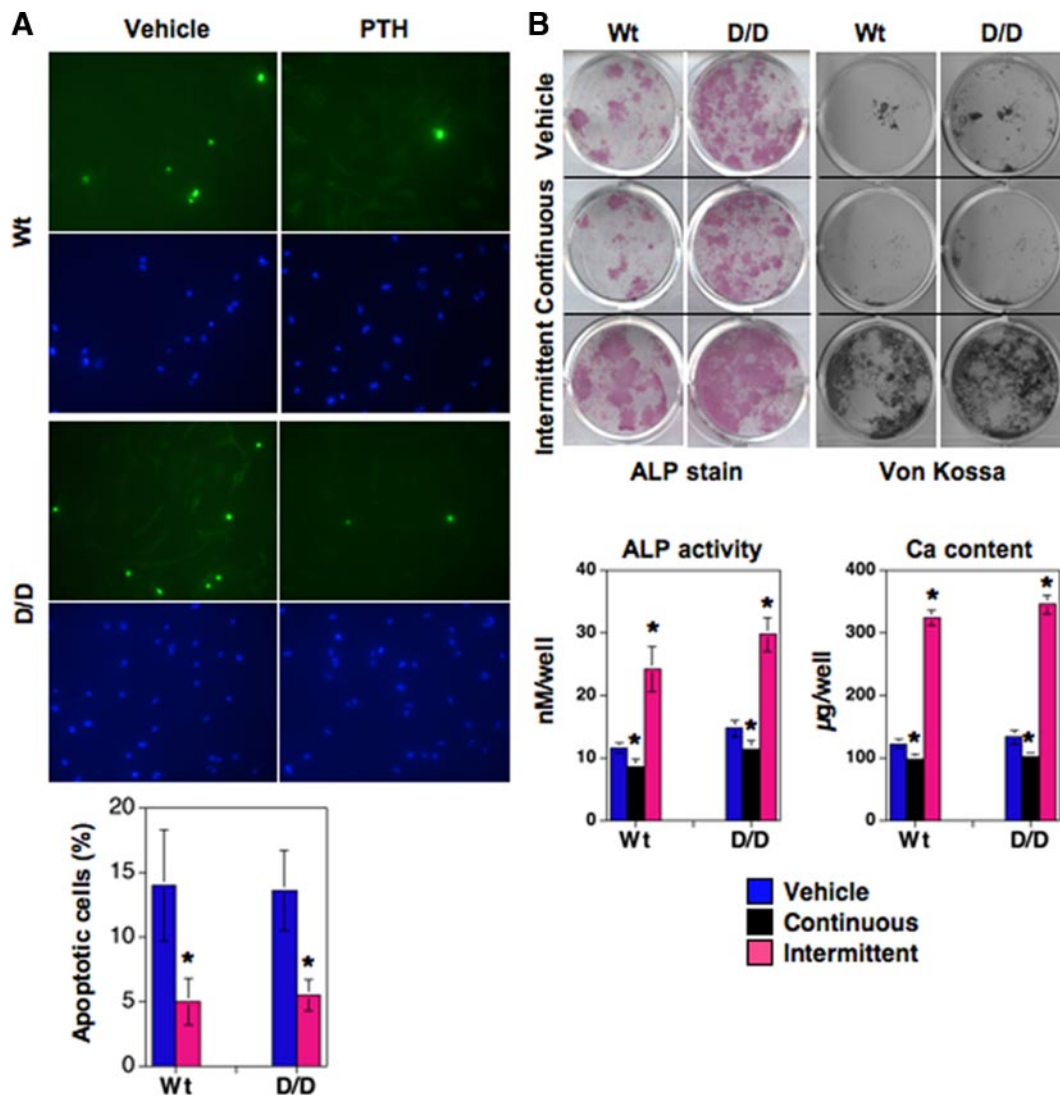


FIG. 6. Effect of PTH on apoptosis and differentiation in primary osteoblastic cells. *A*, *In vitro* osteoblast apoptosis was quantified by TUNEL assay. Primary osteoblasts cultured in an eight-well slide chamber, were treated with vehicle or hPTH(1-34) (100 nM) for 24 h, and then *in situ* fluorescein (TUNEL, green) and 4',6-diamidino-2-phenylindole (DAPI) (blue) stain were performed. TUNEL-positive cells were counted as apoptotic cells (percent, as shown in bar graph). Data are represented as mean \pm SE (two wells per slide in three independent experiments). *, $P < 0.05$ vs. vehicle (*t* test). *B*, Effect of PTH on osteoblast differentiation *in vitro*. Primary osteoblastic cells isolated from 6-wk-old tibiae were cultured in six-well or 12-well plates with α -MEM containing 10% FBS, and after confluence, cells were cultured with differentiation medium and treated continuously (freshly adding PTH every 48 h) or intermittently (adding PTH for 4 h in every 48 h) with 100 nM hPTH(1-34) for 4–6 wk. Mineralization was assessed by von Kossa stain and calcium (Ca) content (bar graph) was measured in acid extracts of the cultures (bar graph). ALP activity was determined by stain and also measured enzymatically (bar graph) (28).

osteoclast surface density at 6 wk of age. Those changes during growth, combined with possible indirect consequences of the growth plate abnormality, might explain the decreased bone mass at 10 wk of age. Alternatively, the modest decrease in serum P1NP, a marker of bone formation, at 10 wk in the DSEL mice may reflect a decrease in bone formation at that age missed in the histomorphometric analysis of the tibial trabecular secondary spongiosa.

DSEL mice at age of 6 wk, when fed a low-calcium diet, developed secondary hyperparathyroidism with elevated levels of serum PTH and substantial cortical bone loss but failed to develop peritrabecular stromal cell and woven bone responses, whereas Wt mice exhibited disorganized bone

and extensive peritrabecular fibroblast-like stromal cell accumulation in response to the low-calcium diet. These stromal cells strongly express mRNAs encoding $\alpha 1(I)$ collagen and osteopontin, markers of the osteoblast lineage, suggesting that they are preosteoblasts, and this observation is very similar to that seen in rat continuously infused with PTH (30). Lotinun *et al.* (30) pulse labeled these stromal cells with thymidine and showed that, after stopping the PTH infusion, these cells disappeared, and radioactive thymidine appeared rapidly in mature osteoblasts. These data suggest that at least some of the stromal cells are osteoblast precursors. The data here suggest that the accumulation of these stromal cells in response to high levels of PTH requires PLC signaling

through the PTHR. Such accumulation could be explained by a variety of mechanisms. Here we show that the Wt mouse responds to continuously elevated levels of PTH by increasing the number of cells capable of forming colonies (CFU-F and CFU-ALP) *in vitro* and that the DSEL mouse fails to demonstrate this response. This finding suggests that one locus of action of the PTHR that requires PLC activation involves early cells in the mesenchymal lineage, directly or indirectly. In UMR106 and primary osteoblastic cells, PTH stimulates proliferation, and such a stimulatory effect was shown to be dependent on activation of PKC (31, 45, 46). Interestingly, PTH failed to increase proliferation in the DSEL-derived primary osteoblastic cells, whereas PTH treatment significantly stimulated proliferation in the Wt-derived primary osteoblastic cells, and such a stimulatory effect of PTH is not mimicked by a PLC/PKC-defective peptide [G¹,R¹⁹]hPTH(1-28). Furthermore, PTH induced expression of cyclin D1 mRNA, an important part of the mitogenic response in the Wt primary osteoblastic cells but failed to increase cyclin D1 mRNA in cells from the DSEL mice. Furthermore, treatment with a PKC activator stimulates cyclin D1 mRNA in cells from DSEL mice. Our data indicate that PLC signaling by the PTHR is important for the stimulatory effect of PTH on cyclin D1 expression and for proliferation in osteoblastic cells. Interestingly, PTH treatment *in vitro* has a similar effect on apoptosis and on differentiation in primary cultured bone cells derived from both Wt and DSEL tibiae. Taken together, these findings suggest that the stromal cell accumulation in response to high levels of PTH in Wt mice results from a proliferative response to PTH that requires activation of PLC.

In summary, our findings *in vivo* indicate that the PLC signaling pathway via the PTHR is essential for normal bone modeling and remodeling and for the stromal cell response to elevations of PTH levels. This *in vivo* evidence for roles of PLC signaling on bone in response to PTH broadens the possible strategies for mimicking or enhancing the effects of PTH in diseases such as osteoporosis.

Acknowledgments

We thank Xiang Zheng for valuable help with the fluorescence microplate reader.

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This work was supported by National Institutes of Health Grant DK11794 (to H.M.K.).

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Disclosure Summary: None of the authors have conflicts of interest in this report.

References

- Iida-Klein A, Guo J, Takemura M, Drake MT, Potts Jr JT, Abou-Samra A, Bringham FR, Segre GV 1997 Mutations in the second cytoplasmic loop of the rat parathyroid hormone (PTH)/PTH-related protein receptor result in selective loss of PTH-stimulated phospholipase C activity. *J Biol Chem* 272:6882–6889
- Guo J, Liu BY, Bringham FR 1997 Mechanisms of homologous and heterologous desensitization of PTH/PTHrP receptor signaling in LLC-PK1 cells. *Am J Physiol* 273:E383–E393
- Guo J, Chung UI, Kondo H, Bringham FR, Kronenberg HM 2002 The PTH/PTHrP receptor can delay chondrocyte hypertrophy *in vivo* without activating phospholipase C. *Dev Cell* 3:183–194
- Lanske B, Karaplis AC, Lee K, Luz A, Vortkamp A, Pirro A, Karperien M, Defize LH, Ho C, Mulligan RC, Abou-Samra AB, Jüppner H, Segre GV, Kronenberg HM 1996 PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science* 273:663–666
- Lee K, Lanske B, Karaplis AC, Deeds JD, Kohno H, Nissenson RA, Kronenberg HM, Segre GV 1996 Parathyroid hormone-related peptide delays terminal differentiation of chondrocytes during endochondral bone development. *Endocrinology* 137:5109–5118
- Miao D, He B, Jiang Y, Kobayashi T, Sorocanu MA, Zhao J, Su H, Tong X, Amizuka N, Gupta A, Genant HK, Kronenberg HM, Goltzman D, Karaplis AC 2005 Osteoblast-derived PTHrP is a potent endogenous bone anabolic agent that modifies the therapeutic efficacy of administered PTH 1-34. *J Clin Invest* 115:2402–2411
- Kobayashi T, Chung UI, Schipani E, Starbuck M, Karsenty G, Katagiri T, Goad DL, Lanske B, Kronenberg HM 2002 PTHrP and Indian hedgehog control differentiation of growth plate chondrocytes at multiple steps. *Development* 129:2977–2986
- Neer RM, Arnaud CD, Zanchetta JR, Prince R, Gaich GA, Reginster JY, Hodsman AB, Eriksen EF, Ish-Shalom S, Genant HK, Wang O, Mitlak BH 2001 Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis. *N Engl J Med* 344:1434–1441
- Dempster DW, Cosman F, Kurland ES, Zhou H, Nieves J, Woelfert L, Shane E, Plavetia K, Müller R, Bilezikian J, Lindsay R 2001 Effects of daily treatment with parathyroid hormone on bone microarchitecture and turnover in patients with osteoporosis: a paired biopsy study. *J Bone Miner Res* 16:1846–1853
- Zhou H, Shen V, Dempster DW, Lindsay R 2001 Continuous parathyroid hormone and estrogen administration increases vertebral cancellous bone volume and cortical width in the estrogen-deficient rat. *J Bone Miner Res* 16:1300–1307
- Yang D, Singh R, Divieti P, Guo J, Bouxsein ML, Bringham FR 2007 Contributions of parathyroid hormone (PTH)/PTH-related peptide receptor signaling pathways to the anabolic effect of PTH on bone. *Bone* 40:1453–1461
- Dobnig H, Turner RT 1997 The effects of programmed administration of human parathyroid hormone fragment (1-34) on bone histomorphometry and serum chemistry in rats. *Endocrinology* 138:4607–4612
- Marx SJ 2000 Hyperparathyroid and hypoparathyroid disorders. *N Engl J Med* 343:1863–1875
- Ishizuya T, Yokose S, Hori M, Noda T, Suda T, Yoshiki S, Yamaguchi A 1997 Parathyroid hormone exerts disparate effects on osteoblast differentiation depending on exposure time in rat osteoblastic cells. *J Clin Invest* 99:2961–2970
- Locklin RM, Khosla S, Turner RT, Riggs BL 2003 Mediators of the biphasic responses of bone to intermittent and continuously administered parathyroid hormone. *J Cell Biochem* 89:180–190

16. Jilka RL 2007 Molecular and cellular mechanisms of the anabolic effect of intermittent PTH. *Bone* 40:1434–1446
17. Dobnig H, Turner RT 1995 Evidence that intermittent treatment with parathyroid hormone increases bone formation in adult rats by activation of bone lining cells. *Endocrinology* 136:3632–3638
18. Jilka RL, Weinstein RS, Bellido T, Roberson P, Parfitt AM, Manolagas SC 1999 Increased bone formation by prevention of osteoblast apoptosis with parathyroid hormone. *J Clin Invest* 104:439–446
19. Bellido T, Ali AA, Plotkin LI, Fu Q, Gubrij I, Roberson PK, Weinstein RS, O'Brien CA, Manolagas SC, Jilka RL 2003 Proteasomal degradation of Runx2 shortens parathyroid hormone-induced anti-apoptotic signaling in osteoblasts. A putative explanation for why intermittent administration is needed for bone anabolism. *J Biol Chem* 278:50259–50272
20. Ma YL, Cain RL, Halladay DL, Yang X, Zeng Q, Miles RR, Chandrasekhar S, Martin TJ, Onyia JE 2001 Catabolic effects of continuous human PTH(1–38) *in vivo* is associated with sustained stimulation of RANKL and inhibition of osteoprotegerin and gene-associated bone formation. *Endocrinology* 142:4047–4054
21. Nakchbandi IA, Mitnick MA, Masiukiewicz US, Sun BH, Insogna KL 2001 IL-6 negatively regulates IL-11 production *in vitro* and *in vivo*. *Endocrinology* 142:3850–3856
22. Hilliker S, Wergedal JE, Gruber HE, Bettica P, Baylink DJ 1996 Truncation of the amino terminus of PTH alters its anabolic activity on bone *in vivo*. *Bone* 19:469–477
23. Rixon RH, Whitfield JF, Gagnon L, Isaacs RJ, Maclean S, Chakravarthy B, Durkin JP, Neugebauer W, Ross V, Sung W, Willick GE 1994 Parathyroid hormone fragments may stimulate bone growth in ovariectomized rats by activating adenyl cyclase. *J Bone Miner Res* 9:1179–1189
24. Mohan S, Kutilek S, Zhang C, Shen HG, Kodama Y, Srivastava AK, Wergedal JE, Beamer WG, Baylink DJ 2000 Comparison of bone formation responses to parathyroid hormone(1–34), (1–31), and (2–34) in mice. *Bone* 27:471–478
25. Murrills RJ, Matteo JJ, Samuel RL, Andrews JL, Bhat BM, Coleburn VE, Kharode YP, Bex FJ 2004 *In vitro* and *in vivo* activities of C-terminally truncated PTH peptides reveal a disconnect between cAMP signaling and functional activity. *Bone* 35:1263–1272
26. Calvi LM, Sims NA, Hunzelman JL, Knight MC, Giovannetti A, Saxton JM, Kronenberg HM, Baron R, Schipani E 2001 Activated parathyroid hormone/parathyroid hormone-related protein receptor in osteoblastic cells differentially affects cortical and trabecular bone. *J Clin Invest* 107:277–286
27. Guo J, Chung UI, Yang D, Karsenty G, Bringham FR, Kronenberg HM 2006 PTH/PTHrP receptor delays chondrocyte hypertrophy via both Runx2-dependent and -independent pathways. *Dev Biol* 292:116–128
28. Yang D, Guo J, Divieti P, Bringham FR 2006 Parathyroid hormone activates PKC- δ and regulates osteoblastic differentiation via a PLC-independent pathway. *Bone* 38:485–496
29. Guo J, Iida-Klein A, Huang X, Abou-Samra AB, Segre GV, Bringham FR 1995 Parathyroid hormone (PTH)/PTH-related peptide receptor density modulates activation of phospholipase C and phosphate transport by PTH in LLC-PK1 cells. *Endocrinology* 136:3884–3891
30. Lotinun S, Sibonga JD, Turner RT 2005 Evidence that the cells responsible for marrow fibrosis in a rat model for hyperparathyroidism are preosteoblasts. *Endocrinology* 146:4074–4081
31. Swarthout JT, Doggett TA, Lemker JL, Partridge NC 2001 Stimulation of extracellular signal-regulated kinases and proliferation in rat osteoblastic cells by parathyroid hormone is protein kinase C-dependent. *J Biol Chem* 276:7586–7592
32. Somjen D, Tordjman K, Katzburg S, Knoll E, Sharon O, Limor R, Naidich M, Naor Z, Hendel D, Stern N 2008 Lipoxygenase metabolites are mediators of PTH-dependent human osteoblast growth. *Bone* 42:491–497
33. Takasu H, Gardella TJ, Luck MD, Potts JT Jr, Bringham FR 1999 Amino-terminal modifications of human parathyroid hormone (PTH) selectively alter phospholipase C signaling via the type 1 PTH receptor: implications for design of signal-specific PTH ligands. *Biochemistry* 38:13453–13460
34. Kim SJ, Kim SY, Kwon CH, Kim YK 2007 Differential effect of FGF and PDGF on cell proliferation and migration in osteoblastic cells. *Growth Factors* 25:77–86
35. Datta NS, Pettway GJ, Chen C, Koh AJ, McCauley LK 2007 Cyclin D1 as a target for the proliferative effects of PTH and PTHrP in early osteoblastic cells. *J Bone Miner Res* 22:951–964
36. Zhang X, Yu S, Galson DL, Luo M, Fan J, Zhang J, Guan Y, Xiao G 2008 Activating transcription factor 4 is critical for proliferation and survival in primary bone marrow stromal cells and calvarial osteoblasts. *J Cell Biochem* 105:885–895
37. Sato T, Abe T, Nakamoto N, Tomaru Y, Koshikiya N, Nojima J, Kokabu S, Sakata Y, Kobayashi A, Yoda T 2008 Nicotine induces cell proliferation in association with cyclin D1 up-regulation and inhibits cell differentiation in association with p53 regulation in a murine pre-osteoblastic cell line. *Biochem Biophys Res Commun* 377:126–130
38. Liu B, Yu HM, Hsu W 2007 Craniosynostosis caused by Axin2 deficiency is mediated through distinct functions of β -catenin in proliferation and differentiation. *Dev Biol* 301:298–308
39. Yadav VK, Ryu JH, Suda N, Tanaka KF, Gingrich JA, Schütz G, Glorieux FH, Chiang CY, Zajac JD, Insogna KL, Mann JJ, Hen R, Ducey P, Karsenty G 2008 Lrp5 controls bone formation by inhibiting serotonin synthesis in the duodenum. *Cell* 135:825–837
40. Partridge NC, Kemp BE, Veroni MC, Martin TJ 1981 Activation of adenosine 3',5'-monophosphate-dependent protein kinase in normal and malignant bone cells by parathyroid hormone, prostaglandin E2, and prostacyclin. *Endocrinology* 108:220–225
41. Abou-Samra AB, Jüppner H, Force T, Freeman MW, Kong XF, Schipani E, Urena P, Richards J, Bonventre JV, Potts Jr JT 1992 Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells: a single receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium. *Proc Natl Acad Sci USA* 89:2732–2736
42. Guo J, Lanske B, Liu BY, Divieti P, Kronenberg HM, Bringham FR 2001 Signal-selectivity of parathyroid hormone (PTH)/PTH-related peptide receptor-mediated regulation of differentiation in conditionally immortalized growth-plate chondrocytes. *Endocrinology* 142:1260–1268
43. Radeff JM, Singh AT, Stern PH 2004 Role of protein kinase A, phospholipase C and phospholipase D in parathyroid hormone receptor regulation of protein kinase C α and interleukin-6 in UMR-106 osteoblastic cells. *Cell Signal* 16:105–114
44. Chase LR, Aurbach GD 1967 Parathyroid function and the renal excretion of 3'5'-adenylic acid. *Proc Natl Acad Sci USA* 58:518–525
45. Sabatini M, Lesur C, Pacherie M, Pastoureaux P, Kucharczyk N, Fauchère JL, Bonnet J 1996 Effects of parathyroid hormone and agonists of the adenyl cyclase and protein kinase C pathways on bone cell proliferation. *Bone* 18:59–65
46. Miao D, Tong XK, Chan GK, Panda D, McPherson PS, Goltzman D 2001 Parathyroid hormone-related peptide stimulates osteogenic cell proliferation through protein kinase C activation of the Ras/mitogen-activated protein kinase signaling pathway. *J Biol Chem* 276:32204–32213