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Intermittent PTH Administration Converts Quiescent Lining Cells to Active Osteoblasts

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Abstract

Intermittent administration of parathyroid hormone (PTH) increases bone mass, at least in part, by increasing osteoblast number. One possible source of osteoblasts might be conversion of inactive lining cells to osteoblasts, and indirect evidence is consistent with this hypothesis. To better understand the possible effect of PTH on lining cell activation, a lineage tracing study was conducted using an inducible gene system. *Dmp1*-CreERT2 mice were crossed with ROSA26R reporter mice to render targeted mature osteoblasts and their descendants, lining cells and osteocytes, detectable by X-gal staining. *Dmp1*-CreERT2(+):ROSA26R mice were injected with 0.25 mg 4-OH-tamoxifen (4-OHTam) on postnatal day 3, 5, 7, 14, and 21. The animals were sacrificed on postnatal day 23, 33 or 43 (2, 12 or 22 days after the last 4-OHTam injection). On day 43, mice were challenged with a subcutaneous injection of human PTH (1–34, 80 µg/kg) or vehicle once daily for 3 days. By 22 days after the last 4-OHTam injection, most X-gal (+) cells on the periosteal surfaces of both the calvaria and tibia were flat. Moreover, bone formation rate and collagen I(α1) mRNA expression were decreased at day 43 compared to day 23. After 3 days of PTH injections, the thickness of X-gal (+) cells increased, as did their expression of osteocalcin and collagen I(α1) mRNA. Electron microscopy revealed X-gal-associated chromagen particles in both thin cells prior to PTH administration and cuboidal cells following PTH administration. These data support the hypothesis that intermittent PTH treatment can increase osteoblast number by converting lining cells to mature osteoblasts *in vivo*.

Keywords

osteoblasts; PTH; lining cells; lineage tracing study; inducible transgene system

Introduction

Intermittent administration of PTH increases bone mass, in part, by increasing osteoblast numbers, but the molecular and cellular mechanisms involved in these anabolic effects have

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not been completely elucidated. The control of osteoblast number is achieved by the balance between cell production and death (1). Multipotent mesenchymal stem cells in the bone marrow give rise to osteoblasts (2). Cells committed to the osteoblast lineage differentiate from osteoblast progenitor cells and exhibit progressive changes in gene expression that are stereotypic for specific stages (3). Mature osteoblasts can ultimately undergo one of three fates. They can 1) become embedded in the mineralized matrix as osteocytes, 2) become quiescent at the surface of the bone as lining cells, or 3) die by apoptosis. Although apoptosis is a critical determinant of osteoblast number, it is very difficult to quantify in vivo (4). A recent study has indicated that osteoblasts on the periosteal surface of murine vertebrae have lower rates of apoptosis than those on cancellous bone (5). Thus, the anti-apoptotic action of intermittent PTH treatment does not appear to substantially contribute to increases in osteoblast number on the periosteal surface, unlike in cancellous bone. One possible source of osteoblasts may be the quiescent lining cells, the very thin cells lining inactive bone surfaces (6). They have flat or slightly ovoid nuclei, synthesize only small amounts of protein, and connect to other lining cells via gap junctions (7). Some indirect data that PTH activates lining cells to differentiate into functioning osteoblasts are consistent with this hypothesis (8,9). Dobnig and Turner showed that osteoblast number on the bone surface increased after intermittent PTH administration, with no evidence of increased proliferation leading to this accumulation (8). Leaffer et al. demonstrated that the fraction of lining cells on the bone surface decreased upon PTH administration, in association with an increase in osteoblast number but no change in the total number of cells on the bone surface, as assessed by electron microscopy (9). Neither study used methods that could show conversion of lining cells into active osteoblasts, however. To better understand the possible effect of PTH on lining cell activation, we conducted a lineage tracing study using an inducible gene system. In this study, we identified and labeled mature osteoblasts, followed the fates of these cells, and demonstrated the effect of PTH on lining cells in vivo.

Methods and Materials

Mice

Temporally-controlled transgene expression was used to trace cells of the osteoblast lineage using *Dmp1*-CreERT2 and ROSA26R mice. The 10kb *Dmp1*-CreERT2 mice were generated by pronuclear injections of a transgene encoding the Cre-ERT2 under the control of 10kb fragment of *Dmp1* in B6C3F1 hybrid mice (Taconic, Hudson, NY, USA). Founders were then backcrossed into C57/BL6, as previously described (10). For the studies described here, one transgenic line (D77) demonstrating expression in both mature osteoblasts and osteocytes was selected for further studies. *Dmp1*-CreERT2 mice were crossed with ROSA26R mice in which expression of *Escherichia coli* β -galactosidase can be induced by Cre-mediated recombination using a universally expressed promoter (11). These studies were approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital.

Genotyping of mice

The genotypes of the mice were determined by PCR analysis of genomic DNA extracted from tail biopsies. For the 10kb-*Dmp1*-CreERT2 transgene, the forward Cre primer (5'-CGCGCGGTCTGGCAGTAAAACTATC-3') and the reverse Cre primer (5'-CCCACCGTCAGTACGTGAGATATC-3') were used to generate a PCR product of approximately 400 bp. PCR primers for genotyping ROSA26R mice were: R1295, 5'-GCGAAGAGTTTGTCTCAACC-3'; R523, 5'-GGAGCGGGAGAAATGGATATG-3' and R26F2, 5'-AAAGTCGCTCTGAGTTGTTAT-3'. Using these primers, a 600 bp PCR product is detected in wild-type mice, a 325 bp PCR product is detected in homozygous

ROSA26R mice, and both 600 and 325 bp PCR products are detected in heterozygous ROSA26R mice (11).

4-OHTam administration

For 4-OHTam injections, 2.5 mg of 4-OHTam (Takeda, Osaka, Japan) was dissolved in 100 μ l of dimethylformamide (Fisher Scientific, Waltham, MA, USA) and then diluted to 2.5 mg/ml in corn oil (Sigma, St. Louis, MO). *Dmp1*-CreERT2:ROSA26R mice were injected with 0.25 mg 4-OHTam on postnatal day 3, 5 and 7 and then day 14 and 21.

PTH administration

Mice were injected subcutaneously with 80 μ g/kg human PTH (amino acids 1–34, Bachem, Bubendorf, Switzerland) for 3 consecutive days starting 22 days after the last 4-OHTam injection.

Histology, X-gal staining and cell thickness measurement

X-gal staining for β -galactosidase activity was performed as reported previously (12). Briefly, calvaria, long bones and vertebrae were dissected from the mice, and all soft tissues were removed. Each sample was rinsed twice with PBS, fixed in 10% formalin for 30 min at 4 °C, washed three times with PBS, and then stained overnight at 37 °C in X-gal solution containing 1 mg/ml X-gal (Takeda, Osaka, Japan), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40. Samples were decalcified with buffered EDTA for 2 weeks, and then embedded and processed for paraffin sectioning. Sections were counterstained with eosin. X-gal (+) cells were examined using 4–6 comparable sections and 8–16 fields from each section under 400 $\times$ from variable numbers of mice per experiment, as indicated in the figure legends. At least 30 and 10 periosteal X-gal (+) cells from each calvaria and tibia, respectively, were analyzed using the imageJ program (NIH) (13).

Mineralized bone histology and bone histomorphometry

Mice were injected with calcein (16 mg/kg of body weight) intraperitoneally at 7 and 3 days prior to euthanization. The calvaria and tibia were isolated, cleaned, and fixed in 4% paraformaldehyde at 48C overnight, and then they were dehydrated, cleared in xylene, and embedded in methyl methacrylate. Static and dynamic histomorphometric analyses were conducted on the calvaria and tibia using the Bioquant program (Bio-Quant, Inc., San Diego, CA, USA).

BrdU labeling and detection

Mice were injected intraperitoneally with BrdU (5-bromo-2-deoxyuridine, Sigma, St. Louis, MO) at 8 h intervals for 24 h and were sacrificed 6 h after the last injection. Tissues were fixed in 4% (vol/vol) paraformaldehyde, processed, and sectioned by standard procedures. BrdU incorporation into DNA was detected using an anti-BrdU antibody staining kit (Zymed Laboratories, San Francisco, CA). The BrdU labeling index was calculated as the ratio of BrdU-positive nuclei relative to the total nuclei on the corresponding periosteal surface of four independent sections of the calvaria from three mice.

In situ hybridization

In situ hybridization was carried out as previously described (14). The antisense probes for *osteocalcin* and *collagen I(α 1)* have been described (14).

Serum biochemistry

Blood was collected by orbital sinus puncture before animals were sacrificed. Serum levels of the N-terminal propeptide of type I procollagen (PINP) was measured with ELISA kits from Immunodiagnostic Systems (Bodon, UK).

Electron microscopic analysis and measurement of cell thickness

After X-gal staining, calvaria were cut transversely at the mid-sagittal suture, fixed in 2.5% glutaraldehyde, 2.0% paraformaldehyde, 0.025% calcium chloride in 0.1 M sodium cacodylate buffer [pH 7.4], and processed for electron microscopy (EM). The LacZ-positive cells were identified by the deposits of X-gal reaction product in the cytoplasm, which appeared as a heterogeneously distributed black amorphous material (15). Samples were examined with a Phillips 301 transmission EM and digital images were captured using an AMT (Advanced Microscopy Techniques) CCD camera at magnifications ranging from 34,500 $\times$ to 319,500 $\times$. Electron micrographs were scanned into the computer, and with imageJ, the mean cell thickness of X-gal-positive cells from each section was determined in a minimum of 10 fields.

Statistics

All data are presented as mean $\pm$ standard error of mean (SEM) as indicated in the figure legends. The statistical significance of differences between groups was determined by Student's t-test or ANOVA. P values < 0.05 were accepted as significant.

Results

Tracing of LacZ⁺ osteoblast descendents on the periosteal surface of the calvaria and tibia

The *Dmp1*-CreERT2 transgene is active in osteocytes and a subset of osteoblasts on the surfaces of calvariae and, to a lesser extent, long bones (10). In this system, the mutated ERT2 domain responds only to the synthetic ligand, 4-OHTam. Administration of 4-OHTam induces transient nuclear translocation and CreERT-mediated gene recombination. A reporter gene, such as the ROSA26R β -galactosidase (LacZ) reporter transgene, can be used to detect the recombination by staining with the chromogenic substrate for β -galactosidase, X-gal, since the stop sequences designed to stop both translation and transcription are removed by the cre-recombinase. The β -galactosidase will then be expressed continuously in the targeted cells and their progeny. Because 4-OHTam activity is detected for only 24 h post-injection (15), this inducible system allows us to mark CreERT2-expressing cells genetically and at a particular time.

To evaluate the fates of mature osteoblasts in vivo, the animals were sacrificed on postnatal day 23, 33 or 43 (2, 12 or 22 days after the last 4-OHTam injection; Fig. 1). When X-gal staining was performed 2 days after the last 4-OHTam injection, many plump osteoblasts and osteocytes in the calvaria and tibia were stained (Fig. 2A & Supplemental Fig. 1SA). At longer times after the last 4-OHTam injection, the cells became flat on the periosteal surface of the calvaria and tibia (Fig. 2C & Supplemental Fig. 1SC). The thickness and number of LacZ⁺ osteoblastic descendants on the periosteal surface of the bones was quantified relative to time since the last 4-OHTam administration. Four comparable sections from the bones were selected, and the thickness and the number of X-gal (+) cells were measured using Image J. As shown in Fig. 2D & 2E, the number and the thickness of X-gal (+) cells on the periosteal surface of the calvaria decreased over time after the last 4-OHTam injection (number: 2 days: $43.3 \pm 4.8/\text{mm}$; 12 days: $25.8 \pm 4.4/\text{mm}$; 22 days: $10.4 \pm 2.1/\text{mm}$; 2 days versus 22 days: $p < 0.01$; thickness: 2 days: $11.6 \pm 0.7 \mu\text{m}$; 12 days: $6.8 \pm 0.5 \mu\text{m}$; 22 days: $4.3 \pm 0.4 \mu\text{m}$; 2 days versus 12 days: $p < 0.01$; 12 days versus 22 days: $p < 0.01$; $n=4/$

group). Similar results were obtained for periosteal X-gal (+) cells in the tibia. By contrast, the number of X-gal (+) osteocytes did not change (Fig. 2F), but the density of X-gal (+) osteocytes significantly decreased as the bones grew (Fig. 2G).

To determine the functional characteristics of cells of the osteoblast lineage on the periosteal surface over time after tamoxifen withdrawal, we performed a calcein double labeling experiment and in situ hybridization analysis for mRNA encoding collagen I(α 1) (col I). As shown in Fig. 2H, the bone formation rate of the calvarial periosteum at day 43 was decreased substantially compared to that at day 23. Similarly, col I mRNA expression at the periosteal surface also decreased over time after the last 4-OHTam injection (Fig. 2I). Collectively, these data reveal that osteoblasts on the periosteal at day 43 are quiescent, functionally supporting the hypothesis that periosteal flat X-gal (+) cells are lining cells. To exclude the possibility that PTH affected endogenous β -galactosidase expression, 4-OHTam and PTH or vehicle was injected into *Dmp1-Cre(-)ERT2:ROSA26R* mice as shown in Fig. 1. As shown in Supplemental Fig. 2SA, no β -galactosidase expression was detected after X-gal staining in these mice. Moreover, to confirm that the promoter is active only after 4-OHTam administration, *Dmp1-Cre(+)*ERT2:ROSA26R mice were not given 4-OHTam and were injected with PTH or vehicle from postnatal day 43 for 3 days, and analyzed for β -galactosidase expression. X-gal (+) cells were not detected in mice that had not received 4-OHTam (Fig. 2SB). Thus, intermittent PTH treatment neither increased endogenous β -galactosidase expression nor induced 4-OHTam-independent leaky expression of β -galactosidase. Very little labeling of the endocortical surface or of trabecular bone was observed in the long bones or vertebrae (data not shown). Consequently, the current methodology could not be used to examine the fates of lining cells on endocortical or trabecular surfaces.

Intermittent PTH treatment increases the thickness of lacZ+ osteoblast descendants on the periosteal surface of the calvaria and tibia

Because most X-gal (+) cells were flat by 22 days after the last 4-OHTam injection (see Fig. 2C and supplemental Fig. 1SC), we injected PTH at this time to determine whether PTH could change the morphology and activity of the flat cells lining the bone. After three PTH injections (80 μ g/kg/d), cuboidal X-gal (+) cells could be detected on the periosteal surface of PTH-treated mice, while in vehicle-treated mice, X-gal (+) cells appeared flat (Fig. 3A & Supplemental Fig. 3S). The thickness of the X-gal (+) cells on the periosteal surface of the bones was quantified before and after PTH or vehicle treatment. PTH markedly increased the thickness of osteoblastic descendants in the calvaria, by almost 50%, compared to the thickness before PTH treatment (pre-PTH: 4.3 ± 0.4 μ m versus PTH: 6.8 ± 0.4 μ m, $p < 0.01$; $n=8$ /group; Fig. 3B). Similar results were obtained for periosteal osteoblasts in the tibia.

Intermittent PTH modulates the fate of lacZ+ osteoblast descendants on the periosteal surface of the calvaria and tibia

The effect of PTH on the number of X-gal (+) osteoblast descendants on the periosteal surface was then quantified. The number of X-gal (+) cells on the periosteal surface of the calvaria treated with PTH for 3 days was comparable to the number before treatment, whereas the number of X-gal (+) cells in vehicle-treated mice decreased markedly compared to those in PTH-treated mice (pre-PTH: 10.4 ± 2.1 /mm; PTH: 14.4 ± 2.0 /mm; vehicle: 3.0 ± 0.9 /mm; pre-PTH versus PTH: $p > 0.05$; pre-PTH or PTH versus vehicle: $p < 0.05$; $n=8$ /group; Fig. 3C). This difference in X-gal (+) cells between vehicle and PTH-treated mice could be due to 1) PTH-induced prolongation of survival of osteoblastic descendants on the periosteal surface or 2) PTH-induced increase in cellular thickness that makes osteoblasts more easily detectable without necessarily changing their number. If the second explanation

is correct, then the changes in cellular thickness shown in Figure 3B are an underestimate of the effect of PTH on cell thickness, since vehicle-treated cells will be, on average, even thinner than the measurements here indicate. Intermittent PTH treatment did not change the actual number and the density of X-gal (+) osteocytes (Fig. 3D & 3E), suggesting that 3 days is too short for PTH stimulation to convert substantial numbers osteoblasts into osteocytes; the X-gal staining density of osteocytes was therefore comparable between samples.

PTH treatment increases the osteoblast activity but not proliferation rate on the periosteal surface of the calvaria

PTH treatment increased serum levels of PINP compared to vehicle (pre-PTH: 5.5 ± 0.4 ng/ml; PTH: 10.5 ± 0.8 ng/ml; vehicle: 5.4 ± 0.8 ng/ml; pre-PTH or vehicle versus PTH: $p < 0.01$; $n=5$ /group; Fig. 4A). Despite this indication that the hormone increased an index of bone formation, it did not increase Brdu incorporation into cells on the periosteal surface of the calvaria (Fig. 4B). To examine the anabolic action of PTH on the periosteum, we performed dynamic histomorphometric analyses. Although PTH increased cortical bone volume of the tibia, the modest increase in bone formation rate and mineral apposition rate were not statistically significant (Fig. 4C). This suggests that 3 days is too short time to directly demonstrate effect of PTH on bone formation at the periosteum. To examine the functional activation of lining cells after PTH treatment, we determined the expression of collagen I($\alpha 1$) (col1) and osteocalcin mRNA by in situ hybridization analysis. Similar to the increase of the blue cell thickness, expression of col1 and osteocalcin mRNA was also greater in cells on the periosteal surface of the calvaria (Fig. 4D). Thus, PTH not only increases the thickness of cells on the periosteal surface, but also changes their gene expression. Collectively, these data support the hypothesis that PTH re-activates quiescent lining cells on the periosteal surface to active osteoblasts.

PTH converts quiescent lining cells to active osteoblasts: ultrastructural criteria

The cells lining the periosteal surface were examined by EM to further characterize the flat cells and to confirm the presence of products of X-gal metabolism ("X-gal particles") in these cells. EM analysis was performed on transverse sections of the calvaria. Consecutive sections were examined by light microscopy and EM. As shown in Fig. 5A, EM of X-gal (+) cells on the periosteal surface of the calvaria from *Dmp1-CreERT2:ROSA26R* mice at 22 days after the last 4-OHTam showed the typical morphology of inactive lining cells. Electron micrographs revealed that PTH resulted in an apparent increase in cell size and in the abundance of intracellular organelles, many of which appeared to be rough endoplasmic reticulum (Fig. 5A; D46:PTH). Quantitative analysis of cell thickness confirmed that PTH-treated X-gal (+) cells were 2.6 times thicker than those before PTH treatment (pre-PTH: 1.4 ± 0.2 μ m; PTH: 3.7 ± 0.3 μ m; vehicle: 1.1 ± 0.1 μ m; pre-PTH or vehicle versus PTH: $p < 0.01$; $n=4$ /group; Fig. 5B). X-gal deposits in the cytoplasm were observed in both, and appeared as randomly distributed black amorphous microcrystalline structures. By contrast, amorphous microcrystalline particles were not observed in Cre negative mice. Thus, the EM analysis supports the hypothesis that intermittent PTH treatment activates lining cells to differentiate into cuboidal osteoblasts.

Discussion

In the present study, we used a lineage tracing strategy to show that PTH can stimulate bone lining cells to become active osteoblasts. Cells of the osteoblast lineage are very heterogeneous depending on their anatomical location and stage of differentiation. Although extensive in vitro studies of osteoblasts have provided a framework for understanding osteoblast differentiation, the direct relevance of this framework to bone must be tested in

intact animals. Recently, our group showed that lineage tracing using CreERT2 mice driven by stage-specific, osteoblast lineage promoters can be used to elucidate the distinct fates of osteoblast precursors and mature osteoblasts in vivo (15). Here we have adapted this technique to examine the fates of bone lining cells after PTH administration.

We employed the 10kb *Dmp1* promoter because it is expressed in late osteoblasts as well as in osteocytes (16,17). We confirmed this pattern of expression when we examined mice 2 days after 4-OHTam administration. Because the CreERT2 protein expressed downstream of this promoter is active in the nucleus during a brief time that administered 4-OHTam is present in sufficiently high concentration (18), all cells found to express β -galactosidase in the chase period must have derived from these initially labeled cells. Importantly, no blue cells were identified in *Dmp1*-Cre(+)ERT2:ROSA26R mice that did not receive 4-OHTam, irrespective of PTH treatment (Supplemental Fig. 2SB). Further, the absence of BrdU incorporation into blue cells demonstrates that these cells are post-proliferative. We found that the *Dmp1* promoter is a marker for many mature osteoblasts on the periosteal surface of the calvaria and long bones, but labels very few osteoblasts in trabecular bone. This pattern may reflect the rapid conversion of late osteoblasts to osteocytes in trabecular bone or, alternatively, may reflect limited penetration of the X-gal staining solution.

After a 22 day chase period, the labeled blue cells were osteocytes or flat cells on the bone surface exhibiting characteristics of bone lining cells. These cells had extremely thin cytoplasm when viewed by light microscopy and had flattened nuclei with very low RER density and sparse secretory granules when viewed by EM. Moreover, they produced only small amounts of col1 and osteocalcin mRNA, as noted previously (19), and their bone formation rates were low. The mean thickness of the blue flat cells observed in our EM analysis is $\sim 1 \mu\text{m}$, similar to findings of others (7). Collectively, these data indicate that the X-gal (+) flat cells are lining cells. Many of X-gal (+) cells disappeared over time after the last administration of 4-OHTam. The disappearance could have resulted from differentiation to osteocytes, from apoptosis, or from differentiation to flat cells that are too thin to be seen by light microscopy.

Three days after daily PTH administration, plump blue cells were found on the periosteum of the calvarium and tibia. Because all blue cells must have derived from the previously observed lining cells (or osteocytes) seen at day 43 of the protocol, we conclude that lining cells can become active osteoblasts after PTH administration. PTH treatment increased the thickness of X-gal (+) cells by only 50% when detected using light microscopy but increased this thickness 260% when detected using EM. This difference might reflect the inability to detect very thin cells using the light microscope. To evaluate better the thickness of lining cells, we measured both the thickness of lining cells and activated osteoblasts using one micron sections. In this analysis, PTH administration increased the thickness of labeled cells by an average of 100% after PTH administration (data not shown). Thus, administration of PTH for 3 days increased the thickness of labeled cells by 50–100% using light microscopy. The more dramatic changes detected with the EM most likely reflect the ability of the EM to detect very thin lining cells missed with light microscopy, even using thin one micron sections. In addition, two-dimensional analysis is limited because changes in the third dimension of cell structure may change, as well. New approaches will be needed to define and evaluate volumetric change before and after osteoblast reactivation.

We characterize the cells after PTH treatment as active osteoblasts because they are cuboidal cells on the bone surface with round nuclei. Further, they have an extensive RER and produce large amounts of col1 and osteocalcin mRNA. We cannot exclude the possibility that osteocytes might become active osteoblasts, but think this is unlikely. The regularity of the bone surface suggests no such dramatic transformation. In addition, the number of blue

cells per length of the periosteal surface did not increase after PTH treatment (Fig. 3C) as it might have, if osteocytes had become active osteoblasts.

In this study, the number of X-gal (+) cells decreased by 3-fold during the 10 day period prior to PTH administration and then decreased by 3-fold further during the 3 days of vehicle administration. The accelerating rate of decrease in cell number suggests again that it is difficult to detect very thin X-gal(+) cells by light microscopy. PTH administration led to detection of significantly more labeled cells on the periosteal surface compared to vehicle. An alternative explanation could be changing rates of cellular apoptosis. In Jilka *et al* (5), however, the investigators found that PTH inhibits osteoblast apoptosis in the cancellous bone but not in the periosteum. In our data, the number of labeled cells on the periosteum of the calvaria and tibia are comparable before and after PTH administration. Thus, the action of PTH to preserve detection of X-gal (+) cells may reflect an action of PTH to delay transformation of osteoblasts into lining cells that can be difficult to detect with light microscopy.

Our results are consistent with earlier findings that lining cell number decreases and osteoblast number increases after PTH administration (5). The current studies demonstrate that this pattern can be explained by conversion of lining cells into active osteoblasts. Further, previous studies have shown that PTH administration increases osteoblast number without evidence of cell proliferation in the mature osteoblasts (8,20). In the present study, 3 days of PTH treatment increased the serum levels of P1NP, but no BrdU incorporation in osteoblasts was observed, consistent with these previous studies. Therefore, X-gal marks post-proliferative cells that do not enter the proliferative cycle even after PTH administration. The idea that lining cells can be activated by other stimuli has also been suggested in other studies. For example, mechanical loading experiments using rats revealed that the osteoblast surface area increased, but the lining cell surface area decreased, 3 days after a single mechanical loading session (21).

PTH-induced reactivation of lining cells in the present study reflects an initial stage of its modeling-based anabolic action along the periosteum of murine calvaria and long bones. This increase in bone formation can result in an increase in bone size and bone strength (22). PTH causes differing anabolic actions on the cancellous and periosteal bone. In humans, Lindsay's group has shown (23) that PTH stimulates bone remodeling and subsequently increased bone turnover through an increase in the number of basic multicellular units, leading to a net gain in bone mass of the cancellous bone. Because we were unable to detect labeling of osteoblasts on the surfaces of cancellous bone, our studies cannot address the question of lining cell activation on cancellous surfaces.

Many questions remain unanswered by the current studies. Whether PTH activates lining cells directly through the PTH/PTHrP receptor in these cells is still uncertain, as are the signaling pathways responsible for osteoblast activation. Further, these studies of lining cells in the calvariae and periosteum in young growing mice need to be extended to other bone surfaces and to older mice.

In conclusion, this osteoblast lineage tracing study demonstrates that PTH can activate quiescent lining cells on the bone surface to differentiate into active osteoblasts. Further studies will be required to ascertain the mechanism of PTH action on lining cell activation as well as to label a substantial number of osteoblasts in trabecular bones of grown subjects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Experimental protocols

Dmp1-CreERT2:ROSA26R mice were injected with 0.25 mg 4-OHTam on postnatal day 3, 5, 7, 14 and 21. To evaluate the fate of mature osteoblasts in vivo, animals were sacrificed on postnatal day 23, 33 or 43 (2, 12 or 22 days after the last 4-OHTam treatment), as well as on day 46 to evaluate the actions of PTH administration.

Figure 2. Tracing of LacZ⁺ osteoblast descendants on the periosteal surface of the calvaria of *Dmp1-CreERT2:ROSA26R* mice

X-gal staining was performed at 2 days (A; D23), 12 days (B; D33) and 22 days (C; D43) after the last 4-OHTam injection. Each panel represents a section from a different mouse. The panels represent sections from four mice at the three time points indicated. The number (D) and thickness (E) of X-gal (+) descendants of osteoblasts significantly decreased over time after withdrawal of 4-OHTam. The actual number of X-gal (+) osteocytes (F) did not change but the density of X-gal (+) osteocytes (G) significantly decreased over time after withdrawal of 4-OHTam. The mean cell thickness and number were determined in 6 comparable sections, viewing 8–16 fields/section under 400× from six mice per each group. Data are expressed as mean ± SEM, a: $p < 0.01$ versus 2 days, b: $p < 0.05$ versus 2 days, c: $p < 0.01$ versus 12 days. Dynamic histomorphometric analysis of calcein-labeled calvaria sections and in situ hybridization analysis indicated that bone formation rate (H) and col1 mRNA expression (I) significantly decreased at day 43 compared to at day 23 (The arrows indicate periosteal surface). Data are representative of experiments performed on sections from three mice for each group. Data are expressed as mean ± SEM, a: $p < 0.05$ versus 2 days

Figure 3. Effects of PTH on LacZ⁺ osteoblast descendants on the periosteal surface of the calvaria of *Dmp1*-CreERT2:ROSA26R mice

(A) X-gal staining was performed at 22 days (D43:Pre-PTH) after the last 4-OHTam injection, or animals were injected with PTH (80 $\mu\text{g/kg/d}$) (D46:PTH) or vehicle (D46:vehicle) for 3 days after day 43. Data are representative of experiments performed on sections from eight mice for each group. Quantitative analysis of the thickness (B) and number (C) of X-gal (+) cells before (pre-PTH) and after 3 days of PTH or vehicle treatment. Data are expressed as mean $\pm$ SEM, a: $p < 0.01$ versus pre-PTH or vehicle, b: $p < 0.05$ versus pre-PTH or vehicle, c: $p < 0.01$ versus pre-PTH or PTH. Quantitative analysis of the actual number (D) and density (E) of X gal (+) osteocytes before and after PTH or vehicle treatment. The mean cell thickness and number were determined in 6 comparable sections, viewing 8–16 fields/section under $400\times$ from eight mice per each group.

Figure 4. The effect of PTH on BrdU incorporation and osteogenic activity

(A) The levels of serum P1NP for *Dmp1-CreERT2:ROSA26R* mice receiving intermittent PTH or vehicle treatment for 3 days. Data are expressed as mean $\pm$ SEM, $n = 5$; a: $p < 0.01$ versus pre-PTH or vehicle treatment after the last 4-OHTam treatment.

(B) BrdU incorporation analysis for *Dmp1-CreERT2:ROSA26R* mice receiving intermittent PTH or vehicle treatment for 3 days. BrdU staining of paraffin sections of the calvaria was performed (left panel). No X-gal (+) cell (asterix) has any BrdU staining. The mean number of BrdU-positive cells (arrows) was determined in 4 comparable sections, viewing 8–16 fields/section under 400 $\times$ from three mice per each group (right panel). Data are expressed as mean $\pm$ SEM. Data are representative of experiments performed on sections from three mice for each group.

(C) Histomorphometric analysis for *Dmp1-CreERT2:ROSA26R* mice receiving intermittent PTH or vehicle treatment for 3 days. Data are expressed as mean $\pm$ SEM. a: $p < 0.05$ versus vehicle treatment. Data are representative of experiments performed on sections from three mice for each group.

(D) In situ hybridization analysis of col1 and osteocalcin (oc) mRNA expression in the calvaria from *Dmp1-CreERT2:ROSA26R* mice receiving intermittent PTH or vehicle treatment for 3 days. Data are representative of experiments performed on sections from three mice for each group.

Figure 5. Electron microscopy analysis of X-gal (+) osteoblastic descendants on the periosteal surface of the calvaria of *Dmp1-CreERT2:ROSA26R* mice

(A) X-gal stained calvarial bones were sectioned at 1μm thickness to visualize tissue morphology and to align X-gal staining with EM imaging. EM analysis was performed at 22 days after the last 4-OHTam (D43, pre-PTH) or after intermittent PTH (D46) or vehicle-treatments for 3 days (D46). Each lower panel shows a magnified image of the red box in the upper panel. X-gal deposits in the cytoplasm of LacZ+ cells were visible by EM as black amorphous material (arrows). The asterisk indicates collagen fibrils. In Cre negative mice, X-gal deposits were not observed. Data are representative of experiments performed on sections from four mice for each group.

(B) Quantitative analysis of the thickness of X-gal (+) cells on the periosteal surface of the calvaria was performed on electron micrographs. The mean cell thickness was determined in four mice sets with 10–15 micrographs per set. Data are expressed as mean ± SEM, a: $p < 0.01$ versus pre-PTH or vehicle.