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Parathyroid Hormone Mediates Bone Growth through the Regulation of Osteoblast Proliferation and Differentiation

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Abstract

PTH (1–34) is the only FDA-approved anabolic agent for osteoporosis treatment in the U.S., but its mechanisms are not completely understood. This study investigated PTH effects on osteogenic cells at various stages of differentiation and proliferation using an engineered bone growth model *in vivo*. Ossicles were generated from bone marrow stromal cells (BMSCs) implanted in immunocompromised mice. Three weeks of PTH (40µg/kg/d) or vehicle treatment initiated 1 day, 1, 2, or 3wks after BMSC implantation resulted in an anabolic response in PTH-treated implants (via histomorphometry and microCT) in all treatment groups. A novel *in vivo* tracking strategy with luciferase tagged BMSCs and weekly bioluminescent imaging of ossicles revealed increased donor cell proliferation in PTH-treated ossicles. The greatest increase occurred during the first week, and the activity remained elevated in PTH-treated implants over time. Zoledronic acid (ZA) was combined with PTH to delineate interactive mechanisms of these bone active agents. Combining ZA with PTH treatment reduced the PTH-mediated increase in luciferase BMSC activity, serum osteocalcin, and serum tartrate resistant acid phosphatase-5b (TRAP-5b) but ZA did not reduce the PTH-induced increase in total bone. Since zoledronic acid reduced PTH-induced proliferation without reducing bone volume, these data suggests that combining PTH and bisphosphonate therapy warrants further investigation in the treatment of bone disorders.

Keywords

parathyroid hormone; zoledronic acid; bone marrow stromal cells; proliferation; tissue engineering

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Introduction

Skeletal integrity is maintained by the process of bone remodeling, which involves the coupling of osteoclast-mediated bone resorption and osteoblast-mediated bone formation. Endogenous parathyroid hormone (PTH) plays a critical role in this process through its action on the skeleton to maintain calcium homeostasis and regulate bone metabolism. Exogenous PTH exerts anabolic or catabolic effects on bone depending on the mode of administration and duration of treatment. It is well accepted that continuous doses of PTH increase bone resorption, whereas intermittent administration of PTH stimulates new bone formation and improves microarchitecture of existing bone. The ability of anabolic doses of PTH to increase bone remodeling and parameters of bone formation has stimulated interest in its current clinical use for the treatment of osteoporosis as well as investigative use for other conditions requiring bone regeneration. Many pharmacologic agents are available for osteoporosis treatment; however, PTH (1–34) is currently the only FDA approved anabolic agent in the U.S. for treatment of this metabolic bone disease.

The biological activity of intact PTH resides in the N-terminal sequence. Several mechanisms have been proposed for PTH anabolic actions, including cAMP activation [1], growth factors such as IGF-1 [2], transcriptional mediators in bone such as c-Fos [3] and Runx2 [4], and inhibition of apoptosis [5]. Although it has been suggested that PTH exerts anabolic actions in bone by reducing osteoblast apoptosis in mice, clinically it has been shown that PTH-mediated bone formation is associated with an increase in osteoblast apoptosis [6]. *In vitro* studies have shown inconsistent effects of PTH on bone cell proliferation [7–9] and evidence to support stimulation of proliferation *in vivo* remains controversial. Studies have demonstrated that intermittent PTH treatment increased osteoblast numbers in adult rats with few proliferating osteoblast progenitors by stimulating differentiation of quiescent bone surface cells, suggesting that the PTH-mediated increase in bone formation was due to the activation of bone lining cells [10–11]. Although a number of biologic mechanisms have been proposed, the effect of PTH on the proliferation and differentiation of cells of the osteoblast lineage is not well understood.

The objective of the current investigation was to explore the effect of intermittent PTH treatment on bone formation using a novel osteoregeneration model. Ectopic ossicles containing trabecular and cortical bone and a hematopoietic marrow were generated from implanted murine bone marrow stromal cells (BMSCs). There are three experimental strategies in the study. In the first strategy, a temporal treatment scheme was used to investigate the effects of PTH on osteogenic cells at various stages of differentiation. In the second, the effect of intermittent PTH treatment on cell proliferation within the ossicles was measured using luciferase tagged BMSCs in conjunction with *in vivo* bioluminescent imaging (BLI). Recent clinical studies conducted to evaluate the benefits of using combination therapy for postmenopausal osteoporosis treatment revealed the surprising findings that bisphosphonates blunted expected additive effects when used concurrently with PTH [12–14]. Hence, in the third strategy, PTH and bisphosphonate treatment were combined to gain new insight of how these two agents affect the pool of bone forming cells that facilitate new bone development. Taken together, the data from these experiments has expanded our understanding of the impact of PTH on cell proliferation, differentiation, and the augmentation of bone regeneration.

Materials and Methods

Ectopic Ossicle Model

BMSCs were isolated from the femoral, tibial, and humeral cavities of 4- to 8-week old C57BL/6 mice, and mice expressing luciferase (B6; C3-Tg (TetTALuc) 1Dgs/J, Jackson Laboratory, Bar Harbor, ME) as previously described [15]. The luciferase transgenic mice contain modified tetracycline controlled transactivator (tTA) and luciferase genes under the control of

tetracycline-responsive promoter elements (TREs) and widely express luciferase in the absence of tetracycline [16]. Briefly, bone marrow was flushed with growth medium (α -modified minimum essential medium (Invitrogen, Grand Island, NY), 2mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin sulfate, supplemented with 20% fetal bovine serum (HyClone, Provo, UT) and 10 nM dexamethasone (Sigma, St. Louis, MO)). The harvested cells were plated in T75 flasks with growth media and the first passage was performed when a confluent adherent layer was observed. BMSCs from each donor were then pooled, plated with growth media in T150 flasks, and expanded for another 5–7 days. BMSCs from all donors were then pooled, resuspended in fresh growth media, and $2\text{--}3.0 \times 10^6$ cells were incorporated into 3–5mm diameter gelatin sponges (Gelfoam®, Sullivan-Schein, a Henry Schein Company Melville, NY) by capillary action. Luciferase expression of luciferase tagged BMSCs was measured using a Monolight® Luminometer (Becton Dickinson Pharmingen, San Diego, CA) prior to BMSC implantation. Immunocompromised 4- to 6-week old male nude mice (NIH III Nude, Charles River Laboratories, Wilmington, MA) were anesthetized and two midlongitudinal skin incisions approximately 1 cm in length were made on the dorsal surface of each mouse. Blunt dissection was used to form subcutaneous pouches and each animal received four implants. All animals tolerated doses of drugs and procedures used in these studies without problems. Mice were maintained in accordance with institutional animal care and use guidelines and experimental protocols approved by the Institutional Animal Care and Use Committee of the University of Michigan.

In vivo treatment regimens

In the temporal impact experiments, which were designed to evaluate the effects of intermittent PTH treatment on osteogenic cells at various stages of differentiation, mice were randomly assigned to one of four groups (4 PTH and 4 vehicle-treated mice per group) (Fig. 1A). Daily injections of either recombinant human PTH (1–34) (40 μ g/kg) (Bachem, Torrance, CA) or vehicle (0.9% sodium chloride, Abbott, IL) were administered to each mouse for 3 weeks, with treatment initiated 1 day (group 1), 1 week (group 2), 2 weeks (group 3), and 3 weeks (group 4) after BMSC implantation. Mice also received an intraperitoneal injection (i.p.) of 100 μ l of calcein (Sigma) (15 μ g/kg) dissolved in PBS containing 2% sodium bicarbonate 10 d and 3 d prior to sacrifice (Fig. 1A). In the luciferase tracking experiments, mice with luciferase positive BMSC implants were treated with PTH (1–34) or vehicle for 3 weeks or 9 weeks (experiment 1) and 1 week or 5 weeks (experiment 2), with treatment initiated 1 week after cell implantation (Fig. 3A). Experiments were also performed using the luciferase tagged system to identify the effects of combining PTH and zoledronic acid (ZA) treatment on developing ossicles. Mice received daily subcutaneous (s.c.) injections of vehicle, PTH (1–34) (40 μ g/kg, s.c.), intraperitoneal injections of ZA (3 μ g/mouse/day, i.p.; Novartis Pharma AG, Basel, Switzerland), or PTH (1–34) (40 μ g/kg, s.c.) in combination with ZA (3 μ g/mouse/day, i.p.) for 3 weeks, with treatment initiated 1 week after implanting BMSCs (same regime as group 2 in Fig. 1A). A high dose of ZA (3 μ g/mouse/day) was based on a dose specified in a previous publication [17] and was chosen to ensure that a maximal response would be observed in the bone regeneration model and thus, the effects of ZA on cell numbers could be effectively evaluated. The dose of PTH (1–34) (40 μ g/kg/day) used in these studies is within the range of anabolic doses commonly used in mouse experiments [2,18]. The average weight of mice in each treatment group was 30g.

Histology and histomorphometry

All dissected bone specimens were fixed in 10% phosphate buffered formalin for 24h at 4°C. For histological analysis, ossicles and vertebrae were decalcified in 10% EDTA (pH 7.4). Some samples were embedded in paraffin and 5- μ m serial sections were prepared and stained with hematoxylin and eosin, while subsets of undecalcified calcein fluorochrome labeled specimens were embedded in methyl methacrylate (MMA), and 5- μ m and 8- μ m sections prepared for

tetrachrome staining and dynamic histomorphometric analysis, respectively. Total bone area was measured and osteoblasts counted from tetrachrome stained sections using Image Pro Plus 5.1 Software (Media Cybernetics, Silver Spring, MD). Osteoblasts were identified as cuboidal shaped cells on the bone surface. The number of osteoblasts/mm bone was enumerated by averaging the number of osteoblasts in five randomly chosen fields in each implant. Rapid tissue formation within the ossicles prevented distinct calcein fluorochrome labeled fronts from forming within ossicles, so the total fluorochrome labeling was determined by measuring total fluorochrome labeling per total ossicle area using Image Pro Plus 5.1 software (Media Cybernetics). The total ossicle area was defined and the percent of fluorochrome labeled tissue was measured. Dynamic parameters of bone formation were measured on sections from vertebrae harvested from mice treated with PTH or vehicle for 3 weeks (group 2). The bone mineralizing surface/bone surface (MS/BS) measurements included double label plus half of the single label surface ($(dL + \frac{1}{2} sL)/BS$). The mineral apposition rate (MAR) was measured as the distance between double labels per labeling interval (7 days), and the fractional volume of trabecular bone formed per unit trabecular surface area was calculated as $BFR/BS = (MS/BS \times MAR)$. Cell proliferation was evaluated in luciferase positive implants by 5-bromo-2'-deoxyuridine (BrdU) staining in tissue sections from mice that were injected i.p. with 50µg/g BrdU (Sigma) 4–8 hours prior to sacrifice. Staining was performed using a Zymed BrdU staining system (San Francisco, CA) following manufacturer's instructions as previously described [19]. BrdU positive cells were enumerated using Image-Pro Plus 5.1 software (Media Cybernetics). The total number of cells and number of BrdU labeled cells were counted in five randomly chosen fields in each ossicle section. A standard 125mm² circular grid was used to count cells in each field. Luciferase immunostaining was performed on luciferase expressing implants using a rabbit antiluciferase antibody (1:1000 dilution on 2wk old implants and 1:100 dilution on 4wk old implants), with an Envision⁺ Rabbit Peroxidase assay system (Dako, Carpinteria, CA).

MicroCT

Ossicles were scanned on a micro-computed tomography (microCT) system (GE Healthcare Preclinical Imaging, London, Ontario) and reconstructed with an 18µm isotropic voxel size. Histograms were then generated to select a global mineralized tissue threshold that delineated bone from all other tissues. The bone mineral content (BMC) and tissue mineral content (TMC) were calculated on the microCT images (MicroView v2, Advanced Bone Application, GE Healthcare) [20], and these data were used in conjunction with a custom MATLAB algorithm to calculate the bone mineral density (BMD), tissue mineral density (TMD) and bone volume fraction (BVF) as previously described [15]. Daily scanning procedures included the use of a phantom for system calibration.

Quantitative RT-PCR

Ossicles were dissected and flash-frozen in liquid nitrogen for gene expression studies. Total RNA was isolated using TRIzol reagent (Invitrogen) and quantified by spectrophotometry. RNA was purified to eliminate DNA contamination using an RNeasy Mini Kit (Qiagen, Inc., Valencia, CA). Double-stranded complementary DNA (cDNA) was synthesized from 2µg of RNA, using Oligo d (T) (Applied Biosystems, Foster City, CA) to prime total RNA samples for reverse transcription using Multiscribe reverse transcriptase (Applied Biosystems). The TaqMan universal PCR master mix (Applied Biosystems) was used for detection of cDNA. cDNA was amplified using customized primers and probes (Applied Biosystems, matrix γ-carboxyglutamic acid protein (MGP): Mm00485009_m1 and osteocalcin (OCN): Part No. 4331348; FAM reporter dye). Rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (VIC reporter dye) was used as an endogenous control. Amplification of cDNA was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Relative

quantitation of data generated using this system was performed using the standard curve method.

In vivo Bioluminescent Imaging (BLI)

Growth of luciferase positive BMSC implants was monitored by weekly BLI at the University of Michigan *In Vivo* Cellular and Molecular Imaging Center as previously described [17,19]. Briefly, mice were injected i.p. with 100 μ l of 40mg/mL luciferin dissolved in sterile PBS prior to imaging. Ventral and lateral images were acquired 12 minutes after injection. Imaging was performed under 1.5% isoflurane/air anesthesia on a cooled CCD IVISTM system equipped with a 50mm lens (Xenogen Corp, Alameda, CA) and coupled to a data-acquisition PC running LivingImage software (Xenogen Corp). Pseudo-color images of photon emissions were overlaid on grayscale images of animals to aid in determining spatial distributions of signals. Photon quantitations were calculated within regions of interest.

In vitro Luciferase Activity Assay

In order to discriminate direct effects of PTH and ZA on luciferase activity versus numbers of luciferase positive cells, an *in vitro* assay was performed. BMSCs were isolated from mice constitutively expressing luciferase as described in the procedure used for generating ectopic ossicles and plated. When a confluent adherent layer of cells was observed, the cells were passaged and plated in 12-well plates at a density of 45,000/cm². BMSC cultures were maintained for 3 days and then treated with 10⁻⁹ M PTH (1–34) or 0.05 μ M, 0.5 μ M, and 5 μ M ZA. Changes in luciferase activity were measured after 0, 1, 4, 8, and 12hrs of treatment using a dual-luciferase reporter assay system (Promega, Madison, WI) following the manufacturer's protocol. Luciferase levels were measured using a Monolight[®] Luminometer (Becton Dickinson Pharmingen).

Biochemical Assays

Terminal serum samples were collected from mice 24 hours after last treatment for biochemical analysis. Changes in serum TRAP 5b, a specific marker of osteoclastic bone resorption, were determined using the Mouse-TRAP assay per the manufacturer's instructions (IDS, Inc., Fountain Hills, AZ). Changes in serum OCN levels, a marker of bone formation, were measured using the Mouse-OCN EIA kit following the manufacturer's protocol (Biomedical Technologies, Inc., Stoughton, MA). Serum calcium was measured using a colorimetric assay with cresolphthalein complexone (Pointe Scientific, Inc., Canton, MI).

Statistical Analysis

Statistical analysis was performed in consultation with the Center for Statistical Consultation and Research (CSAR) at the University of Michigan using analysis of variance (ANOVA) or Student's *t* test with the Graph-Pad Instat biostatistics program (Graph-Pad software, San Diego, CA). In experiments performed to analyze the effects of 3 weeks of intermittent PTH treatment on BMSCs at various stages of differentiation, multiple implants (2–4 implants/mouse) were averaged together for 1 data point for each mouse (3–4 mice/treatment group, hence n=3–4/group). Luciferase *in vivo* studies were performed 2–3 times each with 2–3 mice per group and 4 implants/mouse. In all experiments, avascular implants (qualitatively determined if there was a lack of redish color reflecting vascularization, i.e., implants were completely white, at time of harvest) were excluded before performing analyses. Avascular implants were typically less than 10% of total implants with no predilection for treatment groups. Data are presented as mean $\pm$ SEM of indicated numbers of specimens.

Results

Temporal impact of PTH on BMSC implants at different stages of differentiation

Histomorphometry—In the temporal impact experiments, mice with BMSC implants were treated with anabolic doses of PTH or vehicle for 3 weeks, with treatment initiated at various stages of ossicle development (Fig. 1A). Histomorphometric analysis of tetrachrome stained specimens revealed an increase in total bone area for group 1, 2 and 4 PTH-treated ossicles in comparison to their vehicle-treated counterparts, along with a trend for an increase in group 3 PTH-treated ossicles (Fig. 1B, C). The most significant increase was in group 2 PTH-treated specimens versus vehicle-treated implants. Mice were also injected with calcein fluorochrome 10 days and 3 days prior to sacrifice to evaluate dynamic parameters of bone formation. The rapid tissue growth within the ossicles precluded distinct fluorochrome labeling fronts and quantitation of single and double labels (Fig. 1B); therefore measures of total labeling and labeling per bone area were determined. An increase in total fluorochrome labeling was observed in PTH-treated ossicles in all groups. Total fluorochrome labeling per bone surface was calculated in order to obtain a measure to reflect bone forming activity during the last week of bone growth. Interestingly, there was a tendency towards an increase in labeling per total bone area in PTH-treated ossicles but this did not reach statistical significance, suggesting that PTH increased bone formation in the early stage of ossicle development but at later time points the bone forming activity was not markedly different from vehicle-treated mice (data not shown). The enumeration of osteoblasts in tetrachrome stained ossicle sections revealed a significant increase in osteoblasts/mm bone in PTH-treated implants in groups 1–3 (Fig. 1D). There was a tendency towards an increase in TRAP-positive osteoclasts/mm bone PTH-treated ossicles in groups 2 and 3 and a significant increase in osteoclasts in PTH-treated implants in group 4 (Fig. 1E). Distinct labels in harvested, endogenous vertebrae enabled the dynamic parameters of bone formation to be calculated. An increase in mineralizing surface/bone surface (MS/BS), bone formation rate (BFR), and mineral apposition rate (MAR) was observed in vertebrae treated with PTH for 3 weeks from group 2 mice, validating the expected anabolic response (Fig. 1F–I).

MicroCT—Measurements of bone parameters at the organ level (BVf, BMD, BMC) and tissue level (TMD and TMC) were determined using MicroCT analysis. BVf, BMD, and BMC were statistically increased in PTH-treated ossicles in groups 2 and 3 (Fig. 2A–D). TMD was significantly increased in groups 2 and 3 PTH-treated implants (Fig. 2E), whereas TMC was increased in PTH-treated specimens in groups 2–4 (Fig. 2F).

Gene Expression—Gene expression studies were performed to determine the effects of intermittent PTH treatment on gene expression for the osteoblast phenotypic markers MGP and OCN. Quantitative PCR revealed a tendency towards an increase in MGP expression in PTH versus vehicle treated group 2 ossicles (Table 1). OCN was significantly increased in group 2 PTH-treated ossicles, suggesting either an increase in the number of mature bone forming cells or an increase in their activity (Table 1).

PTH-mediated effects on luciferase tagged BMSC proliferation

In order to track BMSC activity *in vivo*, luciferase positive BMSCs were utilized to generate implants. *In vivo* BLI was used to track the luciferase activity in order to assess the effect of intermittent PTH treatment on BMSC numbers (Fig. 3A). Luciferase activity was similar in all ossicles at baseline (i.e., 0–1 day) and 7 days after BMSC implantation, prior to treatment. A significant increase in luciferase activity was observed after 1 week of treatment with PTH and peaked after 5 weeks of treatment (Fig. 3B–C). Histomorphometric analysis of paraffin embedded sections indicated a significant increase in total percent bone in ossicles harvested after 3 weeks of PTH treatment, but the anabolic impact of PTH was diminished thereafter

(Fig. 4A–B). BrdU and luciferase immunostaining revealed an increase in proliferating luciferase positive cells in the marrow of implants treated with PTH versus vehicle (Fig. 4C–D). Distinct luciferase positivity in osteocytes and cells lining the bone of implants was observed in implants treated with PTH for 3 weeks (Fig. 4E). Enumeration of BrdU positive cells in implants removed from animals after 1 week and 5 weeks of treatment revealed an increase in proliferating cells in the marrow of PTH-treated ossicles at both time points (Fig. 4F–G).

Impact of bisphosphonate on PTH actions on bone growth

Weekly BLI imaging was performed on mice treated with vehicle, PTH only, zoledronic acid (ZA) only, or a combination of PTH and ZA (Fig. 5A). Implants treated with PTH alone exhibited higher luciferase activity over time versus implants treated with vehicle, ZA alone, and PTH+ZA. The luciferase activity of implants treated with vehicle and ZA alone were not statistically different over time. When PTH was administered in conjunction with ZA, reduced luciferase activity was observed (Fig. 5B–C). Interestingly, despite the blunting of increased cell numbers, radiographic and histomorphometric analyses revealed increased evidence of mineralized matrix and total bone area of implants treated with PTH+ZA versus implants treated with PTH only (Fig. 6A–B). TRAP stained histologic sections revealed an increase in osteoclasts/mm bone in PTH- versus PTH+ZA-treated specimens (Fig. 6C). BrdU enumeration also revealed an increase in proliferative cells in PTH-treated implants (Fig. 6D). The number of osteocytes per bone area, which reflects bone formation activity, was significantly increased in specimens treated with PTH, PTH+ZA, and ZA versus vehicle-treated specimens (data not shown). There was also a trend towards an increase in total bone within endogenous vertebrae treated with PTH+ZA and a trend towards an increase in marrow cellularity of PTH-treated vertebrae (data not shown).

Biochemical assays were performed to evaluate the effects of PTH, vehicle, ZA, and PTH+ZA treatment on serum TRAP 5b, OCN, and calcium levels. Serum OCN levels were significantly increased in PTH versus vehicle, ZA, and PTH+ZA-treated mice (Fig. 6E). PTH treatment also significantly increased serum TRAP-5b levels versus levels in mice treated with vehicle, ZA, and PTH+ZA (Fig. 6F). Serum calcium levels were unaltered in all treatment groups (data not shown).

MicroCT analysis revealed an increase in total bone volume of implants treated with ZA, PTH, and PTH+ZA versus vehicle-treated implants (Fig. 7A–B). BMD was also significantly increased in implants treated with ZA and PTH versus vehicle-treated implants and in PTH+ZA versus vehicle-, ZA-, and PTH-treated implants (Fig. 7C). BMC was significantly increased in implants treated with PTH versus vehicle and in implants treated with PTH+ZA versus vehicle-, ZA-, and PTH-treated implants (Fig. 7D). An increase in TMD was observed in implants treated with PTH+ZA versus controls and all other treatment groups (Fig. 7E). TMC was significantly increased in ZA- and PTH-treated ossicles versus ossicles treated with vehicle, and in PTH+ZA-treated implants versus controls and implants treated with ZA and PTH alone (Fig. 7F). The total volume of bone (i.e. the absolute amount of bone formed) was increased over controls in all treatment groups (Fig. 7G).

Luciferase positive BMSCs were treated with PTH and ZA *in vitro* to rule out a direct effect of these agents on luciferase expression levels. The luciferase activity of cells measured at after 0, 1, 4, 8, and 12hrs of treatment was similar, regardless of the type of treatment applied (data not shown), indicating that treatment of BMSCs with PTH and ZA did not directly affect luciferase activity.

Discussion

Despite extensive *in vivo* and *in vitro* studies conducted to investigate the effects of anabolic PTH treatment on bone, the mechanisms of its actions remain unclear. The purpose of the present study was to provide insight into how intermittent PTH treatment affects osteogenic cells at various stages of differentiation and to examine effects on cell proliferation *in vivo*. Utilizing a novel osteoregeneration model, PTH was found to exert differential effects on bone, depending on the stage of development. Previous *in vitro* studies have also shown that PTH exerts temporal effects on osteoblastic cells, depending on when treatment is initiated and the duration of PTH administration [21–23]. In this study, 3 weeks of intermittent PTH treatment induced an anabolic response in ossicles of all groups; however, the anabolic response was the most pronounced in implants with treatment initiated 1 week after BMSC implantation (group 2). MicroCT data also showed an increase in bone parameters in PTH-treated ossicles in all groups, with the greatest increase in total bone volume observed in group 2 ossicles. In addition, levels of OCN, a bone turnover marker, were increased in PTH-treated ossicles. OCN was significantly increased in PTH-treated implants with treatment initiated 1 week after implanting cells and decreased in later treatment groups, confirming that the implanted BMSCs in each group were at different stages of differentiation when PTH treatment was initiated. This is further supported by data from a previous study where early events of PTH action in 1 week and 2 week old BMSC implants were evaluated [15]. OCN mRNA was low in 1 week old ossicles and increased significantly at 2 weeks, confirming that PTH targets cells at the stage of transition from pre-osteoblasts to mature matrix producing osteoblasts. Further, the reduction in numbers of osteoblasts and increase in osteoclasts observed as ossicles matured provided evidence that the implanted BMSCs in each treatment group were at different stages of differentiation.

Bioluminescence was used to track luciferase positive BMSCs in a temporal manner. Since implanted BMSCs were isolated from mice expressing luciferase, observed alterations in bioluminescent-positive signals were due to changes in implanted donor mesenchymal cell numbers, instead of cellular recruitment from the host. This is a novel model system that allows tracking of bone cell activity in real time *in vivo* and to our knowledge has not been reported previously in the field to track bone forming cell proliferation. These data are consistent with our earlier study that showed cellular activity in ossicles diminished with time and the ossicles no longer responded to PTH [18]. Hence, PTH does not appear to act via cell recruitment in this model. In the present study, luciferase activity of PTH-treated implants was increased after 1 week of intermittent treatment with peak activity observed after 5 weeks of treatment. These data were supported by the enumeration of BrdU positive cells, which revealed a significant increase in proliferative cells in PTH-treated specimens. In previous studies we found that PTH increased BrdU positive cells in the bone marrow [17,19] and that PTH increased early osteoblast proliferation *in vitro* with elevated cyclin D1 levels in *in vivo* BMSC implants treated with PTH for 1 week [24]. It has also been reported that low-dose intermittent PTH treatment increased the number of mesenchymal cells in the early stage of chondrogenesis in an experimental fracture healing model [25]. Although evidence has been provided showing that PTH increases cell proliferation, the mechanism by which this occurs remains unclear. Although a contribution of PTH to augment cell numbers via an anti-apoptotic mechanism can not be totally ruled out, the increase in BrdU labeling, and our other studies of increased proliferation via PTH support a pro-proliferative impact of PTH [24]. If the PTH impact was primarily via an anti-apoptotic mechanism, massive apoptosis would have to be the norm for control ossicles since the increase in luciferase tagged cells was so dramatic in the several days after PTH administration. We have also evaluated caspase-3 cleavage products in ossicles treated with PTH for several days and found no difference when compared to vehicle ossicles (data not shown). The sum of these findings suggest that in this model system, PTH is strongly pro-proliferative.

Bisphosphonate treatment increases bone mineral density due to decreased bone turnover at the tissue level, with osteoclast proliferation and recruitment of osteoclast precursors inhibited at the cellular level [26–28]. It was recently reported that treatment with zoledronate (ZA) exerted an antiproliferative effect on immortalized human fetal osteoblast cells and enhanced differentiation and bone-forming activities [29]. In the present study, when ZA alone was administered to mice with luciferase positive BMSC implants, there was no evidence of any alteration in cell numbers. ZA treatment was combined with PTH therapy to evaluate the synergistic effects of these two agents on bone growth. To maximize the potential blocking effects of PTH, a high dose of ZA (3µg/kg/day) was used. When ZA was administered concomitantly with PTH, a reduction in luciferase activity was observed in relation to PTH administration alone, suggesting that ZA hindered the proliferative effects of PTH. Since the effects of PTH on BMSC proliferation were obstructed by ZA, these data suggest that the PTH-mediated increase in cell proliferation is indirect and possibly via a cell responsive to a bisphosphonate such as a hematopoietic cell. It has been reported that bisphosphonates may act on osteoclast precursors and on bone resident macrophages, a source of cytokines stimulating bone resorption, leading to impaired osteoclast recruitment and activity [30–32]. Studies conducted in our laboratory have shown that intact *c-src* mutant mice exhibited increases in bone volume in response to intermittent PTH treatment, suggesting that active bone resorption may not be necessary for the PTH response [36].

Numerous multinucleated TRAP-positive cells are present in the *c-src* mutant mice but these cells do not form ruffled borders and resorption lacunae [34–35]. In the present study, TRAP-positive cells were detected in ossicle sections treated with ZA alone and PTH and ZA. However, it is likely that the ZA hindered the PTH-upregulated activity of these cells since bisphosphonates directly inhibit osteoclast activity and biochemical markers of bone turnover were reduced. Notably, ZA hindered the PTH upregulation of osteoclasts and serum TRAP activity but osteoclasts were still present and ZA-treated groups did not have osteoclast numbers below baseline controls.

ZA hindered the proliferative effects of PTH on BMSCs and blunted the PTH increase in serum OCN activity, which might suggest a diminution of bone turnover. However, seemingly paradoxical, an increase in the percent total bone was observed in implants treated with PTH and ZA. Recent clinical studies conducted to evaluate the potential synergistic effects of administering PTH and a potent bisphosphonate, alendronate, showed that alendronate blunted the anabolic actions of PTH in bone [12–13]. Conversely, it has been shown that combination therapy with PTH and alendronate may be a viable treatment option for postmenopausal osteoporosis when PTH treatment is added to alendronate treatment [36]. Although it has been shown that bisphosphonates may clinically impair the anabolic actions of PTH, this is not always the case in rodent models. Studies have shown that co-treatment with PTH and alendronate increased its anabolic effect [37], whereas pre-treatment with bisphosphonates blunted but did not abolish the anabolic response to PTH on the skeleton of oophorectomized mice [38]. In the present study, the concurrent initiation of PTH therapy and therapy with ZA increased percent bone volume in comparison to ossicles treated with PTH or ZA alone. It is possible that ZA exerted an anti-proliferative effect on implanted BMSCs in early osseous development but increased osteoblastic differentiation, resulting in an increase in total bone volume. Alternatively, the increase in proliferation in response to PTH may be permissive for increased bone formation but not solely sufficient for an increase in bone. Notably, the high dose of the potent bisphosphonate, ZA used here may have different effects than alendronate.

Recent *in vitro* studies suggest that bisphosphonates impact both bone resorption and bone formation [39–42]. It was reported that bisphosphonates inhibit osteoclastic activity and exert an anabolic effect on osteoblasts by altering osteoblast-specific gene expression and enhancing differentiation of human BMSCs [39–42]. Bisphosphonates may also contribute to increasing

the life span of osteoblasts through mechanisms that inhibit osteoblast apoptosis [43]. Data from the present study showed that ZA blocked the PTH-increase in biochemical markers of bone turnover and TRAP-positive osteoclasts and the PTH-mediated increase in marrow cell proliferation. The data points of PTH-induced luciferase activity were blunted most dramatically at days 18 and 24, however the serum and cellular readouts were performed on samples at the study endpoint. Since the ZA had less of an effect in reducing the early rise in PTH-stimulated luciferase activity, it is possible that PTH could have acted before the ZA to increase a wave of osteoblastic cells before ZA blocked the increase. This early wave of increased proliferation could have been sufficient to drive up the bone volume, and then the continued ZA administration was effective at reducing PTH-mediated increases in osteoclasts, with the result being greater bone area in the PTH and ZA samples. It is possible that if the timing of the ZA were altered to evoke an earlier block in the PTH proliferative effects that the end levels of bone area and bone volume would be more reflective of the clear blunting that ZA had on PTH-mediated dynamic and static measures of cell proliferative activity and serum biochemical markers. In addition, since bisphosphonates act via their ability to integrate with the matrix of bone, the early administration of ZA may have not been as effective since very little mineralized matrix is found in the ossicles at 1 week after implantation.

The clear blunting of PTH-mediated increases in cell proliferation without significant impact on percent bone volume raises interesting considerations for combinatorial therapy. In a study performed to evaluate oncogenicity of PTH, rats were administered daily subcutaneous injections of PTH for 2 years, resulting in a substantial increase in new bone formation, altered bone architecture, and osteosarcoma bone proliferative lesions [44–45]. It is reasonable to speculate that combining ZA with PTH treatment could obstruct overly deleterious proliferative effects of PTH on osteogenic cells, while still promoting a desired augmentation in percent bone volume.

In summary, findings from this study suggest that intermittent PTH treatment exerts differential effects on ossicles derived from implanted BMSCs, depending on the stage of osseous development when treatment is initiated. Studies conducted to delineate the impact of PTH on cell proliferation *in vivo* revealed a significant increase in cell numbers in implants treated with PTH, with cell numbers elevated in comparison to vehicle-treated implants over time. In addition, combining ZA with PTH treatment resulted in a reduction in cell proliferation without reducing the PTH-mediated increase in percent bone. In conclusion, results from our studies support the hypothesis that combinatorial therapy with temporal consideration is worth further investigation in the treatment of bone disorders.

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Figure 1.

Experimental design and histomorphometric analysis of undecalcified ossicles. Bone marrow stromal cells (BMSCs) were isolated from C57BL/6 mice, cultured as described, then implanted into athymic mice. (A) 3 weeks (21 days) of anabolic PTH (1–34) or vehicle (0.9% saline) treatment was administered to mice 1 day (group 1), 1 week (7 days) (group 2), 2 weeks (14 days) (group 3), or 3 weeks (21 days) (group 4) after BMSC implantation. (B) Representative tetrachrome stained and calcein fluorochrome labeled sections of ossicles treated with PTH or vehicle for 3 weeks, initiated 1 day (G1), 1 week (7 days) (G2), 2 weeks (14 days) (G3), and 3 weeks (21 days) (G4) after implanting BMSCs. (C) Total bone area was significantly increased in the tetrachrome stained sections for group 1, 2, and 4 PTH-treated

ossicles. (D) PTH significantly increased osteoblasts/mm bone in groups 1–3 implants (n=4/treatment). (E) TRAP-positive osteoclasts/mm bone were significantly increase in PTH-treated implants in group 4 (n=3–4/treatment). (F) Histomorphometric examination of double calcein labeling of vertebrae harvested from mice in group 2 treated with vehicle or PTH for 3 weeks (21 days) (n=4/treatment). (G) Mineralizing surface/bone surface (MS/BS), (H) bone formation rate (BFR), and (I) mineral apposition rate (MAR) were quantitatively increased in PTH-treated vertebrae. Data expressed as mean $\pm$ SEM.

Figure 2.

Micro-computed tomography (microCT) analysis. (A) Representative reconstructed microCT images of ossicles treated with vehicle or PTH for 3 weeks (21 days), initiated 1 day (G1), 1 week (7 days) (G2), 2 weeks (14 days) (G3), or 3 weeks (21 days) (G4) after implanting BMSCs. (B) PTH increased (B) bone volume fraction (BVF), (C) bone mineral density (BMD), and (D) bone mineral content (BMC) of implants from groups 2 and 3. At the tissue level, intermittent PTH treatment increased (E) tissue mineral density (TMD) in ossicles from groups 2 and 3, and (F) tissue mineral content (TMC) of ossicles in groups 2–4 (G1, n=3/treatment; G2, n=6/treatment; G3, n=5–6/treatment; G4, n=6 vehicle and n=2 PTH). Data expressed as mean ± SEM.

Figure 3.

Experimental design and bioluminescent imaging of implants treated with PTH or vehicle. (A) Mice with luciferase expressing BMSC implants received 3 weeks (21 days) or 9 weeks (63 days) and 1 week (7 days) or 5 weeks (35 days) of PTH or vehicle treatment, initiated 1 week (7 days) after cell implantation. (B) Luciferase positive implants in immunocompromised mice at baseline (BL), luciferase activity peak (D43), and on date of sacrifice (D70). (C) The most dramatic increase in BLI-positive signals was observed after 1 week of PTH treatment and peaked on day 43 after 35 days (5 weeks) of PTH treatment. A subset of ossicles was harvested after 3 weeks (21 days) of treatment (n=8 vehicle and PTH) and all animals were sacrificed and the remaining implants removed after 9 weeks (63 days) of treatment (n=8 vehicle and PTH). Data expressed as mean $\pm$ SEM.

Figure 4.

Representative H&E, BrdU, and luciferase stained sections of luciferase positive ossicles. (A) Ossicles treated for 3 weeks (21 days) or 9 weeks (63 days) and 1 week (7 days) or 5 weeks (35 days) with PTH or vehicle (0.9% saline) with treatment initiated 1 week (7 days) after BMSC implantation. (B) The total percent bone was significantly increased in ossicles treated for 3 weeks (21 days) with PTH (n= 8) vs. vehicle (n=8). (C) Representative BrdU and (D, E) luciferase stained sections treated with PTH for 1 week (7 days) or 3 weeks (21 days), respectively. Enumeration of BrdU positive cells in the marrow revealed an increase in the percent positive cells with PTH treatment for (F) 1 week (7 days) (n=4 vehicle and n=3 PTH) and (G) 5 weeks (35 days) (n=3 vehicle and n=5 PTH). Data expressed as mean $\pm$ SEM.

Figure 5.

Luciferase activity of vehicle, zoledronic acid (ZA), PTH, and PTH+ZA treated BMSC implants. (A) Mice with luciferase positive implants were treated with vehicle, ZA, PTH, and PTH+ZA for 3 weeks (21 days), initiated 1 week (7 days) after BMSC implantation. (B) Representative bioluminescent images (BLI) of vehicle, ZA, PTH, and PTH+ZA-treated implants at baseline (D0), D14, and D24. (C) Luciferase activity of PTH-treated implants increased significantly after 1 week (7 days) of treatment and remained higher than the luciferase activity of vehicle, ZA, and PTH+ZA-treated implants over time. When intermittent PTH treatment was combined with ZA treatment, a reduction in luciferase activity was observed. Data expressed as mean $\pm$ SEM (* p <0.05, D14: PTH+ZA vs. Veh, D18: PTH vs. PTH+ZA, PTH vs. Veh, D24: PTH vs. PTH+ZA; ** p <0.01, D14: PTH vs. Veh, D24: PTH vs. Veh).

Figure 6.

Impact of zoledronic acid (ZA) on PTH actions in ectopic ossicles and serum markers of bone turnover. (A) Representative H&E stained images of ossicles treated with vehicle, ZA, PTH, and PTH+ZA for 3 weeks (21 days). (B) Total percent bone was significantly increased in PTH vs. vehicle and PTH+ZA vs. vehicle, ZA, and PTH-treated ossicles (n=7 vehicle, n=11 ZA, n=8 PTH, n=11 PTH+ZA). (C) TRAP positive osteoclasts/mm bone in ossicles (n=6 vehicle, n=10 ZA, n=8 PTH, n=11 PTH+ZA). PTH significantly increased TRAP-positive osteoclasts/mm bone vs. PTH+ZA-treated implants. (D) BrdU enumeration revealed a significant increase in the percent BrdU positive cells in the marrow of PTH-treated implants versus vehicle-treated implants (n=5 vehicle, n=9 ZA, n=6 PTH, n=8 PTH+ZA). (E) Serum OCN levels were significantly increased in PTH vs. vehicle, ZA, and PTH+ZA-treated mice (n=5–7 mice/group). (F) Levels of serum TRAP 5b were also significantly increased in PTH vs. vehicle, ZA, and PTH+ZA-treated mice (n=7–8 mice/group). Data expressed as mean $\pm$ SEM.

Figure 7.

Micro-computed tomography (microCT) analysis of ossicles treated with vehicle, zoledronic acid (ZA) alone, PTH alone, and PTH+ZA for 3 weeks (21 days). (A) Representative reconstructed microCT images of ossicles from each treatment group. (B) Total bone volume (BVF) was significantly increased in ossicles treated with ZA, PTH alone and PTH+ZA versus those treated with vehicle and in implants treated with PTH+ZA versus ZA. (C) Bone mineral density (BMD) was significantly increased in ZA and PTH-treated ossicles versus controls and in PTH+ZA-treated implants versus vehicle-, ZA-, and PTH-treated implants. (D) Bone mineral content (BMC) was significantly increased in ossicles treated with PTH versus ossicles treated with vehicle. There was also an increase in BMC in ossicles treated with PTH+ZA

versus all other treatments. (E) Tissue mineral density (TMD) was significantly increased in ossicles treated with PTH+ZA vs. vehicle-, ZA-, and PTH-treated ossicles. (F) Tissue mineral content (TMC) was significantly increased in PTH- and ZA-treated ossicles versus ossicles treated with vehicle and in PTH+ZA-treated versus vehicle-, ZA-, and PTH-treated implants. (G) ZA, PTH, and PTH+ZA significantly increased total bone volume in implants versus ossicles treated with vehicle. Data expressed as mean $\pm$ SEM (n=15–16 ossicles/group).

Table 1
Quantitative real time RT-PCR analysis of MGP and OCN expression in ectopic ossicles treated with PTH or vehicle for 3 weeks.

		MGP/GAPDH			OCN/GAPDH		
		Vehicle (V/V)	n	PTH (P/V)	n	Vehicle (V/V)	PTH (P/V)
Group 1		1.0 ± 0.51	3	1.93 ± 0.92	3	1.0 ± 0.81	1.50 ± 0.10
Group 2		1.0 ± 0.34	4	2.31 ± 0.43	7	1.0 ± 0.17	3.53 ± 0.35***
Group 3		1.0 ± 0.17	7	0.82 ± 0.28	8	1.0 ± 0.05	1.76 ± 0.41
Group 4		1.0 ± 0.49	6	1.16 ± 0.10	5	1.0 ± 0.33	1.49 ± 0.56

Mean ± SEM

p<0.001, PTH versus Vehicle