Determination of Dual Effects of Parathyroid Hormone on Skeletal Gene Expression in Vivo by Microarray and Network Analysis*

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Parathyroid hormone (PTH) stimulates bone formation when injected daily but causes severe bone loss with continuous infusion. The mechanism of its paradoxical effects is still elusive. In this study, we compared changes in the gene expression profile in bone induced by intermittent or continuous treatment with three different PTH peptides, PTH-(1–34), -(1–31), and -(3–34), in Sprague-Dawley female rats. PTH-(1–34) regulated numerous genes (~1,000), but differentially, in both regimes. PTH-(1–31) regulated a similar number of genes in the intermittent regime but fewer in the continuous regime, consistent with its less potent catabolic effect. PTH-(3–34) regulated very few genes in both regimes, which suggests the protein kinase C pathway plays a limited role in mediating the dual effects of PTH, whereas the cAMP-dependent protein kinase A pathway appears to predominate. In the intermittent treatment, many genes encoding signaling mediators, transcription factors, cytokines, and proteases/protease inhibitors are regulated rapidly and cyclically with each PTH injection; genes associated with skeletal development show a slowly accruing pattern of expression. With continuous treatment, some genes are regulated from 6 h, and the mRNA levels are sustained with a longer infusion, whereas others show a kinetic decrease and then increase later. Significant up-regulation of genes stimulating osteoclastogenesis in the anabolic regime suggests a provocative and paradoxical theme for the anabolic effect of PTH that a full anabolic response requires a transient up-regulation of genes classically associated with a resorptive response. Ingenuity pathway analysis was performed on the microarray data. A novel signaling network was established that is differentially regulated in the two PTH treatment regimes. Key regulators are suggested to be AREG, CCL2, WNT4, and cAMP-responsive element modulator.

PTH,2 administered intermittently, stimulates bone formation in contrast to antiresorptive agents, which reduce bone resorption. Concerns with the limited improvement of antiresorptive treatments in severe osteoporosis led to a search for alternative anabolic agents (1). Teriparatide, recombinant human parathyroid hormone consisting of the first 34 of the 84 amino acids of human parathyroid hormone, has been shown to reduce significantly the risk of both vertebral and nonvertebral fractures in postmenopausal women and to significantly increase bone mineral density (2). It has become the only anabolic agent currently approved for the treatment of osteoporosis in the United States.

Bone is a highly specialized form of connective tissue and dynamic organ in all higher vertebrates; it undergoes continuous regeneration. Bone remodeling occurs at discrete sites within the skeleton and proceeds in an orderly fashion, with coupling of osteoclastic resorption and osteoblastic formation (3). This physiological process is coordinated and tightly regulated by local and endocrine factors to ensure that there is a balance between bone formation and bone resorption (4). PTH is known to regulate bone metabolism directly by its receptor on the osteoblast plasma membrane; binding of PTH to its receptor causes release of cytokines and growth factors from the osteoblast. As a result, it indirectly affects osteoclasts and other bone cells in a paracrine fashion (5, 6). Upon binding to its receptor, early signaling events associated with transient increases in PTH levels are sufficient to induce the anabolic response. However, late events associated with continuously elevated PTH lead to the catabolic response and bone disorganization.
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This approach has been successfully used to analyze changes in blood leukocyte gene expression patterns in human subjects receiving an inflammatory stimulus (17) and other systems (18, 19). In this study, we applied this knowledge-based approach analysis, and for the first time, we demonstrate the interactions and network of signaling elicited by anabolic and catabolic treatment regimes of PTH peptides.

EXPERIMENTAL PROCEDURES

Chemicals

Synthetic PTH-(1–34) (human), PTH-(3–34) (bovine), and PTH-(1–31) (human) were purchased from Bachem (Torrance, CA). All other chemicals used were from Sigma if not specified.

Animals

Sprague-Dawley rats (3-month-old female, weight about 250 g) were purchased from Taconic Farms, Inc. (Germantown, NY). For anabolic models, PTH-(1–34), -(1–31), and -(3–34) peptides dissolved in vehicle (0.9% saline with 1% rat heat-inactivated serum) in a final volume of 200 μl were injected subcutaneously daily for the indicated times at 8 μg/100 g/day to 3-month-old female Sprague-Dawley rats for up to 14 days (10–12, 20, 22; at least four animals per group). To permit measurements of bone apposition rates and the calculation of dynamic parameters in 14-day-treated rats, animals received two subcutaneous injections of calcine, at 9 and 2 days before being killed (2 mg/100 g).

For catabolic models, PTH-(1–34), -(1–31), and -(3–34) dissolved in vehicle (0.9% saline with 1% rat heat-inactivated serum) in a final volume of 200 μl were continuously infused into animals at a nominal pumping rate of 1 μl/h by Alzet osmotic pumps (DURECT Corp.) implanted subcutaneously; continuous infusion of PTH-(1–34), -(1–31), and -(3–34) peptides was at 4 μg/100 g/day for the indicated times to 3-month-old female Sprague-Dawley rats (12, 22; at least four animals per group). The animal protocols were approved by the Robert Wood Johnson Medical School Institutional Animal Care and Use Committee.

Tissue Collection and Processing

At the time of implantation of osmotic pumps into rats with anesthesia, 1 ml of blood was collected by a glass capillary tube being inserted at the median canthus to gain access to the rat orbital venous plexus, the area that offers a readily accessible site for blood collection. Animals were euthanized using CO₂ right after infusion ceased or at the indicated time after injection. Long bones, both pairs of femora and tibiae, were harvested. After the animals were unconscious, a cardiac puncture was performed to collect blood (4 ml) to measure serum calcium, phosphate, exogenous hPTH-(1–34) levels, and markers of bone turnover. For histomorphometry and peripheral quantitative computed tomography, femora or tibiae were preserved in 70% ethanol until they were processed for further fixation and plastic embedding in methylmethacrylate resin, cutting sections, and Goldner’s Trichrome staining for microscopic analysis of the various histomorphometric parameters.
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Blood was left at room temperature for at least 30 min before centrifuging at 200 X g for 10 min to separate sera. Total calcium was determined by a kit from Sigma. Serum phosphate levels were determined using an automatic hemogas analyzer by the Department of Pathology and Laboratory Medicine at UMDNJ-Robert Wood Johnson Medical School.

**RNA Isolation**

Femora were cut, and all connective tissue, including periosteum, completely removed. The distal epiphysis, including the growth plate, was removed, and a subjacent 3-mm wide band of the metaphyseal trabecular primary spongiosa was dissected and kept in RNAlater (Qiagen) at 4 °C until mRNA analyses were performed. Total RNA was extracted by homogenization in TRI Reagent® (Sigma) using a PT 10–35 Polytron homogenizer (Kinematica GA, Switzerland) as recommended by the manufacturer. Isolated RNA was quantitated using spectrophotometry by measuring the absorbance at 260 nm, and the 260/280 nm ratio was calculated, and RNA was kept only when this ratio was 1.9–2.0 to ensure the absence of protein contamination and limited RNA degradation. The integrity of sample RNAs (collected at 1 h after the last injection of 14-day intermittent treatment or right after 14-day infusion ceased) used for microarray analysis was confirmed by capillary electrophoresis resolving 18 S and 28 S ribosomal RNA on an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

**Enzyme-linked Immunosorbent Assay (ELISA)**

The exogenous hPTH-(1–34) levels in sera of rats treated with 14-day infusion were examined by ELISA kit (Immunotopics International, San Clemente, CA). This kit specifically detects bioactive hPTH-(1–34). RatLaps ELISA was performed using sera collected from rats in the 14-day infusion groups to measure collagen type I C-telopeptide degradation products (CTX-I) using a specific monoclonal antibody in a competitive ELISA form (Nordic Bioscience, Diagnostics A/S Herlev, Denmark) according to the manufacturer’s instructions.

**Histology**

Longitudinal undemineralized sections (5 μm thick) were cut from methyl methacrylate plastic-embedded blocks of frontal sections of tibiae or femora, using a Riechert-Jung microtome. These were mounted on gelatin-coated 25 × 75 frosted slides and stained with Goldner’s Trichrome stain for the static measurements, and additional sections were cut at 10 μm and left unstained for dynamic (fluorescent) measurements of calcine injections. A region of interest was selected that was exactly 1 mm distal to the growth plate, and extending 2 mm downward (thereby avoiding the primary spongiosa) through the metaphysis of the tibia or femora. The region of interest is the same size in every animal, regardless of the shape of the section. Standard bone histomorphometry was performed by the methods of Parfitt et al. (21). Image analysis software (SPOT Advanced; Diagnostic Instruments, Inc.) was used for tibial histological analysis. Femoral histological analysis was performed by the Department of Pathology at the University of Alabama, Birmingham.

**Microarray Analysis**

Microarray analyses were performed utilizing 15 μg of total RNA. It should be noted that our analyses are focused on the cancellous and endosteal cortical bone compartments because the periosteum was removed. Affymetrix Rat Genome RAE 230A chips, including probe sets for 5,399 known rat genes and 10,467 ESTs, were used for all intermittent treatment regimen experiments (four replicates in vehicle and PTH-(1–34)-treated samples, three replicates in PTH-(1–31)-treated and PTH-(3–34)-treated samples); RAE 230 2.0. chips (contains over 31,000 probe sets representing more than 30,200 transcripts and variants, including 5,399 known rat genes, the same genes as 230A, and 25,643 ESTs, an upgrade from 230A, and the standard chip available at the time of the continuous experiment) were used for all 14-day continuous treatment regimen experiments (four replicates with all PTH peptides and vehicle-treated samples). Gene expression values were determined using GCOS software (version 1.4 from Affymetrix) with global scaling normalization method (target value 150). The gene expression data after GCOS were further analyzed using GeneSpring software from Agilent or ArrayTools software from the Biometric Research Branch of the NCI, National Institutes of Health.

**Detecting Significantly Changed Genes**—We used the following two methods to determine significantly regulated genes by PTH peptide/administration categories, 1) For the ANOVA test, we conducted ANOVA analysis using GeneSpring software from Agilent with a parametric test, variances not assumed equal (Welch ANOVA). Then we performed multiple testing corrections using the Benjamini and Hochberg False Discovery Rate. 2) For class comparison, we used ArrayTools developed by NCI Biometric Research Group to conduct class comparison to determine significantly regulated genes between different PTH peptide/administration groups.

**Systematic Analysis of Differentially Expressed Genes in Different Peptide Treatments**—Because of the fact that the two sets of experiments were conducted using different Affymetrix chipsets, we used the following steps to determine the significantly regulated genes in each experiment. First we identified the significantly regulated gene probe sets compared with control in each treatment regime, intermittently or continuously, using the class comparison method provided in ArrayTools at a parametric p value cutoff of 0.001. Then we imported the significantly regulated genes into an Access data base. We queried the data base to obtain the commonly regulated genes in both treatment regimens and genes that are uniquely affected by a single treatment regime.

**Real Time RT-PCR**

Two-step real time RT-PCR was performed to confirm the differential expression results obtained by microarray experiments and the expression levels at indicated time points during intermittent and continuous treatments as indicated. As described earlier (9), total RNA was isolated using TRI Reagent® (Sigma) followed by purification with the RNeasy kit (Qiagen). A TaqMan® reverse transcription kit (Applied Biosystems) was used to reverse transcribe mRNA into
TABLE 1
Bone histomorphometric measurements of 14-day intermittent injection of PTH peptides

Femora were removed 1 h after the last injections and were preserved in 70% histological ethanol until they were processed for further fixation, plastic embedding in MMA resin, cutting sections, and Goldner’s Trichrome staining. The abbreviations used are as follows: Ob.S, osteoblast surface; N.Ob, osteoblast number; Oc.S, osteoclast surface; N.Oc, osteoclast number; BS, bone surface; BFR, bone formation rate; BV/TV, bone volume per tissue volume. Data represent the mean ± S.E. n = 4.

|                | Ob.S/BS | N.Ob/BS | Oc.S/BS | N.Oc/BS | BFR/BS | BV/TV |
|----------------|---------|---------|---------|---------|--------|-------|
|                | %       | no./mm  | %       | no./mm  | µm/day | %     |
| PTH-(1–34)     | 45.0 ± 1.4a | 28.6 ± 1.3a | 6.71 ± 1.30 | 1.3 ± 0.16 | 2.2 ± 0.17α | 29.1 ± 2.79b |
| PTH-(1–31)     | 54.3 ± 6.0a | 35.0 ± 3.0a | 7.2 ± 0.53 | 1.3 ± 0.16 | 3.4 ± 0.27α | 26.1 ± 5.09 |
| PTH-(3–34)     | 27.5 ± 7.08a | 18.57 ± 4.7 | 5.01 ± 1.38 | 1.02 ± 0.23 | 1.66 ± 0.34 | 20.5 ± 4.59 |
| Control        | 13.9 ± 4.08  | 9.93 ± 2.74 | 3.88 ± 0.38 | 0.64 ± 0.14 | 1.46 ± 0.18 | 23.8 ± 2.35 |

* α < 0.05
* β < 0.005 versus control by one-way analysis of variance and Tukey’s test.

TABLE 2
Bone histomorphometric measurements of 14-day infusion of PTH peptides

Tibiae were removed after infusion ceased and were preserved in 70% histological ethanol until they were processed for further fixation, plastic embedding in MMA resin, cutting sections, and Goldner’s Trichrome staining. The abbreviations used are as follows: N.Ob, osteoblast number; Ob.S, osteoblast surface; Oc.S, osteoclast surface; N.Oc, osteoclast number; BS, bone surface; BV/TV, bone volume per tissue volume. Data represent the mean ± S.E. n = 4.

|                | Ob.S/BS | N.Ob/BS | Oc.S/BS | N.Oc/BS | BV/TV |
|----------------|---------|---------|---------|---------|-------|
|                | %       | no./mm  | %       | no./mm  |       |
| PTH-(1–34)     | 6.2 ± 0.4a | 5.71 ± 0.1 | 20.13 ± 2b | 5.1 ± 0.6e | 14.78 ± 1e |
| PTH-(1–31)     | 6.4 ± 0.8a | 6.19 ± 0.8 | 29.19 ± 6e | 6.9 ± 1.5a | 20.56 ± 1 |
| PTH-(3–34)     | 3.67 ± 0.3 | 3.57 ± 0.2 | 17.67 ± 3e | 4.02 ± 0.7a | 21.04 ± 2 |
| Control        | 4.70 ± 0.1 | 4.73 ± 0.1 | 8.24 ± 0.7 | 2.18 ± 0.2 | 20.56 ± 1 |

* α < 0.05
* β < 0.005 versus control by one-way analysis of variance and Tukey’s test.

TABLE 3
Bone mineral density of rat tibiae determined by pQCT after intermittent or continuous treatments

Tibiae were removed as described under “Experimental Procedures” and fixed in 70% ethanol prior to the scanning process. Intermittent and continuous control groups were pooled to serve as vehicle values because no differences were found between the two control groups. Data represent the mean ± S.E. One-way ANOVA was performed before Student’s t test. BMD indicates bone marrow density.

|                | Total | Trabecular | Cortical |
|----------------|-------|------------|----------|
| Vehicle (n = 10) | 658 ± 26.0 | 517 ± 18.5 | 774 ± 28.6 |
| PTH-(1–34) injection (n = 6) | 731 ± 21.6e | 612 ± 34.0e | 849 ± 42.8 |
| PTH-(1–34) infusion (n = 4) | 590 ± 38.8e | 482 ± 21.7 | 682 ± 33.4e |

* α < 0.05 by Student’s t test.

cDNA. Following this, PCR was performed on Opticon (MJ Research) using a SYBR® Green PCR core kit (Applied Biosystems). The primers used for the RT-PCR are summarized in supplemental Table 5. β-Actin was used as an internal control.

RESULTS

Efficacy of Different Treatment Regimens (Anabolic Versus Catabolic and Different PTH Peptides)—Prior to performing microarray analyses of femoral RNA from the different treatment regimes, blood chemistry and bone analyses were conducted to ensure that the protocols were effective as reported (10–12, 20, 22) and to compare the effects of the peptides within the two types of administration.

Histomorphometric analyses of the proximal femoral metaphyses confirmed the expected morphological changes that occur with PTH-(1–34) intermittent treatment (22). Substantial increases in bone formation rate, as measured by the labeled surface to bone surface ratio, and in osteoblast parameters were confirmed in intermittent treatment groups relative to control animals (Table 1). Consistently, 14-day intermittent administration of PTH-(1–34) increased tibial total and trabecular bone mineral density level (Table 3). PTH-(1–31) also showed anabolic effects by significantly increasing osteoblast parameters as well as bone formation rate.

Increased serum calcium concentrations were found in animals intermittently treated with PTH-(1–34) and PTH-(1–31) at 2 h after the last injection, which returned to basal within 24 h, but there was no significant elevation of calcium by PTH-(3–34) (Fig. 1A). Phosphate levels in animals treated with 14-day injections of PTH-(1–34) also decreased at 1 h after the last injection (Fig. 1B).

We measured the hPTH-(1–34) level in continuously treated animals three times (at the beginning, the middle, and at the end of infusion) to ensure the performance of the osmotic pumps (Fig. 1C) and found it was elevated at both times. We confirmed the catabolic effect of PTH-(1–34) in the continuous regime by detecting an increase in degraded type I collagen levels (Fig. 1D) and a significant decrease in total bone mineral density level in tibiae (Table 3). Significant increases in osteoclast parameters in tibiae were found in the groups infused with PTH-(1–34) and PTH-(1–31) (Table 2). Although a significant decrease in bone volume only occurred in the group infused with PTH-(1–34), significant increases in bone resorption, indicated by the elevation of the carboxyl-terminal product of type I collagen, were found in both PTH-(1–34) and -(1–31) but not in PTH-(3–34)-infused groups (Fig. 1D).

Moderate but significant serum calcium elevation and phosphate decrease (Fig. 1E) were found in rats treated with a 14-day infusion of PTH-(1–34). Similarly, calcium increased with infusion of PTH-(1–31) and PTH-(3–34), although changes in phosphate were not significant (Fig. 1E).

From the bone sections, we can easily observe the strong similarity in effects of PTH-(1–34) and PTH-(1–31) in both treatment regimes as follows: increasing trabecular bone volume both in tibiae and femora with intermittent injection...
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![Graphs](Image)

**FIGURE 1. Serum chemistry of rats treated with PTH peptides by the intermittent or continuous protocols.** A and B, serum total calcium and phosphate levels in rats receiving PTH peptides by intermittent injections for 14 days. Samples were collected after the animals were unconscious through a cardiac puncture at the indicated time after injection. Data represent the mean ± S.E. (n = 4); *, p < 0.05 compared with control groups according to the time points by Student’s t test. C, exogenous serum hPTH-(1–34) levels in Sprague-Dawley rats before and after 7- and 14-day infusion or 1 h after the last injection with 14-day intermittent injection. Samples were collected either at the time of implantation (day 0) and exchanging (day 7) of osmotic pumps into rats with anesthesia by a glass capillary tube being inserted at the medial canthus to gain access to the rat orbital venous plexus, or after the animals were unconscious (day 14) through a cardiac puncture. Data represent the mean ± S.E. (n = 4); *, p < 0.05 compared with control groups by Student’s t test. D, serum concentration of carboxyl-terminal telopeptide α1 of type I collagen in Sprague-Dawley rats after 14-day PTH peptide infusions. Samples were collected after the animals were unconscious through a cardiac puncture. Data represent the mean ± S.E. (n = 4); *, p < 0.05 (one-tail) by Student’s t test assuming unequal variances. E, serum total calcium and phosphate levels in rats receiving PTH peptides by continuous infusion for 14 days. Data represent the mean ± S.E. (n = 4); *, p < 0.05 compared with the control groups at each time by Student’s t test.

(Tables 1; Fig. 2, A and B; Table 3); strong peritrabecular fibrosis after infusion (Fig. 2C). PTH-(3–34), unlike the other two PTH peptides, failed to induce significant bone responses in either treatment protocol (Fig. 2). Overall, these results suggest that the PKA pathway is the predominant pathway for PTH action in bone, whereas the PKC pathway has a limited role.

**Clustering Analysis Indicates PTH-(1–34)-treated Samples Have Similar Gene Expression to PTH-(1–31)-treated Samples, whereas PTH-(3–34) Is Similar to Vehicle—Microarray analyses were performed as described under “Experimental Procedures.” We used a parametric test, variances not assumed equal (Welch ANOVA), with multiple testing correction by Benjamini and Hochberg False Discovery Rate. Only genes with presence call in at least 3 chips of 14 chips were included in the analysis. We identified 1285 genes showing significant gene expression change with a p value less than 0.05 between any different PTH peptide or vehicle-treated groups in the anabolic (intermittent) experiment, I-control, IP134, IP131, and IP334. Then we used these genes for hierarchical clustering to show their expression value changes across all treatment types by comparing their expression value with the according median for each gene across all samples (Fig. 3A). Consistent with the histomorphometric data that PTH-(3–34) has minor, if any, anabolic effect on bone with intermittent treatment, the gene expression profiles of PTH-(3–34)-treated samples clustered together with vehicle-treated samples. As expected, PTH-(1–31)-treated samples, which have shown significant and similar anabolic effects to PTH-(1–34)-treated samples in Fig. 2A, clustered tightly with samples treated with PTH-(1–34).

Similarly, we identified 3088 genes showing significant gene expression change with p values less than 0.05 between any different PTH peptide and vehicle-treated groups in the continuous treatment experiment. We further trimmed the gene list by filtering for genes with p values less than 0.01 and only included genes with Presence call in at least 3 of 16 chip results. There are 754 genes that passed the filtering criteria to conduct hierarchical clustering with continuously treated samples (Fig. 3B). Our data indicate that continuous treatment with PTH-(3–34) (CP334) induced very limited gene expression changes that cluster together with the controls. CP314 induced the most significant gene expression changes. CP31 also induced some gene expression changes and clusters together with CP314. However, the four CP313 samples were spread on either side of the CP314 samples indicating the variation of gene expression values in CP313-treated animals is relatively high.

**Systematic Analysis of Differentially Expressed Genes in Different Peptide Treatments—**Because of the fact that the two sets of experiments were conducted using different Affymetrix chipsets, we adopted the following steps to determine the significantly regulated genes in each experiment:

Using the class comparison method provided in ArrayTools, we determined PTH-(1–34) intermittent treatment induced 1154 unique probe sets at a parametric p value cutoff of at least 0.001 versus controls. Among them, 345 are known or predicted genes (according to NCBI RefSeq record) with a fold change of at least 1.5-fold uniquely regulated by intermittent treatment with PTH-(1–34) (supplemental Tables 1 and 6); similarly, PTH-(1–34) continuous treatment induced 1342 significantly changed probe sets at a parametric p value cutoff of less than 0.001. There are 462 known or predicted genes uniquely regulated by continuous treatment with PTH-(1–34) by at least 1.5-fold (supplemental Tables 2 and 7). The number of commonly regulated probe sets with both intermittent and continuous PTH-(1–34) treatments is 675. For the commonly regulated probe sets, there are 188 known or predicted genes with at least
1.5-fold change in both treatment regimens (supplemental Tables 3 and 8). Interestingly, all the commonly regulated genes share the same direction of gene expression value change compared with control in both experiments, with the exception of two genes as follows: sfrp2 (secreted frizzled related protein 2) and mepe (matrix extracellular phosphoglycoprotein). The former is proposed to be a secreted Wnt antagonist that directly interacts with Wnt ligands to inhibit signaling (23–25). MEPE was first identified in tumor conditioned medium and osteosarcoma cell lines. Multiple experimental observations support that MEPE is correlated positively to serum Pi and has an important role in negative regulation of mineralization in the skeleton (26, 27).

We repeated these criteria for PTH-(1–31)- and PTH-(3–34)-treated samples. We found intermittent treatment with PTH-(1–31) regulates 1288 probe sets. Among these, 808 probe sets are commonly regulated by intermittent treatment with PTH-(1–34). However, continuous treatment with PTH-(1–31) only causes significant changes in 242 probe sets, much less than the effect on gene expression with continuous treatment with PTH-(1–34). The majority, 176 of 242, of the probe sets can also be found in the continuous PTH-(1–34)-regulated list. PTH-(3–34) has minor effects on regulation of gene expression in either treatment regimen. With intermittent treatment, it only affects 46 probe sets with a significant expression value change at a \( p \) value cutoff of less than 0.001. However, when given continuously, the alteration in gene expression is so minor that no gene with a significant change was detected at a \( p \) value cutoff of less than 0.001. We have plotted the number of the unique regulated (both up- and down-regulated) genes with any two or all PTH peptides in Venn diagrams for intermittent and continuous treatment regimens (Fig. 4).

We conclude that CP134 is more potent in inducing gene expression changes in the rat compared with CP131 and
CP334 as indicated by the number of genes regulated. A notable significant difference between CP134 and CP131 is their effect on the *ctnnb1* gene, which encodes β-catenin, a WNT pathway member that is known to stimulate cell proliferation and repress apoptosis. It is decreased (5-fold) by CP131 treatment and unchanged by the other peptides. This drop in transcription level may indicate CP131 represses cell proliferation.

**Real Time RT-PCR Confirmed Microarray Results**—As expected, many genes that have been demonstrated previously to be PTH-regulated can be found in our lists of genes either as commonly regulated or as uniquely regulated by PTH-(1–34) in the intermittent/continuous treatment regimens. This implies that the microarray results and analyses are reliable. To validate this further, we used quantitative real time RT-PCR to examine the effect of PTH on individual genes, especially on those genes that have never been reported to be regulated by PTH. We examined the following eight genes: *thbs4*, *sfrp4*, *wnt4*, *jun*, *sdc4*, *cxcr4*, *wis2*, and *sfrp2*, which are commonly regulated by both treatment regimens of PTH-(1–34) as shown in supplemental Table 3. We also examined three more genes, *rgs1*, *rgs2*, and *idb4*, which are uniquely regulated by intermittent PTH-(1–34) (supplemental Table 1). Among all the genes we examined, all except *wis2* from the intermittent regimen (1.4-fold) achieve greater than 1.5-fold change in mRNA abundance by real time RT-PCR, although not identical to the stimulation we find in the microarray result (Fig. 5). Hence, the validation rate of our experiment is about 90–100% (one exception out of 11 genes tested with IP134 and zero exceptions in 8 genes from CP134). This further confirmed that we applied very stringent filtering criteria that enabled us to remove false positive genes as much as possible. However, this may also cause us to lose some true positives. The pathway analysis could help us to retrieve these genes because it provides clues as to which pathway is regulated. By performing real time RT-PCR, other members in that pathway could be identified as regulated or unregulated genes. For example, *ctnnb1* was not shown to be regulated in our proposed network. However, its location in the network suggests that it is a key node of the interactions. So we per-
formed real time RT-PCR on ctnnb1 and found it is up-regulated about 1.8-fold both in IP134 and CP134 (Fig. 5A).

**Kinetic Expression of PTH-regulated Genes**—We performed microarray with RNA isolated from animals treated for 14 days to obtain the whole profiles of gene expression in response to anabolic or catabolic effects of PTH treatment regimens. However, the concern may be raised that the gene profiles may contain PTH secondary response genes, which are regulated through some PTH-induced primary response genes or could be the consequence but not the principal contributors of the anabolic or catabolic effects of PTH. To test if the genes are early response genes to either continuous or intermittent PTH administration, we also collected RNA from animals treated with single PTH-(1–34) injection or 6-h infusion (Fig. 5, C and D).

Furthermore, we collected RNA from rats receiving different durations of both intermittent (1, 7, and 14 days) and continuous (6 h and 3, 6, and 14 days) administration of PTH-(1–34) and vehicle. For the intermittent regimen, RNA were also collected at three different time points (1, 2, and 24 h after each injection), which enables us to obtain the kinetic expression pattern of PTH-regulated genes. We found interesting kinetic expression patterns of different genes that we examined by real time RT-PCR. At least two categories of gene expression pattern can be found in both PTH treatment regimens according to their kinetic expression changes. In the intermittent treatment, genes that encode signaling mediators (socs3), transcription factors (c-fos), cytokines (gro1), and proteases/protease inhibitors (spli and tfpi2) are regulated cyclically by each PTH injection; whereas genes associated with skeletal development, such as those indicative of extracellular matrix production and mineralization, show a different pattern of expression. For instance the gene encoding osteocalcin is not up-regulated until after several PTH injections, but then its mRNA level is sustained to the next PTH challenge. Because the stimulation is compared with the vehicle control at each time point instead of the basal for each injection, we are unable to tell if a gene such as osteocalcin has returned to basal at the time of PTH injection. However, we compared all the control vehicle-injected samples at the three time points, 1, 2, and 24 h, and no significant differences in the gene expression levels were found, at least not in the genes we have checked.

In the continuous regimen, similarly extracellular matrix genes (postn and alp) are not stimulated in the 6-h- and 3-day-treated samples but are at 6- and 14-day infusion. The up-regulation of these extracellular matrix genes in the infusion regimen is consistent with an increase in formation activity as reflected by the substantial increases in bone formation rate, as well as bone resorption, and increased bone turnover found in the histomorphometric analyses. The mRNA expression levels of other genes that are involved in different signaling pathways were kinetically regulated by continuous PTH administration. The anti-apoptotic gene bcl2 was up-regulated at the beginning of the infusion and then decreased significantly by 4-fold at 6 days and 6-fold at 14 days. pthl1 was substantially down-regulated after a 3-day infusion but bounced back and was up-regulated 2-fold at 6 days and 14 days of infusion. These results suggest the outcome of the catabolic effects of long term continuous treatment with PTH-(1–34) initiates with a wave of regulation of expression of these genes.

RANKL (tnfsf11) is a well known PTH-regulated gene, which plays a critical role during osteoclastogenesis. It was previously thought to be stimulated by continuous PTH to promote osteoclastic bone resorption. However, to our surprise, we observe a 5-fold increase in the anabolic (intermittent) regime but no significant increase in the catabolic regime by microarray. Consistent with our finding, RANKL is not regulated by continuous PTH treatment in the study from Onyia et al. (12). To confirm this result, we performed real time RT-PCR at different time points for RANKL. A strong and cyclic stimulation of RANKL mRNA is associated with intermittent injections of PTH-(1–34), which increases with increasing injections (Fig. 6A); a moderate but sustained stimulation is associated with continuous infusion of the hormone (Fig. 6D). The level of osteoprotegrin (OPG) and the ratio of RANKL/OPG were also examined because the latter is thought to be more relevant to bone resorption (Fig. 6, B, C, E, and F). With the intermittent PTH-(1–34) regime, the increases in OPG (Fig. 6B) lagged considerably behind the increases in RANKL resulting in a transient stimulation of the RANKL/OPG ratio (Fig. 6C). This may provide the molecular basis for a cyclic sharp up-regulation of bone resorption in the anabolic regime compared with a moderate but constantly elevated level of RANKL/OPG in the catabolic regime (Fig. 6F). The important point for anabolism may be the transient stimulation of osteoclast activity followed by the increase in OPG suppressing osteoclast activation. In contrast, in the continuous treatment, OPG mRNA levels decreased as time went on maintaining an elevated RANKL/OPG ratio.

**Ingenuity Pathway Analysis**—Our Ingenuity pathway analysis (IPA) (see Supplemental Material) indicates IP131 and IP134 induce similar if not identical effects. This conclusion is in agreement with our physiological results showing that they induce similar biological outcomes in the rats. In the network of the signaling pathways regulated by PTH we generated from IPA analysis (see Supplemental Material), potential interactions of three genes are proposed, all of which showed very high levels of gene expression change and are uniquely regulated by intermittent administration of PTH-(1–34) as follows: ccl2 (encodes monocyte chemoattractant protein-1), 142-fold; areg (amphiregulin), 20-fold; and crem (cAMP-responsive element modulator), 20-fold. Based on the IPA network graph, the overexpression of ccl2 and areg appears to be the most outstanding PTH-regulated genes unique to IP134 and IP131, i.e. the anabolic effect of PTH. The network analysis also revealed that differential regulation of Wnt signaling exists in the different PTH treatment regimens. In our accompanying article (42), we proposed a model to explain the importance of the transient stimulation of CCL2 for the dual effects of PTH. We have confirmed the stimulation of these genes in vivo by real time RT-PCR as follows: ccl2, areg, crem, wnt4, and sfrp4 are dramatically changed with intermittent PTH-(1–34) and PTH-(1–31) treatment in vivo (Fig. 7).

**DISCUSSION**

The biological activities of PTH on bone are complex as demonstrated by its catabolic (continuous regimen) and anabolic
(intermittent regimen) effects on the skeleton. Characterizing the genome-wide dynamic regulation of gene expression by PTH-(1–34) is important to investigate the molecular mechanism of its dual actions in bone. However, at the time of the launch of this project, there were no established data for identifying differentially expressed genes in the anabolic versus catabolic animal models. Now, two studies have applied the microarray technique in the study of the effects of PTH in bone in vivo. One compared the transcriptional regulation during anabolic bone formation induced by either estradiol treatment or intermittent PTH treatment. The investigators utilized an ovariectomized mouse model of osteoporosis and transcriptional profiling to identify genes up-regulated by either high dose estradiol or PTH (28). According to their results, several markers of osteoblast activity, including c-fos, RANKL, phex, and pth1r, were consistently up-regulated by intermittent PTH together with the increased expression of cathepsin K, consistent with an increase in osteoclast activity (28). Except for pth1r, all the other genes were also found to be up-regulated by intermittent PTH administration in our model system. With respect to pth1r, we observe a 40% decrease in the intermittent regimen (though it did not meet our stringent criteria to be selected as a regulated gene in supplemental Table 6) but a 2.5-fold increase in the continuous regimen after 14 days (supplemental Table 7). The stimulation of the pth1r mRNA by continuous PTH-(1–34) administration is also reported by Onyia et al. (12). Because pth1r is the receptor for PTH, which initiates and mediates signaling for the hormone in the osteoblast, it is not surprising to see that its mRNA level is kinetically changed in a different fashion with the two different PTH treatments. Two other G protein-coupled receptors, Gprc5c and CXCR4, are also regulated with both PTH administration regimens. Similar to PTH1R, Gprc5c is increased by 2.5-fold in both cases. It belongs to the retinoic acid-inducible orphan G-protein-coupled receptors, but its function is still unknown (29–32). On the contrary, CXCR4 is down-regulated by 2.5-fold in both intermittent and continuous regimes. It is the receptor for CXCL12, stromal cell-derived factor-1, which is up-regulated then down-regulated with continuous PTH treatment (Fig. 5D). Because both the ligand, CXCL12, and the receptor, CXCR4, are down-regulated by continuous PTH treatment, this signaling pathway is likely to be less active with the catabolic PTH protocol at the end of the regime. Previous reports indicated that CXCL12/CXCR4 promoted the chem-
tactic recruitment, development, and survival of osteoclasts (33), and the pathway may be more active early in the PTH-(1–34) infusion.

A recent report on the in vivo study of regulated gene expression profile of PTH studied the anabolic versus catabolic effects of PTH (12). This investigation detailed for the first time the broad profiling of the gene and pathway changes following treatment of intermittent versus continuous PTH-(1–34) in intact female adult rats. They utilized microarray analysis to examine gene expression either 24 h after the last injection or at cessation of the continuous infusion. These results extended previous observations of gene expression changes and revealed the in vivo regulation of bmp3 and multiple neuronal genes by PTH treatment. More than 50% (96 of 174 genes) of the continuous PTH-regulated genes reported in their study can be found in our results. Considering that we used different rat chips in the microarray experiments, and we treated animals for 14 days instead of 6 days, the overlap between the two studies of effects of continuous PTH administration in regulation of gene expression indicates very high reproducibility from one study to the other. Because we also performed microarray with 6-day PTH-(1–34) infused rats (data not shown), we were able to compare our 6-day continuous PTH-(1–34)-regulated gene list with the results from Onyia et al. (12). The overlap is the same as we see with the 14-day results indicating the differences in regulation factors respond to PTH administration very rapidly and thus achieves the optimal time point after PTH administration and thus achieves the most informative and thorough gene expression profile with the continuous treatment regimen. This indicates that the PKC system is likely to be involved in the enhancement of the catabolic effects by interacting with the PKA system. Interestingly, activation of the PKA system is sufficient for the anabolic effects because similar bone phenotypes and very similar changes in gene expression profiling are seen with intermittent administration of either PTH-(1–34) or PTH-(1–31). Intermittent administration of PTH-(3–34) regulated many fewer genes compared with the other two PTH peptides and did not have significant anabolic effects on bone. Thus, we conclude that activation of the PKC system in the intermittent treatment regimen of PTH-(1–34) has a minor role in its anabolic effects. More importantly, PTH-(1–31) demonstrates similar (if not identical) anabolic effects to PTH-(1–34) but is less effective in inducing catabolic effects. This may suggest that PTH-(1–31) could serve as a better anabolic agent than PTH-(1–34) therapeutically.

Besides the complexity of different PTH peptides initiating different signaling pathways and contributing differentially to anabolic and catabolic effects of the hormone, the kinetic profile of PTH-responsive genes is another important factor causing the paradoxical physiological responses to intermittent and continuous PTH administration. We have shown that some extracellular matrix genes changed only after treatment for 1 week, whereas some cytokines, growth factors, and transcription factors respond to PTH administration very rapidly and dramatically but are not sustained when the hormone levels

**Dual Effects of PTH on Gene Expression in Vivo**

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decline. Continuous exposure to the hormone may cause some of the same gene expression changes, but the changes tend to be moderate (usually about 3-fold) and sustained. The different regulation patterns of the same gene provide a clue to understanding the molecular basis of the anabolic and catabolic effects of PTH. It is known that both intermittent and continuous treatment of PTH increase bone remodeling, whereas the anabolic effect is associated with the intermittent protocol, and the catabolic effect is associated with the continuous protocol.

In this study, we demonstrate that a key regulator of osteoclastogenesis and activity, RANKL, is increased with both treatment regimes. It is interesting that the increase in RANKL in the intermittent treatment is very dramatic but transient but increases with increasing injections, whereas in the continuous treatment, its level is moderately up-regulated but sustained throughout the treatment. We think this is because the coupling of osteoclastic bone resorption and osteoblastic bone formation requires transient activated resorption ahead of osteoblasts lining the remodeling sites and building new bone. One of the reasons the anabolic effect is achieved may be because transient up-regulation of RANKL causes short term increased bone resorption that is succeeded by increased bone formation; on the contrary, the catabolic effect produces sustained elevated osteoclastic bone resorption from the sustained up-regulation of RANKL from continuous infusion of PTH. It should be noted that there is a potential contribution of osteocytes to these gene expression data.

Overall, the microarray expression data support a provocative and paradoxical theme, i.e., for PTH therapy to be fully anabolic in nature, there needs to be a transient and cyclic up-regulation of genes classically associated with resorption such as RANKL and interleukin-6. This theme has elements similar to the process of an acute inflammatory response supported by the microarray and kinetic data (apart from RANKL and interleukin-6, CXCL1 and CCL2/MCP-1 are highly increased, see accompanying article Ref. 42). This is in distinct contrast to the catabolic regime where expression of these genes is much lower but, importantly, is sustained.

In summary, we have conducted a comprehensive analysis of the genes that are differentially regulated in vivo by intermittent and continuous treatment with different PTH peptides using a gene array approach. These data support earlier histomorphometric observations in our hands and other publications (37–39). The profiling of gene expression highlights important similarities and differences between intermittent and continuous treatment regimes of PTH. PTH-(1–34) has strong physiological effects and differentially regulates a large number of genes in both treatment regimes. PTH-(1–31) reproduces the effects of PTH-(1–34) in the anabolic regimen but has a lesser catabolic effect in the continuous regimen and thus regulated a lesser number of genes. PTH-(3–34) regulates a very limited number of genes in the intermittent regimen and none in the continuous regimen. We conclude that activation of the PKC system in the intermittent treatment regimen of PTH-(1–34) has a minor role in its anabolic effects but may interact with PKA signaling to enhance the catabolic effect of PTH-(1–34) even though sole activation of the PKC system fails to elicit the catabolic effects.

A large number of genes such as slpi, tfpi2, socs3, and gro1, that were previously not considered to be expressed in bone or to be regulated by either PTH treatment regimen were identified and validated. The kinetic expression patterns of some of the PTH-regulated genes in both treatment regimes were also described. Recently, follicle-stimulating hormone (FSH) was reported to directly regulate bone resorption independent of estrogen (40). Our microarray data found the FSH-responsive gene (fshprh1), which is stimulated by FSH (41), was down-regulated by PTH-(1–34) in both treatment regimes suggesting the interaction of FSH and PTH signaling in bone. The function of these newly identified genes in bone and their precise role, if any, in the anabolic and catabolic bone state deserve further investigation.

Finally, by using the knowledge base of biological networks provided by IPA, we set up a novel signaling network that is differentially regulated in the different PTH treatment regimes and suggest an association of this network with the dual effects of PTH in vivo. This information will be useful for further distinguishing the critical mediators of modeling and remodeling processes and for identification of key targets for therapeutic intervention in disorders of bone metabolism.

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