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Black bear parathyroid hormone and methods of using black bear parathyroid hormone

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Michigan Technological University

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BLACK BEAR PARATHYROID HORMONE AND METHODS OF USING BLACK BEAR PARATHYROID HORMONE

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Assignee:
Michigan Technological University, Houghton, MI (US)

Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

Prior Publication Data

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ABSTRACT
Black bear parathyroid hormone (PTH) and functional fragments thereof are provided. Also provided are methods of using black bear PTH and functional fragments for increasing cAMP in a bone-forming cell; reducing apoptosis in a bone-forming cell; decreasing the ratio of expression levels of the bone protein to Bcl-2 protein in a bone-forming cell; increasing the expression level of one or more of a bone matrix protein, a transcriptional activator, or a transcriptional regulator in a bone-forming cell; enhancing bone mineral density, increasing bone mass, decreasing bone loss, or reducing the incidence of bone fractures, or any combination thereof, in a subject; also provided are antibodies directed against black bear parathyroid hormone (PTH) and functional fragments thereof.

10 Claims, 29 Drawing Sheets


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OTHER PUBLICATIONS


References Cited

Other Publications


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FIG. 1

- Ultimate Stress
  - $R^2 = 0.305$
  - $p = .002$
- Ash Fraction
  - $R^2 = 0.682$
  - $p < .0001$
Osteocalcin (ng/ml) vs. PTH (pg/ml)

FIG. 2

$p = 0.0007$

$R^2 = 0.375$
FIG. 3

3 Month Hibernation Period

Normalized value

ICTP  PICP  Osteocalcin
FIG. 4

PGE2 (pg/cell)

Pre- hibernation

p = 0.058

Hibernation

p = 0.014
FIG. 5

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SEQ ID NO: 5
SEQ ID NO: 6
SEQ ID NO: 7
SEQ ID NO: 8
SEQ ID NO: 9
SEQ ID NO: 10
SEQ ID NO: 11
SEQ ID NO: 12
SEQ ID NO: 13
FIG. 6
FIG. 7

Fold Change (PTH : Vehicle)

Ho: Human PTH 1-34
Be: Bear PTH 1-34

p = 0.047
FIG. 10

Human is 24% higher

p < 0.05

# human vs. vehicle

+ bear vs. vehicle

* Human vs. bear

6 weeks

3 ug/kg  10 ug/kg  30 ug/kg

14 weeks

0.35

0.30

0.25

0.20

0.15

0.10

0.05

0.00

ALP
FIG.
p < 0.05
# human vs. vehicle
+ bear vs. vehicle
* Human vs. bear

10 ug/kg  30 ug/kg

14 weeks
FIG. 16

- sham baseline
- OVX baseline
- OVX vehicle
- hPTH1-34
- bb-PTH1-34

p < 0.05
# human vs. vehicle
+ bear vs. vehicle
* Human vs. bear

% Fatality [%]

6 weeks
14 weeks

3 ug/kg 10 ug/kg 30 ug/kg
Caspase Activity (U)

- Normal culture (not starved)
- Vehicle + starved

Bar graph showing caspase activity levels.
FIG. 19
ANOVA: $p = 0.037$

**FIG. 20**

<table>
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<tr>
<td>B1-84 : H1-84</td>
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<tr>
<td>V : B1-84</td>
</tr>
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Caspase-3/7 Activity By Season

2-Factor ANOVA; p<0.0001
Season p<0.0001
Bear p<0.0001

FIG. 28
FIG. 29

ANOVA: p = 0.034

Ulf Load / BW (N/kg)
BLACK BEAR PARATHYROID HORMONE
AND METHODS OF USING BLACK BEAR PARATHYROID HORMONE

STATEMENT REGARDING FEDERALLY-SPONSORED RESEARCH

This invention was made with United States government support awarded by the National Institutes of Health (NIAMS AR050420, NIDDK DK078407 and AA143990) and the National Science Foundation (IBN-0343515). The United States government has certain rights in this invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority and is a national stage filing under 35 U.S.C. 371 of International Application No. PCT/US2009/066974, filed Dec. 7, 2009. This application is incorporated herein by reference in its entirety.

BACKGROUND

Bone loss diseases are currently a health threat for approximately 44 million Americans, including 10 million with osteoporosis and 34 million with low bone mass and at risk for developing osteoporosis. The number of Americans with osteoporosis is expected to rise by 2020. Consequently, a large number of individuals are at risk for bone fracture due to low bone mass. Approximately 40% of white women and 13% of white men over age 50 are at risk for hip, spine, or forearm fractures within their lifetime. The costs associated with osteoporosis-related fractures were approximately $18 billion dollars in 2002, and are expected to continue climbing. In addition to primary (age-related) osteoporosis, disuse osteoporosis is an important clinical problem, especially for patients chronically immobilized due to stroke or spinal cord injury. Fracture rates double compared to healthy controls in the first year following spinal cord injury and are also elevated compared to healthy controls after the onset of stroke. Disuse increases fracture rates primarily because reduced skeletal loading causes unbalanced bone remodeling which leads to bone loss.

SUMMARY

In certain embodiments, the invention provides an isolated polypeptide comprising at least 10 consecutive amino acid residues of SEQ ID NO: 2 wherein the polypeptide comprises at least one of amino acid residues 1-34 of SEQ ID NO: 2, amino acid residues 1-36 of SEQ ID NO: 2, amino acid residues 1-84 of SEQ ID NO: 2, wherein contacting the bone-forming cell with the polypeptide reduces apoptosis in the bone-forming cell.

In certain embodiments, the invention provides a method of decreasing the ratio of expression levels of Bax protein to Bcl-2 protein in a bone-forming cell comprising contacting the bone-forming cell with an effective amount of at least one polypeptide selected from a polypeptide comprising amino acid residues 1-34 of SEQ ID NO: 2, a polypeptide comprising amino acid residues 1-36 of SEQ ID NO: 2, wherein contacting the bone-forming cell with the polypeptide reduces apoptosis in the bone-forming cell.

In certain embodiments, the invention provides a method of increasing the expression level of a bone matrix protein, a transcriptional activator, or a transcriptional regulator in a bone-forming cell comprising contacting the bone-forming cell with an effective amount of at least one polypeptide selected from a polypeptide comprising amino acid residues 1-34 of SEQ ID NO: 2, amino acid residues 1-36 of SEQ ID NO: 2, wherein contacting the bone-forming cell with the polypeptide increases expression of the bone matrix protein, the transcriptional activator, or the transcriptional regulator in the bone-forming cell.

In certain embodiments, the invention provides a method of enhancing bone mineral density, increasing bone mass, decreasing bone loss, or reducing the incidence of bone fractures, or any combination thereof, in a subject, comprising contacting a bone-forming cell in the subject with an effective amount of at least one polypeptide selected from a polypeptide comprising amino acid residues 1-34 of SEQ ID NO: 2, amino acid residues 1-36 of SEQ ID NO: 2, wherein contacting the bone-forming cell with the polypeptide increases expression of the bone matrix protein, the transcriptional activator, or the transcriptional regulator in the bone-forming cell.
FIG. 2 shows that serum osteocalcin levels are positively correlated with serum parathyroid hormone (PTH) levels (p=0.0007, n=27) in black bears for pooled pre-hibernation, hibernation, and post-hibernation samples.

FIG. 3 shows normalized serum resorption (ICTP) and formation (PICP and osteocalcin) marker concentrations during the 3 month disuse period.

FIG. 4 shows that the amount of PGE2 released by osteoblastic cells was greatest when the cells were treated with serum collected in the post-hibernation period.

FIG. 5 shows the sequence of the mature black bear PTH protein compared to other known PTH sequences.

FIG. 6 shows that both human and black bear PTH 1-34 upregulate osteocalcin (n=2).

FIG. 7 shows the effects of human and black bear PTH 1-34 on apoptosis-related gene expression (n=4).

FIG. 8 shows μCT images of a proximal rat tibia (left), 1.6 mm analysis region of cortical and trabecular bone starting 2 mm distal to the proximal physis (top right), and the trabecular analysis region (bottom right).

FIGS. 9A-9F show μCT images of bones from rats following sham ovariectomy (OVX) (FIG. 9A) or actual OVX followed by six (FIG. 9B) or fourteen (FIG. 9C) weeks of recovery with no PTH treatment, or by fourteen weeks of recovery including eight weeks of treatment with 3 μg/kg (FIG. 9D), 10 μg/kg (FIG. 9E), or 30 μg/kg (FIG. 9F) black bear PTH1-34.

FIG. 10 shows a graph of bone volume as a fraction of total volume (BV/TV) for bones from rats having had sham or actual OVX procedures followed by treatment with vehicle (saline) or human or bear PTH1-34.

FIG. 11 shows a graph of bone mineral apparent density for bones from rats having had sham or actual OVX procedures followed by treatment with vehicle (saline) or human or bear PTH1-34.

FIG. 12 shows a graph of trabecular strut number for bones from rats having had sham or actual OVX procedures followed by treatment with vehicle (saline) or human or bear PTH1-34.

FIG. 13 shows a graph of trabecular thickness for bones from rats having had sham or actual OVX procedures followed by treatment with vehicle (saline) or human or bear PTH1-34.

FIG. 14 shows a graph of trabecular bone mineralization for bones from rats having had sham or actual OVX procedures followed by treatment with vehicle (saline) or human or bear PTH1-34.

FIG. 15 shows a graph of tibial cortical bone volume for bones from rats having had sham or actual OVX procedures followed by treatment with vehicle (saline) or human or bear PTH1-34.

FIG. 16 shows a graph of tibial cortical porosity for bones from rats having had sham or actual OVX procedures followed by treatment with vehicle (saline) or human or bear PTH1-34.

FIG. 17 shows a graph of femoral cortical bone strength for bones from rats having had sham or actual OVX procedures followed by treatment with vehicle (saline) or human or bear PTH1-34.

FIG. 18 shows a graph of serum calcium levels of rats having had sham or actual OVX procedures followed by treatment with vehicle (saline) or human or bear PTH1-34.

FIG. 19 shows the relative caspase-3 activity in MC3T3-S4 cells pretreated with 0-100 nM human or bear PTH peptides and then subjected to serum starvation. LS means with standard error bars are presented. Bars with different letters are significantly (p<0.05) different from each other.

FIG. 20 shows ultimate load of the femur in OVX rats treated with vehicle (V), 2.5 nmol/kg hPTH1-84, or 2.5 nmol/kg bbPTH1-84.

FIG. 21 shows normalized ultimate load of the femur in OVX rats treated with vehicle (V), 2.5 nmol/kg hPTH1-84, or 2.5 nmol/kg bbPTH1-84.

FIG. 22 shows bone volume fraction in OVX rats treated with vehicle (V), 2.5 nmol/kg hPTH1-84, or 2.5 nmol/kg bbPTH1-84.

FIG. 23 shows trabecular number in OVX rats treated with vehicle (V), 2.5 nmol/kg hPTH1-84, or 2.5 nmol/kg bbPTH1-84.

FIG. 24 shows trabecular thickness in OVX rats treated with vehicle (V), 2.5 nmol/kg hPTH1-84, or 2.5 nmol/kg bbPTH1-84.

FIG. 25 shows trabecular separation in OVX rats treated with vehicle (V), 2.5 nmol/kg hPTH1-84, or 2.5 nmol/kg bbPTH1-84.

FIG. 26 shows apparent mineral density in OVX rats treated with vehicle (V), 2.5 nmol/kg hPTH1-84, or 2.5 nmol/kg bbPTH1-84.

FIG. 27 shows material mineral density in OVX rats treated with vehicle (V), 2.5 nmol/kg hPTH1-84, or 2.5 nmol/kg bbPTH1-84.

FIG. 28 shows caspase 3/7 activity in cells cultured in prehibernation, hibernation, and posthibernation bear serum.

FIG. 29 shows ultimate load of femurs in mice treated with vehicle or various PTH peptides.

DETAILED DESCRIPTION

In humans and most other mammals, factors such as aging and extended periods of disuse can lead to osteoporosis and an increased risk of fracture. Disuse due to spinal cord lesion significantly decreases bone mineral density, particularly in the tibia and femur, and significantly reduces the cross-sectional moment of inertia of the femoral diaphysis. Thus, bone bending strength is reduced by spinal cord injury and fracture risk is increased. Disuse due to stroke also increases fracture risk. In addition, mechanical unloading of bone can cause rapid bone loss due to immediate increases in bone resorption in addition to sustained decreases in bone formation. Disuse-induced changes in bone remodeling increase intracortical porosity, and reduce the cross-sectional and mechanical properties of long bone diaphyses. Unloading also considerably reduces trabecular bone mass and microarchitecture.

The deleterious effects of disuse on bone may continue into the remobilization period. Some bone may be recovered during remobilization, but recovery is slow and often incomplete. For example, the rate of bone loss during bedrest is more than three times greater than the rate of bone gain during remobilization, and the recovery of bone lost in spaceflight can be incomplete even after 5 years. When disuse-induced changes in bone can be completely reversed by resumed activity, the remobilization period is often 2 to 3 times longer than the immobilization period. Bone formation decreases and/or bone resorption increases in many situations that reduce mechanical loads on bone. However, both resorption and formation increase during canine forelimb immobilization, yet there is significant bone loss in that case. Likewise, thigh bone turnover occurs in patients with spinal cord injury, which leads to bone loss and increased fracture incidence.

In contrast to this, black bears do not suffer significant bone loss due to aging (FIG. 1) or, more importantly, to the extended periods of disuse that occur during hibernation. Hibernating black bears have immobilization and active periods that can be approximately equal in length in northern
regions. Data on serum markers of bone metabolism (see below) suggest that both resorption and formation increase during disuse in bears, with a normal lag time (i.e., reversal period) between resorption and formation, and that the increase in formation remains coupled and balanced with the increase in resorption. Histological data from black bear iliac crest biopsies also show increased resorption and formation during inactivity. However, bears are unique in that trabecular bone volume, bone mineral density, and bone mineral content do not decrease during hibernation. Moreover, cortical bone strength and ash fraction increase with age, and porosity decreases with age in black bears, despite annual periods of disuse. Cortical bone porosity is significantly lower in hibernating grizzly bears than in active grizzly bears, and femoral cross-sectional geometry and strength are unaffected by hibernation.

Bears have evolved many unique biological mechanisms to survive long periods of immobilization without food. These mechanisms appear to include the recycling of calcium and other products of bone catabolism, since bears increase bone turnover but do not excrete waste during hibernation. In humans, bedrest-induced disuse osteoporosis is caused primarily by increased resorption without a corresponding increase in formation. This results in hypercalcemia and a negative calcium balance brought about by increased urinary and fecal calcium. Since bears do not urinate or defecate during hibernation, it is likely that most of the calcium released from bone by resorption is recycled back into bone via osteoblastic bone formation. Ionized calcium is found to increase by about 23% during hibernation, possibly because of the lag time between resorption and formation. Paradoxically, black bear PTH levels are highest when levels of ionized calcium are highest (Example 2, Table 1). Taken together, these findings suggest that bears have evolved biological mechanisms to avoid osteoporosis.

The mechanisms that uncouple bone formation from resorption during disuse in most animals are unknown, but likely involve both mechanical and biochemical factors. Lack of mechanical strain may lead to increased resorption by initiating osteocyte apoptosis and concomitantly reducing osteoblastic activity. Hormones such as human PTH can sensitize bone cells to mechanical stimulation and synergistically, with mechanical loading, increase bone formation. Human PTH given once daily to humans increases bone mass and decreases fracture incidence. Thus, in black bears, circulating PTH may sensitize bone cells to low levels of mechanical stimulation (possibly due to shivering or repositioning in the hibernaculum) to help maintain bone formation during disuse. PTH may also help maintain bone formation in black bears by stimulating osteoblast differentiation and inhibiting osteoblast apoptosis.

PTH is the primary regulator of blood calcium levels, and thus plays a role in maintaining homeostatic serum calcium levels in black bears during disuse. Serum PTH levels are positively correlated with the bone formation marker osteocalcin in active and hibernating black bears (Fig. 2), and both osteocalcin and PTH increase during hibernation. In addition, black bear PTH concentration is highest when ionized calcium concentration is highest. Since bone resorption increases during hibernation but total serum calcium (Ca) remains unchanged, increased levels of PTH likely cause increased renal reabsorption of calcium, facilitating the recycling of mineral back into the bone with a balanced increase in bone formation. This leads to the observed preservation of trabecular and cortical bone properties like bone mineral density (“BMD”) and cortical porosity. Bone resorption increases during hibernation, but blood calcium concentration remains constant despite the fact that bears do not excrete waste during hibernation. The calcium liberated by bone resorption during hibernation may be recycled and put back into bone by maintaining balanced coupling of bone formation with bone resorption. This supports the idea that PTH has anabolic effects in hibernating black bears and provides an explanation for the bears’ distinctive ability to maintain balanced bone remodeling during hibernation. The anabolic effects of PTH may be enhanced in black bears when physical activity is resumed following arousal from hibernation. Mechanical loading and human PTH have previously been shown to act synergistically to increase bone formation in vivo in rats and biochemical signaling in vitro. During remobilization in the spring, bone formation in the black bear, as indicated by serum osteocalcin, remains higher than prehibernation levels.

The sequence for the polynucleotide that encodes black bear ([Uursus americanus]) parathyroid hormone (PTH) (SEQ ID NO: 1) was discovered as well as the polypeptide sequence for the mature 84 amino acid PTH protein (SEQ ID NO: 2). In addition, the cDNA (SEQ ID NO: 3) which encodes the full length PTH protein (SEQ ID NO: 4), including a 25 amino acid signal peptide (amino acid residues 1-25 of SEQ ID NO: 4) and a 6 amino acid propeptide (amino acid residues 26-31 of SEQ ID NO: 4) has been sequenced. The mature black bear PTH protein differs from other known PTH proteins (Fig. 5). Compared to human PTH, black bear PTH has 9 different amino acid residues out of a total of the 84 amino acid residues of the full-length, mature PTH polypeptide. Also described herein are various methods of use for black bear PTH and functional fragments thereof. It is specifically envisioned that polypeptide subfragments comprising at least 10 consecutive amino acid residues of SEQ ID NO: 2 and including at least one of amino acid residues 41 or 52 can be used to develop antibodies specific for black bear PTH. These antibodies can be used to quantify black bear PTH, e.g., in an ELISA assay.

PTH receptors on the surface of bone-forming cells are coupled to cyclic adenosine monophosphate (cAMP)-dependent second-messenger signaling pathways inside the cells. These signaling pathways, in turn, lead to increased expression of genes involved in bone formation such as those encoding type 1 collagen, osteonectin, and osteopontin. Since the cAMP/protein kinase A pathway is responsible for the majority of PTH-induced increases in histological and serum indices of bone formation, it follows that an increased cAMP response can lead to greater bone formation. A relatively small number of amino acid substitutions in the sequence of a given PTH protein can stimulate greater cyclic adenosine monophosphate (cAMP) production compared to the native form. For example, ovariectomized rats demonstrated a 25% greater bone formation response to daily 25 μg injections of bovine PTH 1-34 than to rat PTH 1-34, where rat PTH 1-34 has 5 amino acid sequence differences compared to bovine PTH 1-34. Injection of bovine PTH 1-34 results in a 37% greater increase in bone volume fraction during treatment.

Thus, it is likely that the amino acid substitutions in black bear PTH cause it to induce greater cAMP production in bone-forming cells than human PTH. PTH, in general, elicits a greater bone formation response by mechanisms such as decreasing osteoblast apoptosis, increasing osteoblast differentiation via RunX2, downregulating SOST-based negative feedback in osteocytes, and increasing production of mRNA for bone matrix proteins, all via cAMP-mediated pathways. Although it is not necessary to understand the mechanism of an invention, it is believed that black bear PTH is likely more osteogenic than other forms of PTH, which explains why
black bears are uniquely able to maintain balanced bone remodeling during disuse. In certain embodiments of the present invention, contacting a bone-forming cell with black bear PTH or a functional fragment thereof increases cAMP levels in the bone-forming cell. In certain embodiments of the present invention, the bone-forming cell is contacted with a polypeptide comprising amino acid residues 1-34 or 1-36 of SEQ ID NO: 2. In certain embodiments of the present invention, the bone-forming cell is contacted with a polypeptide comprising SEQ ID NO: 2.

As used herein, “contacting a cell” with a PTH polypeptide includes adding the polypeptide to the culture solution, in the case of in vitro experiments, or administering the polypeptide to a subject using appropriate administration procedures for polypeptide therapeutic agents. “Contacting a cell” also includes introducing into the subject an exogenous polynucleotide that encodes the desired polypeptide in an expression system so as to synthesize and release the polypeptide in the subject. As used herein, “bone-forming cells” includes, but is not limited to, osteoblasts, osteocytes, bone lining cells, chondroblasts, and chondrocytes. Suitably, the bone-forming cell may be in a subject.

Bone-forming cells regularly turn over, with most of the cells' death being due to programmed cell death, or apoptosis. Given this regular rate of turnover, any mechanism that decreases apoptosis of bone-forming cells will lead to an increased number of bone-forming cells which presumably will promote bone growth. Thus, in certain embodiments of the present invention, contacting a bone-forming cell with black bear PTH or a functional fragment thereof reduces apoptosis in the bone-forming cell. In certain embodiments of the present invention, the bone-forming cell is contacted with a polypeptide comprising amino acid residues 1-34 or 1-36 of SEQ ID NO: 2. In certain embodiments of the present invention, the bone-forming cell is contacted with a polypeptide comprising SEQ ID NO: 2.

Moreover, it is possible that several of the larger C-terminal fragments of endogenous black bear PTH play a role in seasonal bone remodeling processes via binding to CPTHs (C-terminal PTH receptors). Specifically, C-terminal fragments of bear PTH may antagonize the calcemic effects of PTH 1-84 and 1-34 by preventing osteoclastogenesis and possibly by affecting mature osteoclast activity that would normally occur in response to resorptive stimuli such as disease (Divieti, P. et al., 2002, Endocrinology 143(1): 171-6).

The protein Bax promotes apoptosis while the Bcl-2 protein protects cells from apoptosis, and a decrease in the expression ratio of Bax/Bcl-2 is indicative of a decrease in apoptosis in the particular cell population. Thus, in certain embodiments of the present invention, contacting a bone-forming cell with black bear PTH or a functional fragment thereof decreases the ratio of expression levels of Bax protein relative to expression levels of Bcl-2 protein in the bone-forming cell. In certain embodiments of the present invention, the bone-forming cell is contacted with a polypeptide comprising amino acid residues 1-34 or 1-36 of SEQ ID NO: 2. In certain embodiments of the present invention, the bone-forming cell is contacted with a polypeptide comprising SEQ ID NO: 2.

Example 11 shows that black bear PTH 1-34 decreases the expression ratio of Bax/Bcl-2 (FIG. 7). Thus, black bear PTH 1-34 appears to be more effective at preventing apoptosis than human PTH 1-34. Without being bound by theory, this difference may be the result of the two amino acid differences between human and black bear PTH 1-34. These data suggest that bear PTH is more anabolic than human PTH, since decreased osteoblast apoptosis may contribute to the bone formation response induced by PTH treatment.

Contacting a bone-forming cell with black bear PTH or a functional fragment thereof also increases the expression level of bone matrix protein, a transcriptional activator, or a transcriptional regulator in the bone-forming cell. In certain embodiments of the present invention, the transcriptional activator is Runx2. In certain embodiments of the present invention, the transcriptional regulator is c-fos. Exemplary bone matrix proteins include, but are not limited to, osteocalcin, osteopontin, and type I collagen. In certain embodiments of the present invention, the bone-forming cell is contacted with a polypeptide comprising amino acid residues 1-34 or 1-36 of SEQ ID NO: 2. In certain embodiments of the present invention, the bone-forming cell is contacted with a polypeptide comprising SEQ ID NO: 2.

Exogenous human PTH is used to treat post-menopausal and age-related osteoporosis in humans, but it is not an ideal therapeutic. Only recombinant human PTH 1-34 (LY333334, Eli Lilly, Indianapolis Ind.) is currently approved for clinical use, and only one form of recombinant human PTH 1-84 is under consideration for approval by the U.S. Food and Drug Administration (ALX1-11, NPS Pharmaceuticals, Parsippany, N.J.). Though LY333334 and ALX1-11 can stimulate approximately the same magnitude of bone formation in vivo, their biological actions are not identical. For example, PTH 1-34 down-regulates production of procollagen-I mRNA, whereas PTH 1-84 does not (Nasu et al., 1998, Endocr J, 45, 229-34). In addition, it has also been determined that the C-terminal portion of human PTH, when cleaved from the mature hormone, has important biological functions such as inhibition of bone resorption.

Long-term usage of either LY333334 or ALX1-11 generates osteosarcoma in rats, but preliminary results indicate that human PTH 1-84 has a lower rate of carcinogenicity than human PTH 1-34, possibly because C-terminal fragments of exogenous human PTH 1-84 (arising from peripheral proteolytic processing) can bind to C-terminal PTH receptors (CPTHs) and increase osteocyte apoptosis. Thus, though equally anabolic, human PTH 1-84 may be a superior osteoporosis therapy compared to human PTH 1-34. However, human PTH 1-84 cannot completely restore lost bone; it has been suggested that men and women can lose between 20-30% of cortical and cancellous bone due to age-related osteoporosis, but only 8% is recovered using ALX1-11 during its suggested treatment regimen. Therefore, there exists a clinical need for osteoporosis treatments with greater osteogenic capabilities.

In certain embodiments, the present invention is a method of treating osteoporosis comprising administering an effective amount of black bear PTH or a functional fragment thereof to a subject in need thereof. In various embodiments, the osteoporosis is primary or age-related osteoporosis and/or disuse osteoporosis. In certain embodiments of the present invention, contacting a bone-forming cell in a subject with black bear PTH or a functional fragment thereof increases bone mineral density, increases bone mass, decreases bone loss or reduces the incidence of bone fracture in the subject. In certain embodiments of the present invention, the bone-forming cell is contacted with a polypeptide comprising amino
acid residues 1-34 or 1-36 of SEQ ID NO: 2. In certain embodiments of the present invention, the bone-forming cell is contacted with a polypeptide comprising SEQ ID NO: 2.

Suitably, contacting a bone-forming cell in a subject with black bear PTH or a functional fragment thereof increases bone mineral density, increases bone mass, decreases bone loss or reduces the incidence of bone fracture by at least about 5% or at least about 10%. The increase in bone mineral density, increase in bone mass, decrease in bone loss or reduction in the incidence of bone fracture may be at least about 15%, at least about 30%, at least about 50%, at least about 75% or at least about 90%. The increase in bone mineral density, increase in bone mass, decrease in bone loss or reduction in the incidence of bone fracture is determined by measuring the desired characteristic on the same patient before and after treatment by a technique known to one of ordinary skill in the art. For example, bone mineral density can be determined by methods involving taking dual energy x-rays (DEXA) or CT scans of bones in the spinal column, wrist, arm or leg.

Suitably, the subject may be a mammal, including without limitation human, horse, dog, cat, mouse, bear, bovine, pig, or deer. In certain embodiments, the subject is a post-menopausal human female. The subject may have osteoporosis or may be at risk to develop osteoporosis. Risk factors for developing osteoporosis include: personal history of fracture after age 50; current low bone mass; history of fracture in a first-degree relative; being female; being thin and/or having a small frame; advanced age; a family history of osteoporosis; estrogen deficiency as a result of menopause, especially early or surgically induced; abnormal absence of menstrual periods (amenorrhea); anorexia nervosa; low lifetime calcium intake; vitamin D deficiency; use of certain medications (corticosteroids, chemotherapy, anticonvulsants and others); presence of certain chronic medical conditions, such as those that decrease calcium absorption in the gut such as Crohn’s disease; low testosterone levels in men; an inactive lifestyle; current cigarette smoking; excessive use of alcohol; and being Caucasian or Asian; although African Americans and Hispanic Americans are at significant risk as well. Moreover, women can lose up to 20 percent of their bone mass in the five to seven years following menopause, making them more susceptible to osteoporosis.

Black bear PTH or functional fragments thereof are also useful as a preventative (rather than a restorative), or prophylactic, measure to combat disuse osteoporosis or to prevent osteoporosis in a subject at risk for developing osteoporosis. Since bears appear to be the only animals that maintain balanced bone remodeling during disuse, black bear PTH or functional fragments thereof are also useful to prevent bone loss during reduced skeletal unloading that occurs, for example, in astronauts during spaceflight and in spinal cord injury patients after injury.

Black bear PTH or functional fragments thereof may be administered in combination with calcium and/or vitamin D. Suitably, “Vitamin D” refers to the entire Vitamin D class of compounds. Black bear PTH or functional fragments thereof may also be administered in combination with other anabolic or antiresorptive active agents. Suitable anabolic active agents include various fragments of human PTH (e.g. 1-34 and 1-84), fluoride, GH, insulin-like growth factor I, statins, and PKY2 kinase inhibitors. Suitable antiresorptive active agents include, but are not limited to estrogens, selective estrogen receptor modulators, calcitonin, and bisphosphonates. In certain embodiments, an anabolic functional fragment of black bear PTH (e.g. black bear PTH 1-34) is administered in combination with an antiresorptive active agent. In certain embodiment, different functional fragments of black bear PTH may be administered in combination with each other. The different functional fragments may be administered concurrently or in any order that is suitable.

The calcium, vitamin D, anabolic active agent, and/or antiresorptive active agent may be administered concurrently with black bear PTH or functional fragments thereof or may be administered before or after black bear PTH or functional fragments thereof. The calcium, vitamin D, anabolic active agent, and/or antiresorptive active agent may be in a separate composition or may be in the same pharmaceutical composition as the black bear PTH or functional fragments thereof.

Administration of black bear PTH or functional fragments thereof or compositions comprising black bear PTH or functional fragments thereof can be accomplished by any suitable technique. Black bear PTH or functional fragment thereof may be administered by any suitable route including, for example, oral, nasal, rectal, and parenteral routes of administration. As used herein, the term parenteral includes but is not limited to subcutaneous, intradermal, intravenous, intramuscular, intraperitoneal, and intrathecal administration, such as by injection. As is discussed above, administration of a polypeptide includes administration of an exogenous polynucleotide operably connected to a promoter such that the polynucleotide expresses the polypeptide in the subject. Administration of the polypeptide also includes administration of a viral vector comprising a polynucleotide encoding the polypeptide. Suitably, the viral vector is an adenoviral vector.

Black bear PTH or functional fragments thereof, or compositions comprising black bear PTH or functional fragments thereof, can be administered continuously or at discrete time intervals as can be readily determined by a person skilled in the art. An ordinarily skilled clinician can determine a suitable amount of black bear PTH or a functional fragment thereof to be administered to a subject.

The effective dose for any particular subject will depend upon a variety of factors, including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed; the age, body weight, general health, sex and diet of the subject; route of administration; the rate of excretion or inactivation of black bear PTH or functional fragments thereof employed; the duration of the treatment; other pharmaceuticals used in combination or coincidental with black bear PTH or functional fragments thereof and like factors well known in the medical arts. For example, it is well within the level of ordinary skill in the art to start doses at levels lower than those required to achieve the desired effect and to gradually increase the dosage until the desired effect is achieved.

Suitably, the dosage of black bear PTH or functional fragments thereof in certain embodiments is in a range of 0.10 μg/kg per day to 40 μg/kg per day. In certain embodiments, the dosage is in a range of 5 μg/kg per day to 20 μg/kg per day. In certain embodiments, the dosage is 10 μg/kg per day. In certain embodiments, the dosage is in a range of 10 μg/day to 400 μg/day per subject. In certain embodiments, the dosage is in a range of 20 μg/day to 40 μg/day per subject. In certain embodiments, the dosage is 30 μg/day per subject.

For combination administration, one of ordinary skill in the art would be able to determine the proper dosage of both the black bear PTH or functional fragment thereof and the additional active agent, such as calcium, vitamin D, anabolics or antiresorptives. As is discussed above, the effective dose for any particular subject depends upon a variety of factors. In certain embodiments, the dosage of antiresorptives is about 5 to about 100 μg per day; the dosage of calcium is from about
500 mg per day, the dosage of vitamin D is from about 500 to about 1500 mg per day, the dosage of anabolics is from about 0.1 mg to about 0.5 mg per day or about 10 

ug/day to 400 ug/day.

In certain embodiments the subject is a human. Suitably, the daily dosages in certain embodiments are given for one week, in certain embodiments for one month, in certain embodiments for three months, in certain embodiments for six months, in certain embodiments for one year, in certain embodiments for one and a half years, in certain embodiments for two years, and in certain embodiments for three years.

If desired, the effective daily dose may be divided into multiple doses for purposes of administration. Consequently, single dose compositions may contain such amounts or submultiples thereof to make up the daily dose. If desired, a suitable delivery device is loaded with the effective daily dose for more than one day, for example, for seven days, fourteen days, twenty-one days, twenty-eight days or the like, and the delivery device is used to repeatedly administer the desired daily single dose or daily multiple doses for the desired total number of days. As noted, those of ordinary skill in the art will readily optimize effective doses and co-administration regimens as determined by good medical practice and the clinical condition of the individual subject.

Compositions containing black bear PTH or functional fragments thereof useful in the methods of the present invention can be formulated according to known methods for preparing pharmaceutically useful compositions. Formulations are described in detail in a number of sources which are well known and readily available to those skilled in the art. For example, Remington's Pharmaceutical Science, by E. W. Martin, describes formulations which can be used in the disclosed methods. In general, the compositions will be formulated such that an effective amount of the black bear PTH or functional fragment thereof is combined with a suitable carrier in order to facilitate effective administration of the composition.

The compositions used in the present methods can also be in a variety of forms. These include, for example, solid, semi-solid, and liquid dosage forms, such as tablets, pills, powders, liquid solutions or suspension, suppositories, injectable and infusable solutions, and sprays. The form will depend on the intended mode of administration and therapeutic application. The compositions also suitably include conventional pharmaceutically acceptable excipients which are known to those skilled in the art. Examples of excipients include water for injection, ethanol, dimethyl sulfoxide, glycerol, alumina, starch, glacial acetic acid, sodium acetate, mannitol, metacresol, hydrochloric acid and/or sodium hydroxide to adjust the pH of a composition to a suitable value, and equivalent or otherwise suitable carriers and diluents. To provide for the administration of such compositions for the desired application, pharmaceutical compositions will comprise between about 0.1% and 99%, and suitably between about 1% and 15% by weight of the total of one or more of the polypeptides of the present invention based on the weight of the total composition including the carrier or diluent.

As used herein, an "isolated" nucleic acid molecule, polynucleotide, polypeptide, or the like, as the case may be, refers to a composition that is at least partially purified from contaminants (e.g., other species of polynucleotides, polypeptides, or the like) that is found other than in its natural state. An isolated nucleic acid, polynucleotide, or polypeptide may contain less than about 50%, suitably less than about 75%, and most suitably less than about 95%, of the cellular components with which it was originally associated. A polynucleotide amplified using PCR so that it is sufficiently and easily distinguishable (on a gel, for example) from the rest of the cellular components is considered "isolated". The nucleic acid molecules, polynucleotides, and polypeptides of the invention may be "substantially pure"; i.e., having the highest degree of purity that can be achieved using purification techniques known in the art.

As used herein, a "functional fragment" refers to any region or portion of a polypeptide or polynucleotide which is a region or portion of a larger polypeptide or polynucleotide, the region or portion having an activity or function attributable to the larger polypeptide or polynucleotide. For example, a functional fragment of human PTH is the 1-34 region of human PTH. Functional fragments of black bear PTH include, but are not limited to, 1-34 and 1-36.

As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. It should also be noted that the term "or" is generally employed in its sense including "and/or" unless the context clearly dictates otherwise. All publications, patents and patent applications are herein expressly incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent application was specifically and individually incorporated by reference. In case of conflict between the present disclosure and the incorporated patents, publications and references, the present disclosure should control.

It also is specifically understood that any numerical range recited herein includes all values from the lower value to the upper value, i.e., all possible combinations of numerical values between the lowest and highest value of the range. All percentage values are by weight of total composition of the invention. Each and every value or range of values in any column of values in a claims table is to be interpreted as being referenced in each and every other column of values in the same claims table.

Example 1

Genomic DNA Extraction

Blood was collected from a captive female black bear and stored at 4°C. Genomic DNA was extracted from the whole blood samples within 2 weeks, using the GenomicPrep Blood DNA Isolation Kit (Amersham Biosciences, Piscataway, N.J.) according to the manufacturer's instructions.

PCR Cloning and Sequencing

Black bear genomic DNA was used for PCR amplification of PTH, using consensus primers designed based on alignment of all mammalian PTH sequences available in GenBank including bovine (Bos taurus, AA30749), cat (Felis catus, Q9GL67), dog (Canis familiaris, P52212), human (Homo sapiens, NP_000306), macaque (Macaca fascicularis, Q9XT35), mouse (Mus musculus, NP_065648), pig (Sus scrofa, NP_999566), and rat (Rattus norvegicus, NP_058740). PCR amplification was performed using 10-15 ng genomic DNA, 100 μM dNTPs, 0.2 μM each primer, and
1 unit REDTaq (Sigma, St. Louis, Mo.) in 20 µL reaction volume. PCR products were gel-purified using the UltraClean GelSpin Kit (MoBio Carlsbad, Calif.) and cloned into the pCRII vector using the TA cloning kit (Invitrogen, Carlsbad, Calif.). DNA sequencing was performed using the DTCS Quick Start kit and the CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, Calif.), following the manufacturer’s instructions.

Sequence Analysis

Nucleotide sequences were searched against the GenBank protein database using BlastX (Altschul et al., 1997; Nucleic Acids Res., 25, 3389-402) to confirm their putative identity as PTH. Multiple sequence alignment was performed by ClustalW version 1.82 (Chenna et al., 2003; Nucleic Acids Res., 31, 3497-500). Phylogenetic analysis was carried out using the neighbor-joining (NJ) method implemented in the Molecular Evolutionary Genetics Analysis (MEGA) package version 3.0 (Kumar et al., 2004; Brief Bioinform., 5, 150-63), with the pairwise deletion option for handling alignment gaps, and with the Poisson correction model for distance computation.

PTE1 Cloning and Sequence Analysis

Various primer combinations were used for PCR-based cloning of PTH from black bear genomic DNA. Based on the sequencing results of preliminary clones, a gene-specific sense primer corresponding to the start codon was designed and used along with a degenerate antisense primer containing the stop codon to amplify the entire coding region of PTE1. A second antisense primer covering the stop codon was designed to generate a clone for sequence confirmation.

Sequence assembly revealed a precursor PTH protein of 115 amino acids, including a 25 amino acid signal peptide and a 6 amino acid propeptide. The deduced mature protein is 84 amino acids, with a calculated molecular weight of 9,471 Daltons and a pI of 8.1. Black bear PTH shares 84-95% sequence similarity with other mammalian PTHs, and is most similar to dog PTH (91% identity, 95% similarity) (FIG. 5). Interestingly, two amino acid residues, 41 and 52 of the mature hormone, are unique to black bear PTH.

Example 2

Levels of Bone Resorption and Formation Markers During Hibernation

Serum Samples

Blood samples were collected from five black bears (Ursus americanus) held in a captive bear research facility. The Virginia Polytechnic Institute and State University Animal Care Committee approved all bear handling protocols (598-069-FKWS). The bears were anesthetized with a 2:1 mixture of ketamine (100 mg/ml); xylazine (100 mg/ml); the dosage was 1 cc of the mixture per 45.5 kg of body weight. Body temperatures were 4° to 6° C cooler during winter collection, confirming that the bears were in a state of hibernation. No urine or scat was present in the hibernation dens. Stressful behavior was not observed during any of the handling procedures. Blood samples were drawn from the femoral vein while the bears were anesthetized, and the samples were transported to the laboratory in an ice-packed cooler. Immediately on return to the laboratory, the blood was centrifuged to isolate the serum, which was frozen at –20° F. Blood samples were collected from each bear every 10 days from the first of October through the end of May. Hibernation began in early January and ended in early April. Thus, the collection dates encompassed an active pre-hibernation period, a disuse hibernation period, and an active post-hibernation remobilization period.

Black Bear Osteocalcin Purification and RIA Procedures

Black bear cortical bone was broken into small fragments, defatted with a mixture of 3 parts hexane and 2 parts isopropanol, and lyophilized. The dried bone was ground to a fine powder under liquid nitrogen, and the osteocalcin was solubilized as described by Hauser et al. (1989, Physiol. Rev., 69, 990-1047). Osteocalcin was purified from the resulting EDTA extract by a modification of the method of Colombo et al. (1993, J. Bone Miner. Res., 8, 733-43). Briefly, the crude EDTA solution was diluted 2-fold and passed over a bulk column containing 10 g Sephalyte C18 particles (Analytichem International, Harbor City, Calif.) previously activated with methanol and equilibrated with 0.1% trifluoroacetic acid in water (0.1% TFA). An extensive wash with 0.1% TFA was followed by 30% methanol/0.1% TFA until UV absorbance dropped to baseline. Osteocalcin was eluted with 80% methanol/0.1% TFA. Methanol was evaporated under a stream of air and the resulting solution lyophilized. The resulting dried protein was suspended in 0.05 M Tris buffer, pH 8.0 and applied to a 5 ml Biorad Econo-Q column previously equilibrated with the same buffer. The column was developed with a gradient from 0.1 to 0.6 M NaCl in 0.5 M Tris, pH 8.0. Osteocalcin eluted in a symmetric peak, the last to elute from the column. Identity of this peak as osteocalcin was qualitatively verified by reacting fraction aliquots with diazo benzene sulfonic acid yielding a pink color in those fractions containing osteocalcin, with intensity corresponding to peak height. Both the C18 and the Econo-Q column were new and never exposed to protein from other species. Previous experience with other species suggests the final osteocalcin peak is greater than 99% pure. Concentration of black bear osteocalcin in the final elute was determined with BCA reagents from Pierce Chemical (Rockford, Ill.).

Biochemical Assays

The serum was assayed for PTH, 25-OH D, leptin, IGF-1, and osteocalcin (a bone formation marker) using RIA and ELISA.

Highly purified black bear osteocalcin and black bear serum were assayed by radioimmunoassay. The antibody was guinea-pig anti-rat osteocalcin and tracer was 125I-labeled rat osteocalcin. Dose dilutions of both rat osteocalcin standard (Biomedical Technologies, Inc, Stoughton, Mass.) and purified black bear osteocalcin were included in the assay. Aliquots of 10 µl black bear serum per assay tube were assayed in duplicate, and all samples were assayed at the same time. Duplicates varied by less than 5%.

To observe changes in bone formation and resorption markers during disuse, the mean values of osteocalcin for the 5 black bears were calculated for each time point during the hibernation period. These values were normalized by the maximum osteocalcin value during the hibernation period. Similar calculations were done for measurements of PICP (bone formation marker) and ICTP (bone resorption marker). The normalized values of the resorption and formation markers were plotted on the same graph to assess the temporal and relative magnitude changes in bone resorption and formation during disuse.

Using serum samples from hibernating bears obtained as described above, ionized calcium concentration was measured with an ion-selective electrode (Bayer Rapidlab 865, Leverkusen, Germany).

Using serum samples obtained as described above, PTH was assayed with an ELISA kit from Immunitopics International (San Clemente, Calif.); the intra-assay coefficient of
variation was 4.7%. 25-OH D was assayed with an ELISA kit from ALPCO Diagnostics (Windham, N.H.); the intra-assay coefficient of variation was 5%. Leptin was measured by RIA (Linco, St. Charles, Mo.); the intra-assay coefficient of variation was 3.4%. IGF-1 was measured by acid ethanol extraction RIA (Nichols Institute Diagnostics, San Juan Capistrano, Calif.); the intra-assay coefficient of variation was 4.3%.

Serum osteocalcin was measured by RIA as described above. For all the serum metabolites, the mean values (for all bears and all time points within a given season) were calculated for each season (pre-hibernation, hibernation, and post hibernation) and compared by ANOVA. ANOVAs were followed up with Fisher’s PLSD tests for multiple mean comparisons. Natural log transformations were used to correct non-constancy of variance for osteocalcin, PTH, 25-OH D, and IGF-1 to validate the ANOVAs. Linear regressions were used to assess the correlations between osteocalcin and the hormones. The volume of some serum samples was insufficient to run assays; sample sizes for each assay are indicated with the results.

Results

The bone resorption marker (ICTP) began to increase immediately after the onset of hibernation (FIG. 3). Each data point is the mean value from 5 bears. After 10-20 days, the bone formation markers (osteocalcin and PICP) also increased and appeared to remain coupled to the increased resorption for the duration of hibernation. This is consistent with the 1-2 week histological “reversal” period between resorption and formation. These remodeling markers showed trends of increased resorption and formation throughout the hibernation period, and formation appeared to remain coupled and balanced with resorption. Mean osteocalcin levels were higher (p=0.0001) during and after hibernation compared to pre-hibernation (Table 1).

Hibernation ionized calcium levels were significantly higher than the 0.0002 (p=0.0002) higher than the pre-hibernation levels (Table 1). During remobilization following arousal from hibernation, ionized calcium levels did not significantly (p=0.37) increase relative to hibernation levels, but they remained higher (p=0.015) than pre-hibernation levels.

Osteocalcin was positively correlated with PTH (FIG. 2), but not with 25-OH D, leptin, or IGF-1. PTH was significantly higher in the post-hibernation season than in the pre-hibernation season (p=0.006) and hibernation season (p=0.014) seasons. The increase in PTH during hibernation relative to pre-hibernation was not significant (p=0.35). 25-OH vitamin D did not show seasonal variations (p=0.64).

Leptin did not change during hibernation relative to pre-hibernation, but was significantly (p=0.004) lower during post-hibernation remobilization (Table 1). IGF-1 significantly (p=0.0001) decreased during hibernation relative to pre-hibernation and reached its highest value during remobilization (Table 1).

### TABLE 1

<table>
<thead>
<tr>
<th>Metabolite (ng/ml)</th>
<th>Pre-hibernation</th>
<th>Hibernation</th>
<th>Post-hibernation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteocalcin</td>
<td>16.9±(7.4) [15]</td>
<td>76.4±(43.3) [22]</td>
<td>49.3±(26.8) [19]</td>
</tr>
<tr>
<td>Ionized calcium</td>
<td>0.709±(1.50) [12]</td>
<td>0.871±(0.03) [10]</td>
<td>0.969±(0.14) [2]</td>
</tr>
<tr>
<td>PTH (pg/ml)</td>
<td>14.6±(4.5) [6]</td>
<td>25.3±(27.5) [14]</td>
<td>41.4±(20.2) [9]</td>
</tr>
<tr>
<td>25-OH D (nmol/ml)</td>
<td>16.6±(16.3) [9]</td>
<td>11.2±(6.2) [9]</td>
<td>16.0±(1.3) [9]</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>4.0±(0.7) [13]</td>
<td>3.8±(0.7) [22]</td>
<td>3.1±(0.7) [19]</td>
</tr>
<tr>
<td>IGF-1 (ng/ml)</td>
<td>387±(88) [15]</td>
<td>209±(52) [22]</td>
<td>594±(207) [19]</td>
</tr>
</tbody>
</table>

Mean values are given in bold, standard deviations in parentheses, and sample sizes in brackets. For a given metabolite, values with the same superscript are not significantly (p < 0.05) different. 25-OH D did not show significant seasonal differences.
encompassed a pre-hibernation active period, a hibernation disuse period, and a post-hibernation remobilization period. MC-3T3 osteoblastic cells were cultured for 24 hours in media containing 10% serum, after which total RNA was isolated using a BioRad AquePure RNA Isolation Kit (CAT#32-6370, BioRad Laboratories, Hercules, Calif.). To generate cDNA, reverse transcription was performed using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, Calif.), and 0.5 µg Oligo(dT)12-18 primer at 42°C for 20 minutes, 50°C for 10 minutes and 42°C for 1 hour in a gradient thermocycler (Mastercycler gradient, Eppendorf, Westbury, N.Y.). Primers for the pro-apoptotic protein Bax and the anti-apoptotic protein Bel-2 were designed using PrimerQuest software (Integrated DNA Technologies, Coralville, Iowa) and the NCBI gene bank sequences. Semi-quantitative PCR was performed using RedTaq and a protocol consisting of 94°C for 2 minutes, cycles of 94°C for 30 seconds, 69.5°C for 30 sec and 72°C for 1 minute, and a final extension at 72°C for 5 minutes. Band intensity was quantified using the ImageJ software package (National Institutes of Health, Bethesda, Md.) and normalized to the expression of three housekeeping genes (Gadph, β-actin, cyclophilin).

ANOVA with Fisher’s Protected Least Significant Difference (PLSD) post-hoc test was used to compare the ratio of Bax to Bel-2 for the three periods (pre-hibernation, hibernation, post-hibernation). Though it did not achieve statistical significance (p=0.300), the Bax/Bel-2 ratio decreased by approximately 42% during hibernation relative to pre-hibernation. The lack of statistical significance was likely related to the small sample size (n=2 for each group). These data suggest that serum from hibernating bears contains a biological molecule that decreases osteoblast apoptosis. Since endogenous PTH and the bone formation marker osteocalcin both increase during hibernation (Donahue et al., 2006; J. Exp. Biol., 209, 1630-8), it is possible that endogenous bear PTH causes a decrease in osteoblast apoptosis during hibernation, which in turn increases bone formation.

Example 5

Comparison of Effects of Black Bear vs. Human PTH 1-84 or Subfragments Thereof on cAMP in Bone Cell Lines

Full-length recombinant black bear PTH (residues 1-84) is produced and its effects on levels of cyclic adenosine monophosphate (cAMP) concentration in bone cell lines (MC-3T3 osteoblastic cells and MLO-Y4 osteocytes) are investigated and compared to results obtained using recombinant human PTH 1-84. Equivalent experiments are conducted using subfragments of black bear and human PTH, the subfragments including amino acid residues 1-34, 1-36, 7-34, 7-84, 11-84, and 41-52 of the full-length (1-84) mature protein. For some experiments, black bear and human PTH polypeptides are synthesized with solid-phase methods.

To determine the relative ability of black bear and human PTH to prevent osteoblast and osteocyte apoptosis (under pro-apoptotic conditions), cells are incubated with human or black bear PTH 1-84, or one of the subfragments listed above, for one hour. Afterwards, cells are treated for 6 hours with dexamethasone to induce apoptosis. Apoptosis is quantified with an ELISA, as described further below.

For all of the experiments using recombinant polypeptides, the lyophilized peptides are reconstituted to 100 µM stock concentrations in 1 mM acetic acid, and diluted to 10 µM working stock concentrations before use.
run with normal or lower levels of serum. In the experiments reported in this Example, the results are not affected by the amount of FBS that is used. Apoptosis protection studies show that each of the polypeptides tested reduces or prevents apoptosis in MC-3T3 cells.

Cell Culture
MC-3T3 subclone 14 cells (ATCC, CRL-2594) and MLO-Y4 cells (obtained from L. F. Bonewald, University of Missouri, Kansas City, Mo.) are maintained in alpha-minimum essential media, 1% penicillin/streptomycin, and 10% serum (MC-3T3: 10% fetal bovine serum (FBS), MLO-Y4: 5% FBS and 5% bovine calf serum), at 37° C. in 5% CO2. All procedures described herein are repeated with independent cell cultures such that n=6 for all treatment combinations in each assay.

Effects of PTH Treatment on Apoptosis
MC-3T3 cells are seeded at 50,000 cells/cm², and MLO-Y4 cells are seeded at 15,000 cells/cm² in 6-well plates and cultured overnight to reach optimal confluence. The culture media is aspirated and replaced with media containing either 10% serum+vehicle (1 mM acetic acid) or 10% serum+100 nM PTH (human or bear 1-84, or a subfragment thereof). After a one-hour incubation (Jilkia et al., 1999; J. Clin. Invest., 104, 439-46), 10 μM dexamethasone or its vehicle (DMSO) is added to each well and cells are incubated for 6 hours (Bellido, T., et al., 2003, J. Biol. Chem. 278(50), 50259-72; Jilkia et al., 1999, J. Clin. Invest., 104, 439-46). The PTH polypeptide or vehicle is left in situ during apoptosis induction because the suppression of apoptosis by PTH is self-limiting (Bellido et al., 2003). After 6 hours, cells are trypsinized, centrifuged, resuspended, and counted using a hemocytometer. 50,000 cells are removed from the suspension and placed into lysis buffer. The lysate supernatant (following centrifugation) is removed for analysis and stored at -20° C.

Apoptosis is quantified from the lysate supernatant with an ELISA (Cell Death Detection ELISA, #1544675, Roche Applied Science, Indianapolis, Ind.). This assay detects mono- and oligonucleosomes from fragmented cellular DNA in the cytoplasmic fraction of cell lysates, and therefore provides a good measure of the early and middle stages of apoptosis. Briefly, samples are diluted in buffer solution and added to microplate wells coated with an anti-histone mouse monoclonal (clone H11-4) antibody. Lysate supernatant from the vehicle-treated cells serves as a negative control. Optical densities are measured at 405 nm following the addition of a peroxidase-conjugated anti-DNA mouse monoclonal (clone MCA-33) antibody, and the amount of apoptosis in each sample is determined relative to its corresponding negative control. All samples are assayed in duplicate. Each of the polypeptides tested decreased apoptosis of the cells (under pro-apoptotic conditions).

Example 7
Comparison of Effects of Black Bear vs. Human PTH 1-84 or Subfragments Thereof on Gene Expression in Bone Cell Lines

Full-length recombinant black bear PTH (residues 1-84) is produced and its effects on levels of gene expression in bone cell lines (MC-3T3 osteoblastic cells and MLO-Y4 osteocytic cells) are investigated and compared to results obtained using recombinant human PTH 1-84. Equivalent experiments are conducted using subfragments of black bear and human PTH, the subfragments including amino acid residues 1-34, 1-36, 7-84, 11-84, and 41-52 of the full-length (1-84) mature protein. For some experiments, black bear and human PTH polypeptides are synthesized with solid-phase methods.

To determine the effect of black bear and human PTH on regulation of bone matrix, transcriptional regulatory, anti-apoptosis (Bcl-2) genes, and the pro-apoptosis gene Bax, cells are cultured for 1 or 3 hours with human or bear PTH 1-84 or a subfragment. Gene expression is quantified with real-time PCR.

For all of the experiments using recombinant polypeptides, the lyophilized peptides are reconstituted to 100 μM stock concentrations in 1 mM acetic acid, and diluted to 10 μM working stock concentrations before use. Additional experiments are performed with MC-3T3 cells using either 0.1% or 10% FBS. Additional experiments with less (0.1%) than the normal (10%) amount of FBS are run and the results are analyzed to determine whether there is a significantly different response between experiments that are run with normal or lower levels of serum. In the experiments reported in this Example, the results are not affected by the amount of FBS that is used. Real-time PCR is used to assess gene expression levels at 1- and 3-hour time points following addition of the polypeptide, showing in particular that black bear PTH 1-34 upregulates gene expression in MC-3T3 cells.

Cell Culture
MC-3T3 subclone 14 cells (ATCC, CRL-2594) and MLO-Y4 cells (obtained from L. F. Bonewald, University of Missouri, Kansas City, Mo.) are maintained in alpha-minimum essential media, 1% penicillin/streptomycin, and 10% serum (MC-3T3: 10% fetal bovine serum (FBS), MLO-Y4: 5% FBS and 5% bovine calf serum), at 37° C. in 5% CO2. All procedures described herein are repeated with independent cell cultures such that n=6 for all treatment combinations in each assay.

Effects of PTH Treatment on Gene Expression
MC-3T3 cells are seeded at a density of 50,000 cells/cm², and MLO-Y4 cells are seeded at a density of 15,000 cells/cm² in 6-well plates and cultured overnight to reach optimal confluence. The culture media is aspirated and replaced with media containing either 10% serum+vehicle (1 mM acetic acid) or 10% serum+100 nM PTH (human or bear 1-84, or a subfragment thereof). After a one-hour incubation (Jilkia et al., 1999; J. Clin. Invest., 104, 439-46), 10 μM dexamethasone or its vehicle (DMSO) is added to each well and cells are incubated for 6 hours (Bellido, T., et al., 2003, J. Biol. Chem. 278(50), 50259-72; Jilkia et al., 1999, J. Clin. Invest., 104, 439-46). The PTH polypeptide or vehicle is left in situ during apoptosis induction because the suppression of apoptosis by PTH is self-limiting (Bellido et al., 2003). After 6 hours, cells are trypsinized, centrifuged, resuspended, and counted using a hemocytometer. 50,000 cells are removed from the suspension and placed into lysis buffer. The lysate supernatant (following centrifugation) is removed for analysis and stored at -20° C.

Apoptosis is quantified from the lysate supernatant with an ELISA (Cell Death Detection ELISA, #1544675, Roche Applied Science, Indianapolis, Ind.). This assay detects mono- and oligonucleosomes from fragmented cellular DNA in the cytoplasmic fraction of cell lysates, and therefore provides a good measure of the early and middle stages of apoptosis. Briefly, samples are diluted in buffer solution and added to microplate wells coated with an anti-histone mouse monoclonal (clone H11-4) antibody. Lysate supernatant from the vehicle-treated cells serves as a negative control. Optical densities are measured at 405 nm following the addition of a peroxidase-conjugated anti-DNA mouse monoclonal (clone MCA-33) antibody, and the amount of apoptosis in each sample is determined relative to its corresponding negative control. All samples are assayed in duplicate. Each of the polypeptides tested decreased apoptosis of the cells (under pro-apoptotic conditions).
reverse primer, and 2.5 ng total RNA equivalent cDNA template. Gene expression is determined using the relative standard curve method normalized to the geometric mean of the three housekeeping genes. All samples are measured in duplicate, and any samples with a coefficient of variation (CV) greater than 10% are reanalyzed.

The polypeptides cause an upregulation of bone matrix, transcriptional regulatory, and transcriptional activator genes, and a decrease in the expression ratio of Bax/Bcl-2.

**Example 8**

Comparison of the Effects of Black Bear Serum from Different Seasons on Bone Cell Apoptosis and Gene Expression, and Correlation with Serum Levels of PTH and Osteocalcin

Blood samples are collected from at least 3 different female black bears (*Ursus americanus pallas*) held in the Virginia Tech Center for Bear Research between 2004 and 2005. Serum from additional black bears is collected in subsequent years. The Virginia Polytechnic Institute and State University Animal Care Committee approved all bear handling protocols (#98-069-F&WS). The bears are anesthetized with a 2:1 mixture of ketamine (100-mg/ml):xylazine (100-mg/ml); the dosage is 1 cc of the mixture per 45.5 kg of body mass. Blood samples are drawn from the femoral vein while the bears are anesthetized, and the samples are transported to the laboratory in an ice-packed cooler. Immediately on return to the laboratory, the blood is centrifuged to isolate the serum, which is then frozen at ~20°C. Blood samples are collected from each bear every 10 days from the beginning of October until the end of May. Hibernation begins in early January and ends in early April. Thus, the collection dates encompass an active pre-hibernation period, a disuse hibernation period, and an active post-hibernation remobilization period.

Aliquots of 10 µl of bear serum are assayed in duplicate for osteocalcin concentration by radioimmunoassay (Patterson-Allen et al., 1982; Anal. Biochem., 120, 1-7). This assay has previously been validated for bears (Donahue et al., 2006; J. Exp. Biol., 209, 1630-8). The antibody is guinea-pig anti-rat osteocalcin and tracer is 125I-labeled rat osteocalcin. Aliquots of 100 µl of bear serum are assayed in duplicate for PTH concentration (Donahue et al., 2006; J. Exp. Biol., 209, 1630-8) with an ELISA (Pocine Intact PTH ELISA Kit, #60-3305, Immutopics, Inc., San Clemente, Calif.). This assay binds the 39-84 region of PTH, and requires the 13-34 region of PTH to colorimetrically report PTH concentration. Thus, it provides a good measure of intact (1-84) PTH concentration as well as C-terminal subfragments 7-84 and 11-84. This ELISA has been shown to cross-react with bear PTH (Donahue et al., 2006; J. Exp. Biol., 209, 1630-8), and has 100% cross-reactivity with human PTH. To validate this assay for black bears, samples of culture media containing 10 nM recombinant black bear or human PTH 1-84 are assayed in duplicate. The measured concentration determined from the assay’s standard curve. Any potential difference in cross-reactivity determined from these samples is used as a correction for endogenous black bear PTH concentration in the black bear serum samples.

The procedures described above for recombinant black bear PTH apoptosis and gene expression cell culture experiments with cultured bone-type cells are repeated, substituting the 100 nM recombinant PTH-containing media with culture media containing 10% black bear serum (from pre-hibernation, hibernation, or post-hibernation periods). The serum volumes are calculated following the PTH ELISA described above.

From the hibernation and post-hibernation seasons causes a greater prevention of apoptosis compared to pre-hibernation serum, because PTH is higher during hibernation and post-hibernation than in pre-hibernation serum. Endogenous serum PTH concentrations are inversely related to apoptosis levels, in that higher serum PTH levels correspond to lower rates of apoptosis, i.e. serum PTH concentrations are negatively correlated with apoptosis levels.

**Example 9**

In Vivo Testing of Bear PTH

Black bear PTH, either full-length (1-84) or one of several functional subfragments thereof (1-34; 1-36; 7-84; 11-84; 41-52), are tested in vivo for anabolic stimulation of bone cells compared to an equivalent human PTH or subfragment. Each of the PTH polypeptides is synthesized and suspended in a pharmaceutically-appropriate carrier for subcutaneous injection. Full-length PTH or a functional fragment thereof, from either black bear or human, is administered to mice at a dose of 40 µg/kg body weight daily for 7 weeks. Black bear PTH or a functional fragment thereof cause greater increases in bone strength, mass, and mineral content than the equivalent human PTH polypeptide.

**Example 10**

Use of Bear PTH as Prophylactic Agent to Reduce or Prevent Bone Loss

Osteoporosis is induced in mice by hindlimb suspension (a disuse model of osteoporosis) and by ovariectomy (a post-menopausal model of osteoporosis). After the hindlimb suspension begins or the ovariectomy is completed, the mouse is given regular doses of black bear PTH or a functional fragment thereof. The mice treated with black bear PTH or functional fragments thereof show less bone loss than untreated mice.

**Example 11**

Bear and human PTH 1-34 both upregulate gene expression of osteocalcin, but only bear PTH 1-34 decreases the expression ratio of Bax/Bcl-2

MC-3T3 cells were incubated in vehicle or 100 nM synthetic bear or human PTH 1-34 for 3 or 6 hours (n=2 or 4). Total RNA was isolated, and cDNA was generated with reverse transcription. Primers for the bone matrix proteins type I collagen and osteocalcin, the pro-apoptotic protein Bax, the anti-apoptotic protein Bcl-2, and the housekeeping genes Gapdh, β-actin, and cyclophilin were designed using PrimerQuest software (Integrated DNA Technologies, Coralville, Iowa). Real-time PCR was performed using the MX3000P real-time PCR system (Stratagene, LaJolla, Calif.). All samples were measured in duplicate. Gene expression was determined using the relative standard curve method normalized to the geometric mean of the three housekeeping genes (Gapdh, β-actin, and cyclophilin). Apoptosis-related genes were analyzed as the expression ratio of Bax/Bcl-2, since a decrease in this ratio is associated with decreased apoptosis in vitro.
Culture in human or bear PTH 1-34 for 6 hours did not affect expression of type I collagen but substantially upregulated the expression of osteocalcin compared to the vehicle control (FIG. 6). There were no significant differences between human and bear PTH (p=0.09).

Culture in bear PTH 1-34 for 3 hours decreased the expression ratio of Bax/Bcl-2, suggesting decreased apoptosis, but culture in human PTH 1-34 for 3 hours increased the ratio, suggesting increased apoptosis (FIG. 7). The difference between bear and human PTH was statistically significant (p=0.047).

Example 12

In Vivo Rat OVX Dose Response Study

Ninety female, 3-month old Sprague-Dawley rats were obtained. Eighty (80) of the rats were ovariectomized (OVX) and ten (10) underwent a sham OVX procedure (sham OVX) in which the ovaries were left in the animal. The OVX animals were randomly assigned into groups of ten for further experiments.

Following the actual or sham OVX procedure, all animals were untreated for 6 weeks to allow bone loss. After the 6 week period of bone loss, groups of animals were injected for a six- or eight-week period (daily for five days per week) with vehicle (saline) or 3, 10, or 30 μg/kg bear or human PTH1-34. The current dose of PTH1-34 for humans is approximately 0.3 μg/kg while a dose as low as 5 μg/kg has been shown to be carcinogenic in rats (Tashjian et al., J. Bone Mineral Res. 23(6): 803-811 (2008)).

Bone mineral apparent density was determined for each of the samples (FIG. 11) and is shown as milligrams hydroxyapatite per cubic centimeter ("App.Dn (mgHA/ccm)"). The bone mineral apparent density was statistically significantly higher (p<0.05) for bones of OVX rats treated with 3, 10, or 30 μg/kg hPTH or bbPTH than for bones of OVX rats treated with vehicle (saline) alone. The bone mineral apparent density for OVX rats treated with 30 μg/kg hPTH was significantly higher (p<0.05) than OVX rats treated with 30 μg/kg bbPTH.

The number and thickness of trabecular struts were also determined for bones from sham OVX, OVX without PTH treatment, and PTH-treated OVX rats (FIG. 12). The symbols show the trabecular number per millimeter ("Tb.N (mm−1)") for sham OVX rats ("sham baseline," light diamonds), OVX rats treated with vehicle (saline) alone and euthanized at six weeks ("OVX baseline," dark diamonds), OVX rats treated with vehicle (saline) alone for eight weeks and euthanized at fourteen weeks ("OVX baseline," dark diamonds), and OVX rats treated with 3, 10, or 30 μg/kg human ("hPTH1-34," squares) or bear ("bbPTH1-34," circles) PTH1-34 (FIG. 12).

The average thickness of the trabecular struts was also determined (FIG. 13). Treatment with either bear or human PTH significantly (p<0.05) increased the average thickness of the trabecular struts ("Tb.Th (mm)") compared to vehicle-treated controls.

Black bear PTH1-34 showed a trend of greater trabecular bone mineralization ("M.Dn (mgHA/ccm);" FIG. 14) with increasing dose compared to human PTH1-34. Treatment of OVX rats with either bear ("bbPTH1-34," circles) or human ("hPTH1-34," squares) PTH1-34 produced significantly (p<0.05) greater trabecular bone mineralization than OVX rats treated with vehicle (saline) alone.

Human PTH1-34 ("hPTH," squares) is better than bear PTH1-34 ("bbPTH," circles) at increasing tibial cortical bone volume ("BV(mm2);" FIG. 15). Overall, however, changes in the bone cortical region were moderate relative to the changes observed in bone trabeculae.

Tibial cortical porosity in bones of OVX rats was observed to be significantly (p<0.05) decreased when the rats were treated with middle (10 μg/kg) and higher doses (30 μg/kg) of either bear ("bbPTH1-34," circles) or human ("hPTH1-34," squares) PTH1-34 relative to bones from OVX rats treated with vehicle (saline) alone (FIG. 16). However, only higher doses (30 μg/kg) of human or bear PTH1-34 produced significant (p<0.05) increases in femoral cortical bone strength relative to vehicle-treated controls (FIG. 17).

An additional benefit that black bear PTH1-34 may confer over human PTH1-34 is lower serum calcium. Hypercalceemia (elevated serum calcium) is a side effect of PTH treat-
ment in humans. In the present experiments, serum calcium levels were seen to be lower in rats treated with bear PTH1-34 compared to human PTH1-34, although the results are not significant at the p<0.05 level. However, statistical analyses predict that serum calcium concentrations would be significantly (p<0.05) lower in black bear PTH1-34-treated rats if the sample size were doubled. Thus, black bear PTH1-34 treatment may reduce the incidence of this side effect.

The experiments show that black bear PTH1-34 is equally effective as human PTH1-34 at producing dramatic increases in trabecular bone formation. Human PTH1-34 was observed to be more effective than bear PTH1-34 for several parameters at the highest dose (30 μg/kg), although this is 100 times higher than doses given to humans. However, at lower and more clinically-relevant doses, bear PTH1-34 was as effective as human PTH1-34 at increasing important parameters related to reducing bone fracture likelihood.

Example 13

Bear PTH1-34 and Bear PTH1-84 Reduce Caspase-3 Activity in Cultured Mouse Osteoblasts

Cell Culture

MC3T3 subclone 4 cells (ATCC, CRL-2593) were maintained in standard culture media (89% alpha-minimum essential media, 1% penicillin/streptomycin, and 10% FBS) at 37°C in 5% CO2. Cells were not used beyond passage 30.

PTH Attenuation of Serum-Starvation Induced Apoptosis

MC3T3 cells were seeded at a density of 20,000 cells/well in 96-well plates and allowed to attach overnight. Culture media was then aspirated and replaced with 100 μl of culture media containing 10% FBS and either vehicle (PBS) or PTH (10-100 nM) for one hour, after which media was aspirated and cells were washed with PBS to remove residual serum. To induce apoptosis, cells were incubated in control or serum-free media for 6 hours. Replacement media contained 89% alpha-minimum essential media, 1% penicillin/streptomycin, and either 10% FBS (control media) or 0% FBS (starvation media). After starvation, media was aspirated, cells were washed with PBS, and 50 μl of fresh PBS was added to each well. Caspase-3/7 activity was determined by cleavage of a DEVD substrate (Caspase-3/7 Assay, #G8093, Promega Corporation). Measured luminescence values were fit to a standard curve prepared with known concentrations of recombinant human caspase-3 ((#SE-169, BIOMOL Research Laboratories Inc.). Five wells of each treatment were run per 96-well plate, and procedures were repeated on a minimum of 10 plates.

Statistics

Attenuation of caspase-3/7 activation was compared between peptide, vehicle, and control-treated samples with ANOVA with Fisher’s PLSD, blocking by experiment replication. Caspase-3/7 activity values were normalized to the control media (unstarved cells) caspase-3/7 level within each replication, and analysis of covariance (ANCOVA) was used to compare the dose response behavior of the peptides, treating concentration as the covariate.

Serum starvation significantly (p<0.0001) increased caspase-3/7 activity (an early indicator of apoptosis) in the vehicle and PTH1-pretreated samples compared to non-starved cells in this experiment (FIG. 19). All PTH pretreatments significantly (p<0.0001) lowered caspase-3/7 activity compared to vehicle-treated cells. PTH-induced reduction in caspase-3/7 activity did not demonstrate a dose-dependent behavior (p=0.890), therefore, all concentrations were pooled within groups for between group comparisons. Of the four peptide groups, bbPTH 1-34 demonstrated the greatest attenuation of serum starvation-induced apoptosis; mean caspase-3/7 activity in the bbPTH 1-34 group was significantly lower than the hPTH 1-34 group (p=0.006) and either PTH 1-84 group (p=0.018) (FIG. 19). In contrast, the difference in caspase-3/7 activity between the bbPTH 1-84 and hPTH 1-84 groups approached significance (p=0.090) with hPTH 1-84 indicating lower levels of apoptosis (FIG. 19).

Example 14

In Vivo Effects of Bear PTH1-34 and Bear PTH1-84 in Male Swiss-Webster Mice

Animals

All handling procedures and experiments were approved by the Michigan Technological University Institutional Animal Care and Use Committees. Male Swiss-Webster mice were obtained from a commercial breeder (Harlan, Indianapolis, Ind.). All animals were maintained on a 12-h light/12-h dark cycle, and were permitted free access to water and food containing 0.95% calcium (Purina Rodent Diet #5001). In the first experiment, 11 wk old mice received daily (5 days/week) subcutaneous injections of 2.5 nmol/kg PTH 1-34 (hPTH 1-34 or bbPTH 1-34) or acidic saline vehicle (0.15 M NaCl+0.001 N HCl) for 8 weeks (n=10 mice/group). In the second experiment, 12 wk old mice received daily (5 days/week) subcutaneous injections of 2.5 nmol/kg PTH 1-34 (hPTH 1-84 or bbPTH 1-84) or acidic saline vehicle for 8 weeks (n=10 mice/group). All PTH- and vehicle-treated animals received subcutaneous injections of calcein at a dosage of 10 mg/kg on days 12 and 3 before death to label mineralizing bone surfaces. Twenty-four hours following the last treatment injection, mice were sacrificed by carbon dioxide asphyxiation. Right femurs were fixed in 10% neutral buffered formalin. Left femurs were wrapped in 0.15M saline soaked gauze and stored at ~20 deg C. Left tibias were stored in 70% ethanol at 4 deg C.

Trabecular Bone Structure and Mineralization in the Proximal Tibia

Trabecular bone architecture and mineralization were evaluated in the left proximal tibial metaphysis (0.7 mm distal to the growth plate) using micro-computed tomography. Tibias from experiment 1 were scanned on a μCT35 scanner (Scanco Medical AG, Basserdorf, Switzerland) at 3.5 μm (high resolution). Tibias from experiment 2 were scanned on a μCT40 scanner (Scanco Medical AG, Basserdorf, Switzerland) at 7 μm (high) resolution. Bones were scanned in 70% ethanol. Trabecular bone volume fraction (BV/TV, %), trabecular number (Tb.N, mm⁻²), trabecular thickness (Tb.Th, μm), trabecular separation (Tb.Sp, μm), trabecular apparent mineral density (App.Mn.Dn, mgHA/cm³) and trabecular tissue mineral density (Mat.Mn.Dn, mgHA/cm³) were computed using the manufacturer’s software.

Cortical Bone Mechanical Properties of the Femur

Left femurs were loaded to failure in 3-point bending on an Instron mechanical testing system (Model #8872, Canton, Mass.) at a rate of 1 mm/min. Bones were oriented with the anterior surface in compression. The lower testing fixture supports were separated by a span of 10 mm, and all fixtures had rounded contact points (radius=1 mm) to prevent localized stress concentrations in the bone. Ultimate load was calculated using the load-deformation data from testing.

Cortical Bone Mineral Content, Geometrical Properties, and Dynamic Histomorphometry

Following the bending test, the left femoral diaphyses were ashed in a furnace to determine mineral content (ash fraction).
Right femoral diaphyses were embedded in methyl methacrylate and sectioned with a diamond saw to expose the midshaft cross-section. Images of the midshaft cross-sections were captured using a digital camera (Spot Insight QE, Diagnostic Instruments Inc., Sterling Heights, Mich.), and Scion Image analysis software (Scion Corporation, Frederick, Md.) was used to calculate the periosteal area (Ps.Ar), cortical area (Ct.Ar), and endosteal area (Es.Ar) for each sample. Cortical bone thickness (Ct.Th) was calculated in 0.1 mm increments for each anatomical quadrant and for the entire cross-section using image analysis software (Bioquant Osteo, Nashville, Tenn.). Thin sections from the midshaft of the right femur were ground to a thickness of 50 μm and mounted on glass slides. Images of calcein labels were captured at 100x magnification and periosteal and endosteal mineral apposition rates (Ps.MAR and Es.MAR, respectively) and mineralizing surfaces (Ps.MS/BS and Es.MS/MS) were quantified (Bioquant) for each femur.

In Vivo Experiments Statistics

All bone geometrical, architectural, and mineral properties were normalized by body mass to account for potential variation in animal size (31-33). Trabecular bone properties quantified by micro-computed tomography were compared by ANOVA between vehicle, bear, and human PTH-treated mice within each study (PTH 1-34 or PTH 1-84). The microCT parameters were not directly compared between the PTH 1-34 and PTH 1-84 studies because analyses were conducted on different microCT machines. For all other bone properties, all six experimental groups (from both the PTH 1-34 and PTH 1-84 studies) were compared with ANOVA and Fisher’s PLSD. A significance of p<0.05 was used for all comparisons.

Results

Trabecular Bone Structure and Mineralization in the Proximal Tibia

Trabecular bone microCT data are presented in Tables 2 and 3. There were no significant differences between experimental groups in the PTH 1-34 study, although trabecular bone material density tended to be lowest in mice treated with bbPTH 1-34 (Table 2). In contrast, bone volume fraction was greater in the proximal tibia of mice treated with bbPTH 1-84 compared to hPTH 1-84 (Table 3). Differences between the effects of bbPTH 1-84 and hPTH 1-84 on bone volume fraction were significant (p=0.013), with bbPTH being the more efficacious therapy. The increase in bone volume fraction in the bbPTH 1-84 treated mice was likely influenced by trabecular thickness since trabecular thickness tended to be greater in mice treated with bbPTH 1-84 compared to mice treated with hPTH 1-84 (p=0.089). Trabecular number and trabecular separation were not significantly different between groups in the PTH 1-84 study (Table 3). Trabecular apparent mineral density was highest in mice treated with bbPTH 1-84 compared to other treatments; the difference in the effects of bbPTH 1-84 and hPTH 1-84 was statistically significant (p=0.019) (Table 3). Trabecular material mineral density tended to be highest in mice treated with bbPTH 1-84 (Table 3).

### TABLE 1

<table>
<thead>
<tr>
<th>Bone Property</th>
<th>Vehicle</th>
<th>hPTH 1-34</th>
<th>bbPTH 1-34</th>
<th>ANOVA p-value</th>
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<tr>
<td>BV/TV (%/kg)</td>
<td>1.46 (0.68)</td>
<td>1.35 (0.76)</td>
<td>1.34 (0.35)</td>
<td>0.883</td>
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<td>Tb. N (1/mm*kg)</td>
<td>115.2 (27.2)</td>
<td>105.0 (26.8)</td>
<td>107.4 (19.1)</td>
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<td>Tb. Th (mm/kg)</td>
<td>0.96 (0.11)</td>
<td>1.05 (0.10)</td>
<td>1.02 (0.21)</td>
<td>0.446</td>
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<tr>
<td>Tb. Sp (mm/kg)</td>
<td>9.17 (2.48)</td>
<td>9.57 (2.29)</td>
<td>8.76 (2.39)</td>
<td>0.752</td>
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<tr>
<td>App. Mn. Dn (mgHA/(cm³*kg))</td>
<td>1829 (994)</td>
<td>1783 (1052)</td>
<td>1720 (537)</td>
<td>0.363</td>
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<tr>
<td>Mat. Mn. Dn. (mgHA/(cm³*kg))</td>
<td>32563 (2743)</td>
<td>31281 (1957)</td>
<td>30457 (1412)</td>
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Standard deviations are shown in parentheses.

See text for explanations of abbreviations.

### TABLE 2

<table>
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<tr>
<th>Bone Property</th>
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<th>hPTH 1-84</th>
<th>bbPTH 1-84</th>
<th>ANOVA p-value</th>
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<tr>
<td>BV/TV (%/kg)</td>
<td>1.61 (0.75)</td>
<td>1.34 (0.83)</td>
<td>2.26 (0.72)</td>
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<td>Tb. N (1/mm*kg)</td>
<td>97.0 (21.9)</td>
<td>98.7 (20.1)</td>
<td>113.8 (24.3)</td>
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<td>Tb. Th (mm/kg)</td>
<td>1.22 (0.08)</td>
<td>1.26 (0.18)</td>
<td>1.38 (0.17)</td>
<td>0.055</td>
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<td>Tb. Sp (mm/kg)</td>
<td>8.94 (2.35)</td>
<td>9.57 (2.04)</td>
<td>8.47 (2.26)</td>
<td>0.558</td>
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<tr>
<td>App. Mn. Dn (mgHA/(cm³*kg))</td>
<td>2729 (1029)</td>
<td>2513 (1118)</td>
<td>3665 (935)</td>
<td>0.043</td>
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<tr>
<td>Mat. Mn. Dn. (mgHA/(cm³*kg))</td>
<td>28667 (1534)</td>
<td>29810 (2317)</td>
<td>30352 (1374)</td>
<td>0.077</td>
</tr>
</tbody>
</table>

Standard deviations are shown in parentheses.

Different superscripts indicate significant differences (p < 0.05) between groups.

See text for explanations of abbreviations.
Cortical Bone Mechanical Properties and Ash Fraction of the Femur

Ultimate load of the femur was increased by peptide treatment (+3% to +15% compared to vehicle-treated mice) (FIG. 29). The increase in bone strength was significantly (p=0.032) greater in mice treated with bbPTF11-84 compared to hPTF11-84, but was not different (p=0.819) between mice treated with bbPTF11-34 and hPTF11-34. Bone mineral content (ash fraction) was not different between the treatment groups (p=0.180) (data not shown).

Cortical Bone Geometrical Properties in the Femur

Bone periosteal area was highest in vehicle treated mice from the PTH 1-34 study and lowest in vehicle-treated mice from the PTH 1-84 study (Table 3). Mice treated with bbPTF11-84, but not hPTF11-84, had significantly greater values for periosteal area compared to vehicle-treated mice, but mice treated with both PTH 1-34 peptides had lower values for periosteal area compared to vehicle treated mice (Table 3). Neither cortical bone area nor endosteal area were different between treatment groups (p=0.136). Cortical bone thickness was greater in mice from the PTH 1-84 study compared to the PTH 1-34 study, but there were no other differences between treatment groups (Table 3).

**TABLE 3**
Cortical bone geometrical properties in the femoral midshaft of PTH-treated mice.

<table>
<thead>
<tr>
<th>Bone property</th>
<th>Vehicle (1-34)</th>
<th>hPTH 1-34</th>
<th>bbPTH 1-34</th>
<th>Vehicle (1-84)</th>
<th>hPTH 1-84</th>
<th>bbPTH 1-84</th>
<th>ANOVA p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ps.Ar (mm²/kg)</td>
<td>60.5 (7.5)a</td>
<td>55.3 (5.2)b</td>
<td>56.0 (3.6)c</td>
<td>53.7 (3.6)c</td>
<td>54.6 (4.4)c</td>
<td>58.3 (5.0)b</td>
<td>0.042</td>
</tr>
<tr>
<td>Ct.Ar (mm²/kg)</td>
<td>31.3 (2.9)</td>
<td>30.7 (3.0)</td>
<td>31.7 (3.5)</td>
<td>28.9 (2.9)</td>
<td>29.0 (3.7)</td>
<td>31.7 (3.7)</td>
<td>0.194</td>
</tr>
<tr>
<td>Es.Ar (mm²/kg)</td>
<td>29.2 (7.3)</td>
<td>24.7 (3.7)</td>
<td>24.3 (2.0)</td>
<td>24.8 (2.9)</td>
<td>25.6 (3.3)</td>
<td>26.6 (4.6)</td>
<td>0.136</td>
</tr>
<tr>
<td>Ct.Th (mm/kg)</td>
<td>1.97 (0.19)b</td>
<td>2.22 (0.20)b</td>
<td>2.14 (0.26)c</td>
<td>3.29 (0.40)a</td>
<td>3.41 (0.59)a</td>
<td>3.56 (0.42)a</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Standard deviations are shown in parentheses. Different superscripts indicate significant differences (p < 0.05) between groups. See text for explanations of abbreviations.

Cortical Bone Dynamic Histomorphometry in the Femur

There were no significant differences in periosteal or endosteal mineralizing surface or mineral apposition rate between treatment groups (p>0.236) (Table 4).

**TABLE 4**
Cortical bone dynamic histomorphometry in the femoral midshaft of PTH-treated mice.

<table>
<thead>
<tr>
<th>Bone property</th>
<th>Vehicle (1-34)</th>
<th>hPTH 1-34</th>
<th>bbPTH 1-34</th>
<th>Vehicle (1-84)</th>
<th>hPTH 1-84</th>
<th>bbPTH 1-84</th>
<th>ANOVA p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periosteal</td>
<td>20.9 (13.8)</td>
<td>24.0 (13.8)</td>
<td>28.9 (13.6)</td>
<td>22.2 (10.6)</td>
<td>20.2 (16.7)</td>
<td>31.3 (22.5)</td>
<td>0.526</td>
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<tr>
<td>MS/BS</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Endosteal</td>
<td>20.4 (16.7)</td>
<td>39.3 (17.7)</td>
<td>31.2 (25.9)</td>
<td>27.8 (14.3)</td>
<td>32.1 (19.4)</td>
<td>24.5 (17.2)</td>
<td>0.317</td>
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<tr>
<td>MS/BS</td>
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<tr>
<td>Periosteal</td>
<td>0.44 (0.23)</td>
<td>0.53 (0.21)</td>
<td>0.64 (0.21)</td>
<td>0.69 (0.34)</td>
<td>0.60 (0.40)</td>
<td>0.62 (0.33)</td>
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<tr>
<td>Endosteal</td>
<td>0.47 (0.20)</td>
<td>0.56 (0.18)</td>
<td>0.63 (0.26)</td>
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<td>0.62 (0.39)</td>
<td>0.59 (0.42)</td>
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<tr>
<td>MAR</td>
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</table>

Standard deviations are shown in parentheses.
CONCLUSIONS

These Data indicate that bbPTH 1-34 is more anti-apoptotic than hPTH 1-34 and hPTH 1-84 in vitro, however, bbPTH 1-84 increases bone volume and strength in mice more than hPTH peptides. This suggests bbPTH has potential as a new anabolic therapy for osteoporosis.

The hPTH peptides in these studies did not produce an increase in trabecular bone mass compared to vehicle treatment (Tables 1 and 2), likely because of the relatively low peptide dosage administered to the mice. Short-term in vivo studies of PTH’s anabolic effects in mice typically use peptide dosages ≥10 nmol/kg to produce a large increase in bone formation and bone mass over a short period of time. In previous studies, lower peptide dosages are less osteogenic in short-term studies; daily injections of 1 nmol/kg hPTH 1-34 for 6 weeks did not affect trabecular thickness (Th.Tb) or mineral apposition rates (MAR) in the proximal tibia of male mice, whereas a higher molar dosage of hPTH (~10 nmol/kg) increased these properties. See Sakai et al. (1999) J Bone Miner Res 14(10):1691-9. A dosage of 2.5 nmol/kg was used in our studies because it is the lowest peptide dosage shown to increase osteoblast number and decrease osteoblast apoptosis in trabecular bone secondary spongiosa in mice (see Bellido et al. (2003) J Biol Chem 278(50):50259-72), and it is closer to a clinical dosage (20 μg/day, approximately 0.07 nmol/kg for a 70 kg patient) compared to PTH dosages used in other short-term in vivo studies. Although hPTH peptides did not increase trabecular bone mass at 2.5 nmol/kg, mice treated with the same dosage of bbPTH 1-34 showed improvement in trabecular bone properties (Table 2) and cortical bone strength (FIG. 29) compared to hPTH 1-84. Trabecular thickness tended to be elevated in mice treated with bbPTH 1-84 (Table 2). Importantly, trabecular bone volume fraction and apparent mineral density were only significantly increased over vehicle treatment by bbPTH 1-84 (Table 2). Most of the peptide analogs investigated tended to increased femoral ultimate load, but the increase over vehicle treatment was only statistically significant for bbPTH 1-84 (FIG. 29). Taken together, these findings raise the possibility that bbPTH may be an effective osteogenic therapy at a lower dose than hPTH.

All PTH peptides used in the current study attenuated apoptotic signaling induced by serum starvation in vitro (FIG. 19). bbPTH 1-34 reduced apoptotic signaling in pre-osteoblastic cells more than human PTH (FIG. 19). Recently, we found similar results with preliminary studies in human fetal osteoblasts (hFOB). Serum starvation for 24 hours increased cell death in hFOB (confirmed via trypan blue staining), but pretreatment of hFOB cells with 100 nM PTH 1-34 or 1-84 for 1 hour before starvation reduced percent cell death (percentage change in cell death compared to vehicle pretreatment, hPTH 1-34: -18%, bbPTH 1-34: -25%, hPTH 1-84: -17%, bbPTH 1-84: -21%; p<0.0001; data not shown). The enhanced anti-apoptotic effects of bbPTH 1-34 led to the expectation that bbPTH 1-34 would be the most potent osteogenic agent in vivo. However, although bbPTH 1-84 was less anti-apoptotic than bbPTH 1-34 and hPTH 1-34 in vitro (FIG. 19) it caused the greatest increases in cortical and trabecular bone mass in the in vivo studies (Table 1, Table 2).

The disparity between the in vitro and in vivo studies cannot be reconciled at present. Reducing osteoblast apoptosis is a dominant process in hPTH’s anabolic effects on trabecular bone, but it is possible that other biological mechanisms regulate bbPTH’s ability to increase bone mass. For example, increasing osteoblast differentiation (rather than preventing osteoblast apoptosis) is the primary means by which hPTH stimulates periosteal cortical bone formation.
seasonal effects on osteoblast response to apoptotic stimulus, MC3T3-E1 pre-osteoblasts were seeded in Alpha-Modified Minimum Essential Media (–MEM) with 10% fetal bovine serum at 10,000 cells per well in a white-walled tissue culture plate and allowed to attach overnight. Media was then aspirated and replaced with 2% seasonal bear serum in –MEM for a 24 hour treatment. Media was again aspirated and replaced with serum free –MEM for 6 hours to induce apoptosis. Media was aspirated a final time and replaced with 50 μL of PBS and 50 μL of luminescent DEVD substrate (Caspase-glo 3/7, Promega, Madison, Wis.). Luminescence was quantified on a Synergy HT Multi-Detection Microplate Reader (Bio-Tek, Winooski Vt.). As shown in FIG. 2B, caspase-3/7 activity of cells cultured in hibernation sera was lower than those cultured in active sera (p<0.0001). These findings indicate that seasonal changes in circulating factors (e.g., hormones) provide an anti-apoptotic effect to osteoblasts during hibernation.

<table>
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<th>Table 6</th>
<th>Sequences</th>
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<td>black bear PTH-84 (bPTH1-84)</td>
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<td>Polynucleotide encoding full length black bear PTH (bPTH)</td>
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<tr>
<td>4</td>
<td>full length black bear PTH (bPTH)</td>
</tr>
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<td>5</td>
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<td>human PTH1-84 (hPTH1-84)</td>
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### TABLE 6-continued

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acagcgcaca gagatggtgg ttcccagagg cccccagaaaa aggaagacaa tgtgctggtt 180
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aacctgggaag cactcttggag ccaggtggag ggtgggaatt ggtaggctgg 180
gcagcttgag atcttagtct cccaggatgct ccaacagctc agagtagctg tggagccag 240
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Val Ser Glu Ile Gln Phe Met His Leu Leu Gly Lys His Leu Ser Ser
35  40  45
Met Glu Arg Val Glu Trp Leu Arg Lys Leu Gln Asp Val His Ann
50  55
Phe Val Ala Leu Gly Ala Pro Thr Ala His Arg Asp Gly Ser Gin
65  70  75  80
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100  105  110
Lys Ser Gin
115

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Asn Phe Ile Ala Leu Gly Ala Pro Leu Ala Pro Arg Ala Gly Ser
35  40
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Asn Phe Val Ala Leu Gly Ala Pro Leu Ala Pro Arg Ala Gly Ser
35  40
Gln Arg Pro Arg Lys Glu Asp Ann Ile Leu Val Gln Ser His Glu
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35 40 45
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Thr Lys Ser Gln

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20 25 30
Asn Phe Val Ala Leu Gly Ala Pro Ile Ala His Arg Asp Gly Ser Ser
35 40 45
Gln Arg Pro Leu Lys Glu Asp Asn Val Leu Val Glu Ser Tyr Gln
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65 70 75 80
Ala Lys Ser Gln

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1  5  10  15
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20 25 30
Asn Phe Val Ala Leu Gly Ala Pro Ile Ala His Arg Asp Gly Gly Ser
35 40 45
Gln Arg Pro Arg Lys Glu Asp Asn Val Pro Ala Glu Asn His Gln
50 55 60
Lys Ser Leu Gly Glu Ala Asp Lys Ala Asp Val Asp Val Leu Ile Lys
65 70 75 80

Ala Lys Ser Gin

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20 25 30
Asn Phe Val Ala Leu Gly Ala Ser Ile Ala Tyr Arg Aep Gly Ser Ser
35 40 45
Gln Arg Pro Arg Lys Glu Asp Aen Val Leu Val Glu Ser His Gin
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Lys Ser Leu Gly Glu Ala Asp Lys Ala Asp Val Asp Val Leu Ile Lys
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Ala Lys Pro Gin

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Asn Phe Val Ala Leu Gly Ala Ser Ile Val His Arg Aep Gly Gly Ser
35 40 45
Gln Arg Pro Arg Lys Glu Asp Aen Val Leu Val Glu Ser His Gin
50 55 60
Lys Ser Leu Gly Glu Ala Asp Lys Ala Ala Val Asp Val Leu Ile Lys
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35 40 45
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Ala Lys Ser Gln

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35 40 45
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Ser Lys Ser Gln

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Asn Phe

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20 25 30
Asn Phe Val Ala
What is claimed is:

1. A method of increasing cAMP levels in a bone-forming cell comprising administering 0.10 μg/kg per day to 40 μg/kg per day of a polypeptide comprising SEQ ID NO: 2 to a human subject in need thereof.

2. A method of reducing apoptosis in a bone-forming cell comprising administering 0.10 μg/kg per day to 40 μg/kg per day of a polypeptide comprising SEQ ID NO: 2 to a human subject in need thereof.

3. A method of decreasing the ratio of expression levels of Bax protein to Bcl-2 protein in a bone-forming cell comprising administering 0.10 μg/kg per day to 40 μg/kg per day of a polypeptide comprising SEQ ID NO: 2 to a human subject in need thereof.

4. A method of increasing the expression level of one or more of a bone matrix protein, a transcriptional activator, or a transcriptional regulator in a bone-forming cell comprising administering 0.10 μg/kg per day to 40 μg/kg per day of a polypeptide comprising SEQ ID NO: 2 to a human subject in need thereof.

5. A method of enhancing bone mineral density, increasing bone mass, decreasing bone loss, or reducing the incidence of bone fractures, or any combination thereof, in a human subject in need thereof, comprising contacting a bone-forming cell in the subject with 0.10 μg/kg per day to 40 μg/kg per day of a polypeptide comprising SEQ ID NO: 2.

6. The method of claim 5, wherein the subject is a postmenopausal female human afflicted with osteoporosis.

7. The method of claim 5 further comprising administering vitamin D and calcium to the subject.

8. A method of increasing bone volume, increasing bone mineral apparent density, increasing number of trabecular struts, increasing thickness of trabecular struts, increasing trabecular bone mineralization, increasing cortical bone volume, decreasing cortical porosity, or decreasing serum calcium, or any combination thereof, in a human subject in need thereof, comprising contacting a bone-forming cell in the subject with 0.10 μg/kg per day to 40 μg/kg per day of a polypeptide comprising SEQ ID NO: 2.

9. The method of claim 8, wherein the subject is a postmenopausal female human afflicted with osteoporosis.

10. The method of claim 8, further comprising administering vitamin D and calcium to the subject.

* * * * *