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Fgf23/Klotho signaling is not essential for the phosphaturic and anabolic functions of PTH

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Abstract

PTH is widely recognized as a key regulator of mineral ion homeostasis. Daily intermittent administration of PTH is the only currently available anabolic therapy for bone disorders such as osteoporosis. Recent studies have shown that PTH increases transcription and secretion of fibroblast growth factor 23 (FGF23), another important regulator of phosphate homeostasis and skeletal metabolism. However, the full relationship between PTH and FGF23 is largely unknown. This study evaluated the effect of FGF23/Klotho signaling on the phosphaturic and anabolic functions of PTH. Eight-day-old wild-type (*WT*), *Fgf23*^{-/-} and *Kl*^{-/-} mice were injected with 100 µg/kg PTH (1–34) or vehicle daily for a two-week-period and then sacrificed. Intermittent injection of PTH successfully reduced the serum phosphate levels and reversed the hyperphosphatemia of *Fgf23*^{-/-} and *Kl*^{-/-} mice. Bone changes were analyzed in the distal femur metaphysis by pQCT, µCT, and histomorphometry. PTH treatment induced substantial increases in bone mineral density (BMD) and trabecular bone volume in each mouse genotype. Expression of osteoblastic marker genes, including Runx2, Col1, Alp, Ocn and Sost were similarly altered. In addition, primary osteoblasts were isolated and treated with 100nM PTH *in vitro*. PTH treatment similarly induced cAMP accumulation and phosphorylation of ERK1/2 and CREB in the osteoblasts from each genotype. Taken together, our results demonstrate that Fgf23/Klotho signaling is not essential for the phosphaturic and anabolic functions of PTH, suggesting that PTH can function as a therapeutic agent to improve the skeletal quality of patients even in the presence of abnormal serum FGF23 levels.

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Keywords

PTH; FGF23; Klotho; Phosphate homeostasis; anabolism

Introduction

Parathyroid hormone (PTH) is secreted by the parathyroid glands as a polypeptide containing 84 amino acids and plays a central role in regulating mineral ion and bone homeostasis by binding to the PTH/PTH-related protein (PTHrP) receptor (PTH1R), a G protein-coupled receptor that is mainly expressed in kidney and bone ⁽¹⁾. Ligand binding to PTH1R activates multiple intracellular signaling pathways, including the cyclic AMP-dependent protein kinase A (PKA), calcium-dependent protein kinase C signaling pathways and the extracellular signal related (ERK)/mitogen-activated protein kinase (MAPK) pathway, ultimately affecting cell activity ^(2–6).

PTH has both catabolic and anabolic effects on bone depending on the type of administration. Continuous administration of PTH decreases bone mass whereas intermittent administration increases bone mass ^(5,7–10). Studies have shown that intermittent PTH improves bone formation by increasing the number of osteoblasts through multiple processes, including enhanced osteoblast proliferation and differentiation ⁽¹¹⁾, attenuation of osteoblast apoptosis ⁽¹²⁾ and adipogenesis ⁽¹³⁾, activation of quiescent lining cells ⁽¹⁴⁾, as well as indirect effects mediated by the production of growth factors such as insulin-like growth factors ⁽¹⁵⁾ and basic fibroblast growth factor-2 (FGF-2) ⁽¹⁶⁾. Although the induced bone formation is partially antagonized by a stimulation of bone resorption, the net effect of intermittent PTH treatment is an improvement in bone volume and strength ⁽⁵⁾. As a result, intermittent treatment with PTH (1–34) has been approved by FDA as an effective treatment for postmenopausal osteoporosis ⁽¹⁰⁾.

PTH is also a key regulator of phosphate homeostasis. It increases phosphate excretion by reducing apical abundance of sodium-coupled co-transporters NaPi2a and NaPi2c via both PKA- and PKC-dependent pathways ^(17,18), resulting in decreased phosphate re-absorption from the renal proximal tubules. As a consequence, *PTH*^{−/−} mice are hyperphosphatemic ⁽¹⁹⁾. A number of recent studies have demonstrated that PTH increases transcription and secretion of fibroblast growth factor 23 (FGF23) ^(20–22), another important regulator of serum phosphate homeostasis, through increasing the 1,25(OH)₂D₃ levels ⁽²³⁾.

FGF23 is a member of the FGF19 subfamily of fibroblast growth factors and is secreted by osteoblasts and osteocytes as a circulating hormone ^(24–26). FGF23 targets the kidney to induce phosphate wasting by binding to the complex of FGFR1c/Klotho ^(27,28) and acting to decrease the abundance of NaPi2a and NaPi2c ^(29,30). This is similar to the effect of PTH. Genetic disorders involving FGF23, its co-factor Klotho, or genes that alter FGF23 activity lead to either hyper- or hypophosphatemia with severe physical consequences ^(31–33). In addition we and others ^(34,35) have reported that FGF23 has a direct effect on osteoblast function. Treatment with exogenous FGF23 or over-expression of FGF23 in calvarial cells inhibits osteoblastic differentiation and mineralization. Moreover, It has been shown that

Fgf23, as a circulating hormone, could act on the parathyroid gland to decrease PTH transcription and secretion by activating ERK1/2^(36,37).

Although recent reports have indicated that FGF23 is produced in response to elevated PTH^(20–22), and, in turn, decreases PTH expression^(36,37), it is largely unknown whether FGF23 modulates PTH functions, especially the phosphaturic and anabolic effects of PTH. PTH and FGF23 both induce phosphate wasting through sodium-coupled co-transporters, suggesting that they may have interdependent effects on phosphate regulation. However, FGF23 and PTH have opposing effects on osteoblastic differentiation, suggesting an independent effect on osteoblasts. Therefore, the goal of the current study is to evaluate whether the Fgf23 function is essential for the phosphaturic and/or anabolic effects of PTH. Recombinant human PTH (1–34) was administered daily to Fgf23 knockout mice (*Fgf23*^{−/−}) and their wild-type (*WT*) littermates, and their response to PTH was compared. As Klotho is generally accepted to be a required co-factor for FGF23 signaling^(27,28,32), and the phenotype of Klotho knockout mice (*Kl*^{−/−}) closely resembles that of *Fgf23*^{−/−} mice⁽³²⁾, the same experiments were performed in parallel on *Kl*^{−/−} mice.

Material and Methods

Animals

Heterozygous animals were interbred to produce wild-type (*WT*), *Fgf23*^{−/−} and *Kl*^{−/−} animals for subsequent analyses. Routine PCR was used to genotype the mice as described previously^(31,32). The total body weight of each mouse was measured before sacrifice. All studies performed were approved by the institutional animal care and use committee at the Harvard Medical School.

Intermittent PTH (1–34) treatment

Mice were given daily injections of 100 µg/kg human recombinant PTH (1–34) (Polypeptide Group, France) for a two-week-period beginning at the age of eight days. Animals in the vehicle group were injected with an equal volume of sterile saline. Two hours after the final injection, blood was obtained by puncturing the cheek pouch of animals, and mice were subsequently sacrificed for tissue collection.

Biochemical analyses

Total serum calcium and phosphorus levels were determined using Stanbio LiquiColor (Arsenazo III) and LiquiUV kits (Stanbio Laboratory, Boerne, TX), respectively. Serum concentrations of 1,25(OH)₂D₃ and Fgf23 were measured using commercial kits from IDS (Fountain Hills, AZ) and Immunotopics (San Clemente, CA), respectively.

Bone mineral density measurement

Bone mineral density (BMD) of femurs stored in 70% ethanol was measured by pQCT using a XCT Research M+ pQCT instrument (Stratec Medizintechnik, Pforzheim, Germany). The specimens were positioned with the help of small plastic tubes. Slice thickness was 200 µm, and scan time per bone was 15 min. Three slices from the distal femoral metaphysis located 1.5, 2, and 2.5 mm proximal to the articular surface of the knee joint were measured. All

BMD values of the distal femoral metaphysis were calculated as the mean over three slices. A voxel size of 0.070 mm and a threshold of 600 mg/cm³ were used for calculation of cortical BMD. The CV of the whole procedure, including repositioning, for total BMD, trabecular BMD, and cross-sectional area of the distal femoral metaphysis are 1.25%, 5.36%, and 1.61% in our laboratory, respectively.

μCT analysis

μCT analysis was performed according to the recent guidelines⁽³⁸⁾. Briefly, a Scanco Medical μCT 35 system (Scanco, Southeastern, PA) with an isotropic voxel size of 7 μm was used to image the distal femur. Scans were conducted in 70% ethanol and used an X-ray tube potential of 55 kVp, an X-ray intensity of 0.145 mA and an integration time of 600 ms. From the scans of the distal femur, a region beginning 0.28 millimeters proximal to the growth plate and extending 2.1 millimeters proximally was selected for trabecular bone analysis. A second region 0.6 millimeters in length and centered at the midpoint of the femur was used to calculate diaphyseal parameters. A Gaussian filter, with a support of 0.8 and standard deviation of 1, was applied to the femur images. In order to distinguish cortical from trabecular bone, a semi-automated contouring approach was used. A contour was drawn by hand a few voxels from the endocortical surface every 20 slices. Contours on slices in this range were interpolated by the software. The region of interest was thresholded using a global threshold that set the bone/marrow cut-off at 460.1 mgHA/cm³ for trabecular bone and 589.4 mgHA/cm³ for cortical bone. Unbiased, 3-D microstructural properties of trabecular bone and midshaft cortical bone were calculated using methods based on distance transformation of the binarized images.

Bone histology and histomorphometry

Femurs were fixed in 10% buffered formalin at 4°C overnight and stored in 70% ethanol at 4°C before being processed and embedded in methylmethacrylate. Three-μm-thick midsagittal sections of the distal femurs were prepared using a HM 360 microtome (Microm, Walldorf, Germany), and were stained with von Kossa/McNeal. Histomorphometric measurements in the distal femur were made on sections stained with von Kossa/McNeal using a semiautomatic system (OsteoMeasure, OsteoMetrics, Decatur, GA), and a Zeiss Axioskop microscope with a drawing attachment. The area within 0.25 mm from the growth plate was excluded from the measurements. All histomorphometric parameters were calculated and expressed according to the suggestions made by the ASBMR nomenclature committee⁽³⁹⁾.

Quantitative real-time PCR

Total RNA was isolated from fresh femoral cortex using Trizol reagents (Invitrogen) according to the manufacturer's protocol. For qRT-PCR, cDNA was prepared using QuantiTec reverse transcription kit (Qiagen) and analyzed with SYBR GreenMaster Mix (Roche) in an iCycler (Bio-Rad) using specific primers designed for each targeted gene. Relative expression was calculated using the 2^{-Ct} method by normalizing with *Gapdh* housekeeping gene expression, and presented as fold increase relative to control.

Calvarial cell culture and cAMP measurement

Primary osteoblasts from calvariae of 2-day-old *WT*, *Fgf23*^{-/-} and *Kl*^{-/-} mice were isolated as described previously⁽³⁴⁾. Cells were seeded at a density of 5×10^4 /ml on 24-well plate for cAMP measurement or on 6-well plate for Western blot. After confluence, cells were cultured for one week with 50 mg/ml ascorbic acid and 10 mM β -glycerophosphate to induce osteoblastic differentiation. Cells were then treated with vehicle or 100nM human recombinant PTH(1–34) for 15 min. Cells were lysed and subjected to cAMP assay using a cAMP Biotrak Enzyme immunoassay (EIA) kit (GE Healthcare Biosciences Corp, Piscataway, NJ) according to the manufacturer's instructions. Protein for western blot was extracted from the cells cultured on 6-well plates using RIPA buffer.

Western blot

To test the NaPi2a expression in the kidney, we isolated brush border membrane vesicles (BBMVs) by the Ca^{2+} precipitation method as described previously⁽⁴⁰⁾. BBMVs and protein samples from the osteoblast cultures were heated at 95°C for 5 min in sample buffer containing 2% SDS and 1% 2-mercaptoethanol, and were subjected to 10% SDS-polyacrylamide gel electrophoresis. The separated proteins in the gel were transferred via electrophoresis onto Hybond-P polyvinylidenedifluoride transfer membranes. After incubation in blocking solution, the membranes were treated with diluted rabbit NaPi2a antibody (1:2000), a generous gift of Dr. Hiroko Segawa; rabbit phospho-ERK1/2 (P-ERK1/2) antibody (1:1000) (Assay Biotechnology); ERK1/2 (1:1000) (Assay Biotechnology); phospho-CREB antibody (1:1000) (Assay Biotechnology); CREB (1:1000) (Assay Biotechnology), or mouse β -actin monoclonal antibody (1:2000) (SIGMA). Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was utilized as secondary antibody (Jackson ImmunoResearch Laboratories), and signals were detected by the SuperSignal West Pico Chemiluminescent Substrate system (Pierce). The intensity of the bands was quantified using ImageJ.

Statistics

Statistically significant differences between vehicle and PTH-treated groups were evaluated by Student's t-test, and those among vehicle groups of *WT*, *Fgf23*^{-/-} and *Kl*^{-/-} mice were assessed by ANOVA followed by Tukey's multiple comparisons. All values were expressed as mean $\pm$ SD. A p value of less than 0.05 was considered to be statistically significant.

Results

Injection of PTH changes serum phosphate, calcium, 1,25(OH)₂D₃ and Fgf23 levels

We injected PTH (1–34) into *WT*, *Fgf23*^{-/-} and *Kl*^{-/-} mice, comparing the responses in order to test whether the phosphaturic function of PTH is dependent on FGF23/Klotho signaling. Since *Fgf23*^{-/-} mice exhibit abnormal serum phosphate levels as early as 10 days after birth⁽⁴¹⁾, we started daily subcutaneous injection of PTH(1–34) at 8 days of age for a 2-week period (Fig. 1A). At 3 weeks, vehicle-treated *Fgf23*^{-/-} and *Kl*^{-/-} mice were smaller than their *WT* controls. PTH injection did not significantly change their body size (Fig. 1B) and weight (Fig. 1C) when compared to the vehicle-treated animals.

As expected, injection of PTH (1–34) peptide significantly lowered serum phosphate levels in *WT* mice (8.99 ± 0.32 mg/dL vs. 7.62 ± 1.06 mg/dL) (Fig. 2A). Consistent with previous reports^(31,32,34,41,42), both *Fgf23*^{-/-} and *Kl*^{-/-} mice showed hyperphosphatemia (14.95 ± 1.40 mg/dL and 14.73 ± 0.42 mg/dL, respectively). Interestingly, serum phosphate in both *Fgf23*^{-/-} and *Kl*^{-/-} mice was reduced to near-normal levels after PTH injection (10.76 ± 0.62 mg/dL and 9.46 ± 0.88 mg/dL, respectively).

Since PTH primarily controls phosphate homeostasis by regulating the abundance of NaPi2a in the brush border membrane of renal proximal tubule cells^(18,43), we isolated BBMVs from fresh kidneys to analyze NaPi2a protein levels by Western blot analysis. As shown in Fig. 2B, PTH injection reduced the abundance of NaPi2a in *Fgf23*^{-/-} and *Kl*^{-/-} mice as well as in *WT* mice, indicating that PTH is able to regulate phosphate homeostasis even in the absence of FGF23/Klotho signaling. As expected, basal Napi2a expression in mutant mice was increased.

Furthermore, injection of PTH significantly increased serum calcium levels in the *WT* mice (8.84 ± 0.49 mg/dL vs. 10.29 ± 0.57 mg/dL) (Fig. 2C) as predicted. Serum calcium levels in *Fgf23*^{-/-} and *Kl*^{-/-} mice were slightly increased after PTH injection, but the differences did not reach significance. This may be due to the pre-existing hypercalcemia in these mice. Serum 1,25(OH)₂D₃ levels were also increased by PTH treatment in *WT*, *Fgf23*^{-/-} and *Kl*^{-/-} mice (Fig. 2D).

Previous reports have demonstrated that continuous elevation PTH increases transcription and secretion of FGF23^(20–22). To test the effect of intermittent PTH administration, we measured the serum Fgf23 levels. As shown in Fig. 2E, PTH injection significantly increased serum Fgf23 levels in *WT* mice. Serum Fgf23 levels were dramatically elevated in *Kl*^{-/-} mice and were not altered by PTH treatment. Consistent with serum levels, PTH injection also increased *Fgf23* mRNA in the cortical bone of *WT* mice, but not in that of *Kl*^{-/-} mice (Fig. 2F).

Intermittent injection of PTH increases bone mineral density

We then analyzed anabolic responses in bone by comparing the distal end of femurs from PTH treated *WT*, *Fgf23*^{-/-} and *Kl*^{-/-} mice to those of vehicle-treated animals using *ex vivo* pQCT. Intermittent PTH treatment induced similar percentage increases in total BMD in *WT* mice (279.12 ± 36.15 mg/cm³ vs. 218.53 ± 17.55 mg/cm³, 27% increase), *Fgf23*^{-/-} mice (155.51 ± 21.99 mg/cm³ vs. 125.88 ± 8.19 mg/cm³, 24% increase) and *Kl*^{-/-} mice (204.51 ± 15.48 mg/cm³ vs. 161.25 ± 25.34 mg/cm³, 26% increase) (Fig. 3A). PTH also induced increases in trabecular BMD by similar percentages in *WT*, *Fgf23*^{-/-} and *Kl*^{-/-} mice (Fig. 3B).

Intermittent injection of PTH results in an increase in bone mass

Trabecular bone from the distal femoral metaphysis and cortical bone from the femoral midshaft were assessed by μ CT analysis and representative images are shown in Fig. 4A and B. Quantification of trabecular bone volume demonstrated that *Fgf23*^{-/-} and *Kl*^{-/-} mice had markedly decreased (0.64 ± 0.10) and normal (6.73 ± 1.15) bone mass respectively,

compared to the one of *WT* mice (6.98 ± 1.94) (Fig. 4C). The intermittent injection of PTH produced a substantial increase in trabecular volumes in all three genotypes (*WT*: 13.37 ± 2.96 , 91% increase; *Fgf23*^{-/-}: 1.61 ± 0.24 , 151% increase; and *Kl*^{-/-}: 12.84 ± 3.05 , 91% increase). Trabecular number (Tb.N) was also increased in PTH treated *WT* (+47%), *Fgf23*^{-/-} (+51%) and *Kl*^{-/-} (+61%) mice (Fig. 4D). However, no significant changes of cortical area fraction (Ct.Ar/Tt.Ar) (Fig. 4G) and cortical thickness (Ct.Th) (Fig. 4H) were observed in any of the three genotypes.

Consistent with the PTH-induced increases in BMD, histomorphometric analyses of undecalcified sections stained with von Kossa and McNeal blue (Fig. 5A) demonstrated that trabecular bone volumes of *WT*, *Fgf23*^{-/-} and *Kl*^{-/-} mice were all significantly increased after intermittent PTH administration (Fig. 5B), confirming the μ CT results. PTH injection also improved the osteoid volume (OV/TV), osteoblast surfaces/bone surface (Ob.S/BS), trabecular number (Tb.N) and trabecular thickness (Tb.Th) in all three genotypes (Fig. 5B–F). Likewise, the trabecular separation (Tb.Sp) was significantly decreased (Fig. 5G).

We also measured serum CTX, a marker for bone absorption. Intermittent injection of PTH induced a significant increase of CTX in *WT* animals (Fig. 5H). There was also a trend indicating an increase of CTX in *Fgf23*^{-/-} and *Kl*^{-/-} mice, however this did not reach statistical significance, which is likely due to the pre-existing, higher CTX levels in the mutant mice.

PTH-induced gene expression of osteoblast genes is not affected by the lack of FGF23/Klotho signaling

We next determined the effects of FGF23/Klotho deficiency on PTH induction of osteoblast differentiation markers *in vivo*. PTH markedly elevated the expression of genes known to be associated with osteoblastic differentiation in *WT* bone (Figure 6A), including Runx2 (2.3-fold), type I collagen (Col1, 2.9-fold), alkaline phosphatase (ALP, 2.3-fold) and osteocalcin (Ocn, 2.2-fold). Importantly, these PTH-induced increases were also observed in *Fgf23*^{-/-} and *Kl*^{-/-} bone, indicating that FGF23/Klotho functions are not required. Although *Fgf23*^{-/-} and *Kl*^{-/-} mice show reduced basal Ocn mRNA levels, PTH treatment markedly increased the levels 3.1-fold and 3.8-fold, respectively. As expected, expression of *Sost*, an inhibitor of Wnt signaling, was significantly decreased after PTH injection in the *WT* mice. The basal mRNA level of *Sost* in *Fgf23*^{-/-} and *Kl*^{-/-} mice was lower than in *WT* mice, and was further reduced by PTH administration.

The PKA pathway in osteoblasts by PTH treatment remains activated in the absence of FGF23/Klotho Signaling

The anabolic effect of PTH has been clearly associated with the PKA/cAMP-mediated pathway⁽⁵⁾. We therefore examined the PTH-induced intracellular cAMP accumulation in calvarial osteoblasts isolated from *WT*, *Fgf23*^{-/-} and *Kl*^{-/-} pups. Osteoblasts from each genotype showed similar amounts of cAMP accumulation before (baseline) and after PTH treatment (100 nM, 15 min) (Fig. 6B). Furthermore, osteoblasts from *WT*, *Fgf23*^{-/-} and *Kl*^{-/-} mice exhibited similar basal levels of phosphorylation of ERK1/2 and CREB, and

PTH treatment stimulated the phosphorylation of ERK1/2 and CREB in all three types of osteoblasts (Fig. 6C).

Discussion

PTH administered by daily injection is the only currently available anabolic therapy for bone disease ⁽¹⁰⁾. A number of mechanisms, including enhanced osteoblast proliferation and differentiation, attenuation of osteoblast apoptosis and adipogenesis, activation of quiescent lining cells, as well as indirect effects mediated by the production of growth factors ^(11–16) have been proposed to contribute to the anabolic effects of PTH. The goal of this study was to evaluate the role, if any, of FGF23/Klotho signaling in the phosphaturic and anabolic effects of PTH. Using the novel *Fgf23* and *Klotho* knockout mice, we found that intermittent PTH injection correspondingly induced phosphate wasting in WT, *Fgf23*^{−/−} and *Kl*^{−/−} mice. Furthermore, an analogous increase in BMD and BV/TV was detected in all three genotypes. Finally, PTH treatment resulted in both a comparable increase in the expression of osteoblastic marker genes in cortical bone, and in enhanced cAMP accumulation in osteoblasts isolated from all three genotypes. Therefore, this study demonstrates for the first time that FGF23/Klotho signaling is not essential for the phosphaturic and anabolic effects of intermittent PTH(1–34).

FGF23, together with PTH and 1,25D, form an endocrine loop and play a central role in regulating mineral ion homeostasis. Previous studies have shown that both phosphate and 1,25D can act on bone to increase FGF23 secretion ⁽⁴⁴⁾. Recently, several studies have demonstrated that PTH can also directly induce FGF23 transcription and secretion both *in vivo* and *in vitro* ^(20–22,45). Kawata and colleagues ⁽²²⁾ reported that serum FGF23 concentrations were higher in PTH-*cyclin D1* transgenic mice, a model of primary hyperparathyroidism, than in wild-type mice. The serum FGF23 concentration was shown to have a significant and direct correlation with serum PTH. Also, FGF23 levels decreased following parathyroidectomy in these transgenic mice. These results were the first to suggest that PTH may directly affect serum FGF23 levels. Consistent with these data, a clinical experiment performed by Burnett-Bowie and colleagues ⁽²¹⁾ demonstrated that infusion of PTH into healthy humans can increase serum FGF23 levels. There is also a clinical correlate that suggests the complexity of the relationship between PTH action and FGF23. In a child suffering from Jansen's metaphyseal chondrodysplasia, which is the consequence of a constitutive activation mutation of the PTH/PTHrP receptor, serum FGF23 concentration was found to be markedly and persistently elevated despite hypophosphatemia and normal 1,25D levels ⁽⁴⁵⁾. The observations in this case suggested that serum FGF23 could be increased by activation of PTH signaling in bone. The action of PTH on FGF23 is also suggested in the effects of chronic kidney disease (CKD), where both serum PTH and FGF23 levels are markedly elevated ^(46,47). Interestingly, parathyroidectomy prevented an increase in FGF23 levels in experimental kidney failure rats ⁽²⁰⁾ suggesting that the elevated levels of PTH induce the high FGF23 levels observed in these patients. Moreover, PTH treatment increased the levels of *Fgf23* mRNA in osteoblast-like UMR106 cells ⁽²⁰⁾, indicating a direct effect of PTH on *Fgf23* gene expression. Our finding in this study that intermittent PTH treatment increases FGF23 expression in cortical bone is consistent with these reports.

Intermittent PTH injections induced similar bone gain in both *WT* and *Fgf23*^{-/-} mice in spite of opposite levels of FGF23 expression. In fact, there was a 7-fold increase in *Fgf23* mRNA in the femoral cortex of PTH treated *WT* mice compared to controls, yet the bone gain was comparable in PTH treated *Fgf23*^{-/-} mice which lack *Fgf23* entirely. Thus, *Fgf23* is not necessary for an anabolic response to PTH. This was confirmed by the results of similar experiments in *Kl*^{-/-} mice which have extremely high *Fgf23*. The administration of PTH failed to induce a further increase of *Fgf23* mRNA in *Kl*^{-/-} mice, but did result in similar bone gain when compared to *WT* and *Fgf23*^{-/-} animals.

PTH and FGF23 are regarded as the key regulators of phosphate homeostasis. In this study, PTH effectively induced phosphate wasting and reduced NaPi2a abundance in the *Fgf23*^{-/-} and *Kl*^{-/-} animals, suggesting the effects of PTH on phosphate regulation are FGF23-independent. PTH induces phosphate excretion via decreasing apical NaPi2a abundance within minutes, without changing mRNA levels⁽¹⁸⁾. This action is associated with several pathways, including PKA, PKC, and PKG. These pathways may converge at the downstream point of the MAPK (ERK1/2) kinases pathway since an ERK1/2 inhibitor prevents the down-regulation of NaPi2a initiated by PTH⁽⁴⁸⁾. Recently, NHERF1 was also implicated as a mediator of PTH regulation of renal phosphate reabsorption. It forms a complex with NaPi2a and stabilizes the presence of NaPi2a at the apical membrane. PTH treatment disrupts this complex by stimulating the phosphorylation of NHERF1, which then leads to the dissociation and subsequent endocytosis of NaPi2a^(49,50). Although FGF23 also targets the kidney to induce phosphate wasting by decreasing the abundance of NaPi2a and NaPi2c^(29,30), the underlying mechanism for this action is still unclear. FGF23 requires Klotho as a co-factor^(27,28,32), but Klotho is mainly expressed in the distal tubules⁽⁵¹⁾. The signal transduction between the distal and proximal tubules is still poorly understood. Recently, however, Klotho was reported to be expressed in the proximal tubules and it could act independently as an enzyme to inhibit transporter activity⁽⁵²⁾.

We began PTH injections at a very early age (8 days postnatally) in order to start before *Fgf23*^{-/-} mice develop hyperphosphatemia (10 days postnatally). Mice are in a fast growth stage during our selected experimental period, and thus osteoblasts and bone marrow cells are more active than in older, adult mice. Our data showed that the deficiency of FGF23/Klotho does not affect the PTH-induced bone gain in these growing mice, but its effect on older animals cannot be excluded. *Fgf23*^{-/-} and *Kl*^{-/-} mice do not survive beyond 13–15 weeks of age probably due to high mineral ion contents, including increased 1,25(OH)₂D₃ levels, hyperphosphatemia and hypercalcemia.

In this study we have shown for the first time that complete loss of FGF23/Klotho signaling does not attenuate the phosphaturic and anabolic effects of PTH, indicating these functions of PTH do not require the FGF23/Klotho signaling axis. The findings suggest that PTH can still act as a therapeutic agent to improve the skeletal quality of patients even in the presence of abnormal serum FGF23 levels.

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Figure 1.

Intermittent PTH injection does not change the macroscopic phenotype of 3wk-old animals.

(A) Schematic graph of PTH injection strategy. (B) Macroscopic picture of animals injected with PTH (1–34) or vehicle. (C) Body weight at 3wks for mice of all genotypes.

Figure 2.

Serum biochemical measurement and NaPi2a expression in kidney. (A) Injection of PTH decreases serum phosphate levels in WT, *Fgf23*^{-/-} and *Klf*^{-/-} mice. (B) Western blot analysis of NaPi2a using BBMV of the kidney. (C) Measurement of serum calcium. (D) Measurement of serum 1,25(OH)₂D₃. (E) Measurement of serum Fgf23. (F) Expression of *Fgf23* in the cortical bone measured by qPCR. *: p<0.05; **: p<0.01; ***: p<0.001, comparison between matched PTH-treated and vehicle. #: p<0.05; ###: p<0.001, comparison with WT vehicle.

Figure 3.

PTH induced increase of bone mineral density is not affected by loss of FGF23/Klotho function. (A) Total bone mineral density (BMD) of the distal end of femur. (B) Trabecular BMD of the distal end of femur. *: $p < 0.05$; **: $p < 0.01$, comparison between PTH-treated and vehicle.

Figure 4.

μ CT analysis of the femurs. Representative images for the distal metaphysis (A) and midshaft cortical bone (B). Intermittent injection of PTH increased the trabecular volume (BV/TV) and number (Tb.N) in all three genotypes (C–F), but did not change the cortical area fraction (Ct.Ar/Tt.Ar) (G) and cortical thickness (Tb.Th) (H). *: $p < 0.05$; **: $p < 0.01$, comparison between PTH-treated and vehicle.

Figure 5.

Histological and histomorphometric analyses of the distal femoral metaphysis. (A) Undecalcified sections of the femurs stained with von Kossa and McNeal blue. Intermittent injection of PTH induces a similar increase of trabecular bone volumes (BV/TV), osteoid volume (OV/TV), osteoblast surface/bone surface (Ob.S/BS), trabecular number (Tb.N) and trabecular thickness (Tb.Th) in *WT*, *Fgf23*^{-/-} and *Kl*^{-/-} mice (B–F). Likewise, the trabecular separation (Tb.Sp) is significantly decreased (G). (H) Measurement of serum CTX. *: p<0.05; **: p<0.01, comparison between PTH-treated and vehicle.

Figure 6.

PTH-induced expression of osteoblast marker genes and activation of the PKA pathway is not affected by FGF23/Klotho. (A) qPCR analyses of the expression of osteoblast marker genes in the femoral cortex. PTH injection induces similar increases in Runx2, Col1, ALP, and Ocn mRNA, and a decrease in Sost mRNA in all three genotypes. (B) Accumulation of cAMP in the primary osteoblasts isolated from three genotypes. Cells were cultured in 24-well plates with osteogenic medium for 1 week and then treated with or without PTH (100 nM) for 15 min. (C) Western blot analysis of P-ERK1/2, total ERK1/2, P-CREB and CREB. Cells were cultured in osteogenic medium for 1 week and then treated with or without PTH (100 nM) for 15 min before total lysates were collected. PTH treatment increased the phosphorylation of P-ERK1/2 and P-CREB similarly in WT, Fgf23^{-/-} and KI^{-/-} osteoblasts. *: p<0.05; **: p<0.01; ***: p<0.001, comparison between PTH-treated and vehicle.