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Parathyroid hormone receptor signaling in osteocytes increases the expression of fibroblast growth factor-23 *in vitro* and *in vivo*

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Abstract

Mice with constitutive activation of parathyroid hormone (PTH) receptor signaling in osteocytes (DMP1-caPTHR1 transgenic mice) exhibit increased bone mass and remodeling, two of the recognized skeletal actions of PTH. Moreover, similar to PTH administration, DMP1-caPTHR1 mice exhibit decreased expression of the osteocyte-derived Wnt antagonist Sost/sclerostin. We now report that PTH receptor activation also regulates *in vivo* and *in vitro* the expression of fibroblast growth factor 23 (FGF23), an osteocyte product involved in inorganic phosphate (Pi) homeostasis and bone mineralization. Whole bones and osteocytes, but not osteoblasts, from DMP1-caPTHR1 mice exhibit elevated FGF23 expression, which is corrected in double transgenic mice overexpressing Sost in osteocytes. PTH, PTH related protein (PTHrP), or a cAMP stable analog, increase FGF23 transcripts in a time- and dose-dependent manner in osteocyte-containing calvarial cell cultures. Circulating FGF23 is also elevated in DMP1-caPTHR1 mice; however, plasma Pi or renal Pi reabsorption is not altered. Furthermore, the FGF23 receptor complex comprising FGFR1 and KLOTHO is expressed in osteoblastic cells; and FGFR1, GALNT3, as well as downstream targets of FGF23 signaling, are increased in osteocytes but not in osteoblasts from DMP1-caPTHR1 mice. Thus, PTH receptor signaling has the potential to modulate the endocrine and auto/paracrine functions of osteocytes by regulating FGF23 through cAMP- and Wnt-dependent mechanisms.

Keywords

Parathyroid hormone; osteocytes; FGF23; phosphate; KLOTHO

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Introduction

Increasing evidence points to osteocytes, terminally differentiated cells of the osteoblastic lineage and most abundant bone cells, as regulators of several actions of parathyroid hormone (PTH) on the skeleton. Thus, PTH inhibits the expression of sclerostin, the product of the Sost gene that inhibits bone formation by antagonizing the BMP and Wnt pathways [1, 2]. Furthermore, transgenic mice expressing a constitutively active PTH receptor 1 exclusively in osteocytes (DMP1-caPTHR1 mice) exhibit increased bone mass and elevated bone remodeling [3, 4], recapitulating two main actions of PTH on bone.

Osteocytes produce fibroblast growth factor-23 (FGF23) [5-8], a hormone produced in bone that plays a crucial role in phosphate (Pi) homeostasis by inhibiting its renal reabsorption [9]. FGF23 is protected from degradation into N- and C-terminal fragments by glycosylation controlled by the galactosaminyl transferase GALNT3. Inactivating mutations of this enzyme result in reduced intact FGF23 leading to hyperphosphatemia and ectopic calcification in tumoral calcinosis [10]. Conversely, supraphysiologic FGF23 levels found in several genetic disorders and in FGF23-producing tumors cause decreased renal Pi reabsorption and hypophosphatemia resulting in osteomalacia and rickets [10]. Consistent with the findings in the human diseases, transgenic mice overexpressing FGF23 are hypophosphatemic [11] whereas FGF23 knockout mice are hyperphosphatemic, condition that is reversed by FGF23 overexpression in osteoblastic cells [12-14]. Furthermore, FGF23 neutralizing antibodies elevate serum Pi and increase renal expression of the sodium-Pi co-transporter NPT2A in mice [15].

High dietary Pi and the active form of Vitamin D_3, 1,25-dihydroxy-vitamin D_3 (1,25-D_3) are major stimulators of FGF23 secretion [16-19]. FGF23 is a marker of altered phosphate metabolism that increases before detectable changes in PTH in early CKD [20]. However, several pieces of evidence suggest that PTH might also regulate FGF23 production. Thus, the hyperphosphatemia exhibited by chronic kidney disease (CKD) patients is accompanied by elevation in both FGF23 and PTH [10, 21]; and parathyroidectomy prevented the increase in FGF23 in a rat model of kidney failure [22]. FGF23 is also elevated in patients and in a mouse model of primary hyperparathyroidism [23, 24]. Moreover, a patient with Jansen's metaphyseal chondrodysplasia expressing a constitutively active PTHR1 receptor mutant exhibit high FGF23 concentrations in the circulation, despite low Pi and normal 1,25-D_3 levels [25].

In the current study, we present evidence demonstrating that FGF23 production is augmented by direct action of the PTH receptor in osteocytes, *in vivo* in transgenic mice expressing a Jansen's constitutively active PTH receptor under the control of an osteocyte-specific promoter as well as *in vitro* in primary cultures containing osteocytes treated with the ligands of the PTHR1. Although circulating Pi or renal Pi reabsorption is not altered, FGF23 signaling is activated in bones of DMP1-caPTHR1 mice. Thus, regulation of FGF23 in osteocytes represents a potential mechanism by which signaling through the PTH receptor influences systemic and local FGF23 actions.

Materials and Methods

Animals

DMP1-caPTHR1 and DMP1-Sost transgenic mice were generated by microinjection of purified DNA into pronuclei of C57BL/6 mice, as previously described [3, 4]. Mouse colonies were maintained by breeding mice hemizygous for the transgene with wild type C57BL/6 mice. DMP1-caPTHR1 mice were crossed with DMP1-Sost mice to obtain mice expressing both the DMP1-caPTHR1 and the DMP1-Sost transgenes [4]. Mice expressing

green fluorescent protein (GFP) in osteocytes (DMP1-GFP) were provided by Dr. David W. Rowe and were maintained as homozygous [26]. The α-KLOTHO null mice (KLOTHO −/−) were from Lexicon Genetics, Inc. (The Woodlands, TX). Animals were fed a regular diet (Harlan/Teklad #7001, Harlan Laboratories Inc., Frederick, MD), water ad-libitum and exposed to a 12h light/dark cycle. Whole tibiae, ulnae, calvaria, and kidneys from 6-week-old mice (males and females) were snapped frozen at sacrifice and stored at −80°C until used for RNA isolation. Protocols were approved by the Institutional Animal Care and Use Committee of Indiana University School of Medicine.

Cell culture

Osteoblastic cells were obtained from calvarial bones of neonatal C57BL/6 mice and cultured at an initial density of $5\times10^4/cm^2$ for 5-6d in the presence of α-MEM supplemented with 10% fetal bovine serum and 50μg/ml ascorbic acid. Half of the medium was replaced by fresh medium every other day. Cultures were treated with vehicle, 50 nM bovine PTH(1-34) (Bachem, Torrance, CA), 10 nM 1,25-D_3 (Enzo Life Sciences International, Plymouth Meeting, PA), 50 nM human PTHrP (1-34), (Bachem, Torrance, CA), or 1mM DBA (Enzo Life Sciences International, Inc., Plymouth Meeting, PA) for the times indicated in the figures, or with different concentrations of PTH(1-34) for 3h. We have previously shown that these culture conditions promote the formation of osteocytes, as evidenced by the progressive increase in Sost mRNA expression and detection of sclerostin by immunohistochemistry [1]. Furthermore, in cultures established with osteoblastic cells from DMP1-GFP mice, sclerostin expression co-localized with GFP expression in cells exhibiting dendritic morphology [1].

Quantification of gene expression in isolated osteocytes and osteoblasts

Homozygous DMP1-GFP mice were crossed with hemizygous DMP1-caPTHR1 mice to generate DMP1-GFP-positive offspring with and without the DMP1-caPTHR1 transgene. Calvaria cells were isolated from 3-6 day-old mice [3]. In each sorting experiment, cells from 6-12 mice per genotype (WT or DMP1-caPTHR1 mice) were pooled before sorting and GFP-expressing cells (osteocytes) were separated from GFP-negative cells (a population mainly composed of osteoblasts) using a FACSAria flow cytometer (BD Biosciences, Sparks, MD) at the Flow Cytometry Core Facility of Indiana University School of Medicine. RNA from GFP-positive and GFP-negative cells was isolated immediately after sorting [3]. Gene expression is expressed as fold change versus the expression of the corresponding gene in osteocytes from WT mice within each sorting experiment. Results from 3-5 independent sorting experiments are shown.

Quantitative RT-PCR

Total RNA was purified from tissue or cell preparations using Ultraspec reagent (Biotecx Laboratories, Houston, TX) according to the manufacturer's instructions. RNA was reverse-transcribed using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Gene expression was analyzed in triplicates by quantitative PCR using the ΔCt method as previously described [3]. FGF23 and RANKL primer probe sets were purchased from Applied Biosystems (Mm00445621_m1 and Mm00441908_m1, respectively). KLOTHO primer probe set was designed in-house and were as follows: probe: ttgcccacaacctacttttggctcatg, forward primer: cagctccaggctcgggta, reverse primer: aggtgttgtagagatgccagacttt, as reported earlier [27]. Sequencing confirmed that the cDNA PCR product was mouse α-KLOTHO mRNA. For the other genes, primer probe sets were designed using the Assay Design Center of Roche Applied Science (Indianapolis, IN), and were as follows. For FGFR1: probe 66, forward primer: tctggcctctacgcttgc, reverse primer: gaggatgggagtgcatctg; GALNT3: probe 20, forward primer: cgctcacgttcttagatgctc, reverse primer: tatcctggccagcagagg; SOST: probe #16, forward primer: tcctgagaacaaccagacca,

reverse primer: gcagctgtactcggacacatc; EGR1: probe 22, forward primer cctatgagcacctgaccaca, reverse primer tcgtttggctgggataactc; EGR2: probe 60, forward primer: ctacccggtggaagacctc, reverse primer: aatgttgatcatgccatctcc; and ribosomal protein S2 (used as house-keeping gene): probe 45, forward primer: cagaatggtaggaaggtcacg, reverse primer: gatcctgctctggaaatcgt.

Biochemistry measurements and Pi tubular reabsorption measurement

Blood was drawn from the facial vein at the indicated times. Calcium, Pi, and creatinine measurements were performed at the General Clinical Research Center of Indiana University School of Medicine using a Roche Cobas Mira S Chemistry analyzer (Roche Diagnostics, Indianapolis, IN). Plasma 1,25-D_3 concentrations were determined by ELISA (Immunodiagnostic Systems Inc., Scottsdale, AZ). Serum intact FGF23 concentration was measured by ELISA (Kainos Laboratories Inc., Tokyo, Japan) [25]. To determine Pi tubular reabsorption, 4-5 mice were housed in metabolic cages with free access to water and food, and urine was collected over a 24 h-period. Urine samples were centrifuged (3000 g, 5 min, 4°C) and stored at −80°C for further analysis. Blood was drawn from the facial vein at the end of the urine collection time after 3h-fasting and plasma was obtained. Urinary Pi (UP) and creatinine (UCr) and plasma Pi (PP) and creatinine (PCr) were measured using the Roche Cobas Mira S Chemistry analyzer. Pi tubular reabsorption was calculated using the formula 1-[(UP/PP)/(UCr/PCr)] × 100.

Immunohistochemistry

NPT2A was detected in cryosections of kidney using a rabbit anti-mouse NPT2A (gift of Drs. Heini Murer and Jurg Biber) followed by a chicken anti-rabbit AlexaFluor 594-conjugated secondary antibody (Invitrogen, Carlsbad, CA), as published [28]. KLOTHO was detected in paraffin-embedded tibiae sections using a rat monoclonal anti-mouse KLOTHO antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by a goat anti-rat horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Tibial bone sections from KLOTHO knockout mice and non-immune IgG were used as negative controls. KLOTHO −/− bone sections were also stained with goat polyclonal anti-mouse sclerostin antibody (R&D Systems, Minneapolis, MN) followed by rabbit anti-goat conjugated with horseradish peroxidase (Santa Cruz Biotechnology), as previously published [4]. Color was developed with a diaminobenzidine substrate chromogen system (Dako Corp., Carpinteria, CA). Kidney sections from WT and KLOTHO −/− mice were stained with the rat anti-KLOTHO antibody followed by AlexaFluor 488-conjugated chicken anti-rat antibody (Invitrogen, Carlsbad, CA) [28]. Imaging was done with a Leica DM5000B fluorescence microscope (Leica Microsystems, Inc., Bannockburn, IL) with a SPOT camera and analysis software (Diagnostic Instruments, Sterling Heights, MI) or with an Olympus BX51 microscope attached to an OsteoMeasure High Resolution Digital Video System (OsteoMetrics, Inc., Decatur, GA).

Statistical analysis

Data were analyzed using SigmaStat (SPSS Science) and are expressed as means ± standard deviations (SD). Differences between means were evaluated using Student’s t-test or two-way ANOVA.

Results

FGF23 expression is upregulated by PTH receptor signaling in osteocytes *in vivo* and *in vitro*

Prompted by evidence that PTH acts directly on osteocytes to regulate Sost/sclerostin expression [1, 2], we examined whether PTH also regulates the expression of FGF23, a hormone produced by osteocytes that, as PTH, regulates phosphate metabolism [29]. FGF23 mRNA transcripts were elevated in bones from DMP1-caPTHR1 mice with constitutive activation of the PTH receptor in osteocytes compared to littermates not expressing the transgene (WT) (Figure 1A). To distinguish whether osteocytes, osteoblasts, or both cell types were expressing higher FGF23, DMP1-caPTHR1 mice were crossed with DMP1-GFP transgenic mice in which osteocytes are labeled with GFP; and gene expression was examined in GFP-positive (osteocytes) and GFP-negative (osteoblasts) cells separated by fluorescence activated cell sorting of neonatal calvaria cell preparations, as previously reported [3]. The osteocyte-specific Sost gene was highly expressed in GFP-positive cells obtained from mice with or without the DMP1-caPTHR1 transgene, whereas it was practically undetectable in GFP-negative cells, confirming the identity of the cell populations (Figure 1B). Sost expression was lower in osteocytes derived from mice expressing the DMP1-caPTHR1 transgene, consistent with our previous findings in this mouse model [3] and with the reported inhibition of Sost expression by PTH administration to mice [1, 2]. On the other hand, FGF23 transcripts were detected at similar levels in both osteocytes and osteoblasts in WT mice. In addition, only osteocytes derived from DMP1-caPTHR1 mice expressed higher FGF23 levels compared to osteocytes from littermate WT controls, whereas no difference was found in osteoblasts (Figure 1B), demonstrating that the source of the elevated FGF23 in the transgenic mice is the osteocytes. FGF23 expression was not high in bones of double transgenic mice expressing DMP1-caPTHR1 and the human Sost gene under the control of the same DMP1 promoter (Figure 1C), suggesting that FGF23 regulation by PTH receptor signaling in osteocytes requires Wnt signaling.

The addition of 50 nM PTH for 0.5-24 h to osteocyte-containing primary cultures of calvaria cells from WT mice lead to marked increases in FGF23 mRNA (Figure 1D). Maximal effects were observed at 3 h; and in 7 independent experiments the increases varied between 3 to 200-fold compared to vehicle-treated cultures. PTH increased FGF23 mRNA expression in a dose-dependent manner, with significant increases observed with as low as 0.5 nM PTH and maximal effects found with 50 nM (Figure 1E). PTH enhanced FGF23 expression to a similar extent in the presence or absence of 1,25-D_3 (10^{-8} M) (Figure 1F). Treatment with 1,25-D_3 alone did not affect FGF23 expression. However, 1,25-D_3 did stimulate the expression of RANKL, a recognized target gene of this hormone, confirming the functionality of 1,25-D_3 in these culture conditions. PTH also increased RANKL expression, consistent with published evidence [30], and addition of both hormones simultaneously increased RANKL expression to a greater extent than either hormone alone. These data confirm that PTH increases FGF23 expression independently of 1,25-D_3. Similar to PTH, PTHrP (50 nM), the other ligand of the PTH receptor 1, or to the stable analog of cAMP DBA (1 mM), at 3, 8, and 24 h of treatment, upregulated FGF23 mRNA expression (Figure 1G). In contrast, and consistent with our previous findings with PTH [1], Sost expression was decreased by these stimuli.

PTH receptor activation in osteocytes increases circulating levels of FGF23 but does not affect plasma Pi levels or Pi reabsorption in the kidney

Increased FGF23 production in bones and in osteocytes was accompanied by higher levels of the hormone in the circulation of DMP1-caPTHR1 mice compared with WT littermates (Figure 2A). Circulating 1,25-D_3 was also elevated in the transgenic mice (Figure 2B).

Plasma Pi and calcium levels were within normal range (Figure 2C); and Pi reabsorption in the kidney (Figure 2D) or NPT2A protein expression in the renal proximal tubule (Figure 2E) or NPT2A mRNA (not shown) were similar in the transgenic mice compared to WT littermates. We next examined whether the lack of detectable changes in Pi handling in the transgenic mice was due to decreased expression of components of the high affinity FGF23 receptor complex [31], as recently reported for parathyroid cells of CKD rats [32]. However, expression of FGFR1 or KLOTHO were not different in kidneys of DMP1-caPTHR1 mice compared to WT littermates (Figure 2F), excluding a potential resistance to FGF23 in the kidneys of the transgenic mice.

We next examined the possibility that the increase in FGF23 expression in osteocytes would activate signaling locally in bones of DMP1-caPTHR1 mice. FGFR1 is expressed in most cells; whereas KLOTHO, the co-receptor essential for intracellular signaling, is expressed in a limited number of organs including kidney, the primary site of FGF23 action [29, 33]. Binding of FGF23 to FGFR1/KLOTHO triggers phosphorylation of FGFR substrate 2 and the extracellular signal regulated kinases (ERKs), followed by increased expression of early growth response genes (EGR) 1 and 2. We found that both FGFR1 and KLOTHO transcripts are expressed in bone and in osteocytes and osteoblasts of both WT and DMP1-caPTHR1 mice (Figures 3A and B). The level of expression of KLOTHO mRNA in bone lysates as well as in isolated osteocytes and osteoblasts, is approximately 500 times lower than in kidney (Ct for calvaria 31 versus Ct for kidney 22). Nevertheless, the KLOTHO protein is readily detected in osteocytes as well as osteoblast by immunostaining of bone sections (Figure 3C). No changes in KLOTHO were found in the transgenic mice. Specificity of the detection of the protein is confirmed by lack of staining in bone and kidney sections from KLOTHO −/− mice. On the other hand, FGFR1 expression is increased in bones and specifically in osteocytes of DMP1-caPTHR1 mice (Figures 3A and B). Expression of GALNT3, the enzyme that protects FGF23 from degradation, is also elevated in osteocytes of DMP1-caPTHR1 mice (Figure 3D). As for the expression of FGF23, increased GALNT3 expression was not observed in DMP1-caPTHR1 mice also expressing Sost in osteocytes. Consistent with elevated FGF23 and the presence of the FGF23 receptor complex, the downstream targets of FGF23 signaling EGR1 and EGR2 are also high in bones of DMP1-caPTHR1 mice (Figure 3E).

Discussion

The findings of this study demonstrate that activation of PTH receptor signaling *in vivo* in mice expressing a constitutively active PTHR1 in osteocytes or *in vitro* by ligands of this receptor, increases FGF23. A simultaneous increase in GALNT3 stabilizes FGF23, leading to elevation of intact FGF23 in the circulation and locally in bone, where FGF23 binds to the FGFR1/KLOTHO complex expressed in both osteocytes and osteoblasts and activates intracellular signaling (Figure 4).

The elevated FGF23 in the circulation of DMP1-caPTHR1 mice is consistent with a recent report demonstrating marked and persistent high plasma FGF23 in a patient of Jansen's metaphyseal chondrodysplasia expressing ubiquitously the same constitutively active PTHR1 mutant as our transgenic mice [25]. This patient exhibits hypophosphatemia, suggesting that FGF23 was increased by factor(s) other than high Pi levels. Our findings demonstrating that FGF23 is increased by activation of the PTHR1 in osteocytes through cAMP- and Wnt-dependent signaling provide a mechanistic explanation for the phenomenon.

Because FGF23 can act in the kidney and locally in bone, our current findings suggest that, through FGF23, PTH has the potential to modulate not only the endocrine but also the auto/

paracrine function of osteocytes. Although the antibodies available to detect KLOTHO cannot distinguish between membrane-bound and soluble forms of the protein, we found that the FGF23/MAPK target genes EGR1/2 are elevated in bones of the DMP1-caPTHR1 mice. This is consistent with earlier reports demonstrating actions of FGF23 in bone [34, 35]. Analysis of the skeletal phenotype of the DMP1-caPTHR1 mice previously reported shows no apparent overall mineralization defect [3, 4]. However, there is an increase in osteoid perimeter/bone perimeter and in osteoid width in cancellous bone, suggestive of delayed mineralization [4]. This effect might result from increased FGF23 signaling locally in bone cells, independent of changes in circulating Pi, as suggested by previous reports [5, 6]. In contrast, the systemic consequences of FGF23 regulation are not apparent in our transgenic mice. Thus, Pi levels in plasma are normal and Pi tubular reabsorption and NPT2A expression in the kidney are not decreased in DMP1-caPTHR1 mice, as it would be expected in a FGF23 provoked state. The reason(s) for the inability of increased FGF23 to affect Pi handling in the kidney in our transgenic mice is not known. We have excluded the possibility that the expression of components of the FGF23 receptor complex is decreased in the kidney of DMP1-caPTHR1 mice, which could explain the resistance to FGF23 elevation (similar to what it was recently reported for parathyroid cells in a rat model of CKD [32]). A potential explanation for the lack of changes in circulating Pi is that the increase in FGF23 observed in DMP1-caPTHR1 mice is not sufficient to elicit a change in Pi reabsorption in the kidney. Indeed, the elevation in FGF23 levels in DMP1-caPTHR1 mice is relatively lower compared to other models in which changes in Pi reabsorption are detected. Moreover, FGF23 is expressed by both osteocytes and osteoblasts, and although PTH or PTHrP markedly increase FGF23 *in vitro* in the mixed osteoblast/osteocyte cultures, FGF23 expression is elevated modestly and only in osteocytes *in vivo* in our model of PTH receptor activation driven by the DMP1 promoter. Therefore, it is possible that the *in vivo* effect of PTHR1 activation on Pi homeostasis would require the contribution of osteoblast-derived FGF23.

FGF23 has been shown to suppress 1,25-D_3 production in the kidney [36]. However, this previously established inhibitory effect of FGF23 on 1,25-D_3 production is not observed, as circulating levels of 1,25-D_3 are not decreased and 1-α-hydroxylase expression in the kidney was not reduced (not shown) in DMP1-caPTHR1 mice. On the contrary, plasma 1,25-D_3 is elevated in the transgenic mice suggesting that other mechanisms are responsible for the increase. One stimulator of 1,25-D_3 production is PTH. However, PTH levels are normal in the DMP1-caPTHR1 mice. In addition, we have demonstrated that the 8kb fragment of the DMP1 promoter used to drive the expression of the constitutively active PTH receptor is not expressed in kidney [3, 4], thereby excluding the possibility of unwanted activation of PTHR1 signaling that could have increased 1,25-D_3 production in the kidney. A potential scenario that reconciles our findings is one in which increased FGF23 in the DMP1-caPTHR1 mice would have initially caused a drop in Pi levels in the circulation with the resulting increase in 1,25-D_3 production. Elevated 1,25-D_3 could have then increased intestinal Pi absorption and renal Pi reabsorption [37] leading to normalization of circulating Pi levels. This situation could also explain the apparent lack of response of the kidney to increased FGF23 since a FGF23-induced decrease in NPT2A expression and Pi reabsorption would be opposed by a 1,25-D_3-induced increase in these parameters.

1,25-D_3 is one of the major stimulators of FGF23 secretion [16-19]. However, it is unlikely that increased circulating 1,25-D_3 is the cause of elevated FGF23 production in DMP1-caPTHR1 mice since, if that were the case, both osteoblasts and osteocytes should be affected and only osteocytes express more FGF23 in DMP1-caPTHR1 mice. Moreover, PTH, PTHrP or the cAMP analog increase FGF23 expression *in vitro*, in the absence of 1,25-D3, confirming that FGF23 is directly regulated by PTH receptor signaling. Furthermore, the effect of PTH on FGF23 expression in cultured calvaria cells was not

affected by addition of 1,25-D_3. Although we did not observed an effect of short-term treatment with 1,25-D_3 in our experiments, it has been previously shown that 1,25-D_3 increases FGF23 expression in rat calvaria cells and the rat osteosarcoma cell lines ROS17/2.8 and UMR106 [38-40]. Thus, future investigations are warranted to determine whether PTH and 1,25-D_3 have additive or synergistic effects on FGF23 regulation at longer periods of culture.

Our findings showing that the increase in FGF23 expression by PTH receptor signaling is abolished in mice overexpressing Sost in osteocytes suggests that PTH regulates FGF23 through a mechanism that requires elevation of Wnt signaling. This is consistent with previous studies demonstrating that stabilization of β-catenin induced by LiCl increases FGF23 promoter reporter activity in osteoblastic cells [41] and that sclerostin blunts PTH-stimulated FGF23 expression in osteosarcoma cells [22]. The fact that FGF23 expression is increased in osteocytes and not in osteoblasts of the DMP1-caPTHR1 mice suggests that the increase in FGF23 results from a cell autonomous effect of Wnt signaling in the osteocyte and that does not involve paracrine effects of Wnt activation on osteoblasts. Further studies are required to clarify the mechanism of this phenomenon.

It has been long recognized that systemic elevation of PTH induces the synthesis of 1,25-D_3 in the kidney; and that, in turn, 1,25-D_3 inhibits PTH secretion by the parathyroid glands. More recent studies revealed that 1,25-D_3 stimulates FGF23 production in bone [39]; and that, conversely, FGF23 suppresses 1,25-D_3 production as well as PTH secretion [42]. Our findings that PTH stimulates FGF23 expression add a novel feedback loop to the interactive hormonal mechanisms that control mineral homeostasis. Moreover, this evidence points to osteocytes as potential therapeutic targets in the management of conditions exhibiting dysregulation of PTH and FGF23, such as CKD.

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Abbreviations

PTH	parathyroid hormone
DMP1-caPTHR1	dentin matrix protein 1-constitutively active PTH receptor 1 mice
FGF23	fibroblast growth factor 23
PTHrP	PTH related protein
FGFR1	FGF receptor 1
GALNT3	galactosaminyl transferase 3
BMP	bone morphogenetic protein
Pi	inorganic phosphate
NPT2A	sodium-phosphate co-transporter
1,25-D_3	1,25-dihydroxy-vitamin D_3

CKD	chronic kidney disease
GFP	green fluorescent protein
DBA	dibutyrylcyclic AMP
MEM	minimal essential medium
SD	standard deviation
ANOVA	analysis of variance
WT	wild type
ERKs	extracellular signal regulated kinases

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Research highlights

- Parathyroid hormone (PTH) exerts its anabolic and pro-remodeling actions, at least in part, through receptors expressed in osteocytes.
- PTH decreases expression of the osteocyte-derived Wnt antagonist Sost/sclerostin, a potent inhibitor of bone formation.
- We now show that PTH receptor activation in osteocytes increases FGF23, an osteocyte product that inhibits kidney phosphate re-absorption.
- FGFR1/KLOTHO receptor complex is expressed in bone cells and FGF23 signaling is increased in bone by PTH receptor activation.
- Thus, PTH receptor signaling has the potential to modulate the endocrine and auto/paracrine functions of osteocytes through FGF23.

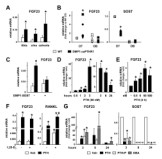

Figure 1. FGF23 expression is upregulated by PTH receptor signaling in osteocytes *in vivo* and *in vitro*

A. FGF23 mRNA expression in bones of 6-week-old DMP1-caPTHR1 mice. Bars indicate means ± SD of 4-6 mice per group; *p < 0.05 vs. WT littermates by Student's t-test.

B. FGF23 and Sost mRNA expression in osteocytes (OT) and osteoblasts (OB) isolated by GFP sorting of neonatal calvaria cell preparations from DMP1-GFP mice expressing or not the DMP1-caPTHR1 transgene. Each symbol represents an independent sorting experiment in which 6-12 mice of each genotype were pooled before sorting. Results from 4 or 5 experiments for FGF23 or Sost expression, respectively, expressed as fold change versus WT OT for each preparation, are shown. *p < 0.05 vs. WT OT, by Student's t-test.

C. FGF23 mRNA expression in ulnar bones of DMP1-caPTHR1, DMP1-Sost, double transgenic, and WT littermates. Bars indicate means ± SD of 4-6 mice each group; *p < 0.05 vs. WT littermates, by Student's t-test.

D and **E.** Time- and dose-response to PTH on FGF23 expression in cultures containing osteocytes and osteoblasts. Bars indicate means ± SD of triplicate determinations; *p < 0.05 versus the respective vehicle treated group, by Student's t-test.

F. Effect of PTH (50 nM), 1,25-D_3 (10 nM) or both for 3 h on FGF23 and RANKL expression in cultures containing osteocytes and osteoblasts. Bars indicate means ± SD of triplicate determinations; *p < 0.05 versus vehicle treated group and #p < 0.05 versus PTH-treated group, by 1-way ANOVA.

G. Effect of PTH (50 nM), PTHrP (50 nM), and DBA (1mM) on FGF23 expression in cultures containing osteocytes and osteoblasts. Bars indicate means ± SD of triplicate determinations; *p < 0.05 versus vehicle treated group by 1-way ANOVA.

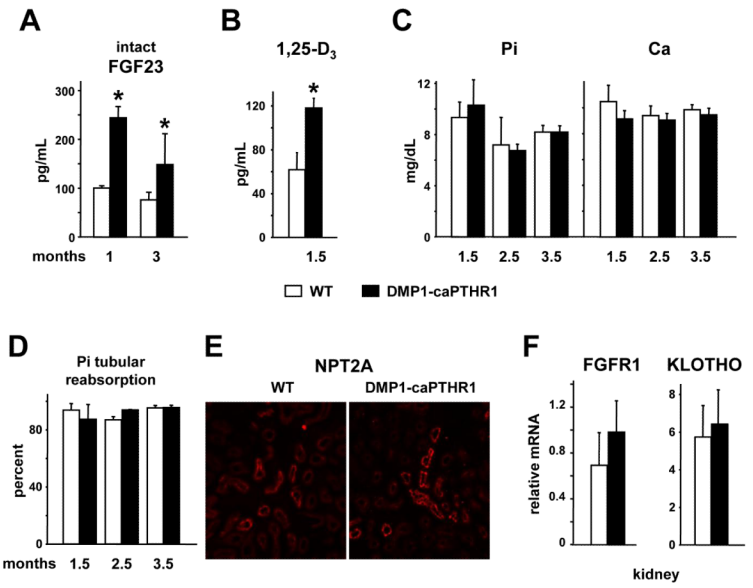

Figure 2. PTH receptor activation in osteocytes increases circulating levels of intact FGF23 but has no detectable effects on Pi tubular reabsorption or Pi or calcium plasma concentrations

A, **B**, **C**. FGF23, 1,25-D3, Pi, and calcium (Ca) concentrations in plasma from DMP1-caPTHR1 mice and WT littermates of the indicated ages. Bars are means ± SD; n=3-5/group; *$p < 0.05$ versus WT littermates of the same age, by Student's t-test.

D. Renal tubular reabsorption of Pi of DMP1-caPTHR mice and WT littermates. Bars are means ± SD; n=4-5/group.

E. NPT2A protein expression measured by immunofluorescence in kidney sections of DMP1-caPTHR1 mice and WT littermates.

F. FGFR1 and KLOTHO expression in kidney from DMP1-caPTHR1 mice and WT littermates. Bars indicate means ± SD; n=4/group.

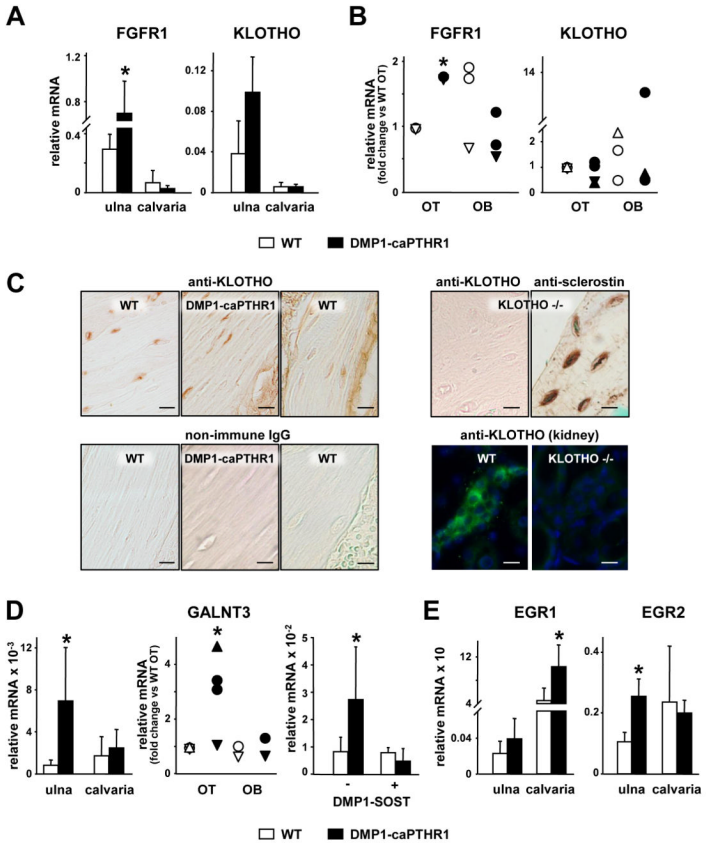

Figure 3. PTH receptor activation in osteocytes enhances FGF23 signaling in bone

A. FGFR1 and KLOTHO mRNA expression in bones from DMP1-caPTHR1 mice and WT littermates. Bars indicate means ± SD; n=5-6 for ulnae, and n=3-4 for calvaria. *p < 0.05 vs. WT littermates, by Student's t-test.

B. FGFR1 and KLOTHO mRNA expression in osteocytes (OT) and osteoblasts (OB) of DMP1-caPTHR1 mice (black symbols) or WT littermates (white symbols). Each symbol indicates an independent sorting experiment. *p < 0.05 vs. WT littermates by Student's t-test.

C. KLOTHO protein expression was detected in bone by immunohistochemistry using anti-KLOTHO antibody; non-immune IgG was used as negative control. Tibial bone sections from WT, DMP1-caPTHR1, and KLOTHO deficient (KLOTHO −/−) mice were used. Anti-sclerostin antibody was used to demonstrate that the KLOTHO −/− bone sections are intact. Kidney sections from WT and KLOTHO −/− mice were used to demonstrate the specificity of the anti-KLOTHO antibody. Bars indicate 10 μm.

D. GALNT3 mRNA expression in bones or OT and OB from DMP1-caPTHR1 mice and WT littermates, or in ulnar bones of DMP1-caPTHR1, DMP1-Sost, double transgenic, and WT littermates. Bars indicate means ± SD of 3-8 mice; *p < 0.05 versus WT littermates, WT OT, or double transgenic mice, by Student's t-test.

E. EGR1 and EGR2 mRNA expression in bones from DMP1-caPTHR1 mice or littermate controls. Bars indicate means ± SD of 3-6 mice per group; *p < 0.05 vs. WT littermates, by Student's t-test.

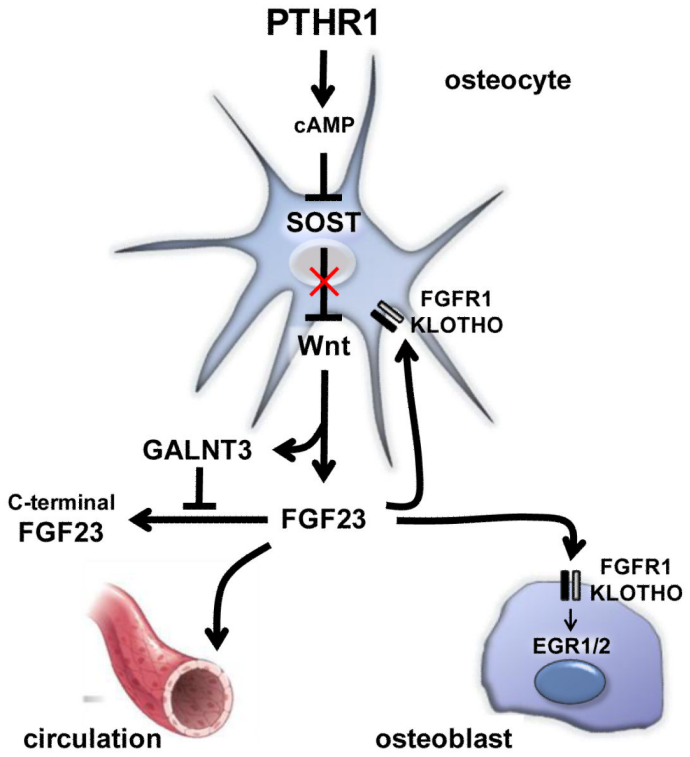

Figure 4. PTH receptor signaling modulates the endocrine and auto/paracrine function of osteocytes by upregulating FGF23 expression

In vivo activation of cAMP by a constitutively active PTHR1 or *in vitro* by the PTHR1 ligands, PTH and PTHrP, or DBA, increases FGF23 and inhibits Sost expression. A simultaneous increase in GALNT3 stabilizes FGF23, leading to increased intact FGF23 in the circulation as well as locally in bone. Binding of FGF23 to the FGFR1/KLOTHO receptor complex expressed in osteocytes and osteoblasts activates intracellular signaling.