Parathyroid Hormone Regulates Circulating Levels of Sclerostin and FGF23 in a Primary Hyperparathyroidism Model

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Abstract

Parathyroid hormone (PTH) increases Fibroblast growth factor 23 (FGF23), mediated by both protein kinase A (PKA) and Wnt signaling, and decreases expression of sclerostin, a Wnt antagonist derived from osteocytes. Patients with primary hyperparathyroidism (PHPT) have lower serum sclerostin levels than healthy controls, consistent with the idea of SOST downregulation by PTH. Nevertheless, the relationship between FGF23 and sclerostin in PHPT is still unclear. We examined this issue in a mouse model of PHPT. PHPT mice had increased FGF23 and decreased sclerostin expression in calvaria and in their serum concentrations, compared with wild-type (WT) mice. In UMR106 osteoblasts, PTH increased Fgf23 expression and decreased Sost expression, as well as forskolin, PKA agonist, whereas inhibition of PKA reversed the changes in Fgf23 and Sost expression, stimulated by PTH. Sclerostin treatment had no effect on Fgf23 expression, but when it was added together with PTH, it significantly abrogated the increase in Fgf23 expression. By contrast, there was no significant correlation between serum FGF23 and sclerostin, whereas PTH was positively and negatively correlated with serum FGF23 and sclerostin, respectively. These results indicate that the high level of PTH in PHPT mice leads to increased FGF23 and decreased sclerostin expression in serum and calvaria. A decrease of sclerostin may further augment FGF23 in vitro, however, there was no significant association between circulating FGF23 and Sclerostin. It is suggested that the pathogenesis of increased FGF23 expression in PHPT mice may be modified by not only sclerostin, but also other regulatory factors modulated by PTH.

Keywords: Parathyroid hormone, Fibroblast growth factor 23, Sclerostin, Protein Kinase A, Wnt signaling
Introduction

Parathyroid hormone (PTH) has calcitropic effects of direct action on bone and kidney and indirect action on the intestine to regulate calcium and phosphate homeostasis (1). The skeletal effect of PTH is an increase of bone remodeling, stimulating osteoblasts and osteocytes via activation of Protein Kinase A (PKA) and Wnt signaling. Furthermore, PTH increases Fibroblast Growth Factor 23 (FGF23), a major phosphaturic factor secreted from mature osteoblasts and osteocytes (2,3). Healthy individuals injected with 1–34 PTH developed hypophosphatemia and an increase of serum FGF23, along with elevated 1,25(OH)2D levels (4). Circulating levels of FGF23 are significantly elevated and positively correlated with serum calcium and PTH levels in a mouse model and patients with primary hyperparathyroidism (PHPT), who are characterized by hypercalcemia and hypophosphatemia due to excessive secretion of PTH from one or more parathyroid tumors or hyperplasia. After parathyroidectomy, FGF23 levels were significantly lower than preoperative levels in these mice and patients with PHPT (5-7). Patients with chronic kidney disease develop secondary hyperparathyroidism (SHPT) accompanying with renal function declines, and their circulating FGF23 levels also increase significantly and positively correlates with serum calcium and PTH levels. (3). Total parathyroidectomy can reverse the increased serum FGF23 levels in patients undergoing hemodialysis who have advanced SHPT (8). According to these observations, excessive secretion of PTH is considered to contribute to the increased FGF23 levels in hyperfunctioning parathyroid diseases (3).

Sclerostin, encoded by the Sost gene, is a secreted Wnt antagonist mainly produced by osteocytes, and is a negative regulator of bone formation (9-11). It abrogates the terminal differentiation of osteoblasts and promotes their apoptosis by binding to low-density lipoprotein receptor-related protein 5/6, resulting in inhibition of the canonical Wnt/β-catenin signaling pathway which plays an essential role in osteoblast differentiation, proliferation, and activity (12,13). PTH decreases Sost mRNA levels in vitro, and in mice treated with continuous and
intermittent administration of PTH (14,15). Similarly, transgenic mice with constitutive activation of parathyroid hormone receptor 1 (PTHR1) specifically in osteocytes exhibit increased bone mass and remodeling and decreased Sost expression (16). Serum sclerostin levels negatively correlate with PTH levels in postmenopausal women (17), and intermittent PTH 1-34 treatment of postmenopausal women is associated with a reduction in circulating sclerostin levels (18). Serum sclerostin levels in patients with PHPT are lower than in healthy controls and negatively correlated with plasma PTH levels (19), whereas serum sclerostin in these patients increased immediately after parathyroidectomy, returned to normal levels by the tenth day postoperatively (7). These findings indicate that the expression and secretion of sclerostin is decreased by PTH stimulation on bone.

Based on these findings, it has been demonstrated that PTH has significant effects on increased FGF23 and decreased sclerostin expression and secretion in bone. In addition, these effects involve both PKA and Wnt signaling, because sclerostin has an inhibitory effect on PTH to increase FGF23 expression (2); however, their mechanistic interrelationships in the setting of hyperfunctioning parathyroid disease remain unclear. To elucidate whether decreased sclerostin levels affect increased FGF23 levels in PHPT, we sought to determine circulating levels of sclerostin and FGF23 and their respective gene expression patterns in bone, and analyze their relationship, using a mouse model of PHPT. We also investigated the involvement of sclerostin and PKA signaling in the regulation of FGF23 expression in the mature osteoblast cell line, UMR106, following PTH stimulation.

**Materials and Methods**

**Experimental Animals**

Transgenic mice overexpressing the human cyclin D1 oncogene specifically in the parathyroids of FVB/N mice were used in this study as a model of PHPT, as previously described (20). PHPT and WT
littermates were previously demonstrated to have no significant differences in morphology, weight, or growth, and alterations of biochemical parameters in PHPT mice are similar between males and females. PHPT mice aged over 60 to 75 weeks, and even 87 to 118 weeks, have obviously higher serum PTH and FGF23 levels than younger mice aged 27 to 33 weeks, while serum PTH levels in these mice at earlier ages is not significantly increased compared to WT mice of the same age (5,21). Thus, we used mice aged 75 to 96 weeks for in vivo experiments. All mice were kept under constant conditions at room temperature (23 ± 2°C) and 50% to 60% relative humidity. They were fed a commercially available rodent diet (CE-2, Crea Japan; Tokyo, Japan) containing 1.03% calcium and 0.97% phosphorus, and were provided food and water ad libitum under specific pathogen-free conditions. As there were no significant sex differences in terms of phenotype, both male and female samples were collected and stored for statistical analysis. All in vivo experiments were approved by the appropriate institutional animal care committees at Osaka City University Graduate School of Medicine (Protocol number 08093).

Measurement of Biochemical Parameters

Blood samples were collected using cardiac puncture of anesthetized mice and were stored at −20°C until chemical analysis. Serum calcium, phosphate, and creatinine levels were determined using the Calcium E-TEST, the Phospha-C TEST, and the LaboAssay Creatinine test (Wako Pure Chemical Industries, Ltd., Osaka, Japan), respectively. Serum PTH, FGF23 and sclerostin were measured using mouse PTH 1-84 ELISA kit (Quidel, previously Immutopics; San Diego, CA), Human FGF23 ELISA Kit (Kinos, Tokyo, Japan) (21) and Mouse/Rat SOST Quantikine ELISA Kit (R&D Systems, Minneapolis, MN), respectively. All kits were used according to the manufacturers’ instructions.
Cell culture

UMR106 rat osteogenic sarcoma cells (CRL-1661; American Type Culture Collection, Manassas, VA) (22) were cultured in α-Minimum Essential Medium (α-MEM; Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco) at 37°C in a humidified atmosphere of 5% CO₂. Cells were seeded into the wells of 12-well plates at 1 × 10⁵ cells/well.

For the analysis of Fgf23 and Sost expression in response to PTH treatment, UMR106 cells were cultured with 1×10⁻⁸ M, 1×10⁻⁷ M and 1×10⁻⁶ M 1-34 PTH for 24 h. In the time-course experiment, UMR106 cells were cultured with 1×10⁻⁷ M 1-34 PTH for 4, 24, 48, 96, 168 hours. For PKA signaling analysis, UMR106 cells were cultured with 10⁻⁶ M, 10⁻⁵ M, and 10⁻⁴ M forskolin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) or with 10⁻⁵ M H89 dihydrochloride (Tocris Bioscience, Bristol, United Kingdom) and/or 10⁻⁷ M 1-34 PTH for 24 h. Further, these cells were also cultured with 4.4×10⁻⁸ M (1 µg/mL) recombinant human sclerostin (R&D Systems) and/or 10⁻⁷ M 1-34 PTH for 24 h. Cells were collected to extract total RNA and/or protein for analyses.

RNA extraction and quantitative real-time RT-PCR

Mouse bone tissues were frozen in liquid nitrogen immediately after surgical resection and stored at −80°C until analysis. Total RNA from bone tissue specimens and cultured cells was extracted using Trizol (Life Technologies, Inc.) and the RNeasy Mini, RNA isolation kit (Qiagen, Hilden, Germany), according to the manufacturers’ instructions. Total RNA was reverse-transcribed to cDNA using the Moloney murine leukemia virus (M-MLV) reverse transcriptase kit (Invitrogen, Carlsbad, CA), as described previously (23). cDNA was analyzed by quantitative real-time RT-PCR, using the StepOnePlus real-time PCR System (Applied Biosystems, Foster City, CA) and specific primers for mouse and rat Fgf23, Sost, and 18S rRNA genes (Applied Biosystems).
**Immunoblotting analysis**

Calvariae were dissected from WT and PHPT mice, and chopped into smaller pieces in RIPA buffer (Santa Cruz Biotechnology, Inc., Dallas, TX) to extract protein. Total protein was also extracted from UMR106 cells with RIPA buffer. Twenty micrograms of protein were loaded into the lanes of 12% SDS gels, and the resolved proteins were transferred onto PVDF membranes (Bio-Rad Laboratories, Inc., Hercules, CA) using the Trans-Blot Turbo System (Bio-Rad Laboratories, Inc.). Membranes were incubated with blocking solution (5% nonfat dry milk in Tris-buffered saline/Tween-20 [TBS-T]) for 1 h. The antibodies used in the current study are listed in Table 1. Primary antibodies against FGF23 (Abcam, Cambridge, MA) (24), Sclerostin (Abcam) (25), β-actin (Abcam) (26) were diluted in 5% BSA in TBS-T, and membranes were incubated overnight at 4°C. Membranes were then washed and incubated with appropriate horseradish peroxidase (HRP)-conjugated immunoglobulin G (IgG) antibodies for 1 h. The blots were washed and visualized with the Immobilon Western Chemiluminescent detection system (Thermo Fisher Scientific, Bremen, Germany), with the signal intensity determined using ImageQuant LAS 4000 (GE Healthcare UK Ltd.; Little Chafont, UK).

**Immunohistochemical analysis**

Calvariae from WT and PHPT mice were fixed with 4% paraformaldehyde in 1× phosphate-buffered saline (PBS), decalcified in 10% EDTA at 4°C for 2 weeks, and then embedded in paraffin. Three-micrometer longitudinal sections were cut and mounted onto glass slides. Deparaffinized sections were treated with 3% hydrogen peroxide solution for 5 min to inhibit endogenous peroxidase activity. Antigen retrieval was performed by L.A.B. Solution (Polysciences; Warrington, PA, USA) for 5 min, followed by blocking with rabbit or goat normal serum, as appropriate to the primary antibody. Sections were incubated with antibodies against FGF23, Sclerostin, goat or rabbit control
IgG (27,28) overnight. After washing with PBS, sections were incubated with anti-goat or anti-rabbit HRP-conjugated antibodies (Histofine, Nichirei Bioscience; Tokyo, Japan), as appropriate, for 60 min at room temperature. Sections were stained using DAB (Vector Laboratories; Burlingame, CA) and counterstained with hematoxylin.

**Statistical analysis**

Data are the mean ± standard deviation (SD). Differences were evaluated using a Student t-test or ANOVA followed by Dunnett’s test or Tukey-Kramer test. P values less than 0.05 were considered statistically significant.

**Results**

*Serum FGF23, Sclerostin and biochemical parameters in WT and PHPT mice*

PHPT mice exhibited typical biochemical features of hyperparathyroidism, such as hypercalcemia (PHPT vs. WT: 11.78 ± 1.20 vs. 9.09 ± 0.34 mg/dL), hypophosphatemia (PHPT vs. WT: 6.74 ± 1.22 vs. 9.02 ± 1.79 mg/dL), accompanying elevated serum PTH levels (PHPT vs. WT: 554 ± 173 vs. 196 ± 84 pg/mL), compared to WT mice. PHPT mice also had significantly higher levels of serum FGF23 than WT mice (PHPT vs. WT: 584 ± 310 vs 167 ± 54 pg/mL), as we reported previously (5) (21), whereas serum sclerostin level was significantly lower in PHPT mice than WT mice (PHPT vs. WT: 85.5 ± 12.3 vs 99.5 ± 7.9 pg/mL). Serum creatinine levels were not significantly different between the two groups (Table 2).
Expression of FGF23 and Sclerostin/Sost in bone tissues in WT and PHPT mice

We next investigated Fgf23 and Sost gene expression in the calvariae, lumbar vertebrae and femurs of WT and PHPT mice using quantitative real-time PCR. In the calvariae, Fgf23 expression was significantly higher whereas Sost expression was significantly lower in PHPT mice as compared with WT mice, but these expression levels in the lumbar vertebrae and femurs were unchanged (Figure 1A and 1B). Similarly, immunohistochemical analyses of calvarial tissues in PHPT mice showed high levels of FGF23 expression in osteoblasts/osteocytes as previously described (21), whereas low levels of sclerostin expression were observed in osteocytes of PHPT mice, compared to WT mice (Figure 1C, 1D, 1F and 1G). There was no positive staining in samples incubated with goat or rabbit control IgG instead of the primary antibody (Figure 1E and 1H). Western blot analysis of calvarial extracts also showed similar changes in protein expression between the two groups (Figure 1I).

Relationship between circulating levels of Sclerostin, FGF23 and PTH in WT and PHPT mice

Serum FGF-23 levels were significantly positively correlated with serum PTH (r = 0.678, P = 0.0010; Figure 2A) and calcium (r = 0.756, P = 0.0001) and negatively correlated with serum phosphate (r = -0.492, P = 0.0274), consistent with previous report. (5) In contrast, serum Sclerostin levels were significantly negatively correlated with serum PTH (r = -0.476, P = 0.0337; Figure 2B) and had borderline significance with serum calcium (r = -0.416, P = 0.0678) and positively correlated with serum phosphate (r = 0.480, P = 0.0321). There was no significant correlation between serum FGF23 and Sclerostin levels (r = -0.238, P = 0.3115).
Effects of PTH on Fgf23 and Sost expression

To study the direct effect of PTH on Fgf23 and Sost expressions, we incubated UMR106 cells with 1-34 PTH. We found that PTH (10⁻⁷ M) treatment led to sustained alterations, with elevated Fgf23 expression and reduced Sost expression (Figure 3A, 3B); Fgf23 expression increased and Sost expression decreased significantly in a dose-dependent manner with 1-34 PTH (10⁻⁸ M to 10⁻⁶ M; Figure 3C, 3D). PTH treatment also increased and decreased protein levels of FGF23 and sclerostin in UMR106 cells, respectively (Figure 3E).

Regulation of Fgf23 and Sost expression via PKA pathways and Effects of Sclerostin treatment

Treatment with Forskolin, an activator of PKA signaling (29), led to an increase in Fgf23 expression and a decrease in Sost expression in UMR106 cells in a dose dependent manner (Figure 4A, 4B). Furthermore, treatment with H89, an inhibitor of PKA (29), led to inhibition of Fgf23 or partial restoration of the alterations in Sost expression, stimulated with 10⁻⁷ M 1-34 PTH (Figure 4C, 4D). Treatment of UMR106 cells with 4.4×10⁻⁸ M sclerostin had no effect on Fgf23 expression, however, sclerostin added together with PTH (10⁻⁷ M) blunted the increased gene expression and protein levels of FGF23 induced by PTH (Figure 4E and 4F).

Discussion

We examined the relationship between FGF23 and sclerostin in PHPT using a mouse model of PHPT and UMR 106 mature osteoblast cell line, and found that in PHPT the excessive secretion of PTH leads to increased FGF23 and decreased sclerostin expression in serum and calvaria.
Our previous studies showed that serum PTH and calcium levels correlated highly with serum FGF23 levels in PHPT mice and that serum FGF23 levels decreased in the PHPT mice after parathyroidectomy (5). There was a positive correlation between Fgf23 expression and the expression of osteoblastic markers, such as Alp and osteocalcin (5), whereas the expression of Dentin matrix protein 1, one of osteocytic marker, was significantly decreased in calvaria in PHPT mice. (21) These studies indicated that excessive secretion of PTH increases calvarial expression of Fgf23 and increases serum FGF23 levels in PHPT, and that activities of osteoblasts and/or osteocytes might be involved in the stimulation of FGF-23 by PTH. In the present study, we demonstrate that in PHPT mice the persistent high level of PTH leads to increased FGF23 and decreased sclerostin expression in serum and calvaria compared with WT mice [Fig. 1 and Table 1]. WT mice had higher expression levels of Sost in calvaria than lumbar spine and femur in accordance with previous findings in vivo (14) and in vitro (30). In contrast, PHPT mice exhibit decreases in Sost/sclerostin expression in calvaria, and have lower circulating sclerostin levels than WT mice [Fig. 1(B), (F), (G) and Table 2], similar to rodent studies in which continuous and intermittent PTH treatment decreased both Sost expression and protein levels of sclerostin in bone tissue (15) (31). No studies in PHPT have investigated the expression of Sost in various parts of bone tissue, despite the higher expression of Sost in cortical bone than trabecular bone. Keller H et al. reported that Sost expression in WT mouse calvaria was higher than femur diaphysis (14), and our results also showed that Sost expression in calvaria was higher than in other bone tissues in WT mice, although there was no significant difference in the expression of Fgf23 or Sost in the femoral or vertebral tissues unlike calvaria, suggesting that mRNA samples extracted from vertebrae and whole femur may not be accurately evaluated due to contamination of trabecular bone and bone marrow. In human studies of PHPT, several studies documented lower sclerostin levels compared to control subjects, and parathyroidectomy normalized circulating sclerostin levels as well as markers of bone turnover (7) (19) (32). These findings suggested that persistent high levels of PTH decrease Sost/sclerostin expression in calvaria and lower circulating levels of sclerostin in PHPT mice.
Stimulation of PTH/PTH-related peptide (PTHRP) type 1 receptor (PTHR1) by PTH, and subsequent activation of PKA signaling is an important pathway to increase Fgf23 expression in the osteoblast/osteocyte (2) (16). Further, PTH mediates the downregulation of Sost expression by PKA signaling in osteoblast/osteocyte in vitro and in vivo (14). Sost expression was lower in osteocytes derived from mice expressing the dentin matrix protein 1 (DMP1)-constitutively active PTHR1 transgene (33) and PTH failed to suppress Sost/sclerostin expression levels in mice defected the PTHR1 in osteocytes (34). We also previously observed an increase in phosphorylation of cAMP-response element-binding protein in protein extracts obtained from calvariae of mice treated with a continuous infusion of PTH (21), suggesting the activation of PKA signaling in the calvaria of these mice. In this study, we found that 1-34 PTH significantly increased Fgf23 expression and decreased Sost expression as well as activation of PKA signaling. [Fig. 3, 4] These results indicate that not only the increase of Fgf23 but also the decrease of Sost expression by PTH in osteoblast/osteocyte is regulated via PKA signaling.

Regarding the relationship between FGF23 and sclerostin in secondary hyperparathyroidism in experimental kidney failure, Lavi-Moshayoff et al. demonstrated that FGF23 expression is increased by PTH via both PKA and Wnt signaling, because sclerostin has an inhibitory effect of PTH to increase FGF23 expression in vitro (2). In the presence of sclerostin, Wnt ligands are blocked from binding the LRP-5/6-Frizzled receptor complex which in turn facilitates the accumulation of β-catenin degradation complex (11). An increase of FGF23 expression by osteocyte specific constitutively active PTH1R is abolished in mice overexpressing Sost in osteocytes, suggesting that PTH regulates FGF23 through a mechanism that requires elevation of Wnt signaling (16). Moreover, activation of Wnt signaling can increase FGF23 promoter activity in a dose-dependent manner and protein and mRNA expression of FGF23 in osteoblastic cell lines (35) (36). Consistent with this inhibitory effect of sclerostin on FGF23 expression, we observed that the treatment of sclerostin added together with PTH prevented an increase in Fgf23 expression [Fig. 4(E) and (F)]. These findings suggest that sclerostin may suppress FGF23 expression via the
inhibition of Wnt signaling and this suppressive effect of sclerostin would disappear when sclerostin expression is attenuated by PTH in osteoblasts/osteocytes, resulting in increase of FGF23 expression, presumably resulting in aggravation of hypophosphateima in patients with PHPT.

However, the possibility remains that PTH may increase FGF23 levels through other pathways, aside from decreasing the inhibitory effect of sclerostin. As shown in Fig. 3(A) and 3(B), there are some differences between the kinetics of Fgf23 and Sost expression. PTH treatment increased Fgf23 expression at day 4 and decreased at day 7, whereas Sost expression decreased at day 2 and almost unchanged thereafter. Sclerostin treatment without PTH did not change Fgf23 expression, consistent with previous report that mice overexpressing Sost in osteocytes did not decrease Fgf23 expression in bone (16). Notably, there was no correlation between serum FGF23 and Sclerostin levels in WT and PHPT mice, although serum PTH had a positive correlation with serum FGF23 and a negative correlation with serum Sclerostin. Mice with continuous administration of PTH for 96 hours decreased expression of sclerostin in bone tissue (15) and Human studies also showed that there was a significant negative correlation between serum PTH and sclerostin in control subject and patients with PHPT (7) (19) (37), but no study have showed the relationship between circulating FGF23 and Sclerostin. FGF23 is also regulated by molecules derived from osteoblast/osteocyte in the process of bone mineralization, and it has been reported that excessive PTH can decrease calvarial DMP1 and phosphate regulating endopeptidase homolog X-linked (PHEX) expression (38), which may be associated with an increase in serum FGF23 levels (21) (39). Moreover, Dickkopf 1 (DKK1), another potent inhibitor of Wnt/β-catenin pathway secreted from osteoblast/osteocyte, was significantly higher in patients with PHPT compared to healthy subjects, whereas decreased levels of serum sclerostin were found in these patients (32). These observations support the notion that the increase in FGF23 caused by PHPT may be mediated, in part, by not only decrease in sclerostin but also PTH-mediated changes of other factors regulating FGF23 expression. Furthermore, high FGF23 levels on bone is considered to contribute to bone loss via direct inhibition of osteoblastic Wnt/β-catenin signaling pathway.
through induction of Dkk1. (40) PTH has the direct effect to increase β-catenin, one of the common mediators of osteoblastic bone formation as well as it suppressed the expressions of Wnt inhibitors in osteoblast. (40) (41) In contrast, mice with continuous administration of PTH exhibited severe bone loss with no increase of β-catenin in osteoblasts. (42) Attenuated sclerostin expression induced by high level of PTH would presumably enhance FGF23 expression in osteoblasts or osteocytes and resultantly exacerbate bone loss in patients with PHPT. Further studies will be required to determine whether regulatory factors derived from osteoblast/osteocyte affect FGF23 expression in bone tissue with PHPT.

In summary, a persistent high level of PTH increases Fgf23/FGF23 expression in calvaria and circulating FGF23 levels, whereas it decreases Sost/sclerostin expression in calvaria and circulating sclerostin levels in a mouse model of PHPT. The continuous activation of PKA signaling in osteoblasts/osteocytes by PTH can alter expression of these genes and a decrease of sclerostin may further augment FGF23 expression in vitro, whereas there was no significant association between circulating FGF23 and sclerostin. Our study proposes that the pathogenesis of increased FGF23 expression in a mouse model of PHPT may be modified by not only sclerostin, but also other regulatory factors modulated by PTH.
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Author Contributions: YN, YI, and ME designed the research; YN, TT and DM performed the research; YN, YI and MK and AA analyzed and interpreted the data; YN and YI wrote the manuscript. YN takes responsibility for the integrity of the data analysis.

Disclosures Summary: All authors state that they have no conflict of interest.

Data Availability

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author upon reasonable request.
Reference

1. Quarles LD. Endocrine functions of bone in mineral metabolism regulation. *J Clin Invest.* 2008;118(12):3820-3828.

2. Lavi-Moshayoff V, Wasserman G, Meir T, Silver J, Naveh-Many T. PTH increases FGF23 gene expression and mediates the high-FGF23 levels of experimental kidney failure: a bone parathyroid feedback loop. *Am J Physiol Renal Physiol.* 2010;299(4):F882-889.

3. Imanishi Y, Kobayashi K, Kawata T, Inaba M, Nishizawa Y. Regulatory mechanisms of circulating fibroblast growth factor 23 in parathyroid diseases. *Ther Apher Dial.* 2007;11 Suppl 1:S32-37.

4. Burnett-Bowie SM, Henao MP, Dere ME, Lee H, Leder BZ. Effects of hPTH(1-34) infusion on circulating serum phosphate, 1,25-dihydroxyvitamin D, and FGF23 levels in healthy men. *J Bone Miner Res.* 2009;24(10):1681-1685.

5. Kawata T, Imanishi Y, Kobayashi K, Miki T, Arnold A, Inaba M, Nishizawa Y. Parathyroid hormone regulates fibroblast growth factor-23 in a mouse model of primary hyperparathyroidism. *J Am Soc Nephrol.* 2007;18(10):2683-2688.

6. Kobayashi K, Imanishi Y, Miyauchi A, Onoda N, Kawata T, Tahara H, Goto H, Miki T, Ishimura E, Sugimoto T, Ishikawa T, Inaba M, Nishizawa Y. Regulation of plasma fibroblast growth factor 23 by calcium in primary hyperparathyroidism. *Eur J Endocrinol.* 2006;154(1):93-99.

7. Ardawi MS, Al-Sibiany AM, Bakhsh TM, Rouzi AA, Qari MH. Decreased serum sclerostin levels in patients with primary hyperparathyroidism: a cross-sectional and a longitudinal study. *Osteoporos Int.* 2012;23(6):1789-1797.
8. Sato T, Tominaga Y, Ueki T, Goto N, Matsuoka S, Katayama A, Haba T, Uchida K, Nakanishi S, Kazama JJ, Gejyo F, Yamashita T, Fukagawa M. Total parathyroidectomy reduces elevated circulating fibroblast growth factor 23 in advanced secondary hyperparathyroidism. *Am J Kidney Dis*. 2004;44(3):481-487.

9. Veverka V, Henry AJ, Slocombe PM, Ventom A, Mulloy B, Muskett FW, Muzyalk M, Greenslade K, Moore A, Zhang L, Gong J, Qian X, Paszty C, Taylor RJ, Robinson MK, Carr MD. Characterization of the structural features and interactions of sclerostin: molecular insight into a key regulator of Wnt-mediated bone formation. *J Biol Chem*. 2009;284(16):10890-10900.

10. Weivoda MM, Youssef SJ, Oursler MJ. Sclerostin expression and functions beyond the osteocyte. *Bone*. 2017;96:45-50.

11. Asamiya Y, K T, Nitta K. Role of Sclerostin in the pathogenesis of chronic kidney disease-mineral bone disorder. *Renal Replacement Therapy*. 2016;2:8.

12. Li X, Zhang Y, Kang H, Liu W, Liu P, Zhang J, Harris SE, Wu D. Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling. *J Biol Chem*. 2005;280(20):19883-19887.

13. Ellies DL, Viviano B, McCarthy J, Rey JP, Itasaki N, Saunders S, Krumlauf R. Bone density ligand, Sclerostin, directly interacts with LRP5 but not LRP5G171V to modulate Wnt activity. *J Bone Miner Res*. 2006;21(11):1738-1749.

14. Keller H, Kneissel M. SOST is a target gene for PTH in bone. *Bone*. 2005;37(2):148-158.

15. Bellido T, Ali AA, Gubrij I, Plotkin LI, Fu Q, O’Brien CA, Manolagas SC, Jilka RL. Chronic elevation of parathyroid hormone in mice reduces expression of
sclerostin by osteocytes: a novel mechanism for hormonal control of osteoblastogenesis. *Endocrinology*. 2005;146(11):4577-4583.

16. Rhee Y, Allen MR, Condon K, Lezcano V, Ronda AC, Galli C, Olivos N, Passeri G, O’Brien CA, Bivi N, Plotkin LI, Bellido T. PTH receptor signaling in osteocytes governs periosteal bone formation and intracortical remodeling. *J Bone Miner Res*. 2011;26(5):1035-1046.

17. Mirza FS, Padhi ID, Raisz LG, Lorenzo JA. Serum sclerostin levels negatively correlate with parathyroid hormone levels and free estrogen index in postmenopausal women. *J Clin Endocrinol Metab*. 2010;95(4):1991-1997.

18. Drake MT, Srinivasan B, Modder UI, Peterson JM, McCready LK, Riggs BL, Dwyer D, Stolina M, Kostenuik P, Khosla S. Effects of parathyroid hormone treatment on circulating sclerostin levels in postmenopausal women. *J Clin Endocrinol Metab*. 2010;95(11):5056-5062.

19. van Lierop AH, Witteveen JE, Hamdy NA, Papapoulos SE. Patients with primary hyperparathyroidism have lower circulating sclerostin levels than euparathyroid controls. *Eur J Endocrinol*. 2010;163(5):833-837.

20. Imanishi Y, Hosokawa Y, Yoshimoto K, Schipani E, Mallya S, Papanikolaou A, Kifor O, Tokura T, Sablosky M, Ledgard F, Gronowicz G, Wang TC, Schmidt EV, Hall C, Brown EM, Bronson R, Arnold A. Primary hyperparathyroidism caused by parathyroid-targeted overexpression of cyclin D1 in transgenic mice. *J Clin Invest*. 2001;107(9):1093-1102.

21. Nagata Y, Imanishi Y, Ohara M, Maeda-Tateishi T, Miyaoka D, Hayashi N, Kurajoh M, Emoto M, Inaba M. Attenuated Dentin Matrix Protein 1 Enhances
Fibroblast Growth Factor 23 in Calvaria in a Primary Hyperparathyroidism Model. *Endocrinology*. 2019;160(5):1348-1358.

22. RRID:CVCL_3617. https://scicrunch.org/resolver/CVCL_3617.

23. Kobayashi K, Imanishi Y, Koshiyama H, Miyauchi A, Wakasa K, Kawata T, Goto H, Ohashi H, Koyano HM, Mochizuki R, Miki T, Inaba M, Nishizawa Y. Expression of FGF23 is correlated with serum phosphate level in isolated fibrous dysplasia. *Life Sci*. 2006;78(20):2295-2301.

24. RRID:AB_880086. https://scicrunch.org/resolver/AB_880086.

25. RRID:AB_10859114. https://scicrunch.org/resolver/AB_10859114.

26. RRID:AB_867494. https://scicrunch.org/resolver/AB_867494.

27. RRID:AB_1163599. https://scicrunch.org/resolver/AB_1163599.

28. RRID:AB_1163659. https://scicrunch.org/resolver/AB_1163659.

29. Chijiwa T, Mishima A, Hagiwara M, Sano M, Hayashi K, Inoue T, Naito K, Toshioka T, Hidaka H. Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p′-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. *J Biol Chem*. 1990;265(9):5267-5272.

30. Miyagawa K, Yamazaki M, Kawai M, Nishino J, Koshimizu T, Ohata Y, Tachikawa K, Mikuni-Takagaki Y, Kogo M, Ozono K, Michigami T. Dysregulated gene expression in the primary osteoblasts and osteocytes isolated from hypophosphatemic Hyp mice. *PLoS One*. 2014;9(4):e93840.
31. Silvestrini G, Ballanti P, Leopizzi M, Sebastiani M, Berni S, Di Vito M, Bonucci E. Effects of intermittent parathyroid hormone (PTH) administration on SOST mRNA and protein in rat bone. *J Mol Histol*. 2007;38(4):261-269.

32. Viapiana O, Fracassi E, Troplini S, Idolazzi L, Rossini M, Adami S, Gatti D. Sclerostin and DKK1 in primary hyperparathyroidism. *Calcif Tissue Int*. 2013;92(4):324-329.

33. O’Brien CA, Plotkin LI, Galli C, Goellner JJ, Gortazar AR, Allen MR, Robling AG, Bouxsein M, Schipani E, Turner CH, Jilka RL, Weinstein RS, Manolagas SC, Bellido T. Control of bone mass and remodeling by PTH receptor signaling in osteocytes. *PLoS One*. 2008;3(8):e2942.

34. Saini V, Marengi DA, Barry KJ, Fulzele KS, Heiden E, Liu X, Dedic C, Maeda A, Lotinun S, Baron R, Pajevic PD. Parathyroid hormone (PTH)/PTH-related peptide type 1 receptor (PPR) signaling in osteocytes regulates anabolic and catabolic skeletal responses to PTH. *J Biol Chem*. 2013;288(28):20122-20134.

35. Liu S, Tang W, Fang J, Ren J, Li H, Xiao Z, Quarles LD. Novel regulators of Fgf23 expression and mineralization in Hyp bone. *Mol Endocrinol*. 2009;23(9):1505-1518.

36. Ma L, Gao M, Wu L, Zhao X, Mao H, Xing C. The suppressive effect of soluble Klotho on fibroblastic growth factor 23 synthesis in UMR-106 osteoblast-like cells. *Cell Biol Int*. 2018;42(9):1270-1274.

37. Kerschan-Schindl K. Bone turnover in hyperparathyroidism. *Wien Med Wochenschr*. 2013;163(17-18):391-396.

38. Alos N, Ecarot B. Downregulation of osteoblast Phex expression by PTH. *Bone*. 2005;37(4):589-598.
39. Yuan B, Takaiwa M, Clemens TL, Feng JQ, Kumar R, Rowe PS, Xie Y, Drezner MK. Aberrant Phex function in osteoblasts and osteocytes alone underlies murine X-linked hypophosphatemia. *J Clin Invest*. 2008;118(2):722-734.

40. Carrillo-Lopez N, Panizo S, Alonso-Montes C, Roman-Garcia P, Rodriguez I, Martinez-Salgado C, Dusso AS, Naves M, Cannata-Andia JB. Direct inhibition of osteoblastic Wnt pathway by fibroblast growth factor 23 contributes to bone loss in chronic kidney disease. *Kidney Int*. 2016;90(1):77-89.

41. Suzuki A, Ozono K, Kubota T, Kondou H, Tachikawa K, Michigami T. PTH/cAMP/PKA signaling facilitates canonical Wnt signaling via inactivation of glycogen synthase kinase-3beta in osteoblastic Saos-2 cells. *J Cell Biochem*. 2008;104(1):304-317.

42. Wan M, Yang C, Li J, Wu X, Yuan H, Ma H, He X, Nie S, Chang C, Cao X. Parathyroid hormone signaling through low-density lipoprotein-related protein 6. *Genes Dev*. 2008;22(21):2968-2979.
Figure Legends

Figure 1. 

Figure 1. 

**Fgf23 and Sost /Sclerostin expression in bone tissues of WT and PHPT mice.** *Fgf23* (A) and *Sost* (B) expression of calvaria, lumbar spine and femur in WT and PHPT mice. Immunohistochemical analysis of FGF23 (C, D) and sclerostin (F, G) expression in calvaria of WT (C, F) and PHPT mice (D, G). Negative controls for FGF23 and sclerostin were performed with (E) goat and (H) rabbit control IgG. (I) Western blot analysis of FGF23 and sclerostin expression in protein extracts from the calvarial tissue of WT and PHPT mice. Results are expressed relative to 18S control, and show fold-change differences in expression as compared with WT mice. Data are shown as the mean ± SD (WT, *n* = 10 (male/female, 6/4); PHPT, *n* = 10 (male/female, 9/1)); *p* < 0.05, by Student *t*-test. Arrowheads and arrows indicate FGF23 and sclerostin-positive osteocytes, respectively. (C–H) Scale bars, 30 µm. WT, wild type; PHPT, primary hyperparathyroidism.

**Figure 2. Relationships between circulating levels of Sclerostin, Fgf23 and PTH in WT and PHPT mice.** Graph shows the relationship between (A) FGF23 and PTH (*r* = 0.678, *P* = 0.0010) and (B) Sclerostin and PTH (*r* = -0.476, *P* = 0.0337), analyzed by Pearson’s correlation. PTH values were log-transformed for skewness. Open circles WT mice, closed circles PHPT mice.

**Figure 3. PTH regulates Fgf23 and Sost expression in UMR106 cells.** (A, B) Time-dependent changes in *Fgf23* (A) and *Sost* (B) expression levels in UMR106 cells after treatment with 10^{-7} M 1-34 PTH at 4, 24, 48, 96, 168 hours. (C, D) Concentration-dependent changes in *Fgf23* (C) and *Sost* (D) expression levels in UMR106 cells treated with 1-34 PTH for 24 h. Results are relative to 18S mRNA, and are presented as the fold-change in expression compared with control (vehicle-treated) cells. Data are shown as the mean ± SD (*n* = 3); *P* < 0.05, by Student *t*-test or ANOVA with Dunnett’s test.
Figure 4. The regulation of Fgf23 by the PTH/PKA pathway and Sclerostin in UMR106 cells. (A) Fgf23 and (B) Sost expression in UMR106 cells after treatment with $10^{-6}$ M to $10^{-4}$ M forskolin for 24 h. (C) Fgf23 and (D) Sost expression in UMR106 cells after treatment with $10^{-7}$ M 1-34 PTH with or without 10 µM H89 for 24 h. (E) FGF23 gene and (F) FGF23 protein expression in UMR106 cells after treatment with $10^{-7}$ M 1-34 PTH with or without $4.4\times10^{-8}$ M sclerostin for 24 h. Results are relative to 18S mRNA, and are presented as the fold-change in expression compared with control (vehicle-treated) cells. Data are presented as the mean ± SD ($n=3$); *$P<0.05$, by ANOVA with Tukey-Kramer test.
Table 1. Antibodies

| Name of Antibody | Source of Antibody | Host; Monoclonal/Polyclonal | Antibody ID               | Dilution for WB and/or IHC |
|------------------|--------------------|-----------------------------|---------------------------|-----------------------------|
| FGF23            | Abcam              | Goat; polyclonal            | RRID: AB_880086 (24)      | WB (1:1000); IHC (1:1000)   |
| Sclerostin       | Abcam              | Rabbit; polyclonal          | RRID: AB_956321 (25)      | WB (1:1000); IHC (1:1000)   |
| β-Actin          | Abcam              | Mouse; monoclonal           | RRID: AB_867494 (26)      | WB (1:20000)                |
| IgG from goat serum | Sigma-Aldrich     | Goat; polyclonal            | RRID: AB_1163599 (27)     | Same concentration of FGF23 for IHC |
| IgG from rabbit serum | Sigma-Aldrich     | Rabbit; polyclonal          | RRID: AB_1163659 (28)     | Same concentration of Sclerostin for IHC |

Abbreviations: FGF23, fibroblast growth factor 23; RRID, Research Resource Identifier; IHC, immunohistochemistry; WB, Western blotting.
Table 2. Serum biochemistries of experimental mice

| Genotype | n  | Calcium (mg/dL) | Phosphate (mg/dL) | PTH (pg/mL) | FGF23 (pg/mL) | Creatinine (mg/dL) | Sclerostin (pg/mL) |
|----------|----|-----------------|-------------------|-------------|---------------|-------------------|-------------------|
| WT       | 1  | 9.0 ± 0.3       | 9.0 ± 1.7         | 19 ± 84     | 16 ± 54       | 0.2 ± 0.2         | 99.5 ± 7.9        |
|          | 0  | 9               | 4                 | 2           | 9             | 6                 | 9 ± 0             |
|          | 78 | 6.7 ± 1.2       | 55 ± 17           | 58 ± 31     | 4 ± 3         | 9 ± 4             | 5 ± 3             |
| PHPT     | 1  | 11.7 ± 1.2      | 4 ± 2             | 16 ± 3      | 4 ± 3*        | 9 ± 4             | 5 ± 3*            |
|          | 0  | 11.7 ± 1.2      | 4 ± 2             | 16 ± 3      | 4 ± 3*        | 9 ± 4             | 5 ± 3*            |

Abbreviations: PHPT, primary hyperparathyroidism; PTH, parathyroid hormone; FGF23, fibroblast growth factor 23.

WT mice at ages of 75, 87, 96 weeks (n=3, 5, 2); PHPT mice at ages of 75, 87, 96 weeks (n=6, 3, 1)

Data are the mean ± SD

*P<0.05 using Student’s t-test
Figure 2.

(A) [Graph showing the relationship between RANKL and log PTH.]

(B) [Graph showing the relationship between sclerostin and log PTH.]
Figure 3.

(A) 

(Fgf23/18s)

Relative gene expression

0 4 24 48 96 168 hours

(B) 

(Sost/18s)

Relative gene expression

0 4 24 48 96 168 hours

(C) 

(Fgf23/18s)

Relative gene expression

Control 10 μM 100 μM 1000 μM

1-34 PTH

(D) 

(Sost/18s)

Relative gene expression

Control 10 μM 100 μM 1000 μM

1-34 PTH

(E) 

| PTH  | Sclerostin | Sclerostin/β-Actin | FGF23 | FGF23/β-Actin | β-Actin |
|------|------------|--------------------|-------|-------------|--------|
| -    | -          | 1.0                | 1.0   | 1.2         |        |
| +    | +          | 0.8                |       |             |        |
Figure 4.

(A)  

(B)  

(C)  

(D)  

(F)  

(E)  

FGF23/18s

Relative gene expression

control 10 + M 10 + M 10 + M

Sost/18s

Relative gene expression

control 10 + M 10 + M 10 + M

FGF23/18s

Relative gene expression

control 10 + M 10 + M 10 + M

Sost/18s

Relative gene expression

control 10 + M 10 + M 10 + M

sclerostin

- + - +

FGF23

1.0 0.9 1.2 1.0

β-Actin

1.0 1.0 1.0 1.0

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