The parathyroid is a target organ for FGF23 in rats

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Phosphate homeostasis is maintained by a counterbalance between efflux from the kidney and influx from intestine and bone. FGF23 is a bone-derived phosphaturic hormone that acts on the kidney to increase phosphate excretion and suppress biosynthesis of vitamin D. FGF23 signals with highest efficacy through several FGF receptors (FGFRs) bound by the transmembrane protein Klotho as a coreceptor. Since most tissues express FGFR, expression of Klotho determines FGF23 target organs. Here we identify the parathyroid as a target organ for FGF23 in rats. We show that the parathyroid gland expressed Klotho and 2 FGFRs. The administration of recombinant FGF23 led to an increase in parathyroid Klotho levels. In addition, FGF23 activated the MAPK pathway in the parathyroid through ERK1/2 phosphorylation and increased early growth response 1 mRNA levels. Using both rats and in vitro rat parathyroid cultures, we show that FGF23 suppressed both parathyroid hormone (PTH) secretion and PTH gene expression. The FGF23-induced decrease in PTH secretion was prevented by a MAPK inhibitor. These data indicate that FGF23 acts directly on the parathyroid through the MAPK pathway to decrease serum PTH. This bone-parathyroid endocrine axis adds a new dimension to the understanding of mineral homeostasis.

Introduction
FGF23 is secreted by osteocytes (1) and osteoblasts (2) in response to high serum phosphate levels and after the administration of the vitamin D metabolite, 1,25(OH)2-vitamin D (2–4). FGF23 acts on its target tissues by binding to and activating its cognate FGFRs in the presence of its obligate coreceptor, Klotho (5, 6). Klotho is a transmembrane protein and acts as a cofactor for FGF23 to facilitate the binding of FGF23 to FGFR1c, -3c, and -4 (5, 6). The expression of Klotho determines the tissue specificity of its ligand FGF23. Klotho is expressed in the renal distal tubule, the parathyroid, pituitary, and sinoatrial node of the heart (7). In the kidney, FGF23 inhibits phosphate reabsorption and decreases the synthesis of 1,25(OH)2-vitamin D (8). Membrane-bound Klotho is present in the distal tubule and inhibits the activity of the Na2Pi cotransporter in the proximal tubule (9, 10), which may be due to the action of a secreted form of Klotho (11). Klotho has weak glucuronidase enzymatic activity and in the renal distal tubule hydrolyzes extracellular sugar residues on the transient receptor potential ion channel V, which leads to increased calcium transport in the kidney (10). In the heart, klotho gene expression is essential for the sinoatrial node to function as a dependable pacemaker under conditions of stress (7). In both the choroid plexus and parathyroid, Klotho protein has been shown to interact with Na+, K+ -ATPase and may be associated with parathyroid hormone (PTH) secretion (12). Klotho also exists as a soluble circulating protein. Administration of FGF23 to rats increased early growth response 1 (Egr-1) mRNA levels in kidney and also in the parathyroid and pituitary (6). We have now studied the function of FGF23 on the parathyroid.

Transgenic mice with FGF23 driven by the β-actin promoter showed all the biochemical and skeletal phenotypes seen in patients with autosomal dominant hypophosphatemic rickets (ADHR) or tumor-induced osteomalacia, diseases that result from marked increases in mutant or intact FGF23 (13, 14). These mice had decreased serum phosphate and 1,25(OH)2-vitamin D levels, which would be expected to increase PTH secretion. However, PTH levels were decreased (15). Three other studies with genetic manipulation of FGF23 showed increased serum PTH levels (16–18). In 2 of these studies, the increase in serum PTH was attributed to the decrease in serum 1,25(OH)2-vitamin D (16, 17). We show that Klotho is expressed in the parathyroid and that FGF23 activates the MAPK pathway and potently decreases PTH gene expression and secretion both in vivo and in vitro. Hence, we portray an endocrinological axis linking bone cells and the parathyroid, in which the parathyroid is a new target organ for FGF23. FGF23 acts directly on the parathyroid to decrease serum PTH in addition to its actions on the kidney to enhance phosphaturia and inhibit vitamin D metabolism. This novel endocrinological axis is important for the physiologic responses of the parathyroid, kidney, and bone in phosphorus homeostasis.

Results
We first demonstrated the expression of Klotho in the parathyroid. Immunoblot with anti-Klotho antibody showed Klotho expression in rat microdissected parathyroid. There was no expression of Klotho protein in the thyroid (Figure 1A) or liver (data not shown). Quantitative RT-PCR (qRT-PCR) for Klotho mRNA showed high expression in parathyroid and kidney with negligible expression in thyroid, duodenum, and liver and no expression at all in the spleen (Figure 1B). Immunohistochemistry confirmed the localization of Klotho to the parathyroid and not the surrounding thyroid tissue.

Nonstandard abbreviations used: ADHR, autosomal dominant hypophosphatemic rickets; CaR, Ca2+ receptor; Egr-1, early growth response 1; FGFR, FGF receptor; PTH, parathyroid hormone; qRT-PCR, quantitative RT-PCR.

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research article

(Figure 1C) and also showed the presence of FGFR1 (Figure 1D) and FGFR3 (Figure 1E) in the parathyroid tissue. These results show that the parathyroid expresses the FGFR-Klotho receptor complex and suggest that the parathyroid is a target organ for FGF23.

To study the effect of FGF23 on parathyroid function, rats were injected i.p. with full-length FGF23 harboring ADHR mutations (FGF23R176Q/R179Q). The ADHR mutations were introduced to inhibit proteolytic inactivation of FGF23 and to increase its half-life. As a negative control, we injected FGF23core, an FGF23 variant lacking C-terminal residues past the 176RXXR179 cleavage site for furin-like proteases (19). This C-terminal truncation inactivates FGF23 by abrogating binding to Klotho (19). FGF23R176Q/R179Q or FGF23core were injected by daily i.p. injections for 5 days. Serum biochemistry showed the expected decrease in 1,25(OH)2–vitamin D levels in FGF23R176Q/R179Q-treated rats (Figure 2A). There was also a significant decrease in serum phosphorus (control, 3.03 ± 0.11 mmol/l (n = 9); FGF23 treated, 2.64 ± 0.02 mmol/l (n = 10), P < 0.01), with no significant changes in serum calcium (data not shown). Importantly, serum PTH was decreased after FGF23R176Q/R179Q administration when compared with FGF23core or HEPES (Figure 2A). FGF23R176Q/R179Q also led to a marked decrease in PTH mRNA levels as measured by northern blot and quantified by qRT-PCR (Figure 2B). Immunoblot
of parathyroid glands from rats given FGF23R176Q/R179Q for 5 days showed an increase in Klotho protein (Figure 2C). FGF23 acts on the Klotho-FGFR through the MAPK pathway in cells transfected with Klotho as well as in the kidney, as shown by an increased phosphorylation of ERK1/2 (5, 20). FGF23R176Q/R179Q led to an increase in phospho-ERK1/2 in the parathyroid gland (Figure 2C), indicating activation of the MAPK pathway in the FGF23-treated rats.

We then performed short-term experiments to better define the FGF23 signal transduction in the parathyroid and its effect on PTH secretion. FGF23R176Q/R179Q decreased serum PTH at 10 and 30 minutes when given i.v. (Figure 3A). FGF23R176Q/R179Q given i.p. led to a decrease in serum PTH at 40 minutes when compared with FGF23core or untreated rats (Figure 3B). Similarly, FGF23R176Q/R179Q given i.p. decreased serum PTH at 24 hours (Figure 3C). Wild-type FGF23 without the R176Q/R179Q mutation also decreased serum PTH (Figure 3C). FGF23R176Q/R179Q decreased PTH mRNA levels at 40 minutes, as shown by qRT-PCR (Figure 3D). Therefore, FGF23 decreased PTH secretion and mRNA levels in short-term experiments. To show that the MAPK pathway is required for the FGF23 effect on PTH secretion, we used the MAPK inhibitor U0126. We devised what we believe to be a novel method where FGF23 was given i.v., and the inhibitor U0126 was added topically.

Rat parathyroid glands were exposed and submerged in PBS with or without the ERK1/2 inhibitor (U0126, 1 μM), immediately followed by i.v. injection of FGF23R176Q/R179Q or HEPS carrier. Serum PTH levels were increased by U0126 at 5 and 20 minutes. FGF23 decreased serum PTH levels at 20 minutes in the absence of the inhibitor (Figure 4), as before (Figure 3A). Importantly, U0126 prevented the FGF23-induced decrease in serum PTH (Figure 4). However, PTH levels after FGF23 administration in the presence of the inhibitor U0126 did not reach the level induced by U0126 alone. These results indicate that the MAPK pathway in the parathyroid is important in maintaining a tonic suppression of PTH secretion. This may represent basal activity of the Ca²⁺ receptor (CaR), FGF23 action, and other factors that decrease serum PTH through the MAPK pathway. To separate the direct effects of the MAPK inhibitor from other humoral factors, we then performed in vitro studies.

In the absence of a parathyroid cell line, we studied the direct action of FGF23 on in vitro organ cultures of rat parathyroids. Microdissected parathyroid glands were incubated in a medium with FGF23R176Q/R179Q or FGF23core. Egr-1 mRNA levels at 10 minutes were increased in the parathyroids incubated with FGF23R176Q/R179Q (Figure 5A). FGF23R176Q/R179Q significantly decreased PTH secretion at 20, 40, and 60 minutes compared with FGF23core control (Figure 5B).
FGF23 decreases PTH secretion in vitro in isolated rat parathyroid glands. (A) Egr-1 mRNA levels. Pairs of parathyroid glands were preincubated for 1 hour and then incubated without or with FGF23\textsuperscript{R176Q/R179Q} for 10 minutes. Glands were extracted and analyzed by qRT-PCR for Egr-1 and β-actin. *P < 0.05. (B) Parathyroid glands from each rat were preincubated in medium for 60 minutes (time 0) and then transferred to medium containing either FGF23\textsuperscript{R176Q/R179Q} or FGF23\textsuperscript{R176Q/R179Q} (n = 13 rats per group). Media were sampled for PTH at the indicated time points. The accumulated PTH level of each parathyroid gland pair was measured and normalized to PTH in the medium of the glands at time 0. PTH secretion was inhibited by FGF23\textsuperscript{R176Q/R179Q} compared with FGF23\textsuperscript{R176Q/R179Q} at 20, 40, and 60 minutes. *P < 0.05. (C) As described above, pairs of parathyroid glands were incubated in control medium or in medium with U0126, FGF23\textsuperscript{R176Q/R179Q} alone, or FGF23\textsuperscript{R176Q/R179Q} with U0126 (n = 7, 6, 7, and 4 rats, respectively). Medium was sampled for PTH at 20 minutes and is presented as fold change in PTH levels compared with time 0. *P < 0.05 for the comparison with control and with the U0126 + FGF23 treatment group.

We next studied the effect of the MAPK inhibitor U0126 on PTH secretion in response to FGF23 (Figure SC). U0126 alone had no effect on PTH secretion, in contrast to the increased PTH secretion it caused when applied topically in vivo. FGF23 decreased PTH secretion as shown in Figure 5. Importantly, U0126 prevented FGF23 from causing decreased PTH secretion (Figure SC). Therefore, FGF23 acted directly on the parathyroid to activate the MAPK pathway and decrease PTH secretion both in vivo (Figure 4) and in vitro (Figure 5).

Discussion

The FGF23-Klotho pathway is crucial to life. Mice with genetic deletion of Klotho age and die prematurely and have diffuse vascular calcification due to increased levels of serum phosphate and 1,25(OH)\textsubscript{2} vitamin D (5). Their serum FGF23 levels are markedly increased. Feeding these Klotho\textsuperscript{-/-} mice with a diet deficient in vitamin D rescues most of the phenotype (21). A recent study using FGF23-null mice showed that a vitamin D-deficient diet improved survival but did not prevent vascular calcification (22). In contrast, when these FGF23-null mice were fed a phosphorus-depleted diet, both survival and vascular calcification were improved, implying that phosphorus retention was the major factor responsible for vascular calcification (22).

The receptor for FGF23 is the heterodimer of the coreceptor Klotho, with the FGFR types 1c, 3c, and 4 (5). We now show that the parathyroid is also a target organ for FGF23. We confirm that Klotho is expressed in the parathyroid (7) and show that FGFR1 and -3 are also expressed in the parathyroid. We show that the expression of Klotho is increased by FGF23. The upregulation of Klotho may be a function of feedback regulation of the FGFR by its ligand, FGF23.

Figure 5

FGF23 decreased PTH mRNA and serum PTH in both short-term and long-term experiments. In short-term experiments, the catalytically stable form of FGF23 and the wild-type FGF23 decreased serum PTH levels, establishing the physiological relevance of the action of FGF23 in decreasing PTH gene expression. Urakawa et al. (6) showed that i.v. FGF23 administration increases parathyroid Egr-1 mRNA levels at 30 minutes, implying that the MAPK pathway is either directly or indirectly involved in the effect that FGF23 has on the parathyroid. We showed that FGF23 acts directly on the parathyroid by using an in vitro organ culture system of microdissected parathyroid glands in medium, using FGF23 or FGF23\textsuperscript{R176Q/R179Q} as control. This organ culture system was used because there is no parathyroid cell line. FGF23 led to an increase in Egr-1 mRNA at 10 minutes and a decrease in secreted PTH that accumulated in the medium at 20, 40, and 60 minutes. This shows that FGF23 acts directly on the parathyroid to activate the MAPK pathway and decrease PTH secretion.

Figure 6

Endocrinological feedback loops in mineral homeostasis. There are endocrinologic feedback loops between PTH, Ca\textsuperscript{2+}, phosphorus, and 1,25(OH)\textsubscript{2} vitamin D. Phosphorus and 1,25(OH)\textsubscript{2} vitamin D levels are both trophic to FGF23, which in turn decreases phosphorus and 1,25(OH)\textsubscript{2} vitamin D levels. We now show that FGF23 decreases serum PTH and PTH gene expression by activating its cognate FGFRs in the parathyroid in a Klotho-dependent fashion.
We studied whether the MAPK system plays a role in the parathyroid in vivo and in vitro by using an ERK1/2 inhibitor. To do this we utilized what is, to our knowledge, a novel in vivo experimental method in which the thyroparathyroid glands were exposed in anesthetized rats. The ERK1/2 inhibitor U0126 was applied topically on the thyroparathyroid glands in vivo. This method maintained the glands in their physiological milieu. FGF23 was then given i.v. and serum PTH levels measured. Interestingly, the ERK1/2 inhibitor alone increased serum PTH levels, implying that MAPK tonically restrains PTH secretion. In part, this may reflect activity of the CaR in the parathyroid (23) as well as other autocrine and humoral factors that decrease PTH secretion, such as endothelin and acidic FGF (24, 25). U0126 prevented the decrease in serum PTH that had resulted from injection of FGF23 but did not restore PTH to the levels found when U0126 was given alone. This suggests that in vivo inhibition of the MAPK pathway partially prevents the decrease in serum PTH that results from FGF23. In vitro, interestingly, U0126 did not increase secreted PTH as it did in vivo. This may be because the isolated parathyroid is not exposed to the other humoral factors that are known to act on the parathyroid to decrease PTH secretion through the MAPK pathway. Therefore, the isolated parathyroid glands in vitro provide a better system to study the role of the MAPK pathway in FGF23 signaling in the parathyroid. Indeed, the effect of FGF23 was abolished by the restoration by U0126 of secreted PTH to basal levels. Together with the FGF23-induced increase in Ergr-1 mRNA levels and ERK phosphorylation, these results indicate that FGF23 acts through the MAPK pathway to decrease PTH secretion.

In the parathyroid, a high-serum Ca\(^{2+}\) leads to activation of the CaR on the parathyroid cell membrane which activates the MAPK pathway as well as PLC\(\gamma\) with the subsequent mobilization of Ca\(^{2+}\) from the endoplasmic reticulum (23, 26). The net result is a decrease in PTH gene expression, secretion and PT cell proliferation (27–29). It is tempting to speculate that the FGF23-Klotho-FGFR and the Ca\(^{2+}\)-CaR systems may act in concert through the MAPK pathway to decrease PTH gene expression and secretion. The parathyroid CaR responds to changes in serum Ca\(^{2+}\) at the levels of PTH gene expression, secretion and PT cell proliferation. In the secondary hyperparathyroidism of chronic renal failure there is down-regulation of the parathyroid CaR (30) and vitamin D receptor (31). 1,25(OH)\(_2\) vitamin D decreases PTH gene transcription together with upregulation of the vitamin D receptor (32, 33).

The finding that FGF23 decreases PTH gene expression and secretion is surprising when one considers that in renal failure there is a consistent increase in both FGF23 and serum PTH. A similar paradox in parathyroid pathophysiology occurs in chronic hypocalcemia, in which there are markedly elevated levels of serum calcitriol, which would be expected to decrease serum PTH levels. Despite the high-serum calcitriol, levels of serum PTH are increased (28). This paradox was explained in part by the finding that calreticulin prevents the binding of the VDR to the VDRE of the PTH promoter (34). The resistance of the parathyroid to elevated levels of FGF23 in uremia remains to be studied. In summary, we propose what we believe to be a new endocrinological bone-parathyroid axis in which FGF23 acts on the parathyroid. As components of endocrinological feedback loops ensuring physiological Ca\(^{2+}\) and phosphate homeostasis, FGF23, 1,25(OH)\(_2\) vitamin D, and Ca\(^{2+}\) all act to decrease PTH synthesis and secretion (Figure 6).

### Methods

**Animal experiments.** FGF23\(R^{176Q/R179Q}\) and FGF23\(_{core}\) (19) and wild-type FGF23 recombinant proteins were prepared in E. coli. The FGF23\(R^{176Q/R179Q}\) mutation is present in patients with excessive activity of FGF23 because of an aberration in the proteolytic cleavage site (13). The FGF23\(_{core}\) lacks the carboxyterminal sequence and is biologically inactive. The FGF23 compounds were injected i.p. or i.v. into Sabra rats (200 g) fed a control diet as a single dose (15 μg/rat) or i.p. daily (7.5 μg/rat/d) for 5 days. For all experiments, 4–5 rats were used in each group. Each experiment was repeated at least 3 times. Serum biochemistry for Ca\(^{2+}\), phosphorus, creatinine, and 1,25(OH)\(_2\) vitamin D were measured as previously described (35). FGF23 (Kainos Lab) (36) and PTH (ImmunoTechs) were measured in sera. Parathyroid glands were microdissected and homogenized. RNA was extracted for northern blots and real-time RT-PCR, and proteins were extracted for Western blots, as previously described (37). In some experiments thyroid, liver, kidney, duodenum, and spleen were also analyzed. All animal experiments were approved by the Hadassah Hebrew University Animal Care and Use Committee.

**In vitro organ culture experiments.** Intact microdissected parathyroid glands were studied using a modification of the method of Rodriguez et al. (38). In brief, intact microdissected parathyroid glands were preincubated for 20 minutes in individual wells on an 8-μm Nucleopore track-etch filter (Whatman catalog no. 110414) floating on 2 ml of incubation medium (1.0 mM CaCl\(_2\), 1 mM Na-pyruvate, 4 mM glutamine, 0.1 IU/ml human insulin, 0.1% bovine serum albumin) in DMEM pH 7.4, at 37°C. The glands were then transferred with the filter medium to the culture medium containing FGF23\(R^{176Q/R179Q}\) or FGF23\(_{core}\) (100 ng/ml) or control medium for the times indicated in Figures 2–4, at which time medium was sampled for PTH analysis and RNA extracted at the end of the incubations.

In vivo FGF23 and local exposure to U0126. The parathyroid glands of anesthetized rats were exposure and submerged in PBS with or without ERK1/2 inhibitor (U0126, 1 μM), immediately followed by i.v. injection of FGF23 (5 ng/rat) or HEPES carrier. Blood samples were taken at 5 and 20 minutes for PTH analysis. In some experiments, U0126 (1 μM) was added to the medium with or without FGF23.

**Protein and RNA analyses and immunohistochemistry.** Microdissected parathyroid tissue from individual rats was homogenized and extracted in TRI reagent (Molecular Research Center), yielding both RNA and proteins from the same sample. Proteins were analyzed by western blots using antibodies for Klotho (Kyowa Hakko Kogyo), phospho-ERK, ERK1/2, and α-tubulin (Sigma-Aldrich). RNA was analyzed by northern blots for PTH mRNA and 18S rRNA and by qRT-PCR for PTH, Ergr-1 mRNA, and control mRNA. Real-time PCR was conducted using ABI Prism 7900 Sequence Detection System (Applied Biosystems) and SYBR Green Mix (Applied Biosystems). Primers for rat PTH and the control genes β-actin and HPRT1, which we found to be similarly expressed across the study groups, spanned large

**Table 1**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>PTH</td>
<td>TTGCTCCTCCATCCAGAGGAT</td>
<td>TTGCCCGAGTGTACATAA</td>
</tr>
<tr>
<td>HPR1</td>
<td>CCAAGCTGTCGAAGATGA</td>
<td>CCAATACCTCGATAATGAGTAT</td>
</tr>
<tr>
<td>β-Actin</td>
<td>CAGCAATGCTGACAGGAT</td>
<td>CTCCAGGAGGCAATGATCTTGAT</td>
</tr>
<tr>
<td>EGR-1</td>
<td>TACGAGCACCCTGACACAGAT</td>
<td>GCTGGGATAACTTGTCCACC</td>
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Real-time PCR was conducted using ABI Prism 7900 Sequence Detection System (Applied Biosystems) and SYBR Green Mix (Applied Biosystems).
intrinsic areas to prevent amplification of genomic DNA. Ampiclon specificity was ascertained by melting curve analysis as well as length evaluation by ethidium bromide staining of agarose gels. The primers used for PCR are shown in Table 1. The primers were designed using Primer Express software version 2.0 (Applied Biosystems). Quantification was done by the standard-curve method.

Immunohistochemistry was performed on paraffin-embedded parathyroid tissue. Following microdissection, parathyroid glands from normal rats were placed in 4% formaldehyde. Paraffin tissue sections (4–6 μm) were deparaffinized and incubated overnight at 4°C with rat anti-Klotho antibody (Kyowa Hakko Kogyo), rabbit anti-Fgf23 antibody, or rabbit anti-Fgr3 antibody (Santa Cruz Biotechnology Inc.). DAB reagent was applied after incubation with the appropriate HRP-bound secondary antibody.

Statistics. Values are reported as mean ± SEM unless stated otherwise. One-way ANOVA was used to assess differences from the control group. For the ex vivo analysis of Egr-1 mRNA, the Mann-Whitney U test was used (SPSS 13.0, SPSS Inc.). A 2-tailed P value was considered significant when less than 0.05.