Fibroblast Growth Factor 23 Is a Counter-Regulatory Phosphaturic Hormone for Vitamin D

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The regulation of the phosphaturic factor fibroblast growth factor 23 (FGF23) is not well understood. It was found that administration of 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) to mice rapidly increased serum FGF23 concentrations from a basal level of 90.6 ± 8.1 to 213.8 ± 14.6 pg/ml at 8 h (mean ± SEM; P < 0.01) and resulted in a four-fold increase in FGF23 transcripts in bone, the predominate site of FGF23 expression. In the Hyp-mouse homologue of X-linked hypophosphatemic rickets, administration of 1,25(OH)2D3 further increased circulating FGF23 levels. In Gcm2 null mice, low 1,25(OH)2D3 levels were associated with a three-fold reduction in FGF23 levels that were increased by administration of 1,25(OH)2D3. In osteoblast cell cultures, 1,25(OH)2D3 but not calcium, phosphate, or parathyroid hormone stimulated FGF23 mRNA levels and resulted in a dose-dependent increase in FGF23 promoter activity. Overexpression of a dominant negative vitamin D receptor inhibited 1,25(OH)2D3 stimulation of FGF23 promoter activity, and mutagenesis of the FGF23 promoter identified a vitamin D–responsive element (−1180 GGAACTcagTAACCT −1156) that is responsible for the vitamin D effects. These data suggest that 1,25(OH)2D3 is an important regulator of FGF23 production by osteoblasts in bone. The physiologic role of FGF23 may be to act as a counterregulatory phosphaturic hormone to maintain phosphate homeostasis in response to vitamin D.


Materials and Methods

1,25(OH)2D3 and PTH Administration

Both Hyp mice (21) and C57BL/6j mice were purchased from Jackson Laboratory (Bar Harbor, ME). Male and female Gcm2+/− mice were mated to generate homozygous Gcm2 null mice that lacked parathyroid glands (22). All mice were maintained and used in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals, prepared by the Institute on Laboratory Animal Resources,
National Research Council (Department of Health & Human Services Publication NIH 86-23, National Academy Press, 1996) and by guidelines established by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center. Mice were fed with Harlan Teklad Rodent Diet (W) 8604 (Harlan Teklad, Madison, WI). Calcitriol (American Pharmaceutical Partners, Inc., Schaumburg, IL) was diluted in 0.9% sodium chloride for intraperitoneal injection. The same volume of 0.9% sodium chloride was injected in the control group. Serum and tissue samples were collected from the mice before injection for baseline measurements and at various time points as indicated.

**Serum Bioassays**

Serum FGF23 levels were measured using an FGF23 ELISA kit (Kairos Laboratories, Inc., Tokyo, Japan). Serum calcium and phosphate were measured, respectively, using Calcium (CPC) Liquicolor (Stabiko Laboratory, Boerne, TX) and the phosphomolybdate–ascorbic acid method as described previously (23). Serum PTH was determined using the Mouse Intact PTH ELISA Kit (Immunotopics, San Clemente, CA).

**RNA Isolation and Quantitative Reverse Transcription—PCR**

Total RNA was extracted from snap-frozen tissues and from cultured cells with Trizol (Invitrogen, Carlsbad, CA) and then treated with RNase-Free DNase using an RNaseasy column (Qiagen, Valencia, CA). First-strand cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). One microgram of total RNA was used in each 20 μl reaction. Two micrograms of cDNA were used to amplify FGF23 from mouse samples. Forward primer 5’-TTGGATGATATCATCTTGAGCTGACAG-3’ and reverse primer 5’-CTGTCGTTCTACACCATGC-3’ were used to amplify rat FGF23 from ROS17/2.8 cells. Forward primer 5’-TTCCCCAGGTTCGACTAGG-3’ and reverse primer 5’-CTGCGAGGTGACTCTCGAAG-3’ were used to amplify FGF23 from mouse samples. Forward primer 5’-GAAAGCGATCAACATGTTGGAAG-3’ and reverse primer 5’-ACAGAAGGAAATGTGGATGGCTAATGC-3’ were used to amplify mouse and rat cyclophilin A. The iCycler iQ Real-Time PCR Detection System was used for real-time PCR analysis (24).

**Isolation of the Murine FGF23 5’-Flanking Region**

A mouse BAC clone that contained the mouse FGF23 gene was purchased from Children’s Hospital Oakland Research Institute. With RP23-195E18 as a template, we amplified a 3550-bp fragment from −1 to −3550, relative to the translation start codon, by PCR with Bovine X-Act DNA polymerase (Bioline USA, Inc., Randolph, MA), using forward primer 5’-GAGGTACCATCATCTATCCAAGACACTTGCCC-3’ and reverse primer 5’-GAGGTACCATCATCTATCCAAGACACTTGCCC-3’. The PCR product was cloned into a pCR II-TOPO vector. The nucleotide sequence was confirmed by DNA sequence analysis.

**FGF23 Promoter/Reporter and Other Constructs**

Luciferase constructs were numbered according to the translation start site of the FGF23 gene. A 3550-bp FGF23 5’-flanking region from −3550 to −1 relative to the translation start site ATG was subcloned into a pGL3-Basic vector (Promega, Madison, WI) between KpnI and Hind III restriction sites (p3550Fgf23-luc) to create FGF23 promoter/luciferase reporter construct. For generation of luciferase constructs that contained different 5’ deletions of the FGF23 promoter, forward primers starting at −2000, −1300, −1000, −600, and the same reverse primer ending at −1 were used for PCR amplification of constructs (Table 1). To confirm the putative vitamin D responsive element (VDRE), we deleted the putative VDRE located in −1180 to −1166 of the FGF23 5’ flanking region using a PCR mutagenesis method. An RL-TK construct (Promega) was used as an internal control for transfection efficiency. pBK-CMV-PTHr expressing the rat PTH receptor driven by a cytomegalovirus promoter was generated as described previously (26). A dominant negative form of vitamin D receptor (VDR)-expressing construct pSG5E420A was provided by Dr. Mark R. Haussler (University of Arizona [27]).

**Mammalian Cell Culture, Transient Transfection, and Promoter/Reporter Assays**

ROS17/2.8 and UMR-106 osteoblasts were grown as described previously (28, 29) in a humidified incubator with 5% CO2 at a temperature of 37°C. Using the FuGENE 6 Transfection Reagent (Roche Applied Science, Indianapolis, IN), we transfected ROS17/2.8 osteoblasts with 1 μg of p3550Fgf23-luc and 0.01 μg of PRL-TK per well in six-well plates. To assess PTH effects, we used 0.6 μg of p3550Fgf23-luc, 0.4 μg of p3550Fgf23-luc, and 0.01 μg of PRL-TK per well in six-well plates.

**Table 1. Primers used in PCR for generation of FGF23 promoter constructs**

<table>
<thead>
<tr>
<th>Pair</th>
<th>Sense Primer/Antisense Primer</th>
<th>Location</th>
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<tbody>
<tr>
<td>1</td>
<td>5’-GCCTGTAATCATATATAACGAAGACACTTG CCC-3’</td>
<td>−600/−1</td>
</tr>
<tr>
<td>2</td>
<td>5’-CAAGCTTCTTGGCAGACACTTGAGTTGGAATGC-3’</td>
<td>−1000/−1</td>
</tr>
<tr>
<td>3</td>
<td>5’-GAGGTACCATCATCTATCCAAGACACTTGCCC-3’</td>
<td>−1300/−1</td>
</tr>
<tr>
<td>4</td>
<td>5’-TGGATGATATCATCTATCCAAGACACTTGCCC-3’</td>
<td>−2000/−1</td>
</tr>
<tr>
<td>5</td>
<td>5’-GAGGTACCATCATCTATCCAAGACACTTGCCC-3’</td>
<td>−3550/−1</td>
</tr>
</tbody>
</table>

*Underlines indicate restriction enzyme sites.*
pHK-PThr, and 0.01 μg of pRL-TK. Different concentrations of 1,25(OH)₂D₃ (Sigma-Aldrich, St. Louis, MO), PTH (1-34 fragment; Sigma-Aldrich), phosphate (potassium phosphate monobasic/sodium phosphate dibasic [pH 7.2]), and calcium chloride were added with fresh culture medium 24 h after transfection, and cells were harvested after another 24-h culture period using Passive Lysis Buffer (Promega). For the FGF23 promoter deletion study, a similar transfection method and 10⁻⁸ M 1,25(OH)₂D₃ concentration were used. The Firefly and renilla luciferase activities were measured with a Dual-Luciferase Reporter Assay System (Promega). To investigate the regulation of the endogenous promoter of the FGF23 gene, we also cultured ROS17/2.8 cells with the same concentration of 1,25(OH)₂D₃, PTH (fragment 1-34), phosphate, and calcium chloride for 24 h as in the transfection study, and then total RNA was isolated for real-time reverse transcription-PCR (RT-PCR).

Statistical Analyses
We evaluated the differences between the two groups with a t test or Wilcoxon test when the distribution was not normal. We used one-way ANOVA for multiple sample comparisons. All values are expressed as mean ± SEM. All computations were performed using the Statgraphic statistical graphics system (STSC, Inc., Rockville, MD).

Results
Effects of 1,25(OH)₂D₃ on Serum FGF23 Concentrations
To determine whether 1,25(OH)₂D₃ increases circulating FGF23 levels, we measured serum FGF23 concentrations 4, 8, and 24 h after intraperitoneal injections of 1,25(OH)₂D₃ (100 ng/kg body wt) into wild-type mice (Figure 1A). We observed that serum FGF23 levels were significantly increased at 4 h from a baseline value of 90.6 ± 8.1 to 141.0 ± 6.9 pg/ml (mean ± SEM; P < 0.01). The increment reached a peak of 213.8 ± 14.6 pg/ml at 8 h and declined to 142.7 ± 7.0 pg/ml at 24 h (Figure 1A), remaining significantly elevated above baseline (P < 0.01). Next, we examined the dose-dependent effects of 1,25(OH)₂D₃ to stimulate serum FGF23 levels (Figure 1B). Intraperitoneal administration of 1,25(OH)₂D₃ at a dose of 10 ng/kg body wt had no effect on circulating FGF23 concentrations, whereas incremental increases in serum FGF23 levels were observed 8 h after the administration of 50, 100, and 1000 ng/kg body wt, achieving a three-fold increase at the highest dose (Figure 1B). Table 2 shows the mean serum FGF23, PTH, calcium, and phosphate concentrations at 8 h after vehicle or 1,25(OH)₂D₃ treatment. The increase in FGF23 levels after 1,25(OH)₂D₃ treatment was associated with a decrease in serum PTH levels from 37.6 ± 7.6 to 19.1 ± 1.8 pg/ml (mean ± SEM; P < 0.05) at 8 h but no changes in calcium or phosphate.

To investigate whether changes in PTH might contribute to the regulation of FGF23 and the potential interrelationships among phosphate, PTH, and 1,25(OH)₂D₃, we examined Gcm2 null mice, which are characterized by hyperphosphatemia and low serum PTH and 1,25(OH)₂D₃ levels (Table 3) as a result of the failure of parathyroid gland development (20). Despite hyperphosphatemia and low serum PTH levels, we observed a three-fold decrease in serum FGF23 levels associated with a four-fold decrease of 1,25(OH)₂D₃ in Gcm2 null mice compared with age- and gender-matched wild-type controls (Table 3). To determine whether low levels of 1,25(OH)₂D₃ contributed to the decreased FGF23 levels in Gcm2 null mice, we measured serum FGF23 concentrations 8 h after intraperitoneal injections of 1,25(OH)₂D₃. We found that administration of 1,25(OH)₂D₃ to Gcm2 null mice resulted in a significant increase in circulating FGF23 levels, attaining maximal response similar to that attained in wild-type mice (Table 2).

We confirmed that Hyp mice, the murine model of XLH, have hypophosphatemia and increased FGF23 as a result of inacti-
vating mutations of the Phex gene (Table 4). To investigate whether calcitriol treatment of Hyp mice further increases FGF23 levels, we administered 1,25(OH)2D3 to Hyp mice for up to 16 d. Chronic administration of 1,25(OH)2D3 resulted in a two-fold increase in FGF23 associated with significant reductions in PTH and nonsignificant increases in serum calcium and phosphate concentrations (Table 4).

Effects of 1,25(OH)2D3 on FGF23 Transcripts in Bone and Osteoblast Cultures

To evaluate whether vitamin D stimulation of circulating FGF23 levels was associated with increased levels of FGF23 transcript in bone, we measured FGF23 message levels by real-time quantitative RT-PCR in the calvaria isolated from mice 8 h after treatment with 1,25(OH)2D3 (Figure 2 A). 1,25(OH)2D3 treatment resulted in a 3.5-fold increase in mRNA levels in calvaria (P < 0.05), consistent with the increased production of FGF23 from bone. To confirm that increased production of FGF23 from bone represents a direct effect on osteoblasts, we evaluated the effects of 1,25(OH)2D3 to stimulate endogenous FGF23 transcripts in cultured ROS17/2.8 osteoblasts by real-time PCR (Figure 2B). Treatment of ROS17/2.8 osteoblasts with 1,25(OH)2D3 (10^{-8} M) for 8 and 24 h increased FGF23 message levels in the cells.

### Table 2. Serum markers at 8 h after injection of calcitriol in wild-type and Gcm2 null mice

<table>
<thead>
<tr>
<th></th>
<th>Vehicle (n = 6)</th>
<th>Calcitriol (n = 6)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGF23 (pg/ml)</td>
<td>113.5 ± 6.2</td>
<td>213.8 ± 14.6</td>
<td>0.0000</td>
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<tr>
<td>PTH (pg/ml)</td>
<td>37.6 ± 7.6</td>
<td>19.1 ± 1.8</td>
<td>0.0413</td>
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<tr>
<td>phosphate (mg/dl)</td>
<td>10.5 ± 0.5</td>
<td>10.4 ± 0.2</td>
<td>0.8691</td>
</tr>
<tr>
<td>calcium (mg/dl)</td>
<td>8.2 ± 0.2</td>
<td>8.4 ± 0.1</td>
<td>0.6536</td>
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</tbody>
</table>

### Table 3. Serum data from Gcm2 null and wild-type mice at 12 wk of age

<table>
<thead>
<tr>
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<th>Wild-Type (n = 4)</th>
<th>Gcm2−/− (n = 4)</th>
<th>P</th>
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<tr>
<td>FGF23 (pg/ml)</td>
<td>94.8 ± 11.5</td>
<td>32.4 ± 3.3</td>
<td>0.0020</td>
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<tr>
<td>1,25(OH)2D3 (pg/ml)</td>
<td>249.8 ± 50.7</td>
<td>58.2 ± 14.9</td>
<td>0.0223</td>
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<tr>
<td>Phosphate (mg/dl)</td>
<td>7.7 ± 0.9</td>
<td>12.3 ± 0.6</td>
<td>0.0042</td>
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<tr>
<td>Calcium (mg/dl)</td>
<td>8.2 ± 0.1</td>
<td>5.1 ± 0.4</td>
<td>0.0007</td>
</tr>
<tr>
<td>PTH (pg/ml)</td>
<td>46.5 ± 8.8</td>
<td>15.6 ± 1.1</td>
<td>0.0131</td>
</tr>
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</table>

### Table 4. Effects of calcitriol on serum markers in Hyp mice

<table>
<thead>
<tr>
<th></th>
<th>Vehicle (n = 6)</th>
<th>Calcitriol (n = 6)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGF23 (pg/ml)</td>
<td>2065 ± 465</td>
<td>4555 ± 415b</td>
<td>0.0025</td>
</tr>
<tr>
<td>PTH (pg/ml)</td>
<td>32.0 ± 13.0</td>
<td>2.6 ± 1.1b</td>
<td>0.0490</td>
</tr>
<tr>
<td>phosphate (mg/dl)</td>
<td>5.3 ± 0.3</td>
<td>6.0 ± 0.4</td>
<td>0.8691</td>
</tr>
<tr>
<td>calcium (mg/dl)</td>
<td>8.0 ± 0.3</td>
<td>8.4 ± 0.2</td>
<td>0.8368</td>
</tr>
</tbody>
</table>

aData shown are serum markers from age- and gender-matched Hyp mice at 8 to 12 wk of age, which were treated by intraperitoneal injection of vehicle or calcitriol (100 ng/kg body wt) daily for 16 d. Data are expressed as mean ± SEM. bSignificant differences at P < 0.05 by t test analysis.
levels approximately eight- and 100-fold, respectively. DNA sequence analysis confirmed that the PCR product was 100% homologous to rat FGF23 mRNA (data not shown).

Cloning of the Mouse FGF23 Promoter and its 5' Untranslated Region

To investigate further the mechanism whereby 1,25(OH)2D3 increases the FGF23 message, we cloned and sequenced the 3550-bp 5' flanking region of the FGF23 gene (Figure 3A). Using a promoter prediction program (Neural Network Promoter Prediction, The Berkeley Drosophila Genome Project, Berkeley, CA), we located two potential transcription start sites at −98 and −108 bp, respectively, upstream of the ATG. The upstream promoter contains a TATA-box. In addition, we found that the FGF23 promoter region is highly conserved between mouse, rat, and human. The mouse FGF23 promoter is 79% homologous to the rat FGF23 promoter over the proximate 2300 bp and is 67% homologous to the human promoter over the proximate 800 bp (data not shown). Predicted transcriptional factor binding sites that are conserved between mouse, rat, and human over the initial 2000 bp of their respective promoter regions are shown in Figure 3A.

We subcloned the region from −3550 to −1 relative to translation starting site ATG into pGL3-Basic to create a promoter/reporter construct (p3550Fgf23-luc) and examined the activity of this promoter/reporter construct in ROS17/2.8 osteoblasts. The relative luciferase activity of p3550Fgf23-luc was approximately six-fold greater than the empty vector in ROS17/2.8 cells (Figure 3B), indicating that the 5' flanking region from −3550 to −1 has promoter activity.

Figure 2. Analysis of FGF23 mRNA expression in bone and ROS17/2.8 cells after treatment with 1,25-dihydroxyvitamin D3 (1,25(OH)2D3). (A) Female C57BL/6J mice were treated with vehicle or calcitriol at 100 ng/kg body wt administered by intraperitoneal injection, and expression in calvaria was assessed by real-time PCR, as described in Materials and Methods. Calcitriol administration increased FGF23 transcripts by approximately 3.5-fold in calvaria after 8 h. (B) ROS17/2.8 cells were stimulated with 1,25(OH)2D3 at concentrations of 10−8 M, and FGF23 transcripts were quantified by real-time PCR at 8 and 24 h. The FGF23 transcript was increased eight-fold at 8 h and 100-fold at 24 h in ROS17/2.8 cells stimulated with 1,25(OH)2D3. (C) ROS17/2.8 cells were stimulated with phosphate (5 mM), calcium (5 mM), or parathyroid hormone (PTH; 10 nM). The FGF23 expressions were measured by real-time PCR 24 h after the addition of phosphate, calcium, or PTH. Phosphate, calcium, and PTH did not stimulate FGF23 transcript in ROS17/2.8 osteoblasts.
Figure 3. Isolation of the FGF23 promoter and its cell-type specificity of the FGF23 promoter activities response to 1,25(OH)2D3.

(A) Nucleotide sequence of the 5’ flanking region of the FGF23 gene. We cloned and sequenced the 3550-bp 5’ flanking region of the FGF23 gene. Shown is the sequence of the 3550-bp 5’ flanking region and partial exon 1. The predicted transcription start sites are shown by △. We identified putative binding sites for GATA-binding factor (GATA), positive regulatory domain I binding factor (PRDF), RAR-related orphan receptor 1 (RORA), ETS1 factors (ETSF), Brn POU domain factor (BRNF), hepatic nuclear factor 4 (HNF4), myelin transcription factor 1 (MYT1) zinc finger protein, GC-Box factors SP1/GC (SP1F), cAMP-responsive element binding protein (CREB), CCAAT/enhancer binding protein (CEBP), ecotropic viral integration site 1–myeloid transforming protein (EVI1), CAS interacting zinc finger protein (CIZF), nuclear factor of activated T cells (NFAT), glioma-associated oncogene homolog (GLI)-Krueppel–related transcription factor (E4FF), gut-enriched Krueppel-like binding factor (GKLF), TATA-binding protein factor (TBPF), enhancer CCAAT binding factors (ECAT), and promoter CCAAT binding factors (PCAT). VDRE, vitamin D responsive element. The primers used to generate promoter/reporter constructs are underlined, and the putative transcriptional factor binding sites conserved between mouse, rat, and human are double underlined. (B) Promoter activity of the 3550-bp 5’ flanking region of FGF23. The promoter/reporter construct p3550Fgf23-luc consists of the sequence from +3550 to −1 relative to translation starting site ATG, which was subcloned into pGL3-Basic vector. The relative luciferase activity of p3550Fgf23-luc is approximately six-fold greater than the empty vector in ROS17/2.8 cells, indicating that the 5’ flanking region from −3550 to −1 has promoter activity in ROS17/2.8 in vitro. *Values that are significantly different from empty vector (P < 0.05).
Effects of 1,25(OH)₂D₃, PTH, Calcium, and Phosphate on FGF23 Promoter Activity

Next, we evaluated the effects of 1,25(OH)₂D₃ (10⁻¹⁰ to 10⁻⁸ M), PTH (1-34; 1 to 100 nM), phosphate (1 to 4 mM), and calcium (1 to 5 mM) on p3550Fgf23-luc activity in ROS17/28 osteoblasts (Figure 4). Compared with vehicle treatments, 1,25(OH)₂D₃ resulted in a dose-dependent stimulation of luciferase activity in ROS17/2.8 cells transfected with p3550Fgf23-luc. The maximal increase was approximately two-fold at 10⁻⁸ M 1,25(OH)₂D₃ (Figure 4A). In contrast, we observed a small (35%) but significant inhibition of FGF23 promoter activity by PTH (Figure 4B). The addition of neither phosphate (1 to 4 mM) nor calcium (1 to 5 mM) to the media affected FGF23 promoter activity in ROS17/2.8 osteoblasts (Figure 4, C and D). To exclude the possibility that the 3500-bp FGF23 promoter lacks necessary cis-acting elements, we examined the effects of PTH (10 nM), phosphate (5 mM), or calcium (5 mM) on endogenous FGF23 expression in ROS17/2.8 osteoblasts by quantitative RT-PCR. In contrast to the stimulation of endogenous FGF23 expression by 1,25(OH)₂D₃ (Figure 2B), we did not observe any stimulation of FGF23 message levels by PTH, phosphate, or calcium (Figure 2C). To determine whether this lack of calcium and phosphate stimulation was a unique feature of ROS17/2.8 osteoblasts, we examined the effects of 1,25(OH)₂D₃ (10⁻⁸ M), phosphate (5 mM), and calcium (5 mM) to stimulate FGF23 expression in UMR-106 osteoblasts. 1,25(OH)₂D₃ but not phosphate or calcium increased FGF23 transcripts by real-time PCR in UMR-106 osteoblasts (data not shown).

Mapping the 1,25(OH)₂D₃ Response Region

Finally, to identify the vitamin D responsive region of the FGF23 promoter, we compared the function of the full-length promoter (p3550Fgf23-luc) with successive 5’ deletion mutants (p2000Fgf23-luc, p1300Fgf23-luc, p1000Fgf23-luc, and p600Fgf23-luc) transfected into ROS17/2.8 osteoblasts. Promoter activity was lower in the p3550Fgf23-luc and p2000Fgf23-luc constructs compared with the more truncated constructs, consistent with the presence of a suppressor region in the distal FGF23 promoter (Figure 5A). 1,25(OH)₂D₃ stimulated the p3550Fgf23-luc, p2000Fgf23-luc, and p1300Fgf23-luc but did not stimulate the promoter activity of the p1000Fgf23-luc or p600Fgf23-luc constructs transfected into ROS17/2.8 cells, indicating localization of the VDRE in the region between −1300 and −1000 bp (Figure 5A).

Figure 4. Effect of 1,25(OH)₂D₃, PTH, phosphate, and calcium on FGF23 promoter activities. ROS17/2.8 osteoblasts were transiently transfected with p3550Fgf23-luc and the pRL-TK construct, which expresses renilla luciferase as an internal control for transfection efficiency. Twenty-four hours after transfection, cells were treated with 10⁻¹⁰ to 10⁻⁸ M 1,25(OH)₂D₃ (A), 1 to 100 mM PTH (1-34; B), 1 to 4 mM phosphate (C), 1 to 5 mM calcium chloride (D), or vehicle as control for 24 h. Data represent relative luciferase activity expressed as the mean ± SEM of at least three independent transfection experiments. *Values that are significantly different from vehicle (P < 0.05).
activities and their response to 1,25(OH)2D3 were assayed as transfected with wild-type and deletion constructs. Promoter analysis of VDRE in p2000Fgf23-luc. ROS17/2.8 cells were each group. *
P
To confirm the vitamin D responsive region, we created serial deletions constructs using p2000Fgf23-luc as a template to generate constructs with different lengths of deletions between −1393 and −1000 using a restriction digestion and PCR methods. Analysis of these constructs further localized the vitamin D responsive region to between −1240 and −1161 (data not shown). Alignment of the mouse and rat sequences that corresponded to this region revealed 83% identity and the presence of a conserved VDRE (−1180 GGAACAgTAACCT −1156). Next, we deleted this 15-bp region from the p2000Fgf23-luc construct using PCR mutagenesis methods to create pDelFgf23-luc. 1,25(OH)2D3 failed to stimulate luciferase activity in ROS17/2.8 cells transfected with pDelFgf23-luc (Figure 5B). Finally, to establish a role for the VDR, we assessed the response to 1,25(OH)2D3 in ROS17/2.8 cells co-transfected p3500Fgf23-luc and pSG5A420E, a dominant negative human VDR expression construct. Overexpression of the human VDR A420E mutant significantly inhibited 1,25(OH)2D3-stimulated FGF23 promoter activity in ROS17/2.8 (Figure 5C).

**Discussion**

This study supports a physiologic role for 1,25(OH)2D3 in the regulation of FGF23 production by osteoblasts in bone. We demonstrate by in vivo administration of calcitriol (Figure 1) and by assessing the effects of direct exposure of osteoblast cultures to 1,25(OH)2D3 (Figure 2B) that 1,25(OH)2D3 stimulates the production of FGF23 in bone and osteoblasts. Additional studies indicate that 1,25(OH)2D3 results in an increase in FGF23 transcripts in calvaria and cultured osteoblasts (Figure 2), as well as activation of a 3500-bp FGF23 promoter-reporter construct transfected into ROS17/2.8 osteoblasts (Figures 4 and 5A).

We identified a VDRE located between −1180 and −1161 that is required for vitamin D effects on the FGF23 promoter/reporter construct in ROS17/2.8 cells (Figure 5B). In addition, expression of a dominant negative VDR inhibited 1,25(OH)2D3 stimulation of FGF23 promoter activity (Figure 5C), indicating that FGF23 is regulated directly by 1,25(OH)2D3 in osteoblasts. It is interesting that 1,25(OH)2D3 also is reported to suppress PHEX mRNA levels in osteoblasts and bone (30,31) and reductions in PHEX can lead to increased FGF23 expression in osteoblasts (32,33). Therefore, there seem to be both direct and indirect mechanisms for 1,25(OH)2D3 to regulate FGF23 production by osteoblasts.

1,25(OH)2D3 regulation of FGF23 production has been reported previously (34–37). In this regard, VDR null mice have low circulating FGF23 levels (35), and 1,25(OH)2D3 administration stimulates FGF23 levels in mice independent of PTH (35,38). Also, 1,25(OH)2D3 has been shown to upregulate FGF23 message expression in UMR-106 osteoblasts (39) and FGF23 promoter activity in K562 erythroleukemia cells (36). These studies more completely characterize the mechanism of vitamin D–stimulated FGF23 production and identified osteoblasts and bone as the principle target for vitamin D effects on this phosphaturic hormone. Moreover, our preliminary findings lead us first to propose that the physiologic role of FGF23 is to act as a counterregulatory hormone for 1,25(OH)2D3 (40).

1,25(OH)2D3 stimulation of FGF23 production by bone and osteoblasts may be important for several reasons. First, FGF23 provides a mechanism to maintain systemic phosphate homeostasis in the setting of 1,25(OH)2D3 inhibition of osteoblast-mediated mineralization of bone (31). The overall physiologic
effect would be to increase renal phosphate clearance under circumstances in which phosphate was not needed for mineralization of extracellular matrix. Second, 1,25(OH)₂D₃ stimulation of FGF23 production by osteoblasts in bone provides a mechanism to maintain phosphate homeostasis in the setting of suppressed PTH secretion (41). In the presence of 1,25(OH)₂D₃ stimulation of calcium and phosphate absorption by the gastrointestinal tract (42), increments in calcium (as well as 1,25(OH)₂D₃ itself) act on the parathyroid gland to suppress PTH, thereby limiting the ability of the kidney to excrete the increased phosphate absorbed from the gastrointestinal tract. 1,25(OH)₂D₃ stimulation of the production of FGF23 by bone and the resulting increase in renal phosphate excretion maintain phosphate homeostasis in the setting of suppressed PTH (Figure 6).

Gcm2 null mice, which have low circulating PTH along with reduced 1,25(OH)₂D₃ levels, hypocalcemia, and hyperphosphatemia (Table 3) as a result of the failure of the parathyroid gland development, also had low serum FGF23 levels (Table 3). That the administration of calcitriol raised serum FGF23 levels to values identical to those in wild-type mice stimulated with calcitriol, and the inability to demonstrate direct stimulation of the FGF23 promoter (Figure 4) or endogenous FGF23 transcription (Figure 2C) in ROS1/7/2.8 cells by PTH, suggest that the low levels of FGF23 are due to decreased 1,25(OH)₂D₃ in Gcm2-deficient mice.

Surprisingly, hyperphosphatemia in Gcm2 null mice did not result in increased circulating FGF23 concentrations (Tables 2 and 3), and the addition of phosphate to osteoblast cultures failed to increase FGF23 transcripts (Figure 4). These observations do not mean that phosphate is not an important regulator of FGF23, because several studies indicate that phosphate administration and restriction, respectively, increase and decrease circulating FGF23 levels in both mice and humans (34,43–45). Also, high phosphate in medium that contains 1,25(OH)₂D₃ has been reported to stimulate FGF23 promoter activity in erythroblast leukemia cells (36). That hyperphosphatemia per se is not associated with increased FGF23 levels, however, suggests possible complex mechanisms whereby serum phosphate regulates FGF23 production or vice versa. Indeed, other factors in Gcm2 null mice, such as decreased PTH, calcium, and 1,25(OH)₂D₃ levels (46), may have prevented phosphate-mediated regulation of FGF23 production, or, conversely, the low FGF23 and PTH levels in Gcm2 null mice may be responsible for the high serum phosphate.

Finally, our findings that calcitriol further increases FGF23 production in Hyp mice raises possible concerns about the use of active vitamin D analogues to treat hypophosphatemia in patients with XLH (Table 4). By further increasing FGF23 levels, such treatment would offset the effects of vitamin D to increase serum phosphate levels. On the basis of these observations, it would be interesting to determine whether treatment with active vitamin D analogues causes similar increments in FGF23 levels in patients with XLH and explains the variation in FGF23 levels that is observed in these patients (7).

Conclusion
1,25(OH)₂D₃ is an important regulator of FGF23 production by osteoblasts through a VDRE in the FGF23 promoter. We propose that FGF23 may provide a means to maintain renal phosphate excretion in the setting of 1,25(OH)₂D₃-mediated stimulation of gastrointestinal absorption of phosphate and suppression of the phosphaturic hormone PTH.

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References

![Figure 6](image-url)


19. Pi M, Spurney RF, Tu Q, Hinson T, Quarles LD: Calcium-sensing receptor activation of rho involves filamin and rho-guanine nucleotide exchange factor. Endocrinology 143: 3830–3838, 2002


Phosphate transport and is a PHEX substrate. Biochem Biophys Res Commun 284: 977–981, 2001


