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

Shiguang Liu, Wen Tang, Jianping Zhou, Jason R. Stubbs, Qiang Luo, Min Pi, and L. Darryl Quarles

Department of Internal Medicine and the Kidney Institute, University of Kansas Medical Center, Kansas City, Kansas

The regulation of the phosphaturic factor fibroblast growth factor 23 (FGF23) is not well understood. It was found that administration of 1,25-dihydroxyvitamin D_3 ($1,25[OH]_2D_3$) 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 $1,25(OH)_2D_3$ further increased circulating FGF23 levels. In *Gcm2* null mice, low $1,25(OH)_2D_3$ levels were associated with a three-fold reduction in FGF23 levels that were increased by administration of $1,25(OH)_2D_3$. In osteoblast cell cultures, $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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.

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Fibroblast growth factor 23 (FGF23) is a recently discovered phosphaturic hormone (1–5). Impaired degradation of FGF23, as a result of mutations in a proprotein convertase cleavage site, accounts for the hypophosphatemia and impaired 1,25-dihydroxyvitamin D_3 ($1,25[OH]_2D_3$) production in autosomal dominant hypophosphatemic rickets (1). Increased production of FGF23 is the cause of phosphaturia and disordered vitamin D metabolism in tumor-induced osteomalacia (6,7), X-linked hypophosphatemic rickets (XLH) (7,8), and McCune-Albright syndrome (9). In addition, transgenic mice that overexpress a cleavage-resistant mutant form of FGF23 have hypophosphatemia (10). Conversely, decrements in FGF23 function, as a result of either loss-of-function mutations of FGF23 in hereditary forms of tumoral calcinosis (11,12) or FGF23 deficiency produced by the deletion of FGF23 in mice (4,13), cause hyperphosphatemia.

FGF23 also suppresses 1α hydroxylase activity in the proximal renal tubule, leading to reduced circulating levels of $1,25(OH)_2D_3$ (2,10,14,15). The significance of FGF23 regulation of $1,25(OH)_2D_3$ production is not clear, but the findings that FGF23 is produced predominantly by osteoblasts in bone and that FGF23 regulates phosphate reabsorption and $1,25(OH)_2D_3$ production by the kidney raise the possibility that FGF23 may be involved in a bone–kidney axis that controls phosphate and vitamin D homeostasis (16,17). How FGF23 is integrated with the vitamin D–parathyroid hormone (PTH) axis, which plays a central role in calcium homeostasis, skeletal development, and mineralization (18), however, is not clear. Understanding the effects of $1,25(OH)_2D_3$ on FGF23 production is important, because vitamin D therapy often is used to treat FGF23-mediated hypophosphatemic disorders, such as XLH (19).

In an effort to understand more fully the regulation of FGF23 expression in osteoblasts and bone, we assessed the effect of $1,25(OH)_2D_3$ administration on circulating levels of FGF23 in wild-type *Gcm2* null (20) and *Hyp* mice (21), as well as the effects of $1,25(OH)_2D_3$ on the FGF23 transcripts in bone. In addition, we investigated the ability of $1,25(OH)_2D_3$ to regulate endogenous FGF23 transcripts and the activity of a transfected murine FGF23 promoter luciferase reporter in osteoblasts. Our findings demonstrate the importance of bone as a target for vitamin D–mediated increments in FGF23 production and suggest that FGF23 production serves as a counterregulatory hormone to enhance renal phosphate clearance in response to vitamin D–mediated increments in gastrointestinal phosphate absorption and decrements in the phosphaturic hormone PTH.

Materials and Methods

$1,25(OH)_2D_3$ 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 *Gcm*2 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,

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Address correspondence to: Dr. Shiguang Liu, Department of Internal Medicine and the Kidney Institute, University of Kansas Medical Center, 3901 Rainbow Boulevard, Room 6020 WHE, MS 3018, Kansas City, KS 66160. Phone: 913-588-0705; Fax: 913-588-9251; E-mail: sliu@kumc.edu

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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 (Kainos Laboratories, Inc., Tokyo, Japan). Serum calcium and phosphate were measured, respectively, using Calcium (CPC) Liquicolor (Stanbio 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 RNeasy 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 of reverse transcription reaction. A total of 400 ng and 20 ng of input RNA were used to amplify FGF23 and cyclophilin A, respectively. The forward primer 5′-TTGGATCGTATCACTTCAGC-3′ and reverse primer 5′-TGCTTCGGTGACAGGTAG-3′ were used to amplify rat FGF23 from ROS17/2.8 cells. Forward primer 5′-TTTCCCAGGTTCGTCTAGG-3′ and reverse primer 5′-CTCGCAGGTGACTCTCAG-3′ were used to amplify FGF23 from mouse samples. Forward primer 5′-GAAGGCATGAACATTGTGGAAG-3′ and reverse primer 5′-ACAGAAGGAATGGTTTGATGGG-3′ were used to amplify mouse and rat cyclophilin A. The iCycler iQ Real-Time PCR Detection System and iQ SYBR Green Supermix (Bio-Rad Laboratories) were 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 BIO-X-ACT DNA polymerase (Bioline USA, Inc., Randolph, MA), using forward primer 5′-GAGGTACCTCATCTATGGAGTAGACTC-3′ and reverse primer 5′-GCAAGCTTTGCACAGCACTGAGTGGCTAATGC-3′. The PCR product was cloned into a pCR II-TOPO vector. The nucleotide sequence was confirmed by DNA sequence analysis.

The putative promoter and transcriptional start site were predicted by Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html), and transcriptional factor binding sites were analyzed by Matinspector and DiAlign TF from Genomatix (http://www.genomatix.de/company/index.html). The nuclear hormone receptor response elements were screened by nuclear hormone receptor–scan (25).

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/firefly 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 mutant 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% CO_2 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

Table 1. Primers used in PCR for generation of FGF23 promoter constructs[a]

Pair	Sense Primer/Antisense Primer	Location
1	5′-GCGGTACCTATGACCATATATCAAGACACTTGCC-3′ 5′-GCAAGCTTTGCACAGCACTGAGTGGCTAATGC-3′	−600/−1
2	5′-TGGTACCGCATGCACAGAGCAATTTCTGC-3′ 5′-GCAAGCTTTGCACAGCACTGAGTGGCTAATGC-3′	−1000/−1
3	5′-GGGTACCATCCATGTGCTTTACACGATGG-3′ 5′-GCAAGCTTTGCACAGCACTGAGTGGCTAATGC-3′	−1300/−1
4	5′-TGGTACCTAGCTGTGGCTATGATTCATCC-3′ 5′-GCAAGCTTTGCACAGCACTGAGTGGCTAATGC-3′	−2000/−1
5	5′-GAGGTACCTCATCTATGGAGTAGACTCC-3′ 5′-GCAAGCTTTGCACAGCACTGAGTGGCTAATGC-3′	−3550/−1

[a]Underlines indicate restriction enzyme sites.

pBK-PTHr, and 0.01 μg of pRL-TK. Different concentrations of $1,25(OH)_2$ D_3 (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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$, 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 $1,25(OH)_2D_3$ on Serum FGF23 Concentrations

To determine whether $1,25(OH)_2D_3$ increases circulating FGF23 levels, we measured serum FGF23 concentrations 4, 8, and 24 h after intraperitoneal injections of $1,25(OH)_2D_3$ (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 $1,25(OH)_2D_3$ to stimulate serum FGF23 levels (Figure 1B). Intraperitoneal administration of $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ treatment. The increase in FGF23 levels after $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$, we examined *Gcm2* null mice, which are characterized by hyperphosphatemia and low serum PTH and $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ in *Gcm2* null mice compared with age- and gender-matched wild-type controls (Table 3). To determine whether low levels of $1,25(OH)_2D_3$ contributed to the decreased FGF23 levels in *Gcm2* null mice, we measured serum FGF23 concentrations 8 h after intraperitoneal injections of $1,25(OH)_2D_3$. We found that administration of $1,25(OH)_2D_3$ 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-

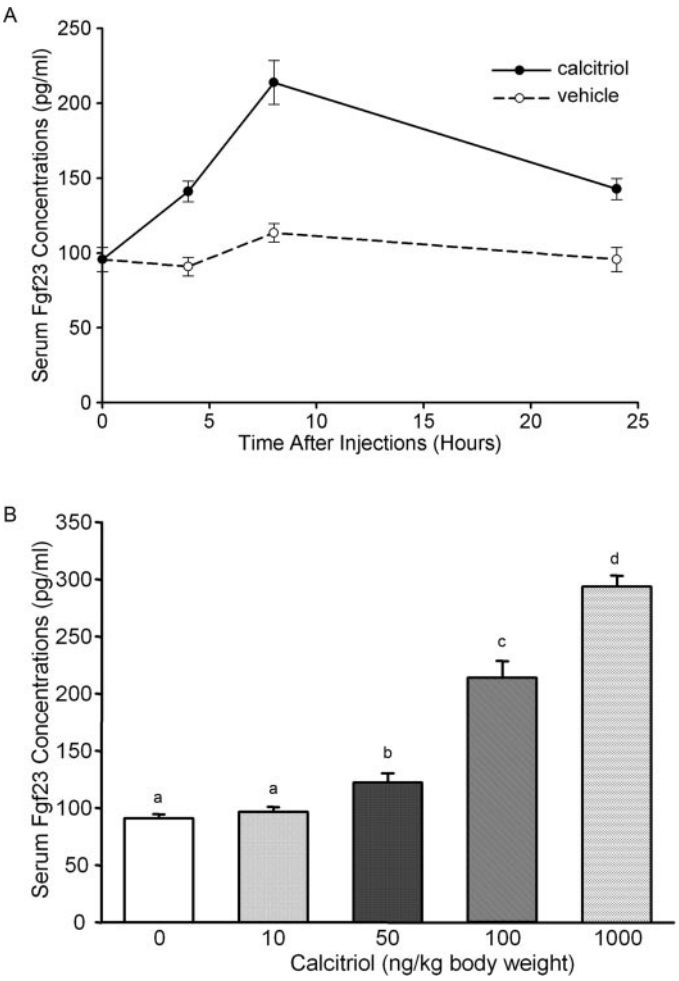

Figure 1. Time- and dose-dependent effects of calcitriol on serum fibroblast growth factor 23 (FGF23) levels in mice. (A) Female C57BL/6J mice received intraperitoneal injections of equal volumes of vehicle or calcitriol (100 ng/kg body wt). Serum samples were collected at 4, 8, or 24 h after injection for FGF23 measurements. We also collected serum from untreated age-matched mice as a baseline. Serum FGF23 concentrations after calcitriol administration are significantly different from vehicle at all time points ($P < 0.05$). (B) Serum FGF23 was measured in C57BL/6J mice 8 h after intraperitoneal administration of calcitriol at doses from 10 to 1000 ng/kg body wt. Values from different doses that share the same superscript letters are not significantly different at $P < 0.05$. Serum FGF23 was measured using an ELISA kit as described in Materials and Methods. FGF23 levels are expressed as mean ± SEM ($n = 6$ in each group).

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

	Vehicle ($n = 6$)	Calcitriol ($n = 6$)	P
Wild-type			
FGF23 (pg/ml)	113.5 ± 6.2	213.8 ± 14.6	0.0000
PTH (pg/ml)	37.6 ± 7.6	19.1 ± 1.8	0.0413
phosphate (mg/dl)	10.5 ± 0.5	10.4 ± 0.2	0.8691
calcium (mg/dl)	8.2 ± 0.2	8.4 ± 0.1	0.6536
Gcm2 null			
FGF23 (pg/ml)	40.8 ± 6.1	212.4 ± 52.0	0.0083
PTH (pg/ml)	13.7 ± 0.5	14.8 ± 0.9	0.2744
phosphate (mg/dl)	13.0 ± 0.6	14.9 ± 1.1	0.1631
calcium (mg/dl)	4.6 ± 0.4	5.6 ± 0.4	0.1051

[a]Data shown are serum markers from 8-wk-old C57BL/6 mice and 8- to 12-wk-old *Gcm2* null mice, which received intraperitoneal injections of vehicle or 100 ng/kg body wt calcitriol. Data were collected at 8 h after injection of vehicle or calcitriol. Data are expressed as mean ± SEM. The statistically significant difference was set at $P < 0.05$ by t test analysis. FGF23, fibroblast growth factor 23; PTH, parathyroid hormone.

Table 3. Serum data from *Gcm2* null and wild-type mice at 12 wk of age[a]

	Wild-Type ($n = 4$)	*Gcm2*−/− ($n = 4$)	P
FGF23 (pg/ml)	94.8 ± 11.5	32.4 ± 3.3	0.0020
$1,25(OH)_2D_3$ (pg/ml)	249.8 ± 50.7	58.2 ± 14.9	0.0223
Phosphate (mg/dl)	7.7 ± 0.9	12.3 ± 0.6	0.0042
Calcium (mg/dl)	8.2 ± 0.1	5.1 ± 0.4	0.0007
PTH (pg/ml)	46.5 ± 8.8	15.6 ± 1.1	0.0131

[a]Data shown are serum marker from 12-wk-old mice. Data are expressed as mean ± SEM. The statistically significant difference was set at $P < 0.05$ by t test analysis. $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3.

vating mutations of the Phex gene (Table 4). To investigate whether calcitriol treatment of *Hyp* mice further increases FGF23 levels, we administered $1,25(OH)_2D_3$ to *Hyp* mice for up to 16 d. Chronic administration of $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ (Figure 2A). $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ to stimulate endogenous FGF23 transcripts in cultured ROS17/2.8 osteoblasts by real-time PCR (Figure 2B). Treatment of ROS17/2.8 osteoblasts with $1,25(OH)_2D_3$ (10^{-8} M) for 8 and 24 h increased FGF23 message

Table 4. Effects of calcitriol on serum markers in *Hyp* mice[a]

	Vehicle ($n = 6$)	Calcitriol ($n = 6$)	P
FGF23 (pg/ml)	2065 ± 465	4555 ± 418[b]	0.0025
PTH (pg/ml)	32.0 ± 13.0	2.6 ± 1.1[b]	0.0490
Phosphate (mg/dl)	5.3 ± 0.3	6.0 ± 0.4	0.8691
Calcium (mg/dl)	8.0 ± 0.3	8.4 ± 0.2	0.8368

[a]Data 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.
[b]Significant differences at $P < 0.05$ by t test analysis.

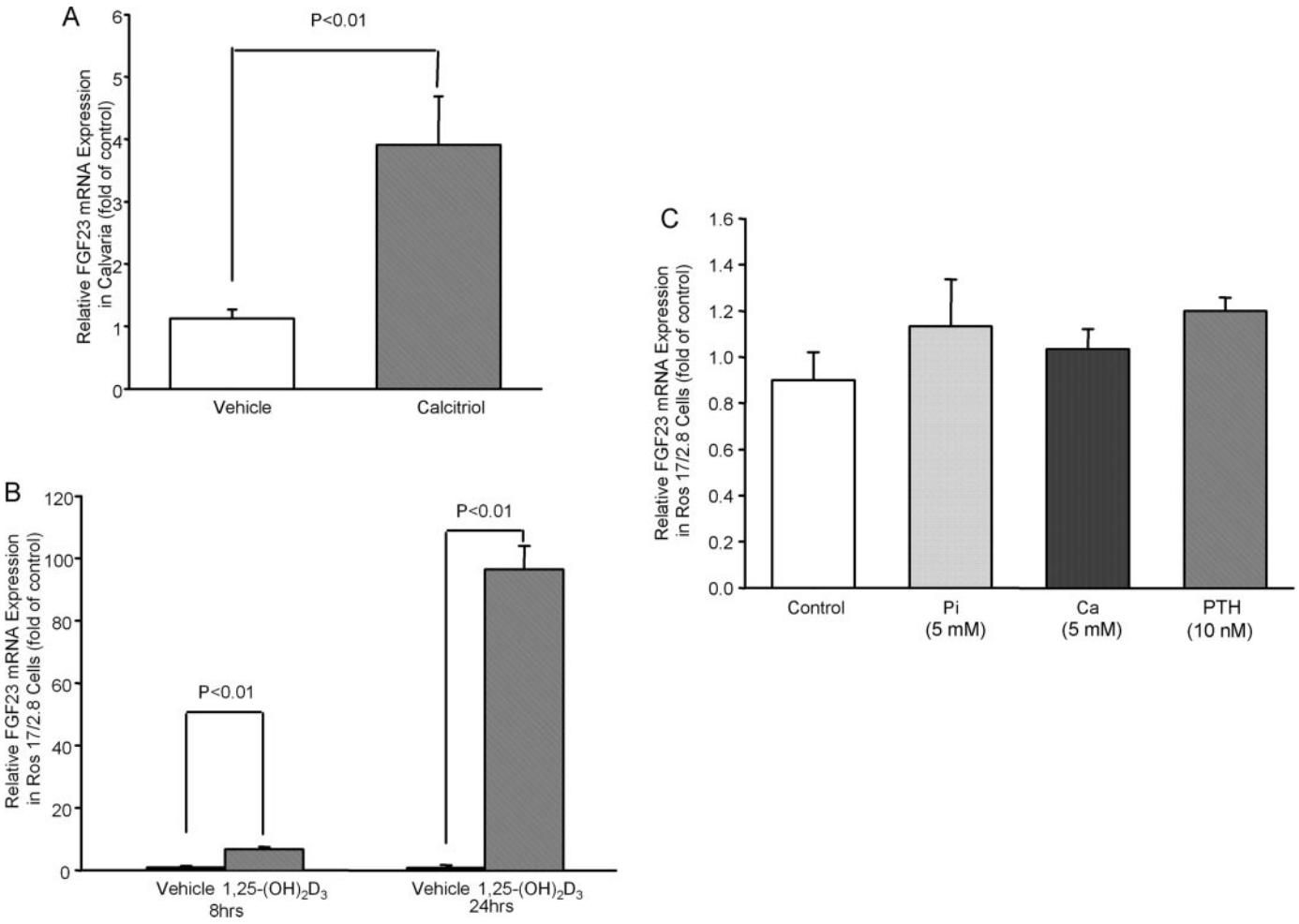

Figure 2. Analysis of FGF23 mRNA expression in bone and ROS17/2.8 cells after treatment with 1,25-dihydroxyvitamin D_3 (1,25[OH]$_2$D$_3$). (A) Female C57BL/6J mice were treated with vehicle or calcitriol at 100 ng/kg body wt administered by intraperitoneal injection, and expression in calvaria was assessed 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) $_2$D$_3$ 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) $_2$D$_3$. (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.

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)$_2$D$_3$ 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.

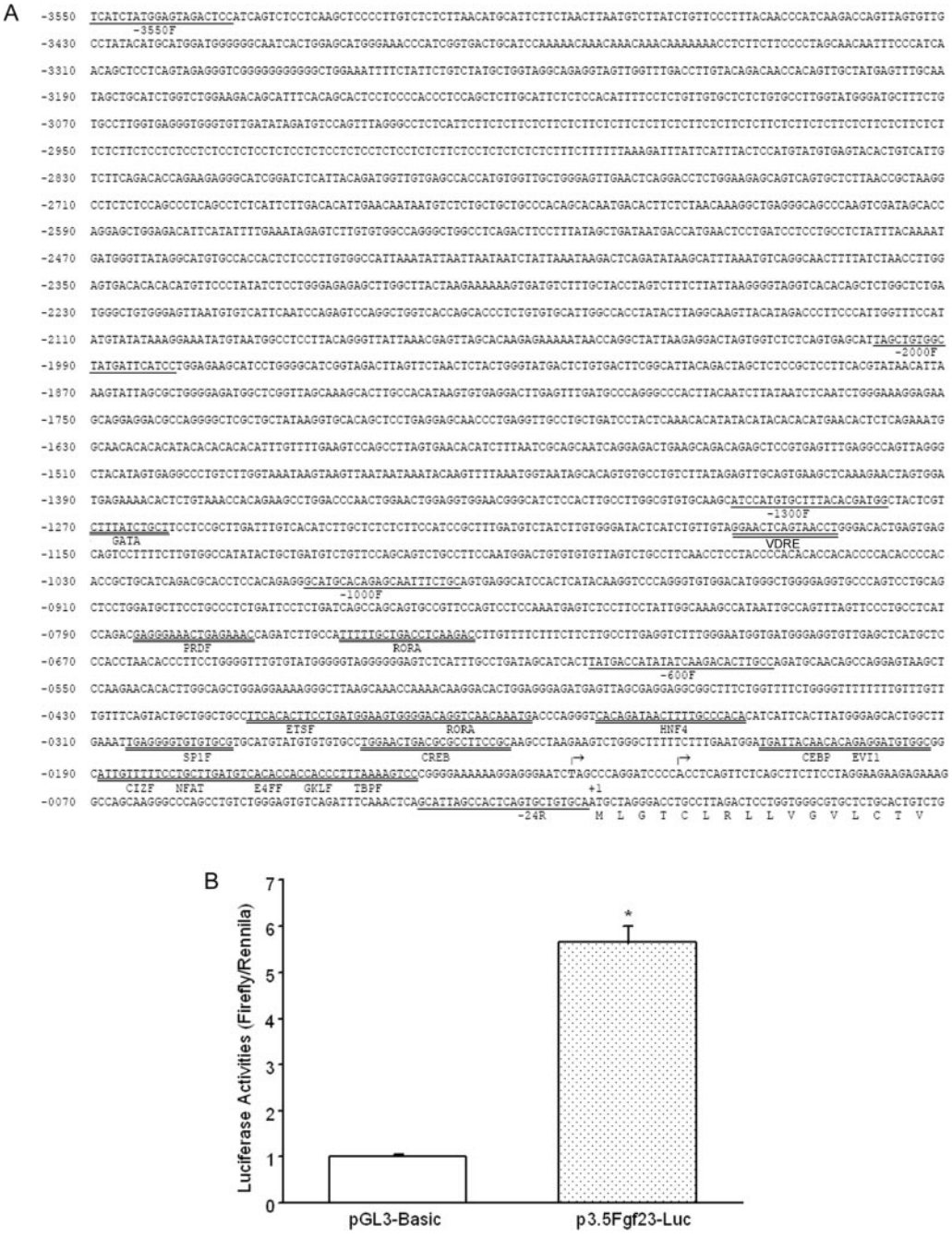

Figure 3. Isolation of the FGF23 promoter and its cell-type specificity of the FGF23 promoter activities response to $1,25(OH)_2D_3$. (A) Nucleotide sequence of the 5′ flanking region of the FGF23 gene. We cloned and sequenced the 3550-bp 5′ flanking region of the FGF23 gene. Shown is the sequence of 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)$_2$D$_3$, PTH, Calcium, and Phosphate on FGF23 Promoter Activity

Next, we evaluated the effects of 1,25(OH)$_2$D$_3$ (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)$_2$D$_3$ 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)$_2$D$_3$ (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)$_2$D$_3$ (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)$_2$D$_3$ (10^{-8} M), phosphate (5 mM), and calcium (5 mM) to stimulate FGF23 expression in UMR-106 osteoblasts. 1,25(OH)$_2$D$_3$ but not phosphate or calcium increased FGF23 transcripts by real-time PCR in UMR-106 osteoblasts (data not shown).

Mapping the 1,25(OH)$_2$D$_3$, 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)$_2$D$_3$ 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).

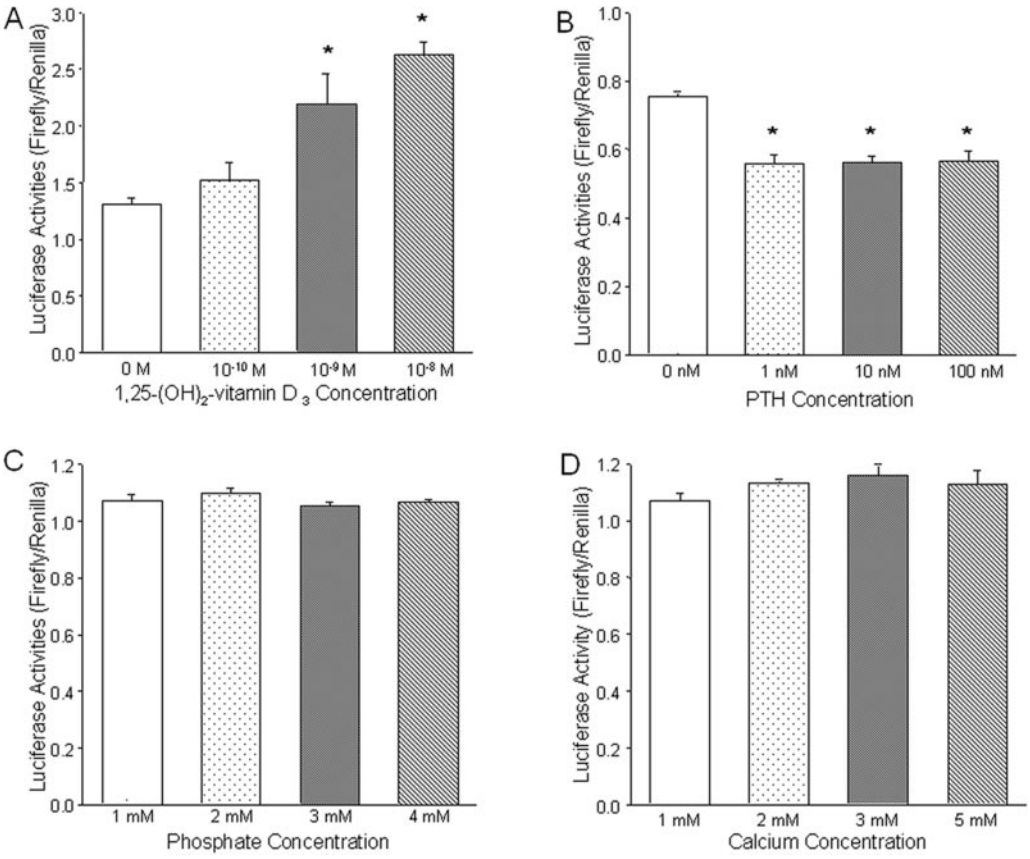

Figure 4. Effect of 1,25(OH)$_2$D$_3$, PTH, phosphate, and calcium on FGF23 promoter activities. ROS17/2.8 osteoblasts were transiently transfected with p3550Fgf23-luc and the pRL-TK construct, which expresses renilla luciferase as an internal control for transfection efficiency. Twenty-four hours after transfection, cells were treated with 10^{-10} to 10^{-8} M 1,25(OH) $_2$D$_3$ (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 ($P < 0.05$).

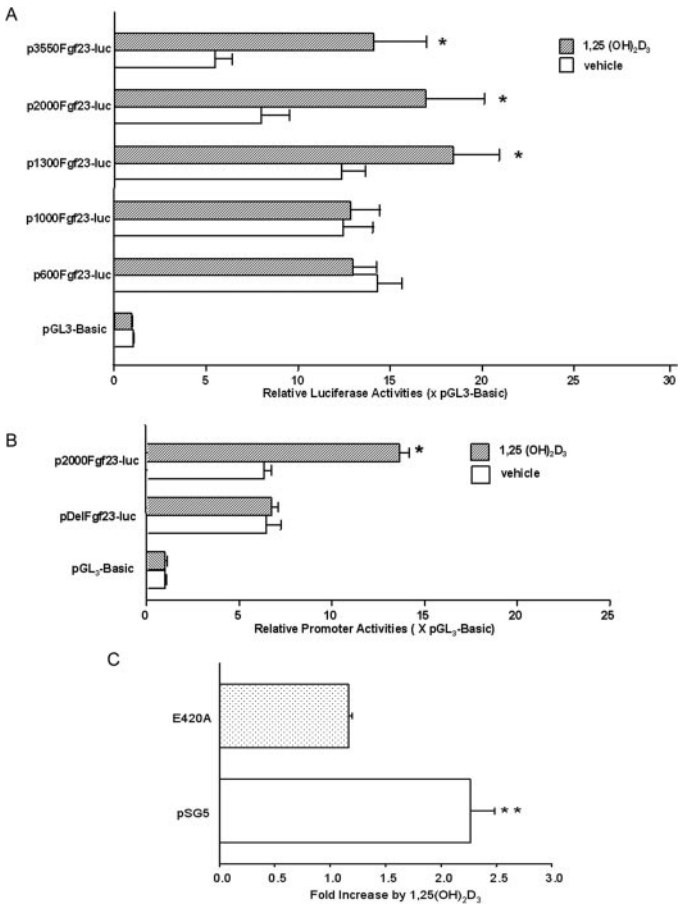

Figure 5. Deletion analysis of the FGF23 promoter activities response to $1,25(OH)_2D_3$ and the response is abolished by dominant mutant vitamin D receptor (VDR). (A) ROS17/2.8 osteoblasts were transiently transfected with a promoterless construct pGL3-Basic or with the indicated FGF23 promoter-luciferase constructs that contained successive 5′ deletions along with the pRL-TK construct, which expresses renilla luciferase as an internal control for transfection efficiency. Twenty-four hours after transfection, the transfected cells were treated with 10^{-8} M $1,25(OH)_2D_3$ or the same amount of ethanol (vehicle) for 24 h. Promoter activities were measured by the Firefly luciferase activities normalized by Renilla luciferase activities and expressed as luciferase activities relative to the activities from pGL3-Basic. All values are mean ± SEM; n = at least 4 in each group. *$P < 0.05$ *versus* vehicle in each group. (B) Deletion analysis of VDRE in p2000Fgf23-luc. ROS17/2.8 cells were transfected with wild-type and deletion construct. Promoter activities and their response to $1,25(OH)_2D_3$ were assayed as described above. (C) Co-transfection of p3550Fgf23-luc with pSG5 vector or pSG5E420A in ROS17/2.8 osteoblasts. E420A dominant mutant VDR expression abolished $1,25(OH)_2D_3$ response in p3550Fgf23-luc promoter/reporter construct. All values are mean ± SEM; n = 3 in each group. *$P < 0.05$ *versus* vehicle in each group.

To confirm the vitamin D responsive region, we created serial deletion 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 GGAACTcagTAACCT −1156). Next, we deleted this 15-bp region from the p2000Fgf23-luc construct using PCR mutagenesis methods to create pDelFgf23-luc. $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ in ROS17/2.8 cells co-transfected p3500Fgf23-luc and pGS5A420E, a dominant negative human VDR expression construct. Overexpression of the human VDR A420E mutant significantly inhibited $1,25(OH)_2D_3$-stimulated FGF23 promoter activity in ROS17/2.8 (Figure 5C).

Discussion

This study supports a physiologic role for $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ (Figure 2B) that $1,25(OH)_2D_3$ stimulates the production of FGF23 in bone and osteoblasts. Additional studies indicate that $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ stimulation of FGF23 promoter activity (Figure 5C), indicating that FGF23 is regulated directly by $1,25(OH)_2D_3$ in osteoblasts. It is interesting that $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ to regulate FGF23 production by osteoblasts.

$1,25(OH)_2D_3$ regulation of FGF23 production has been reported previously (34–37). In this regard, VDR null mice have low circulating FGF23 levels (35), and $1,25(OH)_2D_3$ administration stimulates FGF23 levels in mice independent of PTH (35,38). Also, $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ (40).

$1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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, $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ stimulation of calcium and phosphate absorption by the gastrointestinal tract (42), increments in calcium (as well as $1,25[OH]_2D_3$ 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. $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ has been reported to stimulate FGF23 promoter activity in erythroleukemia 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 $1,25(OH)_2D_3$ 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).

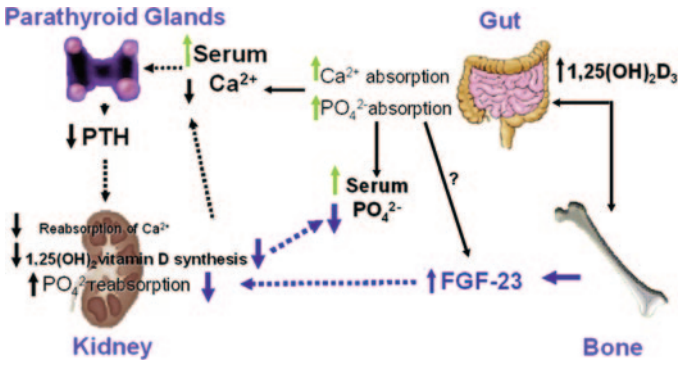

Figure 6. Proposed model showing interrelationship between FGF23 and vitamin D, PTH, calcium, and phosphorus. $1,25(OH)_2D_3$ causes concomitant suppression of the calcemic and phosphaturic hormone PTH as a result of increases in gastrointestinal calcium absorption and directly suppresses PTH production by the parathyroid glands. Consequent to the loss of PTH-mediated phosphaturia, hyperphosphatemia might occur in response to $1,25(OH)_2D_3$-mediated increases in gastrointestinal phosphate absorption if not for the presence of a counterregulatory phosphaturic factor, such as FGF23. Dietary phosphate regulation of FGF23 production by bone likely occurs through unknown intermediate steps, because hyperphosphatemia *per se* does not directly stimulate FGF23 production by osteoblasts.

Conclusion

$1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$–mediated stimulation of gastrointestinal absorption of phosphate and suppression of the phosphaturic hormone PTH.

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