Published in final edited form as:

Calcif Tissue Int. 2013 April ; 92(4): 339–353. doi:10.1007/s00223-012-9683-5.

1,25-Dihydroxyvitamin D₃ Regulation of Fibroblast Growth Factor-23 Expression in Bone Cells: Evidence for Primary and Secondary Mechanisms Modulated by Leptin and Interleukin-6

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Abstract

Fibroblast growth factor-23 (FGF23) is a circulating hormone that acts to correct hyperphosphatemic states by inhibiting renal phosphate reabsorption and to prevent hypervitaminosis D by feedback repressing 1, 25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) biosynthesis. FGF23 gene expression in the osteoblast/osteocyte is induced by the nuclear vitamin D receptor (VDR) bound to 1,25(OH)₂D₃, but cycloheximide sensitivity of this induction suggests

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The authors have stated that they have no conflict of interest.

that it may occur largely via secondary mechanisms requiring cooperating transcription factors. We therefore sought to identify 1,25(OH)₂D₃-regulated transcription factors that might impact FGF23 expression. Although neither leptin nor interleukin-6 (IL-6) alone affects FGF23 expression, leptin treatment was found to potentiate 1,25(OH)₂D₃ upregulation of FGF23 in UMR-106 cells, whereas IL-6 treatment blunted this upregulation. Genomic analyses revealed conserved binding sites for STATs (signal transduction mediators of leptin and IL-6 action) along with transcription factor ETS1 in human and other mammalian FGF23 genes. Further, STAT3, STAT1, ETS1, and VDR mRNAs were induced in a dose-dependent manner by 1,25(OH)₂D₃ in UMR-106 cells. Bioinformatic analysis identified nine potential VDREs in a genomic interval containing human FGF23. Six of the putative VDREs were capable of mediating direct transcriptional activation of a heterologous reporter gene when bound by a 1,25(OH)₂D₃-liganded VDR complex. A model is proposed wherein 1,25(OH)₂D₃ upregulates FGF23 production directly via multiple VDREs and indirectly via induction of STAT3, ETS1, and VDR transcription factors that are then activated via cell surface and intracellular signaling to cooperate in the induction of FGF23 through DNA looping and generation of euchromatin architecture.

Keywords

Fibroblast growth factor-23; 1,25-Dihydroxyvitamin D₃; Hormone responsive elements; Vitamin D receptor; STAT1; ETS1

Vitamin D is metabolized to its circulating hormonal metabolite, 1 α ,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), through two hydroxylation reactions catalyzed by the 25-hydroxylase (CYP2R1) and renal 1 α -hydroxylase (CYP27B1) enzymes [1]. The bioactions of 1,25(OH)₂D₃ are achieved through binding the vitamin D receptor (VDR), followed by heterodimerization of VDR with retinoid X receptor (RXR) and binding to vitamin D responsive elements (VDREs) to regulate gene transcription [2]. The activated 1,25(OH)₂D₃-VDR-RXR complex effects many physiological functions, including calcium and phosphate homeostasis.

Secretion of parathyroid hormone (PTH), stimulated by low plasma calcium, upregulates CYP27B1, thus promoting the final stage of 1,25(OH)₂D₃ synthesis [3]. Through 1,25(OH)₂D₃ action, intestinal calcium and phosphate absorption, bone phosphate and calcium resorption, and renal calcium and phosphate reabsorption are increased, resulting in a rise in the blood calcium and phosphate for bone mineralization [4]. This process corrects hypocalcemia but can easily lead to hyperphosphatemia. Although appreciable phosphate is removed by PTH phosphaturic action, the rapid repression of PTH by calcium and 1,25(OH)₂D₃ dictates the need for a second phosphaturic peptide hormone, namely, fibroblast growth factor-23 (FGF23) [5]. Similar to the role of PTH, FGF23 elicits phosphaturia via suppression of the renal phosphate transporters NaPi-IIa and NaPi-IIc [6].

FGF23 is a secreted protein that is primarily synthesized by osteocytes [7, 8]. Circulating FGF23 acts at the kidney, predominantly in the proximal tubule [9], with a coreceptor complex consisting of the fibroblast growth factor receptor-1c (FGFR-1c) and alpha-klotho [10]. Downstream signaling then exerts an inhibitory effect on phosphate transport in the proximal kidney, leading to phosphate excretion [10].

Regulation of FGF23 production is exerted by several systemic factors, primarily 1,25(OH)₂D₃ and phosphate [8, 11]. Chronic hyperphosphatemia leads to an upregulation of FGF23 synthesis via unknown mechanisms [11]. Further, a central role of 1,25(OH)₂D₃/VDR in FGF23 induction is strongly suggested by the minimal amount of circulating FGF23 found in VDR-null mice [12]. The influence of 1,25(OH)₂D₃ has been confirmed by an 80-

fold rise in serum FGF23 levels in 1,25(OH)₂D₃-treated normal murine models and a 78-fold increase in FGF23 mRNA in 1,25(OH)₂D₃-treated rat osteogenic sarcoma (UMR-106) cells [7]. Conversely, FGF23 downregulates the expression of CYP27B1 in the proximal renal tubule and stimulates the expression of 25-hydroxyvitamin D 24-hydroxylase (CYP24A1), the initial enzyme in vitamin D catabolism [13]. Thus, the activity of FGF23 closes a feedback loop to regulate phosphate and 1,25(OH)₂D₃ levels.

The functional significance of FGF23 has been validated in FGF23 knockout mice, which display hyperphosphatemia, vascular calcification, and disordered bone and soft tissue mineralization, along with increased serum calcium and 1,25(OH)₂D₃ levels [14]. These observations are of clinical significance since elevated serum phosphate levels have been linked to coronary artery disease, left ventricular hypertrophy, and vascular stiffness, among other cardiovascular system-related health issues [15]. Such pathological conditions contribute to the increased mortality rate seen in FGF23-null mice [14]. Finally, several human diseases have also been linked to FGF23, including chronic kidney disease [16], X-linked hypophosphatemia [17], familial tumoral calcinosis [18], autosomal dominant hypophosphatemic rickets [18], and tumor-induced osteomalacia [19].

Treatment and prevention of diseases related to hyper- or hypophosphatemia will be aided by a more thorough comprehension of factors involved in phosphate regulation, including FGF23. It has already been established that FGF23 synthesis is directly regulated by 1,25(OH)₂D₃. However, the precise molecular mechanisms that orchestrate FGF23 transcriptional activation remain unclear. A previous study indicated that a large portion of the regulation of FGF23 is cycloheximide-sensitive, implying a secondary mechanism (e.g., via 1,25(OH)₂D₃ regulation of a transcription factor(s) that then upregulate(s) FGF23 gene transcription) [7]. However, the 1,25(OH)₂D₃ inductive effect on FGF23 gene expression is not totally cycloheximide-sensitive, indicating that primary induction of FGF23 through VDREs remains a viable mechanism. The goal of this study was to explore further the mechanism(s) by which 1,25(OH)₂D₃ controls FGF23 gene transcription in mammals, with the hypothesis that the transcriptional regulation of FGF23 by 1,25(OH)₂D₃ involves both direct and indirect VDRE-mediated mechanisms, likely dependent in part on synthesis of a rapidly turned-over protein.

Methods

Mammalian Cell Culture

Three mammalian cell lines were used in the study: simian kidney cells (COS-7), human embryonic kidney cells (HEK-293), and rat osteogenic sarcoma cells (UMR-106). Each cell line required a different growth medium, and cells were cultured according to the American Type Culture Collection (Manassas, VA) recommendations, including the additives penicillin-streptomycin, and fetal bovine serum (GIBCO, Invitrogen, Grand Island, NY). Crystalline 1,25(OH)₂D₃ was kindly provided by Milan Uskokovic of Hoffmann LaRoche (Nutley, NJ).

UMR-106 cells were cultured in DMEM/F12 (Hyclone, Logan, UT) supplemented with 2.5 mM L-glutamine, 10 % fetal bovine serum (FBS), and antibiotics under a humidified atmosphere of 5 % CO₂ in air at 37 °C. For experiments employing 1,25(OH)₂D₃ and other hormones, 10 % FBS was replaced with 1 % charcoal/dextran-treated FBS to remove endogenous steroids before the cells were exposed to 1,25(OH)₂D₃, leptin (PeproTech, Rocky Hill, NJ), IL-6 (R&D, Minneapolis, MN), or buffer vehicle (phosphate-buffered saline [PBS]) for 24 hours. Normal bone cells were prepared from calvariae of Sprague-Dawley rats. Briefly, calvariae were cut into 1 × 1-mm fragments, which were placed in culture dishes containing DMEM/F12 supplemented with 2.5 mM L-glutamine, 10 % FBS,

and antibiotics (penicillin–streptomycin). Calvarial fragments were incubated under a humidified atmosphere of 5 % CO₂ in air at 37 °C for 7–10 days. The culture medium was changed every 3 days until the outgrowth of osteoblasts from the calvarial fragments reached subconfluence. Then, cells were passaged with 0.125 % trypsin/EDTA in PBS. After the third passage, rat osteoblastic cells at 100 % confluence were incubated in differentiation medium consisting of DMEM/F12 supplemented with 50 µg/mL of ascorbic acid (Spectrum, New Brunswick, NJ), 1 mM beta-glycerophosphate (MP Biomedicals, Solon, OH), and 2 % charcoal/dextran-treated FBS. After 3 days of culture, cells were exposed to 1,25(OH)₂D₃ for 24 hours. Rat calvariae were kindly provided by Dr. Trent Anderson (University of Arizona College of Medicine, Phoenix, AZ).

Candidate VDRE Identification

A bioinformatic search for candidate VDREs was targeted to a genomic region containing the FGF23 gene and bracketed between binding sites for CCCTC-binding factor (CTCF), which may act as insulators [20]. Using both the Human Genome Browser (University of California–Santa Cruz [21]) and the CTCF Binding Site Database (University of Tennessee Health Science Center, <http://insulatordb.uthsc.edu/>), a 140-kb region of DNA on human chromosome 12 surrounding the FGF23 gene was extracted and scanned for VDRE half-elements found in previously published VDREs [see 22 for list of known VDREs]. Only putative VDREs consisting of half-elements arranged in either a direct repeat with a spacer of 3 bp (DR3) or an everted repeat with a spacer of 6 bp (ER6) motifs (see Fig. 3a) were chosen for further testing.

Synthesis of VDRE Oligonucleotides and Plasmid Constructs

Double-stranded, single-copy DNA oligonucleotides of each candidate VDRE, flanked by 4 bp before and after the sequence as found in the online human genome, were obtained from Integrated DNA Technologies (Coralwood, IA) for use in electrophoretic mobility shift assay (EMSA). Four additional nucleotides, 5′-accgNNN...-3′ and 5′-gccanNN...-3′, were added to the 5′ end of the forward and reverse strands, respectively, to create four-base overhangs for ³²P-dCTP labeling.

For those candidate VDREs that displayed VDR binding in the EMSA, double-stranded, dual-copy DNA oligonucleotides of the candidate VDRE sequences were obtained for functional testing. Four-nucleotide base overhangs were added to the 5′ end of both the sense (5′-agctNNN...-3′) and antisense (5′-gatcNNN...-3′) strands to allow for cloning of the VDRE into the pLUC-MCS vector (expressing firefly, *Photinus pyralis*, luciferase) using the *Hind*III and *Bgl*II sites present in this vector. Additional plasmids used for (co-)transfecting mammalian cells included the *Renilla reniformis* luciferase reporter, pRL-null (Promega, Madison WI), and an expression plasmid for human VDR, pSG5-hVDR [23].

Electrophoretic Mobility Shift Assay

Single-copy oligonucleotides of each candidate VDRE were annealed, labeled with ³²P-dCTP (Perkin-Elmer, Waltham, MA) by Klenow fill-in, and analyzed using EMSA as previously described [24]. VDR and RXR proteins were provided via lysates of COS-7 cells previously transfected with the expression plasmids pSG5hVDR and pSG5hRXRα [25]. Since candidate VDREs represented both DR3 and ER6 VDRE motifs, the well-characterized, functional rat osteocalcin DR3 VDRE [26] and an ER6, the proximal everted repeat with a six-nucleotide spacer (PER6) from the human CYP3A4 gene [27], were utilized as positive control VDREs in the appropriate experiments.

Dual Luciferase Reporter Assay

ExpressIN (Thermo Scientific, Lafayette CO) transfection reagent was utilized to transfect HEK-293 cells in 24-well plates (plated at 60,000 cells/well) according to the manufacturer's protocol. Briefly, each well was transfected with 2.0 μ L ExpressIN reagent, 250 ng of the VDRE-containing pLUC-MCS plasmid to be tested, 25 ng of pSG5-hVDR (plasmid expressing human VDR), 20 ng of pRL-null (*R. luciferase* reporter), and 1 μ L of 100 $\times$ sodium pyruvate. Transfection of UMR-106 cells was similar; however, FuGene HD Transfection Reagent (Roche Applied Science, Indianapolis, IN) was used for this cell line. After transfection, each well was treated with either 1×10^{-8} M $1,25(\text{OH})_2\text{D}_3$ or ethanol vehicle for 20 hours at 37 $^{\circ}\text{C}$. Whole-cell lysates were harvested and analyzed sequentially for firefly luciferase and *R. luciferase* activity using a dual luciferase assay kit (Promega) and a Sirius Luminometer (Zylux, Huntsville, AL) according to the manufacturers' protocols.

Bioinformatic Search for Conserved Transcription Factor Binding Sites Flanking the FGF23 Gene

The genomic interval containing the human FGF23 gene between insulators INSUL_ZHA012484 and INSUL_ZHA012487 was scanned using the MatInspector program (Genomatix Software, Munich, Germany). Conservation of these transcription factor binding sites was assessed using the conservation feature in the University of California–Santa Cruz Web browser [28].

Real-Time PCR

UMR-106 cells were plated in 60-mm dishes at 5×10^5 cells/dish, allowed to attach overnight, then treated with 10^{-8} M $1,25(\text{OH})_2\text{D}_3$, or various concentrations of leptin or IL-6. Cells were harvested, and total cellular RNA was isolated utilizing an Aurum Total RNA Mini kit (Bio-Rad, Hercules, CA). RNA was quantified using A260/280 spectrophotometry. DNase-treated RNA (1 μ g) was reverse-transcribed using the iScript cDNA Synthesis kit (Bio-Rad). The cDNA was used in 20 μ L PCRs containing 10 μ L FastStart Universal SYBR Green Master Mix (Roche Applied Science) and primers. Reactions were performed in 96-well PCR plates and read on an ABI 7500 Fast instrument (Life Technologies, Carlsbad, CA). Data were analyzed using the comparative Ct method as a means of relative quantitation, normalized to an endogenous reference (GAPDH) and relative to a calibrator (normalized Ct value from vehicle-treated cells), and expressed as $2^{-\Delta\Delta\text{Ct}}$ according to Applied Biosystems' User Bulletin 2: Rev B, "Relative Quantitation of Gene Expression." Primer sets for real-time PCR were as follows: rat FGF23, forward 5'-AC GGAACACCCCATCAGACTATC-3', reverse 5'-TATCA CTACGGAGCCAGCATCC-3'; rat CYP24, forward 5'-GA TCACCTTTCCAAGAAGGAAGT-3', reverse 5'-AGA GAATCCACATCAAGCTGTTC-3'; rat GAPDH, forward 5'-AGGTCGGTGTGAACGGATTG-3', reverse 5'-CAT TCTCAGCCTTGACTGTGCC-3'; rat STAT1, forward 5'-TCAAGGTCAATCACCAGCCTG-3', reverse 5'-GGG AACAGAACCAATGAGGGTC-3'; rat STAT3, forward 5'-AGCTGCACCTGATCACCTTTGAGA-3', reverse 5'-C CACAGGATTGATGCCCAAGCATT-3'; rat cETS1, forward 5'-CAAGCCGACTCTCACCATCATC-3', reverse 5'-TCAGTGCCTGGGACATCATTTTC-3'; and rat VDR, forward: 5'-GCAAAGGTTTCTTCAGGCGG-3', reverse: 5'-CTTGGTGATGCGGCAATCTC-3'.

Preparation of Total-Cell Lysates

Briefly, cells for total-cell lysate were collected, washed twice with ice-cold PBS, and resuspended in ice-cold lysis buffer (20 mM HEPES-KOH [pH 7.5], 150 mM NaCl, 1 % Triton X-100, 1x protease inhibitor cocktail, 1 mM Na_3VO_4 , and 10 mM NaF). Lysed cells were incubated for 10 minutes, vortexed for 1 minute, and centrifuged at 12,000g at 4 °C for 10 minutes. The supernatant was harvested as total-cell lysate.

Immunoblotting

The protein concentration was determined using the BCA protein assay reagent kit (Thermo Scientific). Proteins were denatured at 95 °C for 3 minutes in SDS sample buffer in the presence of 2-mercaptoethanol, separated by SDS-polyacrylamide gel electrophoresis, and transferred to PVDF membranes. Membranes were blocked with 2 % bovine serum albumin (fraction V; Sigma, St. Louis, MO) or 5 % nonfat milk in Tris-buffered saline with 0.1 % Tween 20 (TBS-T) for 1 hour at room temperature and incubated with the following primary antibodies overnight: anti-STAT3, anti-phospho-STAT3 (Thy705; Cell Signaling, Danvers, MA), and anti-beta-tubulin (Sigma). After binding with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature, reactive bands were visualized with an ECL detection system (Amersham, GE Healthcare Life Sciences, Piscataway, NJ).

Statistical Analysis

Data are expressed as means $\pm$ standard deviation (SD) or in some cases standard error of the mean (SEM). Statistical differences between two groups were determined by a two-sided Student's *t*-test. Differences among multiple groups were analyzed by ANOVA. *p* < 0.05 was considered significant.

Results

Regulation of FGF23 Gene Expression in Bone Cells by 1,25(OH)₂D₃, Leptin, and IL-6

1,25(OH)₂D₃ induction of FGF23 mRNA has been reported to be tissue-localized to bone (calvaria and tibia) in the mouse in vivo [7]. Previous data showing 1,25(OH)₂D₃ stimulation of FGF23 gene expression in cultured cells utilized rat osteosarcoma cells, specifically UMR-106 [7] and ROS 17/2.8 [29]. We tested first whether nonmalignant, normal-outgrowth cells from rat calvariae responded to 1,25(OH)₂D₃ with respect to FGF23 mRNA induction. Indeed, as illustrated in Fig. 1a, normal rat osteoblast-enriched calvarial outgrowth cells display over a fivefold enhancement of FGF23 mRNA when treated with 10 nM 1,25(OH)₂D₃. This result lends biological credence to data obtained in bone cancer cell lines, which are necessarily utilized in multiple experiments requiring abundant numbers of cells. Accordingly, Fig. 1b depicts the 1,25(OH)₂D₃ dose dependence of FGF23 mRNA in UMR-106 cells, which are exquisitely sensitive in this respect, likely because they harbor >10,000 copies of VDR/cell (data not shown) and apparently possess some osteocyte-like character. Next, we examined the influence of leptin on FGF23 mRNA concentrations in UMR-106 cells. Recently, it was reported that leptin is a novel inducer of FGF23 expression in the leptin-deficient (*ob/ob*) mouse [30]. As shown in Fig. 1c, leptin alone has no significant effect on FGF23 mRNA but, in combination with a physiologic concentration of 1,25(OH)₂D₃ (0.1 nM), exerts a dose-dependent stimulation of FGF23 gene expression of approximately twofold. Interestingly, the same trend of leptin enhancement of 1,25(OH)₂D₃-induced gene expression occurs in the case of Cyp24a1, although it is not statistically significant (Fig. 1d), indicating that Cyp24a1 is not modulated directly by leptin signaling (asterisks refer to the effect of 1,25(OH)₂D₃). However, endogenously induced FGF23 could augment Cyp24a1 mRNA levels, as has been reported [13], providing an

indirect mechanism whereby leptin enhances Cyp24a1. Figure 1e, f reveals that leptin is signaling via rapid STAT3 phosphorylation in UMR-106 cells, as it does in vivo.

Finally, we evaluated the influence of IL-6 on FGF23 mRNA levels. IL-6 and FGF23 are directly correlated and associated with inflammation in chronic renal failure [31], and FGF23 and IL-6 are risk factors for left ventricular hypertrophy in peritoneal dialysis patients [32]. Whereas IL-6 alone has no significant effect on FGF23 mRNA levels in UMR-106 cells, in combination with a physiologic concentration of $1,25(\text{OH})_2\text{D}_3$ (0.1 nM) IL-6 exerts a dose-dependent repression of FGF23 gene expression by approximately 40 % (Fig. 1g). This is opposite to the effect of leptin with respect to FGF23, but IL-6 promulgates the same trend, though not a statistically significant effect, as leptin to enhance $1,25(\text{OH})_2\text{D}_3$ -induced gene expression in the case of Cyp24a1 (Fig. 1h). This suggests that, unlike FGF23, Cyp24a1 may be potentiated by proinflammatory signaling.

In Silico Analysis of Conserved Transcription Factor Binding Sites in the FGF23 Gene

Given prior results suggesting that a large component of FGF23 regulation by $1,25(\text{OH})_2\text{D}_3$ is cycloheximide-sensitive and therefore possibly secondary [1, 7], we hypothesized that $1,25(\text{OH})_2\text{D}_3$ may upregulate novel transcription factors involved in FGF23 control. Indeed, based upon the ability of leptin and IL-6 to modulate $1,25(\text{OH})_2\text{D}_3$ /VDR-triggered upregulation of FGF23 mRNA (Fig. 1), candidates would include members of the STAT family of transcription factor mediators of leptin and IL-6 action. A search of conserved transcription factor binding sites was therefore undertaken in the vicinity of the FGF23 gene. As shown in Fig. 2a, conserved consensus binding sites were located for STAT transcription factors, as well as for ETS1, in the vicinity of the human, mouse, and rat FGF23 genes. Those conserved sites that reside in the proximal promoter have been previously described [33] (see sequences inside green outline, Fig. 2a). More recently, ChIP-seq studies in various human cell lines have revealed bona fide binding sites for three of these factors within the genomic interval surrounding the FGF23 gene in human K562 chronic myelogenous leukemia cells [34, 35].

One site, located at -9078 bp relative to the human FGF23, has been shown to be occupied by STAT1 [34] in K562 cells and is conserved in 20 out of 33 mammalian genomes according to the University of California–Santa Cruz Web browser, “Conservation” feature [28]. Unfortunately, conservation of this region could not be assessed for the mouse and rat genomes. Nevertheless, other STAT sites do exist that are conserved among human, mouse, and rat genomes (and other mammals, see Fig. 2a), although the actual occupancy of these sites has not yet been assessed in vivo.

Two sites that bind GATA factors 1 and 2 have been located by ChIP-seq performed in K562 cells [35]. One of these binds both GATA factors and is located at -9.13 kb (Fig. 2a), very near the above-described STAT1 site; the other site, which also binds both GATA factors, is located at +58.8 kb (Fig. 2a). Both sites show partial conservation among mammals (-9.13 kb, 21 of 33 genomes; +58.8 kb, 25 of 33 genomes).

A third category of conserved site is a consensus binding site for human ETS1 (also known as c-Ets-1), located at +58.5 kb, not far from the second GATA site mentioned above. This ETS1 site, which is highly conserved among mammals (27 of 33 genomes), bound its cognate factor as demonstrated in a recent ChIP-seq study in both K562 cells and in human GM12878 lymphoblastoid cells [36]. There is, in fact, a cluster of consensus ETS1 sites in this region (see Fig. 5); but only the site at +58.5 kb is conserved.

It is pertinent that we have recently published [33] data for the rat FGF23 gene which reveal that $1,25(\text{OH})_2\text{D}_3$ treatment of UMR-106 cells elicits a twofold enhancement in histone H4

acetylation in the proximal promoter, specifically between –57 and –249 bp, a region which contains the rat version of the conserved three ETS1 and two GATA sites in the FGF23 gene proximal promoter. These data indicate that distant VDR/VDRE tethered complexes likely influence the architecture of chromatin in the core promoter to favor FGF23 gene transcription.

1,25(OH)₂D₃ Induction of Transcription Factors that Bind Conserved Sites in the FGF23 Gene

Given the existence of conserved sites for transcription factor binding, four of which have been demonstrated to be occupied in various human cells, the ability of 1,25(OH)₂D₃ to influence the expression of the mRNAs encoding these factors was examined. Preliminary results (data not shown) obtained in rat UMR-106 cells indicate that whereas mRNA species for many transcription factors are not upregulated by 1,25(OH)₂D₃ to an appreciable extent (GATA1–3 and -6, STAT4 and -5a,b), mRNAs for STAT1 and ETS1 are upregulated approximately twofold after 24-hour treatment with 1,25(OH)₂D₃. It is noteworthy that ETS1 and STAT1, two of the transcription factors induced by 1,25(OH)₂D₃, are also the two factors for which expression is the highest in UMR-106 cells compared with the other transcription factor mRNAs examined in the present experiments (data not shown). Figure 2b shows the 1,25(OH)₂D₃ dose response of STAT1 mRNA induction in UMR-106 cells, with significant induction by 10 nM 1,25(OH)₂D₃. A similar pattern of ETS1 gene expression stimulation by 1,25(OH)₂D₃ is depicted in Fig. 2c. We also observed that STAT3, the signal transducer of leptin action, is induced by 1,25(OH)₂D₃ (Fig. 2d). Finally, Fig. 2e reveals that VDR mRNA is the most strongly induced by 1,25(OH)₂D₃ of all the transcription factors tested, suggesting that 1,25(OH)₂D₃ apparently amplifies its induction of FGF23 by increasing the copy number of the VDR protein and implicating VDREs as key *cis* elements in the stimulation of FGF23 gene expression.

Candidate Human FGF23 Gene VDRE Sequences

As summarized in Fig. 3a, bioinformatic analysis revealed nine candidate VDREs located within CTCF binding site boundaries for the human FGF23 gene on chromosome 12. Note that our designation “VDRE 3” consists of two exact copies of an ER6 sequence, one positioned at –22.2 kb (VDRE 3) and one located at –16.2 kb (VDRE 3’). Upon analyzing conservation of these human VDREs with other species, only VDRE 8 exhibited conservation among human, rat, and murine species according to the University of California–Santa Cruz Web site [37]. The corresponding VDREs from mouse and rat therefore were examined by EMSA, with the caveat that this VDRE lies within the coding region of CCND2, a gene 3’ of the FGF23 gene. Table 1 lists the candidate DR3 or ER6 VDRE sequences (along with four flanking bases on either side) probed in the present study, with each VDRE half-element underlined and the location given relative to the FGF23 transcriptional start site. Candidate VDREs were synthesized as single-copy oligonucleotides for use in EMSA. For simplicity, only the sense strand is shown (without added overhangs for labeling or cloning).

Ability of Predicted VDREs to Bind VDR and RXR

The annealed, ³²P-labeled candidate VDREs were analyzed via EMSA along with whole-cell lysates containing both human VDR and human RXRα. Results were compared against functional VDREs representing previously characterized DR3 and ER6 VDRE motifs. VDREs 1, 2, 3’, and 4 displayed significant but varying binding affinity for VDR/RXR (Fig. 2b, c). VDREs 6 and 5 also generated shifted bands, indicating significant VDR binding affinity (Fig. 2d, e). Certain candidate VDREs produced shifted bands equal in magnitude to that of the positive controls (e.g., VDREs 4 and 6; Fig. 2b, d, respectively). The presence of VDR within each shifted complex was confirmed by inclusion of the 9A7γ anti-VDR

antibody (α -VDR), causing the disappearance, or supershift, of the band in samples containing VDR. In contrast, candidate VDREs 7 and 8, located at +93.3 and +100.9 kb, respectively, along with the mouse and rat VDREs corresponding to VDRE 8, did not bind VDR/RXR appreciably and were not evaluated further, with one exception. Preliminary data (not shown) in one experiment indicated that two copies of VDRE 8 along with its immediately adjacent C/EPB site (only 2 bp 3') did not respond to $1,25(\text{OH})_2\text{D}_3$ to stimulate transcription when these elements were inserted upstream of a luciferase reporter.

Functional Activity of Candidate VDREs in Mediating $1,25(\text{OH})_2\text{D}_3$ -Stimulated Transcription

To determine transactivation capabilities, candidate VDREs located at -35.7, -32.9, -16.2, and +8.6 kb (VDREs 1, 2, 3', and 4, respectively) were cloned as dual-copy oligonucleotides into the firefly luciferase reporter vector, pLuc-MCS, and cotransfected with VDR and the *Renilla* luciferase vector, pRL-Null, into two mammalian cell lines: HEK-293 (Fig. 4a) and UMR-106 (Fig. 4b). The transactivation capabilities of candidate VDREs located at +78.6 and +83.4 kb (VDREs 5 and 6, respectively) were tested in a similar fashion using HEK-293 (Fig. 4c) and UMR-106 (Fig. 4d). After treatment with 10^{-8} M $1,25(\text{OH})_2\text{D}_3$, luciferase assays performed on cell lysates revealed consistent $1,25(\text{OH})_2\text{D}_3$ responsiveness in four (-35.7, -32.9, +8.6, +83.4; VDREs 1, 2, 4, and 6, respectively) of eight candidate VDREs.

Responsiveness to $1,25(\text{OH})_2\text{D}_3$ by these reporter constructs differed not only by VDRE but also by cell type. All tested VDREs mediated an upregulation in UMR-106 cells (the only bone cell tested). Across three to five independent experiments in UMR-106, of which a representative example is shown ($\pm$ SEM), VDRE 1 yielded an upregulation by $1,25(\text{OH})_2\text{D}_3$ of 1.3 ± 0.19 -fold; VDRE 2, 2.45 ± 0.54 -fold; VDRE 3', 1.57 ± 0.17 -fold; VDRE 4, 1.77 ± 0.29 -fold; VDRE 5 1.57 ± 0.34 -fold; and VDRE 6, 3.91 ± 0.44 -fold. In HEK-293 cells, only VDREs 2, 4, and 6 yielded an appreciable upregulation in three independent experiments (2.56 ± 0.32 , 1.69 ± 0.28 , and 1.68 ± 0.17 , respectively), with VDREs 3' and 5 showing essentially no effect (0.96 ± 0.15 and 0.95 ± 0.07 , respectively) and VDRE 1 only a slight upregulation (1.24 ± 0.25 fold) of reporter gene transcription. However, VDRE 3' is one of the better activators of the luciferase reporter gene in UMR-106 cells and, thus, potentially may play a role in FGF23 regulation in bone-related cells (see Discussion). Thus, the activity of each of the tested VDREs is significant in bone-derived UMR-106 cells but modest in magnitude and somewhat cell-selective. The reason for the difference between the UMR-106 and HEK-293 results is likely that UMR-106 cells possess a higher copy number for VDRE over HEK-293 cells and VDR is markedly induced by $1,25(\text{OH})_2\text{D}_3$ in UMR-106 cells (Fig. 2e). In total, the results indicate that to accomplish the dramatic induction of FGF23 mRNA in UMR-106 cells, either all active VDREs operate in concert or some cooperate with other transcription factors to accomplish full activation of FGF23 transcription. In that vein, Meyer et al. [38] reported that VDR/VDRE regulomes in bone-expressed genes possess RUNX2 and/or C/EBP sites within approximately a 1-kb "neighborhood." As illustrated in Fig. 3a, VDREs 1, 3', 4, and 8 follow that paradigm, implying that these VDREs may be intrinsic to regulomes for FGF23 or nearby genes such as CCND2.

Discussion

The present experiments explore the mechanisms whereby $1,25(\text{OH})_2\text{D}_3$ regulates expression of FGF23 in bone, centered on a bioinformatic examination of the genomic region containing the human FGF23 gene. Nine putative VDREs were discovered within this region, with only one being conserved across species (human, mouse, and rat). This VDRE (denoted 8 in the human, positioned at +100.9 kb) may display sequence conservation because of its location within an exon of the CCND2 gene rather than because

of any role in transcriptional regulation of FGF23. VDREs 1, 2, 3', 4, 5, and 6 displayed significant but varying binding affinity for VDR/RXR (Fig. 3b–e), with VDREs 4 and 6 generating the highest-intensity shifted bands, equal in magnitude to that of the positive control rat osteocalcin VDRE. Human FGF23 VDREs 1 (–35.7 kb), 2 (–32.9 kb), 3' (–16.2 kb), 4 (+8.6 kb), 5 (+78.6 kb), and 6 (+83.4 kb) yielded a 1.3-fold or greater effect on transcription when assayed in UMR-106 bone cells (Fig. 4b, d). The most potent enhancers were VDREs 4 and 6, with the other VDREs being slightly less active.

To discern which of the six transcriptionally active VDREs in the human FGF23 gene are likely functional in vivo, we adopted the paradigm of Meyer et al. [38], who employed ChIP-seq to demonstrate that VDR/VDRE regulomes in bone-expressed genes, such as SP7 (osterix) and RUNX2, possess RUNX2 and/or C/EBP sites within approximately a 1-kb fragment containing the VDRE that is occupied by VDR/RXR in response to 1,25(OH)₂D₃. By these criteria and as summarized in Fig. 3a, VDREs 1 (–35.7 kb), 3' (–16.2 kb), 4 (+8.6 kb), 6 (+83.4 kb), and 8 (+100.9 kb) appear to be the best candidates for anchoring a 1,25(OH)₂D₃/VDR regulome. VDRE 8 (+100.9 kb) can be excluded because it does not bind VDR/RXR to a significant degree via EMSA (Fig. 3e) and because its location 3' distant of FGF23 suggests that if it does mediate 1,25(OH)₂D₃ action on expression, it more likely does so for either the CCND2 gene or the nearby *c12orf5* gene. We therefore conclude (Fig. 5) that VDREs 1 (–35.7 kb), 3' (–16.2 kb), 4 (+8.6 kb), and 6 (+84.3 kb) represent the functional VDREs that anchor VDR regulomes in the primary control of human FGF23 gene expression by 1,25(OH)₂D₃. Because of its proximity to VDRE 1, VDRE 2 could conceivably be embedded in the regulome anchored to VDRE 1. Because much of the present data were derived from studies of rat cells (UMR-106), we searched the rat FGF23 gene region to identify analogous VDR regulomes, keeping in mind that the human FGF23 VDREs are, with the exception of the discarded VDRE 8, not conserved in rodent species. One candidate VDRE/regulome was observed in rat FGF23 at –24 kb, with the sequence AGGGCAatgGAGTCA, surrounded by four RUNX2 sites (not shown). ChIP-seq investigations in human and rat osteocytes will be required to absolutely define the 1,25(OH)₂D₃/VDR regulomes in the FGF23 gene.

Prior unfruitful searches for VDREs were limited by the assumption that transactivation elements for FGF23 would be within 3–5 kb of the promoter. Although the VDREs discovered in this study are located at a considerable distance from the transcription start site, the functionality of such distal enhancers in other 1,25(OH)₂D₃-regulated genes has been previously demonstrated [39, 40]; DNA looping has been proposed to bring these distant VDREs close to the gene promoter in order to form an active transcriptional complex (Fig. 5).

The modest but significant transcriptional activation elicited by the VDREs reported herein suggests that these VDREs are only partially responsible for 1,25(OH)₂D₃-mediated FGF23 transcription. Indeed, a complete picture of FGF23 regulation by 1,25(OH)₂D₃ must account for previous results showing that the major portion of the upregulation of FGF23 mRNA after 1,25(OH)₂D₃ treatment of UMR-106 cells is cycloheximide-sensitive [1, 7], implying secondary regulation.

We hypothesized that secondary regulation of FGF23 by 1,25(OH)₂D₃ would involve conserved binding sites for transcription factors that are regulated directly by 1,25(OH)₂D₃. Bioinformatic analysis of a genomic region surrounding FGF23 identified several conserved binding sites for members of the STAT and GATA families as well as for ETS1 (Fig. 2a). Further literature searches revealed that a subset of these sites actually bound their cognate factor as revealed by ChIP-seq experiments performed mainly in human K562 chronic myelogenous leukemia cells [34–36], in which FGF23 gene expression is cooperatively

induced by $1,25(\text{OH})_2\text{D}_3$ and elevated phosphate [41]. The next step in this analysis was to test whether any of these factors might be upregulated by $1,25(\text{OH})_2\text{D}_3$. The results of real-time PCR experiments in UMR-106 osteocyte-like cells revealed an upregulation of factors STAT1, STAT3, and ETS1, as well as of VDR (Fig. 2), suggesting that $1,25(\text{OH})_2\text{D}_3$ may potentiate the effect of these transcription factors in osteocytes. By upregulating VDR, $1,25(\text{OH})_2\text{D}_3$ would secondarily amplify its primary action to induce FGF23 mRNA. By upregulating STATs and ETS1, $1,25(\text{OH})_2\text{D}_3$ would secondarily enhance VDR/VDRE-mediated control of FGF23 transcription through cooperation between phosphorylated/activated STATs and ETS1. The synthesis of VDR, STAT, and ETS1 would, of course, be sensitive to inhibition by cycloheximide, perhaps accounting for cycloheximide inhibition of the secondary portion of FGF23 induction by $1,25(\text{OH})_2\text{D}_3$.

To prove the biological relevance of the above concept, we determined if extracellular transducers of STAT, specifically leptin and IL-6, which signal the activation of STATs via phosphorylation, affect $1,25(\text{OH})_2\text{D}_3$ -induced FGF23 mRNA concentrations. We observed that whereas neither leptin nor IL-6 alone affects FGF23 expression, treatment of UMR-106 cells with leptin potentiated $1,25(\text{OH})_2\text{D}_3$ upregulation of FGF23, whereas IL-6 treatment blunted this upregulation (Fig. 1). These data lend credence to the model wherein VDR cooperates with other transcription factors in regulating FGF23 gene expression. Cooperation with STATs likely involves *cis* STAT elements within the VDR regulomes and, as summarized in Figs. 2a and 3a, such STAT elements reside near VDREs 1 (−35.7 kb), 2 (−32.9 kb), 4 (+8.6 kb), 5 (+78.6 kb), 6 (+83.4 kb), and 7 (+93.3 kb). In addition, a conserved STAT site exists at −9.1 kb (Fig. 2a), remote from any proposed VDR/VDRE regulomes. This site has been shown to be occupied by STAT1 [34] in K562 cells. According to features on the University of California–Santa Cruz Web browser [28], this element is a “hotspot” of DNase sensitivity and is marked by *PoII*, cMYC, and histone 3 methylation, indicating that, besides the proximal promoter, this *cis* locale is a focus of transcriptional control in the FGF23 gene. We propose that VDR regulomes interact with this locus via DNA looping to govern FGF23 gene expression (Fig. 5).

Like the conserved STAT1 site, none of the conserved ETS1 sites in the FGF23 gene are located in or near VDR regulomes. Instead, they consist of triplets: a conserved triplet in the FGF23 proximal promoter and another triplet (containing one conserved and two nonconserved ETS1 consensus sites) at +54 to +58 kb in a region corresponding to the *c12orf5* gene promoter. This far 3' (+54 to +58 kb) ETS1 triplet could potentially play a role in FGF23 transcriptional regulation (Fig. 5), despite the fact that it resides closer to the *c12orf5* gene.

However, the ETS1 triplet in the FGF23 proximal promoter (Fig. 2a) is no doubt pivotal in the regulation of FGF23 transcription based upon its conserved location and the fact that we have recently published [33] data for the rat FGF23 gene which reveal that $1,25(\text{OH})_2\text{D}_3$ treatment of UMR-106 cells elicits a twofold enhancement in histone H4 acetylation in the proximal promoter, specifically between −57 and −249 bp, a region which contains the rat version of the conserved three ETS1 elements in the FGF23 gene proximal promoter. These data indicate that distant VDR/VDRE tethered complex regulomes likely cooperate with bound phospho-ETS1 dimers to recruit comodulators that influence the architecture of chromatin in the proximal promoter to favor FGF23 gene transcription. Finally, a conserved CRE element resides in the FGF23 proximal promoter at −40 bp [33], analogous to the CRE element within the mouse RANKL regulome at −76 kb that confers PTH responsiveness to this osteoclastogenic gene [42]. The CRE in the FGF23 gene proximal promoter could mediate PTH regulation of the FGF23 gene, which has been reported in bone cells and in vivo [43, 44].

The precise molecular mechanisms of FGF23 regulation by $1,25(\text{OH})_2\text{D}_3/\text{VDR}$, in cooperation with ETS1 and STAT1, are likely to be complex, as suggested by recent studies describing interactions between $1,25(\text{OH})_2\text{D}_3$ signaling and that of either STAT1 or ETS1. One recent report described an interesting interaction between STAT1 and $1,25(\text{OH})_2\text{D}_3$ signaling pathways in which phosphorylated STAT1 bound to nuclear VDR in such a way as to blunt $1,25(\text{OH})_2\text{D}_3$ signaling (consistent with the current results [Fig. 1g]), yet liganded VDR prevented dephosphorylation of STAT1, thereby enhancing STAT1-mediated activity of interferon-gamma [45]. In contrast, phosphorylated ETS1 appears to potentiate the activity of liganded VDR, at least in HeLa and HD2 cells [46]. It should be noted that occupancy of the conserved ETS1 site at +58.8 kb could be by a related factor such as ELF1 or GABPA [36], either of which has been shown to have an overlapping preference for the ETS1 binding site.

In the present study we identify two extracellular factors that signal phosphorylation of STAT3 and STAT1, namely, leptin and IL-6, both of which modulate $1,25(\text{OH})_2\text{D}_3$ -induced FGF23 mRNA levels. However, we do not experimentally identify the extracellular factors that signal the phosphorylation/activation of ETS1. One obvious candidate for this signal is high blood phosphate. However, the identity of a phosphate “sensor” in osteocytes that might detect high blood phosphate levels and signal FGF23 induction has remained elusive, but recently phosphate signaling of FGF23 has been shown to be dominant over that of $1,25(\text{OH})_2\text{D}_3/\text{VDR}$ [47]. Additionally, high phosphate [48], along with PHEX and DMP-1 [49], has been demonstrated to require the FGF23 receptor for regulation of FGF23 gene expression. This implies the existence of a crucial autoregulatory circuit that involves extracellular FGF23 and its cognate receptor on osteocytes. Thus, as depicted in Fig. 5 (upper right), FGFR1, which transduces its signal sequentially via phospho-Erk and phospho-ETS1, may be the focal point wherein osteocytes read extracellular phosphate (through PiT-1 transport) as well as growth factors such as FGF23 itself. As illustrated in Fig. 5, receptors for alternative growth factors that activate the phospho-Erk and phospho-ETS1 axis, such as osteopontin, could also participate in FGF23 regulation. The bottom line is that control of FGF23 gene expression via phospho-ETS1 dimer occupancy of the triplet of conserved ETS1 elements in the proximal promoter is likely fundamental to the regulation of this gene. $1,25(\text{OH})_2\text{D}_3/\text{VDR}$ appears to cooperate with this focal site in the proximal promoter by DNA looping to juxtapose remote regulomes, perhaps bringing into play the second transcriptionally active region centered at -9.1 kb.

In summary, we propose the model shown in Fig. 5 wherein FGF23 gene transcription is regulated by $1,25(\text{OH})_2\text{D}_3$ via both direct, VDRE regulome-mediated transcriptional activation (1°) as well as extracellular factor signaling by leptin and IL-6 to generate phospho-STATs that cooperate directly with VDR in VDRE regulomes and remotely by DNA looping. We also present evidence for secondary (2°) regulation promulgated by $1,25(\text{OH})_2\text{D}_3/\text{VDR}$ -mediated increases in STAT1, STAT3, ETS1, and VDR transcription, followed by activation of these factors by high extracellular phosphate, cytokine, and growth factor signaling or by the $1,25(\text{OH})_2\text{D}_3$ ligand. Thus, in the present study we provide evidence that the $1,25(\text{OH})_2\text{D}_3$ -VDR complex operates to regulate the transcription of the human FGF23 gene by direct, cooperative, and indirect mechanisms. As misregulation of FGF23 leads to a number of diseases involving disordered phosphate homeostasis, a better understanding of FGF23 control could potentially lead to therapeutic interventions for treating disorders of phosphate homeostasis.

Acknowledgments

This work was supported by grants from the National Institutes of Health to M. R. H.

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Fig. 1.

Hormone and cytokine control of FGF23 gene expression in osteocyte-like bone cells. **a** Dose-dependent effect of $1,25(\text{OH})_2\text{D}_3$ on FGF23 mRNA expression in normal rat calvarial outgrowth cells. Real-time PCR analysis of FGF23 mRNA levels after 24 hours of EtOH vehicle or $1,25(\text{OH})_2\text{D}_3$ treatment. * $p < 0.05$ compared with EtOH group. **b** Effect of $1,25(\text{OH})_2\text{D}_3$ on FGF23 mRNA expression in UMR-106 rat osteosarcoma cells. * $p < 0.05$ compared with EtOH group. **c** Effect of leptin on FGF23 mRNA expression in UMR-106 cells. UMR-106 cells were exposed to leptin for 24 hours in the absence or presence of $1,25(\text{OH})_2\text{D}_3$. * $p < 0.05$ compared with EtOH group, # $p < 0.05$ compared with $1,25(\text{OH})_2\text{D}_3$ /zero leptin group. **d** Effect of leptin on Cyp24a1 mRNA expression in UMR-106 cells. * $p < 0.05$ compared with EtOH group. **e** Phosphorylation of STAT3 protein by leptin signaling. Western blotting was performed for evaluation of phosphorylation levels after 10, 30, and 60 minutes of leptin treatment in UMR-106 cells. Total-cell lysates (10 μ g) were loaded into each lane. **f** Histogram quantitating the immunoblot data in e. Values represent means $\pm$ SD of three independent experiments. * $p < 0.05$ compared with 0 minutes of leptin treatment. **g** Effect of IL-6 on FGF23 mRNA expression in UMR-106 cells. UMR-106 cells were exposed to IL-6 for 24 hours in the absence or presence of $1,25(\text{OH})_2\text{D}_3$. * $p < 0.05$ compared with EtOH, # $p < 0.05$ compared with $1,25(\text{OH})_2\text{D}_3$ /zero IL-6 group. **h** Effect of IL-6 on Cyp24a1 mRNA expression in UMR-106 cells. * $p < 0.05$ compared with EtOH group.

Fig. 2.

a Location of conserved transcription factor binding sites in the vicinity of the human FGF23 gene. The interval of the human FGF23 locus shown is between insulators, as explained in the text; and the FGF23 transcriptional start site is denoted in *green*. Consensus binding sites for the indicated factors were located either by manual searches (proximal promoter, indicated by *green outline*) or by analysis using MatInspector (see Methods). Those sites shown in *blue* are confirmed binding sites for the indicated factor as determined by published ChIP-seq studies performed in various human cell lines (see text). Conservation is shown among selected vertebrates: human (*h*), mouse (*m*), rat (*r*), bovine (*b*), dog (*d*), opossum (*Monodelphis domestica*, *o*), and chicken (*c*). Transcription factor induction by 24 hour incubation with the indicated doses of 1,25(OH)₂D₃ in UMR-106 cells was assessed by real-time PCR for STAT1 (**b**), ETS1 (**c**), STAT3 (**d**), and VDR (**e**) mRNAs. **p* < 0.05 compared with EtOH group. Values represent means ± SD of three independent experiments, with each experiment performed in triplicate

Fig. 3.

a Candidate VDREs mapped to their respective locations on human chromosome 12. The genomic interval chosen for analysis is bordered by *CTCF* binding sites flanking the *FGF23* gene [20]. The nine candidate VDREs shown within this interval were identified by bioinformatics methods as described in the text and are numbered starting with the farthest upstream from the *FGF23* transcriptional start site. The VDRE motifs are displayed as two half-elements, either as two direct repeats separated by a (*lowercase*) 3-bp spacer (DR3) or two everted repeats separated by a 6-bp spacer (ER6). Mouse and rat sequences with positional and sequence similarity to human VDRE 8 are labeled *VDRE 8* for their respective species. The code for additional transcription factor sites within each VDRE “neighborhood” of 1 kb (see text) is a *white capital letter in a black solid rectangle* as follows: *R* RUNX2; *C* C/EBP; *S* STAT. The ability of candidate VDREs to bind VDR and RXR was assessed by incubating radiolabeled candidate VDREs with or without whole-cell lysate containing human VDR (and RXRα) as described in “Methods” (EMSA). In the indicated samples, anti-VDR antibody 9A7γ (α-VDR) was added to confirm the presence of the VDR, as indicated by a loss or supershift of the complex. **b** Both VDRE 1 (–35.7 kb) and VDRE 4 (+8.6 kb), which are DR3 VDREs, were tested alongside the rat osteocalcin VDRE (*RatOC*), a well-characterized functional DR3. The presumptive VDRE–VDR–RXR

complex is denoted by an *arrowhead*. **c** VDRE 3' (−16.2 kb) and VDRE 2 (−32.9 kb), which are of the ER6 type, were evaluated alongside the proximal everted repeat 6 (*PER6*) from the human CYP3A4 gene, a published, functional ER6 VDRE [27]. **d** VDRE 6 (+83.4 kb) and VDRE 7 (+93.3 kb), both of the DR3 type, are compared with rat osteocalcin DR3 VDRE. **e** VDRE 5 (+78.6 kb) and human VDRE 8 (+100.9 kb), as well as the mouse (+73.1 kb) and rat (+73.3 kb) VDREs homologous to human VDRE 8, all of the ER6 type, are analyzed alongside human CYP3A4 *PER6*

Fig. 4.

Ability of candidate VDREs to convey responsiveness to $1,25(\text{OH})_2\text{D}_3$ onto a heterologous reporter gene. VDREs were cloned as double copies into the pLUC-MCS reporter vector expressing firefly luciferase and tested alongside a similar vector containing the functional VDRE rat osteocalcin (*ROC*). All transfections included a *R. luciferase* plasmid in order to monitor transfection efficiency. Results using vectors containing VDREs 1, 2, 3', and 4 are shown in **a** and **b**, and those using VDREs 5 and 6 are displayed in **c** and **d**. Two mammalian cell lines were chosen as recipients: human embryonic kidney (HEK-293) and rat osteogenic sarcoma (UMR-106). After 20 hours of treatment with 10^{-8} M $1,25(\text{OH})_2\text{D}_3$, cell lysates were sequentially tested for firefly and *R. luciferase*, as described in Methods. Graphs depict ratios of firefly to *R. luciferase* (measured in arbitrary relative light units) $\times$ 1,000 for each treatment group. Data are the average of three replicates per treatment and represent three independent experiments (see text for composite averages for all three experiments)

Fig. 5.

Hypothetical model of FGF23 transcriptional regulation by 1,25(OH)₂D₃, leptin, IL-6, and potentially other factors. The data suggest that the 1,25(OH)₂D₃–VDR–RXR complex exerts primary (1°) regulation on the expression of the human FGF23 gene predominantly via the five VDREs described herein: VDRE 1 at –35.7 kb, VDRE 2 at –32.9 kb, VDRE 3' at –16.2 kb, VDRE 4 at +8.6 kb, and VDRE 6 at +83.4 kb; the three far downstream VDREs at +79 (VDRE 5), +93.3 (VDRE 7), and +101 (VDRE 8) kb may not be functionally involved in 1,25(OH)₂D₃ signaling of FGF23 induction for the following reasons: (1) they do not bind VDR/RXR in EMSA (VDREs 7 and 8) and (2) they are not surrounded by the requisite number of RUNX2 and C/EBP sites that manifest a VDR/VDRE regulome for bone-expressed genes (VDRE 5). Given that 1,25(OH)₂D₃ has been reported to influence FGF23 transcription through secondary (2°) mechanisms, this model includes conserved transcription factor binding sites for STAT1 (–9.1 kb) and ETS1 (a triplet at –245, –120, and –70 bp in the FGF23 promoter and a downstream triplet at +54, +56, and +58 kb), for which cognate factors are reported herein to be upregulated by 1,25(OH)₂D₃ in UMR-106 cells. The signal(s) that leads to STAT1 and/or ETS1 phosphorylation was not defined in the present experiments, although leptin and IL-6 are known to signal via STAT1 and STAT3, with both modulating 1,25(OH)₂D₃ induction of FGF23 (Fig. 1). Growth factors elicit ETS1 phosphorylation, and high phosphate signaling of FGF23 expression requires the FGF23 receptor [50], suggesting that growth factor and high phosphate signaling converge to activate ETS1 through phosphorylation. This may be crucial to FGF23 induction via occupation of the triplet of conserved ETS1 sites in the proximal promoter of FGF23. Also shown are STAT3 sites adjacent to VDREs 1, 2, 4, and 6, which are proposed to cooperate as enhancers with their companion VDREs. Finally, the model encompasses DNA looping to bring all of these distal regulatory elements into close proximity to the human FGF23 promoter in order to collaboratively regulate FGF23 transcription (see text)

Table 1

VDREs identified bioinformatically near the human FGF23 gene (numbered starting farthest upstream of the transcriptional start site); also shown are mouse (*m*) and rat (*r*) VDREs that share homology with human VDRE 8; VDRE half-sites are *underlined*

Element	Position (kb)	Sequence	Orientation
VDRE 1	-35.7	GGGTGGGAGAATGAGGGCACACG	Antisense
VDRE 2	-32.9	TCTTTGAACTCAAGGGAGGGCAGGAA	Sense
VDRE 3	-22.8	TCAGTAACCCTGCTTTAGTTCAAGGA	Sense
VDRE 3'	-16.2	CAAATAACCCTGCTTTAGTTCAACCT	Sense
VDRE 4	+8.6	ATAGAGGGCAGGAAGGACAAGGT	Sense
VDRE 5	+78.6	AGAGTAACCCAGACTGGGGGCAGGAA	Sense
VDRE 6	+83.4	GGCAAGGTCATCTGGGTTAATGT	Sense
VDRE 7	+93.3	CTGGAGGAGAGGTGGGGCATGCT	Antisense
VDRE 8	+100.9	AGCTTCTCCCGCTGCTGGGGCAGCTT	Sense
m VDRE 8	+73.1	AGCTTCTCCTTTTGCTGGGGCAGCTT	Sense
r VDRE 8	+73.3	AGCTTCTCCTTCTGCTGGGGCAGCTT	Sense