FGF23 gene regulation by 1,25-dihydroxyvitamin D: opposing effects in adipocytes and osteocytes

Ichiro Kaneko^{1,2,*}, Rimpi K Saini^{1,*}, Kristin P Griffin¹, G Kerr Whitfield¹, Mark R Haussler¹ and Peter W Jurutka^{1,2}

¹Department of Basic Medical Sciences, University of Arizona College of Medicine, 425 N. Fifth Street, Phoenix, Arizona 85004, USA

²School of Mathematical and Natural Sciences, Arizona State University, 4701 W. Thunderbird Road, Phoenix, Arizona 85306, USA

*I Kaneko and R K Saini contributed equally to this work and are therefore to be considered co-first authors

Correspondence should be addressed to P W Jurutka
Email
pjurutka@asu.edu

Abstract

In a closed endocrine loop, 1,25-dihydroxyvitamin D₃ (1,25D) induces the expression of fibroblast growth factor 23 (FGF23) in bone, with the phosphaturic peptide in turn acting at kidney to feedback repress *CYP27B1* and induce *CYP24A1* to limit the levels of 1,25D. In 3T3-L1 differentiated adipocytes, 1,25D represses *FGF23* and *leptin* expression and induces *C/EBPβ*, but does not affect *leptin receptor* transcription. Conversely, in UMR-106 osteoblast-like cells, *FGF23* mRNA concentrations are upregulated by 1,25D, an effect that is blunted by lysophosphatidic acid, a cell-surface acting ligand. Progressive truncation of the mouse *FGF23* proximal promoter linked in luciferase reporter constructs reveals a 1,25D-responsive region between –400 and –200 bp. A 0.6 kb fragment of the mouse *FGF23* promoter, linked in a reporter construct, responds to 1,25D with a fourfold enhancement of transcription in transfected K562 cells. Mutation of either an ETS1 site at –346 bp, or an adjacent candidate vitamin D receptor (VDR)/Nurr1-element, in the 0.6 kb reporter construct reduces the transcriptional activity elicited by 1,25D to a level that is not significantly different from a minimal promoter. This composite ETS1–VDR/Nurr1 *cis*-element may function as a switch between induction (osteocytes) and repression (adipocytes) of *FGF23*, depending on the cellular setting of transcription factors. Moreover, experiments demonstrate that a 1 kb mouse *FGF23* promoter–reporter construct, transfected into MC3T3-E1 osteoblast-like cells, responds to a high calcium challenge with a statistically significant 1.7- to 2.0-fold enhancement of transcription. Thus, the *FGF23* proximal promoter harbors *cis* elements that drive responsiveness to 1,25D and calcium, agents that induce *FGF23* to curtail the pathologic consequences of their excess.

Key Words

- ▶ gene regulation
- ▶ hormone receptors
- ▶ adipose
- ▶ transcription factors
- ▶ vitamin D

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Introduction

The vitamin D receptor (VDR) regulates transcription in response to its 1,25-dihydroxyvitamin D₃ (1,25D) ligand by forming a heterodimer with one of the retinoid X

receptors (RXRs) and binding to vitamin D responsive elements (VDREs) near or remote to each target gene (Haussler *et al.* 2013). Fibroblast growth factor 23 (FGF23)

is an osteoblast/osteocyte-elaborated, phosphaturic hormone that feedback represses *CYP27B1* and induces *CYP24A1* to limit the levels of active 1,25D (Haussler *et al.* 2012, Quarles 2012). *FGF23* expression is governed by a complex network of hormones, growth factors, and minerals, including 1,25D, parathyroid hormone (PTH), leptin, cortisol, FGF2, iron, calcium, and phosphate (David *et al.* 2013, Saini *et al.* 2013, Lanske & Razzaque 2014). VDR-null mice possess vanishingly low circulating levels of FGF23 (Yu *et al.* 2005), indicating that the skeletal secretion of FGF23 is dependent on the presence of VDR. The precise mechanism by which 1,25D, via the nuclear VDR, regulates *FGF23* gene transcription is yet to be defined. Kolek *et al.* (2005) originally reported that in osteocyte-like UMR-106 cells, FGF23 mRNA levels are dramatically upregulated by 1,25D. This effect was reproduced, *in vivo*, with 1,25D-injected mice displaying increased FGF23 mRNA in tibia and calvaria, accompanied by a striking enhancement in circulating immunoreactive FGF23 protein (Kolek *et al.* 2005). Induction of FGF23 by 1,25D in UMR-106 cells is sensitive to inhibition by cycloheximide (Kolek *et al.* 2005, Haussler *et al.* 2010), suggesting that the transcriptional effect may be secondary and dependent on the induction of an intermediary transcription factor. However, the time course of FGF23 mRNA upregulation by 1,25D in UMR-106 cells, with the first observable effect at 2 h and the peak effect at 12 h post 1,25D treatment, is essentially identical to that of osteopontin mRNA induction by 1,25D (R K Saini, unpublished). This observation is puzzling because the action of 1,25D on osteopontin transcription is insensitive to cycloheximide (Haussler *et al.* 2010), rendering it a definitive primary effect that occurs in temporal concert with the apparently more complex induction of FGF23.

Employing ROS 17/2.8 osteoblast-like cells, Liu *et al.* (2006) independently confirmed the findings of Kolek *et al.* (2005), reporting a substantial induction of FGF23 by 1,25D that was prevented by cotransfection with a dominant negative VDR plasmid (Jurutka *et al.* 1997). They also identified a candidate VDRE in the form of a direct repeat with a spacer of three nucleotides (DR3) VDRE, AGGTTActgAGTTCC, located at –1124 bp in relation to the transcription start site in the mouse *FGF23* gene (Liu *et al.* 2006). Importantly, site-directed mutagenesis of this VDRE in the context of a mouse *FGF23* promoter–reporter construct compromised the ability of 1,25D to stimulate transcription (Liu *et al.* 2006). The results of Liu *et al.* (2006) provide evidence that 1,25D induces *FGF23* in a primary fashion, mediated by VDR

binding to a VDRE near the proximal promoter of the mouse *FGF23* gene. However, it has been reported that the FGF23 gene region in mouse osteocytes is not marked by detectable 1,25D-dependent VDR/RXR binding sites when ChIP-Seq analysis is carried out (St John *et al.* 2014). Therefore, the *in vivo* relevance of VDREs residing in the mouse *FGF23* gene remains to be confirmed.

Recently, functional VDREs have been identified in the human *FGF23* gene at –35.7 kb (GGGAGAat-gAGGGCA), at –16.2 kb (TAACCCtgccttAGTTCA, an everted repeat with a spacer of six nucleotides), and at +8.6 kb (AGGGCAGgaAGGACA) in relation to the transcription start site (Saini *et al.* 2013). Notably, these human VDREs are located at some distance from the promoter, but each occurs in a cluster of binding sites for C/EBP and RUNX2 (Saini *et al.* 2013), indicating they may lie in *cis*-regulatory modules (CRMs) for control by the vitamin D hormone of osteoblast-expressed genes (Meyer *et al.* 2010). Based on the observation that FGF23 induction by 1,25D is at least partially cycloheximide sensitive (Haussler *et al.* 2010) and the fact that 1,25D upregulates ETS1, a transcription factor that cooperates with VDR (Dittmer 2003), we (Saini *et al.* 2013) previously concluded that 1,25D induces human FGF23 production directly (primarily) via multiple VDREs and indirectly (secondarily) via stimulation of ETS1 expression, with VDR and ETS1 cooperating in the induction of FGF23 through DNA looping and the generation of euchromatin architecture (Saini *et al.* 2013).

The present communication reports that 1,25D represses FGF23 expression in adipocytes, contrasting with the induction of FGF23 by 1,25D in osteocytes. Thus, the directionality of FGF23 regulation by 1,25D is cell selective. Evidence also is provided for an ETS1–VDRE/Nurr1 composite element that is conserved across species in the proximal promoter of the *FGF23* gene. Combined with our previous report that 1,25D induces *ETS1* (Saini *et al.* 2013), we conclude that this ETS1–VDRE/Nurr1 composite element may play a central role in a combined primary and secondary regulation of *FGF23* mRNA by the vitamin D hormone.

Materials and methods

Cell culture

Five mammalian cell lines (from the American Type Culture Collection, Manassas, VA, USA) were employed in this study. Rat osteoblast/osteocyte-like (UMR-106) cells were cultured in DMEM/F12 (Hyclone Laboratories,

Logan, UT, USA) supplemented with 2.5 mM L-glutamine, and human embryonic kidney 293 (HEK293) cells were grown also in DMEM (Hyclone). Mouse embryonic pre-adipose (3T3-L1), preosteoblast (3T3-E1), and human K562 myelogenous leukemia cells were cultured with DMEM/high glucose (Hyclone). All cells were passaged in the above indicated media supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin under a humidified atmosphere of 5% CO₂ in air at 37 °C. K562 cells were maintained as suspensions but received treatments identical to those of adherent cultures. Mouse 3T3-L1 cells were differentiated to adipocytes as follows: attached cells were incubated with Differentiation Medium A (DMEM/high glucose/10% FBS/penicillin/streptomycin + 1 µg/ml insulin + 0.25 µM dexamethasone + 0.5 mM isobutylmethylxanthine) for 3 days, followed by 4 days (with medium change after the first 2 days) in Differentiation Medium B (DMEM/high glucose/10% FBS–penicillin/streptomycin + 1 µg/ml insulin). On day 7, cells were treated with hormone or ethanol vehicle, followed by harvest on day 8.

Oil Red O staining

3T3-L1 cells or adipocytes were washed twice with PBS, fixed in 10% formalin for 10 min at RT and washed again twice with PBS. Cells were then stained with a filtered Oil Red O solution (in 60% isopropanol) for

30 min at RT. Cells were washed twice with PBS and visualized (Fig. 1).

Plasmid constructs

The 1.0 kb mouse FGF23 promoter and truncations thereof (0.6, 0.4, 0.2, and 0.06 kb) in the reporter construct pGL3-basic were a kind gift from Drs Mikiko Ito and Ken-ichi Miyamoto, Tokushima University, Tokushima City, Japan (Ito *et al.* 2005). Mutations of the VDRE and ETS1 binding sites in the 0.6 kb mouse FGF23 promoter construct were carried out using the following oligonucleotides to introduce the mutations: to mutate the ETS1 site, 5'-gtactgctggctgccttcacaCTAAATgatggaagtggg-gac-3' (ETS1 site capitalized, mutated bases underlined); to mutate the VDRE/Nurr1 site, 5'-cctgatggaAGTGGGgacTT-GTCAacaaatgaccagg-3' (VDRE/Nurr1 site capitalized, mutated bases underlined). To create single mutations, the indicated oligonucleotide and its complement were utilized along with the 0.6 kb mouse FGF23 reporter construct and a Quick Change II XL Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. To construct the double mutation, the MutETS1 plasmid was further mutagenized using the VDRE oligonucleotide pair described above. Successful mutation in each case was verified by DNA sequencing of the MutETS1, MutVDRE, and double (2 × Mut) plasmids.

Transfection and dual luciferase reporter assay

Transfection of K562 cells in suspension was performed by plating 2 × 10⁵ cells/well in 24-well plates, then using PolyJet reagent (SignaGen Laboratories, Gaithersburg, MD, USA) according to the manufacturer's instructions. Briefly, each well received 2.0 µl PolyJet reagent, 500 ng of the mouse FGF23 promoter constructs to be tested, 100 ng of pSG5-hVDR (plasmid expressing human VDR), and 20 ng of pRL-null (Renilla luciferase reporter). After transfection, each well was treated with either 1,25D (final concentrations of 10 nM) or an ethanol vehicle for 20 h at 37 °C. Phosphate- or calcium-treated cells were treated with the appropriate dilution of 1 M sodium phosphate or 4.2 M CaCl₂ in water. After incubation, cells were washed with PBS and lysed in 1 × passive lysis buffer (Promega). Lysates were harvested and analyzed sequentially for Firefly luciferase and Renilla luciferase activity using a Dual Luciferase Assay Kit (Promega Corp., Madison, WI, USA) and a Sirius Luminometer (Zylux Corp., Huntsville, AL, USA) according to the manufacturers' protocols.

Figure 1

Oil Red O staining shows differentiation of MC3T3-L1 cells into adipocytes. (A) Undifferentiated 3T3-L1 cells cultured in standard medium. (B) 3T3-L1 cells maintained in growth medium. (C) 3T3-L1 cells cultured in differentiation medium for 7 days and stained with Oil Red O as described in 'Materials and methods'. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-15-0225>.

Quantitative real-time PCR

HEK293 cells were plated at 1×10^6 cells in a 60 mm dish. UMR-106 cells were plated at 5×10^5 cells/well in a six-well plate, allowed to attach overnight, then treated with lysophosphatidic acid (LPA) and analogues, 1,25D, or ethanol vehicle in media containing 1% charcoal/dextran stripped FBS for 24 h. LPA and Ki16425 (Cayman Chemical Co., Ann Arbor, MI, USA) and OMPT (Avanti Polar Lipids, Alabaster, AL, USA) were dissolved in ethanol and used as described in the figure legends.

Cells were harvested and total cellular RNA was isolated utilizing an Aurum Total RNA Mini Kit (Bio-Rad). The RNA obtained was quantified using A260/280 spectrophotometry. DNase-treated RNA (1 µg) was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). The obtained cDNA was used in 20 µl PCRs containing 10 µl FastStart Universal SYBR Green Master (Roche) and primers. Reactions were performed in 96-well PCR plates on an 7500 Fast Instrument (Applied Biosystems, Waltham, MA, USA). Data were analyzed using the comparative Ct method as the 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 $2^{-\Delta\Delta C_t}$ 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'-ACGGAACACCC-CATCAGACTATC-3' and reverse, 5'-TATCACTACGGAGC-CAGCATCCTC-3'; rat *CYP24A1*: forward, 5'-GATCA-CCTTCCAAGAAGGAAGT-3' and reverse, 5'-AGAGAATC-CACATCAAGCTGTTC-3'; rat *GAPDH*: forward, 5'-AGG-TCGGTGTGAACGGATTG-3' and reverse, 5'-CATTCT-CAGCCTTGACTGTGCC-3'; human *GAPDH*: forward, 5'-ACAACCTTGGTATCGTGAAGGAC-3' and reverse, 5'-CAGGGATGATGTTCTGGAGAG-3' or forward, 5'-TGA-CAACTTTGGTATCGTGAAGG-3' and reverse, 5'-AGG-GATGATGTTCTGGAGAGCC-3'; mouse *GAPDH*: forward, 5'-TTCCGTGTTCTACCCCCAATG-3' and reverse, 5'-TGC-CTGCTTACCACCTTCTT-3'; human *CYP24A1*: forward, 5'-CAGCGAACTGAACAAATGGTCG-3' and reverse, 5'-TCT-CTTCTCATACAACACGAGGCAG-3'; mouse *Cyp24a1*: forward, 5'-CGTTCTGGGTGAATACACGCTAC-3' and reverse, 5'-TTCGGGTCTAACTTGTCAGCATC-3'; mouse *leptin*: forward, 5'-GTGCCTATCCAGAAAGTCCAG-3' and reverse, 5'-TGAAGCCAGGAATGAAGTC-3'; mouse *leptin receptor (ObR)*: forward, 5'-GCATGCAGAAT-CAGTGATATTTGG-3' and reverse, 5'-CAAGCTGTA-TCGACACTGATTTCTTC-3'; human *FGF23*: forward, 5'-TGCTGGCTTTGTGGTGATTA-3' and reverse, 5'-TTC-TCCGGGTGCGAAATAGTG-3'; mouse *FGF23*: forward,

5'-GGTGATAACAGGAGCCATGAC-3' and reverse, 5'-TGCTTCTGCGACAAGTAGAC-3'; mouse *C/EBPβ*: forward, 5'-AGCGGCTGCAGAAGAAGGT-3' and reverse, 5'-GGCAGCTGCTTGAACAAGTTC-3'; mouse *PPARγ*: forward, 5'-CGCTGATGCACTGCCTATGA-3' and reverse, 5'-AGAGGTCCACAGAGCTGATTCC-3'; mouse *resistin*: forward, 5'-TCAACTCCCTGTTTCCAAATGC-3' and reverse, 5'-TCTTCACGAATGTCCACGA-3'; and mouse *aP2*: forward, 5'-CATGGCCAAGCCCAACAT-3' and reverse, 5'-CGCCCAGTTTGAAGGAAATC-3'.

Statistical analysis

Data are expressed as means $\pm$ s.d. Statistical differences between two groups were determined by a two-sided Student's *t*-test. Differences among multiple groups were analyzed by ANOVA. A *P* value < 0.05 was considered significant.

Results

1,25D represses the expression of *FGF23* in 3T3-L1 differentiated adipocytes

Previously, only the upregulation of *FGF23* mRNA expression by 1,25D has been reported in mammalian cells (Haussler *et al.* 2012). However, as illustrated in Fig. 2, in differentiated 3T3-L1 mouse adipocytes, *FGF23* is significantly and dose-dependently repressed by 1,25D. When undifferentiated 3T3-L1 cells are transferred from growth medium (GM) to differentiation medium (DM), *FGF23* expression is reduced by 50%, followed by a further suppression of *FGF23* mRNA by 1,25D that is detectable at a hormone concentration as low as 0.1 nM, but first becomes statistically significant at 1.0 nM 1,25D (Fig. 2). The inset to Fig. 2 depicts the average of three independent experiments like that illustrated in the main body of the figure, verifying the 1,25D dose-dependency of *FGF23* suppression in differentiated 3T3-L1 adipocytes.

Vitamin D hormone control of other adipocyte-expressed genes

To determine if the repression of *FGF23* by 1,25D in adipocytes is unique, the effect of the hormone on other adipocyte-expressed genes was investigated. Initially, for reference, after 3T3-L1 cells are transferred from GM to DM, *leptin* is induced sixfold, *leptin receptor* is repressed 20-fold, and *Cyp24a1* is relatively unchanged (data not shown). As shown in Fig. 3A and C, *leptin* expression is downregulated by 1,25D in 3T3-L1 differentiated adipocytes. This effect is statistically significant at 100 nM 1,25D, but because of the

Figure 2

FGF23 mRNA repression by 1,25D in 3T3-L1 adipocytes. 3T3-L1 cells were grown as described in 'Materials and methods' and treated with 1,25D for 24 h at the indicated concentrations. mRNA levels for *FGF23* were determined by real-time PCR. GM, growth medium; DM, differentiation medium. The inset depicts the average of three independent experiments identical to that illustrated in the main body of the figure, except that only doses of 1, 10, and 100 nM 1,25D were tested. Each bar represents the average of three independent experiments performed in triplicate $\pm$ s.d. All statistics are calculated relative to the DM EtOH set at 1.0. ** $P < 0.01$ and *** $P < 0.001$.

high variability of these data, it is difficult to define dose responsiveness. Nevertheless, repression of *leptin* is evident and is unusually rapid in that maximal repression occurs within only 2 h of hormone treatment (data not shown). In contrast, *leptin receptor* mRNA is unaffected by treatment of 3T3-L1 differentiated adipocytes with 1,25D (Fig. 3B).

Figure 3

1,25D represses *leptin* mRNA while inducing *Cyp24a1* in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with 1,25D for 24 h at the indicated concentrations, and mRNA levels for the indicated gene products were determined by real-time PCR (A, B and D) or by gel electrophoresis of PCR products after 40 cycles (C). *Cyp24a1* is a positive control known to be

Cyp24a1 was employed as a highly induced positive control for VDR-mediated 1,25D action to ensure that the tested cells expressed VDR appropriately and responded to the 1,25D ligand in the expected fashion. Indeed, Fig. 3D illustrates a dramatic, dose-dependent enhancement of *Cyp24a1* mRNA concentrations elicited by the vitamin D hormone (nearly 500-fold at the maximal dose of 100 nM), indicating that the differentiated 3T3-L1 mouse adipocyte system represents a valid model for probing VDR-targeted gene expression. Furthermore, as summarized in Fig. 4, among other genes expressed in 3T3-L1 differentiated adipocytes, 1,25D induces *C/EBP β* , whereas *PPAR γ* , *resistin*, and *aP2* are all regulated biphasically, with induction by 1 nM 1,25D and dose-dependent repression by 10–100 nM hormone. Thus, among the genes regulated by 1,25D in adipocytes, *FGF23* stands out as unique with respect to its unequivocal repression by 1,25D in a classic dose-dependent fashion. To assess the physiologic significance of *FGF23* repression by 1,25D in adipose, it will ultimately be necessary to monitor *FGF23* in intact mouse and human fat tissue to investigate possible *in vivo* target pathways affecting metabolism.

1,25D induces the expression of *FGF23* in UMR-106 osteoblasts; modulation by LPA

To contrast *FGF23* regulation in adipocytes vs in its skeletal endocrine source, UMR-106 osteoblast/osteocyte-like cells were treated with 1,25D, as well as with LPA, a known potentiator of 1,25D action to induce osteoblast maturation and alkaline phosphatase expression (Gidley *et al.* 2006, Mansell & Blackburn 2013). As illustrated in Fig. 5A, 1,25D dramatically (1920-fold) induces *FGF23* in UMR-106 cells, as previously observed in our laboratory and by others (Kolek *et al.* 2005, Liu *et al.* 2006, Bergwitz &

upregulated by 1,25D. Statistical significance is depicted in the body of each panel. Each bar represents the average of three independent experiments performed in triplicate $\pm$ s.d. *1,25D-treated groups statistically significantly different from EtOH control ($P < 0.05$).

Figure 4

1,25D induces *C/EBPβ* mRNA and biphasically regulates *PPARγ*, *resistin*, and *aP2* in 3T3-L1 adipocytes. 3T3-L1 cells were grown as described in 'Materials and methods' and the legend to Fig. 2, then treated with 1,25D for 24 h at the indicated concentrations, and mRNA levels were determined by real-time PCR for the following genes: (A) *C/EBPβ*, (B) *PPARγ*, (C) *resistin*, and (D)

aP2. GM, growth medium; DM, differentiation medium. Each bar represents the average of three independent experiments performed in triplicate $\pm$ s.d. All statistics are calculated relative to the DM EtOH set at 1.0. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Juppner 2010). Curiously, LPA and its OMPT agonist blunt the induction of *FGF23*, whereas the LPA antagonist, Ki16425, slightly amplifies the upregulation of *FGF23* by 1,25D (Fig. 5A). A very similar profile of modulation by LPA agonists/antagonists emerges (Fig. 5B) with respect to the induction of the positive control gene, *Cyp24a1*, by 1,25D in UMR-106 cells. In contrast, when HEK293 cells are similarly probed for 1,25D control of *FGF23* gene expression and its modulation by LPA (Fig. 6A), the results differ markedly from those obtained in osteoblasts. Specifically, *FGF23* expression is not affected by 1,25D in HEK293 cells (Fig. 6A), whereas LPA agonists enhance the action of 1,25D to induce *CYP24A1* positive control mRNA (Fig. 6B). The LPA antagonist Ki16425 had no significant effect on 1,25D action in these experiments. Thus, LPA acts more traditionally as a 1,25D potentiator of *CYP24A1* induction in HEK293 cells as opposed to its reverse effect in UMR-106 osteoblasts. This finding indicates that not only is *FGF23* regulation by 1,25D cell selective in terms of its directionality but also modulation of this effect by LPA agonists/antagonists differs according to the cell setting.

Dissection of the mouse *FGF23* promoter by informatics, truncation, and mutagenesis

We have reexamined the mouse *FGF23* proximal promoter (DNA sequence listed in Fig. 7) for transactivator binding sites that might mediate control of *FGF23* mRNA expression. As depicted in Fig. 7, we observed a collection of conserved, consensus *cis* elements between -110 and -347 bp in relation to the start of transcription. Most prominent in this collection are three ETS1, two Nurr1 (bold type), two GATA, and single AP1 and LEF1 sites. It is noteworthy that AP1 and LEF1 *cis* elements are often

associated with VDREs in 1,25D-regulated genes (Ozono *et al.* 1990, Luderer *et al.* 2011). Meir *et al.* (2014) recently provided evidence that PTH induces *FGF23* in osteoblasts via activation of Nurr1, a transcription factor involved in the development of midbrain dopaminergic neurons (Sakurada *et al.* 1999). Strikingly, one of the conserved Nurr1 sites in the *FGF23* promoter (Meir *et al.* 2014) exactly overlaps the 3' putative half element in the proposed new VDRE (Fig. 7). Equally important, ETS1, which was implicated as a partner with VDR in controlling *FGF23* in a previous study (Saini *et al.* 2013), possesses a *cis*-docking site in the mouse *FGF23* promoter that is only 6 bp 5' of a newly discovered candidate VDRE/Nurr1 (AGTGGGgacAGGTCA) site at -334 bp (highlighted in light purple in Fig. 7). This novel VDRE is distinct from the VDRE reported by Liu *et al.* (2006) at -1124 bp (highlighted in dark gray in Fig. 7). We hypothesized that the -110 to -346 bp region of the *FGF23* promoter constitutes a proximal CRM anchored by the adjacent ETS1 and VDRE sites. To test this hypothesis, we dissected the mouse *FGF23* promoter kindly supplied by Drs Mikiko Ito and Ken-ichi Miyamoto of the Tokushima University (Ito *et al.* 2005). By employing progressively truncated promoter-reporter constructs, we obtained evidence that a VDRE is located somewhere between -200 and -400 bp in the mouse *FGF23* promoter (Fig. 8A and B). The activity of the *FGF23* promoter fragments was unaffected by phosphate concentration (Fig. 8A vs B), suggesting that phosphate-responsive elements must reside outside of the 1.0 kb promoter. To determine if the VDRE localized to the -200 to -400 interval corresponds to the newly revealed candidate VDRE shown in Fig. 7, this VDRE/Nurr1 and/or its adjacent ETS1 site were inactivated by point mutation within the context of a -0.6 kb promoter

Figure 5

Induction of *FGF23* and *Cyp24a1* mRNAs by 1,25D in UMR-106 rat osteoblast/osteocyte-like cells and its modulation by lysophosphatidic acid (LPA). UMR-106 cells were treated for 24 h with 20 μ M LPA, 10 μ M OMPT (an LPA agonist), 10 μ M Ki16425 (an LPA antagonist), 10 nM 1,25D, or combinations thereof as indicated. mRNA levels were determined by real-time PCR for the following genes: (A) *FGF23* and (B) *Cyp24a1*. Each bar represents the average of three independent experiments performed in triplicate $\pm$ s.d. All statistics are calculated relative to the EtOH control, set at 1.0. * P <0.05, ** P <0.01, and *** P <0.001.

fragment-luciferase construct and the mutated plasmids evaluated for 1,25D responsiveness. As illustrated in Fig. 8C, the -0.6 kb promoter fragment-luciferase construct yields a fourfold response to 1,25D. This effect is significantly diminished to a 2.2- to 2.4-fold by the mutation of either the VDRE/Nurr1 or ETS1 site, or both

Figure 6

Control of *FGF23* and *CYP24A1* mRNAs by 1,25D in HEK293 embryonic human kidney cells and its modulation by lysophosphatidic acid (LPA) agonists/antagonists. HEK293 cells were treated for 24 h with 20 μ M LPA, 10 μ M OMPT (an LPA agonist), 10 μ M Ki16425 (an LPA antagonist), 10 nM 1,25D, or combinations thereof as indicated. mRNA levels were determined by real-time PCR for the following genes: (A) *FGF23* and (B) *CYP24A1*. Each bar represents the average of three independent experiments performed in triplicate $\pm$ s.d. All statistics are calculated relative to the EtOH control, set at 1.0. * P <0.05 and *** P <0.001.

VDREs: **ACTGGGgacAGGTCA** Sense **GGAACtcagTAACCT** Antisense
ETS1 site **GATA Site** **Lef1 site** **CRE/AP1 site** **Bold type= Nurrl site**

–1158
 TGATGCTATCTTGTGGGATACTCATCTGTTGTA**GGAACtcagTAACCT**GGGACACTGAGTGAGCAGTCCTTTT
 CTTGTGGCCATATACTGCTGATGTCTGTCCAGCAGTCTGCCTTCCAATGGACTGTGTGTGTAGTCTGCCTTC
 AACCTCCTACCCACACACCACACCCACACCCACACCCGCTGCATCAGACGCACCTCCACAGAGGGCATGCAC
 AGAGCAATTTCTGCAGTGAGGCATCCACTCATACAAGGTCCCAGGGTGTGGACATGGGCTGGGGAGGTGCCAG
 TCCTGCAGCTCCTGGATGCTTCTGCCCCTCTGATTCCTCTGATCAGCCAGCAGTGCCGTTCCAGTCTCCAAAT
 GAGTCTCCTTCTATTGGCAAAGCCATAATTGCCAGTTTAGTTCCCTGCCTCATCCAGACGAGGGAACCTGAGA
 AACCAGATCTTGCCATTTTGTGACCTCAAGACCTTGTCTTCTTCTTCTGCTTGGGATGAGTCTTTGGGAATGG
 TGATGGGAGGTGTTGAGCTCATGCTCCCACCTAACACCTTCTCTGGGGTTTGTGTATGGGGGTAGGGGGAGTC
 TCATTGCGCTGATAGCATCACTTATGACCATATATCAAGACACTTGCCAGATGCAACAGCCAGGAGTAAGCTCC
 AAGAACACACTTGGCAGCTGGAGGAAAAGGGCTTAAGCAAAACAAAGGACACTGGAGGGAGATGAGTTAG
 CGAGGAGGCGCTTTCTGGTTTCTGGGGTTTTTTTGTGTTTGTGTTTTCAGTACTGCTGGCTGCCCTTCCACAT
TCCTGATGGA**AGTGGGgacAGGTCA**ACAATGACCCAGGGTCACA**GATAA**CTTTGCCACACATCATTCACTT
 ATGGGAGCACTGGCTTGAATTTGAGGGGTGTGTGCGTGCATGTATGTGTGCTGGAACCTGACGCGC**CTTCCG**
 CAAGCCTAAGAAGTCTGGGCTTTTCTTTGAATGGAT**GATTA**CAACACAG**AGGATC**TGGCGGCATTGTTTTTCC
 TGCT**TGATGTCA**CACCACACCC**TTTAAA**AGTCCCGGGGAAAAAGGAGGGAATCTAGCCACAGATCCCACCT
 CAGTTCTCAGCTTCTTCTAGGAAGAAGAG**AAAGGCCAG**CAAGGGCCAGCCTGTCTGGGAGTGTCAATTCA
 AACTCAGCATTAGCCACTCAGTGTGTGCA**ATC**

Figure 7

Nucleotide sequence of the mouse *FGF23* promoter. The color-coded legend for *cis* elements is depicted above the sequence. Nurrl sites are depicted in bold type. The transcription start site begins at the 5' end of the

underlined sequence. The TATA-box and the ATG start codon for translation are highlighted in light green. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-15-0225>.

simultaneously, with all responses by the mutated promoters not significantly different from the minimal promoter (–0.06 kb) control construct. The observation that the inactivation by site-directed mutagenesis of either the VDRE/Nurrl or ETS1 elements abolishes transcriptional stimulation by 1,25D (Fig. 8C) provides evidence for a novel composite ETS1–VDRE/Nurrl *cis* element that may be central to the CRM in the mouse *FGF23* promoter that mediates at least part of the response of this gene to induction by 1,25D. Finally, we probed the responsiveness of the *FGF23* promoter to calcium, as David *et al.* (2013)

recently called attention to calcium as a stimulator of *FGF23* secretion by osteoblasts. Indeed, as illustrated in Fig. 8D, high calcium significantly (1.7- to 2.0-fold) induces the –1.0 kb mouse *FGF23* promoter–reporter construct when it is transfected into MC3T3-E1 osteoblast-like cells. Therefore, as documented by data in Fig. 8C and D, we conclude that 1,25D and calcium comprise *FGF23* inducers that function by stimulating the proximal promoter of the mouse gene and likely other species, as the CRM components of this promoter are largely conserved across mammalian species.

Figure 8

Dissection of the mouse *FGF23* proximal promoter. (A) A promoter fragment containing 1.0 kb of promoter sequence (1.0K) was linked to a luciferase reporter and transfected into K562 cells. Transcription of the reporter gene was stimulated three- to fourfold by 1,25D in the presence of 0.9 mM (low) phosphate. This transcriptional effect is significantly reduced to less than twofold when progressively 5'-truncated promoter fragments (e.g., 0.4K to 0.2K) are tested. (B) The profile of transcriptional stimulation by 1,25D of *FGF23* promoter fragments, transfected into K562 cells, is essentially unaffected by the 3.0 mM (high) phosphate concentration when compared

to the 0.9 mM phosphate results. (C) When transfected into K562 cells, a –0.6 kb promoter fragment–luciferase construct of the mouse *FGF23* promoter yields a fourfold response to 1,25D. This effect is significantly diminished to 2.2- to 2.4-fold by the mutation of either the VDRE or ETS1 site, or both simultaneously, with all responses not significantly different from the minimal promoter (–0.06 kb) control construct. (D) High (6.0 mM) calcium, but not high (3.0 mM) phosphate, significantly (1.7- to 2.0-fold) induces the –1.0 kb mouse *FGF23* promoter–reporter construct when it is transfected into MC3T3-E1 osteoblast-like cells. **P* < 0.05 and ****P* < 0.001.

Discussion

Like PTH, FGF23 inhibits renal Npt2a and Npt2c to elicit phosphaturia (Shimada *et al.* 2004). In contrast to PTH that is downregulated by 1,25D in parathyroid glands, FGF23 is upregulated by 1,25D in osteocytes (Kolek *et al.* 2005, Liu *et al.* 2006, Bergwitz & Juppner 2010), which are a major source of endocrine FGF23 production by bone. Hyperphosphatemia enhances osteocytic FGF23 production independently of 1,25D, rendering FGF23 the ideal phosphaturic counter-1,25D hormone because it inhibits renal phosphate reabsorption and 1,25D biosynthesis via repression of CYP27B1, while enhancing 1,25D degradation by inducing CYP24A1 in all tissues (Shimada *et al.* 2004). In this fashion, FGF23 allows osteocytes to communicate with the kidney to govern circulating 1,25D as well as phosphate levels, thereby preventing excess 1,25D function and hyperphosphatemia, either of which lead to ectopic calcification.

In the past year, new actions of FGF23 have been revealed via the generation of FGF23-null mice. For example, FGF23 deficiency leads to mixed hearing loss and middle ear malformation in mice (Lysaght *et al.* 2014). FGF23 also regulates renal sodium handling and blood pressure (Andrukhova *et al.* 2014a), as well as cardiac structure and function (Agarwal *et al.* 2014). Additionally, FGF23 is a negative regulator of prenatal and postnatal erythropoiesis (Coe *et al.* 2014) and a stimulator of renal calcium reabsorption through the TRPV5 channel (Andrukhova *et al.* 2014b). Many, but not all, of these novel actions of FGF23 involve the kidney and the vascular system. The function of FGF23 in adipose is unknown, but in the present communication we report a surprising repression of FGF23 expression by 1,25D in adipocytes, contrasting with all previous reports of FGF23 induction by 1,25D in other cell types. The mechanism of this repression is also unknown and is apparently unique for 1,25D control of adipose-expressed genes, differing in direction and 1,25D dose response when compared with the regulation of genes such as *C/EBP β* , *PPAR γ* , *resistin*, *aP2*, the *leptin receptor*, and *Cyp24a1*. The only similarity between the effect of 1,25D on FGF23 and another gene is that of leptin, a gene product that itself induces FGF23. Thus, the influence of 1,25D to repress adipose FGF23 may be related to curbing some deleterious off-target effect of FGF23 in the adipocyte, such as adipose growth (Oldknow *et al.* 2015) or malignancy (Jacobs *et al.* 2011). With respect to obesity, FGF23 has been implicated in the regulation of energy metabolism, and mouse models with elevated FGF23 display increased adiposity (Oldknow *et al.* 2015),

suggesting that 1,25D attenuation of FGF23 may curtail obesity. We (Kolek *et al.* 2005) originally reported that, in osteocyte-like UMR-106 cells, FGF23 mRNA levels are dramatically upregulated by 1,25D. We later determined that this FGF23 induction is potentiated by leptin and inhibited by interleukin 6 (Saini *et al.* 2013), further linking FGF23 homeostasis with adipose hormones and cytokines, possibly implicating FGF23 repression by 1,25D in the modulation of energy metabolism and the prevention of malignancy.

In the current study, we reexamined the mouse FGF23 proximal promoter for transactivator binding sites that might mediate control of FGF23 mRNA expression. Notably, ETS1, which was implicated as a partner with VDR in controlling FGF23 in a previous study (Saini *et al.* 2013), possesses a *cis*-docking site in the mouse FGF23 promoter that is only 6 bp 5' of a newly discovered candidate VDRE/Nurr1 site (AGTGGGGacAGGTCA, highlighted in light purple in Fig. 7). By employing progressively truncated promoter-reporter constructs, we deduced that a 1,25D-responsive region must be present between –200 and –400 bp in the mouse FGF23 promoter (Fig. 8A and B). When two elements within this interval, namely the candidate VDRE/Nurr1 site at –334 bp and/or its adjacent ETS1 site at –346 bp, were inactivated by point mutation, transcriptional activation was diminished to a level not significantly different from that of the minimal promoter (–0.06 kb) control construct (Fig. 8C). Therefore, it was concluded that the –200 to –399 bp region of the mouse FGF23 promoter contains a CRM anchored by the adjacent ETS1 and VDRE/Nurr1 sites.

With respect to the 1,25D induction of murine *FGF23*, there are other considerations for the –399 bp region of the *FGF23* promoter (Fig. 7). In addition to the ETS1–VDRE/Nurr1 composite element, there exist two additional ETS1 sites that are conserved across species, as well as two conserved GATA *cis* elements of unknown significance (Barthel *et al.* 2007). The ETS1 protein is known to complex with VDR (Dittmer 2003) and to confer ligand- and AF2-independent transcriptional activation properties on nuclear receptors such as VDR (Tolon *et al.* 2000). The multiple conserved ETS1 *cis* elements in the FGF23 gene region (Saini *et al.* 2013) therefore render this transcription factor a likely candidate for the recruitment of VDR for the purpose of regulating *FGF23* gene expression. Finally, the ETS1 protein is induced by 1,25D to amplify the response (Saini *et al.* 2013), and this process would be inhibited by cycloheximide, perhaps accounting for the reported cycloheximide sensitivity of the induction of FGF23 by 1,25D in UMR-106 osteocyte-like cells.

Equally interesting is the existence in the *FGF23* of a Lef1 site (Fig. 7), a *cis* element that is often associated with VDREs in 1,25D-regulated genes (Luderer *et al.* 2011). This Lef1 site is yet another target for 1,25D, possibly secondary to the repression of sclerostin (St John *et al.* 2014), a Wnt signaling inhibitor that is crucial in bone remodeling. Moreover, PTH increases *FGF23* mRNA levels, and this effect is mimicked by the protein kinase A (PKA) activator, forskolin. PTH also decreases *SOST* mRNA encoding sclerostin, which is a PTH receptor (PTH1R) target; this action of PTH was abrogated by added sclerostin. Therefore, PTH increases *FGF23* through the PKA and Wnt pathways (Lavi-Moshayoff *et al.* 2010). Thus, by analogy with PTH action on *FGF23* expression, we suspect that the response of *FGF23* expression to 1,25D/VDR may not be a direct one, primarily because VDR has never been observed to associate with putative VDREs in the *FGF23* gene region as monitored by ChIP-Seq analysis (St John *et al.* 2014).

Furthermore, control of *FGF23* transcription by PTH has recently been discovered to be dependent on the induction of *Nurr1* by PTH/PKA signaling (Meir *et al.* 2014). Conserved *Nurr1* elements have been reported (Meir *et al.* 2014) in the *FGF23* proximal promoter and immediately preceding the start site for transcription (as highlighted in bold type in Fig. 7). Although there is a conserved CREB/AP1 site just 11 bp upstream of the TATA-box in the rodent *FGF23* genes (see Fig. 7), Meir *et al.* (2014) demonstrate that the effect of PTH via PKA/cAMP is apparently through induction of *Nurr1*, although it remains possible that the *Nurr1* site just 3' of the TATA-box mediates cooperation between *Nurr1* and CREB to activate transcription. However, Meir *et al.* did not point mutate either of the *Nurr1* sites in their study to demonstrate conclusively that one or both *Nurr1* elements participated in the *FGF23* regulation by PTH (Lanske & Razzaque 2014). In the present experiments, our mutagenesis of the candidate VDRE at -334 bp in the mouse *FGF23* promoter also would inactivate the *Nurr1* site. This maneuver eliminated the response of the promoter to 1,25D (Fig. 7C), but one explanation of this result is that 1,25D/VDR is functioning through *Nurr1* as a secondary transcriptional activation factor as does PTH. We favor the mechanism that 1,25D induces *FGF23* via the primary activation of *Nurr1* expression rather than direct binding of VDR to the *FGF23* promoter. *Nurr1* then would bind to its cognate element adjacent to the *ETS1* element and likely cooperate with *ETS1* to induce *FGF23*. Evidence for this cooperation includes elimination of the 1,25D effect on *FGF23* induction by point inactivation of the *ETS1* site

in question (Fig. 7C). *Nurr1* has, in fact, been shown to interact with *ETS1* in controlling the matrix metalloproteinase 1 promoter in cartilage, but in this case the relationship between the two transcription factors is an antagonistic one that does not require a *Nurr1* consensus element (Mix *et al.* 2007). We do not know if the more proximal *Nurr1* site 3' of the TATA-box is relevant to the (secondary) action of 1,25D but point out that when cells are treated with 1,25D, a significant 1.5- to 2.0-fold increase in reporter expression persists in -0.06 kb truncated promoter-reporter constructs (Fig. 7A, B and C) that retain the proximal *Nurr1* site. This residual effect may indicate that the proximal *Nurr1* site is indeed active. But, more importantly, this new model of 1,25D action is consistent with evidence currently published on the 1,25D induction of *FGF23* as follows: i) the secondary mechanism accounts for the cycloheximide sensitivity of the process (Haussler *et al.* 2010), ii) invoking *Nurr1* is in concert with negative ChIP-Seq results for 1,25D-dependent association of VDR-RXR with putative VDREs in the *FGF23* gene region in osteocytes (St John *et al.* 2014), iii) the secondary mechanism invoking *Nurr1* is entirely consistent with a diminution of the 1,25D effect on the promoter when a *Nurr1* site is altered by mutagenesis, and iv) *Nurr1* expression is reduced in rat brain by developmental vitamin D deficiency (Kesby *et al.* 2013). Moreover, the requirement for a cAMP increase to induce *Nurr1* and, secondarily, *FGF23* in the case of PTH induction of *FGF23*, is likely pertinent to the control of *FGF23* by 1,25D. Notably, we have demonstrated in the present results (Fig. 5A) that LPA, a known activator of G_i to suppress cAMP (Mansell & Blackburn 2013), attenuates the ability of 1,25D to induce *FGF23* in UMR-106 cells. Thus, we conclude that *Nurr1*, in combination with upstream PKA signaling, may be the new key to understanding *FGF23* regulation by the calcemic hormones PTH and 1,25D. Indeed, one of the presentations of McCune-Albright syndrome, in which there is constitutive activation of G_s , is hypophosphatemia and *FGF23* excess (Dumitrescu & Collins 2008). Vitamin D has been associated with the development of the dopaminergic nervous system (Harms *et al.* 2011), a phenomenon dependent on *Nurr1* (Sakurada *et al.* 1999). Therefore, another connection between *Nurr1* and 1,25D action could reside in the CNS. Thus, a more complete characterization of the relationship between 1,25D and *FGF23* could potentially open new vistas far beyond the treatment of bone mineral disorders to include not only energy metabolism and malignancy but also the prevention of neuropsychiatric disease.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Conception and design: I K, R K S, G K W, P W J, and M R H. Development of methodology: P W J, G K W, I K, R K S, and K P G. Acquisition of data: R K S, G K W, K P G, and I K. Writing, review, and/or revision of the manuscript: M R H, G K W, P W J, I K, and R K S. Administrative, technical, or material support: G K W, P W J, and M R H. Study supervision: P W J, G K W, and M R H.

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References

- Agarwal I, Ide N, Ix JH, Kestenbaum B, Lanske B, Schiller NB, Whooley MA & Mukamal KJ 2014 Fibroblast growth factor-23 and cardiac structure and function. *Journal of the American Heart Association* **3** e000584. (doi:10.1161/JAHA.113.000584)
- Andrukhova O, Slavic S, Smorodchenko A, Zeitz U, Shalhoub V, Lanske B, Pohl EE & Erben RG 2014a FGF23 regulates renal sodium handling and blood pressure. *EMBO Molecular Medicine* **6** 744–759. (doi:10.1002/emmm.201303716)
- Andrukhova O, Smorodchenko A, Egerbacher M, Streicher C, Zeitz U, Goetz R, Shalhoub V, Mohammadi M, Pohl EE, Lanske B *et al.* 2014b FGF23 promotes renal calcium reabsorption through the TRPV5 channel. *EMBO Journal* **33** 229–246. (doi:10.1002/embj.201284188)
- Barthel TK, Mathern DR, Whitfield GK, Haussler CA, Hopper HA IV, Hsieh JC, Slater SA, Hsieh G, Kaczmarzka M, Jurutka PW *et al.* 2007 1,25-Dihydroxyvitamin D₃/VDR-mediated induction of FGF23 as well as transcriptional control of other bone anabolic and catabolic genes that orchestrate the regulation of phosphate and calcium mineral metabolism. *Journal of Steroid Biochemistry and Molecular Biology* **103** 381–388. (doi:10.1016/j.jsmb.2006.12.054)
- Bergwitz C & Juppner H 2010 Regulation of phosphate homeostasis by PTH, vitamin D, and FGF23. *Annual Review of Medicine* **61** 91–104. (doi:10.1146/annurev.med.051308.111339)
- Coe LM, Madathil SV, Casu C, Lanske B, Rivella S & Sitara D 2014 FGF-23 is a negative regulator of prenatal and postnatal erythropoiesis. *Journal of Biological Chemistry* **289** 9795–9810. (doi:10.1074/jbc.M113.527150)
- David V, Dai B, Martin A, Huang J, Han X & Quarles LD 2013 Calcium regulates FGF-23 expression in bone. *Endocrinology* **154** 4469–4482. (doi:10.1210/en.2013-1627)
- Dittmer J 2003 The biology of the Ets1 proto-oncogene. *Molecular Cancer* **2** 29. (doi:10.1186/1476-4598-2-29)
- Dumitrescu CE & Collins MT 2008 McCune–Albright syndrome. *Orphanet Journal of Rare Diseases* **3** 12. (doi:10.1186/1750-1172-3-12)
- Gidley J, Openshaw S, Pring ET, Sale S & Mansell JP 2006 Lysophosphatidic acid cooperates with 1 α ,25(OH)₂D₃ in stimulating human MG63 osteoblast maturation. *Prostaglandins & Other Lipid Mediators* **80** 46–61. (doi:10.1016/j.prostaglandins.2006.04.001)
- Harms LR, Burne TH, Eyles DW & McGrath JJ 2011 Vitamin D and the brain. *Best Practice & Research. Clinical Endocrinology & Metabolism* **25** 657–669. (doi:10.1016/j.beem.2011.05.009)
- Haussler MR, Haussler CA, Whitfield GK, Hsieh JC, Thompson PD, Barthel TK, Bartik L, Egan JB, Wu Y, Kubicek JL *et al.* 2010 The nuclear vitamin D receptor controls the expression of genes encoding factors which feed the “Fountain of Youth” to mediate healthful aging. *Journal of Steroid Biochemistry and Molecular Biology* **121** 88–97. (doi:10.1016/j.jsmb.2010.03.019)
- Haussler MR, Whitfield GK, Kaneko I, Forster R, Saini R, Hsieh JC, Haussler CA & Jurutka PW 2012 The role of vitamin D in the FGF23, klotho, and phosphate bone–kidney endocrine axis. *Reviews in Endocrine & Metabolic Disorders* **13** 57–69. (doi:10.1007/s11154-011-9199-8)
- Haussler MR, Whitfield GK, Kaneko I, Haussler CA, Hsieh D, Hsieh JC & Jurutka PW 2013 Molecular mechanisms of vitamin D action. *Calcified Tissue International* **92** 77–98. (doi:10.1007/s00223-012-9619-0)
- Ito M, Sakai Y, Furumoto M, Segawa H, Haito S, Yamanaka S, Nakamura R, Kuwahata M & Miyamoto K 2005 Vitamin D and phosphate regulate fibroblast growth factor-23 in K-562 cells. *American Journal of Physiology. Endocrinology and Metabolism* **288** E1101–E1109. (doi:10.1152/ajpendo.00502.2004)
- Jacobs E, Martinez ME, Buckmeier J, Lance P, May M & Jurutka P 2011 Circulating fibroblast growth factor-23 is associated with increased risk for metachronous colorectal adenoma. *Journal of Carcinogenesis* **10** 3. (doi:10.4103/1477-3163.76723)
- Jurutka PW, Hsieh JC, Remus LS, Whitfield GK, Thompson PD, Haussler CA, Blanco JC, Ozato K & Haussler MR 1997 Mutations in the 1,25-dihydroxyvitamin D₃ receptor identifying C-terminal amino acids required for transcriptional activation that are functionally dissociated from hormone binding, heterodimeric DNA binding, and interaction with basal transcription factor IIB, *in vitro*. *Journal of Biological Chemistry* **272** 14592–14599. (doi:10.1074/jbc.272.23.14592)
- Kesby JP, Cui X, Burne TH & Eyles DW 2013 Altered dopamine ontogeny in the developmentally vitamin D deficient rat and its relevance to schizophrenia. *Frontiers in Cellular Neuroscience* **7** 111. (doi:10.3389/fncel.2013.00111)
- Kolek OI, Hines ER, Jones MD, Lesueur LK, Lipko MA, Kiela PR, Collins JF, Haussler MR & Ghishan FK 2005 1 α ,25-Dihydroxyvitamin D₃ upregulates FGF23 gene expression in bone: the final link in a renal–gastrointestinal–skeletal axis that controls phosphate transport. *American Journal of Physiology. Gastrointestinal and Liver Physiology* **289** G1036–G1042. (doi:10.1152/ajpgi.00243.2005)
- Lanske B & Razzaque MS 2014 Molecular interactions of FGF23 and PTH in phosphate regulation. *Kidney International* **86** 1072–1074. (doi:10.1038/ki.2014.316)
- Lavi-Moshayoff V, Wasserman G, Meir T, Silver J & Naveh-Many T 2010 PTH increases FGF23 gene expression and mediates the high-FGF23 levels of experimental kidney failure: a bone parathyroid feedback loop. *American Journal of Physiology. Renal Physiology* **299** F882–F889. (doi:10.1152/ajprenal.00360.2010)
- Liu S, Tang W, Zhou J, Stubbs JR, Luo Q, Pi M & Quarles LD 2006 Fibroblast growth factor 23 is a counter-regulatory phosphaturic hormone for vitamin D. *Journal of the American Society of Nephrology* **17** 1305–1315. (doi:10.1681/ASN.2005111185)
- Luderer HF, Gori F & Demay MB 2011 Lymphoid enhancer-binding factor-1 (LEF1) interacts with the DNA-binding domain of the vitamin D receptor. *Journal of Biological Chemistry* **286** 18444–18451. (doi:10.1074/jbc.M110.188219)
- Lysaght AC, Yuan Q, Fan Y, Kalwani N, Caruso P, Cunnane M, Lanske B & Stankovic KM 2014 FGF23 deficiency leads to mixed hearing loss and middle ear malformation in mice. *PLoS ONE* **9** e107681. (doi:10.1371/journal.pone.0107681)
- Mansell JP & Blackburn J 2013 Lysophosphatidic acid, human osteoblast formation, maturation and the role of 1 α ,25-dihydroxyvitamin D₃ (calcitriol). *Biochimica et Biophysica Acta* **1831** 105–108. (doi:10.1016/j.bbali.2012.04.005)

- Meir T, Durlacher K, Pan Z, Amir G, Richards WG, Silver J & Naveh-Many T 2014 Parathyroid hormone activates the orphan nuclear receptor Nurr1 to induce FGF23 transcription. *Kidney International* **86** 1106–1115. (doi:10.1038/ki.2014.215)
- Meyer MB, Goetsch PD & Pike JW 2010 Genome-wide analysis of the VDR/RXR cistrome in osteoblast cells provides new mechanistic insight into the actions of the vitamin D hormone. *Journal of Steroid Biochemistry and Molecular Biology* **121** 136–141. (doi:10.1016/j.jsbmb.2010.02.011)
- Mix KS, Attur MG, Al-Mussawir H, Abramson SB, Brinckerhoff CE & Murphy EP 2007 Transcriptional repression of matrix metalloproteinase gene expression by the orphan nuclear receptor NURR1 in cartilage. *Journal of Biological Chemistry* **282** 9492–9504. (doi:10.1074/jbc.M608327200)
- Oldknow KJ, MacRae VE & Farquharson C 2015 Endocrine role of bone: recent and emerging perspectives beyond osteocalcin. *Journal of Endocrinology* **225** R1–R19. (doi:10.1530/JOE-14-0584)
- Ozono K, Liao J, Kerner SA, Scott RA & Pike JW 1990 The vitamin D-responsive element in the human osteocalcin gene: association with a nuclear proto-oncogene enhancer. *Journal of Biological Chemistry* **265** 21881–21888.
- Quarles LD 2012 Skeletal secretion of FGF-23 regulates phosphate and vitamin D metabolism. *Nature Reviews. Endocrinology* **8** 276–286. (doi:10.1038/nrendo.2011.218)
- Saini RK, Kaneko I, Jurutka PW, Forster R, Hsieh A, Hsieh JC, Haussler MR & Whitfield GK 2013 1,25-Dihydroxyvitamin D(3) regulation of fibroblast growth factor-23 expression in bone cells: evidence for primary and secondary mechanisms modulated by leptin and interleukin-6. *Calcified Tissue International* **92** 339–353. (doi:10.1007/s00223-012-9683-5)
- Sakurada K, Ohshima-Sakurada M, Palmer TD & Gage FH 1999 Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain. *Development* **126** 4017–4026.
- Shimada T, Hasegawa H, Yamazaki Y, Muto T, Hino R, Takeuchi Y, Fujita T, Nakahara K, Fukumoto S & Yamashita T 2004 FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis. *Journal of Bone and Mineral Research* **19** 429–435. (doi:10.1359/JBMR.0301264)
- St John HC, Bishop KA, Meyer MB, Benkusky NA, Leng N, Kendzierski C, Bonewald LF & Pike JW 2014 The osteoblast to osteocyte transition: epigenetic changes and response to the vitamin D3 hormone. *Molecular Endocrinology* **28** 1150–1165. (doi:10.1210/me.2014-1091)
- Tolon RM, Castillo AI, Jimenez-Lara AM & Aranda A 2000 Association with Ets-1 causes ligand- and AF2-independent activation of nuclear receptors. *Molecular and Cellular Biology* **20** 8793–8802. (doi:10.1128/MCB.20.23.8793-8802.2000)
- Yu X, Sabbagh Y, Davis SI, Demay MB & White KE 2005 Genetic dissection of phosphate- and vitamin D-mediated regulation of circulating Fgf23 concentrations. *Bone* **36** 971–977. (doi:10.1016/j.bone.2005.03.002)

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