1 α ,25-dihydroxyvitamin D₃ acts predominately in mature osteoblasts under conditions of high extracellular phosphate to increase fibroblast growth factor 23 production *in vitro*

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Abstract

Osteoblasts/osteocytes are the principle sources of fibroblast growth factor 23 (FGF23), a phosphaturic hormone, but the regulation of FGF23 expression during osteoblast development remains uncertain. Because 1 α ,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) and inorganic phosphate (Pi) may act as potent activators of FGF23 expression, we estimated how these molecules regulate FGF23 expression during rat osteoblast development *in vitro*. 1,25(OH)₂D₃-dependent FGF23 production was restricted largely to mature cells in correlation with increased vitamin D receptor (VDR)

mRNA levels, in particular, when Pi was present. Pi alone and more so in combination with 1,25(OH)₂D₃ increased FGF23 production and VDR mRNA expression. Parathyroid hormone, stanniocalcin 1, prostaglandin E₂, FGF2, and foscarnet did not increase FGF23 mRNA expression. Thus, these results suggest that 1,25(OH)₂D₃ may exert its largest effect on FGF23 expression/production when exposed to high levels of extracellular Pi in osteoblasts/osteocytes.

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Introduction

Inorganic phosphate (Pi) contributes to multiple cell pathways and processes by acting as a component of mineralized matrices, nucleic acids and phospholipid bilayers; as a source of energy in the hydrolysis of ATP; as a substrate for various kinases/phosphatases; and as a regulator of intracellular signaling. The 1 α ,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃)–parathyroid hormone (PTH) axis plays a major role in phosphate homeostasis, but clinical features of, for example, vitamin D-resistant rickets suggest the existence of additional phosphaturic factor(s). Recently, intensive studies of several such putative factors (e.g. matrix extracellular phosphoglycoprotein, dentin matrix protein 1 (DMP1), and fibroblast growth factor 23 (FGF23) have provided new insights into phosphate homeostasis (Quarles 2003, Qin *et al.* 2007). Among these molecules, FGF23 has been studied most extensively in both basic and clinical studies, since it was identified as the factor responsible for autosomal dominant hypophosphatemic rickets (The ADHR Consortium 2000). More recently, this newest FGF family member has been shown to be involved in multiple inherited/acquired hypophosphatemic and chronic kidney disease–mineral bone disorders (Yu & White 2005).

FGF23 is expressed primarily in bones, most notably in osteoblasts and osteocytes (Riminucci *et al.* 2003, Kolek *et al.* 2005, Yoshiko *et al.* 2007b). Indeed, analyses of *Fgf23*-null (*Fgf23*^{−/−}) mice on a *Hyp* (a mouse model of X-linked hypophosphatemia with loss-of-function mutations in phosphate-regulating gene with endopeptidase activity on the X chromosome (*Phex*)) background (Sitara *et al.* 2006) and *Dmp1*^{−/−} mice (Feng *et al.* 2006) indicated that osteocytes are the major sources of FGF23 at least under these pathological conditions. Klotho, a 130 kDa single transmembrane protein having β -glucuronidase activity, appears to form a complex with FGF23 and FGF receptors to support FGF23-dependent signaling in target cells (Urakawa *et al.* 2006, Kurosu *et al.* 2007). Indeed, FGF23 is released into the circulation, and it acts on renal proximal tubules to prevent phosphate reabsorption by suppressing the expression of the type IIa and type IIc sodium-dependent phosphate cotransporters (NPT2a,c; Larsson *et al.* 2004). The polypeptide also suppresses the expression of vitamin D 1 α -hydroxylase (1 α (OH)ase) and PTH, resulting in a reduction in serum 1,25(OH)₂D₃ (Shimada *et al.* 2004) and PTH (Ben-Dov *et al.* 2007) levels respectively. Given these effects, an excess of active FGF23 in the circulation causes hypophosphatemia with resultant onset of rickets/osteomalacia (Liu & Quarles 2007).

Thus, elucidation of the mechanisms of the regulation of FGF23 expression may facilitate the development of new therapies for abnormal phosphate metabolism involving FGF23.

1,25(OH)₂D₃ appears to be a stimulator of FGF23 expression/production in humans (Burnett-Bowie *et al.* 2009) and rodents (Shimada *et al.* 2004, Ito *et al.* 2005, Kolek *et al.* 2005, Saito *et al.* 2005). *Klotho*^{-/-} mice show extremely high serum FGF23 levels with increased serum 1,25(OH)₂D₃ and Pi, and decreased serum PTH levels (Yoshida *et al.* 2002), which are traits that are significantly reversed by ablating *1α(OH)ase* as well as *Klotho* (Ohnishi *et al.* 2009), suggesting that 1,25(OH)₂D₃ plays a key role in FGF23 production. However, in *Dmp1*^{-/-} mice, serum FGF23 levels are high, despite low serum Pi and normal 1,25(OH)₂D₃ levels (Liu *et al.* 2008). A contribution of Pi to serum FGF23 levels has been described in rodent models controlled by dietary phosphorus under 5/6 nephrectomized conditions (Saito *et al.* 2005). Normalization of serum Pi levels by diet increases serum FGF23 levels in vitamin D receptor (*Vdr*)^{-/-} mice exhibiting hypocalcemia and hypophosphatemia secondary to hyperparathyroidism (Yu *et al.* 2005).

With respect to *in vitro* studies, Pi at an optimum concentration (3 mM) acts synergistically with 1,25(OH)₂D₃ to increase *FGF23* promoter activity as well as endogenous *FGF23* mRNA expression in the K562 human chronic myelogenous leukemia cell line, but not in the MC3T3-E1 mouse osteoblastic cell line (Ito *et al.* 2005). The 1,25(OH)₂D₃ effect on *FGF23* mRNA expression is observed in UMR-106 osteosarcoma cells (Kolek *et al.* 2005, Barthel *et al.* 2007) and fetal rat calvarial cells (Yoshiko *et al.* 2007b). However, Pi (1–4 mM) alone does not change *FGF23* promoter activity in ROS17/2.8 rat osteosarcoma cells (Liu *et al.* 2006a). PTH has also been indicated as a regulator of FGF23; serum FGF23 levels and *FGF23* mRNA expression in bones increase in transgenic mice with parathyroid-targeted overexpression of the human *cyclin D1* oncogene, a model of primary hyperparathyroidism, and in parathyroidectomized mice (Kawata *et al.* 2007). In contrast, PTH decreases *FGF23* promoter activity in ROS17/2.8 cells (Liu *et al.* 2006a, Barthel *et al.* 2007). Taken together, the data indicate a need for additional studies to clarify whether and how FGF23 expression is regulated during osteoblast development. Herein, we have used a well-established rat calvaria (RC) osteoblast developmental model *in vitro*, and shown that 1,25(OH)₂D₃ acts predominately in mature cells to increase FGF23 expression in response to high levels of extracellular Pi.

Materials and Methods

Reagents

His-tagged human stanniocalcin 1 (STC1) was prepared as described (Yoshiko *et al.* 2003). Human FGF2 were obtained from R&D Systems (Minneapolis, MN, USA). Selective

prostaglandin E receptor subtype EP2 agonist (ONO-AE1-259; Suzawa *et al.* 2000) was a gift from Ono Pharmaceutical Co. (Osaka, Japan). 1,25(OH)₂D₃ and synthetic PTH1–34 peptide were purchased from BIOMOL International (Plymouth Meeting, PA, USA) and BACHEM AG (Bubendorf, Switzerland) respectively. All other chemicals, unless otherwise specified, were purchased from Sigma–Aldrich. Stock solutions were prepared in an appropriate vehicle and diluted with a medium (1000 times or more) before use. We used 1,25(OH)₂D₃ and foscarnet, a competitive inhibitor of NPT at 10 nM and 0.5 mM respectively according to our previous observations (Yoshiko *et al.* 2007a,b).

Animals

Animal use and procedures were approved by the Committee of Research Facilities for Laboratory Animal Science, Hiroshima University.

Cell cultures

Calvariae were obtained from 21-day-old fetal rats as described (Bellows *et al.* 1986). Briefly, calvariae were minced and digested using collagenase (type I) for 10, 20, 30, 50, and 70 min at 37 °C. Cells retrieved from the last four of five digestion fractions were separately grown in αMEM containing 10% fetal bovine serum (HyClone, Logan, UT, USA) and antibiotics. After 24 h, the cells were recovered, pooled, and grown in multi-well plates or 35 mm dishes (0.3 × 10⁴/cm²) in the same medium supplemented additionally with 50 µg/ml ascorbic acid (osteogenic medium). To obtain osteoblast/osteocyte-rich fractions, cells at day 12 were treated with collagenase until cells in osteoid-like nodules were selectively dispersed (Yoshiko *et al.* 2007a). Recovered cell suspension was then replated at a high cell density (~5 × 10⁴/cm²), and grown in osteogenic medium for a week. Cells were treated with or without agents including β-glycerophosphate (βGP) either alone or in different combinations for 2 days; βGP was also used as an inducer of matrix mineralization in this model. Medium was changed every 2–3 days, and cultures were maintained at 37 °C in a humidified atmosphere with 5% CO₂.

Measurement of Pi concentrations

Conditioned media were collected, and Pi concentrations were determined colorimetrically (Phospha-C test, Wako Pure Chemical Industries Ltd, Osaka, Japan) according to the manufacturer's directions.

ELISA

Conditioned media containing cells grown under appropriate conditions were stored at –80 °C until use. Levels of FGF23 were measured using an FGF23 ELISA kit (Kainos Lab, Tokyo, Japan) according to the manufacturer's directions.

RNA extraction and real-time RT-PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen) according to the manufacturer's directions. cDNA was synthesized from $\leq 3 \mu\text{g}$ of total RNA using ReverTra Ace (TOYOBO, Osaka, Japan) at 50°C for 40 min. Primer sets for rat osteoblast markers and ribosomal L32 as the internal control are described elsewhere (Yoshiko *et al.* 2003): alkaline phosphatase (ALP), 5'-GAT AGG CGA TGT CCT TGC AG-3' and 5'-TTA AGG GCC AGC TAC ACC AC-3'; bone sialoprotein, 5'-CGC CTA CTT TTA TCC TCC TCT G-3' and 5'-CTG ACC CTC GTA GCC TTC ATA G-3'; osteocalcin (OCN), 5'-AAC GGT GGT GCC ATA GAT GC-3' and 5'-AGG ACC CTC TCT CTG CTC AC-3'; osteopontin, 5'-AGA GGA GAA GGC GCA TTA CA-3' and 5'-GCA ACT GGG ATG ACC TTG AT-3'; and ribosomal protein L32, 5'-CAT GGC TGC CCT TCG GCC TC-3' and 5'-CAT TCT CTT CGC TGC GTA GCC-3'. Primer sets for rat FGF23, VDR, PHEX and DMP1 were designed using Primer Picking (Primer 3) as follows: FGF23, 5'-TAA TAG GGG CCA TGA CCA GA-3' and 5'-CCT TCC TCT GCA CTC GGT AG-3'; VDR, 5'-ACA GTC TGA GGC CCA AGC TA-3' and 5'-CTG GTC ATC GGA GGT GAG AT-3'; PHEX, 5'-CCG AAC CAG TGA GGC TAT GT-3' and 5'-TCA GAG TCC ACA GAC CAC CA-3'; and DMP1, 5'-AGT TCG ATG ATG AGG GGA TG-3' and 5'-GTC CCT CTG GGC TAT CTT CC-3'. Real-time RT-PCR was carried out using the Light Cycler system (Light CyclerTM DNA Master SYBR Green I; Roche Diagnostics) as described earlier (Wang *et al.* 2008). To confirm the authenticity of DNA products, each amplicon was sequenced, and qRT-PCR melting curve analysis was performed. Controls containing no reverse transcriptase or no cDNA were also used.

ALP/von Kossa staining

Cells were fixed in neutral buffered formalin for 15 min, washed, and incubated with AS MX phosphate/blue LB in 0.1 M Tris-HCl (pH 8.3), followed by incubation with 2.5% silver nitrate solution.

Statistical analysis

Data obtained from at least three samples are expressed as the mean $\pm$ S.D., and minimum two independent experiments were performed. Statistical differences were evaluated by one-way ANOVA and *post hoc* Tukey's test.

Results

FGF23 expression appears to be restricted to osteoblasts and osteocytes in normal human (Mirams *et al.* 2004), rat (Yoshiko *et al.* 2007b) and *Hyp* mouse (Liu *et al.* 2006b) skeletal tissues, but the expression is low throughout osteoblast development

in non-1,25(OH)₂D₃-treated RC cells *in vitro* (Yoshiko *et al.* 2007b). To determine whether 1,25(OH)₂D₃ differentially regulates FGF23 at different osteoblast developmental times, as it does other genes (Gurlek *et al.* 2002), we compared the effect of 1,25(OH)₂D₃ on FGF23 levels in conditioned media containing rat calvarial cells at three typical developmental times/stages: d3 (proliferation stage in primary culture), d9 (differentiation stage in primary culture), s-d7 (cells at d12/mature stage in primary culture subcultured and grown for an additional 7 days). Cells in each developmental time window were either treated or not treated with 1,25(OH)₂D₃ for 2 days in the presence or absence of β GP, the latter was used to stimulate matrix mineralization in the RC model. Based on the relative levels of *DMP1* versus *ALP* and *OCN* mRNAs, we confirmed that cells at d3 were immature, those at d9 were differentiated, and those at s-d7 were most mature/fully differentiated (i.e. exhibited osteocyte-like features; Toyosawa *et al.* 2001, Kalajzic *et al.* 2004; Fig. 1A). FGF23 levels of the cells grown in media without exogenous 1,25(OH)₂D₃ were very low at all stages, and treatment with 1,25(OH)₂D₃ increased FGF23 levels at d9 and s-d7 but not at d3; 1,25(OH)₂D₃-induced FGF23 levels were highest in s-d7 cultures (Fig. 1B).

Figure 1 Effect of 1,25(OH)₂D₃ on FGF23 production during osteoblast development: proliferation stage (d3), differentiation stage (d9) and mature osteoblast/osteocyte stage (s-d7). (A) Profiling of osteoblast/osteocyte marker mRNA expression by qRT-PCR. Total RNA was isolated from cells at each stage, and cDNA was synthesized and subjected to qRT-PCR. Values are relative to d3 set at 1.0. * $P < 0.05$ and ** $P < 0.01$ compared with d3. (B) FGF23 levels in conditioned media determined by an ELISA. Conditioned media were collected from cell cultures fed with fresh medium for 2 days. 1,25(OH)₂D₃, 10 nM. * $P < 0.05$ and ** $P < 0.01$ compared with d3. ## $P < 0.01$ compared with stage-matched vehicle control (–).

To determine whether Pi increases FGF23 production, we treated cells at s-d7 with β GP, a substrate known to be hydrolyzed by ALP in the RC cultures, as described earlier (β GP was hydrolyzed within 8 h (Bellows *et al.* 1992)). In fact, Pi levels in the medium containing s-d7 (mature) cells treated with 10 mM β GP for 2 days were comparable to those when 10 mM Pi was added exogenously (Fig. 2A). β GP alone increased FGF23 levels in the medium, but to a lesser extent than 1,25(OH) $_2$ D $_3$ treatment (Fig. 2B). Cotreatment with both reagents led to FGF23 levels in the medium that were 3 and 500 times higher than those obtained with 1,25(OH) $_2$ D $_3$ alone or β GP alone respectively (Fig. 2B), suggesting that β GP plus 1,25(OH) $_2$ D $_3$ exerted a synergistic effect on FGF23 production. Treatment of s-d7 cells with either β GP or Pi dose-dependently increased FGF23 levels in the medium; the maximum effect (6–9 times higher than those obtained with vehicle alone) was observed at 10 mM in each case (Fig. 2C). Correspondingly, β GP and Pi nearly equally increased *FGF23* mRNA expression, but less effectively than 1,25(OH) $_2$ D $_3$ (Fig. 2D). In contrast to the effects on FGF23 levels in the medium (see Fig. 2B and C), combined 1,25(OH) $_2$ D $_3$ and β GP or Pi treatment increased *FGF23* mRNA expression only slightly; however, the effects were abolished by cotreatment with foscarnet, a competitive inhibitor of NTP (Fig. 2D). Thus, we concluded that both 1,25(OH) $_2$ D $_3$ and Pi increase FGF23 production in RC osteoblast/osteocyte cultures, but the effect of 1,25(OH) $_2$ D $_3$ is much larger than that of Pi alone. However, we also established that Pi potentially enhances the effect of 1,25(OH) $_2$ D $_3$ on FGF23 production.

Consistent with the results showing that 1,25(OH) $_2$ D $_3$ increases FGF23 expression via classical VDR/nuclear receptor-mediated pathways (Ito *et al.* 2005, Liu *et al.* 2006a), we found that *VDR* mRNA levels in RC cells were highest in s-d7 cells (Fig. 3A), a temporal pattern that paralleled the FGF23 expression/production profile in response to 1,25(OH) $_2$ D $_3$ (cf. Fig. 1B). Moreover, treatment of s-d7 cells with β GP increased *VDR* mRNA expression, which was further enhanced by cotreatment with 1,25(OH) $_2$ D $_3$ (Fig. 3B). Therefore, we concluded that RC osteoblasts/osteocytes but not less mature cells produce a large amount of FGF23 when exposed to 1,25(OH) $_2$ D $_3$ concomitant with high levels of extracellular Pi. Because serum FGF23 levels were high in *Hyp* and *Dmp1* $^{-/-}$ mice, even with low or normal levels of serum Pi and 1,25(OH) $_2$ D $_3$, we also assessed whether β GP and/or 1,25(OH) $_2$ D $_3$ down-regulate the expression levels of *PHEX* and *DMP1* mRNAs in s-d7 cells. β GP increased both *PHEX* and *DMP1* mRNA levels, but it lost the stimulatory effect on *PHEX* but not on *DMP1* when combined with 1,25(OH) $_2$ D $_3$ (Fig. 3C and D).

Because PTH is a stimulator of FGF23 expression in a mouse model of primary hyperparathyroidism as well as in parathyroidectomized mice (Kawata *et al.* 2007), we examined whether PTH increases *FGF23* mRNA expression in the RC model. In addition, we tested a number of potential mediators of PTH actions/pathways and factors involved in matrix mineralization. STC1 is suggested to be involved in

Figure 2 Effects of β GP or Pi on FGF23 production/expression in the presence or absence of 1,25(OH) $_2$ D $_3$ in s-d7 cells (mature osteoblasts/osteocytes). (A) Pi concentrations in the culture media in s-d7 cells treated with or without β GP or Pi (10 mM each) for 24 h. No cell indicates Pi concentrations without cells. * P <0.05 compared with vehicle control (-). Pi concentrations were measured colorimetrically. (B) Effect of 10 mM β GP and/or 10 nM 1,25(OH) $_2$ D $_3$ on FGF23 levels in conditioned media. * P <0.05 and ** P <0.01 compared with vehicle control (-). ## P <0.01 compared with 1,25(OH) $_2$ D $_3$ alone. (C) Dose-dependent effect of β GP or Pi on FGF23 levels in conditioned media. * P <0.05 and ** P <0.01 compared with vehicle control (-). (A–C) Conditioned media were collected as described in Fig. 1. (B and C) FGF23 levels were assayed as described in Fig. 1. (D) Effect of β GP or Pi on *FGF23* mRNA expression in the presence of 1,25(OH) $_2$ D $_3$. 1,25(OH) $_2$ D $_3$, 10 nM; foscarnet, 0.5 mM. qRT-PCR was performed as described in Fig. 1. * P <0.05 and ** P <0.01 compared with vehicle control (-). ## P <0.01 compared with 1,25(OH) $_2$ D $_3$ plus β GP.

Figure 3 Regulation of *VDR*, *PHEX* and *DMP1* mRNA expression in rat calvarial cell cultures. qRT-PCR was done as described in Fig. 2. (A) Profiling of *VDR* mRNA expression during osteoblast development. **P* < 0.05 and ***P* < 0.01 compared with d3. (B) Effect of 1,25(OH)₂D₃ and Pi on *VDR* mRNA expression in s-d7 cells (mature osteoblasts/osteocytes). (C) Effect of 1,25(OH)₂D₃ and Pi on *PHEX* mRNA expression in s-d7 cells. (D) Effect of 1,25(OH)₂D₃ and Pi on *DMP1* mRNA expression in s-d7 cells. (B–D) 1,25(OH)₂D₃, 10 nM; βGP, 10 mM. **P* < 0.05 and ***P* < 0.01 compared with vehicle control (–).

FGF23 in cartilage organ cultures (Wu *et al.* 2006) and osteoblast cultures (Yoshiko *et al.* 2003). Prostaglandin E₂ (PGE₂) acts diversely on osteoblasts, for example, by increasing receptor activator of NF-κB ligand (RANKL) secretion (Tat *et al.* 2008). FGF2 inhibits matrix mineralization through the regulation of Pi handling in the MC3T3-E1 mouse calvaria osteoblastic cell line (Hatch *et al.* 2005). Like foscarnet that decreases NPT activity (Yoshiko *et al.* 2007a), some of these factors increase or decrease NPT activity in osteoblasts (see, for example, Selz *et al.* 1989, Veldman *et al.* 1998, Yoshiko *et al.* 2003). We also examined forskolin (not shown) and the prostaglandin E receptor subtype EP2, which, like PTH, activates the adenylate cyclase/protein kinase A (PKA) pathway (Narumiya & FitzGerald 2001). However, in contrast to 1,25(OH)₂D₃, none of these factors increased *FGF23* mRNA expression in s-d7 cells in the presence of βGP (Fig. 4A). Notably, there was also no correlation between FGF23 levels and the changes in mineralization elicited by any of these reagents (Fig. 4B and C).

Discussion

By using the RC cell culture model, we have shown that 1,25(OH)₂D₃ acts mostly on mature cells to increase FGF23 secretion/mRNA expression during osteoblast development. βGP, apparently via its ability to increase extracellular Pi, enhances the 1,25(OH)₂D₃ effect on FGF23 secretion, but has only a small effect on its own. The upregulation of *VDR* mRNA expression in mature cells in response to cotreatment with 1,25(OH)₂D₃ and βGP provides one plausible explanation for the specificity of their effects on mature

osteoblast/osteocyte stages. On the other hand, PTH and other factors involved in NPT activity and/or the PKA pathway did not alter *FGF23* mRNA expression. These results suggest that 1,25(OH)₂D₃ may act specifically on osteoblasts/osteocytes exposed to high levels of extracellular Pi to increase FGF23 production.

Although recent expression profiling of RNA from cortical bones of *Hyp* mice points towards a potential relationship between *FGF23* mRNA expression and molecules involved in Wnt signaling and the FGF family members FGF1 and

Figure 4 Effect of PTH and other agents on FGF23 production in s-d7 cells (mature osteoblasts/osteocytes). Cells were treated with 10 mM βGP in combination with agents tested for 2 days, and conditioned media were collected. 1,25(OH)₂D₃, 10 nM; PGE₂, 10 nM; PTH1–34, 100 nM; FGF2, 20 ng/ml; STC1, 20 ng/ml; EP2 agonist, 10 nM; foscarnet, 0.5 mM. (A) FGF23 levels in conditioned media determined by an ELISA. (B) Representative macroscopic images of cells subjected to ALP/von Kossa staining. (C) Number of mineralized bone nodules. (B and C) Cells were rinsed and fixed for staining. The number of bone nodules in each well was counted under a stereomicroscope. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0058>.

FGF7 (Liu *et al.* 2009), to date, 1,25(OH)₂D₃ and Pi are the most unequivocal stimulators of FGF23 expression/production (Ito *et al.* 2005) (see Introduction). However, FGF23 responses to either 1,25(OH)₂D₃ or Pi are not identical across different osteoblastic/non-osteoblastic cell models (Ito *et al.* 2005, Kolek *et al.* 2005, Liu *et al.* 2006a, Barthel *et al.* 2007, Yoshiko *et al.* 2007b). For example, in some genetically engineered mouse strains, serum FGF23 levels do not respond to increases or decreases in 1,25(OH)₂D₃ and Pi levels (Yu *et al.* 2005, Liu *et al.* 2008); similar discrepancies exist among culture models (Ito *et al.* 2005, Liu *et al.* 2006a) (cf. Introduction). Our data show that there is a synergistic effect of 1,25(OH)₂D₃ and Pi on FGF23 production, but that the effect is much less on *FGF23* mRNA expression. Thus, we speculate that in contrast to 1,25(OH)₂D₃, which enhances transcriptional activation of FGF23 (Barthel *et al.* 2007), Pi may be involved in post-translational control of FGF23. Further analysis of the several signaling pathways activated in osteoblasts by Pi uptake (Beck 2003) is needed to elucidate how Pi contributes to 1,25(OH)₂D₃-induced FGF23 production. Differences in the levels of other systemic and local factors or serum components that participate in Pi homeostasis may also contribute to the variations observed in Pi, 1,25(OH)₂D₃ and PTH effects on FGF23 expression. These include, for example, factors associated with DMP1 (Feng *et al.* 2006), PHEX (Liu *et al.* 2006b) or Klotho as observed in osteoblasts from *Klotho* mutant mice (Kawaguchi *et al.* 1999). It is also worth noting that we did not detect downregulation of PHEX and DMP1 concomitant with increased FGF23 production, as observed in *Hyp* and *Dmp1*-null mice. However, the increased *PHEX* mRNA levels, but not *DMP1* mRNA levels, induced by βGP were completely abolished by cotreatment with 1,25(OH)₂D₃ in our model, which are effects that will require further assessment. In this regard, it is interesting that the *Hyp* bone phenotype is fully rescued by crossing *Hyp* mice with PHEX transgenic mice, despite FGF23 expression remaining high in bones and uncorrected hypophosphatemia (Erben *et al.* 2005). DMP1 decreases 1,25(OH)₂D₃-induced *FGF23* mRNA expression in UMR cells (Samadfam *et al.* 2009). Klotho is not expressed in osteoblasts (Takeshita *et al.* 2004). Thus, our data, taken together with the previous studies (Takeshita *et al.* 2004, Erben *et al.* 2005, Samadfam *et al.* 2009), support the view that neither PHEX, DMP1 nor Klotho is directly involved in the effect of βGP and/or 1,25(OH)₂D₃ on FGF23 production/expression.

Our results showing that the magnitude of FGF23 levels in response to 1,25(OH)₂D₃ varies markedly during osteoblast development may also explain, at least in part, the diverse responses to 1,25(OH)₂D₃, Pi and/or other factors in functionally and phenotypically different osteoblastic models. Our results are also consistent with *in vivo* data showing developmental stage-associated differences in the intensity of FGF23 expression by immunohistochemistry and *in situ* hybridization (Riminucci *et al.* 2003, Yoshiko *et al.* 2007b)

and with the results of *Fgf23*-deficient eGFP reporter activity in *Hyp* mice (Liu *et al.* 2006b).

We reported that not only 1,25(OH)₂D₃ (Yoshiko *et al.* 2007b) but also adenoviral overexpression of FGF23 (Wang *et al.* 2008) inhibits mineralization in osteoid-like nodules when βGP is present in the RC cell model. Osteoblasts may be exposed to high levels of extracellular Pi during bone resorption and formation, and Pi uptake via NPT3 in osteoblasts may be crucial for matrix mineralization (Suzuki *et al.* 2006, Yoshiko *et al.* 2007a). However, old rats over-expressing POU class 1 homeobox 1 via the β-actin promoter exhibit a decrease in bone mineral density with disruption of mineral metabolism (Suzuki *et al.* 2010). Thus, imbalances in serum levels of either 1,25(OH)₂D₃ and Pi or both may lead to an overproduction of FGF23. PTH and other factors are capable of regulating serum 1,25(OH)₂D₃ or Pi levels (Wortsman *et al.* 1986, Hoppe *et al.* 1991, Murer *et al.* 1996, Nakajima *et al.* 2009) whether FGF23 is involved or not. Further studies are needed to dissect these pathways. In any case, our previous data showing the significance of Pi handling by osteoblasts (Yoshiko *et al.* 2007a) suggest that Pi levels not only in the serum but also in the bone microenvironment may be crucial for FGF23 expression/production. Our data on the expression pattern of VDR also support the importance of the microenvironment. In any case, the mechanism(s) underlying the ability of Pi and 1,25(OH)₂D₃ to act cooperatively to increase FGF23 expression/production is unclear. However, taken together with the combined effect of 1,25(OH)₂D₃ and Pi on *FGF23* promoter activity in K562 cells (Ito *et al.* 2005), it seems likely that the mechanisms are not restricted to osteoblasts/osteocytes. In this regard, *VDR* mRNA levels are increased in K562 cells treated with 1,25(OH)₂D₃, but not in those treated with Pi (Ito *et al.* 2005). Thus, intracellular Pi levels in particular cells such as osteoblasts/osteocytes may play a critical role in 1,25(OH)₂D₃-dependent events. Collectively, the data support that FGF23 may act as a phosphaturic factor and/or an inhibitor of bone mineralization under the influence of extracellular Pi and 1,25(OH)₂D₃ locally and systemically.

In summary, the stimulatory effects of 1,25(OH)₂D₃ on *FGF23* mRNA expression/production were observed primarily in mature osteoblasts exposed to high levels of extracellular Pi in the RC cell culture model. *VDR* mRNA expression was also upregulated in an osteoblast developmental stage-specific manner, and expression was further increased by 1,25(OH)₂D₃ and extracellular Pi. However, similar to what has been reported in previous *in vitro* experiments (Liu *et al.* 2006a), we found no stimulatory effect of either PTH or other molecules known or thought to be downstream of PTH and/or Pi uptake on *FGF23* mRNA expression, suggesting that PTH is not a direct stimulator of FGF23 expression at least in cultured RC cells. Thus, we conclude that 1,25(OH)₂D₃ acts predominately in osteoblasts/osteocytes under conditions of high levels of extracellular Pi to increase FGF23 production in the RC cell culture model, an observation worth further evaluation.

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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References

- Barthel TK, Mathern DR, Whitfield GK, Haussler CA, Hopper HA IV, Hsieh JC, Slater SA, Hsieh G, Kaczmarek 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.
- Beck GR Jr 2003 Inorganic phosphate as a signaling molecule in osteoblast differentiation. *Journal of Cellular Biochemistry* **90** 234–243.
- Bellows CG, Aubin JE, Heersche JN & Antosz ME 1986 Mineralized bone nodules formed *in vitro* from enzymatically released rat calvaria cell populations. *Calcified Tissue International* **38** 143–154.
- Bellows CG, Heersche JN & Aubin JE 1992 Inorganic phosphate added exogenously or released from β -glycerophosphate initiates mineralization of osteoid nodules *in vitro*. *Bone and Mineral* **17** 15–29.
- Ben-Dov IZ, Galitzer H, Lavi-Moshayoff V, Goetz R, Kuro-o M, Mohammadi M, Sirkis R, Naveh-Manly T & Silver J 2007 The parathyroid is a target organ for FGF23 in rats. *Journal of Clinical Investigation* **117** 4003–4008.
- Burnett-Bowie SA, Henao MP, Dere ME, Lee H & Leder BZ 2009 Effects of hPTH(1–34) infusion on circulating serum phosphate, 1,25-dihydroxyvitamin D and FGF23 levels in healthy men. *Journal of Bone and Mineral Research* **24** 1681–1685.
- Erben RG, Mayer D, Weber K, Jonsson K, Juppner H & Lanske B 2005 Overexpression of human PHEX under the human β -actin promoter does not fully rescue the Hyp mouse phenotype. *Journal of Bone and Mineral Research* **20** 1149–1160.
- Feng JQ, Ward LM, Liu S, Lu Y, Xie Y, Yuan B, Yu X, Rauch F, Davis SI, Zhang S *et al.* 2006 Loss of DMP1 causes rickets and osteomalacia and identifies a role for osteocytes in mineral metabolism. *Nature Genetics* **38** 1310–1315.
- Gurlek A, Pittelkow MR & Kumar R 2002 Modulation of growth factor/cytokine synthesis and signaling by 1 α ,25-dihydroxyvitamin D₃: implications in cell growth and differentiation. *Endocrine Reviews* **23** 763–786.
- Hatch NE, Nociti F, Swanson E, Bothwell M & Somerman M 2005 FGF2 alters expression of the pyrophosphate/phosphate regulating proteins, PC-1, ANK and TNAP, in the calvarial osteoblastic cell line, MC3T3E1(C4). *Connective Tissue Research* **46** 184–192.
- Hoppe A, Lin JT, Onsgard M, Knox FG & Dousa TP 1991 Quantitation of the Na⁺–Pi cotransporter in renal cortical brush border membranes. [¹⁴C]phosphonoformic acid as a useful probe to determine the density and its change in response to parathyroid hormone. *Journal of Biological Chemistry* **266** 11528–11536.
- 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.
- Kalajic I, Braut A, Guo D, Jiang X, Kronenberg MS, Mina M, Harris MA, Harris SE & Rowe DW 2004 Dentin matrix protein 1 expression during osteoblastic differentiation, generation of an osteocyte GFP-transgene. *Bone* **35** 74–82.
- Kawaguchi H, Manabe N, Miyaura C, Chikuda H, Nakamura K & Kuro-o M 1999 Independent impairment of osteoblast and osteoclast differentiation in klotho mouse exhibiting low-turnover osteopenia. *Journal of Clinical Investigation* **104** 229–237.
- Kawata T, Imanishi Y, Kobayashi K, Miki T, Arnold A, Inaba M & Nishizawa Y 2007 Parathyroid hormone regulates fibroblast growth factor-23 in a mouse model of primary hyperparathyroidism. *Journal of the American Society of Nephrology* **18** 2683–2688.
- Kolek OI, Hines ER, Jones MD, LeSueur LK, Lipko MA, Kiela PR, Collins JE, 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.
- Kurosu H, Choi M, Ogawa Y, Dickson AS, Goetz R, Elisenkova AV, Mohammadi M, Rosenblatt KP, Klierer SA & Kuro-o M 2007 Tissue-specific expression of β klotho and fibroblast growth factor (FGF) receptor isoforms determines metabolic activity of FGF19 and FGF21. *Journal of Biological Chemistry* **282** 26687–26695.
- Larsson T, Marsell R, Schipani E, Ohlsson C, Ljunggren O, Tenenhouse HS, Juppner H & Jonsson KB 2004 Transgenic mice expressing fibroblast growth factor 23 under the control of the α 1(I) collagen promoter exhibit growth retardation, osteomalacia, and disturbed phosphate homeostasis. *Endocrinology* **145** 3087–3094.
- Liu S & Quarles LD 2007 How fibroblast growth factor 23 works. *Journal of the American Society of Nephrology* **18** 1637–1647.
- Liu S, Tang W, Zhou J, Stubbs JR, Luo Q, Pi M & Quarles LD 2006a Fibroblast growth factor 23 is a counter-regulatory phosphaturic hormone for vitamin D. *Journal of the American Society of Nephrology* **17** 1305–1315.
- Liu S, Zhou J, Tang W, Jiang X, Rowe DW & Quarles LD 2006b Pathogenic role of Fgf23 in Hyp mice. *American Journal of Physiology. Endocrinology and Metabolism* **291** E38–E49.
- Liu S, Zhou J, Tang W, Menard R, Feng JQ & Quarles LD 2008 Pathogenic role of Fgf23 in Dmp1-null mice. *American Journal of Physiology. Endocrinology and Metabolism* **295** E254–E261.
- Liu S, Tang W, Fang J, Ren J, Li H, Xiao Z & Quarles LD 2009 Novel regulators of Fgf23 expression and mineralization in Hyp bone. *Molecular Endocrinology* **23** 1505–1518.
- Mirams M, Robinson BG, Mason RS & Nelson AE 2004 Bone as a source of FGF23: regulation by phosphate? *Bone* **35** 1192–1199.
- Murer H, Lötsher M, Kaissling B, Levi M, Kempson SA & Biber J 1996 Renal brush border membrane Na/Pi-cotransport: molecular aspects in PTH-dependent and dietary regulation. *Kidney International* **49** 1769–1773.
- Nakajima K, Nohtomi K, Sato M, Takano K & Sato K 2009 PTH(7–84) inhibits PTH(1–34)-induced 1,25(OH)₂D₃ production in murine renal tubules. *Biochemical and Biophysical Research Communications* **381** 283–287.
- Narumiya S & FitzGerald GA 2001 Genetic and pharmacological analysis of prostanoind receptor function. *Journal of Clinical Investigation* **108** 25–30.
- Ohnishi M, Nakatani T, Lanske B & Razaque MS 2009 Reversal of mineral ion homeostasis and soft-tissue calcification of klotho knockout mice by deletion of vitamin D 1 α -hydroxylase. *Kidney International* **75** 1166–1172.
- Qin C, D'Souza R & Feng JQ 2007 Dentin matrix protein 1 (DMP1): new and important roles for biomineralization and phosphate homeostasis. *Journal of Dental Research* **86** 1134–1141.
- Quarles LD 2003 FGF23, PHEX, and MEPE regulation of phosphate homeostasis and skeletal mineralization. *American Journal of Physiology. Endocrinology and Metabolism* **285** E1–E9.
- Riminucci M, Collins MT, Fedarko NS, Cherman N, Corsi A, White KE, Waguespack S, Gupta A, Hannon T, Econs MJ *et al.* 2003 FGF-23 in fibrous dysplasia of bone and its relationship to renal phosphate wasting. *Journal of Clinical Investigation* **112** 683–692.
- Saito H, Maeda A, Ohtomo S, Hirata M, Kusano K, Kato S, Ogata E, Segawa H, Miyamoto K & Fukushima N 2005 Circulating FGF-23 is regulated by 1 α ,25-dihydroxyvitamin D₃ and phosphorus *in vivo*. *Journal of Biological Chemistry* **280** 2543–2549.

- Samadfam R, Richard C, Nguyen-Yamamoto L, Bolivar I & Goltzman D 2009 Bone formation regulates circulating concentrations of fibroblast growth factor 23. *Endocrinology* **150** 4835–4845.
- Selz T, Caverzasio J & Bonjour JP 1989 Regulation of Na-dependent Pi transport by parathyroid hormone in osteoblast-like cells. *American Journal of Physiology* **256** E93–E100.
- 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.
- Sitara D, Razzaque MS, St-Arnaud R, Huang W, Taguchi T, Erben RG & Lanske B 2006 Genetic ablation of vitamin D activation pathway reverses biochemical and skeletal anomalies in Fgf-23-null animals. *American Journal of Physiology* **169** 2161–2170.
- Suzawa T, Miyaura C, Inada M, Maruyama T, Sugimoto Y, Ushikubi F, Ichikawa A, Narumiya S & Suda T 2000 The role of prostaglandin E receptor subtypes (EP1, EP2, EP3, and EP4) in bone resorption: an analysis using specific agonists for the respective EPs. *Endocrinology* **141** 1554–1559.
- Suzuki A, Ghayor C, Guicheux J, Magne D, Quillard S, Kakita A, Ono Y, Miura Y, Oiso Y, Itoh M *et al.* 2006 Enhanced expression of the inorganic phosphate transporter Pit-1 is involved in BMP-2-induced matrix mineralization in osteoblast-like cells. *Journal of Bone and Mineral Research* **21** 674–683.
- Suzuki A, Ammann P, Nishiwaki-Yasuda K, Sekiguchi S, Asano S, Nagao S, Kaneko R, Hirabayashi M, Oiso Y, Itoh M *et al.* 2010 Effects of transgenic Pit-1 overexpression on calcium phosphate and bone metabolism. *Journal of Bone and Mineral Metabolism* **28** 139–148.
- Takeshita K, Fujimori T, Kurotaki Y, Honjo H, Tsujikawa H, Yasui K, Lee JK, Kamiya K, Kitaichi K, Yamamoto K *et al.* 2004 Sinoatrial node dysfunction and early unexpected death of mice with a defect of *klotho* gene expression. *Circulation* **109** 1176–1182.
- Tat SK, Pelletier JP, Lajeunesse D, Fahmi H, Duval N & Martel-Pelletier J 2008 Differential modulation of RANKL isoforms by human osteoarthritic subchondral bone osteoblasts: influence of osteotropic factors. *Bone* **43** 284–291.
- The ADHR Consortium 2000 Autosomal dominant hypophosphataemic rickets is associated with mutations in FGF23. *Nature Genetics* **26** 345–348.
- Toyosawa S, Shintani S, Fujiwara T, Ooshima T, Sato A, Ijuhin N & Komori T 2001 Dentin matrix protein 1 is predominantly expressed in chicken and rat osteocytes but not in osteoblasts. *Journal of Bone and Mineral Research* **16** 2017–2026.
- Urakawa I, Yamazaki Y, Shimada T, Iijima K, Hasegawa H, Okawa K, Fujita T, Fukumoto S & Yamashita T 2006 Klotho converts canonical FGF receptor into a specific receptor for FGF23. *Nature* **444** 770–774.
- Veldman CM, Schlapfer I & Schmid C 1998 Prostaglandin E₂ stimulates sodium-dependent phosphate transport in osteoblastic cells via a protein kinase C-mediated pathway. *Endocrinology* **139** 89–94.
- Wang H, Yoshiko Y, Yamamoto R, Minamizaki T, Kozai K, Tanne K, Aubin JE & Maeda N 2008 Overexpression of fibroblast growth factor 23 suppresses osteoblast differentiation and matrix mineralization *in vitro*. *Journal of Bone and Mineral Research* **23** 939–948.
- Wortsman J, Haddad JD, Posillico JT & Brown EM 1986 Primary hyperparathyroidism with low serum 1,25-dihydroxyvitamin D levels. *Journal of Clinical Endocrinology and Metabolism* **62** 1305–1308.
- Wu S, Yoshiko Y & De Luca F 2006 Stanniocalcin 1 acts as a paracrine regulator of growth plate chondrogenesis. *Journal of Biological Chemistry* **281** 5120–5127.
- Yoshida T, Fujimori T & Nabeshima Y 2002 Mediation of unusually high concentrations of 1,25-dihydroxyvitamin D in homozygous klotho mutant mice by increased expression of renal 1 α -hydroxylase gene. *Endocrinology* **143** 683–689.
- Yoshiko Y, Maeda N & Aubin JE 2003 Stanniocalcin 1 stimulates osteoblast differentiation in rat calvaria cell cultures. *Endocrinology* **144** 4134–4143.
- Yoshiko Y, Candelieri GA, Maeda N & Aubin JE 2007a Osteoblast autonomous Pi regulation via Pit1 plays a role in bone mineralization. *Molecular and Cellular Biology* **27** 4465–4474.
- Yoshiko Y, Wang H, Minamizaki T, Ijuin C, Yamamoto R, Suemune S, Kozai K, Tanne K, Aubin JE & Maeda N 2007b Mineralized tissue cells are a principal source of FGF23. *Bone* **40** 1565–1573.
- Yu X & White KE 2005 FGF23 and disorders of phosphate homeostasis. *Cytokine and Growth Factor Reviews* **16** 221–232.
- 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.

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