1α,25-dihydroxyvitamin D_3 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 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) 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*. $1,25(OH)_2D_3$-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 $1,25(OH)_2D_3$ increased FGF23 production and *VDR* mRNA expression. Parathyroid hormone, stanniocalcin 1, prostaglandin E_2, FGF2, and foscarnet did not increase *FGF23* mRNA expression. Thus, these results suggest that $1,25(OH)_2D_3$ 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 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$)–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.* 2007*b*). 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 1α-hydroxylase (1α(OH)ase) and PTH, resulting in a reduction in serum $1,25(OH)_2D_3$ (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).

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

$1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ plays a key role in FGF23 production. However, in *Dmp1*$^{-/-}$ mice, serum FGF23 levels are high, despite low serum Pi and normal $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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.* 2007*b*). However, Pi (1–4 mM) alone does not change *FGF23* promoter activity in ROS17/2.8 rat osteosarcoma cells (Liu *et al.* 2006*a*). 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.* 2006*a*, 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 $1,25(OH)_2D_3$ 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-AEI-259; Suzawa *et al.* 2000) was a gift from Ono Pharmaceutical Co. (Osaka, Japan). $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ and foscarnet, a competitive inhibitor of NPT at 10 nM and 0·5 mM respectively according to our previous observations (Yoshiko *et al.* 2007*a*,*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 ($0{\cdot}3\times10^4/cm^2$) 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.* 2007*a*). Recovered cell suspension was then replated at a high cell density ($\sim5\times10^4/cm^2$), 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% CO_2.

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 ≤3 μg 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±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.* 2007*b*) and *Hyp* mouse (Liu *et al.* 2006*b*) skeletal tissues, but the expression is low throughout osteoblast development in non-$1,25(OH)_2D_3$-treated RC cells *in vitro* (Yoshiko *et al.* 2007*b*). To determine whether $1,25(OH)_2D_3$ differentially regulates FGF23 at different osteoblast developmental times, as it does other genes (Gurlek *et al.* 2002), we compared the effect of $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ for 2 days in the presence or absence of βGP, 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 $1,25(OH)_2D_3$ were very low at all stages, and treatment with $1,25(OH)_2D_3$ increased FGF23 levels at d9 and s-d7 but not at d3; $1,25(OH)_2D_3$-induced FGF23 levels were highest in s-d7 cultures (Fig. 1B).

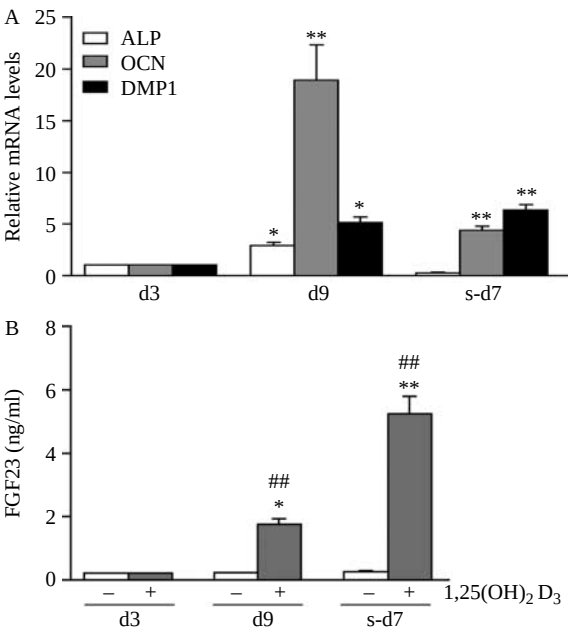

Figure 1 Effect of $1,25(OH)_2D_3$ on FGF23 production during osteoblast development: proliferation stage (d3), differentiation stage (d9) and mature osteoblast/osteocyte stage (s-d7). (A) Profiling of osteoblast/osteocyte marker mRNA expression by qRT-PCR. Total RNA was isolated from cells at each stage, 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. $1,25(OH)_2D_3$, 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 βGP, a substrate known to be hydrolyzed by ALP in the RC cultures, as described earlier (βGP 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 βGP for 2 days were comparable to those when 10 mM Pi was added exogenously (Fig. 2A). βGP alone increased FGF23 levels in the medium, but to a lesser extent than $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ alone or βGP alone respectively (Fig. 2B), suggesting that βGP plus $1,25(OH)_2D_3$ exerted a synergistic effect on FGF23 production. Treatment of s-d7 cells with either βGP 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, βGP and Pi nearly equally increased *FGF23* mRNA expression, but less effectively than $1,25(OH)_2D_3$ (Fig. 2D). In contrast to the effects on FGF23 levels in the medium (see Fig. 2B and C), combined $1,25(OH)_2D_3$ and βGP 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 $1,25(OH)_2D_3$ and Pi increase FGF23 production in RC osteoblast/osteocyte cultures, but the effect of $1,25(OH)_2D_3$ is much larger than that of Pi alone. However, we also established that Pi potently enhances the effect of $1,25(OH)_2D_3$ on FGF23 production.

Consistent with the results showing that $1,25(OH)_2D_3$ increases FGF23 expression via classical VDR/nuclear receptor-mediated pathways (Ito *et al.* 2005, Liu *et al.* 2006*a*), 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 $1,25(OH)_2D_3$ (cf. Fig. 1B). Moreover, treatment of s-d7 cells with βGP increased *VDR* mRNA expression, which was further enhanced by cotreatment with $1,25(OH)_2D_3$ (Fig. 3B). Therefore, we concluded that RC osteoblasts/osteocytes but not less mature cells produce a large amount of FGF23 when exposed to $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$, we also assessed whether βGP and/or $1,25(OH)_2D_3$ down-regulate the expression levels of *PHEX* and *DMP1* mRNAs in s-d7 cells. βGP increased both *PHEX* and *DMP1* mRNA levels, but it lost the stimulatory effect on PHEX but not on DMP1 when combined with$1,25(OH)_2D_3$ (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

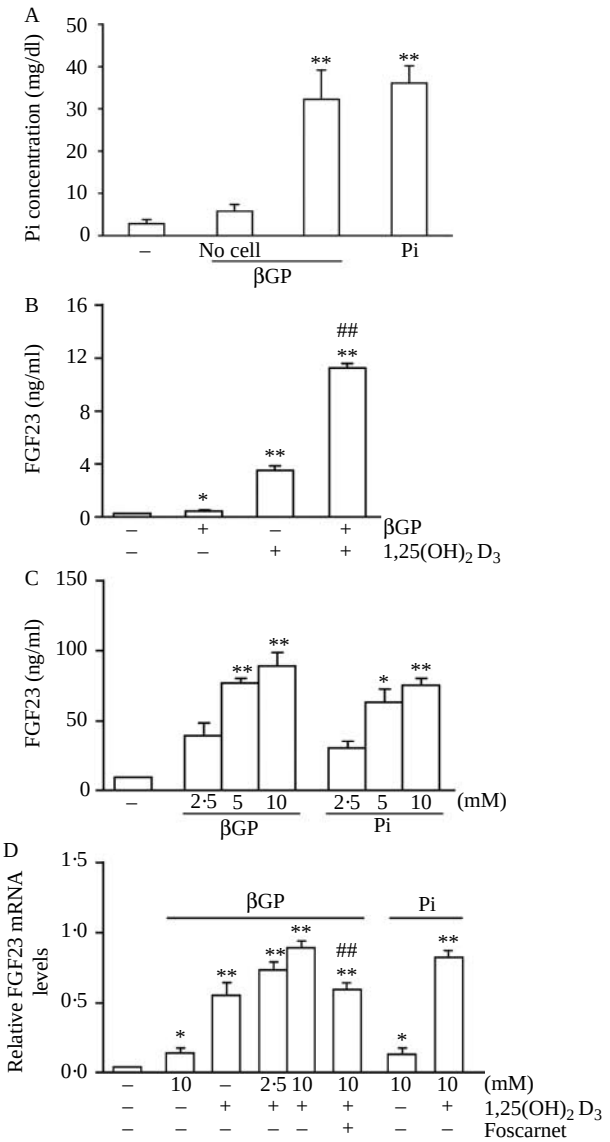

Figure 2 Effects of βGP or Pi on FGF23 production/expression in the presence or absence of $1,25(OH)_2D_3$ in s-d7 cells (mature osteoblasts/osteocytes). (A) Pi concentrations in the culture media in s-d7 cells treated with or without βGP or Pi (10 mM each) for 24 h. No cell indicates Pi concentrations without cells. *$P<0·05$ compared with vehicle control (−). Pi concentrations were measured colorimetrically. (B) Effect of 10 mM βGP and/or 10 nM $1,25(OH)_2D_3$ on FGF23 levels in conditioned media. *$P<0·05$ and **$P<0·01$ compared with vehicle control (−). $^{\#\#}P<0·01$ compared with $1,25(OH)_2D_3$ alone. (C) Dose-dependent effect of βGP 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 βGP or Pi on *FGF23* mRNA expression in the presence of $1,25(OH)_2D_3$. $1,25(OH)_2D_3$, 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 $1,25(OH)_2D_3$ plus βGP.

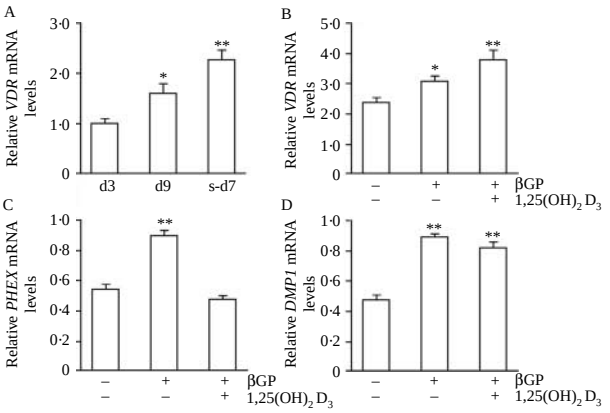

Figure 3 Regulation of *VDR, PHEX* and *DMP1* mRNA expression in rat calvarial cell cultures. qRT-PCR was done as described in Fig. 2. (A) Profiling of *VDR* mRNA expression during osteoblast development. **P*<0·05 and ***P*<0·01 compared with d3. (B) Effect of $1,25(OH)_2D_3$ and Pi on *VDR* mRNA expression in s-d7cells (mature osteoblasts/osteocytes). (C) Effect of $1,25(OH)_2D_3$ and Pi on *PHEX* mRNA expression in s-d7cells. (D) Effect of $1,25(OH)_2D_3$ and Pi on *DMP1* mRNA expression in s-d7cells. (B–D) $1,25(OH)_2D_3$, 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 E_2 (PGE_2) 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.* 2007*a*), 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 $1,25(OH)_2D_3$, 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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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

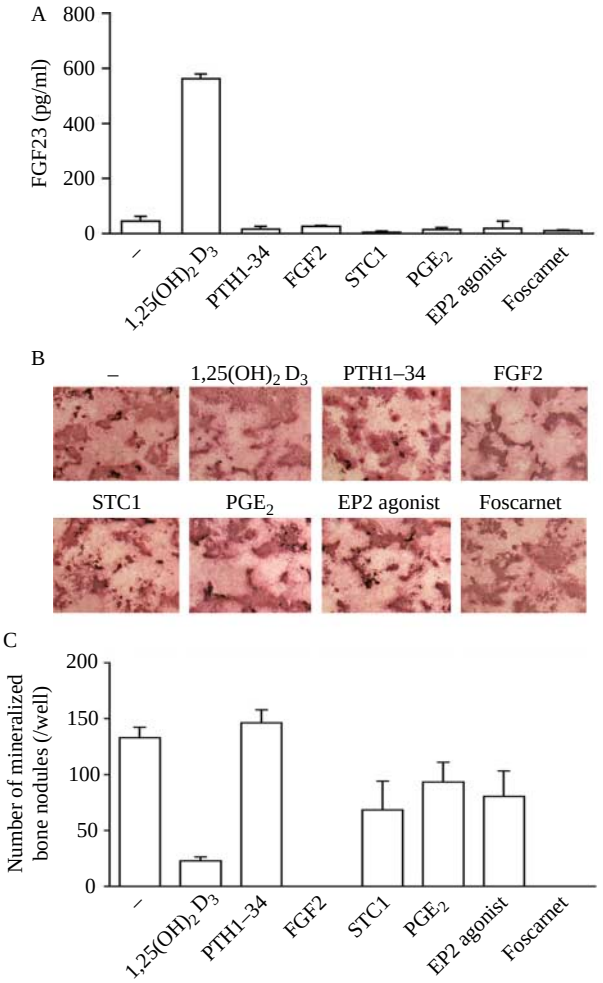

Figure 4 Effect of PTH and other agents on FGF23 production in s-d7 cells (mature osteoblasts/osteocytes). Cells were treated with 10 mM βGP in combination with agents tested for 2 days, and conditioned media were collected. $1,25(OH)_2D_3$, 10 nM; PGE_2, 10 nM; PTH1–34, 100 nM; FGF2, 20 ng/ml; STC1, 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, $1,25(OH)_2D_3$ and Pi are the most unequivocal stimulators of FGF23 expression/production (Ito *et al.* 2005) (see Introduction). However, FGF23 responses to either $1,25(OH)_2D_3$ or Pi are not identical across different osteoblastic/non-osteoblastic cell models (Ito *et al.* 2005, Kolek *et al.* 2005, Liu *et al.* 2006*a*, Barthel *et al.* 2007, Yoshiko *et al.* 2007*b*). For example, in some genetically engineered mouse strains, serum FGF23 levels do not respond to increases or decreases in $1,25(OH)_2D_3$ and Pi levels (Yu *et al.* 2005, Liu *et al.* 2008); similar discrepancies exist among culture models (Ito *et al.* 2005, Liu *et al.* 2006*a*) (cf. Introduction). Our data show that there is a synergistic effect of $1,25(OH)_2D_3$ and Pi on FGF23 production, but that the effect is much less on *FGF23* mRNA expression. Thus, we speculate that in contrast to $1,25(OH)_2D_3$, 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 $1,25(OH)_2D_3$-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, $1,25(OH)_2D_3$ and PTH effects on FGF23 expression. These include, for example, factors associated with DMP1 (Feng *et al.* 2006), PHEX (Liu *et al.* 2006*b*) 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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$-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 $1,25(OH)_2D_3$ on FGF23 production/expression.

Our results showing that the magnitude of FGF23 levels in response to $1,25(OH)_2D_3$ varies markedly during osteoblast development may also explain, at least in part, the diverse responses to $1,25(OH)_2D_3$, 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.* 2007*b*) and with the results of *Fgf23*-deficient eGFP reporter activity in *Hyp* mice (Liu *et al.* 2006*b*).

We reported that not only $1,25(OH)_2D_3$ (Yoshiko *et al.* 2007*b*) 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.* 2007*a*). 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 $1,25(OH)_2D_3$ and Pi or both may lead to an overproduction of FGF23. PTH and other factors are capable of regulating serum $1,25(OH)_2D_3$ 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.* 2007*a*) 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 $1,25(OH)_2D_3$ to act cooperatively to increase FGF23 expression/production is unclear. However, taken together with the combined effect of $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$, 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 $1,25(OH)_2D_3$-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 $1,25(OH)_2D_3$ locally and systemically.

In summary, the stimulatory effects of $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ and extracellular Pi. However, similar to what has been reported in previous *in vitro* experiments (Liu *et al.* 2006*a*), 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 $1,25(OH)_2D_3$ 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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