

Fibroblast growth factor-23 regulates parathyroid hormone and 1 α -hydroxylase expression in cultured bovine parathyroid cells

Tijana Krajsnik¹, Peyman Björklund², Richard Marsell², Östen Ljunggren¹, Göran Åkerström², Kenneth B Jonsson², Gunnar Westin² and Tobias E Larsson¹

Departments of ¹Medical Sciences and ²Surgical Sciences, Uppsala University Hospital, 751 85 Uppsala, Sweden

(Correspondence should be addressed to T E Larsson; Email: tobias.larsson@medsci.uu.se)

Abstract

Fibroblast growth factor-23 (FGF23) is a circulating factor that decreases serum levels of inorganic phosphate (Pi) as well as 1,25-dihydroxyvitamin D₃. Recent studies also suggest a correlation between serum levels of FGF23 and parathyroid hormone (PTH) in patients with chronic kidney disease. It is, however, unknown whether FGF23 directly modulates PTH expression, or whether the correlation is secondary to abnormalities in Pi and vitamin D metabolism. The objective of the current study was therefore to elucidate possible direct effects of FGF23 on bovine parathyroid cells *in vitro*. Treatment of parathyroid cells with a stabilized form of recombinant FGF23 (FGF23(R176Q)) induced a rise in early response gene-1 mRNA transcripts, a marker of FGF23 signaling. FGF23(R176Q) potently and dose-dependently

decreased the PTH mRNA level within 12 h. In agreement, FGF23(R176Q) also decreased PTH secretion into conditioned media. In contrast, FGF23(R176Q) dose-dependently increased 1 α -hydroxylase expression within 3 h. FGF23 (R176Q) did not affect cell viability nor induce apoptosis, whereas a small but significant increase in cell proliferation was found. We conclude that FGF23 is a negative regulator of PTH mRNA expression and secretion *in vitro*. Our data suggest that FGF23 may be a physiologically relevant regulator of PTH. This defines a novel function of FGF23 in addition to the previously established roles in controlling vitamin D and Pi metabolism.

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Introduction

Fibroblast growth factor-23 (FGF23) is a circulating phosphaturic factor that plays a critical role in renal phosphate (Pi) reabsorption and vitamin D metabolism (ADHR-Consortium 2000, Shimada *et al.* 2001). Its physiological importance is exemplified by numerous activating and inactivating mutations in the human *FGF23* gene, causing two clinical disorders of disturbed Pi homeostasis: autosomal dominant hypophosphatemic rickets (ADHR, OMIM#193100; ADHR-Consortium 2000) and hyperphosphatemic familial tumoral calcinosis (HFTC, OMIM#211900; Benet-Pages *et al.* 2005, Larsson *et al.* 2005). Elevated FGF23 levels are also associated with several other disorders of hypophosphatemia or dysfunctional regulation of vitamin D, such as autosomal recessive hypophosphatemic rickets (ARHR; Feng *et al.* 2006, Lorenz-Depiereux *et al.* 2006), X-linked hypophosphatemic rickets (XLH; Liu *et al.* 2003, Riminucci *et al.* 2003), tumor-induced osteomalacia (TIO; Shimada *et al.* 2001, White *et al.* 2001b), fibrous dysplasia (FD; Riminucci *et al.* 2003), and linear nevus sebaceous syndrome (LNSS; Hoffman *et al.* 2005).

The regulation of Pi and the production of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) mainly occur in the kidney, although the physiological source of FGF23

production is bone (Riminucci *et al.* 2003, Mirams *et al.* 2004, Sitara *et al.* 2004). In states of hyperphosphatemia, such as in chronic kidney disease (CKD) and hypoparathyroidism, FGF23 expression is induced, giving rise to increased serum FGF23 levels (Larsson *et al.* 2003a, Gupta *et al.* 2004). The compensatory rise in FGF23 causes a decrease in renal Pi reabsorption and diminished production of 1,25(OH)₂D₃ due to decreased expression of 25-hydroxyvitamin D₃-1 α -hydroxylase (1 α (OH)ase; Shimada *et al.* 2001). Serum levels of FGF23 continuously increase as renal function declines in CKD (Imanishi *et al.* 2004, Larsson *et al.* 2004, Shigematsu *et al.* 2004), at least in part, due to the manifest hyperphosphatemia. In end-stage renal disease (ESRD), FGF23 concentrations are therefore typically increased a 1000-fold or more when compared with healthy individuals (Imanishi *et al.* 2004, Larsson *et al.* 2004, Shigematsu *et al.* 2004). Elevated FGF23 in CKD has been proposed to play a role in the development of secondary hyperparathyroidism (Kazama *et al.* 2005, Nakanishi *et al.* 2005).

Recent studies have shed light on FGF23 signaling. It has been demonstrated that Klotho is a permissive receptor cofactor for FGF23, converting FGFR1 (IIIc) into a native FGF23-specific receptor (Urakawa *et al.* 2006). Klotho null mice also display a marked elevation of serum Fgf23, although

their phenotype closely resembles that of *Fgf23* knockout mice, presenting further evidence of *Klotho* as a regulator of FGF23 signaling (Kurosu *et al.* 2006, Urakawa *et al.* 2006). Importantly, *Klotho* is abundantly expressed in parathyroid glands (Ito *et al.* 2007). This has raised the question whether FGF23 may exert direct effects on parathyroid glands. In a physiological context, a relationship between FGF23 and PTH is also plausible due to their overlapping capacity to diminish renal Pi reabsorption and counter-regulatory effects on $1\alpha(\text{OH})\text{ase}$. Finally, a covariation of FGF23 and PTH has been observed *in vivo* in early and late stages of CKD (Shigematsu *et al.* 2004, Westerberg *et al.* 2007).

In the current study, we sought to elucidate potential regulatory effects of FGF23 on PTH and $1\alpha(\text{OH})\text{ase}$ expression *in vitro*, using bovine parathyroid cells.

Materials and Methods

Preparation of bovine parathyroid glands and cell culture

Bovine parathyroid glands were obtained from healthy adult cattle within minutes after slaughter as previously described (Segersten *et al.* 2002). The glands were minced with scissors and cell suspensions were prepared by collagenase digestion at 37 °C in a shaking incubator in F-10 medium (pH 7.4) containing 1 mg/ml collagenase, 0.05 mg/ml DNase I, 1.25 mM CaCl_2 , and 1.5% BSA. The incubation was carried out for 2 h, interrupted by repeated pipette aspirations. After filtration through a nylon mesh, cells were centrifuged through 25% standard isotonic Percoll (Pharmacia Biotech). Cell viability was determined by Trypan blue staining before seeding into six-well plates. In each well, 0.5×10^6 cells were seeded in Dulbecco's modified Eagle's medium (DMEM)-medium containing 10% fetal calf serum (FCS) and penicillin–fungizone plus L-glutamine (PFL). Cells were grown in an incubator at 37 °C in 5% CO_2 and FGF23(R176Q) treatment was initiated after 24 h. Validations of the experimental system revealed that approximately 95% of cultured cells are stained positive for PTH (Knutson *et al.* 1998) and display an inverse relationship between intracellular calcium concentration and PTH secretion (Segersten *et al.* 2002).

COS-7 cell culture

COS-7 cells were grown in 75 ml flasks in a total volume of 15 ml DMEM medium with 10% FCS supplemented with PFL. The cells were kept in an incubator at 37 °C, 5% CO_2 .

Recombinant FGF23 protein production

The cDNA encoding full-length human FGF23 was cloned into the pcDNA3.1-V5-His-TOPO plasmid (Larsson *et al.* 2003b). A mutation encoding the FGF23(R176Q) protein was introduced into the plasmid using site-directed mutagenesis as previously described (Larsson *et al.* 2003b). FGF23(R176Q) protein was used in all experiments in

order to increase protein stability (White *et al.* 2001a). hFGF23(R176Q)-pcDNA3.1-V5-His-TOPO plasmid and an empty mock pcDNA3.1(+) plasmid as control were transfected into COS-7 cells using FuGENE 6 Transfection Reagent (Roche Diagnostics Scandinavia AB). Conditioned serum-free medium from transfected COS-7 cells was collected 3–4 days after transfection. FGF23 concentrations were determined by ELISA measurements (Yamazaki *et al.* 2002). All FGF23-containing conditioned media were diluted to appropriate experimental concentrations using the mock-transfected control conditioned media as diluent. FGF23 protein was added to the bovine parathyroid cells as diluted serum-free conditioned media. Each experiment was performed in triplicates and repeated two to four times.

RNA extraction

In accordance with EZNA Total RNA Isolation Protocol (Omega Biotek, Doraville, GA, USA), total RNA was extracted and on-membrane DNase I digestion was performed. Electrophoresis on 1% agarose gel was run to check for RNA degradation. NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to estimate concentration as well as to obtain the purity A_{260}/A_{280} ratio (> 1.99). A representative group of samples was further analyzed by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) to confirm adequate RNA integrity. RNA was kept at -70 °C.

cDNA synthesis

Superscript II Reverse Transcriptase and Oligo (dT)-primers (Invitrogen) were used for reverse transcription from 0.6–1 µg total DNased RNA. cDNA was kept at -20 °C.

Real-time PCR analysis

A total volume of 25 µl per PCR was used: 12.5 µl ($2 \times$) iQ SYBR Green Supermix containing fluorescein (Bio-Rad); 2.5 µl (2 µM) Primer Fwd; 2.5 µl (2 µM) Primer Rev; 5.5 µl sterile H_2O ; and 2 µl cDNA. The iCycler, MyiQ Single Color Real-Time PCR Detection System (Bio-Rad) was used and the cycling conditions were: a) 95 °C, 3 min; b) 40 times: 95 °C, 15 s; 54–60 °C (T_a depending on the primers), 1 min; c) 95 °C, 1 min; d) 55 °C, 1 min; and e) 80 times: $+0.5$ °C, starting from 55 °C, 10 s, for melt-curve determination. All samples, including non-template negative controls, were amplified in duplicates. All assays were run three to four times. The sequences of bovine PTH and GAPDH primer pairs have previously been described (Segersten *et al.* 2002). Primer sequences for bovine $1\alpha(\text{OH})\text{ase}$ were as follows: F: 5'-ATC-CCAAAAATACGCTGGT-3' and R: 5'-GCTGGAC-GAAAAGAATTTGG-3'.

Relative gene expression was calculated according to the comparative C_t method ($2^{-\Delta\Delta C_t}$) by Livak & Schmittgen (2001).

ELISA measurements

To determine FGF23 protein concentration, conditioned media from transfected COS-7 cells were analyzed by an intact FGF23 ELISA (Kainos laboratories, Tokyo, Japan; Yamazaki *et al.* 2002). This ELISA detects only the intact, biologically active, portion of the protein. For measurement of secreted PTH protein, a bovine intact PTH ELISA was used (Immutopics, San Clemente, CA, USA) according to the manufacturer's instructions. To determine PTH protein secretion, parathyroid cells were treated at various concentrations of FGF23 for 24 h, followed by two 1×PBS washes and addition of fresh serum-free medium to the cells. After 4 h, conditioned media were collected for subsequent PTH measurements. Secreted PTH concentrations were corrected for total protein content in the wells, determined using a NanoDrop ND-1000 Spectrophotometer. Secreted protein levels were measured from triplicate wells in two separate experiments.

Flow cytometry analysis

Parathyroid cells were trypsinized and diluted with 1×PBS and divided into two 5 ml FACS tubes (measured in duplicates). The cells were centrifuged, washed by, and resuspended in FACS buffer: 2% FCS, 0.05% Na₃N, 2.5 mM CaCl₂, and 1×PBS. After a second centrifugation, the cells were resuspended in 100 µl FACS buffer, and 5 µl anti-Annexin V-FITC-conjugated antibodies were added. They were left for 20–30 min in darkness at room temperature. After a second wash, 5 µl propidium iodide (PI) was added. Non-treated cells were used as FACS controls: a) without FITC or PI, b) only FITC, and c) only PI. Low flow speed and a limit of 50 000 cells were set for each measurement. Identical settings were used at later measurements. Total apoptosis was defined as the sum of early (FITC) and late (FITC and PI) apoptosis. FACS results were derived from two separate experiments.

Tritium-labeled thymidine incorporation

In order to estimate the proliferation of the cells, 1 µCi [³H]thymidine was added to each well 12 h before harvesting. After incubation, medium was removed and unincorporated [³H]thymidine was precipitated with 500 µl cold trichloroacetic acid for 20 min. This procedure was repeated twice. Cells were then lysed in 1 ml 10% NaOH and 0.5% Triton X. The radioactivity was measured in a β-counter. Proliferation data were obtained from three separate experiments and all samples were run in triplicates.

Statistical analysis

GraphPad Prism Version 3.03 software (GraphPad Software Inc., San Diego, CA, USA) was used for statistical data analysis. Unpaired *t*-tests were performed, and the results are shown as arithmetic means ± S.E.M. A *P* value of <0.05 was considered statistically significant.

Results

Homology analysis of human and bovine FGF23 revealed 83% identity at the nucleotide level and 84% identity at the amino acid level (data not shown). Functionally important motifs, such as the ¹⁷⁶RXXR¹⁷⁹ site (ADHR-Consortium 2000), were preserved allowing human recombinant FGF23 protein to be used in the experiments. In addition, we used mutant FGF23(R176Q) protein in all experiments in order to increase FGF23 stability (White *et al.* 2001a). The short-term biological potency of FGF23(R176Q) and wild-type FGF23 are similar based on previous studies on renal Pi transport (Shimada *et al.* 2002). To determine the effect of FGF23 on parathyroid cells, we used a well-established system of primary isolates of bovine parathyroid cells.

As expected, we found by RT-PCR that the FGF23 receptor cofactor Klotho was expressed in the parathyroid cells as previously reported in mice (Ito *et al.* 2007; data not shown). To further validate our assay system, we analyzed the effect of FGF23(R176Q) treatment on the *Egr-1* mRNA expression, a reported marker of FGF23 signaling (Urakawa *et al.* 2006). FGF23(R176Q) treatment evoked an increase in *Egr-1* mRNA after 60 min, suggesting a direct effect of FGF23(R176Q) on parathyroid cells (Fig. 1).

Subsequently, we determined the effect of FGF23(R176Q) on PTH mRNA expression. At 400 pg/ml (equivalent to approximately 286 nM) FGF23(R176Q), the PTH mRNA level was significantly decreased (*P*<0.05) when compared with control-treated cells at 12 h (Fig. 2a) as determined by real-time RT-PCR. The maximal observed effect was detected at 24 h (*P*<0.01) and sustained for at least 48 h

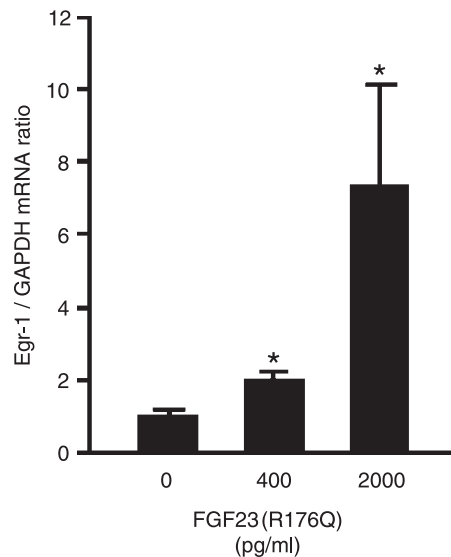


Figure 1 *Egr-1* is an early response gene and a marker of FGF23 signaling. A rise in *Egr-1* mRNA was observed 60 min after treatment with 400 and 2000 pg/ml FGF23(R176Q), supporting a direct effect of FGF23 on parathyroid cells. Error bars indicate mean ± S.E.M.

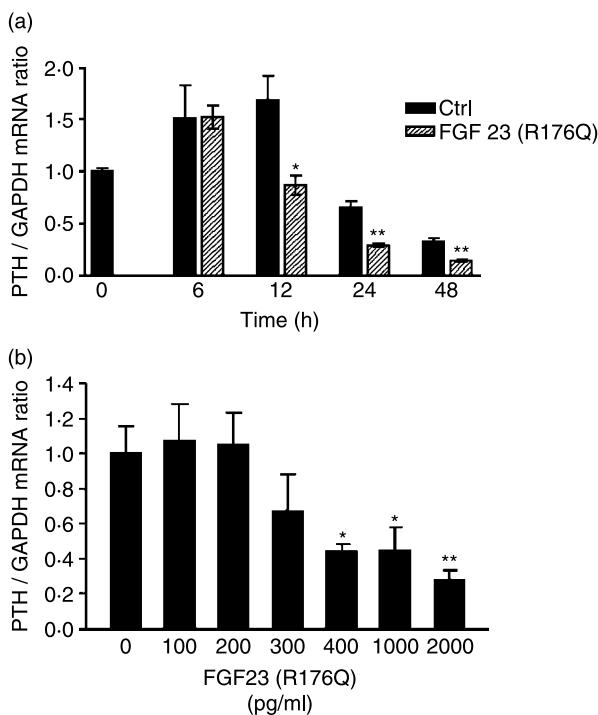


Figure 2 (a) FGF23(R176Q) decreased the expression of PTH mRNA in bovine parathyroid cells when compared with control-treated cells. A significant difference was observed after 12 h of FGF23(R176Q) treatment and sustained for 48 h. Cells were treated with 400 pg/ml FGF23(R176Q). * $P < 0.05$; ** $P < 0.01$. Error bars indicate mean \pm s.e.m. (b) FGF23(R176Q) dose-dependently decreased PTH mRNA in bovine parathyroid cells at 24 h. A significant reduction in PTH mRNA level was observed at 400 pg/ml FGF23(R176Q). * $P < 0.05$; ** $P < 0.01$. Error bars indicate mean \pm s.e.m.

($P < 0.01$; Fig. 2a). In order to determine the concentrations required for reducing the PTH mRNA level, we treated the parathyroid cells for 24 h at various concentrations of FGF23(R176Q), ranging from 100 to 2000 pg/ml (Fig. 2b). FGF23(R176Q) reduced the PTH mRNA level in a dose-dependent manner (Fig. 2b) in the concentration range of 400–2000 pg/ml. To exclude the possibility that control-conditioned media (from mock-transfected cells) regulated the PTH mRNA level, we performed a dose-response treatment with control-conditioned media serially diluted in nonconditioned media. This revealed no effect on PTH mRNA expression (data not shown). In summary, our data suggest that FGF23(R176Q) dose-dependently decreases the PTH mRNA level.

We further examined whether FGF23(R176Q) displayed a regulatory effect on PTH secretion. For this purpose, conditioned media from FGF23(R176Q)- and control-treated parathyroid cells were analyzed for PTH concentrations, using a bovine intact PTH ELISA. Importantly, FGF23(R176Q) dose-dependently decreased the secretion of PTH protein at 24 h when compared with control-treated cells in the concentration range of 700–2000 pg/ml (Fig. 3).

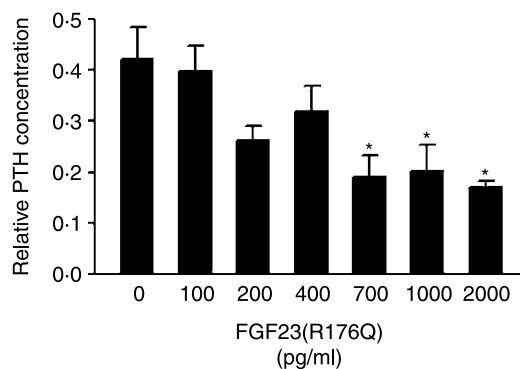


Figure 3 FGF23(R176Q) dose-dependently decreased PTH secretion. A significant reduction in extracellular PTH protein level was detected at 700 pg/ml FGF23(R176Q). PTH was measured using a bovine PTH ELISA after 24 h of FGF23(R176Q) stimulation and subsequent incubation in fresh medium for 4 h. * $P < 0.05$. Error bars indicate mean \pm s.e.m.

Since several previous studies have shown that FGF23 decreases the expression of 1α (OH)ase expression in kidneys (Shimada *et al.* 2001, 2002, 2004a,b,c, Bai *et al.* 2004, Larsson *et al.* 2004, Sitara *et al.* 2004), we investigated the effect of FGF23(R176Q) treatment on parathyroid 1α (OH)ase mRNA expression. Notably, FGF23(R176Q) increased 1α (OH)ase mRNA level within 3 h and the effect sustained for 24 h (Fig. 4a). The effect was dose dependent in the concentration range of 400–2000 pg/ml (Fig. 4b).

We also examined parathyroid cell number, viability, apoptosis, and proliferation. At a FGF23(R176Q) concentration of 2000 pg/ml for 24 h, we did not observe significant effects on total cell number (data not shown) or viability (Fig. 5a) when compared with control-treated cells. Similarly, we did not detect any significant effect on apoptosis (Fig. 5b), whereas a small but significant increase in cell proliferation was observed (Fig. 5c). The proportion of cells expressing PTH was ~ 90 –95% at 0–48 h, independent of FGF23(R176Q) treatment (data not shown).

Discussion

Previous extensive studies have shown that FGF23 is a key regulator of Pi and vitamin D metabolism (ADHR-Consortium 2000, Shimada *et al.* 2001). Maintenance of mineral ion homeostasis is a complex process involving several hormonal systems and target tissues, including bone, kidney, and parathyroid glands. Observational data supporting a correlation between FGF23 and PTH *in vivo* (Shigematsu *et al.* 2004, Kazama *et al.* 2005, Nakanishi *et al.* 2005), and the presence of the FGF23 receptor cofactor Klotho in parathyroid glands (Ito *et al.* 2007) prompted us to elucidate whether FGF23 directly modulates PTH expression in parathyroid cells. Herein, we demonstrate that FGF23(R176Q) induced a dose-dependent decrease in PTH mRNA level and protein secretion. Our data support a previously unidentified direct link between FGF23

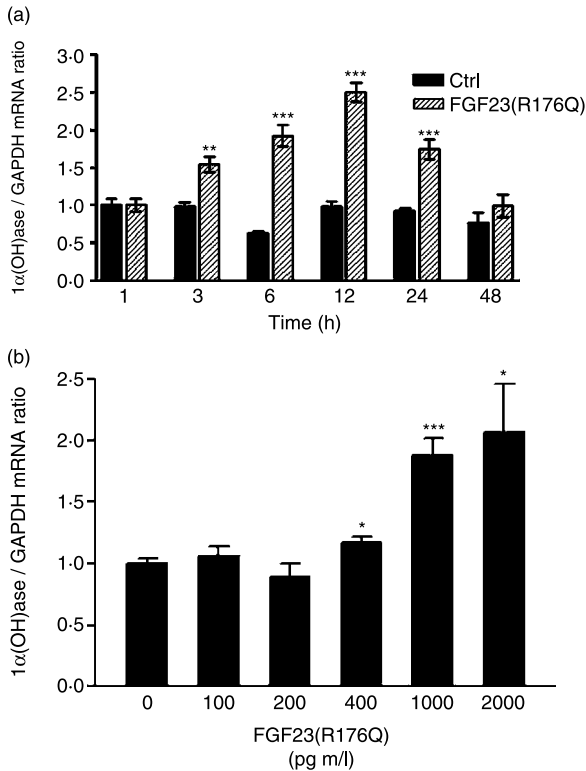


Figure 4 (a) FGF23(R176Q) increased the expression of $1\alpha(\text{OH})\text{ase}$ mRNA in bovine parathyroid cells when compared with control-treated cells. A significant difference was observed within 3 h of FGF23(R176Q) treatment and sustained for 24 h. Cells were treated with 1000 pg/ml FGF23(R176Q). ** $P < 0.01$; *** $P < 0.001$. Error bars indicate mean \pm S.E.M. (b) FGF23(R176Q) dose-dependently increased $1\alpha(\text{OH})\text{ase}$ mRNA level at 24 h. A significant rise in $1\alpha(\text{OH})\text{ase}$ mRNA level was observed at 400 pg/ml FGF23(R176Q). * $P < 0.05$; $P < 0.001$. Error bars indicate mean \pm S.E.M.

and PTH. Because we used an isolated *in vitro* system, we were able to clarify direct FGF23-mediated effects on PTH expression, as opposed to secondary systemic changes in mineral metabolism.

To evaluate direct effects of FGF23 on parathyroid cells, we investigated expression levels of Egr-1, a marker of FGF23 signaling (Urakawa *et al.* 2006). In agreement with previous reports, we observed a rise in Egr-1 as early as 1 h after FGF23(R176Q) treatment. In contrast, we noted a significant decrease in PTH mRNA 12 h after FGF23(R176Q) treatment, indicative of a late response gene. Our data strongly suggest a direct effect of FGF23 on PTH expression, although we cannot rule out the possibility that FGF23 may regulate PTH indirectly, by stimulating the release of other locally produced factor(s).

Notably, neither cell number nor viability or apoptosis was affected by treatment at high concentrations of FGF23(R176Q). A small but significant increase in cell proliferation was observed at 24 h, although the biological significance of this finding is unclear. Our data support the assertion that FGF23 does not

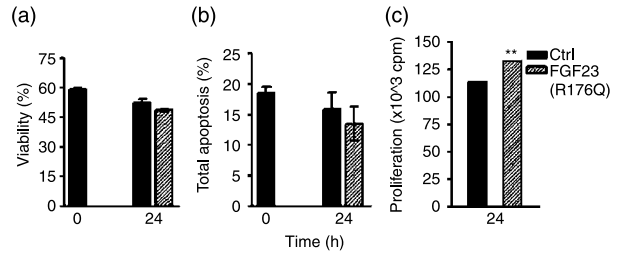


Figure 5 Effects of FGF23(R176Q) treatment on: (a) cell viability; (b) apoptosis; and (c) proliferation. No difference between FGF23(R176Q) and control-treated cells was observed with regard to cell viability and apoptosis, whereas a small increase in cell proliferation was noted. Cells were treated with 2000 pg/ml FGF23(R176Q). All measurements were performed after 24 h of FGF23(R176Q) treatment. ** $P < 0.01$. Error bars indicate mean \pm S.E.M. No error bars are seen in (c) due to small variability.

exert any major stimulatory or inhibitory growth effects on parathyroid cells *in vitro* at the short term. However, they suggest that the negative effect of FGF23(R176Q) on PTH expression is direct and independent of cell growth and/or survival.

In the present study, we used relatively high doses, i.e. 400–2000 pg/ml FGF23(R176Q) in order to observe effects on the PTH mRNA level or protein secretion. These concentrations are well above the reference range for healthy individuals (Yamazaki *et al.* 2002) and are typically found in subjects with ESRD (Larsson *et al.* 2003a) and some TIO and XLH patients (Jonsson *et al.* 2003). Our data may imply that FGF23 regulation of PTH is important in pathophysiology; however, they may also reflect typical *in vivo/in vitro* differences.

The *in vitro* system used in the present study provides a novel tool for the study of potential physiological actions of FGF23. However, a limitation of this system is that primary isolated parathyroid cells lose their ability to secrete PTH protein and display decreased PTH mRNA message over time, independent of FGF23 action. Therefore, one has to be careful when extrapolating our *in vitro* data into *in vivo* physiology. The presence of Klotho in parathyroid cells, as well as independent associations between serum FGF23 and PTH in early CKD (Shigematsu *et al.* 2004, Westerberg *et al.* 2007) are, however, suggestive of a physiological regulation of PTH by FGF23.

Since $1\alpha(\text{OH})\text{ase}$ is expressed in parathyroid glands (Segersten *et al.* 2002), we investigated the effect of FGF23(R176Q) on its mRNA transcript levels. Interestingly, FGF23(R176Q) dose-dependently increased $1\alpha(\text{OH})\text{ase}$ mRNA level. This indicates a differential regulation of $1\alpha(\text{OH})\text{ase}$ in kidneys and parathyroid glands, since FGF23 is known to potently and rapidly reduce renal expression of $1\alpha(\text{HO})\text{ase}$ (Shimada *et al.* 2001, 2002). The reason for this discrepancy is unclear, but may speculatively be an attempt of parathyroid glands to locally compensate for decreased serum calcitriol provoked by high circulating FGF23 levels. Further studies are needed to understand the differential expression and determine whether this has any functional importance *in vivo*.

The etiology of secondary hyperparathyroidism in CKD is multifactorial, but involves alterations in mineral ion homeostasis. The combination of low 1,25(OH)₂D₃ and high Pi in CKD effectively triggers the production of PTH, leading to a gradual development of parathyroid hyperplasia and elevated PTH levels (Fukagawa *et al.* 2006). Another implication of our study is a plausible role of FGF23 in secondary hyperparathyroidism of CKD, supported by recent studies (Bai *et al.* 2004, Larsson *et al.* 2004, Nakanishi *et al.* 2005). Our data may imply that FGF23, at least in part, has an inhibitory role in secondary hyperparathyroidism of CKD. In support, treatment with appropriate doses of calcitriol increases serum FGF23 (Collins *et al.* 2005, Ito *et al.* 2005, Nishi *et al.* 2005), while effectively preventing or postponing secondary hyperparathyroidism in CKD. In fact, one could speculate that the beneficial effects of calcitriol treatment in CKD may partly be attributed to an increase in FGF23. Of note, secondary hyperplastic glands and adenomas of hyperparathyroidism are phenotypically different when compared with normal parathyroid cells used in our *in vitro* system and could therefore respond differently to FGF23. Further studies are needed to clarify the role of FGF23 in secondary hyperparathyroidism.

In conclusion, FGF23(R176Q) is a negative regulator of PTH expression; however, it stimulates parathyroid 1 α (OH)ase expression *in vitro*. Our data support the existence of a physiological skeletal-parathyroid endocrine axis and further provide insights into the complex regulation of calcium and Pi homeostasis.

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Disclosure statements

T E L and Ö Lj receive consulting fees. All other authors have no conflicts of interest.

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