Parathyroid cell resistance to fibroblast growth factor 23 in secondary hyperparathyroidism of chronic kidney disease

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Although fibroblast growth factor 23 (FGF23) acting through its receptor Klotho-FGFR1c decreases parathyroid hormone expression, this hormone is increased in chronic kidney disease despite an elevated serum FGF23. We measured possible factors that might contribute to the resistance of parathyroid glands to FGF23 in rats with the dietary adenine-induced model of chronic kidney disease. Quantitative immunohistochemical and reverse transcription–PCR analysis using laser capture microscopy showed that both Klotho and FGFR1 protein and mRNA levels were decreased in histological sections of the parathyroid glands. Recombinant FGF23 failed to decrease serum parathyroid hormone levels or activate the mitogen-activated protein kinase signaling pathway in the glands of rats with advanced experimental chronic kidney disease. In parathyroid gland organ culture, the addition of FGF23 decreased parathyroid hormone secretion and mRNA levels in control animals or rats with early but not advanced chronic kidney disease. Our results show that because of a downregulation of the Klotho–FGFR1c receptor complex, an increase of circulating FGF23 does not decrease parathyroid hormone levels in established chronic kidney disease. This *in vivo* resistance is sustained in parathyroid organ culture *in vitro*.

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KEYWORDS: chronic renal failure; gene expression; hyperparathyroidism; parathyroid hormone

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Fibroblast growth factor 23 (FGF23) is secreted by osteocytes and osteoblasts in response to hyperphosphatemia and $1,25(OH)_2$ vitamin D and exerts its effect on its receptor, the Klotho–FGFR1c heterodimer, in the kidney where it inhibits the expression of NaPi2 and the 25(OH) vitamin D 1α(OH)ase, CYP27B1.[1,2] The action of FGF23 on the kidney results in phosphaturia and a decreased serum $1,25(OH)_2$ vitamin D level, which acts to correct serum Pi. FGF23 also exerts its effect on the Klotho–FGFR1c complex in the parathyroid to decrease parathyroid hormone (*PTH*) gene expression and secretion in rats with normal kidney function, as well as *in vitro* in rat and bovine tissue.[3,4] Surprisingly, in chronic kidney disease (CKD), there are markedly elevated levels of both FGF23 and PTH.[5] This finding implies resistance of parathyroids to FGF23 in renal failure. The increase in serum FGF23 is found in early CKD[6] and, at least in stage 4 CKD,[7] it precedes the decrease in serum $1,25(OH)_2$ vitamin D, which was found in a large cohort of CKD patients to be the earliest change in mineral metabolism parameters.[8] The decrease in serum $1,25(OH)_2$ vitamin D is considered to be an important factor in the pathogenesis of secondary hyperparathyroidism.[9] The increased FGF23 levels in renal failure patients correlate with the progression of renal failure,[10,11] the prediction of the development of secondary hyperparathyroidism,[12] as well as mortality in patients starting dialysis.[13] Therefore, it is important to understand the mechanisms that prevent the parathyroid from responding to high levels of FGF23 in uremia. In this study, we show that the resistance to FGF23 in parathyroids of rats with experimental CKD correlates with the downregulation of parathyroid Klotho–FGFR1 expression and FGF23 signaling.

RESULTS

Klotho and FGFR1 protein and mRNA levels are decreased in parathyroids of rats with adenine high Pi-induced CKD

Rats fed an adenine high-Pi diet had a fourfold increase in serum creatinine at 2 weeks and a 10-fold increase at 6 weeks, accompanied by matching increases in serum urea (Table 1). Serum Pi was increased at 2 weeks and even more so at 6 weeks. Serum PTH was fourfold increased at 2 weeks and 18-fold increased at 6 weeks together with an increase in serum FGF23 levels (Table 1). Parathyroid staining with

Table 1 | Serum creatinine, urea, calcium, phosphate, and PTH levels in control and CKD rats

	Control	2 Weeks	6 Weeks	*P*-value
Creatinine (μmol/l)	19 ± 2	80 ± 16	225 ± 12	<0.0001
Urea (mmol/l)	7.8 ± 0.3	32.4 ± 2.5	39 ± 2.1	<0.0001
Calcium (mmol/l)	2.43 ± 0.14	2.21 ± 0.11	2.12 ± 0.1	NS
Phosphorus (mmol/l)	2 ± 0.004	2.76 ± 0.17	4.3 ± 0.12	<0.0001
PTH (pg/ml)	207 ± 14	908 ± 161	3764 ± 621	<0.0001
FGF23 (pg/ml)	205 ± 37	552 ± 70	2802 ± 366	<0.0001

Abbreviations: CKD, chronic kidney disease; NS, not significant; PTH, parathyroid hormone.
Values are presented as mean ± s.e.m. (*n*=4).

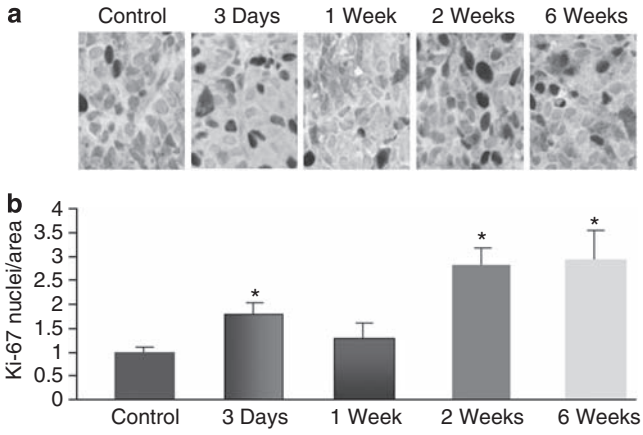

Figure 1 | Parathyroid cell proliferation in experimental chronic kidney disease (CKD) rats. (**a**) Immunohistochemistry (IH) for Ki-67 as a marker of cell proliferation in parathyroid sections from rats fed a control or adenine high-Pi diet for the indicated duration. (**b**) Ariol quantification of Ki-67 staining as in **a** presented as mean ± s.e.m. from 3–4 rats in each group. *$P<0.05$ compared with control.

Ki-67, a marker of cell proliferation, was increased both at 2 and 6 weeks of the adenine high-Pi diet (Figure 1a and b). Parathyroid cell proliferation increased after 3 days of the diet. Intriguingly, at CKD 1 week, Ki-67 staining was transiently reduced to similar levels as in parathyroids of control rats (Figure 1a and b). Therefore, in this model, parathyroid cell proliferation is increased at 3 days, is arrested at 1 week, and is evident again at 2 and 6 weeks of the diet.

Immunohistochemistry (IH) of the parathyroids of rats fed the adenine high-Pi diet for 2 weeks showed an increase in Klotho and a decrease in FGFR1 (Figure 2a and b). There was no significant change in parathyroid VDR and CaR at 2 weeks (Figure 2a and b). Importantly, at 6 weeks, there was a decrease in Klotho and FGFR1 levels, as well as a decrease in VDR and CaR (Figure 2a and b). The parathyroid PTH protein did not change, despite the marked hyperparathyroidism (Figure 2a and b; Table 1). To study the correlation between parathyroid cell proliferation (Ki-67), Klotho, and FGFR1 expression, we stained consecutive parathyroid sections of rats fed the adenine high-Pi diet for 3 days, and for 1 and 2 weeks. Ki-67 was increased at 3 days but not at 1 week and increased at 2 weeks (Figure 2c and d) as before (Figure 1). Klotho was increased at 1 and 2 weeks, whereas FGFR1 was already decreased at 3 days (Figure 2c and d). These results show that in experimental CKD at 6 weeks, there is a decrease in FGFR1 and Klotho protein levels that may contribute to the resistance of the parathyroid to FGF23.

Laser capture microscopy of equivalent areas of parathyroid sections (Figure 3a) from control rats and rats fed the adenine high-Pi diet for 6 weeks showed a decrease in both Klotho and FGFR1 mRNAs (Figure 3b). PTH mRNA levels were increased in CKD rats at 6 weeks (Figure 3b). Therefore, both Klotho and FGFR1 protein and mRNA levels were decreased in these rats with advanced experimental CKD and secondary hyperparathyroidism. We then studied the response of the parathyroids of CKD rats to FGF23.

Administered FGF23 does not decrease PTH expression at 6 weeks of experimental CKD both *in vivo* and *in vitro*

FGF23 administered intraperitoneally led to a decrease in serum PTH in control rats as previously described (Figure 4a).[3] FGF23 also decreased serum PTH in CKD rats at 2 weeks (Figure 4a). However, FGF23 had no effect on serum PTH in CKD rats at 6 weeks (Figure 4a). FGF23 binds to its receptor, the Klotho–FGFR1c heterodimer, to activate the mitogen-activated protein kinase (MAPK) pathway and thereby induces the expression of *Egr1* (early growth response gene 1).[1,3] To study signal transduction after FGF23 in parathyroids of CKD rats, we measured Egr1 mRNA levels. To do this, we developed a novel methodology, whereby we anesthetized rats, exposed the two parathyroid glands, and applied FGF23 topically on one gland and a medium to the contralateral parathyroid as a control in the same rat. After 30 min, we extracted the RNA from each microdissected gland and quantified mRNA levels. FGF23 led to a twofold induction of Egr1 mRNA in control rat parathyroid glands compared with vehicle-treated contralateral glands (Figure 4b). In contrast, there was no induction of Egr1 mRNA by FGF23 in rats fed the adenine high-Pi diet for 6 weeks (Figure 4b). These experiments show that FGF23 does not activate the MAPK pathway in rats fed the adenine high-Pi diet for 6 weeks and subsequently does not decrease serum PTH *in vivo*.

We then studied the effect of FGF23 *in vitro* using rat parathyroid glands maintained in organ culture for 2 h. FGF23 added to the culture medium led to a decrease in PTH secreted into media by parathyroid glands from control and 2-week CKD rats, but not from 6-week CKD rats (Figure 5a). FGF23 also decreased PTH mRNA levels in both control and 2-week CKD rats but not in 6-week CKD rats (Figure 5b). The effect of FGF23 aside, it is noteworthy that the parathyroids of CKD rats that were cultured with control medium secreted more PTH and had higher PTH mRNA levels after 2 h of organ culture compared with control rats (Figure 5b). The increased PTH expression in CKD parathyroids occurred despite being maintained in normal culture medium *in vitro*. Importantly, the decrease in Klotho and FGFR1 mRNA in parathyroids of 6-week CKD rats

(Figure 3b) was also evident in the glands analyzed at the termination of incubation *in vitro* (Figure 5c). Therefore, the parathyroids of CKD rats maintain the increase in PTH expression and decrease in Klotho and FGFR1 mRNA levels of secondary hyperparathyroidism *ex vivo* at least for the 2 h studied here and thus their resistance to FGF23.

DISCUSSION

FGF23 exerts its effect on the parathyroid through its receptor, Klotho–FGFR1c, to activate the MAPK pathway and decrease PTH expression. However, in CKD, there are high levels of both FGF23 and PTH. It was important therefore to understand why in CKD the high levels of FGF23 do not efficiently suppress PTH production and secretion. We now report that there is a decrease in parathyroid gland Klotho and FGFR1 mRNA and protein levels in advanced experimental CKD. These changes may contribute to the resistance of the parathyroid to the high levels of FGF23 in CKD. Accordingly, recombinant FGF23 did not decrease PTH expression in rats fed an adenine high-Pi diet for 6 weeks as opposed to control rats and rats with 2 weeks experimental CKD. Similarly, the parathyroid glands from rats with advanced CKD failed to respond *in vitro* to FGF23, as opposed to the glands from control rats and rats with early CKD.

To study the resistance of the parathyroid to FGF23, we used a rat model of adenine-induced CKD for short term at 2 weeks and for long term at 6 weeks of the diet. The CKD model induced secondary hyperparathyroidism that was greater at 6 than at 2 weeks. We used computerized quantification of Ki-67, Klotho, FGFR1, CaR, VDR, and PTH protein levels in paraffin-embedded parathyroid sections of control and CKD rats. Parathyroid cell proliferation as measured by quantification of Ki-67 staining was increased to the same degree at 2 and 6 weeks. Interestingly, there was already higher cell proliferation at 3 days but not at 1 week. At 3 days of the diet, serum creatinine had already increased 2.5-fold, which was maintained at 1 week when serum PTH levels were increased sixfold.[14] A similar phenomenon was described by others using the 5/6 nephrectomy CKD model, in which it was suggested that cell proliferation was restricted to the first days of experimental CKD.[15] However, they did not report longer time intervals. Our findings show that proliferation resumes after

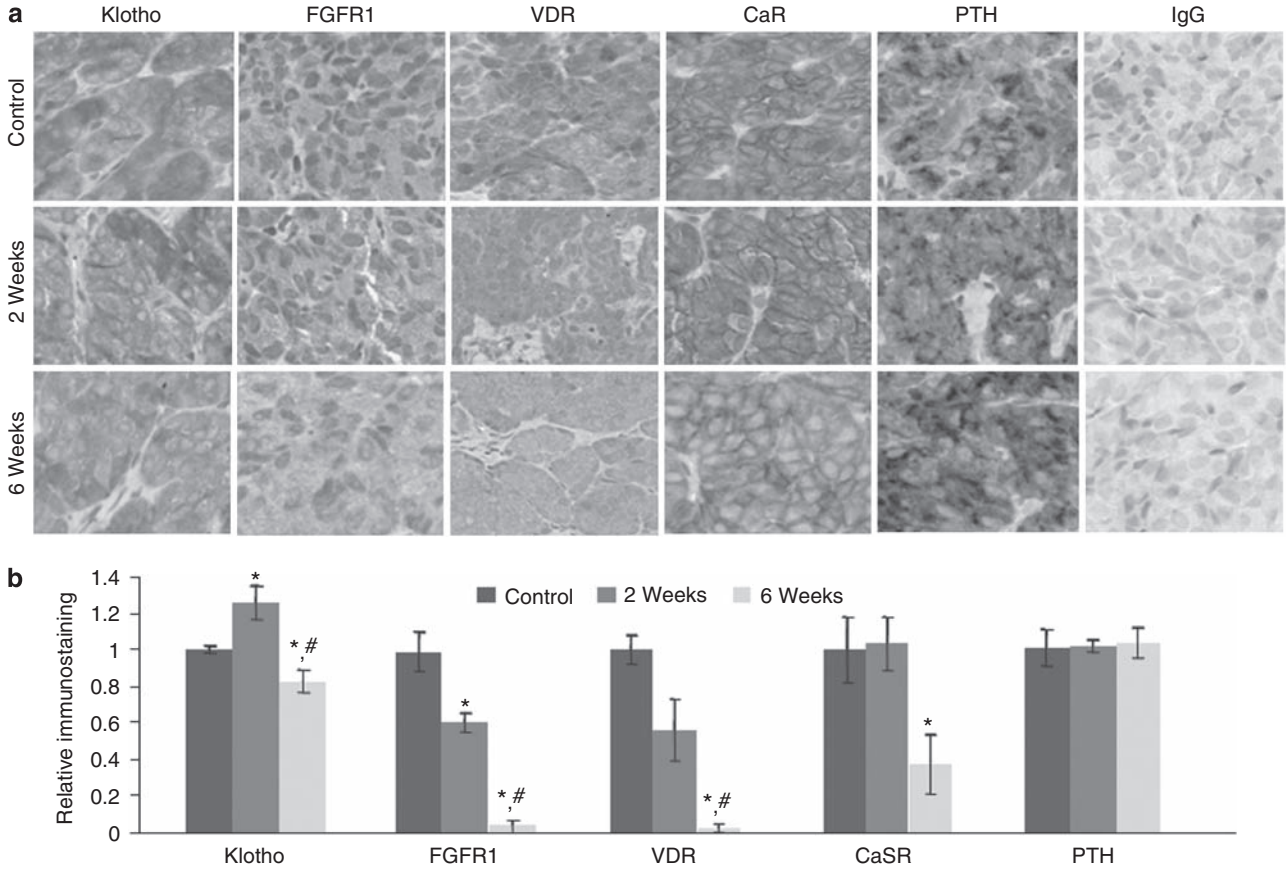

Figure 2 | Ki-67, Klotho, FGFR1, VDR, CaR, and parathyroid hormone (PTH) proteins in parathyroid glands of chronic kidney disease (CKD) rats. (**a**) Immunohistochemistry (IH) of Klotho, FGFR1, VDR, CaR, PTH, and IgG as control of parathyroid sections from control, 2, and 6-week CKD rats. (**b**) Ariol quantification of IH as in **a** for four rats in each group. Results are mean ± s.e.m., $^{*}P<0.05$ compared with control; $^{\#}P<0.05$ compared with 2-w AHP. (**c**) IH of Ki-67, Klotho, and FGFR1 in consecutive parathyroid sections from control, 3-day-, and 1- and 2-week CKD rats. (**d**) Ariol quantification of IH as in **c** for Klotho and FGFR1. Data for Ki-67 are included in Figure 1b. Results are mean ± s.e.m., for 3–5 rats in each group; $^{*}P<0.05$ compared with control.

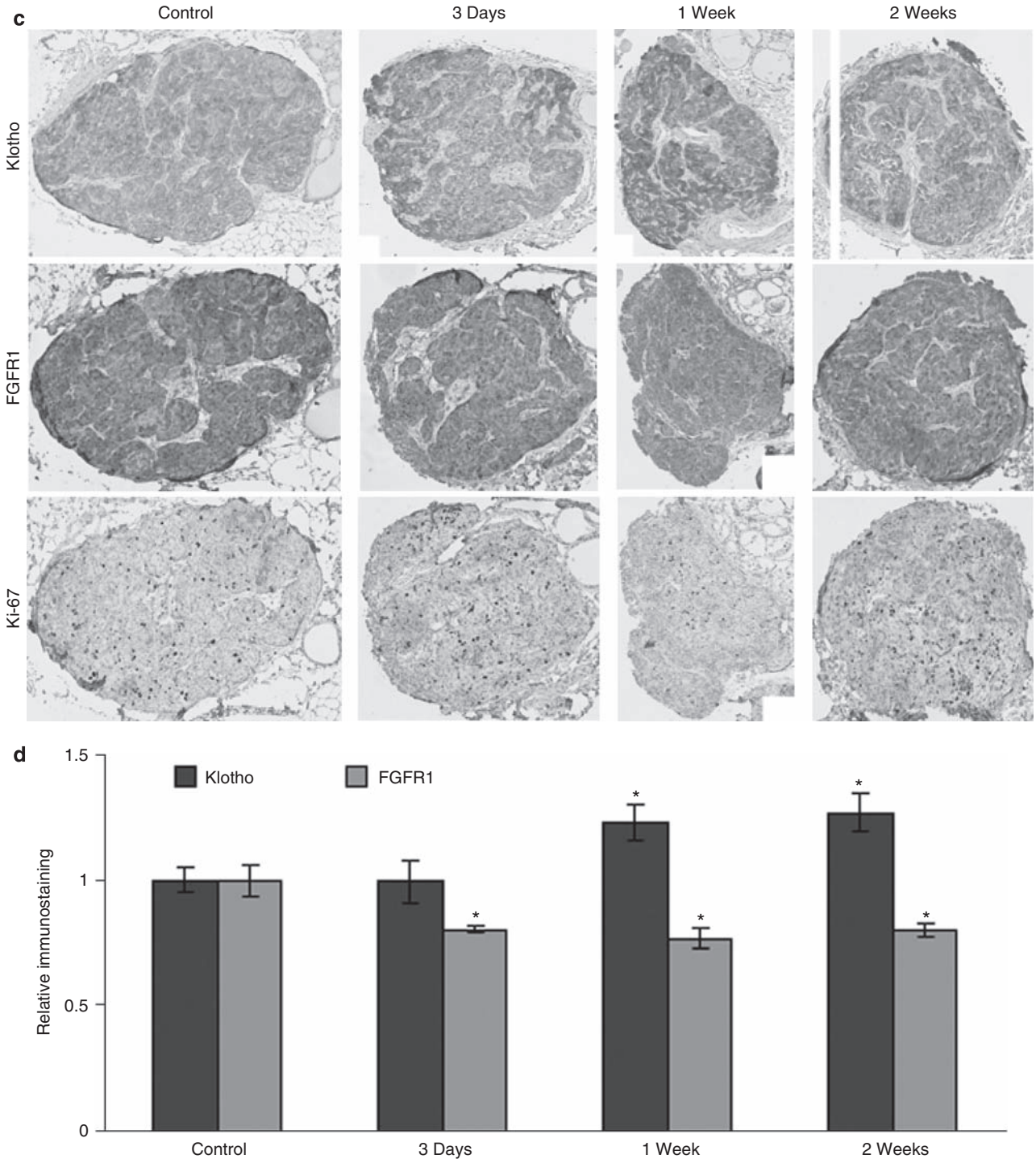

Figure 2 | Continued.

the first week of adenine high Pi-induced CKD. The IH of parathyroid glands from control and 6-week CKD rats showed reduced levels of Klotho and FGFR1, as well as CaR and VDR. The reduction in parathyroid CaR and VDR in CKD has been widely reported.[16,17] Although at 6 weeks of the diet, cell proliferation was increased, whereas FGFR1 and Klotho levels were decreased (Figure 2), the downregulation of the FGF23 receptor complex is not directly related to active cell proliferation because the changes in FGFR1, Klotho, and Ki-67 occur at different time points.

The decrease in Klotho and FGFR1 protein levels is accompanied by a decrease in Klotho and FGFR1 mRNA levels, as detected in parallel sections by laser-captured rat parathyroid tissue of 6-week CKD rats. Laser capture

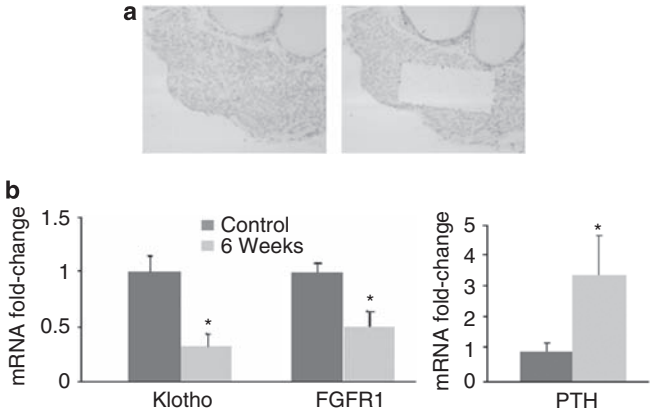

Figure 3 | Klotho and FGFR1 mRNA levels are decreased and parathyroid hormone (PTH) mRNA increased in the parathyroids of rats with chronic kidney disease (CKD) at 6 weeks. (**a**) Histological section of rat parathyroid tissue before and after laser capture showing the defined area excised for RNA extraction. (**b**) Klotho, FGFR1, and PTH mRNAs and β-actin mRNA as control measured by quantitative reverse transcription–PCR from control and 6-week CKD rats. Results are presented as fold change, mean ± s.e.m. from four rats with three areas analyzed from each rat. $^*P<0.05$.

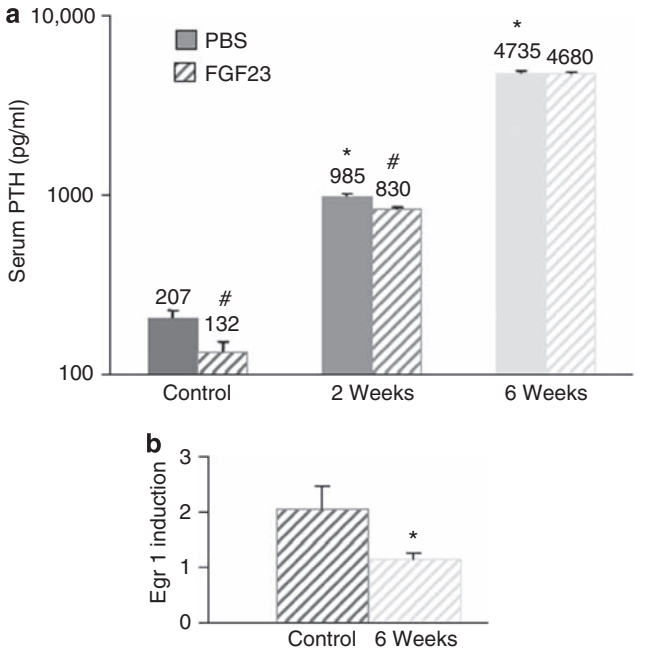

Figure 4 | Fibroblast growth factor 23 (FGF23) decreases serum parathyroid hormone (PTH) in control and 2-week chronic kidney disease (CKD) rats but not in 6-week CKD rats and increases Egr1 mRNA in control but not in 6-week CKD rats. (**a**) Serum PTH levels 1 h after injection of FGF23 or vehicle (phosphate-buffered saline, PBS) into control, 2-week and 6-week CKD rats. Results are presented as mean ± s.e.m. ($n=4$), $^*P<0.05$. (**b**) Egr1 induction. Parathyroid glands from control and 6-week CKD rats were exposed and FGF23 was topically applied to one gland of each rat and control medium to the other gland for 30 min. Quantitative reverse transcription–PCR was performed for Egr1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA from RNA extracted from single glands. Egr1 induction is presented as mean ± s.e.m. of the increase in Egr1 mRNA in the FGF23-treated gland compared with the contralateral untreated gland. $^*P<0.05$, $n=4$–5 rats.

microscopy allows the analysis of the parathyroid and not of the surrounding tissue. PTH mRNA levels were increased in 6-week CKD rats, indicating the active synthesis of the hormone, which is then rapidly translated and secreted as evidenced by the finding that there is no change in PTH protein staining in parathyroid cells. Depletion of hormone stores when there is excess stimulus to the gland is found in other endocrine organs, such as the β-cell in diabetes mellitus type 2.[18,19] In the parathyroids of 2-week CKD rats, there was an increase in Klotho protein but a decrease in FGFR1 expression. The finding that Klotho was increased at 2 weeks may explain the response of the parathyroid to administered FGF23 in early CKD *in vivo* and *in vitro*. The mechanism by which parathyroid Klotho expression is regulated in CKD is not known. It is known that Klotho mRNA levels in bovine parathyroid cells are inversely correlated to serum calcium.[20]

Consistent with the decrease in the Klotho–FGFR1 complex expression in parathyroids in advanced CKD, they also failed to respond to recombinant FGF23 both *in vivo* and *in vitro* at the levels of PTH secretion and gene expression. The failure of parathyroids to respond to administered FGF23 in CKD *in vivo* was reflected in mRNA levels of Egr1, a marker of MAPK activation, which were not increased after FGF23.[1,3] We measured Egr1 mRNA after the topical application of FGF23 to one parathyroid gland, whereas the contralateral gland was used as a control. This methodology allows the maintenance of a physiological milieu and the study of the control and test parathyroid in the same rat. We were able to conclude that FGF23, which led to a twofold induction of Egr1 mRNA in control rats, was ineffective in inducing Egr1 in parathyroids of 6-week CKD rats. This would prevent the effect of FGF23 on *PTH* gene expression.

Glands from 6-week CKD rats secreted higher amounts of PTH *in vitro* in organ culture, despite removal from their uremic surrounding corresponding to the high serum PTH levels *in vivo*. Moreover, there was a progressive increase in PTH mRNA levels in the parathyroid organ cultures from CKD rats corresponding to the severity of the CKD and to PTH mRNA levels *in vivo*.[14] In contrast, Klotho and FGFR1 mRNA levels progressively decreased with CKD *in vitro* as *in vivo*. Thus, characteristics of the parathyroid *in vivo* are sustained *in vitro*, at least for the 2 h studied, despite being removed from the uremic environment and maintained in normal medium. These include both basal expression of PTH and the failure to respond to FGF23. Therefore, the decreased Klotho-FGFR1 and signal transduction, and increased PTH expression are inherent to the parathyroid gland. These changes in secondary hyperparathyroidism may be the result of prolonged changes in any of the parameters of mineral metabolism, such as serum Pi, calcium, vitamin D, FGF23, and perhaps PTH itself, as well as uremic toxins.[21] The limited viability of glands in culture precludes the study of a

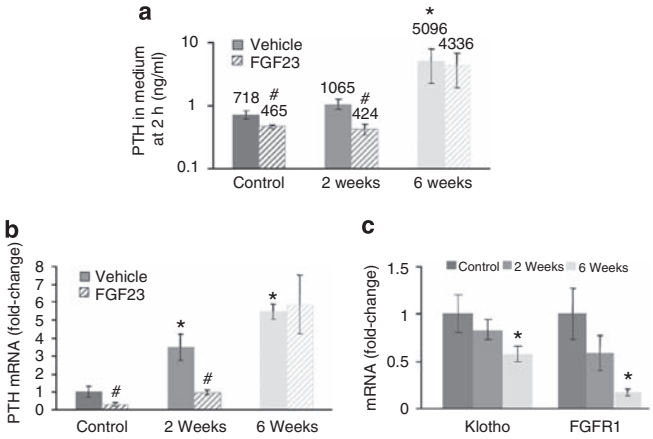

Figure 5 | Fibroblast growth factor 23 (FGF23) decreases serum parathyroid hormone (PTH) and PTH mRNA levels in parathyroid organ cultures from control and 2-week chronic kidney disease (CKD) rats but not in 6-week CKD rats. Pairs of parathyroid glands from control, 2-week or 6-week CKD rats were incubated in culture media without (vehicle) or with FGF23 for 2 h. (**a**) PTH levels in culture media, presented in semilogarithmic scale. $^{*}P<0.05$ compared with glands from control rats; $^{\#}P<0.05$ compared with vehicle-treated glands, $n=4$–8. (**b**) PTH mRNA levels analyzed by quantitative reverse transcription–PCR for PTH and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in parathyroid glands as in **a**. (**c**) Klotho and FGFR1 mRNA levels in vehicle-treated glands as in **a** from control, 2-week and 6-week CKD rats. Results in **b** and **c** are represented as fold change, mean ± s.e.m. ($n=4$–8), $^{*}P<0.05$.

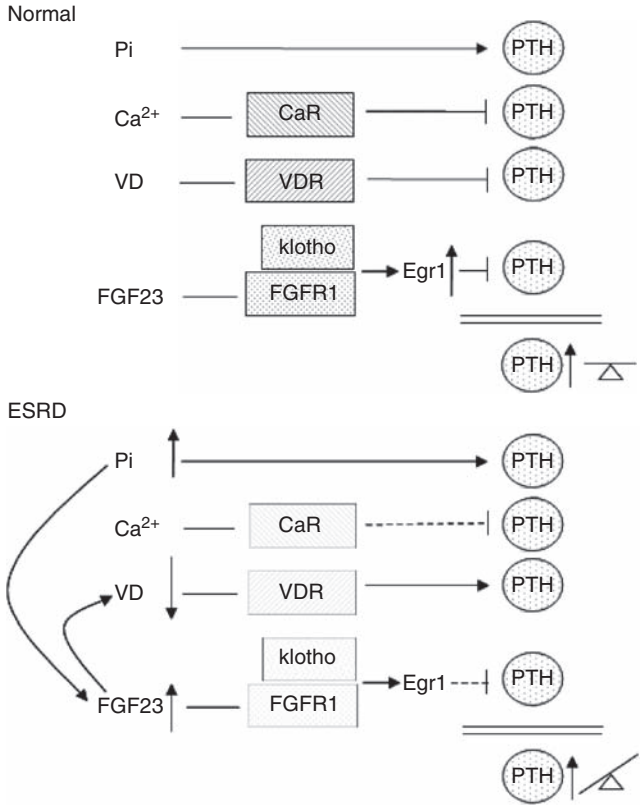

Figure 6 | Model for the regulation of parathyroid hormone (PTH) expression by Pi, Ca^{2+}, vitamin D, and fibroblast growth factor 23 (FGF23) in normal physiology and end-stage renal disease (ESRD). When there is normal renal function, Pi increases serum PTH, and Ca^{2+}, $1,25(OH)_2$ vitamin D, and FGF23 decrease serum PTH by exerting an effect on their respective receptors. The Pi sensing mechanism is not known. In ESRD, the high Pi and low $1,25(OH)_2$ vitamin D lead to an increase in serum PTH. The decrease in parathyroid VDR and CaR also contributes to the lack of response to circulating $1,25(OH)_2$ vitamin D and Ca^{2+}. There are very high FGF23 levels in ESRD, which fail to suppress PTH secretion because of a downregulation of the Klotho-FGFR1 heterodimer and signal transduction through mitogen-activated protein kinase (MAPK). Together, these changes in Pi, Ca^{2+}, $1,25(OH)_2$ vitamin D, and their receptors, as well as the FGF23 receptor, result in secondary hyperparathyroidism.

direct effect of prolonged changes in uremic toxins on the resistance of the parathyroid to FGF23 *in vitro*.

In summary, in advanced CKD, resistance of the parathyroid to FGF23 is associated with a decrease in Klotho-FGFR1 expression both in mRNA and protein levels. As a result, the high FGF23 levels characteristic of advanced CKD fail to signal through the MAPK pathway to increase Egr1 mRNA and decrease PTH (Figure 6). The resistance of the parathyroid to FGF23 in CKD contributes to the secondary hyperparathyroidism of CKD. In addition, FGF23 decreases $1,25(OH)_2$ vitamin D levels, which correlate with increased serum PTH.[10,22] Of interest, specific deletion of the VDR in the parathyroid of mice with normal kidney function leads only to a moderate increase in serum PTH levels.[2] Therefore, in CKD, secondary hyperparathyroidism is caused by a number of factors functioning in concert. These are at least high serum phosphate, decreases in parathyroid CaR, VDR, and Klotho–FGFR proteins, resistance to FGF23, and decrease in serum $1,25(OH)_2$ vitamin D.

MATERIALS AND METHODS

Experimental animals

Adult male Sabra rats (weighing 175–200 g) were fed a control diet or a 0.75% adenine, high-phosphorus (1.5%) diet[23] for the indicated time periods to induce CKD and secondary hyperparathyroidism. Serum calcium, Pi, creatinine, and urea were measured in the study groups (control rats, short-term CKD, and long-term CKD). PTH was measured both before and 1 h after i.p. injection of 4 μg/kg mouse recombinant FGF23 with the R179Q proteolysis-resistant mutation[24] (R&D Systems, Minneapolis, MN, USA, catalog no. 2629-FG-025), or control carrier, phosphate-buffered saline ($n=4$ for each group at each time point). Phosphate-buffered saline was injected daily i.p. into all rats for 2 days before the actual experiment to familiarize the rats with the procedure. After 2 or 6 weeks on the diet, microdissected parathyroid glands were removed under ketamine–xylazine anesthesia for *ex vivo* experiments. In parallel, parathyroid glands were removed from control rats. Parathyroid glands were placed on filter membranes and incubated in cell culture media at 37 °C with constant rocking,[3] with or without ($n=4$ pairs from each group) recombinant mouse FGF23 (100 ng/ml).

In some experiments, parathyroid glands of ketamine-anesthetized rats were exposed. FGF23 was applied topically to one gland and control medium to the contralateral parathyroid gland for 30 min. FGF23 (1 μg/ml) or vehicle (medium) was soaked in Whatmann paper, which was placed on the gland. RNA was then extracted from each microdissected gland. Quantitative reverse

transcription–PCR was performed for *Egr1*and control genes. All animal experiments were approved by the Hadassah Hebrew University Animal Care and Use Committee.

Biochemistry

Serum/medium rat PTH (Immutopics, San Clemente, CA, USA, catalog no. 60-2500) and FGF23 (Kainos Lab, Tokyo, Japan) were measured by enzyme-linked immunosorbent assay. The increased serum FGF23 levels in the CKD rats were also shown using a c-terminal FGF23 Elisa kit (Immutopics, San Clemente, CA, USA, catalog no. 60-6300). Serum calcium, phosphate, creatinine, and urea levels were assayed using appropriate kits (QuantiChrom DICA-500, DIPI-500, DICT-500 and DIUR-500; BioAssay Systems, Hayward, CA, USA).

Immunohistochemistry and its quantification

Rat parathyroid glands were excised and fixed in 4% formaldehyde, embedded in paraffin, and 4 μm sections were prepared. After rehydration, antigens were retrieved by autoclave (120 °C for 3 min) with 20 mM citric acid buffer (pH = 6; for PTH, Klotho, FGFR1, and VDR), EDTA (pH = 8; Zymed Laboratories, San Francisco, CA, USA, for CaR), or 100 mM glycine (pH = 9; for Ki-67). Immunostaining was performed overnight at 4 °C using the following primary antibodies diluted in Cas block (Zymed Laboratories): VDR (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA, catalog no. sc-1009), PTH (1:100; Abd Serotec, Oxford, UK, catalog no. 7170-6216), Ki-67 (1:200; Biocare Medical, Concord, CA, USA, catalog no. CM137A), CaR (1:500; Novus Biologicals, Littleton, CO, USA, catalog no. NB120-19347), FGFR1 (1:100; Santa Cruz Biotechnology), and Klotho 1:100 (Kyowa Hakko Kogyo, Tokyo, Japan). Slides were incubated with the appropriate horseradish-peroxidase-conjugated secondary antibodies and stained by exposure to DAB chromogen (Dako, Glostrup, Denmark), followed by counterstaining with hematoxylin. Light hematoxylin staining was needed to define nuclei and to facilitate some of the quantifications, for example Ki-67. Representative microscopic images are shown. Immunostaining was quantified with an automated system (Ariol, Genetix, New Milton, Hampshire, UK).

Ariol was programmed to identify the spectrum of brown staining and ignore all other colors, including the purple shades of the nuclear hematoxylin and eosin staining. Quantification was as follows for all but the Ki-67 staining: assignment of a threshold intensity value for positive staining; assignment of threshold value for background staining (in such a manner that would include all cells but not intercellular space); and calculation of the ratio between the areas corresponding to these two values (positive area/total tissue area). This method of calculation was in perfect agreement with a variation that included a term for the actual intensity (positive area × intensity/total area). For Ki-67, calculation was determined by positive nuclei/total number of nuclei (which was in excellent agreement with positive nuclei/total area of nuclei and positive nuclei/total tissue area). All parathyroid glands (control, short-, and long-term uremia) were blocked together on the same slide to eliminate differences in staining intensity among slides.

Parathyroid tissue recovery by laser capture microdissection and RNA extraction

Parathyroids from formalin-fixed paraffin-embedded sections were laser microdissected using a PALM Microlaser System (Zeiss, Oberkochen, Germany). RNA was extracted from 2–3 separate areas of identical size from each parathyroid section using peqGold TriFast (peqlabs Biotechnologie, Erlangen, Germany) supplemented with glycogen. cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and PCR analysis was preformed with GoTaq Green PCR Master Mix (Promega, Madison, WI, USA).

Table 2 | PCR primers used for qRT–PCR

	Forward	Reverse
PTH	ttgtctccttacccaggcagat	tttgcccaggttgtgcataa
Klotho	ccatgccgagcaagactca	ccgtccaacacgtaggcttt
FGFR1	ataccaccgacaaggaaatg	ttccaggtacagaggtgagg
Egr-1	tacgagcacctgaccacagagt	gctgggataacttgtctccacc
β-Actin	caggcattgctgacaggatg	ctcaggaggagcaatgatcttgat
GAPDH	gcaactcccattcttccacc	cataccaggaaatgagcttcacaa

Abbreviation: qRT–PCR, quantitative reverse transcription–PCR.

Real-time PCR

RNA was analyzed by quantitative reverse transcription–PCR for PTH, Klotho, FGFR1, Egr1, and control mRNAs. Real-time PCR was performed using an ABI Prism 7900 Sequence Detection System (Applied Biosystems) and SYBR Green Mix (Applied Biosystems). Primers (Table 2) were designed using Primer Express software version 2.0 (Applied Biosystems) to include exon–exon junctions and/or span large intronic sequences to prevent amplification of genomic DNA. Amplicon specificity was ascertained by melting curve analysis and length evaluation was ascertained by ethidium bromide staining of agarose gels.

Statistical analysis

Results were analyzed by one-way analysis of variance with *post hoc* Bonferroni's pairwise comparisons. PTH serum levels after administration of FGF23 were log-transformed and adjusted to preinjection values by means of a general linear model (SPSS 15.0; SPSS Inc., Chicago, IL, USA). Two-tailed *P*-values <0.05 were considered statistically significant. Results are expressed as mean ± s.e.m.

DISCLOSURE

All the authors declared no competing interests.

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