

Parathyroid Hormone Regulates Fibroblast Growth Factor-23 in a Mouse Model of Primary Hyperparathyroidism

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ABSTRACT

The importance of fibroblast growth factor 23 (FGF-23) in the pathogenesis of phosphate wasting disorders has been established, but controversy remains about how parathyroid hormone (PTH), which also stimulates urinary phosphate excretion, regulates the circulating level of FGF-23. We found that the serum FGF-23 concentration was higher in PTH-cyclin D1 transgenic mice, a model of primary hyperparathyroidism, than in wild-type mice. The serum FGF-23 concentration was significantly and directly correlated with serum PTH and calcium, and inversely correlated with phosphate levels in 90- to 118-week-old mice (all $P < 0.005$). Quantitative real-time reverse-transcriptase PCR revealed abundant expression of *fgf23* in bone, especially in calvaria. The *fgf23* expression in calvaria was significantly higher in the transgenic mice compared to the wild-type mice, and correlated well with serum FGF-23 levels. There was a direct correlation between the expression of *fgf23* and the expression of *osteocalcin* and *ALP*, suggesting that activation of osteoblasts is important in the regulation of FGF-23. Serum FGF-23 levels decreased in the transgenic mice after parathyroidectomy. In conclusion, PTH plays a major role in the regulation of serum FGF-23 level in primary hyperparathyroidism, likely via activation of osteoblasts in bone.

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An excess of fibroblast growth factor-23 (FGF-23), a member of the FGF family, is now known to be a major factor in the development of hypophosphatemic rickets/osteomalacia, including X-linked hypophosphatemic rickets and oncogenic osteomalacia.^{1–3} Autosomal dominant hypophosphatemic rickets, a similar disorder characterized by renal phosphate wasting, has been reported to be associated with mutations of the FGF-23 gene⁴ that prevent its cleavage.⁵ Administration of recombinant FGF-23 decreases serum phosphate levels in mice by increasing renal phosphate excretion.⁶ Implantation of Chinese hamster ovary cells stably expressing FGF-23 into mice led to hypophosphatemic rickets *in vivo*,⁶ indicating the importance of FGF-23 in the development of hypophosphatemic rickets.

Plasma FGF-23 levels are directly and significantly correlated with serum parathyroid hormone (PTH), calcium, and phosphate levels in uremic patients who are on maintenance hemodialysis.^{7,8} There is some evidence that plasma FGF-23 levels may be regulated or affected by dietary phosphate. Recently, dietary consumption of phosphate was

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shown to regulate serum FGF-23 levels in both uremic rats^{9,10} and nonuremic mice.¹¹ In addition, 1,25 dihydroxyvitamin D₃ [1,25(OH)₂D₃] upregulates serum FGF-23 level in mice^{12,13} and in thyroparathyroidectomized rats without a corresponding increase in serum phosphate levels⁹, suggesting a role for both dietary phosphate and 1,25(OH)₂D₃ in FGF-23 secretion.

Elevated FGF-23 levels have also been reported in patients with primary hyperparathyroidism (PHPT). FGF-23 concentrations are significantly directly correlated with serum calcium and intact PTH levels and inversely correlated with creatinine clearance and phosphate concentration; of these, creatinine clearance and calcium are independently associated factors.^{14,15} Although both of these clinical studies suggested the importance of PTH action in the regulation of FGF-23 in patients with PHPT, serum FGF-23 levels decreased significantly after parathyroidectomy (PTX) in one study,¹⁵ whereas the decrease was NS in the other.¹⁴

Here we used PTH-*cyclin D1* transgenic (PC2) mice, which exhibit parathyroid-targeted overexpression of the human *cyclin D1* oncogene, as a model of PHPT. These mice develop not only abnormal parathyroid cell proliferation but also chronic biochemical hyperparathyroidism, with characteristic abnormalities in bone and, notably, a shift in the relationship between serum calcium and PTH.^{16,17} These mice eventually exhibited adenomatous-appearing parathyroid region with reduced calcium-sensing receptor expression.¹⁸

In this study, we attempted to determine the roles of PTH in the regulation of serum FGF-23 levels using PC2 mice. To investigate the sources of serum FGF-23 in mice, we analyzed *fgf23* expression in various tissues by quantitative real-time reverse transcriptase-PCR (RT-PCR) and examined the correlation among *fgf23* expression, other biochemical markers, and the circulating levels of the protein. We also performed PTX in PC2 mice to determine the influence of PTH oversecretion on elevated serum FGF-23 levels.

RESULTS

Serum FGF-23 in PC2 Mice

At 27 to 33 wk, the PC2 mice already showed significantly

higher serum calcium levels than age-matched wild-type (WT) mice, and, at older ages, the mice exhibited typical signs of biochemical hyperparathyroidism, such as hypercalcemia, hypophosphatemia, and elevated PTH levels (Table 1). PC2 mice had significantly higher levels of serum FGF-23 than WT mice at all ages examined, and the levels increased significantly with age, reaching three times those of WT mice in 90- to 118-wk-old mice (Figure 1). Serum FGF-23 levels were significantly directly correlated with serum PTH and calcium levels and inversely correlated with serum phosphate levels in 90- to 118-wk-old mice (Figure 2). Serum 1,25(OH)₂D levels were directly correlated with serum FGF-23 levels but not significantly. Serum urea nitrogen levels were not significantly different between PC2 mice and age-matched WT mice at any age (Table 1).

Tissue Expression of *fgf23* and Osteoblastic Markers

For investigation of the sources of serum FGF-23 in mice, *fgf23* expression was analyzed in various tissues from 90- to 118-wk-old PC2 and WT mice using quantitative real-time PCR. High *fgf23* expression was observed in femur and calvaria and was 1.8 and 14.4 times higher than in the thymus of WT mice, respectively (Figure 3A). In PC2 mice, *fgf23* expression was 15.2- and 54.6-fold in the femur and calvaria, respectively, compared with that in the thymus of WT mice (Figure 3A). When *fgf23* expression was examined in more detail in bone, it was found to be 20.2-fold higher in calvaria and 2.7-fold in the femur of PC2 mice than in the same sites of WT mice (Figure 3B). The highest expression was observed in PC2 calvaria. The *fgf23* expression levels in calvaria were significantly directly correlated with serum FGF-23 levels (Figure 4). *fgf23* expression levels in calvaria were also significantly directly correlated with *ALP* and *osteocalcin* expression levels (Figure 5).

Effect of PTX on Serum FGF-23 Levels

For investigation of the effect of PTH on serum FGF-23 levels, PTX was performed on 60- to 75-wk-old PC2 mice. The success of the PTX was confirmed by the presence of significant decreases in serum calcium and PTH levels and a significant

Table 1. Serum biochemistries of experimental mice^a

Age (wk)	Genotype	n	Calcium (mg/dl)	Phosphate (mg/dl)	PTH (pg/ml)	1,25(OH) ₂ D (pg/ml)	SUN (mg/dl)
27 to 33	WT	8	8.1 ± 0.1	7.0 ± 0.6	52 ± 7	138 ± 18	30 ± 4
	PC2	11	9.2 ± 0.1 ^b	8.3 ± 0.5	54 ± 4	159 ± 8	31 ± 2
60 to 75	WT	8	8.0 ± 0.1	8.0 ± 0.5	81 ± 22	141 ± 14	24 ± 1
	PC2	10	9.5 ± 0.2 ^b	5.6 ± 0.4 ^{b,c}	153 ± 21 ^c	113 ± 22 ^c	27 ± 2
90 to 118	WT	6	8.9 ± 0.2 ^c	6.3 ± 0.3	45 ± 9	119 ± 13	24 ± 1
	PC2	18	11.8 ± 0.4 ^{b,c,d}	3.7 ± 0.2 ^{b,c,d}	158 ± 24 ^{b,c}	190 ± 13 ^{b,d}	23 ± 1
<i>P</i>			0.048	<0.001	0.023	0.023	0.760

^aMale/female ratios were similar between PC2 and WT mice. Data are means ± SEM. *P* values of changes in serum parameters were calculated by two-way ANOVA. SUN, serum urea nitrogen.

^b*P* < 0.05 versus same-age WT mice.

^c*P* < 0.05 versus PC2 mice at 27 to 33 wk.

^d*P* < 0.05 versus PC2 mice at 60 to 75 wk by post hoc analysis (Games-Howell).

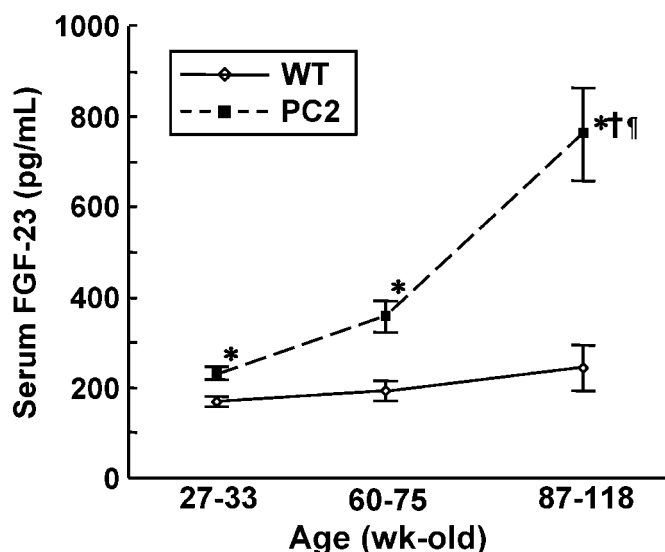


Figure 1. Changes in serum FGF-23 levels in WT and PC2 mice. PC2 mice had significantly higher levels of serum FGF-23 than WT mice at 27 to 33 wk of age. FGF-23 concentrations significantly increased with age in PC2 mice ($P = 0.021$ by two-way ANOVA), reaching three times the level of WT mice at 90 to 118 wk. Data are means \pm SE. * $P < 0.05$ versus same-age WT mice; †versus PC2 mice at 27 to 33 wk; ‡versus PC2 mice at 60 to 75 wk by post hoc analysis (Games-Howell).

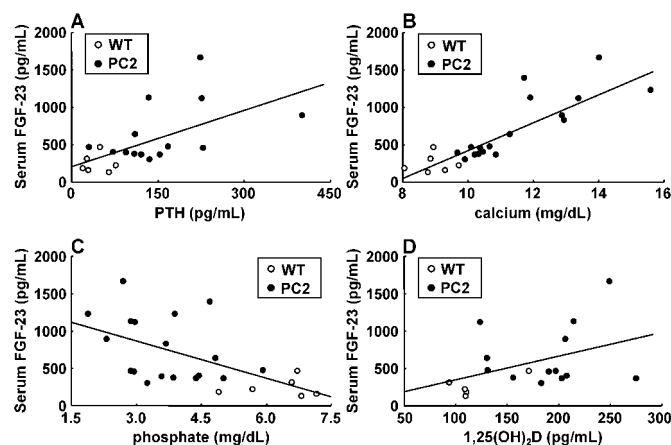


Figure 2. Correlation between serum FGF-23 concentration and other serum parameters in 90- to 118-wk-old mice. ○, WT mice ($n = 6$); ●, PC2 mice ($n = 18$). Serum FGF-23 levels were correlated with serum PTH (A; $r = 0.605$, $P = 0.004$), calcium (B; $r = 0.859$, $P < 0.001$), and phosphate levels (C; $r = -0.583$, $P = 0.002$) by Pearson correlation analysis. Serum $1,25(\text{OH})_2\text{D}$ levels were directly correlated with serum FGF-23 levels but not significantly (D; $r = 0.420$, $P = 0.083$).

increase in serum phosphate level 72 h after the PTX compared with pre-PTX levels (Table 2). By 72 h after PTX, serum FGF-23 levels had significantly decreased from 358 ± 35 to 196 ± 19 pg/ml ($P < 0.001$; Figure 6), reaching the levels of age-matched WT mice (193 ± 23 pg/ml; $P = 0.938$ versus PC2 mice 72 h after PTX).

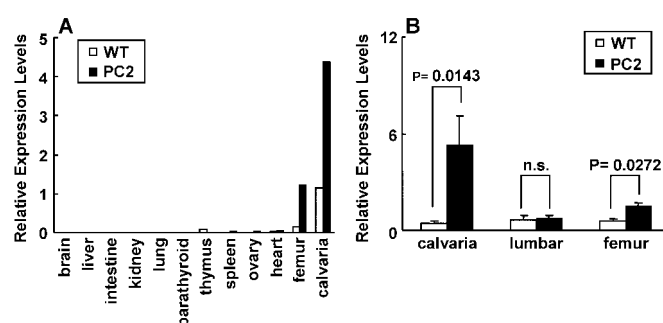


Figure 3. Tissue expression of *fgf23* in 90- to 118-wk-old mice. (A) Quantitative real-time RT-PCR analyses of RNA from tissues of WT and PC2 mice were performed. □, WT mice; ■, PC2 mice. Abundant expression was observed in femur and calvaria from both groups of mice. (B) *fgf23* expression was further analyzed in calvaria, lumbar spine, and femur. Expression of *fgf23* was significantly higher in PC2 calvaria than in WT calvaria ($P = 0.0143$, Mann-Whitney U test). Significant differences between PC2 and WT mice were also observed in the femur ($P = 0.0272$). Data are means \pm SE of three to five WT and eight to 10 PC2 mice.

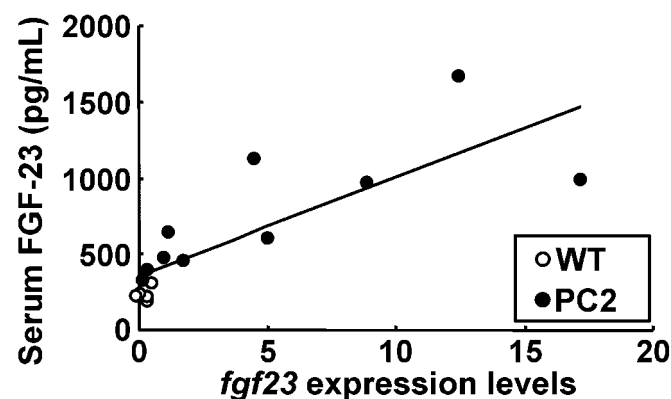


Figure 4. Correlation between serum FGF-23 levels and *fgf23* expression levels in calvaria in 90- to 118-wk-old mice. ○, WT mice ($n = 5$); ●, PC2 mice ($n = 10$). Serum FGF-23 levels were significantly correlated with *fgf23* expression levels in calvaria of PC2 mice ($r = 0.811$, $P < 0.001$) by Pearson correlation test.

DISCUSSION

FGF-23 is a key regulator of serum phosphate and $1,25(\text{OH})_2\text{D}$, but how it is regulated by PTH is still unclear. Our observations suggest that, in a mouse model of PHPT, the calvaria is a major source of circulating FGF-23, likely *via* osteoblast activation. Serum PTH and calcium levels highly correlated with serum FGF-23 levels, whereas serum phosphate seemed to have minor roles in FGF-23 regulation in PHPT mice with normal kidney function. The decrement in serum FGF-23 levels in PC2 mice after PTX suggests that PTH functions to stimulate FGF-23 secretion.

Serum FGF-23 concentrations in PC2 mice were initially elevated at 27 to 33 wk and gradually increased, followed by the

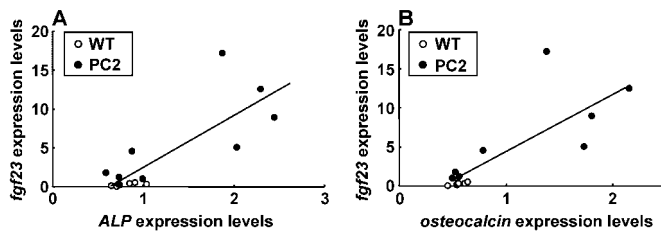


Figure 5. Correlation between expression levels of *fgf23* and osteoblastic markers in calvaria in 90- to 118-wk-old mice. ○, WT mice ($n = 5$); ●, PC2 mice ($n = 10$). *fgf23* expression levels were significantly correlated with the expression levels of both ALP (A; $r = 0.705$, $P = 0.002$) and osteocalcin (B; $r = 0.789$, $P = 0.002$) in calvaria of PC2 mice by Pearson correlation test.

development of PHPT. In 60- to 75-wk-old PC2 mice, mild PHPT was accompanied by significantly higher serum FGF-23 levels than in WT mice. After PTX, FGF-23 levels in PC2 mice significantly decreased to the levels of age-matched WT mice, indicating that PTH is a potential stimulator of serum FGF-23 levels *in vivo* in PHPT. In clinical studies of PHPT, circulating FGF-23 levels decreased significantly after PTX in patients with normal kidney function¹⁵ and decreased—but not significantly—in patients with renal insufficiency.¹⁴ Administration of vitamin D analogs^{9,19} and/or calcium,¹⁵ as well as renal insufficiency,²⁰ may interfere with the decrement of circulating FGF-23 levels after PTX.

Expression of *fgf23* was predominantly observed in bone, especially in calvaria,^{11,21,22} which also showed markedly increased expression in *Hyp*, a mouse homologue of X-linked hypophosphatemic rickets.²¹ In this study, PC2 mice had higher expression of *fgf23* than WT mice in calvaria and femur, where cortical bone content is higher than in cancellous bone. This is in agreement with a previous report of FGF-23 expression in healthy human cortical bones.²³ Expression of *fgf23* in calvaria, which contains an extremely high amount of cortical bone, seemed to be a major determinant of serum FGF-23 levels in PC2 mice. The expression of *fgf23* by cells at the sutures as well as osteoblasts²² may explain the high *fgf23* expression in calvaria. We observed good correlation between *fgf23* expression and the expression of osteoblastic markers, such as ALP and osteocalcin, suggesting that the activities of osteoblasts and/or osteocytes might be involved in the stimulation of FGF-23 by PTH.

We observed advanced PHPT in 90- to 118-wk-old PC2 mice, and serum parameters varied widely, as described previously.¹⁶ In these mice, serum FGF-23 levels were more strongly correlated with serum calcium levels than with PTH levels. The same trend was also reported in patients who had PHPT with¹⁴ and without¹⁵ renal insufficiency. Because PTH mobilizes calcium from the bone to the circulation, the direct correlation of FGF-23 with calcium would likely be a result of PTH activity. A stronger correlation with calcium than PTH was also observed in patients with PHPT^{14,15} and secondary hyperparathyroidism,⁷ but this

does not negate the possibility of a direct effect of calcium on osteoblasts or osteocytes. Calcium has been reported to stimulate osteoblast proliferation directly through calcium-sensing receptor-mediated pathways,^{24,25} and the role of calcium-sensing receptor pathways on *fgf23* expression in bone requires further study.

In previous studies, $1,25(\text{OH})_2\text{D}_3$ was found to upregulate serum FGF-23 levels in mice^{12,13} and in rats.⁹ Overexpression of a dominant negative vitamin D receptor inhibited $1,25(\text{OH})_2\text{D}_3$ stimulation of *fgf23* promoter activity *in vitro* in osteoblasts,²⁶ which suggested that $1,25(\text{OH})_2\text{D}_3$ is an important regulator of FGF-23 production in bone. Elevated serum $1,25(\text{OH})_2\text{D}$ levels induced by oversecretion of PTH were observed in patients with PHPT, and elevated $1,25(\text{OH})_2\text{D}$ levels may enhance FGF-23 production in bone. In this study, serum $1,25(\text{OH})_2\text{D}$ levels were directly correlated with serum FGF-23 levels but not significantly. In addition, a decrease in circulating FGF-23 was observed after PTX. In contrast, the decrease in the level of $1,25(\text{OH})_2\text{D}$ after PTX was not statistically significant. Similar results have been obtained in patients with PHPT.¹⁵ These observations suggest that $1,25(\text{OH})_2\text{D}$ is a positive regulator of serum FGF-23 levels but that its role is limited in PHPT.

FGF-23 has a phosphaturic effect, and its circulating levels may also be regulated or affected by serum phosphate levels. In uremic patients who are on maintenance hemodialysis, plasma FGF-23 levels are elevated and are correlated with inorganic phosphate, PTH, and corrected calcium.^{7,8} Serum FGF-23 levels are also correlated with serum phosphate levels in uremic rats.⁹ Recently, a direct correlation between serum FGF-23 and serum phosphate levels was observed in nonuremic mice consuming dramatically changed amounts of dietary phosphate.¹¹ In contrast, no correlation between FGF-23 and serum phosphate levels was observed in nonuremic healthy humans,^{8,27} and an inverse correlation was seen in patients with PHPT.^{14,15} Even when alimentary intake of phosphorus is excessive, surplus phosphate is excreted very quickly by the kidneys, and hyperphosphatemia is not usually observed in healthy humans. In addition, hyperphosphaturia would be expected to occur in patients with PHPT secondary to elevated PTH levels. In this study, the inverse correlation between FGF-23 and phosphate levels was confirmed *in vivo* in a model of PHPT. Elevated FGF-23 levels in PHPT would presumably enhance the phosphaturia that is already accelerated by elevated PTH levels, resulting in hypophosphatemia.

Our observations suggest that FGF-23 secreted from bone, especially cortical bone such as calvaria, may contribute to elevated serum FGF-23 levels in hyperparathyroidism. PTH is a potential stimulator of the production of FGF-23. The combined reduction in serum phosphate by FGF-23 and PTH may prevent tissue damage, for example by preventing ectopic calcification by lowering the serum calcium-phosphate product in the presence of hypercalcemia caused by oversecretion of PTH in PHPT.

Table 2. Changes in serum parameters after PTX in 60- to 75-wk-old PC2 mice^a

Parameter	Before PTX	After PTX	P
PTH (pg/ml)	153 ± 21	18 ± 3	<0.0001
calcium (pg/ml)	9.5 ± 0.2	7.5 ± 0.3	0.0035
phosphate (pg/ml)	5.6 ± 0.4	7.9 ± 0.3	<0.0001
1,25(OH) ₂ D (pg/ml)	113 ± 22	88 ± 12	0.3348

^aData are means ± SEM of 10 mice. Blood samples were obtained before and 72 h after PTX. P values were calculated by the paired t test.

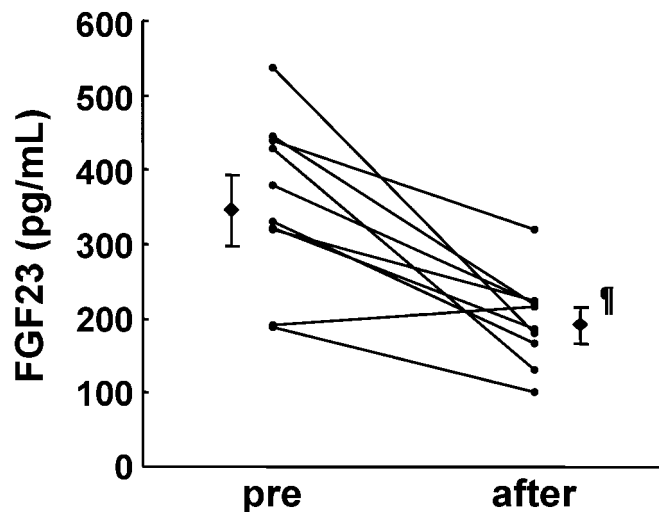


Figure 6. PTX decreases serum FGF-23 levels in PC2 mice. PTX was performed on 60- to 75-wk-old PC2 mice. Blood samples were obtained before and 72 h after PTX. Values for each mouse are represented (●), along with the means ± SE of 10 mice (◆). ‡P = 0.0012 by paired t test.

CONCISE METHODS

Experimental Animals

PC2 mice that overexpress the human *cyclin D1* oncogene specifically in the parathyroid were used in this study as a model of PHPT.¹⁶ For selection of the mice bearing the transgene, offspring were genotyped by Southern blotting using h-*cyclin D1* cDNA as a probe.¹⁶ PC2 and WT mice were used at three ages: 27 to 33, 60 to 75, and 90 to 118 wk. All mice were provided with a commercially available rodent diet, CE-2 (Crea Japan, Tokyo, Japan), containing 1.03% calcium and 0.97% phosphorus, and water *ad libitum*. The studies were approved by the appropriate institutional animal care committees at Osaka City University Graduate School of Medicine.

Measurement of Biochemical Parameters

Blood samples were collected from the orbital cavities of anesthetized mice in the morning (10:00 to 11:00 a.m.). Serum FGF-23 was measured with sandwich ELISA kits (Kainos Laboratories, Tokyo, Japan). Serum PTH was measured with rat PTH IRMA kits (Immutopics, San Clemente, CA). Serum 1,25(OH)₂D concentrations were measured with RIA kits (Immunodiagnostic Systems Ltd, Bolderon, England). Serum biochemistries, including total calcium, phosphate, urea nitro-

gen, and alkaline phosphatase, were determined with a series of TESTWAKO kits (Wako Chemical Co., Ltd., Tokyo, Japan).

PTX of PC2 Mice

PTX was performed on 60- to 75-wk-old male anesthetized PC2 mice. Because the parathyroid glands of PC2 mice are large enough to identify under a stereoscopic microscope, we were able to excise only the parathyroid glands without removing the thyroid glands.

Quantitative Real-Time RT-PCR

Total RNA was isolated from fresh-frozen surgically resected mouse tissues using the standard protocol of the RNA/DNA Kit (QIAGEN, Hilden, Germany). The total RNA was reverse-transcribed to cDNA using a TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA). Amplification and detection were carried out in 96-well optical plates on an ABI Prism 7000 sequence detector (Applied Biosystems) with TaqMan Universal PCR 2× master mix, 20× Assay-on-Demand Gene Expression Assay Mix (Applied Biosystems), and sample cDNA in a final volume of 50 μl per reaction. The *FGF23*, *ALP*, *osteocalcin*, and 18S ribosomal gene sequences were amplified for quantitative PCR with an initial hold of 50°C for 2 min to activate the No AmpErase UNG and a hold of 95°C for 10 min to activate AmpliTaq Gold polymerase, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The products were analyzed with the manufacturer's software, SDS 1.1 (Applied Biosystems).

Statistical Analyses

Differences between the mean values of the groups were evaluated by two-way ANOVA. Correlation coefficients between two parameters were obtained using Pearson correlation analyses. Differences in *fgf23* expression were analyzed with the Mann-Whitney U test. Differences in serum parameters between preoperative and postoperative samples were evaluated by the paired t test. Results are expressed as means ± SE. All statistical analyses were performed by SPSS 14.0J (SPSS Japan, Tokyo, Japan).

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DISCLOSURES

None.

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