

see commentary on page 947

# Direct evidence for a causative role of FGF23 in the abnormal renal phosphate handling and vitamin D metabolism in rats with early-stage chronic kidney disease

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Circulating levels of fibroblast growth factor 23 (FGF23) are elevated in patients with early chronic kidney disease (CKD) and are postulated to cause low blood levels of 1,25-dihydroxyvitamin D, as well as normal phosphate levels. In order to provide more direct evidence for the pathophysiological role of FGF23 in the settings of mineral ion homeostasis typically seen in early CKD, we studied rats with progressive CKD treated with anti-FGF23 neutralizing antibody. Without antibody treatment, rats with CKD exhibited high circulating levels of FGF23 and parathyroid hormone, low 1,25-dihydroxyvitamin D, and normal serum phosphate levels, accompanied by increased fractional excretion of phosphate. Antibody treatment, however, lessened fractional excretion of phosphate, thus increasing serum phosphate levels, and normalized serum 1,25-dihydroxyvitamin D by increased  $1\alpha$ -OHase and decreased 24-OHase expressions in the kidney. These antibody-induced changes were followed by increased serum calcium levels, leading to decreased serum parathyroid hormone. Hence, our study shows that FGF23 normalizes serum phosphate and decreases 1,25-dihydroxyvitamin D levels in early-stage CKD, and suggests a pathological sequence of events for the development of secondary hyperparathyroidism triggered by increased FGF23, followed by a reduction of 1,25-dihydroxyvitamin D and calcium levels, thereby increasing parathyroid hormone secretion.

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Abnormal regulation of mineral ion homeostasis is one of the major problems in patients with chronic kidney disease (CKD). A decrease in the number of functional nephrons has long been thought to lead to impaired urinary phosphate excretion and hyperphosphatemia, and to reduced activity of the renal 25-hydroxyvitamin D- $1\alpha$ -hydroxylase ( $1\alpha$ -OHase) and consequently low 1,25-dihydroxyvitamin D ( $1,25(\text{OH})_2\text{D}$ ) levels. Both changes were thought to result in hypocalcemia, thereby causing an increase in parathyroid hormone (PTH) secretion and thus secondary hyperparathyroidism.<sup>1,2</sup> This interpretation, however, may need to be revised to include fibroblast growth factor 23 (FGF23) actions.

FGF23 is a physiologically important hormone that decreases serum levels of both phosphate and  $1,25(\text{OH})_2\text{D}$ .<sup>3,4</sup> Abnormally increased circulating FGF23 are known to cause hypophosphatemic rickets/osteomalacia accompanied by inappropriately low levels of circulating  $1,25(\text{OH})_2\text{D}$ , such as X-linked hypophosphatemia, autosomal dominant and recessive hypophosphatemia, and tumor-induced osteomalacia.<sup>5–10</sup> Patients with CKD can also present clear elevations of circulating FGF23 levels, which typically develop early in the course of CKD.<sup>11–13</sup> Circulating FGF23 appears to be intact and biologically active in CKD patients, even when circulating levels of the immunoreactive FGF23 are dramatically elevated as in patients with end-stage renal disease.<sup>14</sup> It is therefore likely that increased levels of circulating FGF23 target the remnant nephrons in patients with early-stage CKD, thereby enhancing fractional excretion of phosphate ( $\text{FE}_{\text{P}_i}$ ) and inhibiting the production of  $1,25(\text{OH})_2\text{D}$ . In fact, frank hyperphosphatemia does not develop until later stages of CKD when the glomerular filtration rate drops to  $<30$  ml/min.<sup>15</sup> In addition, recent clinical studies have shown that FGF23 independently correlates with low  $1,25(\text{OH})_2\text{D}$  and normal phosphate levels,<sup>16,17</sup> suggesting the pathophysiological importance of increased FGF23.

The aim of this study is to provide evidence that concludes a pathophysiological role of FGF23 in the abnormal regulation of mineral metabolism. To address this, we developed progressive CKD model rats and analyzed the effect of anti-FGF23 monoclonal antibodies that block the activity of endogenous circulating FGF23.<sup>18,19</sup>

## RESULTS

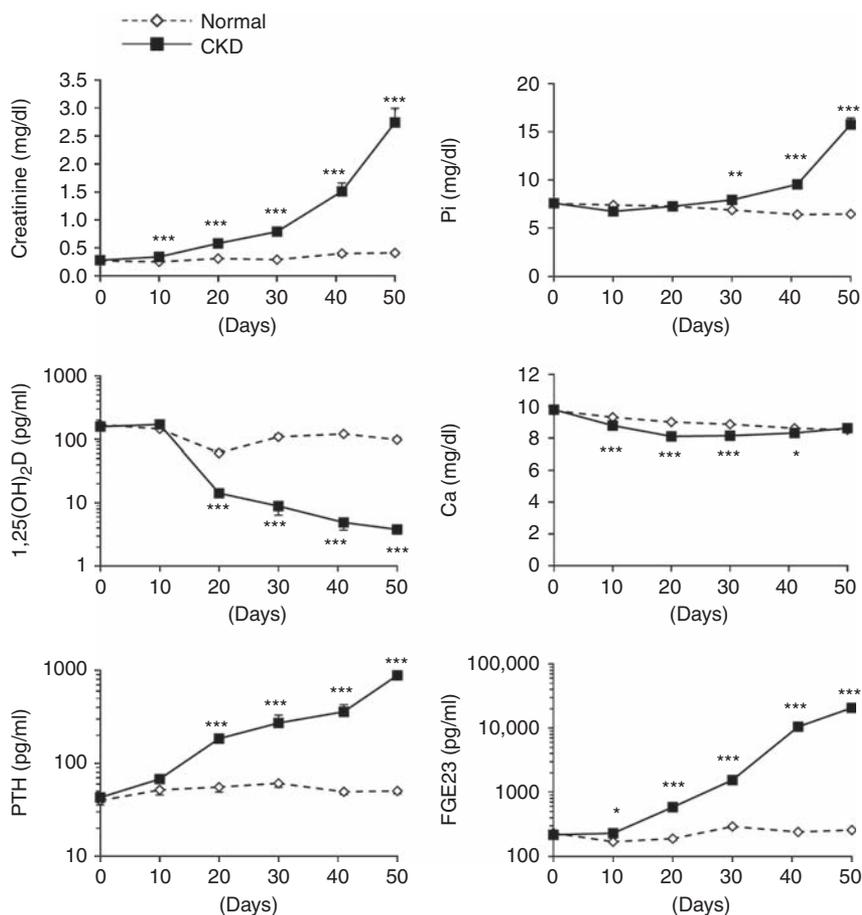
Progressive nephritis was induced in Wistar-Kyoto rats by singly injecting an anti-glomerular basement membrane (GBM) antiserum.<sup>20</sup> Serum creatinine levels in the rats, which had received the anti-GBM antiserum (CKD rats), started to increase from day 10 (10 days after the injection of the anti-GBM antiserum), followed by progressive changes in mineral parameters (Figure 1).

CKD rats did not develop significant hyperphosphatemia until day 30. A major decrease in the circulating levels of 1,25(OH)<sub>2</sub>D (to 10% of baseline) was observed by day 20. This model also exhibited mild but statistically significant hypocalcemia after day 10, which was not progressive and normalized by days 40–50. The low levels of both 1,25(OH)<sub>2</sub>D and calcium presumably caused increased circulating PTH

levels starting on day 20. Serum FGF23 levels showed a moderate but significant increase by day 10 and rapidly increased thereafter. Thus, before day 30, the rats with anti-GBM nephritis mimicked, with the exception of low blood calcium levels, most of the abnormalities in mineral ion homeostasis typically seen in patients with early-stage CKD. Therefore, we focused on this time period in analyzing the actions of FGF23.

Additional CKD rats were developed, and blood and urine analyses on day 28 showed similar laboratory data in mineral metabolism as observed in Figure 1 (Table 1). Of note, CKD rats again did not develop significant hyperphosphatemia, but we found a 65% increase in FE<sub>Pi</sub> (Table 1). These CKD animals were divided into three groups and were then treated with either vehicle (phosphate-buffered saline) or two different doses of anti-FGF23 antibodies (0.1 or 1 mg/kg) as an intravenous single injection on day 32. Subsequently, changes in serum levels of phosphate, calcium, PTH, and 1,25(OH)<sub>2</sub>D, as well as FE<sub>Pi</sub>, were monitored for up to 72 h after the antibody injection.

Treatment with anti-FGF23 antibodies resulted in marked elevations in serum phosphate levels that were dependent on



**Figure 1 | Time course of changes in serum parameters of rats with anti-GBM nephritis.** On day 0, WKY rats were injected with a rabbit anti-rat GBM serum (CKD,  $N = 8$ ) or an equivalent volume of normal rabbit serum (Normal,  $N = 8$ ). On days 0, 10, 20, 30, 40, and 50, blood samples were sequentially collected from tail artery and sera were prepared. Results represent mean  $\pm$  s.e.m. \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$ ; normal vs CKD group on each day. 1,25(OH)<sub>2</sub>D, 1,25-dihydroxyvitamin D; Ca, calcium; CKD, chronic kidney disease; FGF23, fibroblast growth factor 23; GBM, glomerular basement membrane; Pi, phosphate; PTH, parathyroid hormone; WKY, Wistar-Kyoto.

**Table 1 | Blood and urinary parameters in anti-GBM nephritis on day 28**

	Normal (N=8)	CKD (N=30)	P-values
Creatinine (mg/dl)	0.67 ± 0.03	1.22 ± 0.05	< 0.001
Pi (mg/dl)	7.2 ± 0.2	7.2 ± 0.1	0.813
1,25(OH) <sub>2</sub> D (pg/ml)	179.2 ± 23.1	24.9 ± 2.9	< 0.001
Ca (mg/dl)	10.0 ± 0.1	9.5 ± 0.1	< 0.005
PTH (pg/ml)	37.4 ± 3.1	181.1 ± 16.7	< 0.001
FGF23 (pg/ml)	227.2 ± 7.7	640.1 ± 41.3	< 0.001
FE <sub>Pi</sub> (%)	16.6 ± 1.4	27.5 ± 1.3	< 0.001

Abbreviations: Ca, calcium; CKD, chronic kidney disease; FE<sub>Pi</sub>, fractional excretion of phosphate; FGF23, fibroblast growth factor 23; PTH, parathyroid hormone; GBM, glomerular basement membrane; Pi, phosphate.

WKY rats were injected with a rabbit anti-rat GBM serum (CKD, N=30) or an equivalent volume of normal rabbit serum (Normal, N=8). On day 28, blood samples were collected from tail artery and sera were prepared. Urine samples were collected in metabolic cages for 24 h and FE<sub>Pi</sub> was determined. Results represent mean ± s.e.m. and statistical significance was evaluated using Student's *t*-test.

the dose of antibody and lasted, in rats given the higher dose, for >48 h (Figure 2a). In association with the increased serum Pi levels, FE<sub>Pi</sub> decreased within 24 h after the treatment with anti-FGF23 antibodies (Figure 2b).

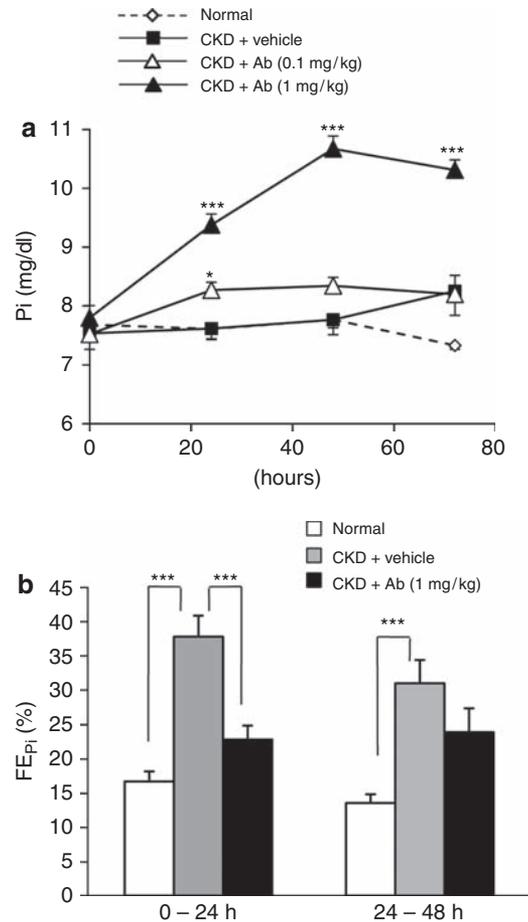
The injection of anti-FGF23 antibodies also induced a dramatic increase in serum 1,25(OH)<sub>2</sub>D levels, such that they reached normal levels, which lasted for at least 72 h in the group that had received the higher dose of the antibodies (Figure 3a). These increases in the biologically active form of vitamin D were accompanied in kidney by equivalent changes in the levels of the mRNAs encoding vitamin D-metabolizing enzymes (Figure 3b). In comparison to vehicle-injected normal animals, CKD rats showed about 50% reduction in the renal expression of the 1 $\alpha$ -OHase, whereas the expression of 25-hydroxyvitamin D-24-hydroxylase (24-OHase) increased by approximately fivefold. The antibody treatment dramatically increased the expression of the 1 $\alpha$ -OHase, whereas decreasing that of the 24-OHase within 8 h.

The recovery in serum 1,25(OH)<sub>2</sub>D levels was followed by increased serum calcium levels after 48 h in the antibody-treated CKD rats (Figure 4). In addition, blocking FGF23 action in CKD rats resulted in moderate suppression of circulating levels of PTH (Figure 4). Because this change occurred after the recovery of both serum 1,25(OH)<sub>2</sub>D and calcium, the suppression of PTH is likely because of the effect of the elevations of 1,25(OH)<sub>2</sub>D and calcium.

## DISCUSSION

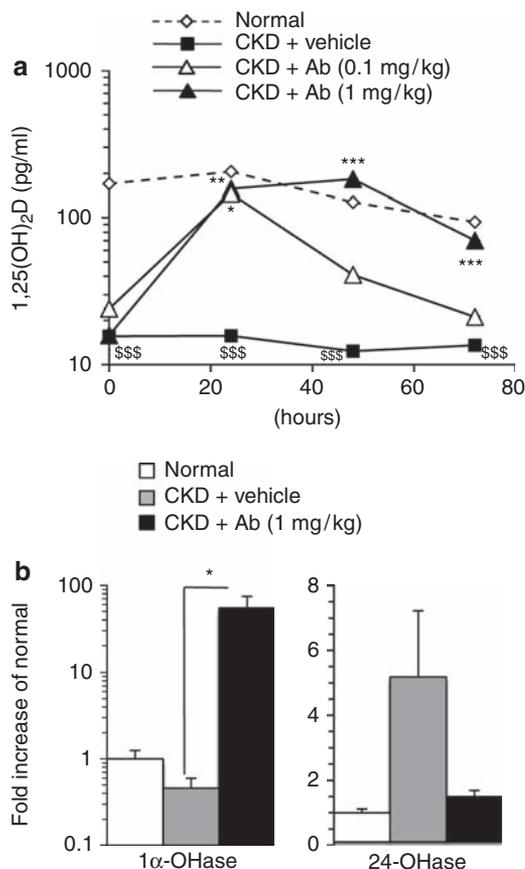
We demonstrated that the inhibition of FGF23 activity in rats with mild CKD resulted in high serum phosphate and normal 1,25(OH)<sub>2</sub>D levels. These findings indicate that normal phosphate and low 1,25(OH)<sub>2</sub>D in these rats are FGF23-dependent changes, and are compatible with the clinical observations that circulating levels of FGF23 correlated well with FE<sub>Pi</sub> or lowered 1,25(OH)<sub>2</sub>D levels in patients with early-stage CKD.<sup>16,17</sup>

FE<sub>Pi</sub> was high in rats with mild CKD and decreased after the treatment with anti-FGF23 antibodies. This indicates that FGF23 inhibits renal phosphate reabsorption in mild CKD,



**Figure 2 | Effects of anti-FGF23 antibodies on phosphate metabolism in rats with anti-GBM nephritis.** On day 32, CKD rats were injected with PBS (CKD + vehicle, N = 10) or anti-FGF23 neutralizing antibodies at 0.1 mg/kg (CKD + Ab (0.1), N = 10) or 1.0 mg/kg (CKD + Ab (1), N = 10). As a reference, normal WKY rats at the same age were treated with an equivalent volume of PBS (Normal, N = 8). (a) Time course of changes in serum phosphate levels. Blood samples were collected from tail artery at 0 (before the injection), 24, 48, and 72 h after the injection. Results represent mean ± s.e.m. \*\*\**P* < 0.001, \**P* < 0.05; CKD-vehicle group vs CKD-Ab groups. (b) FE<sub>Pi</sub> after treatment with antibodies. Rats in reference group (Normal, N = 8), CKD groups treated with vehicle (CKD + vehicle, N = 8) or anti-FGF23 antibodies at 1 mg/kg (CKD + Ab, N = 10) were reared individually in a metabolic cage after the injection and 24-h urine samples were collected for 2 days. FE<sub>Pi</sub> was calculated using the formula (phosphate clearance)/(creatinine clearance). \*\*\**P* < 0.001. CKD, chronic kidney disease; FE<sub>Pi</sub>, fractional excretion of phosphate; FGF23, fibroblast growth factor 23; GBM, glomerular basement membrane; PBS, phosphate-buffered saline; Pi, phosphate; WKY, Wistar-Kyoto.

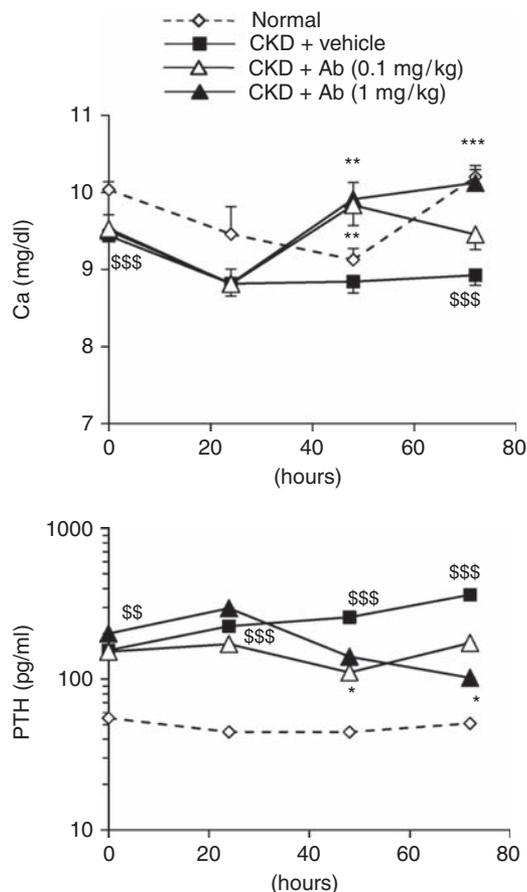
thereby maintaining serum phosphate levels within the reference range. In other words, phosphate retention appeared to be mitigated by compensatory FGF23 actions, although glomerular filtration rate was already significantly reduced. This is compatible with the clinical observations that circulating levels of FGF23 tended to show a better correlation with FE<sub>Pi</sub> than serum phosphate levels in patients with mild CKD.<sup>16,17</sup>



**Figure 3 | Effects of anti-FGF23 antibodies on vitamin D metabolism in rats with anti-GBM nephritis.** (a) Time course of changes in serum 1,25(OH)<sub>2</sub>D levels. Blood samples were collected from tail artery at 0 (before the injection), 24, 48, and 72 h after the injection. Results represent mean ± s.e.m. \*\*\**P* < 0.001, \*\**P* < 0.01, \**P* < 0.05; CKD-vehicle group vs CKD-Ab groups. \$\$\$*P* < 0.001; normal group vs CKD-vehicle group. (b) Quantitative PCR analyses for mRNA levels of renal 1α-OHase and 24-OHase. Anti-FGF23 antibodies (1 mg/kg) or vehicle was injected and kidney samples (Normal, *N* = 3; CKD + vehicle, *N* = 5; CKD + Ab, *N* = 5, respectively) were collected at 8 h after the injection. Results represent mean ± s.e.m. \**P* < 0.05. 1,25(OH)<sub>2</sub>D, 1,25-dihydroxyvitamin D; CKD, chronic kidney disease; FGF23, fibroblast growth factor 23; GBM, glomerular basement membrane.

Because hyperphosphatemia is known to be one of the risk factors for vascular calcification, and the severe vascular calcification seen in *Fgf23* knockout mice has been shown to be caused by hyperphosphatemia,<sup>21</sup> maintaining serum phosphate levels by FGF23 may in part contribute to prevent the development of vascular calcifications in early CKD.

It is of interest that the injection of antibodies increased serum phosphate levels despite continuously high circulating levels of PTH. Although a mild decrease in PTH levels was observed after 48 h, changes in both serum and urinary phosphate occurred before this reduction of PTH. Therefore, the observed hyperphosphatemia resulted from the inhibition of FGF23 action, independently of PTH, suggesting that although further experiments are required, FGF23 has, at least in the early stages of CKD, a more important role in



**Figure 4 | Effects of anti-FGF23 antibodies on calcium and PTH levels in rats with anti-GBM nephritis.** Blood samples were collected from tail artery at 0 (before the injection), 24, 48, and 72 h after the injection. Results represent mean ± s.e.m. \*\*\**P* < 0.001, \*\**P* < 0.01, \**P* < 0.05; CKD-vehicle group vs CKD-Ab groups. \$\$\$*P* < 0.001, \$\$*P* < 0.01; normal group vs CKD-vehicle group. Ca, calcium; CKD, chronic kidney disease; FGF23, fibroblast growth factor 23; GBM, glomerular basement membrane; PTH, parathyroid hormone.

renal phosphate handling than PTH. This is consistent with previous observations in humans showing that the circulating levels of FGF23 but not PTH are correlated with serum phosphate levels.<sup>17</sup>

Our study also provides evidence that increased FGF23 action, in addition to a loss of healthy nephrons, is another driving force by which serum levels of 1,25(OH)<sub>2</sub>D significantly decrease in early-stage CKD. Given that the administration of a phosphate binder reduced circulating levels of FGF23 in CKD rats,<sup>22</sup> the previous observation that dietary phosphate restriction reversed the low 1,25(OH)<sub>2</sub>D levels in patients with moderate CKD<sup>23</sup> can be interpreted as a result of a decrease in circulating levels of FGF23. Another important point illuminated by our study is that the continuously high circulating level of PTH in early CKD, which should increase renal expression of 1α-OHase, cannot maintain normal circulating 1,25(OH)<sub>2</sub>D levels. This implies that in early CKD, FGF23 action on the regulation of 1,25(OH)<sub>2</sub>D is more dominant than that of PTH.

Treatment with antibodies resulted in the mild decrease in serum PTH level, which was probably caused by elevations of both serum calcium and 1,25(OH)<sub>2</sub>D levels. In this regard, our finding suggests the sequence of events in the pathogenesis of secondary hyperparathyroidism in early-stage CKD. Presumably, the elevation of PTH *per se* was necessary to prevent an even more severe hypocalcemia that could be caused by the significantly low serum 1,25(OH)<sub>2</sub>D induced by FGF23. This condition may be consistent with the settings in mice carrying the cells overexpressing recombinant FGF23, where the continuous action of FGF23 caused low serum levels of 1,25(OH)<sub>2</sub>D and calcium, thereby developing secondary hyperparathyroidism.<sup>24</sup> Thus, although the first trigger by which FGF23 increases remains unclear, our finding suggests that the same sequence underlies the enhanced PTH secretion in early CKD, leading to secondary hyperparathyroidism.

Our study raised a question on the impact of direct action of FGF23 on the parathyroid gland. FGF23 has been shown to directly suppress PTH expression/secretion through an essential co-receptor for FGF23 signaling, Klotho.<sup>25,26</sup> However, it is known that circulating levels of FGF23 are positively associated with those of PTH even in mild CKD,<sup>16</sup> which was reproduced in our CKD rats as well. Furthermore, treatment with FGF23 antibodies did not increase circulating PTH, but rather reduced serum PTH levels, probably because of the normalized 1,25(OH)<sub>2</sub>D and increased calcium levels. These suggest that there is a resistance or insensitivity to FGF23 in the parathyroid gland, which may be, in part, explained by the recent finding that Klotho and FGF receptor expressions in this organ were reduced in patients with end-stage renal disease.<sup>27</sup> Even if FGF23 could target the parathyroid gland and regulate PTH secretion in early CKD, our finding suggests that the action of calcium or 1,25(OH)<sub>2</sub>D is more potent than that of FGF23 on PTH secretion. Thus, the FGF23-dependent regulation of PTH is unlikely to have a dominant role at least in early CKD.

In summary, our study provides direct evidence for the conclusion that high levels of circulating FGF23 in early-stage CKD mitigate phosphate retention and cause low levels of circulating 1,25(OH)<sub>2</sub>D, confirming recent clinical observations. Based on these findings, it may be necessary to revise current hypotheses regarding the mechanisms that result in the development of abnormal mineral ion homeostasis in CKD, and to develop strategies to prevent the development of secondary hyperparathyroidism.

## MATERIALS AND METHODS

### Experimental anti-GBM nephritis

The experimental protocol was approved by the experimental animal ethical committee of Kirin Pharma. Rabbit anti-rat GBM serum was prepared in our laboratory according to the previously published method.<sup>20</sup> Anti-GBM nephritis was established by singly injecting 9-week-old male Wistar-Kyoto rats (Charles River, Tokyo, Japan) with rabbit anti-rat GBM serum via tail vein. Normal rats were injected with an equivalent volume of normal rabbit serum (Funakoshi, Tokyo, Japan). Blood samples were sequentially

collected from the tail artery on the indicated days. All rats were fed a standard rodent chow CE-2 (Crea, Japan) containing 1% calcium and 1% phosphate and tap water *ad libitum*.

### Serum and urine chemistries

Serum and urine levels of phosphate and calcium were measured by test Wako kits (Wako Pure Chemical Industries, Osaka, Japan). Serum and urine creatinine levels were measured by CRE-EN kit (Kainos, Tokyo, Japan). Serum PTH and 1,25(OH)<sub>2</sub>D levels were measured using rat PTH-(1–34) immunoradiometric assay (Immutopics, San Clemente, CA, USA) and 1,25(OH)<sub>2</sub>D radioimmunoassay (TFB, Immunodiagnostic System, Tyne and Wear, UK), respectively. Serum FGF23 levels were determined with intact FGF23 assay kit (Kainos).

### Anti-FGF23 antibody

Anti-FGF23 neutralizing antibodies used in this study were 1:1 mixtures of mouse monoclonal antibodies that recognize either the N-terminal receptor-binding domain or the C-terminal Klotho-binding region and have synergistic effects *in vivo*.<sup>18</sup> Antibodies were affinity-purified by protein G sepharose 4FF (GE Healthcare, Buckinghamshire, UK) and stored in phosphate-buffered saline without any other supplements.

### Real-time quantitative PCR analysis

Total RNAs were extracted from kidneys using RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and were used to prepare complementary DNA by reverse transcription using SuperScript III First Strand Synthesis System RT kit (Life Technologies, Carlsbad, CA, USA). Real-time quantitative PCR was performed using the ABI7900HT system and the following TaqMan Expression Assay Primers (Life Technologies): Cyp27b1:Rn00587137, Cyp24:Rn01423141, and β-actin: Rn00667869.

### Statistics

All data were analyzed using SAS analytics software (SAS Institute, Tokyo, Japan). All values represent means ± s.e.m. Statistical significance between CKD and normal groups was analyzed using nested analysis of variance, followed by Student's *t*-test at each time point. Pharmacological effects of anti-FGF23 neutralizing antibodies at each time point were evaluated using parametric Dunnett's test after a nested analysis of variance. The *P*-value of <0.05 was considered statistically significant.

### DISCLOSURE

HH, NN, IU, YY, KI, TY, and TS are employees of Kyowa Hakko Kirin.

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