Role of Hyperphosphatemia and 1,25-Dihydroxyvitamin D in Vascular Calcification and Mortality in Fibroblastic Growth Factor 23 Null Mice

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ABSTRACT
Fibroblastic growth factor 23 (FGF23) regulates renal phosphate reabsorption and 1α-hydroxylase activity. Ablation of FGF23 results in elevated serum phosphate, calcium, and 1,25-dihydroxyvitamin D3 [1,25(OH)2D] levels; vascular calcifications; and early death. For determination of the independent roles of hyperphosphatemia and excess vitamin D activity on the observed phenotypic abnormalities, FGF23 null mice were fed a phosphate- or vitamin D–deficient diet. The phosphate-deficient diet corrected the hyperphosphatemia, prevented vascular calcifications, and rescued the lethal phenotype in FGF23 null mice, despite persistent elevations of serum 1,25(OH)2D and calcium levels. This suggests that hyperphosphatemia, rather than excessive vitamin D activity, is the major stimulus for vascular calcifications and contributes to the increased mortality in the FGF23-null mouse model. In contrast, the vitamin D–deficient diet failed to correct either the hyperphosphatemia or the vascular calcifications in FGF23 null mice, indicating that FGF23 independently regulates renal phosphate excretion and that elevations in 1,25(OH)2D and calcium are not sufficient to induce vascular calcifications in the absence of hyperphosphatemia. The vitamin D–deficient diet also improved survival in FGF23 null mice in association with normalization of 1,25(OH)2D and calcium levels and despite persistent hyperphosphatemia and vascular calcifications, indicating that excessive vitamin D activity can also have adverse effects in the presence of hyperphosphatemia and absence of FGF23. Understanding the independent and context-dependent interactions between hyperphosphatemia and excessive vitamin D activity, as well as vascular calcifications and mortality in FGF23 null mice, may ultimately provide important insights into the management of clinical disorders of hyperphosphatemia and excess vitamin D activity.

Disorders of mineral metabolism are nontraditional risk factors that are associated with high mortality in ESRD.1,2 Epidemiologic studies have linked excessive cardiovascular mortality in ESRD with hyperphosphatemia, hypercalcemia, and elevated parathyroid hormone (PTH) levels.3,4 Vascular calcifications, which are highly prevalent and directly correlated with mortality in this population, are the likely mechanism whereby disordered mineral metabolism contributes to cardiovascular mortality in ESRD.3 Experimental models demonstrate that hyperphosphatemia is a major initiator of extracellular matrix mineralization and vascular calcification.4 In addition, clinical data in ESRD show a strong association between hyperphosphatemia and mortality, as well as between phosphate levels and excess calcium with vascular calcifications.5,6 The relationship among disordered mineral metabolism, the presence of vascular calcifications, and

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increased mortality in ESRD has led to consensus Kidney Disease Outcomes Quality Initiative (K/DOQI) recommendations to control more stringently serum calcium and phosphate levels in ESRD in an effort to minimize vascular calcifications and improve survival.7

Whereas there is general consensus regarding control of serum phosphate and calcium, there is less agreement regarding the optimal use of vitamin D analogues. On the one hand, the high prevalence of nutritional vitamin D deficiency and low circulating levels of 1,25-dihydroxyvitamin D3 [1,25(OH)2D] in ESRD, along with the many salutary effects of vitamin D on various tissues, strongly support the practice of administering active vitamin D analogues to patients with chronic kidney disease. Indeed, epidemiologic studies indicate that vitamin D deficiency is associated with an increased risk for cardiovascular diseases and malignancies, as well as multiple chronic inflammatory and autoimmune disorders.9 Moreover, several studies involving hemodialysis populations have demonstrated a significant survival advantage for patients who were treated with vitamin D analogues.9–12 Although such studies are potentially confounded by intent, resulting from the selection of patients who had worse outcomes and were not treated with vitamin D, the potential of vitamin D analogues to reduce cardiovascular mortality through vitamin D receptor (VDR)-dependent regulation of cardiac hypertrophy, renin-angiotensin system, inflammation, and/or other important homeostatic mechanisms raises questions regarding use of vitamin D even in the face of hyperphosphatemia.9

On the other hand, vitamin D analogues have the potential to induce toxicity from hypercalcemia and hyperphosphatemia through direct actions to increase gastrointestinal absorption of both calcium and phosphate, as well as to stimulate bone resorption.13 Although small differences may exist between calcitriol and its active vitamin analogues paricalcitol and doxercalciferol with regard to incidence of hypercalcemia and hyperphosphatemia, prospective clinical trials show that all can cause hyperphosphatemia and hypercalcemia, especially at high dosages.11,14 Animal and cell culture studies also support a specific role for vitamin D in mediating vascular calcifications, which is an active osteogenic-like process. In this regard, injection of active vitamin D analogues directly promotes vascular calcification in experimental models, even in the face of controlled phosphorus and calcium levels,15–17 whereas administration of vitamin D to vascular smooth muscle cell cultures accentuates calcifications and osteogenic gene expression.18 The fibroblast growth factor 23 (FGF23)-null mouse model is a relatively novel animal model that illustrates the toxic potential of elevated serum 1,25(OH)2D levels. Deletion of FGF23, the bone-derived hormone that regulates serum phosphate and vitamin D homeostasis,19–21 results in hyperphosphatemia, markedly elevated circulating 1,25(OH)2D levels, extensive vascular calcifications, and early mortality.20 Transfer of 1α-hydroxylase deficiency or the VDR null phenotype onto FGF23 null mice corrects the vascular calcifications and high mortality in these animals, implicating toxic effects of high circulating levels of 1,25(OH)2D levels that are mediated through the VDR in this animal model.22,23 The concomitant correction of both hyperphosphatemia and excessive 1,25(OH)2D activity by the addition of either 1α-hydroxylase or VDR deficiency to the FGF23 null background, however, prevents insights into the separate contributions of excess serum phosphate and 1,25(OH)2D on vascular calcifications and increased mortality.

It is unclear whether the dosage of active vitamin D analogues should be limited and/or treatment interrupted in patients who have ESRD with elevated phosphate levels or the potential beneficial effects of vitamin D provides justification for their continued use in the setting of hyperphosphatemia. To address this question, we examined the separate effects of hyperphosphatemia and elevated 1,25(OH)2D on vascular calcifications and mortality in the FGF23 null mouse model. In this study, we placed FGF23 null mice on either a phosphorus-deficient diet, which corrected the hyperphosphatemia but not the elevated 1,25(OH)2D levels, or a vitamin D–deficient diet, which normalized the circulating 1,25(OH)2D levels without correcting the hyperphosphatemia, to determine the individual contributions of serum phosphorus and 1,25(OH)2D to the development of vascular calcifications and early mortality in this mouse model. We found hyperphosphatemia to be the major contributor to vascular calcifications in FGF23 null mice and that in vivo elevations in 1,25(OH)2D in the absence of hyperphosphatemia had no demonstrable toxicity in this model.

RESULTS

High Mortality, Disordered Mineral Metabolism, and Vascular Calcifications in FGF23 Null Mice

Compared with wild-type mice, FGF23 null mice on a control diet displayed an age-dependent increase in mortality beginning at 5 wk of age. No FGF23 null mice that received a regular diet survived beyond 14 wk of age (Figure 1A). Also, FGF23 deficiency resulted in severe growth retardation as evidenced by a significant reduction in body weight (Figure 1B). The shortened survival in FGF23 null mice was associated with hyperphosphatemia, hypercalcemia, and elevated circulating 1,25(OH)2D levels (Table 1), consistent with the known actions of FGF23 to inhibit renal phosphate reabsorption and 1α-hydroxylase.

The FGF23 null mice on a regular diet also displayed extensive soft tissue and vascular calcifications that were apparent by 6 wk of age (Figure 2 through 4). Alizarin red staining of tissues from the FGF23 null mice revealed the presence of widespread calcifications in various organs, including the aorta and kidneys (Figure 2) as well as lung (data not shown). Histologic analyses of these tissues demonstrated calcifications of the elastic lamina in medial layer of the aorta (Figure 2C) along with marked calcifications of the small- and medium-sized vessels of the kidney (Figure 3A).
A vitamin D–deficient diet also resulted in improved survival in FGF23 null mice after dietary manipulation of phosphate and vitamin D. (A) On a control diet, FGF23 null mice exhibited 0% survival \( (n = 9) \) compared with 100% survival of WT mice \( (n = 6) \) at 14 wk of age. A significant improvement in survival of FGF23 null mice was observed on both the phosphate-deficient (PD; \( n = 10 \)) and vitamin D–deficient (DD; \( n = 9 \)) diets, with 90% of the mice surviving to 14 wk of age. WT mice on both the PD \( (n = 6) \) and DD \( (n = 6) \) diets had 100% survival rate at 14 wk of age. (B) WT mice that were fed a PD diet had severe \( (P < 0.01) \) and mild \( (P < 0.05) \) growth retardation, respectively. FGF23 null mice had severe growth retardation on a control diet compared with WT mice \( (P < 0.01) \). This growth retardation was not worsened by feeding a PD diet and was improved with a DD diet \( (P < 0.05) \).

Microcomputed tomography (μCT) analysis of the kidneys revealed extensive cortical calcium deposition that correlated with histologic evidence of calcifications of small- to medium-size arteries (Figure 3, A and B). In addition, Fourier transform infrared (FTIR) microspectroscopic imaging analysis demonstrated focal mineral deposition distributed in a manner similar to that exhibited by Von Kossa staining (Figure 4A). In the spectra of the mineral deposits, there is a broad peak at 1082 cm\(^{-1}\) that is associated with the P-O vibration modes (Figure 4B). The broad, relatively featureless contour that characterizes this peak indicates that the mineral spots contain amorphous calcium phosphate but not highly crystallized apatite.

Low-Phosphate Diet in FGF23 Null Mice Prolongs Survival in Association with Correction of Hyperphosphatemia, Prevention of Soft Tissue Calcifications, and Persistently Elevated 1,25(OH)\(_2\)D Levels

Phosphate restriction of wild-type mice resulted in severe growth retardation as assessed by a 50% reduction in body weight (Table 1) but had no adverse effect on survival (Figure 1). In addition, phosphate restriction in wild-type mice resulted in a marked decrease in serum phosphate levels and a five- to eight-fold increase in serum 1,25(OH)\(_2\)D levels, attaining values similar to FGF23 null mice. The increase in serum 1,25(OH)\(_2\)D is consistent with the known effects of hypophosphatemia on 1α-hydroxylase activity. Phosphate restriction also increased calcium levels and suppressed serum PTH levels compared with wild-type controls that were fed a regular diet (Table 1).

In contrast, FGF23 null mice that were fed a low-phosphate diet displayed marked improvement in survival. In this regard, a phosphate-depleted diet resulted in 90% survival of FGF23 null mice at the end of the 14-wk observation period (Figure 1) compared with no survivors in FGF23 null mice that were fed a regular diet. Phosphate restriction had no effect on the already low body weight in FGF23 null mice. Dietary phosphate restriction, however, significantly reduced serum phosphate levels in FGF23 null mice (Table 1). 1,25(OH)\(_2\)D levels remained six- to 10-fold elevated, and hypercalcemia and suppressed PTH persisted in FGF23 null mice that were fed a phosphate-depleted diet (Table 1).

The soft tissue calcifications associated with the absence of FGF23 were completely corrected by the phosphate-deficient diet (Figures 2 and 3). In this regard, there were no demonstrable calcifications by alizarin red staining, μCT, or histologic analysis of any tissue, including heart, aorta, and kidney, from FGF23 null mice on a low-phosphate diet, despite hypercalcemia and elevated 1,25(OH)\(_2\)D levels.

Vitamin D–Deficient Diet in FGF23 Null Mice Prolongs Survival in Association with Normalization of Serum 1,25(OH)\(_2\)D Levels but Fails to Correct the Hyperphosphatemia or Soft Tissue Calcifications

Wild-type mice that were fed a vitamin D–deficient diet had a slightly lower body weight and 100% survival. By 14 wk of age, wild-type mice that were fed a vitamin D–deficient diet exhibited elevation of serum PTH, consistent with the induction of secondary hyperparathyroidism. Serum calcium and 1,25(OH)\(_2\)D were maintained in the normal range, but serum phosphate was decreased in 14-wk-old wild-type mice that were fed a vitamin D–deficient diet.

A vitamin D–deficient diet also resulted in improved survival of FGF23 null mice, with 90% of the mice surviving at the end of the 14-wk observation period (Figure 1). The improved survival was not associated with correction of either the hyperphosphatemia or vascular calcifications (Table 1, vide infra). A growth advantage was also noted in the FGF23 null mice on the
Table 1. Serum biochemistries in 6- and 14-wk-old WT and FGF23 null mice on control, phosphate-deficient, and vitamin D–deficient diets

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normal</th>
<th>FGF23 Null</th>
<th>Phosphate-Deficient</th>
<th>FGF23 Null</th>
<th>Vitamin D-Deficient</th>
<th>FGF23 Null</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>FGF23 Null</td>
<td>WT</td>
<td>FGF23 Null</td>
<td>WT</td>
<td>FGF23 Null</td>
</tr>
<tr>
<td>serum phosphorus (mg/dl)</td>
<td>8.4 ± 0.3 (n = 7)</td>
<td>14.0 ± 0.5 (n = 5)</td>
<td>3.2 ± 0.5 (n = 8)</td>
<td>2.5 ± 0.3 (n = 5)</td>
<td>7.8 ± 0.3 (n = 7)</td>
<td>12.6 ± 0.6 (n = 5)</td>
</tr>
<tr>
<td>serum calcium (mg/dl)</td>
<td>10.1 ± 0.4 (n = 7)</td>
<td>11.7 ± 0.3 (n = 5)</td>
<td>13.7 ± 0.6 (n = 8)</td>
<td>14.4 ± 0.8 (n = 5)</td>
<td>9.0 ± 0.2 (n = 7)</td>
<td>10.4 ± 0.3 (n = 5)</td>
</tr>
<tr>
<td>serum 1,25(OH)2D (pM)</td>
<td>148 ± 32 (n = 7)</td>
<td>1356 ± 286 (n = 5)</td>
<td>1241 ± 176 (n = 8)</td>
<td>875 ± 80 (n = 5)</td>
<td>177 ± 33 (n = 7)</td>
<td>128 ± 16 (n = 5)</td>
</tr>
<tr>
<td>serum PTH (pg/ml)</td>
<td>15.6 ± 1.3 (n = 7)</td>
<td>5.9 ± 0.4 (n = 5)</td>
<td>6.2 ± 0.5 (n = 8)</td>
<td>6.1 ± 0.6 (n = 7)</td>
<td>29.0 ± 11.2 (n = 7)</td>
<td>10.7 ± 2.0 (n = 4)</td>
</tr>
<tr>
<td>serum phosphorus (mg/dl)</td>
<td>7.1 ± 0.2 (n = 7)</td>
<td>N/A</td>
<td>3.5 ± 0.5 (n = 5)</td>
<td>3.2 ± 0.2 (n = 7)</td>
<td>6.9 ± 0.3 (n = 9)</td>
<td>10.3 ± 0.9 (n = 9)</td>
</tr>
<tr>
<td>serum calcium (mg/dl)</td>
<td>8.5 ± 0.2 (n = 7)</td>
<td>N/A</td>
<td>14.2 ± 0.7 (n = 6)</td>
<td>14.2 ± 0.7 (n = 6)</td>
<td>8.9 ± 0.1 (n = 9)</td>
<td>8.9 ± 0.2 (n = 6)</td>
</tr>
<tr>
<td>serum 1,25(OH)2D (pM)</td>
<td>200 ± 33 (n = 6)</td>
<td>N/A</td>
<td>793 ± 85 (n = 6)</td>
<td>1453 ± 229 (n = 7)</td>
<td>44 ± 14 (n = 7)</td>
<td>128 ± 36 (n = 9)</td>
</tr>
<tr>
<td>serum PTH (pg/ml)</td>
<td>18.5 ± 3.4 (n = 7)</td>
<td>N/A</td>
<td>4.3 ± 1.2 (n = 4)</td>
<td>5.6 ± 0.9 (n = 5)</td>
<td>41.2 ± 8.9 (n = 8)</td>
<td>116 ± 53.9 (n = 7)</td>
</tr>
</tbody>
</table>

aData are means ± SEM from 6- and 14-wk-old mice. 1,25(OH)2D, 1,25-dihydroxyvitamin D; FGF23, fibroblast growth factor 23; WT, wild-type.

*b < 0.01 between WT and FGF23 null mice within each diet group, t test.

*<i>P < 0.01</i> versus mice of the same genotype on a normal diet, t test.

*<i>P < 0.05</i> versus mice of the same genotype on a normal diet, t test.

*<i>P < 0.05</i> between WT and FGF23 null mice within each diet group, t test.

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The vitamin D-deficient diet in FGF23 null mice resulted in significantly smaller that wild-type controls.

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DISCUSSION

In this study, we used dietary manipulation of phosphate and vitamin D to assess separately the contributions of hyperphosphatemia and elevated 1,25(OH)\textsubscript{2}D on vascular calcification and mortality in FGF23 null mice. Similar to other reports,\textsuperscript{20,24} we found that the FGF23 null mouse develops a mineral profile and phenotype similar to patients who have ESRD and are treated with high-dosage vitamin D analogues, namely, elevated serum calcium, phosphate, and 1,25(OH)\textsubscript{2}D levels along with vascular calcifications and decreased survival (Table 1, Figures 1 and 2). By restricting phosphate intake in the FGF23 null mice, we were able to lower selectively serum phosphate levels while maintaining markedly elevated serum levels of 1,25(OH)\textsubscript{2}D and calcium (Table 1). In contrast, feeding FGF23 null mice a vitamin D--deficient diet resulted in normalization of circulating 1,25(OH)\textsubscript{2}D levels while maintaining a state of hyperphosphatemia (Table 1).

Lowering serum phosphate levels by dietary phosphate restriction resulted in a complete resolution of the soft tissue and vascular calcifications and near normalization of survival in FGF23 null mice (Figures 1 and 2). The rescue of vascular calcifications and the lethal phenotype of FGF23 null mice, despite elevated 1,25(OH)\textsubscript{2}D and serum calcium levels, suggests that serum phosphorus is the main determinant of vascular calcifications and a significant contributor to the early mortality in this mutant mouse model. The predominant role of phosphate in vascular calcifications is consistent with recent in vitro and in vivo findings showing that phosphate rather than calcium is the primary factor necessary for mineralization of extracellular matrix.\textsuperscript{4,25} Although applying these results to human populations is somewhat speculative, the apparent association between hyperphosphatemia and vascular calcifications in this model suggest that the clinical observational data linking hyperphosphatemia, vascular calcifications, and mortality in patients with ESRD may be causal associations.\textsuperscript{2,5,6}

The related finding that markedly elevated 1,25(OH)\textsubscript{2}D levels in FGF23 null mice on a phosphate-deficient diet had no apparent adverse effects, including failure to stimulate vascular calcifications, suggests that this potential toxicity of high concentrations of active vitamin D is context dependent, being modified by FGF23 deficiency, vascular calcifications, and hyperphosphatemia or other factors. Several clinical studies have also failed to find a relationship between circulating active vitamin D levels and calcifications.\textsuperscript{26,27} In addition, these find-
ings support the interpretation that the lower mortality associated with the use of less potent vitamin D analogues, such as paricalcitol and doxercalciferol, could be explained by less hyperphosphatemia.\textsuperscript{11,28} Regardless, these studies are the first to show that restriction of dietary phosphate and lowering of the serum phosphate prevent vascular calcifications and improve survival in a hyperphosphatemic model. As such, these animal studies provide further support for the recommendation to treat aggressively hyperphosphatemia as a potential means of improving survival in patients with ESRD.

We also observed context-dependent adverse effects of excessive 1,25(OH)\textsubscript{2}D levels in FGF23 null mice. In this regard, normalization of 1,25(OH)\textsubscript{2}D levels by dietary vitamin D restriction neither fully corrected elevated serum phosphorus levels nor rescued the calcification phenotype in FGF23 null mice but paradoxically improved survival, similar to that of phosphate restriction alone.\textsuperscript{12} Similar to our studies, abolition of vitamin D–dependent activities in FGF23 null mice by creating compound mutant mice that lacked either the 1\textalpha-hydroxylase or VDR gene prevented vascular calcifications and prolonged survival in FGF23 null mice.\textsuperscript{22,29} In these previous studies, however, the 1\textalpha-hydroxylase and VDR null mice resulted in correction of both the hyperphosphatemia and excess activation of vitamin D–dependent signaling in FGF23 null mice, thereby precluding the separate assessment of the contribution of hyperphosphatemia and excess vitamin D.

The ability of decrements in circulating 1,25(OH)\textsubscript{2}D concentrations to have a positive impact on survival without reducing vascular calcifications is opposite to the purported salutary effect of vitamin D treatment in patients with ESRD.\textsuperscript{9} The potential toxicity of high circulating concentrations of active vitamin D analogues, however, is consistent with the observation that the survival benefit of calcitriol therapy in patients with ESRD is attenuated at high dosages.\textsuperscript{12} In any event, this study raises new questions regarding the necessity and potential toxicity of supraphysiologic concentrations of circulating 1,25(OH)\textsubscript{2}D, as well as the potential interactions between hyperphosphatemia and elevated 1,25(OH)\textsubscript{2}D levels.

Previous results from superimposed 1\textalpha-hydroxylase or VDR deficiency in FGF23 null mice that completely corrected the hyperphosphatemia suggested a minor function of FGF23 to regulate phosphate transport across the proximal tu-
bule. The finding of this study, namely that hyperphosphatemia was present in FGF23 null mice (Table 1) in the setting of normal circulating 1,25(OH)2D levels, however, is consistent with an important direct role of FGF23 deficiency to increase renal phosphate reabsorption by the kidney independent of its effects to regulate 1α-hydroxylase activity. The inability to observe the antiphosphaturic effects in FGF23 null mice with ablative 1α-hydroxylase and VDR may be due to the masking of the renal phenotype by decreased gastrointestinal absorption of phosphate.

Finally, the relevance of our findings in FGF23 null mice to the potential toxicity of phosphate and excessive activation of the vitamin D axis in hyperphosphatemic clinical disorders, such as tumoral calcinosis, and hyperphosphatemic patients who have renal failure and are treated with calcitriol analogues, requires further investigation given the limitations of this study. In this regard, the nonuremic FGF23 null mouse model does not address the impact of renal failure, which could contribute additional factors that lead to vascular calcifications and mortality. In addition, although our study clearly demonstrated a survival benefit for both vitamin D and phosphorus restriction in the FGF23 null mice, we are unable to describe the mechanisms behind this improved survival. Last, FGF23 levels are markedly increased in the setting of renal failure, rather than decreased as in the FGF23 null mice. The function of FGF23 in ESRD is not known. It is not clear whether the biologic effects of FGF23 are increased in ESRD or decreased as a result of a loss of kidney function. Because the kidney seems to function as the main target organ for FGF23, it is possible that the loss of a necessary kidney-derived constituent, such as Klotho, is promoting the exponential elevations of serum FGF23 in the setting of ESRD. Regardless, these findings suggest that effective lowering of serum phosphate will have important effects to prevent vascular calcifications. Prospective clinical trials are needed to examine the separate roles of phosphate control and intensity of therapy with active vitamin D analogues on vascular calcifications and mortality in patients with chronic kidney diseases.

CONCLUSION

The results of this study implicate hyperphosphatemia in the pathogenesis of soft tissue and vascular calcifications in FGF23 null mice and support a context-dependent toxic role of excess circulating 1,25(OH)2D. These observations suggest important interactions between hyperphosphatemia and excess 1,25(OH)2D and support the underlying premise of the K/DOQI clinical recommendations to limit the dosage of active vitamin D analogues in the setting of hyperphosphatemia and to strive to achieve lower serum phosphate concentrations. In addition, a better understanding of the interactions between hyperphosphatemia and excessive vitamin D activity in the FGF23 null mouse model may provide insights into the management of chronic kidney disease, where hyperphosphatemia and treatment with vitamin D analogues often coexist.

CONCISE METHODS

Animal Preparation, Study Protocol, and Dietary Modifications

FGF23 knockout mice were generated and genotyped as previously reported. All mice were maintained and used in accordance with the recommendations in the Guide for Care and Use of Laboratory Animals and by guidelines established by the University of Kansas Medical Center Institutional Animal Care and Use Committee.

All diets were purchased from Harlan Teklad (Madison, WI). The phosphate deficient diet contained 0.6% Ca, 0.02% Pi, and 2200 IU vitamin D per kilogram of diet. The vitamin D–deficient diet contained 0.6% Ca, 0.54% Pi, and 1.0 IU vitamin D per kilogram of diet.

Serum Bioassays

Blood samples were obtained by retro-orbital bleeding at both 6 and 14 wk of age. Serum calcium and phosphorus levels were measured, respectively, using Calcium Liquicolor (Stanbio Laboratory, Boerne, TX) and phosphomolybdate-ascorbic acid method as described previously. Serum PTH was determined by using the Mouse Intact PTH ELISA Kit (Immunotopics, San Clemente, CA). Serum 1,25(OH)2D levels were measured using a 1,25 Dihydroxy Vitamin D ELISA Kit (IDS Ltd., Tyne & Wear, UK).

Tissue Collection and Alizarin Red-S Staining

Tissues to be stained for calcifications were collected and stored in 70% ethanol. Alizarin red staining was used to stain soft tissue calcifications as previously described. Briefly, each individual tissue sample was placed in 10 ml of alizarin working solution that contained 0.8% alizarin red S in 0.5% KOH and rotated for 24 h. The tissues were then removed from the alizarin working solution, replaced in 10 ml of 0.05% KOH, and rotated for another 24 h to remove any unbound stain from the tissues; then a photograph was taken for record.

Histologic Analysis

Histologic analysis was harvested and fixed in 4% paraformaldehyde for 24 h and then embedded in paraffin. Five-micrometer tissue sections were used for Von Kossa and Toluidine blue staining as described previously. Ten-micrometer tissue sections were used for FTIR imaging analysis.

FTIR Microspectroscopic Imaging Analysis

FTIR microspectroscopic imaging analysis was completed using the Spectrum Spotlight FTIR imaging system (Perkin Elmer, Waltham, MA) with both single-point and imaging mode. Images were scanned between 4000 and 720 cm−1 at 4 cm−1 spectral resolution with two
scans per pixel. Image sizes were approximately 300 × 300 μm with 6.25-μm pixel resolution. An atmosphere correction was applied to the raw image to subtract the contribution of atmosphere absorbance (water vapor and carbon dioxide). Images were created using Spectrum Spotlight software.

**μCT Analysis**

The kidneys from 6-wk-old mice were collected and fixed in 70% ethanol. High-resolution μCT was used to evaluate mineralization of kidney samples (μCT40; Scanco Medical AG, Basserdorf, Switzerland). A 100-μm-thick area from the middle of each kidney was evaluated for three-dimensional image and quantitative evaluation of the mineralized kidney volume fraction.

**Statistical Analyses**

We evaluated differences between groups by one-way ANOVA for statistically significant. All computations were performed using the GraphPad Prism 4 software (GraphPad Software, San Diego, CA).

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