Direct inhibition of osteoblastic Wnt pathway by fibroblast growth factor 23 contributes to bone loss in chronic kidney disease

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Bone loss and increased fractures are common complications in chronic kidney disease. Because Wnt pathway activation is essential for normal bone mineralization, we assessed whether Wnt inhibition contributes to high-phosphorus-induced mineralization defects in uremic rats. By week 20 after 7/8 nephrectomy, rats fed a high-phosphorus diet had the expected high serum creatinine, phosphorus, parathyroid hormone, and fibroblast growth factor 23 (FGF23) levels and low serum calcium. There was a 15% reduction in tibial mineral density and a doubling of bone cortical porosity compared to uremic rats fed a normal-phosphorus diet. The decreases in tibial mineral density were preceded by time-dependent increments in gene expression of bone formation (Osteocalcin and Runx2) and resorption (Cathepsin K) markers, which paralleled elevations in gene expression of the Wnt inhibitors Sfrp1 and Dkk1 in bone. Similar elevations of Wnt inhibitors plus an increased phospho-β-catenin/β-catenin ratio occurred upon exposure of the osteoblast cell line UMR106-01 either to uremic serum or to the combination of parathyroid hormone, FGF23, and soluble Klotho, at levels present in uremic serum. Strikingly, while osteoblast exposure to parathyroid hormone suppressed the expression of Wnt inhibitors, FGF23 directly inhibited the osteoblastic Wnt pathway through a soluble Klotho/MAPK–mediated process that required Dkk1 induction. Thus, the induction of Dkk1 by FGF23/soluble Klotho in osteoblasts inactivates Wnt/β-catenin signaling. This provides a novel autocrine/paracrine mechanism for the adverse impact of high FGF23 levels on bone in chronic kidney disease.

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Bone loss, increased fractures, cardiovascular complications, and high mortality rates are very common in chronic kidney disease (CKD) patients and are referred to as CKD–mineral bone disorders (CKD-MBD) by Kidney Disease: Improving Global Outcomes (KDIGO) guidelines.[1]

Abnormal phosphorus metabolism plays a key role in the onset and progression of CKD-MBD.[2–5] High serum phosphorus triggers secondary hyperparathyroidism,[6,7] resulting in high serum parathyroid hormone (PTH) and elevations in Fgf23, both hormones with recognized adverse effects on bone metabolism.[8–12] The increases in serum phosphorus, PTH, and Fgf23 also associate with elevations in the circulating levels of Sclerostin (Sost) and Dickkopf 1 (Dkk1),[13,14] 2 potent inhibitors of the Wnt/β-catenin pathway, essential for skeletal development and the maintenance of bone mass.[15,16]

The Wnt pathway plays a key role in the induction of osteoblast activity.[4,5] Briefly, the activation of Frizzled (Frz) and lipoprotein receptor–related peptides (Lrps) inhibits the axin–APC–GSK3 complex, increasing β-catenin translocation to the nucleus to promote preosteoblast differentiation, in part through the induction of Runt-related transcription factor 2 (Runx2).[17,18] Instead, the Wnt inhibitors of Lrps, Dkk1 and Sost, and the secreted Frizzled-related proteins (Sfrps), inhibitors of Frz proteins, reduce osteoblast differentiation and survival.[19] In fact, treatment with anti-Sost or anti-Dkk1 antibodies effectively preserved bone in patients with osteoporosis,[20–22] and in experimental models of diabetic nephropathy[23] or multiple myeloma,[24] respectively. Also, circulating Sost appeared to better predict bone turnover in CKD patients (diagnosed by bone biopsies) than serum PTH.[13] These findings raised interest in strategies to maintain bone Wnt signals in CKD-MBD.

However, recent clinical and preclinical studies have challenged the accuracy of high serum Sost in reflecting high bone Sost and Wnt/β-catenin inhibition.[25] In the jck mouse, a genetic model of polycystic kidney disease, high serum PTH and Fgf23 concurred with inhibition rather than stimulation of bone Sost but with a 2- to 3-fold induction of mRNA levels of the Wnt inhibitors Sfrp1, Sfrp4, and Dkk1,[26,27] which sustained inhibition of β-catenin signaling and higher

osteoclast activity. Accordingly, bone biopsies in hemodialysis patients confirmed a high inhibition of Wnt signals, as measured by increased phospho-β-catenin (p-β-catenin), despite a lower number of osteocytes positive for Sost.[27]

Thus, identifying the progressive impact of increases in serum phosphorus, PTH, and Fgf23 on bone levels of Wnt inhibitors and bone health is essential to design safe and effective strategies to preserve bone in the course of CKD-MBD. Herein, the contribution of the increases in PTH, phosphorus, and Fgf23 to bone Wnt pathway inhibition causing bone loss along chronic renal failure (CRF) progression was examined *in vivo* in a rat model of high-bone-turnover CKD. Furthermore, the independent impact of PTH or a combination of Fgf23 and soluble Klotho on Wnt pathway inhibition was fully delineated *in vitro* in the osteoblastic cells UMR106-01.

RESULTS

High dietary phosphorus–induced changes in renal function and CRF-related markers

All rat groups fed a high-phosphorus diet (HPD) showed higher mortality and a time-dependent impairment of renal function and mineral metabolism as measured by higher serum creatinine, phosphorus, PTH, and Fgf23 levels and lower serum calcium compared to rats fed the normal-phosphorus diet (NPD) (Table 1). The highest serum phosphorus was reached after 16 weeks of uremia, while the highest PTH and Fgf23 (92.5- and 35-fold increases, respectively) only after 20 weeks of uremia (Table 1).

In contrast, serum Klotho (sKlotho) was similar in uremic animals from both dietary groups at weeks 8 and 16. Furthermore, at week 16 both uremic groups had higher sKlotho than rats with normal renal function. Only at week 20, the HPD group had sKlotho lower than that of the NPD group but indistinguishable from that of normal rats (Table 1).

High dietary phosphorus–induced changes in bone mineral density, bone turnover, and the Wnt pathway

The decreases in total and distal tibia bone mineral density (BMD) in the HPD groups (14% and 15%, respectively) only reached statistical significance at week 20. Similarly, cortical porosity progressively increased, reaching by week 20 values almost double in the HPD group compared to the NPD group (Table 2).

In the HPD groups, significant time-dependent increases in the gene expression of osteocalcin and Runx2 (as markers of bone formation) and cathepsin K (as marker of bone resorption) were observed compared to those in time-matched NPD and reference groups (Figure 1a). The greatest increase in mRNA levels was observed for cathepsin K (Figure 1a).

Sost mRNA levels increased similarly in both dietary groups by week 8 after inducing CRF, but then decreased in a time-dependent manner at 16 and 20 weeks in the HPD compared to the time-matched NPD group (Figure 1b). In addition, while gene expression of the Wnt inhibitors Sfrp1, Sfrp4, and Dkk1 was higher in the HPD group compared to time-matched NPD groups, these increments were time-dependent only for Sfrp1 and Sfrp4 (Figure 1b). Sfrp1 and Dkk1 protein levels increased time-dependently in the HPD group, compared to their time-matched NPD group (Figure 2a and b). Immunohistochemical analysis of tibias from HPD-fed uremic rats for 12 weeks after nephrectomy shows the increases in Dkk1 concurring with higher bone formation (osteoblasts and osteocalcin) and resorption (osteoclast) markers (Figure 2c).

Uremic serum inhibits the osteoblastic Wnt pathway *in vitro*

To reproduce an environment similar to that of the early uremic stage examined in the *in vivo* study, UMR106-01 cells were exposed to serum from uremic rats (8 weeks of CRF and HPD), and the expression of Wnt pathway markers was

Table 1 | Cumulative mortality rate and serum biomarkers of the severity of CFR

	8 weeks		16 weeks		20 weeks		
	NPD	HPD	NPD	HPD	NPD	HPD	Reference
Creatinine (mg/dl)	0.9±0.0[a]	1.2±0.2[a,b]	1.1±0.2[a]	1.8±0.5[a,b]	1.4±1.0[a]	2.8±0.7[a,b]	0.4±0.0
Phosphorus (mg/dl)	5.2 [4.9–6.8]	8.6 [7.5–12.5][a,b]	5.2 [4.1–5.4]	12.2 [10.5–13.9][a,b]	5.6 [5.0–6.3]	12.0 [9.7–15.2][a,b]	4.9 [3.8–5.7]
Calcium (mg/dl)	12.1±0.2[a]	11.6±0.4[b]	11.7±0.5	11.3±0.5	12.5±0.6[a]	10.9±0.1[a,b]	11.5±0.6
PTH (pg/ml)	28.0 [17.0–61.0]	139.0 [97.5–975.5][a,b]	66.0 [26.3–124.8]	1238.0 [772.0–2436.0][a,b]	80.0 [37.0–123.0]	2035 [642.5–2867.0][a,b]	22.0 [16.0–59.0]
Fgf23 (pg/ml)	182.8 [99.0–273.7]	424.0 [401.6–424.0][a,b]	214.7 [130.8–741.2][a]	3400.0 [2241.0–3799.8][a,b]	113.9 [102.1–150.8]	4143.7 [3842.0–4300.0][a,b]	119.3 [91.6–149.3]
Klotho (ng/ml)	9.0±0.9	8.7±1.5	10.1±2.4[a]	10.1±1.0[a]	11.0±2.0[a]	8.3±1.3[b]	8.3±0.9
Cumulative mortality (%)	9.5	17	19	33	19	40	0

Serum creatinine, phosphorus, calcium, PTH, Fgf23, and Klotho levels in rats with CRF fed NPD and HPD for 8, 16, and 20 weeks and the reference group. ($N = 6$ rats per group.)
CRF, chronic renal failure; HPD, high-phosphorus diet; NPD, normal-phosphorus diet; PTH, parathyroid hormone.
[a]$P < 0.05$ compared to the reference group.
[b]$P < 0.05$ compared to time-matched NPD group.