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Effects of Klotho deletion from bone during chronic kidney disease

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Abstract

Klotho is a type I transmembrane protein that acts as a permissive co-receptor for FGF23 and helps to maintain proper mineral metabolism. Mice carrying a loss-of-function mutation in either the *Klotho* or *Fgf23* gene develop many similar phenotypes including osteoporosis. Based on these observations it was hypothesized that the bone phenotypes in *Klotho*- and *Fgf23*-null mice may be mediated through a common signaling pathway. Recent improvements in antibody specificity have shown that osteoblasts and osteocytes, which produce FGF23, also express low amount of membrane Klotho. But, the role of Klotho in bone is still largely unclear. In this review we summarize the literature and show that Klotho has an FGF23 dependent and independent effect in bone.

Introduction

Chronic kidney disease (CKD) is a global health burden of growing incidence and prevalence [1]. In the initial stages of the disease, kidney function slows down and adaptive mechanisms attempt to compensate for the reduced number of nephrons in order to maintain serum calcium and phosphate within the normal ranges. But, as renal function further declines, mineral homeostasis becomes deranged. This in turn induces vascular calcification and left ventricular hypertrophy, leading to increased risk for cardiovascular morbidity and mortality [2–11]. Alterations in mineral metabolism also exacerbate the severity and progression of renal failure [12]. Thus, understanding mineral metabolism is of utmost importance for prevention and treatment of CKD.

The regulation of mineral metabolism is achieved through a complex interaction of hormonal factors that utilize crosstalk between endocrine organs to adjust calcium and

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phosphate balance and bone mineralization in response to changing physiological requirements. The primary hormonal factors responsible for mineral metabolism are parathyroid hormone (PTH) from the parathyroid gland, active vitamin D ($1,25(\text{OH})_2\text{D}_3$) from the kidney, and fibroblast growth factor 23 (FGF23) from the bone. A fourth factor called Klotho is expressed in all three of these endocrine organs and has emerged as a unique new regulator of mineral flux and endocrine crosstalk [13–15]. It acts primarily as a required co-receptor for FGF23. However, unlike other endocrine factors, Klotho is a type I transmembrane protein that has a short intracellular domain with no known function and a large extracellular domain that can be shed and secreted into circulation. Another mechanism by which the secreted form of Klotho can be generated is by alternative splicing [13, 16]. This so called soluble Klotho, has exo-glycosidase activity that can hydrolyze β -glycosidic linkages in saccharides, glycoproteins and glycolipids [13, 16]. As a result, it is implicated in diverse biological processes, including protection of endothelial function, inhibition of phosphate-driven vascular calcification and suppression of fibrosis and inflammation [17]. The underlying molecular mechanisms for soluble Klotho actions are not fully elucidated but involve modulation of Wnt, insulin, IGF-1 and TGF- β signaling [18, 19].

However, it is the membrane-anchored Klotho that is an important regulator of mineral homeostasis and it has distinct functions from those of soluble Klotho. It forms a tetrameric complex with FGF receptors (FGFR1c, 3c and 4) and functions as an obligatory co-receptor for FGF23 [20–22]. Mice carrying a loss-of-function mutation in either the *Klotho* or *Fgf23* gene develop similar phenotypes, including shortened life span, growth retardation, osteoporosis, skin and muscle atrophy, defective hearing, ectopic calcifications, pulmonary emphysema, gonadal dysplasia and infertility [13, 23]. In addition, both mouse models exhibit hypercalcemia, hyperphosphatemia, hypervitaminosis D with low to undetectable levels of PTH [13, 23]. Based on these observations it was hypothesized that the bone phenotypes in *Klotho*- and *Fgf23*-null mice may be mediated through a common signaling pathway. We previously challenged *Klotho*^{-/-} and *Fgf23*^{-/-} knockout mice with PTH to test this hypothesis in bone. PTH (1–34) treatment similarly increased trabecular bone density and mineralized bone volume in *Klotho*^{-/-} and *Fgf23*^{-/-} knockout mice [24]. However, ablation of PTH from these mice resulted in different bone phenotypes [25, 26]. Deletion of PTH from *Klotho*^{-/-} mice (*Klotho*^{-/-}*PTH*^{-/-} *dco*) resulted in a complete rescue of the abnormal skeletal phenotype including the severe mineralization defect [25], whereas no improvement could be observed in the skeleton of *Fgf23*^{-/-}*PTH*^{-/-} *dco* mice [26]. These findings were the first to demonstrate that Klotho and Fgf23 exhibit independent functions in bone.

Recently, Murali et al [27] ablated vitamin D signaling in *Klotho*^{-/-} and *Fgf23*^{-/-} knockout mice to investigate whether high $1,25(\text{OH})_2\text{D}_3$ levels are responsible for impairments in bone mineralization. Interestingly, their findings confirmed our previous observations regarding PTH ablation [25, 26]. *Klotho*^{-/-} *VDR* / knockout mice, like *Klotho*^{-/-}*PTH*^{-/-} mice, had complete rescue of the skeletal phenotype, while *Fgf23*^{-/-} *VDR* / knockout mice did not [27]. These data provided a second line of evidence that Klotho and FGF23 have some independent effects in bone.

Mice, rats and humans predominantly express FGF23 in osteocytes [28], which are the most abundant cells in bone. As a result, serum levels of FGF23 are derived mainly from bone. Membrane Klotho, on the other hand, has a much wider expression profile [13–15, 29]. It is most highly expressed in the kidney, the epithelium of the choroid plexus and the parathyroid gland [13, 29]. The kidney and the parathyroid gland are two major organs where FGF23 exerts its endocrine effects by binding to the Klotho-FGFR receptor complex [29–31], whereas the role of Klotho in the choroid plexus is less well characterized. Lower levels of Klotho are found in the pituitary gland, placenta, skeletal muscle, urinary bladder, pancreas, testis, ovary, colon, inner ear, and bone [13–15, 29]. Recent improvements in antibody specificity have helped to demonstrate that osteoblasts and osteocytes, which produce FGF23 also express low amounts of membrane Klotho [14, 15]. In this review, we summarize the literature and show that Klotho has an FGF23 dependent and independent effect in bone.

Effects of global Klotho-deficiency on bone outcomes

The Klotho hypomorphic (*kl/kl*) and the Klotho-knockout (*Klotho*^{−/−}) mice have been used interchangeably to describe the effects of global *Klotho*-deficiency. The *kl/kl* model, generated by Kuro-o et al [13], has an intact *α-klotho*-coding sequence but harbors a severe hypomorphic mutation for *α-klotho* expression. Therefore, it is not a complete null [13]. In contrast, the *Klotho*^{−/−} model has the entire *α-klotho*-coding sequence deleted from its genome and so completely lacks the 1,014 amino acid sequence of the Klotho membrane protein.

Several research groups independently investigated the bone phenotype of *Klotho*-deficient mice and reported radically different phenotypes [13, 25, 32–34] (Table 1). Some groups showed that Klotho-deficient mice had osteoporotic bones despite high serum calcium and phosphate levels [13]; while others reported high bone volume, increased trabecular connectivity and abnormal elongation of trabeculae in the epiphyses of long bones [25, 33, 34]. We have organized the published data by methodological tools used to study bone outcomes in order to better understand the underlying phenotype (Table 1). This reorganization revealed common observations across the studies. Both strains of *Klotho*-deficient mice (*kl/kl* and *Klotho*^{−/−}) had low bone turnover and cortical thickness, but high trabecular bone volume [25, 32–34].

Since cortical parameters are usually analyzed in the mid-shaft of long bones and trabecular parameters are analyzed below the growth plate, it became evident that *Klotho*-deficient mice had different phenotypes in different bone regions (Table 1). In the cortex of the diaphysis, *Klotho*-deficient mice had low bone density and thickness, while in the metaphysis close to the knee, the elongation of trabeculae contributed to lamellar architecture and high cancellous bone volume [13, 25, 32–34]. This difference in bone architecture between trabecular and cortical bone underscores the possible presence of site-specific changes in bone metabolism.

Several reports have suggested that the elongation of trabeculae in *Klotho*-deficient mice may be attributed to the nature of rodents who, unlike humans, have growth plates that never

fuse. It is proposed that the continuous activity in the growth plate, coupled with low bone turnover, allows trabeculae in *Klotho*-deficient mice to extend further into the metaphyseal region and become elongated. However, if this were true, than *Fgf23*^{-/-} knockout mice with a similar endocrine profile and bone turnover rate should also have elongated trabeculae in the metaphysis of long bones. But several published studies that evaluated the bone volume, trabecular thickness and trabecular connectivity demonstrated that they were either unchanged or lower in *Fgf23*^{-/-} knockout mice than wild-type littermates [23, 24, 26, 35]. We therefore believe that the elongation of trabeculae in *Klotho*-deficient mice is not attributable to rodent physiology, but is driven by some unique pathological state of *Klotho*-deficient bone cells.

Histomorphometric analyses of long bones showed that *Klotho*-deficient mice had a significantly lower number and surface area of osteoblasts and osteoclasts than wild-type littermates. (Table 1) [13, 33, 34]. As a result, these mice also had lower bone formation and bone resorption activities. The decrease in bone formation was reported to exceed the decrease in bone resorption by more than two-fold, which begged the question of why *Klotho*-deficient mice had high trabecular bone volume. To better understand this, Yamashita et al [36] investigated the impact of *Klotho* gene deletion on bone remodeling in a bone marrow ablation model. The bone marrow ablation stimulated a build-up of trabecular bone in both wild-type and *Klotho*-deficient mice, but the newly formed trabecular bone was only resorbed in wild-type mice. As a result, two-weeks after bone marrow ablation, wild-type mice had normal trabecular bone volume while *Klotho*-deficient mice had high trabecular bone volume. Quantification of osteoclasts in three distinct regions of trabecular bone showed that *Klotho*-deficient mice had an interesting site-specific reduction in osteoclast number and surface area that was most severe in the distal region of the growth plate where the trabeculae are elongated. Importantly, the site-specific reduction in osteoclast number and surface area was not observed in wild-type mice, confirming that retardation of bone resorption is a specific feature of *Klotho*-deficient mice.

It has long been recognized that factors produced by osteoblasts, including the pro-osteoclastogenic receptor activator of nuclear factor- κ B ligand (RANKL) and the inhibitory decoy receptor osteoprotegerin (OPG), regulate osteoclast formation and differentiation. As a result, several groups investigated the expression levels of *Rankl* and *Opg* mRNA by qRT-PCR using total RNA extracted from long bones of *Klotho*-deficient mice [34, 36]. In the absence of *Klotho*, the mRNA level of *Opg*, but not *Rankl*, was significantly elevated [34, 36], leading to three-fold higher serum OPG levels in these mice [34]. These data suggest that low bone turnover in *Klotho*-deficient mice is in part driven by impaired osteoblast function. We recently used timed pregnancy in wild-type dams to find that *Klotho* is expressed in fetal limb buds at E9.5 [37]. This is the time when mesenchymal cells begin to differentiate into bone forming cells, so there is a possibility that *Klotho* ablation disrupts osteoblast function by reprogramming osteoblast differentiation.

The process of osteoblast differentiation can be characterized by cell proliferation, matrix maturation and matrix mineralization [38]. Primary osteoblasts from *Klotho*^{-/-} knockout mice have been used to show that *Klotho* ablation accelerates osteoblast differentiation without any accompanying increases in cell proliferation. To better understand the role of

Klotho in matrix maturation and mineralization, immune-localization of non-collagenous bone matrix protein was analyzed in long bones from *Klotho*-deficient (*kl/kl*) mice (Table 1) [39]. Findings showed that bone matrix proteins (osteocalcin, dentin matrix protein-1 and matrix Gla protein) were ectopically synthesized in *Klotho*-deficient mice and that osteocytes were surrounded by an unusual microenvironment. First, many osteocytes and some bone cells were surrounded by mineralized matrix expressing an excessive amount of DMP-1 and osteocalcin. Second, the osteoid, which is the unmineralized portion of bone matrix, was filled with mineralized osteocytes in the absence of *Klotho*. These osteocytes had an excessive amount of Ca^{2+} binding molecules (i.e. osteocalcin and DMP-1) in their lacunae [39]. Dr. Feng's group investigated the role of Ca^{2+} binding molecules in *Klotho*-deficient osteocytes by ablating *Dmp1* from *Klotho*-deficient (*kl/kl*) mice [40]. Findings suggested that *Klotho*-deficient osteocytes overproduce bone matrix proteins in order to maintain osteocyte dendrites and increase their viability. If we consider that osteocytes are involved in bone mineral homeostasis, regulation, and transportation, it becomes clear that osteocyte viability is critical for survival. However, since *Klotho*-deficient mice have severe endocrine changes accompanied by pathologically high serum phosphate levels, the observed bone cell changes could be due to cell-autonomous, cell non-autonomous, or systemic effects. We built upon this research to more thoroughly investigate the role of membrane Klotho in bone by ablating *Klotho* in immature (mesenchymal progenitor cells) and mature (osteocytes) bone cells using *Prx1-Cre* and *Dmp1-Cre* mouse lines, respectively. The bone phenotype of these mutant mice under healthy and induced-CKD conditions is discussed below.

Effects of bone-specific Klotho ablation on bone outcomes

The overall appearance, body weight, survival rate and ability to reproduce were indistinguishable up to the age of 6 months between wild-type (*Klotho*^{fl/fl}) and bone-specific Klotho knockout mice (i.e. *Prx1-Cre;Klotho*^{fl/fl} and *Dmp1-Cre;Klotho*^{fl/fl}) [37, 41]. Moreover, there were no differences in circulating levels of Ca^{2+} , Pi, PTH, FGF23 and $1,25(\text{OH})_2\text{D}_3$ between groups. Therefore, unlike *Klotho*-deficient mice (i.e. *kl/kl* or *Klotho*^{-/-}), both *Prx1-Cre;Klotho*^{fl/fl} and *Dmp1-Cre;Klotho*^{fl/fl} mice provided a suitable model to investigate the role of Klotho in bone without interference from systemic disturbances in mineral metabolism.

Histomorphometric analyses of femurs showed that, in the absence of endocrine changes, the timing of Klotho deletion significantly affects bone morphology [37, 41]. *Prx1-Cre;Klotho*^{fl/fl} mice with *Klotho* ablation from immature (mesenchymal progenitor cells) bone cells had similar bone mass, trabecular bone volume and trabecular connectivity to healthy controls at 6 and 16 weeks of age (Table 2) [37]. They also showed no significant changes in osteoblast or osteoclast number, bone formation rate, mineral apposition rate or the amount of mineralized surface. In contrast, *Dmp1-Cre;Klotho*^{fl/fl} mice with *Klotho* ablation from more mature osteoblasts/osteocytes had significantly higher bone mass, trabecular bone volume and trabecular connectivity at 5 weeks of age when compared to healthy controls [41]. One possible explanation for this unexpected finding is an increase in osteoblast activity. Dynamic histomorphometry was performed and showed that *Dmp1-Cre;Klotho*^{fl/fl} mice had also a significantly higher mineralizing surface and bone formation

rate than control mice. qRT-PCR analysis confirmed that osteogenic gene markers, including *collagen type 1 (Col1a1)*, *Runx2*, *osteocalcin* and *Dmp1* were up-regulated in *Dmp1-Cre;Klotho^{fl/fl}* mice, whereas there was no change in the *RANKL/OPG* ratio which is a marker of osteoclastogenesis. Taken together these findings suggest that the deletion of *Klotho* from osteocytes during perinatal and adult stages leads to enhanced osteoblast activity through the lacunocanalicular system.

Biochemical measures of collagen also showed that the timing of *Klotho* deletion significantly affected bone metabolism. *Prx1-Cre;Klotho^{fl/fl}* mice with *Klotho* deletion from mesenchymal progenitor cells had markedly reduced amount of type I collagen fragments (CTX) in circulation than wild-type mice (Figure 1A), similar to *kl/kl* and *Klotho^{-/-}* mice. In contrast, *Dmp1-Cre;Klotho^{fl/fl}* mice with *Klotho* deletion from more mature osteoblasts and osteocytes had markedly increased amounts of serum procollagen type I N-terminal propetides (PINP) than wild-type littermates (Figure 1B) [41]. Since CTX is a measure of bone resorption and PINP is a measure of bone formation these findings highlighted an endocrine independent effect on bone metabolism by *Klotho* ablation. The coupling of bone formation and resorption is regulated by osteoblasts and osteocytes through synthesis of RANKL and OPG. Comparative analyses showed that *kl/kl*, *Klotho^{-/-}* and *Prx1-Cre;Klotho^{fl/fl}* mice had reduced bone turnover due to markedly increased expression of *Opg* in long bones when compared to wild-type mice [34, 36] (Figure 1A). Interestingly, there were no significant changes in *Opg* or *Rankl* expression in *Dmp1-Cre;Klotho^{fl/fl}* mice (Figure 1B), suggesting that membrane-*Klotho* expression plays a role in osteoblast-osteoclast coupling only during early development. The detailed mechanism by which *Klotho* deletion from osteocytes led to increased bone formation is unknown and requires further investigation.

In vitro studies have shown that soluble *Klotho* enables FGF23 signaling, likely through FGFR1 under physiological conditions, whereas highly elevated levels of FGF23 can directly impair bone quality and quantity in the absence of soluble *Klotho* [42]. This result supports the hypothesis that elevated FGF23 in the presence of low serum *Klotho* during CKD may contribute to bone pathologies by directly acting on bone cells. We investigated whether membrane *Klotho* in bone cells can regulate FGF23 expression by challenging *Prx1-Cre;Klotho^{fl/fl}* and *Dmp1-Cre;Klotho^{fl/fl}* with renal failure induced by either adenine-diet or 5/6 nephrectomy (NTX).

Effects of bone-specific *Klotho* ablation on FGF23 synthesis in renal failure

In patients with CKD, circulating levels of FGF23 and PTH increase progressively as kidney function declines [43, 44], presumably to maintain neutral phosphate balance by promoting urinary phosphate excretion. Emerging data suggest that the increase in FGF23 develops earlier than the elevation in PTH and that it plays a more important role in maintaining phosphate balance in patients with CKD [45]. The precise mechanism by which FGF23 levels are increased early in this disease is unclear however. A recent study found that the kidney plays a key role in FGF23 metabolism [46], but there must be additional mechanisms that control serum FGF23 levels in an appropriate range for maintaining a neutral phosphate balance.

There is evidence to suggest that FGF23 expression in bone cells is controlled by FGFR dependent mechanisms. In rodents with high serum FGF23 levels, FGFR inhibition [47] or *Fgfr1* deletion from osteocytes blocks *Fgf23* synthesis and secretion from bone [48]. Conversely, an antibody-mediated activation of FGFR1 induces an opposite effect. Similarly, in osteoglyphonic dysplasia patients, a gain of function mutation in FGFR1 is associated with increased serum phosphate and FGF23 levels [49–51]. Since *Fgf23* uses the Klotho-FGFR1 complex in kidneys and parathyroid glands to regulate its own production through negative feedback loops, it is highly plausible that Klotho binds to FGFR1 in bone to form an autocrine feedback loop controlling *Fgf23* synthesis and secretion.

We hypothesized that the Klotho expressed in bone-forming cells is involved in an autocrine feedback loop regulating *Fgf23* expression during renal failure. Thus, we challenged *Prx1-Cre;KL^{fl/fl}* and *Dmp1-Cre;KL^{fl/fl}* mice with renal failure produced by adenine-induced nephropathy and/or 5/6 NTX with or without high phosphate diet. Uremic *Prx1-Cre;KL^{fl/fl}* mice failed to increase expression of *Fgf23* in osteoblasts or osteocytes from which *Klotho* had been deleted [37]. This resulted in markedly lower levels of circulating FGF23 accompanied by secondary changes in serum PTH and 1,25(OH)₂D₃ when compared to uremic wild-type mice. These effects were not observed in *Dmp1-Cre;KL^{fl/fl}* mice lacking Klotho in only mature osteoblasts/osteocytes [41]. This highlights that the timing of Klotho deletion from bone as well as the type of cells it is deleted from has an important effect on FGF23 synthesis and secretion under CKD conditions. Since both osteoblasts and osteocytes express Klotho and FGF23, it is possible that in *Dmp1-Cre;KL^{fl/fl}* mice Klotho expressing osteoblasts could compensate and synthesis FGF23.

Nonetheless, the extremely high levels of FGF23 in whole body and kidney-specific Klotho knockout mice emphasize that Klotho expression in osteoblasts or osteocytes is not an obligate mechanism for *Fgf23* production. However, in these models it is possible that high levels of serum phosphate, calcium and 1,25(OH)₂D₃ may drive *Fgf23* expression in osteocytes even in the absence of Klotho. Further research is needed to fully elucidate the role of Klotho in osteoblasts and better understand how these cells regulate FGF23 production in both healthy and pathological conditions.

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Abbreviations

1,25(OH)₂D₃	1,25-dihydroxyvitamin D
AKI	acute kidney injury
CKD	chronic kidney disease
FGF23	fibroblast growth factor 23
FGFR	fibroblast growth factor receptor

KL	klotho
NTX	nephrectomy
PTH	parathyroid hormone

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Highlights

- Osteoblasts and osteocytes, which produce FGF23 also express low amounts of membrane Klotho
- Klotho has an FGF23 dependent and independent effect in bone
- Deletion of Klotho from osteocytes leads to enhanced osteoblast activity through the lacunocanalicular system
- Timing of Klotho deletion from bone has an important effect on FGF23 synthesis and secretion under CKD induced conditions

Figure 1. Serum collagen and qRT-PCR analyses of bone metabolism-related genes in femurs from control and bone-specific Klotho knockout mice

(A) Serum collagen type I c-telopeptide (CTX) and procollagen I intact N-terminal (PINP) levels in $KL^{fl/fl}$ and $Prx1-Cre;KL^{fl/fl}$ mice. n=6–8. *: p<0.05. (B) qRT-PCR analyses of

Rankl/Opg transcript in femur of $KL^{fl/fl}$ and $Prx1-Cre;KL^{fl/fl}$ mice. n=5–7. ***: p<0.001. (C) Serum collagen type I c-telopeptide and procollagen I intact N-terminal (PINP) levels in

$KL^{fl/fl}$ and $Dmp1-Cre;KL^{fl/fl}$ mice. N=7. *: p<0.05. (D) qRT-PCR analyses of *Rankl/Opg* transcript in femur of $KL^{fl/fl}$ and $Dmp1-Cre;KL^{fl/fl}$ mice. n=6–8.

Table 1

Summary of bone outcomes in *Klotho*-deficient mouse mice

	Strain & Age	Serum	X-ray	Histomorphometry	Histology	μ CT
Kuro-o et al 1997 [13]	Strain: <i>kl/kl</i> Age: 8 wk	N.D.	Tibia/Femur: ↓ BMD in the mid-region (20%) ↑ BMD in the growth plate	Femur: ↓ Ob.N ↓ Oc.N ↓ C.Th.	N.D.	N.D.
Kawaguchi et al 1999 [34]	Strain: <i>kl/kl</i> Age: 7 wk	↑ Ca ↑ Pi ↑ 1,25(OH) ₂ D ₃ ↑ OPG	Tibia: ↓ BMD (12%) Femur: ↓ BMD (5%) ↑ Trabecular BMD (23%)	Tibia: ↑ BV/TV ↑ Tb.Th ↓ OS/BS ↓ Ob.S/BS ↓ Oc.N/B.Pm ↓ Oc.S/BS ↓ ES/BS ↓ BFR/BS ↓ MAR ↓ C.Th.	Tibia: Elongated metaphyseal trabeculae Lamellar architecter	N.D.
Yamashita et al. 1998 [33]	Strain: <i>kl/kl</i> Age: 4–6 wk	N.D.	Tibia/LV6: ↓ BMD in the mid-region ↑ BMD in the growth plate	Tibia: ↑ BV/TV ↑ Tb.Th ↓ OV/TV ↑ O.Th. ↓ Oc.N	Tibia: Elongated trabeculae	N.D.
Yamashita et al 2000 [32]	Strain: <i>kl/kl</i> Age: 4–6 wk	N.D.	Tibia/LV6: ↓ BMD in the mid-region ↑ BMD on ends	N.D.	N.D.	Femur: -C.Th. Tibia: ↑ BAa/TAr ↑ BV/TV ↑ Tb.Th ↑ Tb.N ↓ Tb.Sp. -C.Th
Liu et al 2007 [52]	Strain: <i>kl/kl</i> Age: 3 mo	N.D.	N.D.	N.D.	N.D.	Tibia: ↑ BV/TV
Yuan et al 2012 [25]	Strain: <i>Klotho</i> ^{+/−} Age: 6 wk	↑ Ca ↑ Pi ↑ 1,25(OH) ₂ D ₃ ↑ FGF23 ↑ CTX ↑ PINP ↑ Opn	Tibia: ↓ radio density ↓ length	Femur: ↑ BV/TV ↑ Tb.Th -Tb.N -Tb.Sp ↑ OV/TV ↑ OS/BS ↑ O.Tb -Oc.N/B.Pm -Oc.S/BS ↓ BFR/BS ↓ MAR ↓ MS/BS	Femur: Unmineralized osteoid in secondary spongiosa	Femur: -BV/TV ↓ C.Th.

	Strain & Age	Serum	X-ray	Histomorphometry	Histology	µCT
Sasaki et al 2013 [39]	Strain: k/k/ Age: 7 wk	N.D.	N.D.	N.D.	Femur: - Defective mineralization with broad un- mineralized areas within the matrix - mineralized osteocytes with excessive Dmp1 expression in lacunae - MGP seen around the cartilaginous cores of the metaphyseal trabeculae	N.D.

Abbreviations: 1,25(OH)2D3 - 1,25 dihydroxyvitamin D; Ca – calcium; BFR/BS – bone formation rate/bone surface; BMD – bone mineral density; BV/TV – bone volume/total volume; C.Th. – cortical thickness; FGF23 – fibroblast growth factor 23; Pi – phosphate; PTH- parathyroid hormone; MAR – mineral apposition rate; MS/BS – mineralized surface/bone surface; N.D. – not determined; Oc.N/B.Pm.- osteoclast number per bone parameter; Oc.S/BS – osteoclast surface per bone surface; OS/BS – osteoid surface/bone surface; O.Tb. – osteoid thickness; OV/TV – osteoid volume/total volume; Tb.Th. – trabecular thickness; Tb.N. - trabecular number; Tb.Sp – trabecular separation.

Table 2

Summary of serum biochemistry and histomorphometric analyses from Klotho-deficient mouse models.

Strain: Age:	kl/kl 6 & 7 weeks	Klotho ^{-/-} 6 weeks	Prx1-Cre; Kl ^{fl/fl} 6 & 16 weeks	Dmp1-Cre; Kl ^{fl/fl} 5 weeks
Serum				
Ca (mg/dL)	↑	↑	–	–
Pi (mg/dL)	↑	↑	–	–
1,25(OH) ₂ D ₃ (pmol/L)	↑	↑↑	–	–
PTH (pg/mL)	↓	↓	–	–
FGF23 (pg/mL)	↑↑	↑↑	–	–
Histomorphometry				
BV/TV. (%)	↑	↑	–	↑
Tb.Th. (μm)	↑	↑	–	–
Tb.N.		–	–	↑
Tb.Sp.		–	–	↓
BFR (μm ³ /μm ² /d)	↓	↓	–	↑
MAR (μm ³ /μm ² /d)	↓	↓	–	–
MS/BS (%)		↓	–	↑
OV/TV (%)	–	↑	–	–
OS/BS (%)	↓	↑	–	–
O.Th. (μm)	↑	↑	–	–
Ob.N. (no.)	↓	N.D.	–	–
Ob.S/BS (%)	↓	N.D.	–	–
Ot/N/B.Ar (no/mm ²)		N.D.	–	–
Oc.N/B.Pm	↓	–	–	–
Oc.S/BS	↓	–	–	–
ES/BS	↓		–	–
C.Th. (mm)	↓	↓	–	–

Abbreviations: 1,25(OH)₂D₃ - 1,25 dihydroxyvitamin D; Ca – calcium; BFR/BS – bone formation rate/bone surface; BMD – bone mineral density; BV/TV – bone volume/total volume; C.Th. – cortical thickness; FGF23 – fibroblast growth factor 23; Pi – phosphate; PTH-parathyroid hormone; MAR – mineral apposition rate; MS/BS – mineralized surface/bone surface; N.D. – not determined; Oc.N/B.Pm.-osteoclast number per bone parameter; Oc.S/BS – osteoclast surface per bone surface; OS/BS – osteoid surface/bone surface; O.Th. – osteoid thickness; OV/TV – osteoid volume/total volume; Tb.Th. – trabecular thickness; Tb.N. - trabecular number; Tb.Sp – trabecular separation.