© 2009 International Society of Nephrology

Reversal of mineral ion homeostasis and soft-tissue calcification of klotho knockout mice by deletion of vitamin D 1α-hydroxylase

Mutsuko Ohnishi[1], Teruyo Nakatani[1], Beate Lanske[2] and M. Shawkat Razzaque[1]

[1]*Department of Oral Medicine, Infection and Immunity, Harvard School of Dental Medicine, Boston, Massachusetts, USA and* [2]*Department of Developmental Biology, Harvard School of Dental Medicine, Boston, Massachusetts, USA*

Changes in the expression of klotho, a β-glucuronidase, contribute to the development of features that resemble those of premature aging, as well as chronic renal failure. Klotho knockout mice have increased expression of the sodium/phosphate cotransporter (NaPi2a) and 1α-hydroxylase in their kidneys, along with increased serum levels of phosphate and 1,25-dihydroxyvitamin D. These changes are associated with widespread soft-tissue calcifications, generalized tissue atrophy, and a shorter lifespan in the knockout mice. To determine the role of the increased vitamin D activities in klotho knockout animals, we generated klotho and 1α-hydroxylase double-knockout mice. These double mutants regained body weight and developed hypophosphatemia with a complete elimination of the soft-tissue and vascular calcifications that were routinely found in klotho knockout mice. The markedly increased serum fibroblast growth factor 23 and the abnormally low serum parathyroid hormone levels, typical of klotho knockout mice, were significantly reversed in the double-knockout animals. These *in vivo* studies suggest that vitamin D has a pathologic role in regulating abnormal mineral ion metabolism and soft-tissue anomalies of klotho-deficient mice.

Kidney International advance online publication, 18 February 2009; doi:10.1038/ki.2009.24

KEYWORDS: FGF23; phosphate; PTH

Correspondence: *M. Shawkat Razzaque, Department of Oral Medicine, Infection and Immunity, Harvard School of Dental Medicine, Room 304, 190 Longwood Avenue, Boston, Massachusetts 02115, USA.*
E-mail: mrazzaque@hms.harvard.edu or razzaquems@yahoo.com

Received 7 October 2008; revised 9 December 2008; accepted 16 December 2008

Vitamin D regulates calcium and phosphate homeostasis, and influences skeletogenesis. 1,25-Dihydroxyvitamin D ($1,25(OH)_2D_3$), the active metabolite of vitamin D, is mostly formed in the kidney by hydroxylation through the enzyme 1α-hydroxylase (1α(OH)ase).[1] Therefore, the effects of $1,25(OH)_2D_3$ can be modified by altering renal 1α(OH)ase enzyme activity. Genetic inactivation of *klotho* from mice resulted in increased serum levels of $1,25(OH)_2D_3$.[2,3] Increased renal expression of the *1α(OH)ase* gene in *klotho*$^{-/-}$ mice was concomitant with elevated serum levels of $1,25(OH)_2D_3$. Such elevated serum levels of $1,25(OH)_2D_3$ were associated with abnormal soft-tissue and vascular calcifications in *klotho*$^{-/-}$ mice.[4,5]

The klotho protein contains a putative signal sequence at its N-terminus and a single transmembrane domain near its C-terminus, which is believed to anchor the protein to the membrane.[6] The *klotho* gene is predominantly expressed in the kidneys, parathyroid glands, and brain.[7] Such restricted expression of klotho is thought to confer the tissue specificity of fibroblast growth factor 23 (FGF23) function. Recently, klotho has been shown to affect the Na+-, K+-ATPase activity by increasing the Na+ gradient, and driving the transepithelial calcium transport in the choroid plexus and the kidney.[6,8] Klotho appears to be actively involved in mineral ion metabolism; however, the molecular regulation of klotho is not yet clearly understood, and studies have shown that vitamin D is a potent inducer of klotho expression.[4]

To determine whether increased vitamin D activities in *klotho*$^{-/-}$ mice are producing abnormal physical, molecular, and/or biochemical phenotypes of these mice, we generated and characterized *klotho*$^{-/-}$*/1α(OH)ase*$^{-/-}$ double-mutant mice, and compared their phenotypes with *klotho*$^{-/-}$ single-mutant mice.

RESULTS AND DISCUSSION

Klotho$^{-/-}$ mice developed normally until 2 weeks of age, and were grossly indistinguishable from their wild-type littermates. However, visible growth retardation was apparent from 3 weeks onwards in *klotho*$^{-/-}$ mice and was associated with sluggish movements. *Klotho*$^{-/-}$ mice remained smaller

during the whole lifespan, developed marked kyphosis, and usually died by 15 weeks of age. *Klotho*$^{-/-}$ mice also showed typical features of emphysema in the lungs, with severe atrophy of the thymus and spleen (Table 1). The serum calcium and phosphate levels were significantly higher in *klotho*$^{-/-}$ mice as compared with the wild-type controls. Furthermore, the renal expression of the *1α(OH)ase* gene was significantly elevated in *klotho*$^{-/-}$ mice, as determined by quantitative real-time PCR (Figure 1). An increased renal expression of *1α(OH)ase* in *klotho*$^{-/-}$ mice was associated with increased serum levels of 1,25$(OH)_2D_3$ in these mice (Figure 1). These increased serum 1,25$(OH)_2D_3$ levels were associated with widespread vascular and soft-tissue calcifications in *klotho*$^{-/-}$ mice. To determine whether increased levels of 1,25$(OH)_2D_3$ in *klotho*$^{-/-}$ mice mediate abnormal physical, biochemical, and morphological phenotypes of *klotho*$^{-/-}$ mice, we generated *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ double-knockout mice by cross-breeding heterozygous *klotho* mutant mice with heterozygous *1α(OH)ase* mutant mice.

In contrast to *klotho*$^{-/-}$ mice, *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ double-knockout mice were larger in size, and regained body weight (Figure 2). At 6 weeks of age, the average body weight of wild-type mice (24.8 ± 1.3 g) was significantly ($P<0.001$) higher than *klotho*$^{-/-}$ mice (13.4 ± 0.4 g) of similar age; compared with the *klotho*$^{-/-}$ mice, the *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ double-knockout mice significantly ($P<0.001$) regained body weight (17.7 ± 0.6 g) by 6 weeks of age (Figure 2). The average body weight of *1α(OH)ase*$^{-/-}$ knockout mice at 6 weeks of age was 19.8 ± 0.4 g. At 9 weeks of age, the body weight of wild-type mice had increased to 28 ± 1.3 g, whereas that of the *klotho*$^{-/-}$ mice was 12.6 ± 0.4 g and that of the *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ double-knockout mice was 20 ± 0.5 g (Figure 2). The body weight patterns of male and female mice of all four genotypes were very similar (Figure S1).

Biochemical analysis showed that increased serum calcium and phosphate levels were reversed, and were relatively low in *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ mice, when compared with *klotho*$^{-/-}$ mice (Figure 3). Extensive vascular and soft-tissue calcifications found in the *klotho*$^{-/-}$ mice were completely eliminated in all the examined tissues, including in the kidney, of *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ mice (Figure 4).

As mentioned above, severe hyperphosphatemia in *klotho*$^{-/-}$ mice was changed to hypophosphatemia in *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ mice (Figure 3). To determine the role of sodium/phosphate cotransporter (NaPi2a) in producing hyperphosphatemia in *klotho*$^{-/-}$ mice, we examined its expression pattern in kidney sections prepared from various genotypes using a polyclonal NaPi2a antibody. We found increased NaPi2a protein expression in the luminal side of

Table 1 | Phenotypes of various mutant mice compared with wild-type mice

	Wild type	Klotho$^{-/-}$	Klotho$^{-/-}$/1α(OH)ase$^{-/-}$	1α(OH)ase$^{-/-}$
Gross appearance				
Body weight	Normal	Reduced (M)	Reduced (S)	Reduced (S)
Growth	Normal	Retarded (M)	Retarded (S)	Retarded (S)
Kyphosis	Absent	Present	Absent	Absent
Generalized atrophy				
Thymus atrophy	Absent	Present	Absent	Absent
Spleen atrophy	Absent	Present	Absent	Absent
Muscle atrophy	Absent	Present	Absent	Absent
Skin atrophy	Absent	Present	Absent	Absent
Intestinal atrophy	Absent	Present	Absent	Absent
Morphological changes				
Atherosclerosis/arteriosclerosis	Absent	Present	Absent	Absent
Ectopic calcifications	Absent	Present	Absent	Absent
Emphysema	Absent	Present	Absent	Absent
Molecular changes				
Renal 1α(OH)ase expression	Normal	Increased	Absent	Absent
Renal NaPi2a expression	Normal	Increased	Decreased	Decreased
Biochemical changes				
Serum 1,25$(OH)_2D_3$	Normal	High	Not done	Not done
Serum phosphate	Normal	High	Low	Low
Serum calcium	Normal	High	Low	Low
Serum PTH	Normal	Low	High	High
Serum FGF23	Normal	High	Low	Low
Overall affect				
Physical activity	Normal	Sluggish	Normal	Normal
Infertility	Absent	Present	Absent	Absent
Lifespan	Normal	Short	Normal	Normal

FGF23, fibroblast growth factor 23; M, markedly; NaPi2a, sodium/phosphate cotransporter; PTH, parathyroid hormone; S, slightly.

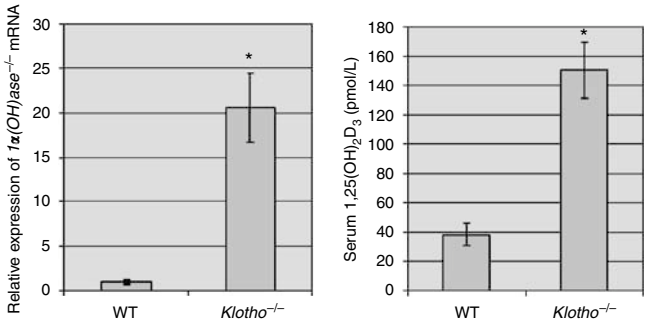

Figure 1 | Analysis of vitamin D components. Renal expression of 1α-hydroxylase enzyme (1α(OH)ase) and serum levels of 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) in wild-type (WT) control and *klotho*$^{-/-}$ mice. Compared with the control mice ($n=6$), expression of 1α(OH)ase, an enzyme that converts inactive vitamin D components to the active $1,25(OH)_2D_3$, was found to be elevated in kidneys obtained from *klotho*$^{-/-}$ mice ($n=10$), as determined by real-time PCR (1 ± 0.27 wild-type vs 20.53 ± 3.9 *klotho*$^{-/-}$). Note that, compared with control mice ($n=8$; 38.3 ± 7.5 pmol/l), serum $1,25(OH)_2D_3$ levels were also significantly elevated in *klotho*$^{-/-}$ mice ($n=8$; 150.25 ± 19.4 pmol/l) ($^{*}P<0.001$ vs wild-type).

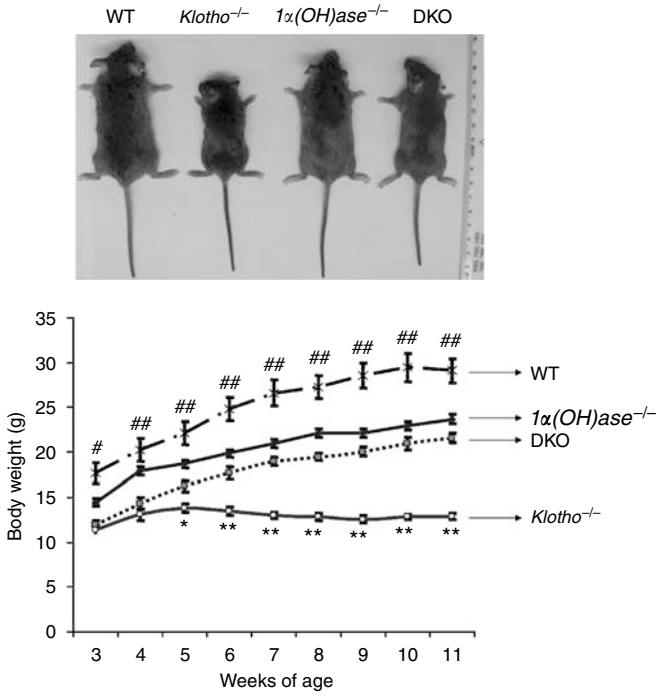

Figure 2 | Macroscopic phenotype of *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ double mutants. Gross phenotype of wild-type, *klotho*$^{-/-}$, *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ (DKO), and *1α(OH)ase*$^{-/-}$ mice at around 9 weeks of age (upper panel). Body weight curves (lower panel) for wild-type (WT; $n=12$), *klotho*$^{-/-}$ ($n=19$), DKO ($n=8$), and *1α(OH)ase*$^{-/-}$ ($n=30$) mice show that DKO mice are smaller than wild-type mice but larger than *klotho*$^{-/-}$ mice. This suggests that inactivation of vitamin D function in *klotho*$^{-/-}$ mice aided in body weight gains in DKO mice ($^{*}P<0.05$, *klotho*$^{-/-}$ mice vs DKO mice; $^{**}P<0.001$, *klotho*$^{-/-}$ mice vs DKO mice; $^{\#}P<0.01$, wild-type mice vs *klotho*$^{-/-}$ mice; $^{\#\#}P<0.001$, wild-type mice vs *klotho*$^{-/-}$ mice).

the proximal tubules of *klotho*$^{-/-}$ mice when compared with wild-type mice (Figure S2). In contrast, NaPi2a expression was markedly decreased in *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ mice, and was similar to its expression in *1α(OH)ase*$^{-/-}$ mice (Figure S2), suggesting that increased activity of NaPi2a may play a role in mediating hyperphosphatemia in *klotho*$^{-/-}$ mice.

Klotho$^{-/-}$ mice showed increased vitamin D activities and abnormal mineral ion homeostasis leading to widespread soft-tissue calcifications.[2,5,9] It has been shown that $1,25(OH)_2D_3$ can induce klotho, as shown by the upregulation of renal expression of *klotho* following $1,25(OH)_2D_3$ injection into wild-type mice.[4] The disappearance of soft-tissue calcifications in *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ mice suggested that at least some of the anomalies found in *klotho*$^{-/-}$ mice were mediated through increased vitamin D activities. In addition, loss of vitamin D activities from *klotho*$^{-/-}$ mice resulted in changes from severe hyperphosphatemia to hypophosphatemia (Figure 3), a pattern possibly attributable to reduced renal expression of NaPi2a protein in *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ mice. Compared with the *klotho*$^{-/-}$ mice, our immunostaining data showed a markedly reduced renal expression of NaPi2a protein in *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ mice (Figure S2).

Earlier studies have shown an inverse correlation between parathyroid hormone and renal expression of NaPi2a;[10,11] we believe that the decreased activity of NaPi2a in *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ double-knockout mice is partly regulated by the elevated serum parathyroid hormone levels in these mice (Figure 5). Such speculation is further substantiated by our preliminary observations that the serum phosphate levels in *klotho*$^{-/-}$ mice can be altered in *klotho*$^{-/-}$/*NaPi2a*$^{-/-}$ mice, and that these were similar to the levels found in *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ mice. Taken together, these results suggest that the hypophosphatemia in *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ is mainly caused by a reduction in renal phosphate reabsorption due to decreased activity of NaPi2a.

Moreover, the thymuses and spleens in *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ mice were larger than those of the *klotho*$^{-/-}$ mice (Table 1). The pathological changes in the lungs (Figure 6), intestines (Figure 7), and skin (Figure S3) of *klotho*$^{-/-}$ mice were dramatically reduced and reversed in *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ mice.

A recent study has found that both *Fgf23*$^{-/-}$ mice and *Fgf23*$^{-/-}$/*NaPi2a*$^{-/-}$ double-knockout mice have high serum

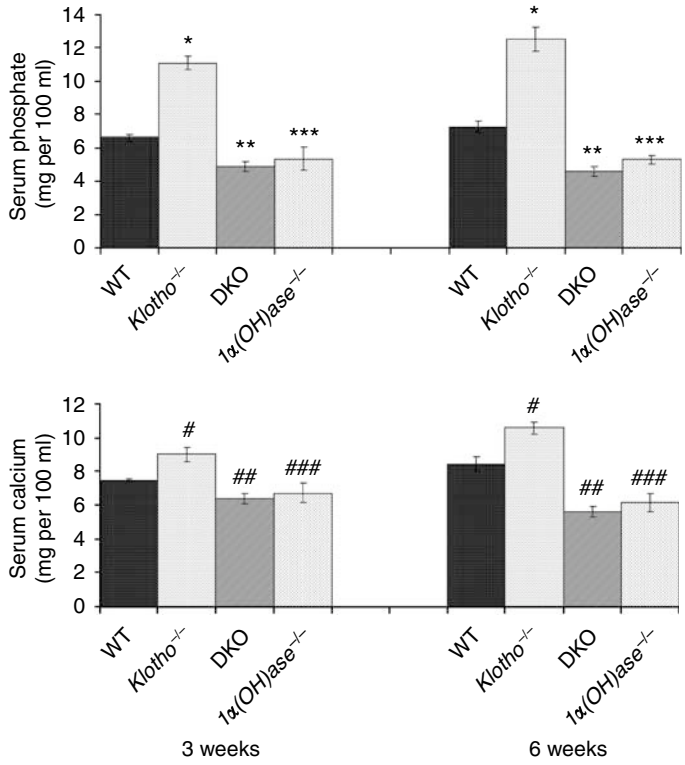

Figure 3 | Biochemical measurement of serum phosphate and calcium in various genotypes. Note that the serum phosphate (upper panel) and calcium (lower panel) levels are higher in *klotho*$^{-/-}$ mice compared with wild-type (WT) mice. Compared with WT mice ($n = 7$; 6.6 ± 0.18), the serum phosphate level was significantly higher in *klotho*$^{-/-}$ mice ($n = 5$; 11.1 ± 0.39) at 3 weeks of age. Similar hyperphosphatemia was also observed in *klotho*$^{-/-}$ mice ($n = 12$; 12.5 ± 0.73) at 6 weeks of age, compared with WT mice ($n = 11$; 7.2 ± 0.35) of same age. In contrast to *klotho*$^{-/-}$ mice, the serum phosphate level was significantly reduced in *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ double-knockout (DKO) mice, both at 3 weeks ($n = 6$; 4.9 ± 0.28) and 6 weeks ($n = 6$; 4.6 ± 0.27) of age. Similar reduction of serum phosphate level was also observed in *1α(OH)ase*$^{-/-}$ mice at 3 weeks ($n = 5$; 5.3 ± 0.67) and 6 weeks ($n = 8$; 5.3 ± 0.26) of age. As for serum calcium (lower panel), compared with the WT mice ($n = 7$; 7.4 ± 0.1), the serum calcium level was significantly higher in *klotho*$^{-/-}$ mice ($n = 6$; 9 ± 0.42) at 3 weeks of age. Similar higher serum level of calcium was also observed in *klotho*$^{-/-}$ mice ($n = 6$; 10.5 ± 0.36) at 6 weeks of age, compared with the WT mice ($n = 9$; 8.4 ± 0.42) of same age. In contrast to the *klotho*$^{-/-}$ mice, the serum calcium level was significantly reduced in DKO mice, both at 3 weeks ($n = 6$; 6.4 ± 0.32) and 6 weeks ($n = 6$; 5.6 ± 0.3) of age. Slightly reduced serum calcium level was also observed in *1α(OH)ase*$^{-/-}$ mice at 3 weeks ($n = 6$; 6.7 ± 0.56) and 6 weeks ($n = 6$; 6.1 ± 0.57) of age (*$P < 0.001$ vs WT; **$P < 0.001$ vs *klotho*$^{-/-}$; ***$P < 0.001$ vs *klotho*$^{-/-}$; $^{\#}P < 0.01$ vs WT; $^{\#\#}P < 0.001$ vs *klotho*$^{-/-}$; $^{\#\#\#}P < 0.001$ vs *klotho*$^{-/-}$).

$1,25(OH)_2D_3$ levels. This study also showed that they have discrepant serum phosphate levels (high serum phosphate in *Fgf23*$^{-/-}$ mice, but low serum phosphate in *Fgf23*$^{-/-}$/*NaPi2a*$^{-/-}$ mice). Despite significantly higher serum $1,25(OH)_2D_3$ levels that were observed in *Fgf23*$^{-/-}$ mice, the soft-tissue calcification was reduced or eliminated in *Fgf23*$^{-/-}$/*NaPi2a*$^{-/-}$ mice.[12] This result suggests that there may be a serum $1,25(OH)_2D_3$-independent calcification process driven by serum phosphate levels.[12] In our study, we have shown that ablation of vitamin D activity from *klotho*$^{-/-}$ mice can completely eliminate soft-tissue and vascular calcifications in *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ mice. As *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ mice also develop hypophosphatemia rather than the severe hyperphosphatemia, it is difficult to estimate whether the elimination of calcification (Figure S4) in these mice is due to inactivation of vitamin D activities or related to reduced serum phosphate levels.

Interestingly, compared with the markedly increased serum levels of Fgf23 in *klotho*$^{-/-}$ mice, the serum levels of Fgf23 were significantly reduced in *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ mice (Figure 5), suggesting a possible *in vivo* role of vitamin D in the induction of Fgf23. It is worth mentioning that elevated serum levels of $1,25(OH)_2D_3$ can induce an increase in serum levels of Fgf23.[13] Conversely, Fgf23 can reduce serum levels of $1,25(OH)_2D_3$ by suppressing the expression of a key converting enzyme, 1α(OH)ase.[11] Whether extremely high serum levels of Fgf23 may exert any toxic effects in *klotho*$^{-/-}$ mice is an important area of research that would indicate whether klotho-independent effects of Fgf23 exist.[14] It is necessary to mention that the injection of bioactive Fgf23 protein into either wild-type or *Fgf23*$^{-/-}$ mice resulted in significant reductions in serum levels of phosphate, however, no such changes were noted in either *klotho*$^{-/-}$ mice or *Fgf23*$^{-/-}$/*klotho*$^{-/-}$ double-knockout mice. This implies that klotho is essential for the *in vivo* systemic regulation of phosphate homeostasis.[3]

In conclusion, we have shown that vitamin D has a pathological role in altered phosphate homeostasis, soft-tissue anomalies, and ectopic calcifications in *klotho*$^{-/-}$ mice. Despite the crucial biological importance of maintaining

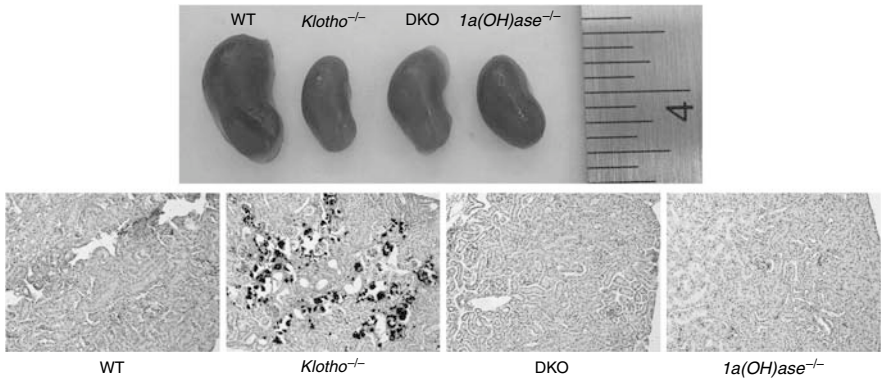

Figure 4 | von Kossa staining of kidney tissues. Renal sections prepared from wild-type (WT), *klotho*$^{-/-}$, *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ double-knockout (DKO), and *1α(OH)ase*$^{-/-}$ mice showing extensive calcifications in the kidneys of *klotho*$^{-/-}$ mice. Inactivation of vitamin D from *klotho*$^{-/-}$ mice completely eliminated calcification from DKO mice (magnification × 20). The macroscopic features of corresponding kidneys are shown in the upper panel.

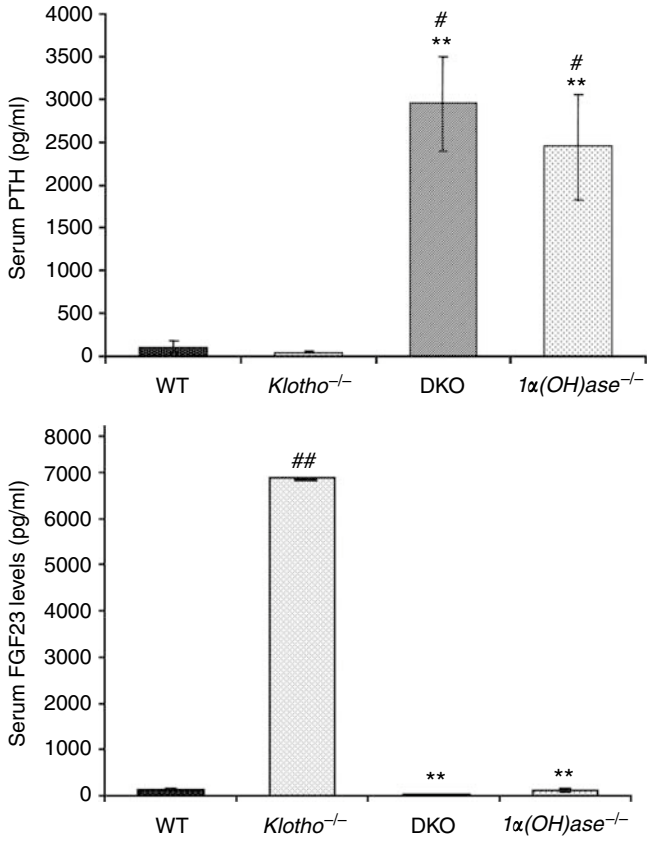

Figure 5 | Biochemical measurements of serum PTH and Fgf23 in various genotypes. Note that compared with wild-type mice ($n = 4$; 102.5 ± 69), serum parathyroid hormone (PTH) levels are markedly reduced in *klotho*$^{-/-}$ mice ($n = 8$; 46.8 ± 16) but are significantly higher in both *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ double-knockout (DKO) mice ($n = 4$; 2945 ± 560) and *1α(OH)ase*$^{-/-}$ mice ($n = 4$; 2443 ± 610). The average serum levels of fibroblast growth factor 23 (FGF23) are higher in *klotho*$^{-/-}$ mice ($n = 5$; 6857 pg/ml), as compared with the wild-type mice ($n = 5$; 134 pg/ml). In contrast to the *klotho*$^{-/-}$ mice, serum FGF23 levels are significantly reduced in DKO mice, ($n = 5$; 6.7 pg/ml). The average serum levels of FGF23 in *1α(OH)ase*$^{-/-}$ mice ($n = 5$; 120 pg/ml) are also lower than in the *klotho*$^{-/-}$ mice ($^{\#}P < 0.01$ vs wild-type; $^{\#\#}P < 0.001$ vs wild-type; $^{**}P < 0.001$ vs *klotho*$^{-/-}$).

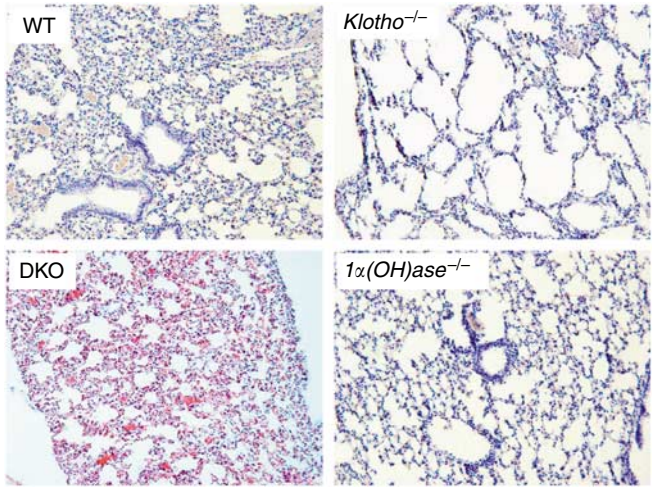

Figure 6 | Histological analysis of lung tissues. Hematoxylin and eosin-stained sections of the lung tissues of 6-week-old wild-type (WT), *klotho*$^{-/-}$, *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ double-knockout (DKO), and *1α(OH)ase*$^{-/-}$ mice. Note that, compared with wild-type mice, there is marked expansion of alveolar spaces (emphysema) in *klotho*$^{-/-}$ mice. Such pulmonary emphysematous changes are seen neither in the DKO mice nor in the *1α(OH)ase*$^{-/-}$ mice (lung, magnification × 10).

mineral ion homeostasis, the precise molecular mechanisms underlying this process are not yet fully understood. Further studies on *in vivo* interactions of vitamin D, parathyroid hormone, FGF23, and klotho will enhance our understanding of the physiological regulation of mineral ion metabolism. Such understanding will help us to fine-tune the existing therapeutic options by manipulating the effects of klotho or its interacting molecules to treat patients suffering from the complications of abnormal mineral ion metabolism.[15,16]

MATERIALS AND METHODS

Generation of double mutant mice

We cross-bred heterozygous *klotho* and *1α(OH)ase* heterozygous mice to obtain compound heterozygous animals,[3,17] which were

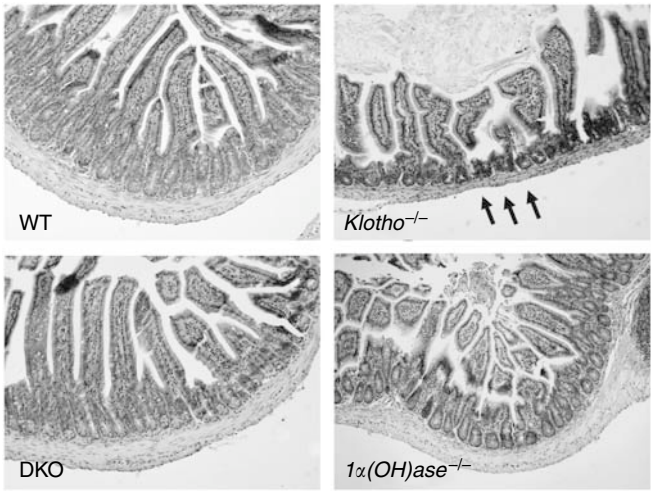

Figure 7 | Histological analysis of intestine. Compared with wild-type (WT) mice, there is marked atrophy of the intestinal wall (*arrows*) in *klotho*$^{-/-}$ mice. Such atrophic changes of the intestine are seen neither in the *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ double-knockout (DKO) mice nor in the *1α(OH)ase*$^{-/-}$ single-knockout mice (intestine, magnification $\times 10$).

then interbred to generate the desired double homozygous mutants (*klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$). Routine PCR using genomic DNA extracted from tail clips, was performed for genotyping of the various groups of mice.[18,19] Mice were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and were employed using protocols approved by the institution's subcommittee on animal care.

Gross phenotype

The total body weight of all mice was taken every 3–5 days starting at 3 weeks of age until death. The survival rates of control, *klotho*$^{-/-}$, and double-mutant *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ mice were recorded until death.

Biochemical measurements

Blood was obtained by cheek pouch bleeding at different time points from wild-type, *klotho*$^{-/-}$, *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$, and *1α(OH)ase*$^{-/-}$ mice. Serum was isolated by centrifugation at 3000 *g* for 10 min and was stored at −80 °C. Serum phosphorus and calcium were determined by colorimetric measurements using the Stanbio Phosphorus Liqui-UV Test and Calcium (Arsenazo) LiquiColor Test, respectively. Serum levels of parathyroid hormone were measured in serum obtained from wild-type, *klotho*$^{-/-}$, *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$, and *1α(OH)ase*$^{-/-}$ mice using a commercial kit (Immutopics Inc., San Clemente, CA, USA). The level of $1,25(OH)_2D_3$ was measured in serum obtained from different genotypes using a kit purchased from Immunodiagnostic Systems Ltd, Fountain Hills, AZ, USA. The serum level of Fgf23 was measured by ELISA (Kinos Inc., Tokyo, Japan), as described earlier.[20]

Histological analyses

Histological sections were prepared from various soft tissues that were routinely fixed in either 10% buffered formalin or Carnoy's solution. The sections were stained with hematoxylin and eosin, periodic acid-Schiff, periodic acid-Schiff methenamine silver, Masson's trichome, and von Kossa.[21,22] Histological changes in various groups were documented under light microscopy.

Calcification analyses

To determine the effects of vitamin D inactivation on soft-tissue and vascular calcification in *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ mice, sections were prepared from lung, kidney, and gastrointestinal tract, and were stained with von Kossa to visualize the mineralized tissue by light microscopy. The von-Kossa-stained sections of *klotho*$^{-/-}$/*1α(OH)ase*$^{-/-}$ mice were compared with similarly stained sections from wild-type, *klotho*$^{-/-}$, and *1α(OH)ase*$^{-/-}$ mice. For quantification of calcification, the relative area of von Kossa staining was determined by calculating the ratio of positively stained pixels and unstained area per high-power microscopic field ($\times 20$) on histological images taken from von-Kossa-stained kidney slides. The von Kossa staining was quantified using an image analyzer (Photoshop software; Adobe Systems, San Jose, CA, USA).

Expression of 1α(OH)ase

Total RNA isolated from the kidneys of each group of mice was used to detect relative expression of *1α(OH)ase* mRNA by real-time PCR, as described earlier.[23,24] Real-time PCR was performed in duplicate, and all reactions were controlled by standards. The sequences of the primers used to detect *1α(OH)ase* were forward 5′-TCA GAT GTT TGC CTT TGC CC-3′ and reverse 5′-TGG TTC CTC ATC GCA GCT TC-3′.

Expression of NaPi2a

Immunostaining was performed as described earlier.[20,25] In brief, OCT-embedded frozen sections were incubated with blocking solution for 30 min and then overnight with polyclonal anti-NaPi2a antibody (dilution, 1:100; Alpha Diagnostic, San Antonio, TX, USA) at 4 °C. The slides were washed with phosphate buffered saline and incubated with fluorescein isothiocyanate-leveled anti-rabbit secondary antibody (dilution, 1:100) for 30 min. After phosphate buffered saline wash, coverslips were placed on slides using 4,6-diamidino-2-phenylindole-containing mounting media. The expression of NaPi2a was visualized under UV light, using immunofluorescence microscopy. Rabbit serum and phosphate buffered saline, instead of primary antibody, were used as negative controls.

Statistics

Statistically significant differences between groups were evaluated by the Student's *t*-test for a comparison between two groups or by one-way analysis of variance followed by Tukey's test for multiple comparisons. All values were expressed as mean ± s.e. A *P*-value of less than 0.05 was considered to be statistically significant. All analyses were performed using Microsoft Excel.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

Part of the research work is supported by a grant (R01-DK077276 to MSR) provided by the NIH. The authors thank Dr Rene St. Arnoud (Shriners Hospital for Children, Montreal, Quebec, Canada) for kindly providing *1a(OH)ase* knockout mice.

SUPPLEMENTARY MATERIAL

Figure S1. Body weight patterns of male and female mice.
Figure S2. Expression of NaPi2a.
Figure S3. Histological analysis of skin tissues.

Figure S4. Quantification of calcification.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

REFERENCES

1. Lanske B, Razzaque MS. Vitamin D and aging: old concepts and new insights. *J Nutr Biochem* 2007; **18**: 771–777.
2. Kuro-o M, Matsumura Y, Aizawa H *et al.* Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature* 1997; **390**: 45–51.
3. Nakatani T, Bara S, Ohnishi M *et al. In vivo* genetic evidence for klotho-dependent, fibroblast growth factor (FGF23)-mediated regulation of systemic phosphate homeostasis. *FASEB J* 2009; **23**: 433–441.
4. Tsujikawa H, Kurotaki Y, Fujimori T *et al.* Klotho, a gene related to a syndrome resembling human premature aging, functions in a negative regulatory circuit of vitamin D endocrine system. *Mol Endocrinol* 2003; **17**: 2393–2403.
5. Memon F, El-Abbadi M, Nakatani T *et al.* Does Fgf23-klotho activity influence vascular and soft tissue calcification through regulating mineral ion metabolism? *Kidney Int* 2008; **74**: 566–570.
6. Nabeshima Y, Imura H. alpha-Klotho: a regulator that integrates calcium homeostasis. *Am J Nephrol* 2008; **28**: 455–464.
7. Li SA, Watanabe M, Yamada H *et al.* Immunohistochemical localization of Klotho protein in brain, kidney, and reproductive organs of mice. *Cell Struct Funct* 2004; **29**: 91–99.
8. Razzaque MS. Klotho and Na+,K+-ATPase activity: solving the calcium metabolism dilemma? *Nephrol Dial Transplant* 2008; **23**: 459–461.
9. Lanske B, Razzaque MS. Premature aging in klotho mutant mice: cause or consequence? *Ageing Res Rev* 2007; **6**: 73–79.
10. Segawa H, Yamanaka S, Ohno Y *et al.* Correlation between hyperphosphatemia and type II Na-Pi cotransporter activity in klotho mice. *Am J Physiol Renal Physiol* 2007; **292**: F769–F779.
11. Shimada T, Kakitani M, Yamazaki Y *et al.* Targeted ablation of Fgf23 demonstrates an essential physiological role of FGF23 in phosphate and vitamin D metabolism. *J Clin Invest* 2004; **113**: 561–568.
12. Sitara D, Kim S, Razzaque MS *et al.* Genetic evidence of serum phosphate-independent functions of FGF-23 on bone. *PLoS Genet* 2008; **4**: e1000154.
13. Saito H, Maeda A, Ohtomo S *et al.* Circulating FGF-23 is regulated by 1alpha,25-dihydroxyvitamin D3 and phosphorus *in vivo*. *J Biol Chem* 2005; **280**: 2543–2549.
14. Razzaque MS. Does FGF23 toxicity influence the outcome of chronic kidney disease? *Nephrol Dial Transplant* 2009; **24**: 4–7.
15. Razzaque MS. Can fibroblast growth factor 23 fine-tune therapies for diseases of abnormal mineral ion metabolism? *Nat Clin Pract Endocrinol Metab* 2007; **3**: 788–789.
16. Razzaque MS. FGF23-mediated regulation of systemic phosphate homeostasis: is klotho an essential player? *Am J Physiol Renal Physiol*. doi:10.1152/ajprenal.90538.2008.
17. Razzaque MS, Sitara D, Taguchi T *et al.* Premature ageing-like phenotype in fibroblast growth factor 23 null mice is a vitamin-D mediated process. *FASEB J* 2006; **20**: 720–722.
18. Sitara D, Razzaque MS, St-Arnaud R *et al.* Genetic ablation of vitamin D activation pathway reverses biochemical and skeletal anomalies in Fgf-23-null animals. *Am J Pathol* 2006; **169**: 2161–2170.
19. Dardenne O, Prud'homme J, Arabian A *et al.* Targeted inactivation of the 25-hydroxyvitamin D(3)-1(alpha)-hydroxylase gene (CYP27B1) creates an animal model of pseudovitamin D-deficiency rickets. *Endocrinology* 2001; **142**: 3135–3141.
20. DeLuca S, Sitara D, Kang K *et al.* Amelioration of the premature ageing-like features of Fgf-23 knockout mice by genetically restoring the systemic actions of FGF-23. *J Pathol* 2008; **216**: 345–355.
21. Zha Y, Taguchi T, Nazneen A *et al.* Genetic suppression of GH-IGF-1 activity, combined with lifelong caloric restriction, prevents age-related renal damage and prolongs the life span in rats. *Am J Nephrol* 2008; **28**: 755–764.
22. Liu D, Nazneen A, Taguchi T *et al.* Low-dose local kidney irradiation inhibits progression of experimental crescentic nephritis by promoting apoptosis. *Am J Nephrol* 2008; **28**: 555–568.
23. Razzaque MS, Foster CS, Ahmed AR. Role of collagen-binding heat shock protein 47 and transforming growth factor-beta1 in conjunctival scarring in ocular cicatricial pemphigoid. *Invest Ophthalmol Vis Sci* 2003; **44**: 1616–1621.
24. Medici D, Razzaque MS, Deluca S *et al.* FGF-23-Klotho signaling stimulates proliferation and prevents vitamin D-induced apoptosis. *J Cell Biol* 2008; **182**: 459–465.
25. Razzaque MS, Taguchi T. Collagen-binding heat shock protein (HSP) 47 expression in anti-thymocyte serum (ATS)-induced glomerulonephritis. *J Pathol* 1997; **183**: 24–29.