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

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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

Vitamin D regulates calcium and phosphate homeostasis, and influences skeletogenesis. 1,25-Dihydroxyvitamin D (1,25(OH)2D3), 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)2D3 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)2D3.2,3 Increased renal expression of the 1α(OH)ase gene in klotho–/– mice was concomitant with elevated serum levels of 1,25(OH)2D3. Such elevated serum levels of 1,25(OH)2D3 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)2D3 in these mice (Figure 1). These increased serum 1,25(OH)2D3 levels were associated with widespread vascular and soft-tissue calcifications in klotho−/− mice. To determine whether increased levels of 1,25(OH)2D3 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−/− mice with heterozygous 1α(OH)ase−/− mice, as determined by cross-breeding heterozygous klotho−/− mice with heterozygous 1α(OH)ase−/− 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 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

<table>
<thead>
<tr>
<th>Gross appearance</th>
<th>Wild type</th>
<th>Klotho−/−</th>
<th>Klotho−/−/1α(OH)ase−/−</th>
<th>1α(OH)ase−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>Normal</td>
<td>Reduced (M)</td>
<td>Reduced (S)</td>
<td>Reduced (S)</td>
</tr>
<tr>
<td>Growth</td>
<td>Normal</td>
<td>Retarded (M)</td>
<td>Retarded (S)</td>
<td>Retarded (S)</td>
</tr>
<tr>
<td>Kyphosis</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Generalized atrophy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thymus atrophy</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Spleen atrophy</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Muscle atrophy</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
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<tr>
<td>Skin atrophy</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
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<tr>
<td>Intestinal atrophy</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Atherosclerosis/arteriosclerosis</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
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<tr>
<td>Ectopic calcifications</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
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<tr>
<td>Emphysema</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
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<tr>
<td>Molecular changes</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Renal 1α(OH)ase expression</td>
<td>Normal</td>
<td>Increased</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Renal NaPi2a expression</td>
<td>Normal</td>
<td>Increased</td>
<td>Decreased</td>
<td>Decreased</td>
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<tr>
<td>Biochemical changes</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Serum 1,25(OH)2D3</td>
<td>Normal</td>
<td>High</td>
<td>Not done</td>
<td>Not done</td>
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<tr>
<td>Serum phosphate</td>
<td>Normal</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
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<tr>
<td>Serum calcium</td>
<td>Normal</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
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<tr>
<td>Serum PTH</td>
<td>Normal</td>
<td>Low</td>
<td>High</td>
<td>High</td>
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<tr>
<td>Serum FGF23</td>
<td>Normal</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Overall affect</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Physical activity</td>
<td>Normal</td>
<td>Sluggish</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Infertility</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Lifespan</td>
<td>Normal</td>
<td>Short</td>
<td>Normal</td>
<td>Normal</td>
</tr>
</tbody>
</table>

FGF23, fibroblast growth factor 23; M, markedly; NaPi2a, sodium/phosphate cotransporter; PTH, parathyroid hormone; S, slightly.
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. It has been shown that 1,25(OH)2D3 can induce klotho, as shown by the upregulation of renal expression of klotho following 1,25(OH)2D3 injection into wild-type mice. 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; we believe that the decreased activity of NaPi2a in klotho−/−/1α(OH)ase−/− double-knockout mouse 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
1,25(OH)₂D₃ 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)₂D₃ levels that were observed in Fgf23⁻/⁻ mice, the soft-tissue calcification was reduced or eliminated in Fgf23⁻/⁻/NaPi2a⁻/⁻ mice. This result suggests that there may be a 1,25(OH)₂D₃-independent calcification process driven by serum phosphate levels. 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)₂D₃ can induce an increase in serum levels of Fgf23. Conversely, Fgf23 can reduce serum levels of 1,25(OH)₂D₃ by suppressing the expression of a key converting enzyme, 1α(OH)ase. 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. 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.

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 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; 9.4 ± 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 klotho⁻/⁻ 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⁻/⁻).
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...
then interbred to generate the desired double homozygous mutants (klotho<sup>−/−</sup>/1x(OH)ase<sup>−/−</sup>). Routine PCR using genomic DNA extracted from tail clips, was performed for genotyping of the various groups of mice. 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<sup>−/−</sup>, and double-mutant klotho<sup>−/−</sup>/1x(OH)ase<sup>−/−</sup> mice were recorded until death.

**Biochemical measurements**
Blood was obtained by cheek pouch bleeding at different time points from wild-type, klotho<sup>−/−</sup>, klotho<sup>−/−</sup>/1x(OH)ase<sup>−/−</sup>, and 1x(OH)ase<sup>−/−</sup> 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<sup>−/−</sup>, klotho<sup>−/−</sup>/1x(OH)ase<sup>−/−</sup>, and 1x(OH)ase<sup>−/−</sup> mice using a commercial kit (Immutopics Inc., San Clemente, CA, USA). The level of 1,25(OH)<sub>2</sub>D<sub>3</sub> was measured in serum obtained from different genotypes using a kit purchased from Immunodiagnostics Systems Ltd, Fountain Hills, AZ, USA. The serum level of Fgf23 was measured by ELISA (Kinos Inc., Tokyo, Japan), as described earlier.

**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. 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<sup>−/−</sup>/1x(OH)ase<sup>−/−</sup> 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<sup>−/−</sup>/1x(OH)ase<sup>−/−</sup> mice were compared with similarly stained sections from wild-type, klotho<sup>−/−</sup>, and 1x(OH)ase<sup>−/−</sup> 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 (×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 1x(OH)ase**
Total RNA isolated from the kidneys of each group of mice was used to detect relative expression of 1x(OH)ase mRNA by real-time PCR, as described earlier. Real-time PCR was performed in duplicate, and all reactions were controlled by standards. The sequences of the primers used to detect 1x(OH)ase were forward 5’-TCA GAT TGT TGC CTT TGC CC-3’ and reverse 5’-TGG TTC CTC ATC ATC GCA GCT TC-3’. (Photoshop software; Adobe Systems, San Jose, CA, USA).

**Expression of NaPi2a**
Immunostaining was performed as described earlier. 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-labeled 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 1α(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