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.

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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). 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. 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.

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. The klotho gene is predominantly expressed in the kidneys, parathyroid glands, and brain. 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. 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.

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
of similar age; compared with the klotho mutant mice with heterozygous 1\(^\text{a}\)klotho levels of 1,25(OH)\(_2\)D\(_3\) in Kidney International (2009).

Generalized atrophy

Gross appearance

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

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>Klotho(^{\text{--}})</th>
<th>Klotho(^{\text{--}})/1(\alpha)(OH)ase(^{\text{--}})</th>
<th>1(\alpha)(OH)ase(^{\text{--}})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gross appearance</strong></td>
<td></td>
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<tr>
<td>Body weight</td>
<td>Normal</td>
<td>Reduced (M)</td>
<td>Reduced (S)</td>
<td>Reduced (S)</td>
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<tr>
<td>Growth</td>
<td>Normal</td>
<td>Retarded (M)</td>
<td>Retarded (S)</td>
<td>Retarded (S)</td>
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<tr>
<td>Kyphosis</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
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<tr>
<td><strong>Generalized atrophy</strong></td>
<td></td>
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<tr>
<td>Thymus atrophy</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
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<tr>
<td>Spleen atrophy</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
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<td>Muscle atrophy</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
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<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>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
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<td><strong>Morphological changes</strong></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><strong>Molecular changes</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Renal 1(\alpha)(OH)ase expression</td>
<td>Normal</td>
<td>Increased</td>
<td>Absent</td>
<td>Absent</td>
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<tr>
<td>Renal NaPi2a expression</td>
<td>Normal</td>
<td>Increased</td>
<td>Decreased</td>
<td>Decreased</td>
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<td><strong>Biochemical changes</strong></td>
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<tr>
<td>Serum 1,25(OH)(_2)D(_3)</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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<td>Serum calcium</td>
<td>Normal</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
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<td>Serum PTH</td>
<td>Normal</td>
<td>Low</td>
<td>High</td>
<td>High</td>
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<td>Serum FGF23</td>
<td>Normal</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
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<td><strong>Overall effect</strong></td>
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<tr>
<td>Physical activity</td>
<td>Normal</td>
<td>Sluggish</td>
<td>Normal</td>
<td>Normal</td>
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<tr>
<td>Infertility</td>
<td>Absent</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
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<tr>
<td>Lifespan</td>
<td>Normal</td>
<td>Short</td>
<td>Normal</td>
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FGF23, fibroblast growth factor 23; M, markedly; NaPi2a, sodium/phosphate cotransporter; PTH, parathyroid hormone; S, slightly.
1,25-dihydroxyvitamin D (1,25(OH)_{2}D_{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)_{2}D_{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)_{2}D_{3} levels were also significantly elevated in klotho^{-/-} mice (n = 8; 150.25 ± 19.4 pmol/l) (*P < 0.001 vs wild-type).

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)_{2}D_{3} can induce klotho, as shown by the upregulation of renal expression of klotho following 1,25(OH)_{2}D_{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
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. 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 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; 6.3 ± 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−/−).

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; 6.3 ± 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)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.12 This result suggests that there may be a serum 1,25(OH)2D3-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)2D3 can induce an increase in serum levels of Fgf23.13 Conversely, Fgf23 can reduce serum levels of 1,25(OH)2D3 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
mineral ion homeostasis, the precise molecular mechanisms underlying this process are not yet fully understood. Further studies on \textit{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.\textsuperscript{15,16}

**MATERIALS AND METHODS**

\textbf{Generation of double mutant mice}

We cross-bred heterozygous \textit{klotho} and \textit{1x(OH)ase} heterozygous mice to obtain compound heterozygous animals,\textsuperscript{3,17} which were

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

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

\textbf{Figure 6} | \textit{Histological analysis of lung tissues.} Hematoxylin and eosin-stained sections of the lung tissues of 6-week-old wild-type (WT), \textit{klotho}\textsuperscript{−/−}, \textit{klotho}\textsuperscript{−/−}/\textit{1x(OH)ase}\textsuperscript{−/−} double-knockout (DKO), and \textit{1x(OH)ase}\textsuperscript{−/−} mice. Note that, compared with wild-type mice, there is marked expansion of alveolar spaces (emphysema) in \textit{klotho}\textsuperscript{−/−} mice. Such pulmonary emphysematous changes are seen neither in the DKO mice nor in the \textit{1x(OH)ase}\textsuperscript{−/−} mice (lung, magnification \times 10).
were measured in serum obtained from wild-type, klotho mice, respectively. Serum levels of parathyroid hormone were determined by colorimetric measurements using the Stanbio Phosphorus Liqui-UV Test and Calcium (Arsenazo) Test, respectively. Mice were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals, 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 ATC GCA GCT TC-3'.

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. 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 ATC GCA GCT TC-3'.

Expression of NaPi2a
Immuno-staining 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-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.

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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