The Kidney Is the Principal Organ Mediating Klotho Effects

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ABSTRACT

Klotho was discovered as an antiaging gene, and α-Klotho (Klotho) is expressed in multiple tissues with a broad set of biologic functions. Membrane-bound Klotho binds fibroblast growth factor 23 (FGF23), but a soluble form of Klotho is also produced by alternative splicing or cleavage of the extracellular domain of the membrane-bound protein. The relative organ-specific contributions to the levels and effects of circulating Klotho remain unknown. We explored these issues by generating a novel mouse strain with Klotho deleted throughout the nephron (Six2-KL−/−). Klotho shedding from Six2-KL−/− kidney explants was undetectable and the serum Klotho level was reduced by approximately 80% in Six2-KL−/− mice compared with wild-type littermates. Six2-KL−/− mice exhibited severe growth retardation, kyphosis, and premature death, closely resembling the phenotype of systemic Klotho knockout mice. Notable biochemical changes included hyperphosphatemia, hypercalcemia, hyperaldosteronism, and elevated levels of 1,25-dihydroxyvitamin D and Fgf23, consistent with disrupted renal Fgf23 signaling. Kidney histology demonstrated interstitial fibrosis and nephrocalcinosis in addition to absent dimorphic tubules. A direct comparative analysis between Six2-KL−/− and systemic Klotho knockout mice supports extensive, yet indistinguishable, extrarenal organ manifestations. Thus, our data reveal the kidney as the principal contributor of circulating Klotho and Klotho-induced antiaging traits.

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Type I membrane-bound α-Klotho (Klotho) was originally identified as an antiaging gene based on the senescent phenotype and reduced life span in mice carrying hypomorph Klotho alleles (kl/kl). kl/kl mice exhibit a large spectrum of organ abnormalities such as soft tissue calcifications, osteomalacia, hypogonadism, lung emphysema, and skin atrophy.1 Although Klotho is expressed in multiple tissues, at the highest level in the kidneys, parathyroid glands, and choroid plexus,2 its tissue-specific role outside the kidney remains elusive.

Membrane-bound Klotho mediates receptor binding and cell signaling by its ligand fibroblast growth factor-23 (FGF23).3 Fgf23 null mice (Fgf23−/−) develop a virtually identical phenotype to kl/kl mice,4 including a serum biochemical profile of elevated 1,25-dihydroxyvitamin D [1,25(OH)2D], hypercalcemia, and hyperphosphatemia consistent with disrupted renal Fgf23 signaling. Membrane-bound Klotho can be released by extracellular cleavage and shedding,5 resulting in its presence in blood, urine, and cerebrospinal fluid, a process at least partially mediated by ADAM10 and ADAM17 (a disintegrin and metalloproteinases 10 and 17, respectively).6 Soluble Klotho and its endocrine signaling are implicated in diverse biologic processes such as protection of endothelial function, inhibition of phosphate-driven vascular calcification, and suppression of fibrosis and inflammation. The underlying molecular mechanisms are not fully elucidated but involve modulation of Wnt, insulin/IGF1 and TGF-β signaling.7–9 In addition, soluble Klotho independently promotes renal phosphate excretion and calcium reabsorption by enzymatic modulation of the sodium-dependent phosphate cotransporter 2a (Npt2a) and transient receptor potential cation channel subfamily V member 5, respectively.10,11
Significant attention was drawn to Klotho biology over the past decade, yet identification of the tissue source of circulating Klotho and the organ-specific contribution to the aging-like phenotype in kl/kl mice remain uncertain. We addressed these issues by generating mice with Klotho deleted throughout the nephron (Six2-KL−/−) using Cre-LoxP recombination (Figure 1, A and B) as described elsewhere.12 In this model, the Six2 promoter targets expression of Cre recombinase to the cap mesenchyme and its derivatives.13 In addition, Six2 expression was reported in restricted parts of the hindbrain, esophagus, stomach, and tendons and ligaments of the limbs during embryonal development.14 Importantly, with the kidney exempt, Six2 expression does not overlap that reported for Klotho. Successful deletion of renal Klotho was confirmed with immunofluorescence and Western blotting (Figure 1, C and D), and renal transcript levels were reduced by approximately 80% compared with wild-type mice (Figure 1E).

Soluble Klotho is present in blood and can be generated by cell membrane shedding or by alternative splicing, the latter presumably less relevant in humans.5 Regulation of its shedding is a critical step for Klotho function because protection against cellular stressors such as inflammation and oxidative stress is mediated by its endocrine signaling.7–9 For obvious reasons, it is not feasible to accurately determine the tissue source of systemic Klotho in humans. Additional challenges in this regard are that Klotho-deficient conditions, such as CKD, are characterized by a parallel reduction of its tissue level in several Klotho-expressing organs15–17 and that serum Klotho is potentially modified by differential shedding, the uremic environment, and concurrent comorbidities and medications.

Herein, we characterized Klotho shedding ex vivo by using kidney explants and noted undetectable Klotho protein in conditioned media from Six2-KL−/− kidneys. By contrast, wild-type kidneys generated substantial amounts of soluble Klotho (Figure 1F). Absent formation of soluble Klotho from Six2-KL−/− kidneys was paralleled by a marked reduction (approximately 80%) in the serum Klotho level (Figure 1G). Given the preserved Klotho expression in other key Klotho-expressing tissues (Supplemental Figure 1), it can be concluded that the kidney is the principal source of circulating Klotho and that

**Figure 1.** Targeted deletion of Klotho in Six2-KL−/− mice. (A) LoxP sequences (red triangles) are introduced in the Klotho gene enabling targeted deletion of exon 2 and disruption of gene function. (B) Gross phenotype of 6-week-old wild-type and Six2-KL−/− mice. Six2-KL−/− mice develop detectable growth retardation and kyphosis at 3 weeks of age. Adult mice display physical inactivity, are infertile, and die prematurely starting at 6–8 weeks of age. (C) Immunofluorescence staining confirms successful deletion of Klotho (red) throughout the nephron in Six2-KL−/− mice. LTL (green) is used as a marker of proximal tubules and DAPI (blue) is used for nuclear staining. (D) Western blot on kidney extracts from Six2-KL−/− mice confirms successful deletion of renal Klotho protein. (E) Renal Klotho transcripts in Six2-KL−/− mice are reduced by approximately 80% compared with wild-type mice as measured with quantitative real-time PCR. (F) Renal Klotho protein shedding is examined ex vivo. Klotho protein is readily detected in wild-type kidney homogenates and in conditioned media in which kidney sections are submerged for 2 hours. By contrast, no tissue or shedded Klotho protein is found in kidney explants or media from Six2-KL−/− mice. (G) Soluble Klotho is analyzed using immunoprecipitation and Western blot and is reduced by approximately 80% in serum from Six2-KL−/− mice. Samples are normalized to IgG. **P<0.01; ***P<0.001. DAPI, 4′,6-diamidino-2-phenylindole; LTL, fluorescein labeled Lotus tetragonolobus lectin; WT, wild-type. Bar, 200 μm in C.
other Klotho-expressing organs are incapable of correcting its systemic deficiency. Notably, a short-term small study in healthy kidney donors demonstrated an approximately 30% reduction in serum Klotho 5 days after unilateral nephrectomy, suggesting that our data are transferable to humans.

Six2-KL−/− mice develop growth retardation and kyphosis at 3 weeks of age (Figure 1B and Supplemental Figure 2). Adult mice were infertile, exhibited reduced activity, and died prematurely starting at 6–8 weeks of age, reproducing the reported phenotype of kl/kl mice. Serum and urine biochemical parameters were examined at 6 weeks of age (Figure 2, A and B). Six2-KL−/− mice had remark-ably elevated Fgf23 and 1,25(OH)2D levels in addition to hyperphosphate-mia. Parathyroid hormone (PTH) was appropriately decreased in the face of hypercalcemia, whereas aldosterone levels were nearly 3-fold increased. The urine profile showed increased excretion of calcium, whereas phosphate excretion was unchanged. Thus, their biochemical abnormalities largely recapitulate those of kl/kl mice and indicate dismantled renal Fgf23 signaling as a result of absent Klotho in the renal tubules. By contrast, heterozygous mice (Six2-KL+/−) had preserved renal expression of Klotho and were phenotypically normal defined by gross appearance and serum biochemistry profile (data not shown).

Data on renal protein expression and relative expression of renal transcripts are shown in Figure 2, C and D. Immunohistochemical analysis revealed increased expression and relative abundance of Npt2a at the brush border membrane in Six2-KL−/− mice, whereas its transcript levels were decreased and dissociated from the protein levels. Transcript levels of the vitamin D activating enzyme Cyp27b1 were increased corroborating with the elevated serum 1,25(OH)2D. The vitamin D inactivating enzyme Cyp24a1 was also increased, presumably as a result of a stimulatory feedback signal from high 1,25(OH)2D, whereas the vitamin D receptor was decreased.

The renal cell proliferation rate (determined by the Ki67 index) was significantly increased in Six2-KL−/− mice compared with wild-type mice (1.47% versus 0.57%, P<0.001; Figure 2C). The pathophysiologic relevance of this finding warrants further exploration, especially in situations of disturbed tubular-repair mechanisms, but supports data pointing to Klotho as an antiproliferative tumor-suppressor agent.

We next determined the effect of renal Klotho ablation on kidney histology and
detected increased cellular density, loss of differentiation between proximal and distal convoluted tubules, as well as loss of cuboidal epithelium lining Bowman’s capsule in male Six2-KL−/− mice (consistent with a female phenotype; Figure 3A). This corroborates with the histopathologic evidence for hypoplastic Leydig cells and tubular atrophy in testis (Figure 3B). Additional histomorphologic changes included moderate interstitial fibrosis (Figure 3A) and widespread nephrocalcinosis (Figure 3C). To note, a mosaic deletion of Klotho in the distal tubules did not reproduce these histopathologic findings in a cell-specific manner (i.e., no fibrosis or calcification was found in Klotho-ablated cells),12 supporting the concept that the renoprotective effect of Klotho is conveyed by endocrine, rather than autocrine, mechanisms.

A central question is whether extra-renal Klotho, locally or systemically, has a protective role against aging-like traits. Six2-KL−/− mice represent a valid model to explore this because they have preserved Klotho expression in other Klotho-expressing organs, such as the parathyroid glands and choroid plexus (Supplemental Figure 1). Accordingly, we conducted an extensive histologic analysis performed by a mouse pathologist to assess potential extrarenal manifestations in Six2-KL−/− mice. Ectopic calcifications in soft tissues such as the kidneys, lungs, and arteries demonstrated by von Kossa staining (Figure 3C) were a prominent feature in Six2-KL−/− mice. Other notable histologic changes in Six2-KL−/− mice included pulmonary emphysema, depletion of periarteriolar lymphoid sheaths, increased red pulp in the spleen, and reduced subcutaneous fat and skin collagen thickness (Figure 3B). These findings are identical with the reported histologic changes in kl/kl mice. Bone histology demonstrated hypomineralization in Six2-KL−/− mice (Figure 3D), corroborating with peripheral quantitative computed tomography data, showing markedly reduced bone mineral density (Table 1). In summary, at the histologic level, we found diverse and severe extrarenal complications presumably elicited by systemic Klotho deficiency as well as abrogated Fgf23 signaling and abnormalities in mineral metabolism. Indeed, the severe phenotype in kl/kl mice is ameliorated by transgenic

Figure 3. Renal and extrarenal histology in Six2-KL−/− mice. (A) Renal morphology is normal in wild-type mice, whereas Six2-KL−/− mice have higher cell density and absence of dimorphic tubules (top). A specific feature in male Six2-KL−/− mice is the loss of cuboidal Bowman’s epithelium of glomeruli (arrowheads). Staining for collagen with Sirius red (bottom) shows a moderate fibrosis in Six2-KL−/− mice. (B) The lungs of Six2-KL−/− mice have moderate dilation of alveolar spaces and occasional rupture of alveolar septa, consistent with emphysema. Testis of Six2-KL−/− mice show marked atrophy, both of tubules and germinative epithelium, and interstitial Leydig cells. Few spermatids and no ripe sperm in Six2-KL−/− mice are observed. In the spleen of Six2-KL−/− mice, red pulp atrophy is accompanied by a mild increase of interstitial fibrosis and marked depletion of both erythroid and myeloid hematopoietic lineages. The skin of Six2-KL−/− mice has no subcutaneous fat with some keratin debris and dysplastic hair shafts. (C) Kidneys (top) from Six2-KL−/− mice display widespread tubular and vascular calcifications when stained according to von Kossa. Disseminated calcifications are also found in lungs (middle) and arteries (bottom) from Six2-KL−/− mice but not in wild-type mice. (D) Six2-KL−/− mice show severe hypomineralization and thickened osteoid seams in the femoral metaphysis (arrowheads) in von Kossa/McNeal-stained, 3-μm-thick, undecalcified sections. H&E, hematoxylin and eosin; WT, wild-type. Bar, 25 μm for testis and skin in B; 50 μm for lung and spleen in B.
Klotho overexpression and by dietary phosphate and vitamin D restrictions, respectively. A limitation of the Six2-KL/mice model is the inability to discriminate effects of membrane-bound versus soluble Klotho. Given the severe and early onset of phenotype in Six2-KL/−/− mice, this issue cannot be appropriately addressed by exogenous Klotho delivery.

The observation that Six2-KL/−/− mice appear phenotypically indistinguishable from kl/kl mice prompted us to perform a direct comparative analysis. To enable this, we generated systemic Klotho knockout mice (β-actin-KL/−/− mice) using mice expressing Cre recombinase under the human β-actin promoter, as previously described. These two strains demonstrated an identical gross phenotype and biochemical profile (Supplemental Figure 3, Supplemental Tables 1 and 2) and extensive histologic analysis did not provide evidence for any differences in renal or extrarenal abnormalities. The hyperaldosteronism in Six2-KL/−/− mice further strengthens the concept that endocrine manifestations in kl/kl mice are caused by the systemic environment, rather than local Klotho expression. Taken together, our data unequivocally point to the kidney as the prime mediator of Klotho function.

Our novel findings have several implications. CKD is a state of Klotho deficiency and we for the first time demonstrate that reduced Klotho in the kidney has a dramatic effect on its systemic availability and presumed endocrine signaling. Reduced Klotho abundance in the kidney may therefore directly contribute to complications such as renal fibrosis, progressive loss of kidney function, and cardiovascular dysfunction frequently observed in CKD. In support of this, systemic Klotho treatment in experimental CKD models improves uremic complications such as matrix deposition, renal fibrosis, osteogenic transformation of vascular smooth muscle cells, and vascular calcification. By combining this knowledge with our present findings, therapeutic Klotho delivery, or augmentation of its endocrine functions, may be a feasible option to mitigate organ damage and slow disease progression in patients with CKD.

CONCISE METHODS

Ethical Statement
All experiments were conducted in compliance with the guidelines of animal experiments at Karolinska Institutet and were approved by Stockholm South Animal Experimental Ethical Committee (permit number S 38-09, S 68-10). Mice were fed standard rodent chow (RM1; SDS Special Diet Services, Essex, UK) containing 0.73% calcium and 0.52% phosphorus. All animals had free access to food and drinking water and were housed in standard makrolon cages with wood chip bedding and a paper house for enrichment, at constant ambient temperature (21°C–22°C) and humidity (40%–50%).

Generation of Kidney-Specific Klotho Knockout Mice
Mice with tissue-specific Klotho deletion were generated using Cre-Lox recombination as previously described. Briefly, loxP sequences were introduced in the flanking regions of exon 2 of the Klotho gene, resulting in a nonfunctioning Klotho protein in tissues expressing Cre recombinase. FloxKlotho mice were crossed with mice expressing Cre recombinase under the Six2 promoter [Tg (Six2-EGFP/cre)1AmcJ]; The Jackson Laboratory] or under the human β-actin promoter [FVB/N-Tg(ACTB-cre)2MrtJ]; The Jackson Laboratory] to generate kidney-specific and systemic Klotho knockout mice, respectively. Floxed Klotho mice not expressing Cre served as wild-type controls. All strains were kept on a C57BL/6 background.

Genotyping
Total DNA was extracted from tail biopsies (ViaGen, Cedar Park, TX) and PCR amplification was carried out (Qiagen, Venlo, The Netherlands).

Serum Biochemistries
Serum calcium, phosphate, and creatinine were measured using quantitative colorimetric assay kits (BioAssay, BioChain Institute, Inc., Newark, CA). Serum PTH was measured using the mouse 1–84 PTH ELISA and FGF23 was measured using the mouse FGF23 (c-term) ELISA (Immutopics, San Clemente, CA). Serum 1,25(OH)2D was measured using the EIA assay (Immunodiagnostic Systems, Scottsdale, AZ). Serum aldosterone was measured using a ELISA (Enzo Life Sciences, Farmingdale, NY).

Transcript Analyses
Kidneys were homogenized (Qiagen) and total RNA was extracted (Omega Bio-Tek, Norcross, GA). DNA was removed (Omega Bio-Tek) and first-strand cDNA was synthesized (Bio-Rad, Hercules, CA). Quantitative real-time PCR analysis was performed using a SYBR Green–based assay (Bio-Rad). The relative gene expression was calculated with the 2−ΔΔCT method normalizing the gene of interest to β-actin in the same sample.

Western Blot Analyses
Kidneys were homogenized and extracts were centrifuged. Protein quantification was carried out before blotting (Thermo Fisher Scientific, Waltham, MA). Forty micrograms of protein was separated and electrotransferred

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Table 1. Bone peripheral quantitative computed tomography in Six2-KL/−/− mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-Type Mice (n=5)</th>
<th>Six2-KL/−/− Mice (n=8)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bone mineral density (mg/cm²)</td>
<td>370.3 (40.7)</td>
<td>272.0 (51.1)</td>
<td>0.001</td>
</tr>
<tr>
<td>Trabecular bone mineral density (mg/cm²)</td>
<td>150.6 (26.0)</td>
<td>101.6 (23.7)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Cortical bone mineral density (mg/cm²)</td>
<td>1017.8 (35.4)</td>
<td>853.6 (74.8)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cortical thickness (mm)</td>
<td>0.172 (0.021)</td>
<td>0.112 (0.020)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are presented as the mean (SD). Femurs from 6-week-old Six2-KL/−/− and wild-type controls were collected for analysis. Six2-KL/−/− mice had significantly reduced cortical and trabecular bone mineral density supporting hypomineralization.
(Invitrogen, Carlsbad, CA). After blocking, the membranes were sequentially incubated with primary and secondary antibodies. Primary antibodies were anti-Klotho (KM2076; TransGenic, Inc., Kobe, Japan) and anti-β-actin (Sigma-Aldrich, St. Louis, MO), and secondary antibodies were from LI-COR Bioscience (Lincoln, NE).

**Immunohistochemistry and Immunofluorescence**

Tissues were dissected, fixed in 4% paraformaldehyde overnight, and subsequently embedded in paraffin. Four-micrometer sections were deparaffinized, rehydrated, and incubated with low-pH antigen unmasking solution (Vector Laboratories, Burlingame, CA). Sections for immunohistochemistry were then immersed in 3% H2O2 in methanol, treated with 4% normal serum, and blocked with avidin and biotin (Vector Laboratories). All sections were incubated with primary antibodies at 4°C overnight. Immunohistochemistry slides were incubated with biotinylated secondary antibodies followed by Vector ABC Reagent and developed with 3,3′-diaminobenzidine substrate (Vector Laboratories). For immunofluorescence, Alexa Fluor conjugated secondary antibodies were used for visualization (Invitrogen) together with fluorescein labeled *Lotus tetragonolobus* lectin, a marker of proximal tubules (Vector Laboratories). For nuclear staining, 4′,6′-diamidino-2-phenylindole (Invitrogen) was used. The primary antibodies used were anti-Klotho (KM2076), rabbit monoclonal anti-Ki67 (SP6; Thermo Fisher Scientific), and rabbit polyclonal anti-Npt2a (NPT27; Alpha Diagnostic, Inc., San Antonio, TX). The proliferation index (%) was calculated as the number of Ki67-positive cells/total number of cells in at least four viewing fields (at ×10 magnification) from two sections of each assessed mouse.

**Histology**

Paraffin sections from wild-type and Six2-KL−/− mice were stained with hematoxylin and eosin and examined in a blinded fashion by a animal pathologist. Renal sections were stained with Picosiris red for fibrosis (stains collagen I and III fibers) and were examined by a blinded renal pathologist. Soft tissues were stained with von Kossa to detect calcification.

**Bone Phenotype**

Processing of undecalcified bone specimens and cancellous bone histology in the distal femoral metaphysis were performed as described elsewhere.24,25 The area within 0.25 mm from the growth plate was excluded from the measurements. Bone mineral density and other parameters of interest were analyzed by peripheral quantitative computerized tomography using a Scanco Medical μCT 35 system (Scanco USA, Inc., Wayne, PA).

**Ex Vivo Study of Secreted Klotho**

Mice were anesthetized and perfused with warm 199 medium (Gibco, Grand Island, NY). Kidneys were rapidly harvested, sliced in four equal pieces, and submerged in prewarmed 199 cell culture medium for 2 hours at 37°C with 5% CO2 and 95% O2, after which the kidneys and medium were harvested.

**Serum Klotho Measurements**

Soluble Klotho in serum was measured by a combination of immunoprecipitation and Western blotting as described elsewhere.16,23

**Statistical Analyses**

GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA) was used for statistical analysis. Gaussian distribution was tested using the D'Agostino and Pearson omnibus normality test. Variables fulfilling the criteria for normal distribution were tested with the two-tailed t test. Non-normally distributed variables were compared using the Mann-Whitney test. P values <0.05 were considered statistically significant.

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**DISCLOSURES**

T.E.L. is a Medical Director at Astellas Pharma.

**REFERENCES**


See related editorial, "In Search of the Fountain of Youth," on pages 2143–2145.

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