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Regulation of stanniocalcin 1 and 2 expression in the kidney by *klotho* gene☆

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Abstract

The *klotho* gene product and stanniocalcin (STC) 1 and 2 are recently identified molecules implicated in calcium and phosphorus homeostasis. In the present study, we investigated the regulation of STC1 and STC2 gene expression in the kidney by *klotho* gene expression. Mice deficient in klotho expression (klotho mice) have hypercalcemia and hyperphosphatemia, and increased renal gene expression of STC1 and STC2 compared with wild-type mice. Administration of vitamin D or $CaCl_2$ to wild-type mice causes upregulation of STC1 but STC2 gene expression is not altered significantly. On the other hand, treatment of *klotho* mice with low phosphorus diet results in partial decrease in STC2 gene expression with normalization of hyperphosphatemia. These findings indicate that *klotho* gene expression plays a crucial role in the regulation of renal stanniocalcin gene expression in vivo, at least partly, through the control of circulating calcium and phosphate concentrations.

Keywords: *Klotho*; *Klotho* mice; Stanniocalcin; Vitamin D; 25-Hydroxyvitamin D_3-1α-hydroxylase; Hypercalcemia; Hyperphosphatemia; Hypercalciuria; Hyperphosphaturia

Klotho mice, which have homologous disruption of *klotho* gene, exhibit a broad spectrum of systemic phenotypes including ectopic calcification, osteopenia, arteriosclerosis, pulmonary emphysema, atrophy of the skin and gonad, and leanness [1–3]. The gene is expressed mainly in the distal convoluted tubules in the mouse kidney [1] and encode soluble and single-pass membrane forms of proteins, which have structural similarities with β-glucosidase [4,5]. As family molecules, lactase-phlorizin hydrolase [6], cytosolic β-glucosidase [7,8], beta*klotho* [9], and *Klotho*-LPH related protein [10] have been cloned in mammals. A recent report indicated that some activity of the *klotho* gene product is mediated by cyclic AMP-protein kinase A signaling pathway [11] but still little is known how the *klotho* gene product exerts its pleiotropic actions.

Stanniocalcin (STC) was first identified in fish as a hormone with activities to inhibit calcium absorption in the gill and intestine and to stimulate phosphate reabsorption in the kidney [12–15]. STC1 has been isolated as the mammalian homologue of fish stanniocalcin and, in rats, STC1 inhibits calcium absorption and stimulates phosphate absorption in the intestine and also stimulates phosphate reabsorption in the kidney, suggesting that STC1 plays an important role in calcium and phosphorus homeostasis [16–20]. Recently another mammalian molecule homologous to STC1 was cloned and termed STC2 [21–23]. Unlike STC1, STC2 attenuates phosphate uptake in cultured renal tubular cells [23], suggesting that STC2 possesses an opposite function against STC1. Fish stanniocalcin is synthesized in endocrine glands called corpuscles of Stannius, which

☆ *Abbreviations:* STC, stanniocalcin; 1α-hydroxylase, 25-hydroxyvitamin D_3-1α-hydroxylase; PTH, parathyroid hormone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ESRD, end-stage renal diseases.

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are located adjacent to the kidney [15,24], and acts as a circulating factor [25,26], whereas mammalian STC1 is produced in various tissues including the collecting ducts in the kidney and appears to act as a local paracrine factor [27,28].

In the present study we investigated the gene expression of STC1 and STC2 in the mouse kidney and its relationship with *klotho* gene expression and blood calcium and phosphate concentrations. Since a recent study revealed that circulating vitamin D levels in *klotho* mice are highly upregulated [29], we also examined the role of vitamin D in the control of STC1 and STC2 gene expression. Here we show that STC1 is upregulated by circulating vitamin D and also directly by blood calcium concentrations, while STC2 is controlled by a different mechanism including blood phosphate concentrations and *klotho* gene expression.

Materials and methods

Animals. By mating heterozygous *klotho* deficient mice (*kl*/+) [1], wild-type (+/+), and *klotho* mice (*kl*/*kl*) were generated and the male littermates were analyzed, which were 5–6 weeks of age at the beginning of treatments described below.

Treatments of animals. Mice were treated and sacrificed following our institutional guidelines for animal care. To examine the effects of vitamin D, subcutaneous injection of 1,25-dihydroxyvitamin D_3 (1.5 μg/kg body weight, 0.15 μg/ml, Wako Pure Chemicals, Osaka, Japan) dissolved in 10% ethanol/90% propylene glycol (Nacalai Tesque, Kyoto, Japan) or vehicle was carried out at 18:00 h for 3 days, and mice were sacrificed at 9:00 h on the fourth day after overnight fasting for 10 h with free access to tap water, to minimize the influence of intestinal food in each animal. In another experiment, mice kept fasted since the previous night were orally given $CaCl_2$ (800 mg/kg, 90 mg/ml, Nacalai Tesque) dissolved in distilled water or vehicle by stomach tubes (1.7 mm × 90 mm, KN349, Natsume Seisakusho, Tokyo, Japan) twice at 10:00 and 14:00 h, and were killed at 16:00 h. To study the effects of diet phosphorus content, mice were given defined diet with normal phosphorus (containing 0.9% calcium and 0.8% phosphorus, CLEA Japan, Tokyo, Japan) or low phosphorus content (0.9% calcium and 0.02% phosphorus) for 7 days, and sacrificed on the eighth day after overnight fasting. Unless otherwise described, animals were given ad libitum standard chow (containing 1.0% calcium and 1.0% phosphorus, CE-2, CLEA Japan).

Sample collection and measurement of parathyroid hormone. Blood was collected by decapitation. Thereafter, the kidneys were resected, snap-frozen by liquid nitrogen, and stored at −84 °C until total RNA was extracted using TRIzol reagent (Life Technologies, Grand Island, NY, USA). Concentrations of intact parathyroid hormone (PTH) were measured by a sandwich ELISA kit using rat intact PTH as standards (Immunotopics, San Clemente, CA, USA). To collect urine, metabolic cages were used and urine pools from two wild-type mice or five *klotho* mice were analyzed, since *klotho* mice are smaller than wild-type mice and easily die if isolated. Urinary calcium and phosphate excretion was expressed in mg per mg creatinine (mg/mgCr), whose value is well proportional to that in mg/day.

Northern blot analysis. Northern blot analysis was performed as previously described [30]. To obtain cDNA probes for mouse STC1 (nucleotides 288–587) [18], STC2 (402–756) [21], and 25-hydroxyvitamin D_3-1α-hydroxylase (1α-hydroxylase, 964–1336) [31], reverse-transcription PCR was performed with mouse kidney total RNA. In each lane of nylon membranes (GeneScreenPlus, NEN Life Science Products, Boston, MA, USA), 40 μg of total RNA was loaded. Hybridization was performed by [^{32}P]dCTP-labeled cDNA probes for STC1, STC2, 1α-hydroxylase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Clontech, Palo Alto, CA, USA). The blots were exposed to BAS-III imaging plate (Fuji, Tokyo, Japan). In the mouse kidney, two transcripts were detected for both STC1 (4.0 and 2.0 kb) and STC2 (4.2 and 1.4 kb). Since the abundance of larger and smaller transcripts for both STC1 and STC2 appeared to change in parallel, we analyzed the larger transcripts. The mean mRNA level in the control kidney in each set of experiments was arbitrarily defined as 1.0 and the relative mRNA levels were normalized by the GAPDH mRNA levels.

Statistics. All values were expressed as means ± SE. Statistical significance of differences was assessed by Student's *t* test or two-way factorial analysis of variance. *P* values less than 0.05 were considered statistically significant.

Results

Blood and urinary data of klotho and wild-type mice

At 5–6 weeks of age *klotho* mice exhibited mild hypercalcemia (10.4 ± 0.2 vs. 9.5 ± 0.2 mg/dl, $P < 0.05$) and severe hyperphosphatemia (16.3 ± 0.3 vs. 11.0 ± 0.4 mg/dl, $P < 0.0001$) in the sera compared with wild-type mice as previously described (Fig. 1) [1,29]. Serum intact PTH levels in *klotho* mice were approximately 30% of the levels in wild-type mice ($P < 0.05$) [29]. Urinary excretion of calcium (2.2-fold, $P < 0.0001$)

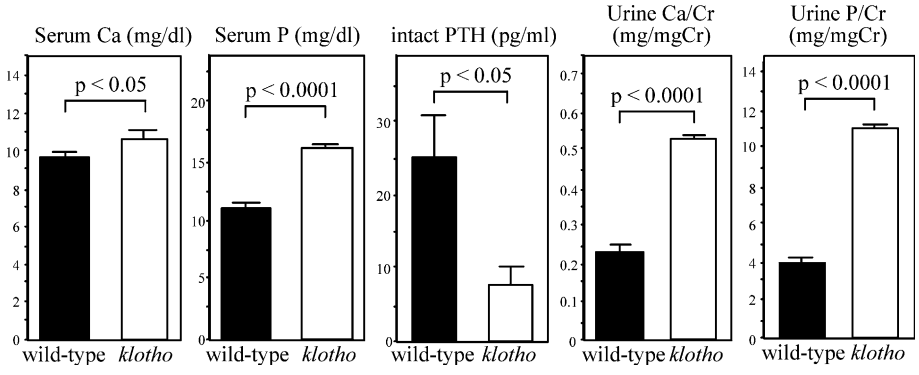

Fig. 1. Serum calcium, phosphate, intact PTH levels and urinary calcium and phosphate levels of *klotho* and wild-type mice ($n = 4$–6).

and phosphate (2.8-fold, $P < 0.0001$) was markedly increased in *klotho* mice.

Effects of vitamin D administration on blood calcium and phosphate concentrations and renal gene expression of STC1, STC2, and 25-hydroxyvitamin D_3-1α-hydroxylase

We examined gene expression levels of STC1 and STC2 in the kidney of *klotho* and wild-type mice, and found that *klotho* mice have 1.5-fold higher STC1 ($P < 0.05$) and fourfold higher STC2 gene expression levels ($P < 0.005$) as compared with wild-type mice (Fig. 2). Since *klotho* mice exhibited mild hypercalcemia and severe hyperphosphatemia, we investigated whether circulating vitamin D or calcium concentrations are involved in the regulation of renal STC1 and STC2 gene expression. Subcutaneous vitamin D administration in wild-type mice caused severe hypercalcemia ($P < 0.0001$) but blood phosphate levels were not changed significantly (Fig. 3A). In wild-type mice given vitamin D, the gene expression of 25-hydroxyvitamin D_3-1α-hydroxylase (the principal enzyme for vitamin D activation) was downregulated [31] and STC1 gene expression was markedly upregulated in the kidney (Fig. 3B). The treatment slightly increased renal STC2 gene expression in wild-type mice, but the level was much lower than that of *klotho* mice. In *klotho* mice, vitamin D administration partially reduced 1α-hydroxylase gene expression, but neither blood calcium and phosphate levels nor STC1 and STC2 gene expression levels in the kidney were altered, indicating that *klotho* mice is resistant to vitamin D treatment [29].

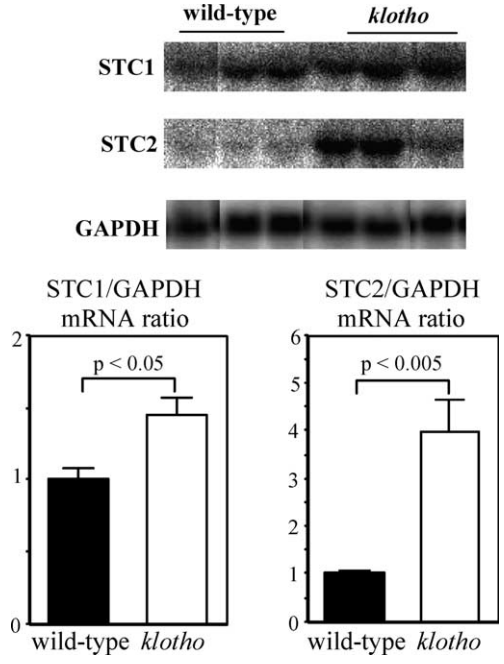

Fig. 2. The gene expression levels of STC1 and STC2 in *klotho* and wild-type kidney ($n = 5$).

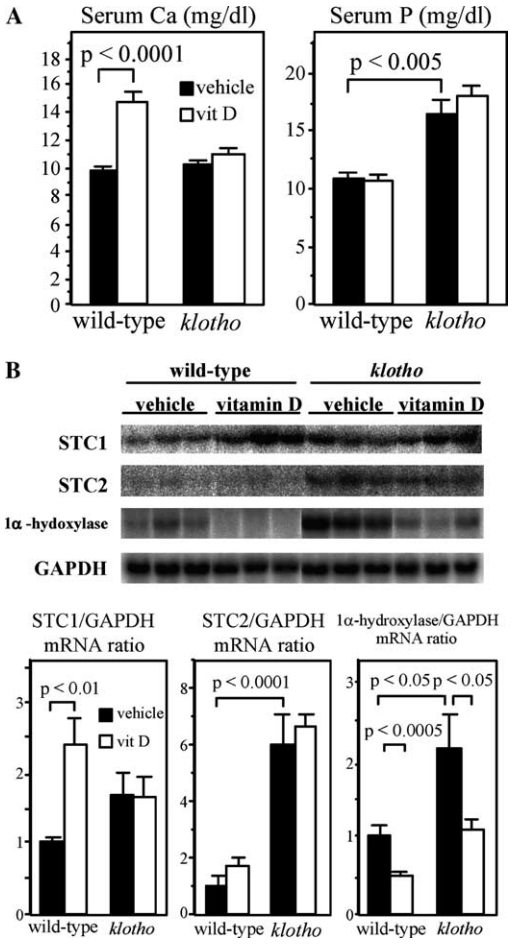

Fig. 3. Effects of vitamin D administration on (A) blood calcium and phosphate concentrations and (B) renal gene expression of STC1, STC2, and 25-hydroxyvitamin D_3-1α-hydroxylase in *klotho* and wild-type mice. 1,25-Dihydroxyvitamin D_3 (1.5 μg/kg) or vehicle was subcutaneously given once a day for 3 days ($n = 5$).

Effects of $CaCl_2$ administration on blood calcium and phosphate concentrations and renal gene expression of STC1, STC2, and 25-hydroxyvitamin D_3-1α-hydroxylase

Oral $CaCl_2$ administration in wild-type mice caused severe hypercalcemia ($P < 0.005$) but blood phosphate levels were not changed significantly (Fig. 4A). The treatment has been shown to decrease renal 1α-hydroxylase activity and circulating concentrations of 1,25-dihydroxyvitamin D_3, the active form of vitamin D [32,33]. Consistently, we found that $CaCl_2$ treatment suppressed 1α-hydroxylase gene expression in wild-type kidney (Fig. 4B). After $CaCl_2$ administration, STC1 gene expression was again significantly upregulated in the wild-type kidney ($P < 0.05$), but STC2 gene expression was not changed. The findings from vitamin D and $CaCl_2$ administration suggest that *klotho* mice have high STC1 gene expression in the kidney because of hypercalcemia, rather than because of direct action of high circulating vitamin D levels [29].

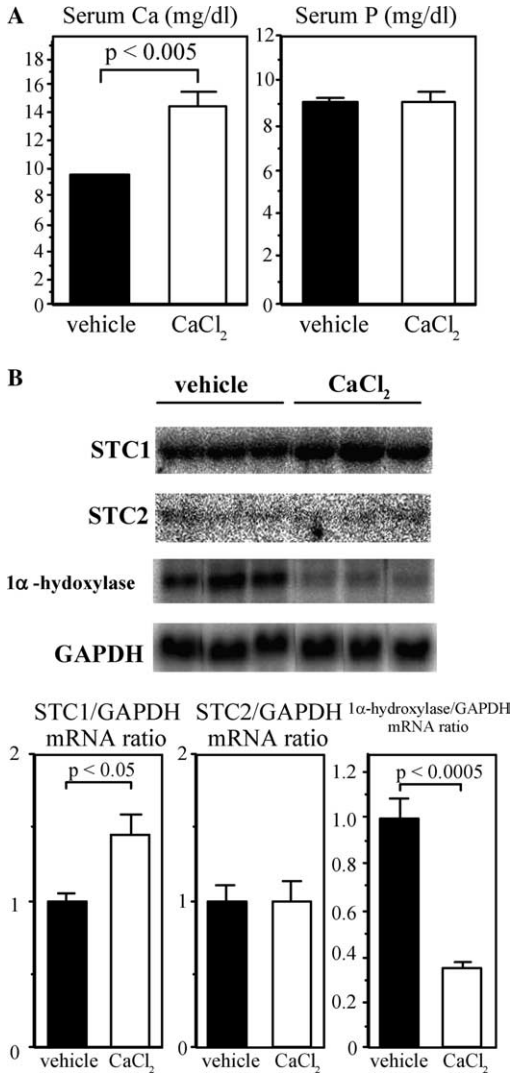

Fig. 4. Effects of $CaCl_2$ administration on (A) blood calcium and phosphate concentrations and (B) renal gene expression of STC1, STC2, and 25-hydroxyvitamin D_3-1α-hydroxylase in *klotho* and wild-type mice. Mice in fasted conditions were orally given $CaCl_2$ (800 mg/kg, twice) or vehicle and analyzed 6 h later ($n = 5$).

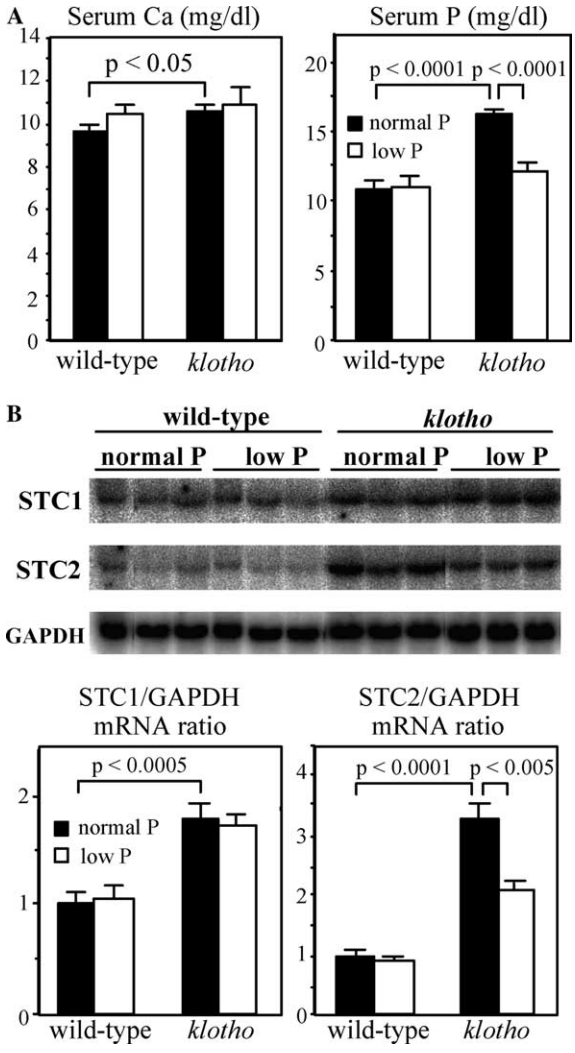

Fig. 5. Effects of low phosphorus diet on (A) blood calcium and phosphate concentrations and (B) renal gene expression of STC1 and STC2 in *klotho* and wild-type mice. Mice were given either low (0.02%) or normal (0.8%) phosphorus diet for 7 days ($n = 5$).

Effects of low phosphorus diet on blood calcium and phosphate concentrations and renal gene expression of STC1 and STC2

To examine the role of hyperphosphatemia in STC1 and STC2 gene upregulation in *klotho* mice, we treated wild-type and *klotho* mice with low phosphorus diet. In wild-type mice, low phosphorus diet tended to increase blood calcium levels without significant effects on blood phosphate levels and STC1 and STC2 gene expression levels (Fig. 5). In *klotho* mice, on the other hand, the treatment decreased the blood phosphate concentrations to the levels comparable to wild-type mice. Furthermore, STC2 gene expression levels in *klotho* mice were partially normalized ($P < 0.005$) but STC1 gene expression level remained high. These findings indicate that STC2 upregulation in *klotho* mice is caused by hyperphosphatemia-dependent and -independent mechanism resulting from lack of *klotho* gene expression.

Discussion

In the present study we have elucidated that renal gene expression levels of STC1 and STC2, which are new hormones implicated in calcium and phosphorus homeostasis, are markedly upregulated in *klotho* mice. Furthermore, we have found that vitamin D or $CaCl_2$ administration to wild-type mice causes upregulation of STC1, and low phosphorus diet for *klotho* mice significantly inhibits STC2 upregulation. These findings reveal the crucial roles of *klotho* gene expression and circulating calcium and phosphate concentrations in the regulation of STC1 and STC2 gene expression in the kidney.

Upregulation of STC1 in *klotho* mice may be exacerbating hyperphosphatemia [20,34], while upregulation of STC2 might play a role to alleviate hyperphosphatemia [23].

Importance of circulating calcium concentrations in STC1 regulation

In rats, vitamin D administration upregulates renal STC1 gene expression [35], but whether this is mediated by increased circulating calcium levels has not been determined. In the present study, we tried $CaCl_2$ administration to wild-type mice, in which high calcium and low 1,25-dihydroxyvitamin D_3 levels [32] co-exist in the blood, and found that STC1 was upregulated. These findings indicate that effects of vitamin D are mediated, at least in part, by hypercalcemia, but a possibility still remains that vitamin D might have some minor positive or negative effects to regulate STC1 expression directly.

The cause of hypercalciuria and hyperphosphaturia in klotho mice

Why is steady-state urinary excretion of calcium and phosphate increased in *klotho* mice? The source of excess calcium and phosphorus could be either release from the bones or increased absorption from the intestine. The osteopenia observed in *klotho* mice is not a developmental abnormality and becomes evident after birth, especially after weaning [1], but bone mineral density in *klotho* mice keeps on increasing between 3 and 7 weeks of age [36], suggesting that calcium and phosphate release from the bones is not occurring during our observation period. As described above, Yoshida et al. [29] clarified that *klotho* mice have high circulating 1,25-dihydroxyvitamin D_3 levels, and vitamin D has been shown to activate not only calcium absorption [37] but also phosphate absorption in the intestine [38,39]. Therefore, it is likely that hypercalciuria and hyperphosphaturia in *klotho* mice are caused by increased calcium and phosphate absorption in the intestine.

Phenotypes in klotho mice which can and cannot be explained by vitamin D overactivation

As described above, increase in vitamin D activity leads to hypercalcemia, hypercalciuria, and hyperphosphaturia. Furthermore, vitamin D may also play a role in the downregulation of circulating PTH levels [40] and the reduction of osteoblast [41] and osteoclast activities [42] observed in *klotho* mice [29,36,43]. However, it should be noted that administration of physiological or pharmacological dose of vitamin D in vivo leads either to net increase in bone mineral [42] or high turnover osteopenia by activation of osteoclasts [44,45], respectively, indicating that low turnover osteopenia in *klotho* mice [36] cannot be explained simply by overactivation of vitamin D. Furthermore, other important abnormalities in *klotho* mice are upregulation of 1α-hydroxylase gene expression in the kidney (Fig. 4) and downregulation of vitamin D receptor protein expression in the kidney and small intestine [29], despite high circulating 1,25-dihydroxyvitamin D_3 concentrations [29,31,46].

Partial vitamin D resistance in klotho mice

In wild-type mice, vitamin D treatment caused remarkable elevation of both blood calcium levels and renal gene expression levels of STC1. In untreated *klotho* mice, by contrast, calcium levels and STC1 expression levels were already elevated compared with wild-type mice, in agreement with vitamin D excess conditions. However, the levels in vitamin D-treated *klotho* mice did not reach the levels of treated wild-type mice. These findings indicate the presence of partial, but not complete, resistance to vitamin D in *klotho* mice [29], which may be caused by high circulating 1,25-dihydroxyvitamin D_3 concentrations and by compensatory changes in other regulatory hormones including downregulation of PTH.

Expression of klotho gene in phosphorus-deprived klotho mice

The defect of *klotho* gene in *klotho* mice resides in the promoter region of the gene and *klotho* mutation is not a null but a severe hypomorph [1]. Furthermore, male *klotho* mice given low phosphorus diet after weaning for 7 days have detectable amount of *klotho* protein expression in the kidney [47]. In our experiments, after low phosphorus treatment of *klotho* mice, *klotho* gene expression in the kidney was still not detected by Northern blot analysis (data not shown), but there is a possibility that effects of low phosphorus diet may be partly mediated by low level restoration of *klotho* expression. However, low phosphorus diet did not affect hypercalcemia and upregulation of STC1 in *klotho* mice, making this possibility unlikely.

Possible difference among species concerning regulation of STC1 and STC2

Here we examined the gene expression of STC1 and STC2 in mouse kidney and the regulation by blood calcium and phosphate levels, but several findings were inconsistent with what have been reported in rats. In mice, we showed that $CaCl_2$ or vitamin D administration increased STC1 gene expression in wild-type mice and low phosphorus diet inhibited STC2 upregulation in *klotho* mice. In rats, by contrast, renal STC1 gene expression has been reported to be decreased by calcium

supplementation or phosphorus deprivation [48] and renal STC2 gene expression is downregulated by vitamin D treatment [35], suggesting that certain species difference may exist as to STC1 and STC2 regulation.

Lessons from klotho mice

Phenotypes in *klotho* mice such as ectopic calcification, osteopenia, arteriosclerosis, and hyperphosphatemia are often observed in patients with end-stage renal diseases (ESRD), raising a possibility that these renal failure-associated changes might be caused by decrease in *klotho* gene expression [49]. However, renal 1α-hydroxylase and circulating 1,25-dihydroxyvitamin D_3 are downregulated in ESRD patients, whereas the opposite is observed in *klotho* mice [29], indicating that conditions in ESRD and in *klotho* mice are clearly distinct. Nevertheless, detailed analysis of *klotho* mice will help us understand the clinical impacts of decreased expression of *klotho* gene, which can be caused either by genetic backgrounds or by acquired diseases [49–52].

Conclusion

In the present study we revealed that the renal gene expression of STC1 and STC2 is upregulated in *klotho* mice. Here we also demonstrated that STC1 is positively regulated by blood calcium concentrations, while STC2 is regulated by a different mechanism involving blood phosphate concentrations.

Acknowledgments

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