Interrelated role of Klotho and calcium-sensing receptor in parathyroid hormone synthesis and parathyroid hyperplasia

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The pathogenesis of parathyroid gland hyperplasia is poorly understood, and a better understanding is essential if there is to be improvement over the current strategies for prevention and treatment of secondary hyperparathyroidism. Here we investigate the specific role of Klotho expressed in the parathyroid glands (PTGs) in mediating parathyroid hormone (PTH) and serum calcium homeostasis, as well as the potential interaction between calcium-sensing receptor (CaSR) and Klotho. We generated mouse strains with PTG-specific deletion of Klotho and CaSR and dual deletion of both genes. We show that ablating CaSR in the PTGs increases PTH synthesis, that Klotho has a pivotal role in suppressing PTH in the absence of CaSR, and that CaSR together with Klotho regulates PTH biosynthesis and PTH growth. We utilized the tdTomato gene in our mice to visualize and collect PTGs to reveal an inhibitory function of Klotho on PTG cell proliferation. Chronic hypocalcemia and ex vivo PTG culture demonstrated an independent role for Klotho in mediating PTH secretion. Moreover, we identify an interaction between PTG-expressed CaSR and Klotho. These findings reveal essential and interrelated functions for CaSR and Klotho during parathyroid hyperplasia.

Significance

Secondary hyperparathyroidism (SHPT) is a severe consequence of chronic kidney disease. A better understanding of the mechanisms controlling the progression of SHPT and the regulation of parathyroid hormone (PTH) production is clinically relevant. Utilizing parathyroid gland (PTG)-specific knockout mouse models, we demonstrated calcium-sensing receptor (CaSR) and Klotho together regulate PTH synthesis and PTG growth, and that Klotho contributes to PTH suppression in the absence of CaSR. Klotho exerts an independent function in mediating PTH secretion under chronic hypocalcemia and in suppressing PTG cell proliferation. Moreover, the results revealed a previously unidentified interaction between PTG-expressed CaSR and Klotho. These findings highlight the essential and interrelated roles for CaSR and Klotho to prevent parathyroid hyperplasia, suggesting potential treatment strategies to control PTH synthesis and hyperparathyroidism.


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fully understood, although some key issues have been identified (18). These include the drop in vitamin D levels due to the high FGF23 and a reduction in the expression of FGF23's required cofactor, Klotho, and its receptor, fibroblast growth factor receptor 1 (FGFR1), in the PTGs, reducing the ability of FGF23 to efficiently control the PTG function (18, 19).

Type 1 membrane-bound alpha-Klotho (Klotho, KL) is a transmembrane protein that has been found in mice, rats, and humans predominantly in the renal distal convoluted tubular cells and to a lesser extent in proximal convoluted tubular epithelial cells and the PTGs (18, 20, 21). It is also expressed in various organ systems, including arterial, epithelial, reproductive, and neuronal tissues (22). The principal role of Klotho is to form the specific receptor complex with FGFR1 that is required for FGF23 signaling (23–25). Activation of the FGFR1–Klotho complex by FGF23 in the kidney regulates phosphate homeostasis by affecting the expression of sodium-phosphate cotransporters, Napi2a and Napi2c, in the proximal tubules. It also inhibits 1,25(OH)2D3 synthesis by altering the vitamin D-metabolizing enzymes CYP27b1 and CYP24a1 (26–29). Noteworthily, Klotho is also highly expressed in the PTGs, but a link between Klotho and PTH in the regulation of serum calcium homeostasis has not yet been investigated. FGF23–Klotho signaling has been shown to inhibit PTH mRNA transcription and hormone secretion in vitro (30) and to negatively regulate PTH secretion in vivo (15). It has also been shown that the calcineurin-mediated FGF23 signaling pathway in PTGs mediates suppression of PTH expression (19). Contrary to these studies, Fgs2+/- mice overexpressing human recombinant FGF23 under the control of the 2.3-kb collagen 1 promoter exhibited high serum PTH despite unchanged serum calcium and 1,25(OH)2D3 levels, and despite hypophosphatemia (32). It has been suggested that FGF23 is a long-term inducer of PTH during CKD (33). Klotho activity has been implicated as fundamental for the stimulation of PTH secretion in hypocalcemia conditions by recruiting Na+, K+-ATPase (34), although the underlying mechanism has been challenged (35).

In this study, we sought to determine the tissue-specific role of Klotho in PTGs in controlling PTH and serum calcium homeostasis and to explore a potential interaction between Klotho and CaSR in proper PTH regulation. We therefore generated mouse strains with a parathyroid-specific deletion of Klotho or CaSR and a parathyroid-specific dual deletion of both genes. The results of our study reveal an independent function of PTG Klotho to regulate PTH production and cell proliferation, as well as a possible interaction between Klotho and CaSR.

Materials and Methods

Animals. Mice with a PTG-specific deletion of Klotho, CaSR, or both together were generated using the Cre-LoxP recombination system. The derivation of the PTHcre;KLfl/g mice was previously described (31). In short, LoxP sequences were introduced into the flanking regions of exon 2 of the Klotho gene. PTHcre;CaSR+/− mice were generated by mating mice in which the PTH promoter drives the expression of Cre recombinase (36) with mice in which the CaSR gene was flanked by LoxP sites (37), featuring a complete loss of CaSR expression in the PTGs (18, 20, 21). It is also expressed in various organ systems, including arterial, epithelial, reproductive, and neuronal tissues (22). The principal role of Klotho is to form the specific receptor complex with FGFR1 that is required for FGF23 signaling (23–25). Activation of the FGFR1–Klotho complex by FGF23 in the kidney regulates phosphate homeostasis by affecting the expression of sodium-phosphate cotransporters, Napi2a and Napi2c, in the proximal tubules. It also inhibits 1,25(OH)2D3 synthesis by altering the vitamin D-metabolizing enzymes CYP27b1 and CYP24a1 (26–29). Noteworthily, Klotho is also highly expressed in the PTGs, but a link between Klotho and PTH in the regulation of serum calcium homeostasis has not yet been investigated. FGF23–Klotho signaling has been shown to inhibit PTH mRNA transcription and hormone secretion in vitro (30) and to negatively regulate PTH secretion in vivo (15). It has also been shown that the calcineurin-mediated FGF23 signaling pathway in PTGs mediates suppression of PTH expression (19). Contrary to these studies, Fgs2+/- mice overexpressing human recombinant FGF23 under the control of the 2.3-kb collagen 1 promoter exhibited high serum PTH despite unchanged serum calcium and 1,25(OH)2D3 levels, and despite hypophosphatemia (32). It has been suggested that FGF23 is a long-term inducer of PTH during CKD (33). Klotho activity has been implicated as fundamental for the stimulation of PTH secretion in hypocalcemia conditions by recruiting Na+, K+-ATPase (34), although the underlying mechanism has been challenged (35).

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Extended methods and information about genotyping, serum and urine measurements, PTH isolation, ex vivo PTG culture, RNA isolation, and qRT-PCR analyses, skeletal preparations and bone histology, and Western blot are described in SI Materials and Methods.

Statistics. GraphPad Prism 6.0 (GraphPad Software Inc.) was used for statistical analysis. Comparisons between groups were evaluated by unpaired two-tailed Student’s t test between two groups or by one-way ANOVA followed by Tukey’s test for multiple comparisons. Two-way ANOVA and Bonferroni posttests were used to evaluate individual samples between control diet and low-Ca2+ diet. All values are expressed as mean ± SEM. P values <0.05 were considered significant for all analyses.

Results

Generation of PTHcre;KLfl/g, PTHcre;CaSRfl/g, and PTHcre;KLfl/g;CaSRfl/g Mice. Mice with PTG-specific deletion of Klotho or CaSR, and mice with specific deletion of both, were generated using Cre-LoxP recombination. Immunostaining and qRT-PCR using PTG RNA confirmed efficient deletion of Klotho and CaSR in the PTG tissue with the according genotype at protein and mRNA levels, respectively (Fig. S1). These mice were born at the expected Mendelian ratio. PTHcre;KLfl/g mice were viable and appeared macroscopically normal in size and weight. However,
PTHCre;CaSR<sup>fl/fl</sup> and PTHCre;KL<sup>Cre/RO</sup>;CaSR<sup>fl/fl</sup> mice were severely growth-retarded (Fig. 1A). These mice had reduced body weight compared with control littermates (KL<sup>Cre/RO</sup>;CaSR<sup>fl/fl</sup>) and died between 1 and 2 wk of age. Moreover, PTHCre;KL<sup>Cre/RO</sup>;CaSR<sup>fl/fl</sup> mice exhibited a significantly lower body weight and shorter life expectancy than PTHCre;CaSR<sup>fl/fl</sup> mice (Fig. 1B and C).

**Serum Biochemistry.** PTHCre;CaSR<sup>fl/fl</sup> and PTHCre;KL<sup>Cre/RO</sup>;CaSR<sup>fl/fl</sup> mice died within 2–3 wk of age; therefore, we compared serum parameters among all four genotypes at P10. We were interested in a potential interaction between Klotho and CaSR in PTG tissues to modulate mineral ion homeostasis. We detected no differences in serum Ca<sup>2+</sup>, Pi, PTH, intact FGF23 (iFGF23), and 1,25(OH)D<sub>3</sub> in PTHCre;KL<sup>Cre/RO</sup> mice compared with controls (Fig. 1D–H). However, both PTHCre;CaSR<sup>fl/fl</sup> and PTHCre;KL<sup>Cre/RO</sup>;CaSR<sup>fl/fl</sup> mice displayed hypercalciuria, hypophosphatemia, and significantly increased serum PTH, iFGF23, and 1,25(OH)D<sub>3</sub> levels (Fig. 1D–H). Most importantly, serum PTH, iFGF23, and 1,25(OH)D<sub>3</sub> levels in PTHCre;KL<sup>Cre/RO</sup>;CaSR<sup>fl/fl</sup> mice were further elevated and significantly higher than those in PTHCre;CaSR<sup>fl/fl</sup> mice, indicating a suppressive function of Klotho on PTH synthesis in the absence of CaSR (Fig. 1F–H).

**Renal and Bone Phenotype.** We investigated potential secondary effects of PTG-specific deletion of the Klotho and/or CaSR genes on the main target organs of PTH signaling, namely kidney and bone. There were no significant alterations in urinary Ca<sup>2+</sup> and Pi excretion of PTHCre;KL<sup>Cre/RO</sup> mice at P10. Both PTHCre;CaSR<sup>fl/fl</sup> and PTHCre;KL<sup>Cre/RO</sup>;CaSR<sup>fl/fl</sup> mice exhibited hypercalciuria and hypophosphatemia compared with control or PTHCre;KL<sup>Cre/RO</sup> mice (Fig. S2A and B). Moreover, these mice had significantly increased renal Cyp27b1 gene expression (Fig. S2C), which was positively correlated with the higher serum 1,25(OH)D<sub>3</sub> levels. Renal Nap2a gene and protein expressions were markedly decreased in PTHCre;CaSR<sup>fl/fl</sup> and PTHCre;KL<sup>Cre/RO</sup>;CaSR<sup>fl/fl</sup> mice (Fig. S2D–F). Radiographs of hind limbs showed no significant changes in bone due to Klotho deletion, while CaSR deletion in PTGs led to markedly increased radiolucency in metaphysis and diaphysis (Fig. S3A). Moreover, the formation of the secondary ossification center in the epiphyses of PTHCre;CaSR<sup>fl/fl</sup> and PTHCre;KL<sup>Cre/RO</sup>;CaSR<sup>fl/fl</sup> mice was dramatically delayed (Fig. S3B). Alizarin Red and Alcian Blue staining showed that these two mouse mutants had severely undermineralized skeletons and the typical rickets-like nodules in the ribs. PTHCre;KL<sup>Cre/RO</sup>;CaSR<sup>fl/fl</sup> mice exhibited a more severe skeletal phenotype compared with single CaSR-deletion mice (Fig. S3C).

**Klotho and CaSR Regulate PTG Growth.** Visualizing and collecting the PTGs from mice for further analyses is difficult due to the small size of the glands. We thus introduced the tdTomato reporter gene (Tm<sup>Cre/RO</sup>) to generate new mouse lines, including PTHcre;Tm<sup>Cre/RO</sup>, PTHcre;Tm<sup>Cre/RO</sup>;KL<sup>Cre/RO</sup>, PTHcre;Tm<sup>Cre/RO</sup>;CaSR<sup>fl/fl</sup>, and PTHcre;Tm<sup>Cre/RO</sup>;KL<sup>Cre/RO</sup>;CaSR<sup>fl/fl</sup>. The presence of the fluorescent reporter facilitated detection and dissection of the PTGs using a fluorescent stereomicroscope. tdTomato fluorescent images from P10 mice suggested that the size of the PTG tissue was significantly increased in PTHcre;Tm<sup>Cre/RO</sup>;CaSR<sup>fl/fl</sup> and PTHcre;Tm<sup>Cre/RO</sup>;KL<sup>Cre/RO</sup>;CaSR<sup>fl/fl</sup> mice. This is especially significant in the presence of the dramatically decreased body weight of those mutant mice. Most notably, PTGs in PTHcre;Tm<sup>Cre/RO</sup>;KL<sup>Cre/RO</sup>;CaSR<sup>fl/fl</sup> mice were even further enlarged compared with PTHcre;Tm<sup>Cre/RO</sup>;CaSR<sup>fl/fl</sup>. There was only a trend toward an increase in PTG size in PTHcre;Tm<sup>Cre/RO</sup>;KL<sup>Cre/RO</sup>;CaSR<sup>fl/fl</sup> mice at P10 (Fig. 2A and B).

We next examined the histology of the PTGs at P10. We generated paraffin sections and used H&E staining to confirm the size difference (Fig. 2C). The results suggested that no significant structural difference could be detected between PTHCre;KL<sup>Cre/RO</sup> and control mice. Both had the typical rope-like structure with dense parathyroid cells. Interestingly, cells in PTHCre;CaSR<sup>fl/fl</sup> and PTHCre;KL<sup>Cre/RO</sup>;CaSR<sup>fl/fl</sup> PTGs appeared to be larger. Also, the glands contained more cosinophilic areas with nodular formations. The morphological structure is more severe and disrupted in double-mutant mice (Fig. 2C). A proliferation analysis revealed a significant increase in the percentage of Ki67-positive cells in
Klotho and CaSR regulate growth of PTGs. (A and B) Tomato fluorescence and size calculations for PTGs from P10 mice showed parathyroid-specific CaSR ablation leads to significantly larger glands relative to body weight. Ablation of both Klotho and CaSR in PTGs resulted in a further enlargement of PTG size over single CaSR-deletion mice. There was a trend toward an increase in PTHCre;Tm$^{fl/fl}$;KL$^{fl/fl}$ PTG size compared with controls at P10. (Scale bar, 500 μm.) (C) H&E staining confirmed the changes of PTG size and showed that CaSR deletion affected parathyroid morphological structure and caused nodular formations. This was more severe for Klotho and CaSR dual ablation. (Scale bar, 200 μm in low magnification, 50 μm in high magnification.) Black arrowheads indicate nodules. (D and E) Immunohistochemical staining of Ki67 and calculation of Ki67-positive cell ratio in PTGs, showing increased cell proliferation in PTHCre;CaSR$^{fl/fl}$ and PTHCre;KL$^{fl/fl}$;CaSR$^{fl/fl}$ mice at P10. n = 6. (Scale bar, 50 μm.) *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. We next investigated a few consequences on gene transcription upon deletion of Klotho/CaSR from PTGs. The results demonstrated that expression of Fgf1, Egr1, and lnt(OH)ase were slightly reduced in PTGs of Klotho-deficient mice. Furthermore, CaSR deletion resulted in increased 24(OH)ase expression levels, which exhibited comparatively lower expression in Klotho-ablated glands. Moreover, mutant mice lacking Klotho, CaSR, or both showed reduced expression of Vdr (Fig. S5 A–E). We next explored the MAP kinase cascade using immunofluorescence staining. As shown in Fig. S5 F and G, there was significantly reduced signal for phosphorylated ERK1/2 in parathyroid tissue from Klotho- and/or CaSR-ablated mice, indicating that the MAP kinase pathway was markedly suppressed in Klotho- and CaSR-deficient PTGs.

Klotho and CaSR Regulate PTH Secretion Under Chronic Hypocalcemia. Our next goal was to determine the role of PTH-specific Klotho and CaSR deletion in development and progression of PTG hyperplasia under conditions of chronic hypocalcemia. We therefore challenged PTHCre;Tm$^{fl/fl}$;KL$^{fl/fl}$, PTHCre;Tm$^{fl/fl}$;CaSR$^{fl/fl}$, and PTHCre;Tm$^{fl/fl}$;KL$^{fl/fl}$;CaSR$^{fl/fl}$ mice with a low-Ca$^{2+}$ diet for 3 wk, starting at weaning. PTHCre;Tm$^{fl/fl}$ mice were used as controls. Serum Ca$^{2+}$ and Pi levels were significantly reduced in all genotypes after being on the low-Ca$^{2+}$ diet (Fig. 4 A and B). As expected, the low-Ca$^{2+}$ diet significantly increased serum PTH levels by around sevenfold in PTHCre;Tm$^{fl/fl}$;KL$^{fl/fl}$, PTHCre;Tm$^{fl/fl}$;CaSR$^{fl/fl}$, and PTHCre;Tm$^{fl/fl}$;KL$^{fl/fl}$;CaSR$^{fl/fl}$ mice, while in PTHCre;Tm$^{fl/fl}$;KL$^{fl/fl}$ and PTHCre;Tm$^{fl/fl}$;CaSR$^{fl/fl}$ mice, the increase in serum PTH was only around twofold and thus not as great as that in controls. Notably, we observed tetracycline dye (Fig. S4 A and B). To investigate whether this could be due to cell death, we performed TUNEL staining. The results suggested that ablation of CaSR leads to increased parathyroid cell apoptosis, which is even more pronounced in PTHCre;KL$^{fl/fl}$;CaSR$^{fl/fl}$ mice that failed to express PTH protein (Fig. S4 A and B). To investigate whether this could be due to cell death, we performed TUNEL staining. The results suggested that ablation of CaSR leads to increased parathyroid cell apoptosis, which is even more pronounced in PTHCre;KL$^{fl/fl}$;CaSR$^{fl/fl}$ mice (Fig. S4 C and D). We next investigated a few consequences on gene transcription upon deletion of Klotho/CaSR from PTGs. The results demonstrated that expression of Fgf1, Egr1, and lnt(OH)ase were slightly reduced in PTGs of Klotho-deficient mice. Furthermore, CaSR deletion resulted in increased 24(OH)ase expression levels, which exhibited comparatively lower expression in Klotho-ablated glands. Moreover, mutant mice lacking Klotho, CaSR, or both showed reduced expression of Vdr (Fig. S5 A–E). We next explored the MAP kinase cascade using immunofluorescence staining. As shown in Fig. S5 F and G, there was significantly reduced signal for phosphorylated ERK1/2 in parathyroid tissue from Klotho- and/or CaSR-ablated mice, indicating that the MAP kinase pathway was markedly suppressed in Klotho- and CaSR-deficient PTGs.

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PTH protein expression under control or low-Ca\textsuperscript{2+} conditions (Fig. 4D). Notably, we observed the PTG nodule formations only in PTH\textsuperscript{Cre};Tm\textsuperscript{fl/+};KL\textsuperscript{fl/fl};CaSR\textsuperscript{fl/+} mice fed a low-Ca\textsuperscript{2+} diet. This indicates that dual deletion of Klotho and CaSR leads to nodule formation with excess PTH production, which is in accord with observation of the highest serum PTH level in this group.

We next confirmed the effect of Klotho on PTH production without the confounding influence of other circulating factors. We dissected the PTGs and performed PTG ex vivo culture. The glands of PTH\textsuperscript{Cre};Tm\textsuperscript{fl/+} and PTH\textsuperscript{Cre};Tm\textsuperscript{fl/+};KL\textsuperscript{fl/fl} mice were subjected to low-Ca\textsuperscript{2+} (0.5 mM Ca\textsuperscript{2+}) or high-Ca\textsuperscript{2+} (3 mM Ca\textsuperscript{2+}) conditions over a 1.5-h period (Fig. 4E) and secreted PTH was measured by ELISA. Regardless of calcium concentration, PTH\textsuperscript{Cre};Tm\textsuperscript{fl/+};KL\textsuperscript{fl/fl} PTGs secreted more PTH than control glands. At low Ca\textsuperscript{2+} concentrations, PTH production was significantly induced in both genotypes but showed a further elevation in Klotho-deficient glands (Fig. 4F).

**Klotho and CaSR Suppress PTG Hyperplasia Under Chronic Hypocalcemia.** PTGs were dissected from mice using Tomato fluorescence as a guide and size was calculated by ImageJ. The results showed that under normal dietary conditions the size of the glands in PTH\textsuperscript{Cre};Tm\textsuperscript{fl/+};KL\textsuperscript{fl/fl}, PTH\textsuperscript{Cre};Tm\textsuperscript{fl/+};CaSR\textsuperscript{fl/+}, and PTH\textsuperscript{Cre};Tm\textsuperscript{fl/+};KL\textsuperscript{fl/fl};CaSR\textsuperscript{fl/+} mice was significantly increased compared with PTH\textsuperscript{Cre};Tm\textsuperscript{fl/+} mice. The effect was even more pronounced when Klotho was deleted (Fig. 5A and B). A low-Ca\textsuperscript{2+} diet induced PTG hyperplasia in all genotypes, but a more severe increase occurred in the absence of Klotho (Fig. 5A and B). Histological analyses of H&E-stained paraffin sections of PTGs showed that under a control diet PTG tissue from PTH\textsuperscript{Cre};Tm\textsuperscript{fl/+};KL\textsuperscript{fl/fl}, PTH\textsuperscript{Cre};Tm\textsuperscript{fl/+};CaSR\textsuperscript{fl/+}, and PTH\textsuperscript{Cre};
Tm<sup>fl/fl</sup>;KL<sup>fl/fl</sup>;CaSR<sup>fl/+</sup> mice appeared to be more eosinophilic compared with that of control glands (Fig. 5). There was a significant increase in the PTG volume in all groups in response to hypocalcemia. Ki67 immunostaining showed that glands with Klotho or Klotho/CaSR deletion exhibit increased cell proliferation under control diet conditions compared with control glands (Fig. 6). Furthermore, chronic hypocalcemia induced by low-Ca<sup>2+</sup> diet resulted in a significant up-regulation of Ki67-positive cells only in Klotho-ablated PTGs, indicating that an increase in cell proliferation contributed to the observed PTG hyperplasia in the absence of Klotho (Fig. 6). TUNEL staining showed that cell apoptosis was not affected by Klotho or partial CaSR deletion or by diet conditions. These results suggest that Klotho and CaSR have an important role in PTG growth by regulating cell proliferation. Deletion of either factor leads to PTG hyperplasia (Fig. S6).

Interaction of Klotho and CaSR in PTGs. We isolated PTGs using tdTomato fluorescence to eliminate any contamination by surrounding thyroid tissue. qRT-PCR analyses and immunostaining showed that deletion of Klotho leads to a tendency of lower CaSR expression on either protein or mRNA levels at P10 (Fig. 7A and B). Interestingly, however, Klotho mRNA expression levels were significantly decreased in CaSR-deleted PTGs (Fig. 7D), which was also confirmed at the protein level by immunostaining (Fig. 7C). This finding suggests a potential interaction between CaSR and Klotho. Dual deletion of Klotho and CaSR resulted in merely undetectable Klotho expression (Fig. 7C). The moderately to severely elevated serum PTH in PTHcre;Tm<sup>fl/+</sup>;KL<sup>fl/fl</sup> and PTHcre;Tm<sup>fl/+</sup>;CaSR<sup>fl/+</sup> mice might relate to Klotho gene dosage (Fig. 1F). We next intended to avoid the high serum calcium levels in PTHcre;CaSR<sup>fl/fl</sup> and PTHcre;KL<sup>fl/fl</sup>;CaSR<sup>fl/fl</sup> mice that could contribute to down-regulation of Klotho expression in the PTGs. We therefore performed PTG ex vivo cultures. The isolated glands of PTHcre;Tm<sup>fl/+</sup> and PTHcre;Tm<sup>fl/+</sup>;KL<sup>fl/fl</sup> mice were subjected to low-Ca<sup>2+</sup> (0.5 mM Ca<sup>2+</sup>) or high-Ca<sup>2+</sup> (3 mM Ca<sup>2+</sup>) conditions. qRT-PCR results showed that high Ca<sup>2+</sup> had no effect on Klotho gene expression in control PTGs (Fig. 7G), suggesting that the deletion of the CaSR itself is responsible for the reduced Klotho expression in PTGs, and that this down-regulation is independent of serum calcium levels.
levels. We investigated the effect of CaSR deletion on Klotho expression at a later stage in 6-wk-old mice. Noteworthy, Klotho expression was markedly decreased in PTHCRE;Tmfl/fl;CaSRfl/+ PTGs compared with controls (Fig. 7E). Concurrently, CaSR expression was also found to be significantly reduced in Klotho-deficient PTGs at 6 wk (Fig. 7F).

Furthermore, PTHCRE;KLfl/fl;CaSRfl/+ mice had slightly higher serum PTH values compared with PTHCRE;KLfl/+ mice. A tendency toward increased PTH levels was observed in PTHCRE;KLfl/+;CaSRfl/+ compared with PTHCRE;CaSRfl/fl. This was correlated with serum Ca2+ levels, suggesting Klotho and CaSR together control PTH synthesis and deletion of only one allele of either protein resulted in more severe phenotype (Fig. S7A and B).

Moreover, we have compared the serum PTH and Ca2+ values of control animals and mice with Klotho ablation alone, with or without heterozygous CaSR deletion, at 6-wk of age (Fig. S7 C and D). The result was similar to what we observed at P10: Serum PTH levels exhibited a tendency to increase among these animals, confirming the synergistic effect of Klotho and CaSR in controlling PTH synthesis.

We further investigated the possibility of an interaction between Klotho and CaSR using coimmunoprecipitation assays. HEK293 cells were transfected with Klotho alone, CaSR alone, or Klotho + CaSR. Klotho and CaSR were then precipitated by Klotho (or Flag) or CaSR (or GFP) antibodies, respectively. The results demonstrated that Klotho communoprecipitates with

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**Fig. 5.** Klotho and CaSR regulate PTG hyperplasia under chronic hypocalcemia. (A and B) Tomato fluorescence and size calculations of PTGs from 6-wk-old mice on a control diet or low-Ca2+ diet. Parathyroid Klotho deletion or heterozygous deletion of CaSR led to increased PTG volume on control diet. The PTG size was further enlarged upon onset of chronic hypocalcemia. Two individual glands are shown as representative in the low Ca2+ diet groups of PTHCRE;Tmfl/fl;CaSRfl/+ and PTHCRE;Tmfl/fl;KLfl/fl;CaSRfl/+ mice. (Scale bar, 500 μm.) (C) H&E staining of PTGs from 6-wk-old control and mutant mice. (Scale bar, 50 μm.) *P < 0.05, **P < 0.01 versus control diet in each genotype.

**Fig. 6.** Effect of Klotho and CaSR deletion on PTG cell proliferation under chronic hypocalcemia. (A and B) Immunohistochemical staining of Ki67 and calculation of Ki67-positive cell ratio in PTGs on control or low-Ca2+ diet at 6 wk of age. Klotho or CaSR deletion, as well as low calcium conditions, increased parathyroid cell proliferation, with the highest elevation of Ki67-positive cells in Klotho-deficient PTGs. n = 3–6. (Scale bar, 25 μm.) ***P < 0.001 versus control diet in each genotype.
CaSR (Fig. 7F), suggesting a physical interaction between Klotho and CaSR.

Discussion

In the current study we generated mice with a PTG-specific deletion of Klotho alone or together with CaSR to better understand their functional mechanisms and determine any potential interactions. Deleting Klotho in PTGs did not affect key serum and urinary parameters. PTHCre;CaSRfl/fl mice displayed hypercalcemia, hypophosphatemia, and significantly elevated serum PTH, iFGF23, and 1,25(OH)2D3 levels, resembling the phenotype of CaSR−/− mice (42). Combined deletion of Klotho and CaSR (PTHCre;KL−/−;CaSR−/−) resulted in significantly higher serum PTH, iFGF23, and 1,25(OH)2D3 levels compared with PTHCre;CaSR−/− mice. There are several ways to interpret these data. First, Klotho deletion could impede the FGF23 feedback loop that suppresses PTH in PTGs. The moderately or severely elevated serum PTH observed in CaSR-deletion versus CaSR+/+Klotho-deletion mice might depend on Klotho gene-dosage effects. Second, PTG hyperplasia developed in both mutants, but mice with dual deletion exhibited the largest effect. Pth mRNA levels were comparable between these mice, but the greater hyperplasia in PTHCre;KL−/−;CaSR−/− mice might lead to an increased parathyroid cell number and thus enhanced PTH synthesis and higher serum PTH levels. Third, PTHCre;KL−/−;CaSR−/− PTGs exhibited a more severe alteration of PTG morphology, accompanied by increased PTG nodule formations, which might also lead to higher PTH secretion. These results collectively indicate that Klotho is a negative regulator of PTH synthesis in the absence of CaSR. Serum PTH levels remained constant in PTHCre;Klotho−/− mice, suggesting that the CaSR has a dominant function in modulating PTH production. Klotho may serve as a supplementary factor in response to changes in serum calcium, especially when CaSR function is diminished.
Renal Klotho is known to enhance renal Ca\textsuperscript{2+} absorption by stabilizing the transient receptor potential vanilloid 5 acting as a β-glucuronidase (43) and is an important factor in the prevention of renal Ca\textsuperscript{2+} loss (44). We investigated the action of parathyroid Klotho in the proper response to serum calcium. A previous study using single injections of EGTA or calcium gluconate to change serum calcium levels demonstrated that PTG Klotho is not essential for PTH secretion in response to acute alterations in serum calcium (31). However, we challenged mice with a Ca\textsuperscript{2+}-deficient diet for 3 wk to investigate the physiological role of Klotho and CaSR during chronic hypocalcemia. The increase in serum PTH was less in PTGCRE;TRL\textsuperscript{−/+} and PTGCRE;TRL\textsuperscript{−/-};CaSR\textsuperscript{−/+} mice compared with control mice under low-Ca\textsuperscript{2+} condition. This result is in accord with a previous report that showed homozygous Klotho knockout mice did not secrete as much PTH as control mice under hypocalcemia (34). Interestingly, ex vivo experiments showed that Klotho-deficient PTGs had more pronounced secretion of PTH under low-Ca\textsuperscript{2+} condition void of the influence by other circulating components, suggesting an independent role for Klotho in suppressing PTH secretion. Therefore, we speculate that the inconsistency of in vivo and ex vivo PTH production might be due to some systemic inhibitory factors preventing PTH secretion in PTGCRE;TRL\textsuperscript{−/-};CaSR\textsuperscript{−/+} mice under chronic hypocalcemia. Thus, additional investigations are required to determine the in vivo regulatory network on PTH secretion.

Accumulating evidence suggests that a chronic increase in PTH production, characteristic of primary or secondary hyperparathyroidism, is accompanied by an increase in PTG size (45–47). Determination of gland size is particularly difficult (48), so we generated mice with a Tomato reporter gene in which red fluorescent protein is selectively expressed in parathyroid cells. Mice with ablation of PTG-CaSR at P10 or heterozygous PTG-specific deletion of CaSR at 6 wk of age had significantly enlarged glands compared with controls, confirming the pivotal function of CaSR in determining PTG growth. Klotho expression is reduced as the PTG tissue becomes hyperplastic (19), as seen in patients with hyperphosphatemic familial tumoral calcinosis (49). It was unclear whether Klotho was a contributing factor to hyperplasia, a consequence of hyperparathyroidism, or some combination of the two. Our results revealed that Klotho has a more prominent role in the development of hyperplasia and/or SHPT. PTG-specific Klotho deletion at P10 reduces the trend of increasing PTG size at P10. However, the combined deletion of Klotho and CaSR caused significant PTG hyperplasia. The additional gland enlargement in PTGCRE;CaSR\textsuperscript{−/−} mice compared with PTGCRE;CaSR\textsuperscript{−/+} mice indicates a role of Klotho in preventing the development of hyperplasia in the absence of a functional CaSR. Interestingly, cell proliferation appears to be more important than cell apoptosis to the increase in PTG volume. It is important to note that the critical role of Klotho in preventing PTG hyperplasia is demonstrated in our prolonged observation that mice with PTG-specific Klotho ablation at 6 wk of age exhibited a substantial increase in PTG size compared with control and PTGCRE;TRL\textsuperscript{−/+};CaSR\textsuperscript{−/-} mice on a control diet. These results emphasize Klotho’s function in suppressing PTG hyperplasia.

An increase in PTG volume, reportedly due to enhanced hyperplasia, has been observed in hypocalcemic animals with normal renal function on a calcium-deficient diet (50). Another finding showed that parathyroid cell hyperplasia largely prevailed over hyperplasia in rats on a low-calcium diet (51). Analyses of human hyperparathyroidism samples found evidence of apoptosis (52, 53). The percentage of PTG apoptotic cells in controls was ∼0.15% in P10 mice and less than 0.1% in 6-wk-old mice, but PTG-specific ablation of CaSR led to significantly increased cell apoptosis. This was more evident in dual deletion of CaSR and Klotho at P10. Nevertheless, cell proliferation prevailed over apoptosis in these mice. Enhanced cell proliferation was observed under chronic hypocalcemia, and cell apoptosis was not altered in 6-wk-old mice. Thus, the higher rate of cell proliferation over apoptosis results in the enlargement of PTG volume, similar to the observations in hyperplastic PTG tissue of uremic patients (53, 54). We also observed that in some extremely hyperplastic PTGs from PTGCRE;CaSR\textsuperscript{−/−} mice, PTH was not expressed in some areas of altered structure, probably due to increased apoptosis. We found that an enlargement of PTG size was detected in all groups under hypocalcemia and revealed that hypocalcemia significantly enhanced cell proliferation. This was more pronounced in mice deficient in PTG Klotho, confirming that reduced Klotho expression could direct PTG hyperplasia by mediating cell proliferation.

Previous studies showed Klotho and CaSR expression were significantly decreased in PTGs of PHTP, SHPT patients, patients after kidney transplantation, and those with end-stage renal disease (55–57). We could demonstrate a binding interaction between Klotho and CaSR that might play a synergistic effect in controlling PTH synthesis and glandular hyperplasia. Several lines of experimental evidence support this tenet. PTG-specific deletion of CaSR, for example, leads to significantly reduced Klotho transcript and protein levels at P10. Moreover, even heterozygous PTG CaSR ablation resulted in markedly reduced Klotho expression. Ex vivo PTG culture revealed that Klotho expression was not affected by changes in calcium levels, indicating that the observed reduction in Klotho expression in vivo was largely due to CaSR ablation. On the contrary, PTG Klotho deletion significantly reduced CaSR expression at 6 wk of age. A trend toward decreased CaSR expression was already noted at P10 in PTGCRE;CaSR\textsuperscript{−/−} PTGs. Moreover, we were able to demonstrate that Klotho and CaSR bind to each other using coimmunoprecipitation. Technical issues related to the small size of the glands limited further investigations of in vivo coimmunoprecipitation experiments. However, we were able to show protein colocalization of CaSR and Klotho by immunostaining (Fig. S7E), consistent with these two proteins acting together to mediate PTH synthesis and PTG growth.

In summary, we demonstrated that the specific deletion of CaSR in the PTGs leads to elevated serum PTH and PTG hyperplasia, and that additional deletion of Klotho in PTGs exacerbated this condition. This suggests a pivotal function for Klotho in suppressing PTH biosynthesis and PTG growth in the absence of CaSR. Moreover, we were able to demonstrate that Klotho exhibits an independent role to modulate PTH production under chronic hypocalcemia and acts as an inhibitory factor on parathyroid cell proliferation, indicating a physiological function for Klotho in modulating the hyperplasia or SHPT. Most importantly, our findings propose a physical interaction between Klotho and CaSR. Nevertheless, the molecular mechanism by which Klotho and CaSR regulate each other requires further investigation. The results suggest that up-regulation or activation of Klotho could provide a potential treatment to control circulating PTH and hyperparathyroidism.

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