The discovery that two recently identified molecules, klotho and fibroblast growth factor 23 (FGF23), played an important role in calcium, phosphate, and vitamin D metabolism has transformed our traditional physiological view in which bone and mineral homeostasis was mainly regulated by parathyroid hormone, vitamin D, and calcitonin, according to mineral body needs. FGF23 is a 251-amino acid secreted protein produced by osteoblasts and osteocytes in bone following the stimulation by phosphate and vitamin D or the inhibition by dentin matrix protein 1. Originally isolated from tumoral cells of patients with tumor-induced osteomalacia and hypophosphatemia, FGF23 inhibits phosphate reabsorption in renal proximal tubular cells and 1α-hydroxylase activity, resulting in decreased synthesis of calcitriol. To exert these actions, FGF23 requires the conversion, by klotho, of the canonical FGF receptor 1 (IIIc) in a specific high affinity FGF23 receptor. On the other hand, klotho is a putative antiaging gene identified in 1997 when a particular mouse strain, created by random insertion mutagenesis, was found to be short-lived and displayed premature atherosclerosis, osteopenia, skin atrophy, pulmonary emphysema, hyperphosphatemia, hypercalcemia, and high serum calcitriol levels. The gene of klotho encodes a 1012-amino acid cell-surface protein with a short cytoplasmic tail and an extracellular domain that consists in tandem duplicated copies of a β-glucuronidase-like sequence, which can be released into the circulation as soluble forms after being cleaved by metallocproteinases such as ADAM10 and ADAM17. By modulating FGF23 action, klotho regulates urinary phosphate excretion and calcitriol synthesis. By virtue of its β-glucuronidase activity, klotho deglycosylates the calcium channel TRPV5 (transient receptor potential vallinoid-5) and regulates urinary calcium excretion. klotho also binds to Na⁺,K⁺-ATPase in parathyroid cells and regulates calcium-stimulated PTH secretion. Finally, klotho extends life span via several mechanisms, including the reduction of calcitriol synthesis, serum calcium, and phosphorus levels; the induction of insulin resistance; and by increasing the resistance to oxidative stress.
artery diseases. Additionally, a homozygous missense inactivating mutation (H193R) in the klotho gene that was found in a 13-year-old girl has been associated with severe tumoral calcinosis with artery calcifications and marked hyperphosphatemia and hypercalcemia as well as elevated serum levels of parathyroid hormone (PTH) and fibroblast growth factor (FGF23). However, a translocation between chromosomes 9 and 13 causing increased circulating klotho levels results in hypophosphatemic rickets and hyperparathyroidism.

All these observations support the assumption that klotho plays an important role in the selective regulation of calcium, phosphate, and active vitamin D levels, in the regulation of parathyroid mass and PTH secretion, and in aging and senescence-related disorders. This article attempts to provide an updated review of these subjects.

Molecular Characteristics of Klotho: Gene, mRNA, and Protein

The human klotho gene is a 5-exon gene located on chromosome 13q12 within a region longer than 50 kb. Two transcripts arise from this single gene: one full-length 5.2-kb encoding a 1012-amino acid (130-kDa), single-pass, membrane-anchored protein. This membrane form is released into the circulation following its cleavage at the membrane level (α-cleavage) and between the two globular glycosidase domains (β-cleavage) by several metalloproteases or sheddases such as ADAM10 and ADAM 17. The main circulating form of klotho is the 130- to 135-kDa, fragment which is ultimately cleaved into two smaller fragments of 65 to 68 kDa. The other transcript, derived from the alternative mRNA splicing, encodes the N-terminal half of klotho, a protein of 549 amino acids with a molecular weight of approximately 65 to 68 kDa.

Klotho protein belongs to the β-glycuronidase family. The human protein shows 86% amino acid identity with its mouse homolog. The extracellular domain of klotho is composed of two internal repeats (KL1, KL2), each of approximately 450 amino acids long with a similarity of 21% to each other. These two domains form a butterfly-shaped molecule at the surface of the cellular membrane. They share 20% to 40% sequence identity to the β-glucosidase of both bacteria and plants and with mammalian lactase glycosylceramidase. Another particularity of klotho proteins is that the secreted form, as well as the membrane form, develops oligomeric complexes, suggesting a posttranslation klotho processing and possible regulatory mechanisms for klotho secretion in vivo.

Klotho mRNA is mainly expressed, in descending order, in kidney, brain, reproductive organs, pituitary gland, parathyroid glands, urinary bladder, skeletal muscle, placenta, thyroid gland, and colon. In the kidney, klotho mRNAs and proteins are mainly localized in the distal convoluted tubule, contrasting with the major site of phosphate reabsorption and the expression of sodium-phosphate cotransporter NPT2a, which take place principally in proximal tubular cells. In these distal tubular cells, klotho colocalizes with other proteins involved in tubular calcium reabsorption such as the epithelial calcium channel TRPV5 and calbindin 28K, suggesting that klotho is intimately implicated in renal calcium homeostasis.

Mode of Action of Klotho

The exact biological role and mode of action of klotho are only partly deciphered. To date, four modes of actions have been described; first, klotho acts as a glucuronidase. This action is supported by several findings demonstrating that klotho deglycosylates synthetic and naturally occurring β-glucuronides such as β-estradiol 3β-D-glucuronide, estrone 3β-D-glucuronide, and estriol 3β-D-glucuronide, as well as proteins such as the calcium channels TRPV5. This enzymatic activity of klotho can be entirely mimicked by purified bovine β-glucuronidase and reduced or blocked by specific inhibitors of β-glucuronidase such as D-saccharic acid 1,4-lactone. Second, klotho can act as a humoral factor since the secreted form and the different fragments released after cleavage of the membrane form are found in the urine, serum, and cerebrospinal fluid. Klotho binds to a high affinity but yet not identified cell-surface klotho receptor and activates the protein kinase C (PKC) pathway in kidney and testicular cells; klotho also stimulates cAMP pathway in several cell types. The activation of this receptor by klotho leads to the inhibition of the intracellular insulin/IGF-1 signalling cascade. This activity likely contributes to the antiaging effects of klotho, because inhibition of
insulin-like signaling is an evolutionarily conserved mechanism for extending life span.2,22 Third, klotho can act as a coreceptor or a cofactor for other proteins such as FGF23. It has been recently demonstrated that klotho directly binds to multiple FGF receptors (FGFRs) and that the klotho/FGFR complex binds to FGF23 with higher affinity than FGFR or klotho alone. Furthermore, klotho significantly enhanced the ability of FGF23 to induce phosphorylation of FGF receptor substrate and extracellular signal-regulated kinase (ERK) in a variety of cell types.23 The interaction between klotho, FGFR, and FGF23 is a new type of receptor modulation, which has been further illustrated in a recent report.24 Indeed, klotho binds to FGFR1(IIIc) and its concerted action constitutes the FGF23 specific receptor; in the absence of klotho, the function of FGF23 is abolished.24 Fourth, klotho interacts physically with Na\(^+\),K\(^+\)-ATPase in parathyroid cells and regulates the calcium-stimulated PTH secretion. In klotho knock-out mice, parathyroid Na\(^+\),K\(^+\)-ATPase activity is decreased, leading also to a reduced PTH secretion.25 However, the molecular mechanisms for this effect of klotho remain to be identified.

Effects of Klotho on Bone Structure and Function

Klotho-deficient mice show low bone formation and bone resorption activities, which results in a radiographic, densitometric, and histomorphometric osteopenia.1 The decrease in bone formation is associated with a diminution in the number of osteoblastic cells and in their ability to produce alkaline phosphatase and to mineralize extracellular matrix.26 On the other hand, cultured osteoclastic cells have normal bone resorption activity and survival rate, but their differentiation process from osteoclast precursor cells is disturbed. Moreover, osteoprotegerin, a secreted factor which inhibits osteoclastogenesis, is upregulated in klotho-deficient mice, suggesting that there is an independent impairment of osteoblast and osteoclast differentiation, which could be the cause of this low bone turnover osteopathy.26,27

In human, several single-nucleotide polymorphisms (SNPs) in the klotho gene have been found associated with changes in bone mineral density in Asian and Caucasian populations. In the Caucasian population, the SNP in the promoter region (G395A) and in exon 4 (C1818T) and their haplotypes are significantly associated with low bone density in postmenopausal women (>65 years) as well as in Japanese postmenopausal women, but not in premenopausal or younger postmenopausal women. The polymorphism G395A substitution in the promoter region affects DNA—protein interaction and may affect the level of expression of klotho.4,7 Another polymorphism in klotho gene (F352V) has been associated with a higher bone mineral density in a Spanish population of postmenopausal women.28

Effects of Klotho on Calcium, Phosphate, Vitamin D, and PTH Metabolism

Mice with klotho gene mutation show disturbed calcium and phosphate homeostasis together with an increased in the serum concentration of active vitamin D [1,25(OH)\(_2\)D\(_3\)]. Interestingly, most of the aging phenotypes of these mice can be lightened, as well as serum calcium and 1,25(OH)\(_2\)D\(_3\) concentrations reduced, with phosphate restriction in the diet,29,30 suggesting that these phenotypes are downstream events resulting from elevated 1,25(OH)\(_2\)D\(_3\) as shown in the FGF23 knock-out mice. Indeed, removal or reduction of 1,25(OH)\(_2\)D\(_3\) in FGF23 and klotho mutant mice, by either dietary restriction or genetic manipulation, rescues premature aging-like features and ectopic calcifications.30–32

Calcium Metabolism

On the one hand, klotho-deficient mice are hypercalcemic mainly because of the hypervitaminosis D and its stimulatory effects on intestinal and renal absorption of calcium. However, concomitantly with the rise in serum calcium concentration, these animals exhibit an increase in urinary fractional excretion of calcium, despite low serum PTH levels, which suggests that the hypercalcemia is not entirely due to abnormal renal calcium handling. Another possible explanation is that the low bone remodeling observed with the lack of klotho could favor the occurrence of hypercalcemia because the skeleton would be unable to provide its buffering action. Moreover, it has been demonstrated that TRPV5 is essential for a proper osteoclastic activity; mice lacking TRPV5 have an increase in osteoclast size and number, but bone
resorption is diminished because of a reduced activity. Klotho could also exert a similar effect on osteoclast TRPV5 expression as in the kidney cells but that remains to be investigated.

On the other hand, there is evidence that klotho directly participates in the regulation of renal calcium reabsorption. Klotho regulates calcium reabsorption in the distal convoluted tubule via a novel molecular mechanism, by deglycosylating and stabilizing the epithelial calcium channel TRPV5 at the cellular membrane surface. Klotho colocalizes with TRPV5 and calbindin-D28K in the distal convoluted and connecting tubule of mouse kidney cells, which are nephron segments responsible for active transepithelial calcium reabsorption. However, the lack of klotho leads to a diminution in the expression of TRPV5 at the cell surface and reduced tubular calcium reabsorption. Likewise, mice lacking TRPV5 have reduced klotho expression and diminished renal calcium reabsorption despite enhanced levels of 1,25(OH)2D3. Although the two proteins together with calbindin-D28K are tightly controlled by vitamin D, suggesting a functional link between these proteins in the maintenance of calcium homeostasis, the renal origin of the hypercalcemia of the klotho null mouse appears to be more unlikely.

**Phosphate Metabolism**

Hyperphosphatemia observed in klotho mutant mice is also principally due to hypervitaminosis D. However, other mechanisms could also be involved because of the major role played by klotho in phosphate metabolism. In this regard, klotho mutant mouse display increased expression and activity of intestinal sodium-dependent phosphate cotransporter type Ib (NPT2b) and increased intestinal phosphate absorption compared with wild-type mice. klotho mutant mice also have increased activity of renal sodium–dependent phosphate cotransporters NPT2a and NPT2c compared with wild-type mice, which corroborates the role played by the kidney, with the increased tubular phosphate reabsorption, in hyperphosphatemia. Moreover, a low phosphate diet results in an increase in renal NPT2a expression in normal mice but not in klotho mice, suggesting that a deregulation of expression and trafficking of NPT2a play a major role in this hyperphosphatemia. Klotho mice have serum FGF23 levels 150- to 2,000-fold higher than wild-type animals, and a low phosphate diet decreases FGF23 levels, which suggests that FGF23 cannot exert its phosphaturic effect correctly in the absence of klotho. The mechanism behind this phenomenon has now been elucidated; klotho binds to FGFR1(IIIc) and its concerted action constitutes the FGF23-specific receptor. Therefore, klotho functions as a cofactor essential for the stimulation of FGFR1(IIIc) by FGF23 and in this way modulates the phosphaturic effect of FGF23.

**Vitamin D Metabolism**

Klotho-deficient mice show low serum 24,25(OH)2D3 and increased 1,25(OH)2D3 concentrations, which are due to an increase in both the renal 25-hydroxyvitamin D-1α-hydroxylase (CYP27b1) and the 24-hydroxylase activities. Of note, in these animals, the normal pathways leading to the upregulation of CYP27b1, such as PTH, calcitonin, and 1,25(OH)2D3, are intact, suggesting the existence of other regulatory pathways. Like 1,25OH2D3, dietary phosphate depletion, a recognized stimulus of CYP27b1 expression, also increases the renal expression of klotho; supporting again the hypothesis that klotho could influence renal CYP27b1 expression.

**PTH Metabolism**

PTH is produced by the parathyroid glands in response to low extracellular ionized calcium concentration and through the stimulation of the G protein–coupled calcium-sensing receptor. However, the molecular mechanisms behind the regulation of PTH secretion by low calcium concentration are not yet fully elucidated and it has been suggested that Na+/K+-ATPase could be one of them. Na+/K+-ATPase is essential in maintaining intracellular Na+ and K+ concentrations and in creating the transmembrane concentration gradient for the transport across the membrane of many substrates. A high Na+ gradient created by the Na+/K+-ATPase activity has been proposed to drive the transepithelial calcium transport in the choroid plexus and the kidney, and probably now in the parathyroid gland. A recent study has demonstrated that klotho interacts physically with Na+/K+-ATPase in parathyroid cells and regulates calcium-stimulated PTH secretion. In addition, klotho mutant mice show a reduction of
27% in the PTH secretion in response to induced hypocalcemia, and the production of PTH by isolated parathyroid cells in a low calcium medium is decreased, similar to the reduction observed in normal parathyroid cells treated by ouabain, a specific inhibitor of Na\(^+\),K\(^+\)-ATPase. However, the addition of ouabain to parathyroid cells from the klotho mutant mice does not further decrease PTH production. These data suggest that klotho may play an important role in the Na\(^+\),K\(^+\)-ATPase-dependent release of PTH; however, the molecular mechanisms for this role of klotho remain to be further investigated.

Klotho may also regulate parathyroid glandular mass and function. This is suggested by the recent observation of elevated circulating klotho levels, hypophosphatemic rickets, and hyperparathyroidism in a young girl with a genomic translocation between chromosomes 9 and 13.\(^{14}\) Interestingly, the increase in klotho level appeared to be sufficient to produce hyperparathyroidism and also PTH-independent hypophosphatemia. As circulating levels of FGF23 were markedly increased in this patient, and given that klotho serves as a cofactor for FGF23, it can be suggested the existence of a very complex interaction between these molecules in the regulation of PTH metabolism.

### Klotho and Longevity

Klotho-deficient mice exhibit a syndrome resembling human premature aging, with multiple pathological phenotypes in tissues including reproductive organs. This phenotype can be rescued by exogenous expression of klotho cDNA\(^{1}\) and, interestingly, klotho gene overexpression extends life span by 20% to 30%\(^{2}\). In humans, in a population-based association study, using two microsatellite markers flanking the klotho gene and DNA sequencing, it was revealed that a functional variant of klotho (KL-VS) was associated with human survival, defined as postnatal life expectancy (>75 years) and longevity.\(^{37}\) In addition, there is a progressive decline with aging of serum klotho levels, as assessed by a recent ELISA using a polyclonal antibody against the C-terminal of human secreted klotho protein.\(^{38}\)

Klotho extends life span by inhibiting the aging process, probably through several mechanisms, including the induction of insulin resistance.\(^{2}\) Indeed, by inducing the inhibition of insulin/IGF-1 signaling, klotho also increases the resistance to oxidative stress at the cellular and subcellular level in mammals. Furthermore, klotho protein activates the FoxO forkhead transcription factors that are negatively regulated by insulin/IGF-1 signaling, thereby inducing expression of manganese superoxide dismutase. This in turn facilitates removal of reactive oxygen species and confers oxidative stress resistance.\(^{32,39}\)

Klotho could also extend life span by protecting the cardiovascular system through endothelium-derived NO production. Many experimental data support this hypothesis: klotho reduces H\(_2\)O\(_2\)-induced apoptosis and cellular senescence in vascular cells\(^{40}\) and the impaired endothelium-dependent vasodilation of the aorta and arterioles of heterozygous klotho-deficient mice are restored by parabiosis with wild-type mice\(^{41}\) or by in vivo klotho gene delivery.\(^{42,43}\) The klotho-induced insulin resistance could prevent cellular lipid overload by reducing insulin-stimulated availability of the lipogenic substrate glucose and thereby could decrease the cellular apoptosis.\(^{44}\)

Finally, the extended life span observed in animal overexpressing klotho may also be due to the reduction of calcitriol synthesis, serum calcium, and phosphorus levels. This is supported by the rescue of the phenotype by removal or reduction of 1,25OH\(_2\)D\(_3\) in FGF23 and klotho mutant mice, by either dietary restriction or genetic manipulation.\(^{30,32}\)

### Conclusion

The discovery of klotho, which is indeed a newly recognized hormone, has opened an extraordinary field of investigation not only because of its implications in human longevity but also because of its implication in a multitude of other biological processes. Although the exact biological role and molecular function of klotho have been only partly deciphered, several functions seem to be clearly established. Klotho is an antiaging molecule, which binds to a not yet identified high affinity receptor and inhibits the intracellular insulin/IGF-1 signaling cascade; klotho functions as a β-glucuronidase that deglycosylates...
steroid β-glucuronidases as well as the calcium channels TRPV5; klotho is a cofactor essential for the activation of FGFRI(IIIc) signalling by FGF23. Circulating klotho plays certainly an important role in a variety of metabolic syndromes including in patients with chronic kidney disease and hyperophosphatemia.

References

KLOTHO IN MINERAL AND VITAMIN D METABOLISM