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Significance of the anti-aging protein Klotho

MIRIBANE DËRMAKU-SOPJANI¹, SELIM KOLGECI², SOKOL ABAZI³ & MENTOR SOPJANI²

¹Department of Chemistry, ²Faculty of Medicine, University of Prishtina, Prishtina, Kosova, and

³Department of Chemistry, University of Tirana, Tirana, Albania

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Abstract

The Klotho gene was identified as an 'aging suppressor' in mice. Overexpression of the Klotho gene extends lifespan and defective Klotho results in rapid aging and early death. Both the membrane and secreted forms of Klotho have biological activity that include regulatory effects on general metabolism and a more specific effect on mineral metabolism that correlates with its effect on aging. Klotho serves as a co-receptor for fibroblast growth factor (FGF), but it also functions as a humoral factor that regulates cell survival and proliferation, vitamin D metabolism, and calcium and phosphate homeostasis and may serve as a potential tumor suppressor. Moreover, Klotho protects against several pathogenic processes in a FGF23-independent manner. These processes include cancer metastasis, vascular calcification, and renal fibrosis. This review covers the recent advances in Klotho research and discusses novel Klotho-dependent mechanisms that are clinically relevant in aging and age-related diseases.

Keywords: Aging, Klotho, fibroblast growth factor, phosphate, calcium homeostasis, parathyroid hormone (PTH), Vitamin D

Introduction

Aging is a complex phenomenon resulting from the interaction between multiple genetic and environmental factors (Tsujikawa et al. 2003, Razzaque et al. 2006). The complex relationship between aging-related genes remains poorly understood. Aging is associated with increased incidence of diabetes, hypertension, and chronic kidney disease (CKD) with impairment of endothelial function (Rakugi et al. 2007, Maekawa et al. 2009) and increased risk of cardiovascular morbidity and mortality (Semba et al. 2011a).

Klotho is a novel anti-aging gene encoding a protein with multiple pleiotropic effects, fortuitously discovered in 1997 by Kuro-o and colleagues, it was named after one of the three Fates in Greek mythology, the goddess who spins the thread of life (Kuro-o M et al. 1997). The Klotho family of proteins consists of three members: α Klotho, β Klotho and γ Klotho. All three are single-pass transmembrane proteins (Kuro-o M et al. 1997). The founder Klotho is designated α Klotho to distinguish it from the other two members.

A defect in mouse Klotho gene expression leads to a syndrome resembling aging, including substantially

decreased lifespan, organ atrophy, infertility, vascular calcification, arteriosclerosis, mitral annular calcification, osteomalacia, hypercalcemia, hyperphosphatemia, osteoporosis, elevated circulating vitamin D hormone, increased peripheral insulin sensitivity and emphysema (Kuro-o M et al. 1997, Utsugi et al. 2000, Yoshida et al. 2002, Manya et al. 2010), whereas overexpression of the gene in mice extends lifespan (Kurosu et al. 2005).

The α Klotho gene is composed of five exons, in humans, mice and rats (Wang and Sun 2009a) expressed predominantly in the distal convoluted tubule (DCT) cells in addition to a lesser expression in the proximal convoluted tubule (PCT) cells of the kidney (Kuro-o M et al. 1997, Hu et al. 2010b) and in the choroid plexus epithelial cells of the brain (John et al. 2011, Zaccchia and Capasso 2011). In addition, but to a lesser extent, α Klotho is also expressed in the pituitary gland, placenta, skeletal muscle, urinary bladder, aorta, pancreas, testis, ovary, colon and thyroid gland (Kuro-o M et al. 1997, Manya et al. 2010, Kuro 2012). Human α Klotho gene (*hKL*) encodes a single-pass transmembrane protein consisting of 1012 amino acids, whereas mouse Klotho (*mKL*) consists of 1014 amino acids

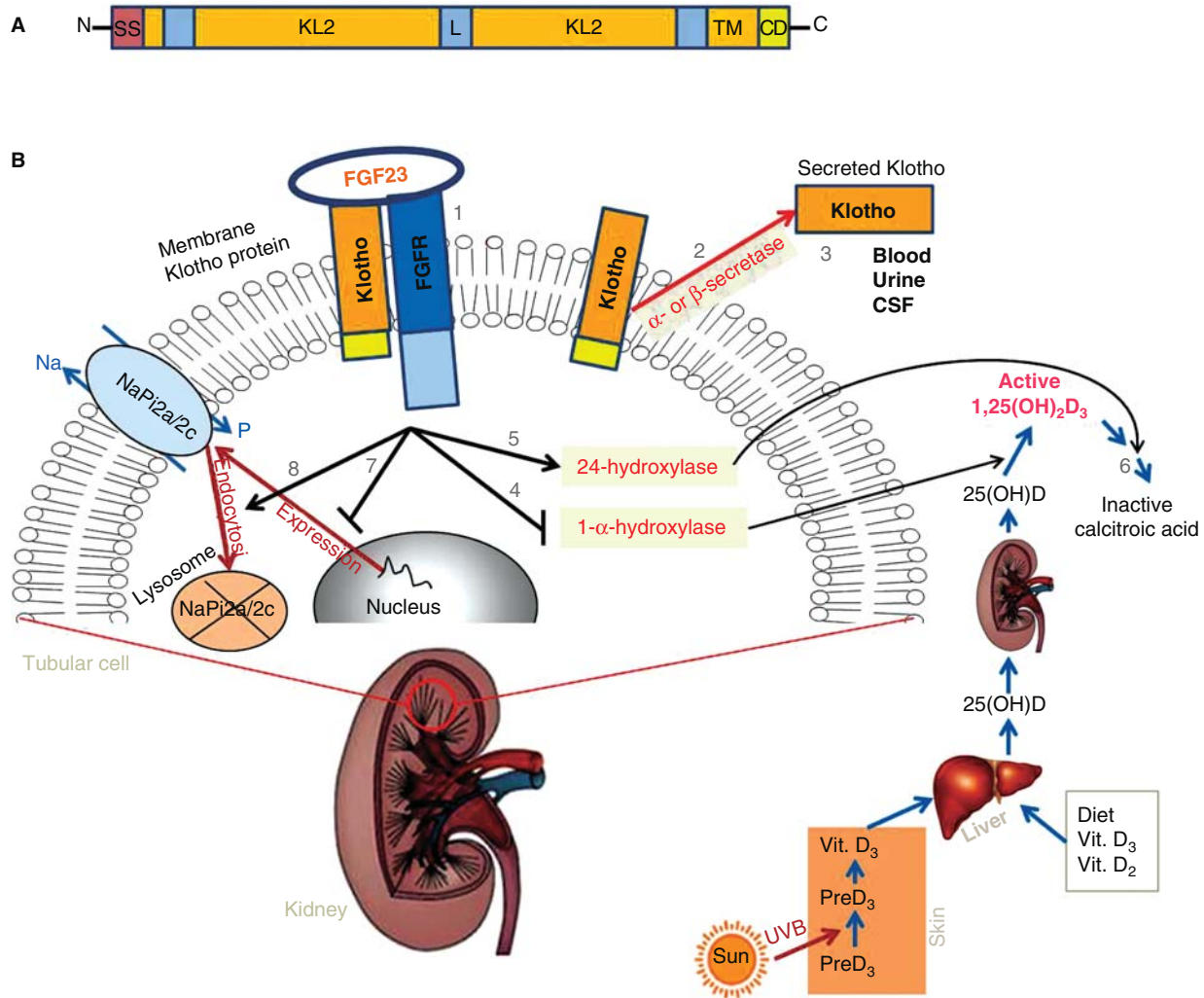


Figure 1. Schematic diagram showing the domain structure, membrane topology and main functions of membrane and secreted Klotho (Jones et al. 2012, Kuro 2012). (A) Human Klotho protein, composed of 1012 amino acids in length, possesses a putative signal sequence (SS) at its N-terminus, two internal repeats (KL1 and KL2) of the extracellular domain that shares sequence homology to the β -glucosidase, linker (L), and a putative transmembrane domain (TM) with a short cytoplasmic domain (CD) at the C-terminus (Not drawn to scale). (B) The transmembrane Klotho forms a complex with the FGFR to create a co-receptor binding site for FGF23 (1), and plays an essential role in renal regulation of phosphate and vitamin D metabolism. Secreted Klotho is created by α - and β -secretases ectodomain shedding of membrane Klotho (2) and released into urine, blood, or cerebrospinal fluid (3). The FGF23/Klotho complex in turn reduces circulating calcitriol ($1,25(\text{OH})_2\text{D}_3$) levels, either by inhibiting the expression levels of enzyme ($1\text{-}\alpha\text{-hydroxylase}$ (*Cyp27b1*)) that participates in calcitriol synthesis (4) or by inducing of another enzyme 24-hydroxylase (*Cyp24a1*) (5) which catalyze the catabolism of calcitriol into inactive form of calcitroic acid (6). Additionally, FGF23/Klotho complex decreases sodium phosphate cotransporters cell membrane expression, either by downregulating their gene expression (7) or by inducing cell membrane protein internalization (8) resulting in their degradation. See text for details. This Figure is to be reproduced in colour in the online version of *Molecular Membrane Biology*.

(Kuro-o M et al. 1997, Tsujikawa et al. 2003). Klotho is composed of three domains with a molecular weight of about 130 kDa: A large extracellular domain with two internal repeats (KL1 and KL2), a transmembrane domain; and a very short intracellular domain with 10 amino acids (Figure 1A). Klotho protein exists in two forms: A membrane and a secreted form (Kuro-o M et al. 1997, Yamamoto et al. 2005). Soluble (secreted) Klotho can arise either by proteolytic cleavage of the Klotho extracellular domain just above the

plasma membrane of the transmembrane form operated by the membrane-anchored proteases ADAM10 and ADAM17, two members of the ADAM (A Disintegrin and Metalloproteinase) family of peptidase proteins (Chen et al. 2007), or as a result of alternative mRNA splicing (isoform 70 kDa) of increased Klotho gene transcription (Matsumura et al. 1998). Secreted Klotho protein is then released from the cell into the extracellular space and is detectable in blood, urine, and cerebrospinal fluid (CSF) (Kuro-o M et al. 1997,

Yamamoto et al. 2005) as well as possibly functioning as an endocrine factor targeting distant organs (Figure 1B). The specific role of ectodomain shedding and alternative splicing to the secreted Klotho protein still remains uncertain. Nonetheless, recent studies have revealed multiple biological functions for membrane and secreted Klotho, these being of importance in several physiology and pathological conditions.

Functions of membrane and secreted Klotho

Studies have shown that the membrane and secreted Klotho have distinct functions (Chen et al. 2007, Cha et al. 2008, Alexander et al. 2009, Camilli et al. 2011, Dermaku-Sopjani et al. 2011, Hu et al. 2011, Kuro 2011, Andrukhova et al. 2012, Anour et al. 2012). The systemic effects of Klotho protein appear to be primarily due to its circulating form of secreted soluble Klotho protein, which operates as a humoral factor (Chang et al. 2005) – potentially through binding to its putative, yet still unknown, cell-surface receptor – and as an enzyme regulating the biological activity of several cell surface glycoproteins and receptors in multiple tissues (Chang et al. 2005, Cha et al. 2008, Hu et al. 2010a, Sopjani et al. 2011, Tang et al. 2011).

The circulating form of Klotho protein in a FGF independent mode regulates, by means of deglycosylation, the activity and membrane abundance of the epithelial calcium (Ca^{2+}) channel, the transient receptor potential vanilloid subtype 5 (TRPV5) in distal tubules of kidney (Chang et al. 2005, Cha et al. 2008), the function of the Na^+ -phosphate transporter type 2 (NaPi2a) in proximal renal tubules (Hu et al. 2010a, Dermaku-Sopjani et al. 2011), the activity of NaPi2b in intestinal brush border membrane (Dermaku-Sopjani et al. 2011), and additionally interferes with insulin/insulin-like growth factor-1 (IGF-1) signalling (Wolf et al. 2008). *In vitro* studies have shown circulating α Klotho to downregulate insulin and IGF-I-induced autophosphorylation of insulin and IGF-1 receptor (Wolf et al. 2008) and subsequently decreasing intracellular oxidative stress (Yamamoto et al. 2005). Inhibiting insulin/IGF-related signaling pathways extend lifespan in a wide variety of species (Yamamoto et al. 2005, Wolf et al. 2008, Li et al. 2012), i.e., in accordance with an anti-aging role for Klotho (Kurosu et al. 2005).

In addition, there are reports suggesting Klotho mediation in parathyroid hormone (PTH) secretion (Yoshida et al. 2002). Studies in mice have shown that isolated parathyroid glands from Klotho-deficient (*Klotho* $-/-$) mice secrete less PTH than wild-type

mice in response to hypocalcemic stimuli in organ culture. This study also provided molecular evidence of α Klotho stimulating cell membrane abundance enhancements of Na^+/K^+ -ATPase in response to hypocalcemic stimuli, indicating involvement of Klotho in PTH secretion in Na^+/K^+ -ATPase-dependent manner. Increased Na^+ gradient by Na^+/K^+ -ATPase might drive the transepithelial transport of Ca^{2+} even as decreased levels of extracellular free calcium rapidly stimulate PTH secretion (Imura et al. 2007). In contrast, another recent *in vivo* study cast doubt on a physiologically relevant role for Klotho, independent of vitamin D and FGF23, in the regulation of glucose metabolism, bone turnover, and steady-state PTH secretion (Anour et al. 2012), suggesting no influence by either the secreted or the transmembrane Klotho on steady-state PTH secretion. However, Klotho role in PTH secretion remains still open, and further studies are needed to clarify this process.

Indeed, secreted α Klotho regulates multiple activities, including suppression of growth factor signaling, regulation of several ion channels, carriers and pumps, and suppression of oxidative stress (Chen et al. 2007, Cha et al. 2008, Wolf et al. 2008, Hsieh et al. 2010, Dermaku-Sopjani et al. 2011, Sopjani et al. 2011, Woudenberg-Vrenken et al. 2012). Some of these effects will be discussed below in detail.

The other form of Klotho, i.e., transmembrane Klotho protein, forms a constitutive binary complex with multiple FGF receptors and works as an obligatory co-receptor for FGF23, a bone-derived hormone (Kurosu et al. 2006, Urakawa et al. 2006), that regulates phosphate excretion into urine (Dermaku-Sopjani et al. 2011) as well as synthesis of the active form of vitamin D in the kidney (Imai et al. 2004). The fact that FGF23 requires cell membrane Klotho to bind for activating FGF receptors explains why *Klotho* $-/-$ mice and FGF23 deficient (*FGF23* $-/-$) mice develop many identical phenotypes (Tsuji-kawa et al. 2003, Razzaque et al. 2006). In addition, *Klotho* $-/-$ mice express higher levels of serum FGF23, hence the inability to promote any phosphaturic effect in the absence of Klotho (Kurosu et al. 2006). Furthermore, increased sodium-dependent phosphate cotransporters activity in both FGF23 and Klotho-mutant mice enhanced phosphate reabsorption in the kidney, thus facilitating calcification. The observed hyperphosphatemia is caused by hypervitaminosis D and increased renal expression/activity of sodium phosphate cotransporters (Kurosu et al. 2006). Vitamin D restriction partially reverses the accelerated aging and early death of *Klotho* $-/-$ mice (Dermaku-Sopjani et al. 2011).

Soluble Klotho also inhibits the highly homologous and ubiquitously expressed transmembrane type III sodium dependent phosphate cotransporters (Pit1 and Pit2) that mediate phosphate uptake (Hu et al. 2010a). Klotho decreases the maximal transport rate of both carriers NaPi2a (*SLC34A1*), expressed on the brush border membrane of renal tubular cells, and NaPi2b (*SLC34A2*), expressed in intestinal cells. The effect was mimicked by the Klotho protein, which is known to be effective as protease (Kuro-o M 2010). Klotho resembles β -glycosidase protein in terms of β -glucuronidase activity (Kuro-o M et al. 1997, Tsujikawa et al. 2003), and its effect on NaPi2a could be reversed by a β -glucuronidase inhibitor (Hu et al. 2010a, Dërmaku-Sopjani et al. 2011).

It has been shown that Klotho/FGF23 complex signaling induces phosphaturia by suppressing the expression of NaPi2a. Moreover, soluble circulating Klotho protein has also been found to promote phosphaturia independently of FGF23, this by directly downregulating renal phosphate transport via deglycosylation of NaPi2a cotransporters in the proximal tubule (Hu et al. 2010a). In this line, Klotho deficiency promotes a state of hyperphosphatemia-induced accelerated aging.

Klotho and intracellular signaling pathways

Several Klotho biological roles have been determined, but the underlying molecular mechanisms are not fully understood and have yet to be clarified. However, many studies have revealed lots of Klotho novel effects to be involved in different intracellular signaling pathways, including: insulin/IGF-1 (Yamamoto et al. 2005), cAMP (Rakugi et al. 2007), PKC (Imai et al. 2004), p53/p21 (Ikushima et al. 2006), and Wnt (Liu et al. 2007) signaling pathway. Most of these signaling pathways involving Klotho are reviewed elsewhere (Wang and Sun 2009a).

FGF23-Klotho complex

FGF23 is a member of the FGF19 subfamily of endocrine fibroblast growth factors, its gene (which exceeds over 8.5 kb) is located in chromosome 12 in humans, and chromosome 6 in mice, encodes a protein composed of 251-amino acids (approx. 32 kDa) with a N-terminal fragment binding domain for the FGF receptor (FGFR) and a novel C-terminal end binding domain for Klotho (Kurosu et al. 2006, Urakawa et al. 2006, Faul et al. 2011).

In humans and mice are found four distinct genes encoding FGFRs (FGFR1-4) (Itoh and Ornitz 2004).

The ectodomain of prototypical FGFRs consists of three immunoglobulin (Ig)-like domains (D1, D2, and D3). Alternative mRNA splicing occurs within the D3 of FGFR 1-3, generating 'b' and 'c' receptor isoforms (FGFR2b and FGFR2c, respectively) with distinct FGF-binding specificities. The unique features of FGF23 require a binary complex of Klotho co-receptor proteins and FGFRs for FGFR activation, thus increasing their affinity to bind circulating FGF23. The α Klotho has different FGFRs binding abilities, including lower affinity to FGFR2 than to the other FGFRs. The FGF23 COOH-terminal domain forms a tertiary complex with α Klotho and the 'c' isoforms of FGFR 1, 3, and 4, reaching its maximum cellular signaling when FGF23-FGFR1c- α Klotho complex is formed (Bai et al. 2004, Kurosu et al. 2006, Urakawa et al. 2006, Ho et al. 2009). As part of a tertiary complex, FGF23 initiates downstream signaling pathways through a variety of intracellular signal transduction proteins, including ERK1/2-SGK1, FRS2, Gab1, Shc, PLC, or STAT1, leading to a potential mechanism for differential and/or gene expression (Urakawa et al. 2006, Sitara et al. 2008, Wolf et al. 2008, Ho et al. 2009, Andrukhova et al. 2012).

FGF23 is predominantly expressed by osteocytes and osteoblasts in bone (which are main sources of FGF23), but it is also expressed in lower concentrations by other tissues, including the thymus, brain, heart, skeletal muscle, spleen, skin, lung, testes, liver, and the mammary and salivary glands (Liu et al. 2003, Urakawa et al. 2006, Sitara et al. 2008, Martin et al. 2012). Synthesis and secretion of FGF23 by osteocytes is upregulated by PTH, 1,25(OH)(2)D as well as dietary and serum phosphate levels (Liu et al. 2006). FGF23 is also downregulated, via still unclear mechanisms, by the dentin matrix protein 1 (DMP1) as well as by a phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX) (Figure 2) (Liu et al. 2008). However, studies using PHEX-deficient mice have demonstrated that PHEX deficiency is necessary, albeit insufficient, to induce FGF23 gene transcription in osteocytes (Sitara et al. 2004, Liu et al. 2006).

FGF23 mutant animals were used to identify the causal role of FGF23. In rodents, FGF23 overexpression or its parenteral administration suppresses both biosynthesis of 1,25(OH)2D and the phosphate reabsorption in the proximal tubules of the kidney (Bai et al. 2004, Shimada et al. 2004b). Conversely, *FGF23* $-/-$ mice or homozygous missense mutation in human FGF23 have shown significantly higher levels of serum 1,25(OH)2D3, leading to severe hyperphosphatemia and causing soft-tissue calcification (Kurosu et al. 2006, Liu et al. 2006, Razzaque et al.

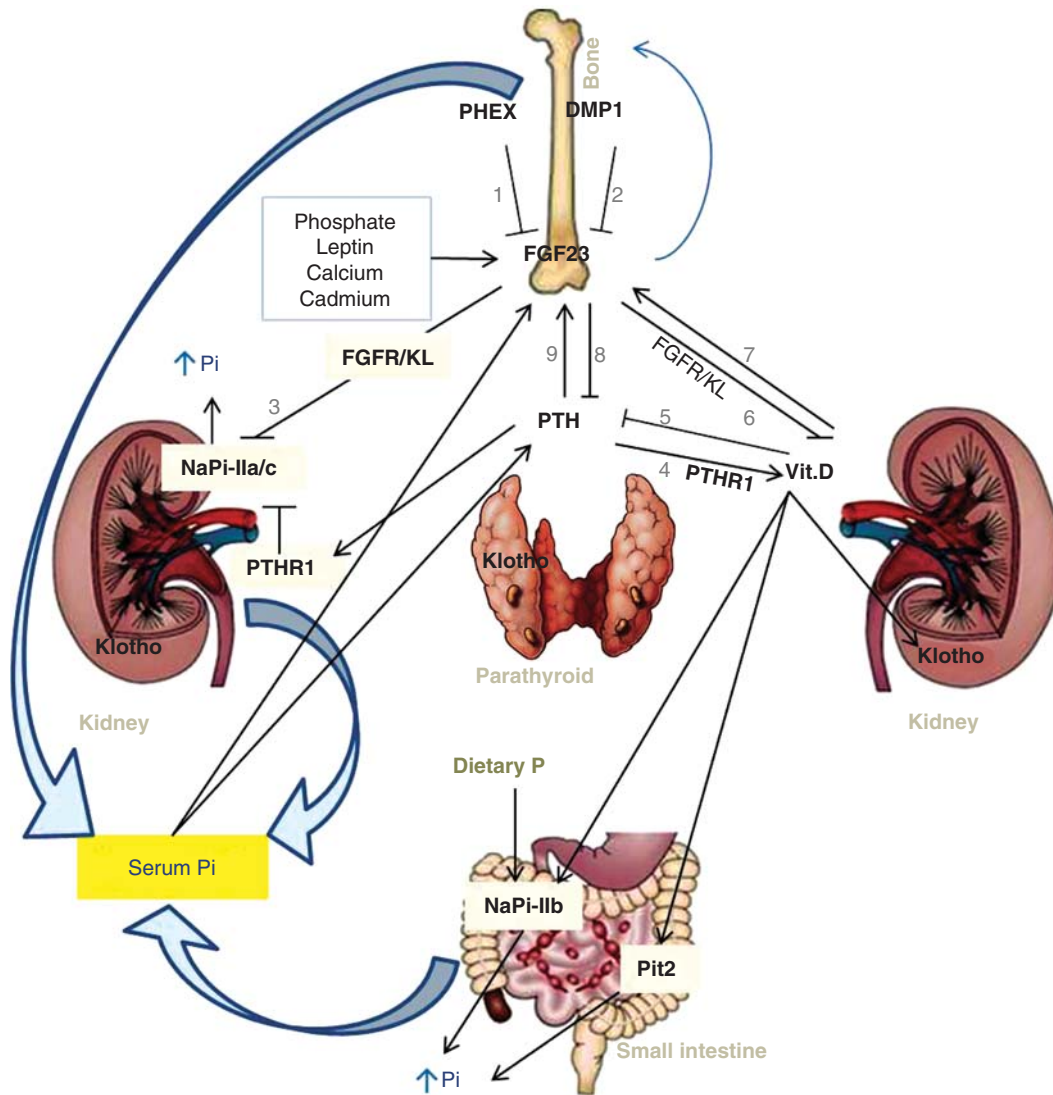


Figure 2. Multi-organ interaction in regulatory mechanisms of phosphate homeostasis (Fukumoto and Yamashita 2002, Wang and Sun 2009a, Blaine et al. 2011, Kuro 2012, Sopjani et al. 2011). Bone proteins, phosphate-regulating neutral endopeptidase (PHEX) (1) and dentin matrix protein-1 (DMP1) (2), downregulate FGF23 activity via still unclear mechanisms. FGF23 is produced in the bone cells and influences bone mineralization. The emerged FGF23-Klotho system is of great importance as the principal phosphate-regulating endocrine axis. FGF23, via FGF23/Klotho pathway, suppresses renal cotransporters NaPi2a and NaPi2c (3) resulting in decreased reabsorption and therefore increased urinary phosphate. PTH, synthesized and secreted by parathyroid glands, stimulates renal synthesis of vitamin D (1,25-dihydroxyvitamin D3) mediated through a PTH receptor (PTHr1) (4). PTH promotes renal phosphate excretion and mobilizes phosphate from the bone. Calcitriol in turn decreases PTH production (5), thus closing a negative feedback loop. FGF23 is secreted from bone cells and acts, independently of PTH, to suppress renal vitamin D synthesis (6). Since vitamin D increases FGF23 expression in bone (7), another endocrine negative feedback loop exists between bone (FGF23) and kidney (calcitriol). FGF23's effect on vitamin D metabolism results in decreased entry of phosphate into the circulation from intestine. Vitamin D induces both FGF23 and Klotho. Likewise, vitamin D can also induce transcriptional upregulation of NaPi2b and Pit2 to increase intestinal phosphate absorption, this leads to increased serum phosphate levels. FGF23 acts directly on the parathyroid to reduce serum PTH levels (8). Because PTH stimulates FGF23 gene expression (9), another negative feedback loop exists between PTH and FGF23. Pi diet results in upregulation of NaPi2b. See text for further details. This Figure is to be reproduced in colour in the online version of *Molecular Membrane Biology*.

2006, Sitara et al. 2006) – in effect indicating numerous FGF23-dependent biological functions.

Membrane expression of both FGFR and Klotho form a higher affinity FGFR- α Klotho necessary cofactor complex for FGF23 and defines the target organs for FGF23 (Kurosu et al. 2006). The

important role of α Klotho membrane protein in FGF23 signaling has been confirmed in both human and mouse genetic disorders where the loss of α Klotho results in end-organ resistance to FGF23 and in abnormalities resembling those of FGF23 deficiency (Kuro-o M et al. 1997,

Ichikawa et al. 2007, Imura et al. 2007, Segawa et al. 2007).

Several findings confirmed a role for putative humoral factors, known as phosphatonins, in regulating of circulating phosphate concentrations independently of calcium (Bai et al. 2004, Sitara et al. 2004). The best characterized of these is phosphatonin FGF23, known to be causally implicated in developing of severe hypophosphatemic diseases, including X-linked dominant hypophosphatemic rickets/osteomalacia (XLH), autosomal dominant hypophosphatemic rickets/osteomalacia (ADHR) and tumor-induced rickets/osteomalacia (TIO) (Fukumoto and Yamashita 2002, Shimada et al. 2004b, Sitara et al. 2004). In this line, many studies have revealed that FGF23 excess is characterized by aberrant vitamin D metabolism, hypophosphatemia, severe growth retardation, and rickets/osteomalacia (Fukumoto and Yamashita 2002, Bai et al. 2004, Larsson et al. 2004), whereas FGF23 ablation in mice results in higher serum phosphate, excess 1,25(OH)₂D activity, and extensive soft tissue calcification (Liu et al. 2006, Razzaque et al. 2006). The FGF23 act as counter regulatory phosphaturic hormone for vitamin D; hence its excess leads to a reduction in circulating 1,25(OH)₂D levels by regulating expression levels of vitamin D-synthesis/degradation renal enzymes, downregulating renal enzyme 25-hydroxyvitamin D 1- α -hydroxylase (*Cyp27b1*) participating in the production of 1,25(OH)₂D, and upregulating 1,25-dihydroxyvitamin D 24-hydroxylase (*Cyp24a1*) catalyzing the catabolism of 1,25(OH)₂D into calcitric acid in the kidney tubules (Perwad et al. 2007), as shown in Figure 1. Hypophosphatemia resulting from the FGF23 excess is mediated by downregulating expression of sodium-dependent phosphate cotransporters, NaPi2a and NaPi2c, in the kidney-proximal tubules (Gattineni et al. 2009), consequently increasing phosphate excretion in the urine. The Klotho/FGFR complex is necessary for eliciting the FGF23-mediated signaling (Bergwitz and Juppner 2010).

Klotho in the regulation of calcium and phosphate homeostasis and toxicity

Klotho in calcium homeostasis

Blood calcium concentration is maintained within a very narrow range at all times despite large variations in episodic food intake and body demand (Chang et al. 2005). Deregulated calcium homeostasis, as one of the observed aging-like phenotypes of *Klotho* $-/-$ mice, has been attributed due to altering of the vitamin D metabolism, but recent studies have indicated alternative

possibilities (Imura et al. 2007) with Klotho being a potential candidate. It has been reported that, in *Klotho* $-/-$ mice, raised Ca^{2+} serum levels, hypercalciuria, and the reduction of vitamin D serum levels by dietary restriction reverts most, but not all, of their abnormal phenotypes, suggesting that they are downstream signaling events resulting from increased vitamin D (Tsujikawa et al. 2003). The role of Klotho, as a necessary cofactor for transducing the FGF23 signal through the FGFR in vitamin D homeostasis regulation, is one explanation. As illustrated in Figure 2, circulating levels of 25(OH)D are directly related to dietary vitamin D intake and exposure of the skin to sunlight. In response to ultraviolet B sunlight exposure, pro-vitamin D₃ (7-dehydrocholesterol) in the skin epidermal basal layers is converted to pre-vitamin D₃ (pre-D₃), and then to vitamin D₃ (cholecalciferol, D₃) (Alexander et al. 2009, Haussler et al. 2012). Vitamin D₃ is transported to the liver where it is hydroxylated by liver 25-hydroxylases (25-OHase). The 25-hydroxycholecalciferol (25(OH) D₃) is 1 α -hydroxylated in the kidney by 25-hydroxyvitamin D₃-1-hydroxylase (1 α -OHase). The resulting active 1,25(OH)₂D₃ (calcitriol) form has different biological effects on various target tissues (Razzaque et al. 2006, Haussler et al. 2012, Woudenberg-Vrenken et al. 2012). Hence it is that Klotho, by suppressing *cyp1b* enzyme expression-which mediates the conversion of 25-hydroxyvitamin D into 1,25(OH)₂D₃ (calcitriol, active form of vitamin D) apart from stimulating another renal enzyme, *cyp27b1* (Perwad et al. 2007, Cha et al. 2008, Woudenberg-Vrenken et al. 2012), that catalyzes the calcitriol hydrolysis-particularly inhibits vitamin D synthesis, and induce its degradation respectively (Yoshida et al. 2002, Tsujikawa et al. 2003). The latter enzyme is increased in *Klotho* $-/-$ mice (Woudenberg-Vrenken et al. 2012). Conversely, the absence of Klotho results in modulation of calcitropic hormones, specifically increased serum levels of vitamin D, and decreased serum concentrations of PTH (Yoshida et al. 2002, Ohnishi et al. 2009). This would cause intestinal hyperabsorption of calcium, raised resorption of Ca^{2+} from bone, resulting in higher Ca^{2+} serum levels and therefore increased renal Ca^{2+} excretion in urine.

Several earlier studies on experimental animals confirmed that both Ca^{2+} and vitamin D can reduce PTH secretion via calcium-sensing receptors (CaSR) (Mizobuchi et al. 2004) as well as by interacting with vitamin D receptor (VDR) (Rodriguez et al. 2007). A complementary hypothesis, involving calcium channels regulated by Klotho, is consistent with this idea (Cha et al. 2008). Moreover, Klotho act as a β -glucuronidase that activates TRPV5 through hydrolyzing extracellular N-linked oligosaccharides residues on TRPV5, entrapping the channel in the

cell membrane and therefore increasing Ca^{2+} influx in renal cells (Chang et al. 2005, Lu et al. 2008). *Klotho* upregulation of the cell membrane TRPV5 expression was confirmed also in *in vitro* studies (Cha et al. 2008). The TRPV5 channel is mainly expressed at the apical membrane of renal epithelial cells in the DCT, and is the main channel for transcellular absorption of calcium (Lu et al. 2008). Thus, deregulation in tubular Ca^{2+} handling might cause calcitriol hypervitaminosis and renal Ca^{2+} wasting, due to disturbed calcium reabsorption as displayed in *Klotho* knockout mice (Woudenberg-Vrenken et al. 2012). The renal Ca^{2+} wasting in *Klotho* $-/-$ mice was determined by modulating the diet, and was found not to be secondary to hypercalcemia and/or hypervitaminosis, a result that underlines the relevance of *Klotho* to the prevention of renal Ca^{2+} loss, secondary hypervitaminosis D, osteopenia, and nephrocalcinosis (Alexander et al. 2009). Similar to its effect on TRPV5, *Klotho* significantly increases the activity of TRPV6, which predominantly mediate Ca^{2+} absorption in the intestine (Lu et al. 2008), further substantiating the role of *Klotho* in maintaining Ca^{2+} homeostasis.

Another very recent study, carried out in double-knockout mice for *Klotho* and for *cyp27b1*, showed that *Klotho* $-/-$ *cyp27b1* $-/-$ mice have phenotypes resembling *cyp27b1* $-/-$ mice, including significantly decreased serum total Ca^{2+} concentrations and normal urinary total Ca^{2+} excretion as compared to wild-type mice. Due to the fact that calcitriol is absent in these mice, the results imply that Ca^{2+} homeostasis in *Klotho* $-/-$ mice is affected by their excessive calcitriol levels (Woudenberg-Vrenken et al. 2012). Thus, demonstrated interplay between *Klotho* and vitamin D and their relative role to the Ca^{2+} homeostasis. *Klotho* is also an important regulator of phosphate metabolism, and when phosphate dysregulation occurs that is primarily responsible for the aging-related phenotypes.

Klotho in phosphate metabolism

Phosphate is a widely distributed mineral ion in the body, being found mainly (app. 80%) in bone and teeth as apatite. To a lesser extent, phosphate is also present in the viscera and skeletal muscle; but also, in very small amounts (<0.1%), in extracellular fluids (Murer and Biber 2010, Ohnishi and Razzaque 2010, Quarles 2012). Proper balance is an essential prerequisite for basic cellular functions and is, moreover, necessary for several processes, including cell signaling, energy metabolism, membrane integrity and transport, lipid metabolism, enzyme activity, nucleic acid synthesis, muscle function and bone mineralization (Berndt and

Kumar 2009, Gattineni et al. 2009, Kempe et al. 2010, Ohnishi and Razzaque 2010). Intracellular phosphate ions are stored mainly in the mitochondria (app. 20%), and ER (endoplasmic reticulum, app. 30%), and are used for oxidative phosphorylation, and for phosphorylation of various proteins. The remaining part of cellular phosphate is located in the nucleus, Golgi complex and lysosomes (Di Marco et al. 2008, Kempe et al. 2010). Not yet fully understood, however, are the exact molecular mechanisms behind the transporting of phosphate in and out of the cell according to the body's needs.

Phosphate is kept in a relatively narrow serum range, with a tightly controlled phosphate balance maintained through hormonal and complex cross-organ communication including the intestine, kidneys, parathyroid glands, and the skeleton, as shown in Figure 2 (Mizobuchi et al. 2004, Liu et al. 2006, Jin et al. 2009, Suyama et al. 2012). Despite nearly 70% of dietary phosphate being absorbed by the intestine, causing transient postprandial increases in the serum concentration of phosphate (Sabbagh et al. 2009), there are many factors influencing phosphate intestinal absorption or its renal reabsorption that are presented elsewhere (Berndt and Kumar 2009).

In general, the body's daily phosphate needs are ensured through intestinal tract absorption from consumed foods, whereas the delicate maintenance of serum phosphate levels is mainly regulated by renal reabsorption/excretion of phosphate through renal phosphate transporters. In addition, bone can also be implicated in phosphate homeostasis by providing phosphate all-out promoting skeletal resorption (Sitara et al. 2008, Sabbagh et al. 2009, Kempe et al. 2010, Dermaku-Sopjani et al. 2011, Foller et al. 2011).

Phosphate absorption

Studies suggest that intestinal phosphate absorption occurs via two pathways: A paracellular transport mechanism driven by passive diffusion that predominates under standard dietary conditions; and a transcellular transport mechanism driven by an active transport through the sodium-dependent phosphate cotransporters (Kempe et al. 2010, Bhandaru et al. 2011, Dermaku-Sopjani et al. 2011, Sabbagh et al. 2011) that constitute the predominant pathway in the intestine (Sabbagh et al. 2009). Sodium-dependent phosphate cotransporters, NaPi2b, Pit1 and Pit2 are localized in the intestine; however, NaPi2b predominates in active transport and, based on *in vitro* analysis performed on ileum segments isolated from wild-type or *NaPi2b* $-/-$ mice, accounts for more than 90% of sodium-phosphate absorption in ileum (Segawa et al. 2007, Sabbagh et al. 2009). Although NaPi2b reaches

maximal expression in the ileum, it is also present in duodenum and jejunum (Sabbagh et al. 2009).

Previous studies have found that both dietary phosphate and calcitriol are involved in NaPi2b activities, thereby regulating intestinal phosphate absorption (Hattenhauer et al. 1999, Katai et al. 1999). For instance, low-phosphate diet, by inducing the 1α (OH)ase renal expression, can upregulate circulating levels of calcitriol, which can, in turn, induce intestinal NaPi2b protein expression in the epithelial cells of the small intestine, resulting in increased intestinal phosphate absorption (Katai et al. 1999). In that regard, *NaPi2b* $-/-$ mice were observed to exhibit approx. 50% less phosphate absorption, compared with wild-type mice, after being fed a low phosphate diet following acute administration of phosphate, thus demonstrating the significant role of NaPi2b in both intestinal phosphate absorption and systemic phosphate homeostasis (Sabbagh et al. 2009). Nevertheless, studies in humans demonstrated that mutations inactivating the human gene *SLC34A2*, encoding NaPi2b, did not show any significant decline in serum phosphate levels (Corut et al. 2006). Thus, further studies are needed to clarify the exact role of NaPi2b in systemic phosphate homeostasis in humans. Despite the importance of phosphate intestinal absorption, its renal reabsorption is critically implicated in maintaining appropriate phosphate homeostasis.

Phosphate reabsorption

The kidney plays a key role in ensuring phosphate balance through renal excretion and reabsorption of phosphate. Its function is to regulate phosphate excretion in the urine and phosphate reabsorption mainly in the kidney proximal tubules via a transcellular pathway, hence the relatively narrow serum phosphate concentration range (Segawa et al. 2007, Andrukhova et al. 2012) that is physiologically important in numerous biological processes, including aging. Three types of sodium-dependent phosphate cotransporters (Bergwitz and Juppner 2010) in proximal tubular cells have been identified: type 1 Na^+ -dependent phosphate transport protein 1, NPT1 (encoded by the *SLC17A1* gene), which is a non-specific phosphate carriers that mediates transport of phosphate and other anions; type 2 family, NPT2, which includes three carriers: NPT2a [NaPi2a) encoded by the *SLC34A1* gene], NPT2b [NaPi2b) encoded by the *SLC34A2* gene] (Xu et al. 1999, Nowik et al. 2008), and NPT2c [NaPi2c) encoded by the *SLC34A3* gene] (Villa-Bellosta and Sorribas 2010); and the type 3 family which comprises of two transporters, PiT1 (*SLC20A1*) (Nowik et al.

2008) and PiT2 (*SLC20A2*) (Villa-Bellosta and Sorribas 2010). NaPi2a is almost exclusively located in the apical membrane of renal proximal tubule cells, and plays a central role in the reabsorption and phosphate balance, while its highly homologous isoform NaPi2c is almost exclusively expressed in the brush-border membrane of the kidney-proximal tubular cells (Sitara et al. 2008). NaPi-dependent phosphate reabsorption is regulated by several molecules. For instance, PTH is a strong inhibitor of NaPi transporters, causing increased renal phosphate excretion in urine (Zhao and Tenenhouse 2000). In the same line, studies have shown that FGF23 and Klotho can also decrease NaPi activities (Kurosu et al. 2006, Hattenhauer et al. 1999, Dërmaku-Sopjani et al. 2011, Gattineni et al. 2009). Renal phosphate reabsorption is dependent on phosphate transporters expression and activities. Many regulators play a significant role in transporters (Bhandaru et al. 2011, Foller et al. 2011), including the amount of dietary phosphate content (Gattineni and Baum 2010). For instance, acute administration of low dietary phosphate stimulates NaPi cotransport activity as well as a protein abundance that occurs independently of *de novo* protein synthesis, this is a result of cellular mechanism mediated by microtubules (tubulin)-facilitating translocation of presynthesized NaPi2a cotransporter protein from intracellular compartments to the apical cell membrane (Lotscher et al. 1997). The most recent data confirmed upregulation of NaPi2b in both the renal cortex and medulla, in response to the active excretion of phosphate in high phosphorus diet-fed rats (Suyama et al. 2012). These results though are not thought to be necessarily contradictory. In fact, the kidney NaPi2b is mainly expressed on the basolateral side of epithelial cells, while phosphate is transported from the outside of the cell to the interior in both cases, therefore increasing levels of basal phosphate excretion in the kidney (Suyama et al. 2012). Within this context, the FGF23-Klotho system provides important mechanistic insights into phosphate homeostatic control (Kurosu et al. 2006), and its manipulation may be used in developing novel strategies to treat diseases associated with abnormal mineral ion metabolism. In addition, an important factor for phosphorus balance is the bone as a major store of both phosphorus and calcium.

Bone in phosphate homeostasis

The skeleton serves multiple functions: Aside from being production site for the major phosphate regulatory hormone FGF23, it is also major storage pool

for phosphate (Sitara et al. 2008). Presence of phosphate is essential for normal bone growth, development and mineralization, while an insufficiency thereof inhibits bone formation and induces rickets and osteomalacia, i.e., undermineralized soft bones (Liu et al. 2003, 2006, 2008, Shimada et al. 2004b, Perwad et al. 2007). Maintenance of adequate inorganic phosphorus concentrations is essential for the osteoblasts and osteocytes activity in the process of extracellular matrix mineralization (Sitara et al. 2008). In that process, phosphorus along with calcium are the main ionic components required for hydroxyapatite formation (Kuznetsov et al. 2000). The enzyme alkaline phosphatase, located at the osteoblast cell membrane with its catalytic unit oriented towards the outside of the plasma membrane, can generate sufficient free minerals by cleaving a phosphorus from beta-glycerol phosphate, allowing free phosphorus via Na^+ -dependent phosphate cotransporters to enter the cell, where its intracellular concentrations is then maintained within a narrow range (Murshed et al. 2005). Lowered serum phosphate levels join with rising alkaline phosphatase activity in bone to impart more phosphate into the bone cells, and may initiate mineralization; moreover, when appropriate phosphate levels are reached the enzyme's activity lessens, as a result of which monitoring bone-specific alkaline phosphatase activity can also be used as a marker indicator of bone phosphate homeostasis and for obtaining information about bone metabolism and bone diseases (Haraikawa et al. 2012). Increased vitamin D levels induce FGF23 production by osteoblasts, which, in turn, protects those cells from higher phosphorus concentrations that may, in practise, lead to cell death (Murshed et al. 2005, Sitara et al. 2008). However, the detailed mechanisms by which these effects on bone are mediated are unknown.

Klotho in phosphate toxicity

Maintaining the physiological balance of phosphate in the body is of key biological significance (Sitara et al. 2008). Excessive retention of phosphate in the body can lead to numerous cellular and tissue injuries and disease. Among these are: Vascular calcification in patients with CKD (Koh et al. 2001), independent risk factors for renal function weakening and high mortality in CKD patients (Voormolen et al. 2007), cardiovascular calcification in *Klotho* $^{-/-}$ mice (Kurosu et al. 2006), premature infertility (Ohnishi and Razzaque 2010), growth of lung tumors in humans mediated by Akt-signalling network, therefore stimulating lung tumorigenesis (Jin et al. 2009),

reduced bone mineralization in rats (Amato et al. 1998), a stimulated apoptosis rate in various tissues (Sugiura et al. 2005, 2010, Ohnishi and Razzaque 2010), impaired cell signalling pathways leading to cellular and tissue damage (Jin et al. 2009, Sugiura et al. 2012), accelerating the aging process in mammalia by causing tissue damage and reducing their cell survival (Ohnishi and Razzaque 2010). Furthermore, phosphate-containing enema can also cause health complications in human patients and animals due to phosphate toxicity from higher serum phosphate levels (Grosskopf et al. 1991).

The exact mechanism of phosphate toxicity in mammalian aging is not clearly defined. However, *Klotho* $^{-/-}$ mice have been shown to increase renal activity of NaPi2a , causing phosphate toxicity to develop that induced premature aging-like features, whereas genetically modified *NaPi2a/Klotho double* $^{-/-}$ mice reduces phosphate toxicity and extends their survival (Ohnishi and Razzaque 2010). In addition, phosphate toxicity can exert cytotoxic effects on various organ systems, compromising their functional ability and ending in dysfunction (Osuka and Razzaque 2012). Thus, identifying factors that regulate phosphate homeostasis can provide a potential therapeutic tool for the controlling of phosphate homeostasis misregulation-dependent diseases (and so of mammalian aging). In this line-up, *Klotho* is an important regulator of phosphate balance.

Clinical relevance of Klotho

Current information suggests that *Klotho* protein has pleiotropic functions, including an emerging role in cardio-renal disease. The expression of the *Klotho* gene is influenced by many physiological and pathological factors (Aizawa et al. 1998). Decreased plasma, urinary and renal *Klotho* levels and increased mortality risk are associated with normal aging (Semba et al. 2011a, 2011b), kidney disease (Satoh et al. 2012), animal models of diabetes, and hypertension diseases (Semba et al. 2011a). Conversely, overexpression of *Klotho* in animals leads to reversal of the aging process, hence to a substantial extension of lifespan, and additionally provides cardiovascular and renal protection (Wang and Sun 2009b), possibly by protecting tissues from oxidative stress damage and by inducing resistance to oxidative stress (Mitobe et al. 2005). *Klotho* clearly has an emerging clinical relevance, especially in nephrology. The expression of the *Klotho* gene, as a key player for phosphate balance in the kidney, is decreased in the distal tubules, blood, and urine of rats subjected to bilateral renal ischemia (Hu et al. 2010b). Animal

Table I. Summary of the partial diseases/conditions owing to dysregulation of Klotho.

Condition/disease	Decreased Klotho activity	
	Species	References
Ageing	Mouse/human	(Hu et al. 2010a, 2010b, 2011, Nagai et al. 2000)
Hypertension	Rat	(Aizawa et al. 1998, Wang and Sun 2009b)
Heart damage	Rat	(Li et al. 2012)
Atherosclerosis	Rat	(Saito et al. 2000, Ikushima et al. 2006)
Anemia	Mouse	(Kempe et al. 2009).
Diabetes	Mouse/rat	(Aizawa et al. 1998, Semba et al. 2011a)
Osteoporosis	Mouse	(Kuro-o M et al. 1997, Sitara et al. 2008)
Renal fibrosis	Mouse	(Satoh et al. 2012)
Acute kidney injury	Mouse/Human	(Hu et al. 2010b)
Chronic kidney disease	Mouse/human	(Koh et al. 2001, Watanabe et al. 2013)
Kidney ischemia	Mouse/rat	(Shimada et al. 2004a, Hu et al. 2010b)
Glomerulonephritis	Mouse	(Haruna et al. 2007)
Renal cell carcinoma	Human	(Zhu et al. 2013)
Gastric cancer	Human	(Xie et al. 2013)
Hepatocellular carcinoma	Human	(Poh et al. 2012, Chen et al. 2013)

studies indicate that the renoprotective effects of Klotho are mostly due to the antioxidant effects of its secreted form (Yamamoto et al. 2005). Studies of Klotho conducted on humans have reported a significant reduction in soluble Klotho expression in the kidneys of patients with CKD, as compared to healthy control (Koh et al. 2001). In the same type of patients, Klotho expression is also decreased in several tissues, contributing to the calcification of soft tissues (Hu et al. 2011). Similarly, a reduction of Klotho protein levels in urine has been observed in acute kidney injury (AKI), while Klotho replacement has protective effects on kidney damage and promotes recovery in AKI (Hu et al. 2010b). Decreased urinary (Hu et al. 2011) and serum levels of Klotho is an early clinical biomarker for CKDs (Shimamura et al. 2012) and for AKI induced by ischemia reperfusion injury (IRI) (Hu et al. 2010b). In experimental ischaemic AKI mice, Klotho reduces apoptosis via stimulating the expression of heat shock protein 70 (HSP70) in mouse kidneys and HK2 cells, and therefore involved in the pathophysiology of IRI (Sugiura et al. 2010). Recently, it has been claimed that replacement of Klotho may potentially be a therapeutic agent for CKD (Hu et al. 2011), since its renal expression is markedly suppressed in CKDs. Experiments performed on mice (Sugiura et al. 2012) and cultured renal cell lines (Doi et al. 2011) suggest that decreased renal Klotho expression is the result of induced renal fibrosis (RF), a final common pathology of CKD, induced by enhancing fibrosis marker activity via, e.g., transforming growth factor- β (1) [TGF- β (1)], which, in turn, reduces renal Klotho expression (Sugiura et al. 2012). Moreover, when overexpressed, Klotho protein binds directly to the type II receptor

(TGF β R2) and subsequently inhibits type I receptor (TGF β R1) activation that phosphorylates Smad2/3 transcription factors, which then regulates expression of TGF- β 1 target genes in nucleus (Doi et al. 2011, Sugiura et al. 2012). Hence, by inhibiting TGF β 1 signalling (Doi et al. 2011), Klotho overexpression increases resistance to RF in mice, even as it suppresses RF caused by chronic glomerulonephritis (Haruna et al. 2007). The addition of Klotho protein decreased mitochondrial DNA damage as well as oxidative stress in renal tissues. Importantly, ameliorations were observed in both the renal tubules and glomerulus, although Klotho exclusively expresses to the tubules, so confirming the Klotho protein effect as a circulating hormone (Haruna et al. 2007). Another study in mice confirmed that soluble Klotho protects against RF on the obstructed kidney model by critically inhibiting Wnt signalling (Satoh et al. 2012). Taken together, RF is associated with Klotho expression, which inhibits multiple growth factor signaling pathways and is additionally involved in the pathophysiology of CKD progression. However, further prospective studies are needed to prove exact diagnostic values of Klotho as an early biomarker of both acute and chronic renal diseases.

Klotho may provide some partial explanation as between inflammation and diseases characterized by accelerated aging of organs, including CKD. Proinflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and TNF-like weak inducer of apoptosis (TWEAK), are involved in renal injury by inhibiting the expression of downstream effector Klotho within kidney cells via a NF κ B-dependent mechanism (Moreno et al. 2011). A summary of

the *Klotho* contribution in partial conditions and diseases is given in Table I.

Klotho^{-/-} mice also display osteoporosis, a skeletal disease, and formation of phenotypic and skeletal abnormalities associated with osteoporosis is prevented by *Klotho* overexpression (Kuro-o M et al. 1997). This suggests at least partial *Klotho* involvement in osteoporosis, via FGF23/*Klotho* pathway that is physiologically needed for maintaining normal bone metabolism, such as has been reported in experiments with *FGF23*^{-/-}/*NaPi2a*^{-/-} double mutant mice, in which bone mineralization seems to be as a phosphate-independent homeostasis effect of FGF23 (Sitara et al. 2008). In addition, among patients with chronic kidney disease-mineral bone disease (CKD-MBD), osteoporosis and adynamic bone are also highly evident. Even though many active antiosteoporotic drugs are now in use, the experience with CKD patient treatment is limited; therefore, its prevention is now accounted of greater importance (Cannata-Andia et al. 2012). Further, maintenance of physiologically optimal phosphate balance, influenced by *Klotho*, is necessary for normal skeletal growth, development and maintenance. Nonetheless, appropriate phosphate level maintenance constitutes of clinical challenge in various osteo-renal diseases, due to the harmful effects of phosphate toxicity and to therapeutic control difficulties with phosphate patients, particularly in those with CKD (Torres et al. 2007, Cannata-Andia et al. 2012). In addition, patients with osteoporosis frequently suffer from vascular calcification, also reported as a *Klotho*-mediated effect as discussed below.

Ectopic vasculature calcification is an age-related characteristic highly prevalent in patients with CKD and deficient in *Klotho* protein (Hu et al. 2011). Reports indicating that *Klotho*^{-/-} animals also develop severe medial vascular calcification, including arteriosclerosis (Kuro-o M et al. 1997), suggest that a deficiency thereof may be a key factor in initiating calcification. Secreted *Klotho* has a particular role in promoting the health of the vasculature, maintaining endothelial wall homeostasis and participating in calcium homeostasis (Hu et al. 2011). *In vitro*, *Klotho* acts as a direct inhibitor of vascular smooth muscle cells (VSMCs) mediated calcification, induced by high phosphate levels through suppressing Na⁺-dependent uptake of phosphate and mineralization by sodium-phosphate cotransporters Pit1 and Pit2 (Hu et al. 2011). Moreover, soluble *Klotho* protects the vascular endothelium (Quyyumi 1998, Nagai et al. 2000, Saito et al. 2000), consequently decreased levels of *Klotho* protein induce endothelial dysfunction, and diffuse vascular calcification (Saito et al. 2000, Ikushima et al. 2006,

Rakugi et al. 2007, Di Marco et al. 2008). Endothelial dysfunction, or the abnormal function of the blood vessels endothelium, is an early condition in the development and progressing of cardiovascular disease (CVD). The cause here is an imbalance between vasodilatation and vasoconstriction factors, due primarily to enhanced inactivation of nitric oxide (NO) through reactive oxygen species (ROS) formation, and resulting, in turn, in decreased NO bioavailability (Deanfield et al. 2007). In human umbilical vein endothelial cells (HUVECs), *Klotho* suppressed TNF- α induced expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1), and it also reversed TNF- α inhibition of endothelial nitric oxide synthase (eNOS) phosphorylation. Moreover, NF- κ B activation and I κ B phosphorylation induced by TNF- α were also inhibited by *Klotho* protein administration (Maekawa et al. 2009). In a rat model of atherosclerosis, adenovirus-mediated *Klotho* gene delivery increased endothelium-derived NO production and ameliorated vascular endothelial dysfunction (Saito et al. 2000), suggesting a therapeutic potential of *Klotho* for atherosclerotic disease. Furthermore, *Klotho* protein inhibits H₂O₂-induced apoptosis and cellular senescence in vascular endothelial cells relevant to the progression of atherosclerosis, therefore *Klotho* participates in atherosclerosis development reduction (Ikushima et al. 2006). Even though *Klotho* is not expressed in blood vessels (Hu et al. 2011), it is reported that its gene delivery decreased elevated blood pressure in rats (Saito et al. 2000). Both angiogenesis (new blood vessel creation by pre-existing mature endothelial cells) and vasculogenesis (blood vessel formation through differentiation of endothelial precursor cells) are impaired with age, and were also impaired in the case of *Klotho* heterozygous mutant mice induced-ischemia, at least partly by endothelium-dependent NO synthesis (Shimada et al. 2004a). Therefore, *Klotho* may serve as a novel target in the therapy of age-related impairment of neovascularization. Moreover, in erythrocytes, *Klotho* is involved in the regulation of suicidal erythrocyte death, or eryptosis, a critical step toward anemia (Sopjani et al. 2008). Observations shown higher eryptosis in *Klotho*^{-/-} mice erythrocytes, as compared to their *Klotho* wild-type littermate erythrocytes (Kempe et al. 2009). However, under vitamin D-deficient diet, *Klotho*^{-/-} mice erythrocytes reversed some of the hallmarks of eryptosis, thus indication a role here for *Klotho* in at least partially vitamin D-dependent regulation in eryptosis (Kempe et al. 2009).

There is a correlation between the level of plasma *Klotho* and CVD in humans. In a population-based

study conducted on adults, it was shown that higher plasma Klotho concentrations are independently related with lower risk of CVD (Semba et al. 2011a). Moreover, it was reported that plasma Klotho can be used as an independent predictor of all-cause mortality risk in older community-dwelling human adults (Semba et al. 2011b). The development of accelerated artery calcification in humans is potentiated in terms of vascular Klotho deficiency through a Runx2 and myocardin-serum response factor-dependent pathway (Lim et al. 2012). Vascular Klotho was shown to have bifunctional role: As a vascular calcification endogenous inhibitor and as a cofactor required for FGF23-dependent vascular signaling (Lim et al. 2012). Taken together, Klotho deficiency causes vascular calcification, itself a predictor of cardiovascular risk, which is a common complication in CKD (Wang and Sun 2009b, Hu et al. 2010b). However, further studies are required to evaluate Klotho as a predictor and novel early clinical biomarker for the diagnosis of CVDs in humans, also, for the better understanding the Klotho's role in vascular diseases and its relation with FGF23 in vascular cells.

Experiments on animals confirmed that suppressing Klotho gene expression and protein production in spontaneous hypertensive rats (SHRs) resulted in progression of spontaneous hypertension and renal damage, while Klotho gene delivery in SHRs prevented progression of spontaneous hypertension and renal damage in rats by increasing plasma IL-10 levels, decreasing renal and aortas superoxide production, and inhibiting Nox2 (NADPH oxidase) activity and expression (Wang and Sun 2009b) – which is the main source for ROS formation involved in the renal disease pathogenesis (Wang and Sun 2009b, Wang et al. 2012). Increased levels of ROS are very common in the pathogenesis of hypertension (Yamamoto et al. 2005, Shimizu et al. 2011). Even though Klotho treatment prevents progression of SHR, its renoprotective effects point, at least in part, to an acute-independent effect of Klotho on blood pressure regulation (Wang and Sun 2009b). Klotho gene deficiency is also associated in progression of heart damage in SHRs. Conversely, its expression can prevent the increase of blood pressure while protecting against myocardial hypertrophy and myocardial fibrosis in rats, an effect attributed to increased Klotho protein and decreased insulin/IGF-1 mediated signaling pathways resulting from inhibition of protein kinase B (PKB) phosphorylation (Li et al. 2012). Thus, Klotho control expression may offer a new therapeutic approach for the long-term handling of hypertension and heart damage, an approach aimed at CVD prevention and renoprotection.

In addition to the Klotho/FGF23 axis, recent studies clearly suggest emerging roles for β Klotho, a homologous single-pass 130 kDa transmembrane protein primarily expressed in the liver, pancreas and adipose tissue. The β Klotho protein shares about 41% amino acid structural identity and trait similarity with α Klotho, and determines the target organs for FGF15/19 (Kurosu et al. 2007, Lin et al. 2007). A key regulator in the FGFR4 pathway is its co-receptor β Klotho, both expressed in mature hepatocytes (Lin et al. 2007, Poh et al. 2012). Increased β Klotho expression in hepatocellular carcinoma (HCC) tissues play an important role in malignant progression; it represents one of the mechanisms of aberrant FGFR4 signaling in HCC, as well as being a novel biomarker (Poh et al. 2012). Therefore, controlling the expression of β Klotho may be of clinical relevance and a potent molecular therapeutic target for HCC patients.

Studies have confirmed a correlation between α Klotho and cancer in humans. New evidence suggests that Klotho have tumor-suppressor activities, either by antagonising Wnt signalling of importance for melanoma metastasis and cervical carcinoma tumorigenesis (Lee et al. 2010, Camilli et al. 2011) or by inhibiting calpain (Camilli et al. 2011) – thus decreasing the filamin cleavage critical for melanoma cell motility. Therefore, very low levels of Klotho expression were revealed during the carcinogenesis process (Lee et al. 2010, Camilli et al. 2011) due to Klotho epigenetic silencing and its resultant functional loss during tumorigenesis. In addition, many studies have reported that oxidative stress can also modulate DNA methylation through both by influencing DNA methyltransferase (DNMT) expression and by altering epigenetic regulation of gene expression involved in carcinogenesis (Franco et al. 2008). DNMTs are the key enzymes for inactivating transcription by DNA hypermethylation (Arai et al. 2006), a key mechanism for epigenetic regulation in critical tumor suppressor genes. An animal study showed that the Klotho gene promoter CpG hypermethylation was associated with decreased Klotho expression in cervical cancer cells (Lee et al. 2010). Recent studies have shown that DNMT 1 inhibitor (5-aza-20-deoxycytidine [5Aza-2dc]) significantly decreased the CpG hypermethylation, resulting in Klotho gene demethylation and therefore increased Klotho expression *in vivo* in mice and *in vitro* in HK2 cells (Sun et al. 2012).

Klotho has been found to act as a tumor suppressor in various other human tumors types (Chen et al. 2013, Xie et al. 2013, Zhu et al. 2013). In a recent study, Klotho was identified as a tumor suppressor gene that is epigenetically silenced by gene promoter methylation in gastric cancer (GC) cells; Klotho gene

restoration stimulated apoptosis, autophagy, while inhibiting GC cell proliferation by downregulating of IGF-1 receptor phosphorylation and subsequent stimulation of IRS-1/PI3K/Akt/mTOR signaling cascade (Xie et al. 2013), thus highlighting the relevance of Klotho in GC cell survival. Furthermore, the overall survival rate of the renal cell carcinoma (RCC) patients has been reported as significantly higher in cases of high Klotho expression compared to cases of low Klotho expression. Experiments confirmed Klotho-mediated suppression of RCC progression through PI3K/Akt/GSK3 β /Snail signaling (Zhu et al. 2013), indicating its therapeutic potential especially for advanced RCC patients. Klotho is endogenously expressed in lung cancer cells, but its expression is lower compared to lung normal cells. The overexpression of Klotho induces apoptosis and inhibits lung cancer cells proliferation and motility by suppressing the activation of Wnt-TCF/ β -catenin signaling, thus participating in Klotho-induced growth inhibition (Chen et al. 2012). This makes Klotho a candidate target for developing novel therapies against cancers. However, another (and most recent) study reported the opposite results in HCC patients: Significant lower overall survival rate in patients with high Klotho expression compared to those with low Klotho expression. Moreover, this study revealed an anoikis resistance induced by Klotho by activating VEGFR2/PAK1 signaling in HCC, which may function as a putative molecular mechanism for tumor metastasis (Chen et al. 2013). Whether this could be used in therapeutics of HCC patients must awaits further clinical studies. Although many Klotho effects have been identified in tumor suppression, findings of a Klotho-mediated role in hepatoma resistance to anoikis in hepatocarcinogenesis suggest various Klotho effects are at work in tumor cells, hence the need for further validation in prospective studies addressing more fully the molecular mechanisms.

Conclusions and perspectives

It seems that anti-aging Klotho can influence various biological processes; in particular, it appears to impact development of several age-related diseases. Klotho functions as a cofactor for FGF23 in FGF23 signaling or, in an independent FGF23 mode, downregulates the expression of vitamin D and phosphate reabsorption (Imai et al. 2004, Anour et al. 2012, Woudenberg-Vrenken et al. 2012). Potential Klotho renoprotective effects suggest an interlinkage between Klotho functions and mitochondria, i.e., a relationship of biological aging with oxidative stress (Yamamoto et al. 2005, Di Marco et al. 2008,

Hsieh et al. 2010, Wang et al. 2012). The latter is known to cause mtDNA mutations that induce mitochondrial dysfunction which appear to promote apoptotic cell death, and possibly induces mtDNA fragmentation (Wu et al. 2011) involved in the pathophysiology of many types of chronic renal disease. Klotho increases oxidative stress resistance, and its protein overexpression, by activating the forkhead transcription factors (FoxO) (Yamamoto et al. 2005), has been found to induce expression of manganese superoxide dismutase (Kops et al. 2002), a mitochondrial enzyme that facilitates removal of ROS. Thus Klotho protects cells and tissues from oxidative damage by inducing the inhibition of insulin/IGF-1 signaling (Chen et al. 2007, Wolf et al. 2008, Doi et al. 2011). Furthermore, Klotho reduces DNA fragmentation (Hu et al. 2010b), oxidative stress, apoptosis, β -galactosidase activity, even as it normalizes cytochrome C oxidase activity; in this way, Klotho ameliorates renal injury by modulating mitochondrial functions (Sugiura et al. 2005, 2010, Hu et al. 2010b). Klotho decreases mitochondrial oxidative stress and so modulates kidney injury (Chen et al. 2007). By acting as a β -glucuronidase Klotho activates TRPV5 and TRPV6 cation channels thus increasing of Ca²⁺ reabsorption and absorption, thus emphasizing the role of Klotho in maintaining Ca²⁺ homeostasis (Chang et al. 2005, Cha et al. 2008, Lu et al. 2008). Tumor-suppressive properties of Klotho have been determined in various malignant transformations (Chen et al. 2013, Xie et al. 2013, Zhu et al. 2013). Endothelial dysfunction as an early condition in progression and development of CVD results primarily from increased inactivation of NO by ROS, leading to decreased in NO bioavailability (Deanfield et al. 2007). Klotho inhibits eNOS phosphorylation (Maekawa et al. 2009), therefore unregulated endothelium-derived NO production ameliorates vascular endothelial dysfunction (Saito et al. 2000), suggesting a therapeutic potential of Klotho in atherosclerotic disease that remains to be clarified. The discovery of Klotho has not (yet) extended our lifespan, but it has opened up new avenues especially in research into the aging process, mineral homeostasis, cancer progression, apoptosis, and cardiovascular and renal diseases.

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