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αKlotho and Chronic Kidney Disease

J.A. Neyra[*,†] and **M.C. Hu**[*,†,1]

[*]University of Texas Southwestern Medical Center, Dallas, TX, United States

[†]Charles and Jane Pak Center for Mineral Metabolism and Clinical Research, University of Texas Southwestern Medical Center, Dallas, TX, United States

Abstract

Alpha-Klotho (αKlotho) protein is encoded by the gene, *Klotho*, and functions as a coreceptor for endocrine fibroblast growth factor-23. The extracellular domain of αKlotho is cleaved by secretases and released into the circulation where it is called soluble αKlotho. Soluble αKlotho in the circulation starts to decline in chronic kidney disease (CKD) stage 2 and urinary αKlotho in even earlier CKD stage 1. Therefore soluble αKlotho is an early and sensitive marker of decline in kidney function. Preclinical data from numerous animal experiments support αKlotho deficiency as a pathogenic factor for CKD progression and extrarenal CKD complications including cardiac and vascular disease, hyperparathyroidism, and disturbed mineral metabolism. αKlotho deficiency induces cell senescence and renders cells susceptible to apoptosis induced by a variety of cellular insults including oxidative stress. αKlotho deficiency also leads to defective autophagy and angiogenesis and promotes fibrosis in the kidney and heart. Most importantly, prevention of αKlotho decline, upregulation of endogenous αKlotho production, or direct supplementation of soluble αKlotho are all associated with attenuation of renal fibrosis, retardation of CKD progression, improvement of mineral metabolism, amelioration of cardiac function and morphometry, and alleviation of vascular calcification in CKD. Therefore in rodents, αKlotho is not only a diagnostic and prognostic marker for CKD but the enhancement of endogenous or supplement of exogenous αKlotho are promising therapeutic strategies to prevent, retard, and decrease the comorbidity burden of CKD.

1. INTRODUCTION

The *Klotho* gene was discovered in 1997 when mice with silencing of this gene developed multiple organ dysfunction and failure with shortened life span resembling human premature aging (Kuro-o et al., 1997). The overexpression of the *Klotho* transgene with a ubiquitous promoter (Kurosu et al., 2005), viral-based transfer (Masuda, Chikuda, Suga, Kawaguchi, & Kuro-o, 2005), or direct parenteral administration (Chen, Kuro, et al., 2013) can extend mouse life span compared to normal mouse and rescue the most phenotypes observed in *Klotho*-deficient mouse (Kurosu et al., 2005). Two other paralogs βKlotho (Ito et al., 2000) and γKlotho (Ito, Fujimori, Hayashizaki, & Nabeshima, 2002) were identified, then *Klotho*

[1]Corresponding author: ming-chang.hu@utsouthwestern.edu.

gene was designated *αKlotho* to distinguish from the other two paralogs (Hu, Shiizaki, Kuro-o, & Moe, 2013).

αKlotho is highly expressed in the kidney, brain, and in lesser extent in other organs (Kato et al., 2000; Kuro-o et al., 1997). Human αKlotho is a single transmembrane 1012 amino acid 130 kDa protein encoded by human *Klotho* gene, while rodent αKlotho is a 1014 amino acid protein (Kuro-o et al., 1997; Matsumura et al., 1998; Shiraki-Iida et al., 1998; Tohyama et al., 2004). The extracellular domain of membrane αKlotho consisting of two repeat sequences (kl1 and kl2) can be shed by secretases and released into the circulation (Bloch et al., 2009; Chen, Podvin, Gillespie, Leeman, & Abraham, 2007; Chen, Tung, et al., 2014; Hu, Shi, Zhang, et al., 2015). This released extracellular domain of membrane αKlotho is referred as soluble or cleaved αKlotho. It is a main functional form in the circulation (Hu, Shi, Zhang, et al., 2015; Hu, Shi, Zhang, Pastor, et al., 2010; Hu, Shi, Zhang, Quinones, et al., 2010; Imura et al., 2004; Kurosu et al., 2005). Soluble αKlotho protein is also present in cerebrospinal fluid (Chen et al., 2015; Degaspari et al., 2015; Emami Aleagha et al., 2015; Imura et al., 2004; Semba et al., 2014) and urine of mammals (Akimoto et al., 2012; Hu, Shi, Zhang, Pastor, et al., 2010; Hu et al., 2011; Lau et al., 2012). Soluble αKlotho functions as a circulating substance exerting multiple systemic biological actions on distant organs and directly protects cells against a variety of insults including hypoxia, hyperoxia, oxidative stress, and cytotoxic medication and suppresses apoptosis (Cheng et al., 2015; Hu, Shi, Cho, et al., 2013; Panesso et al., 2014; Ravikumar et al., 2014; Sun et al., 2015; Wang et al., 2013).

Chronic kidney disease (CKD) is characterized by progressive deterioration of renal function with high risk of end-stage renal disease (ESRD) regardless of whether initial kidney insults have regressed or are continuously present (D'Hoore et al., 2015; Ferenbach & Bonventre, 2015; Rimes-Stigare et al., 2015; Venkatachalam, Weinberg, Kriz, & Bidani, 2015). As expected, CKD risk increases with age, and about half of the CKD stage ≥3 cases occurs in subjects >70 years old. CKD can be viewed as a state of accelerating aging (Kooman et al., 2013; Stenvinkel & Larsson, 2013). The relative risk for cardiovascular (CV) mortality of a 25- to 34-year-old dialysis patient is similar to a non-CKD patient of >75 years of age (Foley, Parfrey, & Sarnak, 1998). The similar phenotypes between αKlotho-deficient mice and CKD subjects also suggest a potential pathogenic role of αKlotho deficiency in CKD development and progression (Hu, Kuro-o, & Moe, 2012, 2013a, 2013b; Hu et al., 2011; Hu, Shiizaki, Kuro-o, et al., 2013; Shi et al., 2015).

In this chapter, we aim to summarize the current state of knowledge on αKlotho biology and pathophysiology in CKD, and provide a possible novel perspective on potential clinical applications of αKlotho in CKD.

2. CKD IS A STATE OF KLOTHO DEFICIENCY

2.1 The Kidney Is the Main Origin for Systemic αKlotho

Compared to the wide distribution of *αKlotho* mRNA in many organs and tissues, αKlotho protein expression is restricted to only a few tissues including the kidney, brain, heart, parathyroid gland, and testis (Kuro-o et al., 1997; Takeshita et al., 2004). αKlotho protein

was also found in vascular endothelial cells and smooth muscle cells in humans and rodents (Fang et al., 2014; Jimbo et al., 2014; Lim et al., 2012; Ritter, Zhang, Delmez, Finch, & Slatopolsky, 2015), but this is still in debate because there is equally convincing evidence that do not support the presence of αKlotho protein in the vasculature (Hu, 2016; Lau et al., 2012; Lindberg et al., 2013; Mencke et al., 2015; Scialla et al., 2013). Therefore, whether αKlotho is expressed in the vasculature remains to be resolved. Among tissues expressing αKlotho protein, the kidney has the highest level. In mammalian kidney, αKlotho is prominently expressed in distal convoluted tubules (DCTs; Kato et al., 2000; Kuro-o et al., 1997), but is also unequivocally found in the proximal convoluted tubules although at lower levels compared to DCT (Hu, Shi, Zhang, Pastor, et al., 2010; Lim et al., 2015).

Despite the fact that the kidney is the organ expressing the highest levels of αKlotho protein, the confirmation that circulating αKlotho in serum mainly derived from the kidney under physiological conditions was demonstrated by Lindberg et al. (2014). The strongest evidence comes from mouse with renal tubule-specific partial deletion of αKlotho. This mouse line has reduced serum αKlotho levels and systemic features resembling the phenotype of global αKlotho deletion or the αKlotho hypomorphic mice, indicating that the kidney may be the principal organ mediating the systemic αKlotho effects (Lindberg et al., 2014). More direct evidence to support this notion is that αKlotho protein in the suprarenal vein is higher than that in infrarenal vein in rodents and humans (Hu, Shi, Zhang, et al., 2015) and circulating αKlotho levels were dramatically and quickly dropped in rodents that underwent bilateral nephrectomy (Hu, Shi, Zhang, et al., 2015) which strongly suggest that the kidney is the main source of αKlotho in the circulation under physiological conditions (Fig. 1). In living human kidney donors (Akimoto et al., 2013; Ponte et al., 2014), αKlotho was shown to drop after nephrectomy but it is difficult to distinguish this from the effect of surgery. However, under pathological renal conditions such as ESRD, circulating αKlotho is low rather absent, suggesting the possibility that αKlotho may come from extrarenal source(s), although its origin is not clear to date (Lau et al., 2012). Establishing extrarenal sources of αKlotho and characterizing how this can be upregulated when renal production fails are of paramount importance.

2.2 Renal αKlotho Deficiency in CKD

With the renal source of circulating αKlotho established (Fig. 1), the next step is to understand why and how αKlotho is drastically reduced in kidney disease. As a general principle, if the organ of origin of an endocrine substance is diseased, it is logical to suspect that endocrine deficiency of that substance ensues. Therefore, it is not surprising to witness the reduction of αKlotho protein in the diseased kidney. However, whether the reduction of αKlotho is due to destruction of kidney, loss ability to produce/secrete αKlotho, or a maladaptive response, remains to be explored.

It has been shown that there is a significant reduction of renal αKlotho transcript and protein in the diseased kidney resulting from a wide variety of etiologies from glomerular and tubulointerstitial diseases, obstructive nephropathy, diabetic nephropathy, ischemic injury, subtotal nephrectomy, oxidative stress, chronic allograft rejection, and exposure to cisplatin, angiotensin II (Ang II), and calcineurin inhibitors in both humans and rodents (Hu et al.,

2012; Hu et al., 2013a, 2013b; Hu, Shiizaki, Kuro-o, et al., 2013; Panesso et al., 2014; Sastre et al., 2013; Shi et al., 2015; Zhou, Li, Zhou, Tan, & Liu, 2013). The mechanisms underlying the relationship between renal αKlotho downregulation and kidney diseases therefore need to be further illustrated.

It is proposed that renal αKlotho deficiency in early stages of CKD may be attributed mainly to suppression of αKlotho expression rather than loss of viable renal tubules. Several intermediates are shown to be involved in the reduction of αKlotho expression: high serum phosphate (Hu, Kuro-o, & Moe, 2014), hypermethylation (Azuma et al., 2012; King, Rosene, & Abraham, 2012; Lee, Jeong, et al., 2010; Sun, Chang, & Wu, 2012; Young & Wu, 2012), and hyperdeacetylation (Moreno et al., 2011) in αKlotho gene promoter induced by inflammatory cytokines and the uremic toxin, indoxyl sulfate (Fig. 2). If these observations are correct, they provide an opportunity to reactivate αKlotho expression by modulation of these factors and thereby correcting circulating and renal αKlotho deficiency in early stages of CKD.

2.3 Circulating αKlotho Deficiency in CKD

αKlotho transcript and protein expression in diseased kidneys from humans and animals is clearly decreased. However, the relationship between renal αKlotho expression and serum and/or urinary αKlotho levels remains to be confirmed.

In a rodent model of CKD from uninephrectomy plus contralateral ischemia reperfusion, serum αKlotho concentration was remarkably decreased, and the degree of its reduction was similar in magnitude to that of decreased αKlotho protein in the kidneys and in the urine (Hu et al., 2011). Thus in rodents, CKD is a state of endocrine (circulating and urinary) αKlotho deficiency in addition to renal αKlotho deficiency (Hu et al., 2011).

In CKD patients, urinary αKlotho levels are significantly decreased at very early stages (stage 1) and sustainably reduced with progression of CKD (Hu et al., 2011), while the reduction of serum αKlotho starts later at stage 2 CKD (Barker et al., 2015). Moreover, human urinary αKlotho excretion is significantly decreased and the amount of urinary αKlotho decrease is directly correlated with estimated glomerular filtration rate (eGFR) decline (Yamazaki et al., 2010). These observations suggest that urinary soluble αKlotho may be a good biomarker for early CKD detection. A growing body of evidence has shown a reduction of circulating αKlotho in CKD and ESRD patients (Devaraj, Syed, Chien, & Jialal, 2012; Fliser, Seiler, Heine, & Ketteler, 2012; Pavik et al., 2013; Scholze et al., 2014; Shimamura et al., 2012; Zhou et al., 2013), therefore identifying the plausible mechanisms and clinical implications of serum and/or urinary αKlotho reduction in CKD/ESRD patients is of paramount importance.

3. αKlotho DEFICIENCY CONTRIBUTES TO CKD DEVELOPMENT AND PROGRESSION

αKlotho deficiency is not only an early biomarker of CKD but also a pathogenic intermediate for CKD development and progression, and extrarenal complications. Compared to wild-type mice, αKlotho-deficient mice have more severe kidney damage and

faster progression to CKD with more fibrosis, and αKlotho overexpressors have milder kidney dysfunction and less fibrosis after exposure to renal insults including ischemic injury (Hu et al., 2011; Hu, Shi, Zhang, Quinones, et al., 2010; Shi et al., 2015), cisplatin (Panesso et al., 2014), Adriamycin (Zhou et al., 2013), and ureteric ligation (Sugiura et al., 2012; Zhou et al., 2013). αKlotho is a multifaceted protein. Different forms of αKlotho may be involved in different biological functions. Membrane αKlotho is confirmed to participate in maintenance of mineral homeostasis, while soluble αKlotho protein plays a more important and systemic role in cytoprotection, antifibrosis, and angiogenesis.

3.1 Increased Cell Senescence and Reduced Ability of Regeneration

Stem cells in most mammalian tissues participate in maintenance of tissue homeostasis and are involved in tissue repair or regeneration (Li & Clevers, 2010; Weissman, 2000). The dysfunction and depletion of stem cells and progenitor cells contribute to aging and aging-associated diseases including kidney disease. CKD can be a consequence of incomplete or failed tubule recovery after AKI (D'Hoore et al., 2015; Ferenbach & Bonventre, 2015; Kramann, Tanaka, & Humphreys, 2014; Polichnowski et al., 2014; Venkatachalam et al., 2015; Zhang et al., 2013). The repeated administration of bone marrow-derived mesenchymal stem cells improved renal function and histology, reduced blood pressure, and attenuated the infiltration of inflammatory cells on a remnant rat kidney (Lee, Lee, et al., 2010). More recently, evidence has shown human-induced pluripotent stem cells derived from any human somatic cell type after the introduction of reprogramming transcription factors contributing to kidney regeneration and improvement in kidney function (Schmitt, Susnik, & Melk, 2015).

The decrease in stem cell number is associated with an increase in progenitor cell senescence, a complicated process present not only in normal aging but also in pathophysiological states (Dmitrieva & Burg, 2007; Haruna et al., 2007; Jennings et al., 2007; Kailong et al., 2007; Nakano-Kurimoto et al., 2009; Yang et al., 2009; Yang & Fogo, 2010). Excessive senescence and subsequent stem cell deletion may decrease the ability of the kidney to defend against renal insults and impair regeneration (Schmitt et al., 2015).

αKlotho deficiency is associated with stem cell dysfunction and depletion which is part of normal aging (Bian, Neyra, Zhan, & Hu, 2015; Liu et al., 2007). αKlotho deficiency in CKD could enhance renal tubular and vascular cell senescence induced by oxidative stress, uremic toxin, and high phosphate (Carracedo et al., 2013; Clements, Chaber, Ledbetter, & Zuk, 2013; de Oliveira, 2006; Niwa & Shimizu, 2012; Small et al., 2012; Tsirpanlis, 2008; Verbeke, Van Biesen, & Vanholder, 2011; Yamada et al., 2015). Wnt signaling activity is significantly increased in tissues from *kl/kl* mice, which can be rescued by genetic αKlotho overexpression (Liu et al., 2007). Administration of exogenous Wnt stimulates Wnt signal transduction, and triggers or accelerates cell senescence both in vitro and in vivo. αKlotho appears to be a secreted Wnt antagonist and may utilize this mechanism to retard mammalian aging. Suppression of cell senescence may be one of many novel strategies for promotion of kidney regeneration after AKI and retardation of CKD progression (Camilli et al., 2011; Liu et al., 2007; Satoh et al., 2012; Zhou et al., 2013).

3.2 Defective Endothelial Function and Impaired Vasculogenesis

In CKD patients, there is endothelial dysfunction and impaired bone marrow-derived endothelial progenitor cells-mediated vascular regeneration and kidney repair (Jie et al., 2010; Mohandas & Segal, 2010), both of which can contribute to progression of CKD and aging of the kidney (Chade et al., 2006; Mu et al., 2009; Reinders, Rabelink, & Briscoe, 2006; Taniyama & Morishita, 2010; Westerweel et al., 2007). Recent studies further indicated a potential causal link between vascular rarefaction and CKD progression. *kl*/*kl* mice do not only have abnormal vasodilatation due to abnormal endothelial function (Nakamura et al., 2002) and low blood flow after hind limb ischemia (Fukino et al., 2002) but also impaired angiogenesis and vasculogenesis (Shimada et al., 2004). The HMG coenzyme A reductase inhibitor, cerivastatin, increases αKlotho levels in cultured kidney cell lines (Narumiya et al., 2004) and mice (Yoon et al., 2012) and also restores impaired neovascularization in *kl*/*kl* mice (Shimada et al., 2004), but the causal relation between increased Klotho and restoration of vasculogenesis remains to be confirmed. The impaired vasculogenesis and angiogenesis might be attributable to downregulation of vascular endothelial growth factor (VEGF) in the aorta (Nakamura et al., 2002). Recent in vivo and in vitro studies showed that αKlotho is associated with VEGF receptor-2 (VEGFR-2) and the transient receptor potential canonical-1 (TRPC-1) Ca^{2+} channel to maintain endothelial integrity because in αKlotho-deficient endothelial cells, VEGF-mediated internalization of the VEGFR-2/TRPC-1 complex is impaired, and surface TRPC-1 expression increases which can be reversed by αKlotho protein (Kusaba et al., 2010). In addition, αKlotho mitigates the increased cell senescence and apoptosis triggered by oxidative stress in endothelial cells (Ikushima et al., 2006); and αKlotho also suppresses TNF-β-induced expression of intracellular adhesion molecule-1 and vascular cell adhesion molecule-1, attenuates NF-kappaB activation, and reverses the inhibition of eNOS phosphorylation by TNF-α. Thus αKlotho protein also protects vascular endothelium by inhibition of endothelial inflammation (Maekawa et al., 2009).

3.3 Promotion of Renal Fibrosis

Renal fibrosis is a histological hallmark of CKD and also believed to be a pathogenic intermediate for CKD progression (Ardura, Rayego-Mateos, Ramila, Ruiz-Ortega, & Esbrit, 2010; Iwano et al., 2002; Kalluri & Neilson, 2003; Liu, 2010; Zeisberg & Duffield, 2010; Zeisberg et al., 2003). *kl*/*kl* mice have more renal tubulointerstitial fibrosis (Sugiura et al., 2012) which is associated with upregulation of TGF-β in the kidneys. The renal fibrosis induced by unilateral ureteral obstruction (UUO) is accompanied by upregulation of TGF-β and fibronectin, and down-regulation of αKlotho mRNA and protein. These alterations are exaggerated in αKlotho-deficient UUO mice compared to *WT* UUO mice. Along the same line, more renal fibrosis was found in αKlotho-deficient mice injected with Adriamycin (Zhou et al., 2013). Soluble αKlotho alleviates renal fibrosis induced by UUO and suppresses expression of fibrosis markers and TGF-β1 target genes (eg, Snail, Twist), but does not reduce TGF-β1 expression in UUO kidney (Doi et al., 2011), suggesting that αKlotho suppresses renal fibrosis primarily through inhibiting TGF-β1 downstream signaling (Doi et al., 2011).

As discussed earlier, αKlotho is an antagonist for Wnt signaling and Wnt is associated with renal fibrosis. αKlotho's suppression of renal fibrosis is conceivably attributable to inhibition of Wnt signal transduction. Mice with UUO and Adriamycin injection have high Wnt levels and β-catenin activity as well as myofibroblast activation which can be suppressed by administration of expression vector encoding the extracellular domain of αKlotho (Zhou et al., 2013).

When renal fibrosis and Wnt signaling are compared between αKlotho overexpression and αKlotho deficiency in UUO, Wnt signaling and tubulointerstitial fibrosis were attenuated in αKlotho-overexpressing compared to *WT* mice. In contrast, Wnt signaling and tubulointerstitial fibrosis were dramatically augmented in αKlotho heterozygous-deficient (*kl*/+) mice after UUO compared with *WT* mice. Interestingly, after transferring plasmid overexpressing αKlotho into skeletal muscle, *kl*/+ mutant mice had much lower Wnt signaling and extracellular matrix deposition. Therefore, αKlotho is a critical negative regulator of Wnt signaling and a suppressor of renal fibrosis in the obstructed kidney model (Satoh et al., 2012). In addition, αKlotho also promotes clearance of collagen I through upregulation of autophagy (Shi et al., 2015). Therefore, αKlotho suppresses fibrosis and enhances removal of collagen. Exogenous αKlotho administration may be a novel therapeutic agent for renal fibrosis.

4. αKlotho DEFICIENCY EXACERBATES DISORDERS OF MINERAL METABOLISM IN CKD

The fact that mice with complete αKlotho deficiency share similar features such as hyperphosphatemia, hyper-FGF23-temia, and high morbidity and mortality from CV disease than CKD subjects suggests that αKlotho deficiency may participate in CKD development (Hu et al., 2013a, 2013b; Hu, Shiizaki, Kuro-o, et al., 2013). Furthermore, αKlotho-deficient mice with AKI progress to CKD more rapidly and exhibit more severe vascular lesions (Hu et al., 2011; Shi et al., 2015) and uremic cardiac remodeling (Hu, Shi, Cho, et al., 2015), supporting the concept that αKlotho deficiency might be a pathogenic intermediate in CKD (Fig. 3). Given that disturbed mineral metabolism contributes to the high morbidity and mortality of CV disease in CKD (Davidovich, Davidovits, Peretz, Shapira, & Aframian, 2009; Fernandez-Martin et al., 2015; Kaisar, Isbel, & Johnson, 2007; Kestenbaum & Belozeroff, 2007; London, Marchais, Guerin, & Metivier, 2005; Obi, Hamano, & Isaka, 2015; Siomou & Stefanidis, 2012; van Ballegooijen, Rhee, Elmariah, de Boer, & Kestenbaum, 2016; Wesseling-Perry, 2015), a better understanding of the molecular mechanisms of how αKlotho deficiency dysregulates mineral metabolism will aid in the exploration of novel therapeutic strategies in CKD.

4.1 Hyperphosphatemia

The role of αKlotho in phosphate homeostasis was recognized as soon as αKlotho was discovered because αKlotho-deficient mice have severe hyper-phosphatemia (Hu, Shi, Zhang, Pastor, et al., 2010; Kuro-o et al., 1997). This was further confirmed by the fact that there is low serum phosphate in αKlotho-overexpressing mice (Kurosu et al., 2005). A patient with homozygous missense mutation (H193R) in the *αKLOTHO* gene had severe

calcinosis, dural and carotid artery calcifications, severe hyper-phosphatemia, hypercalcemia, and high serum 1,25-$(OH)_2$-vitamin D_3 and fibroblast growth factor (FGF23) (Ichikawa et al., 2007). This mutation conceivably destabilizes kl1 domain of αKlotho, thereby attenuating production of membrane-bound and soluble αKlotho protein (Ichikawa et al., 2007). Therefore, in one human, the manifestations are similar to those observed in αKlotho-deficient mice. Phosphate overload suppresses αKlotho expression in the kidney (Hu, Shi, Cho, et al., 2015; Shi et al., 2015; Fig. 2). Normal mice fed a high Pi diet have dramatically decreased αKlotho protein and mRNA in the kidney, while αKlotho hypomorphic mice fed low Pi diet can regain part of their Klotho expression (Hu, Shi, Cho, et al., 2015; Morishita et al., 2001; Shi et al., 2015).

Accumulating evidence showed that kidney disease is a status of αKlotho deficiency. Although the mechanism of reduced renal and circulating αKlotho is not understood, it is conceivable that αKlotho deficiency might be involved in the development of hyperphosphatemia, one of components of CKD–metabolic bone disease (CKD-MBD). αKlotho deficiency impairs phosphaturia (Hu, Shi, Zhang, Pastor, et al., 2010) and consequently accelerates Pi accumulation in CKD. The higher the level of serum Pi, the greater the degree of soft-tissue calcification, and the greater the risk of mortality (Kestenbaum et al., 2005). Higher serum Pi levels have been shown to be associated with high mortality in incident ESRD patients (Gutiérrez et al., 2008) and control of serum Pi may help decrease vascular calcification and suppress proliferation of parathyroid glands (Cannata-Andia & Rodriguez-Garcia, 2002; Isakova et al., 2009; Kestenbaum et al., 2005). αKlotho administration could be a novel strategy for the correction of hyperphosphatemia in CKD patients.

4.2 Increased FGF23 Levels

FGF23, a phosphatonin, is thought to be implicated in the systemic balance of phosphate maintained by the interaction of intestine, bone, and kidneys (Hu, Shiizaki, Kuro-o, et al., 2013) through interplay with αKlotho, parathyroid hormone (PTH), and 1,25-$(OH)_2$-vitamin D_3 (Bian, Xing, & Hu, 2014). One principal stimulus for FGF23 secretion is currently believed to be high serum phosphate caused by dietary phosphate load (Nishida et al., 2006). In CKD, there is an increase in FGF23 levels in parallel with the deterioration of renal function (Fliser et al., 2007) and the increase of serum phosphate and PTH (Ben-Dov et al., 2007; Nagano et al., 2006; Silver & Naveh-Many, 2010). High serum FGF23 in CKD may not only serve as a diagnostic biomarker of early CKD and predictor of CV disease and mortality in CKD/ESRD patients, but recently, it is proposed to be the necessary and sufficient contributor to uremic cardiomyopathy (Faul et al., 2011; Grabner et al., 2015) through activation of FGFR4 and independently from αKlotho. In contrast, recent data also showed that the relationship between FGF23 and cardiac remodeling depends on αKlotho, and the association of FGF23 with cardiac hypertrophy and fibrosis is only evident in the presence of αKlotho deficiency (Hu, Shi, Cho, et al., 2015).

High serum FGF23 levels antedate high serum levels of phosphate, suggesting a disrupted feedback loop resulting in very high levels of serum FGF23. αKlotho-deficient mice have very high serum levels of FGF23 further supporting that αKlotho might be a negative

regulator of FGF23 regardless of the unknown precise mechanisms of how it suppresses FGF23 synthesis in bone (Fig. 3). Currently, there are no experimental data to show direct suppression of FGF23 by αKlotho in osteoblast or osteocytes in vitro. But other animal experiments have shown that extremely high circulating αKlotho with viral delivery can induce severe hypophosphatemia and increase blood FGF23 through stimulation of FGF23 production in the bone although the molecular mechanisms remain to be clarified (Smith et al., 2012). Because phosphate is a potent stimulus for FGF23 production in the bone, therefore, whether αKlotho directly or indirectly increases FGF23 remains to be explored. On the other hand, αKlotho-deficient mice have high levels of serum phosphate and conceivably high FGF23 results from high phosphate due to defective phosphate excretion induced by αKlotho deficiency (Hu, Shi, Zhang, Pastor, et al., 2010; Fig. 3).

4.3 Hypovitaminosis D

Low 1,25-$(OH)_2$-vitamin D_3 is a major component of disorders of mineral metabolism, and is conventionally attributed to cause bone disease and secondary hyperparathyroidism in CKD (Lips, 2001). However, hypervitaminosis D is present in αKlotho deficiency (Kuro-o et al., 1997) and removal of key components involved in vitamin D metabolism or function can rescue these phenotypes in αKlotho-deficient mice (Lanske & Razzaque, 2007; Ohnishi, Nakatani, Lanske, & Razzaque, 2009; Razzaque, 2012) suggesting that αKlotho is a suppressor of vitamin D signaling. If CKD is a state of αKlotho deficiency, why αKlotho deficiency does not raise the serum levels of 1,25-$(OH)_2$-vitamin D_3 in CKD? The increase in plasma FGF23 in CKD is thought to suppress 1α-hydroxylase in the kidney and initiate or accelerate vitamin D deficiency (Gutiérrez, 2010; Liu et al., 2006). Because 1,25-$(OH)_2$-vitamin D_3 induces αKlotho expression in the kidney (Tsujikawa, Kurotaki, Fujimori, Fukuda, & Nabeshima, 2003), it is plausible that low vitamin D levels in CKD may exacerbate renal αKlotho deficiency (Fig. 3).

4.4 Secondary Hyperparathyroidism

Secondary hyperparathyroidism is a common complication of CKD/ESRD and is induced by retention of phosphate as a result of reduced glomerular filtration. The “trade off” hypothesis formulated by Slatopolsky and Bricker has been used for several decades to explain the role of hyperphosphatemia in secondary hyperparathyroidism (Slatopolsky & Bricker, 1973). It was proposed that in the early stages of CKD, an increase in serum phosphate concentrations can be overcome by an increased rate of PTH release, which may be also a result of hypocalcemia. However, as CKD progresses to advanced stages or ESRD, hyperphosphatemia becomes sustained and PTH chronically elevated, suppressing the synthesis of 1α,25$(OH)D_3$ in the kidney. The reduction in the synthesis of 1α,25$(OH)D_3$ also results from hyperphosphatemia and reduced nephron mass. 1α,25$(OH)D_3$ deficiency also contributes to an increase in PTH synthesis (Delmez & Slatopolsky, 1992).

Currently, secondary hyperparathyroidism is considered as part of the syndrome of CKD-metabolic bone disease (MBD; Galitzer, Ben-Dov, Silver, & Naveh-Many, 2010; Khan, 2007). Even mild increments in PTH levels are associated with an increased CV risk, regardless of the serum levels of calcium and phosphorus and whether vitamin D therapy is given, suggesting that decreasing PTH levels may improve mineral metabolism disorders

(Floege et al., 2011; Panichi et al., 2010; Patel et al., 2011; Pontoriero, Cozzolino, Locatelli, & Brancaccio, 2010).

CKD patients have high blood FGF23 levels (Shimada et al., 2010) and low αKlotho and FGFR(s) in parathyroid gland (Canalejo et al., 2010; Krajisnik et al., 2010). In subjects with normal kidney function, FGF23 plays a crucial role, both as a phosphaturic factor (Gattineni & Baum, 2010; Goetz et al., 2010; Weber, Liu, Indridason, & Quarles, 2003) and as a calciotropic hormone to suppress 1,25-$(OH)_2$-vitamin D production in the kidney (Liu et al., 2006) and PTH production in the parathyroid gland (Ben-Dov et al., 2007), and to increase renal calcium reabsorption through modulation of TRPV5 channel (Andrukhova et al., 2014). In contrast, in a CKD setting, FGF23 fails to inhibit PTH production probably due to downregulation of αKlotho and FGFR(s) in parathyroid gland (Fig. 3; Canalejo et al., 2010; Krajisnik et al., 2010). The mechanisms of downregulation of αKlotho and FGFR(s) in uremic parathyroid gland remain to be explored.

Renal and circulating αKlotho deficiency is associated with development and progression of CKD-MBD (Fahrleitner-Pammer et al., 2008; Hruska, Saab, Mathew, & Lund, 2007; Kalantar-Zadeh et al., 2010; Patel et al., 2011). Although whether αKlotho is present in the vasculature is still under debate, it has been shown that even early CKD-MBD may cause a reduction of vascular αKlotho (Fang et al., 2014), stimulate vascular osteoblastic transition, increase osteocytic secreted proteins, and consequently induce vascular calcification. Correction of αKlotho and maintenance of mineral homeostasis do not only benefit bone and mineral metabolism but also may attenuate CV disease and improve the quality of life of CKD/ESRD patients (Fernandez-Martin et al., 2015).

5. αKlotho DEFICIENCY IN CV DISEASE IN CKD

CKD confers significant CV morbidity and mortality (Go, Chertow, Fan, McCulloch, & Hsu, 2004; Gross & Ritz, 2008; Taddei, Nami, Bruno, Quatrini, & Nuti, 2011). A large number of CKD patients die from CV disease even before initiation of dialysis. The main clinical features of CV disease in CKD include uremic cardiomyopathy and vascular calcification.

5.1 Pathological Uremic Cardiomyopathy

Uremic cardiomyopathy or cardiomyopathy of advanced CKD, characterized by cardiac hypertrophy and fibrosis, is a major cause of CV disease, by causing congestive heart failure, cardiac dysrhythmias, and sudden cardiac death (Glassock, Pecoits-Filho, & Barberato, 2009; Go et al., 2004; Gross & Ritz, 2008; Taddei et al., 2011). There are traditional risk factors such as hypertension, coronary disease, atherosclerosis, anemia, and volume overload (Glassock et al., 2009; Gross & Ritz, 2008), and also CKD-specific factors such as hyperphosphatemia (Block, Hulbert-Shearon, Levin, & Port, 1998; Glassock et al., 2009; Gross & Ritz, 2008; Kestenbaum et al., 2005). Recent data have shown an association between elevated FGF23 levels and uremic cardiac remodeling (Faul et al., 2011; Gutiérrez et al., 2009). Soluble αKlotho deficiency may also be an intermediate mediator of the pathological cardiac remodeling observed in CKD (Hu, Shi, Cho, et al., 2015). Furthermore, αKlotho may protect the heart against stress-induced cardiac hypertrophy by inhibiting

TRPC-6 channel-mediated abnormal Ca^{2+} signaling in the heart (Xie et al., 2012; Xie, Yoon, An, Kuro-o, & Huang, 2015) or against uremic solute indoxyl sulfate-induced myocardial hypertrophy probably by suppressing NADPH oxidase Nox2/Nox4-derived reactive oxygen species (ROS) production and its downstream signaling (Yang et al., 2015; Fig. 4).

5.1.1 αKlotho as a Modulator of Pathological Cardiac Remodeling—Uremic cardiomyopathy is a state of pathological cardiac remodeling characterized by left ventricular hypertrophy (LVH) and extensive fibrosis (Foley et al., 1995; Tyralla & Amann, 2003). Both primary genetic αKlotho deficiency (heterozygous αKlotho-deficient, *kl*/+ mice) and secondary αKlotho deficiency (from phosphate loading, aging, and CKD) triggered cardiac hypertrophy and fibrosis in mice, such that higher plasma phosphate and lower plasma αKlotho levels were associated with more severe cardiac hypertrophy and fibrosis (Hu, Shi, Cho, et al., 2015). Furthermore, higher plasma FGF23 levels were associated with more severe cardiac hypertrophy and fibrosis but only in the presence of moderate or low plasma αKlotho levels (Hu, Shi, Cho, et al., 2015). This suggests that FGF23 may not be cardiotoxic unless there is simultaneous αKlotho deficiency.

CKD models of secondary αKlotho deficiency included: (1) unilateral nephrectomy and contralateral ischemic–reperfusion injury followed by high-phosphate diet (2% phosphate) and (2) 5/6th nephrectomy. Both CKD models showed cardiac hypertrophy and left ventricular fibrosis. αKlotho levels in kidney tissue, plasma, and urine were decreased by high-phosphate diet starting at 6 months of age. When high-phosphate diet was given to older mice (12 months of age), additional reductions in plasma and kidney αKlotho were observed (Hu, Shi, Cho, et al., 2015). Cardiac hypertrophy and fibrosis were exaggerated in *kl*/+ mice and lessened in transgenic αKlotho-overexpressing mice (*Tg-Kl*) compared to WT mice and changes were more severe at age 15 months compared with 9 months. Aging exacerbated phosphate or αKlotho deficiency-induced pathological cardiac remodeling. Notably, αKlotho suppressed cardiac fibrosis triggered by high dietary phosphate. αKlotho overexpression (*Tg-Kl*) suppressed phosphorylation of Smad2/3 and extracellular signal-regulated kinase (Erk; Hu, Shi, Cho, et al., 2015), which are known to be involved in uremic cardiac fibrosis (Olson, Naugle, Zhang, Bomser, & Meszaros, 2005).

In vitro, αKlotho blocked TGF-β1- and angiotensin II (Ang II)-induced hypertrophy in cardiomyocytes (primary culture of neonatal rat) by inhibiting Smad2/3 phosphorylation. αKlotho also attenuated TGF-β1-, Ang II-, and high phosphate-induced upregulation of fibrosis markers in cultured cardiac fibroblasts by inhibiting Erk phosphorylation (Hu, Shi, Cho, et al., 2015).

Cardiac hypertrophy and fibrosis scores correlated negatively with plasma αKlotho levels and positively with plasma phosphate levels. In multivariable analysis, adjusting for plasma creatinine, FGF23, PTH, and 1,25-$(OH)_2$-vitamin D_3 levels, only plasma αKlotho and phosphorus levels were independent factors associated with pathological cardiac remodeling (Hu, Shi, Cho, et al., 2015).

5.1.2 αKlotho Protection Against Stress-Induced Cardiac Hypertrophy—Xie and coworkers reported that cardioprotection by αKlotho in normal mice is mediated by

downregulation of TRPC-6 channels in the heart (Xie et al., 2012). In their experiments, deletion of TRPC-6 prevented stress-induced exaggerated cardiac remodeling in αKlotho-deficient mice (*kl*/+). In contrast, mice with heart-specific overexpression of TRPC-6 developed spontaneous cardiac hypertrophy and remodeling. Furthermore, αKlotho overexpression (*Tg-Kl* mice) ameliorated pathological cardiac remodeling and improved long-term survival (Xie et al., 2012). In addition, they proposed that soluble αKlotho inhibits TRPC-6 currents in cardiomyocytes by blocking phosphoinositide-3-kinase-dependent exocytosis of TRPC-6 channels (Xie et al., 2012).

Subsequently, the same investigators inferred that the decrease in soluble αKlotho in CKD is not only an important cause of uremic cardiomyopathy but independent of FGF23 and phosphotoxicity (Xie et al., 2015). They reported that αKlotho levels in αKlotho-deficient mice (*kl*/+) were about one half of those of WT mice, and they further decreased in *kl*/+ CKD mice to barely detectable levels (Xie et al., 2015). Heart weight-to-body weight ratio (a measure of cardiac hypertrophy) was significantly increased in both *WT* and *kl*/+ CKD mice, but the increase in *kl*/+ CKD mice was significantly more prominent than that in WT CKD mice. Similarly, the degree of fibrosis in *kl*/+ CKD was much more severe than that in *WT* CKD heart (Xie et al., 2015). *WT* CKD mice had ventricular hypertrophy, normal chamber size, and preserved contractility (diastolic dysfunction) in contrast to systolic dysfunction with dilated cardiomyopathy and impaired contractility in *kl*/+ CKD mice. Dietary phosphate restriction was successfully utilized to normalize serum phosphate and FGF23 levels in CKD mice (serum phosphate and FGF23 levels were similar in *WT* and *kl*/+ CKD mice compared with sham controls). Notably, dietary phosphate restriction did not significantly alter the pattern of cardiac hypertrophy in *WT* or *kl*/+ CKD mice (Xie et al., 2015). Moreover, viral-based deliver of αKlotho transgene significantly ameliorated cardiac hypertrophy and fibrosis in *kl*/+ CKD mice when compared with empty vector-injected mice. Functional TRPC-6-mediated currents were increased in cardiac myocytes isolated from CKD mice (vs sham), and the increase was more pronounced in *kl*/+ CKD vs WT CKD mice. Extracellular application of soluble αKlotho decreased these currents, confirming that αKlotho directly affects TRPC-6 functionality (Xie et al., 2015). The mechanisms of how the increase in TRPC-6 induces cardiomyopathy are not currently known.

5.1.3 αKlotho Protection Against Indoxyl Sulfate-Induced Myocardial Hypertrophy—Yang and colleges studied 86 patients with CKD and showed higher levels of indoxyl sulfate (Yang et al., 2015), a uremic solute derived from dietary protein and excreted by the kidney. Indoxyl sulfate accumulates with progressive loss of kidney function and can induce vascular endothelial cell dysfunction by enhancing oxidative stress (Tumur & Niwa, 2009; Tumur, Shimizu, Enomoto, Miyazaki, & Niwa, 2010). They showed a negative correlation between serum levels of indoxyl sulfate and αKlotho ($r = -0.59$, $p<0.001$). Importantly, serum levels of indoxyl sulfate and αKlotho were independently associated with LVH (Yang et al., 2015). This was further confirmed by experiments in normal mice in which intra-peritoneal injection of indoxyl sulfate for 8 weeks induced LVH, accompanied by substantial renal αKlotho downregulation. Notably, indoxyl sulfate-induced LVH was more severe in heterozygous αKlotho-deficient (*kl*/+) mice relative to *WT* mice,

indicating that αKlotho deficiency may exacerbate indoxyl sulfate-mediated LVH (Yang et al., 2015) and αKlotho supplementation may be a strategy to counteract indoxyl sulfate-mediated LVH.

In vitro experiments showed that indoxyl sulfate induces cardiomyocyte hypertrophy through activation of Nox/ROS/MAPK (p38 and Erk1/2) signaling pathways and this activation can be attenuated by pretreatment with αKlotho protein, possibly through inhibition of ROS signaling. Indoxyl sulfate-induced cardiomyocyte hypertrophy is not mediated through TRPC-6 signaling pathway (Yang et al., 2015). Interestingly, the in vivo administration of exogenous αKlotho protein significantly alleviated the development of LVH in a mouse model of CKD-associated LVH characterized by high serum indoxyl sulfate levels, which confirmed in vitro findings (Yang et al., 2015). Moreover, indoxyl sulfate has been shown to suppress αKlotho deficiency in mice (Adijiang, Shimizu, Higuchi, Nishijima, & Niwa, 2011) and downregulate αKlotho expression in cultured cells (Shimizu et al., 2011; Sun et al., 2012). Therefore, indoxyl sulfate has a dual effect: induction of cardiomyocyte hypertrophy and induction of αKlotho deficiency to enhance cardiomyocyte hypertrophy.

5.2 Vascular Medial Calcification

Apart from traditional risk factors, the high CV morbidity and mortality in CKD have been linked to CKD-specific mechanisms of vascular calcification through modulation of the endothelium–vascular smooth muscle network (Hu et al., 2014; Vervloet, Adema, Larsson, & Massy, 2014). Calcium and phosphate play an important role in the initiation of osteochondrogenic changes of cellular elements in the arterial wall, and also in the final common pathway of alleged ectopic bone formation (Vervloet et al., 2014). Although the expression of αKlotho in the vasculature is highly controversial, there are data associating changes in circulating αKlotho levels with uremic vasculopathy (Hu et al., 2014; Vervloet et al., 2014). Blood vessels are composed of endothelial cells, mural cells (smooth muscle cells and pericytes), their shared basement membrane, and extracellular matrix. Vascular smooth muscle cells (VSMCs) and endothelial cells work synergistically for maintenance of the integrity of the vasculature (Heydarkhan-Hagvall et al., 2003; Fig. 5).

5.2.1 αKlotho and Endothelium—Endothelial dysfunction is associated with CV morbidity and mortality in CKD (Ravani et al., 2005). Endothelial cells damaged by high phosphate or uremic solutes show increase in apoptosis, ROS, proinflammatory cytokines, profibrotic and proangiogenic growth factors, and impaired nitric oxide production (Carracedo et al., 2013; Di Marco et al., 2008). Vascular endothelium can be a source of osteoprogenitor cells in vascular calcification (Di Marco et al., 2008). Moreover, the endothelium could also stimulate VSMCs to initiate or participate in vascular calcification (Yao et al., 2013).

A functional vascular tone and low levels of oxidative stress are maintained by releasing nitric oxide, prostacyclin, and endothelin-1, and by controlling local angiotensin II activity. In addition, the endothelium also regulates vascular permeability, platelet and leukocyte adhesion and aggregation, and thrombosis (Sitia et al., 2010). Elevated asymmetric dimethylarginine, a known inhibitor of nitric oxide synthase, and consequent reduced nitric

oxide production and reduced flow-mediated dilatation (FMD) of the vessels have been characterized in CKD (Schwedhelm & Boger, 2011; Yilmaz et al., 2006). High phosphate impairs FMD in experimental CKD and FMD is inversely related to serum phosphate level in humans (Shuto et al., 2009; Van et al., 2012). Similarly, oxidative stress and inflammation are implicated in the development of endothelial dysfunction in CKD (Recio-Mayoral, Banerjee, Streather, & Kaski, 2011).

Interestingly, circulating αKlotho regulates vasodilation through modulation of nitric oxide production in vascular endothelium (Nagai et al., 2000; Saito et al., 1998; Six et al., 2014; Yamagishi et al., 2001). In addition, treatment of cultured endothelial cells with αKlotho alleviates tumor necrosis factor α-mediated ROS activity, cell apoptosis, and induction of adhesion molecules (Carracedo et al., 2012; Maekawa et al., 2009; Yang et al., 2012). Importantly, αKlotho-deficient mice have increased VEGF-mediated calcium influx, downregulation of cadherin surface expression, increased apoptosis, and increased permeability (Kusaba et al., 2010). It is suggested that the *Kl2* domain of αKlotho protein binds directly to VEGFR-2 and endothelial TRPC-1 Ca^{2+} channel and promotes their cointernalization and consequent reduction of cellular Ca^{2+} influx limiting the activity of Ca^{2+}-dependent proteases that disrupt endothelial integrity (Kusaba et al., 2010). αKlotho protein is also capable of attenuating indoxyl sulfate-induced endothelial dysfunction, partly through inhibition of ROS/p38 mitogen-activated protein kinase and downstream nuclear factor-κB signaling pathways (Yang et al., 2012).

It is possible that αKlotho may act on the endothelium and induce a secondary effect via endothelial-VSMC crosstalk, or that VSMC-resident αKlotho regulates VSMC function in an autocrine mode or even endothelium in a paracrine mode (Fig. 5). The role of αKlotho protein in the disturbed endothelium–vascular smooth muscle network in CKD requires further investigation.

5.2.2 αKlotho and Vascular Smooth Muscle—Measurement of aortic αKlotho mRNA expression has not been consistent (Hu et al., 2014; Lim et al., 2012; Mencke et al., 2015; Navarro-Gonzalez et al., 2014; Ritter et al., 2015; Six et al., 2014). However, the association between αKlotho deficiency and medial vascular calcification has been well documented in hypomorphic αKlotho mice (Kuro-o et al., 1997), a phenotype rescued by transgenic overexpression, viral delivery of αKlotho, or recombinant αKlotho protein (Chen, Kuro, et al., 2013; Masuda et al., 2005; Shiraki-Iida et al., 2000). Furthermore, transgenic mice over-expressing αKlotho had significantly less vascular calcification after CKD induction in comparison to αKlotho-haploinsufficient mice with CKD that exhibited more severe vascular calcification (Hu et al., 2011).

The potential mechanisms underpinning the association between high serum phosphate and vascular calcification have been described in experimental models of CKD (Jono et al., 2000; Lomashvili, Cobbs, Hennigar, Hardcastle, & O'Neill, 2004; Mathew et al., 2008). The beneficial effect of αKlotho on vascular calcification in CKD is thought to be a result of more than its effect on amelioration of renal dysfunction and hyperphosphatemia. In vitro experiments have shown that αKlotho suppresses type III Na^{+}-dependent uptake of phosphate (Pit-1 and Pit-2 cotransporters) and mineralization induced by high phosphate in

VSMCs (Hu et al., 2011). Runt-related transcription factor-2 (Runx2) expression, an early marker of ectopic osteogenesis, was decreased in the aortas of overexpressing αKlotho mice (Hu et al., 2011). The contractile phenotype of VSMCs was lost with exposure to high phosphate or resident αKlotho knockdown (Lim et al., 2012). Resident αKlotho knockdown in VSMCs accelerated the development of vascular calcification through Runx2 and myocardin-serum response factor-dependent pathway (Lim et al., 2012). Therefore, αKlotho may regulate VSMCs differentiation under CKD procalcific stressors (Hu et al., 2011; Lim et al., 2012). However, other experiments have resulted in conflicting evidence. One study did not show any effect of αKlotho protein on FGF23 and high phosphate-mediated vascular calcification in human or mouse VSMCs (Scialla et al., 2013). Another study performed in uremic rats showed that FGF23 augmented phosphate-induced aortic calcification in αKlotho-overexpressing but not naive VSMCs through Erk1/2 phosphorylation pathway (Jimbo et al., 2014). The conflicting in vitro data may be, besides plausibility, a reflection of differences in VSMCs or αKlotho protein preparations utilized.

Vitamin D receptor agonists (eg, calcitriol or paracalcitol) were shown to increase serum and urine αKlotho levels and abate aortic calcification in CKD mice likely through modulation of osteopontin, an anticalcification factor in VSMCs (Lau et al., 2012). Importantly, no αKlotho mRNA expression was found in the aorta in these in vivo experiments (Lau et al., 2012). One independent study further confirmed that there is no membrane αKlotho expression in either healthy or uremic vessels in humans (Mencke et al., 2015). In contrast, Lim and colleagues showed that calcitriol effectively restored mRNA αKlotho expression in VSMCs (Lim et al., 2012). In a different experiment, αKlotho mRNA was detected in mouse aorta but specific deletion of αKlotho in mouse VSMCs did not induce vascular calcification (Lindberg et al., 2013) challenging the principal role of αKlotho in VSMCs. Moreover, αKlotho protein was found to be increased in atherosclerotic arteries (Donate-Correa et al., 2013) contradicting the suggested protective role of resident αKlotho in vasculature. The existence and role of resident αKlotho protein in the vasculature need to be clarified and further investigated (Table 1).

In contrast to the inconclusive evidence of vascular αKlotho expression, the administration of exogenous recombinant αKlotho, αKlotho gene delivery, and increased endogenous circulating αKlotho significantly reduced vascular calcification and improved endothelial function, suggesting that soluble αKlotho may play a pivotal role in the protection of vasculature integrity as an endocrine factor (Chen, Kuro, et al., 2013; Lau et al., 2012; Masuda et al., 2005; Saito et al., 2000; Utsugi et al., 2000; Fig. 5).

It was shown that αKlotho protein attenuates endothelial cell damage from high phosphate and oxidative stress, and inhibits osteogenic transformation of VSMCs induced by high phosphate. The administration of exogenous αKlotho or modulators of αKlotho expression may represent novel therapies for the management of vascular calcification in CKD patients. Studies of endothelial cells or VSMCs in isolation may not fully represent the vascular system to dissect out the role of the individual players. Coculture of endothelial cells and VSMCs may be a viable intermittent system to further elucidate the role of αKlotho in the vasculature under normal and pathological conditions (Hu et al., 2014).

6. αKlotho DEFICIENCY AS A BIOMARKER OF CKD

CKD is a global public health problem that affects over 20 million people in the United States (Snyder, Foley, & Collins, 2009). The major complications in this population are progression to ESRD and CV morbidity and mortality (Eckardt et al., 2013; Hemmelgarn et al., 2010; Levey et al., 2011).

There has been intense search for highly sensitive (diagnostic value) or highly specific (treatment effect value) biomarkers of CKD onset and/or prognosis of progression. An ideal prognostic CKD biomarker should be able to predict CKD onset and progression, characterize the severity of CKD stage, display similar reliability across multiple species (particularly humans), and be accessible in readily available body fluids or tissues.

In the following sections, novel functional (detecting primarily loss of kidney function) and injury biomarkers (with or without loss of kidney function) of CKD and the potential role of FGF23 and αKlotho as early diagnostic and prognostic biomarkers of CKD will be discussed.

6.1 Functional Biomarkers in Human CKD

New filtration markers such as β-trace protein (BTP), β2 microglobulin (β2M), and cystatin C were associated with mortality risk in a representative sample of 6445 US adults from the Third National Health and Nutrition Examination Survey (Foster et al., 2013). The highest quintile for cystatin C, BTP, and β2M were associated with increased all-cause mortality risk, whereas the association was weaker for serum creatinine-based eGFR (Foster et al., 2013). A >50% decline in serum creatinine-based eGFR is an established surrogate marker for ESRD in clinical trials but a >30% decline in kidney function assessed using novel filtration markers (cystatin C and β2M) has been strongly associated with ESRD (Rebholz, Grams, Matsushita, Selvin, & Coresh, 2015).

6.2 Injury Biomarkers in Human CKD

The assessment of the plasma proteome through mass spectrometry analysis identified three fragments of high-molecular-weight kininogen associated with early progressive renal function decline in microalbuminuric patients with type 1 diabetes (Merchant et al., 2013). The performance of urine neutrophil gelatinase-associated lipocalin (NGAL) was analyzed in a cohort of 3386 patients with CKD in the Chronic Renal Insufficiency Cohort (CRIC) study. Urine NGAL was independently associated with 50% decreased eGFR or incident ESRD development over a mean follow-up of 3.2 years. However, it did not improve prediction models for CKD progression (Liu et al., 2013). More recently, urine NGAL has been independently associated with ischemic atherosclerotic events but not heart failure events or death (Fufaa et al., 2015). In a cohort of 124 patients with type 1 diabetes and proteinuria, serum kidney injury molecule-1 (KIM-1) levels at baseline strongly predicted rate of eGFR loss and risk of ESRD during 5–15 years of follow-up, after adjustment for baseline urinary albumin-to-creatinine ratio, eGFR, and hemoglobin A1C (Sabbisetti et al., 2014). Similarly, urinary NGAL and liver fatty acid-binding protein (L-FABP) were independently associated with incident ESRD and mortality but did not meaningfully

improved clinical prediction models of CKD progression in a cohort of 260 Pima Indians with median follow-up of 14 years (Fufaa et al., 2015).

6.3 FGF23 in Human CKD

Current evidence favors a direct pathogenic role for dysregulated FGF23-α Klotho in CKD-related adverse outcomes, in particular CV disease. The relationship between baseline serum intact FGF23 and incident ESRD was evaluated in 13,448 Atherosclerosis Risk in Communities (ARIC) study participants during a median follow-up of 19 years. After adjustment for demographics, baseline eGFR, and traditional CKD risk factors, the highest FGF23 quintile (>54.6 pg/mL) compared with the lowest quintile (<32.0 pg/mL) was associated with risk of developing ESRD (Rebholz, Grams, Coresh, et al., 2015). Similarly, elevated FGF23 has been proposed as an early indicator of kidney injury or CKD progression (Fliser et al., 2007; Wolf, 2012). Elevated serum FGF23 has been independently associated with LVH and a causal inference through Klotho-independent activation of FGF receptor-dependent activation of the calcineurin–NFAT signaling pathway in rat cardiomyocytes has been proposed (Faul et al., 2011). Increased FGF23 was independently associated with mortality among incident hemo-dialysis patients (Gutiérrez et al., 2008) and has been linked to mortality and higher risk of ESRD or CV disease in patients with CKD during a median follow-up of 3.5 years (Faul et al., 2011; Ix et al., 2012). In contrast, FGF23 was not associated with arterial calcification in 1501 patients from the CRIC study (Scialla et al., 2013).

6.4 The Role of αKlotho as a Marker of Adverse Outcomes in Human CKD

CKD is a state of αKlotho deficiency in multiple tissues. αKlotho mRNA levels in parathyroid gland declined in parallel with decreasing eGFR over CKD stages (Krajisnik et al., 2010). Similarly, αKlotho mRNA expression in kidney tissue was greatly reduced and positively and significantly correlated with eGFR in CKD patients (Asai et al., 2012,; Koh et al., 2001). In a larger sample of 236 CKD patients with available kidney biopsies, αKlotho mRNA levels were significantly and positively correlated with eGFR ($p<0.001$) in multiple regression analysis including CKD-MBD parameters (Sakan et al., 2014). Most importantly, αKlotho mRNA in the kidney was the only independent contributing factor to serum αKlotho across all strata of CKD patients. Renal αKlotho was significantly correlated with serum calcium, serum phosphorus, 1,25-$(OH)_2$-vitamin D_3, FGF23, and intact PTH (Sakan et al., 2014). Correspondingly, circulating serum αKlotho levels were progressively lower with each CKD stage when compared to healthy controls (Pavik et al., 2013). This was also observed in kidney transplant recipients vs healthy controls (Sawires, Essam, Morgan, & Mahmoud, 2015). Furthermore, adjusted mean serum αKlotho decrease was 3.2 pg/mL for each 1 mL/min eGFR decrease in adult CKD patients (Pavik et al., 2013). A positive correlation between αKlotho levels (serum and urine) and eGFR has been further characterized in adult CKD patients (Akimoto et al., 2012; Hu et al., 2011; Kim et al., 2013; Kitagawa et al., 2013; Ozeki et al., 2014), although only 24-h urine αKlotho but not serum αKlotho has been shown to be independently associated with eGFR change (Akimoto et al., 2012). However, questions about the stability of αKlotho in the urine have emerged (Adema, Vervloet, Blankenstein, & Heijboer, 2015). Therefore, a standardized urine protocol is required. A similar positive correlation between plasma αKlotho levels and eGFR was

shown in CKD children without kidney transplant (Wan et al., 2013). Moreover, serum αKlotho levels in children on chronic peritoneal dialysis were significantly lower (~41%) than healthy controls (Cano et al., 2014). The progressive decline of serum αKlotho in adults with early stages of CKD (eg, stage 2) has been subsequently demonstrated, suggesting that the decrease in serum αKlotho may antecede high FGF23, PTH, and hyperphosphatemia (Barker et al., 2015; Kim et al., 2013; Rotondi et al., 2015; Shimamura et al., 2012; Table 2).

6.4.1 α lotho and CV Disease in CKD—A cross-sectional study of 114 CKD patients (mean eGFR 48±29 mL/min/1.73 m^2) revealed that serum αKlotho was a significant marker of arterial stiffness measured by ankle–brachial pulse waive velocity (Kitagawa et al., 2013). In contrast, a large cohort study of 444 patients with CKD stages 2–4 showed that plasma αKlotho levels (highest vs lowest tertile) did not predict atherosclerotic events or death at 2.6 years follow-up. Serum αKlotho was also significantly reduced in hypertensive (essential and renovascular) patients with mild CKD when compared to healthy controls, even after adjustment by eGFR (Park et al., 2015). The proposed cross talk between the renin–angiotensin–aldosterone system and the vitamin D–FGF23–α Klotho pathways supports the concept that modulation of one system can have positive effects on the other (de Borst, Vervloet, ter Wee, & Navis, 2011). In this context, a post hoc analysis of the ESCAPE trial in children with CKD (all received fixed dose of ramipril 6 mg/m^2 per day) showed that 25(OH)D ≥50 nmol/L was associated with greater preservation of renal function. Interestingly, ACEI therapy significantly increased serum αKlotho levels without any associated changes in serum calcium or phosphate (Shroff et al., 2016; Table 2).

6.4.2 αKlotho and Progressive CKD—The most conclusive evidence thus far about the role of αKlotho as a predictor of adverse outcomes in CKD is based on a post hoc cohort study of 243 adult patients with CKD. In this study, serum αKlotho levels independently predicted the composite outcome of doubling S_{Cr}, ESRD, or death after multivariable adjustment. If serum αKlotho was ≤396.3 pg/mL, 35.2% reached the composite outcome vs 15.7% if >396.3 pg/mL (adjusted HR 2.03, 95% CI 1.07–3.85, $p = 0.03$). The areas under the curve (a measure of discrimination, that is, the ability of αKlotho to correctly classify those with and without the outcome) for 1/serum αKlotho to predicate the composite outcome (doubling S_{Cr}, ESRD, or death) were 0.81, 0.78, and 0.72 at 12, 24, and 36 months (Kim et al., 2013; Table 2). Further studies are needed to corroborate these findings.

Taken together, CKD is a state of αKlotho deficiency and dysfunctional vitamin D–FGF23–αKlotho pathways. Plasma αKlotho level positively correlates with eGFR and negatively with S_{Cr} and FGF23 and is a promising biomarker for the prediction of adverse outcomes (eg, CKD progression, CV morbidity, and death) in CKD patients. Therefore, αKlotho may represent a novel therapy for CKD patients that needs to be further investigated.

7. αKlotho AS A PROMISING TREATMENT STRATEGY FOR CKD

The kidney is confirmed as the major site that contributes to circulating αKlotho (Hu, Shi, Zhang, et al., 2015; Lindberg et al., 2014) and dysfunctional or decreased number of αKlotho-producing cells contribute to αKlotho deficiency leading to accelerated aging (Hu,

Shi, Zhang, et al., 2015; Lindberg et al., 2014). Therefore, αKlotho deficiency may not only be a pathogenic intermediate for accelerating CKD progression but also a main promoter of complications such as secondary hyperparathyroidism and CV disease in CKD. Conceivably, any therapy that restores or stimulates endogenous αKlotho or administration of exogenous αKlotho might provide a novel treatment strategy in CKD.

7.1 Epigenetic regulation of αKlotho Expression

αKlotho deficiency in the kidney of hypomorphic αKlotho-deficient mice was thought to result from interruption of the promoter of *αKlotho* gene by the exogenous transgene (Kuro-o et al., 1997). But recent data do not entirely support this notion because there was aberrant αKlotho promoter methylation in *kl*/*kl* mice. The in vitro study showed that *αKlotho* gene promoter methylation reduced promoter activity by 30–40%, whereas DNA demethylating agents increased *αKlotho* expression 1.5- to 3.0-fold (Azuma et al., 2012). Similarly, uremic toxins—indoxyl sulfate or *p*-cresyl sulfate—induced hypermethylation of the *αKlotho* gene, and decreased αKlotho expression in renal tubules and kidney cell line, which can be reversed by demethylation of the *αKlotho* gene. Therefore, hypermethylation may be one of the mechanisms of *αKlotho* gene expression inhibition in CKD (Chen, Zhang, et al., 2013; Sun et al., 2012; Young & Wu, 2012). In addition, promoter histone acetylation was also proposed as a possible mechanism for αKlotho silencing in many types of cancer cell lines (Rubinek et al., 2012; Xie et al., 2013). Furthermore, TNF and TNF-like weak inducer of apoptosis (TWEAK)-induced downregulation of αKlotho expression in the kidney and kidney cell lines can be blunted by inhibition of histone deacetylase (Moreno et al., 2011). Therefore, demethylating agents and deacetylase inhibitors may be agents to reactivate αKlotho expression in the kidney and consequently increase circulating αKlotho.

7.2 Reactivation of Endogenous αKlotho Expression Independently of Epigenetics

To date, several categories of drugs in the market including peroxisome proliferator-activated receptors-gamma (PPAR-γ) agonists (Chen, Cheng, Ku, & Lin, 2014; Yang et al., 2009; Zhang et al., 2008; Zhang & Zheng, 2008), angiotensin II type I receptor antagonists (Karalliedde, Maltese, Hill, Viberti, & Gnudi, 2013; Yoon et al., 2011; Zhou et al., 2010), 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitors (statin; Narumiya et al., 2004), and vitamin D active derivatives (de Borst et al., 2011; Forster et al., 2011; Lau et al., 2012; Lim et al., 2012; Ritter et al., 2015) have been shown to upregulate αKlotho expression in vivo and in vitro. The effect of upregulating αKlotho is definitely not associated with their well-identified original pharmacological targets, and pharmacological mechanisms remain to be explored.

7.3 Administration of Soluble αKlotho Protein

αKlotho gene delivery is shown to effectively rescue many phenotypes observed in αKlotho-deficient mice (Shiraki-Iida et al., 2000), attenuating the progression of hypertension and kidney damage in spontaneous hypertensive rats (Wang & Sun, 2009, 2014), improving kidney function in acute kidney injury (Sugiura et al., 2005), ameliorating angiotensin II-induced kidney injury (Mitani et al., 2002), improving endothelial function (Saito et al., 2000), and protecting from uremic cardiomyopathy (Xie et al., 2012, 2015). Although gene therapy is effective in animal studies, its safety is still questionable and

human clinical application is not in the proximity. There are only few clinical trials testing gene therapy in specific human diseases including genetic diseases (Williams, 2014) and some types of cancers (Heller & Heller, 2015).

In contrast, administration of exogenous αKlotho protein is more direct, safer, and an easier modality to restore endocrine αKlotho deficiency. Animal studies have already provided prove-of-concept encouraging data that soluble αKlotho protein administration is safe and effective (Hu, Shi, Zhang, Quinones, et al., 2010; Shi et al., 2015). Soluble αKlotho protein attenuates kidney damage and preserves kidney function in an ischemia-reperfusion injury model causing acute kidney injury, which is a state of acute αKlotho deficiency (Hu, Shi, Zhang, Quinones, et al., 2010). Furthermore, αKlotho protein inhibited renal fibrosis in a UUO kidney injury model (Doi et al., 2011), and effectively extended the life span of homozygous αKlotho-deficient mice, ameliorating premature aging-related phenotypes (Chen, Kuro, et al., 2013). Although there are no clinical data showing αKlotho protein administration to CKD patients, the preclinical data clearly support the therapeutic potential of soluble αKlotho protein in CKD patients.

8. CONCLUSION AND FUTURE DIRECTIONS

Although progress has been made in understanding that αKlotho is not only an aging suppressor involved in longevity and aging, but also a renoprotective factor which has a central impact on renal physiology and renal pathophysiology, the utility of αKlotho in clinical practice is still under development (Table 3). First, αKlotho could serve as an early and sensitive biomarker of CKD, although its specificity and its prognostic value require further exploration in humans. Similarly, whether urine or serum αKlotho is a better biomarker remains to be confirmed. Second, exogenous αKlotho supplementation may represent a novel therapy to retard or block progressive CKD, post-AKI transition to CKD, as well as preventing and reversing CV complications associated with renal disease as shown in animal studies. The therapeutic efficacy of αKlotho in kidney disease has been unequivocally demonstrated in animal models. One needs to validate the efficacy of αKlotho therapy in different stages of kidney disease.

Although the effects of αKlotho protein on the kidney will invariably be pleiotropic, how αKlotho exerts its renoprotection is largely inconclusive. High levels of FGF23 have been found to be associated with high mortality and morbidity in CKD (Arnlov et al., 2013; Desjardins et al., 2012; Faul et al., 2011; Fliser et al., 2007; Grabner et al., 2015; Greenhill, 2011; Guo & Yuan, 2015; Hanks, Casazza, Judd, Jenny, & Gutierrez, 2015; Hasegawa et al., 2010; Krupp & Madhivanan, 2014; Mencke et al., 2015; Mirza, Larsson, Melhus, Lind, & Larsson, 2009; Razzaque, 2009a, 2009b; Rotondi et al., 2015; Sawires et al., 2015; Silswal et al., 2014; Silver, Rodriguez, & Slatopolsky, 2012; Sinha et al., 2015; Wolf, 2010; Wright et al., 2014; Zhang, Yan, Zhu, & Ni, 2015; Zhang, Yang, et al., 2015). Given that administration of exogenous αKlotho has a favorable effect on CKD animals in terms of improvement of renal function, better maintenance of phosphate homeostasis, and attenuation of vascular calcification and cardiac hypertrophy, whether synergistic utilization of FGF23 antagonist or inhibitor and αKlotho can enhance αKlotho therapeutic efficacy needs to be tested.

Some therapeutic modalities including ACE inhibition, HMG-CoA reductase inhibition, vitamin D derivatives, and antioxidants could sustain or increase endogenous αKlotho expression. It is conceivable that a multi-targeted regimen including αKlotho protein, FGF23 antagonist, and endogenous αKlotho inducers may enhance the beneficial effect of αKlotho on retardation of CKD progression and prevention or reduction of CV morbidity and mortality in CKD. The immediate challenge is to test whether human CKD resembles the rodent counterpart and if so, how to more efficiently increase αKlotho levels in patients with CKD, either by stimulating endogenous αKlotho or by administering recombinant αKlotho. Finally, αKlotho has shown to be effective in experimental CKD models such as renal ablation (Xie et al., 2015), hypertensive kidney damage (Tang et al., 2011; Wang & Sun, 2009), UUO (Doi et al., 2011; Guan et al., 2014; Satoh et al., 2012; Sugiura et al., 2012), but few experimental data showed its effect on CKD secondary to diabetes or other glomerular diseases which account for large part of the CKD population. More animal studies and clinical observational studies are required to consider the administration of exogenous αKlotho protein a potential novel therapeutic strategy for CKD.

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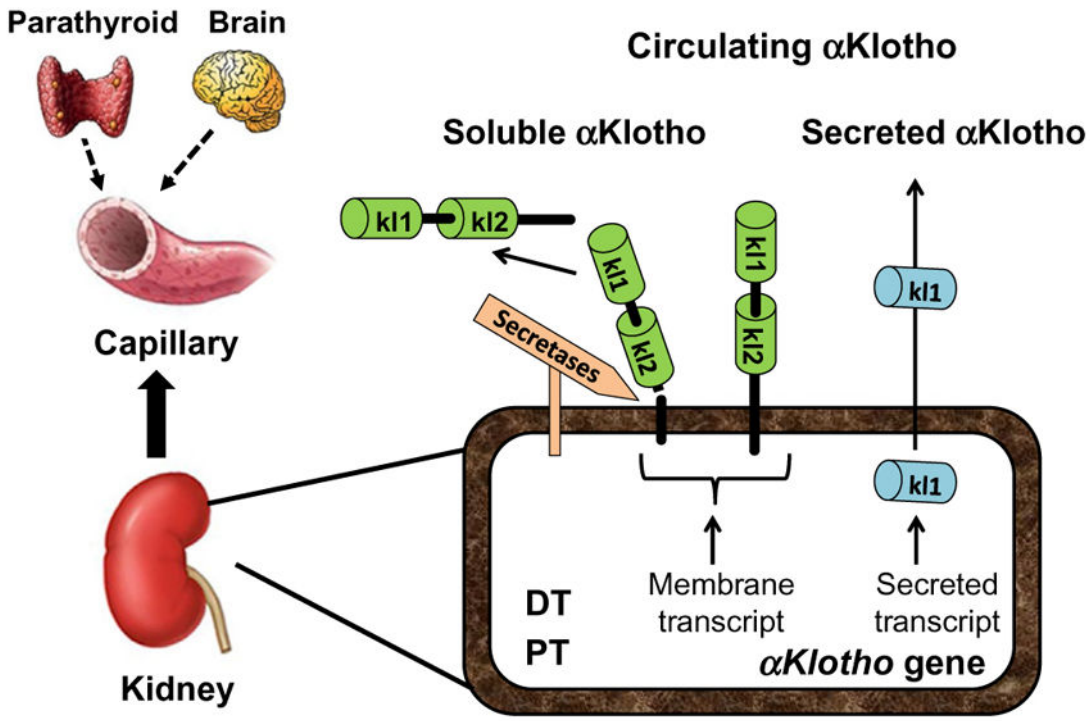

Fig. 1.

Source of circulatory αKlotho. αKlotho protein is expressed in a few organs, but the kidney is a main resource of circulating αKlotho under physiological conditions. The contribution of parathyroid gland and brain is not clear. Both renal proximal (PT) and distal tubules (DT) express membrane αKlotho protein and may also produce a secreted αKlotho protein which only contains kl1 domain and is directly secreted into the blood circulation. Extracellular domain of membrane αKlotho-containing kl1 and kl2 repeats is shed and cleaved by α and β-secretases, and released into the blood circulation.

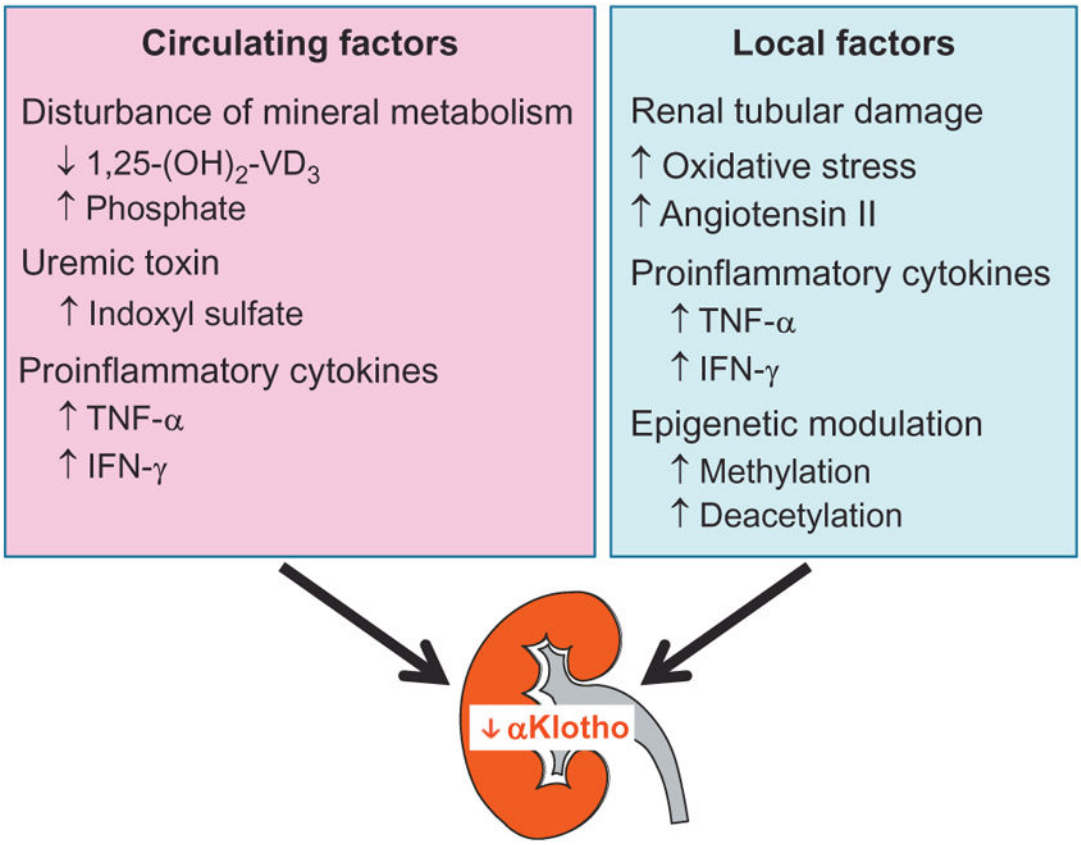

Fig. 2.

Circulating and local renal factors involved in the reduction of αKlotho expression in the kidney. In acute and chronic kidney disease, a variety of circulating factors including disturbed mineral metabolism, and accumulation of indoxyl sulfate and proinflammatory cytokines (*left panel*), can downregulate renal αKlotho expression. On the other hand, the elevation of reactive oxygen species, Ang II, and inflammatory cytokines in the diseased kidney can also downregulate renal αKlotho expression (*right panel*). Epigenetic modulation of αKlotho promoter via hypermethylation and deacetylation can reduce αKlotho expression and contribute to αKlotho deficiency in chronic kidney disease.

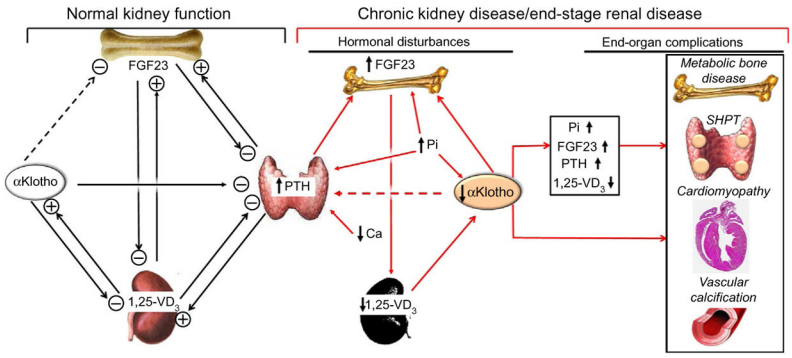

Fig. 3.

Proposed physiological role of αKlotho in mineral metabolism and pathophysiological consequences of αKlotho deficiency in CKD. In the setting of normal kidney function with normal αKlotho levels (*left panel*), αKlotho may suppress FGF23 production and release from the bone. But there is no direct evidence to prove it. αKlotho functions as coreceptor of FGFR to allow FGF23 to suppress PTH production and release from parathyroid gland. PTH stimulates and increases plasma levels of FGF23 and 1,25-$(OH)_2$-vitamin D_3. Increased 1,25-$(OH)_2$-vitamin D_3 further stimulates FGF23, and directly and indirectly suppresses PTH levels. Increased 1,25-$(OH)_2$-vitamin D_3 also stimulates αKlotho production in the kidney. Taken together, through several negative- or positive-feedback loops, αKlotho functions as both a phosphate and calcium regulatory hormone to directly or indirectly suppress PTH, 1,25-$(OH)_2$-vitamin D_3, and FGF23 production and release. αKlotho's action on the kidney is to prevent renal Pi retention and to prevent renal Ca loss. In CKD and ESRD (*right panel*), the network is deranged (*red arrows*). Renal αKlotho is decreased followed by decrease in plasma αKlotho. The downregulation of αKlotho increases FGF23 production via unknown mechanism, which in turn suppresses 1,25-$(OH)_2$-vitamin D_3 production in the kidney. Whether low plasma αKlotho renders parathyroid gland resistant to the suppressive effect of FGF23 on PTH production is not proven. However, decreased FGFR1/3 and αKlotho expression in the uremic parathyroid gland could make the gland resistant to FGF23, and triggers and/or promotes secondary hyperparathyroidism (SHPT). Low plasma Ca also participates in SHPT development. Hyperphosphatemia amplifies the high FGF23 and PTH levels, and low αKlotho levels in the blood. The high plasma PTH, Pi, and FGF23, and low plasma 1,25-$(OH)_2$-vitamin D_3 and αKlotho in concert contribute to the development of complications such as metabolic bone disease, SHPT, cardiomyopathy, and vascular calcification. *Dash line*: unproven putative roles of αKlotho. *Ca*, ion calcium; *CKD*, chronic kidney disease; *ESRD*, end-stage renal disease; *FGFR*, FGF receptor; *Pi*, phosphate; *PTH*, parathyroid hormone; *SHPT*, secondary hyperparathyroidism; *1,25-VD3*, 1,25-$(OH)_2$-vitamin D_3.

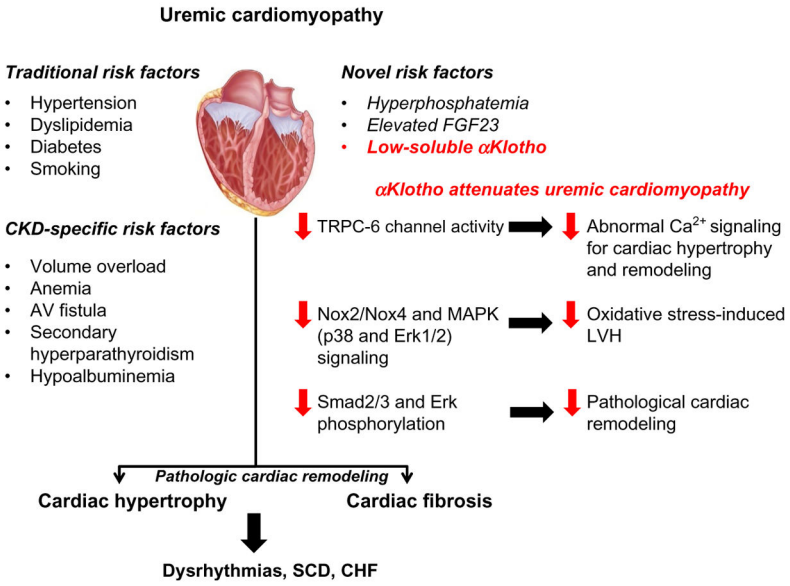

Fig. 4.

Risk factors for uremic cardiomyopathy and proposed mechanisms of attenuation of pathological cardiac remodeling by αKlotho. αKlotho deficiency is a novel risk factor for uremic cardiomyopathy. Soluble αKlotho deficiency is an intermediate mediator of the pathological cardiac remodeling observed in CKD. Experimental αKlotho overexpression (*Tg-Kl*) suppressed phosphorylation of Smad2/3 and Erk, which are known to be involved in uremic cardiac fibrosis. Furthermore, αKlotho may protect the heart against stress-induced cardiac hypertrophy by inhibiting TRPC-6 channel-mediated abnormal Ca^{2+} signaling in the heart or against uremic solute indoxyl sulfate-induced myocardial hypertrophy probably by suppressing NADPH oxidase Nox2/Nox4-derived reactive oxygen species production and its downstream signaling. *CHF*, congestive heart failure; *Erk*, extracellular signal-regulated kinase; *LVH*, left ventricular hypertrophy; *MAPK*, mitogen-activated protein kinases; *Nox*, NADPH oxidase; *SCD*, sudden cardiac death; *TRPC-6*, transient receptor potential canonical-6.

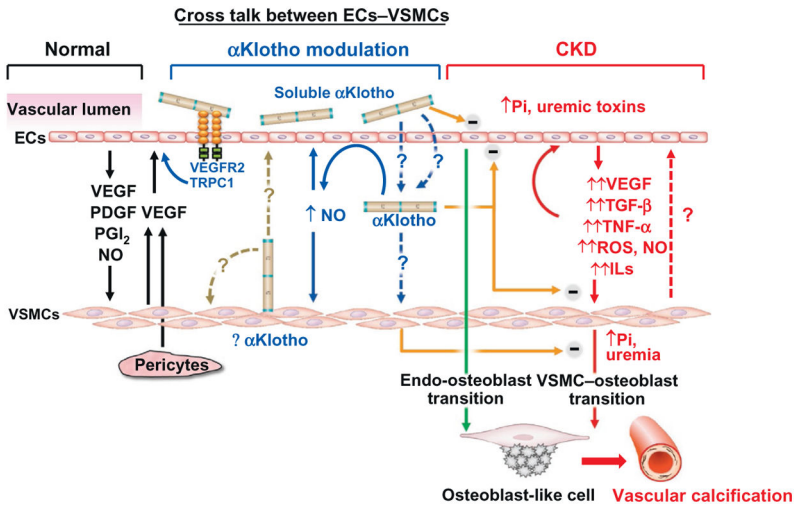

Fig. 5.

Proposed model of αKlotho as intermediate of endothelial cells (ECs)–vascular smooth muscle cells (VSMCs) cross talk. *Left panel*: Normal ECs–VSMCs cross talk. Normal ECs modulate VSMCs growth via release of growth factors (*black line* in *left panel*). *NO*, nitric oxide; *PDGF*, platelet-derived growth factor; *PGI_2*, prostaglandin I_2; *VEGF*, vascular endothelial growth factor. VEGF released from pericytes and/or VSMCs could also regulate endothelial cell. *Right panel*: In CKD, uremic toxins including high plasma Pi, damage ECs and induce release of growth factors, proinflammatory cytokines, and profibrotic factors, which exacerbate ECs injury and also induce VSMCs transition to osteoblast and promote vascular calcification in medial layer (*red solid lines* in *right panel*). Impaired ECs also directly contributes to vascular calcification through endo-osteoblast transition (*green line* in *right panel*). Whether dedifferentiated or damaged VSMCs could further modulate function of endothelial cells is speculative (*red dash line* in *right panel*). *Central panel*: αKlotho is a vascular protective protein. Whether resident aortic αKlotho protein in VSMCs functions in autocrine and/or paracrine mode to modulate VSMCs and/or ECs remains to be clarified (*brown dash line* in *central panel*). The mechanisms of how αKlotho is able to reach the VSMCs from the circulation and function as an endocrine factor remain to be defined (*blue dash line* in *central panel*). αKlotho, regardless of source, could increase NO production from ECs and NO consequently modulates VSMCs and ECs function in an autocrine mode (*blue solid line* in *middle panel*). αKlotho could protect EC from high phosphate and other uremic toxins and also attenuate oxidative stress and proinflammatory cytokines-induced cell senescence and apoptosis in VSMC (*orange line* in *central panel*). αKlotho also directly inhibits osteoblast transition induced by hyperphosphatemia and uremic milieu (*orange line* in *central panel*). Current experimental and clinical observations suggest that both ECs and VSMCs endothelium may be potential targets of soluble αKlotho to protect the vasculature from vascular calcification in CKD. *ECs*, endothelial cells; *Pi*, phosphorus; *VSMCs*, vascular smooth muscle cells.

Table 1

The Determination of Aortic αKlotho mRNA Expression Has Been Inconsistently Reproduced in Different Studies

Aortic αKlotho mRNA Expression = Yes	**Aortic αKlotho mRNA Expression = No**
Klotho mRNA and protein is present in aorta and vascular smooth muscle cells Resident αKlotho knockdown induced vascular calcification through *Runx2* and myocardin-serum response factor-dependent pathway Calcitriol effectively restored mRNA αKlotho expression in VSMCs (Lim et al., 2015, 2012)	No αKlotho mRNA expression was found in the aorta in in vivo experiments of aortic calcification in CKD (Lau et al., 2012)
αKlotho mRNA was detected in mouse aorta but specific deletion of αKlotho in mouse VSMCs did not induce vascular dysfunction and vascular calcification (Lindberg et al., 2013)	No membrane αKlotho expression in either healthy or uremic human vessels was found (Mencke et al., 2015)
αKlotho protein was found to be increased in atherosclerotic arteries (Donate-Correa et al., 2013)	
Human umbilical vein endothelial cells (HUVECs) express αKlotho. The decline in αKlotho preceded the manifestations of cell aging induced by repeated passage and those of senescence by TNF-α. The exogenous αKlotho administration prevented TNF-α-mediated senescence (Carracedo et al., 2012)	

Table 2

Clinical Studies Demonstrating the Implication of Kidney and Circulating αKlotho in Different Strata of Chronic Kidney Disease and Complications of Chronic Kidney Disease

Study	Design	Sample	Exclusion	Measurement	Observations	Comments
Koh et al. (2001)	Cross sectional	10 CKD and 15 controls	None	Kidney tissue *kl* mRNA expression	αKlotho expression was greatly reduced in CKD vs controls	Controls included healthy parts of resected kidneys with renal cell carcinoma or angiomyolipoma
Asai et al. (2012)	Cross sectional	31 pts with DN, 31 IgAN, and 7 MCD	None	Kidney tissue *kl* mRNA expression	Kidney *kl* mRNA expression levels were positively and significantly correlated with eGFR ($r = 0.35$, $p = 0.003$)	Different slopes depending on CKD cause were observed, particularly in patients with eGFR >60
Pavik et al. (2013)	Cross sectional	87 CKD pts (stages 1–5) and 21 controls	PKD, kidney transplantation	Serum αKlotho	Adjusted mean αKlotho decrease was 3.2 pg/mL for each 1 mL/min eGFR decrease	Age and eGFR were independently associated with αKlotho
Wan et al. (2013)	Cohort	154 children with CKD (1–5, 28 on dialysis, 44 posttransplant)	None	*Predictor*: Plasma αKlotho *Outcome*: eGFR Follow-up: 1 year	αKlotho levels decreased with decreasing eGFR in CKD children without kidney transplant	No independent association between plasma αKlotho and eGFR. Decreased αKlotho was associated with increased FGF23 and PTH levels in CKD children without kidney transplant
Kitagawa et al. (2013)	Cross sectional	114 CKD pts	Patients with established atherosclerotic complications (CAD, CHF, PVOD) or those treated with vitamin D or phosphate binders	Serum αKlotho	αKlotho was a significant determinant of arterial stiffness (adjusted OR per 100 pg/mL increase 0.60, 95% CI 0.39–0.98, $p = 0.008$)	Positive correlation between serum αKlotho and eGFR. Arterial stiffness was determined by ankle– brachial pulse wave velocity
Kim et al. (2013)[a]	Cohort	243 pts with CKD (stages 1–5), post hoc analysis	RRT, organ transplantation, heart failure, cirrhosis, malignancy, pregnancy, acute coronary syndrome, or ischemic stroke within 3 months prior to the study, progressive CKD within 3 months prior to the study	*Predictor*: Serum αKlotho *Outcome*: composite of doubling SCr, ESRD, or death Follow-up: median 29.7 months	αKlotho level independently predicted the composite outcome after multivariate adjustment (adjusted HR per 10 pg/mL increase 0.96, 95% CI 0.94–0.98, $p<0.001$). If serum αKlotho was ≤396.3 pg/mL, 35.2% reached the composite outcome vs 15.7% if >396.3 pg/mL ($p = 0.03$)	αKlotho levels were lower at more advanced CKD stages (p for trend <0.001) and correlated positively with eGFR and negatively with FGF23 and phosphate level. In multivariate linear regression analysis, αKlotho was independently associated with eGFR ($p<0.001$)
Akimoto et al. (2012)	Cross sectional	131 CKD pts (stages 1–5)	RRT	Serum and urine αKlotho	Positive correlation between serum and urine αKlotho and eGFR	24-h urine αKlotho was independently associated with eGFR
Sakan et al. (2014)	Cross sectional	236 CKD pts (stages 1–5), 43 on dialysis	None	Kidney tissue *kl* mRNA expression and serum αKlotho	Kidney *kl* mRNA levels were significantly and positively correlated with eGFR ($p<0.001$) in multiple regression analysis including CKD-MBD parameters	Kidney *kl* mRNA was the only independent contributing factor to serum αKlotho across all strata of CKD patients ($p<0.001$)
Seiler et al. (2014)	Cohort	444 CKD pts (stages 2–4)	None	*Predictor*: plasma αKlotho *Outcome*: (1) composite of	Plasma αKlotho levels (highest vs lowest tertile) did not predict atherosclerotic events/death (HR	Events were adjudicated by two independent nephrologists

Study	Design	Sample	Exclusion	Measurement	Observations	Comments
				atherosclerotic event or death or (2) time until ADHF admission or death Follow-up: median of 2.6 years	0.75, 95% CI 0.43–1.30, $p = 0.30$) or ADHF/death	
Ozeki et al. (2014)	Cross sectional	185 CKD pts (stages 1–5)	RRT	Serum αKlotho	eGFR was an independent predictor of serum αKlotho levels in multivariate linear regression analysis, specifically when eGFR was <60	Serum αKlotho was not significantly correlated with serum calcium or phosphate
Cano et al. (2014)	Cohort	31 children on chronic PD and 45 healthy controls	None	*Predictor*: CKD-MBD parameters *Outcome*: serum αKlotho Follow-up: 1 year	Baseline αKlotho levels were lower than controls (132±58 vs 320±119 pg/mL, $p<0.001$) and remained virtually unchanged throughout the observation period	αKlotho levels did not correlated with FGF23 and phosphorus levels
Park et al. (2015)	Cross sectional	24 HTN pts (12 with RVH and 12 EH) and 12 HV controls	eGFR<30, uncontrolled BP, diabetes, recent CV event (within 6 months), pregnancy, kidney transplant	Serum αKlotho	Serum αKlotho was significantly reduced in HTN pts vs HV controls after adjustment by eGFR	eGFR correlated directly with serum αKlotho levels
Sawires et al. (2015)	Cross sectional	40 CKD pts (stages 2–5), 44 pts with ESRD on HD, 40 kidney transplant recipients, and 40 healthy controls	Marked hypocalcemia, dysfunctional HD access, combined organ transplantation, surgical parathyroidectomy, sarcoidosis	Serum αKlotho	Using multivariate regression analysis, only serum calcium was an independent predictor of serum αKlotho in all groups with kidney disease	There was an inverse significant correlation between serum calcium and αKlotho
Shroff et al. (2016)	Cohort	ESCAPE cohort post hoc analysis, 167 children with CKD on ACEI	None	*Predictor*: ACEI therapy *Outcome*: serum αKlotho Follow-up: 8 months	ACEI therapy significantly increased serum αKlotho levels without any associated changes in serum calcium or phosphate	αKlotho levels did not correlate with eGFR at baseline ($r = 0.02$, $p = 0.82$) or at 8-month follow-up ($r = 0.06$, $p = 0.45$)

[a] Highlighted as an important observational study.

Table 3

Potential Applications of αKlotho in Chronic Kidney Disease

Biomarker		**Therapeutic Agent**	
Diagnostic	Early detection of CKD	Prevention	• CKD progression • CV comorbidity and complications
Prognostic	Prediction of progression to ESRD	Treatment	• Anti-Pi toxicity • Attenuation of CV

CKD, chronic kidney disease; *CV*, cardiovascular event; *ESRD*, end-stage renal disease.