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SECRETED KLOTHO AND CHRONIC KIDNEY DISEASE

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Abstract

Soluble Klotho (sKl) in the circulation can be generated directly by alterative splicing of the Klotho transcript or the extracellular domain of membrane Klotho can be released from membrane-anchored Klotho on the cell surface. Unlike membrane Klotho which functions as a coreceptor for fibroblast growth factor-23 (FGF23), sKl, acts as hormonal factor and plays important roles in anti-aging, anti-oxidation, modulation of ion transport, and Wnt signaling. Emerging evidence reveals that Klotho deficiency is an early biomarker for chronic kidney diseases as well as a pathogenic factor. Klotho deficiency is associated with progression and chronic complications in chronic kidney disease including vascular calcification, cardiac hypertrophy, and secondary hyperparathyroidism. In multiple experimental models, replacement of sKl, or manipulated up-regulation of endogenous Klotho protect the kidney from renal insults, preserve kidney function, and suppress renal fibrosis, in chronic kidney disease. Klotho is a highly promising candidate on the horizon as an early biomarker, and as a novel therapeutic agent for chronic kidney disease.

INTRODUCTION

As one approach almost one and one-half decade of research since its discovery in 1997, Klotho biology is eventually entering into the public health domain where its dysregulation is implicated in human pathophysiology, and its diagnostic and therapeutic potential are beginning to enter the realm of reality. We will take a preclinical viewpoint of the status of Klotho in this aspect using kidney disease as a vehicle. Klotho is a single transmembrane 130 KDa protein encoded by the *Klotho* gene. A secreted form of Klotho of 70 kD is a product of alternative splicing[4–7] and the extracellular domain of membrane Klotho can be released into blood[8,9] thus functioning as a circulating substance to exert multiple systemic biological actions on distant organs.[10–13] This cleaved extracellular domain of membrane Klotho is referred as soluble Klotho (sKl) in this chapter. sKl could function as a β-glucuronidase[7,14] and sialidase.[15,16] Klotho is principally synthesized in kidney and brain although it is expressed in multiple organs.[6,17] Klotho-deficient mice ($Kl^{-/-}$) manifest multi-organ premature aging while over-expression of Klotho through virus-mediated delivery or genetic overexpression can rescue the Klotho-deficient phenotype at baseline[6,19,20] and enhance mice's resistance to oxidant stress and ischemic damage.[6,21–23]

One important biological function of Klotho is to maintain mineral homeostasis, which is both fibroblast growth factor-23 (FGF23)-dependent and FGF23-independent. From a renal point of view, sKl regulates urinary calcium,[16,24] potassium[15] and phosphorus excretion.[14] In addition, Klotho suppresses 1α-hydroxylase in the kidney to regulate calcium

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metabolism[25,26] and participates in the regulation of PTH synthesis in parathyroid gland by FGF23.[27,28]

The study of Klotho in human biology has been limited to association studies of clinical features with Klotho polymorphisms with potential but yet to be determined significance.[29–36] The association of genetic variations in the *KLOTHO* gene with high mortality in hemodialysed patients was shown, but this link could be modified by activated vitamin D supplementation.[38] This modification of association of nucleotide polymorphisms in the *KLOTHO* gene may result from epigenetic upregulation of Klotho expression by Vitamin D[26] and subsequently affects the outcome of CKD patients. Interestingly, unlike the myriads of phenotypic features described in the rodent models of Klotho deficiency or excess, both Klotho deficiency caused by loss-of-function missense mutation[45] and presumed Klotho overexpression due to translocation in human *Klotho* gene[47] display disturbances in mineral metabolism but surprisingly both deficient and excess states have high FGF23 and hyperparathyroidism. These findings support the important role of Klotho in mineral homeostasis and suggest that there is still higher level of complexity that is beyond our comprehension at the moment.

In this book, there are several comprehensive reviews addressing Klotho's effect on aging, renal ion channels and transporter, and Pi homeostasis. This chapter will be mainly focused on the role of sKl in chronic kidney diseases and potential clinical implications.

KIDNEY: THE MAJOR SITE FOR KLOTHO SYNTHESIS

The association of kidney disease with Klotho can be view as intuitive considering the expression pattern of Klotho. The distribution of *Klotho* mRNA is restricted to few tissues with the strongest expression in the kidney and weaker ones in the brain, heart, parathyroid gland and testis.[6,48] RT-PCR detects *Klotho* expression in more tissues including aorta, colon, pituitary gland, thyroid gland, pancreas, and gonads but the kidney still has the strongest Klotho expression. From the onset, the findings suggest that the kidney might be a major source of endocrine Klotho. The higher Klotho protein in the suprarenal vein than that in infrarenal vein in rodents (personal observation) and the low circulating plasma levels observed in rodent with renal failure[2] strongly suggests that the kidney is a main source for Klotho in the blood circulation (Fig. 1 A).

In mammalian kidney including mouse, rat and human, Klotho is prominently expressed in distal convoluted tubules (DCT),[17] but Klotho is also unequivocally found in the proximal convoluted tubule (PCT) although in lower levels compared to DCT.[14] The presence of *Klotho* mRNA is confirmed by RT-PCR in micro dissected rat nephron segments, and in OK cells, a renal proximal tubule-like cell line. The PCT mRNA rules out Klotho protein uptake as an explanation for Klotho antigen in the PCT (Fig. 1B).[14]

Klotho protein is present in cerebrospinal fluid (CSF),[49] blood[2,49,50] and urine of mammals.[2,14,50] This soluble Klotho protein of 130 kDa by SDS-PAGE[49] is different from the secreted Klotho encoded by a spliced transcript of Klotho with a predicted size of 70 kDa.[4,5] The extracellular domain of Klotho is shed from full length membrane Klotho protein by cc-secretase, ADAM (a disintegrin and metalloprotease) 10 and 17 and β-secretase, β-APP cleaving enzyme 1, and released into blood circulation (Fig. 1C).[9] The cleavage and release of the extracellular domain of Klotho by ADAM 10 and ADAM 17 is stimulated by insulin and inhibited by metalloproteinase inhibitors.[8] ADAM 10 is expressed in the DCT and is potentially perfectly poised to cleave Klotho.[51] To date, whether other molecules are also able to regulate membrane Klotho shedding is not known. The substrates for ADAM 10/17 are massively broad and what regulates ADAM 10/17-mediated ectodoamin shedding requires much more studies. This is a serious void in the database as

the control of release from the parental pre-molecule is a crucial step for any endocrine substance.

sKl plays important roles in a variety of physiological and pathological processes including modulation of Wnt signal transduction,[11] anti-oxidation[52] and renal ion channels[15,16,24] and transporters.[14] Another possible role of the cleaved extracellular domain of Klotho may act to modify the signal transduction role played by membrane Klotho as a coreceptor for FGF23 but there is no data to date to support Klotho's ability to quench soluble FGF23 (as a decoy) or to block the Klotho-FGF receptor complex (as a competitive inhibitor).

In addition to the final urine, Klotho protein is detected in the proximal tubule urine.[14] This location of Klotho is absolutely necessary for it to function as a phosphaturic substance.[14] Currently, the origin of this proximal luminal Klotho is unclear. It may be directly cleaved from proximal tubular cell or derived from the pertibular blood circulation via transcytosis (Fig. 1B). The only route that cannot be true is glomerular filtration as Klotho is too large for the glomerular basement membrane selectivity filter. Indeed, there is no detectable Klotho antigen in the glomerular Bowman space proto-urine (personal observation).

The kidney is a principal organ in calcium[16,24,53] and phosphate[14] homeostasis and directly modulates blood 1,25-$(OH)_2$ Vitamin D_3 and indirectly PTH, and FGF23 levels.[25,27,28,54–56] In addition to being commanded by calciotropic hormones as an operative, the kidney is endowed with the calcium sensing receptor[57] which renders it a self-sufficient afferent and efferent organ for calcium homeostasis. Although the identity of the phosphate sensor is elusive, the proximal tubule is clearly responding to ambient phosphate concentrations[58] and exerts it effect by regulating Na-phosphate cotransporters directly[59] and 1α-hydroxylase expression and activity.[60] Although 1α-hydroxylation is present in many organs,[60] the proximal tubule activity is the most important and regulated.[61] Klotho protein is poised in the central place of this network of mineral metabolism homeostasis where it is in direct apposition with the sensors and effectors. The physical proximity and functional coupling of the glomerulus, proximal, and distal tubule renders this ensemble a perfect locale for a paracrine, autocrine, and endocrine hormone.

When normal rats were subjected to ischemia-reperfusion injury, adenovirus-mediated Klotho gene delivery results in significantly improved serum creatinine, dramatically ameliorated renal histological changes, and remarkably diminished apoptotic cells in the kidneys.[23] It is important to note that the gene transfer occurred only in the liver and not in kidney. This experimental finding strongly suggests that Klotho functions as a circulating substance to exert renoprotection.

CHRONIC KIDNEY DISEASE: A STATE OF KLOTHO DEFICIENCY

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. There are many similar features between the clinical manifestations of CKD with the phenotypes of $Kl^{-/-}$ mice (Table 1), which is the first suggestion that CKD might be a state of Klotho deficiency. Experimental data and clinical findings have thus far supported this view. There is a significant reduction of renal Klotho transcript and protein in CKD with varied etiology including ischemic perfusion injury, subtotal nephrectomy, oxidant stress, and exposure to angiotensin II (Ang II) and calcineurin inhibitors, and also in human CKD including chronic glomerulonephritis, obstructive nephropathy, diabetic nephropathy, and chronic graft rejection (Table 2). Hyperphosphatemia is a prominent abnormality in CKD, and is a major contributor to cardiovascular disease, which accounts for significant mortality in this population. Severe hyperphosphatemia is also found in Klotho-deficient mice.[6,14] High FGF23 level in the blood is a feature in patients with CKD,[62,63] and could contribute to

decline of the 1α-hydroxylase activity, hyperparathyroidism, bone disease and cardiac hypertrophy in CKD.[63,64] This can be viewed as a revisited version of the "trade-off hypothesis" in CKD. High plasma FGF23 is also observed in Klotho-deficient mice.[65] In patients with CKD, ectopic calcification is frequently encountered.[66,67] Interestingly, *Kl*$^{-/-}$ mice have very severe and wide-spread ectopic calcifications including vascular calcification.[2,6]

A hypothesis can be raised that the Klotho deficiency may be one of the factors triggering complications in CKD and correction of Klotho deficiency may be a therapeutic possibility for treatment of kidney diseases.

Renal Klotho Deficiency

Aging Kidney—Aging is a complicated and chronic degenerative biologic process that affects kidney function and morphology. Aging is associated with decline in kidney function[68] coincident with a progressive decrease in the number of functioning nephrons and increase in glomerular and tubulointerstitial scarring.[69] These changes may cause secondary abnormal systemic hemodynamics, and disturbed mineral and hormonal homeostasis.[70]

The aged kidney is more prone to ischemic injury and nephrotoxin.[71,72] Epidemiological data clearly shows that CKD is common in the elderly and associated with higher morbidity and mortality. More than 40% Canadian residents at 65 years or older has moderate CKD (estimated GFR < 60 ml/min/1.73 m^2). Among American CKD patients, about 30% are from age 75 to 84 and 50% of patients of age 85 to 100 have at least moderate CKD.[73]

Aged mice (29 months) have low renal Klotho protein expression compared to young mice (4 weeks).[46] Furthermore, aged rats (male, 27 months) have significantly higher serum creatinine than that of young rats (12-months). Notably, aged rats have significantly decreased renal Klotho protein levels along with increase in oxidative stress, overproduction of proinfiammatory cytokine and activation of endothelin signal transduction.[44]

In one report using a newly developed human plasma Klotho assay, an inverse relationship was found between the plasma Klotho levels and age.[43] One possibility is that the physiologic decrease in renal function with age reduces Klotho expression and shedding into circulation, which consequently promote multi-organ senescence. On the other hand, age-related renal Klotho decline renders the kidneys more susceptible to insults such as hypertension, ischemia, and nephrotoxins. However regardless of how Klotho is down-regulated with aging, Klotho supplementation has potential beneficial impact in renoprotection and slowing of aging.

Rodent CKD from Ablation of Renal Mass—CKD rats with subtotal (5/6) nephrectomy have hypertension, proteinuria, azotemia, anemia, lower urinary concentrating ability[74] and vascular calcification.[75] 5/6th nephrectomized rats have low *Klotho* mRNA expression in the kidney at 8 weeks after surgery.[3] Low renal Klotho protein and mRNA expression is also demonstrated in another rodent model generated by unilateral nephrectomy plus contralateral ischemic reperfusion injury followed by high dietary phosphate intake.[2] Renal histology reveals the same changes as those in the 5/6th subtotal ablation model. In addition, animals have high plasma creatinine concentration, hyperphosphatemia, anemia, and ectopic calcification in soft tissues including the kidney, aorta, heart and stomach.

Similar findings are observed in apolipoprotein E-deficient (apo-E$^{-/-}$) uremic mice induced by nephrectomy plus electrocautery.[37] Furthermore, apo-E$^{-/-}$ uremic mice have lower blood Klotho than apo-E$^{-/-}$ non-uremic mice.[37]

Immune-Mediated Chronic Glomerulonephritis—In addition to CKD model by ablation of renal mass, immune-mediated chronic glomerulonephritis, which is a major cause of CKD in humans, also have reduced renal Klotho mRNA.[18] Imprinting Control Region (ICR) strain-derived mice with spontaneous chronic glomerulonephritis due to mutation in *Tensin2* have shorter life span, which is reversed by Klotho overexpression. Klotho overexpression improves renal function, and ameliorates renal histology. The improvements are associated with less superoxide anion generation and lipid peroxidation, and with decreased levels markers of cell senescence, mitochondrial DNA fragmentation, and apoptosis. Thus Klotho protein might serve as a renoprotective factor by diminishing oxidative stress, cell senescence and apoptosis.[18]

Metabolic Syndrome and Diabetes—The association of kidney disease with the metabolic syndrome is known in humans and rodents.[76–78] OLETF rat is a rodent model for the metabolic syndrome.[79,80] OLETF rats have lower level of *Klotho* mRNA in the kidneys than age-matched control rats.[3,80]

The administration of thiazolidinedione to OLETF rats significantly increases renal *Klotho* mRNA expression, and attenuates abnormal lipid and glucose metabolism and reduces systolic blood pressure.[80] The effects of thiazolidinedione on OLETF rats is also observed in OLETF rats overexpressing the *Klotho* gene, suggesting that the capacity of thiazolidinedione to restore the phenotypes in OLETF rats may results from an increase in renal Klotho expression.[80]

In rats with streptozotocin-induced diabetes,[40] Klotho protein in the kidneys is notably decreased along with kidney destruction.[40] Both insulin and phloridzin corrects hyperglycemia, reverses the reduced renal Klotho expression, and improves kidney function and histology of diabetic rats. Klotho protein in Madin-Darby Canine Kidney cell (MDCK) is reduced in vitro by incubation in high glucose medium. Insulin has been shown to participate in the shedding of extracellular domain of Klotho,[8] which may increase the blood Klotho concentrations.

Hemodynamic Effect in Animal Models—Emerging data suggest a role of*Klotho* in hypertension. In spontaneous hypertensive rats and in rats administered deoxycorticosterone acetate plus high salt intake, which is a volume-dependent type of hypertension, renal *Klotho* mRNA expression is significantly reduced compared to normotensive control rats.[3] Hypotension *per se* does not seem to affect renal Klotho, because *Klotho* gene and protein are not decreased in the kidneys of rats with low systolic blood pressure due to myocardial infarction or phlebotomy.[3,81] However, decreased renal Klotho expression is observed in rats with hypotension induced by lipopolysaccharide (LPS) injection.[81] Thus the upstream regulator is more likely inflammatory mediators including TNF-α and IFN-γ released during LPS injection than hypotension (Table 3).[82]

Calcineurin Inhibitor Related Nephrotoxicity—Calcineurin inhibitors (CNI's) such as cyclosporine A (CsA) and tacrolimus (also called FK-506, FK) were introduced for immunosuppression in organ transplantation.[83,84] CNI's are also widely used for treatment of immune-mediated nephrotic syndrome and glomerulonephritis.[85,86] But CNI-induced nephrotoxicity is frequently a cumbersome limitation for its clinical utilization.[87,88] Mice treated with CsA or FK have reduced renal *Klotho* mRNA, and protein, and increased urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG), an oxidative stress marker, excretion compared with vehicle-treated mice. In addition, there is a strong inverse correlation between Klotho protein levels and urinary 8-OHdG excretion. The CNIs-induced down-regulation of renal Klotho expression is correlated with increased renal angiotensinogen and renin expression, tubulointerstitial fibrosis and urinary 8-OHdG excretion, which are

reversed by angiotensin II Type I receptor blocker, Losartan.[42] These results suggest that Ang II-induced Klotho down regulation in the kidneys may be involved in CsA nephropathy.

Moreover, combination of CNI's with sirolimus (SRL), commonly used to enhance immunosuppression for renal transplantation[89] accelerates CNI-induced oxidative stress and down-regulates renal Klotho expression in the kidney.[41] Those results may alarm the higher nephrotoxicity induced by mTOR and CNI's combination.

Human CKD—Thus far, measurements of blood Klotho in human CKD have been limited. Renal expression of the *Klotho* gene is reported to be markedly decreased in patients with CKD (Table 2). The levels of Klotho mRNA and protein are reduced in nephrectomy samples from patients with CKD or end stage renal disease (ESRD), obstructive nephropathy, rejected transplanted kidneys, diabetic nephropathy and chronic glomerulonephritis.[1]

Klotho transcript and protein expression in kidneys from human and animal is clearly decreased. However, the relationship between the renal Klotho expression and the plasma Klotho levels remains to be studied and the mechanisms underlying the relationship between renal Klotho down-regulation and kidney diseases in humans remain unclear. These are studies of the highest priority at this juncture.

Endocrine Klotho Deficiency in CKD

The apo-$E^{-/-}$ 5/6th nephrectomized mice have decreased plasma Klotho and this decline increases with age. Furthermore, the reduction of plasma Klotho was more dramatic compared to non-uremic apo-$E^{-/-}$ mice.[37] In another CKD model of uninephrectomy plus contralateral ischemia reperfusion in mice and rats, plasma Klotho concentration was remarkably decreased in CKD compared to Sham animals. The degree of decreased plasma Klotho is similar in magnitude to that of decreased Klotho protein in the kidneys and in the urine.[2] Transgenic mice with Klotho overexpression also have reduction in their renal, blood and urine Klotho levels with CKD but still maintain levels comparable to wild type mice without kidney disease (Fig. 2) and have better kidney function and less vascular calcification.[2] Soft tissue calcium content is inversely related to Klotho levels. Klotho overexpressing mice have lower, and haploinsufficient $Kl^{+/-}$ mice have higher calcium contents than WT mice do at baseline and after induction of CKD.[2] Thus in rodents, CKD is a state of endocrine Klotho deficiency in addition to renal Klotho deficiency, the systemic Klotho levels efficiently affect the renal function preservation as well as calcification development in CKD animals.[2]

Equivalent data in human plasma Klotho are yet to be acquired. Urinary Klotho levels of CKD patients are significantly decreased and this decrease starts at very early stage and sustain ably reduced with progression of CKD (Fig. 2).[2] Thus far, rodent plasma, kidney and urine Klotho appears to covary rather tightly.[2] To date, one ELISA kit for plasma Klotho measurement is available and using this kit, plasma Klotho levels are found to be negatively related to the plasma creatinine concentration in children and adults without kidney disease.[43] Further confirmation is required.

ROLE OF KLOTHO DEFICIENCY IN PROGRESSION AND COMPLICATIONS OF CKD

In rodents, endocrine and renal Klotho deficiency is striking in CKD. The most important question is whether this is a mere biomarker of the presence of CKD or whether the Klotho deficiency is a pathogenetic factor for CKD development, progression, and complications.[2]

Potential Mechanism of Klotho Deficiency

Renal, plasma, and urine Klotho are decreased very acutely upon ischemic-reperfusion injury[50] and in CKD, it is one of the earliest abnormality.[2] This begs the question as to how Klotho is decreased with kidney injury.

Phosphotoxicity—Klotho is a phosphaturic hormone. Klotho deficiency impairs phosphaturia,[14] and consequently accelerates Pi accumulation in CKD. The higher level of serum Pi, the greater the degree of soft tissue calcification, and the greater risk of mortality.[90] Control of serum Pi significantly improves survival in CKD and ESRD patients, decreases vascular calcification, and suppresses proliferation of parathyroid glands.[90–92] There is little doubt that the prevailing thought is that Pi is one of “uremic toxins” but the concept of phosphotoxicity extends beyond just CKD.[93]

Phosphate overload suppresses Klotho expression in the kidney. Normal mice fed high Pi diet have dramatically decreased Klotho protein and mRNA in the kidney, while Klotho hypomorph mice fed low Pi diet regain part of their Klotho expression.[94] Whether the blood Klotho concentration is regulated by physiologic changes in dietary Pi is yet to be determined.

Vitamin D Deficiency—Low 1,25-$(OH)_2$-vitamin D_3 is a major component of disturbance of mineral metabolism, and is conventionally attributed to cause bone disease and secondary hyperparathryoidism in CKD.[95] The increase in plasma FGF23 in CKD plays an important role in suppression of 1α-hydroxylase in the kidney and initiates or accelerates vitamin D deficiency.[96,97] Importantly, administration of 1,25-$(OH)_2$-vitamin D_3 induces klotho expression in the kidney.[26] It is plausible that low vitamin D level in CKD further worsens renal Klotho deficiency (Table 3).

Angiotensin II—Long-term infusion of Ang II down-regulates renal Klotho mRNA and protein. Administration of norepinephrine,[21,39] or L-NAME, an inhibitor of NO synthesis,[81] cause a comparable hypertensive effect as that of Ang II, but does not affect renal Klotho expression. Continuous infusion of low dose of Ang II in rats that does not affect systolic blood pressure, also down-regulates Klotho mRNA in the kidney[21,39] suggesting that the Ang II-induced Klotho down-regulation is not dependent on blood pressure. In addition, both losartan, an angiotensin Type 2 receptor antagonist, and hydralazine block the rise in blood pressure by Ang II, but only losartan completely blocks the Ang II-induced decrease in Klotho indicating that the Ang II effect on Klotho is via an Ang II Type 2 receptor.[21] This suggests a significant role of Ang II in the regulation of Klotho expression in the kidney and infers important therapeutic possibilities.

Oxidative Stress—Oxidative stress in the kidney originates not only from infiltrating inflammatory cells, but also from damaged kidney cells. Oxidative stress does not only trigger acute kidney injury but also accelerates chronic progression of kidney disease.[98,99] H_2O_2 suppresses Klotho mRNA and protein expression in cultured $mIMCD_3$ cells.[22] Adenovirus-mediated overexpression of the *klotho* gene reduces apoptosis in $mIMCD_3$ cells,

suggesting that klotho may be protective against oxidative stress injury and apoptosis at least in this cell line.[22]

Indoxyl Sulfate Toxicity—Indoxyl sulfate is one of uremic toxins, and significantly increased in the blood of CKD.[100,101] It is not only a biomarker for kidney damage,[101] but also seems to promote progression of CKD and cardiovascular disease in CKD.[101] Indoxyl sulfate reduces renal Klotho expression, and contributes to cell senescence in the kidneys and enhances renal fibrosis of hypertensive rats.[102] The mechanism of Klotho down-regulation in the kidney of CKD is complicated and is summarized in Table 3.

Potential Pathogenetic Roles of Klotho Deficiency in Kidney Disease

The fact that Klotho-deficient mice have more severe kidney damage and fibrosis, and Klotho overexpressors have milder kidney dysfunction and fibrosis after CKD induction surgery, strongly supports the notion that Klotho might be a pathogenic intermediate for CKD development.[2]

Reduced Ability of Regeneration—Klotho deficiency is associated with stem cell dysfunction and depletion, which is part of normal aging.[11] The decrease in stem cell number is associated with an increase in progenitor cell senescence. Secreted Klotho binds to various Wnt family members and inhibits their biological activity. Wnt signaling activity is significantly increased in tissues from $Kl^{-/-}$ mice, while suppressed by genetic Klotho overexpression.[11] Administration of exogenous Wnt could stimulate Wnt signal transduction, and trigger or accelerate cell senescence both in vitro and in vivo. Thus, Klotho appears to be a secreted Wnt antagonist and antagonizes mammalian aging.

Cell senescence is a complicated process present not only in normal aging but also in several pathophysiological states.[18,103,108] Secondary Klotho deficiency in kidney diseases could enhance cell senescence accompanying oxidative stress.[18,102,109] Cell culture studies confirm that Klotho deficiency directly promotes senescence of renal epithelial cells.[110] Klotho supplementation could block senescence induced by oxidative stress.[52,111] Excessive senescence or apoptosis and secondary stem cell deletion might decrease the ability of kidney to defend against renal insults and impair regeneration.

Abnormal Endothelial Function and Angiogenesis—Abnormal endothelial function and impairment of angiogenesis and vasculogenesis can delay kidney regeneration post injury and contribute to progression of CKD and aging of the kidney.[112–116] There are two abnormal aspects in the vasculature of the $Kl^{-/-}$ mice. One is abnormal vasodilatation due to abnormal endothelial function;[117] another is impaired angiogenesis and vasculogenesis.[118] The aortas of $Kl^{+/-}$ mice has exaggerated contractile response to norepinephrine and attenuated responses to acetylcholine and superoxide dismutase. $Kl^{+/-}$ mice have low urinary NO metabolites (NO_2^-/NO_3^-) and cGMP concentrations, but normal the prostaglandin.[117] Low NO which is as result of low expression of NO synthase[117] may be one of causes of hypertension in $Kl^{+/-}$ mice (personal observation).

The defect in angiogenesis and vasculogenesis is well known with aging, but its mechanism is not completely understood. $Kl^{+/-}$ mice have delayed angiogenesis and vasculogenesis and low blood-flow after hind limb ischemia.[119] The HMG co-enzyme A reductase inhibitor cerivastatin increases Klotho levels and restores the impaired neovascularization.[118] The low vasculogenesis and angiogenesis in $Kl^{+/-}$ mice might be attributable to down-regulation of vascular endothelial growth factor (VEGF) in the aorta.[117]

Klotho incubation mitigates increased cell senescence and apoptosis in endothelial cells triggered by oxidative stress or by Klotho deficiency.[111] In addition, Klotho suppresses

TNF-α-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 Klotho protein may have another role in protection of vasculature by inhibition of endothelial inflammation.[120]

Renal Fibrosis—Renal fibrosis is one striking histological characteristic in CKD, and is generally attributed to epithelial-mesenchymal transition (EMT).[121,126] TGF-β1, a major fibrotic progression protein, plays a key role in EMT in the kidneys.[125,127,129] $Kl^{-/-}$ mice have more appreciable renal tubulointerstitial fibrosis,[130] which is associated with up-regulation of TGF-β1 in the kidneys. The renal fibrosis in unilateral ureteral obstruction (UUO) is accompanied by up-regulation o f TGF-β1 and fibronectin, and down-regulation of Klotho mRNA and protein. These alterations are more exaggerated in $Kl^{+/-}$ UUO mice than in *WT* UUO mice. HK2 cells incubated with TGF-β1 have down-regulation of Klotho expression which is attenuated with TGF-β1 receptor inhibitor (ALK5 inhibitor).[130] Secreted Klotho alleviates renal fibrosis induced by UUO and suppresses expression of fibrosis markers and TGF-β1 target genes (Snail, Twist), but does not reduce TGF-β1 expression in UUO kidney.[131] Furthermore, Klotho suppresses TGF-β1 induced Smad2 phosphorylation in a rat tubular epithelial cell line (NRK52E), suggesting that Klotho protein suppresses renal fibrosis primarily through inhibiting TGF-β1 signaling.[131]

Plasminogen activator inhibitor-1 (PAI-1) is a member of the serine protease inhibitor family and regulates fibrinolysis and proteolysis through inhibiting plasminogen activation.[132,133] PAI-1 levels are increased in the kidneys of human with glomerulonephritis, hypertensive nephrosclerosis, diabetic nephropathy, and chronic allograft nephropathy, CysA nephrotoxicity[134] and lupus nephritis in female MRL *Ipr/lpr* mice.[132,133] PAI-1 mRNA and activity are strikingly elevated in multiple tissues in $Kl^{-/-}$ mice compared with *WT* mice. This increase in PAI-1 is age-dependent and linked to the development of ectopic calcification, and glomerular fibrin deposition in the kidneys of $Kl^{-/-}$ mice.[135] Thus, the lack of Klotho increases PAI-1 mRNA and activity.

Potential Contribution of Klotho Deficiency to Complications of CKD

The emerging view is that Klotho deficiency not only worsens renal disease but also exacerbates extra renal complications in CKD.

Ectopic Calcification—Cardiovascular calcificaion is a heterogeneous disorder with overlapping yet distinct mechanisms of initiation and progression.[67,136,137] Vascular calcification is a dynamic process resulting from the imbalance between promoters and inhibitors.[136,138] Abnormal FGF23 and Klotho can contribute to ectopic calcification in soft tissue.[2,6,65]

The fact that $Kl^{-/-}$ mice have extensive ectopic calcification in soft tissue, which is also observed in CKD subjects strongly suggests a pathogenetic association between Klotho deficiency and calcification. CKD animals and human patients have significantly low Klotho protein and mRNA in the kidneys (Table 2) and CKD animals have low plasma and urinary Klotho protein.[2] Administration of exogenous Klotho protein (personal observation) or increasing Klotho level via genetically engineered manipulation[2] significantly inhibits vascular calcification in CKD animals suggesting that Klotho deficiency is associated with vascular calcification in CKD.

Elevated plasma Pi is associated with vascular calcification in experimental animals and in CKD patients and cellular Pi influx is believed to be mediated by the NaPi-3 group of Na^+-coupled transporters (also named Pit1 and Pit2) in vascular smooth muscle cells (VMSC).[139] $Kl^{-/-}$ mice have high levels of Pit1 and Pit2 mRNA and higher tissue calcium contents

compared to *WT* litter mates. In addition, $Kl^{+/-}$ CKD mice have higher and *Tg-Kl* CKD mice have lower Pit1/2 mRNA in the aortas than *WT* CKD mice. Upregulation of expression and activity of Pit1/2 in the aortas which may result from high ambient Pi and/or unknown uremic toxin (s) could increase intracellular Pi concentration, induce VSMC de-differentiation, and increase or trigger vascular calcification.[2]

FGF23-deficient mice have ectopic calcification in soft tissues including vasculature, raising the possibility that FGF23 may be protective for the vasculature. However, the high plasma FGF23 in CKD patients challenges this notion. One possible explanation might be that the target organ loses the response to FGF23 in CKD. Klotho and FGFR1/3 expression are down-regulated when human aorta derived-SMCs are incubated with high phosphate and calcium. These cells exhibit concomitant osteo/chondrocytic transformation and loss of contractile phenotype. De-differentiation of SMC and loss of the ability to respond to FGF23 may result from down-regulation of Klotho-FGFRl/3 expression in the arteries.[140]

Cardiac Hypertrophy—Cardiac hypertrophy in CKD, also referred to as “uremic cardiomyopathy” by some, is another pathological feature of cardiovascular complication. Clinically, it is characterized by cardiac arrest or sudden death, left ventricular hypertrophy, and congestive heart failure, which may be distinct from hypertensive and ischemic cardiomyopathy.[141,142] Left ventricular hypertrophy is more frequent in CKD patients than the general population when age and gender are matched, and has a negative impact on cardiovascular prognosis.[143] In addition to traditional risk factors such as smoking, hypertension, dyslipidemia, diabetes, anemia, AV fistula, volume overload, hypoalbuminemia, oxidative stress, chronic inflammation, and secondary hyperparathyroidism,[144] additional risk factors include inappropriate activation of the RAS (renin-angiotensin-aldosterone system),[145,146] vitamin D deficiency,[147] high FGF23 in blood,[148] and more recently, low Klotho in blood.[2] We have observed left ventricular hypertrophy in Klotho-deficient animals without kidney disease (personal observation).

In the heart, Klotho is expressed solely at the sinoatrial node.[48] The high rate of sudden death of $Kl^{-/-}$ mice under restraint is likely caused by sinoatrial node dysfunction.[48] Intrinsic heart rate after pharmacological blockade of autonomic nerves in $Kl^{-/-}$ mice was significantly lower than that in *WT* mice. The sinus node recovery time after overdrive pacing is significantly longer in $Kl^{-/-}$ mice than in *WT* mice; but there is no degenerative structural change in the sinoatrial node, suggesting that normal Klotho gene expression is essential for the sinoatrial node to function as a dependable pacemaker under conditions of stress.[48] Whether sKl also functions as regulator of the pacemaker is still unclear. Recently, we have observed cardiac fibrosis in $Kl^{-/-}$ mice, and this change is progressive with aging (personal observation). These findings suggest that Klotho may suppress intrinsic fibro genesis in heart, but whether the mechanism of fibrosis is same between the heart and the kidney is not known. Since there is normally no Klotho expression in the ventricles, the cardiac phenotype in $Kl^{-/-}$ mice is mostly likely due to deficiency in circulating Klotho or other factors. There is no data on Klotho expression in the heart of CKD patients. Thus the direct role of Klotho deficiency on the high cardiovascular mortality and morbidity in CKD patients remains to be confirmed.

Secondary Hyperparathyroidism—Secondary hyperparathyroidism is part of the CKD-Metabolic bone disease (MBD) spectrum.[149,150] The role of PTH in CKD-MBD is well known and the role of FGF23 and Klotho in CKD-MBD is attracting attention. CKD subjects have high blood level and over activity of FGF23[62] and low Klotho and FGFR(s) in parathyroid gland[151,152] although some studies have argued for up-regulation of Klotho protein in uremic parathyroid gland.[153] In normal subjects with normal kidney function, FGF23 plays a crucial role, both as a phosphaturic factor[12,64,154] and as a suppressor of

active vitamin D (1,25D) production in the kidney,[97] and of PTH production in the parathyroid gland.[28] In contrast, FGF23 fails to inhibit PTH production probably due to down-regulation of Klotho and FGFR(s) in parathyroid gland in the uremic setting.[151,152]

Bone Disease—Renal bone disease is the skeletal component of CKD-MBD (Mineral and Bone Disorder), and is characterized by either high or low turn-over and either presence or absence of mineralization defect.[155,156] Secondary hyperparathyroidism, poor calcium intake, Pi retention, deficiency of 1,25-dihydroxy vitamin D, high FGF23 and low Klotho may all potentially contribute to CKD-MBD.[157–159] *Kl*$^{-/-}$ mice have high blood FGF23, and low turn-over form of osteopenia[160,161] associated with reduced number of osteoblast progenitors and low activity of osteoblastic cells in ex vivo bone marrow cultures derived from *Kl*$^{-/-}$ mice. This suggests independent impairment of both osteoblast and osteoclast differentiation leading to low-turnover osteopenia observed in *Kl*$^{-/-}$ mice.[160,161] The bone disease in *Kl*$^{-/-}$ mice is not the same as those in CKD patients. The direct effect of Klotho on CKD-MBD remains to be clarified.

MECHANISMS OF HOW SECRETED KLOTHO PRESERVES KIDNEY FUNCTION AND AMELIORATES COMPLICATIONS IN CKD

Induction of Phosphaturia

Hyperphosphatemia results mainly from renal phosphorus retention,[14,26] and is a prominent feature in the *Kl*$^{-/-}$ mice.[6,14] The restoration of Klotho levels via genetic manipulation,[20] or viral-based delivery[162] successfully normalizes blood phosphate level. *Kl*$^{-/-}$ mice display increased activity of Na-coupled phosphate (NaPi) cotransport and elevation of NaPi-2a and NaPi-2c cotransporter proteins compared with wild-type (*WT*) mice.[163] This suggests that the hyperphosphatemia at least in part is of renal origin. Moreover, NaPi-2b protein and mRNA in gut are higher in *Kl*$^{-/-}$ mice than in *Wt* mice[163] indicating that increased intestinal phosphorus absorption may exacerbate augmentation of blood Pi concentration in *Kl*$^{-/-}$ mice. Transgenic Klotho overexpressing mice (*Tg-Kl*) have lower blood Pi, while renal fractional excretion of phosphorus (FE_{phos}) is increased indicating a renal leak of Pi.[14] Injection of sKl significantly increases FE_{phos} and decreases blood Pi in the normal rat.[14]

Klotho protein inhibits NaPi cotransport activity in renal brush border membrane (BBM) and in OK cells.[14] Klotho dramatically reduces NaPi-2a abundance in apical NaPi-2a protein in kidney and OK cells after 4 or more hours in vivo and in vitro respectively, indicating that the more sustained effects of Klotho on NaPi involves the canonical pathway of NaPi-2a internalization.[14]

The extracellular domain of Klotho contains two tandem repeats with 20%–40% amino acid identity with members of the glycosidase family including β-glucosidase, and has β-glucuronidase-like enzymatic activity.[67] NaPi-2a is a glycosylatedprotein.[164] The direct and acute inhibition of NaPi transport by Klotho can be mimicked by recombinant β-glucuronidase but not by sialidase. The substrate of this glycuronidase activity in the BBM is not known. While protease inhibitors abolish the proteolysis, they do not reverse the Klotho-induced inhibition of transport, indicating that Klotho-induced deglycosylation is sufficient and that subsequent proteolysis is not required to suppress Na-dependent Pi transport. Klotho modulates NaPi-2a in a biphasic fashion with dual distinct mechanisms. It acutely (<4 hrs) decreases its intrinsic transport activity via removal of glucuronate from some yet to be identified substrate, followed by proteolytie cleavage, and in a second phase (>4 hrs) induces changes in cell surface NaPi-2a.[14]

Hyperphosphatemia is universally observed in CKD patients, and is a potent predictor of cardiovascular morbidity and mortality.[90,165] Controlling blood phosphate by restriction of intake,[166] phosphate binder,[92] and more efficient dialysis[167] all improve clinical outcome in CKD patients. Undoubtedly, lack of the phosphaturic action of Klotho protein is an important pathogenic factor in CKD and means of restoring Klotho is of potential benefit.

Antivascular Calcification: Inhibition of Type III Na-Phosphate Cotransporter

Klotho can decrease soft tissue calcification by lowering plasma phosphate levels through promotion of phosphaturia. In addition, overexpression of Klotho improves kidney function after CKD induction surgery. Both of these actions can reduce the calcium content in soft tissues. The third most important and direct effect of Klotho on inhibition of calcification is the modulation of the NaPi-3's-Pit1 and Pit2, key modulators for Pi influx into vascular smooth muscle cells (VSMC).[2]

While there is abundant calcification in the multiple organs of both *WT* and $Kl^{+/-}$ CKD mice, *Tg-Kl-CKD* animals have very few or no calcification.[2] Calcium content is higher in the aortas and the kidneys of CKD than Sham in both the *Wt* and $Kl^{+/-}$ mice. This increase is ameliorated by overexpression of Klotho.[82] *Pit1, Pit2,* and *Runx2* (a marker of osteoblast-like phenotype) mRNA are increased and *SM22* (a marker of contractile smooth muscle cell) is decreased in $Kl^{-/-}$, while overexpression of Klotho has the opposite effect. Klotho may control the balance between differentiation and de-differentiation of VMSC.[2] CKD induces a similar pattern as Klotho deficiency and Klotho overexpression completely blocked the changes induced by CKD. When rat VMSC are grown in vitro, Klotho inhibits Na-dependent Pi influx and minimizes the mineralization induced by high ambient Pi.[2] The up-regulation of *Runx2,* and down-regulation of *SM22* by high Pi is reversed by recombinant Klotho protein, suggesting that Klotho directly blocks Pi-induced de-differentiation of rat VMSC.[2]

Anti-Oxidation

The pathogenic effect of reactive oxygen species in initiation or exacerbation of kidney damage is well-known.[22,168–171] Part of the anti-aging effect of Klotho is thought to be mediated by anti-oxidation or increased resistance to oxidant stress.[172]

$Kl^{-/-}$ mice have high level of oxidative stress and damage, while *Tg-Kl* mice have lower levels as compared with *WT* mice.[172] Klotho-transfected $mIMCD_3$ cells exposed to H_2O_2, show fewer apoptotic cells.[22] Paraquat-induced increase in lipid peroxidation is suppressed in HeLa cells treated with Klotho protein.[172] These results suggest that the Klotho may be involved in prevention of oxidative injury and apoptosis possibly related to activation of FOXO transcription signal and stimulation of MnSOD.[172]

Anticell Senescence

The permanent and irreversible growth arrest of cell and cell senescence are central paradigms of aging[173] and diseases secondary to ischemia, toxin, inflammation, and so on.[18,103,104,106,108,109,174] senescent cells may secrete altered levels of growth factors, increase susceptibility to apoptosis, and delay the repair and regeneration in the aging kidney. Thus prevention of cell senescence may provide an important beneficial impact on aging as well as kidney disease.[106] Klotho functions as a secreted Wnt antagonist by directly binding to Wnt3/5 proteins klotho and inhibits cell senescence.[11]

Cell senescence and oxidative stress are closely associated and implicated in acute and chronic kidney disease. Mice with spontaneous chronic glomerular disease carrying a mutation in *Tensin2* have low renal Klotho, high level of lipid peroxidation, superoxide

anion production, mitochondrial oxidative stress, and severe cell senescence in the kidney.[18] Genetic Klotho overexpression ameliorates renal injury associated with a dramatic improvement in mitochondria damage, reduction in senescent cells, decreased oxidant stress, and reduced apoptosis in the kidney.[18]

Suppression of Angiotensin II Effects

It is known that the angiotensin II (Ang II) activity and production are significantly upregulated in a variety of kidney diseases. Ang II is a proinflammatory mediator and oxidant.[175–179] Animal experiments clearly revealed that Ang II decreases Klotho mRNA and protein expression in the kidney which could be blocked by Angiotensin Type I receptor antagonist.[21] Moreover, Klotho gene transfer also blocks Ang II-induced kidney damage, suggesting that the Ang II may act upstream of Klotho and produce pathogenic effects by reducing Klotho.[21]

In addition, Ang II receptor antagonists do not only block the downregulation of Klotho, but also abrogate the induction of TGF-β, p-p38 and p53 expression in cultured NRK cells by Ang II. Therefore, blocking Ang II action causes upregulation of Klotho and exerts a cytoprotective role.[180] Similarly HMG-CoA reductase inhibitors effectively blunt Ang II-induced reduction of Klotho expression in $mIMCD_3$ cell line.[181] Upregulation of Klotho may be one of therapeutic mechanisms of HMG-CoA reductase inhibitor action.

VALUE OF MEASURING KLOTHO IN CKD

CKD is one of major diseases affecting human life span and quality of life. Early diagnosis is paramount for one to initiate early and effective treatment. Extensive effort has been devoted to search for early biomarker for kidney diseases focusing mostly on acute kidney disease and less on CKD by transcriptomics, proteomics, metabolomics, lipidomics, and gene arrays.[182–184] In addition to traditional clinical parameters such as eGFR, proteinuria, albuminuria, N-acetyl-beta-D-glucosaminidase, and cystatin c,[185–190] novel proteins as adiponectin,[191,192] γ-Glutamyltransferase,[193,194] endothelin-1,[195,196] uric acid,[197–199] and FGF23[200] have been proposed as biomarkers but remained to be validated in population studies.

Klotho has two potentials in terms of its role as a biomarker. First is that fall in plasma or urinary Klotho can be one of the earliest abnormality in CKD from a variety of causes. The most promising data thus far is that urinary Klotho is reduced at a very early stage CKD (Stage 1 and 2) and is progressively lowered with declining eGFR (Table 2).[2] Hopefully, urinary Klotho protein could be an ideal early biomarker for CKD; as it declines much earlier than other parameters (Fig. 3). Second, in the presence of CKD, Klotho levels may bear prognostic value in predicting progression and complications of CKD. Judging from the rodent model, for the same degree of renal insufficiency and hyperphosphatemia, the lower the Klotho level, the more severe the soft tissue calcification.[2] A longitude study is required to reveal the prognostic value of Klotho in CKD patients.

To date, there are limited assays available to determine the blood Klotho in humans. Immunoprecipitation from human plasma have proven to be not readily reproducible.[47] Recently, one sandwich ELISA became available to detect soluble Klotho in human blood.[43] This first report states that human serum Klotho ranges from 239 to 1266 pg/ml with a mean of 562 ± 146 pg/ml in normal adults. It is inversely related to blood creatinine and age. Normal children (7.1 ± 4.8 years) have significantly higher blood Klotho (952 ± 282 pg/ml). In addition, plasma Kl is correlated negatively with age and serum Ca, and positively with serum Pi.[43] To date, there is no publication on plasma Klotho levels in CKD patients.

POTENTIAL STRATEGIES TO INCREASE KLOTHO PROTEIN

The animal data to date is overwhelmingly strong to indicate that Klotho is not merely an early biomarker for CKD, but also a pathogenetic intermediate for accelerating CKD progression and for development of complications. Restoration of endogenous Klotho or administration of exogenous Klotho might provide novel treatment strategies for CKD patients. There are two ways to increase soluble Klotho-administering exogenous Klotho or enhancing endogenous Klotho (Fig. 4).

Administration of Exogenous Klotho

Ideally, administration of exogenous Klotho to CKD subjects is one simple and effective means to treat an endocrine deficiency similar to replacement of erythropoietin and vitamin D. Klotho can potentially reverse or retard the progression of CKD. Even in advanced stages of CKD, Klotho supplementation can alleviate extra renal complications of CKD.

Delivery of Klotho Gene to CKD Subjects—Gene therapy is broadly defined as the insertion, alteration, or removal of genes within an individual's to change the phenotype. One form of gene therapy involves the insertion of functional genes into an unspecified genomic location to replace a mutated gene or enhance or silence the target gene. Other forms involve directly correcting the mutation or modifying normal gene via a viral carrier. Although the technology is still rather experimental, it has been used with some success.

Theoretically, gene therapy has distinct potential to treat CKD and complications at the most fundamental level. The present viral vector systems seem to have limitations for clinical use because of uncertainties regarding their toxicity and immunogenicity. But animal experiments have shown encouraging results. Adenovirus-mediated gene transfer has succeeded in gene expression in the kidney. Adeno-associated virus has a potential to be utilized as a vector targeting both kidney and skeletal muscle. Non-viral vectors such as the haemagglutinating virus of Japan (HVJ)-liposome method and cationic liposome are possibilities, but their efficiency needs to be improved. Electric pulse is emerging as a new and less harmful strategy of gene transfer to various tissues, including the kidney.[201,202]

Klotho gene delivery via adeno-associated virus (AAV) carrying mouse klotho full-length cDNA (AAV.mKl) efficiently attenuates the progression of spontaneous hypertension and renal damage in spontaneous hypertensive rats (SHR). A single dose of AAV. mKl prevents the progression of spontaneous hypertension for at least 12 weeks and reverses reduced Klotho expression in SHR rats. AAV.mKl attenuates renal tubular atrophy and dilation, tubular deposition of protein aceous material, glomerular collapse, and collagen deposition seen in SHRs, indicating that *Klotho* gene delivery limits renal damage induced by hypertension.[203]

Administration of Soluble Klotho Protein—Administration of exogenous Klotho protein is more direct, safe, and easier modality to correct endocrine Klotho deficiency in CKD compared with delivery Klotho gene. Following the history of use of Calcitriol and active derivative,[204–207] and erythropoiesis-stimulating agents[208–210] for CKD patients, Klotho protein may be a viable option in the horizon.

Compared with viral delivery system, fewer studies were reported for Klotho protein delivery. Recombinant Klotho protein of the full length of extracellular domain is able to inhibit IGF signal transduction to prolong animal life span,[20] suppress Wnt signal pathway to decrease cell senescence and retain more stem cells,[11] modulate renal ion channel or transporter[10,14–16] or control FGF23 signal transduction.[211] Klotho administration has been proven successful in the setting of acute kidney injury in animals which is a state of acute

Klotho deficiency.[50] Klotho protein was injected intraperitoneally into rats after ischemia-reperfusion injury. Rats given Klotho had better renal function, less kidney damage, and lower neutrophil gelatinase-associated lipocalin.[50] More interestingly, this Klotho preparation inhibits renal fibrosis in UUO model by suppressing expression of fibrosis markers (α-Smooth muscle actin, Vimentin, Collagen-1, Metalloproteases) and TGF-β1 target genes (Snail, Twist) in a dose dependent manner. Klotho does not reduce TGF-β1 production but rather inhibits TGF-β1 signaling suggesting that Klotho prevents renal fibrosis primarily through inhibiting TGF-β1 signaling.[131] The authors' laboratories found that administration of Klotho protein does not only ameliorate kidney functions and histology, but also alleviates vascular calcification in CKD rats (personal observation). Thus, exogenous Klotho protein supplementation is a potentially feasible way of replacement therapy in Klotho deficient states (Fig. 4).

Up-Regulation of Endogenous Klotho Protein

In instances where endogenous Klotho-producing cells are not destroyed but simply suppressed, strategies to increase its production will be of therapeutic benefit, especially to early CKD patients (Fig. 4).

Blockage of Angiotensin II—Ang II is known to be an important mediator for initiation and progression of kidney diseases. Ang II contributes to hypertension, intraglomerular hyper filtration, oxidant stress and lipid peroxidation and tubulointerstitial inflammation and fibrosis in acute or chronic kidney diseases.[212–222] Angiotensin converting enzyme inhibitor and angiotensin receptor antagonist are extensively used in the treatment of chronic kidney diseases. Recent studies have demonstrated that Ang II contributes to the pathogenesis of kidney diseases by reducing Klotho expression in the kidney.[21] Induction of endogenous Klotho protein expression may have potential as a therapeutic agent in treating Ang II-related kidney disease.[21,223] Similar therapeutic effect was observed in Ang II-perfused rats when treated with angiotensin receptor antagonist.

Angiotensin II Type I receptor antagonist, Losartan, doesnot only block the down-regulation of Klotho in the kidney of rats perfused with Ang II, but also in the kidney of rats treated with Cyclosporin A with improvement of kidney function, histology and less oxidative stress,[42] suggesting that (1) CsA triggers oxidative stress and downregulates Klotho expression; both amplifying each other to cause nephropathy; (2) angiotensin Type I receptor antagonist interrupts the vicious cycle and attenuates kidney damage induced by CsA.

Free radical scavenger is able to suppress Ang II-induced downregulation of Klotho in the kidneys of rats, to decrease plasma oxidative stress marker, and to block the decline in creatinine clearance, indicating oxidative stress is involved in downregulation of renal Klotho induced by Ang II.[39]

HMG-CoA Reductase Inhibitor—3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitors are effective in lowering plasma concentration of LDL-cholesterol and are widely used in patients with hypercholesterolemia. Recently, this group of drugs called statins, have been shown to decrease the incidence of myocardial infarction and other ischemic vascular events independent of their lipid lowering properties.[119] An ischemic hind limb model in mice achieved by ligation of femoral and saphenous arteries showed higher percentage of leg loss in $Kl^{+/-}$ mice compared to WT mice suggesting that lower angiogenesis and impairing blood flow recovery and diminishing capillary growth in $Kl^{+/-}$ mice.[119] A HMG CoA reductase inhibitor, cerivastatin, enhances angiogenesis and restores the impaired neovascularization in $Kl^{+/-}$ mice after ischemic lind limb illustrated by similar

blood perfusion between $Kl^{+/-}$ mice treated with cerivastatin and *WT* mice.[118] The mechanism by which statins accelerate angiogenesis and vasculogenesis in Klotho-deficient mice is at least partly related to maintenance of Klotho,[181,224] and secondary to improvement of endothelial function through endothelium-derived NO production.[117]

An in vitro study reveals that pretreatment with atorvastatin ameliorates the Ang II-induced reduction of *Klotho* mRNA.[181] However, whether this in vitro effect of HMG CoA reductase inhibitors translates to an in vivo effect on the kidneys of whole animals or humans needs to be further defined.

PPAR-γ Agonists—Peroxisome proliferator-activated receptors (PPAR) are a group of nuclear receptors that function as transcription regulators of metabolic pathways. PPAR-gamma (PPAR-γ), also known as the glitazone receptor, is the molecular target of thiazolidinediones (TZDs), and has been used for treatment of Type II diabetes mellitus (DM) in human.[225]

In addition to therapeutic benefits of improved insulin sensitivity, thiazolidinediones upregulate Klotho expression both in vivo and in vitro. The induction of both Klotho mRNA and protein expression in HEK293 cells by PPAR-γ agonist is blocked by PPAR-γ antagonists or siRNA-mediated gene silencing of PPAR-γ. This induction is mediated by binding of PPAR-γ to 5′-flanking region of *Klotho* gene. Moreover, thiazolidinediones or adenovirus-mediated overexpression of PPAR-γ increases Klotho expression in mouse kidneys while renal klotho expression was attenuated in mice treated with PPAR-gamma antagonists. These results demonstrate that Klotho is a downstream target gene along PPAR-γ signal transduction.[226] This effect may be one of mechanisms of PPAR-γ action on aging, DM and bone disease.[227]

Oral administration of troglitazone for 10 weeks significantly up-regulates renal *Klotho* mRNA expression, enhances endothelium-dependent aortic relaxation, and reduces systolic blood pressure, plasma glucose and triglyceride levels in OLETF rats, suggesting that improvement of the vascular endothelium function and dyslipidemia by troglitazone might be accompanied by up-regulation of renal Klotho.[80] Pioglitazone not only improves metabolic abnormalities of diabetes and consequent diabetic nephropathy, but also protects against nondiabetic chronic kidney disease in experimental models of aging kidney. It reduces proteinuria, improves GFR, decreases sclerosis, and alleviates cell senescence in the kidneys of aged rats. A similar effect is observed by increased the expression of PPAR-γ in the kidney.[108] Proposed underlying mechanisms include increased expression of Klotho, decreased systemic and renal oxidative stress, and decreased mitochondrial injury.[108]

PPAR-α is another member of the PPAR family and is highly expressed in the kidney. PPAR-α activation is able to protect the kidney from acute injury either by cisplatin or ischemic reperfusion.[228,229] But whether PPAR-α modulates renal Klotho as PPAR-γ does has not been studied yet.

Anti-Oxidant and Free Radical Scavenger—Oxidative stress in the kidney plays an important role in the development and progression of kidney disease. Oxidative stress directly suppresses Klotho expression in a kidney epithelial cell line in vitro[22] and in the kidney in vivo.[22,39,230] Oxidative stress is also implicated in Ang II[39] or CsA-induced Klotho downregulation.[42] The moderate efficacy of antioxidants used for treatment of acute and chronic kidney disease has been shown most in animal studies and not in CKD patients.[39,168,169,231]

Animals with genetic or secondary Klotho deficiency have low anti-oxidants or/and over production of free radicals and lipid peroxidation in the kidneys.[23,39,40,170,172] In contrast, Klotho-overexpressing mice and animals who received Klotho gene, or treated for up-regulation of endogenous Klotho have exactly the opposite changes.[18,42,108,172,232]

Thus it appears that Klotho deficiency increases oxidative stress or makes cells more prone to oxidative stress induced injury, and oxidative stress further down-regulates Klotho expression. Anti-oxidants are potentially useful in interrupting the deterioration spiral.

CONCLUSION AND PERSPECTIVES

In animal models, CKD is a sustained state of pan Klotho deficiency in the kidney, plasma, and urine. This fact remains to be established in humans. Klotho plays a pathogenetic role in kidney disease progression, and development of disturbed mineral metabolism such as secondary hyperthyroidism and vascular calcification. As such, it is more than a biomarker. Early administration of exogenous Klotho protein, delivery of Klotho gene, or enhancement of endogenous Klotho could correct Klotho deficiency and improve kidney function in CKD (Fig. 4). The potential utility of Klotho in clinical practice is at least two-fold. First, Klotho could serve as an early and sensitive biomarker of presence of kidney diseases. But its specificity and its prognostic value and differential diagnostic value remain to be studied in humans. Second, Klotho supplementation may provide novel therapy for AKI patients to retard or block its progression to CKD and for CKD by slowing progression as well as preventing and reversing complications.

The therapeutic efficacy of Klotho in kidney disease has been unequivocally demonstrated in several animal models. One needs to validate the efficacy of Klotho in larger variety of kidney diseases and most importantly how Klotho exerts its effects which in variably will be pleiotropic. The mechanism of decline of renal Klotho in kidney diseases is not completely clarified. The upstream regulators of Klotho need to be identified. However, some therapeutic modalities including ACE inhibitor, HMG Co A reductase inhibitor, and anti-oxidants could sustain or increase endogenous Klotho expression to normal levels. The immediate challenge is to test whether human CKD resembles the rodent counterpart and if so, how to more efficiently increase Klotho levels in patients with kidney disease by stimulating endogenous Klotho or giving recombinant Klotho.

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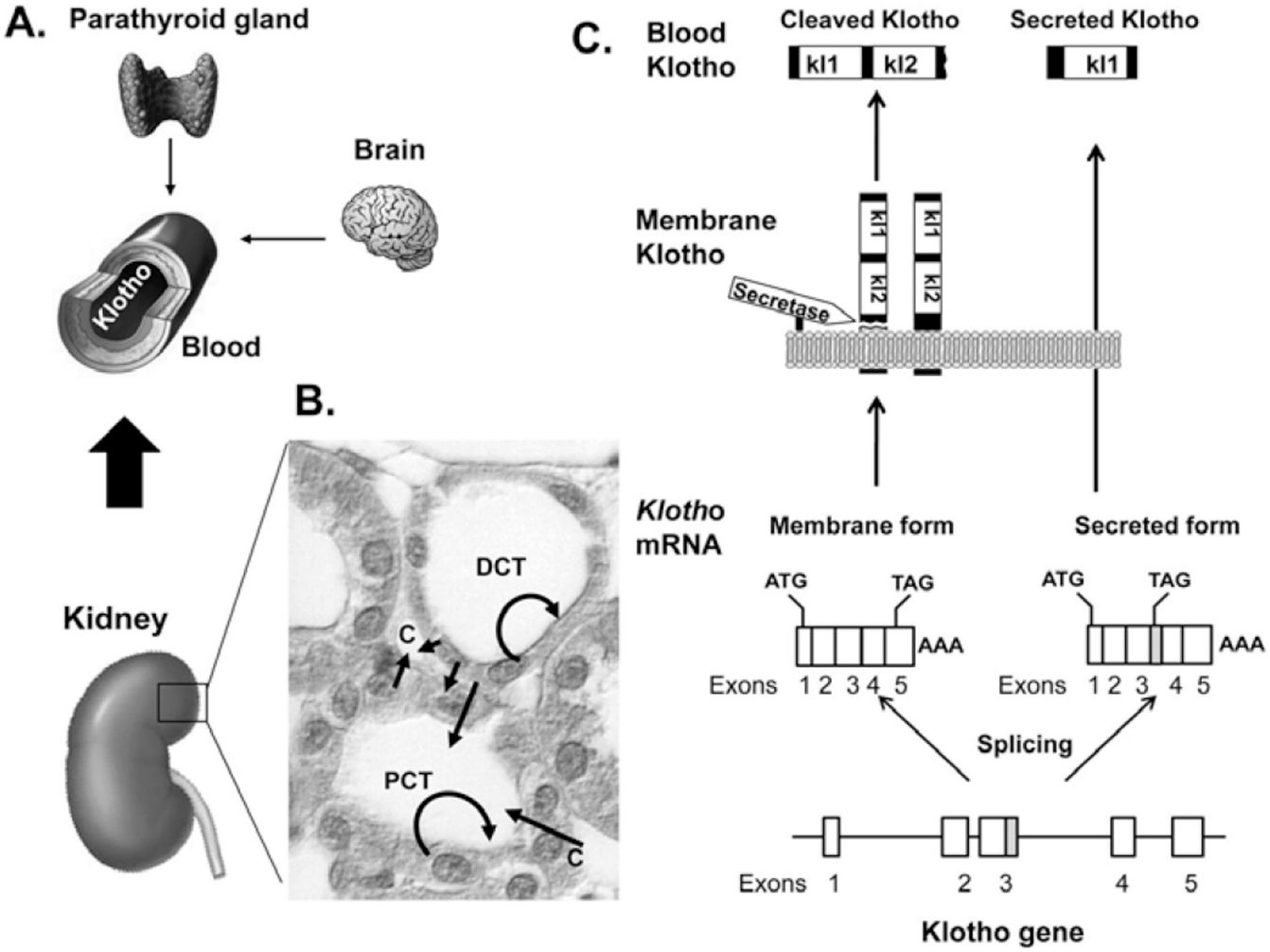

Figure 1.

Circulating Klotho. A) Klotho is synthesized in few organs but the kidney is main resource of circulating blood Klotho. Whether parathyroid gland and brain contribute significantly to circulating Klotho is not clear. B) Both renal proximal convoluted tubules (PCT) and distal convoluted tubules (DCT) produce Klotho. Klotho is released into the capillaries (C) and systemic circulation. The relative contribution of these two segments to circulating Klotho is unknown. Conversely, Klotho from the blood may enter the urine potentially via transcytosis of the tubular epithelium. Luminal Klotho from the PCT can travel axially down the nephron to the DCT lumen. Klotho from PCT cells and/or DCT cells may also exert paracrine actions on tubular epithelium. C) Membrane form of Klotho transcript arises from Klotho gene. Secreted Klotho be derived from alternative RNA splicing. The internal splice donor site is in exon 3 of Klotho gene. The resultant alternatively spliced transcript contains 50 bp insertion after exon 3 (grey) with an in-frame translation stop codon introduced. The product contains only Kl1 and is released into blood circulation. On the other hand, the Klotho protein encoded by membrane form of Klotho transcript is a plasma membrane-anchored protein in Klotho producing cells. Extracellular domain of membrane Klotho containing Kl1 and Kl2 repeats is shed and cleaved by α and β-secretases, and released into blood stream. Thus in blood circulation, there are two forms of Klotho, one is derived from cleavage of the extracellular domain of membrane Klotho. Another one is secreted membrane derived from an alternatively spliced Klotho transcript.

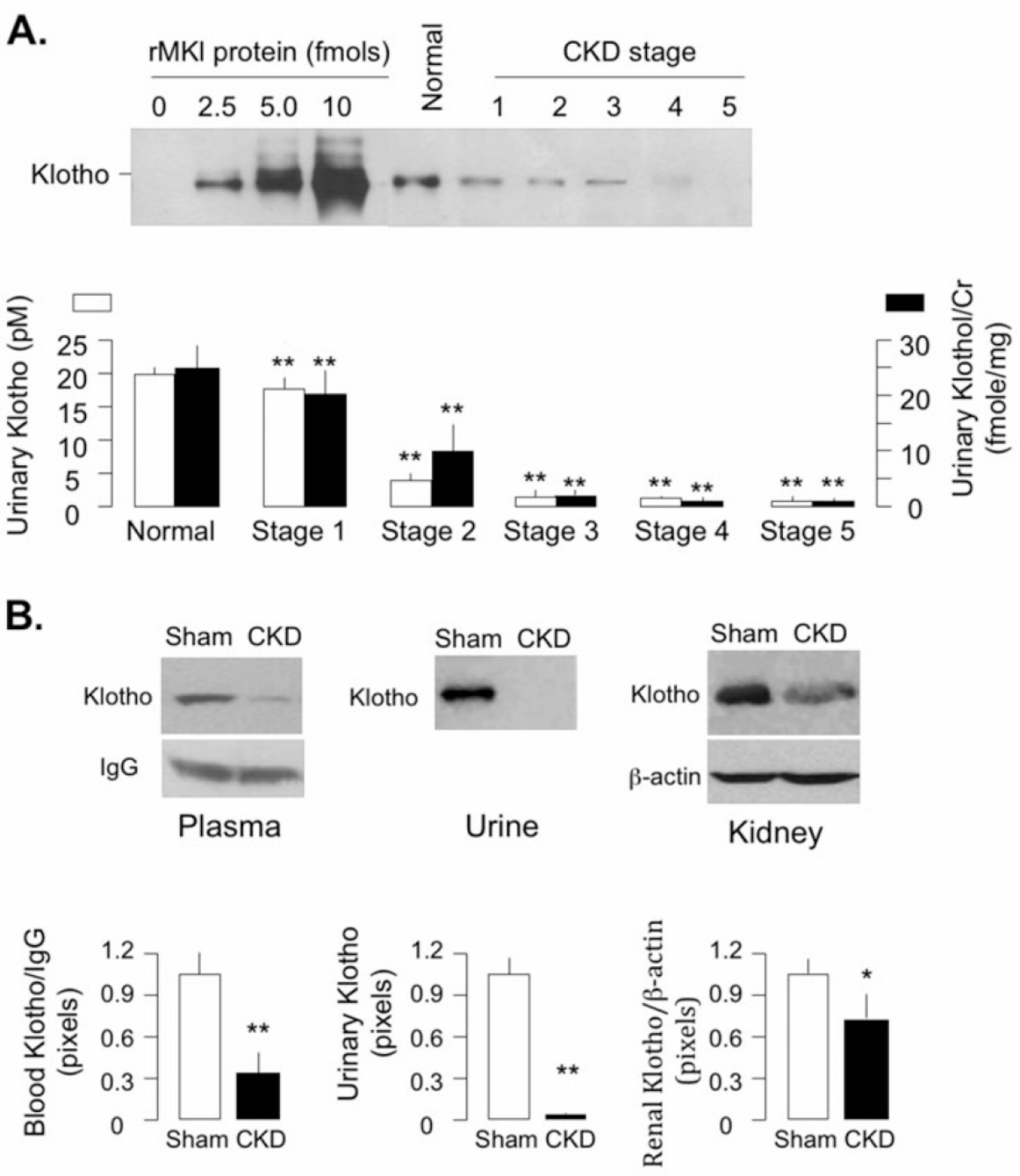

Figure 2.

Klotho protein in humans and rodents with CKD. A) Representative immunoblot for Klotho protein with serial dilutions of known concentration of recombinant mouse Klotho (rMKI) as standard and concentrated human urine samples of identical amount of creatinine. Lower panel shows summary of urinary Klotho protein concentration (open bars) and of Klotho normalized by creatinine (black bars) of normal subjects and CKD patients. Significant difference when $P < 0.05$ between groups analyzed by one-way ANOVA followed by Student-Newman-Keuls test. *: $P < 0.05$, **: $P < 0.01$ vs. Normal. B) Klotho levels in plasma, urine, and kidney in a murine model of CKD. Representative blots of Klotho protein in plasma, urine and kidney in CKD mice. Co-IP of Klotho in 100 μl of mouse serum was

followed by immunoblot. IgG heavy chain = loading control. Urine Klotho was examined by directly immunoblotting ~40 μl of animal fresh urine with identical amount of creatinine. Klotho protein in the kidney was analyzed by immunoblotting 30 μg of the total kidney lysate. Lower panel shows summary of blood, urinary and renal Klotho protein of Sham (open bars) and CKD (black bars) mice. Quantitative data represented as means ± SEM and analyzed by the unpaired t-test. $*P < 0.05$, $**P < 0.01$ between Sham and CKD mice.

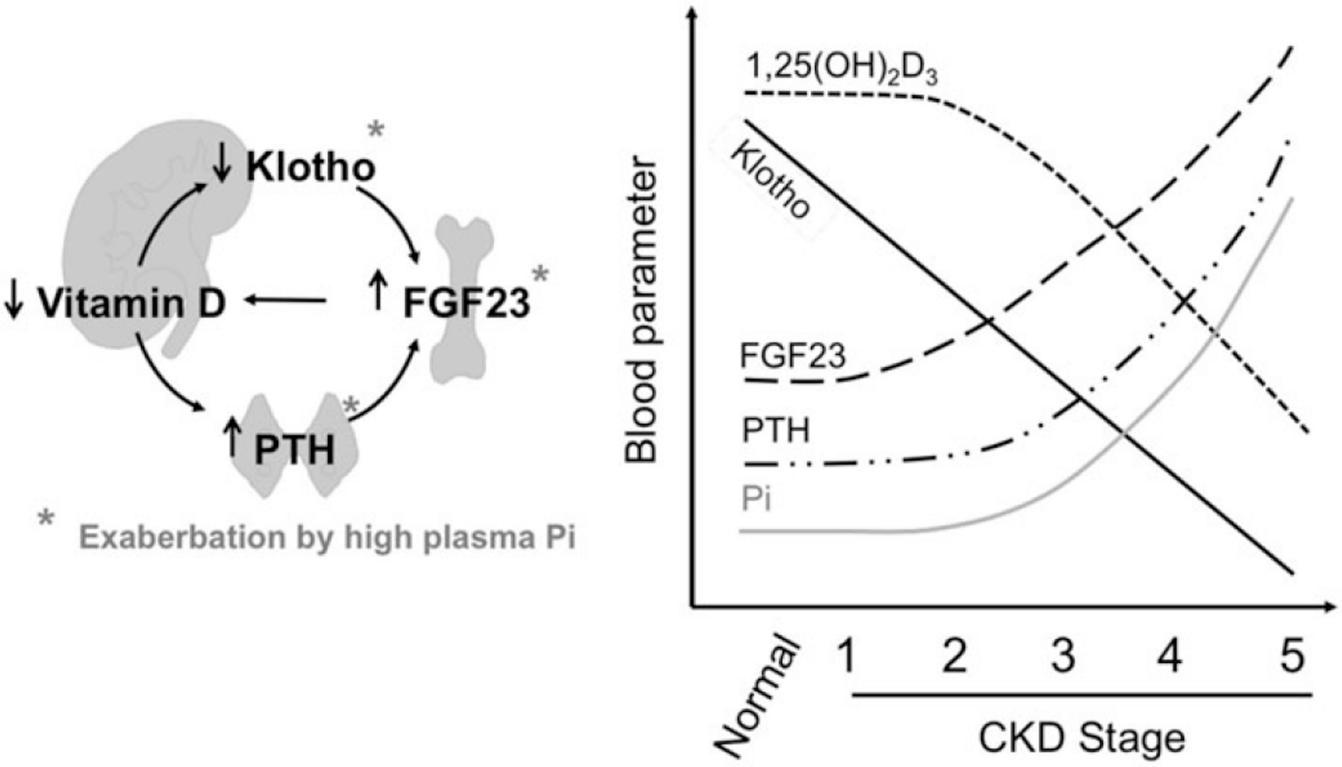

Figure 3.

Proposed model and time profile of changes in Klotho and hormones relevant to mineral metabolism in CKD. Left panel depicts a model where the decline in Klotho is an early event which then leads to other secondary changes. The secondary changes constitute a downhill spiral that exacerbates each other. The decline of Klotho protein in the kidney, urine and blood is an early event, and continues to decrease with CKD progression. Low Klotho consequently induces FGF23 resistance causing compensatory increase in blood FGF23 levels to maintain Pi homeostasis in CKD. The compensatory increase in FGF23 then suppresses vitamin D synthesis. In addition, low vitamin D and high blood Pi increases PTH. The high PTH may contribute to high FGF23 at late stage of CKD. The proposed time profile is shown on the right.

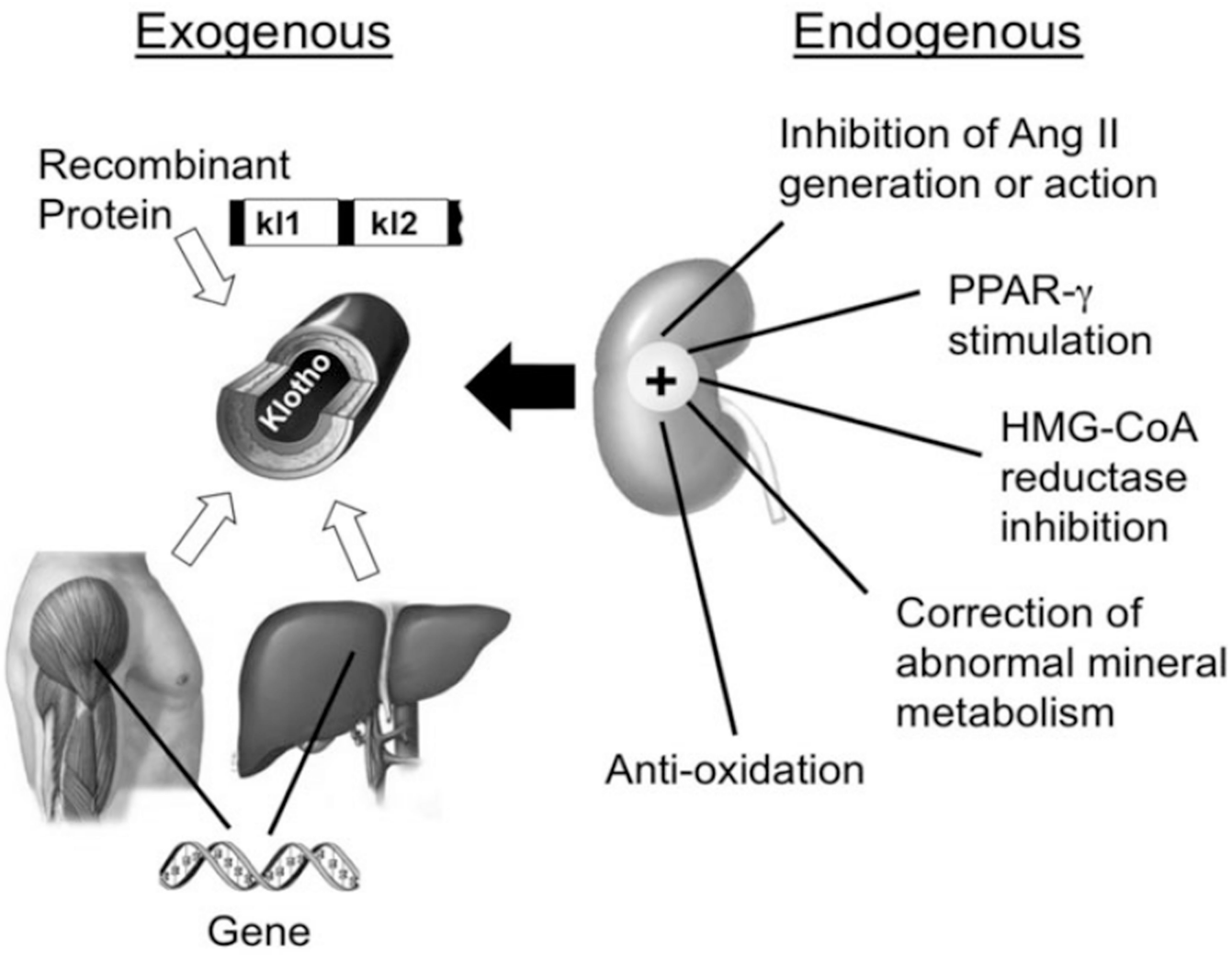

Figure 4.

Potential strategies to increase Klotho protein. Prevention and retardation of progression of CKD could theoretically block the decline of endogenous Klotho protein in the kidney. Administration of exogenous Klotho protein or Klotho gene could directly provide high levels of Klotho in the blood. This is potentially useful for advanced CKD or ESRD. For early or moderate CKD patients where endogenous Klotho-producing cells are not destroyed completely or simply suppressed, stimulation of endogenous renal Klotho is of potential therapeutic benefit.

Table 1

Similar clinical characteristics between *Klotho*$^{-/-}$ animals and CKD subjects

	***Klotho*$^{-/-}$**	**CKD**
Blood creatinine	↑	↑↑↑
Renal Klotho expression	↓↓↓	↓↓
Serum Pi	↑↑↑	↑↑
Serum FGF23	↑↑↑	↑↑↑
Ectopic calcification	Present	Present
Artherosclerosis	Present	Present
Growth	Severe retardation	Retardation in children
Anemia	No or mild	Severe
Life span	↓↓↓	↓↓

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Table 2

Endocrine and Renal Klotho deficiency in chronic kidney disease in humans and in rodent models

Category of Kidney Disease	Cause or Animal Model	Renal Klotho Protein		Renal Klotho mRNA		Blood Klotho	Urine Klotho	Human or Rodent			Citation
		IB	IHC	NB	qPCR	Assay	Assay	Mouse	Rat	Human	
CKD	CGN	✓	✓	✓						✓	1
CKD	DN	✓	✓	✓						✓	1
Graft rejection	CGR	✓	✓	✓						✓	1
CKD	Npx+IRI	✓	✓	✓	✓	IP + IB	IB	✓	✓		2
CKD	5/6 Npx			✓					✓		3
CKD	ICGN				✓						18
CKD	Npx in apo-E$^{-/-}$	✓			✓	ELISA		✓			37
Hypertension	SHR			✓					✓		3
Hypertension	DOCA			✓					✓		3
Hypertension	AngII	✓	✓	✓					✓		21
Hypertension	AngII	✓		✓					✓		39
DM	OLETF			✓					✓		3
DM	Streptozotocin	✓						✓			40
Nephrotoxicity	CsA	✓	✓		✓			✓			41
Nephrotoxicity	FK	✓	✓		✓			✓			41
Nephrotoxicity	SRL+CsA or FK	✓	✓		✓			✓			41
Nephrotoxicity	CsA	✓	✓		✓						42
Childrn/adults	Normal					ELISA				✓	43
Age/aged kidney	Normal		✓						✓		44
Age/aged kidney	Normal	✓						✓			46

CGN: Chronic glomerulonephritis; CsA: cyclosporine A; DM: Diabetes mellitus; DN: Diabetic nephropathy; DOCA-salt: Deoxycorticosterone acetate and high salt intake (DOCA-Salt); FK: FK-506, or called Tacrolimus; IB: Immunoblot; ICGN: ICR-derived spontaneous glomerulonephritis; IHC: Immunohistochemistry; IP: immunoprecipitation; NB: Northern blot; Npx: nephrectomy; OLETF: the Otsuka Long-Evans Tokushima Fatty Rat; RT: Reverse transcription; SHR: Spontaneous hypertension; SRL: Sirolimus; qPCR: quantitative or real time PCR.

Table 3

Factors that downregulate renal Klotho in CKD setting

Loss of functional kidney mass
Abnormal cytokine production
TNF-α ↑
IFN-γ ↑
Oxidative stress
H_2O_2 ↑
Lipid peroxidation ↑
Over activation of renal RAS
Ang II ↑
Abnormal hormone
1,25-VD_3 ↓
Disturbed mineral metabolism
Pi overload
Blood Pi ↑
Small uremic toxin
Indoxyl sulphate ↑