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Effects of Klotho on fibrosis and cancer: A renal focus on mechanisms and therapeutic strategies☆

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ABSTRACT

Klotho is a membrane-bound protein predominantly expressed in the kidney, where it acts as a permissive co-receptor for Fibroblast Growth Factor 23. In its shed form, Klotho exerts anti-fibrotic effects in several tissues. Klotho-deficient mice spontaneously develop fibrosis and Klotho deficiency exacerbates the disease progression in fibrotic animal models. Furthermore, Klotho overexpression or supplementation protects against fibrosis in various models of renal and cardiac fibrotic disease. These effects are mediated at least partially by the direct inhibitory effects of soluble Klotho on TGFβ1 signaling, Wnt signaling, and FGF2 signaling. Soluble Klotho, as present in the circulation, appears to be the primary mediator of anti-fibrotic effects. Similarly, through inhibition of the TGFβ1, Wnt, FGF2, and IGF1 signaling pathways, Klotho also inhibits tumorigenesis. The Klotho promoter gene is generally hypermethylated in cancer, and overexpression or supplementation of Klotho has been found to inhibit tumor growth in various animal models. This review focuses on the protective effects of soluble Klotho in inhibiting renal fibrosis and fibrosis in distant organs secondary to renal Klotho deficiency. We also discuss the structure-function relationships of Klotho domains and biological effects in the context of potential targeted treatment strategies.

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Abbreviations: ACE, angiotensin converting enzyme; ADAM, a disintegrin and metalloproteinase; AKI, acute kidney injury; ANGII, angiotensin II; AT1, angiotensin II receptor type 1; AMPKα, AMP-activated protein kinase α; α-SMA, α-smooth muscle actin; CKD, chronic kidney disease; CsA, cyclosporine A; CTGF, connective tissue growth factor; DLBCL, diffuse large B cell lymphoma; DNMT1, DNA methyltransferase 1; ECM, extracellular matrix; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; ESRD, end-stage renal disease; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; FOXO1, forkhead box protein O1; HDAC, histone deacetylase; IGF1, insulin-like growth factor 1; IGF1R, insulin-like growth factor receptor; IL-6, interleukin 6; IRI, ischemia/reperfusion injury; JNK, c-Jun N-terminal kinase; LRP6, low-density lipoprotein receptor-related protein 6; MME, mesangial matrix expansion; MMP, matrix metalloproteinase; Mn-SOD, manganese superoxide dismutase; mTOR, mammalian target of rapamycin; NaPi2a, sodium/phosphate co-transporter 2a; PAI-1, plasminogen activator inhibitor-1; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; PI3K, phospho-inositide 3-kinase; PPARγ, peroxisome proliferator-activated receptor γ; PWV, pulse wave velocity; Rac1, Ras-related C3 botulinum toxin substrate 1; RIG-1, retinoic acid-inducible gene 1; S100A4, S100 calcium-binding protein A4; SIRT1, sirtuin 1; TGFβ1, transforming growth factor β1; TGFβR, transforming growth factor β receptor; TRPC1, transient receptor potential cation channel, subfamily C, member 1; TRPC3, transient receptor potential cation channel, subfamily C, member 3; TRPC6, transient receptor potential cation channel, subfamily C, member 6; TRPV5, transient receptor potential cation channel, subfamily V, member 5; UUO, unilateral ureteral obstruction; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; Wnt, wntless-related integration site; WISP1, Wnt1-inducible signaling pathway protein 1.

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1. Introduction

Fibrosis can be defined as an exaggerated response to tissue damage leading to excessive deposition of extracellular matrix. This process may become maladaptive and impair organ or tissue function. Renal fibrosis, for instance, is a shared feature of chronic kidney disease (CKD) irrespective of primary etiology, providing a rationale for the development of currently lacking anti-fibrotic drugs. One promising protein uniquely poised to provide the basis for anti-fibrotic treatment strategies is the renal anti-ageing protein Klotho. Deficiency of Klotho in mice leads to a phenotype resembling human ageing, including a short lifespan, kyphosis, osteoporosis, vascular calcification, pulmonary emphysema, gonadal atrophy, and cognitive dysfunction [1]. Overexpression of Klotho, on the other hand, extends lifespan by 20–30% [2] and protects to a large extent from renal disease [3–8], cardiac disease [8–12], pulmonary damage [13,14], neurodegenerative disease [15–19], vascular disease [20–22], and diabetes [23,24].

Klotho is a membrane-bound protein primarily expressed in the kidney, mostly in the distal tubule and at a low level in the proximal tubule

(see Fig. 1B), as well as in the parathyroid gland, choroid plexus, and sinoatrial node [1,25,26]. Membrane-bound Klotho is a single-pass transmembrane protein with a 10 aa intracellular domain that has not been found to have a function in signal transduction. The extracellular part of Klotho contains two homologous domains, termed KL1 and KL2, that share a high degree of sequence similarity [27–29]. Both below KL2, just above the membrane, and in between KL1 and KL2, cleavage sites are targeted by ADAM10 and ADAM17, producing soluble Klotho proteins that either contain KL1, KL2, or both [30–32] (see Fig. 1A). It appears that the predominant soluble Klotho protein is the one of 130 kDa, containing both KL1 and KL2 [33], and that further cleavage is dependent on the generation of this 130 kDa soluble Klotho protein [34], although it should be noted that neither secondary cleavage product has been detected in human serum so far, only in *in vitro* systems. Finally, an alternatively spliced Klotho mRNA transcript has been hypothesized to code for a secreted Klotho protein [27,28], which would amount to the KL1 domain with a unique 10 aa tail, but this putative protein has proven rather elusive and has not been identified.

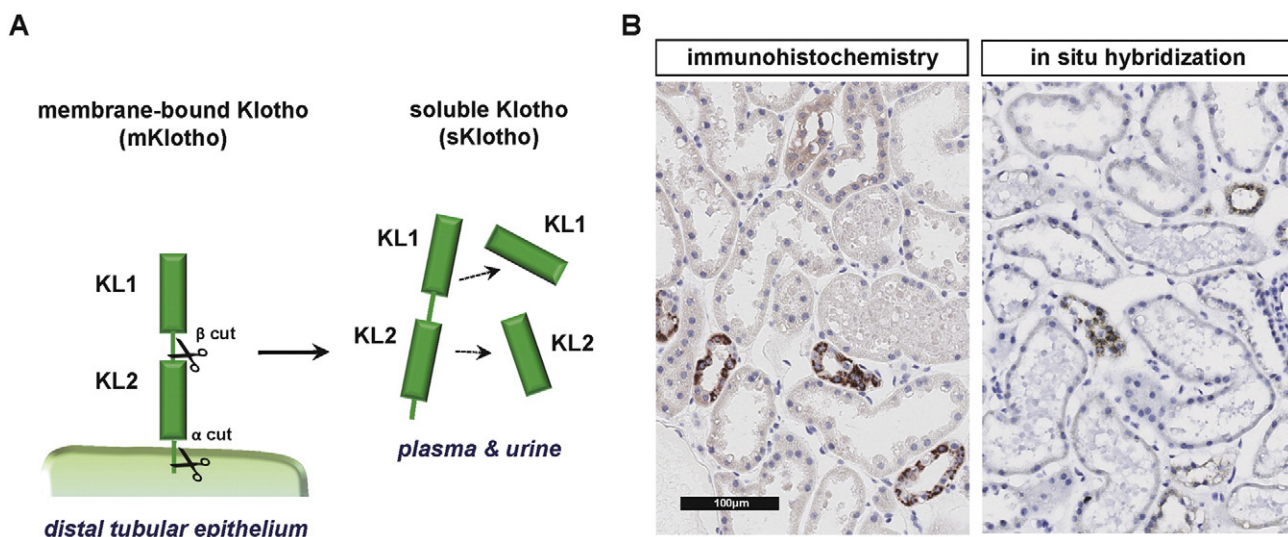


Fig. 1. Klotho protein forms and Klotho expression pattern. A) Schematic representation of Klotho proteins. Membrane-bound Klotho contains a small intracellular domain, a transmembrane domain, and a large extracellular domain consisting of two homologous domains termed KL1 and KL2. Proteolytic cleavage occurs at the indicated α cut and β cut sites, giving rise to soluble Klotho proteins, comprising the entire extracellular domain, or the single KL1 or KL2 domains. B) Immunohistochemistry and *in situ* hybridization for Klotho protein (using antibody KM2076) and Klotho mRNA, respectively, on human kidney tissue. Klotho is expressed predominantly in the distal convoluted tubule, with lower expression in the proximal tubule. No positivity is observed in tubulointerstitial cells. Original magnifications: 200 \times .

The anti-ageing functions ascribed to Klotho have been partially attributed to its function as a membrane-bound co-receptor for Fibroblast Growth Factor (FGF)23, promoting phosphaturia and inhibition of 1-alpha-hydroxylation of 25-hydroxyvitamin D [35,36]. However, there is mounting evidence that several of the phenotypic traits in Klotho deficient mice are mediated by soluble Klotho, which is derived from shedding of the membrane-bound protein and can be found in the circulation and the urine as a humoral factor [30,33] (see Fig. 1A). Soluble Klotho effects are mediated by direct modulation of several fundamental signaling pathways, including TGF β 1 [37], Wnt [38,39], IGF1 [2,40], and FGF2 signaling [41]. It is *via* these pathways that Klotho is thought to exert its marked anti-fibrotic effects. Furthermore, as many of these pathways affect both the development of fibrosis and tumorigenesis, a number of studies have identified Klotho as a tumor suppressor gene [40,42,43]. Klotho is primarily derived from the kidney [44] and any damage to the kidney will result in depression of Klotho expression [20,45,46]. This places the kidney in a central position as a source of soluble Klotho, a protein with demonstrated anti-fibrotic effects on several distant organs. We will discuss the current state of the evidence with regard to Klotho and fibrosis, and explore the possibilities for future treatment strategies based on Klotho delivery.

2. Soluble Klotho as an anti-fibrotic agent

When Klotho deficiency was discovered to lead to a phenotype resembling human ageing [1], it was not immediately clear that Klotho would become a subject of interest in fibrosis research. Over the years, as various strategies of Klotho overexpression and supplementation had been employed in various models of fibrotic diseases, it became increasingly apparent that Klotho is an endogenous inhibitor of the pathological fibrotic response. Interestingly, experiments using models such as unilateral ureteral obstruction (UUO) and ischemia-reperfusion injury (IRI) have been replicated many times, providing us with a solid scientific basis for discussion of the anti-fibrotic effects exerted by Klotho. We will herein focus first on establishing this relationship between Klotho and fibrosis, before addressing the molecular mechanisms.

2.1. Klotho and renal fibrosis

2.1.1. Klotho deficiency induces and promotes renal fibrosis *in vivo*

As a primarily renal protein with extensive renoprotective effects, it is perhaps surprising that Klotho deficiency only leads to a relatively mild renal phenotype. Fully Klotho-deficient mice display both vascular and tubular calcification [47], a decline in renal function [20], and a mild degree of interstitial fibrosis, as indicated by increased collagen deposition on Masson Trichrome staining, and an increase in α -smooth muscle actin (SMA) expression [48]. As expected, heterozygous Klotho mice (Klotho^{+/-}) have a less striking phenotype compared to full Klotho knockout mice (Klotho^{-/-}) mice and few pathological changes at a comparable age [49–51], but they also have a lifespan longer than the 8–10 weeks Klotho^{-/-} mice generally experience. At around 16 weeks of age, Klotho^{+/-} mice have been shown to develop both glomerulosclerosis and interstitial fibrosis, accompanied by albuminuria and a decline in renal function [52,53]. Interestingly, these mice also develop mesangial matrix expansion (MME), which is another typically ageing-related renal lesion characterized by increased extracellular matrix deposition by mesangial cells [49,52], as well as collapsing of glomeruli and tubular dilation, atrophy, and cast formation [52]. As a general observation, it is probably best to regard Klotho^{-/-} mice as a model for the premature ageing that occurs in severe human Klotho deficiency, like end-stage renal disease (ESRD), in which the development of vascular calcification is also a dominant feature. On the other hand, the milder Klotho^{+/-} phenotype appears to be more akin to partial human Klotho deficiency, like in physiological ageing or mild to moderate chronic kidney disease (CKD). As a rule, Klotho^{-/-} mice are too fragile to withstand the demands of anaesthesia and surgery, which only

allows us to establish that Klotho^{+/-} mice display an increased susceptibility to the development of a pathological response to injury, including fibrosis, which is also characteristic of ageing. For example, after UUO in Klotho^{+/-} and wild-type (WT) mice, Sugiura et al. found that Klotho^{+/-} mice exhibited more renal fibrosis, as well as markedly higher expression levels of α -SMA, fibronectin, TGF β 1, and S100A4, coupled with a more pronounced loss of E-cadherin and endogenous Klotho expression [48]. Satoh et al. describe a similarly exaggerated response after UUO, cementing that a “second hit” will readily expedite fibrosis development in Klotho^{+/-} mice [54], as do Sun et al. [55]. Paradoxically, however, the latter authors also report less fibrosis after UUO in hypomorphic Klotho (*kl/kl*) mice, which is at odds with other reports that indicate that these almost fully Klotho-deficient mice generally do not survive surgery [48,56]. Extending the discussion to other models, Shi et al. found that mice with one hypomorphic allele for Klotho (*kl/+*), develop more extensive renal fibrosis and have higher expression levels of α -SMA, CTGF, and collagen I, 20 weeks after bilateral IRI, compared to WT mice [7]. Overall, it appears that both Klotho^{-/-} and Klotho^{+/-} mice are prone to the development of renal fibrosis, although the complexity and timeline of the Klotho deficiency phenotype constitute a challenge.

Acquired rather than genetic Klotho deficiency is also associated with fibrosis, notwithstanding that causal relationships cannot be inferred from associations. In various models of renal fibrosis, including UUO [39,41,48,54,57–59], adriamycin nephropathy [39,60], cyclosporine A nephropathy [61–65], IRI [7,8], 5/6th nephrectomy [66], doxorubicin nephropathy [67], hypertension [22] (also with the addition of indoxyl sulfate) [68], uremic toxemia [69], renal artery constriction [70], adenine nephropathy [59,71–73], and diabetic nephropathy [74], fibrosis has been shown to develop while Klotho expression is concurrently decreased, possibly potentiating or exacerbating the development of fibrosis. More evidence supporting this notion of causality stems from *in vivo* RNA interference as a model of acquired Klotho deficiency. In both UUO- and adenine-induced renal failure models, *in vivo* Klotho siRNA treatment exacerbates the development of renal fibrosis and even potentiates the spontaneous development of fibrosis in sham or control mouse kidneys [57,59,71–73]. Similarly, in all stages of CKD but especially in ESRD patients in which we know renal fibrosis is present, we also know that Klotho expression is extremely decreased [20,45,46]. It is currently unknown whether mutations that confer an impairment of Klotho function also induce renal fibrosis [75].

Taken together, it is well-established that Klotho deficiency is both associated with renal fibrotic disease, as well as induces and exacerbates the development of renal fibrosis in many different models and in different species.

2.1.2. Klotho protects against the development of renal fibrosis *in vivo*

In addition to studies of fibrosis in Klotho deficiency, studies on experiments with Klotho overexpression or supplementation provide evidence from which we can gauge its possible therapeutic potential. One early indication that soluble Klotho is important in mediating the anti-fibrotic effects is a study by Chen et al., in which *kl/kl* mice were injected with soluble Klotho protein and developed less renal fibrosis, indicating that soluble Klotho protein can ameliorate fibrosis induced by Klotho deficiency [76].

More systematically, though, multiple UUO experiments supply evidence that various strategies are successful in attenuating the development of renal fibrosis. First of all, constitutive Klotho overexpression in transgenic mice was shown to inhibit fibrosis, as well as collagen III, CTGF, TGF β 1, fibronectin, cMyc, WISP1, β -catenin mRNA expression levels, in addition to attenuating the decline in renal mass [54]. Fibronectin protein levels, β -galactosidase activity, activated Rac1 levels, and phosphorylated JNK levels were also decreased compared to WT mice. Interestingly, a similar effect was achieved by overexpressing Klotho in Klotho^{+/-} mice that underwent UUO using skeletal muscle electroporation, resulting in less fibrosis than in WT mice, also

demonstrative of its potential [54]. The finding that ectopically overexpressed Klotho protein can prevent the development of renal fibrosis suggests that a humoral factor, like soluble Klotho, is responsible for the anti-fibrotic effects. Also illustrative of the therapeutic anti-fibrotic potential is the finding that induction of Klotho overexpression both at 1 day before and even 3 days after UUO resulted in a marked reduction of fibrosis (including fibronectin and α -SMA protein expression), demonstrating how Klotho is capable of preventing fibrosis even after the damage response has started to develop [39]. The hypothesis that these anti-fibrotic effects are mediated by soluble Klotho is further substantiated by three studies in which UUO was performed in mice that were then treated with various concentrations of recombinant Klotho protein [37,41,77]. Assessing the effects of soluble Klotho on fibrosis-related gene and protein expression after UUO, Doi et al. found that Klotho dose-dependently decreased α -SMA and collagen I mRNA and protein levels, as well as vimentin, Snail, Twist, MMP-2, MMP-3, and MMP-9 mRNA levels, whereas, interestingly, TGF β 1 mRNA levels were unaffected [37]. Similarly, Wu et al. found that mRNA levels of α -SMA, collagen I, CTGF, MMP-2, and vimentin were decreased by Klotho, without an effect on TGF β 1 mRNA [77]. Perhaps the effects of partial Klotho deficiency and increased Klotho levels on TGF β 1 are different, but this is currently not clear. In the study by Guan et al., it was found that protein levels of FGF2, fibronectin, and α -SMA were decreased by soluble Klotho treatment, while E-cadherin protein expression was preserved [41]. Finally, one UUO study in rats indicates that Klotho protein treatment may have similar effects also in other species [78].

Another line of evidence that has started to explore the therapeutic potential for Klotho as a treatment for renal fibrosis, is a series of studies on IRI (in which Klotho overexpression had already been shown to be protective [3]). Bilateral IRI in mice that constitutively overexpress Klotho lead to less fibrosis and expression of fibrosis-related proteins, like α -SMA, collagen I, and CTGF [7], compared to in WT mice. Interestingly, similar effects were found at 2, 4, and even 20 weeks after AKI if mice were treated with soluble Klotho for only 4 days after induction of bilateral IRI, attesting to the therapeutic potency of a hypothetical Klotho-based treatment, even after the induction of renal damage [7, 8]. Another example of this is the finding that starting soluble Klotho treatment 4 weeks after the induction of CKD (uninephrectomy + 30 min of contralateral IRI + high phosphate diet) and continuing Klotho treatment for 3 months in these mice with established CKD, renal fibrosis was reduced in both the Klotho-treated CKD mice and Klotho-treated sham mice (that had received a high phosphate diet), compared to vehicle-treated controls [8]. These studies also indicate that to prevent renal fibrosis, treatment with Klotho protein is potentially beneficial even if it is not administered before or directly after the occurrence of a renal ischemic insult.

In addition to UUO and IRI, other renal disease models have been used as well to test the effects of overexpression or supplementation of Klotho on renal fibrosis. For instance, Klotho gene delivery markedly reduced fibrosis in adriamycin nephropathy in mice, coupled with lower expression levels of β -catenin, Snail1, PAI-1, and fibronectin [39]. Klotho protein treatment in adriamycin nephropathy completely prevented the development of renal fibrosis as well as up-regulation of fibronectin and loss of E-cadherin on the mRNA level [60]. In another model, Klotho gene delivery prevented the development of fibrosis induced by 5/6th nephrectomy after 6 weeks in mice [66] and, extending the discussion to other species as well, in diabetic streptozotocin-injected rats Klotho gene delivery also prevented renal fibrosis, as well as fibronectin and vimentin protein expression [74]. In 24-week-old spontaneously hypertensive rats (SHR), Klotho gene delivery at 12 weeks of age also prevented the development on hypertension-induced renal fibrosis [22]. Finally, Klotho gene delivery has been shown to reduce cyclosporine A (CsA) nephropathy-induced renal fibrosis both in rats, including reduced α -SMA and TGF β 1 and increased E-cadherin mRNA and protein levels [61], and in mice [62].

A different approach that has been studied is to increase endogenous Klotho levels in order to decrease renal fibrosis. This is most commonly accomplished by employing strategies targeting epigenetic regulation of gene expression. This approach, however, does not allow for differentiation between the effects of different Klotho proteins. Sun et al. were the first to show that the Klotho promoter is hypermethylated by uremic toxins, which concurrently resulted in renal fibrosis [69]. Using 5-Aza-2dc as a DNA methyltransferase 1 (DNMT1) inhibitor, Klotho expression was increased *in vivo*, but the effect on fibrosis was not reported. Yin et al., however, recently performed a similar experiment and found that inhibition of DNMT1 both increased Klotho expression and decreased renal fibrosis in UUO. This attenuation was abrogated by Klotho siRNAs, indicating that Klotho rather than any other DNMT1-demethylated gene is essential in preventing renal fibrosis [59]. The same group reported similar results after using a different compound, rhein, which also demethylated the Klotho promoter, resulting in less fibrosis (and more fibrosis after RNA interference for Klotho) in both UUO and adenine-induced renal failure models [57,71]. A similar approach has been tried successfully with a histone deacetylating agent that also increase Klotho expression, showing that in adenine-induced CKD, HDAC inhibitor trichostatin A both decreased renal fibrosis and increased Klotho expression, an effect that was abolished in the presence of Klotho siRNAs, illustrating the key role Klotho plays in this process [72]. More specifically, HDAC3 inhibitor RGFP966 de-repressed Klotho expression *via* PPAR γ and prevented renal fibrosis, but only in the absence of Klotho siRNAs [73]. Klotho expression was also increased by inhibition of H3K9 methyltransferase G9a either pharmacologically using BIX01294 or after RNA interference, coincident with less α -SMA, fibronectin, and collagen-I protein expression [58]. Other strategies that have shown that up-regulation of Klotho coincides with a halted development of fibrosis include losartan treatment [79], pravastatin treatment [64], N-acetylcysteine treatment [63], and curcumin treatment [65], all in cyclosporine A nephropathy, as well as aliskiren treatment in chronic ischemic kidney injury *via* renal artery constriction [70] and TGF β RI inhibitor SB431542 treatment in adenine nephropathy [73]. No causality, however, between an up-regulation or a retention of Klotho expression and the outcome of a reduction in renal fibrosis can be inferred from these studies.

To summarize, reports indicate that soluble Klotho, either directly supplemented as recombinant protein or derived from induced or constitutive overexpression, is capable of inhibiting the development of renal fibrosis in various models and multiple species. Details from the studies in which Klotho overexpression or supplementation has been used in models of renal fibrosis, are summarized in Table 1.

2.2. Klotho and cardiac fibrosis

2.2.1. Klotho deficiency induces and promotes cardiac fibrosis *in vivo*

The effects of Klotho on fibrosis in the heart have been the subject of a number of studies. As Klotho is not expressed in the heart except for in the sinoatrial node [25], most effects Klotho exerts on the heart are expected to be mediated by kidney-derived soluble Klotho present in the circulation [44]. Klotho deficiency leads to sinoatrial node dysfunction and consequently to arrhythmias [25], as well as to an increase in heart weight/body weight according to some [12,80], but not all accounts [10,11]. Left ventricular ejection fraction, stroke volume, and cardiac output were all found to be reduced in *kl/+* mice compared to WT mice [9]. With regard to cardiac fibrosis, reports differ a bit: Hu et al. describe that *kl/kl* mice have more spontaneous cardiac fibrosis at 6 weeks, and even more at 12 weeks of age, coupled with more collagen-I and α -actinin and β -myosin heavy chain expression [9]. Heterozygotes were not found to have more spontaneous fibrosis at these ages. Xie et al., however, did not find more spontaneous cardiac fibrosis in *kl/+* mice [11] or in *kl/kl* mice [10]. This discrepancy with the study by Hu et al. may be due to the use of a low-phosphate diet that increases Klotho expression and generally improves the phenotype of *kl/kl* mice [81,82].

Table 1
Studies using Klotho treatment in animal models of renal fibrosis.

Klotho intervention	Treatment regimen	Fibrosis model	Treatment duration/time points	Recombinant Klotho source	Severity of model	Effect on fibrosis	Effect size on fibrosis	Reference
Klotho protein treatment (i.p.)	20 µg/kg/48 h	<i>Kl/k</i> mice	3–8 weeks of age	Self-made, rat Klotho	Mild	Decreased	Moderate	[76]
Genetic Klotho over expression		UUO in Klotho–Tg mice	Day 3, 7, 14		Severe	Decreased	Large	[54]
Induced Klotho over expression		UUO in <i>kl/+</i> mice	Day 14		Severe	Decreased	Large	[54]
Induced Klotho over expression	1 day before UUO	UUO in mice	Day 7		Severe	Decreased	Large	[39]
Induced Klotho over expression	3 days after UUO	UUO in mice	Day 7		Severe	Decreased	Large	[39]
Klotho protein treatment (i.p.)	10 or 20 µg/kg/48 h	UUO in mice	Day 3, 7	Self-made, rat Klotho	Severe	Decreased	Large	[37]
Klotho protein treatment (i.p.)	10 µg/kg/48 h	UUO in mice	Day 3, 7, 14	R&D Systems, mouse Klotho	Severe	Decreased	Large	[41]
Klotho protein treatment (i.p.)	10 µg/kg/48 h	UUO in mice	Day 7	R&D Systems, human Klotho	Severe	Decreased	Large	[77]
Klotho protein injection (i.p.)	20 µg/kg/48 h	UUO in rats	Day 14	?, rat Klotho	Severe	Decreased	Large	[78]
Genetic Klotho over expression		Bilateral IRI in mice	20 weeks	Self-made, mouse Klotho	Moderate	Decreased	Large	[7]
Klotho protein treatment (i.p.)	10 µg/kg for 4 days	Bilateral IRI in mice	2, 4, 20 weeks	Self-made, mouse Klotho	Severe	Decreased	Large	[7, 8]
Klotho protein treatment (mini-pump, i.p.)	300 µg/kg/month	Uninephrectomy + IRI + HPD	3 months	Self-made, mouse Klotho	Severe	Decreased	Large	[8]
Induced Klotho over expression		Adriamycin nephropathy	3 weeks		Severe	Decreased	Large	[39]
Induced Klotho over expression		5/6th nephrectomy	6 weeks		Moderate	Decreased	Large	[66]
Induced Klotho over expression		STZ-induced diabetic nephropathy	12 weeks		Mild	Decreased	Moderate	[74]
Induced Klotho over expression		Hypertension in SHR	12 weeks		Moderate	Decreased	Large	[22]
Klotho protein injection (i.p.)	10 µg/kg/48 h (?)	CsA nephropathy in rats	4 weeks	R&D Systems, mouse Klotho	Severe	Decreased	Large	[61]
Induced Klotho over expression		CsA nephropathy in mice	4 weeks		Moderate	Decreased	Moderate	[62]

UUO, unilateral ureteral obstruction; IRI, ischemia-reperfusion injury; HPD, high-phosphate diet; STZ, streptozotocin; CsA, cyclosporin A; SHR, spontaneously hypertensive rats.

Conversely, this is substantiated by the finding that a high-phosphate diet does spontaneously induce cardiac fibrosis in *kl/+* mice at later ages (9 months and 15 months) [9]. Furthermore, ageing itself substantially exacerbated the development of cardiac fibrosis in these mice. Xie et al. as well, using a “second hit”, do describe a marked increase in cardiac fibrosis in *kl/kl* mice compared to WT littermates after administration of isoproterenol, a model for stress-induced cardiac hypertrophy [10]. In *kl/+* mice as well, it was found that cardiac fibrosis secondary to 5/6th nephrectomy was dramatically increased compared to WT littermates [11], indicating an increased susceptibility to the induction of cardiac fibrosis. It should be noted that 5/6th nephrectomy in *kl/+* mice also exacerbated their Klotho deficiency. Taken together, these findings indicate that complete Klotho deficiency may be accompanied

by a mild degree of cardiac fibrosis and that partial Klotho deficiency leads to an increased propensity to developing cardiac fibrosis upon a “second hit”.

2.2.2. Klotho protects against the development of cardiac fibrosis in vivo

Analogous to the kidney, different approaches and different models have been employed to assess the effects of Klotho on cardiac fibrosis. Hu et al. show that in Klotho-overexpressing mice fed a high-phosphate diet until the age of 9 months and until the age of 15 months, there was less cardiac fibrosis than in WT littermates [9]. Only one study, by Xie et al., has examined whether induction of Klotho expression can inhibit the development of cardiac fibrosis. They used *kl/+* mice and induced cardiac fibrosis by 5/6th nephrectomy. Klotho gene delivery resulted

Table 2
Studies using Klotho treatment in animal models of cardiac fibrosis.

Klotho intervention	Treatment regimen	Fibrosis model	Time points	Recombinant Klotho source	Severity of model	Effect on fibrosis	Effect size on fibrosis	Reference
Genetic Klotho over expression		5/6th nephrectomy	4 weeks		Severe	Decreased	Large	[11]
Genetic Klotho over expression		HPD in ageing mice	9, 15 months		Moderate	Decreased	Mild	[9]
Genetic Klotho over expression		Angiotensin II in mice	4 weeks		Very mild	Increased	Small	[83]
Klotho protein treatment (i.p.)	10 µg/kg/48 h	Isoproterenol in mice	Day 2, 5, 9	R&D Systems, mouse Klotho	Moderate	Decreased	Large	[84]
Klotho protein treatment (i.p.)	10 µg/kg for 4 days	Bilateral renal IRI in mice	20 weeks	Self-made, mouse Klotho	Moderate	Decreased	Large	[8]
Klotho protein treatment (mini-pump, i.p.)	300 µg/kg/month	Uninephrectomy + renal IRI + HPD	3 months	Self-made, mouse Klotho	Severe	Decreased	Large	[8]

HPD, high-phosphate diet; IRI, ischemia-reperfusion injury.

in circulating Klotho levels still well below normal WT Klotho levels, but the development of cardiac fibrosis 35 days after 5/6th nephrectomy was markedly lower than in vector-treated *kl/+* mice [11]. Surprisingly, one study, on angiotensin II infusion in Klotho-overexpressing mice, describes that there was actually a bit more fibrosis in these mice, for unclear reasons [83]. More experiments, however, have been performed to investigate the effects of soluble Klotho protein on cardiac fibrosis. Song et al. treated mice with isoproterenol and Klotho and found that cardiac fibrosis, both within the myocardium and associated with intramyocardial arteries, was decreased at days 5 and 9, compared to isoproterenol-treated mice [84]. Collagen I and III mRNA levels were also decreased by Klotho treatment at 9 days after the start of isoproterenol treatment [85]. Hu et al. used Klotho treatment in models of AKI-induced cardiomyopathy and CKD-related cardiomyopathy [8]. After bilateral IRI, mice were treated with Klotho for 4 days and cardiac fibrosis was found to be much less extensive 20 weeks after surgery. Protein levels of α -actinin and α -SMA were also decreased. It should be noted that progression from AKI to CKD was prevented in these mice, so it is not immediately clear whether the inhibition of fibrosis is the direct result from Klotho protein effects exerted on the heart, or whether the prevention of cardiac fibrosis secondary to CKD is prevented by Klotho effects on the kidney. In their CKD study, using uninephrectomy, contralateral IRI, and a high-phosphate diet, Klotho treatment was started 4 weeks after surgery for 12 subsequent weeks. In these mice as well, Klotho markedly inhibited the development of cardiac fibrosis, as well as lowered the protein levels of α -actinin and α -SMA. As this experiment again begs the question whether the heart is protected directly from developing fibrosis, or is protected by the prevention of renal disease-induced fibrosis, the points should be made that Klotho itself is at least one of the kidney-derived factors that may prevent cardiac fibrosis and that Klotho also protects against cardiac fibrosis in the isoproterenol model, which is not dependent on renal injury. Finally, as uremic toxins like indoxyl sulfate, the accumulation of which is the result of renal disease, are known to induce cardiac fibrosis, in part due to down-regulation of Klotho [69], many of the effects that those uremic toxins exert on the heart are also found to be prevented by Klotho protein administration [12], although fibrosis has not yet been assessed in such a study.

To summarize, it is generally found that Klotho prevents the development of cardiac fibrosis and the fact that administration of the soluble protein has this effect coupled with the absence of Klotho in cardiomyocytes, lets us conclude that soluble Klotho is likely to directly modulate these effects. Since Klotho is primarily kidney-derived, an

implication of these recent studies on cardiac fibrosis is that the renal Klotho supply is integral to the prevention of cardiac fibrosis. Details from the studies in which Klotho overexpression or supplementation has been used in models of cardiac fibrosis, are summarized in Table 2.

2.3. Klotho and fibrosis in other tissues

2.3.1. Arteries

Most studies on the vasculature in Klotho deficiency have focused on the calcification phenotype that plagues the full Klotho knockout. The predominance of this pathological process is likely the reason that it was not recognized until very recently that Klotho^{+/-} mice develop arterial stiffening, characterized by an increase in pulse wave velocity (PWV) and deposition of extracellular matrix in the media [86–88]. Notably, the development of arterial stiffening does not appear to be secondary to the development of hypertension, as arterial stiffening precedes the rise in blood pressure. The increased collagen deposition can be found in the aorta, but not in other large arteries like the carotids and femoral arteries, at least in this age range. The same group then reported that in the high-fat diet model of arterial stiffening, PWV and aortic collagen I protein expression were dramatically increased in mice that were heterozygous for Klotho, indicating that Klotho deficiency exacerbates arterial fibrotic processes as well [87]. No experiments have been performed so far using transgenic mice that overexpress Klotho, using Klotho gene delivery, or administering Klotho protein to assess whether Klotho can prevent the development of arterial stiffening. However, treatment with eplerenone, inhibiting aldosterone signaling [86], SRT1720 treatment, activating SIRT1 [88], and treatment with AMPK α activator AICAR [87] have all been reported to prevent the development of arterial stiffening in Klotho^{+/-} deficient mice. Although it is a controversial topic, there is currently no solid evidence supportive of membrane-bound Klotho expression in arteries [89–91], so the vasculature should, like the heart, be considered a target tissue for soluble Klotho.

Taken together, it has been shown that partial Klotho deficiency in mice both induces and exacerbates fibrotic changes in the aorta, leading to a higher pulse wave velocity. It is yet to be determined whether an increased Klotho level has beneficial effects on arterial fibrotic processes.

2.3.2. Aortic valve

While full Klotho deficiency induces aortic valve calcification [92–94], the aortic valve of Klotho^{+/-} mice has only recently been examined. While there does not appear to be any fibrosis at baseline, a

high-fat diet resulted in marked fibrosis of the aortic valve cusps, including collagen I deposition, primarily on the aortic side [95], indicating that Klotho deficiency may play a role in the pathogenesis of aortic valve stenosis, affecting both aortic valve calcification and fibrosis. It is yet to be investigated whether Klotho overexpression or protein supplementation can counteract aortic valve fibrosis.

2.3.3. Lungs

There are currently two studies in which a relation between Klotho and pulmonary fibrosis has been investigated. Firstly, Kim et al. recently found that although *kl/+* mice do not exhibit spontaneous pulmonary fibrosis at 11–13 weeks of age, pulmonary fibrosis induced by tracheal instillation of asbestos is exacerbated in *kl/+* mice as assessed histologically, with an increase in pulmonary collagen content, compared to WT mice [96], fitting with the overall hypothesis that Klotho deficiency renders organs more prone to developing fibrosis. Shin et al. report that ovalbumin-induced pulmonary fibrosis, which was progressive over the course of 4 weeks, was negatively associated with pulmonary Klotho protein expression [97]. However, whether Klotho is expressed in airway epithelium or in lung tissue in general, is not generally accepted [98,99] so this observation warrants further analysis. There are currently no reports on whether Klotho overexpression or supplementation affects pulmonary fibrosis.

2.3.4. Skin

Given that the previous observations have established that Klotho exerts anti-fibrotic effects in *in vivo* models, it is important to address how Klotho affects wound healing. There is limited data on this topic, but a few studies are able to provide us with at least partial answers. Liu et al. were the first to describe that wound healing is impaired in

Klotho-deficient mice 4 days after wounding [38]. Another group compared *kl/kl*, *kl/+*, and WT mice and found repeatedly that after inflicting a standardized wound, *kl/kl* mice displayed slower wound healing [56, 100]. On day 7, when *kl/+* and WT wounds were still 20% open, but *kl/kl* wounds were still 80% open, collagen I and III mRNA levels were lower in *kl/kl* mice, in line with a lower collagen content on both days 4 and day 7 in these mice [56]. However, Klotho-deficient mice generally develop a thinner dermis with hardly any subcutaneous adipose tissue, compatible with their progeroid phenotype. The delay in wound healing could be attributed to non-intrinsic dermal factors or the influence thereof (such as circulating Klotho?), since grafting of WT skin or *kl/kl* skin on WT mice resulted in an undistinguishable wound healing response [100]. Additionally, Klotho expression was not detected in skin, ruling out effects of locally expressed Klotho. Although Klotho-deficient mice apparently do not react to wounding by excessively producing ECM during the process of wound healing, it would be interesting to examine the morphology and composition of healed wounds as it is possible that the resultant scar remodelling and turnover of ECM proteins is impaired, leaving these mice with more fibrosis long-term. It is also possible that other factors that influence wound healing, like angiogenesis, which is impaired in Klotho-deficient mice [101], play a role in delaying wound healing. A final possibility is that there is a mechanistic discrepancy between Klotho effects in “physiological” fibrotic processes as opposed to pathological fibrotic processes. There are currently no studies examining wound healing in Klotho-overexpressing mice or treatment of wounded mice with soluble Klotho.

2.3.5. Underexplored fibrosis models

As fibrosis is a feature of many diseases in many different organs and tissues and Klotho has been firmly established to exert anti-fibrotic

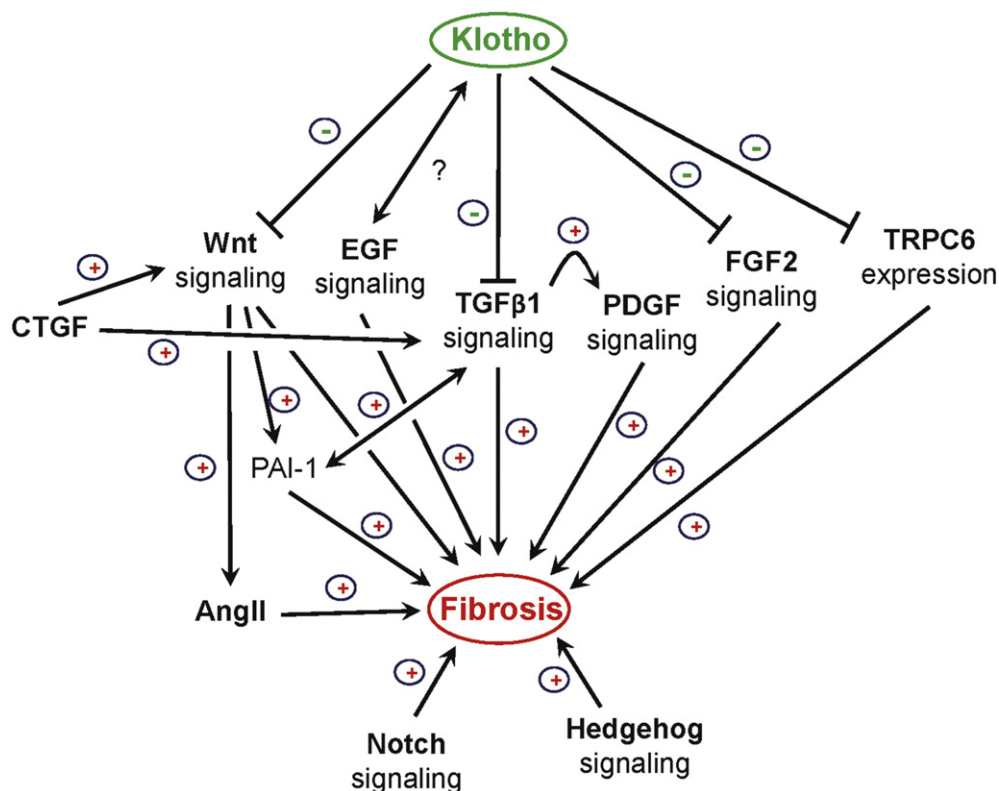


Fig. 2. Fibrosis-related growth factor pathways and their link to Klotho. Many pathways, including CTGF (connective tissue growth factor), TGFβ1 (transforming growth factor β1) signaling, Wnt signaling and downstream plasminogen activator inhibitor 1 (PAI-1) activity, epidermal growth factor (EGF) signaling, platelet-derived growth factor (PDGF) signaling, fibroblast growth factor 2 (FGF2) signaling, renin-angiotensin system activation resulting in high angiotensin II (AngII) levels, transient receptor potential cation channel subfamily C, member 6 (TRPC6) overactivation (downstream of growth hormone signaling), Hedgehog signaling, and Notch signaling have been implicated in fibrosis. Klotho has been shown to inhibit TGFβ1 signaling, Wnt signaling, RAS activation, FGF2 signaling, and TRPC6 expression. The link between Klotho and the EGF pathway is unclear and it is unknown whether a direct link exists between Klotho and PDGF, CTGF, Hedgehog, and Notch signaling. (–): inhibitory effect of Klotho on respective pathway; (+) stimulatory effect of respective pathway on fibrogenesis.

were inhibited as well, and ultimately this resulted in a decrease in high-glucose-induced cell hypertrophy and fibronectin expression. In porcine fibroblasts, transgenic Klotho overexpression increased IGF1 mRNA expression, as well as expression of anti-oxidant defense factors FOXO1, Mn-SOD, and catalase [117]. Expression of p53 and p16, genes encoding proteins that promote cellular senescence, caspase 3, and DNA methyltransferases was decreased. Interestingly, using the nuclei of these transgenic fibroblasts increased blastocyst formation, possibly pointing towards a better cellular health resulting in improved survival.

In general, however, not many studies have addressed the effects of Klotho on fibroblasts in terms of proliferation, migration, differentiation, and synthesis of extracellular matrix. A study by Liu et al. found differential effects depending on whether cardiac fibroblasts were treated with the 130 kDa Klotho protein, leading to higher collagen-I and α SMA expression, ERK phosphorylation, and proliferation, or whether cells were treated with KL1, leading to a decrease in proliferation and collagen-I production. While it is possible that different Klotho proteins exert different effects, Hu et al. investigated neonatal cardiac fibroblasts and did find that Klotho treatment (the 130 kDa protein) inhibited TGF β 1-induced CTGF expression, Ang-II-induced collagen-I expression, and phosphate-induced expression of both CTGF and collagen-I [9]. Although Smad2/3 phosphorylation was not found to be induced or inhibited by any treatment in these cells, Klotho did prevent ERK phosphorylation induced by either TGF β 1, AngII, or phosphate. Taken together, these data indicate that Klotho may protect fibroblasts from senescence and may inhibit the synthesis of extracellular matrix, although some conflicting data complicate the overall picture. As it appears that Klotho can inhibit the pathological de-differentiation of vascular smooth muscle cells [20], and prevent epithelial-to-mesenchymal transition of renal HK-2 cells [41], a central question for fibroblast research remains what the effect of Klotho is on fibroblast differentiation to myofibroblasts, as well as on subsequent ECM synthesis.

5. Klotho in cancer

When it was found that Klotho extends lifespan at least in part by inhibiting IGF1/insulin signaling, it was quickly hypothesized that Klotho may have anti-tumor effects [2,123]. Not long after, experiments were performed that supported anti-tumor effects by Klotho through targeting of IGF1/insulin signaling as well as other oncogenic pathways [40]. As many of the pathways addressed in the context of fibrosis are also relevant in cancer biology, it is of particular interest to discuss the effects of Klotho on tumors in this review. It should be noted that Klotho $^{-/-}$ mice are not known to develop tumors, although a potential increased propensity to develop cancer might be obfuscated by their short lifespan. Notably, Klotho $^{+/-}$ mice are also not known to develop tumors spontaneously despite their near-normal lifespan. An interesting relationship to mention is the one between p16 Ink4a , a well-established tumor suppressor that induces cellular senescence, and Klotho, which is down-regulated by the former [82].

5.1. Klotho expression in tumors

It should be noted that Klotho expression is generally considered to be extremely low in most tissues (aside from the kidney, parathyroid gland, and choroid plexus), so any biological relevance of any further down-regulation, if established, may indicate that our current views on Klotho expression levels require some thorough evaluation. On the other hand, it should be noted that different anti-Klotho antibodies are known to yield discrepant results. This is likely due to unspecific antigen binding, which may produce both false-positive and false-negative results [98]. As a general rule, though, the Klotho promoter is hypermethylated in tumor tissue and Klotho expression is consequently decreased. This was first demonstrated by Lee et al. who showed that the Klotho promoter is frequently hypermethylated in cervical carcinoma [124]. This was later also shown to be the case in colorectal

carcinoma [125–127], gastric carcinoma [128,129], mamma carcinoma [130–132], hepatocellular carcinoma [133], pancreatic adenocarcinoma [134], and even chordoma [135]. Klotho expression is also silenced by histone deacetylation in various tumors [124]. As a result, Klotho gene and protein expression have been shown to be decreased in esophageal carcinoma [136], gastric carcinoma [128,137], pancreatic carcinoma [42, 134], breast cancer [40,130,132], colorectal carcinoma [125,138], cervical carcinoma [139,140], hepatocellular carcinoma [133,141], renal cell carcinoma [142], ovarian carcinoma [143,144], glial tumors [145], urothelial carcinoma [146], oral squamous cell carcinoma [147], and diffuse large B cell lymphoma (DLBCL) [148]. In contrast, Klotho expression may be increased in multiple myeloma [149]. Taken together, the available data indicate that Klotho is nearly universally silenced upon oncogenesis. Of note is the observation that it was generally found to be the case that residual Klotho expression, however little, was still associated with a better outcome [128,133,134,136–138,141–143,148, 150–152].

5.2. Klotho effects on cancer cells *in vitro* and *in vivo*

As stated, Klotho inhibits many pathways involved in carcinogenesis, including IGF1R (with downstream PI3K, Akt, and mTOR signaling), Wnt/ β -catenin signaling, FGF2 signaling (generally affecting ERK1/2 signaling), and TGF β 1 and downstream Smad2/3 signaling. Generally, *in vitro* anti-tumor effects were found to include the induction of apoptosis [125,128,129,141,145,148,153–158], inhibition of proliferation [40,42,43,124,125,128,138,141,143–145,148,153–159], induction of autophagy [129,159], inhibition of autophagy [155], and inhibition of migration [37,138,139,142,154,159,160]. Klotho effects were found to be mediated by inhibition of activation of IGF1R (and/or downstream PI3K and Akt signaling) [40,42,43,129,138,142,144,148,153,157,159], ERK1/2 signaling [42,128], Wnt/ β -catenin signaling [124,139,141,154, 160,161], and TGF β 1 signaling [37]. Notably, the EGF pathway has not been found to be modulated by Klotho [40], although Klotho-deficient mice are known to have low EGF levels [111]. Interestingly, Klotho treatment appears to interact favourably with cytostatics in resistant cell lines [42,144,155,157].

In vivo, it was shown that Klotho significantly inhibited tumor growth and/or improved survival in athymic mice xenotransplanted with lung cancer [37], pancreatic carcinoma [42], colorectal carcinoma [162], breast cancer [43], hepatocellular carcinoma [141], ovarian carcinoma [143], melanoma [161], and diffuse large B cell lymphoma [148]. These studies have used different approaches to Klotho treatment. While the pancreatic carcinoma, breast cancer, hepatocellular carcinoma, and melanoma experiments were performed with recombinant soluble Klotho treatment, Klotho was overexpressed in the models of colorectal and ovarian carcinoma. The DLBCL experiments did both and the lung cancer experiments by Doi et al. also employed both approaches, in addition to injecting lung cancer cells into WT and Klotho-overexpressing mice, all of which resulted in fewer metastases after exposure to higher Klotho levels, likely through an effect on the TGF β 1 pathway [37]. Additionally, an *in vivo* study in which Klotho was down-regulated in melanoma cells using a short hairpin RNA showed that mortality and tumor growth were increased [152]. Finally, a similar approach using short hairpin RNAs against Klotho in cisplatin-resistant lung cancer cells also led to an increase in tumor volume, further establishing a role for Klotho as a tumor suppressor [157]. The available data indicate that Klotho acts as a universal tumor suppressor and that there may be a role for Klotho in the treatment of cancers.

6. Strategies for Klotho treatment

Currently, there are no Klotho-based treatments available, although a number of commonly used compounds do either directly up-regulate Klotho *in vitro*, like PPAR γ agonists [73,163], vitamin D [164], testosterone [165], and resveratrol [166], or otherwise appear to up-regulate or

at least de-repress down-regulation of *Klotho in vivo*, like ACE inhibitors/AT1R blockers [79,167], statins [64], and *N*-acetylcysteine [63]. Establishing higher renal *Klotho* expression levels or systemic soluble *Klotho* levels could therefore be achieved by treatment with these compounds. However, in the presence of factors that actively down-regulate *Klotho*, like uremic toxins [69], or in case of advanced renal disease, in which tubular cells may no longer possess the ability to express *Klotho*, the effect of up-regulating endogenous *Klotho* levels may be mild to absent. It is therefore interesting, given the established *in vivo* effects of soluble *Klotho* protein and for the potential treatment of ESRD patients, to also explore the possibilities for exogenous soluble *Klotho* treatment.

A few things should be noted on *Klotho*-based treatments. First of all, if the goal is to maintain *Klotho* levels at a physiological level, which is expected to confer a certain degree of protection compared to disease states in which *Klotho* is either locally or systemically down-regulated, then there are currently no indications that such a treatment would be expected to cause undesirable or adverse side effects. Raising endogenous *Klotho* levels to about twice the normal level, as present in the transgenic *Klotho*-overexpressing mouse, also does not appear to induce unwanted side effects. Given the general beneficial effects of high circulating *Klotho* levels on the entire organism, it could be argued that the full protein with all of its functions intact would be suitable for

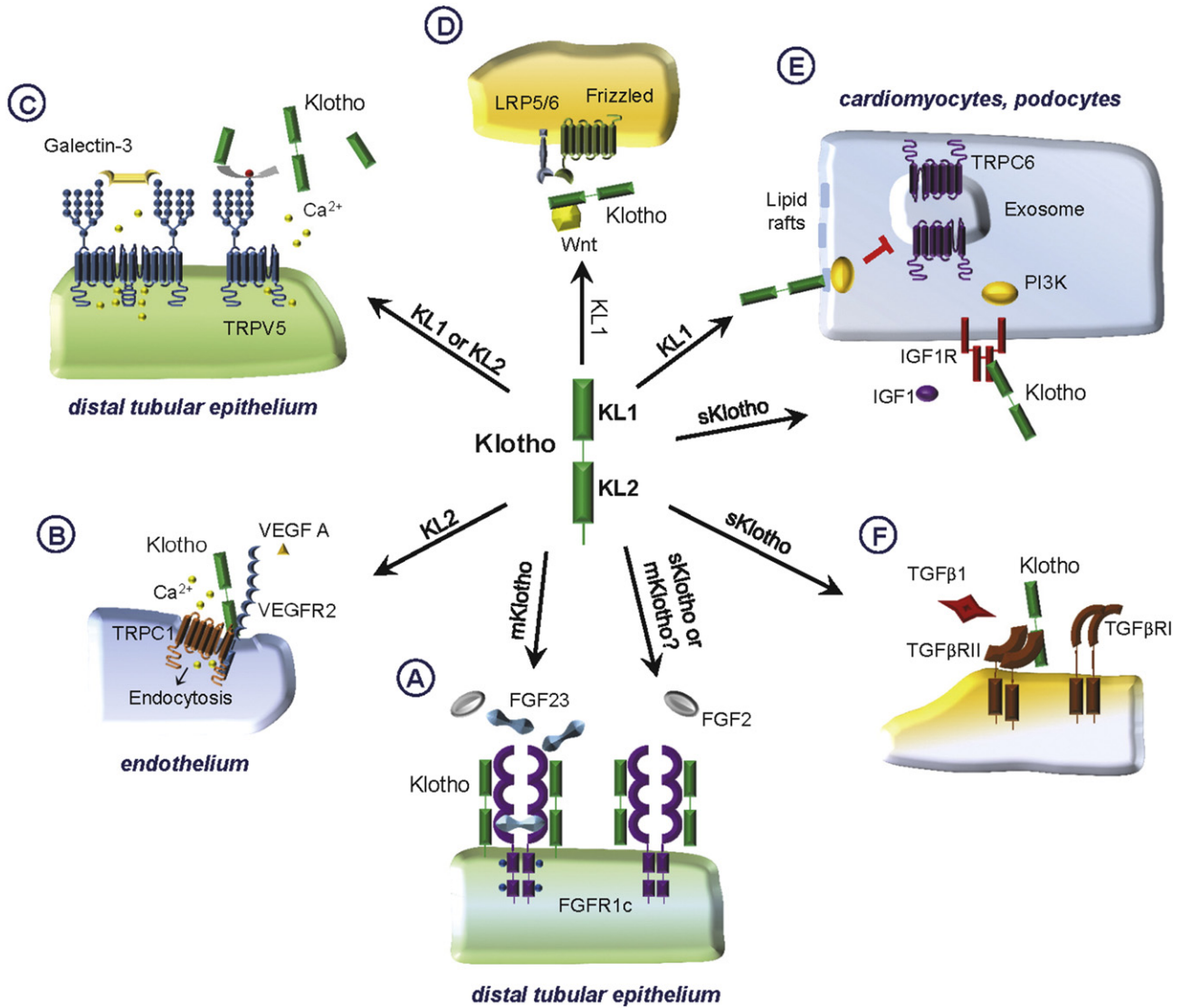


Fig. 3. Direct Klotho-mediated molecular mechanisms of action. Klotho (depicted in the centre) directly interacts with other pathways. Clockwise from the bottom panel: (A) on renal distal tubule epithelial cells, membrane-bound Klotho binds to fibroblast growth factor receptor 1c (FGFR1c) to form a ternary complex with FGF23, facilitating phosphaturia in the proximal tubule. Binding of Klotho to FGFR1c reduces the affinity for FGF2, inhibiting FGF2 signaling. (B) On endothelial cells, the KL2 domain binds to Ig6 and 7 from vascular endothelial growth factor receptor 2 (VEGFR2) and to the fifth loop of transient receptor potential cation channel, subfamily C, member 1 (TRPC1), forming a complex that is taken up by endocytosis upon stimulation by VEGF, protecting the cell from calcium-induced μ -calpain overactivation. (C) On distal tubule epithelium in the kidney, Klotho enzymatically modifies the N-linked glycan on transient receptor potential cation channel, subfamily V, member 5 (TRPV5), which allows stabilization of TRPV5 molecules by galectin-3 on the cell membrane, promoting calcium reabsorption from the urine. Klotho acts as a sialidase or β -glucuronidase on other ion channels and transporters as well, including sodium and phosphate transporter NaPi2a and potassium channel ROMK1. (D) The KL1 domain is capable of sequestering soluble Wnt molecules, preventing Wnt signaling. (E) The KL1 domain also inhibits PI3K signaling, either by inhibiting insulin-like growth factor 1 (IGF1) signaling by binding to IGF1R, or by binding monosialogangliosides on lipid rafts in the cellular membrane. Inhibition of downstream PI3K and Akt signaling will prevent exocytosis of transient receptor potential cation channel, subfamily C, member 6 (TRPC6) on cardiomyocytes and podocytes. (F) Soluble Klotho binds to transforming growth factor β 1 receptor II (TGF β RII), reducing the affinity for TGF β 1 and preventing the heterodimerization of TGF β RII and TGF β RI, thereby inhibiting downstream Smad2/3 signaling. Abbreviations: FGF: fibroblast growth factor; FGFR: fibroblast growth factor receptor; IGF1: insulin-like growth factor 1; IGF1R: insulin-like growth factor receptor; LRP6: low-density lipoprotein receptor-related protein 6; mKlotho: membrane-bound Klotho; PI3K: phospho-inositide 3-kinase; sKlotho: soluble Klotho; TGF β 1: transforming growth factor β 1; TGF β R – transforming growth factor β receptor; TRPC1: transient receptor potential cation channel subfamily C, member 1; TRPC6: transient receptor potential cation channel, subfamily C, member 6; TRPV5: transient receptor potential cation channel, subfamily V, member 5; VEGF: vascular endothelial growth factor; VEGFR2: vascular endothelial growth factor receptor 1; Wnt: wingless-related integration site.

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