Chapter 15

General discussion and future perspectives

This thesis centers around the relationship between the renal ageing suppressor Klotho and the vasculature, focusing on vascular Klotho expression and on the role of Klotho in arterial remodeling. **Chapter 2**, in which we summarize the existing literature on Klotho expression and Klotho-mediated effects on the vasculature, served as a starting point. We discuss what is known with regard to Klotho in relation to vascular calcification, as well as to endothelial dysfunction, endothelial hyperpermeability, intimal hyperplasia, arterial stiffening, hypertension, and impaired angiogenesis. A critical review of the literature reveals a few interesting and novel concepts. Firstly, all of the morphological differences and differences in gene and protein expression observed in Klotho-deficient mice that have also been investigated in human ageing, are remarkably similar. Secondly, it appears that soluble Klotho is the most important effector in mediating Klotho effects on the vasculature. Furthermore, while hyperphosphatemia is a pre-requisite for the development of vascular calcification, a number of interventions that were used to target pathways associated with cellular senescence resulted in a reduction of vascular calcification without a reduction in serum phosphate levels, indicating that cellular susceptibility to vascular calcification is a limiting factor and that hyperphosphatemia alone is not enough. Finally, we conclude that the experimental Klotho effects on the vasculature that have been reported establish Klotho as a very promising target for possible therapeutic strategies.

Part I focused on Klotho expression. With **Chapter 3**, we first started with another review of the literature on Klotho expression, describing that circulating, soluble Klotho derives from the kidney and where else Klotho is expressed in which organs, tissues, and cell types, qualifying the level of Klotho expression and how certain we are of these data. We also tried to provide an assessment of anti-Klotho antibodies, detailing to what extent anti-Klotho antibodies in the literature have been validated. While multiple, independent lines of evidence leave us as certain as we can be of the renal Klotho expression pattern, the topic of vascular Klotho expression is highly controversial and the field is riddled with contradictory data. Therefore, in **Chapter 4**, we investigated whether membrane-bound Klotho is expressed in human arterial tissue. First, we focused on renal membrane-bound Klotho expression, for which we wanted to validate our detection methods. We used multiple antibodies, most notably KM2076, which we validated using Western blotting (WB), with recombinant Klotho as a control, using a competition assay with recombinant Klotho, and using Klotho knockout tissue as a negative control. Having validated that KM2076 is specific for renal Klotho, which we detected in both human and mouse kidney in the distal tubules, we could subsequently not detect Klotho in arterial tissue from healthy donors or CKD patients using WB or immunohistochemistry (IHC). Despite the fact that we detected an extremely low expression of Klotho mRNA in the vasculature using qRT-PCR, in addition to not detecting membrane-bound Klotho protein in the vasculature we also found that there was no activation of FGF23 signaling in the aorta after FGF23 administration in mice, while the kidney did exhibit down-stream FGF23 signaling in the local presence of Klotho as a co-receptor for FGF23. These data indicate that membrane-bound Klotho, as expressed in the kidney, cannot be detected in the vasculature, nor is it functional as a co-receptor in facilitating FGF23 signaling.

We further assessed vascular Klotho expression in **Chapter 5**. Because many studies detail that Klotho is expressed as a protein of 116 kDa (as opposed to the 130 kDa attributed to renal Klotho) in smooth muscle cells (SMCs), we identified an anti-Klotho antibody, Ab69208, that detected a protein at 116 kDa and exhibited a vascular staining pattern. Ab69208, however, did not detect renal tubular Klotho and the renal Klotho staining pattern using KM2076 and the vascular staining pattern using Ab69208 were mutually exclusive. After using both systemic Klotho knockout mice and artery-specific Klotho knockout mice, which both displayed the vascular staining pattern with Ab69208 like in WT mice, we concluded that this staining pattern was non-specific for Klotho. This is a strong indication that other anti-Klotho antibody-generated staining patterns in the vasculature and 116 kDa bands on WB are also likely non-specific, especially if renal Klotho is not detected with these antibodies. In line with our previous PCR findings that Klotho is expressed at an extremely low level in the vasculature, we could detect Klotho expression in SMCs using RT-PCR, but not using RNA-ISH. To assess whether this low expression level of Klotho is relevant to the vasculature, we further investigated artery-specific Klotho knockout mice and found that while they have no overt morphological phenotype, functionally SM22α-Cre/Klotho$^{flox/flox}$ mice display endothelial dysfunction and impaired contractility, compared to WT mice. The impaired endothelium-dependent vascular relaxation could indicate that a very small amount of SMC Klotho affects SMC-to-EC cross-talk from the basolateral side. Because ECs are continuously exposed to Klotho on the apical side, we hypothesize that trace amounts of SMC Klotho likely affect SMCs in an autocrine fashion and modulate SMC-to-EC cross-talk mediated by other factors. The impaired contractility could indicate that local Klotho is important in maintaining SMC differentiation and a contractile phenotype.

Next, in **Chapter 6**, we wanted to investigate the expression of the putative secreted Klotho protein, long thought to be translated from the alternatively spliced Klotho mRNA transcript. Again, we first focused on the kidney and, lacking any methods to specifically detect secreted Klotho protein, we started at the gene and mRNA levels. The human alternative Klotho mRNA contains a premature stop codon between exons 3 and 4, which would theoretically trigger the degradation of the transcript by nonsense-mediated mRNA decay. By interfering with this degradation pathway in HK-2 cells *in vitro*, as well as by using RNA immunoprecipitation, polysome fractionation, and RNA *in situ* hybridization, we concluded that the alternative Klotho mRNA is indeed subject to nonsense-mediated mRNA decay and that it is not translated to a protein. This would mean that the putative secreted Klotho protein does not exist and we could indeed not detect a putative secreted Klotho in conditioned medium. There does, however, appear to be a clinical relevance to this finding, as the relative abundance of the alternative Klotho transcript increases greatly in patients with acute kidney injury (AKI). This likely dysregulation of splicing efficacy would essentially result in a down-regulation of membrane-bound Klotho protein, which is highly relevant, since Klotho is greatly down-regulated in AKI, but maintenance of Klotho expression would protect the kidney against AKI. Although other mechanisms, like promoter hypermethylation, are also known to down-regulate Klotho, we hypothesize that down-regulation of Klotho by loss of splicing efficacy in

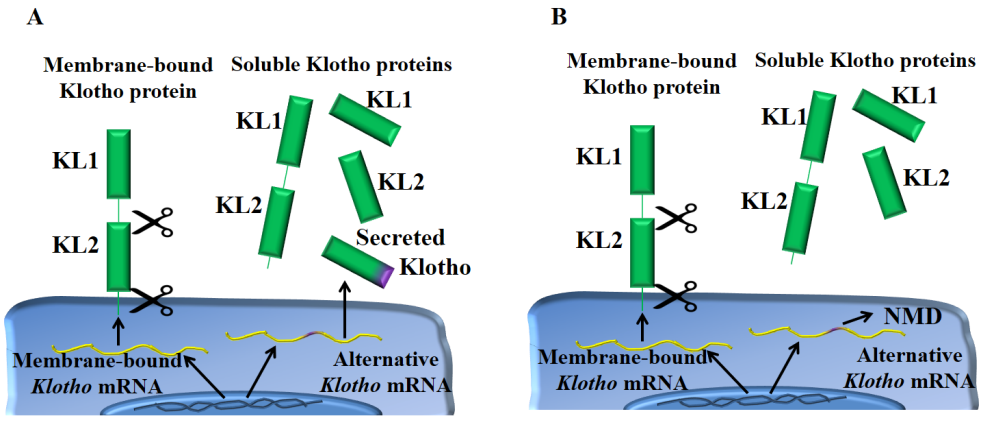

Figure 1. Paradigm of Klotho protein expression. (A) Schematic overview of old paradigm: mRNA for membrane-bound Klotho and an alternatively spliced Klotho mRNA transcripts are transcribed. The normal transcript is known to code for the membrane-bound Klotho protein, containing KL1 and KL2 regions and two sites for proteolytic cleavage, which generate full-length soluble Klotho and separate KL1 and KL2 domains. Secreted Klotho is thought to be translated as a splice variant. **(B)** Schematic overview of proposed paradigm. The alternative human Klotho mRNA is degraded by nonsense-mediated mRNA decay and is not translated to a protein.

favor of a non-translated transcript, resulting in less membrane-bound Klotho protein, constitutes an additional mechanism by which Klotho expression is lost and by which the kidney enters a vicious cycle of Klotho down-regulation and continuously incremental kidney damage. Having concluded that secreted Klotho produced by the kidney does in fact not exist, we have not specifically investigated its expression in the vasculature.

Overall, we conclude from the chapters in **Part I** that

- membrane-bound Klotho protein is not detectable in the vasculature
- secreted Klotho does not exist
- what has been considered to be vascular Klotho is likely non-specific
- an extremely low amount of local vascular Klotho expression is nonetheless important for vascular function.

A new paradigm for Klotho expression and Klotho proteins is depicted in Figure 1.

In **Part II**, about the role of Klotho in vascular remodeling, we further explored the effects of Klotho on SMC differentiation and the aberrant SMC behavior that emerges in the absence of Klotho, like vascular calcification (Figure 2). Vascular calcification is an active, cell-mediated process in which the SMCs de-differentiate and start expressing an osteochondrogenic gene signature. In **Chapter 7**, we studied the development of vascular calcification in Klotho

deficiency using NaF-18, a bone tracer now explored for the study of vascular calcification, in µPET-CT and autoradiography. While the µPET-CT scans did not reveal a signal, autoradiography images indicate that vascular calcification in Klotho deficiency first develops in the aortic arch and in the abdominal aorta. Since vascular calcifications were only very sporadically detected using histochemistry, the widely detected NaF-18 signature indicates that NaF-18 is a suitable tracer for microcalcifications. Mechanistically, we found for the first time that there is already a greatly increased serum calcification propensity in Klotho$^{-/-}$ mice at a stage without overt histological calcifications, which suggests a role for the fetuin A system and the maturation of secondary calciprotein particles (CPPs) in the etiology of Klotho deficiency-induced vascular calcification. We indeed found that Klotho could inhibit CPP-induced SMC calcification *in vitro*.

In **Chapter 8**, we investigated the renal vasculature of Klotho$^{-/-}$ mice, which generally lacked overt calcifications, as mentioned. Instead, we observed arteriolar hyalinosis, a lesion found in ageing, hypertension, and after transplantation, which has not been described before in relation to Klotho deficiency. We could detect plasma proteins in the hyalinous lesions,

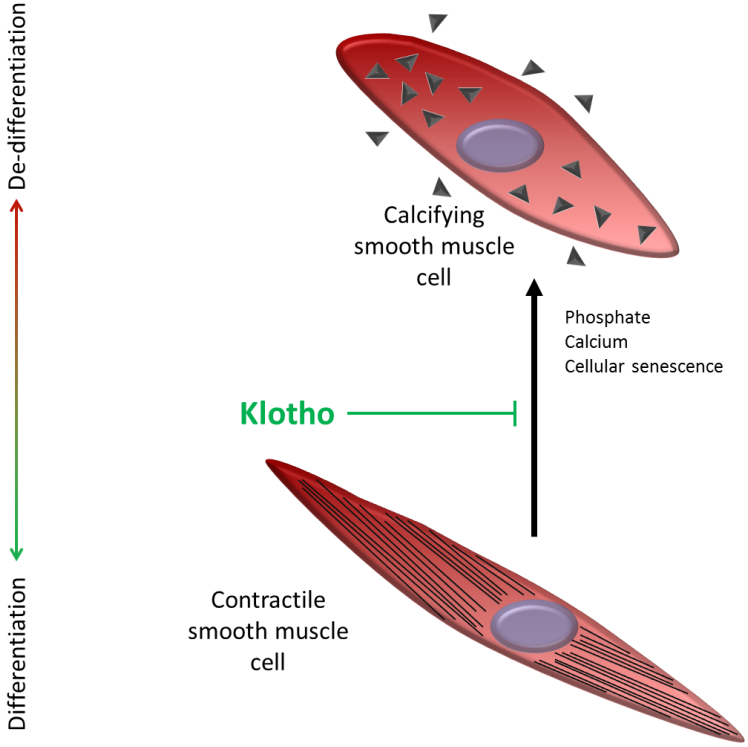

Figure 2. Old paradigm of Klotho inhibiting smooth muscle cell (SMC) de-differentiation from a contractile phenotype to a calcifying phenotype.

indicative of endothelial hyperpermeability, as well as an increase in extracellular matrix proteins, indicative of increased deposition by SMCs. Lesional SMCs had also lost their differentiated, contractile phenotype, while gaining markers associated with a more synthetic phenotype. To assess whether the presence of arteriolar hyalinosis in Klotho$^{-/-}$ mice was something that should in general be considered part of Klotho deficiency, we studied other Klotho knockout strains, which displayed arteriolar hyalinosis to a variable extent. In *kl*/*kl* mice (the original mice with a disruption in the Klotho promoter) in particular, there was a predominance of vascular calcification, which, however, was partially replaced by arteriolar hyalinosis when the development of vascular calcification was inhibited by spironolactone. These findings provide novel indications that Klotho deficiency may be principally characterized not by calcification, but by de-differentiation of SMCs, followed by a phenotypic transition in one of multiple directions.

Chapter 9 provides further evidence in support of this paradigm. After it had been originally reported that Klotho-deficient mice display intimal hyperplasia, observations or further investigations of this phenomenon have not been described. Because intimal hyperplasia is a process in which medial SMCs de-differentiate and migrate to the intima, where they proliferate and obstruct the lumen, we were interested in assessing whether Klotho$^{-/-}$ SMCs would assume a migratory phenotype as well. Not having observed spontaneous neointima formation in Klotho$^{-/-}$ mice, we did find that Klotho$^{+/-}$ mice display an exaggerated response compared to WT mice with increased stenosis and/or neointimal surface area after endovascular wire injury or cuffing, although in the latter model, this could have been due to a lack of arterial wall thinning in partial Klotho deficiency. Furthermore, *kl*/*kl* mice did display spontaneous intimal hyperplasia, which became more apparent after inhibition of vascular calcification using spironolactone. The hypothesis that Klotho inhibits SMC de-differentiation is further supported by the finding that SMC migration was inhibited by recombinant Klotho *in vitro*. The resultant paradigm is depicted in Figure 3.

In **Chapter 10**, we investigated vascular function in Klotho deficiency. We first confirmed that Klotho$^{-/-}$ mice display endothelial dysfunction, which, surprisingly, was not the case in Klotho$^{+/-}$ mice, not even at 1 year of age. We used different substances for aortic ring pre-constriction because phenylephrine sensitivity was also altered in Klotho$^{-/-}$ mice, which could be a reflection of the Klotho deficiency premature ageing phenotype. Finally, we provide preliminary indications that soluble Klotho itself may ameliorate Klotho deficiency-induced endothelial dysfunction. Interestingly, while the phenylephrine response was increased in Klotho$^{-/-}$ aortic rings, it was decreased in artery-specific Klotho knockout mice in mesenteric artery rings, which is akin to the differential response in conduit *vs.* resistance arteries also observed in hypertensive rats (likely with a relative Klotho deficiency), compared to normal rats (1). In addition to a functional consequence of Klotho deficiency in the form of altered contractility, these data affirm the profound effects Klotho has on the endothelium.

Continuing this focus on the endothelium, in **Chapter 11**, we investigated the role of Klotho in angiogenesis and tumor angiogenesis in glioblastoma (GBM), a highly vascularized primary brain tumor. We found that Klotho inhibited tube formation in microvascular endothelial cells *in vitro*, while there was no effect of Klotho in angiogenesis in the chorioallantoic membrane model (*in ovo*). These data do not materially elucidate the direct effects of Klotho on angiogenesis, aside from lending more credence to the notion that perhaps the effects of Klotho on angiogenesis are different in different tissues or depending on the type of endothelial cells. Further studies using additional models are required to shed light on the relationship between Klotho and angiogenesis. We found, however, that Klotho inhibited angiogenesis induced by various glioblastoma cell lines both *in vitro* and *in ovo*, although we did not detect an effect of Klotho on tumor growth or tumor angiogenesis in mice with GBM xenografts, which we think may have been an excessively pro-angiogenic model. Despite these *in vivo* results, we think that our data establish that Klotho can act as an inhibitor of tumor angiogenesis and we hypothesize that the anti-tumor effects mediated by Klotho, as described in other studies, are likely in part due to inhibition of tumor angiogenesis.

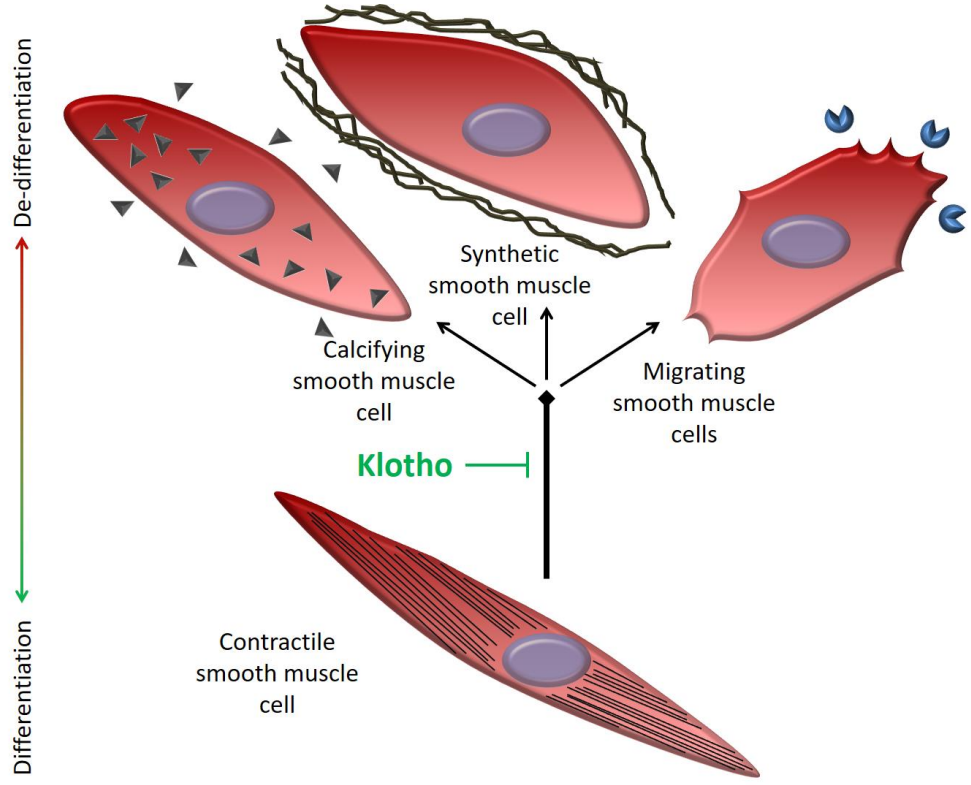

Figure 3. Proposed paradigm of Klotho inhibiting smooth muscle cell (SMC) de-differentiation from a contractile phenotype to either a calcifying phenotype, a synthetic phenotype, or a migratory phenotype.

We conclude from the chapters in **Part II** that

- Klotho is a quintessential factor in governing arterial remodeling
- Klotho deficiency can spontaneously lead to vascular calcification, arteriolar hyalinosis, and intimal hyperplasia.
- Klotho deficiency induces endothelial dysfunction,
- Klotho is a novel inhibitor of tumor angiogenesis.

In **Part III**, we try to start paving the way towards clinical applications of Klotho. Therefore, we first reviewed in **Chapter 12** the Klotho-based therapeutic strategies that have been used experimentally, both broadly in models of fibrosis and cancer, and specifically using novel delivery methods. We discussed which domains of the Klotho protein are known to interact with which pathways, which could prove useful in isolating Klotho-mediated effects from each other. We also discussed which (technical) problems are still to be overcome in devising Klotho-based therapies.

In **Chapter 13**, we discuss whether there are any other aspects of Klotho that can be used clinically, for example in contributing to diagnostics or prognostics. Various SNPs in the Klotho gene have been studied in relation to vascular disease and KL-VS allele heterozygosity may protect against cardiovascular disease, while KL-VS homozygosity could predispose to the development of CVD. Additionally, while many studies exist on serum Klotho levels in relation to renal or vascular disease, the currently available ELISAs are not reliable enough to base meaningful conclusions on data produced with these assays. In short, there appears to be no role yet for Klotho SNPs as a risk factor or for soluble Klotho level measurements in the clinic.

In **Chapter 14**, we find that intronic Klotho SNPs rs577912 and rs553791 in kidney transplant recipients are negatively associated with graft survival. The finding that recipient SNPs, rather than donor SNPs, in the Klotho gene are potentially clinically relevant points to a potential role for Klotho in the immune system of the recipient and its interaction with the kidney graft. This possibility again points to a potential role for Klotho in cell types that express Klotho in very low amounts.

From **Part III**, we can surmise that

- there are both a lot of opportunities and challenges ahead for Klotho-based therapeutics
- the potential uses of Klotho SNPs as a risk factor and of soluble Klotho as a biomarker are currently insufficiently supported by the available evidence
- a future role for Klotho in diagnostics can certainly be envisioned.

On the whole, this thesis describes work broadly exploring the relationship between Klotho and the vasculature, during which we happened upon a number of interesting and novel concepts in Klotho biology. The first would be the finding that the putative secreted Klotho protein does not exist at all, which is contrary to a 20-years-long-held paradigm of the

different forms of Klotho proteins. The second is that the existence of the alternative Klotho mRNA transcript may interfere with Klotho protein expression. Thirdly, the phenotype we detect in artery-specific Klotho knockout mice indicates that the almost undetectably low level of Klotho expression is still locally relevant, which has lately been shown to be the case in other cell types with very low Klotho expression levels. This includes two strains of bone-specific Klotho knockout mice (2-4) and three strains of proximal Klotho knockout mice (5), which all develop a subtle phenotype, while conversely, restoration specifically of down-regulated Klotho expression in muscle tissue in mice with muscle dystrophy ameliorates the muscle dystrophy phenotype (6). Fourthly, this begs the question of what the "normal" Klotho expression level is and raises the possibility that a very low expression level (*i.e.* an undetectable protein expression level) of Klotho, which is constitutively present in almost all cell types, could be a normal and functional Klotho expression level, while the distal tubule and choroid plexus epithelia may express levels of Klotho that should be regarded as extremely high, as they are considered the production sites that generate soluble Klotho for large compartments of the body. Fifthly, as it becomes clear that Klotho deficiency does more to SMCs than just causing them to calcify, in showing that SMCs in the absence of Klotho can also assume a synthetic or a migratory phenotype, our data suggest that Klotho may be a crucial factor in maintaining SMCs in their contractile, differentiated state. We conclude that Klotho deficiency renders SMCs supremely vulnerable to undergoing a phenotypic transition towards any of a number of pathological SMC phenotypes and that the balance between these pathological "lineage fates", while generally tilted towards the development of vascular calcification, can easily be swayed to intermediates or extremes of what is apparently a spectrum of Klotho deficiency-associated vascular lesions in which one common denominator is the role of de-differentiated SMCs. Sixthly, as it has become clear that there is a degree of phenotypic variability in mice as a function of the amount of Klotho present (full deficiency, gradations of partial deficiency, Klotho "sufficiency"), our data also indicate that there is to an important extent phenotypic variability in full Klotho deficiency, suggesting that factors like the composition of the diet and genetic background interact with (patho)physiological factors and that a slight change in this interplay can result in a different phenotype. We therefore took extra care to confirm the presence or emergence of vascular lesions like arteriolar hyalinosis in multiple types of Klotho knockout mice and investigated the modifiability of phenotypic variability by additional treatments.

Furthermore, although not a novel concept, our data are in agreement with the long-hailed characterization of Klotho as an anti-ageing protein. While a discussion on whether Klotho actually is an ageing suppressor (aside from the lifespan-extending effects (7, 8)) will generally focus on whether Klotho deficiency is phenotypically and molecularly consistent with ageing, this discussion is complicated by both model-related factors like the short lifespan (and window to develop conditions) of Klotho knockout mice and by a paucity in our understanding of the molecular mechanisms of ageing and how ageing should be defined. Therefore, we will settle for the observation that, for example, arteriolar hyalinosis is a common ageing-

associated vascular lesion that we determined is also a prominent characteristic of the Klotho deficiency phenotype.

One limitation of our work with Klotho$^{-/-}$ and *kl*/*kl* mice is that because of the pleiotropic and profound effects Klotho has, a change in the serum Klotho level will result in a multitude of dysregulations in many pathways, which makes it very difficult to ascertain whether any changes are the direct result of the loss of Klotho, or an indirect result. In a way, this distinction may not matter, since Klotho acts up-stream in both cases. However, to determine whether Klotho directly affects a molecular process, experiments with direct modulation of Klotho levels *in vitro*, like we did in our SMC calcification and migration assays, are crucial. Complicating this matter, it is likely in many disease processes that there is an interaction between the direct and indirect effects of Klotho. For instance, vascular calcification in Klotho deficiency develops because Klotho deficiency leads to hyperphosphatemia (by disinhibiting NaPi2a-mediated phosphate reabsorption via distal FGF23 signaling (9, 10), independent regulation of NaPi2a by soluble Klotho from the circulation (11, 12), and independent regulation of NaPi2a by Klotho expressed in the proximal tubules (5), as well as by increasing vitamin D levels (13) which mobilizes phosphate from the bone and increases intestinal phosphate absorption). Klotho also protects against vascular calcification by preserving renal function and therefore by preventing the accumulation of uremic toxins, and there are additional, directly protective effects on SMCs, which involve driving SMC differentiation (14). While even this summary is overly simplified, it illustrates the challenges of Klotho research, while also attesting to the potential of addressing a myriad of dysregulations found in Klotho deficiency by restoration of only one factor.

Future perspectives

It is clear that despite the progress made over the past couple of years, there is still a lot we do not know about Klotho and the molecular mechanisms by which Klotho exerts its (vascular) effects. The aforementioned Klotho deficiency-induced dysregulation in a great number of physiological processes therefore provides a large puzzle, rather than simple answers. An increasing number of studies, however, is employing ways to slowly unravel this puzzle, including the use of tissue-specific Klotho knockout mice (2, 3, 15-18), tissue-specific Klotho overexpression mice (19-21), and the reversal of epigenetic silencing specifically of Klotho (6). Generally speaking, coupled with the broadening of the disease processes in which various Klotho treatments are being investigated, this approach will slowly but surely determine the scope and scale of potential indications for Klotho-based therapies. The recent X ray crystallography data describing the protein structure of Klotho (22), as well as recent

investigations into specific structure-function relationships (23-25), are expected to provide an impetus to the general field.

More specifically focusing on the vasculature, many questions raised in or by our work are left to be answered. This includes first and foremost what the molecular relationship is between Klotho and smooth muscle cells and which pathways are activated or inhibited down-stream. Secondly, while we provide an expanded description of what vascular lesions occur in Klotho deficiency, more experiments are to be performed to determine whether Klotho treatment can ameliorate the occurrence of these vascular lesions. Broadly speaking, a mystery that continues to pose a challenge to the field is what the mechanism is behind the observed transcytosis of Klotho through the endothelium (11). While the molecular mechanisms responsible for Klotho-mediated vasculoprotection are largely yet to be elucidated, we consider the available experimental data highly auspicious for the development of Klotho-based therapies for the treatment of ageing- and CKD-related vascular disease.

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