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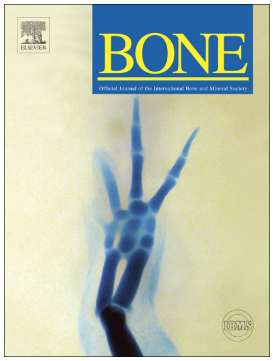

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Tissue expression and source of circulating αKlotho

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Abstract
αKlotho (Klotho), a type I transmembrane protein and a coreceptor for Fibroblast growth factor-23, was initially thought to be expressed only in a limited number of tissues, most importantly the kidney, parathyroid gland and choroid plexus. Emerging data may suggest a more ubiquitous Klotho expression pattern which has prompted reevaluation of the restricted Klotho paradigm. Herein we systematically review the evidence for Klotho expression in various tissues and cell types in humans and other mammals, and discuss potential reasons behind existing conflicting data. Based on current literature and tissue expression atlases, we propose a classification of tissues into high, intermediate and low/absent Klotho expression. The functional relevance of Klotho in organs with low expression levels remain uncertain and there is currently limited data on a role for membrane-bound Klotho outside the kidney. Finally, we review the evidence for the tissue source of soluble Klotho, and conclude that the kidney is likely to be the principal source of circulating Klotho in physiology.

Introduction

αKlotho (hereafter referred to as Klotho) is a 130 kDa type I membrane-bound protein containing two large extracellular domains (KL1 and KL2) with a short single-pass transmembrane and a short intracellular domain in its C terminus[1]. On the cell surface Klotho forms dimeric complexes with Fibroblast Growth Factor Receptors (FGFRs) that function as high-affinity receptors for Fibroblast Growth Factor 23 (FGF23)[2,3]. Klotho is also released from the cell membrane by proteolytic cleavage, a process mediated by the alpha-secretases ADAM10 and 17[4]. A first cut near the cell membrane (alpha-cut) produces a 130 kDa circulating protein, and a second cut (beta-cut) between the KL1 and KL2 domains produces two smaller proteins with molecular sizes of approximately 60-70 kDa[5]. In addition, a truncated Klotho variant containing only the KL1 domain may be produced through alternative splicing, although the physiological relevance of this splicing variant is unclear[6]. Both the full-length and the shorter forms of soluble Klotho are hormones, with distinct but overlapping effects. The detailed functions of the membrane-bound and soluble forms of Klotho are discussed in detail elsewhere in this issue.

The reigning paradigm is that Klotho expression is confined to a small number of tissues, most importantly the renal tubules, parathyroid glands and choroid plexus; an expression pattern determined by epigenetic regulation[1,7]. Although controversies exist, the kidney is commonly assumed to be the principal tissue source of circulating Klotho given that it is the largest organ that expresses Klotho at high levels. The aim of this paper is to systematically review available data on tissue expression of Klotho, primarily in rodents and humans, and address the discrepancies found in the literature. Additionally, we will discuss the tissue source of soluble Klotho.

Discovery of Klotho

In the original study identifying the mouse *Kl* gene (encoding for Klotho protein), high mRNA levels were reported in kidney, brain and pituitary gland, and lower levels in placenta, skeletal muscle, urinary bladder, aorta, pancreas, testis, ovary, colon, and thyroid gland[1]. No expression was detected in lung, liver, spleen and a number of other tissues. Shortly thereafter, the human *KL* gene was identified, demonstrating over 80% homology to its mouse ortholog[6]. Similar to in mice, gene expression was high in kidney and also in placenta, and lower expression was seen in brain, prostate, and small intestine. To this point the expression pattern of Klotho had been characterized solely by RNA-based methods. In 2000 the first monoclonal antibodies specifically detecting mouse and human Klotho were established, allowing investigation of protein expression[8]. Subsequent studies demonstrated that Klotho mRNA and protein expression largely overlapped, albeit protein expression in tissues with low gene expression could not be validated with immunostaining techniques. In 2004, a novel mouse strain with a reporter gene expressed under the *Kl* promoter was developed, permitting precise investigation of Klotho localization during embryonic development and in adult mice[9]. Analyses of this model led the authors to conclude that Klotho was exclusively expressed in the distal tubules of the kidney, parathyroid gland, sinoatrial node and choroid plexus. Over the next years, Klotho expression was reported in additional tissues that were not included in the initial screens, such as the inner ear[10], bone-forming cells[11], breast tissue[12], and monocytes[13]. More recent studies have provided conflicting data on Klotho in the arterial wall [14-16] and proximal tubule[17,18]. Also, a study from 2015 by Lim *et al.* using mass spectroscopy and immunohistochemistry reports widespread expression of Klotho throughout a number of examined human tissues[19].

Klotho in tissue atlases

To get an unbiased and systematic overview of Klotho expression in different tissues, we compiled data from publicly available RNA-Seq and mass spectrometry databases. RNA-Seq data of human tissues from four databases (The FANTOM5 project, The Human Protein Atlas, The GTEx Consortium and Illumina Body Map. Data available at http://www.ebi.ac.uk/gxa/home) demonstrated high Klotho expression in kidney (normalized to 100%), followed by placenta, lung, pancreas, breast and adipose tissue (10-25% of that for kidney)(Figure 1A). One database contained data for parathyroid gland, which compared to the kidney had approximately 2.5 fold higher expression of Klotho. Expression was low but detectable (<10%) in all other examined tissues except for liver. Importantly, none of the databases contained expression data for sinoatrial node or choroid plexus. To exclude unspecific background expression, we compared the expression with that of SLC34A1 (NPT2A), a known kidney-specific gene. Indeed, expression of SLC34A1 was almost completely confined to the kidney in all four databases (Supplemental Figure 1). For protein expression, mass spectrometry data from The Human Proteome Map showed strong expression in kidney, and no detectable expression in any of the other investigated tissues, including placenta, brain, and prostate (Figure 1B). For mouse tissues we examined three RNA-Seq databases (The FANTOM5 project, Blencove *et al.* and Kaessman *et al*. Data available at http://www.ebi.ac.uk/gxa/home) showing high expression in kidney, low expression in brain and cerebellum, and no expression in heart, liver, skeletal muscle, and testis (Figure 1C). Of note, none of the RNA-Seq databases differentiate between expression of the full-length transcript and the truncated transcript.

Based on the current literature and publicly available gene and protein atlases, we suggest a classification into three categories; tissues with 1) high Klotho expression: kidney, parathyroid gland, choroid plexus, and sinoatrial node; 2) intermediate-low Klotho expression: brain, eye, inner ear, endocrine system, lung, part of the gastrointestinal and genitourinary tracts, and placenta; and 3) low-absent Klotho expression; bone, cartilage, skin, adipose tissue, liver, spleen, heart, blood and immune cells, and part of the gastrointestinal and genitourinary tracts. Klotho expression in arteries is discussed in a separate section due to the current controversies in the field. The detailed evidence for Klotho expression in these various tissues is discussed in the following sections and summarized in Table 1.

Tissues with high Klotho expression

Kidney

In both humans and rodents, the kidney is consistently the organ with the highest mRNA and protein levels of Klotho. Initially, Klotho was reported to be exclusively expressed in the distal tubules[1]. This was paradoxical given Klotho's role as a permissive co-receptor for FGF23, the chief function of which is to regulate phosphate reabsorption and vitamin D metabolism in the proximal tubule. Nevertheless, studies examining the initial response to FGF23 injections corroborated this observation, with activation of the MAPK pathway only in distal tubules[20]. To explain the effects by FGF23 on phosphate and vitamin D metabolism in the proximal tubule, a putative paracrine interplay between the proximal and distal tubule was proposed. This theory was later substantiated by mice with a distal nephron-specific deletion of Klotho, which displayed hyperphosphatemia and elevated FGF23 levels[21]. By contrast, Hu *et al.* showed expression of Klotho transcripts in microdissected proximal tubules from mouse kidney, at approximately a third of the levels in the distal tubule[17]. Using

immunoelectron microscopy, they further report Klotho protein in the basolateral membrane and the apical brush border, as well as the cytoplasm of the proximal tubule. Similarly, Andrukhova *et al*. showed Klotho mRNA and protein expression in microdissected mouse proximal tubular segments, and also report that FGF23 directly targets the proximal tubule through activation of the ERK1/2-SGK1 pathway[18]. More recently, it was reported that Klotho is expressed also by podocytes and ameliorates proteinuria by targeting TRPC6 channels[22]. Finally, Klotho has been reported not to be expressed in mesangial cells[7].

To gain a more detailed understanding of Klotho expression in the different tubular segments and in podocytes, we gathered data from a recently published RNA-Seq dataset on segment-specific gene expression in rat[23]. Klotho expression in the S2 segment of the proximal tubule was around 30% of that in the distal tubule, supporting the concept of lower but distinct expression of Klotho in the proximal tubule (Figure 2A). Of note, its expression in microdissected glomeruli was low or undetectable (0-4% relative to distal tubule). Next, we employed a commercial *in situ* hybridization technique called RNAScope to determine the expression pattern in mouse and human kidney sections. In mouse, probes were targeted to nucleotides 879 – 1844, and in human 797 – 1768, thus targeting both the KL1 and KL2 domains. Mouse kidney displayed an abundant number of transcripts in distal tubules, and a lower but distinctly positive signal in cortical proximal tubules (Figure 2B). The straight S3 segments of the outer medulla were also positive but at a markedly lower level, whereas the collecting ducts were negative. Importantly, we could not validate expression of Klotho in intrarenal arteries (Figure 2B, insert). Samples from healthy parts of nephrectomized human kidneys stained in a similar way (Figure 2C). Positive staining was seen in some glomeruli, although most were negative. No distinct positive staining was found in human intrarenal arteries (Figure 2C, insert). Staining with the anti-Klotho antibody KM2076 generated a very

similar expression pattern as for *in situ* hybridization (Figure 2D). High resolution images of mouse and human kidney sections co-stained for Klotho and LTL can be found in Supplemental data (Supplemental Figure 2A and B).

To shed further light on Klotho's role in the proximal tubule, we recently generated three different strains of proximal tubule-specific Klotho knockout mice[24]. All three strains had reduced urinary phosphate excretion and increased abundance of Npt2a protein in the brush border of the proximal tubule, but remained normophosphatemic under unchallenged conditions. Effects on vitamin D appeared strain-specific, but were overall modest. These data indicate a distinct but functionally limited role for Klotho in the proximal tubule. FGF23-Klotho signalling in the kidney is discussed more extensively elsewhere in this issue.

Parathyroid gland

Klotho expression in parathyroid gland was reported already in 2004[9], but it took until 2007 before it was examined in detail[25,26]. Using primary isolated bovine parathyroid cells, we reported that parathyroid cells are a target for FGF23 signalling, and that FGF23 directly suppresses parathyroid hormone (PTH) synthesis and secretion[26]. These results were supported *in vivo* in a study by Ben-Dov *et al.* the same year[27]. A number of subsequent studies in humans and rodents have shown that parathyroid Klotho is suppressed in both primary and secondary hyperparathyroidism, a mechanism at least partly mediated by hypermethylation of the promoter region[28-41]. The reduction in Klotho expression was initially believed to confer a parathyroid tissue resistance to FGF23 signalling, explaining the concurrently high PTH and FGF23 levels observed in both primary and secondary hyperparathyroidism. However, we showed in mice with a parathyroid-specific Klotho

deletion that FGF23 is still able to suppress PTH through a Klotho-independent mechanism[42]. The exact function(s) of parathyroid Klotho thus remains unclear, and further studies are warranted.

Choroid plexus

Klotho is expressed widely throughout the brain in mouse and rat, with the highest levels in the choroid plexus[21,25,43-50]. Expression of Klotho in human choroid plexus has not yet been examined. The exact functions of Klotho in choroid plexus are largely unknown but presumably entail calcium transport across the blood-brain barrier, and a role as a tissue source for soluble Klotho in cerebrospinal fluid (CSF).

Sinoatrial node

Despite high expression of Klotho in the sinoatrial node in rodents, only a few studies so far have examined its expression pattern and function in this tissue[9,51]. In a study by Takeshita *et al.*, the authors demonstrate an essential role of Klotho for sinoatrial node function during conditions of cardiac stress[9]. Expression of Klotho in human sinoatrial node has not yet been examined.

Tissues with intermediate Klotho expression

Head and nervous system

Originally, significant Klotho mRNA levels were found in murine and rat brain [1,52-54] but not in human brain[6,55]. Klotho mRNA is detected rather consistently in mouse or rat brain[56-62] and in most[63,64], but not all studies[40], at the protein level. Subsequent studies uncovered several distinct areas of the brain that express Klotho. Cerebellar Purkinje cells were quickly identified as a cell type that expresses Klotho[19,47], whereas other sites with reported expression are the hypothalamus, thalamus, striatum, substantia nigra, amygdala, cerebellum, cerebral cortex, dentate gyrus, medulla oblongata, optic nerve, corpus callosum, and spinal cord[19,61,65-76]. Fon Tacer *et al.*, however, did not detect any Klotho expression in the cerebellum, olfactory bulb, brain stem, or spinal cord[65]. Hippocampal Klotho expression was also reported by several groups[66,70,71,77-83]. Many investigators have used Klotho *null* mice tissue as control and found that brain Klotho protein and spinal cord Klotho mRNA were detectable in wild-type (WT) mice, but not in Klotho *null* mice, attesting to the specificity of their findings[74,76]. Additionally, Klotho expression is suppressed in multiple CNS-derived malignancies including glioblastoma, oligodendroglioma, and astrocytoma, indicating that Klotho may have tumor suppressor functions in primary brain tumors[84]. Finally, brain pericytes have also been reported to express Klotho mRNA[85].

The function of Klotho in brain has gained significant attention. Massó *et al*. found that Klotho expression declined during ageing in mice, most prominently in prefrontal cortex[86]. Duce *et al*. found similar down-regulation of Klotho protein in white matter in rhesus monkey

brains during ageing[87,88]. Klotho deficiency in CNS has been implicated in cognitive dysfunctions and dementia[72,77].

Only a few studies have examined Klotho expression in the eye. Klotho was initially not detected in mouse eye by qRT-PCR[65], however, Reish *et al*. reported retinal Klotho expression in the ganglion cell layer, inner nuclear layer, and at a lower level in the outer nuclear layer, using a polyclonal antibody (AF1819)[89]. This staining pattern was not present in Klotho *null* retina. In a different study a nuclear staining pattern was demonstrated for Klotho in the retina[90]. Another study described detectable Klotho transcript and protein levels in human retinal pigmented epithelium (RPE) that could be silenced by anti-Klotho siRNA[91]. Finally, Jin *et al*. reported Klotho mRNA and protein expression in lens epithelium, which was down-regulated during ageing and cataract formation[92].

The inner ear was also found to be a site of moderately high Klotho expression by RT-PCR and Western blotting[10]. Most notably, the stria vascularis (producing endolymph) was found to be positive for Klotho protein[10,93]. Speculatively, Klotho may influence endolymph composition by modifying activities of various ion channels. The organ of Corti, outer and inner hair cells, and to a lesser extent, spiral ganglion cells, all expressed Klotho protein as well[93]. Finally, an auditory cell line was also found to express Klotho mRNA and protein[94].

Endocrine system

The anterior pituitary was early pinpointed as a Klotho-expressing tissue[1], which was corroborated by later studies[52,57,65,95]. Notably, Growth Hormone (GH)-producing adenomas express lower levels of Klotho mRNA than normal pituitary tissue, whereas non-functional adenomas have higher Klotho expression[96]. Neidert *et al*. showed that Klotho protein was expressed diffusely in adenoma and in lobular fashion in normal pituitary, only partially overlapping with GH-positive cells[97]. Speculatively, Klotho may play a role in regulation of GH production and/or secretion and this field merits further investigation.

Relatively high amounts of Klotho in the thyroid gland were reported in some studies [1,65] although accidental contamination with parathyroid gland tissue has to be considered as a potential source or error. Other studies employing RNA-Seq detect no or very little thyroid Klotho (a Fragments Per Kilobase Of Exon Per Million Fragments Mapped (FPKM) value of 1.93, which amounts to roughly 2 transcripts per cell)[52,55]. Follicular thyroid carcinoma cells express Klotho *in vitro*, and Klotho was suggested to inhibit cell proliferation and survival[98]. This observation is consistent with a tumour suppressor function of Klotho. In the study by Lim *et al*., Klotho protein is reported in the thyroid gland[19]. By contrast, we detected high expression of Klotho in the parathyroid gland but no expression in adjacent thyroid gland using IHC and *in situ* hybridization on normal mouse and human tissue [42] (Figure 3A).

Contrasting most other endocrine tissues, the adrenal glands express low or undetectable Klotho levels[1,52,65], albeit some studies indicate Klotho expression in human and murine adrenal gland, and in phaeochromocytoma cell lines[55,58,99]. Lim *et al*. localize Klotho protein expression in adrenal gland to catecholamine-producing medullary cells[19]. Finally,

Klotho expressed in the zona glomerulosa cells in the adrenal cortex was recently suggested to inhibit aldosterone synthesis by down-regulating *Cyp11b2* expression[100].

Klotho expression in mammary glands was investigated in 2008 by Wolf *et al*. who found that ductal epithelium and other breast cell lines expressed Klotho mRNA and protein (using the monoclonal antibody KM2076) and that its expression decreased during carcinogenesis[12]. Later reports are in line with these initial findings[7,19,101-106].

Klotho expression is evident in human and murine pancreas [1,6,52,55,57,62,107], although sometimes below the detection limit[65]. The islets of the endocrine pancreas appear to be the main site of Klotho expression, although one study reported higher expression in the exocrine pancreatic ducts[108]. Abramovitz *et al*. noted that pancreatic islets exhibit low but detectable Klotho expression[108], which has been speculated to protect against the development of type 1 and type 2 diabetes[109-111]. As for other endocrine tissues its expression has been reported to be lower in carcinomas compared to in healthy tissue[108,112].

Respiratory system

Klotho expression was originally not detected by RNA-based methods in mouse, rat or human lung[1,6,52-54,65]. In subsequent analyses, lung tissues of mice and pigs were found to express low levels of Klotho[56], around 300-fold lower than in kidney[57,113]. Consistent with these findings, Klotho protein was also not detected in lung tissue lysates by several groups, using the monoclonal antibody KM2076[40,114]. In contrast, one study identified alveolar macrophages as a cell type that express detectable levels of Klotho protein[115]. The

same group also described that airway epithelium produces Klotho[116], a notion that previously had been reported by immunohistochemistry and Western blotting[117]. Subsequent analyses of pulmonary cells and cell lines indicate that Klotho may indeed be expressed at a low level[103,118-122] and immunohistochemical analysis of lung tumor samples (small cell lung cancer and large neuroendocrine carcinoma) have provided evidence that loss of Klotho expression is associated with lower survival rates[123,124].

Gastrointestinal tract, (oesophagus, stomach, small and large intestine)

Esophageal Klotho protein expression has been investigated in one study, in which Klotho expression was detected in the esophageal epithelium, and suppressed in carcinoma[125]. Additionally, RNA sequencing has provided a rather low FPKM value of 1.45 in esophagus[55].

Klotho expression is not detected in gastric tissue in most studies[1,52,54,65], or at a very low levels[57,126], and an RNA-Seq analysis yielded an FPKM value of 1.39[55]. However, Izbeki *et al*. found that WT mice express significantly more gastric Klotho mRNA than Klotho *null* mice, indicating that it may be of relevance[126]. Using immunofluorescence they revealed Klotho expression in the epithelium, smooth muscle cells, and enteric neurons, although it is unclear what antibody was used. Focusing on gastric epithelium, Xie *et al*. also report some Klotho mRNA and protein expression, and further show that expression was decreased in gastric carcinoma cell lines compared to normal gastric epithelial cells[127]. He *et al*. obtained similar results, which is in line with the common finding that Klotho is down-regulated in cancers[128]. In conclusion, gastric Klotho expression appears to be low but might be of relevance for gastric malignancies.

The small intestine is not particularly well studied concerning Klotho expression. Early studies either showed no[1,52,54] or little Klotho mRNA expression in rats, mice and humans [6,53,129]. Later studies in mice largely echo these results, showing no Klotho mRNA in whole lysate of small intestine [56], nor in duodenum, jejunum, or ileum [65], or levels several hundred-fold below that of the kidney[57]. One report also includes negative immunohistochemistry results for Klotho in murine duodenal epithelium [130]. An RNA-Seq analysis yielded an FPKM value of 3.01 for small intestine (and 2.74 specifically for duodenum and 1.05 for appendix) [55]. Low expression of Klotho in the small intestine is supported by data showing that Klotho mRNA levels in WT jejunum and ileum were higher than in Klotho *null* mice [131]. The authors pinpointed Klotho protein expression to mucosal epithelial cells, smooth muscle cells, and in deep muscular plexus interstitial cell of Cajal (ICC), but not myenteric plexus ICCs or myofibroblasts[131,132]. Finally, using different antibodies, an epithelial staining pattern for Klotho was observed in human jejunum and ganglionic cell bodies[19]. With multiple studies confirming low expression, it is likely that Klotho is indeed expressed by a number of cell types in the small intestine.

The large intestine appears to express Klotho at levels similar to the small intestine [52,54,57,65], with slightly higher expression in some [1,53,56], and slightly lower expression in other studies[6,55,131]. Importantly, Asuzu *et al*., report similar Klotho transcript levels in WT and Klotho *null* mice, indicating that this might be unspecific background expression [131]. However, they do detect a similar expression pattern for Klotho protein as in the small intestine. Other authors also report Klotho mRNA and protein in colon, mostly in epithelial cells[19,133-135].

Genitourinary tract, (testis, ovaries, cervix, Fallopian tube, prostate, urinary bladder, placenta)

Testicular Klotho mRNA expression was identified in mice in by Kuro-o *et al*.[1], although later studies indicated low[6,52,54,56] or even absent gene expression[53,55,58,65]. Immunohistochemistal analyses indicate that Klotho protein is expressed in Sertoli cells and in elongating spermatids[46,136], as well as in Leydig cells[19].

In the female reproductive system, Klotho mRNA has consistently been found at moderate levels in ovaries from mice, rats, and humans[1,6,52-54,56,57,65]. Similar to the testis, Klotho protein is expressed in mature germ cells, i.e. mature oocytes, on the membrane and in the cytosol[46]. Reduced Klotho expression has been found in many ovarian carcinomas and ovarian carcinoma cell lines, again suggesting a potential tumour suppressor role of Klotho[102,137].

In addition to the ovaries, the cervix may also express some Klotho. PCR data indicate that Klotho is expressed in cervix and in cervical cell lines[138,139], although some studies were negative[7,140]. At the protein level, however, both endocervix and ectocervix appear to express Klotho protein[138,140].

The Fallopian tube has been studied very little and only at the protein level. Lojkin *et al*. report Klotho expression in normal Fallopian tube and reduced expression in carcinoma originating from the Fallopian tube[102]. Lim *et al*. show Klotho expression along the epithelial lining of healthy human Fallopian tube[19].

The prostate was originally thought to be a tissue with moderate Klotho expression as Matsumura *et al*. detected Klotho mRNA in human prostate by Northern blot[6]. This was later confirmed using RT-PCR in both normal and carcinomatous prostate cell lines[141,142]. Also, RNA-Seq analysis showed relatively high expression of Klotho (10.9 FPKM) in human prostate[55]. Lim *et al*. recently reported that Klotho protein is expressed in human prostate epithelium[19]. In contrast, studies of murine prostate could not confirm Klotho expression[56,65]. All in all, the prostate is likely to express above average levels of Klotho, at least in humans.

The urinary bladder was initially shown to express Klotho mRNA in mice[1]. This finding has been backed by additional data in both mice and humans[55,57]. To the best of our knowledge, there are no data available on Klotho protein expression in the urinary bladder.

After the kidney, parathyroid and choroid plexus, the placenta is one of the organs reported to express the highest levels of Klotho. Klotho mRNA in placenta was originally detected by Kuro-o *et al*.[1] and confirmed by additional studies[6,52,55,139,143,144]. Placental expression of Klotho is also detected by *in situ* hybridization (Figure 3B). At both mRNA and protein level, Klotho is expressed predominantly in syncytiotrophoblasts[95,145-148]

Skeletal muscle

Skeletal muscle was initially shown to express moderate amounts of Klotho mRNA in mice[1], although subsequent data from human, murine, and rat skeletal muscle revealed that the expression was very low or absent[6,53,54,56,65]. Of note, data from Murata *et al*. revealed that Klotho mRNA expression was higher in WT mice than in Klotho *null* mice, indicating a specific gene product[149]. More recent analyses show that Klotho mRNA indeed is detectable but expressed several hundred-fold below the level of renal Klotho[57,58,61,62,113,150]. Recent attempt to detect Klotho protein in skeletal muscle yielded negative results[57,151,152]. Notably, a publication from this year reported low levels of Klotho mRNA and protein in skeletal muscle, both of which were decreased in a mouse model of Duchenne muscular dystrophy[62]. Skeletal muscle may therefore express Klotho at a low but detectable level; speculatively, it may be important in maintaining skeletal muscle viability and regenerative capacity.

Tissues with low or no Klotho expression

Connective tissue and skin

Bone was originally identified as a tissue without Klotho expression[1,65,153]. Subsequent studies indicate that murine and porcine cortical bone do expresses Klotho mRNA at levels of 400 to 1000-fold lower than the renal cortex[113,154]. Similarly, analyses of Klotho expression in isolated bone cells have shown that osteoblasts express Klotho at a level of around 800-fold below kidney cortex level[154]. In some studies, Klotho expression was below detection level[153,155,156], however, Yuan *et al*. found that Klotho mRNA levels in whole bone and isolated osteoblasts were still around 5- to 10-fold higher in WT than in Klotho *null* mice[154]. Klotho protein analysis equally supports a specific albeit low signal in bone[157]. Importantly, deletion of Klotho in long bones points to a functional role of Klotho in the regulation of FGF23 production and secretion under uremic conditions[11].

Cartilage is a rather unexplored field in Klotho research. Klotho mRNA was shown to be expressed in the growth plate and articular cartilage at a level around 300- to 1,000-fold lower than in renal cortex in pigs[113]. Kawai *et al.* also report very low levels of Klotho mRNA in both primary chondrocytes and in a chondrogenic cell line during chondrogenesis[95]. At the protein level, however, data are discrepant. Raimann *et al.* yielded positive immunohistochemistry results with an unspecified antibody[113], whereas Kawai *et al.* did not detect Klotho with an unspecified antibody and also showed that chondrocytes are not responsive to FGF23, suggesting that Klotho is not present as a functional co-receptor[95]. Finally, nucleus pulposus cells were reported to express Klotho mRNA and protein (using the commercially no longer available ab75023 antibody)[158].

Adipose tissue is believed to express Klotho mRNA at low levels. One RNA-Seq study showed expression at 3.1 FPKM[55], which is in agreement with the low levels detected by qRT-PCR in epididymal white adipose tissue[61]. In contrast, qRT-PCR of white and brown adipose tissue[65] and RT-PCR of various (inguinal, visceral, subscapular) white adipose tissues[58] have yielded negative results in other studies. However, adipose tissue expression was substantially higher in RNA-Seq data from The GTEx Consortium and Illumina Body Map (Figure 1A). In adipocyte cell lines, Klotho was found to be expressed at very low levels, which increased during differentiation[159,160]. Klotho protein was detected using the KM2076 antibody and decreased after anti-Klotho siRNA transfection, suggesting that the immunoreactivity on Western blot was specific.

Fibroblasts in connective tissue have only occasionally been investigated. While renal embryonic fibroblasts were found not to express Klotho by RT-PCR[7] and Klotho mRNA expression was very low in synoviocytes[161], a number of studies indicate that skin fibroblasts[162], tenocytes[163], mouse embryonic fibroblasts and fibroblast cell lines[164,165] express Klotho protein. In these studies, the molecular size of the detected protein are highly variable. Liu *et al*. detect a 64 kDa protein in a fibroblast cell line, De Oliveira *et al*. do not indicate a protein size[164], and Xie *et al*. detect a 116 kDa protein[162,165]. It is yet to be properly determined whether fibroblasts produce physiologically relevant amounts of Klotho.

Skin is another tissue that has not received much scrutiny regarding Klotho expression. Originally, skin from mice was found to be negative for Klotho by RT-PCR[1,52], a finding corroborated by subsequent studies assessing mRNA and protein levels[56,57,65]. The large

RNA-Seq study by Fagerberg *et al.* yielded a FPKM value of 0.59 for skin, which is below one transcript per cell[55]. However, *in vitro* studies paint a different picture. Kim *et al*. report Klotho mRNA and protein expression in human keratinocytes[166] and Liu *et al*. detect Klotho in human primary keratinocytes and in a keratinocyte cell line[167]. One study that focuses on melanoma reports that Klotho expression is inversely proportional to malignant behavior of melanoma cell lines[168]. Finally, Lim *et al*. report high expression of Klotho protein in the epidermis, hair follicles, sebaceous glands, and cultured keratinocytes, using antibodies ab69208 and/or ab181373[19].

Cardiovascular system

Initial studies reported absence of Klotho in hearts from mice[1,52,54], which was also the case in rat[53] and only a very faint Northern blot band was detected in human heart[6]. Only one study indicates a faint RT-PCR band representing a low amount of Klotho transcripts in mouse heart[58]. Subsequent studies using either qRT-PCR or RT-PCR approaches confirm low or absent levels of Klotho in mouse heart[56,57,60,65,169], and cardiac Klotho expression was found to be over 10,000-fold lower than renal Klotho expression levels in pig hearts[113]. In an RNA-Seq study, the human heart yielded a very low FPKM value of 1.11[55]. On the protein level, using antibody KM2076, Lau *et al*. could not detect Klotho expression in mouse heart[40]. A recent article reports that there is no detectable Klotho mRNA in heart biopsies from deceased pediatric patients[170]. However, Klotho protein was detected by Western blot, at the same molecular size as renal Klotho. This leads the authors to raise the question whether cardiomyocytes are able to scavenge and absorb soluble Klotho from circulation.

Blood and immune system

Bone marrow has consistently been found to express virtually no Klotho in humans and in mice[1,52]. Vadakke Madathil *et al*. reported that murine bone marrow Klotho levels were around 1,000-fold lower than in kidney[171]. Similarly, RNA-Seq analysis of human bone marrow yields a negligible FPKM value of 0.11[55], and normal human bone marrow samples were found not to express Klotho protein[105]. However, Raimann *et al*. estimate porcine bone marrow Klotho expression to be only around 10-fold lower than in kidney[113], which is highly discrepant from the other observations.

The thymus has never received much interest in the Klotho field, even though it involutes in Klotho *null* mice. Thymic Klotho expression has been found to be undetectable in human, mouse, and rat samples[1,6,52,53,56,57,65]. It was detected in 4- to 6-weeks-old pigs as around 30,000-fold lower than in kidney[113].

Splenic Klotho expression levels have also relatively consistently proven to be negative by Northern blotting, RT-PCR, and qRT-PCR in mouse[1,54,56,57,65,65]. In rat spleen, Klotho mRNA could be detected at an extremely low level[53], which was also the case in human spleen[6]. Quantitative analysis revealed Klotho to be expressed in spleen at a level of around 10,000-fold below averaged renal expression levels[113,171], further validating the notion that splenic Klotho expression is negligible.

Lymph node Klotho expression has only been investigated in one large RNA-Seq study, yielding a very low FPKM value of 1.1[55]. To our knowledge, lymph vessels and lymphatic endothelium have never been studied in this context.

Blood cells have received more scrutiny in recent years. Human peripheral blood mononuclear cells (PBMCs) were found not to express Klotho mRNA[6,7], nor were platelets[172] or dendritic cells[173], while a number of analyses indicates that Klotho mRNA is at least detectable in lymphocytes[174] and in lymphocyte subsets [175,176]. Bacchetta *et al.* show that in PBMCs from 35 different donors, the average Ct value was 21 cycles higher than the Ct value of 18S, which equals > 200,000 times lower expression[13]. They do, however, detect Klotho protein in PBMCs using immunofluorescence, which is unexpected based on their mRNA results. Chronic myeloid leukaemia cells were found not to express Klotho mRNA[7] and acute myeloid leukaemia cells were found to express Klotho mRNA and protein, but the quantity could not be inferred[177]. Furthermore, macrophages were also shown to express Klotho at an indiscernible expression level, which was higher in M1 macrophages as compared to M0 and M2 macrophages[178].

Gastrointestinal tract, (salivary glands, liver, gall bladder)

The salivary glands have not been a major focus in Klotho research and only a few studies have assessed Klotho expression. Using RT-PCR, no Klotho mRNA expression was found in murine submandibular gland[1,52]. Similarly, RNA-Seq of human salivary gland yielded a very low FPKM value of 0.61[55]. Amano *et al.* used immunohistochemistry and showed that there was no Klotho protein in acini, striated ducts, or intercalated ducts, in either the parotid,

sublingual, or submandibular gland in mouse. However, they found Klotho expression on the basal side of granular duct cells[136].

The liver, together with adipose tissue, expresses high levels of β-Klotho, an obligate co-receptor for Fgf15/FGF19[179]. In contrast, the liver is generally regarded as one of the organs that are completely devoid of Klotho. But even the topic of hepatic Klotho expression is not without conflicting data. A number of studies report negative data for Klotho mRNA in murine, rat and human liver[1,6,52-54,56-58,61,65,67]. More sensitive analyses indicate that Klotho mRNA is expressed in liver at a level 1,000- to 10,000-fold lower than in kidney[113,171], and RNA-Seq analyses yield very low or undetectable levels (Figure 1A and [55]). The liver has also repeatedly been found not to express Klotho protein[19,40]. However, there are some studies indicating that normal liver tissue and cell lines express Klotho mRNA and protein[180,181] and that the levels are decreased in hepatocellular carcinoma (HCC) samples or cell lines[182-185], and in steatotic liver tissue[186]. Whether low levels of Klotho predict poor outcome in HCC is also a matter of debate[182-185,187]. All in all, we conclude that the levels of Klotho in the liver is extremely low, and is unlikely to play a major role in physiology.

The gall bladder and biliary epithelium have so far only been examined in one study of mouse tissue, in which no expression was found in the bladder by RT-PCR[65], and in one human RNA-Seq study, in which an FPKM value of 1.86 was reported[55]. This is slightly higher than in some other tissues, but the expression needs to be validated by additional studies.

Genitourinary tract, (epididymis, uterus)

With regards to the epididymis, one qRT-PCR analysis indicates that Klotho is not expressed at all[65], whereas another study reports that Klotho is expressed at a level several hundred-fold lower than the kidney[57]. No Klotho expression could be detected in seminal vesicles, preputial gland, and vas deferens in mice[65].

For uterus, only negative data have been reported in mice and in rats[1,52,53,57,65]. RNA-Seq analysis yielded a FPKM value of 1.39 for human endometrium[55]. Interestingly, Lim *et al*. report distinct positive Klotho protein expression in human endometrium, which stands in sharp contrast to mRNA data[19].

Expression of Klotho in arteries

Arterial Klotho expression is a matter of debate and current literature is contradictive. Given the link between FGF23 and vascular complications in CKD, it is of principal interest to elucidate whether FGF23 modifies structural or functional properties of the vasculature via Klotho-dependent mechanisms. In aorta and in other arteries, Klotho mRNA is either not detected[40,52,65,188-190] or detected at an extremely low level[1,16,191-199]. This holds true also for vascular smooth muscle cells[15,189,196,200-204] and endothelial cells[202,205-211] when analyzed separately. However, at the protein level, data are conflicting. Many authors do not detect Klotho protein in aorta[15,16,40,188,212], smooth muscle cells[15,196,200,201], or endothelial cells[206,211,213] whereas others do[14,19,19,165,195,199,199,207-210,214]. It is also uncertain whether arterial Klotho is down-regulated in CKD or not[14,41,193,194,196,214,215]. There is also some evidence that Klotho expression is induced at sites of vascular calcification[195,197].

Another unresolved issue is that the detected protein in vasculature is usually around 115-120 kDa, which is smaller than the renal 130 kDa protein. Analysis of the renal and vascular samples led us to conclude that the full-length membrane-bound Klotho protein is not expressed in arterial tissue or smooth muscle cells[15]. The existence of an alternatively spliced Klotho variant in vascular tissue cannot be excluded. A functional role of vascular Klotho also remains to be proven. In this regard, mice with a specific deletion of Klotho in vascular smooth muscle cells did not display any apparent vascular phenotype[16]. Furthermore, acute and chronic FGF23 infusions do not elicit the down-stream Klotho dependent signaling response observed in kidney, suggesting that the Klotho receptor complex is not functionally present[15,16]. Consistent with these findings, FGF23 treatment did not alter the dynamic properties of isolated mouse arteries *ex vivo*[16].

Tissue source of circulating Klotho

Both the full-length and the shorter forms of soluble Klotho are detected in serum, urine, and CSF[43,216]. Since the kidney is the largest organ with high abundance of Klotho, it is also likely to be the principal contributor to circulating Klotho. However, little is known about the mechanism(s) that regulate cleavage of Klotho, and the tissue source remained undetermined until recently. To address this question, we generated whole-nephron Klotho knockout mice[21]. This model recapitulated the severe phenotype of systemic Klotho *null* mice, emphasizing the importance of renal Klotho. Importantly, kidneys harvested from whole-nephron Klotho knockout mice do not secrete soluble Klotho *ex vivo*, and soluble Klotho is markedly reduced in serum in these mice. Thus, we established the kidney as the chief source of serum Klotho in mouse.

Notably, mice with a proximal-tubule specific deletion of Klotho did not have reduced serum Klotho level[24]. There might be several reasons for this. First, Klotho expression in the proximal tubule is markedly lower than in the distal tubule, and deletion only in the proximal tubule might not be sufficient to lower the serum levels. Second, a deletion of Klotho in the proximal tubule might trigger a compensatory upregulation in other Klotho-expressing tissues that maintain a constant shedding rate of Klotho. Technical limitations in Klotho measurements (based on immunoprecipitation and immunoblotting) might also contribute. Another potential explanation is that Klotho in the proximal tubule is shed from the apical side into urine, and does not contribute to blood levels.

In a recent paper by Hu *et al*., the renal production and handling of Klotho was examined in greater detail[217]. The authors first measured soluble Klotho in blood collected from the infra-renal and supra-renal vena cava, in rats and humans, and demonstrate an infra-to-supra-renal Klotho ratio greater than one, indicating that Klotho is released from the kidney into circulation. In bilaterally nephrectomized rats, the levels of Klotho in serum had dropped to

half within one day, providing further evidence that the kidneys are the main tissue source of soluble Klotho. Finally, the renal handling of soluble Klotho was evaluated by injecting rats with fluorescently-labelled recombinant Klotho. Clearance of recombinant protein was markedly slower in anephric rats, indicating that the kidney actively contributes to the clearance of Klotho.

Except from the aforementioned reports, data on the tissue source of serum Klotho in humans are largely lacking. Patients with CKD have markedly decreased tissue level of Klotho in the kidney paralleled by reduced serum concentrations[218,219]. However, CKD is associated with a global reduction in tissue Klotho expression and therefore the relative organ-specific contributions cannot be inferred from this setting[220]. Similarly, a case report of a patient with a inactivating mutation in the *KL* gene and undetectable serum levels of Klotho does not permit further dissection of this question[221].

Both full-length and the truncated forms of soluble Klotho can also be found in CSF[43]. Due to the intrinsic properties of the blood-brain-barrier, only small amounts of Klotho can enter the CNS from circulation, and a vast majority is instead produced locally in the CNS. Although never assessed in detail it is reasonable to assume that soluble Klotho in CSF is mainly produced and shed from the choroid plexus. A recent study of Klotho in CSF from pediatric patients undergoing lumbar puncture to exclude inflammatory neurological disease showed that the levels of full-length soluble Klotho in CSF were circa 30% lower than in serum[222].

Discussion

Tissue profiling of Klotho expression is at first glance a simple task, yet several conflicting data and controversies have emerged as a consequence of its promiscuous low-grade expression pattern and different splice variants. In addition, the widespread use of newer polyclonal antibodies, with unproven sensitivity and specificity, is likely to precipitate false positive as well as false negative results. In this regard, a number of studies employing polyclonal antibodies report positive staining for several tissues expressing very low Klotho mRNA levels, contrasting the first monoclonal Klotho antibodies (KM2019 and KM2076), which generate consistent results when comparing protein and mRNA data. A detailed list of the most commonly used anti-Klotho antibodies can be found in Table 1. Furthermore, a recent mass spectrometry-based study demonstrated Klotho protein expression in a wide panel of human tissues, some which were not previously categorized as 'Klotho positive'[19]. However, this study provides no quantitative data, and detected an amino acid sequence that is present also in soluble Klotho, thus preventing conclusions on amounts, form (membrane-bound or soluble) or tissue source of the detected protein. To enhance data quality and homogeneity, it is recommended that studies reporting on Klotho expression should more precisely define the specificity of the antibodies used (i.e. a size indicator and positive and negative controls should be included in all analyses, preferentially using recombinant protein and tissue from Klotho *null* mice). Second, protein and mRNA expression should be adequately quantified and contextualized in all studies presenting positive data, especially for 'new' tissues. Expression should also be differentiated between the full-length and the truncated transcripts. Third, the source of the detected protein (i.e. locally expressed or absorbed from circulation) should be examined whenever feasible.

Another unresolved issue is that the molecular size and subcellular localization of Klotho differ from the expected (i.e. 60-116 kDa instead of 130 kDa, and nuclear instead of membrane-bound/cytoplasmic) in some studies. Whether these findings are equally attributable to technical limitations remains to be determined. An alternative, and more exciting, explanation is the existence of different Klotho protein forms derived from local RNA splice variants or structurally modified full-length Klotho. There is however currently limited evidence to support such a hypothesis.

We herein propose a classification for Klotho expression in tissues based on current evidence for the basal mRNA and protein expression levels: high expression (distal tubules, parathyroid gland, sinoatrial node and choroid plexus), intermediate expression (e.g. proximal tubules, brain, eye, inner ear, endocrine system, lung, parts of the genitourinary and gastrointestinal tracts, and placenta), and low/absent expression (e.g. bone, cartilage, skin, adipose tissue, liver, spleen, heart, arteries, blood and immune cells, and parts of the gastrointestinal and genitourinary tracts). The expression level of Klotho in different tissues/cell types and the level of evidence are summarized in Table 2. A key discriminator of what constitutes a relevant Klotho expression signal is in our view determined by the degree of signalling/functionality that can be accredited to Klotho in specific cell types. In this regard, there are rather limited data on tissues with reported low-grade expression, whereas the evidence for organs with high expression such as kidney and parathyroid glands are robust and consistent. Nevertheless, as described herein, organs with very low Klotho expression might promote Klotho signalling under certain conditions. Further elucidation of relevant tissues and associated conditions is therefore important to adequately portray the details of the expanding story of Klotho.

It should also be recognized that the tissue-specific Klotho level (and the significance thereof) is likely to vary substantially depending on stimulatory or repressive signals in the systemic or cellular environment. For example, Klotho expression in kidney is reduced in acute and chronic kidney injury[223,224]; CNS-derived Klotho is diminished in states of cognitive decline[72,225]; and suppressed Klotho is frequently observed in tumours of various origin[12,108]. All such confounders must be carefully considered when interpreting measured Klotho levels. In fact, in addition to strain and species differences, they may account for some of the underlying discrepancies found in the literature on this topic.

Recent genetic and experimental data from us and other groups uncovered the kidney as the principal source of soluble Klotho[21]. The simple observation that a renal Klotho deletion recapitulates the systemic Klotho *null* mouse phenotype, whereas other targeted tissue Klotho deletions reported so far do not elicit any discernable abnormalities[16,42,226], substantiates a pivotal role of the kidney in Klotho biology.

Table 1. Characteristics of commonly used anti-Klotho antibodies.

Antibody	Company	Described in	Clonality	Host species	Immunogen	Validation assessment*	Validation references	Remarks
KM2076 (KO063)	Kyowa Hakko Kirin / TransGenic Inc	[8]	Monoclonal (IgG2a)	Rat	Human KL1 (aa 55-261)	Excellent	[5,15,17,21,24,42,43,95,114, 160,196,216,217,223,226-238]	Best characterized antibody, most reliable in most applications
KM2119		[8]	Monoclonal (IgG2b)	Rat	Human KL2 (aa 801-954)	Excellent	[9,43,238,239]	Known to be specific, but not as well-studied as KM2076
KM2365		[8]	Monoclonal (IgG1)	Mouse	Human KL1 peptide	Poor		
Mink1		[43]	Monoclonal (IgG1)	Mouse	Recombinant mouse KL1	Good	[43,95]	
KL-115		[48]	Monoclonal	Rat	Human KL1 (aa 55-261)	Very good	[48]	
KL-234		[48]	Monoclonal	Rat	Human KL1 (aa 51-261)	Very good	[48]	
Sb106		[219]	Monoclonal	Synthetic		Very good	[219]	
AF1819	R&D Systems		Polyclonal	Goat	Mouse recombinant Klotho	Excellent	[50,67,89,240,241]	Best suited for mouse studies.
MAB1819 (236214)	R&D Systems		Monoclonal (IgG2a)	Rat	Mouse Klotho (aa 23-550 and 35-982)	Poor	[131]	
SAB3500604	Sigma-Aldrich		Polyclonal	Rabbit	Internal 16 aa peptide, human Klotho	Poor		
SC-22220 (E-21)	Santa Cruz Biotechnology		Polyclonal	Goat	Internal region, human Klotho	Very good	[15]	No longer available
SC-22218 (T-19)	Santa Cruz Biotechnology		Polyclonal	Goat	Internal region, human Klotho	Moderate	[165]	No longer available
SC-74205 (27Y-1)	Santa Cruz Biotechnology		Monoclonal (IgG2a)	Rat	Mouse recombinant Klotho	Moderate	[242]	
423500	Merck Millipore		Polyclonal	Rabbit	17 aa peptide near the C terminus, mouse Klotho	Poor		
Ab75023	Abcam		Polyclonal	Rabbit	Peptide between aa 150-250, human KL1	Poor		No longer available
Ab69208	Abcam		Polyclonal	Rabbit	Peptide between aa 800-900, human KL2	Poor		No longer available
Ab181373 (EPR6856)	Abcam		Monoclonal (IgG)	Rabbit	Peptide between aa 400-500	Poor		
Ab154163	Abcam		Polyclonal	Rabbit	Peptide between aa 100-200, mouse Klotho	Poor		
KL11-A	Alpha Diagnostic		Polyclonal	Rabbit	17 aa peptide, mouse recombinant Klotho	Good	[2,243]	

*The current antibody validation status is summarized as:

- Poor: the antibody has not been validated or studies report highly discrepant findings
- Moderate: the antibody plausibly detects Klotho (it produces the staining pattern that specific anti-Klotho antibodies and RNA *in situ* hybridization produce (i.e. staining in renal distal tubules >> proximal tubules, if tested choroid plexus or parathyroid gland positivity) and produces a band on Western blot of 130 kDa);

- Good: the antibody was also shown to detect purified recombinant or tagged Klotho protein (the staining pattern disappears after pre-incubation of antibody and recombinant protein, or recombinant protein is detected on Western blot, or tagged Klotho is detected on Western blot after (preferably reciprocate) immunoprecipitation; immunoprecipitated proteins are shown to include Klotho using mass spectrometry);
- Very good: the antibody was also shown be specific for native Klotho protein without aspecific detection of other proteins (the staining pattern is not present in knockout tissue, and positive immunostaining or a specific band on Western blot emerges only after transfection with Klotho plasmid in Klotho-negative cells/tissue or the staining pattern disappears after RNAi for Klotho in Klotho-expressing cells);
- Excellent: the aforementioned experiments have been replicated independently in multiple studies.

Table 2. Expression of Klotho in different tissues and cell types

Organ system	Organ/tissue	Specific cell type/structure	Klotho expression	Robustness of evidence*
Head and central nervous system	CNS	Neurons	Intermediate	Excellent
		Purkinje cells	Intermediate – higher than most neurons	Moderate
		Choroid plexus	High	Excellent
		Pericytes	Low	Poor
	Eye	Ganglion layer	Intermediate	Moderate
		Outer nucleated layer	Intermediate – lower than other layers	Moderate
		Inner nuclear layer	Intermediate	Moderate
		Retinal pigmented epithelium	Intermediate	Moderate
		Lens epithelium	Intermediate	Moderate
	Ear	Stria vascularis	Intermediate - high	Moderate
		Hair cells	Intermediate	Moderate
		Organ of Corti	Intermediate	Moderate
		Ganglion cells	Intermediate	Moderate
Endocrine system	Anterior pituitary		Intermediate	Good
	Thyroid gland	Follicular epithelium	Intermediate	Poor
	Adrenal gland	Medullary cells	Low - intermediate	Moderate
	Mammary gland	Ductal epithelium	Intermediate	Moderate
	Endocrine pancreas		Intermediate	Good
	Parathyroid gland		High	Excellent
Cardiovascular system	Heart	SA node	High	Good
		Cardiomyocytes	Absent or very low	Good
	Arteries	Smooth muscle cells	Absent or very low	Good
		Endothelial cells	Absent or very low	Moderate
Respiratory system	Lung	Airway epithelium	Intermediate	Poor
		Alveolar macrophages	Intermediate	Moderate
Gastrointestinal tract	Salivary gland		Absent	Poor
	Esophagus		Low - intermediate	Poor
	Stomach		Low - intermediate	Moderate
	Small intestine	Epithelial cells	Low - intermediate	Moderate
		Deep muscular plexus interstitial cells of Cajal	Low - intermediate	Moderate
		Smooth muscle	Low - intermediate	Poor
		Ganglion cells	Low - intermediate	Poor
	Colon		Low - intermediate	Poor
	Liver		Absent	Good
	Gall bladder		Absent	Poor
	Exocrine pancreas		Intermediate	Good
Genitourinary tract	Kidney	Podocytes	Low	Moderate
		Proximal tubule cells	Intermediate	Excellent
		Distal convoluted tubule cells	High	Excellent
	Urinary bladder		Low	Poor
	Prostate		Intermediate	Moderate
	Seminal vesicles		Low	Poor
	Epididymis		Absent or very low	Poor
	Testis	Elongating spermatids	Intermediate - high	Moderate
		Sertoli cells	Intermediate	Moderate
		Leydig cells	Intermediate	Poor
	Ovary	Oocyte	Intermediate - high	Moderate
	Cervix		Intermediate	Moderate
	Fallopian tube		Intermediate	Poor
	Uterus		Low	Poor
	Placenta	Trophoblasts	Intermediate	Moderate
Musculoskeletal system and skin	Skeletal muscle		Very low	Moderate
	Bone		Very low	Good

	Cartilage	Chondrocytes	Very low	Moderate
		Nucleus pulposus cells	Low	Poor
	Adipose tissue		Low	Poor
	Fibrous tissue	Fibroblasts	Low	Poor
	Skin	Keratinocytes	Low	Moderate
		Melanocytes	Low	Poor
Blood and immune system	Bone marrow		Absent or very low	Moderate
	Thymus		Absent or very low	Poor
	Spleen		Absent or very low	Poor
	Lymph nodes		Absent or very low	Poor
	Blood	Lymphocytes and monocytes	Low	Moderate
		Dentritic cells	Absent or very low	Poor
		Platelets	Absent or very low	Poor
		Macrophages	Low	Poor

*The robustness of evidence is summarized as

- Poor: only mRNA or only protein data, not yet independently replicated, lacking controls
- Moderate: solid mRNA and/or protein data, not yet independently replicated or difficult to reconcile with other studies, or lacking controls
- Good: multiple studies, assessed at both the protein and mRNA levels, use of validated antibodies, but some data are conflicting or controls are lacking
- Excellent: numerous studies, assessed at both the protein and mRNA level using independent methods and validated antibodies, important controls have been performed, supported by genetic animal models

Figure 1. Expression of Klotho in tissue atlases.

A) Expression of Klotho in various human tissues from four RNA-Seq databases. Values have been normalized to renal expression (100%) for each database. B) Klotho protein expression in various tissues from the Human Proteome Map. C) RNA-Seq data for Klotho from three databases of mouse tissues. Raw data available at http://www.ebi.ac.uk/gxa/home and http://www.humanproteomemap.org/.

Figure 2. Klotho expression in the kidney

A) Klotho expression in the rat tubule, using data derived from an RNA-Seq study of microdissected tubular segments (Ref: [23]). B) Left panel: *In situ* hybridization for Klotho in mouse kidney reveals high expression in distal tubule, high-moderate expression in the proximal tubule, and low-absent expression in glomeruli and intra-renal artery. Right panel: Fluorescent *in situ* hybridization for Klotho (red) and co-staining with the proximal tubular marker LTL (green). Colours have been inverted for improved visibility. C) Left panel: *In situ* hybridization for Klotho in human kidney. Expression is high in distal tubule, low-intermediate in proximal tubule, and low-absent in glomeruli and intra-renal artery. Right panel: Fluorescent *in situ* hybridization for Klotho (red) and co-staining with the proximal tubular marker LTL (green). Colours have been inverted for improved visibility. D) Immunohistochemistry for Klotho in human kidney reveals a nearly identical expression pattern as for mRNA.

Figure 3. Klotho expression in human parathyroid gland/thyroid and placenta.

A) *In situ* hybridization reveals distinct Klotho expression in normal human parathyroid gland, and no detectable expression in adjacent tissue. B) *In situ* hybridization reveals distinct Klotho expression in certain cell types in healthy human placenta

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Figure 1A.

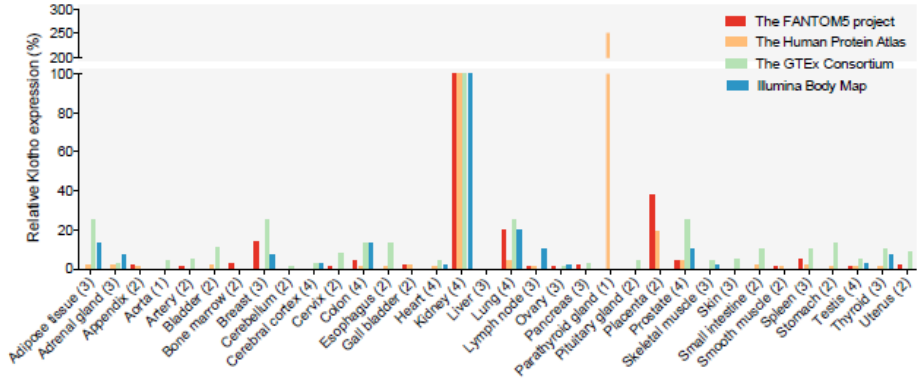


Figure 1B.

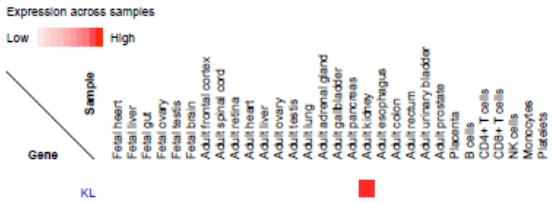


Figure 1C.

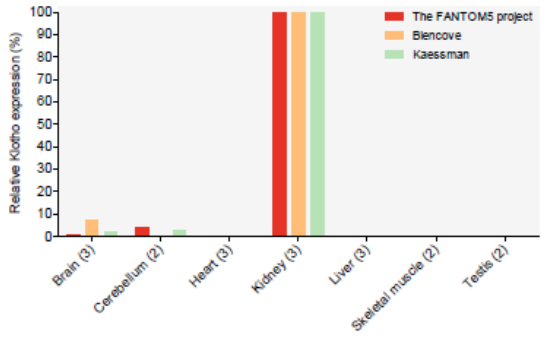
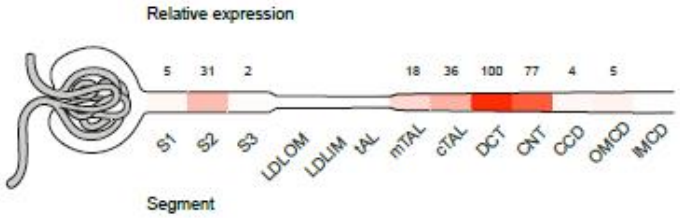


Figure 2A.



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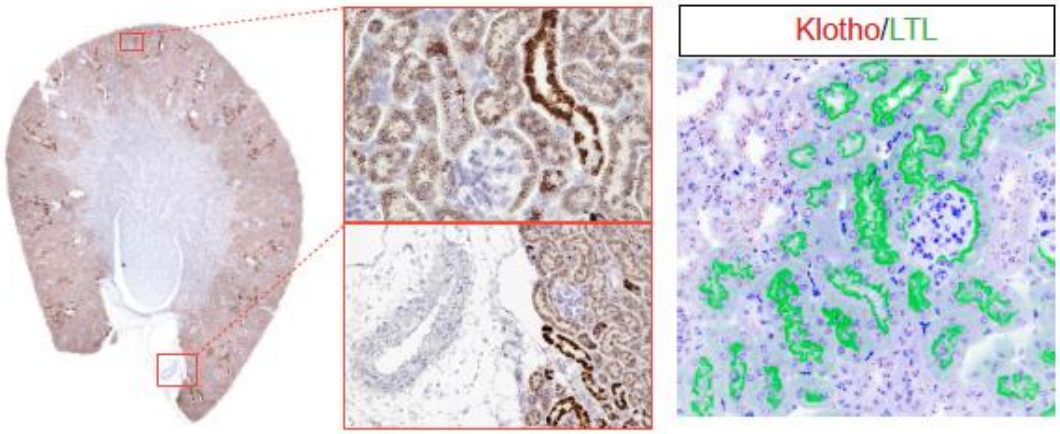

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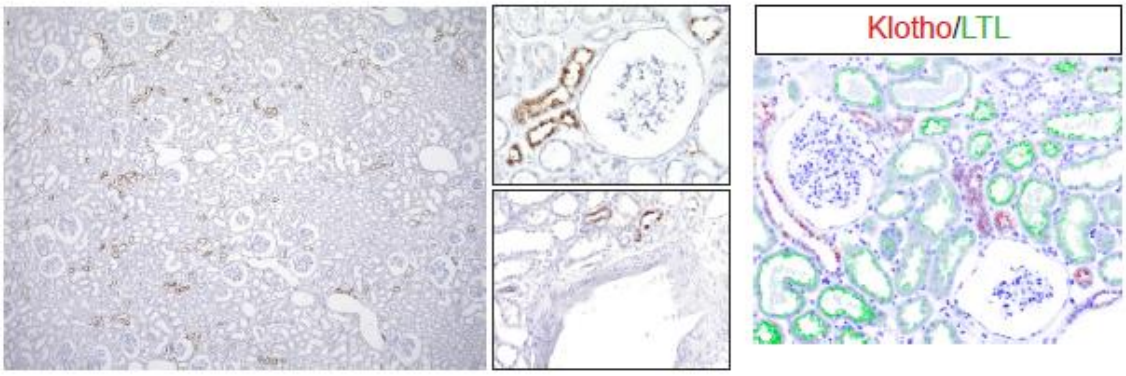

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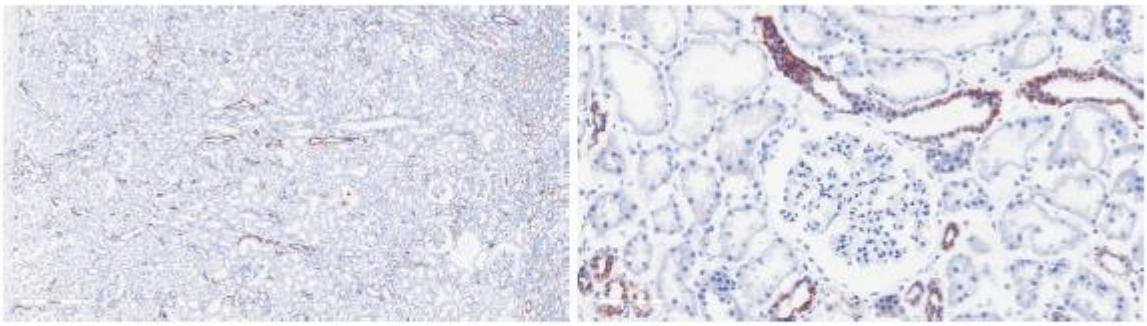


Figure 3A.

In situ hybridization of human parathyroid/thyroid

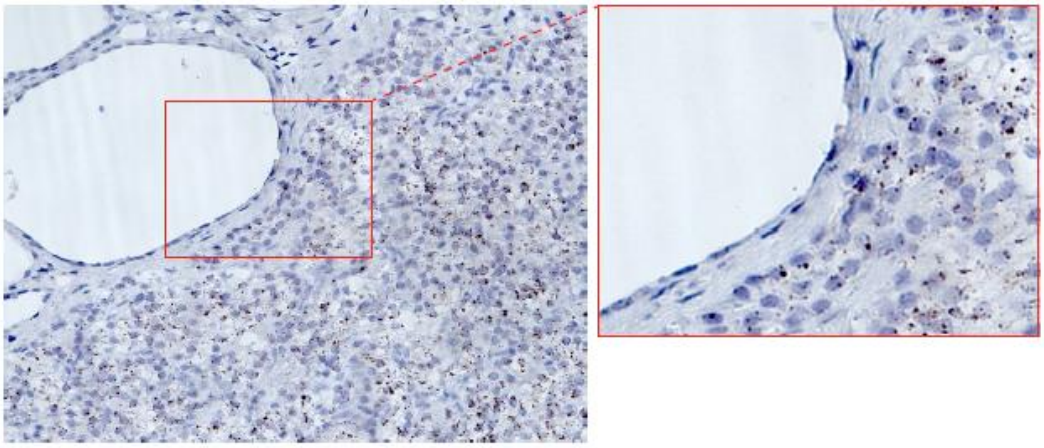
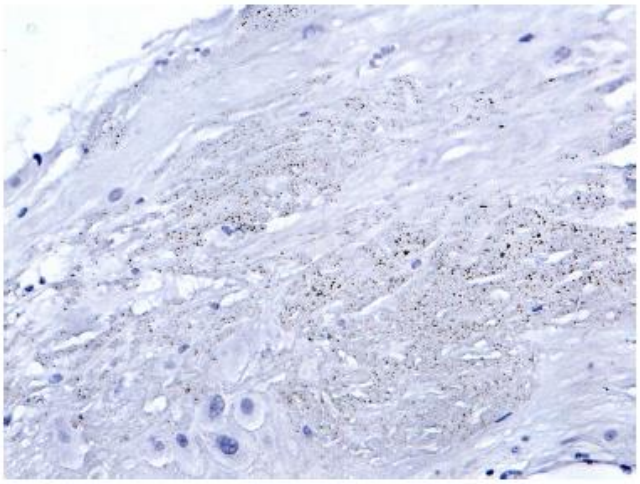


Figure 3B.

In situ hybridization of human placenta



Highlights

- High expression of Klotho is restricted to a few tissues, most importantly the kidney
- Emerging evidence suggest low to intermediate Klotho expression in a number of tissues
- The kidney is the principal source of soluble Klotho
- Use of different Klotho antibodies is a major source of discrepancies in the field
- We propose a classification based on current evidence for Klotho RNA and protein levels