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REVIEW

Klotho Acts as a Tumor Suppressor in Cancers

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Abstract The *klotho* gene is a classical "aging suppressor" gene. Its roles in the pathology of chronic kidney diseases have been well documented. However, the role of Klotho in tumorigenesis, cancer progression, and prognosis is attracting more and more attention. Recent studies have shown that Klotho participates in the progression of several types of human cancers. Klotho functions as a tumor suppressor by inhibiting insulin/IGF1, p53/p21, and Wnt signaling. Silencing *klotho* gene expression is mainly mediated through promoter hypermethylation and histone deacetylation in cancer. Klotho has been proposed to take part in cell proliferation, survival, autophagy, and resistance to anti-cancer therapies.

Keywords Klotho · Insulin/IGF1 axis · Autophagy · Wnt signaling · ROS

Introduction

The *klotho* gene was first identified as an anti-aging gene in mice. Its disruption and overexpression have been associated with accelerated aging and extended life span, respectively. Researchers have found that a defect in *klotho* gene expression in mice leads to multiple aging-like phenotypes [1]. In contrast, transgenic mice that overexpress the *klotho* gene live longer than wild-type mice [2]. The role of klotho in chronic kidney diseases has been well documented [3, 4]. Recently, the involvement of Klotho in tumorigenesis, cancer progression, and prognosis has been attracting increased attention [5–7]. Translation of the *klotho* gene yields a 1014-amino acid single-pass transmembrane protein, containing both membrane bound Klotho and secreted Klotho with distinct functions. Membrane bound Klotho forms a complex with fibroblast growth factor (FGF) receptors and functions as an obligate co-receptor for FGF23, a bone-derived hormone that induces urinary phosphate excretion [4, 6, 7]. Mice lacking Klotho not only exhibit phosphate retention but also display premature-aging syndrome, suggesting an unexpected link between phosphate metabolism and aging. Secreted Klotho functions as a humoral factor to regulate the activities of multiple glycoproteins on the cell surface, including ion channels and growth factor receptors such as insulin/insulin-like growth factor-1 receptors. It also acts as a circulating hormone to inhibit critical signal pathways in cancer such as insulin/IGF1, p53/p21, ROS, and Wnt signaling [8–17]. These signaling pathways have been widely recognized to be involved in cell proliferation, survival, autophagy and resistance to anti-cancer therapies in a variety of cancers. More and more studies have shown that Klotho participates in the progression of several different human cancers including breast cancer, lung cancer, gastric cancer, pancreatic adenocarcinoma, and recently reported hepatocellular carcinoma [6, 9, 11, 12, 18, 19]. In this review, we will discuss the role of Klotho in insulin/IGF-1, Wnt, and ROS signaling axis as well as the effect of Klotho on autophagy.

Klotho Inhibits Insulin/IGF-1 Axis in Cancer

Cancer is one of many age-related disorders and often has enhanced signaling of the GH/IGF-1/insulin axis [20]. The insulin-like growth factor pathway plays a crucial role in cancer cell proliferation, survival, autophagy and resistance to anti-cancer therapies in many human malignancies [21] (Fig. 1). IGF-1R is a key signal transduction receptor of the insulin-like growth factor pathway. In tumor cells, the IGF-1

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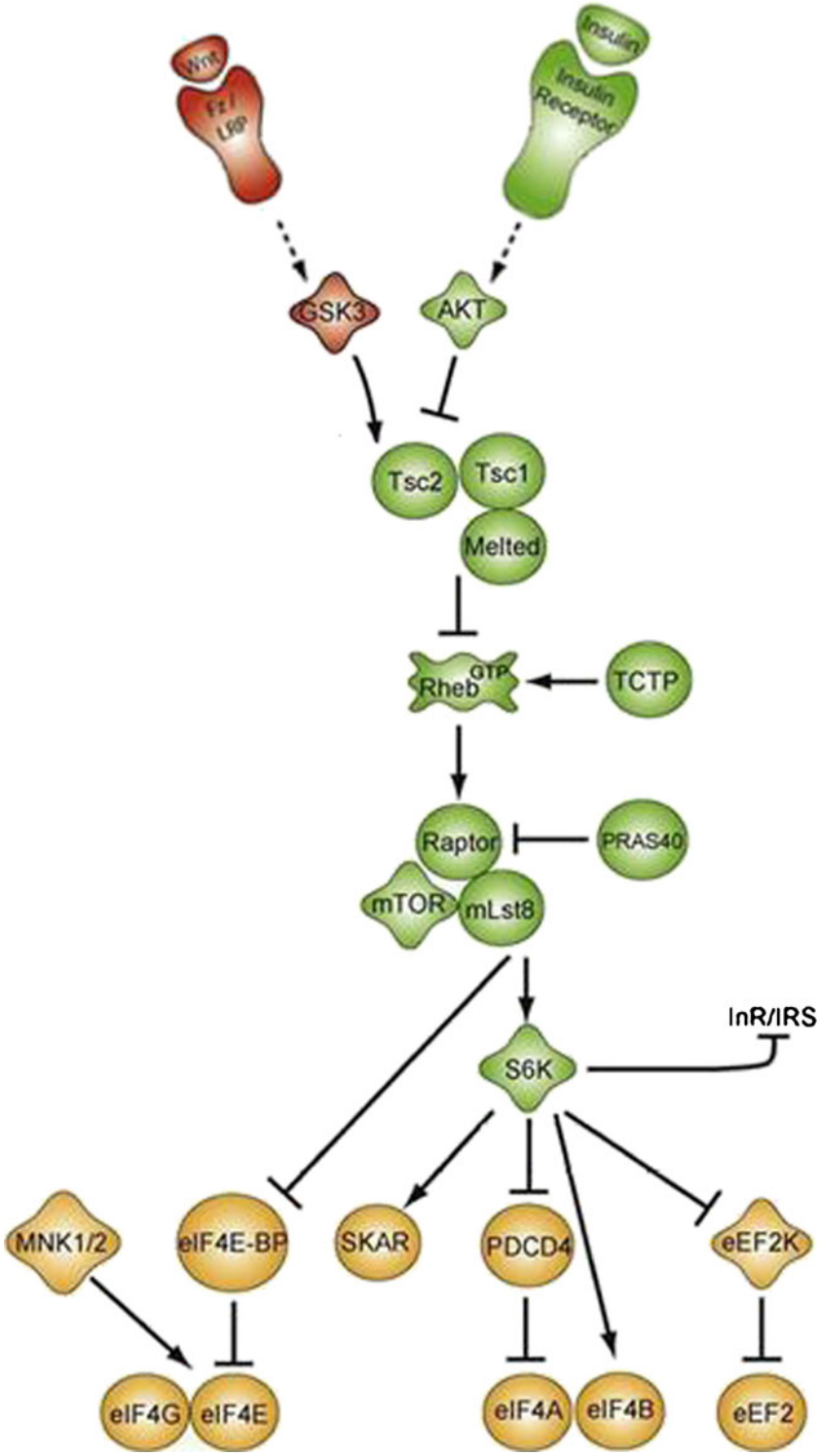

Fig. 1 Klotho as a candidate tumor suppressor gene that inhibits the insulin/IGF1 and Wnt signaling pathway, which plays a major role in cancer cell proliferation, survival, autophagy and resistance to anti-cancer therapies in many human malignancies

receptor (IGF-1R) is often amplified, upregulated and/or hyperactivated. Forced overexpression of IGF-1R induced tumor development and enhanced tumor metastasis in animal models [22, 23]. Furthermore, increased circulating IGF-1 level is also considered a significant risk factor for the development of various types of cancers, including breast, prostate, colon, and lung cancer [20, 24]. In IGF-1 deficient mice, tumor growth and metastasis were significantly inhibited [25]. These oncogenic properties are mediated through the signal transduction crosstalk between two IGF-R activated pathways: Ras/Raf/MEK/ERK/MAPK (Ras pathway) and PI3K/AKT (AKT pathway).

As a tumor suppressor gene, *klotho* has been associated with many types of neoplasia [8–17]. Clinical observation revealed that loss of *klotho* gene expression is positively related to poor prognosis of gastric cancer and lung cancer patients [11, 26, 27]. Klotho expression is associated with epithelial ovarian cancer progression and can serve as an independent marker for ovarian cancer prognosis [5]. Klotho expression is markedly downregulated in carcinoma tissues compared to adjacent tissues. For example, the expression of the *klotho* gene is significantly lower in breast cancer [8], gastric cancer [11, 28], pancreatic cancer [12], cervical carcinoma [10], rectal cancer [14], and hepatocellular carcinoma [19] tissues than in adjacent tissues. Forced overexpression of the *klotho* gene in tumor cells inhibited tumor cell proliferation, reduced invasion, and increased tumor cell apoptosis. Some controversial results have also been observed. For example, high expression of secreted Klotho was associated with increased risk of disease progression and death from ovarian cancer [5]. This association was independent of the patient's clinical and pathological characteristics.

Further studies have demonstrated that the *klotho* gene exerts an anti-cancer role mainly through inhibition of tumor related signal transduction pathways, such as insulin and insulin receptor (insulin/IGF-1R), Wnt/β-catenin, and especially the insulin/IGF-1 signaling pathway, thereby inhibiting tumor cell growth, migration, and proliferation [6]. Forced overexpression of Klotho can not only inhibit IGF-1 signaling by reducing IGF-1 receptor and insulin receptor substrate 1 expression, but also phosphorylate extracellular signal-regulated kinase expression [9]. The inhibitory role of Klotho is also associated with the enhancement of CCAAT/enhancer binding protein expression and the expression of apoptotic proteins [29]. Knockout of endogenous *klotho* gene expression in breast cancer MCF-7 cells can promote the proliferation of tumor cells through upregulating IGF-1 serine/threonine protein kinase phosphorylation [6]. Overexpression of Klotho protein was found to reduce proliferation and promote apoptosis in lung cancer A549 cells, whereas silencing of the *klotho* gene in A549 cells enhanced proliferation. The role of *klotho* gene overexpression in A549 cells is associated with reduced IGF-1/insulin-induced phosphorylation of IGF-1R (IGF-1 receptor)/IR (insulin receptor). In contrast, Lorenzi et al. study found that soluble Klotho protein does not inhibit IGF-1 and/or insulin signaling in HEK293, L6, and HepG2 cells, arguing against the direct role of Klotho in insulin signaling [2, 30]. These studies certainly indicate Klotho's different roles in different tumors.

The loss of *klotho* gene expression is closely related to promoter methylation and histone deacetylation. *klotho* gene expression has been found to be absent or reduced in many cancer cells, which can be reversed by treatment with the demethylation agent 5- aza-2′-deoxycytidine (Aza) or histone deacetylase inhibitor trichostain A. Methylation of the Klotho gene promoter was frequently detected in primary tumor tissues when compared to adjacent normal colon tissues. However, it is unlikely that methylation is the sole mechanism responsible for decreased Klotho mRNA and protein expression [31]. Other factors may also affect the expression of the *klotho* gene. For example, Egr-1 regulates *klotho* gene transcription [32]. In addition, changes in the expression of microRNAs (miRNA) have been widely observed in cancers [33]. Global gene expression profiling of miRNA reveals that more miRNAs are upregulated or downregulated in tumors than in normal tissues [34, 35]. Currently, no correlation has been documented between miRNA and *klotho* gene silencing in cancer.

The Function of Klotho on Wnt Signaling Pathway in Cancer

The Wnt signaling pathway controls cell proliferation and differentiation, and its deregulation is involved in many different diseases, including cancer. Wnt signaling contributes to tumorigenesis and progression with β-catenin playing a key role. Aberrant activation of Wnt/β-catenin signaling pathways has been regarded as a generic pathway in a variety of human malignancies [36, 37]. Epigenetic gene silencing of soluble Wnt antagonists, such as secreted Frizzle related proteins (SFRPs), has been observed to result in constitutive activation of the canonical Wnt pathway in many tumors [38–41]. Biochemical evidence shows that the TOR pathway receives stimulation from the Frizzled family of Wnt receptors, leading to the activation of Wnt signaling and subsequent shortening of lifespan. Klotho acts as a checkpoint in Wnt5A signaling, filamin cleavage, and metastatic progression. The progressive loss of Klotho in an aging microenvironment could potentially allow for an increase in Wnt5A expression, calpain activity, and subsequent filamin cleavage, which is associated with melanoma progression [42].

The *klotho* gene encodes a transmembrane protein that appears to act as a humoral factor that regulates multiple pathways, including the Wnt signaling pathway [43]. Klotho has been shown to be a secreted antagonist of the Wnt signaling pathway [43]. Secreted Klotho protein has been reported to bind to several Wnt ligands and inhibit Wnt signaling by preventing Wnt from binding to its cognate cell-surface receptor [44]. Studies found that Klotho also acts as a Wnt antagonist, and *klotho* gene mutants displayed enhanced Wnt signaling along with stem cell depletion [43]. It may appear counterintuitive that Wnt signaling, a process known to be involved in stem cell self-renewal [37, 45], can result in stem cell depletion and accelerated aging. However, continuous exposure to Wnt has been shown to induce cellular senescence of both stem cells and differentiated cells [43, 46]. Although activation of Wnt signaling is essential for stem cell proliferation and survival, continuous and prolonged

Wnt signaling activation may exhaust and deplete stem cells [47, 48]. Since stem cell dysfunction limits tissue regeneration and potentially affects aging processes, the ability of secreted Klotho protein to inhibit Wnt signaling may also contribute to aging-like phenotypes in Klotho-deficient mice. The reduced number of epidermal stem cells may also explain poor wound healing observed in Klotho-deficient mice.

The Function of Klotho on ROS

ROS not only contribute to oxidative stress accumulated during aging, but also play a significant role in the regulation of different signaling pathways [49]. ROS mediate diverse biological functions, including cell proliferation, adhesion, migration and apoptosis. For example, it has been shown that an oxidative burst is required for the modulation of the insulin/IGF-1 pathway [50] and vascular endothelial growth factor (VEGF) signaling through inducing reversible protein modifications, such as disulfide bonds or oxidation of cysteinyl thiols [51]. A decrease in *klotho* gene expression has been associated with an increase in Nox2 expression and superoxide production in kidneys of spontaneous hypertensive rats (SHRs) [52]. Oxidative stress was revealed to decrease the expression of Klotho in a mouse inner medullary collecting duct (mIMCD3) cell line [53]. *Klotho* gene delivery suppressed Nox2 expression, reduced superoxide production, and attenuated kidney damage in SHRs [52]. There is growing evidence that the Klotho protein induces the expression of MnSOD [54] and suppression of NADPH oxidases [48, 52] to protect against oxidative stress.

ROS initiate and promote the development of tumors [55]. They can also activate the PI3K/AKT and MEK/ERK signal transduction pathway [56, 57], which can promote tumor growth and inhibit tumor cell apoptosis. Physiological levels of ROS are closely related to cell proliferation. However, when intracellular ROS content exceeds a certain threshold, ROS cause cell cycle inhibition, and higher concentrations of ROS can even lead to DNA damage, apoptosis, and necrosis [58]. Tumor cells maintain a higher oxidation state and lower antioxidant enzyme levels [59]. The level of ROS in tumor cells increases under the hypoxic state [60]. ROS are able to oxidize target molecules involved in invasion of tumor cells, such as protein kinase C (PKC) and protein tyrosine phosphatase (PTP). ROS may also trigger angiogenesis.

ROS also take part in autophagy. Scherz-Shouval et al. demonstrated that mammalian cells produce ROS during starvation, which seem to be essential for autophagosome formation and autophagic degradation. They also show that treatment of mammalian cells with hydrogen peroxide stimulates autophagy by redox regulation of ATG4 [61]. These findings, together with the analysis of autophagy deficient mutants [62–64], strengthen the idea that ROS act as important signaling molecules, controlling the induction of critical catabolic pathways. Yamamoto et al. [65] study on signaling pathways responsible for Klotho-induced increase in resistance to oxidative stress demonstrated that membrane bound Klotho inhibits FOXO3a phosphorylation and promotes its nuclear translocation. Nuclear FOXO3a then binds to the MnSOD promoter and upregulates its expression, resulting in suppression of ROS formation. Saito et al. [66] investigated the effects of iron overload and iron chelation on renal expression of Klotho in untreated rats and rats treated with angiotensin II. Their findings suggested that iron overload and increased ROS overproduction were involved in angiotensin II-mediated modulation of Klotho expression. Tumor cells maintain a higher oxidation state, and lower antioxidant

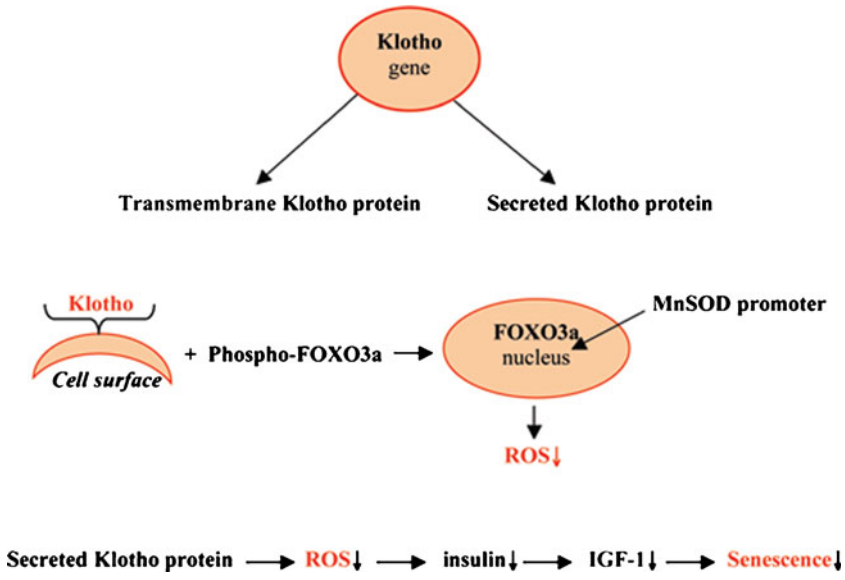

Fig. 2 The mechanisms underlying the antiaging effects of klotho (kuro-043 and yamamoto et al. 44)

enzyme levels [59]. Klotho may be involved with ROS via the following pathway: Klotho → ROS↓ → insulin↓ → IGF-1↓ → autophagy and apoptosis [2, 65, 67](Fig. 2). Currently, no study has investigated the association between Klotho and ROS in cancer. It is also unclear whether klotho acts on cellular ROS in tumors through the insulin/insulin-like growth factor signal pathway. Klotho may affect cell autophagy in tumors through a ROS-mediated signal pathway. Therefore, ROS can activate important signaling pathways in tumor cells that promote tumor cell proliferation and resistance.

The Role of Klotho in Autophagy in Cancer

The GH/insulin/IGF-1 axis has also been demonstrated to prevent autophagic clearance of cellular waste material and apoptotic cleaning of senescent cells [68–70]. Under normal conditions, autophagy helps maintain cell homeostasis by eliminating damaged organelles [71]. Autophagy starts with the formation of double membrane vesicles (autophagosomes) which engulf organelles. The autophagosomes then fuse with lysosomes, forming the autophagolysosome where the contents are degraded [71]. It has been suggested that autophagy is induced under pathological conditions and functions as an adaptive cell response, protecting cells from bioenergetic stress [72]. However, extensive or persistent autophagy also results in cell death [73]. Thus, autophagy is an important factor for maintaining the balance between cell death and survival.

Autophagy is commonly observed to be induced by reduced growth factor signaling. mTORC1 is a key mediator of growth factor signaling involved in autophagy. The growth factor signaling that regulates mTORC1 is mainly activated through the insulin/insulin-like growth factor (IGF-1)-PI3K (phosphoinositide 3-kinase class I)-Akt pathway, which negatively regulates autophagy induction. The insulin/IGF-1 pathway involves regulation of PDK1 and Rheb expression, the positive regulators of mTORC1 signaling, and PTEN and TSC2 expression, the negative regulators of mTORC1 signaling. TSC2, which forms a complex with TSC1, acts as a GTPase activating protein (GAP) for the small GTPase Rheb, and the GAP activity is regulated by Akt-mediated phosphorylation of TSC2 [74, 75]. PRAS40, a component of mTORC1, also plays an important role in mediating mTORC1 signaling by serving as a substrate for Akt [76, 77]. It has been previously reported that the activation of insulin/IGF signaling suppresses the autophagic-lysosomal pathway [78, 79], and the klotho protein functions as a circulating hormone that binds to a cell-surface receptor to repress intracellular signals of insulin and IGF-I [2, 54].

Constitutive activation of insulin/IGF signaling under Klotho deficient conditions represses the autophagic lysosomal pathway in *klotho* mutated kl/kl mice [84]. However, the autophagic-lysosomal pathway in kl/kl mice was activated in the masseter and tongue but not in the gastrocnemius, where the ubiquitin–proteasomal pathway was not altered. No significant difference in the phosphorylation levels of insulin/IGF-I signaling components, such as insulin/IGF-I receptor, Akt, or FoxO, was found between kl/kl and k^+/k^+. However, phosphorylation levels of signaling components downstream of mTOR, such as 4E-BP1 and p70 S6K, were downregulated in the masseter and tongue of kl/kl compared to k^+/k^+ mice. Recently, we found that klotho has also been involved in autophagy in hepatocacinoma and gastric cancer cells [19, 28]. Klotho may also affect tumor cell autophagy through the insulin/insulin-like growth factor signal pathway or through other pathways associated with autophagy (Fig. 1).

In conclusion, klotho is a new tumor suppressor gene epigenetically inactivated in cancer. Promoter hypermthylation and histone deacetylation can silence the expression of the *klotho* gene. Klotho is a candidate tumor suppressor which inhibits insulin/IGF1, p53/p21 and Wnt signaling and subsequently plays a crucial role in cancer cell proliferation, survival, autophagy and resistance to anti-cancer therapies. Blockade of IGF-1 signaling could be potentially useful in the clinic for extending the duration of clinical responses to cytotoxic therapies when used in gene therapy. The therapeutic potential of targeting the IGF-1 signaling pathway rests in its ability to effectively inhibit tumor growth. For example, observations in patients with breast cancer have revealed that tumors with high IGF-1R expression are much less likely to respond to neoadjuvant therapies, including hormonal therapies such as tamoxifen and herceptin, than non-IGF-1R expressing tumors [23]. However, as a major inhibitor of IGF-1 signaling, the therapeutic potential of *klotho* targeting should be validated.

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