KL1 internal repeat mediates klotho tumor suppressor activities and inhibits bFGF and IGF-1 signaling in pancreatic cancer

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Translational relevance:

We have recently identified klotho as a tumor suppressor in breast cancer. In this study we show that klotho expression is reduced in human pancreatic adenocarcinoma, and treatment with klotho or with KL1 internal repeat effectively slow growth of pancreatic cancer cells in vitro and in vivo. Moreover, long-term administration of KL1 to mice shows a favorable toxicity profile. As klotho is an endogenous hormone, its administration is potentially feasible and may serve as a novel therapy for pancreatic, as well as other cancers. As diagnosis of pancreatic cancer is often cytology-based, the identification of reliable histological markers is of utmost importance. Our expression analysis suggest that loss of klotho may serve as novel diagnostic marker for this disease.
Abstract

Purpose: Klotho is a transmembranal protein which can be shed, act as a circulating hormone and modulate the insulin growth factor (IGF)-1 and the fibroblast growth factor (FGF) pathways. We have recently identified klotho as a tumor suppressor in breast cancer. Klotho is expressed in the normal pancreas and both the IGF-1 and FGF pathways are involved in pancreatic cancer development. We, therefore, undertook to study the expression and activity of klotho in pancreatic cancer.

Experimental design: Klotho expression was studied using immunohistochemistry and quantitative RT-PCR. Effects of klotho on cell growth were assessed in the pancreatic cancer cells Panc1, MiaPaca and Colo357, using colony and MTT assays, and xenograft models. Signaling pathway activity was measured by Western blotting.

Results: Klotho expression is downregulated in pancreatic adenocarcinoma. Overexpression of klotho, or treatment with soluble klotho, reduced growth of pancreatic cancer cells in vitro and in vivo, and inhibited activation of the IGF-1 and the bFGF pathways. KL1 is a klotho subdomain formed by cleavage or alternative splicing. Compared to the full-length protein, KL1 showed similar growth inhibitory activity but did not promote FGF23 signaling. Thus, its administration to mice showed favorable safety profile.

Conclusions: These studies indicate klotho as a potential tumor suppressor in pancreatic cancer, and suggest, for the first time, that klotho tumor suppressive activities are mediated through its KL1 domain. These results suggest the use of klotho or KL1 as potential strategy for the development of novel therapeutic interventions for pancreatic cancer.
Introduction

Pancreatic adenocarcinoma is the fourth leading cause of cancer death in the Western world, and despite recent advances, the median survival time after diagnosis of metastatic disease remains less than six months (1, 2). The insulin-like growth factor (IGF)-1 and the fibroblast growth factor (FGF) pathways play important roles in the development of this cancer (3, 4). The IGF-1 pathway promotes tumorigenesis of various malignancies, and excessive activation of the IGF-1 receptor (IGF-1R) is associated with malignant transformation, increased tumor aggressiveness, and protection from apoptosis (3, 5, 6). IGF-1 and IGF-1R are often overexpressed in human pancreatic tumors and cell lines; and inhibition of the IGF-1 pathway suppresses tumorigenicity and increases sensitivity of pancreatic tumors to radiation and chemotherapy (7, 8). Targeted therapies directed against the IGF-1R are currently being tested for the treatment of pancreatic cancer in clinical trials (http://clinicaltrials.gov/). The FGF pathway is involved in various stages of cancer development, including mitogenesis, cell differentiation and angiogenesis (4, 9). Increased expression of FGF receptors (FGFRs) and the ligands FGF-1,-2,-5 and 7 was noted in pancreatic cancer samples and cell lines, and high expression of FGF-2 (also termed bFGF) was associated with a more advanced tumor stage and shorter survival (10). Moreover, exogenous FGF-2, -5 and -7 exert mitogenic activity toward pancreatic cells (10, 11).

Human klotho is a 1012-amino acid single-pass transmembrane protein, which is abundantly expressed in the distal convoluted renal tubules of the kidney and the choroid plexus in the brain, and also present in various exocrine and endocrine tissues, including the pancreas (12-15). The extracellular domain of klotho is composed of two internal repeats, KL1 and KL2, that share amino-acid sequence homology to β-glucosidase but lack glucosidase activity (15). KL1 may also be transcribed through an alternative splicing (15). Klotho knockout mice exhibit phenotype resembling human premature aging, while
overexpression of klotho in mice extends lifespan (15, 16). Klotho is a potent inhibitor of ligand-dependent activation of the insulin and IGF-1 pathways (12, 16, 17). Importantly, intra-peritoneal injection of soluble klotho to mice effectively reduced their response to insulin treatment (16). Klotho is also an important regulator of the FGF pathway. Klotho -/- and FGF23 -/- share similar phenotype, and klotho is a mandatory co-factor for the interaction of FGF23 with FGFRs 1,3 or 4. On the other hand, klotho inhibits activation of the FGFRs by bFGF in human embryonic kidney (HEK) 293 and COS7 cells (12, 18, 19).

We have previously identified klotho as a tumor suppressor in breast cancer (12). We noted high klotho expression in normal breast tissue and low klotho expression in breast cancer and discovered that overexpression of klotho specifically inhibited growth of breast cancer cells in vitro (12). We also discovered that a less active functional variant of KLOTHO, which contains two amino acid substitutions F352V and C370S (KL-VS), is associated with increased breast cancer risk among BRCA1 mutation carriers (20).

We aimed to study klotho expression pattern and activities in pancreatic cancer. Our results reveal high expression of klotho in normal exocrine pancreas and reduced expression in pancreatic adenocarcinoma. Klotho inhibited the IGF-1 and FGF pathways in pancreatic cancer cells and further studies revealed the KL1 domain as a potent inhibitor of these pathways. Treatment with either klotho or KL1 effectively reduced growth of pancreatic cancer cells in vivo. Compared to the full-length protein, KL1 did not affect renal functions and did not enhance FGF23 signaling. These findings indicate different physiologic roles for klotho and KL1 and may suggest KL1 as a safe and effective therapy for pancreatic adenocarcinoma.
MATERIALS AND METHODS

Chemicals, antibodies and constructs: The chemicals used were bFGF (Biological industries, Kibbutz Beit Haemek, Israel), IGF-1 and soluble human KL1 (hKL1) (PeproTech Inc, Rocky Hill, NJ), 5-fluorouracil (5-FU) (Sigma, St. Louis, MO), soluble human klotho (hKL) (R&D Systems, McKinley Place NE, MN) G418 from (Invitrogen, Carlsbad, CA), and gemcitabine (Eli Lilly). Antibodies: phospho-AKT1 (S473), phospho-IGF-1R (Y1131), phospho-FRS2α (Y196), total pan-AKT (Cell Signaling Technology, Danvers, MA), diphosphorylated and -total ERK1/2 (Sigma), phospho-IRS1 (Invitrogen), total IRS1 (Upstate, Temecula, CA), HA (Covance, Princeton, NJ), klotho (Calbiochem, CA). Klotho expression vector was a generous gift of Y. Nabeshima (Kyoto University, Japan).

Immunohistochemistry analysis: Pancreatic cancer sections were created, after IRB approval, from 18 pancreatic cancer samples and 5 normal pancreas samples, diagnosed at Sheba Medical Center and Cedars-Sinai Medical Center. Staining was performed as described (12) and scored by percent of positive tumor cells and staining intensity. Negative and low expressions were categorized low, while intermediate and high expressions were categorized as high.

Cells and transfections: Panc1, MiaPaCa2 and Colo357 pancreatic adenocarcinoma cell lines, the pancreatic neuroendocrine tumor (NET) cancer cell line BON-1 and the non-cancerous HEK-293 were obtained from the American Type Culture Collection (Manassas, VA). All transfections used LipofectAMINE 2000 (Invitrogen).

Quantitative Reverse Transcription-PCR (RT-PCR): Total RNA extraction, cDNA production and quantitative RT-PCR were performed as described (12). Total RNA of human normal pancreas was obtained from BioChain (Hayward, CA, USA). cDNA from pancreatic adenocarcinoma tumor and normal tissue adjacent to the tumor were obtained from the Institutional Tissue Bank of the Sheba Medical Center after IRB approval.
Western blot analysis: Protein extraction and Western blotting was performed as described (12).

Colony assays: Two days following transfection with the indicated plasmids, G418 (900 μg/ml for Panc1, 1000 μg/ml for MiaPaCa2 and 750 μg/ml for Colo357, HEK-293 and BON1) was added to the culture media. At day 14, cells were stained using crystal violet (Sigma) and quantitated as described (12).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay: 3000 cells/well were plated in 96-well plates, cultured in the appropriate culture media, and treated for 48 hrs as indicated. MTT assay was carried out as done previously (12).

Soft agar assay: To determine the effect of klotho on anchorage-independent growth, 15,000 Panc1 cells per well were cultured in Noble agar (Sigma) at a final concentration of 0.3%, on top of 0.5% base agar. Cells were seeded in triplicate wells of 6-well plates, and re-fed twice a week with growth medium containing hKL (0.8 pM) or control vehicle. Colonies were counted after 21 days, following staining with 0.005% crystal violet.

Generation of soluble mouse klotho: Soluble mouse klotho was generated as described (16).

Genetic analyses for the KL-VS variant: Genotyping the KL-VS variant was carried out as described (20).

Animal studies: Mice maintenance and experiments were performed under institutional guidelines of the Sheba Medical Center. Panc1 cells were prepared as described (21). For the experiment with mouse klotho (mKL): six-week-old female athymic nude mice were injected with cells (0.5x10^6) subcutaneously into both flanks. After five days mice were treated with daily intra-peritoneal injections of mKL: 10μg/kg, 25μg/kg or saline control (N=5 per group). Tumor size was measured with a digital caliper for up to 4 weeks, and the volume was estimated using the equation V = (a x b²) x 0.5236, where ‘a’ is the larger dimension and ‘b’
the perpendicular diameter. Human klotho treatment experiment was performed as described above with minor exceptions. One flank was injected, and mice were treated with either 2.5μg/kg hKL, 10, 25μg/kg hKL1 or vehicle control (N=6-7 for each treatment group, N=12 for the control group). For the experiment with MiaPaCa2 cells, 0.75x10^6 cells were injected into both flanks. Mice (N=8 per group) were treated with either 2.5μg/kg hKL, 10μg/kg hKL1 or vehicle control.

**Statistical analysis:** Results are presented as mean ± standard deviation (SD). Categorized variables were compared between the study groups using Fisher's exact test and continuous variables were compared using t-test. All significance tests were two-tailed and a P-value of <0.05 was considered as statistically significant. The nature of interaction between klotho and 5-FU or Gemcitabine was analyzed using the additive model (22). A ratio between the observed and the predicted viability was calculated for all combinations and a ratio <0.8 for the interaction was considered to be synergistic.
RESULTS

Expression pattern of klotho in normal pancreas and pancreatic adenocarcinoma

While high klotho mRNA expression was noted in whole pancreas extracts (23), the expression pattern of klotho in different components of the normal pancreas or in pancreatic cancer are currently unknown. We conducted immunohistochemistry analysis of klotho expression in pancreatic tissues, using an antibody directed against the intracellular domain of klotho, as previously described (12). Normal human kidney sections served as control and positive staining was noted only in the distal convoluted tubules (12). Analysis of five normal pancreas samples revealed high klotho expression in the exocrine pancreatic ducts, lower expression in the islet cells and no expression in the surrounding stromal tissue (Fig.1a). Analysis of 18 pancreatic adenocarcinoma samples revealed reduced klotho expression in 15 (83%) of the samples: no expression in nine and low expression in six samples (Fig.1b, Table 1, p=0.002 for the comparison between normal and cancer samples).

Klotho expression was also analyzed using quantitative RT-PCR in normal human pancreatic tissue and three pancreatic adenocarcinoma cell lines: Panc1 and MiaPaCa2, which represent poorly differentiated adenocarcinoma, and Colo357, which represent moderately differentiated cancer (24). Significantly lower klotho mRNA levels were observed in the cancer cells: 12% in Panc1 and less than 1% in MiaPaCa2 and Colo357 cells compared to the normal tissue (Fig.1c). Klotho mRNA levels were then measured in five adenocarcinoma samples and adjacent normal pancreatic tissue. Samples were obtained during pancreatic surgery and the presence of adenocarcinoma was verified by an experienced pathologist. Reduced klotho expression in the tumor compared to normal pancreas was noted in 4 (80%) of the samples (Fig 1d).

Recently, silencing of klotho by promoter hypermethylation was noted in cervical cancer (25); and we noted epigenetic silencing of klotho in breast cancer cells and clinical
samples (Rubinek et al, submitted for publication). We tested the presence of methylation in the promoter of klotho using methylation-specific PCR (MSP) directed against a region within the CpG island in the klotho promoter. In correlation with the expression pattern (Fig.1c), we noted the klotho promoter to be fully methylated in Colo357 and MiaPaCa2 and hemi-methylated in Panc1 cells (Fig.S2). Genetic analysis (data not shown) revealed that while wild-type klotho gene was found in MiaPaCa2 and Colo357, only the less active klotho variant, KL-VS (20), was detected in Panc1.

**Klotho inhibits growth of pancreatic cancer cells in vitro and in vivo**

The effect of klotho over-expression on growth of pancreatic cancer cells was assessed using colony formation assays. Colo357, MiaPaCa2 and Panc1 pancreatic cancer cells, pancreatic NET cell line BON1 (26), and the noncancerous HEK-293 cells were transfected with either a HA-tagged klotho expression vector (pcDNA3-HA-KL) or an empty vector (pcDNA3). Transfected cells were cultured in media containing G418 for two weeks and stained to determine the number of surviving colonies. Klotho expression reduced the number and size of surviving colonies of the pancreatic adenocarcinoma and BON1 cells (Fig. 2a), but did not affect growth of HEK-293 cells ((12) and data not shown).

Klotho may be shed and act as a circulating hormone, and we and others have shown that either soluble klotho or conditioned medium taken from klotho-over-expressing cells is active (12, 15, 23, 27). We, therefore, tested the ability of soluble klotho to inhibit proliferation of pancreatic cancer cells. Cells were treated with mouse klotho (mKL), generated as described (12), or commercially available human klotho (hKL). Cells were seeded on 96-well plates, treated with either mKL or hKL for 48 hrs, and viability was assayed by MTT assay. Both mKL and hKL were found to be active and reduced viability of Panc1 cells by 70% and 55% respectively (Fig. 2b, *p<0.005 for the comparison between either compound and the control). mKL was also tested against Colo357 and MiaPaCa2 and
found to be active, albeit to a lesser extent (40% reduction in viability, p<0.01, Fig. 2e,f).

Anchorage-independent growth is a hallmark of the neoplastic phenotype. Importantly, soft agar assays revealed that treatment with klotho significantly inhibited anchorage-independent growth of Panc1 (Fig. 2c).

5-fluorouracil (5-FU) and gemcitabine are chemotherapeutic drugs commonly used for the treatment of pancreatic cancer (28). The ability of klotho to enhance their activity on pancreatic cancer cells was examined. Panc1, MiaPaCa2 and Colo357 cells were treated for 48 hrs with either mKL, 5-FU or gemcitabine, their combination, or a control vehicle, and viability was assayed. The addition of klotho significantly increased the activity of 5-FU (Fig. 2d-f). The nature of the interaction between mKL and 5-FU was analyzed using the additive model (22). The combination of mKL (6 nM) and 5-FU (500 µM) showed a sub-additive effect in MiaPaCa2 and Colo357 whereas a synergistic effect was noted in Panc1 cells (Fig. 2d and supp. Table 1). The combined treatment of hKL (0.8 pM) with gemcitabine (100 nM) had a minor effect on Panc1 cells (Fig. 2g) and Colo357 (data not shown), showing a sub-additive effect (supp. Table 2), whereas a synergistic effect was noted for the combination of 0.8 pM hKL with 1 nM Gemcitabine in MiaPaCa2 cells (Fig. 2h and supp. Table 2).

In order to test the in vivo activity of klotho, Panc1 cells were injected into both flanks of nude mice (0.5x10^6 cells per injection), and the mice were treated with intraperitoneal injections of either a control vehicle (N=5) or mKL 10µg/kg (N=5) or 25µg/kg mKL (N=5), five days a week for four weeks. Treatment with klotho did not affect weight (Fig. 2i) or general health of the mice, but significantly reduced tumor size (Fig. 2j, p<0.05 for comparison between treated and control groups).

**Klotho inhibits activation of the IGF-1 pathways in pancreatic cancer cells**

The IGF-1 pathway plays an important role in the development of pancreatic cancer and klotho inhibits activation of this pathway (8, 12). Panc1, MiaPaCa2 and Colo357 cells
express high levels of the IGF-1R and show enhanced proliferation following IGF-1 treatment (7, 8). We examined the ability of klotho to inhibit activation of the IGF-1 pathway in these cells. For these studies cells were transfected with either pcDNA3-HA-KL or an empty vector, starved for 48 hrs, treated with IGF-1 (12.5 nM, 15 min), and analyzed using Western blotting for the expression and phosphorylation of the IGF-1R and downstream components of the IGF-1 pathway (Fig. 3a). Klotho over-expression reduced phosphorylation of the IGF-1R and its downstream targets IRS-1, AKT1 and ERK-1 and -2 in all three cell lines. Similarly to klotho overexpression, soluble mKL also inhibited activation of the IGF-1R and its downstream effectors by IGF-1 in Panc1 cells (Fig. 3b).

**Klotho inhibits the bFGF pathway in pancreatic cancer cells**

bFGF is over-expressed in pancreatic cancer cell lines and is associated with a more advanced tumor stage and poor prognosis (29, 30), and klotho is a well-established modulator of the FGF pathway (18, 19). In order to study the effects of klotho on the bFGF pathway in pancreatic cancer, pcDNA3-HA-KL or an empty vector were over-expressed in Panc1, MiaPaCa2 and Colo357 pancreatic cancer cells. Cells were starved for 48 hours, treated with bFGF (10 ng/ml), and analyzed for the expression and activity of ERK1/2, the downstream targets of the FGF pathway. Klotho significantly inhibited activation of the bFGF pathway in all three cell lines (Fig. 3c).

**KL1 inhibits pancreatic cancer growth**

The predominant circulating forms of klotho in humans are the full-length protein (hKL), KL1 cleaved product (hKL1), and a splice variant composed only of KL1 (13, 14, 23). We explored the ability of KL1 to inhibit growth of pancreatic cancer cells, compared to full length klotho. Panc1 cells were transfected with either a full length mouse klotho expression vector (mKL), mouse KL1 (mKL1) or an empty vector (pcDNA3). Similar growth inhibitory activities were noted for KL1 and the full-length protein (Fig. 4a). The activity of soluble
KL1 was also tested. Panc1 cells were treated with either hKL or hKL1 for 48 hrs and viability was evaluated by MTT assay. Reduction of viability by 50% was observed following treatment with hKL1 at concentrations 100-times higher than hKL (Fig. 4b, *p<0.005 for comparison between treated and control cells).

We then examined the in vivo growth inhibitory activity of hKL1, compared to hKL, toward pancreatic cancer cells. Panc1 cells were injected to one flank of athymic mice and mice were treated with intraperitoneal injections of either control vehicle (N=12), hKL (2.5µg/kg, N=7), or hKL1 (10 µg/kg, N=6; 25 µg/kg, N=7), five days a week for five weeks. Klotho treatment significantly inhibited tumor growth (Fig. 4d-f, p < 0.05 for comparison of tumor volume and weight between the control and klotho treated groups). Immunohistochemistry analysis of the proliferation marker Ki67 revealed a significant reduction in the percent of proliferating cells in four tumors harvested from klotho treated mice compared to four controls (50% compared to 70%, p=0.02 Fig 4g). Similar experiment, with minor exceptions, was also performed using MiaPaCa2 cells. Cells were injected into both flanks of athymic mice and mice were treated with either a control vehicle (N=8), hKL (2.5µg/kg, N=8) or hKL1 (10 µg/kg, N=8). After four weeks of treatment, all the injected tumors were observed in the control group, while two tumors (12%) of the hKL-treated mice and four (25%) of the hKL1-treated mice completely regressed (fig.4i). Moreover, hKL1 inhibited tumor growth more effectively than hKL (Fig. 4h-i).

To our knowledge, the safety of long-term klotho or KL1 administration has not been determined. No changes in weight (Fig. 2i, 4c and data not shown) or general health were noted in mice treated with either hKL or hKL1. The effects of hKL and hKL1 on blood counts and chemistry were also studied. For these studies, blood was withdrawn from control, hKL-treated (2.5 µg/kg) or hKL1-treated (10 µg/kg) mice, at the same day of killing and tumor harvesting, following five weeks of treatment. Treatment with either hKL or KL1 did
not affect blood counts or liver function tests (data not shown). Importantly, full-length klotho but not KL1, altered blood urea, cholesterol and phosphate levels (Table 2). Glucose levels were determined after fasting in the MiaPaCa2 xenograft experiment. While klotho treatment mildly increased glucose levels, hKL1 did not have any significant effect on glucose level (Table 2).

We next analyzed the effects of KL1 on IGF-1, bFGF and FGF23 signaling. Panc1 cells were transfected with either full length klotho (pCDNA3-HA-KL), KL1 (HA-KL1) or an empty vector, starved for 48 hours, treated with IGF-1 (12.5 nM, 15 min) or bFGF (10 ng/ml), and analyzed using Western blotting for the level of expression and phosphorylation of AKT or ERK1/2. KL1 reduced phosphorylation of these proteins to a greater extent than the full length klotho (Fig. 4j). Yet, KL1 had no effect on the phosphorylation of ERK1/2 following FGF23 stimulation in Mouse Distal Convoluted Tubules (MDCT) cell line (Fig. 4k). Thus, hKL and hKL1 present cell-type and pathway-dependent differential activities.
Discussion

Our observations indicate klotho as a tumor suppressor in pancreatic cancer. High klotho expression was observed in normal pancreas, while low expression was noted in pancreatic adenocarcinoma. Klotho, either over-expressed or soluble, inhibits activation of the IGF and FGF pathways and slow growth of pancreatic cancer cells in vitro and in vivo. Furthermore, we found that KL1 domain was as active as the full length protein but had more favorable safety profile when administered to mice.

Reduced expression of klotho was noted in most pancreatic adenocarcinoma samples and cell lines. In accordance with recent studies showing repression of klotho by epigenetic mechanisms in human cervical carcinoma (25) and breast cancer [Rubinek et al submitted], we noted methylation of the klotho promoter in pancreatic cancer cell lines. Panc1 cells and some of the clinical samples do express klotho. However, our genetic analysis revealed that Panc1 carry only the less active KL-VS variant. Thus, we observed two mechanisms that may be involved in reducing the activities of klotho in pancreatic cancer cells - epigenetic silencing and expression of a less active protein.

As tissue diagnosis of pancreatic cancer is often based on cells obtained by fine needle aspiration and is based on cytology rather than on architecture of the tissue (31), sensitive and specific markers that will aid differentiating normal and tumor cells are eagerly sought. We noted universal expression of klotho in normal pancreatic cells and loss of expression in most cancer tissues and cells. Thus, if validated by additional studies, low expression of klotho may serve as a novel diagnostic tool for the differentiation between normal and cancerous pancreatic cells.

The presence of KL-VS in pancreatic cancer cells may suggest a role for the variant in pancreatic cancer development. BRCA1 and BRCA2 mutation carriers have an increased risk of developing pancreatic cancer (32-34). We have recently shown that the KL-VS functional
variant exhibits reduced activity and is associated with increased breast cancer risk in BRCA1 mutation carriers (20). As klotho may serve as a potential tumor suppressor, the association of a less active variant with increased risk of pancreatic cancer, especially in high-risk populations, should be further explored.

The IGF-1 pathway is active in pancreatic NETs (35). Klotho expression was noted in pancreatic islets and expression of klotho inhibited colony formation in the NET cells BON1. The role of klotho as a tumor suppressor in NET should be further explored.

Soluble klotho compounds and also a conditioned medium obtained from klotho-overexpressing cells effectively inhibit growth of breast (12) and pancreatic cancer cells. Thus, secreted klotho may be involved in mediating growth-inhibitory activities. Yet, the expression of klotho in the normal pancreas tissue surrounding the cancer cells does not impair cancer development. Possible explanations for this phenomenon can be reduced shedding of klotho from normal pancreatic cells or reduced penetrance of klotho to the tumor due to the presence of desmoplastic stroma. This stroma is typical to pancreatic cancer and is well-known to confer resistance to cancer therapies (36).

Several mechanisms may govern klotho growth-inhibitory activities. The IGF-1 pathway plays an important role in pancreatic cancer development (37, 38) and klotho is a potent inhibitor of the IGF-1 pathway in breast (12), lung (39, 40) and pancreatic cancers. Mechanisms other than inhibition of the IGF-1 pathway may mediate the growth-inhibitory activities of klotho in pancreatic cancer. A possible such mechanism is inhibition of the bFGF pathway (4). Klotho is an inhibitor of the bFGF pathway in HEK293 cells (12, 18, 19) and in pancreatic cancer cells (Fig. 3c and 4j). Moreover, modulation of the bFGF pathway by klotho and β-klotho has also been shown in breast (12) and hepatic cancers, respectively (41), pointing to bFGF as an important mediator of klotho activities. The ERK cascade is a downstream pathway of several signaling pathways, including the IGF-1 and bFGF
pathways. We observed only partial correlation between inhibition of ERK1/2 activation and inhibition of pancreatic cells proliferation by klotho. For example, while klotho only slightly inhibited bFGF-induced ERK1/2 in MiaPaCa2 (Fig 3c), it effectively inhibited their growth in vitro and in vivo. Thus, additional downstream targets of the IGF-1 and bFGF pathways may participate in mediating klotho growth inhibitory activities.

Our in vivo studies indicate klotho as a potential novel therapy against pancreatic cancer. As the in vivo activity was observed following treatment with either human or mouse klotho, produced and purified by different methods, the possibility of a non-specific effect is unlikely. Currently, there is no reliable and readily available method to determine klotho levels in the blood. Therefore, the pharmacokinetic properties of klotho could not be determined. Preliminary data using Western blotting indicate detectable levels of HA-tagged klotho in the blood six hours following intraperitoneal injection (data not shown). While low molecular mass plasma proteins of less than 60 kDa poses a short half life, larger proteins exhibit half-life of several days (42). For example, half life of injected antibodies may be as long as several weeks. It is therefore possible that the half lives of injected klotho (~125 kDa) or KL1 (~68 kDa) are relatively long. While we injected klotho five days a week, a less intense schedule of injection may also be effective.

Structure-function relationships of klotho have not been fully elucidated yet. Klotho is composed of two internal repeats, KL1 and KL2, which share amino-acid sequence homology to family 1 glycosidases and can be cleaved, shed and act as a circulating hormone. Cleavage can be mediated by ADAM10 and ADAM17, members of the A disintegrin and metalloproteinase (ADAM) family (27, 43). The cleavage products can be either a 130 kDa protein composed of both KL1 and KL2 or 68 kDa and 64 kDa proteins representing KL1 and KL2, respectively. Moreover, klotho gene can transcribe a secreted form of klotho by alternative RNA splicing encoding only the KL1 domain (13, 14, 23).
full-length klotho can function as a co-receptor for FGF23 (19, 44). Indeed, KL1 did not affect signaling by FGF23 in MDCT cells (Fig. 4k). Accordingly, we observed hypophosphatemia, which may be attributed to the activity of FGF23, in mice treated with klotho, but not in mice treated with the KL1 domain. These data are supported by a recent clinical observation, where genetic overexpression of klotho in a young girl resulted in hypophosphatemia attributed to abnormal regulation of FGF23 signaling (45). On the other hand, each domain can independently regulate cell-surface expression of TRPV5 and ROMK ion channels (46, 47), and we show here, for the first time, that KL1 is a potent inhibitor of bFGF and IGF-1 signaling. Taken together, these observations imply differential activities of klotho and KL1 toward different signaling pathways and in different cell lines. These findings may have implications regarding the ability to use klotho as a therapy. While KL1 may be as effective as the full-length protein for the treatment of cancer, it may have a better safety profile.

In summary, this study identified klotho as a potential tumor suppressor and indicated the full-length protein, as well as the KL1, as growth inhibitors and modulators of the IGF-1 and bFGF pathways in pancreatic adenocarcinoma. Klotho and KL1 are endogenous circulating hormones and their administration to mice was safe and effectively inhibited growth of pancreatic cancer cells in vivo. The role of klotho and KL1 as novel therapeutic approaches for the treatment of pancreatic adenocarcinoma, as well as other malignant diseases, should be further explored.
REFERENCES


FIGURE LEGENDS

Figure 1. Reduced klotho expression in pancreatic cancer.
(a) Immunohistochemistry staining of klotho in normal pancreatic tissues, left panel: x400 magnification, and zoom-in x2.5, right, showing high klotho expression. (b) Adenocarcinoma (x400 magnification, left and x1000 magnification, right), showing very low expression of klotho. (c,d) Klotho mRNA levels were determined by quantitative RT-PCR in (c) pancreatic cancer cell lines relative to the expression in normal pancreatic tissue and (d) pancreatic adenocarcinoma clinical samples and adjacent normal tissue.

Figure 2. klotho inhibits growth of pancreatic cancer cells.
(a) Pancreatic adenocarcinoma cell lines and the NET cell line BON1 were transfected with either a HA-klotho expression vector (pcDNA3-HA-KL) or an empty vector (pcDNA3). Transfected cells were cultured in media containing G418 for two weeks. Colonies were stained with crystal violet and photographed. (b) Panc1 cells were plated in 96-well plates and treated with either soluble mouse klotho (mKL), human klotho (hKL) or a vehicle control. Viability was assessed after 48 hrs using MTT assay. Data are shown as mean ± s.d. of three independent experiments. * p<0.005 and ** P<0.05 for comparison between treated cells vs. control. (c) soft agar assay with colony counts plotted (below). Numbers reflect one representative experiment done in triplicate. The experiment was repeated thrice with the same results. Representative image of a soft agar assay done with Panc1 cells treated with hKL (left) or control vehicle (right). Klotho treatment (0.8 pM) dramatically suppressed anchorage independent growth. Quantitative summary of the result of a representative.

Soluble klotho increases sensitivity of pancreatic cancer cells to 5-FU: (d) Panc1 (e) MiaPaCa2 and (f) Colo357 cells were treated with either mKL, 5-FU, their combination or a control vehicle. (g) Panc1 and (h) MiaPaCa2 cells were treated with either mKL,
Gemcitabine, their combination or a control vehicle. MTT assay was conducted at 48 hrs. Data are shown as mean ± s.d. of three independent experiments. * p<0.05 for comparison between treated cells and control, and (**) between klotho and chemotherapeutic drug vs. drug alone. (i,j) Panc1 cells were injected into both flanks of female athymic nude mice. The mice (N=5 per group) were treated with daily intra-peritoneal injections of either mKL (10 μg/kg or 25 μg/kg) or vehicle control (saline), for 4 weeks. Mice weight (i) and tumor size (j) were monitored weekly. (p<0.05, for tumor volume between treated and control groups by the end of the study).

**Figure 3. Down-regulation of the IGF-1 and FGF pathways by klotho in pancreatic cancer cells.** (a) Cells were transfected with either HA-klotho expression plasmid (HA-KL) or a control vector (pcDNA3); after 24 hrs, cells were serum starved for 48 hrs and treated with IGF-1. Following treatment, cells were harvested and proteins were resolved and immunoblotted using antibodies as indicated. (b) Panc1 cells were starved for 48 hrs, treated with klotho (10min) followed by IGF-1 stimulation. Expression and phosphorylation of the IGF-1R, AKT and ERK1/2 were determined. (c) Cells were transfected as detailed in (a) starved for 48 hrs and stimulated with bFGF. Cell lysates were immunoblotted as indicated. Experiments were repeated at least three times, band intensities were quantified, and fold increase is specified for each treatment, relative to pcDNA3 without bFGF treatment and SD was calculated. * P<0.05.

**Figure 4. KL1 inhibits growth of pancreatic cancer cells.** (a) Panc1 cells were transfected with HA-KL, HA-KL1 or pCDNA3 and colony assay was performed as in Fig 2a. (b) Viability assay for Panc1 cells treated with either soluble hKL or hKL1. Viability was assessed after 48 hrs using MTT assay. Data are shown as mean ± s.d. of three independent experiments. * p<0.03 for comparison between treatment and control. (c-f) Panc1 cells were injected subcutaneously into the right flank of female athymic nude
mice. The mice were treated with daily intra-peritoneal injections of either 2.5 μg/kg hKL, 10, 25 μg/kg hKL1 or vehicle control (saline), for 5 weeks. 12 mice were used in the control group and 6-7 in the klotho treated groups. Mice weight (p<0.05 for tumor volume in control vs. klotho treatment by the end of the study) (c) and tumor size (d) were monitored weekly (e,f) and tumors weight was measured on day of sacrifice. p<0.05 for comparison of tumor weight between the control group and the mice treated with klotho.  (g) H&E and Ki67 immunostaining (X400 magnification).  (h) MiaPaCa2 cells were injected into both flanks of athymic mice and treated with daily intra-peritoneal injections of either 2.5 μg/kg hKL, 10 μg/kg hKL1 or vehicle control, for 5 weeks 8 mice per group (16 tumors). Tumor size (h) was monitored weekly and tumors weight (i) was measured on day of sacrifice. p<0.05 for comparison of tumor weight between the control group and the mice treated with klotho.  (j) Panc1 cells were transfected as described above, and stimulated with IGF1 or bFGF. Cell lysates were immunoblotted with indicated antibodies. Phosphorylated to total ratio is calculated relative to pCDNA.  (k) MDCT cell line was transfected as described above and stimulated with FGF23. Cell lysates were immunoblotted with phospho and total ERK1/2.
Figure 1
Table 1
klotho expression in normal pancreas and pancreatic adenocarcinoma samples.

<table>
<thead>
<tr>
<th>Klotho expression</th>
<th>Normal Pancreas (N=5)</th>
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<tr>
<td>Low</td>
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<td>6</td>
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<td>Absent</td>
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Figure 1
Figure 2

a. Ha-klotho expression in different cell lines.

b. Viability of cells treated with different concentrations of mKL and hKL.
Figure 2
Figure 2
<table>
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<tr>
<th>Weeks</th>
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<th>mKL 25</th>
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<tr>
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**Figure 2**
### a.

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<td>pcDNA3</td>
<td>HA-KL</td>
<td>pcDNA3</td>
<td>HA-KL</td>
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<td>+</td>
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### b.

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<td>p-ERK1/2</td>
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**Figure 3**
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Panc-1

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MiaPaCa2

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Colo357

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Figure 3
Figure 4
Figure 4
h. 

![Graph showing weight (gr) over weeks for control, hKL, and hKL1 treatments.]

i. 

<table>
<thead>
<tr>
<th>Treatment</th>
<th>control</th>
<th>hKL</th>
<th>hKL1</th>
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</thead>
<tbody>
<tr>
<td>N tumors (%)</td>
<td>16 (100%)</td>
<td>14 (88%)</td>
<td>12 (75%)</td>
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<tr>
<td>Tumor weight (mg ± SD)</td>
<td>513 ± 304</td>
<td>226 ± 201</td>
<td>127 ± 93</td>
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</table>

Figure 4
**Figure 4**

**j.**

<table>
<thead>
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<td>IGF1</td>
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<tr>
<td>p/t</td>
<td>1</td>
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**pAKT**

**tAKT**

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<td>bFGF</td>
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<tr>
<td>p/t</td>
<td>1</td>
<td>0.82</td>
<td>0.59</td>
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**pERK1/2**

**tERK1/2**

**k.**

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<td>FGF23</td>
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<td>p/t</td>
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**pERK1/2**

**tERK1/2**
Table 2
Comparison between blood chemistry levels of the control and mice treated with hKL (2.5 µg/kg) or hKL1 (10 µg/kg).

<table>
<thead>
<tr>
<th></th>
<th>Control (2.5 µg/kg)</th>
<th>hKL (10 µg/kg)</th>
<th>P value</th>
<th>hKL1 (10 µg/kg)</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(C vs. hKL)</td>
<td>(C vs. hKL1)</td>
<td></td>
<td>(C vs. hKL1)</td>
<td></td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>54 ± 8</td>
<td>39 ± 7</td>
<td>0.014</td>
<td>44 ± 3</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>172 ± 14</td>
<td>129 ± 12</td>
<td>0.001</td>
<td>162 ± 17</td>
<td>0.3</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>172 ± 14</td>
<td>129 ± 12</td>
<td>0.001</td>
<td>162 ± 17</td>
<td>0.3</td>
</tr>
<tr>
<td>Phosphorous (mg/dl)</td>
<td>8 ± 1</td>
<td>5 ± 1</td>
<td>0.004</td>
<td>8 ± 2</td>
<td>0.99</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>103 ± 9</td>
<td>78 ± 8</td>
<td>0.003</td>
<td>106 ± 12</td>
<td>0.8</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.26 ± 0</td>
<td>0.25 ± 0</td>
<td>0.5</td>
<td>0.31 ± 0</td>
<td>0.03</td>
</tr>
<tr>
<td>Bilirubin (mg/dl)</td>
<td>0.1 ± 0</td>
<td>0.1 ± 0</td>
<td>0.9</td>
<td>0.1 ± 0</td>
<td>0.5</td>
</tr>
<tr>
<td>Uric acid (mg/dl)</td>
<td>3.40 ± 1.22</td>
<td>3.46 ± 0.3</td>
<td>0.9</td>
<td>5 ± 1</td>
<td>0.1</td>
</tr>
<tr>
<td>SGOT (ATS) (IU/l)</td>
<td>426 ± 225</td>
<td>384 ± 258</td>
<td>0.8</td>
<td>416 ± 290</td>
<td>0.96</td>
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<tr>
<td>SGPT (ALT) (IU/l)</td>
<td>70 ± 39</td>
<td>57 ± 39</td>
<td>0.61</td>
<td>103 ± 70</td>
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<tr>
<td></td>
<td>89 ± 17</td>
<td>106 ± 19</td>
<td>0.08</td>
<td>91 ± 15</td>
<td>0.8</td>
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</tbody>
</table>

* Glucose (mg/dl)

* glucose values of mice after fasting from experiment shown in figure 4h-i.
KL1 internal repeat mediates klotho tumor suppressor activities and inhibits bFGF and IGF-1 signaling in pancreatic cancer

Lilach Abramovitz, Tami Rubinek, Hagai Ligumsky, et al.

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