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[9]. Laboratory animals that do not express Klotho exhibit a shorter lifespan and cognitive impairment.

In contrast, mice that overexpress Klotho have a longer lifespan and enhanced cognition and memory [10]. Klotho significantly inhibited the growth of lung cancer [11], pancreatic carcinoma [12], colorectal carcinoma [13], breast cancer [14], hepatocellular carcinoma [15], ovarian carcinoma [16], melanoma [17], diffuse large B cell lymphoma [18]. Several molecules, such as PPARγ agonists [19], testosterone [20], and resveratrol [21], either directly promote Klotho expression in vitro or inhibit Klotho regulation in vivo. Jung et al reported a novel molecular mechanism by which a small molecule (N-(2-chlorophenyl)-1H-indole-3-caboxamid) induces Klotho expression [22].

Other studies [23] have used CRISPR methodology to upregulate Klotho transcription and production in two different cell lines, one of which was a neuron-like cell line. Pharmaceutical companies have been developing Klotho agonists that up-regulate Klotho expression and are of significant interest in treating diseases. For example, Klotho Therapeutics has developed a patent-pending treatment based on Klotho that affects aging [24]. Kleenex has been working on a two-pronged approach to target endogenous Klotho's natural production and regulation and deliver Klotho genetic material directly to patient cells, thus enabling them to produce the protein [25].

Table 1: List of diseases associated with low levels of Klotho

Acetylcholine and Nitric Oxide Dysregulation	Hypertension
Aging (highly accelerated)	Impaired Cognition (such as Alzheimer's Disease)
All-Cause Mortality	Hyperphosphatemia
Anemia	Lung Damage
Anorexia	Multiple System Atrophy
Atherosclerosis (as well as calcification of the arteries)	Pseudo exfoliation Syndrome
Bone Loss (such as osteoporosis and low bone mass)	Rheumatoid Arthritis
Cancers Bone Brain Breast Colon Stomach Kidney Liver	Sarcopenia Skin Atrophy (such as scleroderma) Stroke Vascular Disease (such as coronary artery disease) Hyperparathyroidism Inflammatory Bowel Disease

Previously, we showed that treatment of the pancreatic cancer cell lines PANC1, MIA-PACA, and COLO-205, combined with Metadichol, a lipid emulsion consisting of long-chain alcohols at 1-100 pg/mL concentrations, resulted in a 4- to 15-fold increase in Klotho expression as determined by qRT-PCR [26]. In the present study, we have extended our original work to determine the effects of metadichol in many other cell lines, including primary cancer cell lines, stem cell lines, and fibroblasts (Figure1).

Methods

Experimental procedures

This work was carried out by a service provider: Skanda Life Sciences, Bangalore, India.

Chemicals and reagents

The A549, Colo-205, PANC1, MDAMB31, Hela, HepG2, and human cardiac fibroblast cells were purchased from the ATCC (USA). Primary breast cancer cells were obtained from BIOIVT (Detroit, Michigan, USA). Primary antibodies were purchased from AB clonal (Woburn, Massachusetts, USA) and E-lab Science (Maryland, USA). Primers were obtained from Saha Gene, Hyderabad, India (Table 2). All other molecular biology reagents were purchased from Sigma Aldrich.

Cell line maintenance and seeding

The cells were cultured in a suitable medium with or without supplements in the presence of 1% antibiotics in a humidified atmosphere of 5% CO_2 at 37°C. The medium was changed periodically until the cells reached confluency. Cell survival was determined using a hemocytometer. When the cells reached 70–80% confluency, single-cell suspensions were seeded into 6-well plates at a density of 10^6 cells per well and incubated for 24 h at 37°C in 5% CO_2. Afterward, we rinsed the cell monolayer with a serum-free medium and added metadichol at predefined concentrations.

Cell treatment

Metadichol was prepared at various concentrations (1 pg/mL, 100 pg/mL, one ng/mL, and 100 ng/mL) in serum-free media, and the mixture was added to predesignated wells. The control cells received media without drugs. The cells were incubated, then gently rinsed with sterile phosphate-buffered saline (PBS) solution. Quantitative RT-PCR (qRT-PCR) and western blot analysis were done as described below.

RNA isolation

RNA was isolated from each treatment group using TRIzol reagent (Invitrogen). Cells (10^6) were collected into 1.5-mL microcentrifuge tubes and centrifuged at 5,000 rpm for 5 min at 4°C. Then, 650 µL of TRIzol was added to the pellet, mixed, and incubated on ice for 20 min. Subsequently,

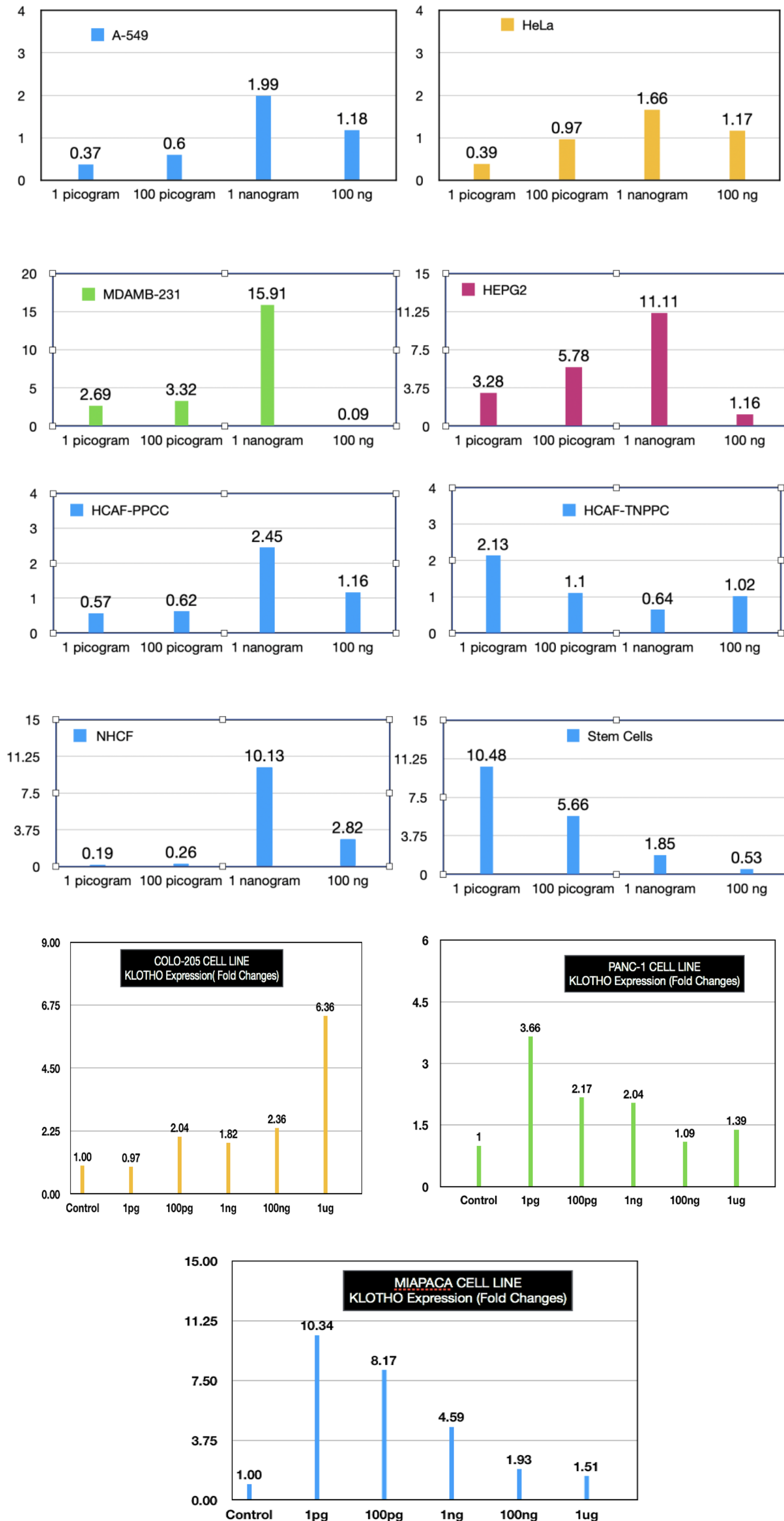

Figure 1: Klotho fold expression changes in various cell lines

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Table 2: Primers used for qPCR

Gene	Primer pair	Sequence	Tm	Product size (base pairs)
β–Actin	FP	TCCTCCTGAGCGCAAGTACTCT	62.1	153
	RP	GCTCAGTAACAGTCCGCCTAGAA	62.4	
KLOTHO	FP	GGGAGGTCAGGTGTCCATTG	55.88	152
	RP	TGCTCTCGGGATAGTCACCA	53.83	

we added 300 µL of chloroform, mixed the samples with gentle inversion for 1–2 min, and set them on ice for 10 min. The samples were centrifuged at 12,000 rpm for 15 min at 4°C. The upper aqueous layer was transferred to a new sterile 1.5 mL centrifuge tube, and an equal amount of prechilled isopropanol was added. The samples were incubated at −20°C for 60 min, then centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was carefully removed, and the RNA pellet was washed with 1.0 mL of 100% ethanol and 700 µL of 70% ethanol by centrifugation as previously described. The RNA pellet was air-dried at room temperature for approximately 15–20 min and resuspended in 30 µL of DEPC-treated water. RNA concentration was measured using a Spectra drop (Spectramax i3x, USA) spectrophotometer (Molecular Devices).

cDNA synthesis

Complementary DNA (cDNA) was synthesized from 2 µg of total RNA using the Prime Script cDNA synthesis kit (Takara) with oligo dT primers following the manufacturer's instructions. The reaction volume was 20 µL, and cDNA synthesis was performed at 50°C for 30 min, then incubated at 85°C for 5 min using an Applied Biosystems instrument (Veritii). The resulting cDNA was used as a template for qPCR.

Primers and qPCR

A final reaction volume of 20 µL of PCR mixture was prepared, consisting of 1 µL of cDNA, 10 µL of SYBR green Master Mix, and one µM complementary forward and reverse gene-specific primers. The samples were run under the following conditions: initial denaturation at 95°C for 5 min, followed by 30 cycles of secondary denaturation at 95°C for 30 seconds, annealing at the optimized temperature for 30 seconds, and extension at 72°C for 1 min. We defined the number of cycles that allowed amplification in the exponential range without reaching a plateau as the optimal number of cycles. The results were obtained using CFX Maestro software. We calculated fold-change using the comparative CT method and determined the relative expression of each target gene relative to a housekeeping gene (β-actin) and untreated control cells. ΔCT was calculated for each treatment using the following formulas: ΔCt = Ct (target gene)–Ct (reference gene), ΔΔ, Ct = ΔCt (treatment group)–ΔCt (control group). The fold change was calculated for target gene expression for each treatment using the formula: Fold change = 2^ (−ΔΔCt).

Protein isolation and western blot analysis

Total protein was isolated from 10^6 cells using radioimmuno precipitation assay buffer supplemented with the protease inhibitor phenylmethyl sulfonyl fluoride. The cells were lysed for 30 min at four °C with gentle inversion, centrifuged at 10,000 rpm for 15 min, and the supernatant was transferred to a new sterile tube. The Bradford method (BIO-RAD USA) was used to measure the protein concentration. Protein (25 µg) was mixed with 1X sample loading dye containing SDS and loaded onto a polyacrylamide gel. The proteins were separated under denaturing conditions using a Tris-glycine running buffer. The proteins were transferred to PVDF membranes (Invitrogen) using a Turbo transblot system (Bio-Rad, USA), blocked with 5% BSA for one h, incubated with the respective primary antibody overnight at four °C, followed by a species-specific secondary antibody for one h at RT. After washing, the membranes were incubated with ECL substrate (Merck) for 1 min in the dark. The images at suitable exposure settings were captured using the ChemiDoc XRS system (Bio–Rad, USA).

Discussion

The highest Klotho expression was observed in MDAMB-231 (Triple-Negative Breast Cancer cells developed at MD Anderson Institute Houston), followed by stem cells, then cardiac fibroblasts (NHCFs) (Figure 1). Although the expression of Klotho induced by small molecules is elevated in kidney cells [27], we found that Metadichol increased Klotho expression in many different cell types and thus may represent a universal Klotho agonist. Because Klotho exhibits anticancer activity, its level of expression in cancer cells is significant. Down regulation of Klotho has been observed in several cancers, such as pancreatic cancer, HCC, and others [28, 29]. Epigenetic modulation, such as promoter methylation and histone deacetylation, also contributes to the dysregulation of Klotho in cancer. Forester et al (30, 31). Suggested that the liganded vitamin D receptor (VDR) upregulates Klotho via vitamin D response elements (VDRE). Metadichol is a VDR inverse/protein agonist [32, 33]. Among the consequences of the enhanced expression of Klotho is an increase in telomerase activity. Similarly, Metadichol can upregulate telomerase [34] and thus potentially prevent stem cell aging [35]. In addition, the nuclear receptor PPAR gamma regulated Klotho expression. [35]. We have recently

shown that Metadichol increases PPAR gamma expression 2-3-fold, increase in Stem and human dermal Fibroblast cells [36].

Consequently, the downregulation of Klotho enhances proliferation and reduces apoptosis in cancer cells. Conversely, the over expression of Klotho results in cancer cell inhibition [37-40]. Similarly, our results suggest that metadichol could be useful in inducing apoptosis in cancer cells by increasing Klotho expression, which also has benefits in other diseases. The current study and previously published results [41-44] suggest that the observed results could have been due to increased Klotho expression [45]. The potential therapeutic utility of metadichol in elevating Klotho levels warrants further study in vitro and in vivo.

Abbreviated Cell Lines Description

PANC1: human pancreatic cancer cell line isolated from a pancreatic carcinoma of ductal cell origin

HCAF-PPCC: (Human Cancer-associated Fibroblast primary prostate cancer cell.

MIA-PACA: Human pancreatic cancer cell line

HCAF-TNBCC; Human Cancer-associated Fibroblasts Triple Negative Breast Cancer Panel

COLO-205: The cell line is made up of epithelial cells isolated from ascitic fluid derived from a 70-year-old male with colon cancer.

A-549: Adenocarcinomic human alveolar basal epithelial cell

HEPG2: human liver cancer cell line

HELA: Immortalized cell line used in scientific research. It is the oldest and most commonly used human cell line. The line is derived from cervical cancer cells

hESC BGO1V: cells are pluripotent and can differentiate to representatives of the three primary germ layers.

MDAMB 231: isolated at M D Anderson Institute, Houston, USA, from a pleural effusion of a patient with invasive ductal carcinoma) is commonly used to model late-stage breast cancer

Author Contributions

All work was planned and supervised by the author (PPR), who is solely responsible for its content.

Conflicts of Interest: None

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Competing Interests: None

Availability of data and materials

All data are in the manuscript and the supplementary material provided.

Supplementary material: Western Blot data and Klotho Diseases Network

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