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Klotho RNAi induces premature senescence of human cells via a p53/p21 dependent pathway

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Abstract **Klotho has recently emerged as a regulator of aging. To investigate the role of Klotho in the regulation of cellular senescence, we generated stable MRC-5 human primary fibroblast cells knockdown for Klotho expression by RNAi. Downregulation of Klotho dramatically induces premature senescence with a concomitant upregulation of p21. The upregulation of p21 is associated with cell cycle arrest at G1/S boundary. Knockdown of p53 in the Klotho attenuated MRC-5 cells restores normal growth and replicative potential. These results demonstrate that Klotho normally regulates cellular senescence by repressing the p53/p21 pathway. Our findings implicate Klotho as a regulator of aging in primary human fibroblasts.**

Keywords: Klotho; Senescence; Aging; p53; Human lung fibroblasts; Insulin/IGF-1 signaling

1. Introduction

Aging is a fundamental process, affecting all organisms from yeast to mammals. The aging process can be regulated by multiple genetic pathways that have emerged as conserved among evolutionary distinct species. One of many proteins that have been implicated in the regulation of aging is Klotho.

Overexpression of Klotho extends mouse lifespan by 20% while loss of Klotho function (kl/kl$^{-/-}$) results in the accelerated onset of progeriod traits [1,2]. kl/kl$^{-/-}$ mice manifest infertility, arteriosclerosis, skin atrophy, osteoporosis, and other age-associated infirmities three to four weeks after birth [2]. Soon thereafter, kl/kl$^{-/-}$ mice exhibit growth retardation and gradually become inactive; dying prematurely around two month of age [2].

Mouse Klotho is expressed in the distal renal tubules and in the choroid plexus of the brain; existing in both a membrane-bound and secreted form [2–4]. Membrane bound Klotho is a single-pass transmembrane protein containing an N-terminal signal sequence, an extracellular domain containing repeated sequences (KL1 and KL2), and a short intracellular domain [4]. Secreted Klotho, lacks the KL2 sequence, transmembrane and intracellular domains, and arises as a consequence of alternative splicing [4]. Functionally, Klotho overexpression in mice has been shown to attenuate the insulin/insulin growth factor-1 (IGF-1) 1 signaling pathway [1]. It has been shown that Klotho increases resistance to oxidative stress both in vitro and in vivo [5]. Klotho also regulates calcium metabolism by hydrolyzing extracellular sugar residues on the transient receptor potential ion channel TRPV5, entrapping the channel in the plasma membrane [6].

Human Klotho is mainly expressed in the kidney; it is also detectable in small intestines, hippocampus, placenta and prostate. With the secreted form being the predominant form of the protein [3,4]. Reduced production of this protein has been observed in patients with chronic renal failure. Klotho has also been implicated in human aging. Single nucleotide polymorphisms in the human Klotho gene are associated with altered lifespan, osteoporosis, stroke and altered risk for coronary artery disease [7–12]. The molecular basis of these effects is yet to be determined.

Non-transformed animal cells have a finite proliferative capacity in culture, known as the Hayflick limit, that results in irreversible proliferative arrest named cellular senescence [13]. Phenotypic changes of senescent cultured cells include enlargement, flattened morphology, expression of acidic β-galactosidase (β-Gal) and a permanent cell cycle arrest at G1 [14]. The pro-apoptotic protein p53 regulates the transcription of genes involved in cell-cycle arrest and apoptosis and is a well-characterized regulator of cellular senescence [15]. Transient or irreversible p53-mediated cell cycle arrest in the transition from the G1 to S phase of cell replication is mediated by the transcriptional activation of the cyclin-dependent kinase (CDK) inhibitors such as p16 and p21 [16]. As cells exhaust their replicative potential, increased p53 activity activates CDK's, triggering growth arrest and senescence [17].

In this study, we have sought to determine if Klotho regulates human cellular senescence and to elucidate the molecular pathway of its function in human cells. We find that reduction of Klotho expression by RNAi induces premature senescence of primary human fibroblasts.

2. Materials and methods

2.1. Cell culture

MRC-5 and WI-38 cells supplemented with fetal bovine serum (FBS; 10% v/v), L-glutamine (2 mM), penicillin (100 U/ml), and

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Abbreviations: IGF-1, insulin growth factor-1; CDK, cyclin-dependent kinase; β-gal, β-galactosidase; IRS1, insulin receptor substrate 1; HDF, human dermal fibroblasts

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streptomycin (100 μg/ml). 293T cells were grown in DMM medium, supplemented with FBS (10% v/v), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were cultured at 37 °C in a humidified incubator containing 5% CO_2 (g).

2.2. β-Gal staining

Cells for β-Gal staining were plated in six well plates. One day after plating, cells were washed in PBS, fixed for 5 min in 2% formaldehyde/0.2% glutaraldehyde. Fixed cells were washed with PBS and incubated at 37 °C with senescence associated 6-Gal staining solution (1 mg/ml of X-Gal, 40 mM citric acid, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$, pH 6.0). Staining was detected by light microscopy following an overnight incubation.

2.3. Plasmids

RNAi retroviral plasmids were constructed using the plasmid pSUPER.retro (OligoEngine, Seattle, WA). The RNAi sequences used were: Klotho (1) 5′ aagttacaaatagttctgaac 3′ and Klotho (2) 5′ aaggaatatgacatacagtgc 3′. The p53 RNAi was obtained from Dr. R. Agami (Amsterdam, The Netherlands).

2.4. Viral production

Viral stocks were obtained by co-transfecting 293T cells with the pSUPER.retro, VSV-G and pCMVC3 using Fugene 6 (Roche Applied Science) transfection reagent. Retroviruses were harvested 48 h post transfection. Stable MRC-5 cells expressing RNAi were obtained by viral infection performed in the presence of polybrene and selection with puromycin. The third day after infection was designated day zero in all of the experiments.

2.5. Lifespan assay

Proliferative lifespan was determined by growing cells continuously until senescence. Every five days cells were assayed for cell number and visual morphology and subsequently sub-cultured with exactly 3×10^5 cells. The number of population doublings (PDL) for each culture was determined by the formula $(\log N - \log N_0)/\log 2$, where N is the cell number at each five day interval and N_0 is the cell number at the time of seeding (3×10^5).

2.6. Western blots

Cells were lysed in protein sample buffer, vortexed, boiled for 10 min, and centrifuged at $17000 \times g$ for 5 min. Proteins concentrations were determined by Bio-Rad DC protein assay kit (Bio-Rad).

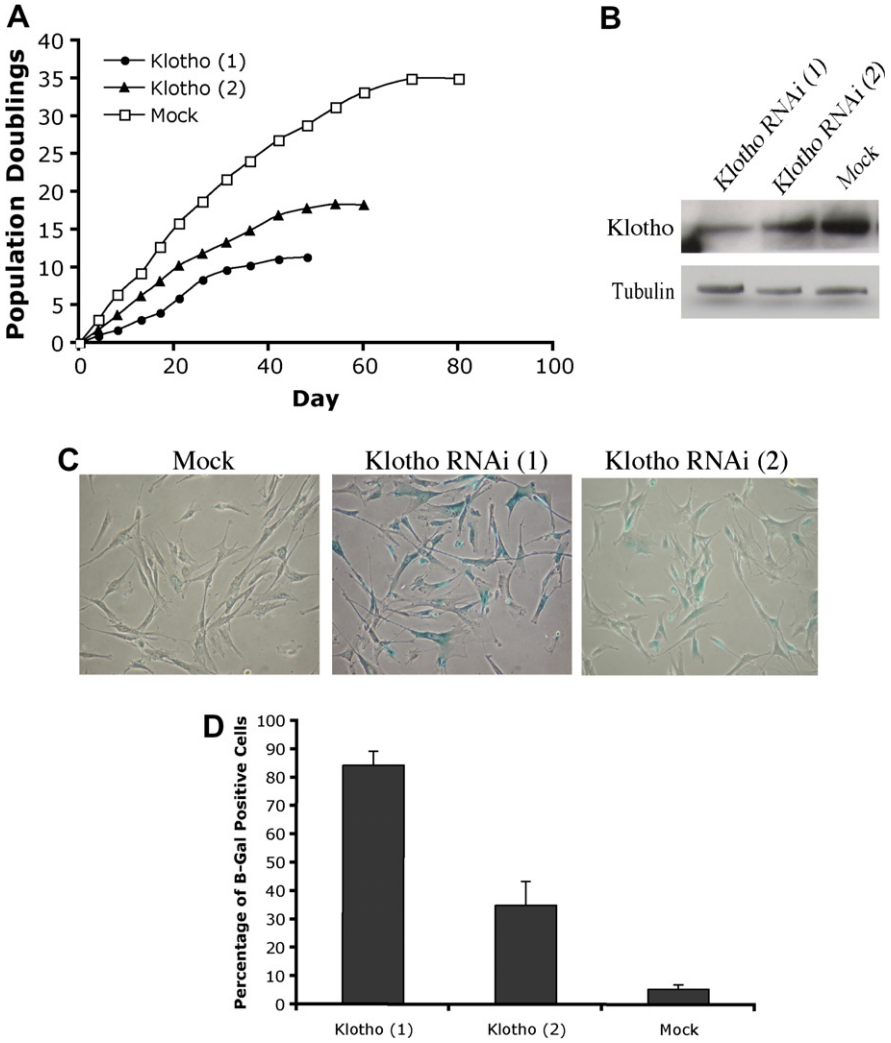

Fig. 1. Klotho RNAi triggers senescence (A) Human lung fibroblasts cells (MRC5) infected with pSUPER (Mock) or Klotho RNAi were selected with puromycin. Cells were then passage until they underwent senescence, and the number of cumulative population doubling was determined. Similar results were obtained from three independent experiments. (B) Whole cell lysates of each-infected population (processed as in (A)) were examined by Western blot for expression of Klotho. Tubulin was used as a loading control. (C) Senescence associated β-galactosidase staining in MRC5 cells infected with pSUPER or Klotho RNAi. The photographs were taken at day 8 of lifespan. (D) Positive β-galactosidase cells (from B) were counted in three random fields and the standard deviation is indicated.