Supporting Online Materials

Suppression of aging and insulin/insulin-like growth factor-1 signaling by the hormone Klotho

Hiroshi Kurosu, Masaya Yamamoto, Jeremy D. Clark, Johanne V. Pastor, Animesh Nandi, Prem Gurnani, Owen P. McGuinness, Hirotaka Chikuda, Masayuki Yamaguchi, Hiroshi Kawaguchi, Iichiro Shimomura, Yoshiharu Takayama, Joachim Herz, C. Ronald Kahn, Kevin P. Rosenblatt, & Makoto Kuro-o

Materials and Methods

Mice. Generation of $KL^{-/-}$, $KL^{+/-}$, and $KL^{+/+}$ mice and transgenic mice that overexpress Klotho (*EFmKL46* and *EFmKL48*) was described previously (*1*). Briefly, $KL^{-/-}$ mice were generated by crossing $KL^{+/-}$ mice carrying an insertional mutation at 5' promoter region of the *klotho* gene. Transgenic mice (*EFmKL46* and *EFmKL48*) were generated by microinjection of a human elongation factor 1 promoter and membrane form of a mouse Klotho cDNA fusion gene (EFmKL) into fertilized mouse eggs obtained from C57BL/6 x C3H F1 females mated with C3H males. To generate $KL^{-/-}$ $IRS\text{-}1^{+/-}$ mice, male $IRS\text{-}1^{-/-}$ mice (*2*) were crossed to female $KL^{+/-}$ mice to obtain $KL^{+/-}$ $IRS\text{-}1^{+/-}$ mice. These mice were crossed to each other to obtain $KL^{-/-}$ $IRS\text{-}1^{+/-}$ mice and their littermate controls ($KL^{-/-}$ $IRS\text{-}1^{+/+}$ mice).

All the mice were housed under a specific-pathogen free condition during the study. Sentinel mice sera were tested every two months for antibodies against MHV, MVM, PVM, EDIM, MPV, Reo-3, GD-7, Sendai virus, LCMV, MAV, ECTRO, K, POLY and M. pulmonis and confirmed as seronegative. All sentinel mice were also tested for the internal and external

parasites (pinworm and fur mite) and confirmed as negative. All the mice were fed *ad libitum* on a standard diet and housed in a room with a time-controlled lightening system set for 12 hours dark (6PM-6AM) and 12 hours light (6AM-6PM). Temperature and humidity set points were 72°F and 50%, respectively. All animal experiments were approved by the Institutional Animal Care and Research Advisory Committee of the University of Texas Southwestern Medical Center at Dallas or Vanderbilt University School of Medicine.

Physiological analysis. All data of physiological studies were compared between age- and sex-matched wild-type mice and transgenic mice that overexpress Klotho (*EFmKL46* and *EFmKL48*) by ANOVA.

Insulin and IGF-1 tolerance tests: Human insulin (Humulin R, Ely Lilly, 0.5 U/kg) or IGF-1 (a kind gift from Genentech, 0.75 mg/kg) was administered into 5-h fasted 20-week-old mice by intraperitoneal injection. Blood glucose was measured immediately before the injection and at 15, 30, 60 minutes after the injection using One Touch Basic Meter (Lifescan).

Hyperinsulinemic euglycemic clamp: Hyperinsulinemic euglycemic clamp was performed using conscious mice with catheters chronically implanted in the jugular vein and carotid artery as previously reported (*3*). Briefly, mice were continuously infused with insulin (4 mU/kg/min) and their glucose levels were monitored in arterial samples every 5-10 minutes. Glucose (0.5 g/ml) was infused in the jugular vein catheter at rates necessary to maintain euglycemia, based on feedback from arterial glucose measurements.

Food intake: Food intake was assessed using an automated feeding apparatus that continuously measured feeding behaviour for 24 hours by allowing animals free access to food cups that were mounted on balances.

Oxygen consumption: Whole body oxygen consumption was measured continuously for 24 hours in conscious mice using the Oxymax System (Columbus Instruments).

Production and purification of recombinant Klotho protein. Soluble Klotho protein was generated using Drosophila Expression System (Invitrogen). A cDNA fragment encoding the entire extracellular domain of mouse Klotho protein (amino acid number 31-982, gil2618594) was inserted into pMT/BiP/V5-His expression vector, allowing inducible expression from the metallothionein promoter and secretion of the recombinant protein with V5 and 6xHis tags at the C-terminus into conditioned medium. The expression vector was transfected into Drosophila Schneider cells by the calcium phosphate method together with pCoHYGRO selection vector. Stable transformants were selected with hygromycin B and cloned by limiting dilution to obtain clones with high expression levels. Expression of soluble Klotho protein was induced in serum-free medium containing 1 mM $CuSO_4$ for 14 days. Klotho protein secreted in the conditioned medium was purified by affinity column chromatography using anti-V5 antibody (Sigma). Purified Klotho protein and known concentrations of albumin were subjected to SDS-PAGE followed by Sypro Ruby staining (Molecular Probe) to check the concentration and purity of the protein.

Glucose uptake. L6 cells cultured to confluence in 12-well plates were incubated in DMEM medium with 2% fetal bovine serum for 48 hours. The cells were treated with or without 10 nM insulin and 100 pM Klotho at 37°C for 20 min and then incubated with the further addition of D-[2-^{3}H]-Glucose (1.5 □Ci, 0.1 mM, Amersham Biosciences) for 10 min. The reaction was terminated by washing the cells three times with ice-cold PBS. Cell-associated radioactivity was determined by lysing the cells with 0.05 N NaOH, followed by liquid scintillation counting.

Iodine labeling of Klotho protein. Iodine labeling of purified Klotho protein was performed using IODO-GEN (Pierce) and $Na^{125}I$ (Amersham Biosciences) according to the protocols provided by the manufacturers. Specific activity of [^{125}I] Klotho was determined by trichloroacetic acid precipitation.

Quantitative ligand binding assay to cultured cells. H4IIE cells and L6 cells cultured to confluence in 12-well-plates with DMEM containing 10 % FBS were starved in serum-free DMEM for overnight. The wells were washed with binding buffer (PBS containing 0.5% BSA) and then incubated for 60 minutes at room temperature with [^{125}I] Klotho or [^{125}I] Insulin (Amersham) or [^{125}I] IGF-1 (Amersham) in binding buffer. The cells were rinsed 3 times with ice-cold binding buffer, lysed in 0.2 ml of lysis buffer (PBS containing 1% NP-40 and 1% Triton X-100), and counted in a scintillation counter. Specific binding was determined by taking the difference for assays with only labeled ligand (total binding) and assays that also contained unlableled ligand in excess of 100-fold of the labeled ligand (non-specific binding).

Production of anti-Klotho antiserum. Anti-Klotho antiserum was produced by immunizing rabbits with a part of mouse Klotho protein (amino acids 55-261) expressed in *E. coli* and tested by immunoblot analysis for the ability to recognize the recombinant Klotho protein. Specificity of the antiserum to Klotho protein was further confirmed by immunoblot analysis using mouse kidney membrane fraction. Briefly, kidney from wild-type mice and *KL*$^{-/-}$ mice were homogenized in 0.25% sucrose with Polytrone and centrifuged at 800 x g for 5 minutes. The supernatant was centrifuged at 40,000 x g for 2 hours and the pellet was suspended in PBS. The kidney membrane fraction was subjected to SDS-PAGE, transferred to a nitrocellulose membrane (Hybond-C, Amersham), and incubated with the antiserum. The membrane was washed with TBS containing 0.1% Tween 20, incubated with anti-rabbit IgG

conjugated with horseradish peroxidase (Amersham), and detected with SuperSignal West Dura system (Pierce). A single band at 130 kDa corresponding to the endogenous membrane form of Klotho protein was detected in the wild-type mouse kidney, whereas no signal was detected in the *KL*$^{-/-}$ mouse kidney, indicating that the antiserum specifically recognized endogenous Klotho protein as well as the recombinant Klotho protein.

Immunoblot analysis using anti-Klotho antiserum. Mouse plasma (3 μl) was subjected to SDS-PAGE and transferred onto a PVDF membrane (Immobilon-FL, Millipore). The membrane was probed with the anti-Klotho antiserum and detected by biotinylated anti-rabbit IgG (BA-1000, Vector Laboratories), a tyramide-based avidin/biotin system (PerkinElmer), and Quantum dots (Qdot 655 nanocrystals, Quantum dots Corporation).

Radioimmunoassay. Plasma (10 μl) or recombinant Klotho protein of known concentration was incubated with the anti-Klotho antiserum (final dilution of 1:256,000) in 75 μl of RIA buffer (PBS containing 0.5% bovine serum albumin and 5 mM EDTA) at 4 °C. After 20-h incubation, [^{125}I] Klotho (120 pg in 25 μl of RIA buffer) was added. After additional 22-h incubation, 100 μl of anti-rabbit IgG goat serum and 100 μl of carrier rabbit IgG (Secondary Antibody Precipitating System; Linco Research) were added. After 2-h incubation, bound [^{125}I] Klotho was separated from free [^{125}I] Klotho by centrifugation at 3,000xg for 30 minutes and counted in a scintillation counter. The minimal detectable quantity was 0.5 fmol/tube. Plasma Klotho protein in *KL*$^{-/-}$ mice was below the detectable level. Within and between assay coefficient of variation was 6.0% and 11.5%, respectively, in four determinations in four separate assays.

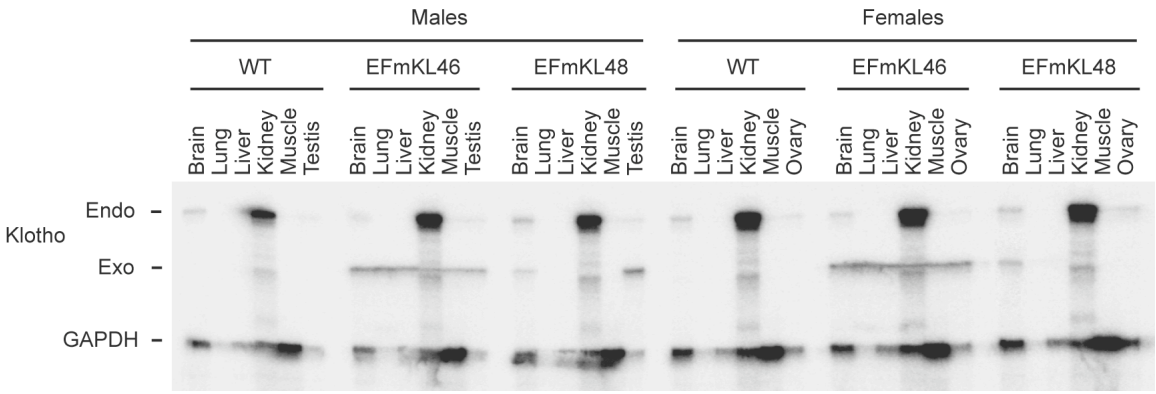
Analysis of the insulin/IGF-1 signaling activity. L6 cells and H4IIE cells were cultured to confluence in 10 cm plates in DMEM containing 10 % fetal bovine serum and then starved

with serum-free DMEM for overnight before use. The cells treated as indicated were frozen in liquid nitrogen and lysed in the lysis buffer containing inhibitors for phosphatase and proteinase as previously described (*4*, *5*, *6*). A part of the cell lysates were immunoprecipitated with anti-insulin receptor β-chain (Santa Cruz), anti-IGF-1 receptor β-chain (Santa Cruz), anti-IRS-1 (Upstate Biotechnology), or anti-IRS-2 antibody (Upstate Biotechnology). The precipitants were subjected to SDS-PAGE, transferred onto Hybond-C membranes, and blotted with the same antibodies used in the immunoprecipitation or with anti-phosphotyrosine (pY20, Santa Cruz) or with anti-p85 (Upstate Biotechnology) antibody. The rest of the cell lysates were immunoblotted with anti-Akt antibody (Santa Cruz) and anti-phospho-Akt antibody (Cell Signaling). The membranes were incubated with HRP-linked secondary antibodies and detected with SuperSignal West Dura system.

RNase protection assay. Total RNA samples of brain, lung, liver, kidney, muscle, testis, and ovary were prepared from 20-week-old mice using TRI reagent (Invitrogen). A riboprobe for mouse Klotho was generated from a PCR-amplified 175 bp DNA fragment of 3' UTR of the *klotho* cDNA (sense primer: 5'-CAG GAA GAA GGA GGA GCT CTC-3', antisense primer: 5'-TCA CAG AAG CAC ACG GTA GAT C-3'). The amplified fragment was subcloned into pCRII vector (Invitrogen), excised with *EcoRI* digestion, and subcloned into pBluscript II SK(-) (Stratagene). A riboprobe for mouse GAPDH was prepared from a PCR-amplified 127 bp cDNA fragment (sense primer: 5'-CCC TCG AGG CCT TCC GTG CTT CC-3', antisense primer: 5'-TTC AGT GGG CCC TCA GAT GC-3'). The fragment was digested with *XhoI*/*ApaI* and subcloned into pBluscript II SK(-). The radioactive antisense riboprobes were generated using *XbaI*-digested template plasmids, T7 RNA polymerase (Maxi Script Kit, Ambion), and ^{32}P UTP (Amersham). The riboprobes were hybridized with 20 μg of total RNA at 42 ºC overnight. The

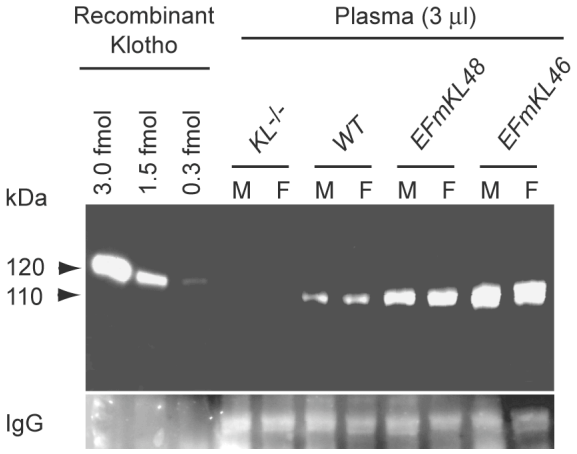
hybridization mixture was digested with RNase A/T1 using RPA Kit (Ambion) and subjected to 8 M urea/8% polyacrylamide gel electrophoresis. Predicted bands and their size were as follows: the riboprobe for Klotho mRNA (283 nt), endogenous Klotho mRNA (175 nt), exogenous Klotho mRNA originated from the EFmKL transgene (145 nt), the riboprobe for GAPDH mRNA (196 nt), GAPDH mRNA (113 nt).

Figure S1



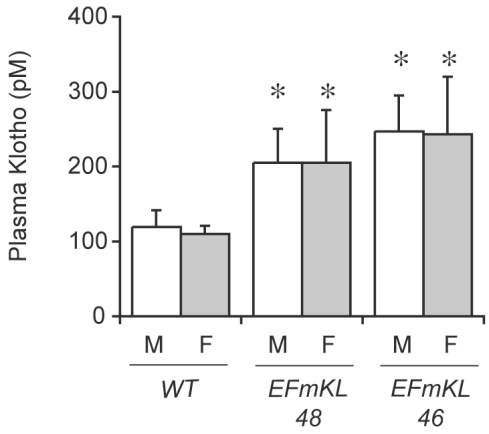
Klotho gene expression at the mRNA level in wild-type, *EFmKL46*, and *EFmKL48* mice detected by the RNase protection assay. Endogenous Klotho mRNA (**Endo**) was detected predominantly in the kidney and faintly in the brain, testis and ovary. Exogenous Klotho mRNA originated from the transgene (**Exo**) was detected in all tissues examined in *EFmKL46*, but absent in the lung, liver, kidney, muscle, and ovary in *EFmKL48*. GAPDH mRNA was indicated as a loading control (**GAPDH**).

Figure S2



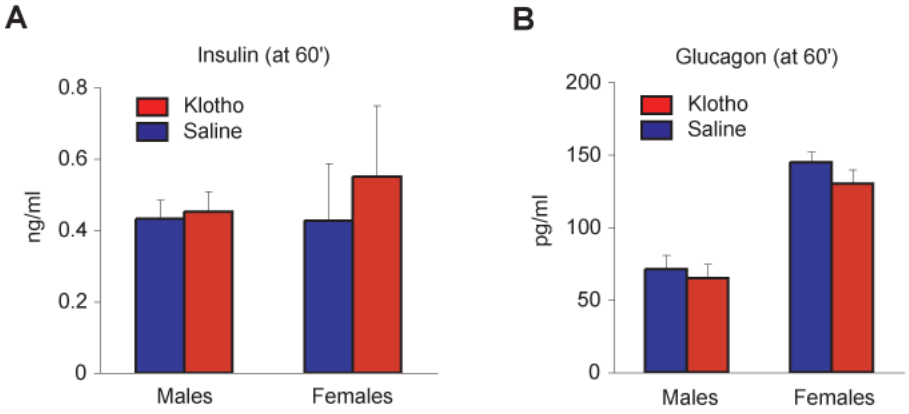
Immunoblot analysis of plasma samples from male (M) and female (F) mice with different Klotho expression levels using rabbit anti-Klotho antiserum. Upper panel: The molecular weight of plasma Klotho is ~110 kDa. Lower panel: Non-specific signals from IgG in a long exposure as a loading control.

Figure S3



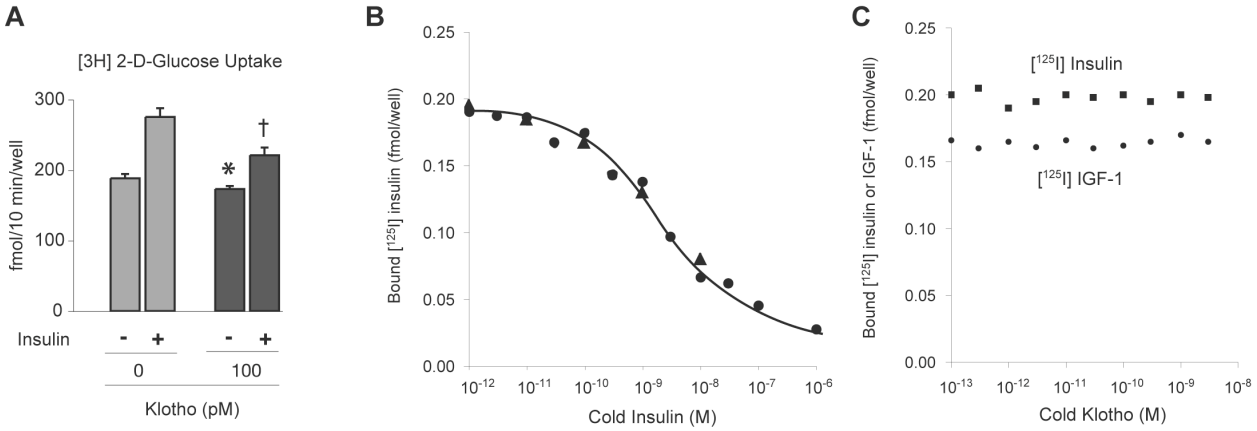
Plasma Klotho protein levels determined by radioimmunoassay ($n = 6$ for each group) in age-matched (8-weeks of age) male (M) and female (F) wild-type (*WT*), *EFmKL48*, and *EFmKL46* mice. Error bars indicate s.d. *$P < 0.05$ vs. wild-type mice by ANOVA.

Figure S4



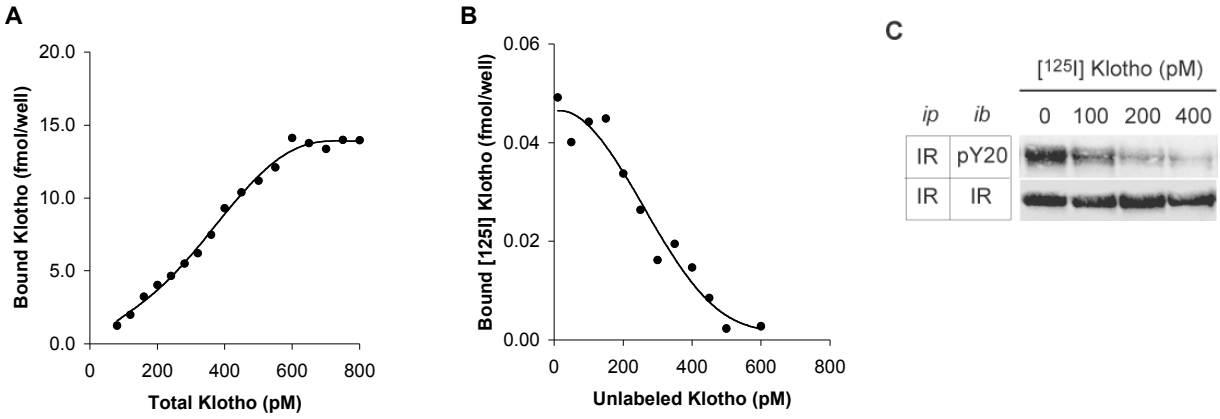
Effect of Klotho protein injection on blood insulin and glucagon levels. Plasma insulin (**A**) and glucagon (**B**) levels in wild-type mice 60 minutes after the injection of saline (n = 6 for both males and females, blue bars) or Klotho (10 □g/kg, n = 8 for both males and females, red bars) were determined using rat/mouse insulin ELISA kit and glucagon RIA kit (Linco Research), respectively, according to the manufacturer's protocols. Error bars indicate s.d. Although Klotho tended to increase insulin and decrease glucagon, which may be a compensatory response to the Klotho-induced increase in blood glucose levels, ANOVA detected no statistical difference.

Figure S5



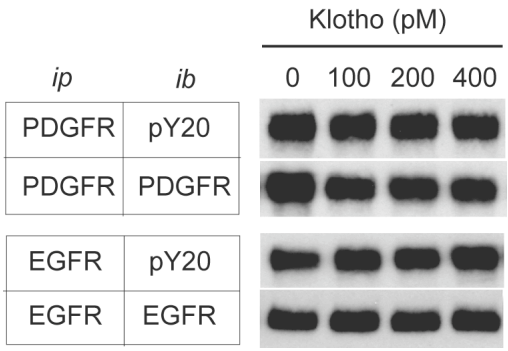
Klotho protein inhibits insulin action without inhibiting insulin binding to cells. (**A**) The effect of Klotho protein on glucose uptake in L6 cells. Recombinant Klotho protein (100 pM) suppressed both basal and insulin-induced (10 nM) glucose uptake. Error bars indicate s.d. **P* < 0.05 and †*P* < 0.01 vs. glucose uptake in the absence of Klotho by ANOVA. (**B**) L6 cells cultured to confluence in 12-well-plates were incubated for 60 minutes at room temperature with 5.6 pM of [^{125}I] Insulin and various concentrations of unlabeled insulin in the absence (●) or presence of 1 nM unlabeled Klotho protein (▲). Klotho did not affect the binding kinetics of insulin. (**C**) L6 cells were incubated with 5.6 pM of [^{125}I] insulin (■) or [^{125}I] IGF-1 (●) and various concentrations of unlabeled Klotho protein. Binding of [^{125}I] Insulin and [^{125}I] IGF-1 to the cells was not inhibited by Klotho protein.

Figure S6



Klotho binds to cell-surface in a saturable and reversible manner. (**A**) A quantitative analysis of specific binding of Klotho to H4IIE cells. A sigmoidal curve suggests positive cooperativity in the interaction between Klotho and putative Klotho receptor. The average number of Klotho binding sites on the cell surface is estimated at ~ 1 x 10^5/cell in H4IIE cells. (**B**) Competitive inhibition of [^{125}I] Klotho protein binding to H4IIE cells with unlabeled Klotho protein. H4IIE cells cultured to confluence in 12-well-plates were incubated with 10 pM of [^{125}I] Klotho protein together with increasing concentrations of unlabeled Klotho protein. The concentration of unlabeled Klotho protein necessary to obtain half-maximal inhibition of [^{125}I] Klotho binding is 300 pM. (**C**) As in Fig. 3, except that [^{125}I] Klotho protein was used instead of unlabeled Klotho protein. The [^{125}I] Klotho protein used in the binding assay has an activity that inhibits tyrosine phosphorylation of insulin receptor.

Figure S7



Klotho protein does not inhibit activation of PDGF receptor and EGF receptor. L6 cells were incubated with the indicated concentrations of Klotho protein for 15 minutes and then stimulated with recombinant PDGF-BB (1 nM) or EGF (100 nM) for 15 minutes. The cell lysates were immunoprecipitated with anti-PDGF receptor antibody (PDGFR, Santa Cruz) or anti-EGF receptor antibody (EGFR, Santa Cruz) and then immunoblotted with the same antibody or anti-phosphotyrosine antibody (pY20) as in Fig. 3. Klotho protein at 200-400 pM can inhibit insulin/IGF-1-induced tyrosine phosphorylation of insulin/IGF-1 receptors (Fig. 3) but cannot inhibit PDGF and EGF receptors.

Table S1. Food intake and oxygen consumption in wild-type mice and transgenic mice that overexpress Klotho (*EFmKL46* and *EFmKL48*). Food intake and oxygen consumption were determined using mice at 32-36 weeks of age.

	Wild-type		*EFmKL46*		*EFmKL48*	
	Male	Female	Male	Female	Male	Female
Food intake (g/day)	3.11 ± 0.18 (n = 5)	2.42 ± 0.95 (n = 11)	3.86 ± 0.73 (n = 4)	2.43 ± 0.79 (n = 10)	2.71 ± 0.45 (n = 4)	2.63 ± 0.62 (n = 10)
Oxygen consumption (l/kg/hr)	3.62 ± 0.39 (n = 7)	2.98 ± 0.30 (n = 11)	3.75 ± 0.42 (n = 5)	3.17 ± 0.37 (n = 11)	3.51 ± 0.33 (n = 5)	2.94 ± 0.27 (n = 10)

References

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