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Extended lifespan, reduced body size and leg skeletal muscle mass, and decreased mitochondrial function in *clk-1* transgenic mice

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ABSTRACT

Mutational inactivation of *clk-1*, which encodes an enzyme necessary for the biosynthesis of coenzyme Q (CoQ), extends the lifespan of *Caenorhabditis elegans*. However, whether mammalian *clk-1* regulates the lifespan of mice is not known because *clk-1*-deficiencies are embryonic lethal. Here, we investigated the lifespan of *clk-1* transgenic mice (Tg96/I), which were rescued from embryonic lethality via the transgenic expression of mouse *clk-1*. Tg96/I mice lived longer and had smaller bodies than wild-type mice, but Tg96/I mice had CoQ levels equivalent to wild-type mice. The small-sized Tg96/I mice exhibited reduced whole-body oxygen consumption (VO_2) during the dark period, and lean leg skeletal muscles with reduced mitochondrial VO_2 and ATP content compared with wild-type mice. These findings indicate a close relationship between lifespan extension and decreased mitochondrial function, which was induced by the transgenic expression of *clk-1*, in leg skeletal muscles that exhibit high metabolic activity.

1. Introduction

The most important function of coenzyme Q (CoQ) is to transport electrons in the respiratory chain from mitochondrial complexes I and II to complex III (Brandt, 1997; Friedrich et al., 1994; Guenebaut et al., 1997; Scheffler, 1998) or within the respiratory supercomplex composed of complexes I, III, and IV (Genova and Lenaz, 2011; Schägger, 2001; Schägger and Pfeiffer, 2000), which facilitates the release of protons into the mitochondrial intermembrane space to generate membrane potential and ATP (Crane et al., 1957; Mitchell, 1961). The major source of CoQ is biosynthesis (Artuch et al., 2009), which is mediated by at least six enzymes of known function encoded by CoQ biosynthesis genes (Artuch et al., 2009; Kawamukai, 2009; Marbois and Clarke, 1996). Mutational inactivation of the *clk-1* gene (Wong et al., 1995) or silencing of other CoQ biosynthesis genes (Asencio et al., 2003) results in lifespan extension in nematodes. The *clk-1* gene is conserved among several species (Asaumi et al., 1999; Ewbank et al., 1997; Vajo et al., 1999), and the transgenic expression of the mouse *clk-1* in long-lived *clk-1* nematodes reverted their extended lifespan to a lifespan comparable to wild-type nematodes (Takahashi et al., 2001). This result suggests a functional commonality of the *clk-1* gene in the regulation of lifespan, which prompted our examination of the ability of *clk-1* to regulate mammalian lifespan.

We previously created *clk-1*-deficient mice, which were embryonic lethal (Nakai et al., 2001) due to frequent apoptosis throughout the embryonic body (Takahashi et al., 2008). Similarly, *clk-1* mutant nematodes undergo developmental arrest in the L2 larval stage, and these mutants are larval lethal when fed a diet lacking CoQ (Jonassen et al., 2001). Taken together, these results indicate that *clk-1* is indispensable during early animal development, and that lifespan is extended only when the CoQ supply is limited. However, investigating the role of *clk-1* in the regulation of the adult lifespan of *clk-1*-deficient mice has been challenging.

We created transgenic mice (Tg96) to overcome the embryonic lethality of *clk-1*-deficient mice, which lack the CLK-1 protein or endogenous CoQ (Levavasseur et al., 2001; Nakai et al., 2001). Tg96 mice rescue *clk-1*-deficient mice from embryonic lethality via the transgenic expression of mouse *clk-1*, which decreases CoQ levels (Nakai et al., 2004). We crossed Tg96 mice with wild-type ICR mice to increase litter size and generate Tg96/I mice, and we determined the adult lifespan of these mice. We found that CoQ levels in Tg96/I mice reverted to levels comparable to the wild-type counterpart mice. However, male and female Tg96/I mice lived longer and had smaller bodies and leaner leg skeletal

Abbreviations: CoQ, coenzyme Q; Q, oxidized coenzyme Q; QH_2, reduced coenzyme Q; Tg mice, transgenic mice; VO_2, rate of oxygen consumption; Quad, quadriceps; Sol, soleus; GA, gastrocnemius; TA, tibialis anterior; EDL, extensor digitorum longus; SD, standard deviation of the mean.

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muscles than wild-type mice. The long-lived and small-sized Tg96/I mice exhibited a reduced rate of absolute whole-body oxygen consumption (VO_2) during the dark period and reduced mitochondrial VO_2 and ATP content in leg skeletal muscles compared with wild-type mice. These findings indicate a close relationship between lifespan extension and decreased mitochondrial function in leg skeletal muscles, which actively consume and produce substantial amounts of energy.

2. Materials and methods

2.1. Animals

The *clk-1*-deficient (*clk-1*$^{-/-}$) mice expressing the mouse *clk-1* transgene (Tg96/I) were obtained by mating the Tg96 mice (C57BL/6NCr) (Nakai et al., 2004) with wild-type ICR mice to increase litter size, followed by crossing the *clk-1*$^{+/-}$ transgenic mice with *clk-1*$^{+/-}$ mice. The genotypes of the mice were determined as described previously (Nakai et al., 2001, 2004).

Mice were weaned under pathogen-free barrier conditions, fed standard chow ad libitum on a 12-h dark/light cycle, and permitted to die of natural causes. Five female mice were housed in a cage throughout the experiment, and five male mice that had been housed first in a cage were not gathered later. All protocols for animal use and experiments followed the Principles of Laboratory Animal Care (NIH publication No. 86-23, revised 1985) and were reviewed and approved by the Animal Care Committee of the Tokyo Metropolitan Institute of Gerontology.

2.2. Lifespan analysis

Male and female mice were caged separately following sexual maturation. The survival curves of male (n = 50) and female (n = 50) Tg96/I mice and male (n = 47) and female (n = 46) wild-type mice were plotted using the Kaplan–Meier method.

2.3. Preparation of mitochondria

Tissues and organs were harvested from mice aged 6–7 months old. After measurement of its wet weight, the liver was minced into small pieces with scissors and homogenized with 20 strokes of a Dounce homogenizer (Wheaton, Milleville, NJ) on ice in 20 volumes of a homogenizing buffer consisting of 0.25 M sucrose, 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The thigh muscles were homogenized in a Polytron homogenizer (Kinematica AG, Luceme, Switzerland) prior to Dounce homogenization. The homogenates were centrifuged at 800 × *g* for 10 min at 4 °C, and the precipitates were resuspended in a homogenizing buffer for centrifugation at 800 × *g* for 5 min. The combined supernatant was centrifuged at 12,000 × *g* for 10 min at 4 °C. Centrifugation at 800 × *g* and 12,000 × *g* was repeated once more, yielding a mitochondrial fraction. Aliquots of the mitochondrial fraction were then used to measure the protein content with a protein reagent kit (Thermo Fischer Scientific, Pierce, Waltham, MA) with bovine serum albumin (BSA).

2.4. Measurement of the CoQ content

The mitochondrial fractions obtained from frozen livers or thigh muscles in liquid nitrogen were added to four volumes of 2-propanol and centrifuged at 12,000 rpm for 5 min at 20 °C. The supernatant was then filtered using a syringe-driven filter unit (Millex® LH, Millipore, Temecula, CA), and subjected to chromatography in a reverse-phase high performance liquid chromatography (HPLC) system (LC-10Advp, Shimadzu, Kyoto, Japan). An HPLC column (Capcell Pak C18 MG 100, 4.6 × 50 mm, 3 μm; Shiseido, Tokyo, Japan) with a mobile phase of methanol/1-propanol/1.0 M ammonium acetate (pH 4.4) (78:20:2, v/v) and a flow rate of 1.0 ml/min at 32 °C was used. CoQ was detected by an electrochemical detector (ECD) (ESA Coulochem II, Thermo Fisher Scientific) with the following settings: guard cell, −700 mV; analytical cell, E1 = −700 mV, E2 = +400 mV. Additionally, reduced forms of CoQ_9 (Q_9H_2) and CoQ_{10} ($Q_{10}H_2$) standards were prepared from oxidized CoQ_9 (Q_9) and CoQ_{10} (Q_{10}) (Sigma-Aldrich, St. Louis, MO) via incubation in 5 mM $NaBH_4$ for 30 min in the dark. CoQ_6 (Avanti Polar Lipids, Alabaster, AL) was used as an internal standard.

2.5. Measurement of whole-body oxygen consumption (VO_2), respiratory quotient (RQ), and mitochondrial VO_2

Whole-body oxygen consumption (VO_2) and the respiratory quotient (RQ) were determined with an O_2/CO_2 metabolism measurement system (MK-5000RQ, Muromachi Kikai, Tokyo, Japan). Mice were allowed to acclimate to their metabolic cage for 4 days, and measurements were obtained on the fifth day. O_2 and CO_2 were measured in the exhaust gases from individual cages by a computer-controlled sequential analysis that repeated the cycle every 3 min and used a paramagnetic O_2 analyzer and an infrared CO_2 analyzer. For daily calibration, we used pure N_2 and standards of 20% O_2 and 5% CO_2. RQ was calculated by the ratio of VCO_2 to VO_2.

The rate of mitochondrial VO_2 was determined for fresh organs or tissues at 37 °C by high-resolution respirometry (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria). DatLab software (Oroboros Instruments) was used for the data acquisition and analysis, according to the manufacturer's protocols. Resting respiration (state 2, absence of adenylates) was measured after the addition of 50 μl of isolated mitochondria (100–300 μg protein) to glass chambers containing 10 μM cytochrome *c* in 2 ml of the mitochondrial respiration medium (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 1 g/l BSA, pH 7.1). Complex I respiration was assessed by the addition of 2.5 mM ADP (state 3) after adding of 5 mM malate and 10 mM glutamate as the complex I substrate followed by titration of 5 mM succinate as the complex II substrate to measure complex I and II respiration.

2.6. Measurement of the ATP content

The quadriceps muscles were frozen in liquid nitrogen, minced with scissors, and homogenized in homogenizing buffer (0.25 M sucrose, 10 mM Tris–HCl, pH 7.5, 1 mM PMSF) using a Polytron homogenizer followed by a Dounce homogenizer, as described above. The lysates were centrifuged at 800 × *g* for 10 min at 4 °C, and the cytoplasmic supernatant was used to measure the protein content, as described above, and ATP content using an ATP assay kit (Toyo Ink, Tokyo, Japan) with a luminometer (EnVision 2104, Perkin Elmer, Waltham, MA).

2.7. Statistical analyses

The data for the male and female mice were analyzed separately. Differences between the Kaplan–Meier survival curves were evaluated by the Cox–Mantel log rank test using IBM SPSS Statistics 20 software. Statistical analyses were conducted using a one-way ANOVA followed by a two-tailed Student *t*-test for unpaired values for all two-sample comparisons reported other than the survival data. The differences between the data were considered significant at $P < 0.05$.

3. Results

3.1. Extended lifespan of Tg96/I mice

Kaplan–Meier curves were analyzed to determine whether Tg96/I mice lived longer than wild-type mice. The survival curves of Tg96/I mice differed significantly from wild-type mice for males and females ($P < 0.0005$ and $P < 0.005$, respectively; Cox–Mantel log rank test; Fig. 1A and B). The mean lifespan of male Tg96/I mice (744 ±

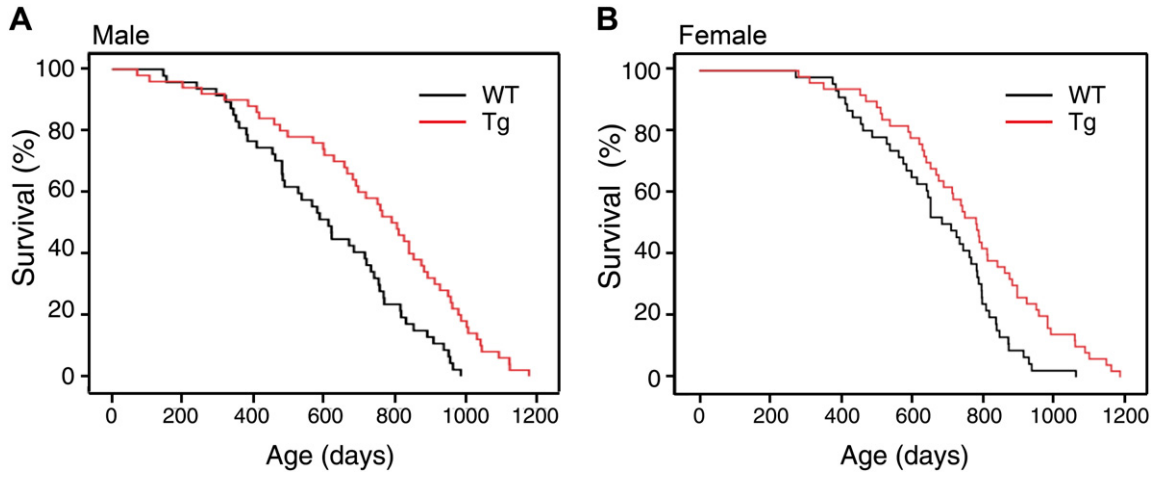

Fig. 1. Increased lifespan of Tg96/I mice. (A) Male Tg96/I mice (n = 50) lived longer than male wild-type mice (n = 47; $P < 0.0005$; Kaplan–Meier curves analyzed by the Cox–Mantel log-rank test). (B) Similarly, female Tg96/I mice (n = 50) lived longer than female wild-type mice (n = 46; $P < 0.005$; Cox–Mantel log-rank test).

39 days) was significantly (23%) longer than male wild-type mice (607 ± 34 days; $P < 0.01$, unpaired *t*-test). Similarly, the maximal lifespan of male Tg96/I mice (1183 days) was 20% longer than male wild-type mice (989 days; Table 1). The mean lifespan of female Tg96/I mice was 770 ± 31 days, which was 14% longer than the 675 ± 27 day lifespan of female wild-type mice ($P < 0.03$). The maximum observed lifespan of female Tg96/I mice was 1190 days, which was 12% longer than the maximum lifespan of 1065 days for female wild-type mice (Table 1). These results indicate that male and female Tg96/I mice lived significantly longer than their wild-type counterparts.

3.2. Low body weight and reduced whole-body oxygen consumption (VO_2) in Tg96/I mice

A representative male Tg96/I mouse was notably smaller than a representative male wild-type mouse at 30 weeks of age (Fig. 2A). Measurements of body weight every fourth week, when the mice were between 8 and 52 weeks of age, revealed that the mean body weight of male Tg96/I mice was significantly lower than age-matched male wild-type mice (all analyses, $P < 0.03$; unpaired *t*-test; Fig. 2B). In addition, the mean body weight of male Tg96/I mice was significantly lower than male wild-type mice at 72 weeks of age ($P < 0.002$). Similarly, the mean body weight of female Tg96/I mice was significantly lower than female wild-type mice between 12 and 52 weeks of age (all analyses, $P < 0.02$), and at 72 weeks of age ($P < 5 \times 10^{-7}$), respectively (Fig. 2C). In contrast, the mean body weights of male and female wild-type mice at 96 weeks of age were reduced and comparable to male and female Tg96/I mice, respectively. These results indicate that male and female Tg96/I mice had significantly lower body weights than wild-type mice until 72 weeks of age.

Table 1
Lifespan of Tg96/I and wild-type mice.

	Mean lifespan (mean ± SD days)	Maximal lifespan (days)	Number of animals
Male			
Wild-type	607 ± 34	989	47
Tg96/I	744 ± 39 ($P < 0.01$)	1183	50
Female			
Wild-type	675 ± 27	1065	46
Tg96/I	770 ± 31 ($P < 0.03$)	1190	50

Statistical analyses were conducted using ANOVA followed by a two-tailed Student *t*-test for unpaired values for all two-sample comparisons.

The differences between certain long-lived *clk-1* mutant and wild-type nematodes have tentatively been attributed to differences in metabolic rates (Lee et al., 2012; Van Voorhies, 2001, 2002; Van Voorhies and Ward, 1999). We measured whole-body VO_2 during light and dark periods to examine whether the small size of Tg96/I mice was due to their metabolism. Measurement of whole-body VO_2 in 6- to 7-month-old individual wild-type and Tg96/I mice revealed that absolute whole-body VO_2 in male Tg96/I mice was 1.75 ± 0.23 ml/min during the dark period, but not during the light period or a whole day, which was significantly lower than the value of 1.95 ± 0.09 ml/min observed in male wild-type mice ($P < 0.05$; Fig. 3A). In contrast to the males, the absolute whole-body VO_2 of female Tg96/I mice was significantly lower than female wild-type mice during the dark ($P < 0.001$) and light ($P < 0.03$) periods and throughout a whole day ($P < 0.002$; Fig. 3B). In contrast to the absolute whole-body VO_2 values, the whole-body VO_2 relative to body mass during the dark and light periods and a whole day was not significantly different between male Tg96/I and wild-type mice (Fig. 3C). However, the whole-body VO_2 values relative to body mass of female Tg96/I mice during the light period were significantly higher than female wild-type mice ($P < 0.05$; Fig. 3D).

The mean respiratory quotient (RQ) in the two mouse groups during dark and light periods was not significantly different in males (Fig. 3E) or females (Fig. 3F) despite the difference in the absolute whole-body VO_2 between Tg96/I and wild-type mice during the dark period. These results indicate that the absolute whole-body VO_2 was significantly lower in male and female Tg96/I mice during the dark period and in female Tg96/I mice during the light period than for their wild-type counterparts.

3.3. Low mitochondrial oxygen consumption (VO_2) in the liver of female Tg96/I mice

The differences in lifespan (Fig. 1) between Tg96/I and wild-type mice were similar than the observed differences between long-lived *clk-1* heterozygous mice and wild-type mice, which were tentatively attributed to differences in their mitochondrial function (Lapointe and Hekimi, 2008). The liver is the largest visceral organ, and it plays a major role in metabolism. Therefore, we measured the wet weight and rate of mitochondrial oxygen consumption (VO_2) in the livers of 6- to 7-month-old wild-type and Tg96/I mice. The mean liver weights of male wild-type and Tg96/I mice were 2.27 ± 0.32 and 2.12 ± 0.18 g, respectively; these values were not significantly different (Fig. 4A). The mean liver weight of female wild-type mice (1.56 ±

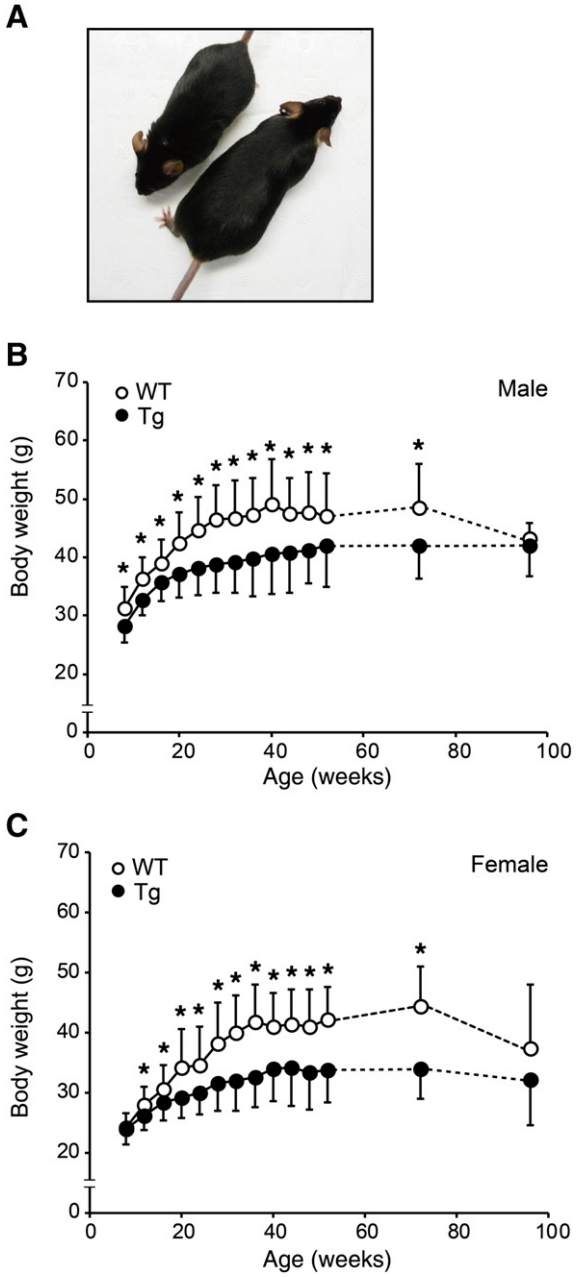

Fig. 2. Low body weight of Tg96/I mice. (A) Representative small Tg96/I male mouse (upper left) compared with a representative wild-type male mouse (lower right), both at 30 weeks of age. The body weight of the (B) male and (C) female mice was determined every fourth week when the mice were between 8 and 52 weeks of age and at 72 and 96 weeks of age. The recorded body weight was significantly lower in Tg96/I mice (Tg) compared with those in wild-type mice (WT) between 8 and 52 weeks of age (all analyses $P < 0.03$, unpaired *t*-test), at 72 weeks of age ($P < 0.002$) for the males, between 12 and 52 weeks of age (all analyses, $P < 0.02$) and at 72 weeks of age ($P < 5 \times 10^{-7}$) for the females, respectively. Vertical bars indicate the SD of the mean values shown.

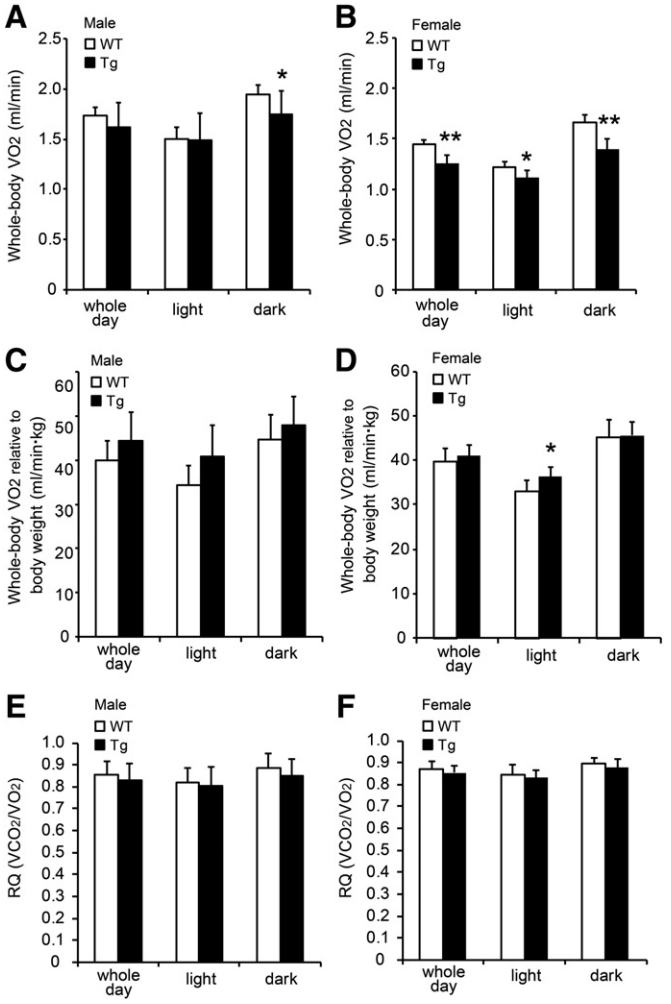

Fig. 3. Low whole-body VO_2 in Tg96/I mice during the dark period. The rates of absolute whole-body VO_2 of (A) male (n = 6) Tg96/I (Tg) mice during the dark period and (B) female (n = 5) Tg96/I mice during the light and dark periods and whole day were significantly lower than the corresponding rates in male (n = 8) and female (n = 5) wild-type (WT) mice, respectively. The rates of whole-body VO_2 relative to body mass in (C) male Tg96/I and wild-type mice were equivalent, but the rate was significantly higher in (D) female Tg96/I mice than those in female wild-type mice during the light period. The respiratory quotient (RQ) was equivalent between Tg96/I (Tg) and wild-type (WT) mice of (E) males and (F) females during dark and light periods. Vertical bars indicate the SD of the mean values. $^{*}P < 0.05$, $^{**}P < 0.002$ vs. wild-type mice.

0.27 g) was not significantly different from that of female Tg96/I mice (1.50 ± 0.21 g; Fig. 4B).

The measurement of mitochondrial VO_2 in livers revealed that the rates of complex I- and II-mediated VO_2 in male wild-type mice were 0.10 ± 0.05 and 0.26 ± 0.09 nmol/s·mg protein, respectively (Fig. 4C). These rates were not significantly different from the corresponding rates in male Tg96/I mice, which were 0.08 ± 0.01 and 0.19 ± 0.05 nmol/s·mg protein, respectively (Fig. 4C). In contrast, the rates of complex I- and II-mediated VO_2 in the livers of female Tg96/I mice (0.08 ± 0.02 and 0.16 ± 0.03 nmol/s·mg protein, respectively) were 57% ($P < 0.003$) and 48% ($P < 0.002$), respectively, of the corresponding rates in female wild-type mice (0.14 ± 0.04 and 0.33 ± 0.09 nmol/s·mg protein, respectively; Fig. 4D). These results indicate that the liver weights were equivalent between wild-type and Tg96/I mice in males and females, but the rates of complexes I- and II-mediated VO_2 in liver mitochondria were significantly reduced only in female Tg96/I mice compared with those in female wild-type mice.

3.4. Lean leg skeletal muscles with low mitochondrial VO_2 and ATP content in Tg96/I mice

We examined the physiological status of leg skeletal muscles in 6- to 7-month-old wild-type and Tg96/I mice because skeletal muscles account for much of the body's energy consumption (Heymsfield et al., 2002). Comparisons of the wet weights of quadriceps (Quad), soleus (Sol), gastrocnemius (GA), tibialis anterior (TA), and extensor digitorum longus (EDL) in wild-type and Tg96/I mice revealed that all

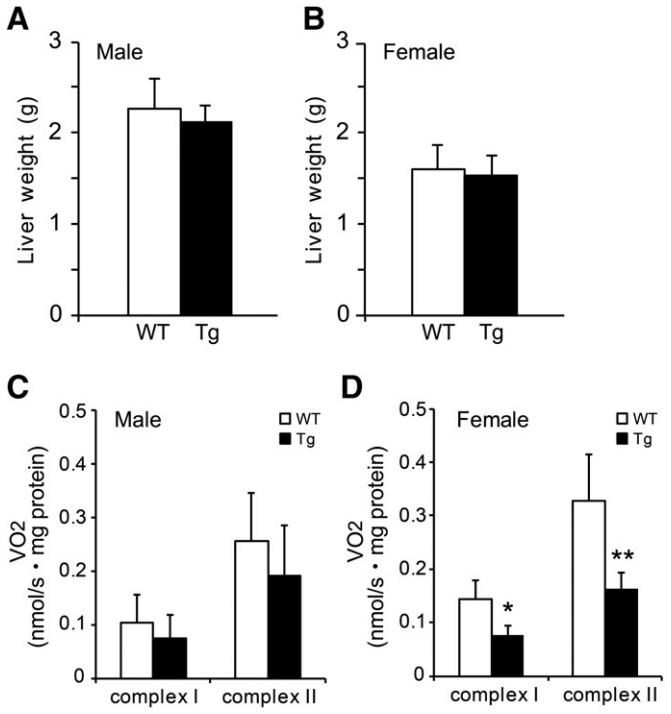

Fig. 4. Equivalent liver weights of Tg96/I and wild-type mice and low mitochondrial VO_2 in the liver of female Tg96/I mice. The liver weights were equivalent between Tg96/I (Tg) mice (6–7 months old) and wild-type (WT) mice (6–7 months old) for (A) males (n = 7) and (B) females (n = 8). The values are given as the mean ± SD. (C) The rates of mitochondrial complex I- and II-mediated VO_2 in the livers of male Tg96/I (Tg) mice (n = 8) were equivalent to male wild-type (WT) mice (n = 8). (D) The rates of complex I- and II-mediated VO_2 in the liver were significantly reduced in female Tg96/I (Tg) mice (6–7 months old; n = 8) compared with those in female wild-type (WT) mice (6–7 months old; n = 8). Vertical bars indicate the SD of the mean values shown. $^{*}P < 0.003$, $^{**}P < 0.002$ vs. wild-type mice.

leg muscles examined were significantly smaller in weight in male (Fig. 5A) and female (Fig. 5B) Tg96/I mice than in male and female wild-type mice, respectively (all analyses, $P < 0.03$). In contrast to the absolute weights of leg muscles, the wet weight of each leg muscle relative to body weight was significantly higher in the Quad, GA, and TA, but not in EDL and Sol, of male Tg96/I mice than male wild-type mice, respectively (all analyses, $P < 0.03$; Fig. 5C). Relative values of all leg muscles in females were comparable between Tg96/I and wild-type mice (Fig. 5D).

The rates of mitochondrial VO_2 were determined in Quad muscles to test mitochondrial function in leg muscles. The rates of complex I- and II-mediated VO_2 in the Quads of male Tg96/I mice were 2.2 ± 1.1 and 0.94 ± 0.52 nmol/s·mg protein, respectively (Fig. 6A). These rates were significantly lower than the corresponding rates in male wild-type mice, which were 3.6 ± 1.4 and 1.6 ± 0.50 nmol/s·mg protein, respectively (both analyses, $P < 0.04$; Fig. 6A). Similarly, the rates of complex I- and II-mediated VO_2 in the Quads of female Tg96/I mice were 1.8 ± 0.42 and 0.74 ± 0.33 nmol/s·mg protein, which were significantly lower than the corresponding rates in female wild-type mice, which were 4.0 ± 2.0 and 1.5 ± 0.82 nmol/s·mg protein, respectively ($P < 0.01$ and $P < 0.04$ for complexes I and II, respectively; Fig. 6B).

We next examined whether reduced mitochondrial VO_2 affected ATP content in Quad muscles. ATP content in the Quads of male Tg96/I mice was 2.4 ± 0.8 nmol/mg protein, which was significantly lower than in male wild-type mice (4.6 ± 1.2 nmol/mg protein; $P < 0.02$; Fig. 6C). Similarly, the ATP content in the Quads of female Tg96/I mice was 9.6 ± 4.4 nmol/mg protein, which was 56% of wild-type mice (17.0 ± 5.2 nmol/mg protein; $P < 0.04$; Fig. 6D). The reason for the greater than 3-fold higher ATP content in the Quads of female mice compared with those in male mice is not clear, but the present results indicate that the rates of complex I- and II-mediated mitochondrial VO_2 and ATP content in Quad muscles were significantly lower in male and female Tg96/I mice compared with those in wild-type mice.

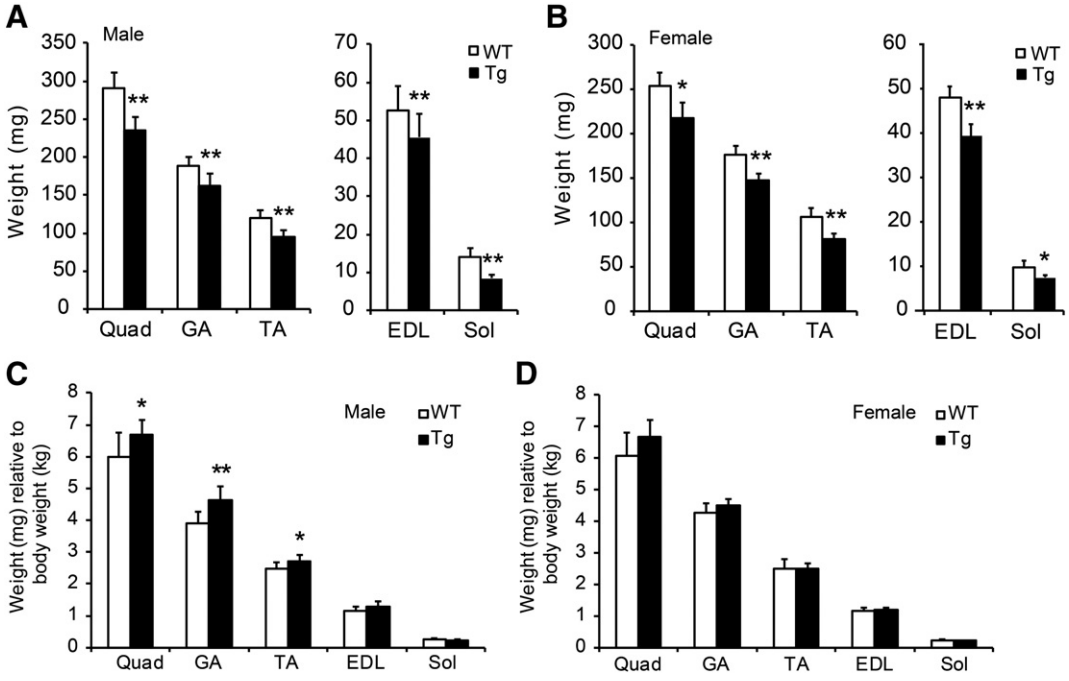

Fig. 5. Low leg muscle weight of Tg96/I mice. The weights of skeletal leg muscles in (A) male and (B) female mice. The wet weights of quadriceps (Quad), gastrocnemius (GA), tibialis anterior (TA), extensor digitorum longus (EDL), and soleus (Sol), were significantly lower in male (n = 8) and female (n = 8) Tg96/I (Tg) mice compared with male (n = 8) and female (n = 8) wild-type (WT) mice, respectively. The wet weight of leg muscles relative to body weight in (C) male and (D) female mice. The relative values of the Quad, GA, and TA were significantly higher in male Tg96/I mice. The vertical bars indicate the SD for the mean values shown. $^{*}P < 0.03$, $^{**}P < 0.01$ vs. wild-type mice.

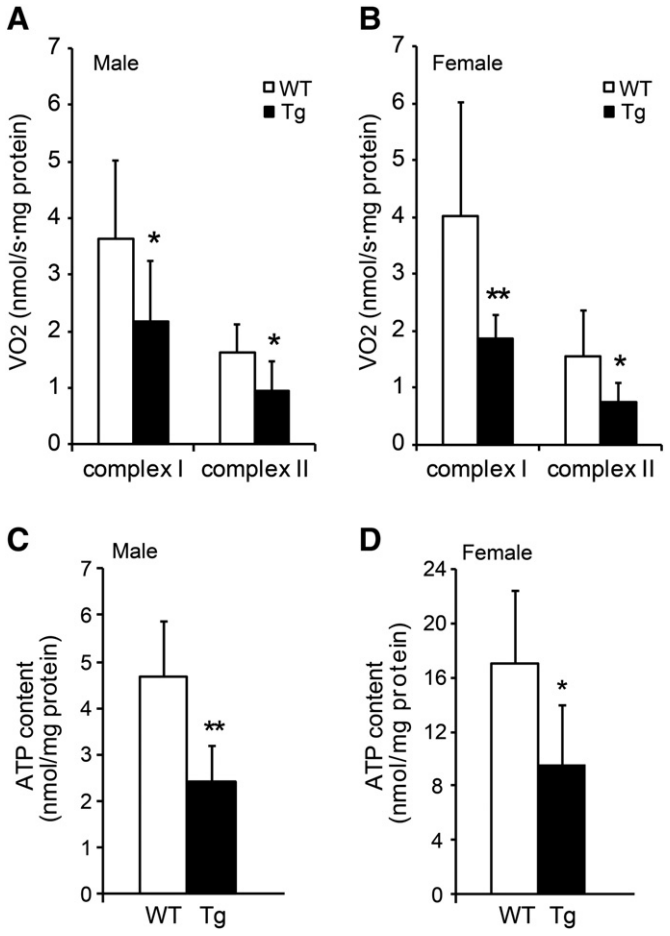

Fig. 6. Low mitochondrial VO_2 and ATP content in thigh muscles of Tg96/I mice. The rates of complex I- and II-mediated VO_2 in the Quads of (A) male (n = 8) and (B) female (n = 8) Tg96/I (Tg) mice were significantly lower than the corresponding rates in male (n = 8) and female (n = 8) wild-type (WT) mice, respectively. Vertical bars indicate the SD of the mean values shown. $^{*}P < 0.04$, $^{**}P < 0.01$ vs. wild-type mice. ATP content in the Quads of (C) male (n = 6) and (D) female (n = 6) Tg96/I (Tg) mice was significantly lower than male (n = 6) and female (n = 6) wild-type (WT) mice, respectively. Vertical bars indicate the SD for the mean values shown. $^{*}P < 0.04$, $^{**}P < 0.02$ vs. wild-type mice.

3.5. Equivalent levels of CoQ in Tg96/I and wild-type mice

Finally, we measured CoQ content in liver and thigh muscle mitochondria of 6- to 7-month-old wild-type and Tg96/I mice to determine whether the observed phenotypes characteristic of Tg96/I mice were accompanied by reduced levels of CoQ. CoQ_9, which is the predominant form of CoQ in mice, and CoQ_{10} represent the oxidized forms of CoQ (Q_9 and Q_{10}), whereas the fully reduced forms are Q_9H_2 and $Q_{10}H_2$. Q_9H_2 and $Q_{10}H_2$ levels in liver mitochondria were not significantly different between wild-type and Tg96/I male mice (Table 2). Similarly, Q_9 and total Q_9 (Q_9H_2 plus Q_9) and Q_{10} and total Q_{10} ($Q_{10}H_2$ plus Q_{10}) levels in liver mitochondria did not differ significantly between male Tg96/I and wild-type mice (Table 2). Q_9H_2, Q_9, total Q_9 and $Q_{10}H_2$, Q_{10}, and total Q_{10} levels in liver mitochondria did not differ significantly between female wild-type and Tg96/I mice (Table 2).

Similar to CoQ levels in the liver, Q_9H_2 and $Q_{10}H_2$ levels in thigh muscle mitochondria were very low, but not significantly different, in male wild-type and Tg96/I mice. Q_9 and total Q_9, Q_{10} and total Q_{10} levels in thigh muscle mitochondria were comparable between male wild-type and Tg96/I mice (Table 2). Similarly, levels of the reduced and oxidized forms of Q_9 and total Q_9 and both forms of Q_{10} and total Q_{10} in thigh muscle mitochondria did not differ significantly between female wild-type and Tg96/I mice (Table 2). These results indicate that male and female Tg96/I and wild-type mice had equivalent CoQ content in liver and thigh muscle mitochondria.

4. Discussion

This article presents data showing that male and female *clk-1* transgenic mice, Tg96/I, live longer than wild-type mice in terms of mean and maximal lifespan. The magnitude of lifespan extension in Tg96/I mice was larger in males than females. The reason for this difference between the sexes is not clear, but similar phenomena are occasionally observed. For example, overexpression of *sirt6* significantly extends the lifespan of male mice, but not female mice (Kanfi et al., 2012). Small body size was another characteristic of male and female Tg96/I mice between 12 and 72 weeks of age. However, at 96 weeks of age, no difference in the body weights of male and female Tg96/I mice were observed compared with those of wild-type mice. This observation may be due to an early onset of body weight loss during aging in wild-type mice because of their relatively shorter lifespan compared to Tg96/I mice. The small body size of Tg96/I mice was accompanied by low absolute whole-body oxygen consumption (VO_2) during the dark period compared with wild-type mice. However, the equivalent RQ between Tg96/I and wild-type mice suggests that the difference in absolute whole-body VO_2 between Tg96/I and wild-type mice was not due to differences in respiration substrates. Furthermore, the small body and low absolute whole-body VO_2 values during the dark period were reflected by leaner leg skeletal muscles with reduced mitochondrial VO_2 in male and female Tg96/I mice compared with age- and sex-matched wild-type

Table 2
CoQ content in the liver and thigh muscle mitochondria.

	Q_9H_2[a]	Q_9	Total Q_9	$Q_{10}H_2$	Q_{10}	Total Q_{10}
	(pmol/mg mitochondrial protein)					
Liver						
Male						
Wild-type (n = 4)	164.5 ± 50.5	308.5 ± 70.0	473.1 ± 73.3	7.8 ± 1.0	18.0 ± 6.3	25.8 ± 6.4
Tg96/I (n = 4)	152.9 ± 66.5	268.2 ± 79.3	421.1 ± 114.0	8.3 ± 7.3	14.2 ± 6.6	22.5 ± 12.8
Female						
Wild-type (n = 4)	376.2 ± 76.1	442.7 ± 88.4	819.0 ± 137.0	12.9 ± 1.9	15.0 ± 1.4	27.6 ± 2.7
Tg96/I (n = 4)	339.6 ± 130.5	476.8 ± 120.4	816.5 ± 231.4	12.2 ± 3.6	13.1 ± 1.4	25.3 ± 4.5
Thigh						
Male						
Wild-type (n = 8)	231.4 ± 47.8	2241.6 ± 610.6	2473.0 ± 602.7	21.9 ± 5.2	132.9 ± 27.6	154.8 ± 25.6
Tg96/I (n = 8)	244.5 ± 67.2	2172.9 ± 535.1	2417.5 ± 544.5	26.5 ± 8.9	139.3 ± 44.9	165.8 ± 49.3
Female						
Wild-type (n = 8)	176.5 ± 28.3	2435.4 ± 540.2	2611.9 ± 550.1	10.9 ± 5.6	107.9 ± 31.9	118.8 ± 35.8
Tg96/I (n = 8)	244.3 ± 50.6	2435.1 ± 216.9	2679.5 ± 256.7	16.1 ± 4.3	119.2 ± 15.2	135.4 ± 19.4

Values expressed as mean ± standard deviation (SD).
[a] Q_9H_2, reduced CoQ_9; Q_9, oxidized CoQ_9; total Q_9, Q_9H_2 plus Q_9; $Q_{10}H_2$, reduced CoQ_{10}; Q_{10}, oxidized CoQ_{10}; Total Q_{10}, $Q_{10}H_2$ plus Q_{10}.

mice. In addition, the decreased rate of mitochondrial VO_2 in thigh muscles was accompanied by a significant reduction in ATP content, which indicates a significant reduction in leg mitochondrial function and energy production in Tg96/I mice. The skeletal muscles along with the cerebrum and peripheral nerves consume more energy than most other organs or tissues in the body (Heymsfield et al., 2002). Therefore, these results indicate that the lifespan extension of Tg96/I mice cannot be separated from energetic and metabolic tradeoffs in one of the major energy producing tissues in the body. In *Caenorhabditis elegans*, long-lived mutants also display reduced metabolic rates (Lee et al., 2012; Van Voorhies, 2001, 2002; Van Voorhies and Ward, 1999). Similarly, long-lived mutant mice exhibited mitochondrial dysfunction and reduced energy metabolism, such as a lower rate of mitochondrial ATP synthesis and lower cellular ATP levels than wild-type mice (Lapointe and Hekimi, 2008; Lapointe et al., 2009).

Transgenic expression of the *clk-1* gene restored CoQ content to levels comparable to wild-type mice despite the differential lifespan and mitochondrial function between these groups of mice. The reason for this restoration of CoQ levels is not clear, but this result may be primarily due to the crossing of Tg96 mice (Nakai et al., 2004) with ICR mice to increase litter sizes. A similar uncoupling of lifespan extension and mitochondrial dysfunction with CoQ content has been observed between long-lived *clk-1* heterozygous mutant mice and wild-type mice (Lapointe and Hekimi, 2008; Lapointe et al., 2009). A decline in mitochondrial function as assessed by mitochondrial VO_2 was observed in aged wild-type mouse brains with no significant reduction in mitochondrial CoQ content (Takahashi and Takahashi, 2013).

Therefore, the underlying mechanism behind mitochondrial-mediated longevity is not clear. A modest inhibition of respiratory complexes extends lifespan in a wide variety of species (Chin et al., 2014; Copeland et al., 2009; Dell'Agnello et al., 2007; Dillin et al., 2002; Kayser et al., 2004; Lapointe and Hekimi, 2008). These findings suggest a close relationship between mitochondrial respiratory activity and longevity. However, the molecular and cellular mechanisms by which a reduction in mitochondrial function extends lifespan are not yet known for any species. One possibility is that alterations in the genes that encode a respiratory complex-related protein are induced in Tg96/I mice by the transgenic expression of *clk-1*. Mutational inactivation of *surf-1*, which encodes a putative cytochrome *c* oxidase (complex IV) assembly factor, causes lifespan extension and reduces mitochondrial function (Dell'Agnello et al., 2007). Alternatively, alterations in the assembly of respiratory complexes or CoQ distribution within mitochondrial membranes may possibly be induced in Tg96/I mice. In mammals, nearly all enzymes of respiratory complex I are assembled into supercomplexes (Genova and Lenaz, 2011; Schägger, 2001; Schägger and Pfeiffer, 2000), which enhance respiratory activity (Schägger and Pfeiffer, 2000). Little is known regarding the mechanism of respiratory complex assembly regulation, but another possibility is that some unknown events were induced in Tg96/I mice that reduced the recruitment of complex I to supercomplexes, which reduced mitochondrial function. Moreover, one recent study indicated that CoQ levels were decreased in the inner membranes of mitochondria but increased in the outer membrane, with no change in CoQ content in long-lived *clk-1* heterozygous mice compared with wild-type mice (Lapointe et al., 2012). These observed phenomena hint at a mechanism to explain the reduced mitochondrial function with no significant decrease in CoQ content in the Tg96/I mice.

In conclusion, our results indicate that male and female Tg96/I mice exhibit lifespan extension compared with their wild-type counterparts. The long-lived Tg96/I mice had small body mass, low whole-body VO_2, and decreased mitochondrial function in lean leg skeletal muscles, which actively consume and produce substantial amounts of energy. Further studies are needed to determine the mechanisms underlying the decrease in mitochondrial function, which leads to lifespan extension in mice.

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