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RESEARCH ARTICLE

Decline in muscle strength and running endurance in klotho deficient C57BL/6 mice

Michael Phelps · Christina Pettan-Brewer · Warren Ladiges · Zipora Yablonka-Reuveni

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Abstract Alpha klotho (known as klotho) is a multifunctional protein that may be linked to age-associated decline in tissue homeostasis. The original klotho hypomorphic (klotho^{hm}) mouse, produced on a mixed C57BL/6 and C3H background, is short lived and exhibits extensive aging-like deterioration of several body systems. Differently, klotho^{hm} mice on a pure C57BL/6 background do not appear sickly nor die young, which has permitted us to gain insight into the effect of klotho deficiency in adult life. First, analyzing klotho transcript levels in the kidney, the main site of klotho production, we demonstrated a 71-fold decline in klotho^{hm} females compared to wildtype females versus only a 4-fold decline in mutant males. We then examined the effect of klotho deficiency on muscle-related attributes in adult mice, focusing on 7–11 month old females. Body weight and forelimb grip strength were significantly reduced in klotho^{hm} mice compared to wildtype and klotho overexpressing mice. The female mice were also subjected to voluntary wheel running for a period of 6 days. Running endurance was markedly reduced in klotho^{hm} mice, which exhibited a sporadic running pattern that may be characteristic of repeated bouts of exhaustions. When actually running, klotho^{hm} females ran at the same speed as wildtype and klotho overexpressing mice, but spent about 65 % less time running compared to the other two groups. Our novel results suggest an important link between klotho deficiency and muscle performance. This study provides a foundation for further research on klotho involvement as a potential inhibitor of age-associated muscle deterioration.

Keywords Aging · Alpha klotho · EFmKL46 transgene · Hypomorph · Klotho · Muscle strength · Running endurance · Sarcopenia · Skeletal muscle

M. Phelps · Z. Yablonka-Reuveni (✉)
Department of Biological Structure, School of Medicine, University of Washington, Health Sciences Building, Room G520, 1959 NE Pacific Street, Box 35740, Seattle, WA 98195, USA
e-mail: reuveni@uw.edu

C. Pettan-Brewer · W. Ladiges
Department of Comparative Medicine, School of Medicine, University of Washington, Seattle, WA 98195, USA

Introduction

Aging is a complex degenerative process characterized by the diminished capacity for tissue maintenance and gradual decline in organ systems. In the context of skeletal muscle, there is an age-associated decline in both muscle mass and strength, a condition termed sarcopenia (Rosenberg 1997; Ryall et al. 2008; Thompson 2009; Janssen 2010). More recently, the definition of sarcopenia has been broadened, not only to indicate a decline in muscle strength, but an overall decline in muscle function (Fielding et al. 2011;

Rosenberg 2011). In humans, sarcopenia can begin as early as the 4th decade of life (Walston 2012). At the histological level, as seen in humans and in animal models, muscle aging is associated with a gradual elimination of myofibers, atrophy of remaining myofibers, and the progressive replacement of skeletal muscle with adipose and connective tissue (Faulkner et al. 2007; Zamboni et al. 2008; Serrano and Muñoz-Cánoves 2010). The regenerative capability of skeletal muscle also decreases with age (Rader and Faulkner 2006; Barberi et al. 2013). This may be due in part to the decline in number and function of myogenic stem cells (i.e., satellite cells) in old muscle tissue as seen in the context of limb muscles (Renault et al. 2002; Brack et al. 2005; Conboy et al. 2005; Shefer et al. 2006, 2010, 2013; Yablonka-Reuveni 2011). Overall, sarcopenia represents one of the most dramatic declines in tissue function seen in human health (Rosenberg 1997), leading to increased frailty and loss of independence with age (Evans et al. 2010; Walston 2012). Understanding the factors contributing to this condition is essential for developing therapies to slow age associated muscle deterioration.

Both systemic and muscle tissue intrinsic factors are thought to contribute to the age-associated decline in muscle quality (Carlson and Faulkner 1989; Brack et al. 2007; Walston 2012; Barberi et al. 2013; Bucci et al. 2013). Indeed, age-associated changes in the cellular environment can be detrimental to satellite cell performance (Conboy et al. 2005; Shefer et al. 2006; Chakkalakal et al. 2012; Barberi et al. 2013). Once isolated from the aging muscle and maintained ex vivo in a rich mitogenic environment or transplanted into a young host environment, satellite cells from old rodents exhibit good regenerative potential (Carlson and Faulkner 1989; Conboy et al. 2005; Shefer et al. 2006; Collins et al. 2007; Carlson et al. 2009). Parabiotic pairing between young and old mice has identified circulating factors in young animals that are capable of improving muscle regeneration in aged tissue (Conboy and Rando 2005). Such parabolic studies have shown that both Notch and growth differentiation factor 11 (GDF11) decline with age and have the capacity to reduce age-associated phenotypic changes in skeletal and cardiac muscle, respectively (Conboy et al. 2005; Loffredo et al. 2013). Conversely, transforming growth factor β1 (TGF-β1) and Wnt increase with age and promote fibrotic changes in skeletal muscle (Brack et al. 2007; Carlson et al. 2008).

One circulating multifunctional factor that has broad effects on an array of tissues and interacts with a number of growth factor pathways is alpha klotho (klotho) (Liu et al. 2007; Cha et al. 2008). Klotho is one of two members of the klotho gene family (i.e., alpha and beta) (Kurosu and Kuro-o 2009). The main site of klotho expression is the kidney, but it is also expressed, albeit at a much lower level, in other tissues (Kuro-o et al. 1997; Takeshita et al. 2004; Fon Tacer et al. 2010; Zhou et al. 2013). Klotho functions as both a transmembrane protein and a secreted humoral factor (Chen et al. 2007; Bloch et al. 2009; Tomiyama et al. 2010). The membrane-bound form of klotho is an obligate co-receptor for some of the endocrine members of the fibroblast growth factor (FGF) super family (Wu et al. 2007; Tomiyama et al. 2010). Klotho is particularly important for the function of FGF23 signaling, which regulates vitamin D metabolism and subsequent phosphate excretion in the kidney (Razzaque 2009). The secreted form, which is shedded from the transmembrane protein, is believed to be responsible for the systemic influence of the klotho protein, since it is capable of regulating various growth factor signaling pathways throughout the body (Imura et al. 2004, Kuro-o 2009).

The secreted klotho protein has been shown to directly inhibit the pro-fibrotic TGF-β1 and Wnt pathways in renal tissue (Doi et al. 2011), and could potentially play a similar function in muscle tissue. This makes klotho particularly attractive as a potential regulator of age associated increases in muscle fibrosis. TGF-β1 not only is a master regulator of tissue fibrosis, but also has been shown to inhibit satellite cell activation and myoblast differentiation (Allen and Boxhorn 1987; Yablonka-Reuveni and Rivera 1997; Shefer and Yablonka-Reuveni 2008). Evidence that aging muscle contains higher amount of TGF-β1 and that inhibition of TGF-β1 signaling enhances muscle regeneration, have established a possible role of TGF-β1 in sarcopenia (Carlson et al. 2008; Burks et al. 2011). The secreted form of klotho interacts directly with the TGF-β1 type II receptor (TGF-βR2), reducing the affinity of the endogenous TGF-β1 ligand (Doi et al. 2011). Klotho has also been shown to bind and directly inhibit several Wnt family members in murine small intestine and hair follicle cells (Liu et al. 2007). Like TGF-β1, Wnt family proteins have been implicated in enhancing age related muscle fibrosis (Brack et al. 2007). By targeting TGF-β1 and Wnt pathways,

secreted klotho is capable of reducing kidney fibrosis after ureteral obstruction (Doi et al. 2011; Zhou et al. 2013) and has been proposed as a therapy for reducing fibrosis during chronic kidney disease (Sanchez-Niño et al. 2013). Should similar anti-fibrotic effect of klotho extend to muscle tissue, the klotho protein could become an intriguing candidate for reducing the age-associated decline in muscle tissue.

The klotho hypomorphic (klotho^{hm}) mutant mouse has reduced klotho expression and develops advanced aging-like symptoms, at a young age, that resemble conditions such as arteriosclerosis, ectopic calcification, osteoporosis, skin atrophy, and emphysema (Kuro-o et al. 1997). These mice are also smaller in size, have decreased physical activity and reduced life span (Kuro-o et al. 1997). The severely compromised healthspan and lifespan features exhibited by the klotho^{hm} mouse can however, be rescued by crossing the mutant with a transgenic mouse overexpressing klotho (Kuro-o et al. 1997; Kurosu et al. 2005). With the presence of advanced aging-like symptoms in the klotho^{hm} mouse, klotho has gained a reputation of being an anti-aging factor (Kuro-o 2009) but this topic has remained a subject of debate (Miller 2007).

Despite documented effects of klotho deficiency in a diverse range of tissues and evidence of its ability to attenuate tissue damage in the kidney, little is known of the influence of klotho on skeletal muscle (Iida et al. 2011). In this study we have examined the effect of klotho deficiency on muscle strength and running endurance in klotho^{hm} mice compared to klotho overexpressing transgenic and wildtype mice. We specifically used klotho^{hm} mice from the C57BL/6 background, due to the extensive use of this strain background in aging studies (Goodrick 1975; Turturro et al. 2002; Pettan-Brewer and Treuting 2011). Moreover, as further discussed in the "Results and discussion" section, klotho^{hm} C57BL/6 mice do not die at a young age, which has permitted studies with older mice. Our research shows that female klotho^{hm} mice on the C57BL/6 background exhibit significantly lower levels of klotho gene expression in the kidney compared to klotho^{hm} males. Female klotho^{hm} mice also have a significant reduction in body weight, muscle strength and running endurance compared to wildtype females. Our findings offer in-vivo physiological evidence of the effect of klotho deficiency in a muscle context and provide the foundation for further studies on the involvement of this factor in the development of sarcopenia.

Materials and methods

Mouse strains

All mice were from colonies maintained at the University of Washington. Mice were housed in micro-isolator cages in a pathogen-free facility under 12/12-h light/dark cycle and were fed ad libitum Lab Diet 5053. All animal procedures were approved by the University of Washington Institutional Animal Care and Use Committee. Klotho deficient (Klotho^{hm}, homozygous males, C57BL/6) and transgenic klotho overexpressing (*EFmKL46,* homozygous, males and females, C57BL/6) breeders, were generously provided by Dr. Makoto Kuro-o (UT Southwestern Medical Center). Both lines were maintained as homozygous lines for analysis. To establish a local homozygous klotho^{hm} line, the original klotho^{hm} breeders were first crossed out one generation to wildtype C57BL/6 females and the resulting heterozygous females were backcrossed to the original male breeders.

All mice were genotyped at weaning and again prior to experimentation. Genomic DNA was purified from ear punches with a Qiagen DNeasy, DNA extraction kit and amplified using HotStarTaq (Qiagen) polymerase. Mutant and wildtype alleles were analyzed in separate PCR reactions using a common reverse primer: GGA AGA TTG GAA GTG GAC G and the following allele specific forward primers: mutant, CAA GGA CCA GTT CAT CAT CG; wildtype, TTA AGG ACT CCT GCA TCT GC (Kuro-o et al. 1997). The *EFmKL46* mouse was genotyped using the fwd/rev primers: CCT GGT CGA CCA TTT CAG/AGC ACA AAG TCG ACA GAC TTC TGG C (Kuro-o et al. 1997). Genotyping PCR reactions were performed on a C1000 thermal cycler (BioRad) using a protocol of: 95 °C for 15 min, followed by 36 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min 30 s with a final extension of 72 °C for 10 min. Presence or absence of an amplicon was determined by running the products on a SYBR Safe (Life Technologies) 1 % agarose gel.

Gene expression analysis

Klotho expression levels were quantified from kidney and gastrocnemius muscle using SYBR Green reverse transcription quantitative PCR (RT-qPCR) analysis.

Total RNA was extracted from the tissue samples using the Qiagen RNeasy Kit, with on column DNase digest, then quantified on a NanoDrop spectrophotometer (Thermo Scientific) and reverse transcribed into cDNA with iScript reverse transcriptase (Bio-Rad). The iTaq Universal SYBR Green Supermix (BioRad) was used for RT-qPCR analysis of 25 ng of total cDNA per sample. The RT-qPCR reactions were performed on an ABI 7300 Real Time PCR machine (Life Technologies) with 500nM of forward and reverse primers under the conditions; 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 63 °C for 30 s and 72 °C for 30 s. Eukaryotic translation elongation factor 2 (*Eef2*) expression was determined for each sample (mean ± SEM, Ct: kidney 17.12 ± 0.09; gastrocnemius muscle 18.51 ± 0.34). Klotho expression was then normalized by subtracting the respective *Eef2* expression value for each sample to obtain the ΔCt values used for analysis. All RT qPCR reactions were performed in triplicate with appropriate no template controls. The RT-qPCR primer sets used were (fwd/rev): klotho (398 bp) TAT GCC ACT CGA AAC CGT CCA TGA/CGA CTA CCC AGA GAG TAT GAA G, and *Eef2* (123 bp) TGT CAG TCA TCG CCC ATG TG/CAT CCT TGC GAG TGT CAG TGA [PrimerBank ID: 33859482a1, (Spandidos et al. 2010)]. The efficiency for each primer set was validated using a standard curve produced from purified PCR product and RT-qPCR reaction specificity was monitored each reaction by performing a melting curve analysis. Based on the RT-qPCR assay efficiency, gene amplification in gastrocnemius samples at a level higher than 34 cycles (ΔCt of 15) was considered to have no expression.

Body composition

Total lean and fat mass were measured using quantitative magnetic resonance (QMR). Live mice were weighed to the nearest 0.1 g and QMR readings were recorded using an EchoMRI QMR machine (Echo medical systems). The percent lean and fat mass was determined by normalizing to body weight.

Grip strength

Forelimb grip strength was analyzed using a force tension apparatus (San Diego Instruments). Prior to the test, each mouse was weighed to the nearest 0.1 g. Once mice gripped the stationary bar with their forepaws, they were stretched horizontally while held at the base of their tails. Mice were pulled gradually until they let go of the bar. The process was repeated at least 8 times to determine the peak grip force value (gram-force) used for analysis. For an image depicting the procedure go to http://depts.washington.edu/compmed/ivs/grip.html.

Running wheel

Low-profile wireless running wheels (Med Associates, Inc.) were used to compare continuous voluntary running activity. Mice were housed in individual cages and allowed to acclimate to a fixed running wheel for three days after which the wheels were activated to enable rotation. Total revolutions were recorded every min for 6 days. For further details go to http://depts.washington.edu/compmed/ivs/running_wheels.html.

Statistics

Statistical analysis of gene expression data was performed on normalized ΔCt values in order to avoid potential artifacts introduced during ΔΔCt calculation, especially with low expression levels. A *t* test was used to analyze expression levels and weight data and p values less than 0.05 were considered significant. When comparing physiological outcomes between wildtype, klotho^{hm} and *EFmKL46* mouse strains, data were first analyzed using a one-way ANOVA, followed by post hoc analysis with individual *t* tests and Bonferroni correction ($p < 0.017$ considered significant). All average values stated in the text or shown in figures represent mean ± SEM.

Results and discussion

Characterization of the klotho^{hm} C57BL/6 mice

Klotho^{hm} mice have a deficiency in klotho production due to a transgene insertion, which deleted approximately 8 kb of the promoter region of the klotho gene (Kuro-o et al. 1997, Imura et al. 2004). These mice, which were originally produced on a mixed strain background of C57BL/6J and C3H/J, show an accelerated deterioration of many of their organs and are short-lived, with an average life span of around

60 days. In the current study we used klotho^{hm} mice (homozygous for the klotho mutant allele; i.e., klotho$^{hm/hm}$) on a pure C57BL/6 mouse background, which have no noticeable deterioration in health or lifespan. The original male breeders we received ($n = 3$), lived to an age of around 22 months before being harvested for tissue sampling. To date, klotho^{hm} progeny generated in our lab have been followed for up to 14 months of age, and both males and females appear healthy. Notably, klotho^{hm} mice on the C57BL/6 background are fertile, while mice on the original strain have been maintained by crossing heterozygous parents (Kuro-o et al. 1997). To our knowledge the lifespan of klotho^{hm} mice on the C57BL/6 background has not been reported, and it is possible based on our pilot observation with the original breeders, that their lifespan may not differ significantly from wildtype mice.

We validated the genotype of our klotho^{hm} colony founders and of their progeny using established PCR assays (Kuro-o et al. 1997). All homozygous klotho^{hm} mice were found to be negative for the wildtype klotho allele (Fig. 1a) and positive for the klotho mutant allele (Fig. 1b). Heterozygous mice were positive for both wildtype and mutant alleles (Fig. 1a, b). We then characterized our local colony of klotho^{hm} C57BL/6 mice for endogenous klotho mRNA expression compared to wildtype mice using RT-qPCR. This analysis was done with RNA isolated from the kidney, the main site of klotho production (Kuro-o et al. 1997). Klotho expression values (ΔCt) were calculated by normalizing klotho expression to endogenous *Eef2* expression, determined for each individual RNA sample (mean ± SEM, Ct: kidney 17.12 ± 0.09; gastrocnemius muscle 18.51 ± 0.34).

The RT-qPCR analysis of kidney cDNA revealed no differences in klotho expression between wildtype C57BL/6 males and females ($p = 0.76$, Fig. 2a; mean ± SEM, ΔCt: male, $n = 3$, 0.34 ± 0.6; female,

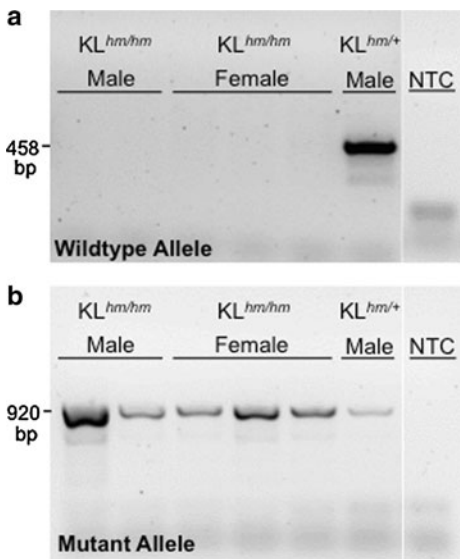

Fig. 1 Genotyping of the klotho^{hm} mouse. PCR analysis of **a** wildtype and **b** mutant alleles in klotho^{hm} mice (klotho$^{hm/hm}$) compared to a heterozygous control mouse (klotho$^{hm/+}$). PCR no template control samples (NTC) are included for each primer set

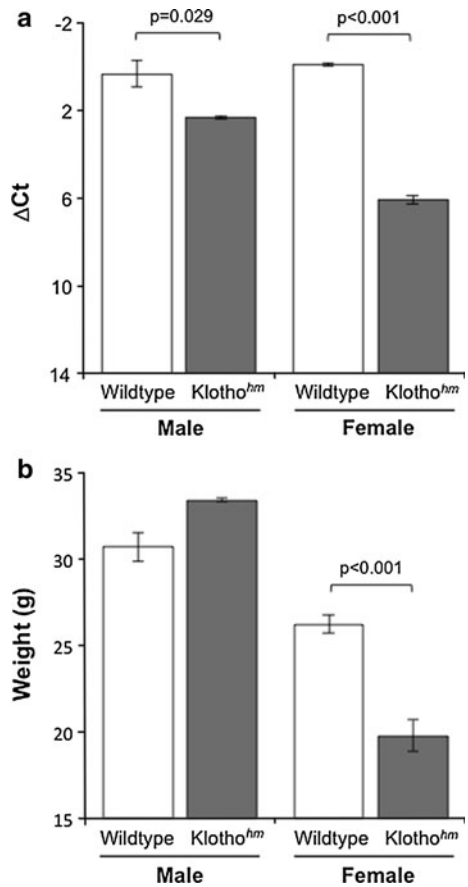

Fig. 2 Characterization of the klotho^{hm} mouse. **a** Klotho expression level (ΔCt, mean ± SEM) in the kidney of wild type (male $n = 3$, female $n = 6$) and klotho^{hm} (male $n = 3$, female $n = 3$) mice. All samples were independently normalized to the expression of the *Eef2* reference gene. **b** Body weight (mean ± SEM, grams) of wildtype (male $n = 9$, female $n = 6$) and klotho^{hm} (male $n = 4$, female $n = 7$) mice between the ages of 7 and 11 months. *Brackets* represents significant differences with the indicated p value

$n = 6, -0.05 \pm 0.06$). Both male and female klotho^{hm} mice, showed significantly reduced klotho expression in the kidney compared to the wildtype mice (Fig. 2a, mean ± SEM ΔCt: male, $n = 3, 2.34 \pm 0.34$; female, $n = 3, 6.09 \pm 0.2$). Surprisingly, there was a significant difference in the extent of decline in klotho expression between male and female klotho^{hm} mice. Whereas klotho^{hm} males exhibited only 4-fold less klotho expression in the kidney when compared to wildtype male mice, the female klotho^{hm} mice had 71-fold less expression than wildtype females (Fig. 2a).

In addition to their drastic decline in kidney klotho expression, female klotho^{hm} mice were significantly smaller than wildtype females (Fig. 2b, data shown for 7–11 months old animals; mean ± SEM, grams: Klotho^{hm} 19.77 ± 0.90; WT 26.2 ± 0.52). There was however, no difference between the weight of mutant and wildtype males (Fig. 2b, 7–11 months old animals; mean ± SEM, grams: Klotho^{hm} 33.4 ± 0.09; WT 30.67 ± 0.81). The fact that male klotho^{hm} mice did not differ in weight from wildtype males could suggest that the relatively low knockdown of klotho mRNA in male klotho^{hm} C57BL/6 mice was insufficient to trigger changes in body weight. Indeed, in the original klotho^{hm} mouse, both males and females are significantly smaller than the wildtype mice (Kuro-o et al. 1997).

Characterization of the klotho overexpressing, *EFmKL46* C57BL/6 mice

Two transgenic lines that overexpress klotho (driven by the ubiquitous human elongation factor 1 promoter) were previously produced and both showed increased lifespan compared to wildtype mice (Kuro-o et al. 1997; Kurosu et al. 2005). These mice were created on the same mixed mouse background as that described above for the original klotho^{hm} mouse. From these two transgenic lines, we focused in the current study on the transgenic strain *EFmKL46,* which was reported to have transgene expression in skeletal muscle (Kurosu et al. 2005). Klotho serum protein levels have been previously shown to be elevated in *EFmKL46* mice concurrent with increased transcript expression (Kurosu et al. 2005). The skeletal muscles of *EFmKL46* mice are therefore likely exposed to the direct effects of both secreted and membrane-bound klotho forms.

All *EFmKL46* mice used in this study were confirmed to harbor the transgene based on genotyping (see "Materials and methods"). Klotho expression level was then analyzed in both the kidney and gastrocnemius muscle from one male and one female *EFmKL46* mouse, to verify klotho overexpression. Per each tissue, no difference in klotho expression was observed between the male and female mice, so genders were combined for analysis. Klotho overexpression in the gastrocnemius muscle of *EFmKL46* mice was 51-fold lower than expression levels found in the kidney (mean, ΔCt: gastrocnemius 5.78, kidney 0.10). Nevertheless, the gastrocnemius klotho expression level in *EFmKL46* mice was 70-fold higher compared to the low levels of expression present in the gastrocnemius muscle of wildtype mice (mean ± SEM, ΔCt: WT gastrocnemius 11.90 ± 0.65, WT kidney 0.08 ± 0.19, based on mice presented in Fig. 2a). Klotho expression was not detected in the gastrocnemius muscle of klotho^{hm} mice (see "Materials and methods"). The low level of klotho expression detected in the gastrocnemius muscle of wildtype mice is consistent with earlier studies that have shown little to no klotho expression in various limb muscles (Kuro-o et al. 1997; Fon Tacer et al. 2010; Stuelsatz et al. 2012, supplemental Fig. S3). It is important to note that klotho gene expression in the muscle tissue does not necessarily arise from myogenic cells. Our unpublished expression data with myogenic and non-myogenic cell populations isolated from adult mouse muscles (Day et al. 2007) attribute low-level klotho expression only to the non-myogenic cell population (K. Day and Z. Yablonka-Reuveni).

Assessment of muscle strength and running endurance in female mice

In order to examine the effect of klotho deficiency and overexpression in the physiological context of muscle performance, klotho^{hm}, *EFmKL46,* and wildtype C57BL/6 adult females (7–11 month old), were subjected to standard protocols for measuring forelimb grip strength and voluntary wheel running. These physiological assays were preferentially performed on female mice in view of the distinctive effect of klotho deficiency on klotho^{hm} females (i.e., a large decline in klotho kidney expression and reduced weight) compared to only a slight effect in klotho^{hm} males (i.e., only a small decline in kidney klotho expression). Notably, the reduced body weight of the klotho^{hm} females was not due to a change in lean/fat body composition, as shown by QMR analysis

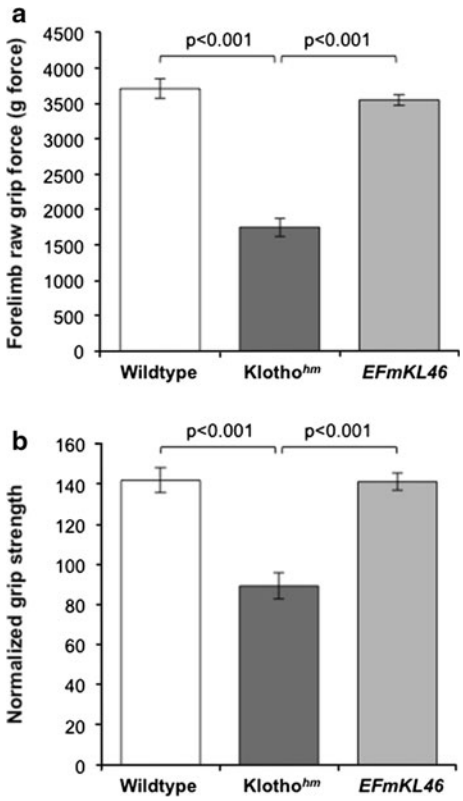

Fig. 3 Analysis of forelimb grip strength in adult (7–11 months old) female mice. **a** Maximum grip strength force (mean ± SEM, grams-force) and **b** normalized force to body weight ratio (mean ± SEM) of wild type ($n = 7$), klotho^{hm} ($n = 7$), and klotho transgenic, *EFmKL46* mice ($n = 8$). Significant differences identified by *brackets* with respective p values

(mean ± SEM, %lean, %fat: Klotho^{hm,} 76.99 ± 0.46, 11.99 ± 0.66; WT, 74.81 ± 1.57, 14.47 ± 1.41; *EFmKL46*, 78.07 ± 1.07, 13.83 ± 1.16).

Klotho^{hm} mice had significantly reduced grip strength compared to wildtype and *EFmKL46* mice (Fig. 3). The maximum forelimb grip force measured in female klotho^{hm} mice was 53 % and 51 % lower than wildtype and *EFmKL46* mice, respectively (Fig. 3a). After normalizing raw grip force to body weight, klotho^{hm} mice exhibited 37 % less strength than both wildtype and *EFmKL46* mice (Fig. 3b). These results were consistently reproduced when assayed 4 independent times with the same animals over a 4-month period.

The female mice were additionally examined for their endurance level by submitting the animals to voluntary wheel running for 6 days (Fig. 4). Consistently for all 3 experimental groups, the active running period was during the dark cycle. While there was no difference in the total revolutions ran between wildtype and *EFmKL46* mice, the klotho^{hm} mice ran 66 and 63 % less than wildtype and *EFmKL46* mice, respectively (Fig. 4a). A detailed analysis of klotho^{hm} mouse running trends revealed that during their actual running activity there was no difference in the average running speed (i.e., revolutions per minute) compared to wildtype and *EFmKL46* mice (Fig. 4b). Klotho^{hm} mice however, spent 66 and 62 % less time running than wildtype and *EFmKL46* mice, respectively (Fig. 4c). This is readily apparent when analyzing the overall running profile actograms from each mouse strain, which show a sporadic running pattern in klotho^{hm} mice (Fig. 4d). Similar results were observed in 2 additional running wheel experiments conducted when the mice were younger in age.

Changes in physical activity were indeed evident in the original klotho^{hm} mouse from the mixed strain background. These mice demonstrated an altered gait with significantly reduced stride lengths (Kuro-o et al. 1997). They also exhibited 50 % less horizontal and rearing activity than control mice when assayed in an open field experiment (Kuro-o et al. 1997). Interpretations about physical activity in the original klotho^{hm} mouse strain however, have been limited by the fact that these mice do not strive and die at a young age from a number of complications. We did not observe any obvious changes in routine cage activity or gait disturbances in klotho^{hm} C57BL/6 mice, but these parameters were not measured directly.

A number of conditions could be responsible for the observed decreases in muscle strength and running endurance in klotho^{hm} mice. In addition to potentially influencing skeletal muscle, klotho may influence muscle strength by its effect on bone density. Bone mineral density has been consistently shown to be associated with muscle strength in humans (Arden and Spector 1997). The original klotho^{hm} mouse strain exhibited a significant reduction in bone mineral density (Kuro-o et al. 1997). This decrease in bone mineral density is believed to be due to the effect of klotho on osteoblast and osteoclast differentiation as well as on calcium and phosphate homeostasis (Kawaguchi et al. 1999; Kuro-o 2006; Nakatani et al. 2009). While further studies would be needed to establish the mechanism involved in the reduced grip strength exhibited by klotho^{hm} females, it is interesting to note that a positive correlation between klotho levels and

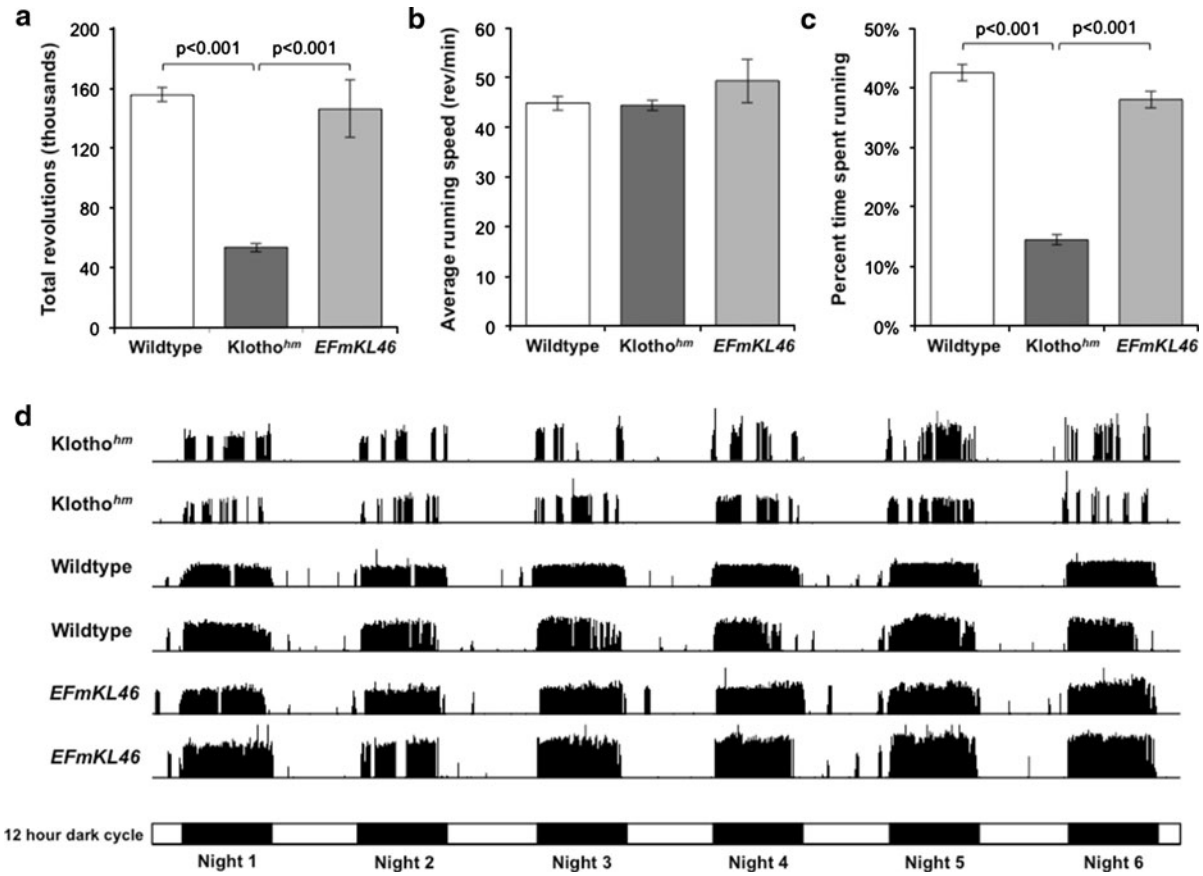

Fig. 4 Mouse activity over 6 days of voluntary wheel running. Studies were performed with the adult female groups described in Fig. 3. **a** Klotho^{hm} mice ran significantly less than wildtype and *EFmKL46* mice as shown by the total revolutions ran over the 6 days analyzed. **b** While klotho^{hm} mice ran at the same rate as wildtype and *EFmKL46* mice (i.e., speed in revolutions/min, per actual time spent running), as shown in **c** the percentage of time spent running was significantly less compared to the other two groups. **d** Typical running frequency actograms (showing 2 independent examples per each mouse group), demonstrating a unique sporadic running pattern in klotho^{hm} mice characterized by frequent gaps in the nightly running routine. A schematic representation of the light and dark cycle (12/12-h), over the 6 experimental days, is shown below the actograms. **a–c** Significant differences are noted by *brackets* and respective *p* values

grip strength has been established in a longitudinal study of elderly humans (Semba et al. 2012).

The sporadic running trend exhibited by klotho^{hm} mice could also suggest changes in the cardiovascular or respiratory systems, which could induce early onset exhaustion in klotho deficient mice. Cardiovascular and respiratory changes have been documented in the original klotho^{hm} mouse strain (Kuro-o et al. 1997). These mice developed arteriolosclerosis, pulmonary emphysema and ectopic calcification of several tissues including bronchial mucosa, alveolar cells, and cardiac muscle (Kuro-o et al. 1997; Suga et al. 2000). Even heterozygous klotho^{hm} mice were shown to exhibit endothelial dysfunction (Saito et al. 1998; Takeshita et al. 2004).

There are many intriguing possibilities for why female klotho^{hm} mice exhibit such a dramatic decline in muscle-associated functions compared to wildtype and *EFmKL46* animals. Whether it involves the musculoskeletal, cardiac or respiratory systems or a combination of factors needs to be further investigated at multiple ages and at the histological level.

Conclusion

Klotho has been shown to have broad reaching effects on a number of body systems. Insight into the role of klotho in skeletal muscle is however, extremely limited. Our results demonstrate that klotho deficiency

has a dramatic effect on both muscle strength and running endurance in mice. While it is currently unclear as to the exact underlying cause of the decline in muscle function in klotho^{hm} mice, our data provide a critical first step to understanding the impacts of klotho on skeletal muscle. This research also reinforces the importance of mouse strain background and gender in influencing the phenotypic effect of specific genetic modifications. The mouse strain effect is highlighted in this study with the use of klotho^{hm} mice from the C57BL/6 mouse background, which do not show any overt pathology and live to an old age. Our research demonstrates that the underutilized klotho^{hm} C57BL/6 mouse is a unique model to study the effects of klotho in adult mice without the experimental limitation of an ailing phenotype and a short lifespan. Overall, our findings indicate that klotho deficiency influences muscle strength and running endurance in mice. As the decline in muscle-associated performance is also the hallmark of muscle aging, future research should examine klotho as a potential inhibitor of age-associated muscle deterioration with the goal of understanding the complex mechanisms underlying sarcopenia.

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