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Klotho Enhances Postnatal Skeletal Muscle Growth

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Klotho Enhances Postnatal Skeletal Muscle Growth

A thesis submitted in partial satisfaction of the requirements for the degree

Master of Science in Physiological Science

by

Catherine Claire Lindsay

2015

ABSTRACT OF THE THESIS

Klotho Enhances Postnatal Skeletal Muscle Growth

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Catherine Claire Lindsay

Master of Science in Physiological Science

University of California, Los Angeles 2015

Professor James G. Tidball, Chair

Klotho is a pleiotrophic gene that regulates homeostasis in various tissues. Although Klotho function has been typically viewed in the context of an "anti-aging" molecule, we have observed that its expression in muscle decreases during postnatal muscle development. Here, we report that *Klotho* has an important role in modulating early muscle growth. In actively growing skeletal muscle, loss of Klotho reduces postnatal myofiber size and overexpression of *Klotho* increases the size above wild-type levels. Furthermore, the presence of Klotho is required to

maintain satellite cell numbers. Klotho enhances skeletal muscle growth by increasing protein content in differentiated myotubes and promoting proliferation of satellite cells. Despite the well-characterized inhibitory relationship between Klotho and IGF-1 signaling in age-related disease models, we find that Klotho upregulates IGF-1 signaling in postnatal skeletal muscle. We conclude that *Klotho* is not solely a gene that prevents premature aging, rather, it also has an important role in modulating early muscle growth.

The thesis of Catherine Claire Lindsay is approved.

Reggie Edgerton

Alan Grinnell

James G. Tidball, Committee Chair

University of California, Los Angeles

2015

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Introduction

Muscle growth during the first month of age is a rapid and dynamic process. Whole body mass doubles every week and muscle mass increases seven-fold by increasing in both length and cross-sectional area within the first 4 weeks of life (Gokhin et al., 2008). At birth, muscle stem cells, termed satellite cells, comprise nearly 30% of the total nuclei and they proliferate extensively throughout the early growth period (Moss and Leblond, 1971). The postnatal period provides an excellent model for understanding the normal process of muscle growth and discovering how to enhance the rate of growth. Modulating muscle growth during the postnatal period has important implications for skeletal muscle disorders, regeneration following acute injury, and aging.

Skeletal muscle is formed through a process called myogenesis in which satellite cells undergo activation, proliferation, differentiation, and fusion to form mature myofibers. Each stage of myogenesis is marked by the expression of important myogenic transcription factors that regulate gene expression. Satellite cells that express the transcription factor Paired box 7 protein (Pax7) are quiescent muscle stem cells that when activated, also express the transcription factor MyoD and enter the cell cycle. Once activated, these proliferative myoblasts can continue to self-renew or withdraw from the cell cycle and undergo differentiation (Collins and Partridge, 2005). Myoblasts that proceed through the differentiation process first express myogenin and later, transiently express MRF4 as they fuse with existing myofibers (Cornelison and Wold, 1997; Yablonka-Reuveni and Rivera, 1994).

Postnatal muscle growth in mice occurs primarily by myofiber hypertrophy. The progression of postnatal muscle growth can be divided into two stages: the early stage, in which growth involves the addition of myonuclei to each myofiber, and the late stage, in which growth in cross-sectional area occurs independent of satellite cell fusion (White et al, 2010). The early stage occurs until about 3 weeks of age in mouse skeletal muscle and is a period of growth dependent on the activation of satellite cells to proliferate and eventually fuse with the myofiber, a process termed myonuclear accretion. The late stage occurs from 21 days to at least two months of age, is independent of myonuclear accretion, and is most likely due to a positive shift in the protein anabolism-catabolism balance (Schiaffino et al., 2013; White et al., 2010).

With age, there is a gradual loss of muscle mass and decreases in myofiber size and number (Lexell, 1995). Additionally, reductions in satellite cell numbers and functional capacity with age have been reported, although the extent that aging satellite cells contribute to sarcopenia is controversial (Day et al., 2010; Fry et al., 2015). Among various proteins that change with age and affect muscle mass, there are few data on the role of these proteins during normal postnatal growth. Examining the function of anti-aging factors in the context of a normal growth process can assist in elucidating their role for muscle mass maintenance and preventing functional impairments with age. Thus, we chose to investigate the role a previously characterized anti-aging factor, Klotho, during the process of normal muscle growth.

The gene, *Klotho*, was named for the Greek goddess who spins the thread of life. Makoto Kuro-o was the first to report that an insertional mutation in a single locus corresponding to the *Klotho* promoter region gave rise to systemic defects, including features such as reduced body size, skin

atrophy, osteoporosis, arteriosclerosis, and reduced physical activity (Kuro-o et al., 1997). Since the loss of this gene results in a phenotype that resembles premature systemic aging, Klotho has been typically considered an anti-aging factor.

Klotho is a pleiotrophic gene and can regulate homeostasis in various tissues. The *Klotho* gene encodes a transmembrane protein that functions as a co-receptor for fibroblast growth factor 23 (FGF23), although its extracellular domain can also be cleaved and function as an endocrine factor (Chen et al., 2007; Kurosu et al., 2006). While *Klotho* is primarily expressed in the kidney where it functions with FGF-23 to enhance the elimination of phosphate from the body, it is found in low levels in other tissues (Kuro-o et al., 1997; Nakatani et al., 2009). Klotho protein can inhibit insulin/IGF-1 signaling and subsequently activate the FoxO transcription factors, inducing superoxide dismutase expression and conferring the tissue with increased resistance to oxidative stress (Kurosu et al., 2005; Yamamoto et al., 2005). One of the primary functions of soluble Klotho is in cleaving sugar groups with its sialidase activity. Klotho can cleave terminal sialic acid motifs from the transient receptor potential ion channel 5 (TRPV5), exposing the ligand for galectin-1, which holds TRPV5 on the cell membrane (Cha et al., 2008; Chang et al., 2005). TRPV5 is a functional calcium channel and its increased accumulation in the membrane results in an elevated calcium flux.

Although *Klotho* is expressed in skeletal muscle at relatively low levels, numerous data suggest that it may serve an important functional role. Other investigators have demonstrated that systemic loss of Klotho causes a reduction in muscle weight and fiber size in the tongue, masseter, and gastrocnemius (Iida et al., 2011). Functional limitations including decreased

overall treadmill running time and reduced grip strength have been reported with the loss of Klotho (Phelps et al., 2013). In addition, our laboratory has shown that Klotho levels are low in dystrophic and aging muscle, models in which there is a loss of muscle mass and decline of regenerative capacity. Furthermore, unpublished data from our laboratory indicate that following acute injury, loss of Klotho slows muscle fiber regeneration. Taken together, these data suggest that Klotho may play a role in maintaining muscle mass.

Klotho may also have a role in altering the functional capacity of satellite cells. Mice with reduced *Klotho* expression display increased Wnt signaling and it has been shown that Klotho can directly inhibit Wnt signaling molecules in hair follicle cells (Liu et al., 2007). Increased Wnt signaling can promote a conversion of satellite cells to a fibrogenic fate and reduce muscle regeneration (Brack et al., 2007). Taken together, these data suggest that Klotho could play a positive role in regeneration by down-regulating Wnt signaling.

Our goal was to elucidate Klotho's role in postnatal muscle growth. We investigated the effects of overexpression and loss of Klotho on myofiber size, satellite cell numbers, protein balance, and expression of myogenic regulatory factors. We further assayed for changes in the IGF-1 signaling pathways that are involved in controlling skeletal muscle growth. We hypothesize that *Klotho* is not solely a gene that prevents premature systemic aging, rather, it also has an important role in modulating early muscle growth.

Materials and Methods

Mouse model

All experiments involving animals were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Animal Research Committee of the University of California, Los Angeles approved this protocol. Mice were housed in a pathogen-free facility under 12-hour light and 12-hour dark cycle. Dr. Makoto Kuro-o generously provided Klotho deficient (KL-) and transgenic Klotho overexpressing (KLtg+) mice. The Klotho hypomorphic line contained an insertional mutation in the 5' flanking region of the Klotho gene and the Klotho transgenic mouse line over-expressed Klotho under control of the ubiquitous human elongation factor-1alpha promoter, EFmKL46. We backcrossed the mutant mice onto the C57BL/6J background and genotyped mice at weaning to confirm the presence of mutant alleles. We utilized only male mice in this study.

Tissue preservation

Male mice at 2 weeks, 3 months, and 5 months of age were euthanized by isofluorane and weighed prior to dissection. We carefully dissected and recorded the weights of selected muscles. We calculated and compared muscle mass to body mass ratio. Tissues were embedded in Tissue Tek O.C.T. and flash-frozen in isopentane precooled in liquid nitrogen.

Myofiber cross-sectional area analysis

Ten μm sections of tibialis anterior muscles were cut using a cryostat and tissue sections were stained with filtered hematoxylin (Gill's formula, Vector H-3461). Cross-sectional myofiber area was measured for all intact fibers in the section on 100 x magnification (for 3 month) and 200 x

magnification (for 2 week) with BioQuant digital imaging system. We plotted the mean and SEM of each group for representation.

Immunohistochemistry

For the Pax7 immunohistochemical labeling, 10 μm sections of tibialis anterior muscles were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and then subjected to a standard antigen-retrieval protocol. Sections were heated at 95°C in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) then incubated with mouse on mouse (M.O.M.) blocking reagent (M.O.M kit, Vector Laboratories PK-2200). Sections were washed with 0.1% Tween-20 in PBS buffer and subsequently incubated with Pax7 primary antibody (Aviva Systems Bio ARP32742_P050) at a 1:50 dilution for 3 hours at room temperature. Following primary incubation, sections were incubated with 0.3% hydrogen peroxide in PBS to quench endogenous peroxidases. Anti-mouse secondary antibody (M.O.M kit, Vector Laboratories PK-2200) was utilized at 1:250 in PBS for 30 minutes. Following washes with PBS, sections were incubated with the ABC reagent (M.O.M kit, Vector Laboratories PK-2200). Slides were developed with A.E.C. kit (Vector Laboratories SK-4200) and sealed with VectaMount (Vector Laboratories H-5501).

RNA extraction

For the 3 and 5 month-old mice, 100 mg of quadriceps muscle were homogenized in 1 ml of Trizol reagent (Life Technologies) for 30-second intervals with a Sorvall omni-mixer. Samples were spun at 12,000 g for 10 minutes at 4°C to clear insoluble material. We incubated the supernatant at room temperature for 5 minutes to allow for disassociation of nucleoprotein

complexes and added chloroform (20% of original Trizol volume) to lyse cells. RNA was separated into the aqueous phase after spinning at 12,000 g for 15 minutes at 4°C. We precipitated the RNA with isopropanol (50% of original Trizol volume) and spun at 12,000 g for 10 minutes at 4°C. The pellet was washed in 75% ethanol, spun at 8,400 g for 5 minutes at 4°C, and resuspended in 50 µl RNase-free water.

RNA was further purified and cleaned using Qiagen RNeasy Mini Kit (Qiagen #74106). RNA concentration was read on a Beckman DU730 spectrophotometer. All samples achieved an OD260:OD280 ratio of 1.8 or greater and demonstrated good RNA integrity as verified by the presence of both ribosomal subunits on a 1.2% agarose gel.

For the small 2-week quadriceps muscles, we homogenized the entire muscle in 0.8 ml Trizol reagent and adjusted the RNA isolation protocol for the different starting Trizol volume as previously noted. The RNA pellet was resuspended in 20 µl RNase-free water and further purified with the Zymo DNA-free RNA kit (Zymo Research #R1014), which is useful for small samples with low RNA yields. The "General Procedure" protocol was followed and RNA eluted in a final volume of 20 µl RNAse-free H20. All samples had an OD260:OD280 ratio of 1.8 or greater.

Quantitative real-time PCR

2 µg of total RNA from each sample were reverse transcribed to cDNA in a 20 µl total reaction volume using Super Script Reverse Transcriptase II (Invitrogen). Following the reaction, we diluted cDNA in a 1:1 ratio in water.

We utilized real-time PCR (qPCR) to quantitatively measure the expression of our genes of interest. 100 ng of cDNA were mixed with Biorad SYBR Green Supermix in a 25 µl total reaction volume and placed in a Biorad My iQ 96-well cycler with the following temperature cycles:

Cycle 1:	**1x**	95°C for 2 mins (Denaturation and enzyme activation)
		95°C for 30 sec (Denaturing)
		62°C for 30 sec (Annealing)
Cycle 2:	**50x**	72°C for 15 sec (Extension)
Cycle 3:	**81x**	Ramping 55-95°C (in 0.5°C increments for 10 sec)

Table 1 lists the primers used for qPCR.

We selected the housekeeping genes SRP14 and HPRT1 after verification of similar expression in all samples. After standardizing expression values of each sample to the values for each of the two housekeeping genes, the corrected threshold cycle number dictated the relative expression of individual transcripts. The natural log of the relative quantification was utilized for comparison. Mean and SEM expression values were plotted in Prism and used for determination of significance.

Western blotting technique

Whole muscle samples were homogenized on ice in 30 volumes of reducing sample buffer (80 mM Tris pH 6.8, 0.1 M dithiothreitol, 70 mM sodium dodecyl sulfate) with protease inhibitor (at a dilution of 1:100, Sigma). Steaming for 2 minutes denatured the proteins and the sample was then centrifuged at 10000 g for 10 minutes in 4°C. A filter paper assay in reference to bovine

serum albumin standards determined the protein concentration of the supernatant, according to a standardized method (Minamide and Bamburg, 1990).

We loaded 30 micrograms of protein from each sample in 10% polyacrylamide gels with a 5% stacking gel. The SDS-PAGE gel was run at 80 V for approximately 1 hour and 180 V for 2 hours. The proteins were then electrophoretically transferred for 3 hours at 4°C onto nitrocellulose membranes using a transfer apparatus (Trans-Blot cell, Biorad). Ponceau S solution (Sigma P7170) confirmed equal protein loading for all samples and the membrane incubated with blocking buffer (0.5% Tween-20, 0.2% gelatin and 3% dry milk) for 1 hour at room temperature to inhibit non-specific binding. We then washed the nitrocellulose membranes with 0.1% Tween-20 in PBS buffer and incubated them with 1:500 dilution of rabbit anti-Klotho (Abcam #ab154163, 20 μg/ml) overnight. After primary incubation, membranes were again washed with 0.1% Tween-20 PBS buffer and incubated for 1 hour with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (GE Healthcare #NA934) at 1:10,000 in PBS-Tween 20. An enhanced chemiluminescence kit (Protein Simple Chemi-Glow) allowed visualization of protein bands. We took images of the membranes with the Alpha Innotech imaging system.

Cell culture stimulation

For the Klotho stimulation of myoblasts, C2C12 cells were plated at a concentration of 133,000 cells per well in complete medium (Dulbecco's Modified Eagle Medium, 10% fetal bovine serum, and 1% penicillin and streptomycin, Sigma) and media was changed every day. We stimulated the C2C12 myoblasts at 24 and 48 hours with one of the 4 treatment conditions: 1) heparin, 2) Klotho, FGF-23, and heparin, 3) heparin and anti-tumor necrosis factor [TNF], and 4)

heparin, FGF-23, Klotho, and anti-TNF. Cells were collected sub-confluent at 72 hours after plating. Final concentrations for the reagents were as follows: heparin (10 μg/ml), FGF-23 (0.5 μg/ml, Klotho (1 μg/ml, anti-TNF (6 μg/ml), isotype control (6 μg/ml). FGF-23 has a weak affinity for FGF receptors and thus requires the cofactor heparin in the absence of Klotho to activate FGF signaling (Yamashita et al., 2002). To ensure that the effects of FGF-23 would be measured in the absence of Klotho, we added heparin as a cofactor in the Klotho-stimulation experiments.

For the Klotho stimulation of myotubes, C2C12 cells were plated at a concentration of 133,000 cells per well in complete medium (Dulbecco's Modified Eagle Medium, 10% fetal bovine serum, and 1% penicillin and streptomycin, Sigma) and media was changed every day. On day 3 we incubated the cells with DMEM and penicillin/streptomycin only to induce differentiation. Cells were stimulated at 24 and 48 hours following transfer to differentiation medium with one of the 5 treatment conditions: 1) FGF-23 and heparin, 2) FGF23, heparin and anti-TNF 3) FGF23, heparin, and Klotho 4) FGF23, heparin, Klotho, and isotype control 5) FGF23, heparin, Klotho, anti-TNF. N=5 for each group. Cells were collected at 72 hours after starvation in 400 μl reducing sample buffer and protease inhibitor (Sigma). We triturated the cells with a 22-gauge needle, boiled them for 2 minutes, and conducted a filter paper assay compared to bovine serum albumin standards to measure the protein concentration, according to a standardized method (Minamide and Bamburg, 1990).

Statistical methods

Significance was measured between means assessed with a Student's unpaired t-test unless otherwise noted. If one-way ANOVA were utilized, it was followed by post hoc analysis with Tukey's test. Values stated in the text and figures are mean +/- SEM unless otherwise indicated. Differences were considered significant at $P< 0.05$. Graphs made with GraphPad Prism, with the exception of the myofiber frequency distributions that were made with Excel 2011.

Table 1: List of primers used in quantitative PCR

	Forward primer	Reverse primer
SRP14	GAGACGAGCAGTTCCTGAC	CGGTGCTGATCTTCCTTTTC
HPRT1	GCTGACCTCGTGGATTACATTAAAG	CCACCAATAACTTTTATGTCCCC
Klotho	GTCTCGGGAACCACCAAAAG	CTATGCCACTCGAAACCGTC
FGF-23	GCACTGCTAGAGCCTATCCG	GCACTGTAGATGGTCTGATGG
Pax7	CTCAGTGAGTTCGATTAGCCG	AGACGGTTCCCTTTGTCGC
MyoD	GAGCGCATCTCCACACACAG	AAATCGCATTGGGGTTTGAG
Myogenin	CCAGTACATTGAGCGCCTAC	ACCGAACTCCAGTGCATTGC
MRF4	ATCAGCTACATTGAGCGTCTACA	CCTCCAATCATCCCAAACACTTG
IGF-1	CTCACCTTCACCAGCTCCAC	GCAACACTCATCCACAATGCC
IGF-1R	GGACATTGGAAGGAGAAGCC	CCACGCAGGTTGTGTTGTC
CD206	GGATTGTGGAGCAGATGGAAG	CTTGAATGGAAATGCACAGAC

Results

Modulation of Klotho expression during early postnatal muscle growth

Klotho expression is highest during the period of rapid postnatal muscle growth. Quantitative PCR showed that *Klotho* transcript levels in wildtype mice peak at 2 weeks of age and decline throughout postnatal muscle development (Figure 1B). As expected, the *Klotho* hypomorphic mice (KL-) had a significantly reduced expression of *Klotho* and the *Klotho* overexpressing mice (KLtg+) had a greater than 15-fold increase in *Klotho* expression compared to C57 control mice of the same age.

Modulation of *Klotho* at 3 months of age did not alter FGF-23 expression (Figure 1C). However, 2 week-old KL- mice have significantly elevated FGF-23 expression levels (Figure 1D), which is perhaps a compensatory measure to account for the loss of FGF-23 signaling in the absence of its co-receptor Klotho. Western blot confirmed the presence of Klotho protein (Figure 1E).

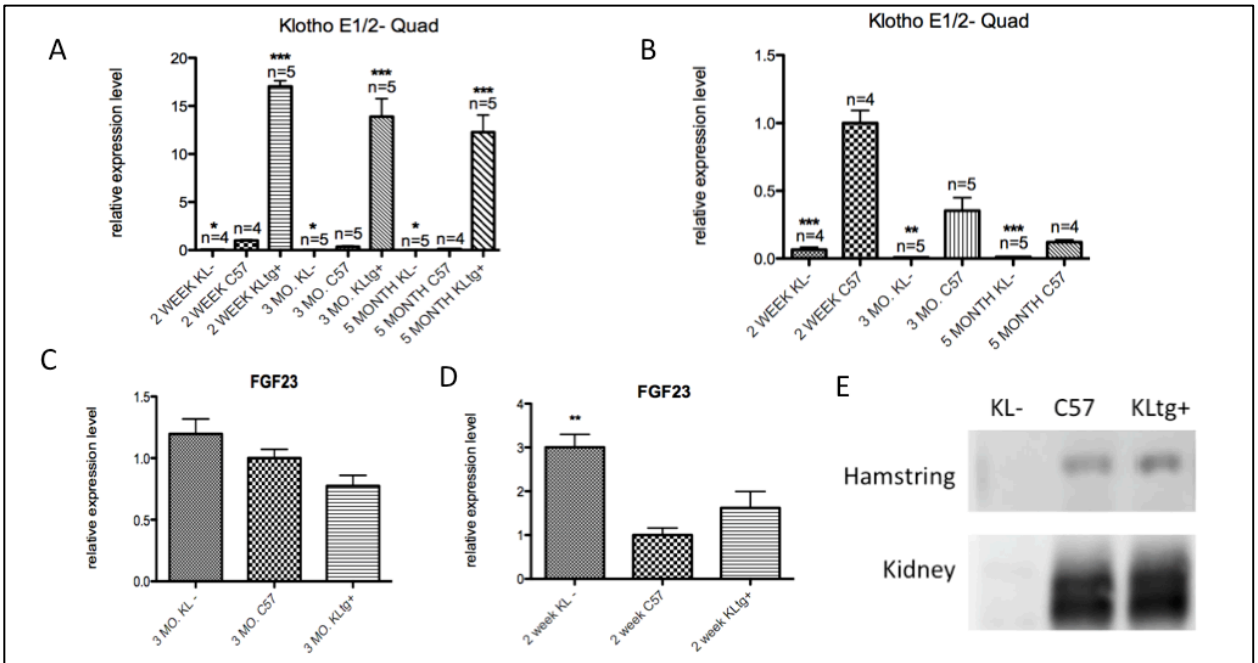

Figure 1: Klotho and FGF-23 expression levels
(A) *Klotho* mRNA expression was analyzed by quantitative PCR in the quadriceps muscle and expressed relative to the 2 week wildtype C57 expression level, which was set at 1.0. (B) *Klotho* mRNA expression with only wildtype C57 and KL- genotypes to visualize significant difference at each timepoint. (C) *FGF-23* mRNA expression was analyzed by quantitative PCR in the 3-month quadriceps muscle and expressed relative to the wildtype C57 expression level, which was set at 1.0. (D) *FGF-23* mRNA expression was analyzed by quantitative PCR in the 2-week quadriceps muscle and expressed relative to the wildtype C57 expression level, which was set at 1.0. (E) Western blot for Klotho protein in 3-month hamstring and kidney samples.
Each bar represents the mean and SEM for each group. N=5 where not specified. * P< 0.05, ** P< 0.01, ***P< 0.001 by one-way ANOVA.

Loss of Klotho prevents normal muscle mass accumulation during postnatal growth

Loss of Klotho reduces muscle mass in early postnatal skeletal muscle. Although body mass did not differ significantly between the 3 groups at a given age (Figure 2A), loss of Klotho caused significant reductions in muscle mass at 2 weeks and 3 months of age (Figure 2B, 2D). By 5 months of age, average muscle masses of KL- mice were no different from the controls. Muscle mass was also standardized for overall body mass and compared between genotypes within each age group. At 2 weeks, 3 months, and 5 months, Klotho hypomorphs had significantly reduced muscle mass: body mass ratios compared with their age-matched controls (Figure 2C, 2E). Klotho transgenic mice had a similar TA mass: body mass ratio compared to the controls.

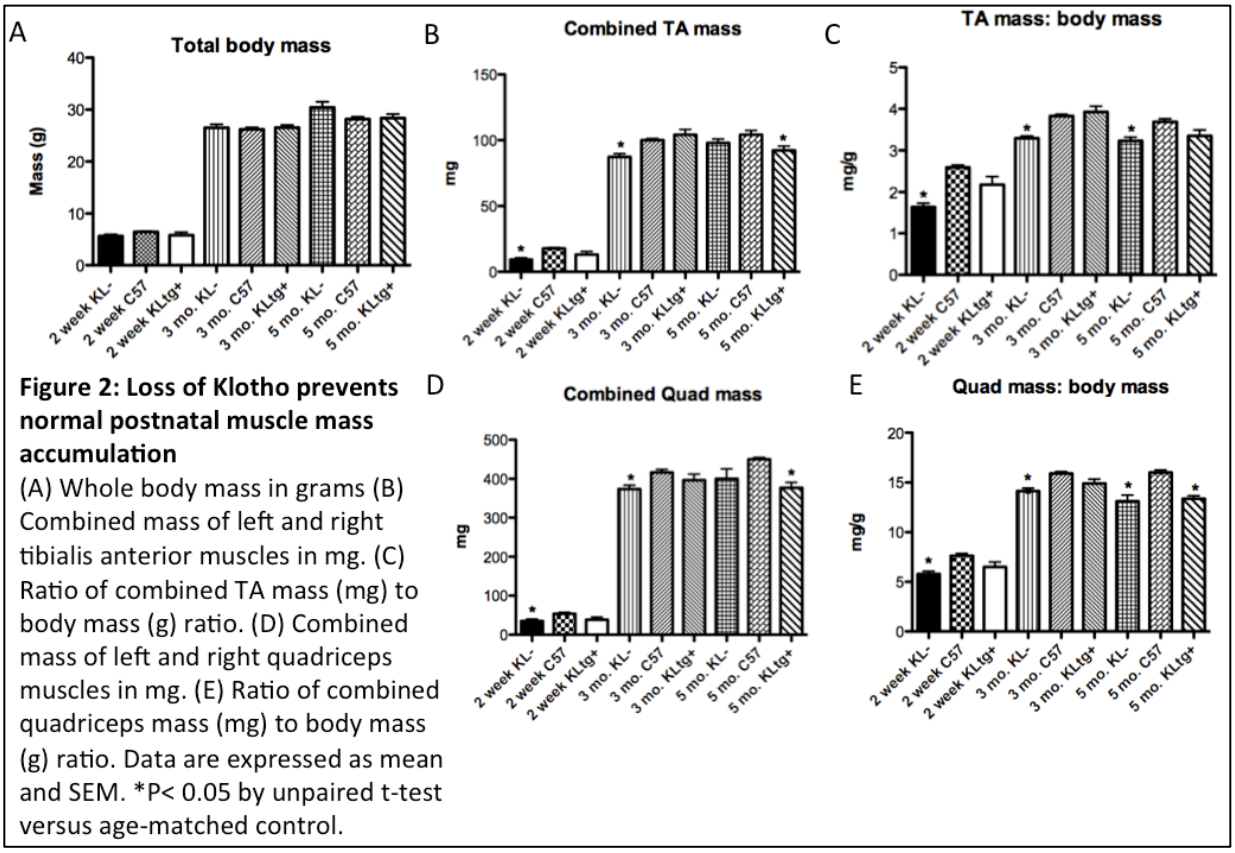

Figure 2: Loss of Klotho prevents normal postnatal muscle mass accumulation
(A) Whole body mass in grams (B) Combined mass of left and right tibialis anterior muscles in mg. (C) Ratio of combined TA mass (mg) to body mass (g) ratio. (D) Combined mass of left and right quadriceps muscles in mg. (E) Ratio of combined quadriceps mass (mg) to body mass (g) ratio. Data are expressed as mean and SEM. *P< 0.05 by unpaired t-test versus age-matched control.

Klotho increases myofiber cross-sectional area

Myofiber cross-sectional area measurements from tibialis anterior muscles revealed that loss of Klotho slows muscle growth and Klotho overexpression can increase fiber area above wildtype levels. At 2 weeks of age, we found a strong trend towards a larger fiber area in the KLtg+ mice and a smaller area in the Klotho hypomorphs (Figure 3). At three months of age, KLtg+ mice exhibit a 30% increase in fiber area compared with age-matched controls (Figure 4B, 4C, 4D) and the Klotho hypomorphs have significantly smaller mean fiber area (Figure 4A, 4B, 4D). The frequency distribution demonstrates the shift toward a larger fiber area in the KLtg+ (Figure 4F). By 5 months of age, there is no significant difference in myofiber cross-sectional area between the three groups, supporting the importance of Klotho during the early postnatal period of muscle

growth (Figure 4E). Indeed, the rate of early muscle growth is dependent on relative *Klotho* expression levels.

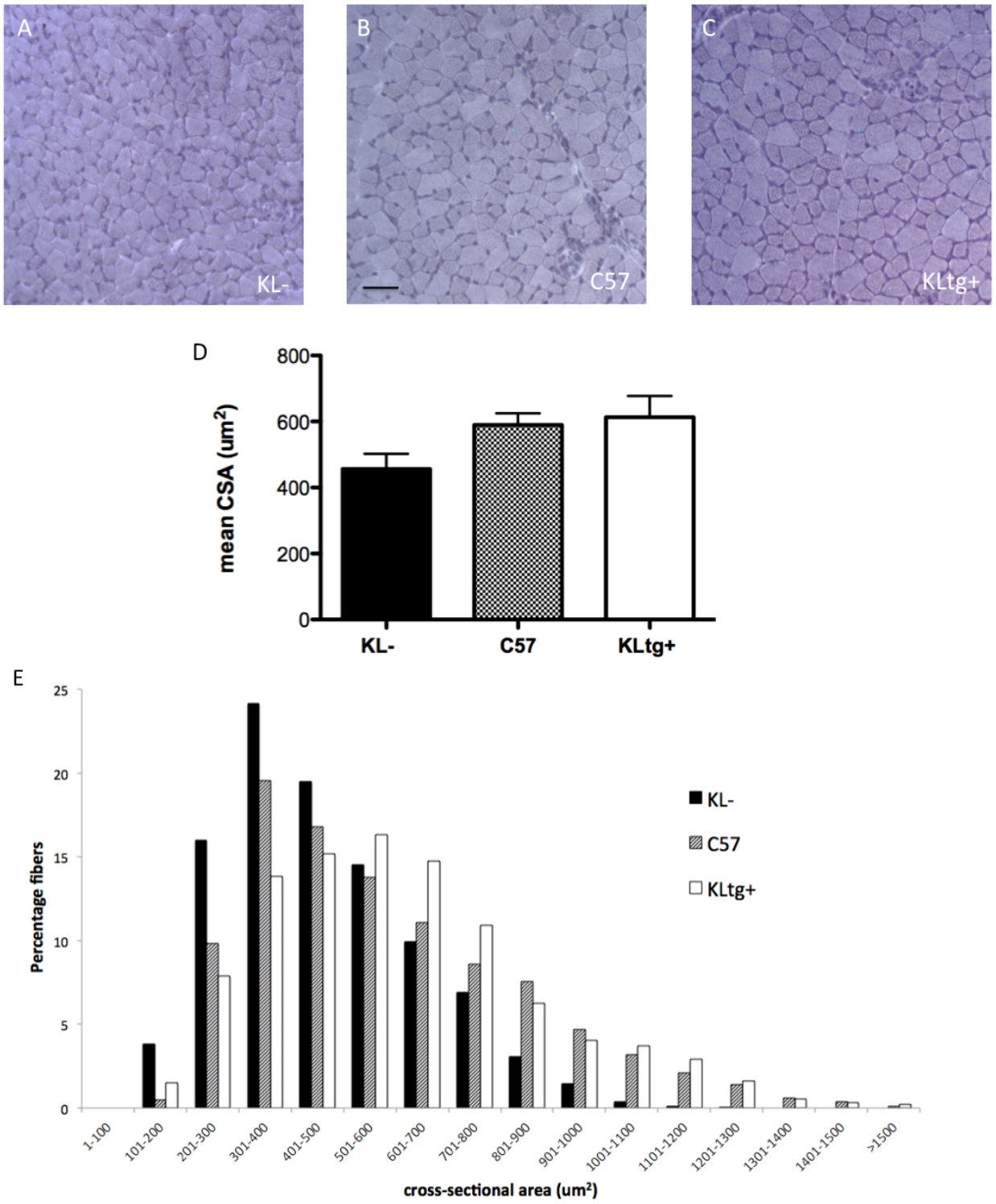

Figure 3: Myofiber cross-sectional area of 2-week tibialis anterior muscles
2-week tibialis anterior muscle sections were stained with hematoxylin. Representative images of myofiber area of KL- (A), C57 (B), and KLtg+ (C). (D) Cross-sectional area was quantified and values expressed as mean and SEM (E) Frequency distribution for the percentage of fibers within a particular cross-sectional area. All images are at the same magnification, bar = 50 µm.

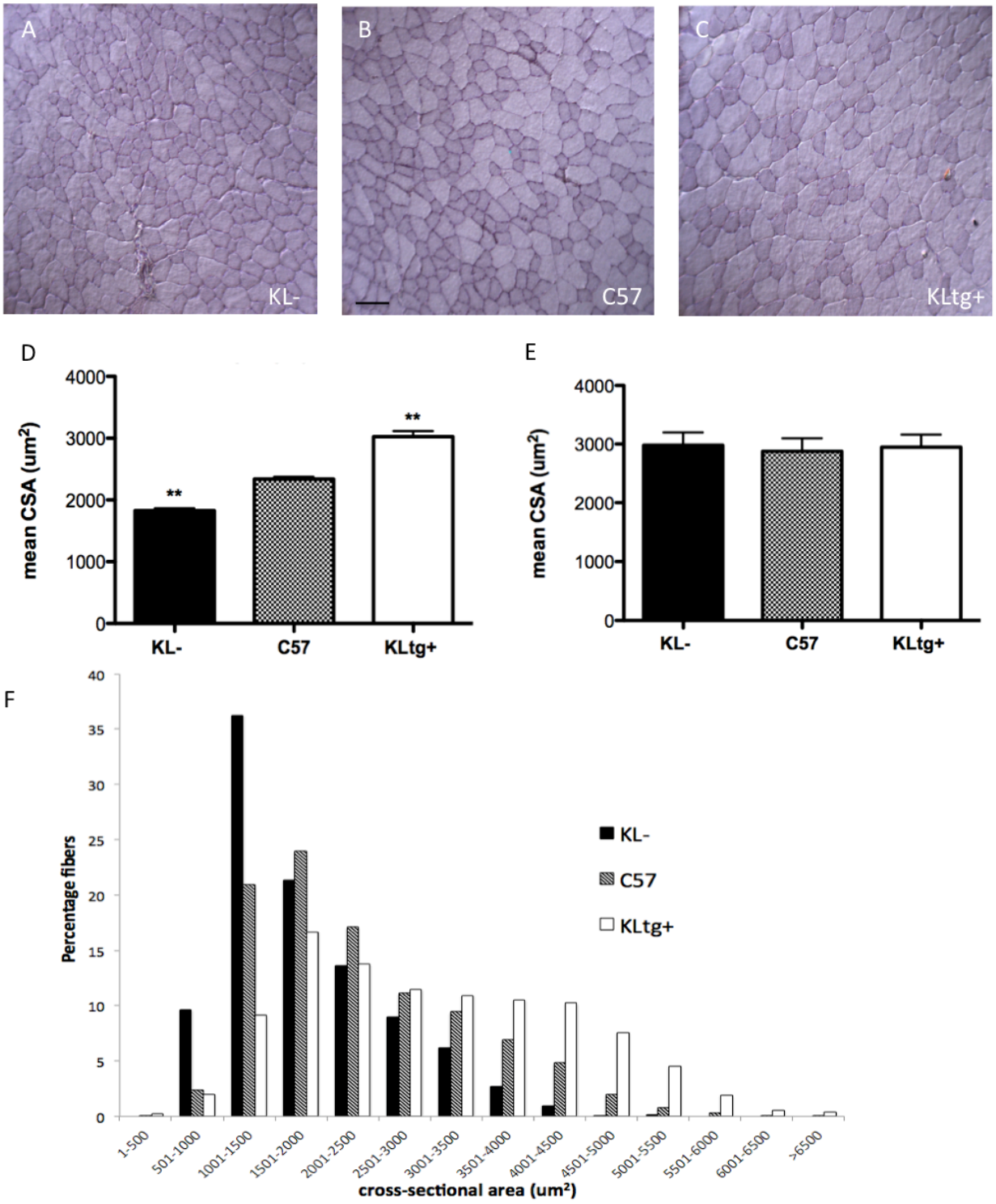

Figure 4: Myofiber cross-sectional area of 3-month and 5-month tibialis anterior muscles
3-month tibialis anterior muscle sections were stained with hematoxylin. Representative images of myofiber area for male KL- (A), C57 (B), and KLtg+ (C). (D) 3-month cross-sectional area was quantified and values expressed as mean and SEM (E) Cross-sectional area at 5 months of age expressed as mean and SEM. **P< 0.01 in comparison to wildtype control. *Data utilized with permission from Dr. Steven Welc.* (F) Frequency distribution for the percentage of fibers within a particular cross-sectional area at 3 months of age. All images are at the same magnification, bar = 100 µm.

Klotho modulates the expression of myogenic transcription factors

Klotho can modulate the expression of myogenic transcription factors. Given that modulating Klotho results in significant differences in myofiber area, we next investigated whether Klotho can alter the patterns of expression of myogenic transcription factors. At 2 weeks of age, all groups had similar expression of *MyoD* and myogenin although both the KL- and KLtg+ mice demonstrated significantly reduced *MRF4* levels (Figure 5). Because *MRF4* expression is normally transiently elevated during myotube fusion (Montarras et al., 1991), the general trend toward a decreased *MRF4* expression in Klotho transgenic mice may suggest that a greater proportion of developing muscle cells already fused with existing mature myofibers and thus down-regulated *MRF4* expression. This explanation is consistent with the larger myofiber cross-sectional area in the KLtg+ mice. At 3 months of age, KLtg+, KL-, and C57 exhibit no significant differences in *MyoD, myogenin,* or *MRF4* expression. However, a trend in the data indicates that KL- may have lower *myogenin* expression and KLtg+ has lower *MRF4* expression. Cyclin-dependent kinase inhibitor *p21* is a cell cycle exit regulator that promotes the differentiation of myoblasts (Andres and Walsh, 1996). The decrease in *p21* in the KL- mice supports the idea that loss of Klotho restricts myoblast differentiation in the process of normal muscle growth. KLtg+ mice demonstrate a trend to also have lower *p21* levels, but this may reflect an increase in proliferation of satellite cells. These data suggest that Klotho has a complex role in altering myogenesis and may affect both proliferation and differentiation of skeletal muscle.

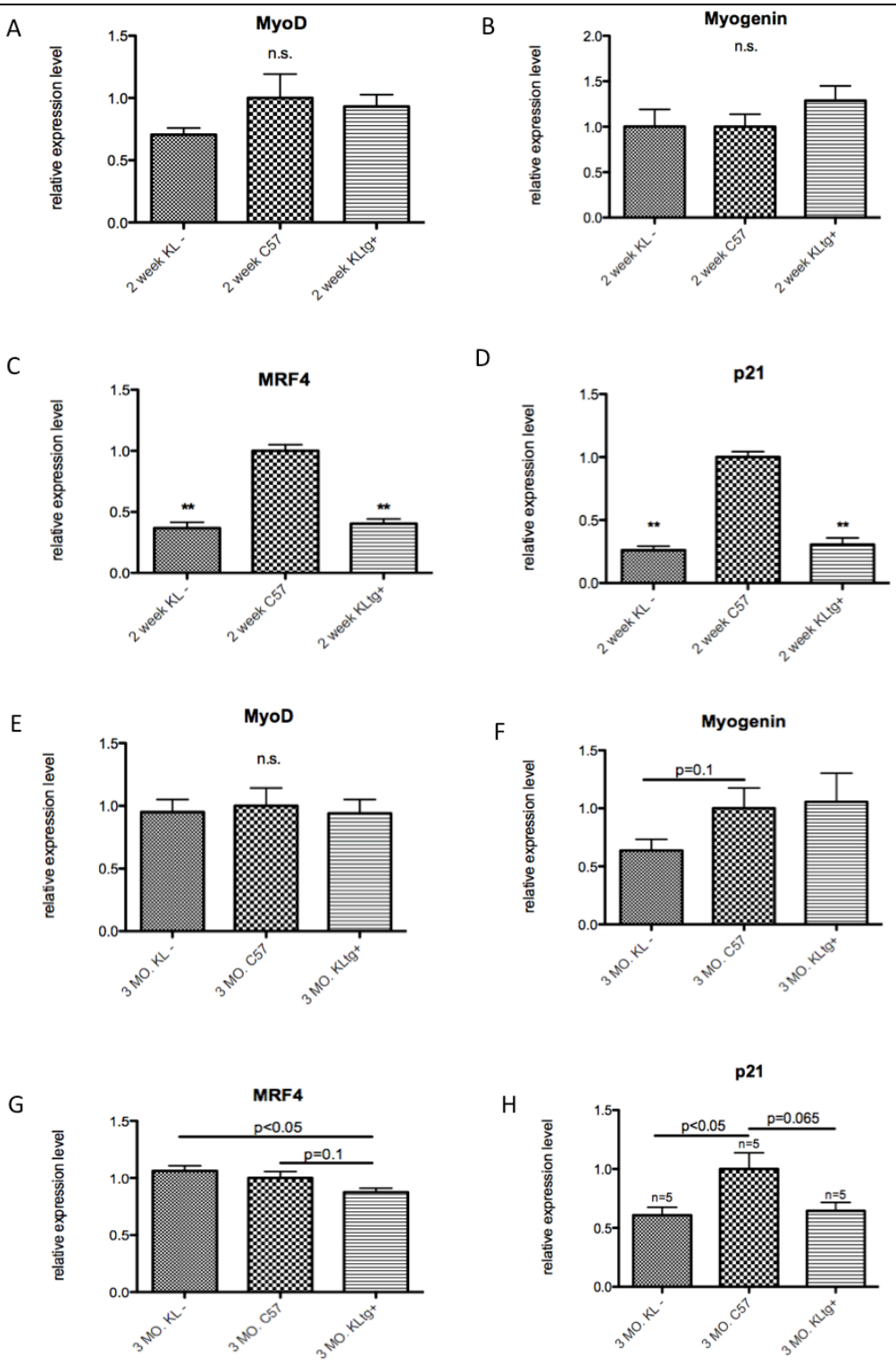

Figure 5: Modulation of myogenic transcription factor expression by Klotho
mRNA expression was analyzed by quantitative PCR in the quadriceps muscle and expressed relative to the wildtype C57 expression level, which was set at 1.0. Each bar represents the mean and SEM for each group. (A-D) For the two-week mRNA transcript levels, N=3-5. (E-H) For the three-month mRNA transcript levels, N=5. **P< 0.01 in comparison to wildtype control.

Klotho maintains the satellite cell population

The presence of Klotho is required to maintain the satellite cell population. Given the significant myofiber hypertrophy with overexpression of *Klotho*, we examined how Klotho modulates skeletal muscle growth. The existence of a quiescent satellite cell population is necessary for the ability of skeletal muscle to regenerate following an injury, and thus reflects the regenerative potential of a muscle (Relaix and Zammit, 2012). Therefore, we investigated whether modulation of Klotho could alter the satellite cell population by measuring Pax7 transcript levels and quantifying the Pax7+ satellite cells present in the tibialis anterior muscle after immunolabeling. At 2 weeks of age, KL- mice have a significant increase in Pax7 expression in comparison to the control (Figure 6A) but their total satellite cell numbers are not significantly different from the wildtype. KLtg+ mice have significantly greater numbers of satellite cells per 100 myofibers (Figure 6B). At three months of age, Pax7 transcript levels between the three groups are not significantly different, although the loss of Klotho resulted in a significantly reduced total number of satellite cells (Figure 6C and 6D). Thus, loss of Klotho results in a decline in satellite cell number, suggesting that Klotho has a role in maintaining a normal satellite cell population.

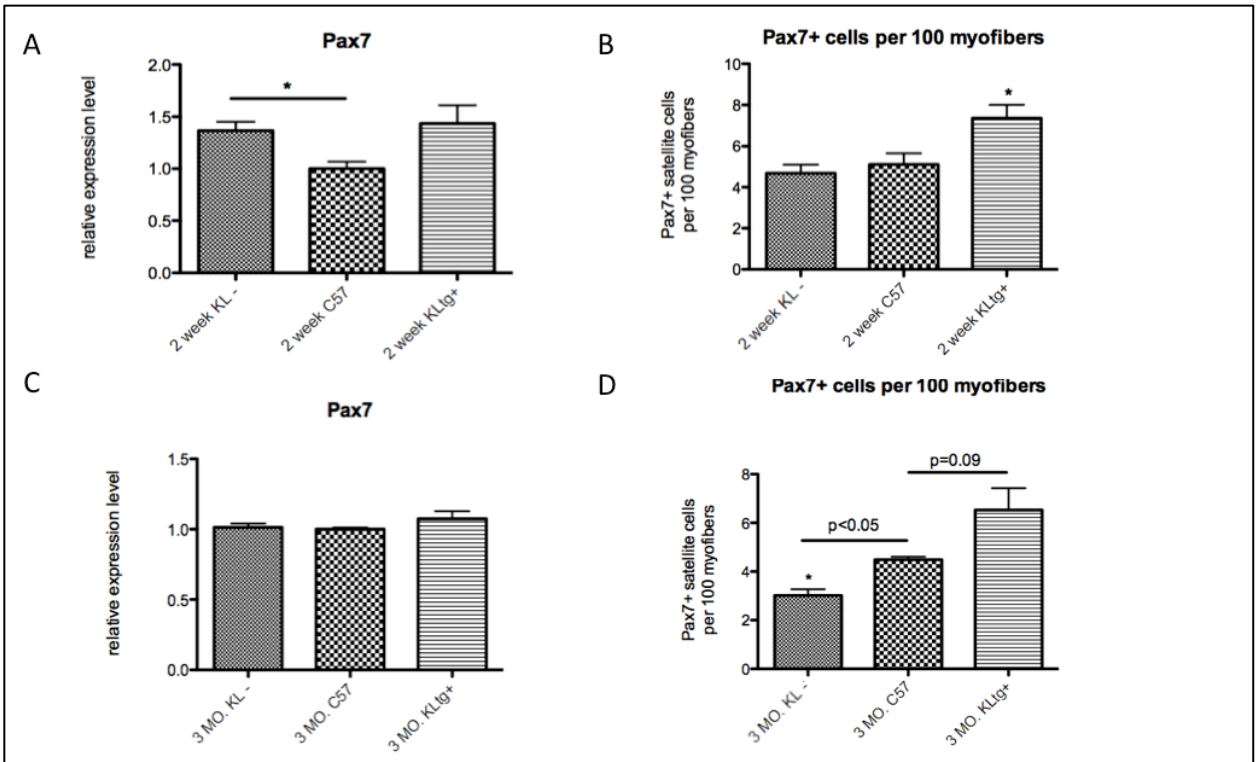

Figure 6: The loss of Klotho reduces the number of Pax7+ satellite cells
(A, C) *Pax7* mRNA expression was analyzed by quantitative PCR in the quadriceps muscle and expressed relative to the wildtype C57 expression level, which was set at 1.0. Each bar represents the mean and SEM for each group. For the two-week mRNA transcript levels, N=4-5. For the three-month mRNA transcript levels, N=5. (B, D) 2-week and 3-month tibialis anterior muscle sections were immunolabeled for Pax7 and positive cells were quantified per 100 myofibers. N=5. *P< 0.05 in comparison to wildtype control.

Klotho promotes proliferation in C2C12 myoblasts

Because the presence of Klotho is required to maintain the satellite cell population in skeletal muscle, we investigated the direct effects of Klotho on C2C12 myoblast proliferation *in vitro*. Stimulation of myoblasts with Klotho significantly increased the proliferation (Figure 7A).

Klotho's pro-proliferative effects in myoblasts are dependent on TNF-α. Muscle-derived TNF-α has been shown to activate satellite cells and promote proliferation in skeletal muscle (Li, 2003). Quantitative PCR performed on the Klotho-stimulated C2C12 myoblasts indicated that Klotho treatment modulates TNF-α expression level (Figure 7B). We further tested whether the addition

of a neutralizing antibody to TNF-α would inhibit the Klotho-induced proliferation of myoblasts. Indeed, the neutralizing antibody significantly blocked the pro-proliferative effects of Klotho+FGF-23-stimulated myoblasts (Figure 7A). Thus, Klotho's proliferative effects on myoblasts are mediated in part by TNF-α.

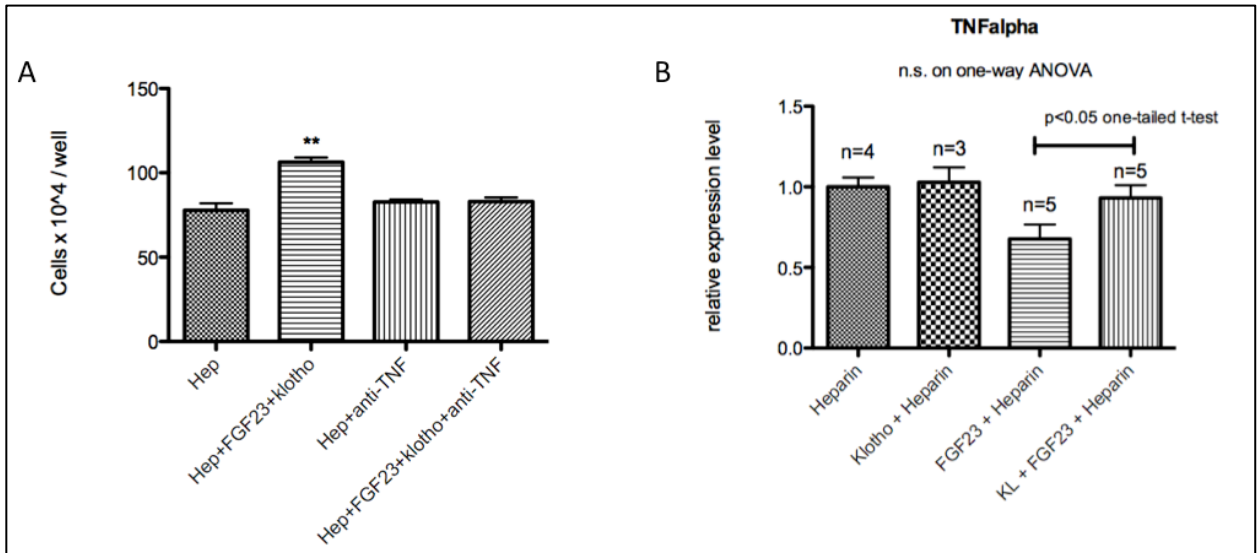

Figure 7: Klotho promotes proliferation of C2C12 myoblasts
(A) C2C12 cells were stimulated at 24 and 48 hours with one of the 4 following treatment conditions: 1) heparin (10 µg/ml); 2) FGF-23 (0.5 µg/ml), heparin, and Klotho (1 µg/ml); 3) heparin and anti-TNF (6 µg/ml); 4) FGF23, heparin, Klotho, anti-TNF. N=5 for each group. Cells were collected sub-confluent at 72 hours after plating and the protein concentration was measured by filter paper assay compared to bovine serum albumin standards. (B) C2C12 cells were stimulated at 24 and 48 hours with one of the 4 following treatment conditions: 1) heparin (10 µg/ml); 2) heparin and Klotho (1 µg/ml); 3) heparin and FGF-23 (0.5 µg/ml); 4) heparin, FGF23, and Klotho. We collected the cells at 72 hours after plating and measured relative expression of TNFalpha transcript by quantitative PCR. **P< 0.01 compared to all other groups by ANOVA.

Data utilized with permission from Dr. Michelle Wehling-Henricks.

Klotho increases protein content in differentiated myotubes

Klotho can also directly increase protein content in fully differentiated C2C12 myotubes *in vitro,* supporting our data for Klotho-induced myofiber hypertrophy. We repeated our Klotho stimulation experiment utilizing differentiated C2C12 myotubes and investigated whether Klotho contributes to muscle hypertrophy by affecting the protein balance in skeletal muscle.

The protein content in Klotho+FGF-23+heparin-treated myotubes was significantly higher than the control culture with FGF-23+heparin only, supporting Klotho's ability to increase growth of skeletal muscle even after the myocytes are differentiated (Figure 8). Because Klotho increases myoblast proliferation through a TNFα-dependent mechanism, we tested whether addition of a netutralizing antibody to TNF-α would affect the increase in protein content in Klotho-stimulated myotubes. However, we found that the presence of anti-TNF-α at a concentration sufficient to block the mitogenic activity of Klotho had no effect on Klotho-mediated increases in protein content. Thus, TNF-α is not a mediator of Klotho's positive regulation of protein content in myotubes.

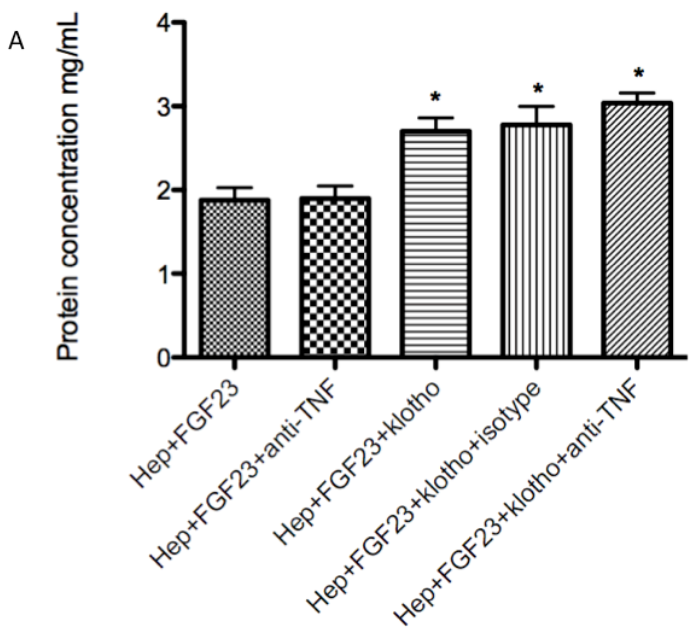

Figure 8: Klotho can increase protein content in C2C12 myotube cultures
(A) C2C12 cells were stimulated at 24 and 48 hours following transfer to differentiation medium with one of the five following treatment conditions: 1) FGF-23 (0.5 µg/ml) and heparin (10 µg/ml); 2) FGF23, heparin and anti-TNF (6 µg/ml); 3) FGF23, heparin, and Klotho (1 µg/ml); 4) FGF23, heparin, Klotho, and isotype control (6 µg/ml); 5) FGF23, heparin, Klotho, anti-TNF. N=5 for each group. Cells were collected at 72 hours after starvation and the protein concentration was measured by filter paper assay compared to bovine serum albumin standards. *P< 0.05 compared to first two groups by ANOVA.

Klotho and the insulin/IGF-1 signaling pathway

We tested whether the Klotho-mediated increase in myotube protein content and increase in myofiber area during early postnatal muscle growth was reflected by changes in expression of IGF-1 or its receptor. IGF-1 signaling is crucial for increasing skeletal muscle growth and hypertrophy (Liu et al., 1993; Musarò et al., 2001). In 3 month-old quadriceps muscles, KL- and KLtg+ had significantly reduced expression of IGF-1 compared to control mice. However, the effect was greater for the Klotho hypomorphs (Figure 9C). Furthermore, IGF-1 receptor expression was significantly upregulated in Klotho transgenic mice, suggesting that despite the reduced IGF-1 expression, the greater number of receptors augments the skeletal muscle IGF-1 signaling that leads to muscle hypertrophy (Figure 9D).

Because phosphorylation of the IGF-1 receptor is an early event in activation of IGF-1 signaling, we tested whether changes in KL expression affected IGF-1R phosphorylation levels. We chose to focus on muscles from 3 month-old mice since Klotho-mediated effects on hypertrophy were the most evident at this time. KLtg+ mice have greater phosphorylation of IGF-1R, indicating that the pathway is more highly activated with the overexpression of Klotho (Figure 10). Furthermore, KL- mice have lower levels of IGF-1Rbeta than the wildtype controls or KLtg+ samples. Taken together, these data suggest that during postnatal muscle growth, Klotho can upregulate IGF-1 signaling that leads to muscle hypertrophy.

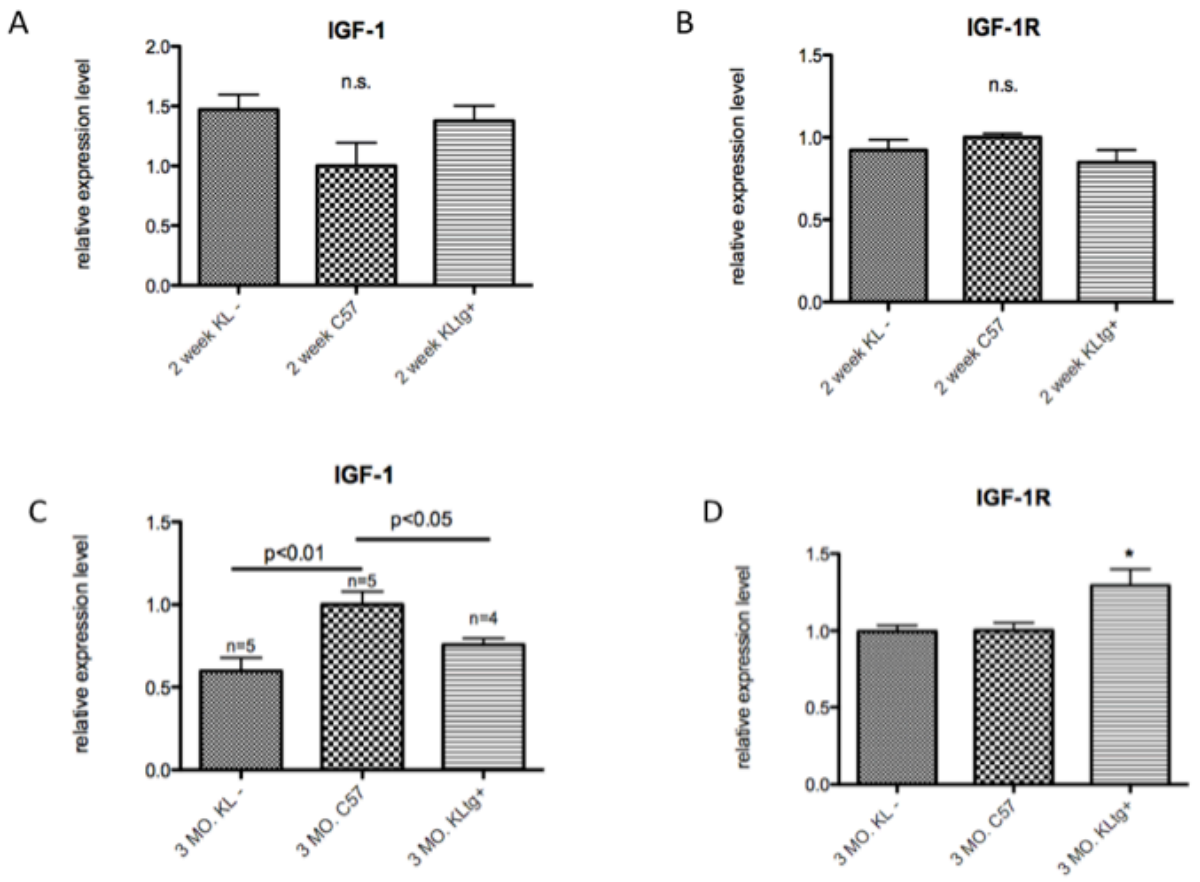

Figure 9: Klotho modulates IGF-1 and IGF-1R expression
(A, C) *IGF-1* and (B, D) *IGF-1R* mRNA expression were analyzed by quantitative PCR in the quadriceps muscle and expressed relative to the wildtype C57 expression level, which was set at 1.0. Each bar represents the mean and SEM for each group. For the 2-week mRNA transcript levels, N=3-5. For the 3-month mRNA transcript levels, N=5. *P< 0.05 in comparison to wildtype control.

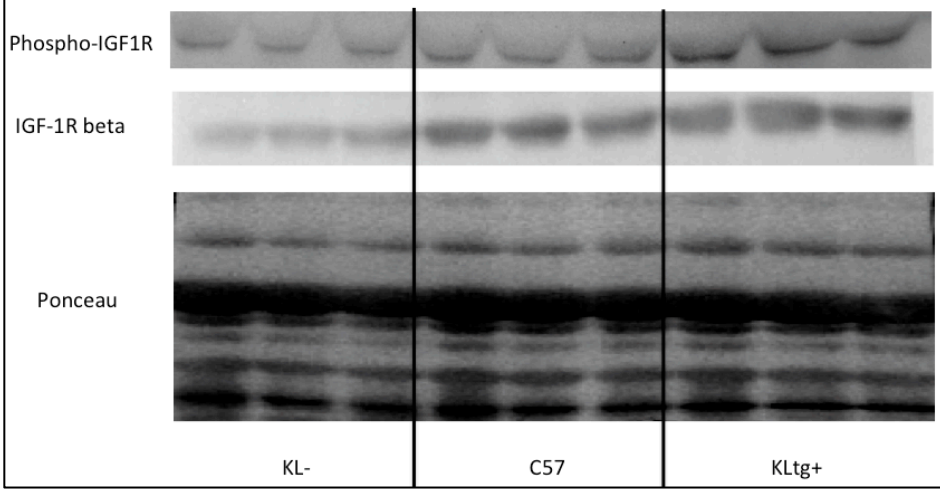

Figure 10: Klotho modulates activation of the IGF-1 receptor

Western blot of 3-month quadriceps muscles was performed to measure IGF-1R and phosphorylated-IGF1R protein. Ponceau red staining of the Western blot membrane was used to standardize sample loading.

Discussion

In this study, we show a previously unknown role for Klotho in promoting postnatal muscle growth. Loss of Klotho reduces muscle mass, decreases satellite cell populations, and slows the rate of myofiber growth. Klotho has direct effects on skeletal muscle to increase myoblast proliferation and increase protein content in myotubes. Although Klotho has previously been considered an anti-aging molecule and there are numerous data demonstrating its role in aging and age-related diseases, our data show that Klotho affects both stages of postnatal muscle growth: the early, satellite-cell mediated period that relies on proliferation and fusion of muscle progenitors, and the late, stem-cell independent period in which myofiber hypertrophy occurs independent of satellite cell fusion.

The discrepancy between Pax7 transcript levels and Pax7+ cell counts can be explained in consideration of the heterogeneous population of satellite cells. Other investigators have shown that satellite cells expressing low levels of Pax7 can also express high levels of MyoD and myogenin, which represent the cells that can proceed to myogenic commitment and differentiation (Olguin and Olwin, 2004; Rocheteau et al., 2012). However, satellite cells that express high Pax7 do not express MyoD or myogenin and thus are capable of self-renewal and a return to quiescence following activation. KL- mice displayed a high expression of Pax7 in proportion to the number of Pax7+ cells, suggesting that loss of Klotho leads to an increase in the Pax7 high population. However, the number of Pax7+ cells was significantly reduced at 3 months of age, indicating that the loss of Klotho prevents the return of satellite cells to a quiescent state once they have been activated. Taken together, these data suggest that loss of Klotho results in the functional deficiency of satellite cells to self-renew in skeletal muscle.

Our data suggest that loss of Klotho may also alter the fate of satellite cells. When activated, satellite cells undergo an asymmetric division resulting in one daughter cell remaining as self-renewing entity and the other proceeding to myogenic commitment (Kuang et al., 2007). MRF4 has a role to direct satellite cells into the myogenic lineage, in addition to other transcription factors such as MyoD and Myf5. Loss of Klotho results in a low MRF4 expression level and a trend towards decreased MyoD expression, suggesting impaired commitment of satellite cells to the myogenic lineage. Furthermore, satellite cells can be directed to various fates such as myogenic, fibrogenic, or adipogenic based on signals from the microenvironment (Asakura et al., 2001; Brack et al., 2007). Klotho has been shown to inhibit Wnt signaling. Because increased Wnt signaling can promote a conversion of satellite cells to a fibrogenic fate (Brack et al., 2007), loss of Klotho may result in greater proportion of satellite cells converting to a fibrogenic fate. This would prevent normal myonuclear accretion and impair muscle growth, an assumption that is supported by comparing 2 week and 3 month numbers of satellite cells: C57 and KLtg+ maintain their mean satellite cell numbers with age, but KL- reduce their number of satellite cells by nearly half. While the reduction in satellite cells could be due to increased differentiation, our qPCR data suggest that loss of KL does not promote greater differentiation of satellite cells, but rather, a loss of commitment to the myogenic phenotype. Our data indicate that the presence of Klotho is required to maintain the self-renewing satellite cell population in skeletal muscle and Klotho has direct pro-proliferative effects on C2C12 myoblasts *in vitro*. Taken together, these data support that Klotho promotes proliferation of self-renewing satellite cells to maintain the stem cell pool and promotes the myogenic commitment of satellite cells that proceed to differentiation. Loss of Klotho results in a drastic reduction in the stem cell pool, suggesting that

KL- mice have limited regenerative capacity with increasing age. Figure 11 contains a summary of our suggested role for Klotho in satellite cell function.

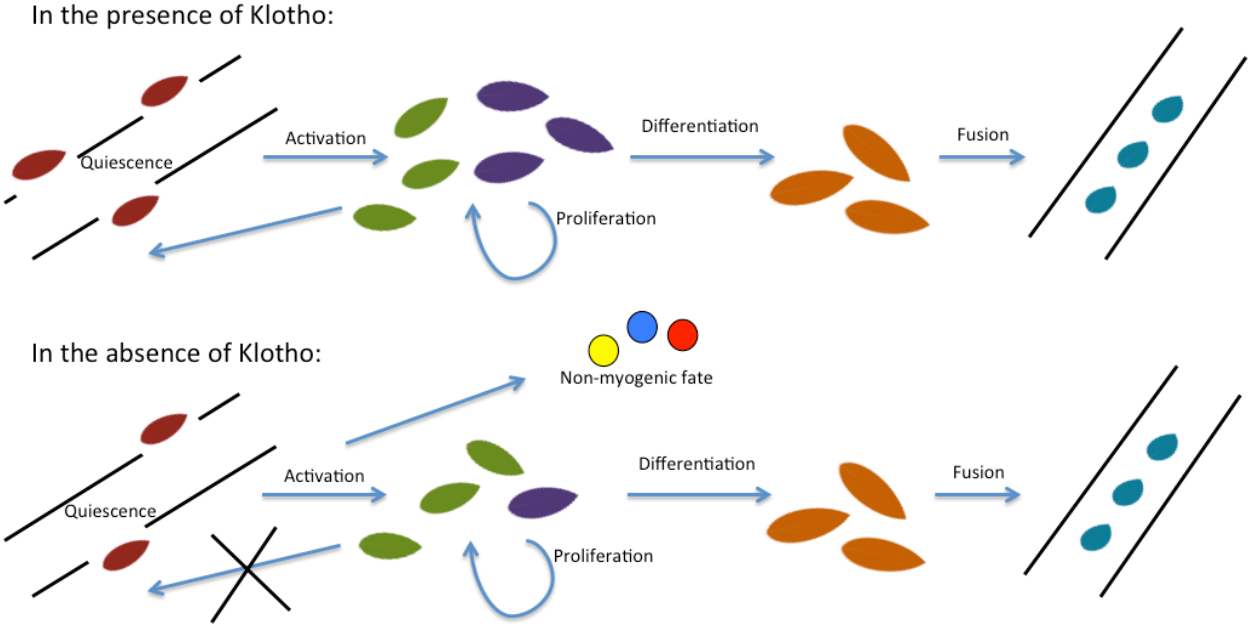

Figure 11: Schematic of Klotho's suggested role in satellite cell function. (A) Klotho is required to maintain the satellite cell pool. Once quiescent satellite cells are activated, they express MyoD and enter the cell cycle. This heterogeneous population of satellite cells are comprised of Pax7 high and Pax7 low-expressing cells. Those that are Pax7 high will return to a quiescent state. Those that are Pax7 low will exit the cell cycle, proceed to differentiation, and eventually fuse to form multinucleated myofibers. This system allows for the maintenance of the stem cell pool that are crucial for regeneration following an injury while allowing some myoblasts to fuse with existing myofibers. (B) Our data indicate that the loss of Klotho results in a reduction of total Pax7+ cells. We propose that loss of Klotho results in an increase in cells highly expressing Pax7 but an impaired return to quiescence, leading to an reduction in total numbers by 3 months of age. Furthermore, our data suggest that satellite cells may be converted to a non-myogenic fate, which prevents normal myonuclear accretion and impairs muscle growth.

The enhanced expression of Pax7 and maintenance of satellite cell numbers in 2 week-old KL- mice may also be linked to their compensatory upregulation of FGF-23. FGFs have a well-characterized mitogenic role on skeletal muscle myocytes (Allen and Boxhorn, 1989). In 2 week KL- mice, high expression of FGF-23 may be able partially rescue the reduction in satellite cell numbers due to the loss of Klotho. However, by 3 months of age KL- mice show no difference in FGF-23 expression and their satellite cells are significantly reduced compared to the control.

Our transgenic model for a systemic overexpressing or knockdown does present some limitations to our interpretation. We must consider the possibility that the effects on skeletal muscle in our KLtg+ model are a result of elevated Klotho expression in other tissues. Overexpressing Klotho systemically may cause increased soluble Klotho from other body tissues to affect skeletal muscle directly. Skeletal muscle does express Klotho, but we cannot make conclusions about the actions of muscle-derived Klotho specifically based on this systemic model. However, our *in vitro* experiments indicate that Klotho can have a direct effect on myoblast proliferation and myotube protein synthesis, highlighting the importance of soluble Klotho on muscle growth. The utilization of a muscle-specific genetic model in the future will clarify some of the roles of the locally expressed Klotho in muscle.

The EFmKL46 promoter we utilized for Klotho overexpression in our model is claimed to be a ubiquitous promoter, but the expression of human elongation factor-1α isoforms vary during early postnatal muscle development. In rat skeletal muscle, EF1α-1 decreases drastically within the first month and EF1α-2 increases to take over the majority of expression (Khalyfa et al., 2001). Expression of EF1α-2 is exclusive to terminally differentiated cells (Lee et al., 1992). Certainly in skeletal muscle, the time course differences in isoform expression could affect the extent of Klotho overexpression.

Our data support that Klotho-mediated effects on skeletal muscle growth occur through activation of IGF-1 signaling, which can be integrated into the well-characterized inhibitory relationship of Klotho and IGF-1. Although Klotho can suppress IGF-1 signaling, this has been demonstrated only in models of disease or aging *in vivo*. Klotho suppresses IGF-1 signaling in

breast and lung cancer cells, which prevents proliferation and growth of the tumor cells (Chen et al., 2010; Wolf et al., 2008). Klotho can also modulate compensatory renal hypertrophy by suppression of IGF-1 signaling (Nagasu et al., 2011). IGF-1 signaling is associated with increased oxidative stress and a shortened lifespan, and thus, Klotho's inhibition of IGF-1 may be evolutionarily conserved mechanism to limit the undesirable effects of IGF-1-induced oxidative stress through the FoxO pathway (Tatar et al., 2003). Indeed, our data agree with the inhibition of IGF-1, as KLtg+ mice demonstrated reduced levels of IGF-1. However, Klotho's relationship to IGF-1 during the normal growth process, one that is not impacted by an environment of oxidative stress, has not been investigated until now.

We report for the first time in vivo evidence that Klotho enhances IGF-1 signaling during postnatal skeletal muscle growth. Studies have shown that muscle-derived IGF-1 has a unique role to enhance hypertrophy that cannot be replicated by systemic IGF-1 overexpression (Mathews et al., 1988; Musarò et al., 2001). Our data show that KLtg+ mice have significantly reduced expression of IGF-1, but higher expression of IGF-1R and greater IGF-1R activation. KL may suppress systemic IGF-1 signaling by downregulating IGF-1 levels, but can increase IGF-1 receptor expression in skeletal muscle and thus promote local IGF-1 signaling that leads to myofiber hypertrophy. Future studies should focus on elucidating the relationship of Klotho and IGF-1 in aging skeletal muscle, not during an active growth state. Overall, Klotho's relationship with IGF-1 is likely tissue-specific and sensitive to local environmental cues.

Klotho could be a potential therapeutic benefit for patients with muscle wasting disorders that have an early onset, such as Duchenne muscular dystrophy (DMD), one of the most common

sex-linked lethal disorders. Unpublished data from our laboratory indicate that muscle biopsies from patients with DMD have significantly reduced Klotho levels in comparison to healthy controls. We show that overexpressing Klotho increases fiber cross-sectional area while maintaining the satellite cell pool, implicating a role for Klotho in maintaining a muscle's regenerative capacity. Given its high expression at early time points in skeletal muscle growth, modulation of Klotho may be therapeutically beneficial for early onset musculoskeletal disorders.

Although Klotho has been previously characterized as an anti-aging factor, our study not only shows that Klotho is highly expressed within the first month, but additionally demonstrates that modulation of Klotho during this time can affect myocyte proliferation and fiber growth. Despite the well-characterized inhibitory relationship between Klotho and IGF-1 signaling in age-related disease models, here we demonstrate that Klotho can upregulate IGF-1 signaling in postnatal skeletal muscle to induce myofiber hypertrophy. Klotho's designation as an anti-aging factor should be re-evaluated given its role in muscle development and growth, with implications for therapeutic approaches beyond age-related disorders.

REFERENCES

Allen, R.E., and Boxhorn, L.K. (1989). Regulation of skeletal muscle satellite cell proliferation and differentiation by transforming growth factor-beta, insulin-like growth factor I, and fibroblast growth factor. J. Cell. Physiol. *138*, 311–315.

Andres, V., and Walsh, K. (1996). Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis. J Cell Biol *132*, 657–666.

Asakura, A., Komaki, M., and Rudnicki, M. (2001). Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. Differentiation *68*, 245–253.

Brack, A.S., Conboy, M.J., Roy, S., Lee, M., Kuo, C.J., Keller, C., and Rando, T.A. (2007). Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. Science *317*, 807–810.

Cha, S.-K., Ortega, B., Kurosu, H., Rosenblatt, K.P., Kuro-o, M., and Huang, C.-L. (2008). Removal of sialic acid involving Klotho causes cell-surface retention of TRPV5 channel via binding to galectin-1. PNAS *105*, 9805–9810.

Chang, Q., Hoefs, S., van der Kemp, A.W., Topala, C.N., Bindels, R.J., and Hoenderop, J.G. (2005). The beta-glucuronidase klotho hydrolyzes and activates the TRPV5 channel. Science *310*, 490–493.

Chen, B., Wang, X., Zhao, W., and Wu, J. (2010). Klotho inhibits growth and promotes apoptosis in human lung cancer cell line A549. Journal of Experimental & Clinical Cancer Research *29*, 99.

Chen, C.-D., Podvin, S., Gillespie, E., Leeman, S.E., and Abraham, C.R. (2007). Insulin stimulates the cleavage and release of the extracellular domain of Klotho by ADAM10 and ADAM17. Proc. Natl. Acad. Sci. U.S.A. *104*, 19796–19801.

Collins, C.A., and Partridge, T.A. (2005). Self-renewal of the adult skeletal muscle satellite cell. Cell Cycle *4*, 1338–1341.

Cornelison, D.D., and Wold, B.J. (1997). Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. Dev. Biol. *191*, 270–283.

Day, K., Shefer, G., Shearer, A., and Yablonka-Reuveni, Z. (2010). The depletion of skeletal muscle satellite cells with age is concomitant with reduced capacity of single progenitors to produce reserve progeny. Dev. Biol. *340,* 330–343.

Fry, C.S., Lee, J.D., Mula, J., Kirby, T.J., Jackson, J.R., Liu, F., Yang, L., Mendias, C.L., Dupont- Versteegden, E.E., McCarthy, J.J., et al. (2015). Inducible depletion of satellite cells in adult, sedentary mice impairs muscle regenerative capacity without affecting sarcopenia. Nat Med *21,* 76–80.

Iida, R., Kanko, S., Suga, T., Morito, M., and Yamane, A. (2011). Autophagic-lysosomal pathway functions in the masseter and tongue muscles in the klotho mouse, a mouse model for aging. Mol. Cell. Biochem. *348*, 89–98.

Khalyfa, A., Bourbeau, D., Chen, E., Petroulakis, E., Pan, J., Xu, S., and Wang, E. (2001). Characterization of Elongation Factor-1A (eEF1A-1) and eEF1A-2/S1 Protein Expression in Normal and wasted Mice. J. Biol. Chem. *276*, 22915–22922.

Kuang, S., Kuroda, K., Le Grand, F., and Rudnicki, M.A. (2007). Asymmetric Self-Renewal and Commitment of Satellite Stem Cells in Muscle. Cell *129*, 999–1010.

Kuro-o, M. (2009). Klotho and aging. Biochim Biophys Acta *1790*, 1049–1058.

Kuro-o, M., Matsumura, Y., Aizawa, H., Kawaguchi, H., Suga, T., Utsugi, T., Ohyama, Y., Kurabayashi, M., Kaname, T., Kume, E., et al. (1997). Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature *390*, 45–51.

Kurosu, H., Yamamoto, M., Clark, J.D., Pastor, J.V., Nandi, A., Gurnani, P., McGuinness, O.P., Chikuda, H., Yamaguchi, M., Kawaguchi, H., et al. (2005). Suppression of aging in mice by the hormone Klotho. Science *309*, 1829–1833.

Kurosu, H., Ogawa, Y., Miyoshi, M., Yamamoto, M., Nandi, A., Rosenblatt, K.P., Baum, M.G., Schiavi, S., Hu, M.-C., Moe, O.W., et al. (2006). Regulation of Fibroblast Growth Factor-23 Signaling by Klotho. J Biol Chem *281*, 6120–6123.

Lee, S., Francoeur, A.M., Liu, S., and Wang, E. (1992). Tissue-specific expression in mammalian brain, heart, and muscle of S1, a member of the elongation factor-1 alpha gene family. J. Biol. Chem. *267*, 24064–24068.

Lexell, J. (1995). Human aging, muscle mass, and fiber type composition. J. Gerontol. A Biol. Sci. Med. Sci. *50 Spec No,* 11–16.

Li, Y.-P. (2003). TNF-alpha is a mitogen in skeletal muscle. Am. J. Physiol., Cell Physiol. *285*, C370–C376.

Liu, H., Fergusson, M.M., Castilho, R.M., Liu, J., Cao, L., Chen, J., Malide, D., Rovira, I.I., Schimel, D., Kuo, C.J., et al. (2007). Augmented Wnt Signaling in a Mammalian Model of Accelerated Aging. Science *317*, 803–806.

Liu, J.P., Baker, J., Perkins, A.S., Robertson, E.J., and Efstratiadis, A. (1993). Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell *75*, 59–72.

Mathews, L.S., Hammer, R.E., Behringer, R.R., D'Ercole, A.J., Bell, G.I., Brinster, R.L., and Palmiter, R.D. (1988). Growth enhancement of transgenic mice expressing human insulin-like growth factor I. Endocrinology *123*, 2827–2833.

Minamide, L.S., and Bamburg, J.R. (1990). A filter paper dye-binding assay for quantitative determination of protein without interference from reducing agents or detergents. Anal. Biochem. *190,* 66–70.

Montarras, D., Chelly, J., Bober, E., Arnold, H., Ott, M.O., Gros, F., and Pinset, C. (1991). Developmental patterns in the expression of Myf5, MyoD, myogenin, and MRF4 during myogenesis. New Biol. *3*, 592–600.

Moss, F.P., and Leblond, C.P. (1971). Satellite cells as the source of nuclei in muscles of growing rats. Anat. Rec. *170*, 421–435.

Musarò, A., McCullagh, K., Paul, A., Houghton, L., Dobrowolny, G., Molinaro, M., Barton, E.R., Sweeney, H.L., and Rosenthal, N. (2001). Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle. Nat Genet *27*, 195–200.

Nagasu, H., Satoh, M., Kuwabara, A., Yorimitsu, D., Kidokoro, K., Nishi, Y., Tomita, N., Sasaki, T., and Kashihara, N. (2011). Overexpression of klotho protein modulates uninephrectomy-induced compensatory renal hypertrophy by suppressing IGF-I signals. Biochem. Biophys. Res. Commun. *407*, 39–43.

Nakatani, T., Sarraj, B., Ohnishi, M., Densmore, M.J., Taguchi, T., Goetz, R., Mohammadi, M., Lanske, B., and Razzaque, M.S. (2009). In vivo genetic evidence for klotho-dependent, fibroblast growth factor 23 (Fgf23) -mediated regulation of systemic phosphate homeostasis. FASEB J *23*, 433–441.

Olguin, H.C., and Olwin, B.B. (2004). Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. Dev Biol *275,* 375–388.

Phelps, M., Pettan-Brewer, C., Ladiges, W., and Yablonka-Reuveni, Z. (2013). Decline in muscle strength and running endurance in klotho deficient C57BL/6 mice. Biogerontology *14*, 729–739.

Relaix, F., and Zammit, P.S. (2012). Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. Development *139*, 2845–2856.

Rocheteau, P., Gayraud-Morel, B., Siegl-Cachedenier, I., Blasco, M.A., and Tajbakhsh, S. (2012). A subpopulation of adult skeletal muscle stem cells retains all template DNA strands after cell division. Cell *148,* 112–125.

Schiaffino, S., Dyar, K.A., Ciciliot, S., Blaauw, B., and Sandri, M. (2013). Mechanisms regulating skeletal muscle growth and atrophy. FEBS J *280*, 4294–4314.

White, R.B., Biérinx, A.-S., Gnocchi, V.F., and Zammit, P.S. (2010). Dynamics of muscle fibre growth during postnatal mouse development. BMC Dev Biol *10*, 1–11.

Wolf, I., Levanon-Cohen, S., Bose, S., Ligumsky, H., Sredni, B., Kanety, H., Kuro-o, M., Karlan, B., Kaufman, B., Koeffler, H.P., et al. (2008). Klotho: a tumor suppressor and a modulator of the IGF-1 and FGF pathways in human breast cancer. Oncogene *27*, 7094–7105.

Yablonka-Reuveni, Z., and Rivera, A.J. (1994). Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibers. Dev. Biol. *164*, 588–603.

Yamamoto, M., Clark, J.D., Pastor, J.V., Gurnani, P., Nandi, A., Kurosu, H., Miyoshi, M., Ogawa, Y., Castrillon, D.H., Rosenblatt, K.P., et al. (2005). Regulation of Oxidative Stress by the Anti-aging Hormone Klotho. J Biol Chem *280*, 38029–38034.

Yamashita, T., Konishi, M., Miyake, A., Inui, K., and Itoh, N. (2002). Fibroblast growth factor (FGF)-23 inhibits renal phosphate reabsorption by activation of the mitogen-activated protein kinase pathway. J. Biol. Chem. *277*, 28265–28270.