Title
The Effect of Klotho on Muscle Regeneration after Acute Muscle Injury

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The Effect of Klotho on Muscle Regeneration
after Acute Muscle Injury

A thesis submitted in partial satisfaction
of the requirements for the degree Master of Science
in Physiological Science

by

Kimberly Hwang
Skeletal muscle function can be compromised by injury or trauma. Muscle regeneration requires the activation and proliferation of satellite cells. The anti-aging protein Klotho (KL) has been shown to enhance regeneration of dystrophic muscle by stimulating satellite cell production. However, it is not known whether exogenous KL treatment can be used to improve muscle regeneration after acute injury. Using C57BL/6 mice that received intraperitoneal injections of recombinant KL prior to muscle injury and throughout recovery, we found that KL did not affect muscle regeneration or expression of inflammatory and fibrogenic transcripts. We also tested whether KL affected Wnt signaling in myogenic cells. Our investigation confirms that KL is an antagonist of Wnt signaling. Although our findings do not support the therapeutic use of KL in
young muscle after acute injury, our results suggest that KL modulates Wnt signaling and therefore could be therapeutic in conditions of aberrant Wnt-signaling in muscle.
The thesis of Kimberly Hwang is approved.

Victor R Edgerton

Alan D Grinnell

James G Tidball, Committee Chair

University of California, Los Angeles

2018
Dedication

This thesis is dedicated to:

My parents, Lingo and C.C. Hwang, for their endless love and support. Thank you for always being there for me and making sure I am happy and healthy.

My sister, Vicki, for introducing me to science and showing me just how fun it can be.

My boyfriend, Jesse Chin, for his unwavering support. You pushed me to be a better student, scientist, and person.
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I would like to thank Dr. James Tidball for his guidance and support over the past 2 years. Thank you for pushing me to become a better scientist and for being so understanding and with my teaching schedule.

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INTRODUCTION

Acute skeletal muscle injuries are common and occur through a variety of mechanisms. Due to its superficial location and mechanical functions, skeletal muscle can be injured mechanically due to damaging loads or by contusions and lacerations during contact sports as well as severe trauma, like in automobile accidents. Regardless of the etiology, skeletal muscle responds to injury with a highly-coordinated reparative response. The muscle’s capacity for regeneration is vital to restore muscle function. However, the ability for muscle to regenerate can be complicated by several factors, including muscle disease and aging\textsuperscript{1,2}. Thus, it is important to understand the underlying processes involved in muscle regeneration. Developing therapeutics that improve muscle regeneration can improve independence and quality of life by improving an individual’s ability to return to normal daily activities after trauma or injury.

The remarkable ability of skeletal muscle to regenerate is dependent on muscle stem cells, also known as satellite cells. Satellite cells are quiescent under basal conditions and are located under the basal lamina on the surface of muscle fibers. Following trauma, satellite cells are activated and re-enter the cell cycle to proliferate. A subset of satellite cells will return to quiescence and replenish the satellite cell pool. The remaining activated satellite cells will proliferate, differentiate, and fuse to repair or form new muscle fibers, a process called myogenesis. Each stage of myogenesis can be characterized by the sequential expression of transcription factors responsible for the transition from a quiescent satellite cell to a differentiated myofiber. Quiescent satellite cells express Pax7. Once activated, Pax7 expressing satellite cells express MyoD. Once fully committed to the differentiating myocyte, Pax7 expression decreases while expression of transcription factors MyoD, Myogenin, and Mrf4 increase\textsuperscript{3}. Myocytes fuse together to form new myotubes or to damaged muscle fibers to
facilitate repair. Disruption of myogenic transcripts such as *myod* impairs regeneration\(^4\)\(^-\)\(^6\). In young healthy muscle, the satellite cell pool is maintained allowing for quick and efficient muscle regeneration. However, aging or repetitive muscle injuries can cause an imbalance in self-renewal and differentiation, resulting in a depleted satellite cell pool and impaired regeneration\(^7\)\(^,\)\(^8\). This imbalance can also cause muscle degeneration which is marked by replacement of contractile tissue with noncontractile tissue like fat and connective tissue to impair muscle function.

Recent discoveries from our lab identified Klotho as a novel molecule that regulates maintenance of the satellite cell pool and regeneration in dystrophic muscle. We showed that Klotho expression is reduced in human Duchenne muscular dystrophy (DMD) skeletal muscle biopsies and the *klotho* gene is silenced through epigenetic modifications in the *mdx* mouse model of DMD at the onset of pathology. DMD is an X-linked recessive disorder characterized by progressive muscle weakness and wasting. Restoring Klotho expression with a systemic transgene improved muscle regeneration, at least in part by preserving the pool of satellite cells in *mdx* mice\(^1\).

The *klotho* gene was discovered by a point mutation that resulted in a phenotype that resembled human aging, characterized by traits such as a shortened life span, tissue atrophy, and osteoporosis\(^9\). The *klotho* gene encodes a single-pass transmembrane protein that functions as an obligate co-receptor with fibroblast growth factor (FGF) receptors for FGF23 signaling. Klotho is predominantly expressed in the kidneys, but also in other tissues including skeletal muscle. Klotho can also affect tissues that do not produce Klotho because the extracellular domain of the transmembrane protein can be cleaved and released into circulation to function like a hormone\(^10\).
In addition to our studies demonstrating that Klotho affects dystrophic muscle pathology, other studies showed that reduced Klotho expression is associated with decreased muscle strength, endurance and compromised regeneration due to impaired myogenesis\textsuperscript{11,12}. The effects of Klotho on myogenesis could be due to the ability of Klotho to bind to Wnt ligands and inhibit Wnt-signaling\textsuperscript{12,13}. The activation of Wnt-signaling in satellite cells is associated with the induction of differentiation and an important regulator of postnatal muscle regeneration\textsuperscript{14}. Furthermore, Wnt-signaling can also affect satellite cell fate and promote fibrogenesis\textsuperscript{15}.

In this investigation, we explore the potential therapeutic role of exogenous Klotho (KL) protein treatment in muscle regeneration after acute injury. While previous studies have established a role for Klotho in the regulation of muscle health during postnatal development and muscular dystrophy, this investigation tests the hypothesis that exogenous KL can be used therapeutically to improve muscle regeneration after an acute muscle injury\textsuperscript{1,9}. To do this, we treated mice with exogenous KL or vehicle control treatments and assessed regeneration and the expression of myogenic transcription factors throughout the reparative process. We also investigated the Wnt pathway an important regulator of myogenesis. We seek to find the relationship between KL and Wnt in order to understand the mechanism in which KL acts on satellite cells in an acute injury model.
MATERIALS AND METHODS

Animals and treatment

Figure 1: Diagram showing the injection schedule of acutely injured C57BL/6 mice. Mice were started with intraperitoneal injections of KL or a vehicle solution 3 days prior to induction of a sterile muscle injury via the intramuscular injection of a BaCl₂ solution. These injections continued every other day until tissue collection (purple lines: I.P. injections of KL or Vehicle before injury; blue lines: injections of KL or Vehicle after injury). Samples were collected at 3, 7, and 21-days post-injury (green vertical lines).

C57BL/6 mice were kept in a vivarium at the University of California, Los Angeles. All animals were handled according to guidelines approved by the Chancellor’s Animal Research committee at the University of California, Los Angeles. Six-month old mice were assigned to either a saline treated group (Vehicle) or a KL treated group (n = 6 at each time point). The groups received intraperitoneal (I.P.) injections with either saline solution or KL solution (20 µg/kg) respectively three days before injury. We elected to inject mice with recombinant KL protein at a dosage of 20 µg/kg because this dosage was shown to prevent renal pathology caused by ureter obstructions¹⁶. I.P. injections continued to be administered every other day until tissue was collected. No injections were performed on the day of tissue collection (Fig.1).
Injuries were performed with \( \text{BaCl}_2 \) three days after the start of treatment. \( \text{BaCl}_2 \) injury has been shown to result in extensive muscle cell necrosis within 18 hours of injection with complete regeneration within 1-month post-injury. In addition, satellite cells numbers return to similar non-injured values by 3 months post-injury\(^{17}\).

Mice were briefly anesthetized with isoflurane inhalation in a chamber (5% isoflurane mixed with pure oxygen for induction) then moved to a nose cone (1.5% isoflurane). The left leg of the mouse was wiped down the 70% ethanol before 50 \( \mu \)l injections of barium chloride (\( \text{BaCl}_2 \): 1.2%) in the tibialis anterior (TA) as well as the quadriceps muscle. Food pellets moistened with water were put in the cage after injury to ensure that food intake was not affected due to injury.

Whole TA and quadriceps muscles were collected at 3-days, 7-days and 21-days post-injury. All quadriceps were stored in liquid nitrogen for further RNA analysis while TAs were frozen in optimal cutting temperature compound (OCT) and saved in isopentane at -80°C until used for histology.

**RNA isolation and qPCR**

Whole quadriceps were homogenized in Trizol and RNA was extracted and isolated using the Qiagen RNeasy kit (Germantown, MD). Using a spectrophotometer, the absorbance of the RNA sample was read to measure the RNA concentration while the A260/280 ratio was used to assess its purity. RNA was also run on a 1.2% agarose gel to check 28s and 18s bands to assess RNA integrity.

RNA was then reverse transcribed to generate cDNA using SuperScript Reverse Transcriptase II and oligo dTs (Invitrogen). cDNA was used to assay the expression of selected
myogenic, fibrogenic, inflammatory, and Wnt pathway transcripts using SYBR green QPCR
Supermix according to the manufacturer’s protocol. Real-time PCR was performed on an iCycler
thermocycler system equipped with iQ5 optical system software (BioRad). Multiple reference
genases were empirically tested for stability. Reference genes RPNS1 and RPLO were chosen
because of their stable expression across treatment and used as controls when assessing
myogenic, fibrogenic, and inflammatory transcripts in 3, 7, and 21-day quads. Reference genes
RPNS1 and SRP14 were used as controls when evaluating the effects of KL on Wnt signaling in
vitro. Sample cycle threshold (Ct) values were then normalized by setting the control samples to
1 and then scaling the genes of interest values to the controls. Primers used for QPCR are listed
in Table 1.

<table>
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<th>Direction (5’ to 3’)</th>
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<tr>
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<tr>
<td>CCND1</td>
<td>Fwd: CGAGAAGCTGCTGCAAAATG&lt;br&gt;Rev: GGTTGAAAAATGAACCTCACTC</td>
</tr>
<tr>
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<td>Fwd: GAAAGACTGCGAGCTGAGG&lt;br&gt;Rev: GTCCAAATCACAGCAGAGGAG</td>
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<tr>
<td>CD68</td>
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<tr>
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</tr>
<tr>
<td>Col3</td>
<td>Fwd: ATCCCTTTTGAGAAGATCTTGC&lt;br&gt;Rev: GGAATGTTACAGCAGATCAGAG</td>
</tr>
<tr>
<td>Col5</td>
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</tr>
<tr>
<td>Mrf4</td>
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</tr>
<tr>
<td>Myc</td>
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<td>MyoD</td>
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<td>Myogenin</td>
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<tr>
<td>Pax7</td>
<td>Fwd: CTGAGTGGTTCTGAGTAACG&lt;br&gt;Rev: AGAGCCTCCTTTTGTGGCC</td>
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<tr>
<td>PRLD</td>
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</tr>
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<td>SRP14</td>
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<tr>
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<td>Wisp2</td>
<td>Fwd: GACACCACTTCTGCGCTT&lt;br&gt;Rev: CGAGTGGCGAGAATGCTGTC</td>
</tr>
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Table 1. Primer sequences used in this investigation.
Immunohistochemistry

Cryosections 10 μm thick were collected from TA muscles. Sections were air dried for 30 minutes, fixed in ice cold acetone, endogenous peroxidases were quenched with 0.3% H.O., and blocked for an hour using blocking buffer from a mouse on mouse (MOM) detection kit (Vector laboratories, Burlingame, CA). Sections were then incubated overnight at 4℃ with mouse anti-developmental myosin heavy chain (dMHC; 1:100). The next day, sections were washed with PBS and then incubated with a biotinylated anti-mouse secondary antibody (Vector Labs, 1:250) for 30 minutes. Afterwards, sections were washed with PBS and incubated for 30 minutes in ABC reagents provided by the MOM kit. Stains were visualized using 3-amino-9-ethylcarbazole (Vector Labs).

Staining for Pax7, MyoD, and Myogenin was performed similarly by air drying sections for 30 minutes. Sections were then fixed in 4% paraformaldehyde (PFA) and submerged in antigen retrieval buffer at 95-100℃ for 45 minutes. They were then rinsed with PBS with 0.05% Tween 20, quenched in 0.3% H.O., and blocked using MOM blocking reagent for one hour (Vector). Sections were washed with phosphate buffered saline solution (PBS) and incubated with mouse anti-MyoD (BD Biosciences, 1:50), mouse anti-Myogenin (BD Biosciences, 1:50), or mouse anti-Pax7 (DSHB Hybridoma, 1:500) overnight at 4℃. Sections were washed with PBS and incubated with biotinylated anti-mouse secondary antibody (Vector, 1:250). Stains were visualized using 3-amino-9-ethylcarbazole.

Hemotoxylin stains were started by air drying sections for 30 minutes. Sections were then stained with hematoxylin for 10 minutes. Slides were then washed with water until the rinsed water ran clear.
**Immunofluorescence of Pax7 and β-catenin**

Cryosections of 3d post-injury TA muscles were air dried for 30 minutes and then fixed with 4% PFA. Next, they were washed with PBS and submerged in antigen retrieval buffer (10 mM sodium citrate, 0.05% Tween-20, pH = 6) at 95-100°C for 45 minutes. Sections were rinsed in PBS and 0.05% Tween-20 and then blocked using MOM blocking reagent for one hour (Vector). Sections were washed with PBS and then incubated with mouse anti-Pax7 (1:500) and rabbit anti-active β-catenin (Cell Signaling Technology, 1:1600) overnight at 4°C. The active β-catenin antibody specifically detects non-phosphorylated β-catenin (Ser45) making it a useful readout for stabilized β-catenin protein, which is functionally active in mediating transcriptional activity via the canonical Wnt signaling pathway. Slides were then rinsed in PBS and incubated with a fluorescent horse anti-mouse Dylight-594 (Vector, 1:200) and horse anti-rabbit Dylight-488 (Vector, 1:100) for 30 minutes. After washing with PBS, slides were cover-slipped with Prolong Gold (Invitrogen).

**Cell counts**

Cell counts were determined by counting the number of positively labeled cells from cross-sections of the muscle. Due to inconsistencies in the location of injury, cell counts for MyoD, Myogenin, and dMHC were only obtained from regions that were most representative of a regenerating area. In addition, counts were collected from 7-day post-injury tissue, a time point of active regeneration, a time in which it is difficult to distinguish myotubes and fibers. MyoD and Myogenin counts were represented by expressing the number of total cells over the total injured area. dMHC counts were expressed as total number of dMHC expressing fibers within the injured area of each muscle.
Pax7 counts were collected by counting the total number of Pax7 positive cells and dividing it over the total number of fibers. Since Pax7 counts were collected from 21-day post-injury tissue with vast regeneration, it was easier to obtain accurate fiber counts. The data are represented by the number of Pax7 counts/100 fibers.

Co-labeled β-catenin and Pax7 counts were collected by counting the total number of Pax7 counts and determining if the cells were also β-catenin positive. Cell counts are expressed as the number of Pax7 positive cells co-expressing β-catenin.

Cell culture and treatment

Sub-confluent myoblasts: C2C12 myoblasts were seeded at 6 x 10^4 cells/plate in 6 cm dishes with DMEM containing 10% FBS. Media was changed 24 hours after plating. 48 hours after plating, cells were stimulated once with Wnt-3a (R&D Systems, 0.1µg/ml). This dose of 0.1µg/ml was chosen because it was shown to activate Wnt signaling in C2C12 cells[18]. 72 hours after plating, cells were collected at 60-70% confluence. RNA was extracted and isolated from collected cells.

Confluent myoblasts: C2C12 myoblasts were treated similarly with an exception that they were seeded at 1.2 x 10^5 cells/plate. At time of collection, cells were 90-100% confluent.

Myotubes: C2C12 myoblasts were seeded at 1.2 x 10^5 cells/plate in 6 cm dishes with DMEM containing 10% FBS. 48 hours after plating, the cells were serum starved for 24 hours by switching to DMEM only in order to induce differentiation and promote myotubes formation. After 24 hours of serum starvation, the media was changed back to DMEM with 10% FBS. Media was changed 48 hours after switching back to complete media. 24 hours after the latest
media change, cells were stimulated with Wnt-3a (0.1 µg/ml) for 24 hours and then collected for RNA.

In experiments in which we co-treated myotubes with Wnt-3a and KL, we seeded and treated the cells the same as described for myotube formation. The treatment groups were as follows: control, Wnt-3a (0.1 µg/ml), KL (1 µg/ml), and KL & Wnt-3a. Because KL can bind and inhibit Wnt ligands, we first co-incubated the two proteins together and rotated them overnight at 4°C. There were triplicates of each treatment group. After 24 hours of treatment, cells were collected for RNA isolation.

_Fiber size and minimum feret diameter_

Cryosections of TA muscles were collected for cross-sectional area and minimum feret diameter measurements. Sections were stained with hematoxylin and imaged using Bioquant. Measurements of cross-sectional areas and minimum feret diameters were collected on ImageJ. Cross-sectional areas were a representation of the fiber size while minimum feret diameters reflected fiber size while accounting for any inconsistencies in fiber diameter due to oblique sectioning. Approximately 300 fibers were circled for each muscle section.

“Small fibers” were defined as fibers for which the mean cross-sectional area was more than three standard deviations less than the mean cross-sectional area in the untreated group for both 7-days and 21-days post-injury. Similarly, “large fibers” were defined as those fibers for which the mean cross-sectional area was more than three standard deviations greater than the mean cross-sectional area in the untreated group. The ranges at 7-days post-injury were < 305um² for small fibers and > 1700um² for large fibers. For 21-days post-injury, the ranges were < 315um² for small fibers and > 3300um² for large fibers.
Similar standards were determined using the minimum feret diameter. The range for 21-days post-injury were < 17.5um for small fibers and > 40um for large fibers. At 21-days post-injury, the range was < 19.5um for small fibers and > 57.5um for large fibers.
RESULTS

**KL treatments did not affect the regenerating muscle fiber size**

Histology of muscle from 6-month old mice after an acute muscle injury showed no significant difference in muscle regeneration between those treated with the vehicle or those treated with KL. At 3, 7, and 21-days post-injury, there was no significant visual difference between the vehicle treated and the KL treated muscles (Fig. 2A). Both treatment groups were marked by a large number of inflammatory cells within the muscle at 3-days post-injury, small regenerating muscle fibers with residual inflammatory cells at 7-days post-injury, and centrally-nucleated muscle fibers in close proximity at 21-days post-injury. Quantitatively, muscle regeneration was measured by cross-sectional area and minimum feret diameter of centrally-nucleated regenerating muscle fibers in the central lesion of injured muscles at 7- (Fig. 2D, H) and 21-days post-injury (Fig. 2F, J). There was no significant difference in the average fiber size (Fig. 2B, C) or in the proportions of small or large muscle fibers at 7- (Fig. 2E, I) or 21-days post-injury (Fig. 2G, K).

**KL treatments did not affect the number of newly formed regenerating fibers**

Although we did not see a significant difference in muscle fiber size, regeneration could also be measured by the number of newly formed muscle fibers. Developmental myosin heavy chain is expressed in regenerating myotubes making it a commonly used marker for regeneration\(^{19}\). We assayed for KL-mediated effects on muscle regeneration by quantifying the number of dMHC positive cells 7-days post-injury. We found that KL treatment did not affect the number of dMHC positive fibers at 7-days post-injury (Fig. 3A, B).
**KL treatments decreased expression of myogenic transcripts**

Although we did not detect differences in muscle regeneration with KL treatment, we further tested whether KL affects myogenesis by assaying for changes in the expression of myogenic transcripts pax7, myod, myogenin, and mrf4 at 3- (Fig. 4A-D), 7- (Fig. 4E-H) and 21-days post-injury (Fig. 4I-L). KL expression did not affect the expression of myogenic transcripts 3-days post-injury. At 7-days post-injury, KL treatment reduced myod and myogenin transcript expression (Fig. 4F, G). By 21-days post-injury, KL treatment reduced mrf4 gene expression (Fig. 4L). These data suggest that KL did not enhance myogenesis after muscle injury.

**KL treatments did not affect the concentration of MyoD, Myogenin, or Pax7 expressing cells after injury.**

Because we saw reduced myod or myogenin transcript expression levels at 7-days post-injury (Fig. 4F, G), we used immunohistochemistry to test whether changes in gene expression coincided with reductions in the number of cells expressing MyoD and Myogenin at this stage of regeneration. We found that KL does not affect the density of MyoD (Fig. 5A, B) or Myogenin (Fig. 5C, D) expressing cells at 7-days post-injury. We also quantified the number of Pax7+ cells at 21-days post-injury since KL was shown to affect the pool of Pax7+ cells in dystrophic muscle\(^1\) and since early reductions in myod or myogenin suggest changes in myogenesis and could reflect an increase in the number of satellite cells. There was no change in the number of Pax7 expressing cells at 21-days post-injury (Fig. 5E, F).
Figure 2: KL treatment did not improve muscle fiber growth. (A) Hematoxylin stained TA sections at 3-days, 7-days, and 21-days post-injury of KL treated and PBS treated groups. Images show the progression of muscle regeneration after injury. At 3-days, most fibers are necrotic, with a large infiltration of inflammatory cells. At 7-days, the number of regenerating muscle fibers increased with many inflammatory cells remaining in between fibers. By 21-days, muscle morphology is nearly normal. The centrally nucleated muscle fibers are in close proximity with a
decrease in the number of inflammatory cells located between fibers. Bars for scale are 100 µm. 
(B) Average CSA at 7-days post-injury. (C) Average CSA at 21-days post-injury. (D, F) Cross-
sectional areas of muscle fibers at 7-days and 21-days post-injury. (black bars: PBS treated; 
white bars: KL treated). (E, G) Frequency distribution of fibers cross-sectional area was 
compared between treatment groups at 7- (E) and 21-days post-injury (G). There were no 
significant differences between the percentages of small or large fibers between treatment groups 
at any time point. (H, J) Frequency distribution of minimum feret muscle fiber diameter 
measurements at 7 (H) and 21-days post-injury (J) (black bars: PBS treated; white bars: KL 
treated). (I, K) Percentages of small and large fibers based on minimum feret diameter were 
compared between treatment groups at each time point. There were no significant differences 
between the percentages of small or large fibers between treatment groups at any time point.

**Figure 3:** KL treatment did not affect dMHC expression 7-days post-injury. (A) Histological 
analysis of a cross section of a TA muscle 7-days post-injury. Black arrows indicate a positively 
labeled fiber. (B) dMHC counts were collected by counting the total number of dMHC positive 
cells per muscle section. KL treatments caused no significant difference in the number of dMHC 
positive cells.
Figure 4: QPCR analysis of RNA collected from quadriceps of C57BL/6 mice 3-, 7-, and 21-days post-injury. KL treatment reduced expression of myogenic transcription factors *myod* and *myogenin* at 7-days post-injury. *Mrf4* expression was also reduced in KL treated muscle at 21-days post-injury. *indicates a significant difference as compared to vehicle treated mice collected at the same time point p<0.05.
Figure 5: KL treatment did not affect the concentration of MyoD, Myogenin, or Pax7 expressing cells in muscle after injury. (A-D) MyoD and Myogenin counts of TA sections 7-days post-injury. Cells were only counted from regions that were most reflective of a regenerating area. Counts were then divided by the total regenerating area in order to give the density. There were no significant differences in MyoD or Myogenin cell concentrations when comparing treatments. (E,F) Pax7 counts were collected from TA sections 21-days post-injury. Total cell counts were divided by the total number of muscle fibers. There was no significant difference in the number of Pax7+ cells based on treatment. Black arrows indicate positively labeled cells.
KL reduced Wnt-signaling in myogenic progenitor cells

The decrease in *myod*, *myogenin*, and *mrf4* expression due to KL treatment suggested that KL most greatly affected the transition from an activated and proliferating satellite cell to a differentiating myocyte. We further explored this possibility by assaying KL effects on the Wnt signaling pathway. The Wnt pathway is thought to play a critical role in myogenesis as the onset of Wnt signaling is associated with the start of differentiation\textsuperscript{14}. Interestingly, KL has been shown to interact with and inhibit Wnt-ligands, activators of the Wnt pathway\textsuperscript{13}. We used QPCR of whole muscle lysates to test for changes in Wnt-signaling by measuring the expression of *axin2*, a known downstream Wnt signaling transcript that is induced by the activation of the canonical pathway\textsuperscript{20}. At 3-, 7-, and 21-days post-injury, there was no significant difference in *axin2* expression in the KL treated group compared to the vehicle treated group (Figure 6A-C).

Despite not detecting a difference in the downstream Wnt signaling transcript *axin2* in whole muscle lysates, we tested whether KL could be modulating Wnt signaling specifically in Pax7 expressing myogenic progenitor cells using immunofluorescence. β-catenin is an important mediator of Wnt signaling\textsuperscript{21}. We double labeled muscle sections with antibodies to Pax7 and a specific antibody for active β-catenin. Because previous investigators have shown that KL can inhibit Wnt signaling, we measured the effects of KL treatment by quantifying the proportion of Pax7 positive cells co-expressing active β-catenin. We focused on the 3-day post-injury time point because Brack *et al.* previously demonstrated that Wnt signaling was particularly important for muscle regeneration at 3-5 days post injury\textsuperscript{14}. Histological analysis showed that there was a significant reduction in the number of cells co-expressing Pax7 and active β-catenin in the KL treated group (p = 0.031) (Figure 6D, E).
Figure 6: KL treatment reduced Wnt signaling after muscle injury. (A-C) RNA collected from quadriceps of C57BL/6 mice at 3, 7 and 21-days post-injury did not show any differences in axin2 expression levels due to KL treatment. (D) Immunofluorescence for active β-catenin (green) and Pax7 (red). White arrows indicate co-labeled cells. (E) Histogram quantifying the proportion of cells co-expressing β-catenin and Pax7. The number of Pax7+ cells co-labeled with β-catenin were counted and then divided by the total number of Pax7+ cells. There was a significant decrease in the number of β-catenin+/Pax7+ cells in the KL treated group (p = 0.0305).

Wnt-3a treatment decreased myogenin expression in sub-confluent C2C12 myoblasts

The reduction in satellite cells expressing active β-catenin with KL treatment suggested that KL modulates Wnt signaling during regeneration. In order to better understand the role of Wnt signaling in myogenesis, we treated myoblasts at varying confluences with Wnt-3a. We first treated myoblasts that were 60-70% confluent then collected them for RNA extraction. Sub-
confluence ensured that there was little to no myotube formation. QPCR analysis of the RNA collected from myoblasts suggested that Wnt-3a did not have a large effect on axin2, pax7, or myod expression in myoblasts. However, myogenin expression was significantly decreased in cells treated with Wnt-3a (p = 0.0009) (Figure 7A).

**Wnt-3a treatment induced the Wnt pathway in confluent myoblasts and affected early myogenic transcripts**

Wnt3a treatment unexpectedly reduced myogenin expression, a marker for muscle cell differentiation in sub-confluent C2C12 myoblasts. Because this result was counter to the previously described role of Wnt promoting muscle cell differentiation *in vivo*, we tested whether the confluency of myoblasts also affected Wnt-mediated effects on myogenesis. We continued our investigation by stimulating confluent myoblasts with Wnt-3a. These myoblasts were 90-100% confluent upon collection. QPCR analysis showed a large increase in axin2 expression in Wnt-3a treated cells showing that Wnt-3a treatment induced the Wnt pathway (p = 0.0036). Early myogenic transcripts, myod and myogenin, showed a trending decrease in expression with Wnt-3a treatment with p-values of 0.0827 and 0.0951 respectively. Late myogenic transcript mrf4 and satellite cell marker pax7 were unaffected by Wnt-3a treatment (Figure 7B).

**Wnt-3a treatment induced the Wnt pathway and affected myogenic transcripts in myotubes**

To complete our investigation of the effect of Wnt-3a on myogenesis in C2C12 cells, we induced myotube formation from C2C12 myoblasts. Here, we tested the role of Wnt-3a treatments on differentiated myotubes. QPCR analysis of RNA collected from myotubes showed
an increase in *axin2* demonstrating Wnt pathway induction (*p* = 0.0001). *Myod* and *myogenin* decreased with Wnt-3a treatment (*p* = 0.0287, *p* = 0.0177), however, *mrf4* increased with Wnt-3a treatment (*p* = 0.0037). *Pax7* expression did not change with Wnt-3a treatment (Figure 7C). Together, these findings suggest the exogenous Wnt3a treatment of C2C12 cells does not have the predicted effect of promoting muscle cell differentiation, except possibly in differentiating myotubes reflected by increased *mrf4* transcript expression.

**Figure 7:** QPCR analysis of RNA collected from Wnt-3a treated, sub-confluent C2C12 myoblasts, confluent C2C12 myoblasts, and myotubes. (A) In C2C12 myoblasts that were 60-70% confluent, Wnt-3a treatment did not significantly increase *axin2* expression. There was a significant decrease in *myogenin* but no significant changes in *myod* or *pax7* due to Wnt-3a expression (*p* = 0.0009). (B) In C2C12 myoblasts that were 90-100% confluent at collection, Wnt-3a treatment significantly increased *axin2* expression, indicating an active Wnt pathway. There were no significant treatment effects on *pax7*, *myod*, *myogenin*, or *mrf4*. (C) Myotubes
showed a significant increase in axin2 expression when treated with Wnt-3a (p=0.0001). Myod and myogenin expression levels were significantly decreased (p = 0.0287, p = 0.0177) while mrf4 expression was significantly increased (p = 0.0037). There was no significant difference in pax7 expression. *indicates a significant difference as compared to untreated myoblasts or myotubes at p < 0.05.

**KL inhibited Wnt-3a induced Wnt signaling**

The induction of the Wnt pathway and subsequent changes in myogenic transcripts suggested that the Wnt pathway plays a role in myogenesis in myotubes. Because KL can inhibit Wnt signaling, we tested whether KL affects myogenesis by inhibiting the Wnt pathway. We treated myotubes with Wnt-3a, KL, or both, for 24 hours and then collected the cells for RNA isolation. QPCR analysis showed that axin2 expression was induced by Wnt-3a. However, axin2 expression was significantly reduced in the Wnt-3a and KL treated groups (Fig. 8A). Interestingly, myogenesis markers myod and myogenin showed similar decreases in transcript expression with Wnt-3a, KL, or KL & Wnt-3a treatment (Fig. 8B, C) while late myogenesis marker mrf4 was unaffected by treatment (Fig. 8I). Satellite cell marker pax7 was also unaffected by treatment (Fig. 8D). Contrary to our expectations, downstream Wnt pathway markers such as ccnd1 or wisp2 did not show any significant changes due to Wnt-3a treatment (Fig. 8E, F). However, myc, a known Wnt pathway target gene, increased in expression when treated with Wnt-3a alone or KL and Wnt-3a (Fig. 8G). Because previous studies showed an increase in fibrosis with the introduction of Wnt-3a into young regenerating muscle, we also assayed for fibrogenic marker Coll15. Coll1 was found to be unaffected by any treatment (Fig. 8H).
Figure 8: QPCR analysis of RNA collected from myotubes treated with Wnt-3a, KL, or co-treated with both. (A) Axin2 is significantly decreased in the KL & Wnt-3a group when compared to the group treated with Wnt-3a alone. (B,C) Wnt-3a and KL have similar effects on early myogenesis markers *myod* and *myogenin* with significant decreases in expression levels with Wnt-3a, KL, as well as KL & Wnt-3a treatment. (G) Myc expression increased significantly with Wnt-3a treatment, compared to control or KL treated myotubes. (D, E, F, H, I) Pax7, ccnd1, wisp2, col1, and mrf4 were not significantly affected by any treatment. Brackets indicate a significant difference between the groups *p < 0.05*.

**KL treatments did not affect inflammatory transcripts at 3-days, 7-days, or 21-days post-injury**

Previous investigations have shown that KL can affect inflammatory pathways in muscle cells. Wehling-Henricks *et al.* demonstrated that the introduction of KL through a *klotho* transgene into the *mdx* mouse model of DMD increased CD206+ cell concentrations, and reduced CD68+ macrophage numbers. We tested whether recombinant KL treatment in acutely
injured mice would have similar effects, by assaying for macrophage markers \textit{cd68}, \textit{cd163}, and \textit{cd206}, as well as pro-inflammatory cytokine \textit{tnfa}. QPCR analysis was run on RNA collected from 6-month old C57BL/6 mice at 3-days, 7-days, and 21-days post-injury. There was no significant difference in inflammatory transcripts (\textit{cd68}, \textit{cd163}, \textit{cd206}, \textit{tnfa}) at any of these points (Figure 9).

Figure 9: QPCR analysis of RNA collected from quadriceps of C57BL/6 mice at 3-, 7-, and 21-days post-injury. There were no significant differences in inflammatory (\textit{cd68}, \textit{cd163}, \textit{cd206}, \textit{tnfa}) transcripts at any of the time points.

KL treatments did not affect fibrogenic transcript expression after muscle injury

Although we did not detect a robust effect of KL on muscle regeneration, KL could affect other aspects of muscle repair, like fibrosis, which often manifests during skeletal muscle repair
post-injury. The introduction of Wnt-3a and activation of the Wnt pathway have been associated with connective tissue deposition during regeneration\textsuperscript{15}. Furthermore, Wnt ligands have been shown to be inhibited by KL\textsuperscript{13}. Thus, we further assessed KL’s effects on connective tissue proteins by assaying for connective tissue protein transcripts (\textit{col1}, \textit{col3}, \textit{col5}) at 3-, 7-, and 21-days post injury. KL treatment did not affect the expression of these transcripts at any time point sampled (Figure 10).

\textbf{Figure 10:} QPCR analysis of RNA collected from quadriceps of C57BL/6 mice 3-, 7-, and 21-days post-injury. There were no significant differences fibrogenic markers (\textit{col1}, \textit{col3}, and \textit{col5})
at any time point. *indicates a significant difference as compared to the vehicle treated mice collected at the same time point p < 0.05.
DISCUSSION

The results of this investigation demonstrate that exogenous KL treatment did not improve adult mouse muscle regeneration after acute injury. Our findings show that KL did not affect the formation of new muscle fibers or muscle fiber size. Our QPCR results showed reduced expression of *myod*, *myogenin* and *mrf4* transcripts in KL treated muscle after injury. However, the reduction did not correlate with a reduction in the proportion of cells expressing their respective proteins. Additionally, transcripts for connective tissue proteins and important mediators of the inflammatory response to muscle injury were unaffected by KL treatment. Although our findings do not support our hypothesis that KL treatment has a pro-regenerative effect on muscle, our *in vitro* experiments confirm that KL is an antagonist of Wnt-signaling in muscle cells and show that exogenous KL can inhibit Wnt signaling in satellite cells in regenerating muscle.

KL treatment reduced the expression of myogenic transcripts, but the decrease in gene expression did not correlate with changes in muscle regeneration, such as the number of newly formed myotubes expressing dMHC or regenerating myofiber size. While we did not detect differences in muscle fiber size at 7-days or 21-days post-injury, it is possible that the rate of muscle fiber growth was still affected. For example, KL treatment appeared to result in a leftward shift in myofiber size distribution 7-days post-injury indicating a larger proportion of smaller fibers; however, by 21-days post-injury, the KL treated group is more shifted right with greater proportions of large myofibers. These data suggest that a shift in the kinetics of our KL treatment regimen may be therapeutic. For instance, early KL treatment reduced Wnt signaling in myogenic progenitor cells during a time in which Wnt signaling is critical for the induction of differentiation and regeneration\(^1\). Thus, it is feasible that muscle from the KL treated group
overcame an early delay in myogenesis caused by the inhibition of Wnt signaling and that
treatment beginning after the onset of myogenic differentiation (days 3-5) would have been
beneficial.

We identified the Wnt signaling pathway as a potential KL target affecting muscle repair
as it has been shown to bind to multiple Wnt ligands to impair Wnt-signaling\(^\text{13}\). A previous study
inhibiting Wnt signaling using sFRP3 showed impaired muscle regeneration in young muscle
when the inhibitor was specifically administered during the switch between proliferation to
differentiation in satellite cells\(^\text{15}\). This specified time point of administration may have produced
a robust and targeted response on myogenesis. While the inhibition of different Wnt ligands can
affect different signaling pathways, we focused on the Wnt-3a ligand as it had been shown to
promote myogenesis in injured muscle\(^\text{14}\). Wnt-3a inhibits the degradation of β-catenin, a
molecule that is involved in the activation of Wnt genes\(^\text{24}\). However, the role of Wnt-signaling
in muscle regeneration is complex and incompletely understood, as both the conditional
disruption or activation of β-catenin can impair muscle regeneration\(^\text{25}\). We found that KL
treatment decreased the number of Pax7+ cells that co-expressed active β-catenin, suggesting
that KL treatment reduced Wnt signaling in satellite cells. Indeed, our \textit{in vitro} experiments
showed that Wnt-3a treatment of confluent myoblasts increased expression of the Wnt target
gene \textit{axin2} and treatment with both Wnt3a and KL attenuated the induction of \textit{axin2} expression.
This indicates that KL inhibits Wnt3a-mediated induction of Wnt signaling in muscle cells.

Our experiments suggest that the effect of Wnt-3a treatment on C2C12 muscle cells is
different in myoblasts and differentiating myotubes. For example, Wnt-3a treatment was not
shown to induce the expression \textit{axin2} in sub-confluent myoblasts. The lack of Wnt-pathway
activation could be due to a small data set. Some trends may have been found to be significant
with a larger sample size. Another explanation could involve the stage of myogenesis of the cells. Fusing or nearly fusing confluent myoblasts and differentiating myotubes responded to Wnt3a with a robust increase in \textit{axin2} expression. Thus, our data supports muscle cells differentially responding to Wnt stimulation depending on the stage of myogenesis. Intriguingly, others have observed different outcomes on muscle regeneration depending on the stage in which Wnt-signaling was disrupted\textsuperscript{15}.

Overall, our \textit{in vivo} and \textit{in vitro} findings show that KL is an antagonist of the Wnt signaling pathway in muscle cells. This further supports previous studies that showed suppressed Wnt activity with KL treatment in kidney cells\textsuperscript{13}. Others have also described reduced Wnt activity when treating KL-deficient muscle cells stimulated with KL. Additionally, when isolated muscle fibers and their adjacent satellite cells were cultured and stimulated with Wnt-3a there was a decrease in Pax7+ cells\textsuperscript{12}. In our \textit{in vitro} experiments using C2C12 myoblasts, we did not detect differences in \textit{pax7} transcript expression. We also saw an unexpected reduction in \textit{myod} and \textit{myogenin} expression even though we saw increased expression of \textit{axin2} in confluent myoblasts and myotubes. This contrasts with previous findings where \textit{myogenin} expression was increased and \textit{pax7} expression was decreased in primary myoblasts treated with Wnt-3a\textsuperscript{26}. This difference could be explained by the types of cells used. We used an immortalized cell line that may not be representative of \textit{in vivo} processes.

Although we did not see a robust effect of KL on myogenesis, we also examined whether KL affected the inflammatory response to muscle injury and the expression of genes encoding connective tissue proteins. We have shown previously that KL affects the inflammatory response in muscle from dystrophic \textit{mdx} mice. Introduction of a KL transgene into \textit{mdx} mice increased CD206+ cell concentrations and reduced CD68+ macrophage numbers\textsuperscript{23}. We also found that KL
reduces the expression of connective tissue proteins in dystrophic muscle, which could be due to changes in inflammation or Wnt-signaling\(^1\). Both inflammation and Wnt-signaling promote fibrosis in dystrophic muscle\(^{27,28}\). In our investigation, we did not detect any significant effects of KL on inflammatory or connective tissue transcripts. These differences could be due to different injury models. The \textit{mdx} mouse is representative of a chronic muscle wasting disease while the intramuscular injection of BaCl\(_2\) in C57/BL6 mice is an acute injury model and representative of a one-time sterile chemical induced injury. \textit{Mdx} mice were also shown to have muscle specific silencing of the \textit{Klotho} gene at the onset of pathology\(^1\). It is likely that the positive effects of Klotho on regeneration, inflammation and fibrosis in \textit{mdx} mice was due to the expression of a transgene restoring KL in dystrophic muscle. An alternative explanation is the potential effect of increased KL expression in mice throughout their lifetime having a more robust effect than KL treatments on alternating days starting 3 days prior to injury.

The findings from this study are important because they show that excess exogenous KL treatment does not have a therapeutic effect on muscle regeneration, at least under the treatment conditions we used in our investigation. However, previous studies have established that rescuing KL expression in dystrophic muscle or treating KL deficient muscle cells has beneficial effects on myogenesis and regeneration\(^{1,12,23}\). It would be interesting to test whether exogenous KL has a therapeutic effect on muscle regeneration following acute injury when KL expression is reduced, like in old wild-type muscle\(^{23}\). We also show that a supraphysiological dose of KL may impair regeneration by inhibiting differentiation due to its effects on Wnt-signaling. A change in the kinetics of our KL treatment so that KL stimulation begins after the induction of differentiation could have beneficial effects since we previously found KL induces a positive protein balance in differentiating myotubes \textit{in vitro}\(^1\). It is also possible that KL is therapeutic
during conditions of elevated Wnt signaling like in muscular dystrophy and aging\textsuperscript{1,13}. For example, KL treatment could attenuate the fibrogenic conversion of myogenic cells in aging muscle caused by elevated Wnt-signaling\textsuperscript{15}.

In conclusion, we demonstrate that introduction of recombinant KL after an acute muscle injury under the conditions used in the present investigation does not affect muscle regeneration or the expression of inflammatory and fibrogenic transcripts. Although we saw muscle specific decreases in myogenic transcripts, this was not reflected in the fiber size nor in the proportion of cells expressing their respective proteins. However, our investigation supports the concept that KL is an antagonist of Wnt signaling in muscle cells. Therefore, our findings suggest the interaction of KL and the Wnt signaling pathway may be relevant to mechanisms of myogenesis and should be further explored for therapy development.
REFERENCES


