UCLA
UCLA Electronic Theses and Dissertations

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

Permalink
https://escholarship.org/uc/item/1mf3j8f5

Author
Hwang, Kimberly

Publication Date
2018

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA

Los Angeles

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

2018

© Copyright by

Kimberly Hwang

2018

ABSTRACT OF THE THESIS

The Effect of Klotho on Muscle Regeneration

after Acute Muscle Injury

by

Kimberly Hwang

Master of Science in Physiological Science

University of California, Los Angeles, 2018

Professor James G Tidball, Chair

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.

TABLE OF CONTENTS

ABSTRACT ii

COMMITTEE PAGE iv

DEDICATION v

TABLE OF CONTENTS vi

LIST OF FIGURES vii

ACKNOWLEDGMENTS viii

INTRODUCTION 1

MATERIALS AND METHODS 4

ANIMALS AND TREATMENT 4

RNA ISOLATION AND QPCR 5

IMMUNOHISTOCHEMISTRY 6

IMMUNOFLUORESCENCE OF PAX7 AND β-CATENIN 7

CELL COUNTS 8

CELL CULTURE AND TREATMENT 9

FIBER SIZE AND MINIMUM FERET DIAMETER 10

RESULTS 12

DISCUSSION 27

REFERENCES 32

LIST OF FIGURES

FIG. 1: Diagram showing the injection schedule of acutely injured C57BL/6 mice 4

FIG. 2: KL treatment did not improve muscle fiber growth 14

FIG. 3: KL treatment did not affect dMHC expression 7-days post-injury 15

FIG. 4: QPCR analysis of RNA collected from quadriceps of C57BL/6 mice 3-, 7-, 21-days post-injury 16

FIG. 5: KL treatment did not affect the concentration of MyoD, Myogenin, or Pax7 expressing cells in muscle after injury 17

FIG. 6: KL treatment reduced Wnt signaling after muscle injury 19

FIG. 7: QPCR analysis of RNA collected from Wnt-3a treated, sub-confluent C2C12 myoblasts, confluent C2C12 myoblasts, and myotubes 21

FIG. 8: QPCR analysis of RNA collected from myotubes treated with Wnt-3a, KL, or co-treated with both 23

FIG. 9: QPCR analysis of RNA collected from quadriceps of C57BL/6 mice 3-, 7-, 21-days post-injury 24

FIG. 10: QPCR analysis of RNA collected from quadriceps of C57BL/6 mice 3-, 7-, 21-days post-injury 25

ACKNOWLEDGMENTS

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.

I would also like to thank my mentor Dr. Steve Welc for his endless patience and guidance. I am forever grateful for all the time you took to thoroughly explain concepts and teach me how to become a better scientist.

Lastly, I would like to thank all the members of the Tidball Laboratory for all their advice and support. It was a joy working with each and every single one of you.

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[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[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[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[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[14]. Furthermore, Wnt-signaling can also affect satellite cell fate and promote fibrogenesis[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[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

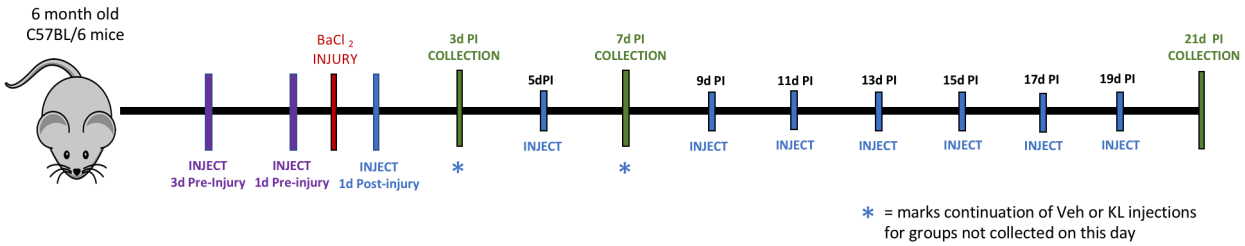

Figure 1: Diagram showing the injection schedule of acutely injured C57BL/6 mice. Mice were started with intraperitoneal injections of KL or a vehicle solution 3 days prior to induction of a sterile muscle injury via the intramuscular injection of a $BaCl_2$ solution. These injections continued every 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 obstructions[16]. 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 $BaCl_2$ three days after the start of treatment. $BaCl_2$ 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 μl injections of barium chloride ($BaCl_2$: 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 genes 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.

Gene		Direction (5' to 3')
Axin2	Fwd	GACGCACTGACCGACGATTC
	Rev	CTGCGATGCATCTCTCTCTGG
CCND1	Fwd	CGAGGAGCTGCTGCAAATG
	Rev	GGGTTGGAAATGAACTTCACATC
CD163	Fwd	GCAAAAACTGGCAGTGGG
	Rev	GTCAAAATCACAGACGGAG
CD206	Fwd	GGATTGTGGAGCAGATGGAAG
	Rev	CTTGAATGGAAATGCACAGAC
CD68	Fwd	CAAAGCTTCTGCTGTGGAAAT
	Rev	GACTGGTCACGGTTGCAAG
Col1	Fwd	TGTGTGCGATGACGTGCAAT
	Rev	GGGTCCCTCGACTCCTACA
Col3	Fwd	ATCCCATTTGGAGAATGTTGTGC
	Rev	GGACATGATTCACAGATTCCAGG
Col5	Fwd	CGGGGTACTCCTGGTCCTAC
	Rev	GCATCCCTACTTCCCCCTTG
Mrf4	Fwd	ATCAGCTACATTGAGCGTCTACA
	Rev	CCTGGAATGATCCGAAACACTTG
Myc	Fwd	CCCTATTTCATCTGCGACGAG
	Rev	GACGTAGCGACCGCAACATAG
MyoD	Fwd	GAGCGCATCTCCACAGACAG
	Rev	AAATCGCATTGGGGTTTGAG
Myogenin	Fwd	CCAGTACATTGAGCGCCTAC
	Rev	ACCGAACTCCAGTGCATTGC
Pax7	Fwd	CTCAGTGAGTTCGATTAGCCG
	Rev	AGACGGTTCCCTTTGTCGC
RPLO	Fwd	GGACCCGAGAAGACCTCCTT
	Rev	GCTGCCGTTGTCAAACACC
RPNS1	Fwd	AGGCTCACCAGGAATGTGAC
	Rev	CTTGGCCATCAATTTGTCCT
SRP14	Fwd	AGAGCGAGCAGTTCCTGAC
	Rev	CGGTGCTGATCTTCCTTTTC
TNFa	Fwd	CTTCTGTCTACTGAACTTCGGG
	Rev	CACTTGGTGGTTTGCTACGAC
Wisp 2	Fwd	GACACCAACTTTCTGCCCTTG
	Rev	CCAGTTGGCAGAATCGGTTC

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_2O_2, 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_2O_2, 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℃ 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℃. 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℃[13]. 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 < 305um^2 for small fibers and > 1700um^2 for large fibers. For 21-days post-injury, the ranges were < 315um^2 for small fibers and > 3300um^2 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).

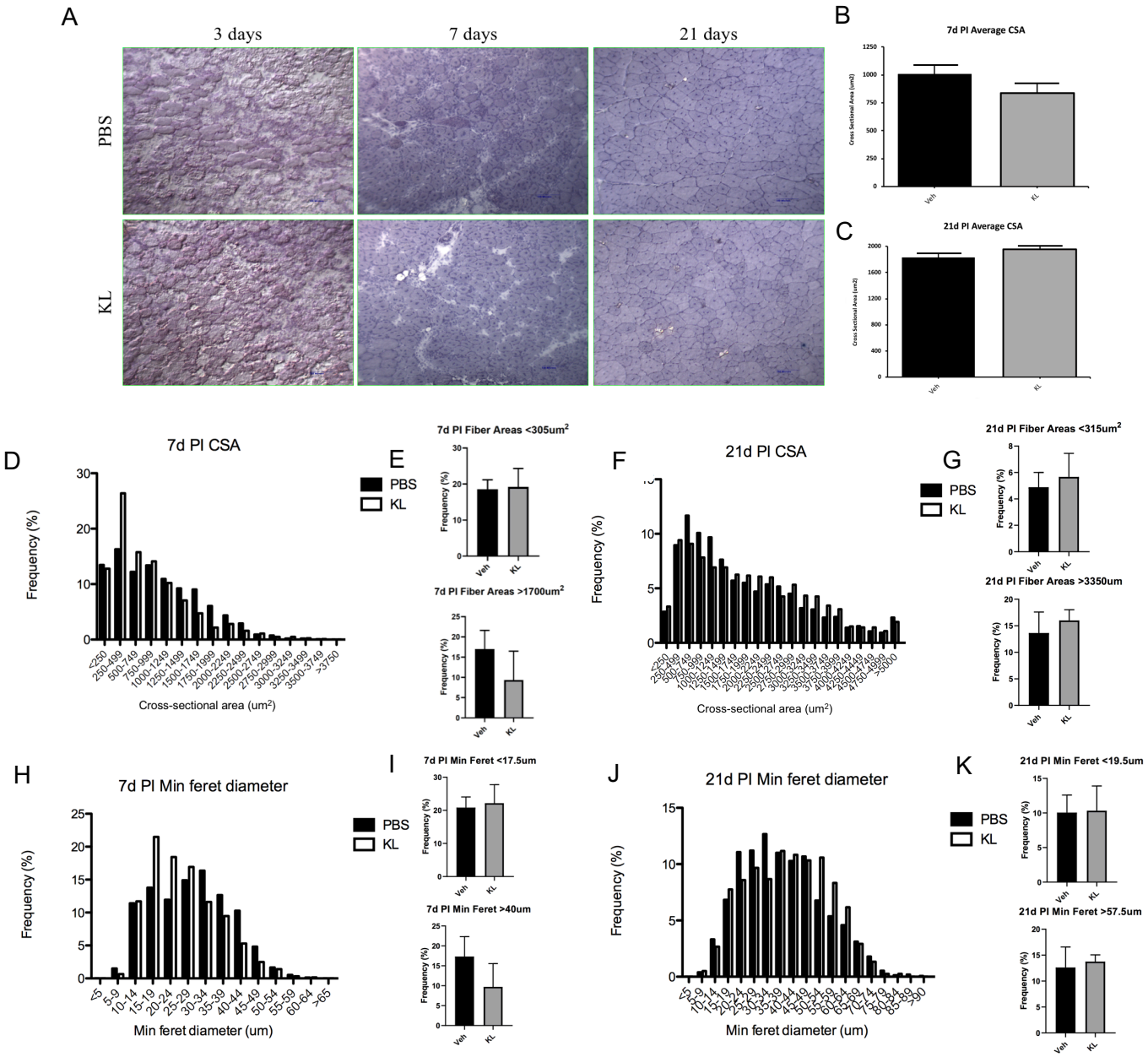

Figure 2: KL treatment did not improve muscle fiber growth. (A) Hematoxylin stained TA sections at 3-days, 7-days, and 21-days post-injury of KL treated and PBS treated groups. Images show the progression of muscle regeneration after injury. At 3-days, most fibers are necrotic, with a large infiltration 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.

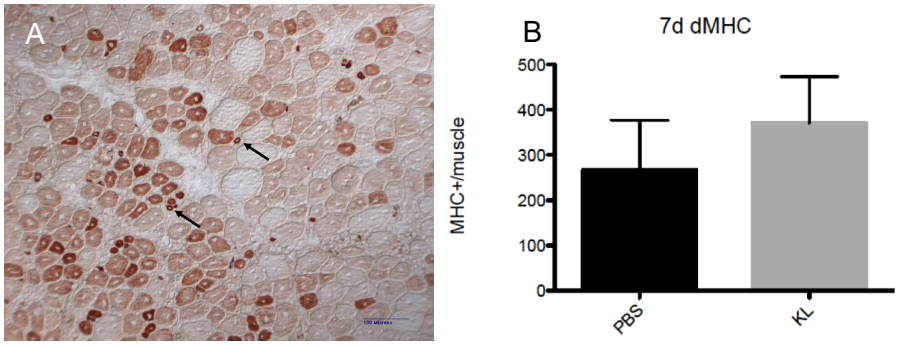

Figure 3: KL treatment did not affect dMHC expression 7-days post-injury. (A) Histological analysis of a cross section of a TA muscle 7-days post-injury. Black arrows indicate a positively labeled fiber. (B) dMHC counts were collected by counting the total number of dMHC positive cells per muscle section. KL treatments caused no significant difference in the number of dMHC positive cells.

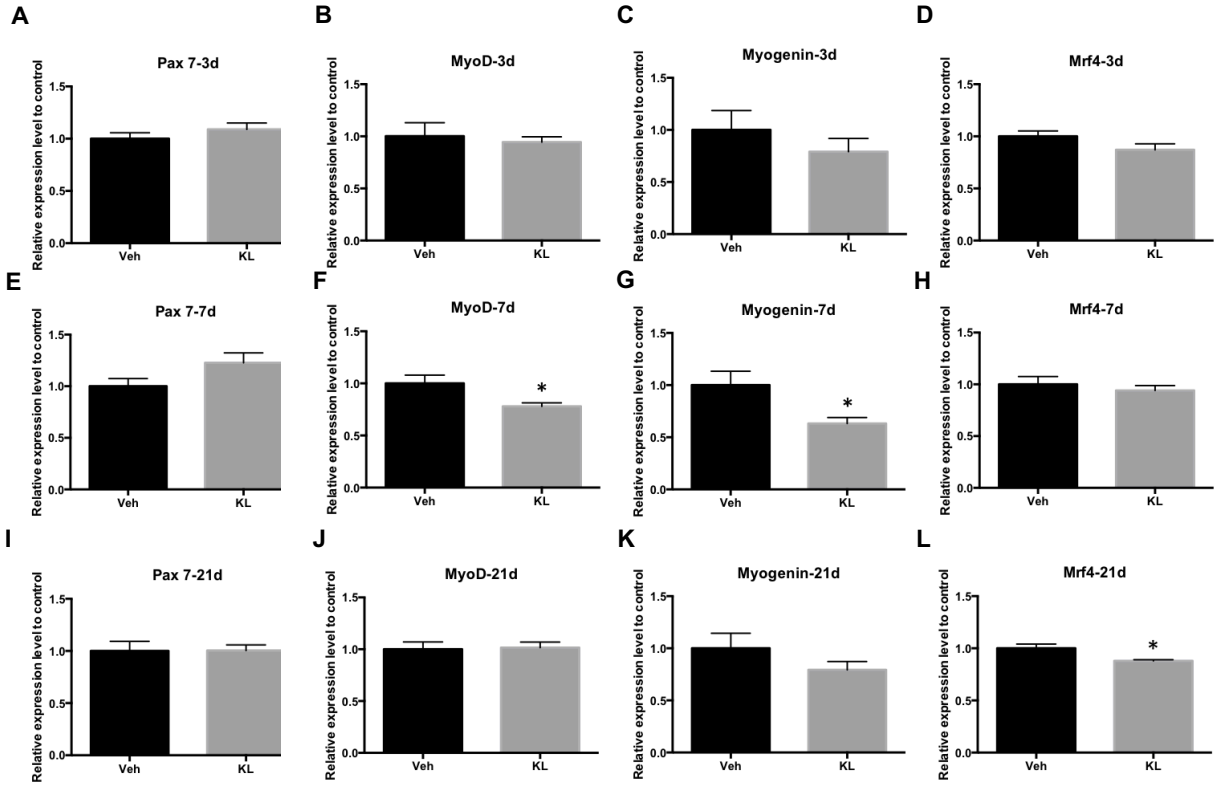

Figure 4: QPCR analysis of RNA collected from quadriceps of C57BL/6 mice 3-, 7-, and 21-days post-injury. KL treatment reduced expression of myogenic transcription factors *myod* and *myogenin* at 7-days post-injury. *Mrf4* expression was also reduced in KL treated muscle at 21-days post-injury. *indicates a significant difference as compared to vehicle treated mice collected at the same time point $p<0.05$.

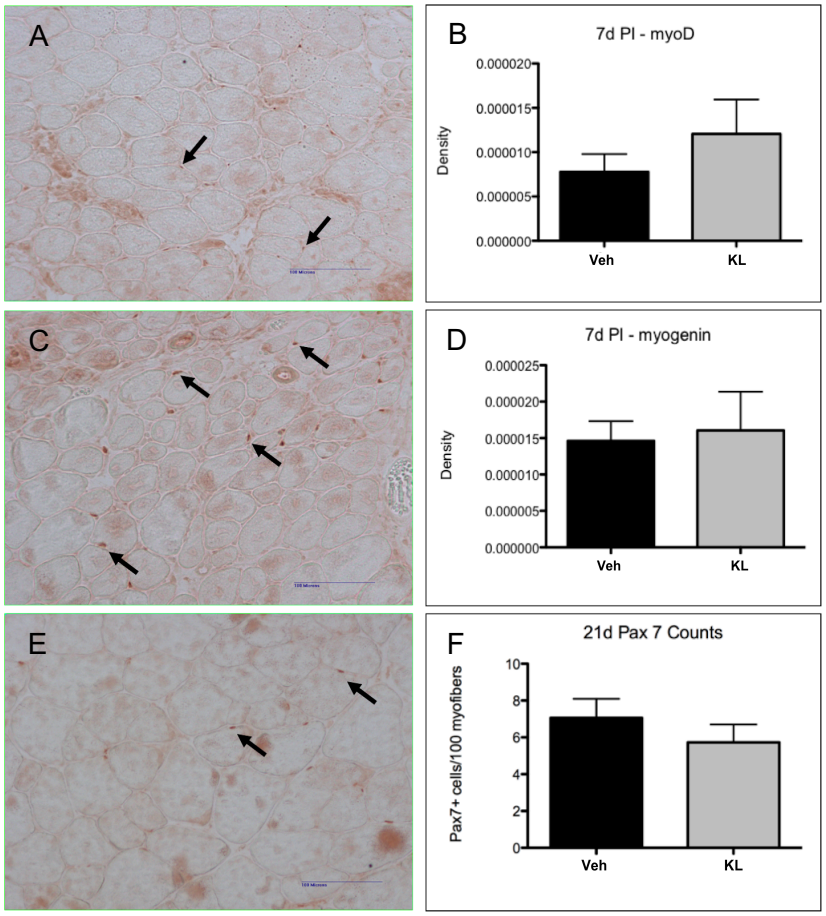

Figure 5: KL treatment did not affect the concentration of MyoD, Myogenin, or Pax7 expressing cells in muscle after injury. (A-D) MyoD and Myogenin counts of TA sections 7-days post-injury. Cells were only counted from regions that were most reflective of a regenerating area. Counts were then divided 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[14]. Interestingly, KL has been shown to interact with and inhibit Wnt-ligands, activators of the Wnt pathway[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[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[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[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).

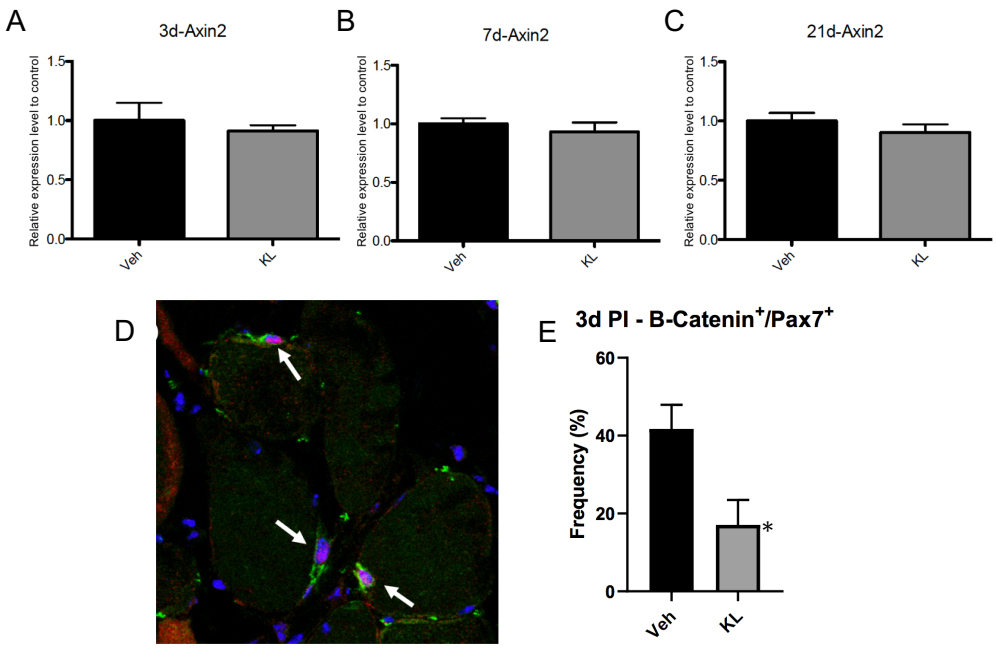

Figure 6: KL treatment reduced Wnt signaling after muscle injury. (A-C) RNA collected from quadriceps of C57BL/6 mice at 3, 7 and 21-days post-injury did not show any differences in *axin2* expression levels due to KL treatment. (D) Immunofluorescence for active β-catenin (green) and Pax7 (red). White 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.

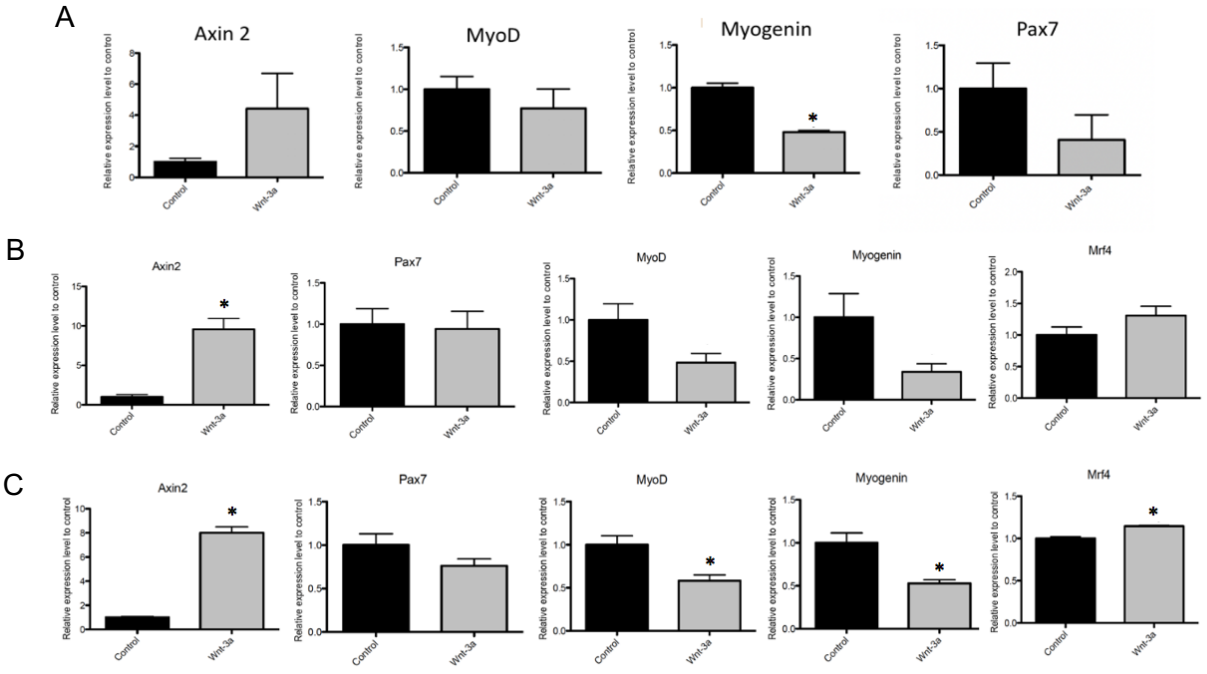

Figure 7: QPCR analysis of RNA collected from Wnt-3a treated, sub-confluent C2C12 myoblasts, confluent C2C12 myoblasts, and myotubes. (A) In C2C12 myoblasts that were 60-70% confluent, Wnt-3a treatment did not significantly increase *axin2* expression. There was a significant decrease in *myogenin* but 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[22] (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 *Col1*[15]. *Col1* was found to be unaffected by any treatment (Fig. 8H).

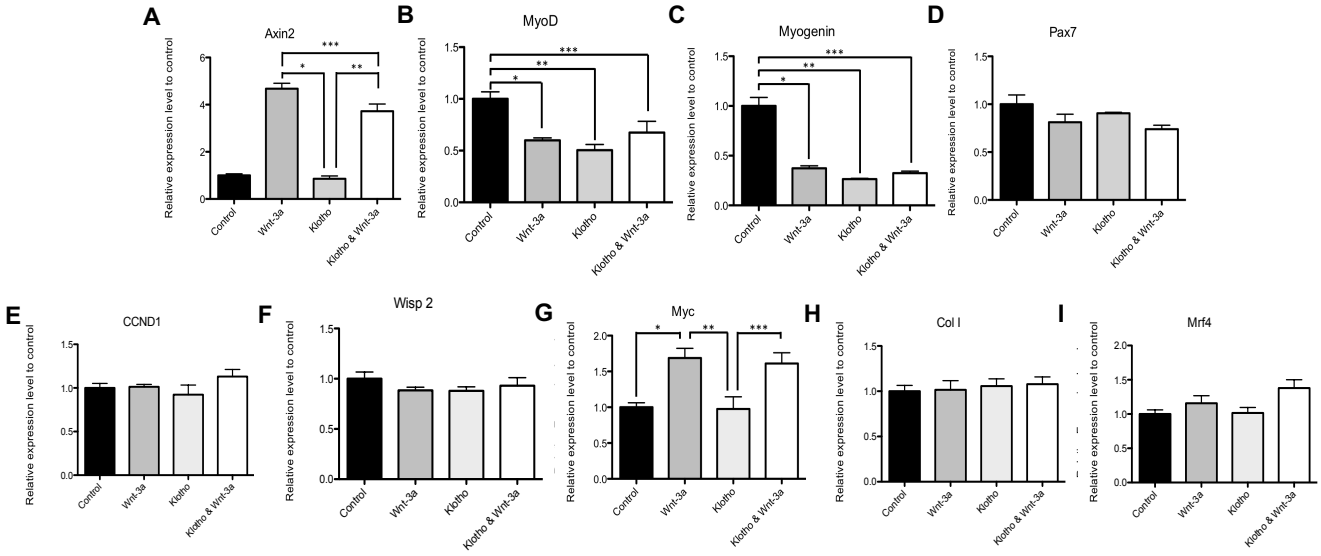

Figure 8: QPCR analysis of RNA collected from myotubes treated with Wnt-3a, KL, or co-treated with both. (A) *Axin2* is significantly decreased in the KL & Wnt-3a group when compared to the group treated with Wnt-3a alone. (B,C) Wnt-3a and KL have similar effects on early myogenesis markers *myod* and *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[23]. We tested whether recombinant KL treatment in acutely

injured mice would have similar effects, by assaying for macrophage markers *cd68*, *cd163*, and *cd206*, as well as pro-inflammatory cytokine *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 (*cd68, cd163, cd206, tnfa*) at any of these points (Figure 9).

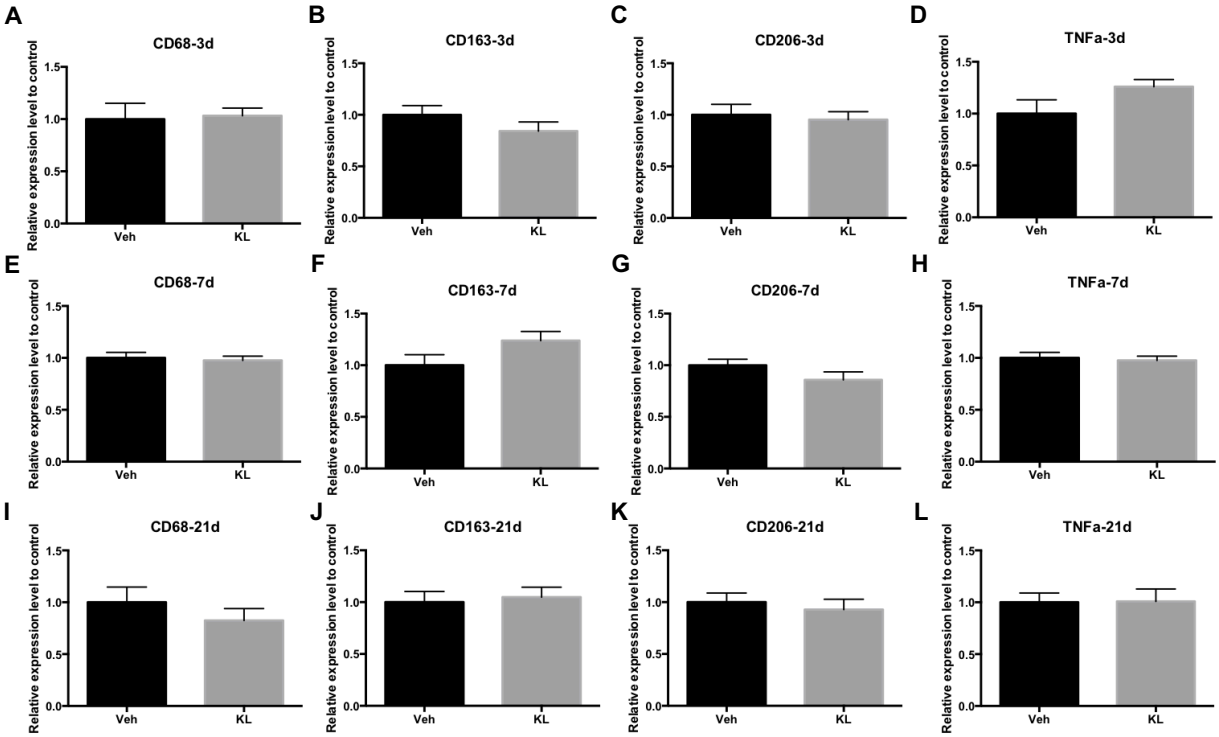

Figure 9: QPCR analysis of RNA collected from quadriceps of C57BL/6 mice at 3-,7-, and 21-days post-injury. There were no significant differences in inflammatory (*cd68*, *cd163*, *cd206*, *tnfa*) transcripts at any of the time points.

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[15]. Furthermore, Wnt ligands have been shown to be inhibited by KL[13]. Thus, we further assessed KL's effects on connective tissue proteins by assaying for connective tissue protein transcripts (*col1*, *col3*, *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).

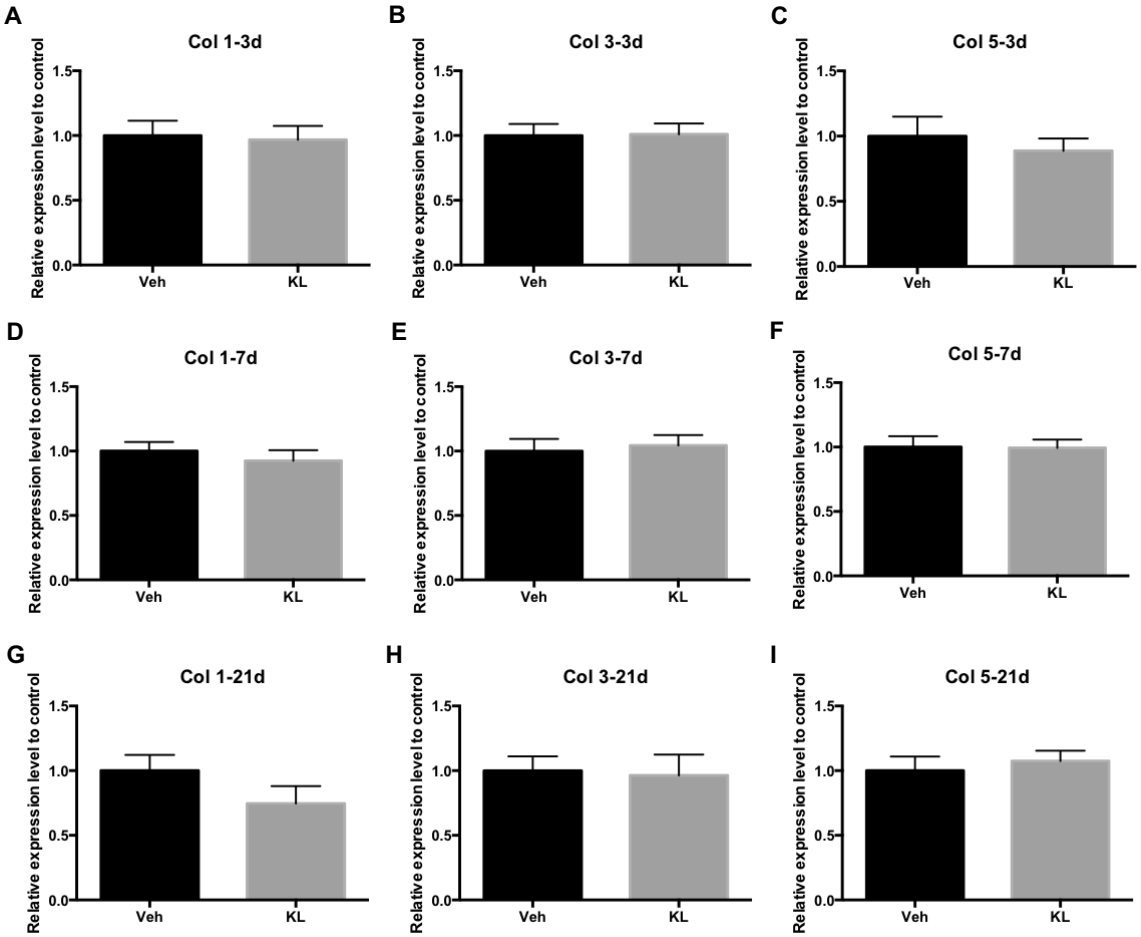

Figure 10: QPCR analysis of RNA collected from quadriceps of C57BL/6 mice 3-, 7-, and 21-days post-injury. There were no significant differences fibrogenic markers (*col1*, *col3*, and *col5*)

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[14]. 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[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[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[14]. Wnt-3a inhibits the degradation of β-catenin, a molecule that is involved in the activation of Wnt genes[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[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 *in vitro* experiments showed that Wnt-3a treatment of confluent myoblasts increased expression of the Wnt target gene *axin2* and treatment with both Wnt3a and KL attenuated the induction of *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 *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 *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[15].

Overall, our *in vivo* and *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[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[12]. In our *in vitro* experiments using C2C12 myoblasts, we did not detect differences in *pax7* transcript expression. We also saw an unexpected reduction in *myod* and *myogenin* expression even though we saw increased expression of *axin2* in confluent myoblasts and myotubes. This contrasts with previous findings where *myogenin* expression was increased and *pax7* expression was decreased in primary myoblasts treated with Wnt-3a[26]. This difference could be explained by the types of cells used. We used an immortalized cell line that may not be representative of *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 *mdx* mice. Introduction of a KL transgene into *mdx* mice increased CD206+ cell concentrations and reduced CD68+ macrophage numbers[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 *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. *Mdx* mice were also shown to have muscle specific silencing of the *Klotho* gene at the onset of pathology[1]. It is likely that the positive effects of Klotho on regeneration, inflammation and fibrosis in *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 *in vitro*[1]. It is also possible that KL is therapeutic

during conditions of elevated Wnt signaling like in muscular dystrophy and aging[1,13]. For example, KL treatment could attenuate the fibrogenic conversion of myogenic cells in aging muscle caused by elevated Wnt-signaling[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

1. Wehling-Henricks M, Li Z, Lindsey C, et al. Klotho gene silencing promotes pathology in the *mdx* mouse model of Duchenne muscular dystrophy. *Hum Mol Genet*. 2016;25(12):ddw111. doi:10.1093/hmg/ddw111
2. Domingues-Faria C, Vasson M-P, Goncalves-Mendes N, Boirie Y, Walrand S. Skeletal muscle regeneration and impact of aging and nutrition. *Ageing Res Rev*. 2016;26:22-36. doi:10.1016/J.ARR.2015.12.004
3. Tidball JG, Dorshkind K, Wehling-Henricks M. Shared signaling systems in myeloid cell-mediated muscle regeneration. *Development*. 2014;141(6):1184-1196. doi:10.1242/dev.098285
4. Sambasivan R, Yao R, Kissenpfennig A, et al. Pax7-expressing satellite cells are indispensable for adult skeletal muscle regeneration. *Development*. 2011;138(17):3647-3656. doi:10.1242/dev.067587
5. Lepper C, Partridge TA, Fan C-M. An absolute requirement for Pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration. *Development*. 2011;138(17):3639-3646. doi:10.1242/dev.067595
6. Megeney LA, Kablar B, Garrett K, Anderson JE, Rudnicki MA. MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes Dev*. 1996;10(10):1173-1183. http://www.ncbi.nlm.nih.gov/pubmed/8675005. Accessed December 2, 2018.
7. Day K, Shefer G, Shearer A, Yablonka-Reuveni Z. The depletion of skeletal muscle satellite cells with age is concomitant with reduced capacity of single progenitors to produce reserve progeny. *Dev Biol*. 2010;340(2):330-343. doi:10.1016/j.ydbio.2010.01.006

8. Heslop L, Morgan JE, Partridge TA. Evidence for a myogenic stem cell that is exhausted in dystrophic muscle. *J Cell Sci*. 2000;113 (Pt 12):2299-2308. http://www.ncbi.nlm.nih.gov/pubmed/10825301. Accessed December 2, 2018.

9. Kuro-o M, Matsumura Y, Aizawa H, et al. Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature*. 1997;390(6655):45-51. doi:10.1038/36285

10. Kuro-o M. Klotho. *Pflügers Arch - Eur J Physiol*. 2010;459(2):333-343. doi:10.1007/s00424-009-0722-7

11. Phelps M, Pettan-Brewer C, Ladiges W, Yablonka-Reuveni Z. Decline in muscle strength and running endurance in klotho deficient C57BL/6 mice. *Biogerontology*. 2013;14(6):729-739. doi:10.1007/s10522-013-9447-2

12. Ahrens HE, Huettemeister J, Schmidt M, Kaether C, von Maltzahn J. Klotho expression is a prerequisite for proper muscle stem cell function and regeneration of skeletal muscle. *Skelet Muscle*. 2018;8(1):20. doi:10.1186/s13395-018-0166-x

13. Liu H, Fergusson MM, Castilho RM, et al. Augmented Wnt signaling in a mammalian model of accelerated aging. *Science*. 2007;317(5839):803-806. doi:10.1126/science.1143578

14. Brack AS, Conboy IM, Conboy MJ, Shen J, Rando TA. A temporal switch from notch to Wnt signaling in muscle stem cells is necessary for normal adult myogenesis. *Cell Stem Cell*. 2008;2(1):50-59. doi:10.1016/j.stem.2007.10.006

15. Brack AS, Conboy MJ, Roy S, et al. *Increased Wnt Signaling During Aging Alters Muscle Stem Cell Fate and Increases Fibrosis*. Vol 317.; 2007:807-810. doi:10.1126/science.1144090

16. Doi S, Zou Y, Togao O, et al. Klotho inhibits transforming growth factor-beta1 (TGF-

beta1) signaling and suppresses renal fibrosis and cancer metastasis in mice. *J Biol Chem*. 2011;286(10):8655-8665. doi:10.1074/jbc.M110.174037

17. Hardy D, Besnard A, Latil M, et al. Comparative Study of Injury Models for Studying Muscle Regeneration in Mice. *PLoS One*. 2016;11(1):e0147198. doi:10.1371/journal.pone.0147198

18. Suzuki A, Pelikan RC, Iwata J. WNT/β-Catenin Signaling Regulates Multiple Steps of Myogenesis by Regulating Step-Specific Targets. *Mol Cell Biol*. 2015;35(10):1763-1776. doi:10.1128/MCB.01180-14

19. Kirk SP, Oldham JM, Jeanplong F, Bass JJ. Insulin-like Growth Factor-II Delays Early but Enhances Late Regeneration of Skeletal Muscle. *J Histochem Cytochem*. 2003;51(12):1611-1620. doi:10.1177/002215540305101205

20. Jho E, Zhang T, Domon C, Joo C-K, Freund J-N, Costantini F. Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. *Mol Cell Biol*. 2002;22(4):1172-1183. doi:10.1128/MCB.22.4.1172-1183.2002

21. He X, Semenov M, Tamai K, Zeng X. LDL receptor-related proteins 5 and 6 in Wnt/ -catenin signaling: Arrows point the way. *Development*. 2004;131(8):1663-1677. doi:10.1242/dev.01117

22. He TC, Sparks AB, Rago C, et al. Identification of c-MYC as a target of the APC pathway. *Science*. 1998;281(5382):1509-1512. http://www.ncbi.nlm.nih.gov/pubmed/9727977. Accessed December 2, 2018.

23. Wehling-Henricks M, Welc SS, Samengo G, et al. Macrophages escape Klotho gene silencing in the mdx mouse model of Duchenne muscular dystrophy and promote muscle growth and increase satellite cell numbers through a Klotho-mediated pathway. *Hum Mol*

Genet. 2018;27(1):14-29. doi:10.1093/hmg/ddx380

24. Xiong Y, Kotake Y. No exit strategy? No problem: APC inhibits beta-catenin inside the nucleus. *Genes Dev*. 2006;20(6):637-642. doi:10.1101/gad.1413206

25. Rudolf A, Schirwis E, Giordani L, et al. β-Catenin Activation in Muscle Progenitor Cells Regulates Tissue Repair. *Cell Rep*. 2016;15(6):1277-1290. doi:10.1016/j.celrep.2016.04.022

26. Jones AE, Price FD, Le Grand F, et al. Wnt/β-catenin controls follistatin signalling to regulate satellite cell myogenic potential. 2015. doi:10.1186/s13395-015-0038-6

27. Desguerre I, Mayer M, Leturcq F, Barbet J-P, Gherardi RK, Christov C. Endomysial Fibrosis in Duchenne Muscular Dystrophy: A Marker of Poor Outcome Associated With Macrophage Alternative Activation. *J Neuropathol Exp Neurol*. 2009;68(7):762-773. doi:10.1097/NEN.0b013e3181aa31c2

28. Biressi S, Miyabara EH, Gopinath SD, Carlig PMM, Rando TA. A Wnt-TGFβ2 axis induces a fibrogenic program in muscle stem cells from dystrophic mice. *Sci Transl Med*. 2014;6(267):267ra176. doi:10.1126/scitranslmed.3008411