THE EFFECT OF ELECTRICAL STIMULATION ON AGED SKELETAL MUSCLE REGENERATIVE POTENTIAL

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Increasing age typically results in a decreased skeletal muscle regenerative capacity after injury, a decreased muscle mass and weakness, ultimately resulting in an increased likelihood for falls, susceptibility to recurrent injury, and a prolonged recovery. In response to injury, aged muscle displays a decreased capacity to regenerate damaged myofibers. Instead, the repair response is characterized by fibrotic accumulation, a response that has been attributed to dysfunction of the muscle stem cell (MuSC) population. MuSCs are a reserve cell population that plays a central role in dictating muscle regeneration. Muscle contractile activity, elicited by exercise or electrical stimulation, for example, has been suggested to be an important intervention to increase blood flow to the injury area and to promote the activation of muscle MuSCs. The purpose of this study is to test the hypothesis that neuromuscular electrical stimulation (Estim) inhibits activation of the muscle progenitor cell (MPC) fibrogenic molecular program to enhance skeletal muscle

regeneration in aged animals. MPCs represent a heterogenous myogenic cell population, the majority of which are MuSCs. Our *in vivo* studies demonstrate that two weeks of Estim enhances myofiber regeneration and increases tetanic force recovery in aged muscles after an acute injury. *In vitro*, we find that improved muscle regeneration in aged muscle following the application of Estim is concomitant with a rejuvenated MPC phenotype. Our results further suggest that the beneficial effect of Estim on MPC regenerative potential may be mediated by up-regulation of the anti-aging protein, Klotho. Taken together, these data provide evidence that Estim is a safe and effective method and to improve functional recovery after an acute injury in aged muscle. It is anticipated that findings from this study will aid in the development of clinically relevant rehabilitation programs to enhance the muscle healing response in a geriatric population.

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PREFACE

After these long and intense years of doctoral training, I learned one important lesson: that I was never alone during this journey. I had the opportunity to meet and work with people from different countries, with diverse personalities and thoughts. I learned a lot. I was disappointed many times. But, in general, the happy moments far outnumbered the sad moments. During this seemingly infinite road, several people walked with me and helped to make this dream come true. For this reason, I would like to say 'thank you' to each of you for your kindness, motivation, and love.

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Thank you God for being a lamp unto my feet and a light unto my path.

1.0 INTRODUCTION

1.1 STATEMENT OF THE PROBLEM

Age-related skeletal muscle weakness and impairment in regenerative capacity following injury is a major contributor to declines in functional mobility and is associated with an increased morbidity in an elderly population (1-4). Muscle wasting and weakness resulting from sarcopenia can increase the risk for subsequent injury, leading to dramatic declines in mobility that may ultimately deprive a person of functional independence (1, 5). In addition to sarcopenia—an age-related decrease in skeletal muscle mass—the capability of skeletal muscle to repair itself after injury is typically impaired, and functional recovery is often impeded by fibrotic tissue formation (1). As the number of elderly individuals in the United States grows at an escalating rate, such detriments increasingly represent an important public health burden. For an elderly individual who is already weak, a failed regenerative response following an injury may mean the difference between independence and going to a nursing home.

Age-related impairment- and functional-level declines are a clear manifestation of underlying tissue and cellular dysfunction. After muscle injury, muscle stem (satellite) cells (MuSCs) exit their normally quiescent state, proliferate, and fuse to form new myofibers. However, there is a decline in the activation, proliferation, and differentiation of MuSCs over time (6-8).

An increased understanding of the cellular signaling pathways responsible for regenerative decline in aged muscle is of considerable interest toward the development of clinical programs designed to counteract the effect of age on skeletal muscle healing capacity.

Recent studies have demonstrated that MuSC behavior, and, ultimately, skeletal muscle regenerative capacity is largely dependent on characteristics of the microenvironment, or niche. Data from *in vitro* studies have demonstrated that, when MuSCs are isolated from aged muscle and cultured in serum obtained from young animals, MuSCs demonstrate an enhanced myogenicity (9). Conversely, when MuSCs isolated from young muscle are cultured in the presence of serum derived from aged animals, MuSCs demonstrate an increased fibrogenic fate (10). These data suggest that systemic factors play an important role in the functional capacity of MuSCs, further implicating the microenvironment as a target for the development of approaches to enhance aged muscle regeneration.

Modulation of the microenvironment through muscle loading—via endurance exercise training, for example—has been shown to be beneficial for skeletal muscle adaptations such as changes in muscle fiber type and fiber area, angiogenesis, and the secretion of myogenically favorable growth factors (11, 12). Evidence further suggests that mechanical stimuli via downhill treadmill running promotes MuSCs myogenicity and may be an effective and practical intervention strategy to prevent and/or reverse the cellular and molecular alterations associated with impaired repair of aged muscle (13). However, little is known about the ability of muscle contractile activity to reverse the effect of age on declines in muscle regenerative capacity. A better mechanistic understanding of how muscle loading may optimize MuSC functioning is an essential step in the development of rehabilitation approaches to prevent and/or improve muscle healing in aged population.

1.2 BACKGROUND

1.2.1 Skeletal muscle regeneration

Skeletal muscle injuries are a common problem in trauma and orthopedic surgery. In cases of relatively minor injuries, young healthy skeletal muscle possesses a great ability to fully recover the architecture and contractile function (14). The regeneration of skeletal muscle after injury comprises several coordinated and overlapping phases, including (1) degeneration, (2) inflammation, (3) regeneration and, finally, (4) fibrosis (15).

The Degenerative Phase

The degenerative phase of the muscle repair process starts in the first few days post-injury. It is characterized by disruption of sarcolemma, which results in an influx of calcium and cell death (10). Increased vascular permeability and blood flow results in hematoma formation and edema accumulation (16). Within a few hours after injury, inflammatory cells (leukocytes, monocyte-lymphocyte, and macrophages) invade to the injury area. Macrophages initiate phagocytosis of the injured region, resulting in a “ghost-like” and “moth-eaten” appearance of the muscle fiber, (17). These ghost fibers are important to guide and recruit the regenerating cells into position (18). In addition, degrading myofibers become denervated through destruction of intramuscular nerve branches (19).

The Inflammatory Phase

The inflammatory phase generally peaks within 24-48 hours after injury. During the inflammatory phase, macrophages and neutrophils are activated to eliminate the necrotic tissue and prepare the tissue for the regeneration phase. During this phase, growth factors are released, which affect the regenerative microenvironment and contribute to the symptomatology of muscle injuries, including pain and swelling (20). Inflammation is a critical step in the regenerative process, and studies have demonstrated that inhibition of the inflammatory phase, through non-steroidal anti-inflammatory drugs (NSAIDs) administration, for example, dramatically impairs muscle-healing (20, 21). Though studies using NSAIDS demonstrate a decrease in inflammation and pain shortly after muscle injury, investigators have observed long-term residual deficits in muscle functional capacity and muscle healing (21). Clearly, prostaglandins play a well-established role in dictating MuSC proliferation and regenerative potential, and their inhibition is problematic for effective muscle healing (22, 23).

It is hypothesized that the interruption of growth factor secretion associated with inhibition of inflammation, such as Insulin-like Growth Factor-1 (IGF-1), basic Fibroblast Growth Factor (bFGF), Hepatocyte Growth Factor (HGF), Epidermal Growth Factor (EGF), and Transforming Growth Factor Beta-1 (TGF-β1), contributes to the regenerative defect following NSAIDs administration (20). However, supplementation with growth factors, including IGF-1, bFGF, and nerve growth factor (NGF) has been shown to dramatically enhance the healing process after muscle injury (24, 25). These growth factors have also been shown to play a critical role in regulating muscle stem cell (MuSC) proliferation and differentiation during regeneration (26)

The Regenerative Phase

The regenerative phase is subsequently triggered by growth factor secretion. The primary cells regulating the skeletal muscle regenerative response are MuSCs. MuSCs are located between the basal lamina and the plasma membrane, and normally reside in a quiescent state (27)(Figure1). Upon activation, MuSCs enter a phase of proliferative expansion. A critical aspect of MuSC activation is that a portion of the activated MuSCs will return to a quiescent state, a process called "self renewal". Self-renewal is critical for maintenance of the stem cell reservoir, such that a pool of stem cells is available for future rounds of regeneration. Another portion of the activated cells will fuse to the existing damaged myofibers, or will fuse to produce nascent myofibers. The regenerating myofiber is characterized by centralized nuclei, which eventually migrate to the myofiber periphery. The regenerative phase typically peaks within 2 weeks after injury, and then gradually decreases.

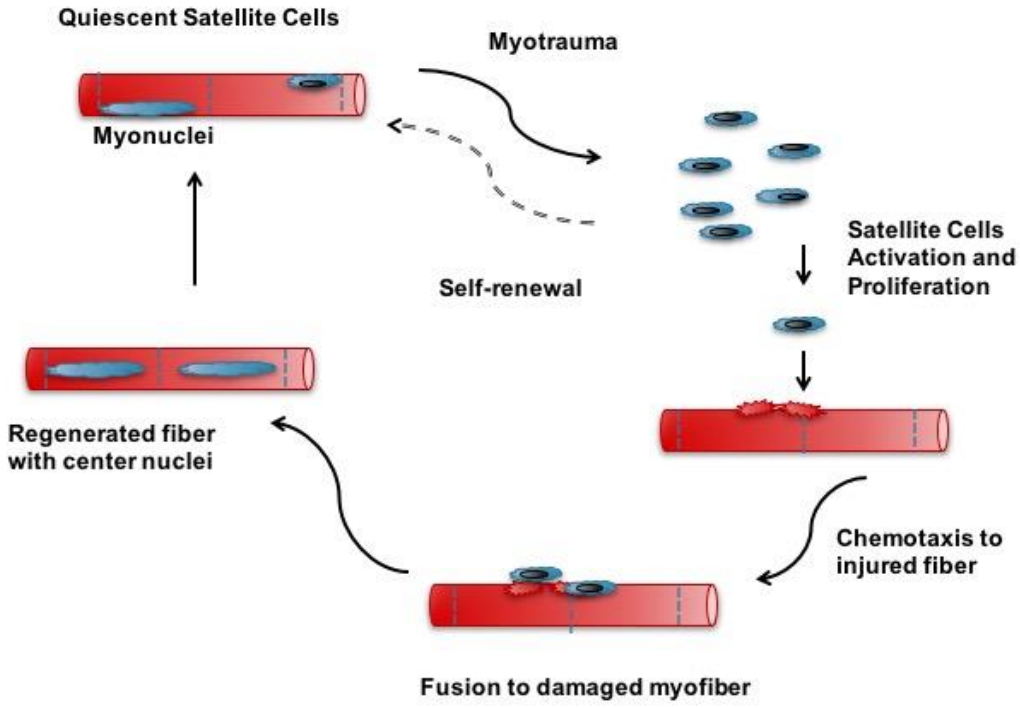

Figure 1. MuSC responses after myotrauma.

The Fibrotic Phase

In cases of severe injury or when the regeneration process fails, such as with disease or aging, the formation of fibrosis is initiated. Fibrosis serves to fill in the gap and minimize further injury to the tissue. Fibrosis generally begins between the second and third weeks post injury (15). Although this scar tissue provides early support for the injury area, it becomes progressively dense over time, and ultimately may restrict the formation of regenerating myofibers (16). Transforming Growth Factor Beta-1 (TGF-β1) is considered to be a "master regulator" of fibrosis, and plays a role in the differentiation of myoblasts into a myofibroblast lineage (28). Several studies have demonstrated that inhibition of TGF-β1 promotes an enhanced myofiber regeneration and muscle recovery (24, 29, 30). Sato and colleagues demonstrated that a combination treatment of IGF-1 and Decorin (a TGF-β1 antagonist), administered 1, 3, or 5 days post-laceration, improved both histological and contractile characteristics of skeletal muscle 4 weeks after injury (24). Another study demonstrated that scar tissue formation after muscle laceration was effectively inhibited with the use of Suramin, another TGF-β1 inhibitor (31). These are just a few of the several existing studies demonstrating that inhibition of skeletal muscle fibrosis significantly improves functional regeneration after a severe muscle injury.

Vascularization and reinnervation of injured muscle

Most traumatic muscle injuries will also involve vascular and neural tissues. As such, the importance of restoration of these structures after an acute injury cannot be discounted. Both nerves and vessels have been shown to play a critical role in the regenerative cascade and

maturation of myotubes into myofibers (32). Nascent capillaries are necessary to provide adequate oxygen supply to area of injury area, subsequently supporting aerobic energy metabolism for the regenerating myofibers. Christov and colleagues (2007) observed a tight relationship between MuSCs and capillaries, and found that most resident MuSCs are juxtaposed with capillaries (33). In cases of myopathy, which display a decrease in myofiber capillarization, there is a concomitant decrease in the number of MuSCs per myofiber (34). Conversely, the skeletal muscle of healthy athletes displays an increased myofiber capillarization that is strongly correlated with an increased number of MuSCs (33).

Finally, another important aspect of muscle regeneration is the myofiber reinnervation. Reinnervation is required both to prevent myofiber atrophy and restore functional capacity (15). Indeed, non-innervated regenerating fibers remain both functionally and histochemically immature (33). During regeneration, the neuromuscular junction is a tightly regulated and consists of two principal phases of repair. First, the ingrowth of nerves is stimulated by either outgrowth from the cut ends of nerves leading to the muscle, or by the sprouting of nerves into regenerating fibers (33). In the second phase, the regenerating nerves form functional neuromuscular junctions (33). Ingrowing nerve fibers have a strong predilection for settling down at the sites of previous neuromuscular junction (35).

1.2.1.1 Aged-related declines in skeletal muscle regeneration

Like most tissues, aging results in a dramatic decrease in skeletal muscle regenerative capacity, and the regenerative response becomes characterized by an increased fibrosis and adipose tissue accumulation (8). In addition, aging has been associated with a decrease in skeletal muscle

capillarity, owing, at least in part, to decreased expression of Vascular Endothelial Growth Factor (VEGF) levels, important for both for angiogenesis and for guiding MuSC responses (36).

As described above, the ability of skeletal muscle to repair itself after injury is largely dependent on the action of MuSCs. However, with aging, a multitude of compromised MuSC responses ensue. Almost four decades ago, Snow (1977) observed a dramatic decrease in the number of MuSCs in aged skeletal muscle, when compared to young adult counterparts (37). This is consistent with more recent studies demonstrating that aged MuSCs demonstrate a dramatically decreased proliferative potential (38). In addition, MuSCs derived from aged skeletal muscle display a predisposition to differentiate toward a fibrogenic lineage, as opposed to the desirable myogenic cell fate (39). It is this myogenic-to-fibrogenic conversion that is hypothesized to underlie the increased fibrosis formation following injury in aged skeletal muscle. Taken together, these age-related alterations lead to a dramatically impaired regenerative capacity and functional recovery after a severe muscle injury (40).

1.2.1.2 The molecular basis for age-related declines in skeletal muscle regeneration after injury

Considerable progress was observed in the last decades in regards to our understanding of the molecular mechanisms underlying an age-related reduction in MuSCs function. There are many signaling pathways that have been implicated as playing major role in driving in age-related declines in muscle regenerative capacity. For example, Mitogen-Activated Protein Kinase (MAPK) is an important pathway that is stimulated in response to cytokines, growth factors, and cellular stress (41, 42). A recent study by Cosgrove et al (2014) demonstrated that aged MuSCs display an elevated p38α/β MAPK activity, but that small molecule inhibition of p38α/β MAPK inhibits

MuSCs senescence (33). Importantly, the beneficial effect of p38α/β MAPK inhibition was only observed when cells were cultured in a soft matrix substrate. These findings suggest that both biochemical and biophysical cues are important for defining MuSC behavior.

Notch signaling, important for MuSCs activation, proliferation, and cell lineage specification, is another important pathway that has been shown to be dysregulated with increasing age. Decreased Notch activation contributes to the diminished regenerative capacity of aged muscle through an impaired MuSC proliferative capacity (8, 9). The progression of MuSCs from a phase of proliferative expansion to one of terminal differentiation is reliant on inhibition of the Notch signaling pathway by activation of the Wnt signaling pathway (33).

Activation of the canonical Wnt signaling pathway has been suggested to be essential for dictating MuSC fate (43-45). The canonical Wnt signaling cascade is activated when soluble Wnt ligands interact with Frizzle receptors as well as low-density lipoprotein receptor-related protein co-receptors (LRP) at the cell surface. Frizzle receptor activation subsequently stimulates the phosphorylation of Disheveled and inactivates GSK3β phosphorylation of β-catenin. These events lead to inhibition of Axin-mediated β-catenin phosphorylation, leading to the stabilization of β-catenin, which then accumulates and translocates to the nucleus to form complexes with TCF/LEF for the activation of Wnt target gene expression (Figure 2).

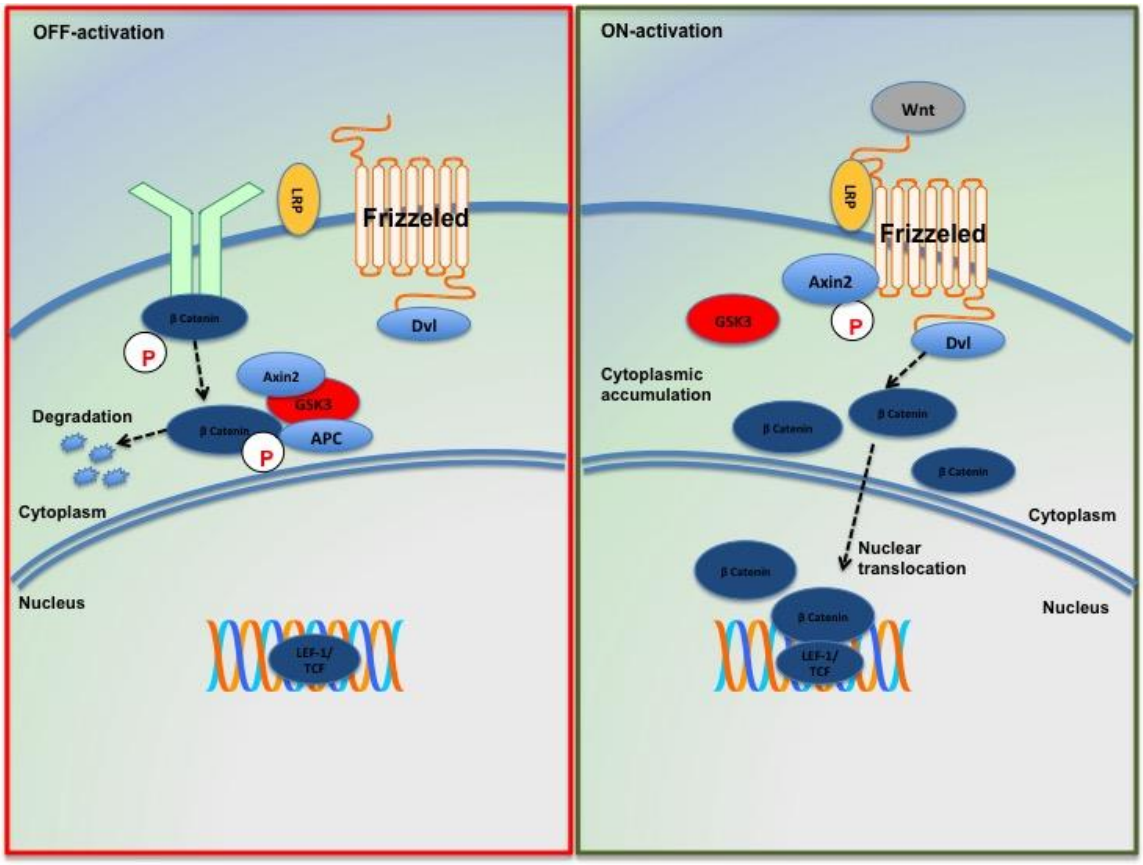

Figure 2. Canonical Wnt signaling pathway

Schematic representation of the canonical Wnt/β-Catenin pathway. Off activation (left): In the absence of Wnt signal, β-Catenin is recruited into the APC/Axin2/GSK3 complex and phosphorylated by GSK3. As the result, β-Catenin is degraded, and β-Catenin fails to enter the nucleus. **On Activation (right):** Wnt binds the Frizzeled receptor and LRP co-receptor. Next, activated Disheveled leads to the inhibition of APC/Axin2/GSK3. Accumulated β-Catenin translocates to the nucleus and complexes with TCF/LEF, activating the canonical Wnt-target gene expression.

Recently, Murphy and colleagues demonstrated that, though Wnt signaling/β-catenin pathway is active in amplifying myoblasts, silencing of this pathway is absolutely critical for effective regeneration (46). Accordingly, with aging, MuSCs demonstrate an excessive activation of the Wnt signaling pathway, which has been shown to ultimately result in a dysfunctional myogenic capability and enhanced fibrosis deposition at the injury site (7). The ligands contributing to this

increased Wnt signaling activation in aged muscle have yet to be elucidated, but are believed to be present in the circulation (47).

1.2.1.3 Modulation of the microenvironment and its role in aged-related declines in muscle regeneration

It is clear that the microenvironment, or niche, is a potent regulator of MuSC regenerative potential within skeletal muscle. The niche is necessary for the maintenance of MuSC quiescence and it provides critical support of tissue homeostasis and regenerative capacity (32). In addition, the niche includes vascular components that are important to deliver homeostatic signals to MuSCs, such as secreted molecules and cellular contacts (48).

Indeed, modulation of microenvironment has the potential to dramatically alter the behavior of aged MuSCs. Studies have demonstrated that when aged MuSCs are exposed to a youthful microenvironment, regenerative potential is dramatically improved to levels comparable to young counterparts (7, 9). Moreover, this enhanced MuSC behavior is concomitant with an improved healing response (9, 39, 49). Carlson & Faulkner performed a series of experiments in which the extensor digitorum longus (EDL) muscle was heterochronically transplanted between young and old mice (ie. young muscle was transplanted into old hosts and old muscle was transplanted into young hosts) (49). The authors observed a significant improvement in contractile force and regeneration when old muscles were transplanted into young hosts (49). Conversely, both young and old muscles grafted in old hosts resulted in a dramatically decreased regeneration and force producing capacity of the transplanted muscle (33). Authors concluded that the poor regeneration of muscles in old animals may be related to an unsupportive microenvironment.

More recently, Brack et al demonstrated that MuSCs exposed *in vitro* to serum derived from young mice display a decreased Wnt signaling activation (as evidenced by an increased GSK3β, decreased Axin 2 and increased nuclear β-Catenin), as well as a concomitant increase in myogenic differentiation (7). Conversely, MuSCs exposed to old mouse serum display an increased Wnt signaling and increased fibrogenic conversion. The findings suggest that some serum component that is upregulated in the circulation of aged animals may be responsible for excessive Wnt signaling activation. Conversely, it is possible that some serum component found within young animals may be responsible for inhibition of the Wnt signaling cascade.

Translating these *in vitro* findings to an *in vivo* model, investigators next turned to a heterochronic parabiotic approach (7). In this approach, aged animals are surgically joined with young animals, resulting in a chimeric circulatory system. Consistent with *in vitro* findings where aged MuSCs exposed to young serum display an enhanced myogenicity, *in vivo* studies demonstrated decreased fibrosis and improved myogenesis of aged heterochronic parabionts. Interestingly, exposure of aged mice to a young circulation was concomitant with an increased Notch signaling (49) and a decreased Wnt signaling activation (7). Findings were further confirmed by Wagers *et al*. who similarly demonstrated the ability of heterochronic parabiosis to improve muscle healing in aged mice (33). In their study, Wagers and colleagues suggested that the beneficial effect of exposure of aged mice to a youthful circulation was owing to the circulating protein Growth Differentiation Factor 11 (GDF11) (33). Specifically, they found that mice displayed a decrease in circulating GDF11 with increasing age, but that supplementation with GDF11 in aged mice resulted in significantly improved muscle structure after injury, as well as an improved capacity for endurance exercise (50). These findings were recently refuted by Egerman et al., who observed that GDF11, a part of the TGF-β1 superfamily, actually increases in the

circulation of aged mice (51). Moreover, they found that GDF11 significantly inhibits MuSC proliferation and promotes fibrosis formation after injury (52).

Despite the ongoing debate, it is clear that systemic factors play a critical role in supporting MuSC regenerative potential and skeletal muscle healing (7, 9). Though the circulating factor(s) responsible for mediating the beneficial effect of exposure to a youthful systemic environment is/are still unknown, heterochronic parabiosis studies confirm that MuSCs display downstream, autonomous alterations that support a rejuvenated phenotype in aged muscle. Elucidation of the circulating protein(s) that is (are) modulated through the aging process would, therefore, be a major advance toward the development of approaches to enhance skeletal muscle regenerative capacity in an aged population.

1.2.2 Klotho as a mediator of tissue regeneration and stem cell functionality

Klotho is circulating hormone that has been extensively investigated as playing an important role in regulating aged-declines. Klotho is an anti-aging protein predominantly produced in the kidney, but can be found small amounts in the brain, placenta, skeletal muscle, urinary bladder, aorta, colon, and pancreas (53-56). It serves as an aging suppressor protein through a variety of mechanisms, including anti-oxidation, anti-senescence, anti-autophagy, as well as modulation of signaling pathways associated with longevity, including Insulin Growth Factor (IGF) (53). Klotho has been found in the circulatory system of both animals and humans (57, 58), although it's serum concentration gradually declines with increasing age (57, 58). Mice deficient for Klotho display premature aging, and die at proximally 8-9 weeks of age (the typical lifespan of wild type mice is

approximately 2.5-3 years) (54, 58). This decreased longevity is consistent with decreased physical activity, ectopic calcification, pulmonary emphysema, osteoporisis, atrophy of skin, intestine, lipodystrophy (54), impaired wound repair process (59), and chronic inflammation in many age-related chronic disease, such as kidney disease, degenerative disease, diabetes, arteriosclerosis, skeletal muscle wasting, and hypertension (55, 60-64). Genetic up-regulation of Klotho, on the other hand, markedly delays age-related functional declines in various tissues and extends lifespan to levels ~31% greater than wild type counterparts (65, 66).

Among the aged phenotypes associated with a decreased expression of Klotho is an impaired tissue healing response. A recent study by Liu and colleagues (2007) (58), reported that impaired skin and small intestinal regenerative response in Klotho knockout mice is associated with a decreased stem cell number, proliferation and resistance to stress, as well as an impaired angiogenesis (58). However, supplementation with Klotho rescues epithelial stem cell function to improve tissue repair and suppress fibrosis (67). Interestingly, Liu and colleagues (2007) observed that the Klotho protein binds to multiple Wnt ligands and inhibits the biological activity of Wnt proteins to enhance stem cell function (58). *Do age-related declines in circulating Klotho similarly play a role in the fibrogenic conversion of MuSCs through a de-repression of Wnt signaling activation?*

1.2.3 Modulation of the skeletal muscle microenvironment through mechanical stimulation

Exercise is a commonly implemented intervention to promote muscle repair in aged muscle and to stimulate the activation of molecular mechanisms critical for functional muscle repair (68), and

emerging evidence indicates that the application of mechanical stimuli increases MuSC activation and proliferation (69). Upon muscle loading, muscle produces chemical signals that may be essential for MuSC behavior and myogenicity (70). Studies have demonstrated that resistance training and treadmill exercise elevate MuSC numbers and increase myogenin (a transcription factor associated with myogenesis) levels in aged skeletal muscle (71-73). Mackey and collaborators (2007) reported that resistance training, performed 3 times a week for 12 weeks, was effective for enhancing the MuSC pool in the skeletal muscle of health elderly men and women (74). In addition, resistance training restores the proliferative capacity of MuSCs isolated from aged individuals to levels comparable to younger counterparts (75). Similar results were found in a study by Dreyer et al, in which investigators demonstrated a significantly increased number of MuSCs within 24 hours after maximal eccentric knee extensor exercise in both young and older men, though the older men displayed an attenuated response (76). Accordingly, Shefer and colleagues (2010) observed a significant increase in the number of satellite cells/myofiber after completion of a running protocol (71). Importantly, mechanical loading upregulates the gene expression of angiogenic factors (77, 78), induces growth factor secretion (79), increases neural recruitment (33), increases blood flow and increases leukocyte and monocyte infiltration to the injury area (80), all of which are critical for adequate regeneration.

Although, there are many studies demonstrating an increased number, activation, proliferation and differentiation of MuSCs, as well as an increased MuSC myogenicity and angiogenesis, the molecular mechanisms underlying these alterations remain poorly understood. One study suggested that application of a high-intensity exercise protocol to mice of varying different ages increases Wnt signaling activation in MuSCs (81, 82). Sakamoto and colleagues (2004) performed a study that included eight healthy human subjects. Subjects were submitted to

30 minutes of cycling exercise at both 75% of maximum intensity and 125% maximum intensity. Investigators found that exercise, both at submaximal and maximal intensities, deactivated GSK3 and decreased phosphorylation of β-catenin in MuSCS (83). Another study by Armstrong and collaborators described that mechanical overloading of the plantaris muscle through bilateral synergist ablation significantly increased in Wnt/β-catenin pathway in plantaris muscle of young animals. Finally, Fujimaki *et al.* (2014) performed voluntary wheel running in adult and aging mice for 4 weeks and observed that voluntary wheel running induced the activation of canonical Wnt/β-catenin signaling pathway. Unlike observations by Brack *et al.* (2007), in these cases, increased Wnt signaling was not associated with increased fibrosis formation, even in aged skeletal muscle (84). Taken together these data confirm that mechanical loading is a potent regulator of the Wnt/β-catenin signaling pathway. However, the mechanisms underlying these effects remain unresolved.

Recently, Avin and colleagues (2014) proposed the intriguing possibility that Klotho might be regulated in response to skeletal muscle contractile activity (85). Though a direct relationship between muscle activity and Klotho expression has never been investigated, a number of studies support the hypothesis that there is a tight relationship between skeletal muscle activity and Klotho expression (reviewed in (85)). Phelps and colleagues evaluated grip strength and running endurance between Klotho-deficient mice, transgenic Klotho overexpressing mice (EFmKL46), and wild-type control mice. They observed Klotho-deficient mice displayed ~50% less force, when compared to both Klotho overexpressing and wild-type control mice. Furthermore, Klotho deficient mice also presented ~60% less endurance capacity when compared to EFmKL46 and control mice (33). These murine observations have clinical correlates, and it has been shown through epidemiological studies that decreased circulating Klotho is associated with decreased muscle strength (86).

The emerging evidence that mechanical stimuli promotes MuSC myogenicity, potentially through regulation of Wnt signaling activation and/or Klotho expression, provides support for the hypothesis that muscle contractile activity may be an effective and practical intervention strategy to reverse the effect of age on cellular and molecular mechanisms associated with an impaired regenerative response after injury.

1.2.4 Electrical stimulation as a viable modality to promote MuSC myogenicity and counteract muscle regenerative deficits in aged animals

Electrical Stimulation (Estim) is a modality that has been adopted in sports medicine and in rehabilitation settings as a complement and/or substitute to voluntary strength training (87). Studies have demonstrated that Estim may be used to recover contractile function, prevent atrophy associated with disuse of the skeletal muscle, and stimulate MuSC activation (88). Moreover, Estim effectively induces changes in the skeletal muscle microenvironment and, specifically, promotes skeletal muscle angiogenesis and the release of growth factors (89, 90). One study demonstrated that 5, 10, or 20 days of chronic low-frequency stimulation increased MuSC content in hypothyroidic muscles, suggesting that it may be an effective modality for enhancing activation, proliferation, and/or the fusion of MuSCs (91). Additionally, five days of Estim increases VEGF secretion and blood flow within the muscle in a rat model ischemia (77), further confirming the ability of Estim to be a potent modulator of the skeletal muscle microenvironment.

Caggiano and colleagues compared the training effects of electrical stimulation and voluntary isometric contraction protocol (traditional exercise on the quadriceps strength) of 65 year old males (92). They observed that Estim had the same capacity to increase torque as

traditional exercise in older males. At molecular level, Kern et al, demonstrated that 9 weeks of Estim administered to bilateral anterior quadriceps in old sedentary males increased the expression biomarkers of activated MuSCs and myoblasts, such as IGF-1, and promoted the remodeling of myofibers and extracellular matrix (ECM) (93). These results suggest that Estim attenuates the functional decline associated with aging, and improves muscle strength and mass. Moreover, Guo et al. demonstrated that Estim provides an effective stimulus to prevent the loss of myonuclei and MuSCs in models of disuse muscle atrophy (88). Although several studies have demonstrated and elucidated skeletal muscle responses to Estim (94-97), the ability of Estim to modulate the aged skeletal muscle microenvironment and promote a rejuvenated MuSC phenotype is unknown. We hypothesize that targeted muscle contractile activity, elicited by Estim, reverses the age-related decline in skeletal muscle regenerative capacity at the tissue, cellular and molecular levels (Figure. 3)

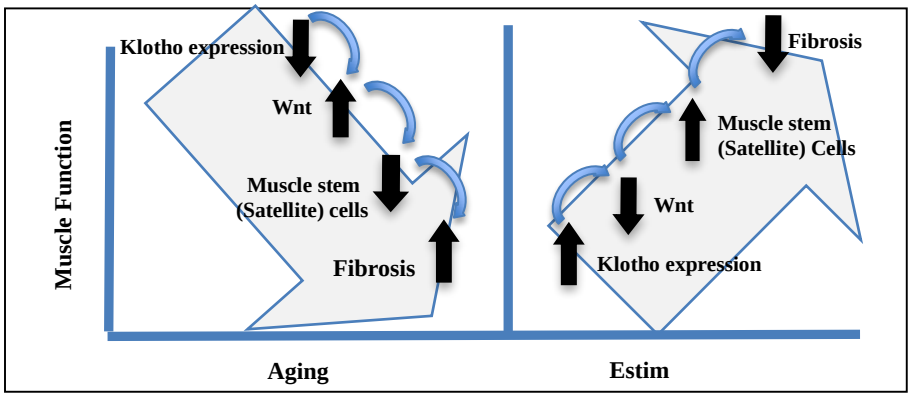

Figure 3. Hypothesis schematic

Figure 3. Decreased muscle function that typically follows the aging process (left). We hypothesize that Estim reverses age-related declines in muscle stem (satellite) cell (MuSC) regenerative potential, and, ultimately, muscle function.

1.3 SIGNIFICANCE

In an elderly population, muscle weakness and atrophy occurs soon after the onset of decreased mobility, and previous studies have shown that lower extremity strength is an important determinant of fall risk (98, 99). Targeted interventions to prevent such a devastating cycle have the potential to hasten an elderly individual's return to previous level of functioning. We anticipate that completion of these studies will serve as an important first step in the development of therapies designed to counteract and/or reverse the effect of age on skeletal muscle regeneration after injury. An improved understanding of molecular and cellular mechanisms controlling tissue-healing response may reveal novel pharmacological targets to increase enhance skeletal muscle functional capacity in an aged population.

We propose that muscle contractile activity is an effective tool for modulating skeletal muscle regenerative potential, and the expansion of these studies will be important steps towards the development of targeted treatment interventions to prevent muscle wasting and weakness following periods of prolonged immobility, such as after a severe muscle injury, following an intensive care unit stay, or post-operatively. In addition, we propose that Estim that may be a beneficial modality to rejuvenate the aged skeletal muscle environment *prior* to undergoing an elective surgical procedure that involves significant muscle trauma, such as a total hip arthroplasty or spinal surgery. Such procedures result in considerable muscle damage, which may lead to decreased tissue blood flow, muscle atrophy, and decreased muscle strength (100); the resulting degenerative changes are oftentimes long-lasting (101). We propose that pre-operative treatment with Estim may better position aged skeletal muscle for an accelerated regenerative response post-operatively, ultimately contributing to a faster return to functional mobility.

1.4 INNOVATION

This study is innovative because it implements a clinically-relevant and cost-effective modality, Estim, as a means to target MuSC responses and promote enhanced regeneration of aged skeletal muscle. Although Estim has been investigated in many studies in the context of muscle hypertrophy and healing, further investigation is needed to analyze the underlying mechanisms by which Estim may modulate MuSC behavior in aged muscle.

This research is also innovative because it integrates basic science principles with rehabilitation approaches, an integration that may aid in the development of targeted protocols designed to enhance muscle healing in a geriatric population. This project will allow us to discriminate the effects of mechanical loading at the molecular, cellular, histological and functional levels with the goal of better understanding how mechanical stimulation may attenuate, prevent or reverse muscle impairments in a geriatric population.

1.5 AIMS AND HYPOTHESIS

The overall objective of this work is to investigate the ability of Estim to reverse the effect of age on skeletal muscle healing process. To test our hypothesis that Estim may reverse age-related declines in myofiber regeneration after injury, Specific Aim 1 will examine histological evidence of muscle regeneration, and physiological strength recovery among Young Control (YC), Young Injury (YI), Aged Control (AC), Aged Injury (AI), and Aged Estim (AEI) groups.

In Specific Aim 2, we will analyze the effect of Estim on *in vitro* muscle progenitor cell (MPC) characteristics, including myogenic differentiation and activation of the myogenic molecular program. For *in vitro* characteristics, MPCs from all experimental groups, Young Control (YC), Aged Control (AC), and Aged Estim (AE), will be isolated in order to investigate the effect of Estim on cellular terminal differentiation and senescence. Moreover, we will employ a gain- and loss-of function paradigm to test our hypothesis that the beneficial effect of Estim on MPC myogenicity is a result of inhibition of the Wnt signaling cascade by Klotho.

1.5.1 Specific aims and hypothesis of study 1

To determine the ability of Estim to improve the regeneration and functional recovery of aged skeletal muscle after acute muscle injury.

Hypothesis: Estim will improve myofiber regeneration (as evidenced by the number and size of regenerating fibers), and decrease fibrosis formation (as evidenced by a decreased collagen deposition) after injury in aged skeletal muscle. This enhanced tissue regeneration will be associated with a significantly enhanced functional recovery (as determined by *in situ* contractile testing).

1.5.2 Specific aim and hypothesis of study 2

To determine the ability of Estim to rejuvenate the regenerative potential of MuSCs through a Klotho-mediated inhibition of Wnt signaling

Hypothesis: Estim will increase the viability (as evidenced by an enhanced metabolism and senescence) and myogenic capacity (as evidenced by the expression of myogenic marker, desmin) of aged MPCs. These alterations will be associated with a down-regulation of Wnt signaling activation, as evidenced by a decreased expression of nuclear β-catenin and increased expression of GSK3. Finally, we hypothesize that Estim induces an increase in local Klotho expression, thereby inhibiting Wnt signaling activation in aged MPCs.

2.0 NEUROMUSCULAR ELECTRICAL STIMULATION REJUVENATES THE REGENERATIVE POTENTIAL OF AGED MUSCLE PROGENITOR CELLS

2.1 SUMMARY

Increasing age typically results in a decreased skeletal muscle regenerative capacity and increased fibrosis after injury, ultimately contributing to a weakness and declines in physical functioning. With age, stem cells display cell-autonomous alterations that predispose the muscle to an impaired regenerative potential. However, many of these age-related changes have been shown to be reversible through rejuvenation of aged stem cell responses. Here, we test the hypothesis that mechanical stimulation via neuromuscular electrical stimulation (Estim) boosts the regenerative potential of aged muscle progenitor cells (MPCs) and improves skeletal muscle regeneration after injury. Estim is an attractive modality since it has been shown to enhance secretion of myogenic and angiogenic growth factors, as well as inhibit fibrogenic pathways. Our results demonstrate that two weeks of Estim enhances myofiber regeneration and increases tetanic force recovery in aged muscles after an acute injury. Improved muscle regeneration in aged muscle following the application of Estim is concomitant with a rejuvenated muscle progenitor cell (MPC) phenotype, as demonstrated by a decreased activation of the pro-fibrogenic pathway, Wnt signaling, as well as a decreased senescence. Finally, our results suggest that the beneficial effect of Estim on MPC regenerative potential is concomitant with an up-regulation of the anti-aging protein, Klotho. Taken together, these data provide evidence that Estim may be a great mechanical stimulus to improve

functional recovery after an acute injury in aged muscle and activate important pathways to modulate the anti-aging declines.

2.2 INTRODUCTION

Age-related skeletal muscle weakness and impairment in regenerative capacity following damage is a major contributor to declines in functional mobility and is associated with an increased morbidity in an elderly population (1). After injury, the skeletal muscle of aged individuals demonstrates increased skeletal muscle fibrosis formation and adipose tissue accumulation (8, 15, 24). These are concomitant with an impaired myofiber regeneration and subsequent decrease in muscle contractility (103-106). A better understanding of the cellular mechanisms controlling age-related skeletal muscle dysfunction will aid in the development of clinically relevant approaches to address this significant problem.

The ability of skeletal muscle to repair itself after injury is largely dependent on the response of muscle stem (satellite) cells (MuSCs). After injury, MuSCs are activated to become myoblasts, which subsequently fuse to form myofibers (107). However, aging typically culminates in a multitude of compromised MuSC responses. Several decades ago, it was proposed that a dramatic decrease in the absolute number of MuSCs in aged skeletal muscle may underlie the observed age-related impairment of healing after injury (37). More recently, studies have demonstrated that autonomous defects within the resident MuSC population may primarily explain the deficit in muscle regeneration of aged muscle. Aged MuSCs display an impaired ability to respond to an injury stimulus, owing in large part to a reduced capacity for myogenic

differentiation (108). Specifically, aging disrupts MuSC lineage specification, as demonstrated by a myogenic-to-fibrogenic conversion (7, 39).

The skeletal muscle microenvironment is an important regulator of MuSC function. Studies using heterochronic parabiosis, in which the circulatory systems of young and aged animals are surgically joined, have demonstrated that exposure of aged MuSCs to a young microenvironment restores myogenic capacity to youthful levels. This enhanced MuSC behavior is concomitant with an improved healing response after an acute injury (9, 39, 49). Importantly, it has been shown that the improved muscle healing of aged parabiotic partners is not the result of a physical contribution of the young cells within the circulation (109), suggesting that systemic niche factors play a critical role in dictating MuSC behavior. *In vitro* models revealed that, when MuSCs were exposed to mouse serum derived from aged mice, young MuSCs display an increased fibrogenic conversion. This increased fibrogenic conversion was further associated with an increased Wnt signaling activation. Whereas canonical Wnt signaling is typically activated in young healthy muscle (43-45), aged MuSCs demonstrate an excessive activation of the Wnt signaling pathway, which has been shown to ultimately result in a dysfunctional myogenesis and increased fibrosis at the injury site (7). Intriguingly, when aged MuSCs are exposed to serum derived from young mice, the cells display a decreased Wnt signaling activation and an enhanced myogenic potential(7). These findings suggest that some circulating factor found in young serum may inhibit or functionally neutralize activation of the Wnt signaling cascade.

Similar to the rejuvenating effects of heterochronic parabiosis, physical activity induces a myriad of anti-aging effects, including prevention of muscle wasting, cardiovascular diseases, hypertension and diabetes. There has been mounting evidence to suggest that muscle contractile activity, by exercise or electrical stimulation (Estim), may also enhance muscle progenitor cell

(MPC) regenerative potential (110, 111). In both animals and humans, it has been shown that muscle loading induces the activation and proliferation of MPCs, promotes skeletal muscle angiogenesis, and stimulates the release of myogenically favorable growth factors (reviewed in (90, 112)). In the current study, we tested our hypothesis that skeletal muscle contractile activity mitigates the effect of age on MPC function and skeletal muscle healing capacity. Our results demonstrate that two weeks of Estim restores MPC myogenicity to youthful levels, and that this enhanced cellular functioning is consistent with a decreased Wnt signaling activation and decreased cellular senescence. We further present evidence to suggest that the beneficial effect of Estim on MPC senescence is mediated by an up-regulation of the circulating longevity protein, Klotho. Importantly, the rejuvenating effects of Estim on MPC behavior are concomitant with evidence of enhanced histological and functional recovery two weeks after injury.

2.3 METHODS

2.3.1 Skeletal muscle injury

For the *in vivo* study, a total of forty animals (n=16, 14-16 weeks old mice; n=24, 22-24 months old mice) were divided into the following groups: Young control (YC), Aged Control (AC n=8), Young + Injury (YI), Aged + Injury (AI n=8), and Aged+Estim+Injury (AEI n=8). For the *in vitro* study, a total of fifty animals were divided in 3 groups: (YC n=15), (AI n=20), and (AE n=15). All experiments were performed with prior approval from the Institutional Animal Care and Use Committee of the University of Pittsburgh. Mice were anesthetized with 2% isofluorane,

administered by inhalation during muscle injury, functional contractile testing, and euthanasia. Cardiotoxin (CTX) was intramuscularly injected into bilateral tibialis anterior (TA) muscles in order to induce an acute muscle injury in all experimental groups after 5 sessions of Estim (performed over the course of 10 days). Young Injury and Aged Injury animals that were not been treated with Estim were similarly injured bilaterally.

For the injury, mice were anesthetized with 2% isoflurane (Abbott Laboratories, North Chicago, IL) in 100% O2 gas. The hair was shaved and the skin was cleaned with isopropyl alcohol. CTX from *Naja mossambica mossambica venom* (Sigma C9759) was prepared with phosphate-buffered saline (PBS) at a concentration of 1μg/μl. Ten microliters of CTX were injected into bilateral TAs. Since there was no significant difference in the muscle weights across age groups (Young mice: 58.5±1.3 mg; Aged mice: 58.8±1.5 mg), an equal volume of CTX was injected into all muscles.

2.3.2 Electrical stimulation (Estim) protocol

Neuromuscular electrical stimulation was performed using a Neuromuscular Stimulator (Empi 300 PV, St Paul, USA) and modified surface electrodes, as we previously described (113). Prior to stimulation, the anterior lower limb was shaved and cleaned with alcohol. Location of the peroneal nerve was confirmed when stimulation resulted in a full hindlimb dorsiflexion and digit extension, indicating stimulation of the anterior compartment muscles, including the TA and extensor digitorum longus (EDL) muscles. Estim was performed bilaterally for two sets of ten contractions per session, for a total of five sessions and at least one day of rest between each session. The intensity was started at 9mA and was increased by 1.0mA when the animal was able to complete

two full sets (typically 2 days). We have found that this progression of stimulation does not contribute to additional muscle injury and does not result in any visible gait impairment as observed in our previously study using the same Estim protocol as described by Distefano (2013) (111). The parameters used include: pulse duration of 150ms, frequency of 50Hz, time on: 5 seconds, time off: 10 seconds, 0.5-second ramp and 0.5-second ramp down. This protocol was based on clinical protocols designed to increase muscle strength according to the principle of increased functional load (114, 115).

2.3.3 *In situ* contractile testing

Contractile testing was performed 14 days after injury using an *in situ* testing apparatus (Model 809B, Aurora Scientific Inc, Canada), a stimulator (Model 701C, Aurora Scientific Inc, Canada), and a force transducer (Aurora Scientific Inc, Canada). The method used allows for the determination of muscle contractile properties of a muscle of interest, while maintaining normal muscle orientation, innervation and vascular supply (111). Briefly, the peroneal nerve of anesthetized animals was isolated through a small incision lateral to the left knee. Mice were then placed supine on a 37^{o}C-heated platform and the foot being tested is positioned on the footplate. The left hindlimb used for testing was stabilized with cloth tape on the knee and foot. Muscles were stimulated through the peroneal nerve by a hook electrode inserted beneath the skin. Muscle peak twitch, time to peak twitch and half-relaxation time with the ankle positioned at 20^{o} of plantarflexion, the position that we determined to result in the greatest force output (data not shown), were quantified. Tetanic contractions at 10, 30, 50, 80, 100, 120, 150 Hz were elicited to obtain a force- frequency curve, with a 2-minute rest between each contraction. The muscles were

then subjected to a 7 minute high-frequency fatigue protocol consisting of a series of short 350ms tetanic contractions at 100 Hz, with 4-second intervals between contractions (116). Force recovery was analyzed at 5 and 10 minutes following completion of the fatiguing protocol, as we previously described (111, 117). Right TA muscles from mice in each group were then harvested and frozen in nitrogen-cooled 2-methylbutane for subsequent histological analysis by blinded investigator.

2.3.4 Histology and immunofluorescence

Frozen muscles were serially sectioned (10 μm sections) at -30^{o}C on a Thermo Scientific cryostat and mounted onto slides. Histological assessment of the recovery process was performed in groups of control and injured mice 14 days after injury.

Laminin Immunofluorescence: Slides were washed 3x with 1x PBS and permeabilized with 0.03% Triton X-100 in PBS for 20 min at room temperature. Slides were then blocked with 5% Normal Goat Serum (Vector S-1000) in PBS for 60 min at room temperature. Slides were washed 3x with 1x PBS and 3x with 0.5% Bovine Serum Albumin (BSA). Slides were then incubated with Rat anti-Laminin primary (1:100, Abcam 11576) for 60 min at room temperature. Slides were washed 3x with 0.5% BSA and incubated with Goat anti-Rat Alexa Fluor 488 secondary (1:500, Life Technologies A-11006) for 60 min at room temperature. Slides were washed 3x with 0.5% BSA, 3x with 1x PBS, and incubated with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies 1370421) for 1 min at room temperature. Slides were washed 6x with 1x PBS and cover slips were mounted with Cytoseal XYL (Richard-Allen Scientific 8312-4). Slides were imaged (20x magnification) using a Nikon 90i motorized upright fluorescent microscopy. Myofiber diameter and the total number fibers were determined utilizing an automated macro

written in Nikon Elements AR (Nikon Elements AR, Nikon, Tokyo, Japan). To calculate the myofiber diameter we calculated how many individual bundles of fibers there were. After, the average area of everything divided by the total number of fibers gave us the average area per fiber.

Fibrogenesis: Masson's Trichrome staining was performed to quantify the percent of collagen content and muscle fiber in a muscle section. Slides were processed using Masson's Trichrome Stain Kit as per manufacturer's instructions (K7228; IMEB, Chicago, IL). This process stains skeletal muscle fibers red, collagen blue, and nuclei black. For each sample, three random sections were selected and photographed using Nikon 90i motorized upright fluorescent microscopy and NIS-Elements. The percentage of the total collagen-positive area relative to the total cross-sectional area was calculated. For determination of collagen content, sections were analyzed using MetaMorph Offline.

2.3.5 MPC isolation

TA and Extensor Digitorum Longus muscles from bilateral hindlimb muscles of 5 animals per experimental group (young, aged, and aged+Estim) were harvested for MPC isolation using a modified pre-plate technique, as previously described (26, 118, 119). Briefly, after hindlimb muscle enzymatic digestion, the homogenate was serially plated onto collagen-coated flasks. It has been previously shown that cells that rapidly adhere to the collagen-coated flasks (passage 1 and 2) are predominantly of a fibroblastic lineage (26). On the other hand, those late adhering cells, isolated after three or four serial passages, have been previously characterized as having a myogenic lineage (26). All cells were expanded in high-serum proliferation medium (Dulbecco's modified Eagle's

medium, 10% fetal bovine serum, 10% horse serum, 1% penicillin/streptomycin, and 0.5% chick embryo extract). Analyses were performed on MPCs that had been passaged fewer than three times in order to minimize the effect of chronic culture on MPC characteristics. Whenever possible, MPC analyses were performed by an investigator blinded to groupings.

2.3.6 Metabolic activity (MTS) assay

MPC viability was measured using CellTiter 96 Aqueous One solution (MTS: 3-(4,5-di-methylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium); Promega, Sunnyvale, CA) according to manufacturer's instructions. In brief, 1,000 cells were seeded in each well of a 96-well plate and allowed to attach for 24 hours. Twenty μL of MTS solution was then added to the cells at 0, 1, 2, 3, or 7 days, incubated at 37 ^{0}C for 1 hour and quantified using a spectrophotometer (Tecan, San Jose, CA) at 490 nm. This assay is based on the conversion of a color reagent by NADPH or NADH, which is produced in metabolically active cells and is, therefore, indicative of the number of cells that are metabolically active.

2.3.7 Senescence associated SA-β-Galactosidase (SA-β-Gal) activity assay

This assay followed the method described by Debacq-Chainiaux et al.,(120) which is based on a histochemical stain for β-galactosidase activity (pH 6.0). In this assay, senescent cells are stained blue/green. Cells were washed with PBS and subsequently fixed in 2% PFA+0.2% glutaraldehyde in PBS for 5 min at room temperature, washed twice with PBS twice, and then incubated with a

staining mixture containing 5-bromo-4-chloro-3-indolyl β-d-galactoside, potassium ferricyanide, potassium ferrocyanide in citric acid–sodium phosphate buffer (pH 6.0) at 37℃ overnight. Cells were then washed with PBS and mounted with DAPI-fluoromount-G mounting media. Cells expressing SA-β-Gal were manually counted, and expressed as a percentage of the total cell count.

2.3.8 MPC myogenicity

To determine whether Estim can reverse the effect of age on MPC terminal differentiation, we compared *in vitro* myogenicity across groups. Cells from each population were seeded onto a 12-well collagen-coated plate, and expanded to confluence in proliferation media (typically 3-4 days). Once cells were 70% confluent, the media was changed to a low-serum media in order to promote differentiation. After 3-5 days, immunofluorescence was performed on fixed cells (4% PFA, 10 min) after permeabilization (0.2% PBT; 10 min) and blocking (5% GS in PBT). MPCs were then incubated in a primary antibody against desmin (myogenic marker). Cells were incubated in primary the primary antibody against desmin (1:1000) (overnight at 4°C at the following dilutions. Next, cells were washed and blocked in 5% GS/PBS then incubated in fluorophore-conjugated antibody (goat anti-rabbit Alexafluor 488 at 1/1000) and DAPI to visualize nuclei for one hour at room temperature. The percentage of cells positively stained cells for desmin was calculated as a function of total cell number (as determined by quantification of the total nuclear count).

2.3.9 Wnt signaling activation

Flow cytometry was used to quantify the effect of Estim on Wnt signaling in MuSCs. MPC populations isolated from young and old animals were labeled with a fluorescent antibody specific to a substrate of Wnt signaling, β-catenin. Cells were fixed with 4%PFA for 10 minutes at 37^0C and then permeabilized in 100% methanol for 30 minutes in ice and after that cells were kept in -20^0C overnight. On the next day, 5ml of PBS was added and cells were centrifuged at 10000 rpm for five min. Cells were resuspended in 100 ml of PBS and 5 µl of first anti-body anti-human β-Catenin Alex Fluor 488 (ref#53-2567) was added and incubated at room temperature for one hour. Afterwards, cells were centrifuged, rinsed, and resuspended in 100 µl of PBS. Cells were subsequently incubated in 5µl of second anti-body mouse IgG1 K Isotype Control Alex Fluor 488 (ref#53-4714) for 30 minutes at room temperature. Finally, cells were rinsed with PBS, centrifuged, and resuspended in 0.5ml of phosphate buffered saline (PBS). Cells were analyzed using fluorescence activated cell sorting (FACS) and gated to include cells with fluorescence for MuSCs markers, CD31-, Sca1-, CD45- and VCAM+ (Fig 4). β-Catenin expression was evaluated in a MuSC sub-population and the percentage of positively stained cells for each variable was compared across groups.

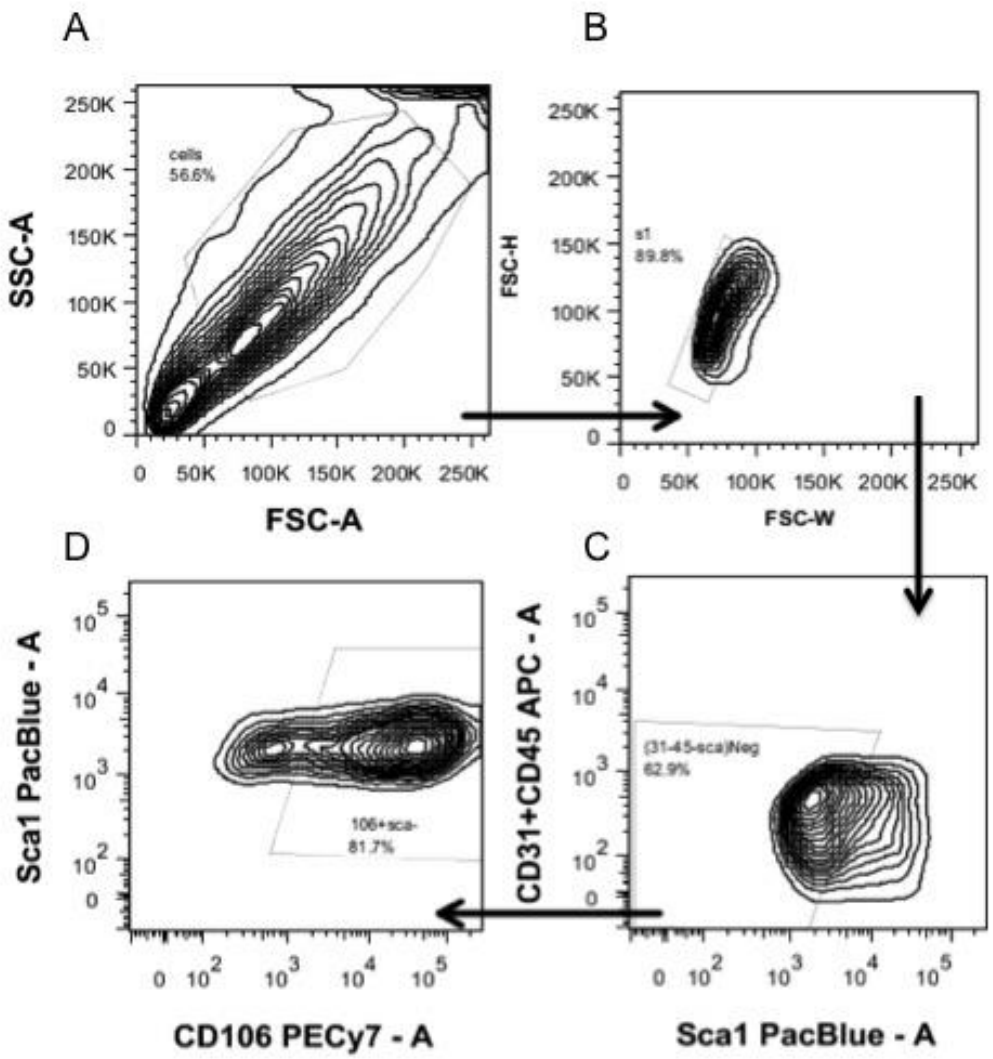

Figure 4 - Gating strategy for sorting of MuSCs from the MPC population

In this sample gating strategy, cells were isolated from TAs and EDLs and analyzed by flow cytometry. (A-D): Gating strategy for analysis of 106+ population. (A) Gating for cells based on forward and side scatter. (B-C) Cells were first gated for singlets versus doublet populations, and then cells were gated for CD31- and 45-, and Sca1-. (D) Only triple negative cells were gated for 106+. Abbreviations: FSC-A, Forward Scatter Area; SSC-A, Side Scatter Area; FSC-H, Forward Scatter Height; FSC-W, Forward Scatter Width.

For GSK-3β analysis, MPCs were plated in 12 well-coated plates and after 24 hours they were incubated with primary antibody (anti-GSK3 beta antibody (ab2602) at 1:100 overnight at

4^0C). Cells were incubated with second antibody (488 goat anti rabbit, respectively) at 1:100 for 1 hour following the manufacturer's recommendation. The average intensity of fluorescence was quantified across group.

2.3.10 Immunocytochemistry protocol to evaluate Wnt and Klotho

Samples were incubated with primary antibodies rat anti-Klotho (R&D, MAB1819) and mouse anti β-catenin (Thermofisher scientific, MA1-2001) overnight in 4C at 1:1000 dilution in 3% Goat serum (Jackson Laboratories, Product no. 005-000-121). After a triple wash with PBS, the cells were incubated with their respective secondary antibodies, goat-anti Rat Alexa Fluor 488 (Life Technologies, A11006), goat anti-mouse Alexa Fluor 594 (Life Technologies, A11005) and Phalloidin 647 in 3% Goat serum at 1:500 dilution for 60 minutes. The chamber slides were mounted with DAPI Fluoromount-G media (SouthernBiotech, 0100-20) with a glass coverslip after washing with PBS three times. Imaging was performed using a Nikon Confocal All images were obtained at the same exposure for each channel. ImageJ was used to analyze confocal images for intensity of Klotho and β-catenin in the region of interest.

2.3.11 siRNA Klotho knockdown

MPCs were plated to 70% confluence, after which time they were transfected with 50nM of pools of siRNA specific for Klotho or with 25nM of both specific siRNA pools (siGENOME SMART pool, Dharmacon, Lafayette, CO) using Dharmafect 1 reagent (Dharmacon) according to the

supplier's protocol. As a negative control, cells were transfected with 50nM of Non-Specific Pool #2 siRNA (siGENOME SMART pool, Dharmacon. Four days post transfection, the cells were placed in differentiation media for 2 days, and nuclear β-Catenin expression and senescence were subsequently evaluated, as described above.

2.3.12 Statistical analysis

Analyses were performed using SPSS v22.0 software (Armonk, NY). Shapiro-Wilk and Levene's tests were initially performed to assess normality of data and equality of variances, respectively. If assumptions of normality and homogeneity of variances were met, a one-way ANOVA followed by *post-hoc* Tukey tests were performed to compare differences across groups. However, because normality conditions were not met and the distribution was not normal, *Post hoc* comparisons between groups were performed using the non-parametric Kruskal-Wallis test followed by Mann-Whitney U tests. Multiple linear regressions were performed for the *in situ* contractile data, to investigate the slopes of muscular fatigue and recovery in different time points. Non-linear regression using exponential growth modeling on normalized data was performed for the proliferation assay results to evaluate the cells metabolic activity in different time points. All results were expressed as mean ± standard error. Statistical significance was established, *a priori*, at $p \leq 0.05$.

2.4 RESULTS

2.4.1 Estim enhances myofiber regeneration and force recovery in aged skeletal muscle after an acute injury

As expected, acutely injured aged muscle displayed a significant impairment in myofiber regeneration, as determined by a decrease in total number of myofibers (TNF) and the myofiber cross-sectional area (CSA), when compared to young injured counterparts ($p=0.009$ and $p<0.001$; respectively) (Figure 5). However, two weeks of Estim significantly increased regeneration in acutely injured aged muscle, when compared to age-matched controls (TNF: $p=0.009$, CSA: $p<0.001$) (Figure 5). There was no difference in the regeneration of aged muscle that had been exposed to Estim and young counterparts (TNF: $p=0.465$, CSA: $p=0.785$) (Figure 5).

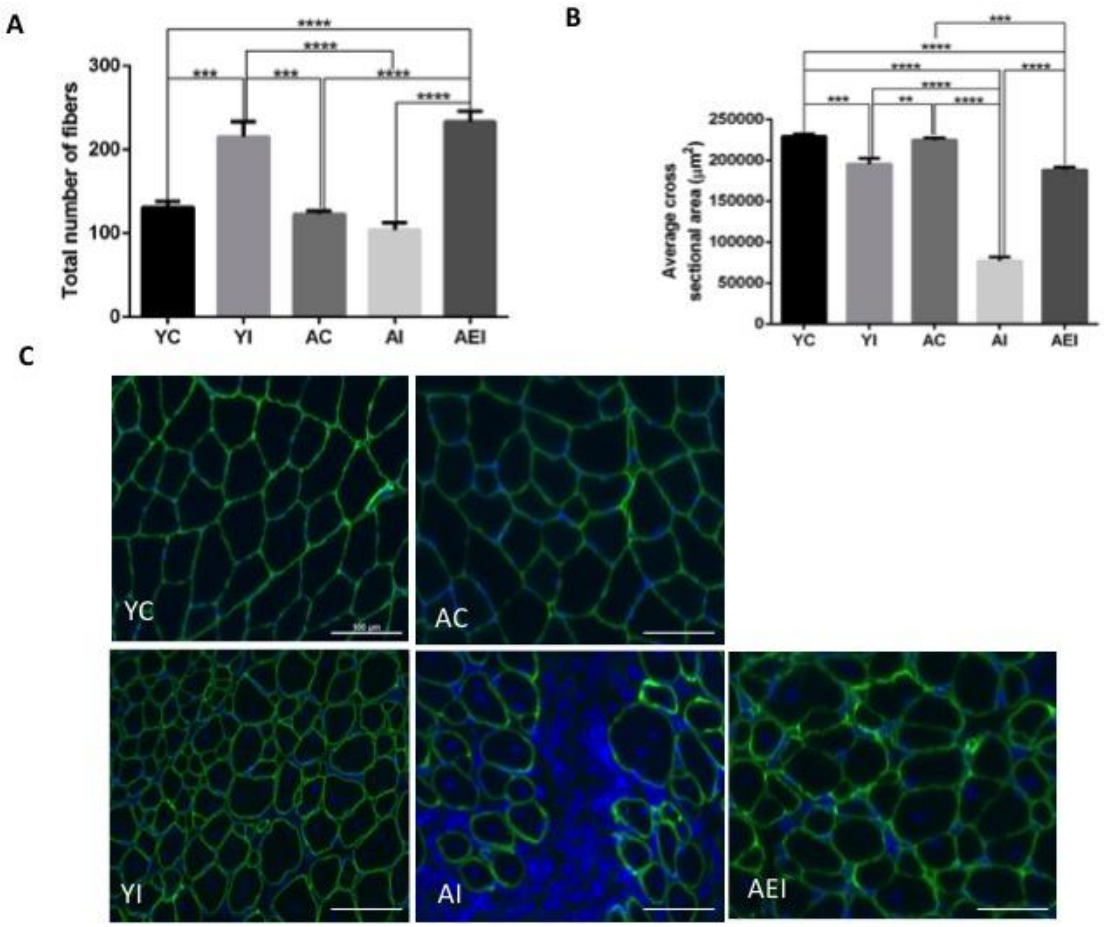

Figure 5. Effect of pre-treatment Estim on the total number fiber and cross sectional area in aged mice.

(A) Total number fibers in all experimental groups 14 days after cardiotoxin injury (n=7-8/group). (B) Cross sectional area of TA muscles in across the experimental groups: Young control (YC); Aged control (AC); Young injured (YI); Aged injured (AI); and Aged injured + Estim (AEI). (C) Laminin staining in the tissue cross-sections of TA muscles in all groups (Green=Laminin, Blue= DAPI). n=7-8/group, (20x magnification, scale bar=100μm).**Denotes significantly different p=0.0019, ***Denotes significantly different p<0.0005, ****Denotes significantly different p<0.0001.

Analysis of collagen content revealed no significant difference in fibrosis formation between any of the injury groups evaluated (Figure 6).

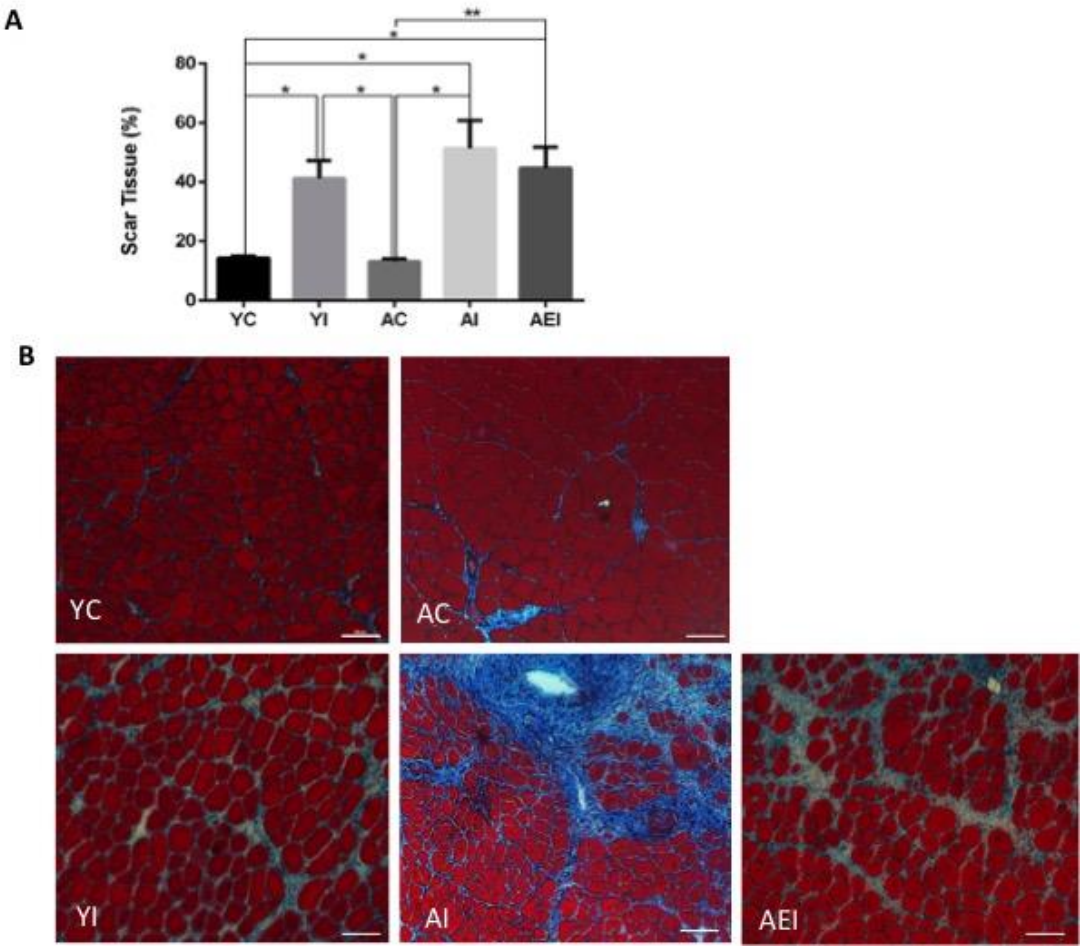

Figure 6 Effect of pre-treatment Estim on the percentage of scar tissue formation in aged mice

(A) Percent of collagenous tissue across experimental groups. (B) Masson's Trichrome stain (Fibers (Red); Collagen (Blue), and Nuclei (Black)) in the tissue cross-sections of TA muscles of Young Control (YC), Aged Control (AC), Young Injury (YI), Aged Injury (AI), and Aged Estim Injury (AEI) 14 days after cardiotoxin injury (CTX) (10x magnification, scale bar = 100 μm). *Denotes significantly different $p<0.05$; **Denotes significantly different $p<0.01$.

To examine whether the enhanced regenerative response following the application of Estim in aged animals was concomitant with an improved force recovery after injury, we performed *in situ* contractile muscle testing. Fourteen days post injury, the specific tetanic force tested at 100Hz in aged injury muscle was significantly lower when compared young injury counterparts ($p=0.006$) (Figure 7A). However, completion of an Estim protocol restored force recovery in aged muscles to levels comparable to young counterparts ($p=0.509$; Figure 7A; Table 1), and significantly greater than age-matched injured muscles ($p=0.046$; Figure 7A and C; Table 1). There was no difference in the fatigue resistance or recovery from a fatiguing protocol at 5 or 10 minutes recovery between injured groups (Figure 7 B), but all injured muscles displayed a decreased recovery from a fatiguing protocol when compared to uninjured controls at 5 and 10 minutes recovery ($p\leq0.001$). Analysis of temporal contractile characteristics revealed no differences in time to peak twitch or half relaxation time between groups (Table 1).

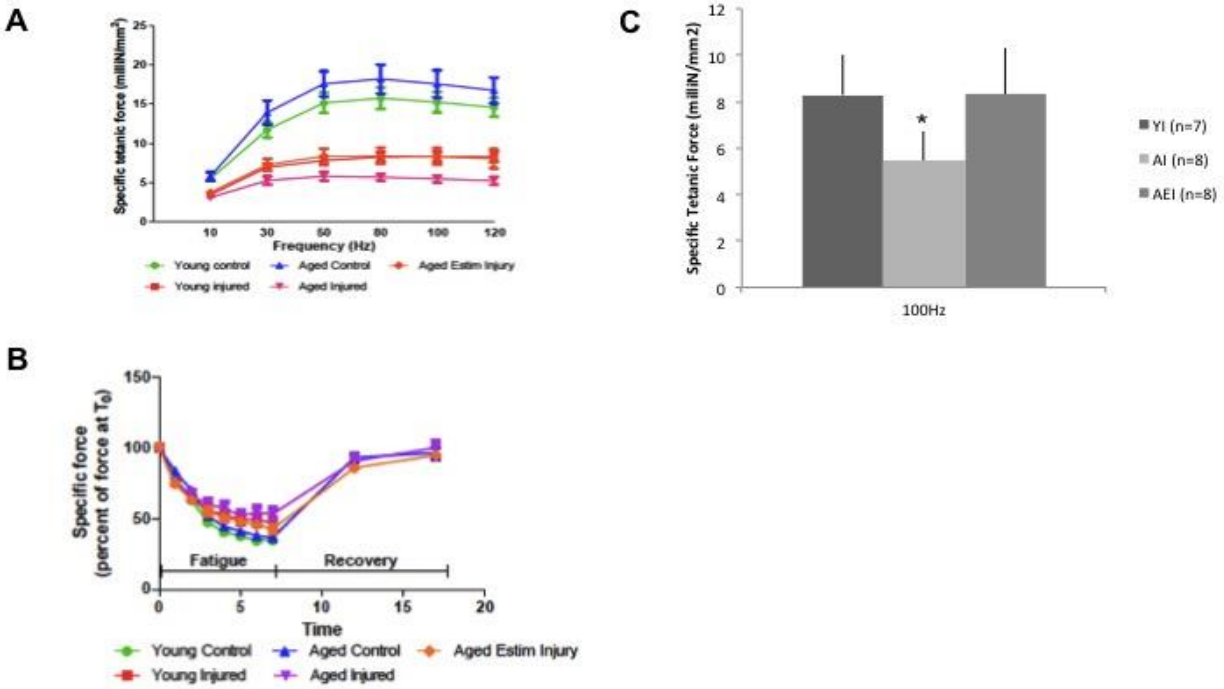

Figure 7 *In situ* contractile testing of the lower leg anterior compartment

Fourteen days after cardiotoxin injury, *in situ* contractile testing of tetanic force production in the anterior leg muscle compartment was performed in Young Control (YC), Young Injury (YI), Aged Control (AC), Aged Injury (AI), and Aged Estim Injury (AEI). (A) Force Frequency Curve; (B) Quantification of fatigue resistance and force recovery after a fatiguing protocol; (C) Peak tetanic force at 100Hz. Data are mean ± standard error of the mean (SEM) for all experimental groups. * $p<0.05$ when comparing to AEI.

Table 1. Contractile characteristics

Measurement	YC	YI	AC	AI	AEI
Mice (n)	8	7	8	8	8
Muscle weight (mg)	58.5±1.3	48.5±1.2** #	58.8±1.5	40.8±2.5 #	44.3±3.1 #
Muscle length (mm)	12.6±0.1	12.4±0.1	12.8±0.2	12.7±0.1	12.7±0.2
Cross-sectional area (mm^2)	4.3±0.1	3.6±0.08** #	4.3±0.1	3.0±0.2 #	3.3±0.2 #
Maximum twitch torque (milliNm)	0.71±0.02	0.34±0.02 #	0.73±0.06	0.25±0.04 #	0.36±0.06 #
Maximum tetanic torque, 100 Hz (milliNm)	1.97±0.15	0.91±0.07** #	2.25±0.21	0.47±0.04 #	0.85±0.16 ** #
Specific twitch force ($milliN/mm^2$)	5.48±0.19	3.14±0.22 #	5.71±0.55	2.53±0.54 #	3.58±0.34 #
Specific tetanic force, 100 Hz ($milliN/mm^2$)	15.2±1.2	8.2±0.65** #	17.5±1.7	5.4±0.44 #	8.3±1.0 ** #
Frequency for maximum specific tetanic force (Hz)	125.8±1.35	59.0±0.67** #	142.7±1.83	45.8±0.45 #	72.6±1.00 ** #
Time to peak twitch (s)	0.43±0.02	0.37±0.04	0.39±0.03	0.41±0.07	0.40±0.07
Half-relaxation time (s)	0.001±0.00002	0.008±0.005	0.01±0.005	0.001±0.0004	0.003±0.001

Data are presented as the mean ± SEM and statistical differences were determined by one-way ANOVA ($p<0.05$). #Significantly different when compared to YC and AC; **Significantly different when compared to AI.

2.4.2 Estim rejuvenates the aged MPCs phenotype

We next tested whether the enhanced regenerative response following Estim was associated with an altered MPC phenotype. Flow cytometry analysis revealed that ~68% of the young and 50% of aged populations were CD106+, Sca1-,CD31, and CD45-, an expression profile show to be highly selective for MuSCs (118, 119). Interestingly, aged animals that completed an Estim protocol displayed a MuSC profile that closely paralleled that of young counterparts, and approximately 65% of cells isolated from were CD106+, Sca1-, CD31, and CD45- (Figure 8).

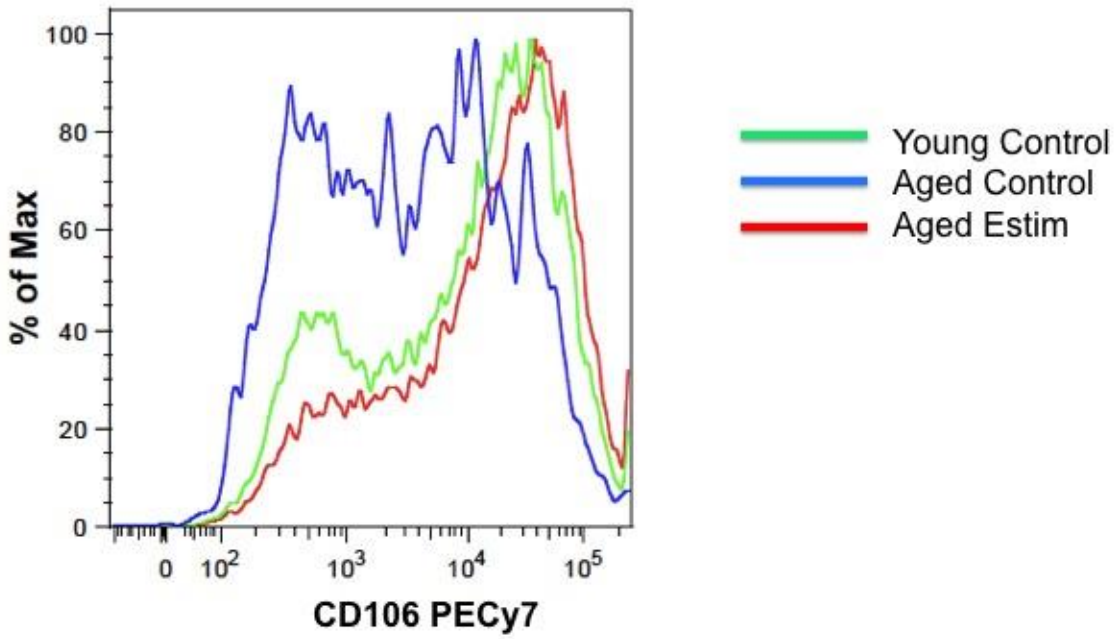

Figure 8 FACS sorting for 106+ cells to isolate pure MPCs population

Cells were isolated from TA and EDLs muscle of Young Control (YC), Aged Control (AC), and Aged Estim (AE). Cells were stained for CD31-, CD45-, Sca1-, and CD106+ (indicative of an MuSC subpopulation), and analyzed by FACS. Fluorescent staining of sorted cells from all experimental groups shows the percentage (y-axis) and intensity (x-axis) of MuSCs.

Consistent with previous studies (121), MPCs isolated from aged animals demonstrated a significantly decreased myogenesis, when compared to young controls (Figure 9). However, following Estim, aged MPCs displayed a myogenic differentiation comparable to young counterparts, * p=0.03 (Figure 9 A and B).

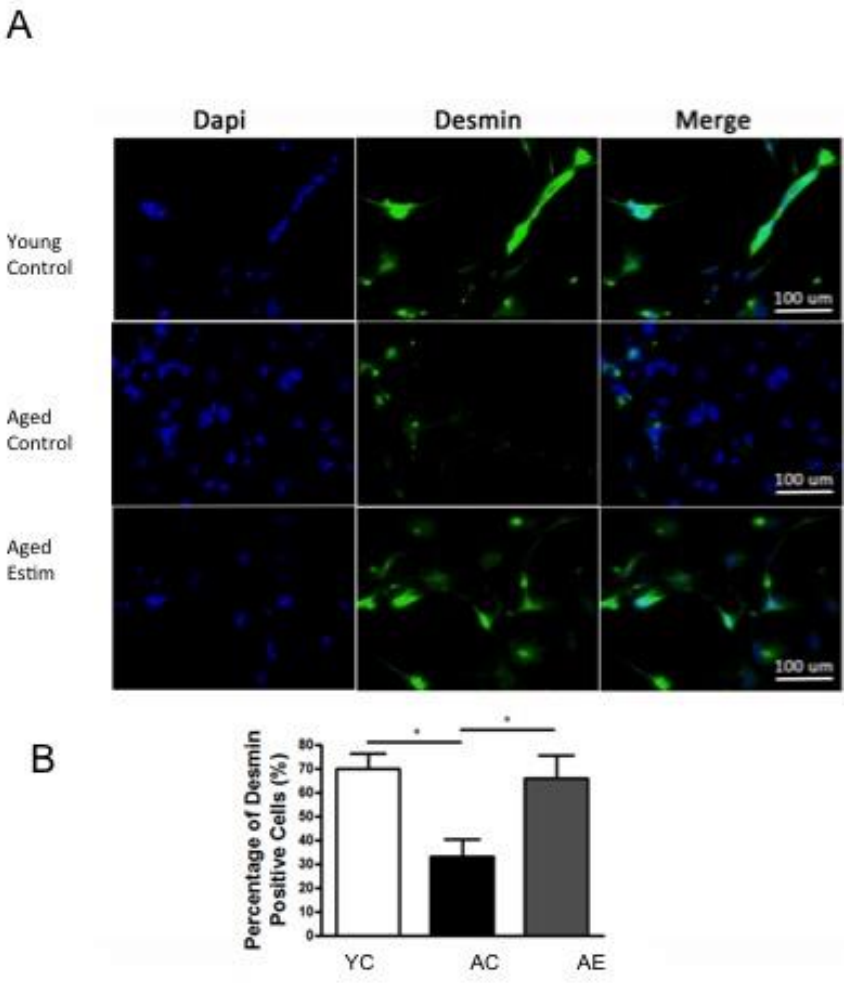

Figure 9 Effect of Estim on MPC myogenicity

(A) MPCs were fixed and immunostained for Desmin, a myogenic marker (Green) and the nuclear stain, DAPI (Blue) in Young Control (YC), Aged Control (AC), and Aged Estim (AE). *, p=0.03. (B) Graph showing the percentage of cells positive for Desmin. Scale bar=100 μm

When we examined MPC viability across experimental groups using an MTS assay, we observed a significant decrease in the metabolic activity of aged MPCs relative to young counterparts ($p<0.0001$), but the metabolic activity of aged MPCs was restored to youthful levels following completion of an Estim protocol ($p<0.001$) (Figure 10).

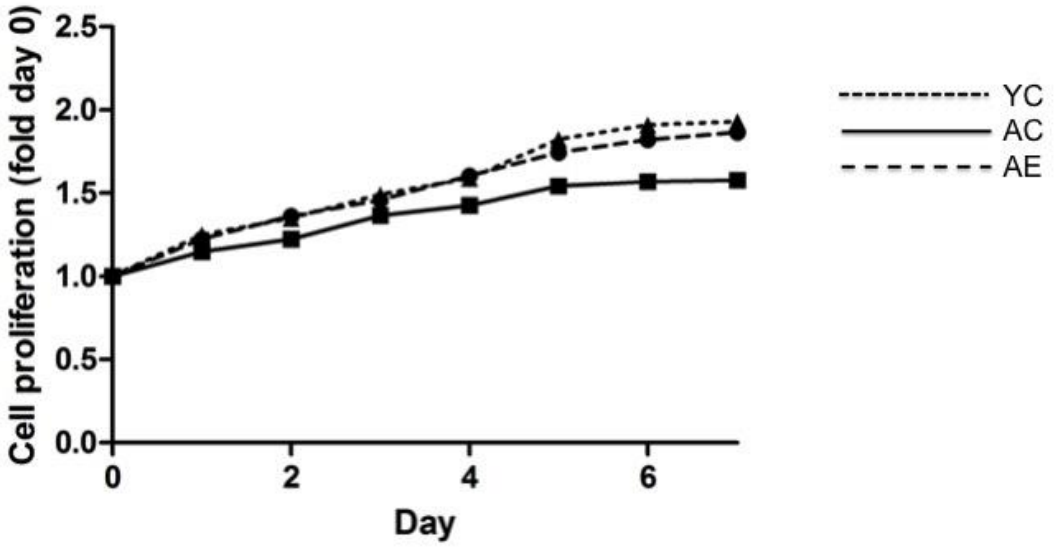

Figure 10. Metabolic viability assay of MPCs

Line graph showing an increased in cell viability assay in Young Control (YC) and Aged Estim (AE), when compared to Aged Control (AC).

When we analyzed cellular senescence across the three groups, we observed a significant increased in senescence-associated beta-galactosidase (SA-β-gal) staining of MPCs isolated from the skeletal muscle of aged mice, relative to young controls (Figure 11A, B). These results are consistent with previous findings reporting an increased cellular senescence of aged MuSCs, as determined by $p16^{Ink4a}$ and $p21^{Cip1}$(123). Following completion of Estim protocol in aged animals,

however, the percentage of senescent MPCs resembled levels comparable to young counterparts, demonstrating an improved capacity of Estim to rejuvenate aged MPC (124, 125) (Figure 11A, B).

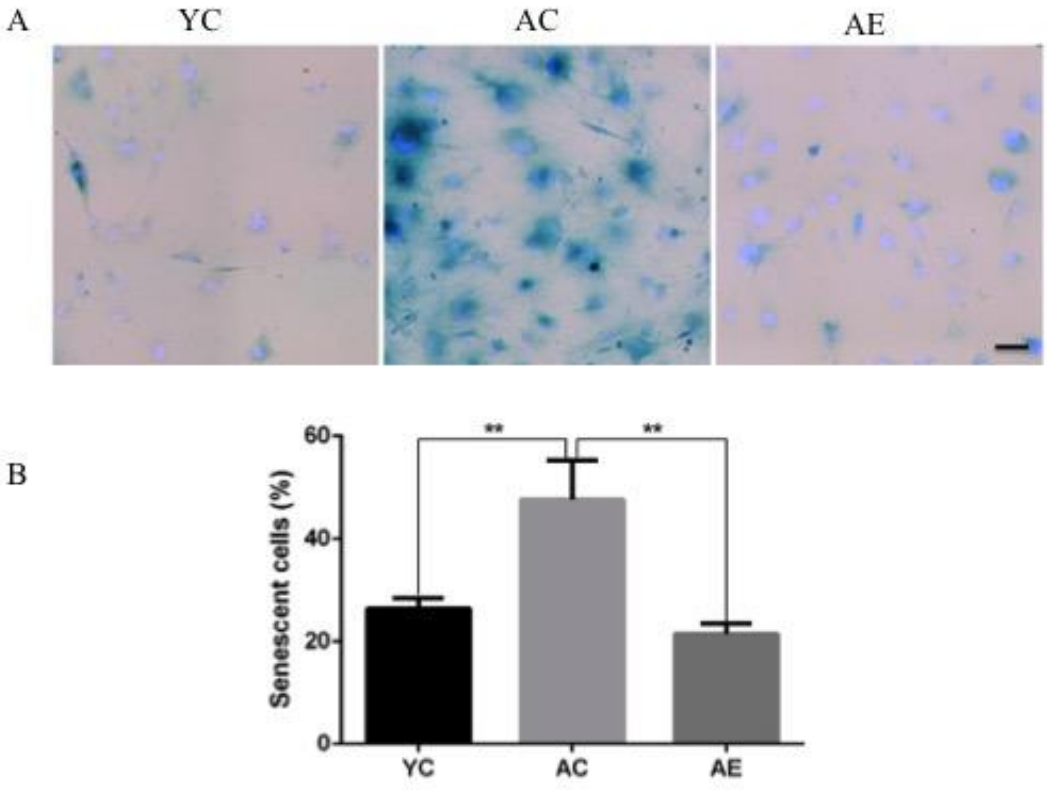

Figure 11 Estim decreases the percentage of senescent cells in aged MPCs

(A) SA-β-gal staining (blue) of MPCs isolated from the skeletal muscle of Young Control (YC), Aged Control (AC) and Aged Estim (AE) mice. Scale bar=50μm. (B) Graphic representation of the percentage of senescence cells across each of the three experimental groups. The number of SA-β-gal positive cells is significantly increased in AC cells, when compared to YC. However, the percentage of senescent cells is significantly decreased in AE, when compared to AC group. ** $p \leq 0.005$.

2.4.3 Estim modulates Wnt signaling/β-Catenin pathway in aged MPCs

Given previous reports demonstrating a role for Wnt signaling in age-related declines in MuSC function, we next tested whether Estim may affect MPC myogenicity through modulation of Wnt signaling activation. Though flow cytometry analysis revealed that the aged MuSC subpopulation display an increase in Wnt signaling activation relative to young counterparts (Figure 12 A), consistent with previous reports (7, 58), the application of an Estim protocol decreased the percentage of nuclear β-catenin positive cells when compared to aged controls (Figure 12A). These findings were further confirmed by immunocytochemical analysis of nuclear β-catenin (Figure 12D and E). Finally, analysis of glycogen synthase kinase 3β (GSK3β), important for maintenance of the β-catenin degradation complex and, therefore, inhibition of β-Catenin nuclear translocation, revealed that Estim mitigated the age-related decrease in the percentage of GSK3β expression to values comparable to young controls ($p=0.002$) (Figure 12 B and C). Taken together, these findings suggest that the application of an Estim to aged skeletal muscle decreases Wnt signaling activation in aged MPCs.

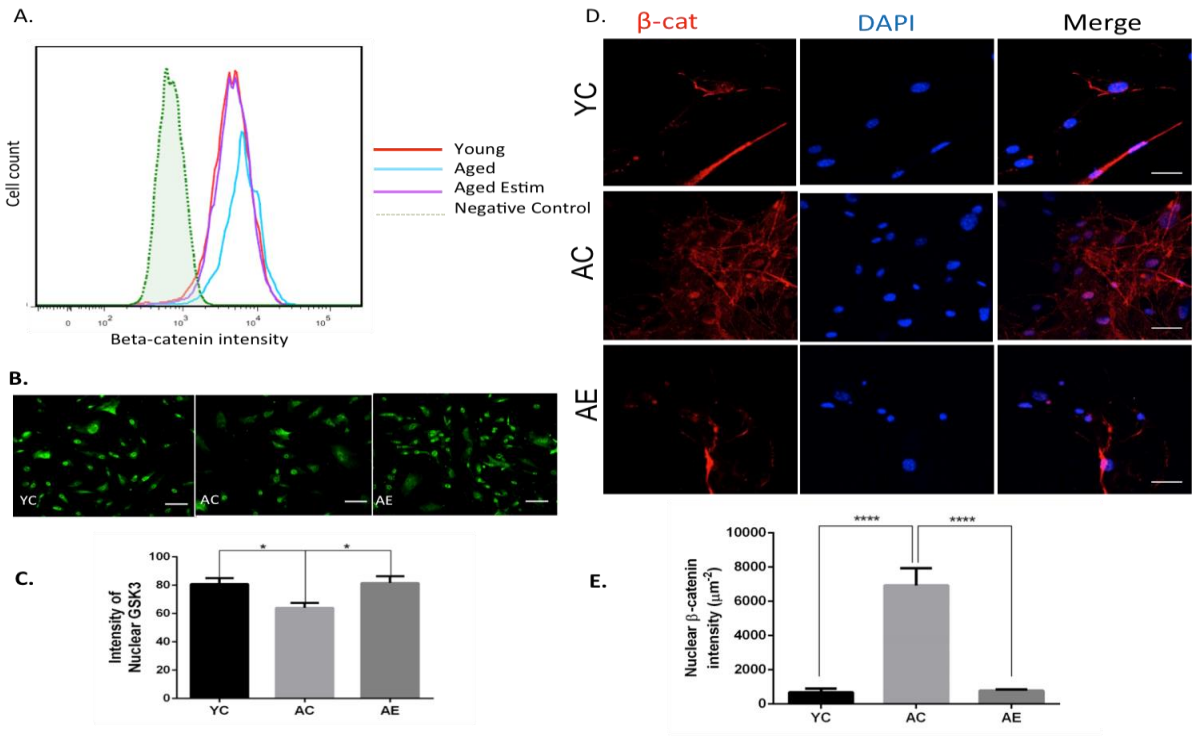

Figure 12 Estim modulates Wnt/β-Catenin pathway

(A) Flow cytometry analysis of the intensity of β-Catenin present in the MuSC population of Young Control (YC), Aged Control (AC), and Aged Estim (AE). (B) Immunocytochemestry for GSK3 in MPCs from across the three groups (Green). Scale bar =100 μm. (C) Percentage of GSK3 is decreased in Aged Control MPCs, when compared to Young controls (YC). However, Aged Estim (AE) display a significant increase in GSK3 when compared to Aged Control (AC). * denotes $p<0.005$ (D) Cells were fixed and stained for β-Catenin (Red) and DAPI for nuclei (Blue). Scale bar = 50μm (E) Quantification demosntrates significantly increased levels of nuclear β-Catenin in AC MPCs when compared to YC and AE, **** $p<0.0001$.

2.4.4 MPCs display an aged-related decrease in Klotho expression, but Estim restores expression to youthful levels

Heterochronic parabiosis experiments suggest that the introduction of youthful factors into the circulation of aged partners is sufficient to inhibit MuSC Wnt signaling activation and restore myogenic potential (47). However, the primary protein responsible for eliciting such rejuvenating effects is still unclear. One potential candidate of interest is the circulating hormone, Klotho. In tissues such as the skin and small intestine, declines in Klotho have been associated with inhibition of Wnt signaling activation and stem cell dysfunction (126). We therefore wondered if the beneficial effect of Estim on MPC function and regenerative potential in aged muscle might be a result of an up-regulation of Klotho expression.

To test this hypothesis, Klotho expression in injured young, aged and aged skeletal muscle exposed to Estim was quantified using immunofluorescence. Klotho expression was highly expressed at the site of acutely injured young skeletal muscle, but expression was markedly attenuated in the injured muscle of aged muscle (Figure 13). Strikingly, Klotho expression at the injury site of aged muscle was restored to youthful levels following completion of an Estim protocol (Figure 13).

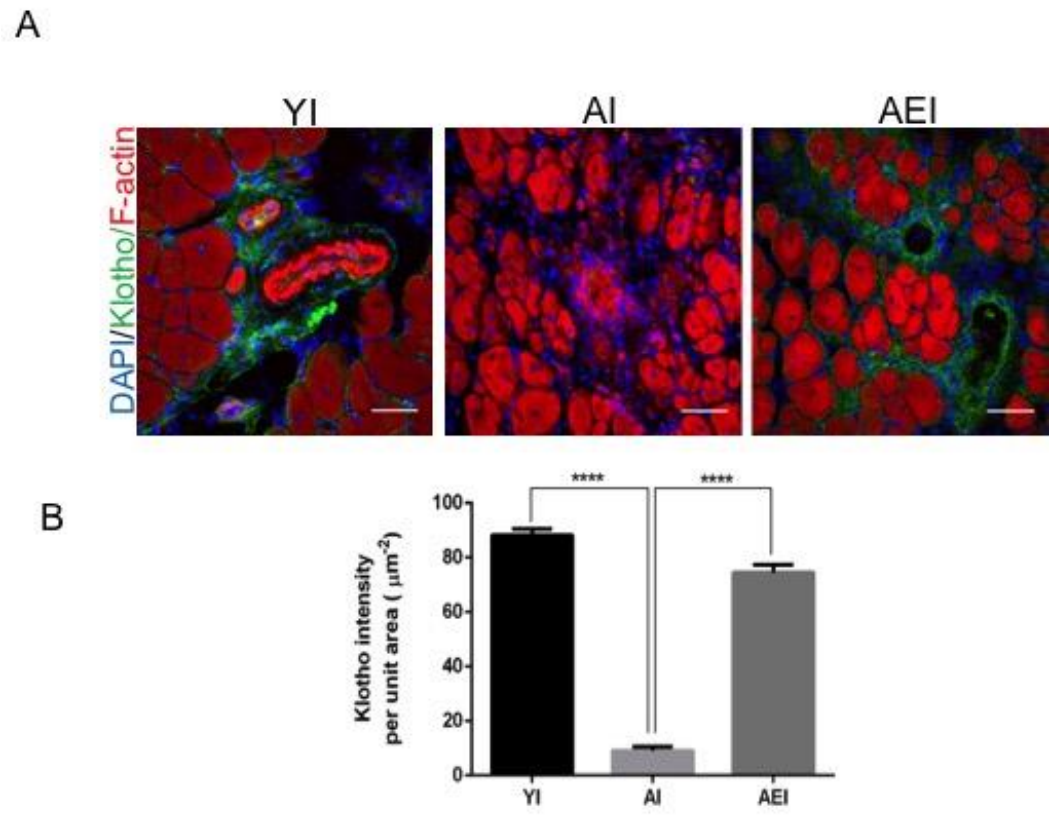

Figure 13 Klotho expression after skeletal muscle injury

(A) Tibialis Anterior (TA) muscles were stained for Klotho expression (Green), myofibers (phalloidin; Red), and DAPI for nuclei (Blue). (B) Immunocytochemistry shows abundant Klotho expression after 14 days of CTX in Young Control (YC), but expression is significantly decreased in Aged muscle (AI). However, Aged Estim (AE) muscles displayed a Klotho expression significantly greater than age-matched controls, and to levels comparable to young counterparts. Scale bar 50 μm. ****p<0.0001.

We next evaluated Klotho expression in MPC isolates from the three groups using confocal microscopy. Consistent with histological findings, young MPCs highly expressed Klotho throughout the nucleus and cytoplasm. Although Klotho expression was markedly decreased in aged MPC counterparts, when MPCs were isolated from aged muscle following completion of an Estim protocol, Klotho expression resembled that of young counterparts (Figure 14).

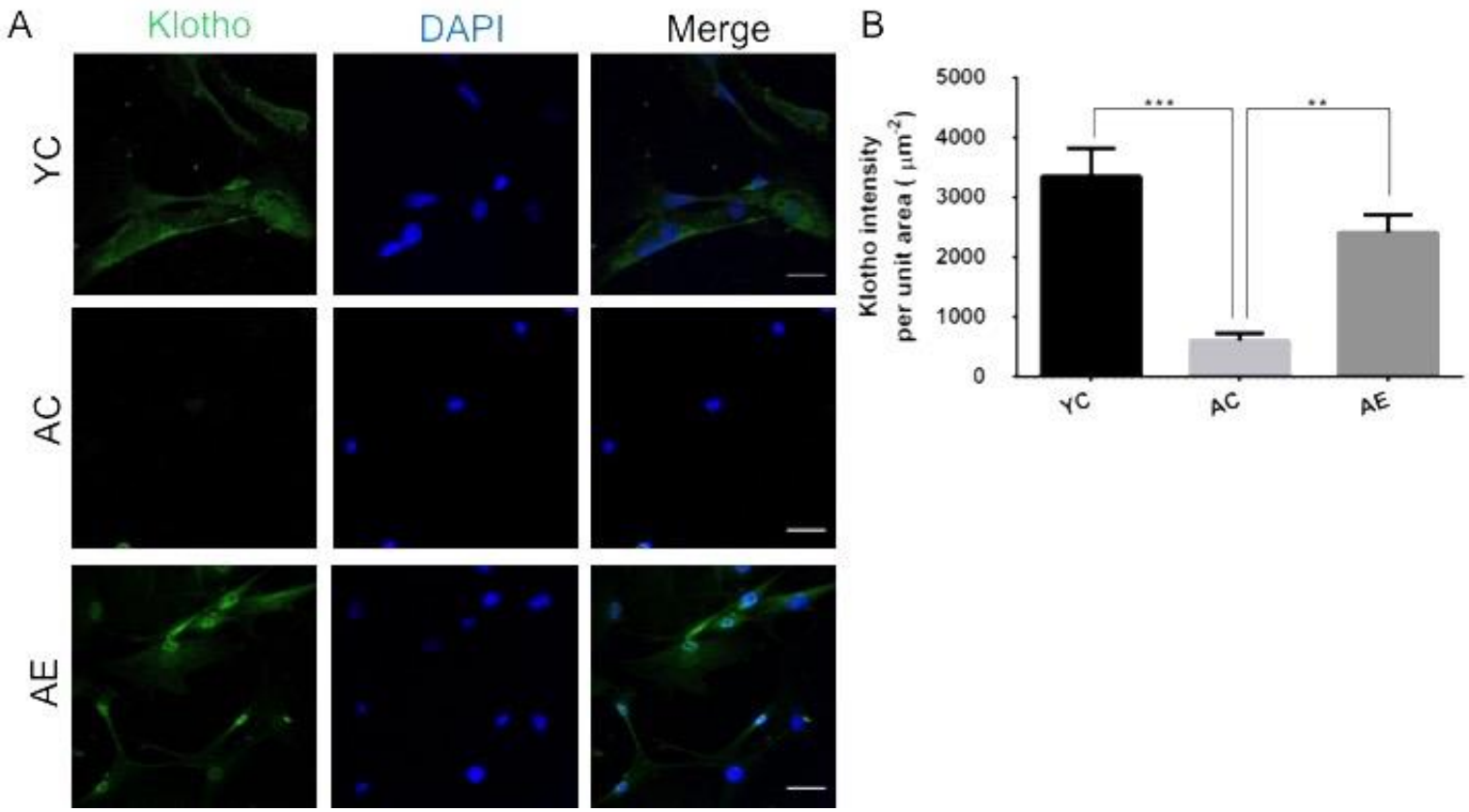

Figure 14 **Estim restores the expression of longevity protein, Klotho, in aged MPCs**

(A) Immunocytochimistry for Klotho expression (Green) and nuclear stain (DAPI; blue). Scale bar = 50 μm. (B) Aged Control (AC) MPCs display significantly decreased expression of Klotho as compared to Young Control (YC) MPCs, *** denotes $p<0.001$. However, Estim increases Klotho expression in Aged Estim (AE) MPCs, as compared to Aged Control (AC). ** denotes $p<0.01$.

To further interrogate a potential role for Klotho in modulating MPC function, Klotho expression was inhibited using siRNA to Klotho in YC MPCs and AE MPCs; an inhibition which resulted in MPC Klotho levels comparable to that observed in aged MPCs (Figure 15A). Unexpectedly, we found that inhibition of Klotho in young MPCs and aged MPCs MPCs did not significantly increase nuclear β-catenin levels, as compared to YC or AE MPCs treated with a scramble control (Figure 15 B).

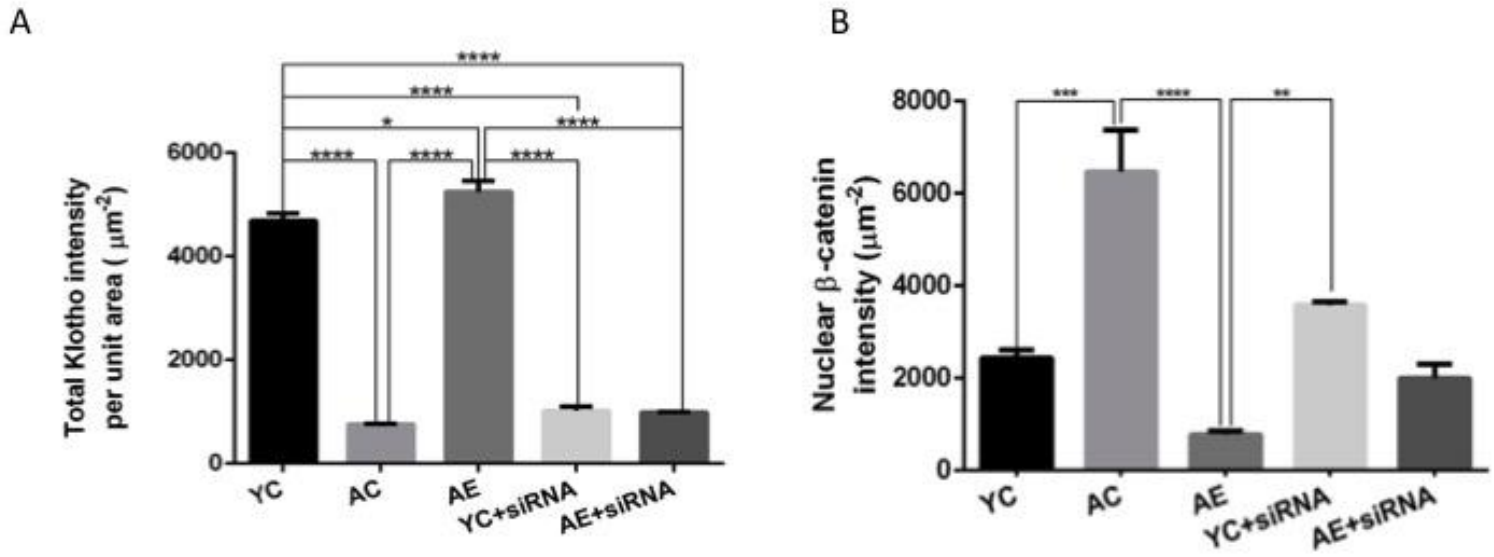

Figure 15 Silencing Klotho in Young Control and Aged Estim MPCs increases levels of β-catenin

Cells from Young control (YC) and Aged Estim (AE) were cultured and treated with siRNA to Klotho. Counterparts were treated with a non-coding (scramble) vector. Cells were fixed and immunostained for β-catenin and Klotho. (A) Klotho intensity decreases with silencing Klotho in the same levels of Aged Control, **** $p<0.0001$ (B) β-catenin increases in both Aged Control and Young+siRNA, ****$p<0.0001$, ***$p=0.0003$, **$p=0.005$.

Since we did not observe the expected increase in nuclear β-catenin when Klotho was inhibited in our MPC populations, we next evaluated the effect of Klotho expression on cellular senescence across groups using a loss-of-function paradigm. There was no significant difference between the percentage of senescent cells in YC and AE groups ($p>0.05$), therefore, the data was pooled across these two populations. Our data showed that inhibition of Klotho significantly increases the percentage of senescent MPCs in a dose-dependent manner, when compared to control counterparts treated with a non-coding vector (Figure 16).

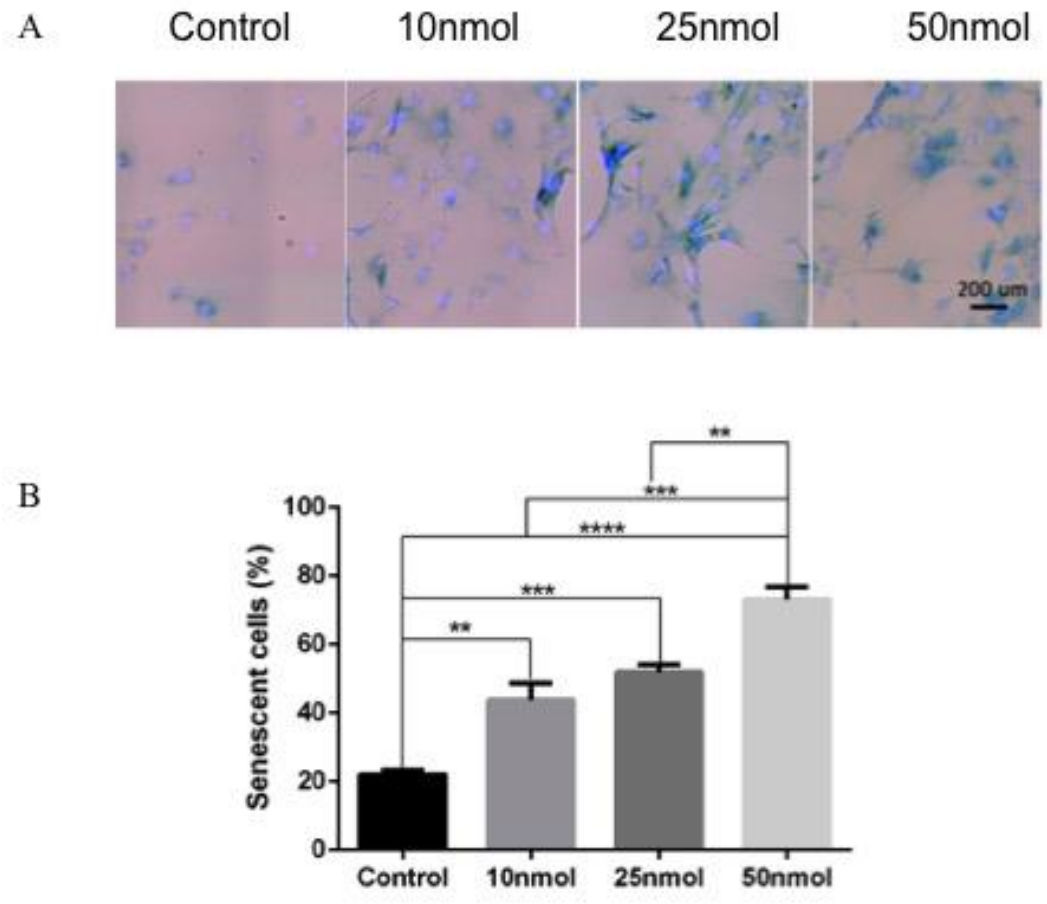

Figure 16 Silencing Klotho affects the percentage of senescence cells in a dose-dependent manner

(A) Representative images showing SA-β-gal staining (Blue) of MPCs with silencing Klotho at varying doses (0, 10nmol, 25nmol, and 50nmol). Scale bar=200 μm. (B) Bar graph showing quantification of the percentage of senescent MPCs across the different siRNA doses.

2.5 DISCUSSION

With advancing aging, the regenerative process of most tissues declines, and these declines are, in large part, attributed to dysfunction of tissue progenitor cells (9, 127). To better understand these declines with aging, it is necessary to identify the cellular and molecular mechanisms contributing to this impaired tissue function with age. Recently, studies have demonstrated the critical role of extrinsic factors within the niche for dictating MuSC regenerative potential (9, 128). In the present study, we investigated the influence of Estim on skeletal muscle regeneration in aged animals. Following two weeks of Estim, aged muscles displayed a myofiber regeneration and functional strength recovery comparable to young counterparts. Histological and functional improvements were concomitant with improvements in MPC characteristics, as evidenced by decreased in Wnt/β-Catenin signaling activation. Moreover, two weeks of Estim was sufficient to restore the percentage of senescent MPCs to youthful levels. Finally, we provide evidence to suggest that the beneficial effect of Estim on MPC regenerative potential may be a result of increased Klotho expression within aged skeletal muscle.

Many studies have demonstrated that mechanical loading, via exercise or Estim, for example, increases progenitor cell proliferative capacity and myogenicity (68, 81, 82, 93, 129-131). One study using microcurrent electrical neuromuscular stimulation (MENS) demonstrated enhanced regeneration and an increased number of MuSCs following cardiotoxin injury in 7 week-old mice (131). Another study compared leg press to Estim in 70 year-old subjects, and demonstrated that the application of Estim significantly increased the diameter of fast muscle

fibers and the number of Pax7 (a canonical MuSC marker) positive cells (132). Fujimaki and colleagues investigated the effect of functional overloading (FO) (via resistance exercise) on the plantaris muscle of 12 weeks old rats. Following FO, they found an increase in MuSC proliferation and differentiation, further confirming the ability of loading to modulate MuSCs responses (82).

Consistent with these previous studies, we observed a significantly increased myogenicity of aged MPCs following completion of an Estim protocol. Moreover, this increased myogenicity was accompanied by a decreased activation of the Wnt signaling cascade, as evidenced by a decreased β-Catenin and an increased GSK3. Wnt/β-Catenin signaling is upregulated during normal myogenesis (45, 133). However, in aged mice, the molecular signatures underlying the skeletal muscle regenerative cascade are dramatically altered, and aged muscle displays excess activation of the Wnt/β-catenin signaling cascade, resulting in fibrosis formation at the expense of myogenesis (7, 9). In contrast to our findings, Fujimaki *et al.* (2014) demonstrated a *decreased* GSK-3 expression and *increased* Wnt signaling activation in skeletal muscle of adult and old mice after 4 weeks of voluntary wheel running (84). There are some key differences in the previous studies and the current study that may help explain the conflicting results. First, Fujimaki et al used implemented a 4-week voluntary wheel running protocol. It is possible that longer duration protocol eventually promotes up-regulation of Wnt signaling, perhaps through the onset of myofiber microinjury. In the case of the Estim training protocol used in the current study, we chose a low intensity protocol, which we previously demonstrated does not result in injury, but does modulate the microenvironment through an increased angiogenesis (111). In addition, the molecular responses to voluntary wheel running versus Estim are likely to be different, as wheel running represents a whole body exercise protocol, whereas Estim is highly localized to the muscle of interest.

Our study demonstrated that the enhanced regenerative response in aged muscle following two weeks of Estim was associated with an increased expression of the longevity protein, Klotho. Using the skin and small intestine as models, Liu and colleagues demonstrated that Klotho enhances stem cell regenerative potential and promotes tissue healing through an inhibition of Wnt signaling activation (58). These latter findings were confirmed in recent studies demonstrating that Klotho is able to directly bind to Wnt ligands extracellularly, thereby inhibiting renal fibrosis formation (134). In the light of these previous findings, we originally hypothesized the decreased Wnt signaling following Estim in aged muscle might be a result of increased Klotho expression. However, though we observed an increase in Klotho expression in the aged animals exposed to Estim, loss-of-function studies for Klotho did not drive the expected increase in β-catenin nuclear translocation. These findings suggest that other signaling pathways, independent of MPC Klotho expression, may mediate the observed decrease in Wnt signaling activation of aged MPCs following an Estim protocol.

In contrast, we did observe a direct relationship between Klotho expression and the percentage of senescent MPCs. When Klotho was inhibited through siRNA in young MPCs and aged MPCs exposed to an Estim protocol, we observed a significantly increased percentage of senescence cells. These findings suggest that Klotho is inversely associated with senescence cells, and that Estim modulates Klotho expression in aged MPCs. Indeed, there is precedence to suggest that Klotho plays a role in inhibiting cellular senescence. Ikushima and colleagues demonstrated that Klotho has anti-senescent and anti-apoptotic roles in Human Umbilical Vein Endothelial Cells (HUVEC) through suppression of the p53/p21-signaling pathway (135), an important intracellular signal pathway of senescence. A follow up study by Maekawa *et al.* demonstrated that Klotho attenuates cellular senescence and apoptosis in HUVEC through inhibition of p53/p21 via the

Extracellular signal-Regulated Kinase MEK/ERK pathway, a subfamily of Mitogen-Activated Protein Kinase (MAPK)(136).

A number of studies have demonstrated that, with exercise, muscles promote the secretion of a number of cytokines (137-139). Studies suggest that cytokines and other peptides that are expressed and released by muscle fibers, aka "myokines", which have the capacity to promote paracrine and/or endocrine effects (140, 141). Indeed, several myokines, such as interleukin (IL)-6 (142), AMP-activated protein Kinase (AMPK) signaling, etc. have been shown to be stimulated by exercise (143). Importantly, many of these exercise-induced myokines have been shown to affect MuSCs proliferation, regulation of fat oxidation, and induced protection against several chronic disease (138, 140, 141).

Like heterochronic parabiosis and Klotho, physical activity induces a myriad of anti-aging effects, including prevention of muscle wasting, cardiovascular diseases and diabetes. Unfortunately, also like Klotho, physical activity levels have been shown to decline in both aged mice and men, which can limit the effectiveness of exercise and the benefits from it. Our findings suggest that Estim is a potent modality to modulate age-related declines in MPCs function and aged skeletal muscle regenerative capacity, possibly through regulation of Klotho expression. An important unresolved question is how does Estim regulate Klotho expression, and whether the Klotho expressed in acutely injured muscle originates from the circulation or the muscle itself? This would be an important line of future research.

2.5.1 Limitations

To our knowledge, this is the first study to investigate the effect of Estim in aged skeletal muscle healing and the association of Wnt signaling, cellular senescence and Klotho expression in MPCs. While our results suggest that Estim is a promising intervention to promote muscle healing in aged animals, several limitations should be noted. First, cells used for analysis were expanded in culture prior to *in vitro* analyses. Because only muscles directly exposed to Estim (ie. muscles of the anterior compartment) were used for cell isolation, the available muscle samples used for cell isolation were relatively small. As such, cells were necessarily expanded *in vitro* in order to obtain sufficient cell numbers for the analyses. Although all analyses were performed using cells that had undergone less than three passages, it cannot be discounted that amplification of cells in culture alters cellular characteristics and yields a heterogenous population (a phenomenon known as proliferation artifact). Therefore, isolated MPCs may not represent the behavior of cells in their native environment. Nevertheless, the fact that we demonstrate a significant improvement in force recovery after injury in aged mice following Estim, consistent with cellular and tissue findings, supports our hypothesis that Estim rejuvenates the regenerative response of aged muscle.

Another limitation of this study is that we investigated only a single Estim protocol. Though we observed a significant improvement in the regenerative capacity of aged muscle using a low intensity, five-day protocol, it would be very informative for future studies to investigate how timing, duration and frequency of Estim may optimize outcomes. This could serve as important insight to ultimately guide the development of clinical rehabilitation protocols. Along these lines, the current study only compared two age groups, young versus aged. However, under ideal conditions, this study would compare multiple age groups so as to have better idea of the

trajectory of declines in regenerative potential over time, and how this may be modulated, or even prevented, by the application of mechanical stimulation protocols.

Finally, in our study, the effect of Estim on muscle regeneration was only evaluated in aged male mice. Future studies should consider whether the effect would be the different in the case of aged female mice. Indeed, Deasy *et al.* (2007) demonstrated that MPCs display sexual dimorphism, and that female mice display an enhanced regenerative potential when compared to male counterparts, owing to an increased resistance to oxidative stress (144). To the best of our knowledge, there are no studies that have investigated the role of sex on declines on MPC regenerative capacity over time.

2.6 CONCLUSION

Though aging is generally associated with a decreased healing response after injury, these age-related effects are clearly reversible. Our findings suggest that mechanical loading may restore the metabolic activity of MPCs and exert a beneficial effect on progenitor cell fate through an inhibition of Wnt signaling activation and cellular senescence. Moreover, we provide evidence to suggest that the beneficial effect of Estim on inhibition of cellular senescence may be attributed to an increased expression of the longevity protein, Klotho. Future studies are needed to further elucidate the mechanisms by which Klotho expression in MPCs is reduced with aging, as well as the role of muscle loading as a potential treatment to retard declines in skeletal muscle vitality.

3.0 SIGNIFICANCE AND DIRECTION OF FUTURE RESEARCH

The results presented here demonstrate that muscle contractile activity, administered via Estim, may be an effective method to promote tissue healing after injury in a geriatric population. Estim is often used in the clinic to promote muscle hypertrophy and healing after injury. However, a thorough understanding of how Estim may regulate molecular mechanisms to enhance MPC function in an elderly population has heretofore been lacking.

This study investigated the ability of Estim to reverse age-related declines in the skeletal muscle healing and function through improved MPC regenerative potential. Specifically, we provided evidence to suggest that Estim enhances MPC myogenicity, and that this enhanced myogenicity is concomitant with a decreased Wnt/β-Catenin activation and cellular senescence. Although previous studies have demonstrated the mechanosensitivity of the Wnt signaling pathway by myogenic cell populations, these studies were primarily conducted using young animal models in which Wnt signaling occurs at physiological levels (82, 145). Here, we show that a low-intensity Estim protocol restores Wnt signaling activation to the physiological levels found in young counterparts. This decreased Wnt signaling activation following Estim was concomitant with a decreased expression of senescent markers in aged MPCs, further confirming a restoration of a MPC young phenotype. Still unclear is whether there is a direct relationship between Wnt signaling activation and cellular senescence in MPC populations. Moreover, to the best of our

knowledge, our study is the first to suggest that MPC senescence may be regulated by mechanical stimulation.

Our data suggest that the decreased cellular senescence following Estim may be attributed to an increased expression of the longevity protein, Klotho. Klotho has been detected in the circulatory system in both animals and humans, and has been shown to play a role in the attenuation of an aging phenotype (33). Epidemiological studies have demonstrated a strong relationship between plasma Klotho expression and skeletal muscle strength and functioning (86, 146). Semba and colleagues related plasma Klotho levels with grip strength in 805 adults greater than 65 years old, and they found that decreased grip strength was associated with lower plasma Klotho levels (86). Another study by Semba and colleagues measured plasma Klotho and knee extension strength in 2734 aged adults (71-80 years old) (146). They observed that older adults with higher baseline levels of plasma Klotho presented a more gradual decline in knee strength over time, when compared to those with lower baseline level of Klotho. These findings support the hypothesis that there is a strong relationship between Klotho expression and skeletal muscle vitality. However, our study is the first to suggest that Klotho expression may be critical for skeletal muscle regeneration and may be modulated by skeletal muscle contractile activity.

Following an acute muscle injury in young animals, we observed a significant increase in local Klotho expression at the site of acutely injured young skeletal muscle, but that this response is markedly attenuated with increasing age. Intriguingly, local expression was restored to youthful levels with the application of just two weeks of Estim. Still unclear is whether an increased local expression of Klotho within the injured muscle is concomitant with an increase in circulating Klotho levels. Avin et al recently provided preliminary evidence to suggest that, indeed, circulating Klotho levels are upregulated following an acute exercise bout (33). Specifically, young and old

animals trained for 45 min on the treadmill running and immediately after exercise, display an increase in circulating Klotho levels (85). Taken together, an interesting direction of future research would be to explore whether evaluation of circulating Klotho levels may be useful as a predictive biomarker of muscle healing capacity. If so, might Estim be indicated to enhance Klotho expression in cases where baseline levels suggest a less-than-optimal healing response? (147, 148).

Recent murine investigations have demonstrated the health benefits of increased circulating Klotho levels, and genetic overexpression of Klotho in mice results in a significantly improved healthspan and lifespan (65, 66, 149). Our findings that muscle contractile activity enhances Klotho expression may shed insight into a potential mechanism by which exercise has been shown to confer systemic anti-aging effects, such as on cognitive or cardiovascular functioning. As an example, exercise has been shown to reverse age-related declines in cognition, and to promote neurogenesis in the brain (150, 151). Recently a study from Dubal and colleagues demonstrated that increased levels of Klotho similarly improve synaptic and cognitive functions in human amyloid precursor protein (hAPP) transgenic mice (which simulate phenotypes characteristic of Alzheimer's disease). The results suggested that Klotho administration might be a promising treatment for Alzheimer's disease (151). This raises the intriguing question, could the beneficial effect of exercise on brain health be mediated by up regulation of Klotho?

Exercise also has well-established benefits in the preservation of cardiovascular function (152, 153). Likewise, Klotho is inversely related to cardiovascular pathology. Studies have demonstrated that absence of Klotho in murine models causes atherosclerosis, vascular calcification, and defects in angiogenesis, all of which are reversed by Klotho administration (154-157). Could exercise-induced increases in Klotho play a role in the beneficial effects of exercise on cardiovascular functioning?

The results of this dissertation study provide novel insights into the association between cellular senescence and Klotho in MPCs, as well as the influence of Estim to modulate the expression of senescent markers in aged MPCs. Although, muscle contractile activity and Klotho expression represents a fascinating relationship that may help us better understand the anti-aging effects of exercise, further investigations are necessary to better understand the molecular basis for these interactions. For example, it would be interesting to further explore the relationship of different age-associated pathways, such as Notch, Transforming Growth Factor Beta (TGFβ-1), and Mitogen-Activated Protein Kinase (MAPK), and how these may be regulated by Klotho expression in MPCs. A better understanding of Klotho signaling pathway may reveal a new understanding of aging declines and aging-related disease.

More broadly, these findings demonstrate that Estim may be a useful modality to increase stem cell regenerative potential, even while still within their native niche, and these results speak to the ability of rehabilitation modalities to tap into the intrinsic regenerative potential of resident stem cells. Of course, this study also brings new questions and new ideas to the forefront, and paves the way for cellular- and molecular-based investigations to guide clinical rehabilitation protocols. Despite the promising results, it should be noted that the Estim protocol used in this study was not optimized, and it is possible that a protocol with long duration or different intensity would further enhance the benefit of Estim on aged muscle healing capacity. Moreover, there are controversies in the literature related to the parameters and protocols of Estim used and the ability of murine responses to effectively mimic responses observed in humans. This potential disconnect may create confusion and a lack of general agreement regarding to specific parameters necessary for each specific pathology or treatment. Further translational studies investigating Estim protocol refinement are needed to determine the ability of Estim to enhance regeneration in a clinical setting.

Taken together, this study brings to light an important and needed intersection between the fields of rehabilitation and regenerative medicine. The results collected here suggest that Estim can be applied to people and modulates similar factors associated with physical exercise, and attenuates the functional decline with aging by improving muscle strength, activating satellite cells, and, finally, regulating muscle progenitor cell regenerative potential. We anticipate that these findings will be useful in the design of effective and novel therapeutic strategies to counteract aged skeletal muscle declines.

APPENDIX A

COMPLEMENTARY TABLES

Table 2 *In vivo* outcomes

Outcomes	YC	YI	AC	AI	AEI
TNF	130.7±7.9	215.1±18.3	123±3.7	103.8±8.8	233.4±12.6
CSA	229097±2837.9	195211.3±7320.9	224445.9±3190.4	77141±4360.2	187778.2±3988.2
Fibrosis	14.4±0.6	37±2.5	15.6±2.5	45.7±9.1	41.2±6.4
Klotho		88.19979±2.3		9.040618±1.4	74.49656±2.9

Values are Mean±SEM. Abbreviations: TNF (Total Number Fibers), CSA (Cross Sectional Area), YC (Young Control), YI (Young Injury), AC (Aged Control), AI (Aged Injury), and AEI (Aged Estim Injury)

Table 3 *In vitro* outcomes

Outcomes	YC	AC	AE
Desmin	68.1±8.4	32.6±7.4	65.9±10.9
Senescence	26.41171±1.959855	47.69041±7.629873	21.46615±1.989951
GSK-3	80.7±4.1	64±3.5	81.5±4.7
B-Catenin	689.62±216.13	6926.61±1000.80	779.67±75.56
Klotho	3349.30±467.86	607.98±124.0012	2496.17±173.7837

Values are Mean±SEM. Abbreviations: YC (Young Control), AC (Aged Control), and AE (Aged Estim)

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