Estradiol deficiency impairs satellite cell function and causes muscle weakness via an estrogen receptor alpha mediated mechanism

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Dedication

I dedicate this dissertation to my parents for their constant support and unconditional love throughout this entire academic endeavor. They have always told me to strive for greatness and that nothing was impossible. They gave me everything I needed to succeed in life and I am forever grateful for all they have sacrificed for my dreams.

Abstract

Overall, my dissertation work has shown that estradiol is a critical extrinsic factor in females that regulates muscle stem cell (i.e. satellite cell) and skeletal muscle function (Chapters 3 and 4) and estrogen receptor alpha (ERα) is the main receptor estradiol utilizes for these functions (Chapters 3 and 4). I identified that the loss of ovarian hormones resulted in impaired satellite cell functions such as maintenance and self-renewal, while estradiol treatment rescued the detrimental effects on satellite cell maintenance and self-renewal (Chapter 3). Further experiments utilized a transgenic mouse that specifically ablated ERα in satellite cells, the results of which indicated that ERα is necessary for proper satellite cell function (Chapter 3). In agreement with my work on satellite cells, I identified that ERα is necessary for overall skeletal muscle function (Chapter 4). I utilized a transgenic mouse model that deleted ERα specifically from skeletal muscle fibers which resulted in impairments in strength, power, and fatigability of skeletal muscle (Chapter 4). The work of my dissertation highlights a novel mechanism for estradiol and ERα in skeletal muscle.

Table of Contents

List of Tables

List of Figures

Chapter 1

Introduction

The loss of skeletal muscle strength with aging is disadvantageous to the ability to carry out activities of daily living. This leads to an increased incidence of falls and recurrent injury which in turn affects strength and function [1-3]. Both men and women experience the detriment of strength loss with aging, but at differing rates [3, 4]. The sex difference is most robust at the time women experience menopause, and has been associated with the simultaneous loss of the sex hormone, estradiol. It is also suggested that cycles of muscle injury followed by inadequate repair contribute to muscle weakness in the aged [2, 3]. Most importantly, estradiol has been implicated to play a role in the inflammatory and regenerative processes following injury [5, 6]. Aged female mice and young adult estradiol-deficient mice have incomplete recovery of strength following contraction-induced skeletal muscle injury [7]. The reason why regeneration of injured muscle is impaired with estradiol deficiency is still not clear and is the emphasis of my dissertation.

The main focus of my dissertation is on investigating mechanisms whereby estradiol affects muscle regeneration. The remarkable regenerative capacity of skeletal muscle is greatly dependent on the activation, proliferation, differentiation, and self-renewal of muscle stem cells (i.e. satellite cells). The reason why regeneration of injured muscle is impaired with estradiol deficiency is not clear, but I hypothesize that satellite cell maintenance is involved. Satellite cell maintenance refers to the ability of the cell to appropriately balance quiescence, self-renewal and commitment to ensure life-long preservation of skeletal muscle. Investigating the mechanisms of why the satellite cell pool

becomes depleted with aging has become an important question since it has been shown that satellite cells from aged mice function the same following injury as those from young mice [20, 21]. Yet, we know that in aged muscle regeneration following an injury is impaired [24-26], but the reasons are still not understood. There has been speculation that the environment is not optimal and therefore causes intrinsic changes in the satellite cells such that they no longer self-renew and the satellite cell pool becomes depleted due to a loss in quiescence, increased susceptibility to apoptosis, and increased cellular senescence [12, 24, 26]. The concept of circulating factors impacting satellite cell function has been shown by studies in mice using heterochonric parabiosis [8-10]. These classical studies primed the field to discover factors in the young and old environment that alleviate the effect of age on satellite cells [11, 12]. Such studies support the concept that sex hormones (i.e. estradiol), which differ between the young and old environment in females and their subsequent signaling pathways, may contribute to age-associated decrements in satellite cell maintenance and muscle regenerative capacity.

The following sections summarize the current understanding of 1) skeletal muscle biology related to regeneration 2) skeletal muscle aging 3) estradiol effects on skeletal muscle regeneration. The first aim of this dissertation is to determine the mechanism(s) whereby estradiol deficiency impairs satellite cell function. The second aim is to determine the role of estrogen receptor α in muscle regeneration following injury. The third aim is to determine the role of estrogen receptor α in muscle strength. Thus, the studies in this dissertation have been conducted to test the overarching hypothesis that an estradiol

deficient environment negatively impacts satellite cell function through impairment of maintenance and self-renewal via an ER-α mediated pathway.

Chapter 2

Literature Review

2.1 Skeletal muscle components

Skeletal muscle is the largest organ in the human body and responsible for limb movements, as well as providing the body with the majority of its energy consumption [13]. Skeletal muscles are striated, composed of multi-nucleated cells with a microstructure and macrostructure which all together establish the structural hierarchy of skeletal muscle (Figure 1). Skeletal muscle tissue is composed of numerous fascicles of muscle fibers. Muscle fibers are composed of myofibrils; the largest functional unit of skeletal muscle [13]. Myofibrils are composed of sarcomeres in series. The sarcomeres are composed of thin (actin) and thick (myosin) filaments. The sarcomere is the home of the cross-bridge which powers muscle contraction [13]. Since skeletal muscle cells are highly specialized to produce force and movement, the cellular components are also highly specialized. Muscle fibers are cylindrical in nature surrounded by scaffolding known as the sarcolemma which surrounds the basal lamina [13]. Skeletal muscle fibers are surrounded by the endomysium, made of connective tissue in which the fiber is intimately associated [13]. As stated above, muscle fibers are multi-nucleated with the myonuclei located at intervals along the cell length. The myonuclei are responsible for the cellular material that is distributed throughout the fiber. There is evidence to support that myonuclei form internuclear communications while retaining some degree of autonomy. The sarcoplasmic reticulum in skeletal muscle is associated with the myonuclei and responsible for the transportation of proteins as well as having specialized functions in muscle contraction,

namely storage and release of calcium [13, 14]. Skeletal muscle fibers house mitochondria, responsible for cellular metabolism and produce energy in the form of adenosine triphosphate [13, 15].

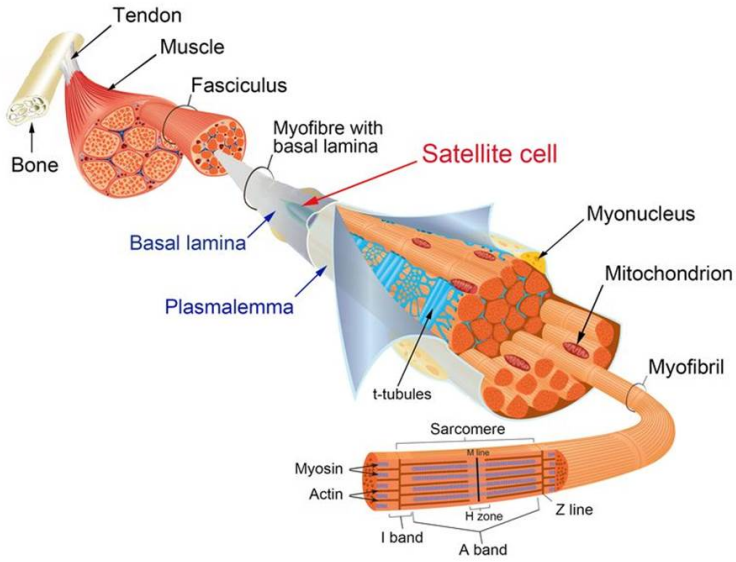

Figure 1. Components of skeletal muscle. Skeletal muscle is composed of a specialized hierarchy that is made up of myofibrils that are packaged with myofibers that contain all the supporting structures of muscle; adapted from [16].

2.2 Embryonic myogenesis

Skeletal muscle (limb, diaphragm, and body wall) is derived from the paraxial mesoderm cells originating from somites in the myotome [13, 17, 18]. During myogenesis, the specialization of the mesoderm precursor cells to the myogenic lineage is regulated by permissive and repressive signals from various surrounding tissues, leading to Pax3/pax7 expression and MyoD/Myf5 expression. The basic helix-loop-helix (bHLH) family of transcription factors including myoblast determination protein (MyoD), myogenic factor 5 (Myf5), muscle-specific regulatory factor 4 (MRF4), and myogenin are responsible for the cascade of signaling events involved in muscle development [13, 17]. These factors regulate gene expression through forming DNA-binding complexes with other downstream bHLH transcription factors. It was shown through single and double knockout mice that Myf5 and MRF4 lie upstream of MyoD and myogenin, respectively [17]. Myf5, MyoD, and MRF4 are considered to be determination genes involved in the undifferentiated proliferating myoblast cells [17, 19], while, myogenin is necessary for the terminal differentiation of myoblasts [17, 19]. These core myogenic factors are expressed in all muscle cells, but the upstream signals of these factors differ between the various anatomical locations [17]. Overall, clusters of myoblasts fuse to form primary myotubes and the nuclei of the cells are forced to the periphery of the fiber in order for the formation of the sarcomeres to form secondary myotubes [13, 17]. Secondary myotubes mature into muscle fibers and the motor nerve forms the neuromuscular junction [13, 17].

2.3 Satellite cells

During myogenesis some myoblasts fail to fuse together and remain closely positioned to maturing myotubes [16, 17, 20]. These cells become encased by the basal lamina of mature myofibers hence the name satellite cell [17]. The satellite cell was first identified in 1961 using electron microscopy by Alexander Mauro [13, 15-17, 21, 22] (Figure 2). Satellite cells are responsible for generating myoblasts in postnatal skeletal muscle during basal turnover and following injury [16, 20]. These muscle progenitor cells have been inextricably linked to the paired box transcription factor 7 (Pax7) [23-26]. Pax7 is solely expressed in quiescent and activated satellite cells in adult skeletal muscle and inactivation of Pax7 results in depletion of satellite cells [20, 23-27]. In brief, satellite cells are activated through an external stimulus (e.g. injury) proliferate into myoblasts [27]. A subpopulation of activated satellite cells does not commit to terminal differentiation and re-enters quiescence to replenish the satellite cell pool [20, 27]. Quiescence does not necessarily equate to dormancy for satellite cells; recent evidence suggests they contribute to muscle fibers in the absence of injury, and may be required for the life-long maintenance of skeletal muscle [28, 29]. However, the mechanism that signals for the contribution to muscle fibers as well as the reason for contribution is still not known. But, this gives rise to the concept that satellite cells have functions in adult skeletal muscle beyond skeletal muscle repair following injury.

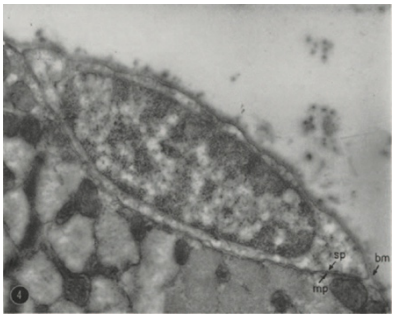

Figure 2. The first identified satellite cell. The electron micrograph of a satellite cell visualized by a transverse muscle section from a rat sartorius muscle (adapted from Alexander Mauro's 1961 paper; [22]).

Muscle satellite cells depend on a specialized environment termed the "niche" [17, 27, 30-32]. This environment is the compartment in which the satellite cells are provided support for self-renewal while preventing them from differentiation which is critical for life-long maintenance [16, 17]. The niche is also responsible for the signaling (e.g. Wnt and Notch pathways) that activates the satellite cells to commit to the muscle lineage [20, 31, 33, 34]. In their niche, satellite cells sit in close proximity to capillaries, known to be critical for supply of essentials such as growth factors (e.g. vascular endothelial growth factor) and soluble proteins (e.g angiopoietin 1) [17, 35, 36]. Additionally, numerous extracellular matrix components and receptors are present either on the surface of the sarcolemma, satellite cell, or contained within the basal lamina which allows for rapid changes in satellite cell behavior [37]. It is becoming more and more apparent that satellite cells are dependent upon their systemic environment and niche and disruptions to these extrinsic factors can highly impact satellite cell function [17, 20, 30, 31].

2.4 Skeletal muscle regeneration

Skeletal muscle is remarkable in the fact that it has the ability to repair itself after damage whether it is from exercise, disease, or injury. This process is called muscle regeneration. Muscle regeneration involves complex regulatory pathways in order to have a well-innervated, fully vascularized, contractile skeletal muscle fiber. The regeneration process is broadly characterized by two phases; a degenerative phase and regenerative phase.

2.4.1 Degenerative phase

Initially, there is an injurious event that disrupts the structure of the sarcolemma resulting in myofiber permeability [16, 20, 25-27, 38, 39]. This leakage of muscle proteins, such as endogenous enzymes from lyososomes, triggers the activation of inflammatory cells at the damaged site [16, 20, 25-27, 38, 39]. Neutrophils are known to be the first cells to invade the injury site and are responsible for chemotaxis of other neutrophils, cytokines, and myogenic cells (Figure 3) [26, 38, 40-42]. Macrophages succeed neutrophil infiltration and predominantly phagocytose cellular debris (Figure 3) [26, 38, 41]. Recent evidence has established that the infiltration of pro-inflammatory macrophages (M1) plays a role in the induction of myoblast cell proliferation, but represses differentiation (Figure 3) [26, 38]. Specifically, cytokines such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) have been implicated in promoting cell cycle progression and destabilizing transcripts needed for muscle to terminally differentiate [26]. As necrosis disappears from the injury site the macrophages

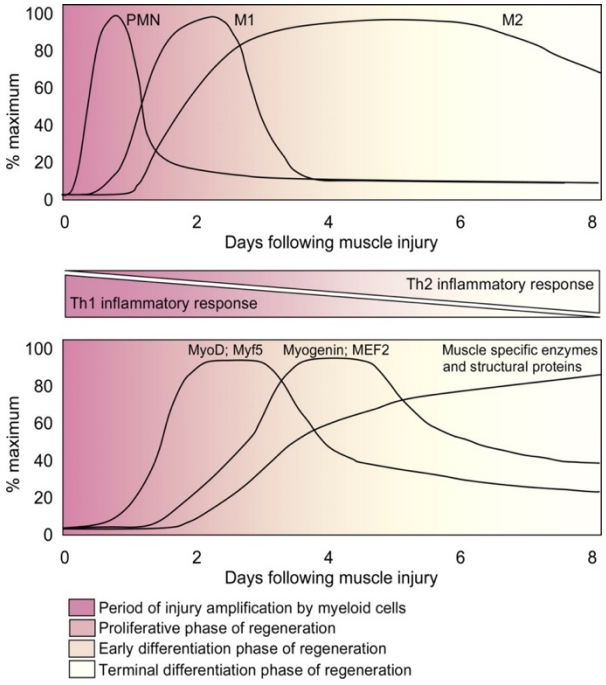

Figure 3. Interaction between the inflammatory process and myogenic response following injury. Time course of changes in myeloid cell populations (top) and changes in the expression levels of muscle-specific transcription factors, enzymes, and structural proteins in muscle (bottom) following injury; adapted from [26]. Th1, pro-inflammatory; Th2, anti-inflammatory. PMN, neutrophils; M1, M1 macrophages; M2, M2 macrophages.

are in a more anti-inflammatory state (M2) and anti-inflammatory cytokines dominate (IL-10) which can stimulate fusion of muscle cells (Figure 3) [38]. In fact, muscle of M2 null mice displayed delayed regeneration and growth after cardiotoxin injury, however, when transplanted with wild-type mice bone marrow the M2 invasion was restored along with muscle regeneration [26]. Additionally, this injury- induced inflammatory response is responsible for the release of growth factors that assist in the muscle repair process. These growth factors include fibroblast growth factor (FGF), insulin-like growth factor 1 (IGF-1), and hepatocyte growth factor (HGF) and each play a specific role in the regeneration process [20, 26, 30]. FGF and HGF are known to promote proliferation of myogenic cells by inhibiting MyoD, while IGF-1 promotes differentiation through the activation of the translation factor 4E binding protein (4E-BP) [26, 33, 43].

2.4.2 Regenerative phase

The regenerative phase is the second phase of muscle regeneration. It includes the involvement of growth factors, as well as, many other extrinsic factors. The regenerative phase is highly dependent upon the degenerative phase that precedes it and as stated there is recent evidence to show that the two phases are inherently linked with one another [26, 38, 41, 42]. The cellular proliferation and differentiation that occurs in muscle regeneration in essence mimics embryonic myogenesis, however, the microenvironment in which muscle regeneration takes place vastly differs from embryonic myogenesis. The capacity for muscle to regenerate following injury is primarily due to the mononuclear Pax7 positive satellite cell population that resides beneath the basal lamina in skeletal muscle (Figure 4) [16, 21, 24]. These normally quiescent cells are activated following damage to the muscle tissue, re-enter the cell cycle, and proliferate to form a source of myogenic cells that differentiate into myofibers to repair the tissue [16, 20, 25-27, 33, 44]. The cellular proliferation and differentiation of myogenic cells during muscle regeneration are regulated by the same bHLH proteins in embryonic myogenesis along with members of the ubiquitous E2A and myocyte enhancer factor-2 (MEF2) families [19]. In concert with one another, these proteins are known to regulate the commitment to the myogenic lineage and the terminal differentiation program by inducing transcription of regulatory and structural muscle-specific genes.

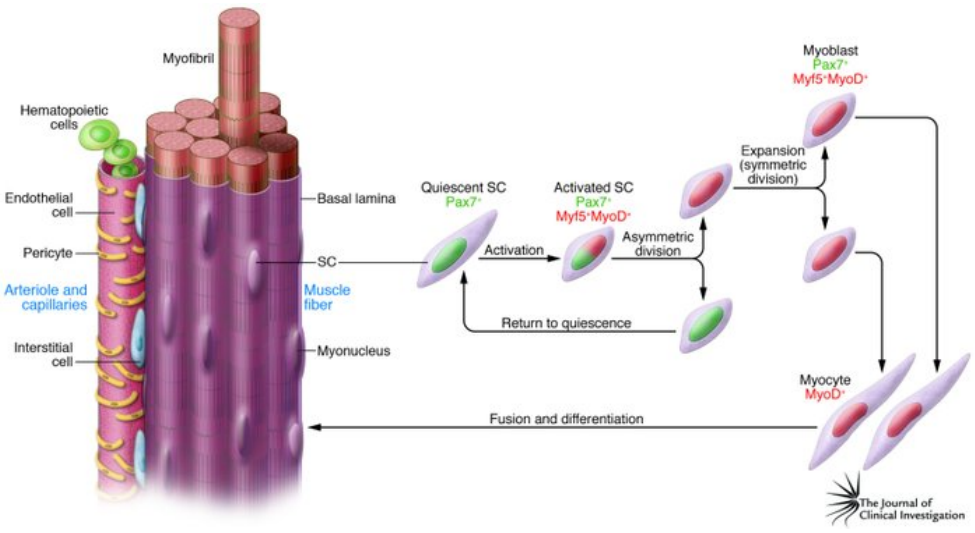

Figure 4. Satellite cell activation pathway. Once activated, satellite cells (SC) undergo assymetric division. This assymetric division allows for the commitment to the myogenic lineage as well as replenishment of the satellite cell pool; adapted from [45].

Once satellite cells are no longer quiescent and enter the cell cycle they are termed myoblasts and express transcription factors MyoD and Myf5 (Figure 6) [16, 19, 20, 25, 26]. Phosphorylation of MEF2 proteins is known to potentiate the activity of MyoD and in turn promote the differentiation program of muscle cells (Figure 4) [19]. Myogenin and MRF4 become active once the cells exit the cell cycle and enter the differentiation stage governing the transition of myoblasts to myocytes and myocytes to myotubes [19, 20, 46]. Lastly, muscle-specific genes (e.g. α-actin, myosin heavy chain) are expressed at high levels in order for the muscle cell to differentiate into a mature muscle fiber [19, 20, 26]. In addition to the cell proliferation and differentiation, a minor fraction of the activated satellite cells divide and give rise to cells that do not express MyoD and only express Pax7 [20, 27]. There has been recent evidence that quiescent cells may also express Myf5 [37]. These cells revert back to a quiescent state and replenish the satellite cell pool (i.e. self-renewal) which is essential for continuous regeneration throughout a lifetime [16, 27, 34].

There have been several studies that have aimed to discover the signaling pathways that are interconnected with MRFs, the master regulators of satellite cells and muscle regeneration. Most of the attention has been to the p38α/MAPK pathway which is known to be a positive regulator in myogenic differentiation [19, 46-48]. Liu et. al., used comparative expression profiling of p38α/MAPK and myogenin to identify that in the absence of p38α, myogenin inhibited cell cycle progression, meaning myoblasts were unable to form multi-nucleated myotubes. Muñoz-Cánoves and colleagues also established that p38α had a defining role in myoblast fusion through the regulation of NF-kB signaling and was a potent activator during the differentiation of myocytes [47].

2.5 Skeletal muscle aging

2.5.1 Age-related muscle loss

It is well known that skeletal muscle mass is lost at a rate of approximately 1% per year past the age of 50 [1, 13, 15, 49, 50]. The loss of skeletal muscle mass is attributed to the loss of skeletal muscle fibers, type 1 and 2 [1, 34, 49, 51]. Muscle fibers contain the functional apparatus of skeletal muscle and therefore the loss of fibers translates to a loss of function. The loss of function is attributed to the decline in force production and muscle power [1, 49]. The decline in skeletal muscle mass paired with the decline in force production is known as sarcopenia, which is one of the major causes of loss of mobility with age [1, 49]. These changes lead to increased risk of falls and frailty seen with increasing age [52].

Denervation of muscle fibers is a major cause for loss of fibers with sarcopenia. [13, 51]. The remaining intact motor units recruit the denervated fibers which may change their fiber-type as an adaptive response to the increased burden of work on the motor units that have survived [1, 13, 49]. The consequence of re-innervation is fewer but larger motor units overall resulting in major decrements on muscle power and movements [49]. Fibers that do not get re-innervated die, contributing to the decrease in muscle mass and function.

In addition to the loss of motor units, there is fiber atrophy; loss of fiber cross-sectional area that contributes to sarcopenia. Historically, it was understood that type 1 muscle fibers were less sensitive to atrophy than type 2 fibers, however, this finding has since been refuted [51]. It was shown that both type 1 and type 2 fibers undergo severe

atrophy as a result of age. Carter et. al. [51] recently discovered that type 2 fibers atrophied 60% in rat soleus muscle and 31% in the rat gastrocnemius while type 1 fibers atrophied 40% and 38% in the soleus and gastrocnemius, respectively. The loss of cross-sectional area in muscle fibers contributes significantly to the loss of skeletal muscle mass and force production. Carter et. al. [51] showed that when normalized for muscle mass, tetanic force declined by 58% and 36% in rat soleus and gastrocnemius muscle, respectively. These results redefine the impact of aging on type 1 fiber atrophy and emphasize the importance of addressing the atrophy in both fiber-types.

2.5.2 Age-related strength loss

Another consequence of aging skeletal muscle is the decline in muscle strength that happens at a greater rate than the decline in muscle mass described above [1, 49, 53]. This is supporting evidence that there are additional contributors other than the loss of muscle mass that impact the strength loss observed with aging. Specifically, changes in the contractile unit of skeletal muscle associated with the loss of force production has been demonstrated [54]. In brief, the interaction of actin and myosin through a strong binding state forms a cross-bridge which in turn causes the muscle to contract, thus producing force [13]. However, with aging there is a reduction in the strong binding of myosin during muscle contraction, resulting in a lower force production from the muscle fiber [54, 55]. Additionally, there is evidence supporting that the rate of cross-bridge cycling is decreased in the aged, as well as, a decrease in the amount of myosin protein [56]. These age-related changes that are associated with strength loss are observed in both sexes, with strength

notably declining at 50 years of age and older [4]. At this time-point there is an even more rapid decline in muscle strength in women [4], which will be discussed in the next chapter.

It is important to note that the majority of the human studies have used a cross-sectional study design. With aging comes high variability therefore cross-sectional studies may underestimate the strength deficits observed with aging and longitudinal studies should be used to evaluate these measures. However, longitudinal studies in this area are scarce and the few that exist focus on males [57, 58]. Since we know that muscle strength in women declines at a more rapid rate around the age 50, it suggests the mechanisms by which each sex loses skeletal muscle strength with age differ.

2.5.3 Age-related decline in regenerative capacity

The regenerative potential of skeletal muscle is impaired in aged skeletal muscle [2, 3, 7, 59]. The inability of the skeletal muscle to fully recover from damage plays a major role in the age-related loss of muscle mass and force production [2, 13, 27, 31, 49]. Specifically, there are changes in the satellite cell environment that impair homeostasis and regeneration of skeletal muscle. These changes in the satellite cell environment can include the dysregulation of growth factor release which causes an imbalance in the Notch and Wnt signaling pathways [27, 30-33, 44, 60].

It has been shown that with aging, Notch signaling is repressed while Wnt signaling is activated tipping the balance of cells toward fibrogenesis, not myogenesis causing an impaired regenerative potential of the skeletal muscle [30, 32]. Specifically, FGF signaling is increased in homeostatic aged skeletal muscle. This directed signaling from the aged

satellite cell niche leads to a loss of quiescence and self-renewal, ultimately depleting the resident satellite cell population in uninjured skeletal muscle [30, 48, 61]. However, satellite cells that express sprouty 1 inhibit FGF signaling and retain their quiescent phenotype and normal satellite cell function [30]. The dysregulation of the FGF/Sprouty1 axis explains the difficulty of satellite cells from aged mice to maintain quiescence, making one of the first connections of changes in the satellite cell niche with satellite cell autonomous function. In addition, there is heightened pro-inflammatory signaling with advancing age, through cytokines and stress signaling proteins, that may contribute to a reduction in myogenesis [2, 26, 32, 62, 63].

Along with extrinsic signaling processes, intrinsic signaling has been attributed to modulation of satellite cell function and impairing regeneration in aged muscle. As with many other cells in the body, intrinsic aging effects of satellite cells are associated with accumulation of oxidative damage, altered metabolic properties, increase in apoptotic factors, and impaired autophagy [32, 34, 64, 65]. For instance, aged satellite cells have an increased expression of atrophy-related FOXO-regulated genes and altered expression of genes related to protein folding and mitochondrial function [32, 34, 63-67]. Recent publications have introduced evidence that a cell-autonomous increase in the activity of the p38α/MAPK pathway leads to an impairment of the self-renewal capacity of aged muscle stem cells that causes cell senescence by dysregulation in $p16^{INK4a}$ signaling [48, 68, 69]. This cell autonomous signaling of senescent pathways was not reversed when aged cells were transplanted into a young host environment [68]. However, it must be noted that the aged cells were considered “very old” in mice terms and irradiation was not used in the host

mice which means there could be competition from host cells in this experiment. Despite this, these studies give evidence that there may be age-related changes with satellite cells that are not dependent upon the niche in which they reside.

Another important point to consider is the majority of the literature on age-related decline in regenerative capacity has been completed in male rodents. This is important because sex differences in the regeneration of skeletal muscle following injury have been reported [59, 70, 71]. As discussed above, aging itself plays a part in the regenerative process, as all aged mice have an incomplete recovery of strength following injury, however, the strength recovery is worst in the old females [7, 59, 72]. This point will be discussed further in the next section.

2.6 Estrogen and skeletal muscle

2.6.1 Estrogen and estrogen receptors

Estrogens are the main female sex steroid hormones synthesized predominantly in the ovaries, however, can be produced locally in some tissues (e.g. brain, arterial, adipose) [73-75]. There are three endogenous estrogens: estrone (E1), estradiol (E2), and estriol (E3). Estradiol (E2) is the primary form in premenopausal women while E1 is predominant in postmenopausal women and E3 is predominant during pregnancy [75]. The most biologically active form of E2 in the body is 17β-estradiol [73-75]. The plasma concentration of 17β-estradiol in premenopausal women can range from 0.1-2.2 nM depending on the phase of the 28 day menstrual cycle [75]. The local concentrations in specific tissues can be much higher than the plasma levels. 17β-estradiol is hydrophobic; therefore, it can passively diffuse through cell membranes to reach intracellular targets (i.e. estrogen receptors).

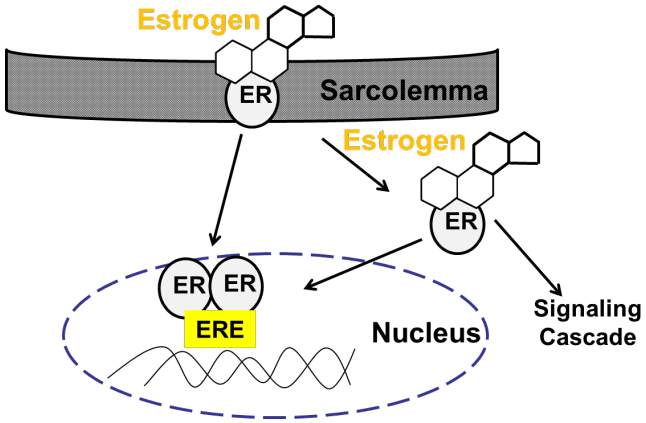

Figure 5. Estradiol signaling through ER's. Estradiol signals through genomic or non-genomic mechanisms throughout the body.

The main action of 17β-estradiol is thought to be through estrogen receptors (ER). There are three known ER's: ER-α, ER-β, and G-protein coupled receptor (GPER) [73-76]. ER-α is the first and best described ER followed by ER-β and GPER, respectively [73-75]. ER-α and ER-β are primarily nuclear transcription factors that bind estrogen response elements (ERE) in the promoter and enhancer regions of genes, however, they

can shuttle between the cytoplasm and the nucleus [75, 76]. In addition to the classical mechanism of genomic signaling (that is, gene transcription and subsequent protein synthesis), 17β-estradiol has non-genomic mechanisms (Figure 5). These non-genomic mechanisms are associated with the activation of numerous protein-kinase cascades that can lead to indirect changes in gene expression due to phosphorylation of transcription factors [75] (Figure 5). This form of 17β-estradiol signaling can be mediated by the membrane bound forms of ER-α and ER-β, as well as the more recently identified GPER [74, 75] (Figure 5). GPER is a transmembrane protein that is thought to be involved in many of the extracellular signal regulated kinase signaling pathways [74]. Each of these receptors has been identified in numerous tissues throughout the body, including skeletal muscle [74-77]. 17β-estradiol has also been shown to have effects in many of the physiological systems of the body (i.e. reproductive system, immune system, nervous system, cardiovascular system, and musculoskeletal system); however, the mechanistic actions seem to be highly tissue specific and not fully understood.

2.6.2 Estradiol and aging

17β-estradiol levels in women fluctuate depending on the phase of the menstrual cycle up until menopause. The majority of estrogens in premenopausal women are synthesized in the ovaries while in postmenopausal women and men estrogens are solely produced in extragonadal tissues such as breast adipose tissue.[75, 78, 79]. The clinical definition of menopause is 12 consecutive months without menstruation. The menopausal transition is an on-going process defined as the stage of ovarian aging and starts approximately at the age of 46 in women and lasts around 6 years. The last menstrual cycle (menopause) occurs at an average age of 51 years in women. During this transition there are many changes in the levels of several hormones in which estrogen is the most drastic. In postmenopausal women 17β-estradiol plasma levels decline to 0.04 nM or less [75]. This decline in 17β-estradiol levels is known to adversely affect numerous tissues (e.g. bone, adipose).

Mice also have a similar fluctuation of estradiol levels which is termed the estrous cycle and lasts 4-5 days [80, 81]. The estrous cycle begins at sexual maturation (3 months of age) and continues until ovarian senescence (18-20 months of age) [81, 82]. The estrous cycle in mice is similar to the menstrual cycle in that estradiol, progesterone, inhibin, and follicle stimulating hormone levels fluctuate in a regular, cyclic manner [80, 81, 83, 84]. As in menopause, female mice undergo ovarian aging with irregular cycling and concomitant decline in estradiol levels [85]. However, it must be noted that a percentage of mice transition into a polyfollicular anovulatory state of constant estrus which is characterized by a sustained, high levels of estradiol levels that can last 10-110 d before transitioning to the anestrus state with consistent low levels of estradiol [85]. Ovarian

senescence is similar to that of humans in that the driving force for this transition is reduction in the number of follicles [81, 83-86].

The ovariectomy mouse model is commonly used to mimic menopause in adult female mice [5, 77, 87-94]. This approach allows for the study of estradiol deficiency without the confounding factors associated with aging. However, this induction of ovarian senescence involves an abrupt reduction in estradiol levels while the menopausal transition in women involves fluctuating levels of estradiol followed by a relatively slow decline in the later stages [83-86]. Some limitations of the ovarian model are that outcomes derived from short-term ovariectomy do not necessarily generalize to long-term ovarian hormone deprivation as well as the removal of the ovaries does not always generalize to adaptive responses in the body that occur with menopause [85]. Despite these translational barriers, ovariectomy is still the most widespread model of menopause in preclinical translational research [85]. Therefore, a common approach used by our lab and in this dissertation work is the ovariectomy model to study the effects of estradiol deficiency and the replacement of estradiol by implantation of a time release 17β-estradiol pellet in order to mimic hormone replacement therapy in women [5, 77, 87-93, 95-98].

2.6.3 Estradiol and skeletal muscle strength

Skeletal muscle of females is even more affected by age, compared to males, due to the simultaneous loss of ovarian hormone production. Muscle strength of females declines at a robust rate around the time of menopause [4, 99, 100]. Estradiol-based hormone therapy in post-menopausal women has been shown to maintain muscle strength [4, 101, 102] (Figure 6). This is further established by a meta-analyses showing that post-menopausal women on estradiol-based hormone therapy are stronger than those not on hormone therapy and this effect is also true in rodent models [103]. As discussed previously, muscle force-generating capacity is possible through the strong-binding of myosin to actin. Therefore, it was hypothesized that estradiol influenced muscle strength by directly affecting the contractile apparatus, specifically myosin. Our lab's initial studies used electron paramagnetic resonance, which discovered there was a reduction in the strong-binding of myosin to actin with estradiol deficiency and it was restored with estradiol treatment [93, 95, 104]. Moreover, this mechanism has since been confirmed in aged women as force-generating capacities and myosin function was better in single, permeabilized fibers from those on hormone replacement therapy to those not on hormone replacement therapy [105]. Current

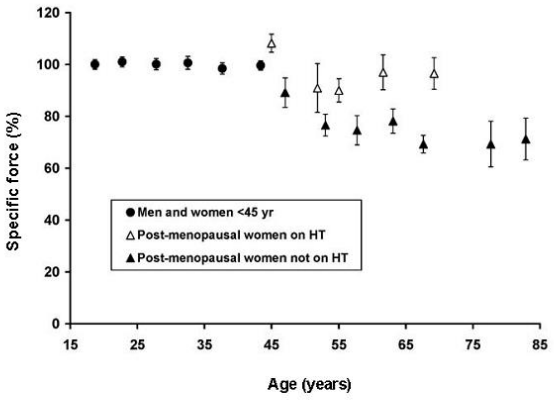

Figure 6. Estradiol maintains muscle strength in women. Hormone therapy (HT) restored muscle strength (specific force) in post-menopausal women; adapted from [4]

efforts in our lab include elucidating the mechanism(s) in which the strong-binding of myosin is altered thereby impacting force generation, i.e. muscle strength. One myosin-based mechanism is estradiol modulation of phosphorylation of the regulatory light chain [106]. Further research in our lab has been focused on identifying the primary estrogen receptor for estradiol's effect on skeletal muscle strength. The majority of our efforts have emphasized ERα, as it is the most abundant ER in skeletal muscle and is the receptor estradiol utilizes for its effects on skeletal muscle metabolic and mitochondrial function [77, 107-109].

2.6.4 Role of estradiol following skeletal muscle injury

The existence of sex differences in the process of skeletal muscle regeneration are not a new concept and was first demonstrated in the 80's using serum creatine kinase (CK) levels as an indirect marker of muscle injury. Cumulatively, the results of those studies found that CK leakage was increased in male and female rats after eccentric contraction induced injury, however male and ovariectomized (OVX) female rats showed elevated levels when compared to ovary intact female rats [88, 110, 111]. These initial findings were the first to suggest that female sex hormones may play a vital role in protecting muscle membranes from damage. Since then, it has been shown that CK leakage is not the best marker for comparisons for muscle damage because the values can be arbitrary and highly variable between subjects. However, there has been little effort to determine if sex hormones, such as estradiol, influence membrane stability and therefore provide a protective mechanism against damage.

A recent study from our laboratory is the only study to date that has shown a functional impact of estradiol deficiency on skeletal muscle strength following injury [112] (Figure 7). In this study, we showed that ovarian hormone deficiency impaired the functional recovery of muscle strength 14 and 21 days following eccentric-contraction induced injury [112] (Figure 7). The majority of the ovarian hormone effects in skeletal muscle research up until our lab's study have been on energy homeostasis and overall skeletal muscle strength, not on skeletal muscle recovery following injury.

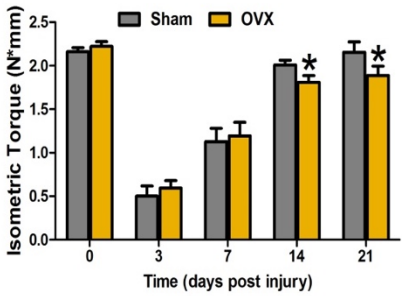

Figure 7. Incomplete recovery of muscle strength occurs with ovarian hormone deficiency. Our results from ovariectomized mice (OVX) show a 20% deficit in strength recovery 14 and 21 days post-injury; adapted from [112].

2.6.5 Role of estradiol in skeletal muscle inflammation following injury

The evidence of estrogen modulating the degenerative phase or inflammatory response preceding muscle regeneration after damage has not been heavily studied, but thus far shows very conflicting results. As mentioned above neutrophils are the first to invade an injured area, followed by phagocytic macrophages then non-phagocytic macrophages. Estradiol has been shown to affect this cycle specifically at the point of neutrophil response. Multiple studies, mostly from Tiidus and co-workers, have demonstrated that neutrophil response is exacerbated by estradiol deficiency (Ovx) and can be attenuated by estradiol treatment [6, 96, 113-115]. These studies concluded that although neutrophil infiltration to skeletal muscle following injury is an essential process for muscle regeneration they lack

the ability to differentiate between "good" intact tissue and "bad" damaged tissue; therefore, the increased neutrophil response seen in Ovx rodents is thought to be harmful not beneficial [6]. In contrast, preliminary experiments in our lab have shown a blunted response of neutrophils in Ovx mice compared to estradiol-treated (Ovx+E_2) [116]. Our interpretation of the blunted neutrophil infiltration following traumatic muscle injury is that estradiol deficiency causes an altered inflammatory response resulting in a delayed or incomplete regeneration of skeletal muscle. Major differences in our studies and Tiidus' included the type of muscle injury as well as the time-points used following the injury. Tiidus and co-workers consistently use downhill running as a method of exercise-induced skeletal muscle damage while we use freeze-injury as a method of traumatic muscle injury [6]. It is well established that each method of skeletal muscle damage triggers similar regeneration pathways, but they also differ in major ways [41, 42]. For example, the regeneration timeline for a freeze injury is prolonged compared to eccentric-contraction induced injury which is similar to downhill running in mice [42]. Although these different methodologies have been used, the estradiol influence on the transition that occurs in macrophages during regeneration is more consistent. Our lab along with others has found that estradiol deficiency blunts the activation of anti-inflammatory macrophages (M2) and cytokines as well as elevating pro-inflammatory cytokines [96, 117-119]. Similar to neutrophils, macrophage response can be harmful in some circumstances, but the entire process is necessary for a complete regeneration process of skeletal muscle. Some studies have shown that if the inflammatory process is negatively affected, then the myogenic response during regeneration is consequently negatively affected [26, 38, 42, 120].

2.6.6 Role of estradiol in skeletal muscle regeneration following injury

Studies from Tiidus and co-workers have indicated that satellite cell activation and proliferation is lower in muscle of Ovx compared to Ovx+E_2 rats 72 hours after exercise-induced damage using immunohistochemical methods for detecting BrdU, Pax7, and MyoD [5]. Velders et. al. [121] reported similar results 3 days post notexin injury in that MyoD and proliferating cell nuclear antigen (PCNA) were significantly low in muscle of Ovx rats and estradiol treatment rescued this decrement. Velders et. al. also showed that measurements of differentiation (e.g. embryonic myosin heavy chain and IL-10) were low in notexin-injured muscles of Ovx rats and levels were restored with estradiol treatment. Additionally, *in vitro* studies using C2C12 muscle cells provide additional evidence for estradiol regulation of proliferation as well as the early phases of differentiation of myoblasts [122-124]. Specifically, there is an estradiol dose-dependent increase in BrdU incorporation [124], myogenin protein expression [122], and myosin heavy chain protein expression [122]. Similarly, cultured bovine satellite cells incorporated ^{3}H-Thymidine, a measurement of cell divisions (i.e. proliferation), in the presence of estradiol [125]. It is also established that estradiol positively impacts muscle growth by stimulating protein synthesis through increasing immediate early genes [124], growth factors [126, 127], and AKT pathway signaling [122]. Studies in post-menopausal women with hormone replacement therapy have showed a decrease in protein degradation [125], abrogation of apoptosis [128-131], and induction of differentiation through MRF's in skeletal muscle compared to those not on hormone therapy [132]. Taken together, there is substantial evidence that estradiol impacts skeletal muscle regeneration as well as affecting pathways

of skeletal muscle growth and atrophy which have major implications in muscle regeneration. Although, some mechanisms for estradiol action in regeneration have been uncovered, there are still many unknowns such as how satellite cell function is altered with estradiol deficiency. Furthermore, studies with better methods of measuring satellite cell number, proliferation, and differentiation *in vivo* are necessary.

2.6.7 Role of ERs in skeletal muscle regeneration

Although there has been some progress in understanding the effects of estradiol on skeletal muscle regeneration, there have only been a few studies that address whether the effects are ER-mediated. In many other tissues, there is evidence that estradiol works through ERs; therefore, it follows that at least some of the changes in skeletal muscle could be from ER involvement. As already discussed, there are three known estrogen receptors in skeletal muscle ER-α, ER-β, and GPER [74-77, 89, 121, 122, 125, 126, 128, 133-135].

The studies involving ERs in skeletal muscle regeneration are somewhat limited and the majority have only been completed with *in vitro* models. For instance, Dayton and co-workers have established that estradiol enhancement of protein synthesis and inhibition of protein degradation during fusion was stimulated through ER-α or ER-β, but not GPER [125, 136]. However, estradiol increase of IGF-1 during proliferation was mediated through GPER [127, 137]. Moreover, additional *in vitro* skeletal muscle studies have uncovered potential roles for ER-α and ER-β in the signaling pathways that regulate satellite cell function by modulating TNF-α, p38, MAPK, FGF-1, MEF2c, MyoD, Pax7 and MRF-4 [121, 122]. Thomas et. al. [135] used an ER-α agonist to augment Pax7 and

MyoD expression following exercise-induced muscle damage in Ovx and Ovx+E_2 rodents. Velders et. al. [121] utilized whole-body knockouts of each ER-α and ER-β and found that ER-β was the main receptor involved in Pax7 and MyoD expression following notexin injury. Although there has been few studies that have investigated estradiol mediated effects through ERs there is commonality in that the estrogen responses during skeletal muscle regeneration are stimulated through ERs, however, investigations into which ER is predominant in regulating estradiol's effects have generated conflicting results. Therefore, further experiments need to determine which ER is responsible for estradiol's effect on regeneration. Additionally, the identification and mechanisms of ERs in relation to each phase of skeletal muscle repair; degeneration and regeneration, and their specific cells (i.e. neutrophils, macrophages, fibroblasts, and satellite cells) is necessary.

Chapter 3

Estradiol is critical for the regulation of satellite cell maintenance and self-renewal in females

As one can appreciate, the literature review on estradiol's effect on skeletal muscle regeneration, specifically in relation to satellite cells, is sparse and from a small number of laboratory groups. Our lab was the first to establish that the loss of estradiol impacts the functional recovery of skeletal muscle following injury [112] and we have more recently recapitulated this in a different model of injury and rescued the detrimental effect with estradiol treatment (unpublished), but the mechanism of estradiol's beneficial effect is still unknown. Moreover, it has yet to be determined which estrogen receptor estradiol utilizes to elicit its effects during muscle regeneration and if satellite cells express estrogen receptors. Thus the aim of this study and chapter is to determine the mechanism whereby estradiol affects satellite cell function.

The contents of this chapter are under final preparation to be submitted to *Nature Medicine*.

Brittany C. Collins, Robert W. Arpke, Alexie A. Larson, Cory W. Baumann, Nardina L. Nash, Espen E. Spangenburg, Michael Kyba, and Dawn A. Lowe

Abstract

Loss of skeletal muscle mass and strength occurs with aging and the rate of loss is accelerated in women at menopause, coinciding with the decline of sex hormones (i.e., estradiol). There is also evidence indicating that muscle regenerative capacity is impaired with estradiol-deficiency, however the mechanism for the impairment is unknown. Here we show satellite cell maintenance, self-renewal and differentiation into muscle fibers are severely compromised in muscle that does not contain estradiol, primarily through regulation of apoptosis and autophagy. We further demonstrate that treatment with estradiol mitigates these detrimental effects and that estradiol utilizes its receptor, ERα in satellite cells, to elicit its beneficial effects. Taken together these results indicated that estradiol is a key extrinsic factor that alters skeletal muscle's regenerative capacity by regulating the number and function of satellite cells the loss of which can ultimately detrimentally affect the quality of life in aged females.

Introduction

The maintenance and regenerative capacity of skeletal muscle is highly dependent on its resident stem cell, i.e., the satellite cell. In fact, genetic ablation of satellite cells completely abolishes the ability of skeletal muscle to regenerate following injury [12, 138, 139]. Satellite cells are located on the periphery of the muscle fiber between the sarcolemma and the basal lamina, where they remain in a quiescent state, which is characterized by the expression of the transcription factor Pax7 [12, 23, 139]. Satellite cells become activated through external stimuli, such as a muscle injury, and transition from quiescence into the cell cycle, proliferate, differentiate into myoblasts, and then fuse together or to existing fibers to regenerate the damaged muscle [140, 141]. During the proliferative phase, satellite cells undergo asymmetric division through which a subpopulation of the daughter satellite cells do not differentiate but instead return to quiescence to repopulate the satellite cell pool (i.e., self-renewal) [140, 141]. The balance of this asymmetric division process is critical and necessary in an attempt to ensure life-long preservation of satellite cells in skeletal muscle.

The satellite cell pool declines with age and the regenerative capacity following an injury is impaired in skeletal muscle from older male animals compared to younger [28, 30, 31, 142, 143]. Recent studies have shown that age-associated changes in the satellite cell environment, in combination with cell intrinsic alterations, disrupt the balance of asymmetric division, ultimately impacting satellite cell maintenance [48, 68, 69]. The concept that extrinsic, environmental factors affect the functionality of satellite cells is has been established by studies in mice using heterochronic parabiosis [8, 10]. Such studies

support the concept that circulatory factors, including hormones, which differ between the young and old systemic environment and their subsequent signaling pathways, may contribute to age-associated decrements in satellite cell maintenance and muscle regenerative capacity.

A hormone that changes with age is estradiol, the main circulating sex hormone in adult females. Serum estradiol concentration declines dramatically between the ages of about 45 and 50 in women, corresponding to the time of menopause [86]. Estradiol impacts skeletal muscle contractility in women [4, 102, 103, 144, 145] and female rodents [93, 95, 146] and may also contribute to skeletal muscle's ability to regain strength following an injury. Specifically, there is evidence that the loss of ovarian hormones blunts recovery of force following eccentric-contraction induced injury [7, 59, 112]. It has been hypothesized that estradiol's influence on muscle inflammation[6, 96, 112, 116] and protein synthesis [96, 118, 125] contributes to the impairment in force recovery, but has yet to be resolved.

There are three known estrogen receptors in skeletal muscle: estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), and g-protein coupled receptor (GPER). In non-skeletal muscle tissue the main ER in which estradiol elicits its effects is ERα [147, 148]. ERα is the most abundant ER in skeletal muscle [77] and the loss of ERα has a multitude of detrimental effects in muscle metabolism [107, 108]. Cell culture studies in C2C12 cells have also pointed towards ERα as mediating estradiol functions for cell differentiation and growth [122]. However, there have been other reports showing that ERβ i**s** the main receptor to elicit estradiol's effects during myogenic cell differentiation [121].

Furthermore, it has yet to be determined if satellite cells express estrogen receptors and if so, to what extent estrogen receptor signaling is essential for optimal satellite cell function.

Here, we show that ovarian hormone deficiency leads to a defect in self-renewal and differentiation, substantially diminishing the satellite cell pool, and that this is rescued by estradiol treatment. We demonstrate that satellite cells express ERα and it is indeed the main receptor through which estradiol signals to elicit its effects. Thus, our data identify a critical pathway that regulates satellite cell function and highlights the potential therapeutic opportunity for hormone therapy and selective estrogen receptor modulators (SERMS) in post-menopausal women.

Methods

Mice

Animal experiments in this study were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Minnesota. All experiments were conducted on female mice when they were young adults (3-4 mo of age). Satellite cells were harvested from C57/BL6 (Purchased from Jackson Laboratories, 000664) and Pax7ZsGreen mice that were either control or ovariectomized (bilateral removal of the ovaries) and sacrificed 2, 3.5, or 7 mo post-surgery (n=5 for each group). $ER\alpha^{fl/fl}$ mice were ovariectomized (Ovx) and half received a 60-day slow-release 17β-estradiol (Ovx+E_2) pellet via trochar implantation (n=3-4) [93]. For transplant experiments, female Pax7ZsGreen and $Pax7^{CreERT2/+}$;$R26R^{tdTomato}$ mice were donors, and were age-matched to female C57/BL6 recipient mice (n=6-15 for each experiment); a subset of recipients were ovariectomized. $Pax7^{CreERT2/+}$;ZsGreen;$ER\alpha^{fl/fl}$ and ZsGreen;$ER\alpha^{fl/fl}$ were generated in-house. Female $Pax7^{CreERT2/+}$;ZsGreen;$ER\alpha^{fl/fl}$ and ZsGreen;$ER\alpha^{fl/fl}$ mice were treated with tamoxifen for 5 d consecutively [12, 28]. Two mo post tamoxifen treatment, mice were used for satellite cell harvests (n=6), transplantation (n=6), or cardiotoxin injury (n=3-5) experiments. For all experiments, the estrous cycle was tracked for 3-5 d consecutively via vaginal cytology to confirm mice had normal estrous cycles or had ceased cycling for the Ovx mice [81]. Uterine mass was measured at the time of sacrifice as a second verification of successful ovariectomy surgery or estradiol treatment [149]. Uterine mass across all experiments for control, Ovx, and Ovx+E_2 mice averaged (SEM): 101.7 (5.1), 13.5 (0.7), and 207.9 (27.0) mg, respectively.

Mouse Satellite Cell Isolation

Isolation of satellite cells from single skeletal muscles (e.g., gastrocnemius) was performed as described previously [150]. Muscles were carefully dissected and chopped in parallel with muscle fibers using a razor blade and forceps to separate the fibers. Muscles were incubated shaking for 75 min in 0.2% collagenase type II (17101-015, Gibco, Grand Island, NY) in high glucose Dulbecco's modified Eagle's medium (DMEM) without phenol red containing 4.00 mM L-glutamine, 4,500 mg/L glucose, and sodium pyruvate (SH30284.01, Hyclone, Logan, UT) supplemented with 1% Pen/Strep (15140122, Gibco) at 37^{o}C. Samples were washed with Rinsing Solution (F-10+), Ham's/F-10 medium (SH30025.01, HyClone) supplemented with 10% Horse serum, 1% HEPES buffer solution (15630080, Gibco) and 1% Pen/Strep (Gibco) and centrifuged at 1500 rpm x 5min at 4^{o}C. Samples were washed and centrifuged a second time. Samples were pulled into a sheared Pasteur pipette, centrifuged and washed again. Following aspiration, samples were resuspended in F-10+ with collagenase collagenase type II and dispase (17105-041, Gibco), vortexed and incubated shaking at 37^{o}C for 30 min. Samples were vortexed again, drawn and released into a 3 mL syringe with 16-gauge needle four times then with a 18-gauge needle four times and passed through a 40-μm cell strainer (Falcon, Hanover Park, IL). 3 mL of F-10+ was added to each sample and centrifuged at 1500 rpm x 5min 4^{o}C. Following aspiration, samples were resuspended in FACS staining medium. Bulk isolation (hindlimb muscles excluding soleus, tricep muscles, and psoas muscles) of satellite cells was performed similarly and as described previously [151].

FACS Analysis and Cell Sorting

Muscle samples were stained using an antibody mixture of PE-Cy7 rat anti-mouse CD31 (clone 390), PE-Cy7 rat anti-mouse CD45 (clone 30-F11), Biotin rat anti-mouse CD106 (clone 429(MVCAM.A)) and PE Streptavidin from BD Biosciences (San Diego, CA); and Itga7 647 (clone R2F2) from AbLab (Vancouver, B.C., Canada). Antibody cocktail was added to samples and incubated on ice for 30 min. Samples were washed and resuspended with FACS staining media containing propidium iodide for FACS analysis on the FACSAriaII (BD Biosciences, San Diego, CA). Total satellite cells (lineage negative; VCAM,alpha7double positive cells or ZsGreen+) (Fig.8) were analyzed while draining the entire sample from each skeletal muscle sample. For transplanted tibialis anterior (TA) muscles, the number of donor (ZsGreen + or tdTOM+) satellite cells were examined as previously described [151].

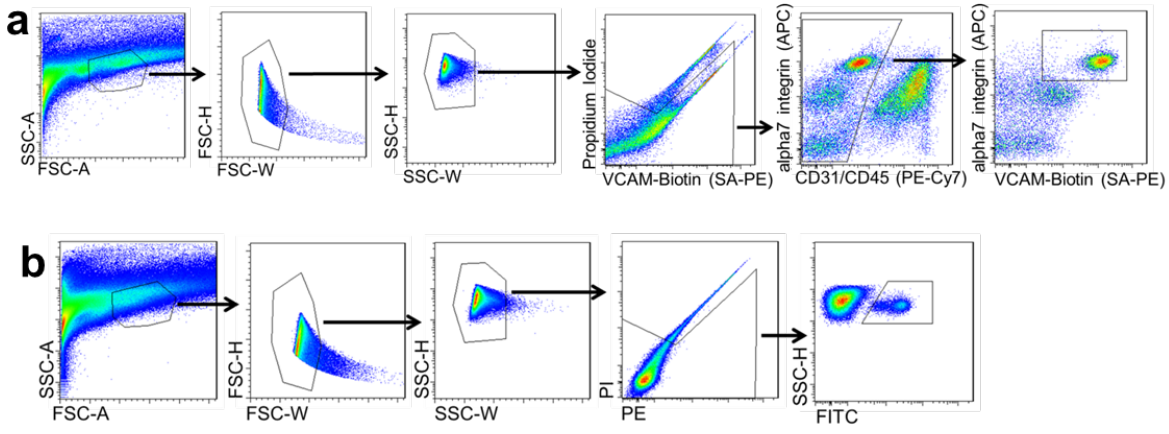

Figure 8. Satellite cells identified by surface marker staining and ZsGreen expression. **(a)** Gating scheme for lineage negative; VCAM,alpha7 double positive cells **(b)** Gating scheme for ZsGreen+ cells

Cardiotoxin Injury and Transplantation

Transplant recipient mice were anesthetized with 150 mg/kg ketamine plus 10 mg/kg xylazine and both hind limbs were subjected to a 900 cGy dose of irradiation using an RS 2000 Biological Research Irradiator (Rad Source Technologies, Inc., Suwanee, GA). Lead shields limited exposure to the hind limbs only. 24 h following irradiation, 15 μL of cardiotoxin (10 μM in PBS, Sigma-Aldrich, Saint Louis, MO) was injected into both TA muscles of each mouse with a Hamilton syringe. 24 h following cardiotoxin injection, 600 ZsGreen+ cells were resuspended into 10 μL of sterile saline and injected into both TA's. Both TA's were harvested 1 mo post-transplantation and prepared for FACS analysis as described above. When tdTom+ cells were transplanted, one TA was harvested and prepared for FACS analysis and the contralateral TA was harvested and prepared for sectioning and staining.

Immunofluorescence Microscopy

TA muscles were removed and placed in OCT compound, frozen in 2-methylbutane (Sigma-Aldrich) cooled by liquid nitrogen and stored at -80°C until use. For visualization of satellite cells and apoptotic cells in skeletal muscle, Pax7 and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed on 7 μM cyrosections (CM 1850, Leica Microsystems, Buffalo Grove, IL). TUNEL was detected using In Situ Cell Death, Fluorescein Detection Kit according to manufacturer's instructions (11684795910, Roche Diagnostics, Mannheim, Germany). Following TUNEL, Pax7 staining was completed as previously reported [12, 28]. In brief, slides were

incubated in citrate buffer for 10 min and then antigen retrieval was performed by microwaving at 20 sec intervals for a total of 3 min in citrate buffer. Slides were washed two times in 1x PBS for 2 min each and blocked in 3% bovine serum albumin (BSA) for 1 h at room temperature. Slides were incubated with anti-pax7 mouse IgG1 primary antibody (PAX7, Developmental Hybridoma Bank at Iowa University, 1:20 in 3% BSA) overnight at 4ºC. Slides were incubated with goat anti-mouse biotin-conjugated secondary antibody (115-065-205, Jackson Immuno Research Laboratories Inc, West Grove, PA, 1:1000 in 3% BSA) for 1 h at room temperature. Visualization of the primary antibody was performed using the Vectastain ABC kit (PK-6100, Vector Laboratories, Burlingame, CA) and Tyramide Signal Amplification (TSA) Plus Cyanine 3 kit (NEL744, PerkinElmer, Waltham, MA, 1:50 in diluent buffer). Slides were washed again and then prolong gold antifade mountant with DAPI (P36931, Life technologies, Grand Island, NY) was applied and coverslip added.

For visualization of fiber engraftment following transplantation, 10 μM sections of TA muscles were cryosectioned. Samples were fixed in 2% paraformaldehyde (PFA) for 5 min, washed with 0.01% triton in 1x PBS, permeabilized with 0.2% triton for 5 min, and then blocked with 1% BSA in wash buffer for 30 min at room temperature. Sections were incubated overnight at 4ºC with primary antibodies diluted in 1% BSA in wash buffer simultaneously; rabbit polyclonal anti-RFP (600-401-379, Rockland Immunochemicals Inc, Pottstown, PA, 1:200) and mouse monoclonal anti-laminin (Clone LAM-89; Sigma-Aldrich, 1:250). Slides were washed with wash buffer and secondary antibodies were applied for 1 h at room temperature simultaneously, Alexa 555 goat anti-mouse and Alexa

480 goat anti-rabbit IgG (A-21422 and A-11034, 1:500; Life Technologies). Slides were washed and then mounted with prolong gold anti-fade mountant media (P10144, Life Technologies).

Image Processing and Analysis

All images were processed and analyzed in a blinded manner with samples being de-identified as to treatment. Samples were examined and imaged using a Leica DM5500B microscope (Leica Microsystems) at 5x-20x magnification. Images were stitched using the automated tile-scan tool to construct an image of the entire cross-section of the TA muscle. For transplantation experiments, donor cells were quantified by counting RFP+ (Red) fibers using the Image J software package (NIH, Bethesda, MD, USA). Muscle cross-sectional area (CSA) in square millimeters was measured by tracing the muscle section border in Image J. The number of myofibers per muscle was counted using laminin staining to delineate each myofiber border. TUNEL/Pax7/DAPI staining was normalized to cross-section and myofiber number determined by laminin staining of serial sections. Nuclei undergoing apoptosis were identified as TUNEL+ and DAPI+. Satellite cells were identified as Pax7+ and DAPI+ residing along the myofiber border. Satellite cells undergoing apoptosis were identified as Pax7+, TUNEL+ and DAPI+ residing along the myofiber border.

RNA-seq Preparation and Analysis

RNA from freshly FACS-isolated satellite cells (70,000 – 200,000 satellite cells per mouse) was isolated using direct-Zol MicroPrep kit (R2061, Zymo Research, Irvine, CA). On-column DNase digestion was performed according to manufacturer protocols to prevent genomic DNA contamination. Total RNA was quantified using a fluorimetric RiboGreen assay. RNA and library preparation integrity were verified using a 2100 BioAnalyzer system (Agilent, Wokingham, UK), generating an RNA integrity number (RIN). All samples were at least 250 pg total RNA and RIN values of 3. Libraries were prepared using the Smarter Stranded Total RNA_Seq Kit (Clontech) following the pico input protocol using n =3 for biological replicates. In summary, between 250 pg – 10 ng of total RNA was fragmented and then reverse transcribed into cDNA using random primers. The Template Switching Oligo (TSO) was incorporated during cDNA synthesis and allowed for full length cDNA synthesis and strand specificity to be retained. Illumina sequencing adapters and barcodes were then added to the cDNA via limited PCR amplification. Next, mammalian ribosomal cDNA was enzymatically cleaved. Uncleaved fragments were PCR enriched 12-16 cycles. Final library size distribution was validated using capillary electrophoresis and quantified using fluorimetry (PicoGreen). Indexed libraries were then normalized and pooled for sequencing. Illumina libraries were hybridized to a paired end flow cell and individual fragments were clonally amplified by bridge amplification on the Illumina cBot (Illumina, Saffron Walden, UK). Once clustering was complete, the flow cell was loaded on the HiSeq 2500 and sequenced using Illumina's SBS chemistry. Upon completion of read 1, an 8 base pair index read for Index 1 was performed. The Index 1 product was then removed and the template re-anneals to the flow

cell surface. The run proceeds with 7 chemistry-only cycles, followed by an 8 base pair index read to read Index 2. Finally, the library fragments were resynthesized in the reverse direction and sequenced from the opposite end of the read 1 fragment thus producing the template for paired end read 2. Base call (.bcl) files for each cycle of sequencing are generated by Illumina Real Time Analysis (RTA) software. The base call files and run folders are then exported to servers maintained at the Minnesota Supercomputing Institute. Primary analysis and de-multiplexing are performed using Illumina's CASAVA software 1.8.2.

qRT-PCR

RNA from freshly FACS-isolated satellite cells was isolated using direct-Zol MicroPrep kit according to manufacturer's instructions. cDNA was synthesized from 10 ng RNA according to directions in SUPERVILO cDNA Synthesis Kit (11756050, ThermoFisher, Waltham, MA). Relative quantitation of the ERα, p53, atrogin-1, p16, p38, beclin-1 and caspase-3 transcripts were determined using Taqman probes (ThermoFisher) for ESR1 (Mm00433149_m1), Trp53, atrogin-1, CDKN2A, Mapk14, BCN1 (Mm01265461_m1), CASP3 (Mm01195085_m1), and house-keeping gene gapdh (Mm99999915_g1).

Gastrocnemius Muscle Injury and *In Vivo* Function

Pax7$^{CreERT2/+}$;ZsGreen;ERα$^{fl/fl}$ and ZsGreen;ERα$^{fl/fl}$ mice were anesthetized via isoflurane inhalant (1%) with oxygen at a flow rate of (125 mL/min) and maintained under

anesthesia on a 37 ^{o}C heating pad for the duration of the injury and testing. 25 μL of cardiotoxin (10 μM in PBS, Sigma-Aldrich) or sterile PBS was injected into 3 precise locations of the left gastrocnemius muscle (lateral head, medial head, and center of muscle) of each mouse with a Hamilton syringe similar to Call et al [64]. As previously described in detail, for gastrocnemius testing the peroneal branch of the sciatic nerve was severed to avoid recruitment of antagonistic muscles [152, 153]. The ankle joint was then positioned such that the foot was perpendicular to the tibia and the knee was stabilized by a clamp to inhibit movement of the lower limb. The foot was secured to a footplate that was attached to the shaft of a servomotor (300B-LR; Aurora Scientific, Aurora, Ontario, Canada). Contraction of the gastrocnemius was elicited via stimulation of the sciatic nerve through platinum percutaneous electrodes attached to a stimulator (E2-12 and S48; Grass Telefactor, Warwick, RI). Peak isometric torque was determined by stimulating the nerve for 200-ms with 0.1-ms square wave pulses at a frequency of 300 Hz and voltage increasing from 4-12 volts until maximum torque was achieved. Torque as a function of stimulation frequency was measured at 10-300 Hz. All functional testing was completed in a blinded manner.

Histology

Gastrocnemius muscles were sectioned on 10 μM thick and hematoxylin and eosin–phloxine staining was performed. These sections were used to count the percentage of fibers with central nuclei [154].

Statistical Analysis

Data are presented as means ± SE. Two-way analysis of variance (ANOVA) were used to determine differences among time and treatment. If an interaction was significant, Holm-Sidak post-hoc tests were used. All other data were analyzed with independent *t*-tests for determining differences between groups. Analyses were conducted using SigmaPlot (version 12.5, Systat Software, Inc) and significance was set at $p<0.05$.

Results

Estradiol regulates satellite cell maintenance

To comprehensively determine how ovarian hormone deficiency affects the satellite cell compartment, we used FACS to count the total number of satellite cells in the TA, extensor digitorum longus (EDL), gastrocnemius, soleus, and diaphragm muscles from control and Ovx C57/BL6J mice following 2, 4, and 7 mo of intervention (Fig. 9). Satellite cells (lineage negative; VCAM,alpha7 double positive) were 30-60% fewer in TA muscles of Ovx than control mice and the longer duration of hormone deficiency resulted in a further decline of satellite cell number (Fig. 10a). Ovarian hormone deficiency also resulted in low numbers of satellite cells in the EDL, gastrocnemius, and diaphragm, though the longer duration did not cause further declines (Fig. 10a). Interestingly, satellite cell number in the soleus muscle was unaffected by hormone manipulation (Fig. 10a). The density of satellite cells was calculated by dividing cell number (Fig. 10a) by the mass of each muscle (Fig.

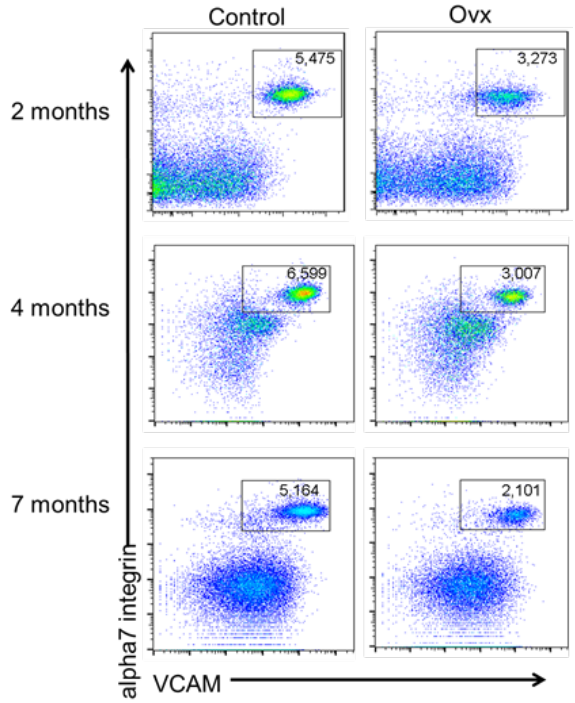

Figure 9. FACS plots for the quantification of the total number of satellite cells in the tibialis anterior muscle from control and ovariectomized (Ovx) mice at different durations of hormone deficiency. Gating for lineage negative;VCAM,alpha7 double positive cells.

10b,c). Satellite cell density recapitulated the cell number declines with ovarian hormone deficiency in all muscles except the diaphragm, in which normalizing for muscle size resulted in low satellite cell density in Ovx samples at all time points studied (Fig. 10c), whereas total number was only affected at 2 mo.

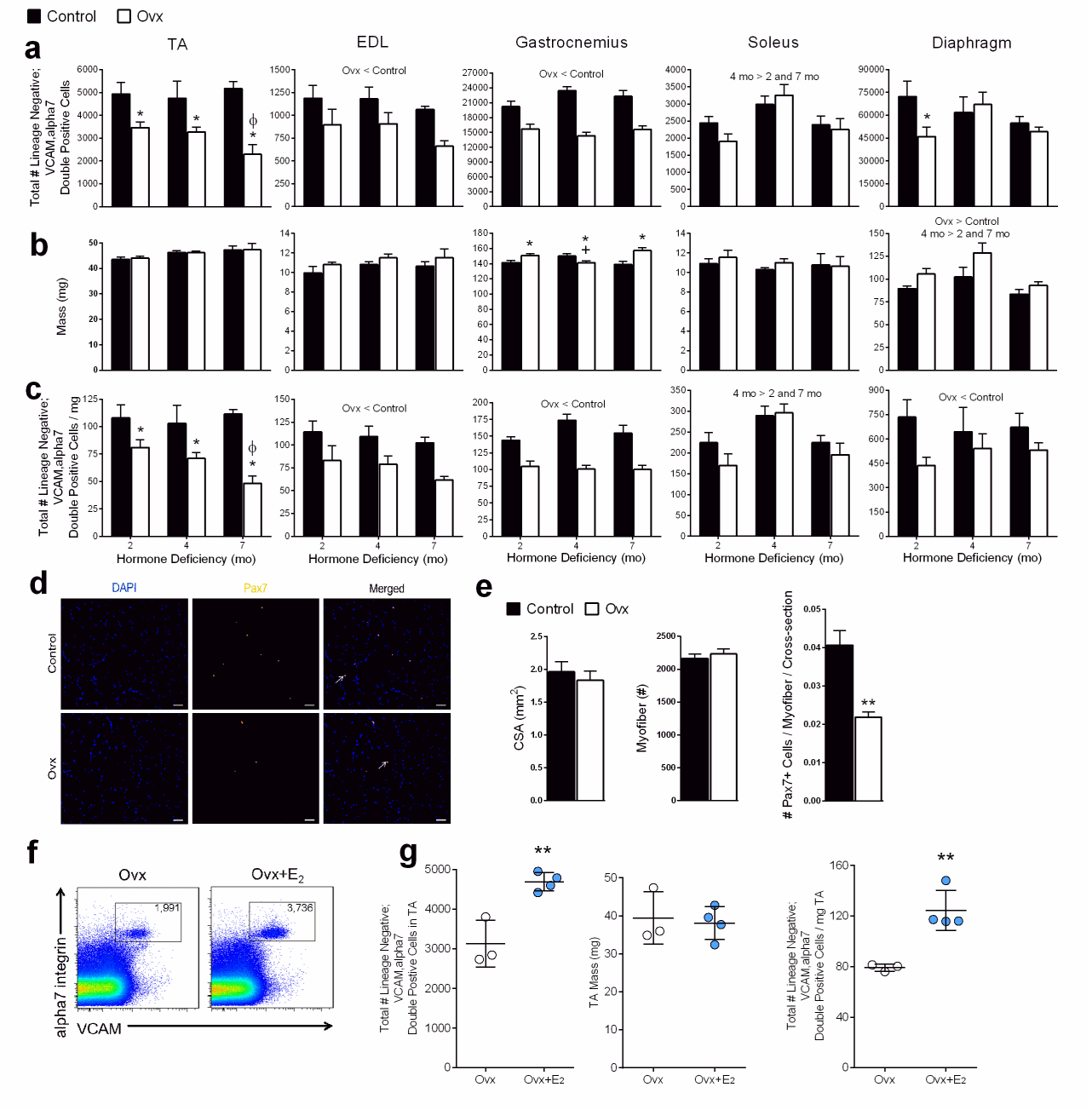

Figure 10 . Estradiol maintains the satellite cell pool in skeletal muscles of females. **(a)** Total number of satellite cells quantified by lineage negative;VCAM,alpha7 double-positive cells in five discrete muscles from Control (n=15) and ovariectomized (Ovx) (n=15) mice. Muscles were harvested and analyzed 2, 4, or 7 mo after Ovx and in age-matched controls. **(b)** Muscle masses. **(c)** Total number of satellite cells normalized to muscle masses. **(d)** Satellite cells quantified by immunohistochemistry of Pax7+ cells in TA muscles from Control (n=4) and Ovx (n=4) mice at 2 mo of hormone deficiency. Arrows indicate localization of DAPI;Pax7 double-positive cell. Scale bars = 50 µm. (e) Cross-sectional area and number of fibers in TA muscles from Control and Ovx mice ($P \geq 0.193$), and Pax7+ cells per TA myofiber per cross-section. **(f)** Representative FACS plots of cells isolated from TA muscles of Ovx mice without (n=3) and with 17β-estradiol (n=4) treatment (Ovx+E_2). **(g)** Total number of satellite cells and those relative to TA muscle mass. Significant main effects of two-way ANOVA ($P<0.05$) are indicated above the bars (abc) and when interactions occurred ($P<0.05$), Holm–Sidak post hoc tests are indicated by *different than Control at corresponding duration (abc), $^{\phi}$different than 2 and 4 mo Ovx (ac), and $^{+}$different than 2 and 7 mo Ovx (b). **$P<0.005$ by student t-tests (eg).

To confirm the effect of the loss of ovarian hormones on satellite cell number using an independent marker, we employed the Pax7ZsGreen mouse model in which quiescent satellite cells fluoresce green. ZsGreen+ cells were measured in all 5 skeletal muscles of control and Ovx Pax7ZsGreen mice at 2 mo of hormone deficiency (Fig. 11a-c). Similar to the results with surface marker staining, ZsGreen+ cells were significantly lower in the TA, EDL and gastrocnemius muscles of Ovx mice, with the soleus again being unaffected (Fig. 11a-c). To independently verify the results of FACS quantification, we counted Pax7+ cells in immunostained TA muscle sections (Fig. 10d) which showed that TA muscles from Ovx mice have 46% fewer satellite cells than controls when Pax7+ cells are expressed per myofiber and cross-section (Fig. 10e).

Ovariectomy results in deficiencies of all ovarian hormones and thus to determine if 17β-estradiol (E_2) was sufficient to impact the satellite cell number, a subset of Ovx mice was treated with E_2. Treatment with E_2 rescued satellite cell numbers restoring maintenance of the satellite cell pool (Fig. 10f-g).

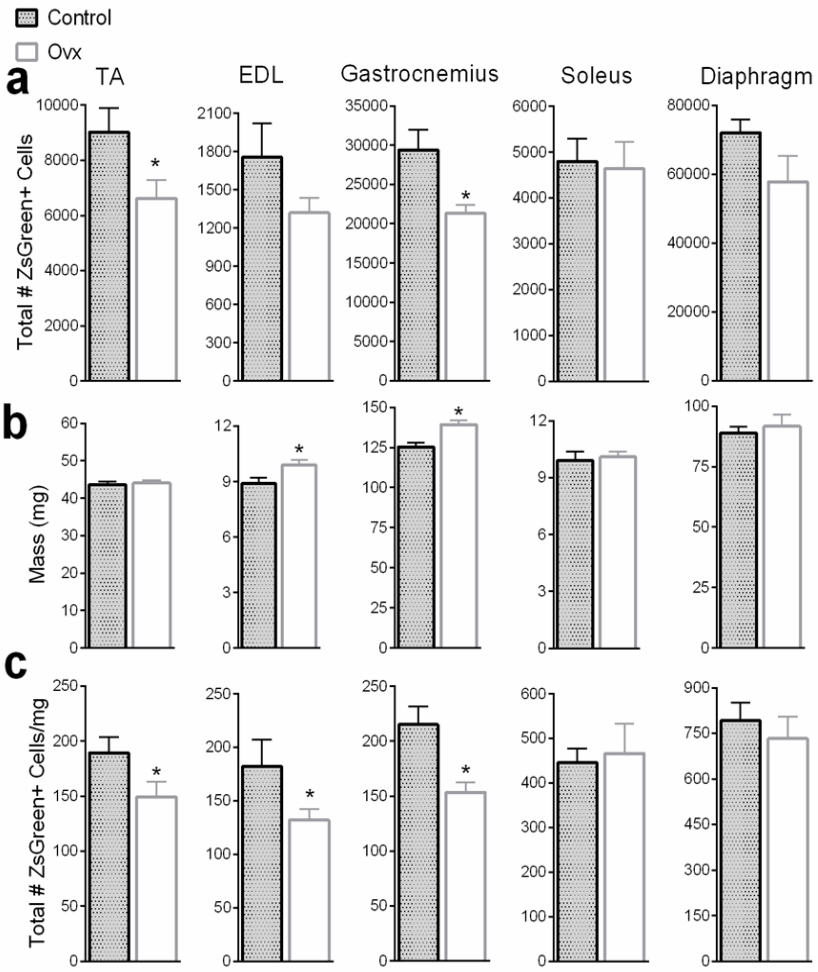

Figure 11. Lack of estradiol results in lower satellite cell numbers in Pax7ZsGreen female mice. Quantification of **(a)** Total number of ZsGreen+ cells **(b)** Muscle mass **(c)** Total number of ZsGreen+ cells normalized to muscle mass in control and ovariectomized (Ovx) Pax7ZsGreen female mice. * $P < 0.05$ by student t-tests.

Estradiol deficiency impairs self-renewal and differentiation *in vivo*

The disruption of satellite cell maintenance by the loss of estradiol led us to next determine if self-renewal and/or differentiation were affected as these cellular processes have direct implications in replenishment of the satellite cell pool. Therefore, we assayed self-renewal and differentiation *in vivo* via transplantation. Initially we aimed to address this question utilizing previously published methods by Arpke et al using immune/dystrophin-deficient NSG-mdx^{4cv} mice as recipients [151]. That approach would have allowed us to show satellite cell and fiber engraftment in the same mouse by FACS analysis of donor ZsGreen+ cells and dystrophin+ fibers in the transplant recipient. Unexpectedly, we determined that our recipient mouse model, NSG-mdx^{4cv}, were not undergoing normal estrous cycles which confounded our results (Fig. 12a). A critical component of our study design is based on comparing different hormone manipulations to normally cycling control mice, thus we began optimizing our transplant technique in female C57/BL6J mice (Fig. 12b). Following verification of normal cycling in the C57/BL6J recipients, satellite cells were bulk harvested from female Pax7ZsGreen mice

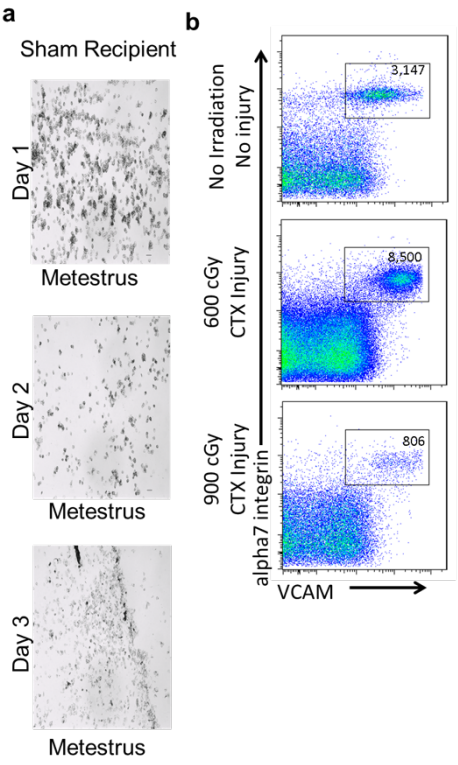

Figure 12. **(a)** Representative vaginal swab cytology from NSG-mdx^{4cv} sham recipient mouse 3 days prior to transplantation. **(b)** Representative FACS plots from irradiation dosage experiment in wild-type C57/BL6 female mice

and transplanted into previously irradiated, cardiotoxin-injured TA muscles of control or Ovx recipient C57/BL6J mice (Fig. 13a). Satellite engraftment was measured by FACS analysis of the donor ZsGreen+ cells in the recipient tissue (Fig. 13b). Estradiol deficiency of the recipient (i.e., deficiency in the muscle environment) resulted in 75% less satellite cell engraftment compared to control recipients (Fig. 13c).

To address the contribution of transplanted satellite cells to fibers, in the next experiment we utilized the Pax7 $^{CreERT2/+}$;R26R;tdTom mice as donors (Fig. 13d). This study again confirmed the deleterious effect of estradiol deficiency in the environment on satellite cell engraftment (Fig. 13e,f) and further demonstrated that all satellite cells contributed to fibers in muscle of control recipients but contribution to fibers was minimal when estradiol was not present (Fig. 13g,h). Because transplanted satellite cells were highly impaired in engraftment and differentiation in an environment lacking estradiol, we next aimed to determine if an environment containing estradiol could rescue satellite cells isolated from a hormone-deficient environment (Fig. 13i). ZsGreen+ satellite cells were isolated from the TA and gastrocnemius muscles of control and Ovx donor mice and transplanted into control recipients (Fig. 13i). The presence of estradiol in the environment (i.e., control recipient) did in fact rescue the deleterious cell autonomous effects attributed to estradiol deficiency (Fig. 13j,k).

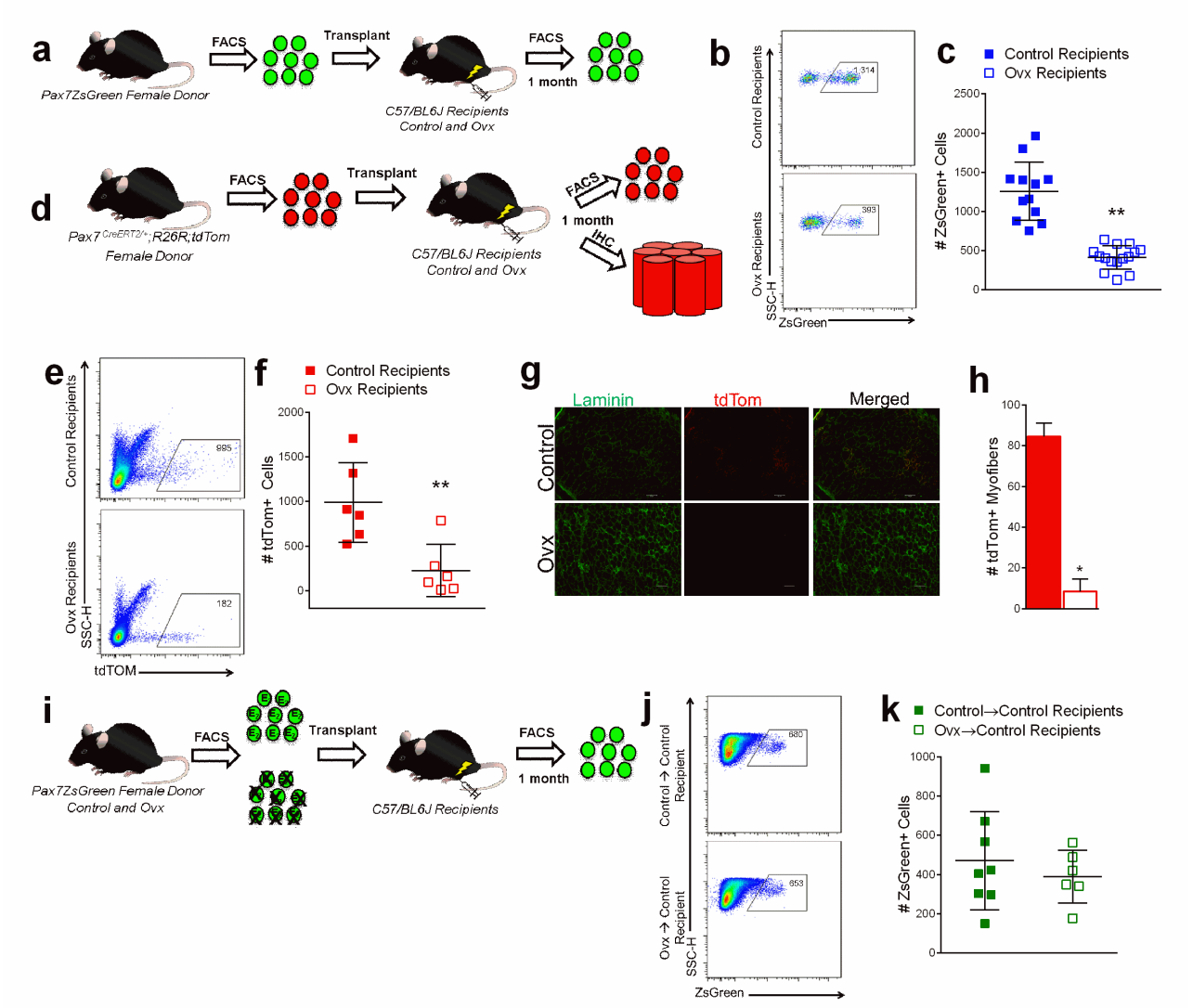

Figure 13. Loss of estradiol in the environment negatively affects satellite cell and fiber engraftment that can be rescued by the presence of estradiol. (a) ZsGreen+ cells transplantation scheme into control and ovariectomized (Ovx) recipients (b) Representative FACS plots of ZsGreen+ donor satellite cells in tibialis anterior (TA) muscle 1 mo following transplant in control and Ovx recipient mice. (c) Total number of ZsGreen+ donor satellite cells in control (n=12) and Ovx (n=15) recipient mice 1 mo following transplant. (d) tdTom+ cells transplantation scheme into control (n=6) and Ovx (n=6) recipients (e) Representative FACS plot of tdTOM+ donor satellite cells in TA muscle 1 mo following transplant in control and Ovx recipient mice. (f) Total number of tdTOM+ donor satellite cells in control (n=6) and Ovx (n=6) recipient mice 1 mo following transplant. (g) Representative images of tdTom+ fibers in the engrafted region of TA muscle from control and ovx recipient mice. Scale bar = 100 μm (h) Quantification of total number tdTom+ fibers from control and Ovx recipients. (i) ZsGreen+ cells from control and Ovx donors transplantation scheme (j) Representative FACS plots of ZsGreen+ control and Ovx donor satellite cells in TA muscle 1 mo following transplant in control recipient mice. (k) Total number of ZsGreen+ control (n=8) and Ovx (n=6) donor satellite cells in control recipient TA muscles 1 mo following transplantation. * $P < 0.05$ by student t-tests

Apoptosis of satellite cells with estradiol deficiency

In non-skeletal muscle tissues, estradiol is known to protect against apoptosis (e.g., [128]) and more recently protection has been observed in C2C12 cells [155]. Moreover, it has been suggested that apoptosis plays an important role in skeletal muscle health, ultimately affecting strength [156], which prompted us to determine if the lower satellite cell numbers measured in estradiol-deficient muscles was due to cell apoptosis. First, we measured overall apoptosis *in vivo* by identifying TUNEL+ cells in the TA muscles of Control and Ovx mice (Fig. 14a). Estradiol-deficient muscles had 3.7-fold more TUNEL+ cells, trending toward significance, compared to Controls (Fig. 14b).

Next, we determined the percent of Pax7+TUNEL+ double-positive cells in control and Ovx TA muscles. Loss of estradiol resulted in 4-fold more Pax7+TUNEL+ double-positive cells (Fig. 14c). Due to estradiol's dramatic effect on satellite cell apoptosis, we performed qRT-PCR on freshly isolated satellite cells from control and Ovx Pax7ZsGreen mice to determine specific apoptosis-related genes regulated by estradiol. Estradiol deficiency resulted in 3- to 1500-fold upregulation of p53 and p38, beclin-1, and caspase-3 gene expression as well as a down-regulation of atrogin-1 (Fig. 14d).

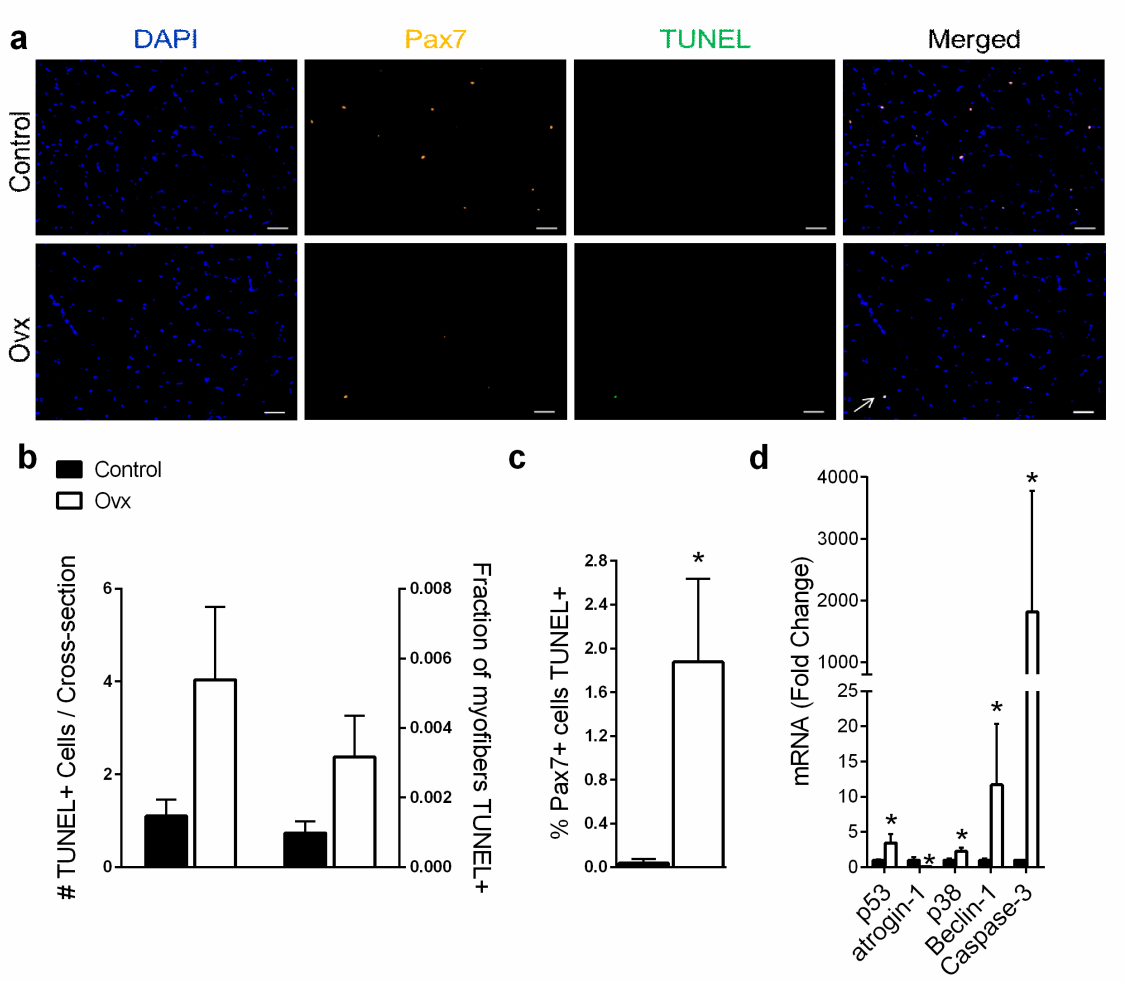

Figure 14. Loss of estradiol results in satellite cell apoptosis. (a) Representative images of DAPI stained nuclei (blue), Pax7 satellite cells (gold), TUNEL cells (green), and merged image from cross-sections of the tibialis anterior (TA) muscle of control (n=4) and Ovariectomized (Ovx) (n=4) mice. Arrows indicate DAPI;pax7;TUNEL triple-positive cell. Scale bar = 100 µm **(b)** Quantification of total number TUNEL+ cells per cross-section and per number of myofibers **(c)** Percent of TUNEL+ cells that were also Pax7+. **(d)** mRNA gene expression of p53, atrogin-1 p38, Beclin-1, and Caspase-3 in isolated ZsGreen+ satellite cells from gastrocnemius muscles of control (n=4) and Ovx mice (n=4). * $P < 0.05$ by student t-tests.

Estradiol signals through ERα to regulate satellite cell function

Recently, our lab demonstrated that estradiol signals through skeletal muscle ERα to elicit its effects on contractility [157]. These results in combination with other work showing ERα is the main estrogen receptor involved in regulating muscle metabolism [107, 109, 158] and oxidative stress [77, 130], led us to determine 1) if satellite cells express ERα and 2) the receptor's role in mediating estradiol effects on satellite function. To our knowledge, we are the first to report that satellite cells express ERα (Fig. 15a). Therefore we developed a satellite cell-specific ERα knockout mouse (Fig. 15b) in order to determine the mechanism of estradiol's effects in satellite cells. We isolated satellite cells for FACS analysis from multiple muscle groups of female Pax7$^{CreERT2/+}$;ZsGreen;ERα$^{fl/fl}$ and ZsGreen;ERα$^{fl/fl}$ mice (Fig 16a-e).

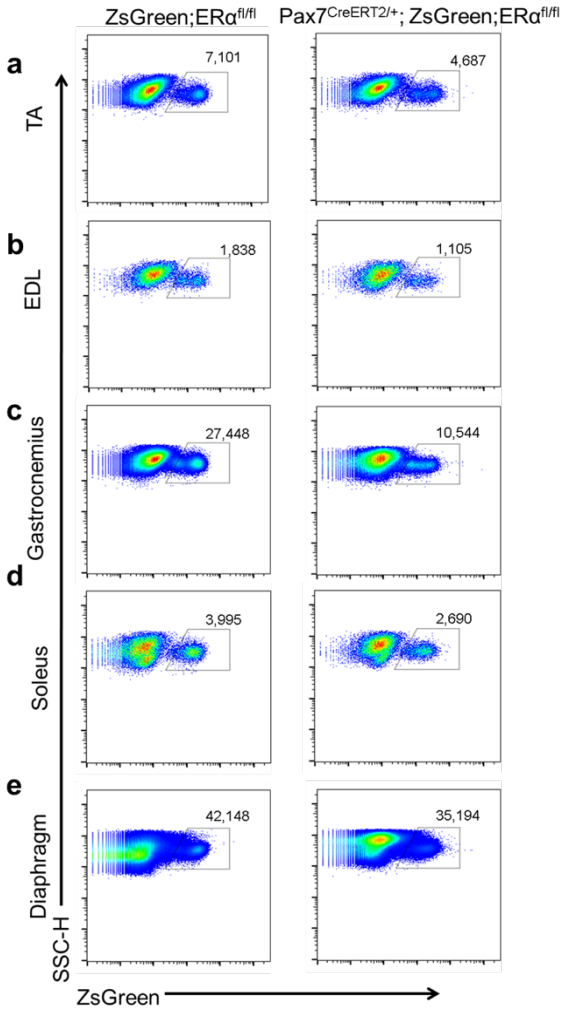

Figure 16. FACS plots for the quantification of the total number of satellite cells in different muscle groups from ZsGreen;ERα$^{fl/fl}$ and Pax7$^{CreERT2/+}$;ZsGreen;ERα$^{fl/fl}$ female mice. Gating for ZsGreen positive cells from five different muscle groups – **(a)** Tibialis anterior (TA), **(b)** Extensor digitorum longus (EDL), **(c)** Soleus, **(d)** Gastrocnemius, **(e)** Diaphragm

Ablation of ERα in satellite cells resulted in 40-60% fewer satellite cells in all 5 muscles analyzed, again both expressed in absolute cell numbers and normalized to muscle mass (Fig. 15c-e).The magnitude of the reductions were similar to those of estradiol deficiency (Fig. 10a-c), indicating that satellite cell ERα is the main receptor estradiol signals through to impact maintenance of the satellite cell pool in muscles of females.

Analogous to our estradiol-deficient transplant experiment, we next aimed to determine if the loss of ERα in satellite cells would impair self-renewal using our *in vivo* transplantation assay (Fig. 15f). Satellite cells from female $Pax7^{CreERT2/+}$;ZsGreen;$ER\alpha^{fl/fl}$ and ZsGreen;$ER\alpha^{fl/fl}$ mice were transplanted into control C57/BL6J recipient mice in which satellite engraftment was measured by FACS analysis of total number of donor ZsGreen+ cells (Fig. 15g). The resulting loss of satellite cell engraftment in the absence of ERα in donor cells (Fig. 15h) suggests ERα plays a transcriptional role in the self-renewal signaling pathway.

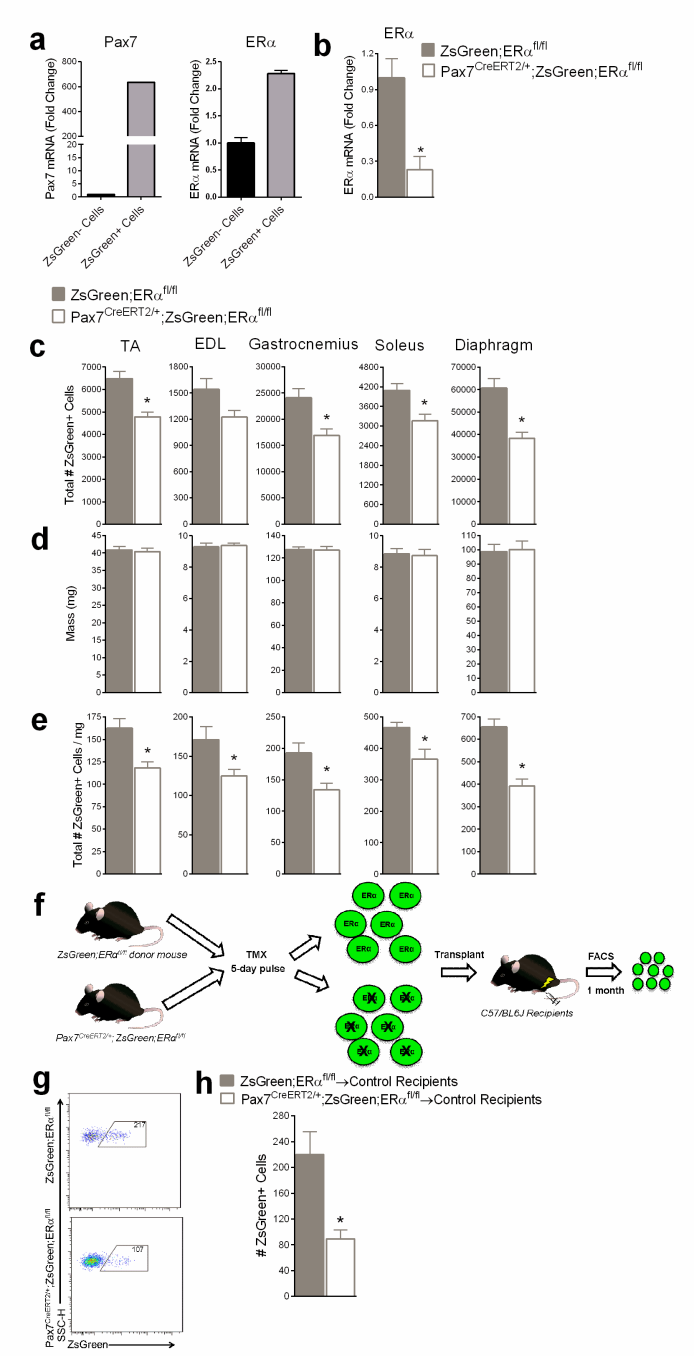

Figure 15. Estradiol utilizes ERα to regulate satellite cell maintenance and self-renewal. (a) mRNA gene expression of Pax7 and estrogen receptor alpha (ERα) in isolated ZsGreen+ satellite cells from Pax7ZsGreen female mice (n=4). (b) mRNA gene expression of ERα in ZsGreen;ERα$^{fl/fl}$ (n=4) and Pax7$^{CreERT2/+}$; ZsGreen;ERα$^{fl/fl}$ (n=4) female mice. (c) Total number of ZsGreen+ satellite cells in muscles of ZsGreen;ERα$^{fl/fl}$ (n=6) and Pax7$^{CreERT2/+}$; ZsGreen;ERα$^{fl/fl}$ (n=6) mice. (d) Muscle masses. (e) Total number of ZsGreen+ cells normalized to muscle mass. (h) ZsGreen;ERα$^{fl/fl}$ and Pax7$^{CreERT2/+}$; ZsGreen;ERα$^{fl/fl}$ transplantation scheme. (g) Representative FACS plots of ZsGreen+ donor satellite cells from ZsGreen;ERα$^{fl/fl}$ (n=3) and Pax7$^{CreERT2/+}$; ZsGreen;ERα$^{fl/fl}$ (n=3) mice that were transplanted into control recipients (n=12). (h) Quantification of ZsGreen+ donor satellite cells in control recipient TA muscles following transplantation. * $P < 0.05$ by student t-tests.

Due to the role of ERα in self-renewal, we hypothesized that estradiol signals through satellite cell ERα to prevent apoptosis of that cell. To test this hypothesis, we measured apoptotic-related genes in satellite cells isolated from Pax7$^{CreERT2/+}$;ZsGreen;ERα$^{fl/fl}$ and ZsGreen;ERα$^{fl/fl}$ mice. In the absence of satellite cell ERα, we observed a similar upregulation of apoptotic-related genes to that of estradiol deficiency (Fig. 17, Fig. 14d). These results further indicate that ERα is the main estrogen-receptor by which estradiol regulates satellite cell maintenance and function *in vivo*. To determine if the lack of ERα in satellite cells affected regeneration after injury, we subjected the gastrocnemius to a single injury and measured peak torque 3, 7, and 21 days following cardiotoxin injury. Torque and centrally-nucleated fibers did not differ between muscles from Pax7$^{CreERT2/+}$;ZsGreen;ERα$^{fl/fl}$ and ZsGreen;ERα$^{fl/fl}$ mice ($p>0.09$) (Fig. 18a-d).

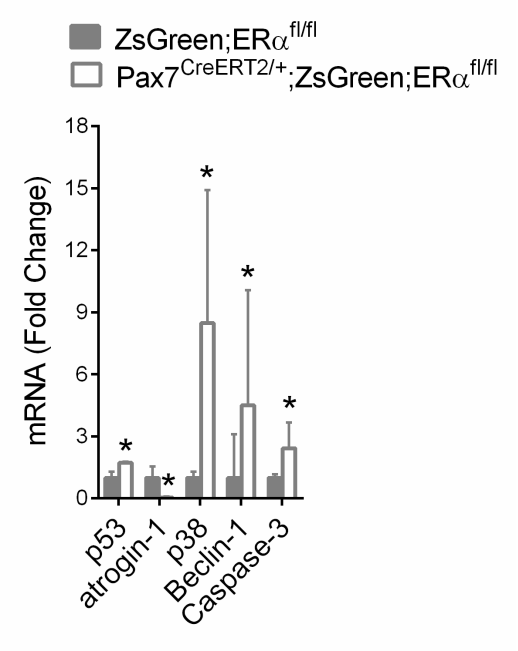

Figure 17. **Lack of ERα results in satellite cell apoptosis.** mRNA gene expression of p53, atrogin-1 p38, Beclin-1, and Caspase-3 in isolated ZsGreen+ satellite cells from gastrocnemius muscles of ZsGreen;ERα$^{fl/fl}$ (n=4) and Pax7$^{CreERT2/+}$; ZsGreen;ERα$^{fl/fl}$ (n=4) mice. * $P < 0.05$ by student t-tests.

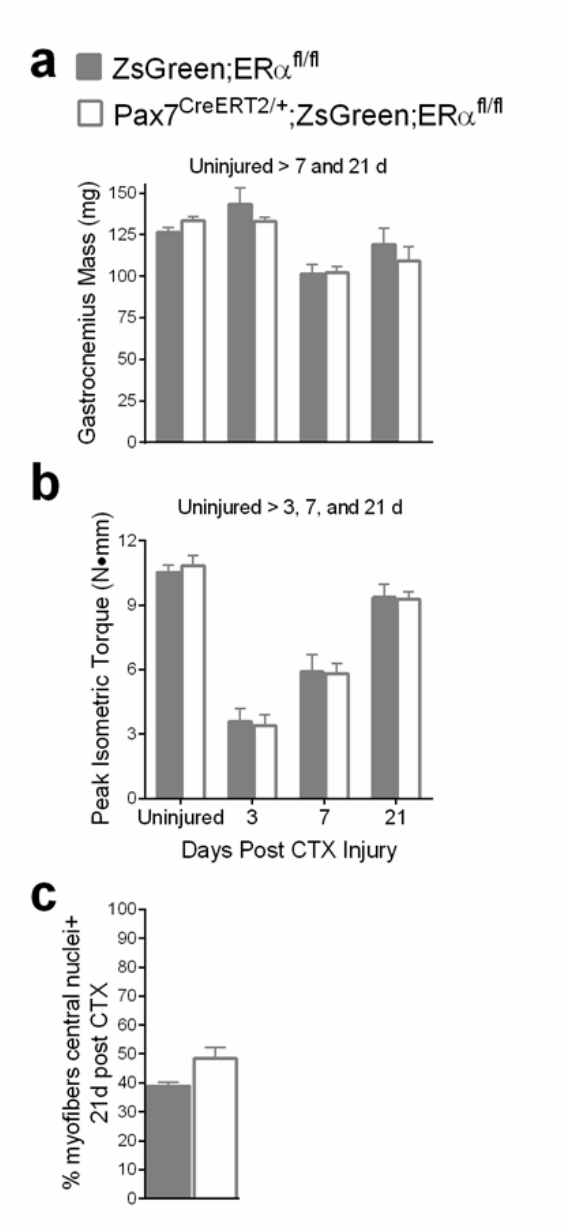

Figure 18. Strength recovery of the gastrocnemius muscle following 1 bout of cardiotoxin injury in ZsGreen;ERα$^{fl/fl}$ and Pax7$^{CreERT2/+}$; ZsGreen;ERα$^{fl/fl}$ mice (a) Muscles masses **(b)** Peak torque **(c)** Percent of fibers with centralized nuclei. ZsGreen;ERα$^{fl/fl}$ (n=5) and Pax7$^{CreERT2/+}$; ZsGreen;ERα$^{fl/fl}$ (n=5). Significant main effects of two-way ANOVA ($P<0.05$) are indicated above the bars (ab)

Discussion

Here we demonstrated that the female sex hormone, estradiol, substantially affects the satellite cell. We show that satellite cells in several skeletal muscles are lost, by ~30-50%, in the absence of ovarian hormones. Estradiol treatment rescued the satellite cell deficit, pinpointing estradiol as a key regulator for the maintenance of the satellite cell pool in females. We show that satellite cells harvested from an estradiol-rich environment failed to self-renew when transplanted into a host environment that lacked estradiol, but when satellite cells from a hormone-deficient environment were transplanted into a host environment with estradiol self-renewal was robust. In addition to the loss of self-renewal, satellite cells from an estradiol-deficient environment failed to differentiate into muscle fibers following transplantation. These results demonstrate that estradiol is a crucial extrinsic factor in the satellite cell environment in females and support the concept that age-associated deficits in skeletal muscle of aged females is driven by extrinsic factors that differ between young and aged environments [30, 31].

After demonstrating that estradiol plays a key role in the maintenance of the satellite cell pool through regulation of self-renewal in females, we questioned if the hormonal effect was driven by estrogen receptor signaling in satellite cells. We first established that ERα is indeed present in satellite cells. Our experiments then identified ERα as the main receptor for estradiol's actions on satellite cell function. We show that when estradiol is removed from the satellite cell environment through removal of the hormone or its receptor, satellite cells undergo apoptosis demonstrated by elevated levels of TUNEL and by the upregulation of apoptosis-related genes; p53, beclin-1 and caspase-3. This finding

coincides with the failure of self-renewal and lower levels of differentiation into myofibers with the lack of estradiol. We also demonstrated that the removal of the hormone or its receptor resulted in elevated levels of p38/MAPK activity which has been shown to drive satellite cells out of quiescence towards myoblast differentiation and promote cell apoptosis and senescence [48, 69, 159]. Even though satellite cell maintenance and self-renewal was impaired in the absence of ERα (i.e., there were ~50% less satellite cells), the muscle retained its ability to recover force generation following a single injury. Because muscle encounters repetitive injuries throughout a lifetime which is purported to cause age-related muscle weakness [2], it is logical to speculate that loss of satellite cells would contribute to muscle weakness in aged, hormone-deficient in females

Our findings demonstrate that estradiol is a key factor regulating *in vivo* satellite cell function and provide evidence that its downstream pathway contains potential therapeutic targets to mitigate skeletal muscle deficits observed with aging in women. For example, selective estrogen-receptor modulators that are agonists for ERα in skeletal muscle and antagonists for ERα in reproductive tissues could be investigated more thoroughly across multiple tissues when considered clinically to mitigate menopausal symptoms. The results also highlight the importance of biological sex differences in skeletal muscle, particularly regarding age-related deficits.

Chapter 4

Deletion of estrogen receptor alpha in skeletal muscle results in impaired contractility in female mice

After showing that estradiol deficiency results in a depletion of satellite cells through the loss of self-renewal and differentiation, ultimately impacting satellite cell maintenance, and the treatment of estradiol restores these processes, I went on to examine the mechanism of estradiol in regard to satellite cell function. In brief, I found that ERα is the main receptor for estradiol signaling in satellite cells. This provoked me to ask the question if ERα was the main receptor for estradiol's effect on skeletal muscle fiber force generation that is muscle strength, since we have established that treatment with estradiol in mice results in 10-20% greater strength compared to estradiol deficient mice. The aim of this study and chapter was to determine if estradiol mediates effects on skeletal muscle contractility through ERα. The approach used was to generate a mouse model in which ERα is not present in the muscle fibers.

The content of this chapter is under review at *Journal of Applied Physiology*.

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Abstract

Estradiol deficiency in females can result in skeletal muscle strength loss, and treatment with estradiol mitigates the loss. There are three primary estrogen receptors and estradiol elicits effects through these receptors in various tissues. Ubiquitous estrogen receptor α (ERα) knockout mice exhibit numerous biological disorders, but little is known regarding the specific role of ERα in skeletal muscle contractile function. The purpose of this study was to determine the impact of skeletal muscle specific ERα deletion on contractile function, hypothesizing that ERα is the main receptor through which estradiol effects muscle strength in females. Deletion of ERα specifically in skeletal muscle (skmERαKO) did not affect body mass compared to wild-type littermates (skmERαWT) until 26 wk of age at which time body mass of skmERαKO began to increase disproportionally. Overall, skmERαKO mice had low strength, similar in magnitude to estradiol deficient female mice. Isolated extensor digitorum longus muscles from skmERαKO mice produced 16% less eccentric and 16-26% less submaximal and maximal isometric forces and isolated soleus muscles were more fatigable with impaired force recovery relative to skmERαWT. *In vivo* maximal torque productions by plantar- and dorsi-flexors were 16 and 12% lower in skmERαKO than skmERαWT mice and skmERαKO muscles had low phosphorylation of myosin regulatory light chain. Plantarflexors also generated 21-32% less power, submaximal isometric and peak concentric torques. Data support the hypothesis that ablation of ERα in skeletal muscle results in muscle weakness. Furthermore, results indicate that the beneficial effects of estradiol on muscle strength are receptor mediated through ERα.

New and Noteworthy

We comprehensively measured *in vitro* and *in vivo* contractility in a female estrogen-receptor α (ERα) skeletal muscle-specific knockout mouse to support the hypothesis that the primary mechanism through which estradiol elicits its effects on strength are mediated by ERα. Estradiol signaling through ERα appears to modulate myosin-actin binding via post-translational modifications of the myosin regulatory light chain.

Introduction

Aging is associated with decreased skeletal muscle strength and contributes to the inability to carry out activities of daily living (e.g., [52]). Both men and women experience strength loss with aging, but at differing rates [4, 53, 160-162]. The sex difference is most robust at the time women experience menopause (e.g., [4]) coinciding with a sharp decline in the sex hormone, estradiol. There are reports of enhanced muscle strength in post-menopausal women on hormone therapy (HT) [4, 101, 102, 163-165], but those results are not consistent with all other studies. To address discrepancies in the literature, a meta-analysis of 23 papers comparing strength in post-menopausal women on estrogen-based HT versus not was conducted and concluded an overall positive effect of HT on muscle strength [103].

Similarly, ovarian hormone deficiency in adult female mice results in decrements in skeletal muscle force generation and the decrements are reversed by estradiol treatment [93, 103, 104, 166, 167]. Moran et al. [93, 104] showed that force-generating deficits with ovarian hormone deficiency resulted from impaired muscle quality; specifically there was less strongly bound (force generating) myosin to actin during contraction which was reversed with estradiol treatment. Similarly, Qaisar et al. [102] showed that HT in post-menopausal twins improved fiber force generating capacity, without affecting fiber size, compared to those of twin pairs not on HT. Phosphorylation of the regulatory light chain of myosin is estradiol sensitive [106, 168, 169], thus providing additional evidence for estradiol's critical role in modulating myosin-actin interactions in skeletal muscle,

ultimately impacting how muscle contracts and produces force. Moreover, studies on both women and female rodents show that estradiol has regulatory roles in energy metabolism and mitochondrial function, which can also affect muscle contractility [134, 158, 170-175]. What is less clear is if estradiol mediates its effects on contractility through estrogen receptors or via a non-receptor mediated mechanism.

The primary action of estradiol in reproductive tissues is regulated by estrogen receptors (ER) through classical ligand-activated transcription factor mechanisms. In this process, two ERs dimerize upon activation by estradiol, translocate to the nucleus, bind to estrogen response elements in target gene promoters and elicit a transcriptional response [73, 76]. However in non-reproductive tissues, ERs can elicit responses using non-classical mechanisms [73], as well as classical ligand-activated mechanisms, deeming the need to determine estradiol mechanisms in specific tissues, such as skeletal muscle.

To date, there are three recognized estrogen receptors: estrogen receptor alpha (ERα), estrogen receptor beta (ERβ) and g-protein coupled receptor (GPER). Tissue specific receptor content and actions through which estradiol elicits effects particularly in non-reproductive tissues are being realized. For example, estradiol's role in metabolism specifically through ERα has been demonstrated in transgenic mice lacking ERα [108, 176, 177]. Whole-body deletion of ERα in mice affects multiple metabolic tissues (i.e., pancreas, skeletal muscle, liver, and adipose tissue) resulting in hyperinsulinemia, insulin resistance [147, 176], impaired oxidative metabolism [108], increased inflammation [108], impaired glucose tolerance [147, 176, 178, 179], decreased physical activity [180, 181], and increased fat and body masses [108, 182, 183]. Thus, ubiquitous knockout of ERα

results in a phenotype similar to that of metabolic syndrome [108, 184, 185]. This creates a situation in which it is difficult to directly assess skeletal muscle autonomous effects of ERα because skeletal muscle contractility can be impaired secondarily to reduced physical activity, obesity and metabolic disorders. Therefore, analysis of an ERα skeletal muscle-specific knockout mouse is critical to determine the skeletal muscle autonomous effects of ERα deficiency.

A skeletal muscle-specific ERα knockout mouse developed by Ribas and co-workers was reported to have metabolic deficits similar to those observed in the whole-body ERα knockout mice including impaired glucose tolerance, insulin resistance and impaired fatty acid oxidation [107]. Modest fatigability of the soleus muscle was suggested but thorough assessment of skeletal muscle contractility was not presented such that the impact of ERα deficiency on muscle contractile function remains unclear. The purpose of this study was to employ a mouse model that is deficient of ERα specifically in the skeletal muscle fibers to comprehensively measure contractile properties of several hindlimb skeletal muscles. This model allows us to overcome a number of experimental limitations that have previously hampered accurate interpretation of the role ERα plays in influencing skeletal muscle. We hypothesize that contractility will be impaired because ERα is the primary mode through which estradiol affects skeletal muscle.

Methods

Generation of transgenic mice

Generation of ERα floxed mice has been previously described [186, 187]. Human skeletal actin cre (HSAcre) mice were obtained (strain 006149 originated from The Jackson Laboratory, Bar Harbor, ME). Human skeletal actin is the promoter of choice to drive gene expression specifically in skeletal muscle [188]. To generate a skeletal muscle specific knockout of ERα, homozygote ERα floxed females were crossed with male HSAcre mice. Heterozygote ERα floxed males that were HSAcre positive were subsequently crossed with homozygote ERα floxed females to generate homozygote ERα floxed mice positive for HSAcre (skmERαKO) and homozygote ERα floxed mice negative for HSAcre (skmERαWT). Genotype was determined by PCR using primers (F: TCACATGGAGTGAATGCTCTG
R: AGTGAACACCAGGCCAGTTT) for floxed ERα and HSAcre [188, 189].

Female mice were used in experiments across a wide age range (15-35 wk) as described for each experiment. Mice of the same sex were housed in groups of 3-5 and had access to phytoestrogen-free food (Harlan-Teklad; #2019) and water *ad libitum*. The room was maintained on a 14:10 light:dark cycle. All animal procedures were in accordance with standards set by the Institutional Animal Care and Use Committees at the University of Minnesota and by guidelines put forth by the American Physiological Society.

Verification and specificity of skeletal muscle ERα knockout

qRT-PCR. RNA from mouse tissues was isolated using TRIzol Reagent according to manufacturer's instructions. cDNA was synthesized from 1 ug RNA according to directions in SUPERVILO cDNA Synthesis Kit (11756050, ThermoFisher, Waltham, MA). Relative quantitation of the ERα transcript was determined using Taqman probes (ThermoFisher) for ESR1 (Mm00433149_m1) and house-keeping gene18S rRNA (4333760F).

Characterization of transgenic mice

Vaginal cytology. The estrous cycle in female mice is 4-5 days long [81]. Therefore, vaginal swabs were taken from a subgroup of female skmERαWT and skmERαKO mice for 5 consecutive days in order to verify mice were cycling normally. In brief, a cotton swab was soaked in sterile PBS and inserted in the vaginal canal. Cells were smeared onto a glass microscope slide. Cells were stained with hemotoxylin for 5 min and washed in tap water. Estrous cycle was determined by the type of cell present [81].

Physical activities

Cage activities. Physical activities of individual mice were measured over a 24-h period using open-field activity chambers (Med Associates, St. Albans, VT). Immediately prior to measurement of activity, each mouse was placed in a mock chamber for a 24-h familiarization period. Activity data was acquired using Activity Monitor version 5 software (Med Associates) with a box size set to "3" (4.8 cm^2). Description of activity

counts and box size rationale have been previously described [82]. Food intake for each mouse was measured during the 24 h activity period.

Wheel running. Voluntary running wheel distance was measured in a subset of female mice (n= 11 and 10 for skmERαWT and skmERαKO, respectively). Mice were individually housed in wheel running cages (Mouse Single Activity Wheel Chamber model 80820, Lafayette Instrument Company, Lafayette, IN), with wheel diameter of 12.7 cm. Revolutions were counted via optical sensor connected to a USB interface for 14 consecutive days. Data was recorded via Activity Wheel Monitor Software model 86065. Data reported are only those from days 8-14 in order to avoid the variability that occurs during the first week of wheel running due to the mice learning and becoming familiar with the wheel.

Skeletal muscle physiology

In vitro soleus and extensor digitorum longus muscle contractile functions. Mice were weighed and anesthetized by an intraperitoneal injection of sodium pentobarbital (100 mg/kg body mass) with supplemental doses given as required. The soleus muscle from one hindlimb and the EDL muscle from the contralateral hindlimb of each mouse were studied to determine muscle contractile function. The isolated muscle preparation used was similar to that described previously [82, 95, 190]. Briefly, after a muscle was excised, it was mounted in a 1.2 ml bath assembly filled with Krebs-Ringer bicarbonate buffer maintained at 25ºC and the proximal tendon was attached to a dual-mode muscle lever system (300B-LR; Aurora Scientific Inc., Ontario, Canada). Muscles were set to their anatomic L_o and

measured using digital calipers. Muscles remained quiescent in the bath for 10 min and then a protocol for testing contractile function began. First, muscles underwent 3-8 isometric tetanic contractions (400 ms stimulation at 120 Hz for soleus muscles and 200 ms at 180 Hz for EDL muscles) with 3 min rest in between each contraction until maximal isometric tetanic force had plateaued. Three min later, peak twitch force was elicited by stimulating the muscle with a 0.5-ms pulse at 150 V, followed by a second twitch 30 s later. A maximal isometric tetanic contraction was again performed 30 s later. After 3 min of rest, a second maximal tetanic contraction was elicited and at peak force a sinusoidal length oscillation of 0.01% L_o at 500 Hz was imposed to determine active stiffness [190]. Then a series of 10 isometric contractions was performed at increasing frequencies: 5, 10, 15, 20, 25, 30, 40, 50, 70, and 110 Hz for soleus muscles and 10, 20, 25, 30, 40, 50, 60, 80, 120, and 160 Hz for EDL muscles. Contractions were separated by 3 min rest. Muscles were then measured for maximal concentric force elicited by passively lengthening the muscle to 110% of L_o over 3 s, and stimulating tetanically for 133 ms as the muscle was shortened to 90% of L_o. Three minutes later, eccentric force was elicited by passively shortening the muscle to 90% of L_o and then stimulating tetanically as it lengthened to 110% of L_o at 1.5 L_o/s. In a subset of soleus and EDL muscles, fatigability was assessed 3 min following the eccentric contraction. Soleus muscles were stimulated for 1000 ms at 40 Hz every 5 s for 5 min [82]. EDL muscles underwent 200 ms stimulations at 60 Hz every 10 s for 5 min. Recovery from fatigue was assessed by eliciting one peak isometric tetanic contraction immediately following the fatigue protocol and every 5 min for 20 min thereafter. Additional parameters measured from isometric twitch and tetanic contractions include

time to peak twitch (TPT), twitch one-half relaxation time ($RT_{1/2}$), maximal rate of isometric tetanic force development (+d*P*/dt), and maximal rate of relaxation (-d*P*/dt).

Following contractility testing, each soleus and EDL muscle was removed from the bath, trimmed at the myotendinous junctions, blotted, weighed, and snap-frozen in liquid nitrogen and stored at -80ºC until further analysis. Contralateral soleus and EDL muscles were dissected and mounted in OCT, tibialis anterior (TA), gastrocnemius, and quadriceps muscles, visceral and subcutaneous fat pads, and uterus were collected from each mouse and stored at -80ºC until further analysis. Mice were euthanized with an overdose of sodium pentobarbital (200 mg/kg) after all tissues were collected.

In vivo plantarflexor muscle function. While several measures of contractility can be measured in isolated muscles, only small muscles such as soleus or EDL are viable for extensive testing due to oxygen diffusion limitations [191]. Testing of larger muscle groups such as the ankle plantar- and dorsi-flexors are important because such groups of muscles are more functionally relevant, e.g., for ambulation. Thus, we next measured contractility of those muscle groups *in vivo*. Mice were anesthetized via isoflurane inhalant (1%) with oxygen at a flow rate of (125 mL/min) and maintained under anesthesia for the duration of testing on a 37 ^{o}C heating pad. We narrowed these experiments to only females because minimal sex by genotype interactions were determined for soleus and EDL muscle contractility measures. As previously described in detail, for plantarflexor testing the peroneal branch of the sciatic nerve was severed to avoid recruitment of antagonistic dorsiflexor muscles [152, 153]. The ankle joint was then positioned such that the foot was perpendicular to the tibia (defined as neutral position 0^{o}) and the knee was stabilized by a

clamp to inhibit movement of the lower limb. The foot was secured to a footplate that was attached to the shaft of a servomotor (300B-LR; Aurora Scientific, Aurora, Ontario, Canada). Passive torque about the ankle was measured by passively moving the foot -20, -15, -10, -5, 0, 5, 10, 15, and 20^{o} in randomized order. Contraction of the plantarflexor muscle group (gastrocnemius, soleus, and plantaris) was elicited via stimulation of the sciatic nerve through platinum percutaneous electrodes attached to a stimulator (E2-12 and S48; Grass Telefactor, Warwick, RI). Peak isometric torque about the axis of the ankle was determined by stimulating the nerve for 200-ms with 0.1-ms square wave pulses at a frequency of 300 Hz and voltage increasing from 4-12 volts until maximum torque was achieved. Peak isometric torque at nine ankle angles was measured by stimulating at 5 ^{o} intervals from 20 ^{o} plantar- to 20 ^{o} dorsi-flexion [152]. Torque as a function of stimulation frequency was measured at 10-300 Hz. Peak concentric torque was measured by passively moving the foot from 0 to 19 ^{o} of dorsiflexion followed by 38 ^{o} of plantarflexion at an angular velocity of 2 ^{o}/ms during stimulation. Peak eccentric torque was measured by passively moving the foot from 0 to 19^{o} of plantarflexion followed by 38^{o} dorsiflexion at an angular velocity of 2^{o}/ms during stimulation. To assess the relationship between torque and velocity, a series of concentric contractions were performed at set velocities from 0-1200 ^{o} /sec. Power was calculated by multiplying the torque generated at each velocity by the velocity in radians/sec.

In vivo dorsiflexor muscle function. In brief, a separate cohort of mice were anesthetized and positioned on the *in vivo* apparatus similarly as stated previously in the

plantarflexor muscle function section. Muscle function of the dorsiflexor muscles (TA, EDL, and extensor hallicus longus) was assessed by stimulation through platinum percutaneous electrodes attached to a stimulator, as described previously [112, 192]. Peak isometric torque about the axis of the ankle was determined by stimulating the nerve for 200-ms with 0.1-ms square wave pulses at a frequency of 300 Hz and voltage increasing from 2-8 volts until maximum torque was achieved.

Skeletal muscle histomorphology and protein analyses

NADH-TR histology. Frozen 10 μm cross-sections were cut at the mid-belly of the muscle and placed on microscope slides. Slides were thawed at room temperature for 10 min, incubated with NADH-TR staining solution (0.2 M Tris, 1 mg/ml β-nicatinomide adenine dinucleotide, and 1.3 mg/ml Nitro Blue Tetrazolium) in a 37 ^{o} C water bath for 5 min, dehydrated in graded alcohols, and mounted. Dark stained fibers were counted as NADH-TR positive. All images were processed and analyzed in a blinded manner with samples being de-identified as to treatment. Sections were imaged using a Leica DM5500B microscope (Leica Microsystems, Buffalo Grove, IL) at 10x magnification. Images were stitched using the automated tile-scan tool to construct an image of the entire cross-section of the soleus muscle. Anatomical muscle cross-sectional area (CSA) was measured by tracing the muscle section border in Image J (NIH, Bethesda, MD). The total numbers of light and dark fibers in each muscle cross section were counted.

Fiber types and cross-sectional areas. Skeletal muscle fiber typing was performed in soleus and gastrocnemius muscles by immunofluorescence staining of myosin heavy

chain positive individual fibers as previously described [193]. Frozen 10 μm cross-sections were cut and fixed in ice-cold 4% paraformaldehyde/PBS for 5 min. Sections were stained with primary antibodies for myosin heavy chain (MHC) I (DSHB-#BA-F8, mouse IgG2b), IIa (DSHB-#SC-71, mouse IgG1), IIb (DSHB-#BF-F3, mouse IgM), and dystrophin (ThermoFisher, RB-9024). Following overnight primary antibody incubation, slides were washed with PBS for 5 min x 3. The samples were then incubated for 1 h with a secondary antibody solution containing Alexa Fluor 350 goat-anti-mouse IgG2b (#A-21140, Life Technologies), Alexa Fluor 488 goat-anti-mouse IgG1 (#A-21121 Life Technologies), and Alexa Fluor 546 goat-anti-mouse IgM (#A-21045, Life Technologies). The proportion of Type I (blue fluorescence), Type IIa (green fluorescence), Type IIb (red fluorescence), and Type IIx (unstained) fibers were quantified. Dystrophin was visualized using the following secondary Alexa Fluor 647 goat anti-rabbit IgG. Fiber CSA was assessed using Image J software on dystrophin positive stained fibers.

Total and contractile protein content. A subset of tibialis anterior and gastrocnemius muscles were homogenized in RIPA lysis buffer containing protease inhibitor tablet (11836170001, Roche Diagnostics, Indianapolis, IN) and phosphatase inhibitor II and III cocktails (P5726 and P0044, Sigma Aldrich, St. Louis, MO) using bullet blender according to the manufacturer's instruction (Midwest Scientific, St. Louis, MO). Homogenates were centrifuged at 664 *g* for 10 min at 4 °C to remove connective tissue and the supernatant was collected. Protein lysates were assayed in triplicate for total protein content using Pierce BCA protein assay kit (23225, ThermoFisher) and quantitative gel electrophoresis was performed to determine contractile protein content of each muscle

[104]. Contractile protein content is defined here as the content of myosin heavy chain plus actin for each muscle.

Western blots. pRLC: Protein lysates that were used for contractile protein content were used for measuring phosphorylation of myosin regulatory light chain (RLC) [106]. Loading buffer was added to protein lysates (50 μg), heated for 5 min at 95 °C, loaded on 4-20% SDS-PAGE gels, and run at 90 V for 90 min. Proteins were transferred to nitrocellulose membranes and probed for RLC (1:1000, ab48003, Abcam, Cambridge, United Kingdom), phosphorylated RLC (pRLC) (1:1000, ab2480, Abcam) and GAPDH (G8795, Sigma Aldrich) over night at 4 °C. Membranes were washed, incubated with a secondary antibody for 1 h, washed, and then scanned and quantified using the Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

Oxidative damage. Frozen muscles were homogenized using mortar and pestle in liquid nitrogen and resuspended in a solution containing 20% trichloroacetic acid to prevent oxidation [194, 195]. Samples were fractionated into myofibrillar and cytosolic fractions via centrifugation [196]. Protein concentration was determined by nano drop spectrometer. Samples were diluted to a concentration of 1 mg/mL protein in biotin hydrazide coupling buffer and treated with Biotin Hydrazide as adapted from Grimsrud et al [194]. Duplicate gels were run; one gel was transferred to PVDF membrane for quantification of oxidized proteins and the second gel was stained with coomassie to quantify total protein as a loading control. Following transfer, the PVDF membrane was blocked in PBS-based Odyssey blocking buffer and probed with IR800-conjugated Streptavidin. Membranes were imaged on LICOR and the entire signal of each lane was

quantified. The bands for contractile proteins, myosin heavy chain and actin, were also quantified individually and identified by myosin:actin standard . Oxidized protein signal was normalized to the protein loading control within each lane.

Statistical analyses

Data are presented as means ± SE. Independent *t*-tests were used to for determining differences between skmERαWT and skmERαKO female mice. Significance was set at $p<0.05$.

Results

Validation and specificity of skmERαKO

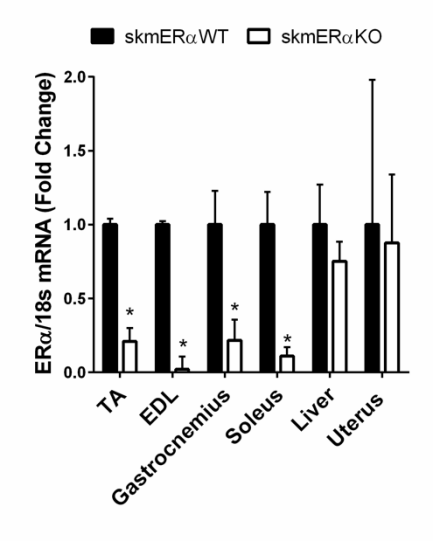

Figure 19. Verification of knockdown of ERα protein specifically in skeletal muscle. ERα mRNA expression in female skmERαWT and skmERαKO mice. *significantly different from skmERαWT ($p \leq 0.017$)

To examine the role of ERα specifically in skeletal muscle, female ERα fl/fl mice were crossed with male HSA-cre mice. In order to confirm knockout of ERα in HSAcre-homozygote lox3ER$\alpha^{fl/fl}$ mice (skmERαKO), mRNA expression of ERα was measured and showed little to no expression in five skeletal muscles of female skmERαKO mice compared to wild-type littermates (skmERαWT) with normal ERα mRNA expression in liver and uterus (Figure 19). The lack of ERα in skeletal muscles did not appear to disturb estrogenic responses in reproductive organs as shown by uterine mass (Figure 20A) or estrous cycle of the mice monitored by vaginal cytology (data not shown).

Physical activity levels

To determine whether the loss of skeletal muscle ERα was detrimental to the ability and/or motivation for physical movement, multiple measurements of physical activity were made. skmERαWT and skmERαKO female mice not differ in any of the eight cage activities measured (Table 1). Food consumption was also measured during the same time as the 24 h cage activities and did not differ between skmERαWT and skmERαKO mice (Table 1). An additional form of physical activity, voluntary wheel running, was measured in a separate cohort of female mice and the distance run by skmERαWT and skmERαKO mice averaged 8 km/24 h for both groups (Table 1).

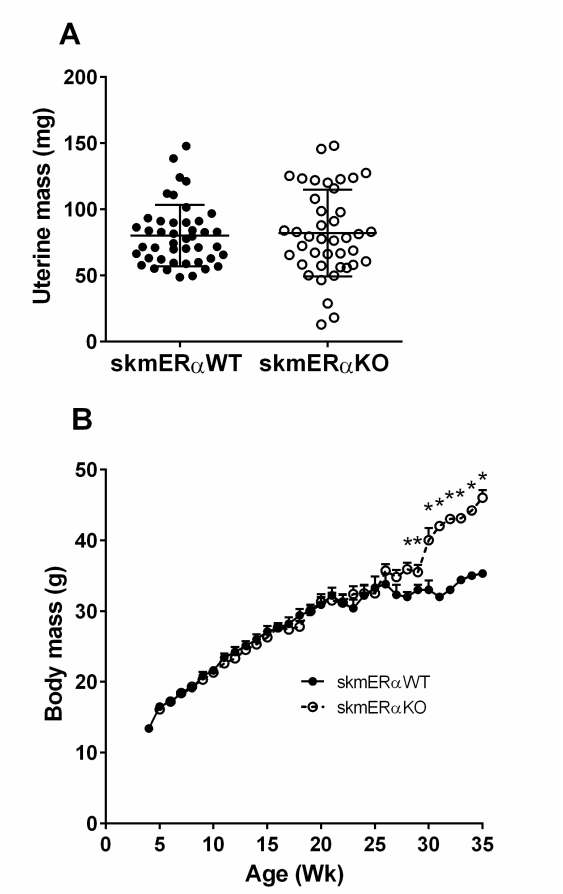

Figure 20. Phenotypic characteristics of female skmERαWT and skmERαKO mice. A) Uterine mass of skmERαWT and skmERαKO mice did not differ (p=0.177). B) Weekly body masses of skmERαWT and skmERαKO female mice from weaning until sacrifice were not different between skmERαWT and skmERαKO mice until the age of 26 wk. *significantly different from skmERαWT at corresponding age (p≤0.034)

Table 1. Physical activities and food intake by female skmERαWT and skmERαKO mice at 22 weeks of age.

	skmERαWT	**skmERαKO**	***P-value***
24 h Cage Activities	(n=12)	(n=13)	
Vertical count (#)	5823 (745)	6701 (704)	0.400
Stereotypic count (#)	48076 (3406)	49987 (2675)	0.660
Jump count (#)	1219 (185)	1696 (263)	0.159
Ambulation distance (m/24 h)	995 (239)	901 (162)	0.745
Ambulation time (min/24 h)	118 (14)	129 (12)	0.538
Stereotypic time (min/24 h)	125 (6)	128 (6)	0.682
Jump time (min/24 h)	7 (1)	10 (2)	0.163
Total active time (min/24 h)	250 (15)	268 (14)	0.375
Food intake (g/24 h)	3.4 (0.8)	3.2 (1.1)	0.630
Wheel Running	(n=11)	(n=10)	
Distance during 2nd week (km/24 h)	7.97 (0.42)	8.06 (0.38)	0.475

Values are means (SEM).

Body and tissue masses

Body mass was measured weekly in female mice from wean until sacrifice (Figure 20B). No differences between skmERαWT and skmERαKO mice were measured until 26 wk of age when skmERαKO mice became heavier ($p \leq 0.034$; Figure 2B). By 35 wk of age, female skmERαKO mice weighed 30% more than skmERαWT littermates.

Table 2 shows hindlimb muscle masses, fat pad and uterine masses from the cohort of 21 wk-old female mice analyzed for soleus, EDL, and *in vivo* posterior muscle contractility. The presence or absence of skeletal muscle ERα had no effect on the mass of any hindlimb skeletal muscle examined (Table 2). Subcutaneous and visceral fat pad masses did not differ between skmERαWT and skmERαKO mice nor did uterine masses (Table 2).

Table 2. Tissue masses of female skmERαWT and skmERαKO mice.

	skmERαWT (n=21)	**skmERαKO** (n=20)	***P-value***
Tibialis anterior mass (mg)	39.9 (0.8)	38.8 (0.7)	0.604
Extensor digitorum longus mass (mg)	9.2 (0.2)	8.9 (0.2)	0.995
Gastrocnemius mass (mg)	129.7 (2.6)	126.7 (2.6)	0.786
Soleus mass (mg)	10.3 (0.3)	11.2 (0.4)	0.038
Subcutaneous fat pad mass (mg)	1390 (115)	1301 (183)	0.687
Visceral fat pad mass (mg)	1823 (181)	1563 (201)	0.351
Uterine mass (mg)	76.4 (3.2)	67.7 (8.1)	0.383

Values are means (SE).

Contractility of isolated muscles

The size of the soleus muscle (mass, L_o, CSA) did not differ between female skmERαWT and skmERαKO mice (Table 3). Force production also did not differ (Table 3 and Figure 21A). Force tracings from twitch and tetanic contractions were analyzed to

determine if contraction kinetics differed between soleus muscles with and without ERα. Neither twitch contraction or relaxation times or tetanic rates of contraction or relaxation differed between female skmERαWT and skmERαKO mice (Table 3).

Table 3. *In vitro* contractile properties of soleus and extensor digitorum longus muscles of female skmERαWT and skmERαKO mice.

	skmERαWT	**skmERαKO**	***P-value***
Soleus Muscle	(n=15)	(n=16)	
Mass (mg)	9.9 (0.5)	10.9 (0.4)	0.114
Optimal muscle length (mm)	8.9 (0.2)	9.3 (0.2)	0.240
Physiological CSA (mm^2)	1.47 (0.066)	1.56 (0.051)	0.275
Peak twitch force (mN)	20.0 (0.9)	21.6 (1.3)	0.310
Twitch time-to-peak force (ms)	31.2 (0.7)	34.2 (0.8)	0.013
Twitch one-half relaxation time (ms)	39.5 (1.7)	37.9 (1.7)	0.563
P_o (mN)	152.9 (5.0)	157.7 (7.3)	0.599
+d*P*/d*t* (N/s)	1.7 (0.05)	1.8 (0.09)	0.172
-d*P*/d*t* (N/s)	2.1 (0.07)	2.3 (0.2)	0.147
Active stiffness (N/m)	326.6 (10.9)	324.6 (17.7)	0.926
Peak concentric force (mN)	44.1 (2.8)	40.7 (2.9)	0.423
Peak eccentric force (mN)	292.0 (12.2)	310.7 (13.1)	0.309
Freq50 (Hz)	21.4 (0.6)	21.6 (1.0)	0.921
Extensor Digitorum Longus Muscle	(n=13)	(n=15)	
Mass (mg)	9.2 (0.2)	9.1 (0.3)	0.743
Optimal muscle length (mm)	10.5 (0.2)	10.7 (0.3)	0.688
Physiological CSA (mm^2)	1.87 (0.042)	1.80 (0.058)	0.582
Peak twitch force (mN)	87.2 (5.1)	89.5 (5.0)	0.766
Twitch time-to-peak force (ms)	20.4 (1.0)	22.3 (0.4)	0.182
Twitch one-half relaxation time (ms)	20.1 (1.2)	24.6 (1.3)	0.034
P_o (mN)	319.7 (8.9)	296.6 (11.9)	0.091
+d*P*/d*t* (N/s)	9.0 (0.3)	8.8 (0.4)	0.675
-d*P*/d*t* (N/s)	15.2 (1.2)	11.2 (1.04)	0.024
Active stiffness (N/m)	614.0 (29.7)	610.9 (34.1)	0.947
Peak concentric force (mN)	130.7 (8.4)	114.7 (11.1)	0.057
Peak eccentric force (mN)	558.2 (19.5)	478.9 (27.8)	0.031
Freq50 (Hz)	31.7 (2.0)	26.1 (1.3)	0.034

Values are means (SE). CSA = cross-sectional area, P_o=maximal isometric tetanic force, +d*P*/d*t* = maximum rate of tetanic force development, -d*P*/d*t* = maximum rate of tetanic relaxation, Freq50 = frequency at which 50% isometric tetanic force is generated.

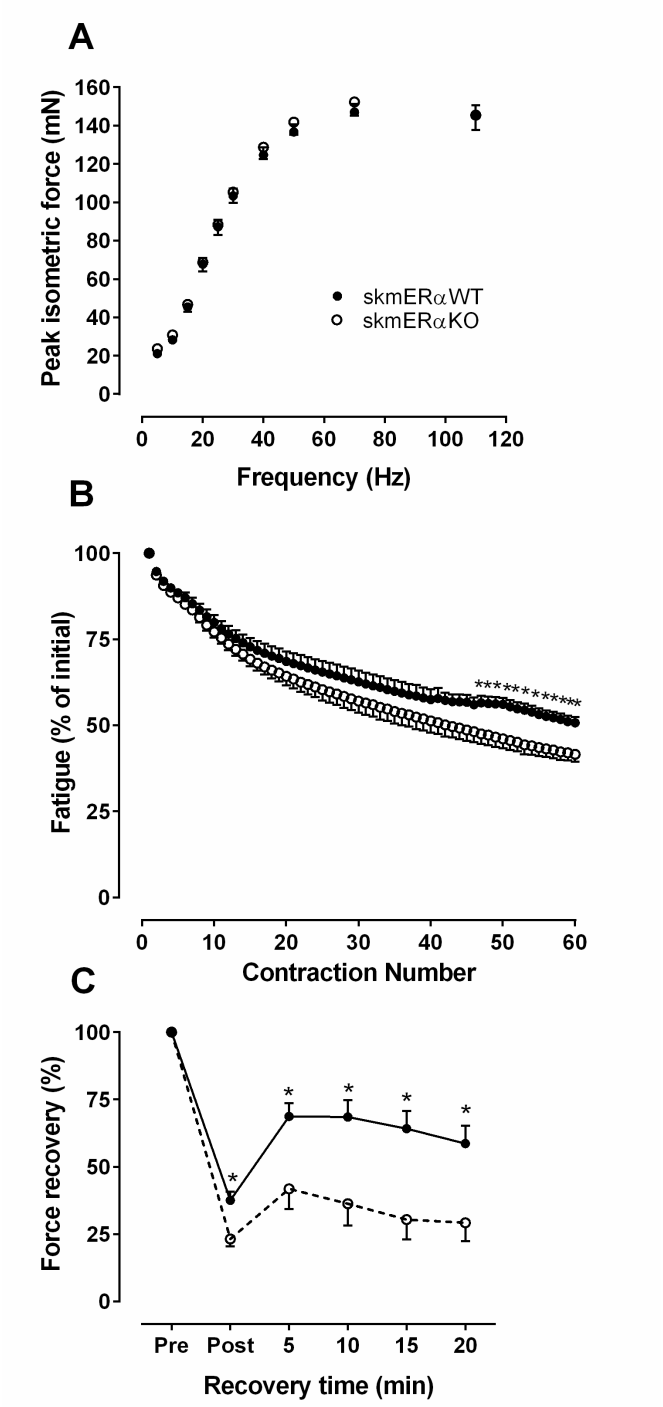

Figure 21. Contractility of isolated soleus muscles from female skmERαWT and skmERαKO mice. A) Submaximal and maximal isometric forces did not differ between skmERαWT and skmERαKO mice ($p \geq 0.133$). B) Fatigue is expressed as the percentage of force relative to that of the first contraction of the protocol. Beginning at contraction 46, muscles lacking ERα generated less force. C) Force recovery was calculated as the percent change from pre P_o. The recovery of soleus muscle P_o was measured following the fatiguing contraction protocol. At every 5 minute interval, including Post which was immediately after the fatiguing contractions, soleus muscles from skmERαKO mice recovered less P_o than did those from skmERαWT mice ($p \leq 0.043$). *significantly different from skmERαWT

We previously showed that estradiol can protect soleus muscle from fatigue [82], therefore, to determine if estradiol works through ERα to elicit this effect, soleus muscles from a subset of female mice completed a bout of submaximal fatiguing contractions. There was greater force loss by skmERαKO than skmERαWT mice from contraction 46 to 60 ($p \leq 0.043$; Figure 21B). Similarly, maximal isometric force was lower in soleus muscles from skmERαKO than skmERαWT mice immediately following the fatigue contractions (37.6 ± 3.2 and 23.2 ± 2.7. mN, respectively; $p=0.032$; Figure 21C) Soleus muscles from skmERαKO mice recovered significantly less force from contraction-induced fatigue compared to skmERαWT mice 5, 10, 15, and 20 min following the fatigue protocol ($p \leq 0.039$; Figure 21C).

Mass, length, and CSA of the EDL muscle did not differ between genotypes (Table 3). Although muscle size was not affected in EDL muscles from skmERαKO mice there were impairments in force generation. Isometric forces at the four highest stimulation frequencies were lower in skmERαKO than skmERαWT mice (Figure 22A) and Freq50 was significantly lower in EDL muscles from skmERαKO mice (Table 3). EDL muscles from skmERαKO mice also produced 16% less eccentric force compared to those from skmERαWT mice (Table 3). Peak concentric torque was not significantly different though tended to be lower in skmERαKO mice (Table 3). Twitch and tetanic contraction kinetics of the EDL muscle did not differ between genotypes, except for $-dP/dt$ which was slow in muscles from skmERαKO mice (Table 3).

A fatiguing protocol of 30 contractions was completed on EDL muscles and induced ~50% force loss that did not differ between skmERαWT and skmERαKO (Figure

22B). The recovery of force after the fatiguing protocol also did not differ between groups (Figure 22C).

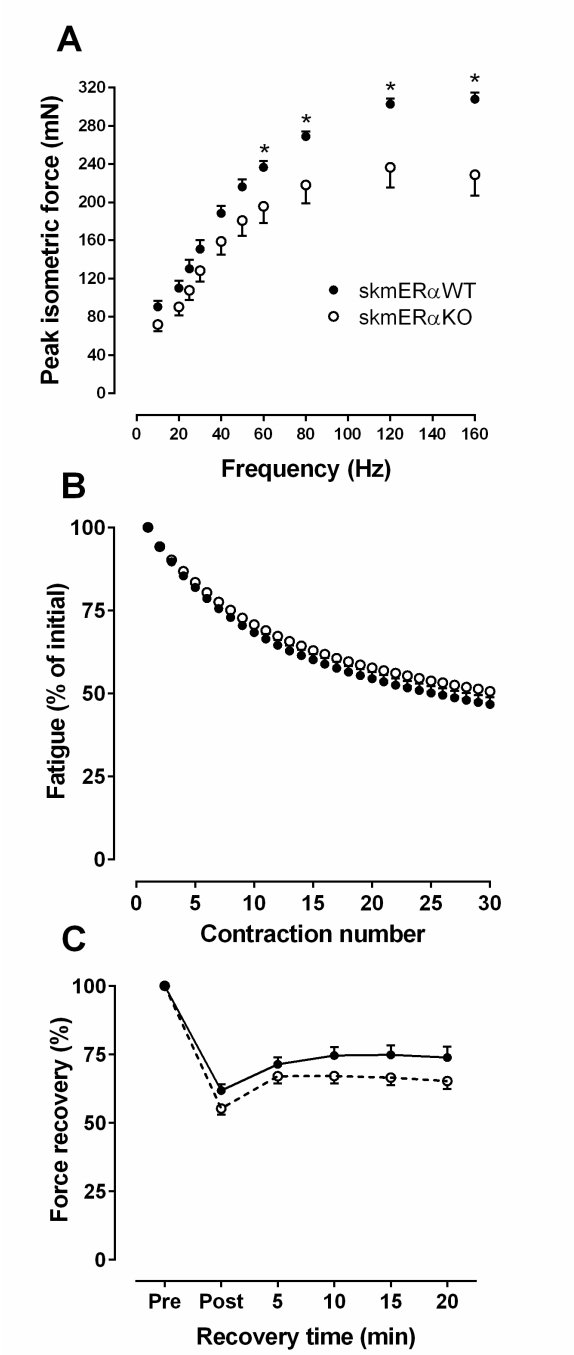

Figure 22. Contractility of isolated EDL muscles from female skmERαWT and skmERαKO mice. A) Submaximal forces were lower in skmERαKO mice compared to skmERαWT mice at 60, 80, 120 Hz stimulation frequencies ($p \leq 0.047$). Maximal force, at 160 Hz, was also lower in skmERαKO compared to skmERαWT mice ($p < 0.001$). B) Fatigue is expressed as the percentage of force relative to that of the first contraction of the protocol. There were no differences between skmERαWT and skmERαKO mice for any of the 30 fatiguing contractions ($p \geq 0.108$). C) EDL muscle recovery of P_o did not differ between genotypes ($p \geq 0.070$). *significantly different from skmERαWT

Soleus muscle composition

To begin to explore if the greater fatigability and impaired recovery from fatigue were related to alterations in the metabolic profile of the soleus muscle from skmERαKO mice, NADH-TR histology was performed (Figure 23A). The percent of fibers positive for NADH was 11% greater in soleus muscles from skmERαKO compared to skmERαWT mice ($p=0.015$; Figure 23C). Soleus muscle anatomical CSA and fiber number did not differ between genotypes ($p \geq 0.669$; Figure 23B).

Soleus muscle fiber-type distribution and size were also measured (Figure 23D-F). There were no statistical differences in type II fiber composition of soleus muscle between skmERαWT and skmERαKO mice ($p \geq 0.147$), though there was a trend for less type I fibers in skmERαKO mice ($p=0.055$). Soleus fiber CSA's did not differ for any fiber type between skmERαKO and skmERαWT mice ($p>0.132$).

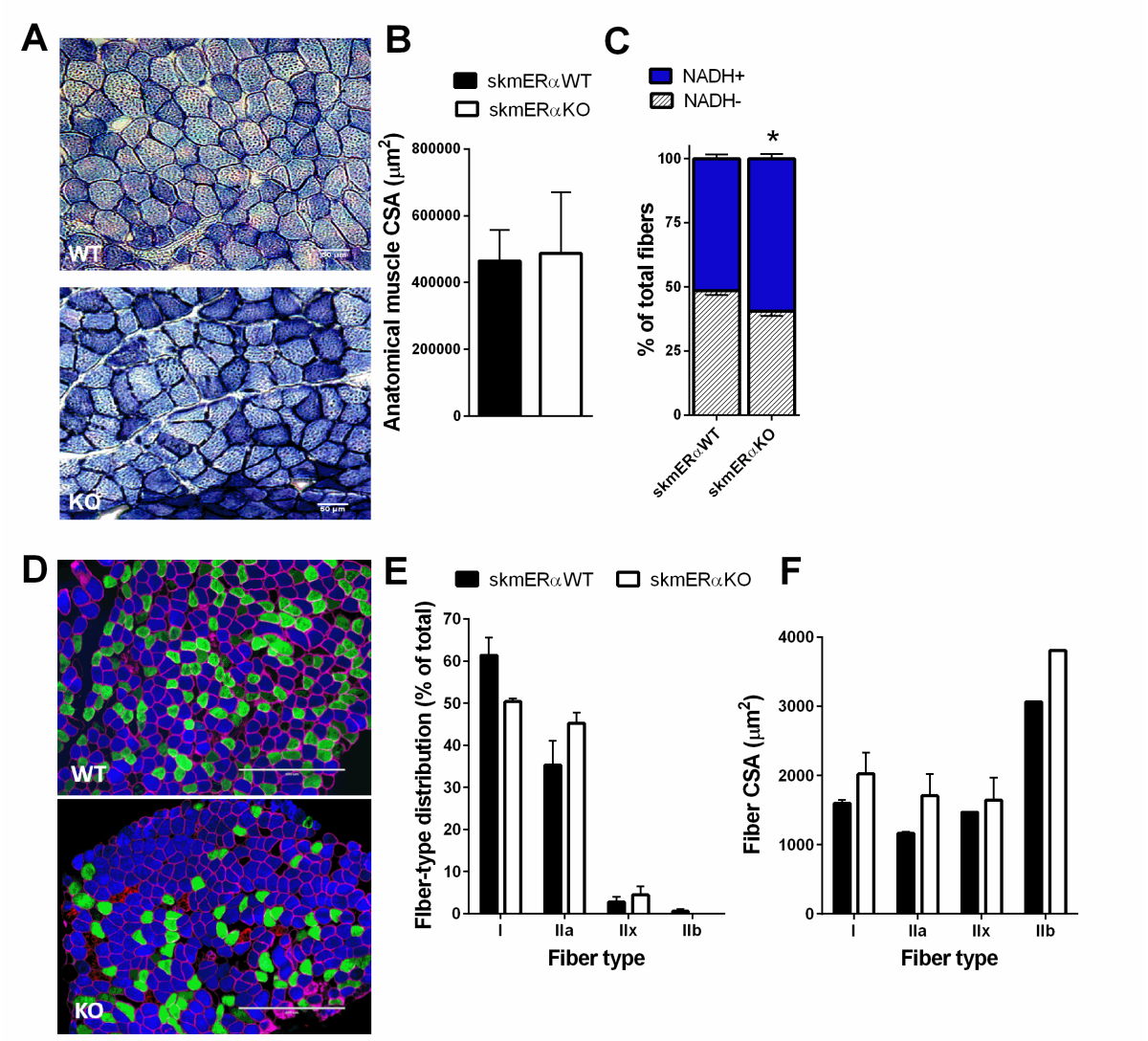

Figure 23. Factors that could influence fatigability of soleus muscle. A) Representative images of NADH-TR staining of soleus muscle cross-sections from skmERαWT and skmERαKO mice. NADH+ fibers (dark blue) NADH- fibers (light blue). Scale bar = 50 μM. B) Anatomical muscle cross-sectional area (CSA) did not differ between skmERαWT and skmERαKO mice ($p>0.834$). C) Percent of fibers NADH+ was greater in skmERαKO than skmERαWT mice ($p=0.015$) D) Representative images of skeletal muscle fiber-typing in cross-sections of soleus muscles from skmERαWT and skmERαKO mice. Myosin heavy chain isoform staining is as follows: Type I (blue), Type IIa (green), Type IIb (red), and Type IIx (unstained) fibers. Muscle fiber border depicted by dystrophin (pink). Scale bar = 400μM. E) Fiber-type distribution in soleus muscles from skmERαWT and skmERαKO mice did not differ ($p>0.055$). F) Cross-sectional area (CSA) by fiber type in skmERαWT and skmERαKO soleus muscles did not differ ($p>0.132$). *significantly different from skmERαWT

In vivo skeletal muscle contractility

To further analyze contractility of muscle groups critical for ambulation, *in vivo* maximal and submaximal muscle strength and power of the plantarflexors were measured. Peak isometric torque was 16% less in the skmERαKO compared to skmERαWT littermates ($p=0.001$; Figure 24A). skmERαKO mice also produced lower maximal concentric torque than skmERαWT mice ($p<0.001$) and maximal eccentric torque was not significantly different though tended to be lower in skmERαKO mice ($p=0.084$; Figure 24A). Moreover, when the plantarflexor muscles were stimulated at submaximal frequencies, skmERαKO mice produced less submaximal torque at every frequency compared to skmERαWT mice ($p<0.05$; Figure 24B). SkmERαKO mice also require a greater stimulation frequency to generate 50% of maximal isometric torque than skmERαWT mice ($p=0.042$; Figure 24C). skmERαKO mice produced less concentric torque from 0 to 1200 degrees per second (Figure 24D), generated 20-30% less power across all velocities tested (Figure 24E), and generated less peak isometric torque at nine ankle angles (Figure 24F) compared to skmERαWT mice.

In a separate group of mice, peak isometric torque of the dorsiflexors was measured. Consistent with the plantarflexors, the dorsiflexors produced 12% less torque in the skmERαKO compared to the skmERαWT female mice ($p<0.047$; Figure 25).

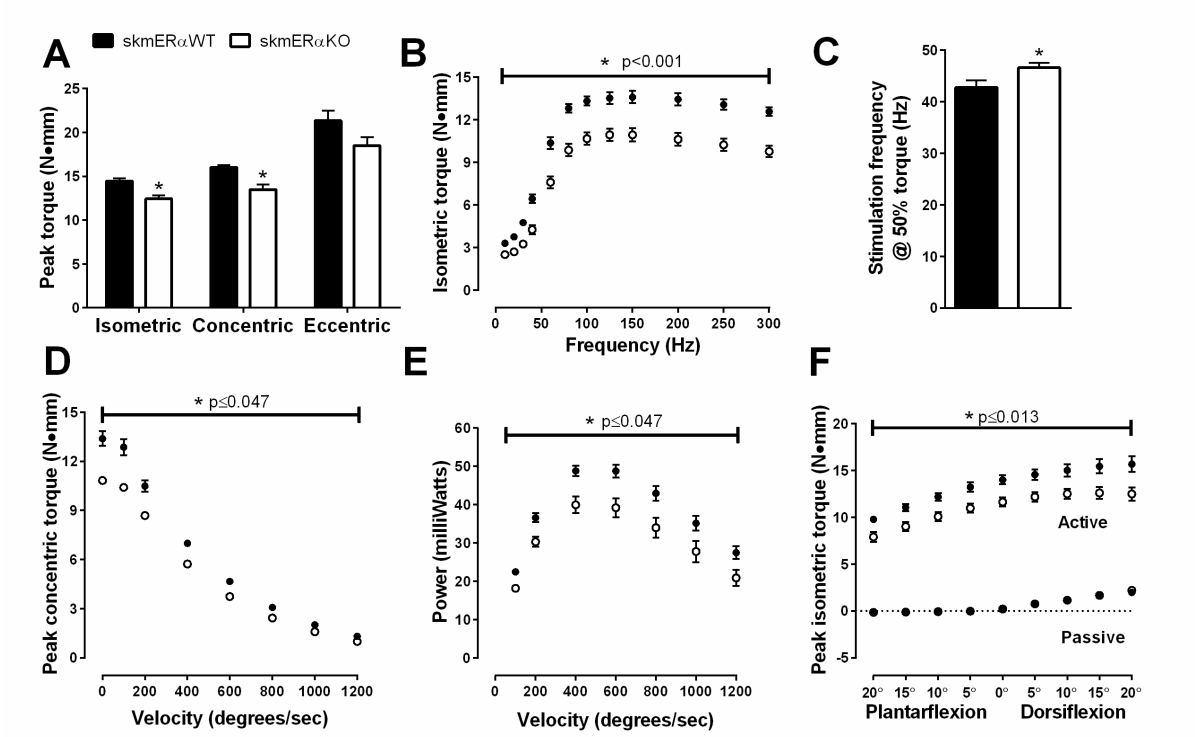

Figure 24. *In vivo* plantar flexor muscle contractility by female skmERαWT and skmERαKO mice. A) Peak isometric and peak concentric torques were lower in skmERαKO mice compared to skmERαWT mice ($p=0.001$). Peak eccentric torque did not differ between groups ($p=0.080$). B) Submaximal and maximal torques were lower in skmERαKO mice compared to skmERαWT mice at each stimulation frequency ($p<0.001$). C) Frequency at which plantarflexor muscles produced 50% torque was greater in skmERαKO mice than skmERαWT mice ($p<0.001$). D) skmERαKO mice produced less concentric torque at all velocities between 0 (isometric) and 1200 degrees/sec compared to skmERαWT mice ($p\leq0.047$). E) Power produced at each velocity was lower in skmERαKO mice than skmERαWT mice ($p\leq0.047$). F) Active torque at different ankle angles was lower in skmERαKO mice compared to skmERαWT mice ($p\leq0.013$). Passive torque did not differ between the groups ($p\geq0.264$). *significantly different from skmERαWT

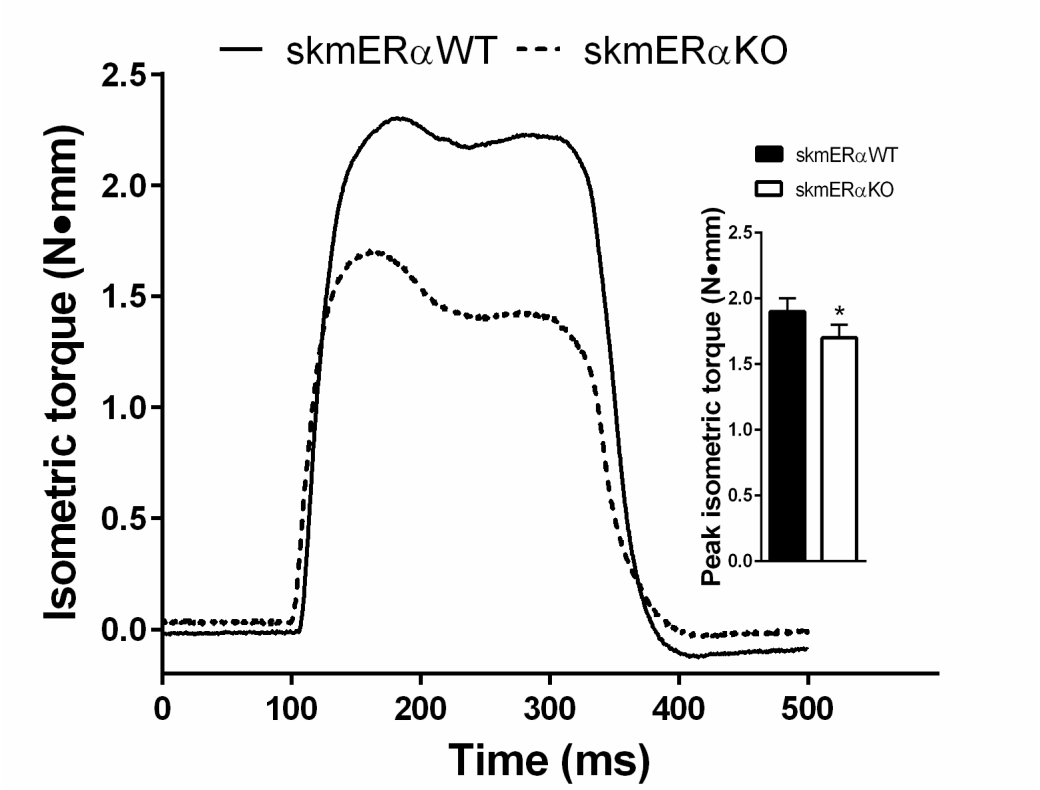

Figure 25. Peak isometric in vivo dorsi flexor muscle torque by female skmERαWT and skmERαKO mice. Representative isometric tetanic torque tracings of dorsiflexor muscles by skmERαWT and skmERαKO mice measured in vivo. Peak isometric torque was lower in female skmERαKO than skmERαWT mice. *significantly different from skmERαWT ($p=0.047$)

Gastrocnemius muscle composition

To determine if the low torque and power of the skmERαKO plantarflexors were related to alterations in fiber type or size, or contractile protein modifications, gastrocnemius muscles in a subset of female mice were analyzed further. There were no differences in fiber-type composition of the gastrocnemius muscle between skmERαKO and skmERαWT mice ($p>0.100$; Figure 26A). Type I and IIb fibers from gastrocnemius muscle of skmERαKO mice had 21% and 14% smaller CSA's, respectively, compared to those from skmERαWT mice ($p\leq0.047$; Figure 26B).

Total protein and contractile protein contents were measured in gastrocnemius muscles, but no differences were observed between those from skmERαKO and skmERαWT mice (p=0. 960 and p=0.429, respectively; Figure 26C). Similarly, RLC was measured in gastrocnemius as well as TA muscles and did not differ between skmERαWT and skmERαKO mice (p = 0.103 and p= 0.075, respectively; Figure 26D). RLC phosphorylation, however was lower by ~90% in muscles from skmERαKO compared to skmERαWT mice ($p \leq 0.047$; Figure 26D,E).

To further probe possible mechanisms that could underlie the differences in muscle contractility, oxidative damage to gastrocnemius muscle proteins was measured by Biotin Hydrazide tagging (Figure 26F). Oxidative damage to proteins in the myofibrillar and cytosolic fractions did not differ between skmERαKO and skmERαWT mice ($p \geq 0.504$; Figure 26G). Oxidative damage to specific contractile proteins, myosin heavy chain and actin did not differ between groups (data not shown $p \geq 0.316$).

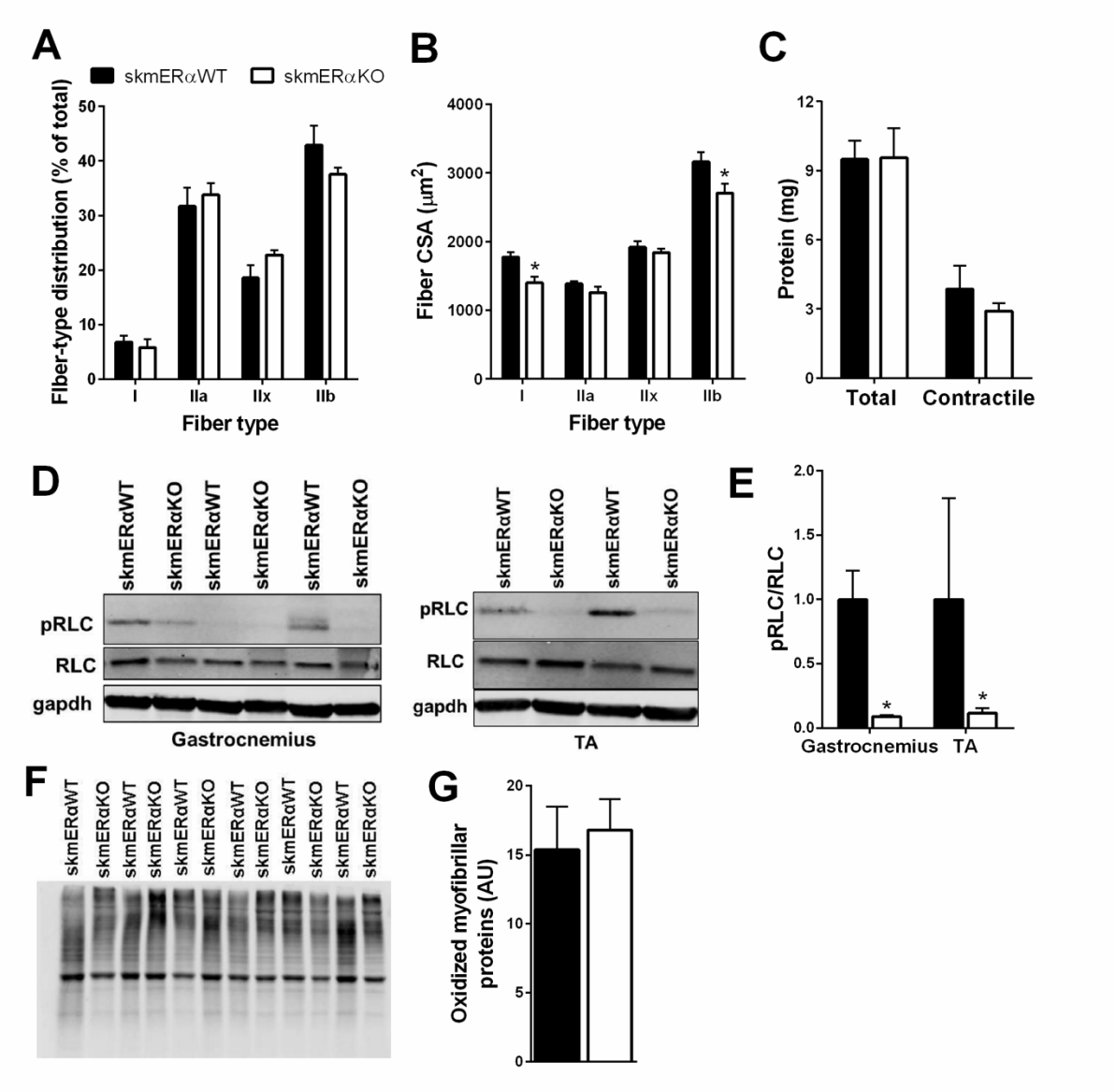

Figure 26. Factors that could influence muscle contractility of the gastrocnemius and tibialis anterior muscles. A) Fiber-type distribution in gastrocnemius muscles from skmERαWT and skmERαKO mice did not differ ($p \geq 0.100$). B) Cross-sectional area (CSA) by fiber type from skmERαWT and skmERαKO mice showed that Type I and Type IIb fibers are smaller in gastrocnemius muscles of skmERαKO mice compared to those in skmERαWT mice. C) Total and contractile protein content did not differ between skmERαWT and skmERαKO mice ($p \geq 0.429$). D) Representative western blots of phosphorylated myosin regulatory light chain (pRLC), myosin regulatory light chain (RLC) and gapdh as loading controls in gastrocnemius and tibialis anterior (TA) muscles from skmERαWT and skmERαKO mice. E) pRLC relative to total RLC is lower in gastrocnemius and TA muscles of skmERαKO mice compared to skmERαWT mice ($p \leq 0.047$). F) Biotin-hydrazide tagged oxidized myofibrillar protein blot G) Oxidized myofibrillar proteins did not differ between skmERαWT and skmERαKO mice ($p = 0.716$) *significantly different from skmERαWT

Discussion

Estradiol impacts skeletal muscle contractility, and here we tested the hypothesis that ERα is the primary estrogen receptor in muscle fibers which estradiol utilizes to elicit its beneficial effects on contractile functions. Our data support the hypothesis that ERα is critical for optimal muscle contractility by demonstrating that mice lacking ERα specifically in skeletal muscle fibers are weaker and produce less power. Our results also indicate that ERα may play a role in fatigability of skeletal muscle and collectively are in agreement with the work by Ribas and coworkers demonstrating that ERα is necessary for overall skeletal muscle health [107]. Our data provide new insight into the physiological actions of estradiol in that the lack of skeletal muscle ERα detrimentally affects force generation indicating that estradiol utilizes a receptor-mediated mechanism to modulate contractility.

A major finding of this study was that the ability of skeletal muscle to generate force was impaired in plantar- and dorsi-flexor muscle groups as well as isolated soleus and EDL muscles in female skmERαKO mice. The plantarflexors in these mice were particularly affected having low submaximal and maximal isometric torques, low peak concentric torque across a range of velocities and at multiple ankle angles, and reduced power compared to skmERαWT littermates (Figure 24). These are physiologically relevant measurements of contractility, meaningful beyond those restricted to maximal isometric contractions, and furthermore were made *in vivo* permitting analyses of muscles' contractile function in its natural environment. Peak isometric torque of the dorsiflexors was similarly low (Figure 25), as were submaximal (Figure 22A) and peak eccentric tetanic

forces of isolated EDL muscles from skmERαKO mice (Table 3). Collectively, these results demonstrate that deficiency of ERα in skeletal muscle fibers detrimentally impacts force generation, consistent with a previous report that isometric force was affected in some skeletal muscles of whole-body ERα knockout mice [197]. Beyond hindlimb skeletal muscle, ERα has been implicated as a key estrogen receptor in rodent cardiac [198-201] and genioglossal muscle contractile function [202, 203].

Maximal and submaximal force generation was not affected in soleus muscles from female skmERαKO mice, but the ability to sustain force during repetitive contractions, that is during a fatigue protocol, was impaired (Figure 21B). This result is consistent with a previous report indicating soleus muscle lacking ERα had low fatigue-resistance [107]. Our data expand on that result by not only linking ERα to muscle fatigability, but also with the recovery from fatigue. Soleus muscle lacking ERα did not recover the ability to generate force following a series of repetitive fatiguing contractions to the same extent as soleus muscle containing ERα (Figure 21C), even though soleus muscles from skmERαKO had a greater percentage of oxidative muscle fibers as indicated by those positive for NADH (Figure 23C). Such outcomes may be related to ERα's role in muscle metabolism such as optimal mitochondrial function. [107, 108]. More directed experiments specifically designed to address these connections in skeletal muscle fibers are warranted, ideally including *in vivo* fatigue protocols and investigation of other estrogen receptors.

Skeletal muscle contractility can be affected by physical (in)activity and estradiol status is a strong regulator of physical (in)activity in female rodents (e.g., [204, 205]). Therefore, it was important to measure possible effects of manipulating skeletal muscle

ERα on physical activity of the mice. We comprehensively show that physical activities in the cage and voluntary wheel running are unaffected by the elimination of ERα in skeletal muscle (Table 1). These results provide evidence that changes we measured in skeletal muscle contractility, such as force and power, are not due to physical inactivity and consequential muscle disuse in the skmERαKO mice, but rather to intrinsic effects on the muscle. A limitation in previous studies using whole body ERα knockout models is that those mice are physically inactive, obese, and diabetic [108], which can detrimentally impact skeletal muscle. Thus, such secondary impacts could not be distinguished from any direct effects of ERα deficiency on muscle contractility. Here, because physical activities of skmERαKO and control littermates did not differ (Table 1), impaired muscle contractility can be directly attributed to lack of ERα in skeletal muscle fibers and presumably diminished estradiol signaling.

In addition to physical activities of female mice not being affected by the lack of ERα in skeletal muscle fibers, at 22 wk of age, food intake (Table 1), body mass (Figure 2B), and subcutaneous and visceral fat pad masses (Table 2) did not differ between skmERαKO and skmERαWT female mice. However, at about 24 wk of age body mass of skmERαWT mice plateaued while that of skmERαKO mice continued to increase, and by 34 wk of age skmERαKO mice weighed 30% more (Figure 20B). A similar delayed increase in body mass has been reported in whole body ERαKO female mice [176, 206] and in skeletal muscle ERαKO female mice [107].

Considering factors that may contribute to low skeletal muscle force generation, wet masses of hindlimb skeletal muscles did not differ between skmERαKO and

skmERαWT female mice (Table 2). We further assessed the gastrocnemius muscle because it is the major muscle comprising the plantarflexors which showed impaired torque and power in the skmERαKO mice. In addition to wet mass of this muscle not differing between skmERαKO and skmERαWT female mice, total protein, contractile proteins (myosin heavy chain and actin) and total RLC contents also did not differ (Figure 26C,D). Fiber type distribution based on myosin heavy chain isoforms did not differ between gastrocnemius muscles of skmERαKO and skmERαWT female mice (Figure 26A). Thus, the amount of contractile proteins and myosin isoform composition does not help to explain contractility impairments in the skmERαKO mice such as power. This is consistent with another report demonstrating that estradiol mediates effects on myosin heavy chain isoform expression in skeletal muscle through estrogen receptor β rather than ERα [207]. Cross-sectional areas of myosin heavy chain type IIa and IIx fibers also did not differ between gastrocnemius muscles from skmERαKO and skmERαWT female mice, although type I and IIb fibers had smaller cross-sectional areas in those from skmERαKO than skmERαWT mice (Figure 26B). Because type I and IIb fibers together compromise about one-half of the fibers in mouse gastrocnemius muscle, this may explain a portion of the reduced force generating capacity of skmERαKO plantarflexors but not plausible to explain the impairment in full.

The capacity of myosin to bind strongly to actin and generate force in skeletal muscle of mice [93, 95, 104] and women [102] is impaired with estradiol deficiency and is mediated at least in part through post-translational modification of contractile proteins [102]. In particular, phosphorylation of the myosin regulatory light chain is reduced in

skeletal muscle of ovariectomized mice [106] and in fibers of aged women [168] and is related to impaired force generation. Consistent with those reports, we measured a substantial reduction in phosphorylation of the myosin RLC in muscles of skmERαKO mice indicating estradiol utilizes ERα to phosphorylate RLC, which in turn allows for a greater capacity of myosin to bind strongly to actin and generate force. Previously it was suggested the estradiol mediates RLC phosphorylation through ERβ and GPER, but those experiments were conducted on C2C12 cells [106]. Results here support the hypothesis that in muscle fibers ERα is the primary mode through which estradiol affects pRLC and skeletal muscle contractility. Furthermore, the magnitude of force deficits we have previously measured in muscles of estradiol-deficient female mice has been 10-20% [93, 95, 104, 208] and is similar to the magnitude of force, torque, and power deficits measured in muscles with an ERα deficiency.

Early evidence that estradiol mediates its effects in skeletal muscle through estrogen receptors came from Dieli-Conwright and co-workers [209]. Results presented here show that ERα is the specific receptor necessary for optimal contractile function in female muscle, further substantiating that mechanisms underlying estradiol's beneficial impact on skeletal muscle is important to elucidate to understand relationships between hormone deficiency and sarcopenia. The maintenance of strength across the life span and with the loss of ovarian function is critical for women to maintain functional independence. This point is further emphasized by the fact that women live nearly one-third of their lives after menopause. Due to the controversial results of the Women's Health Initiative, standard approaches for pharmacological activation of ERα are not employed with the

same frequency as they were prior to that initiative; however our data strongly suggest that ERα is critical for normal physiological function of skeletal. Thus, identification of novel compounds that could activate ERα might provide alternative therapeutic interventions to help women maintain strength and independence throughout their lifespan.

Chapter 5

Summary statement

The contents of this dissertation add significant and novel findings to the fields of satellite cell biology, estradiol hormone actions, hormone receptor signaling, and skeletal muscle physiology. During the time of menopause, women experience the loss of ovarian hormones, specifically estradiol. Previous research from our lab has established that the loss of estradiol causes a deficit in the strength recovery from injury. Prior to my studies, the mechanism for the delay in recovery was unknown. My studies suggest that the baseline differences in satellite cell number, as well as, impaired satellite cell function (self-renewal and differentiation) present as potential mechanisms. Moreover, there has been little research completed *in vivo* to establish which estrogen receptor estradiol utilizes during the regeneration process of skeletal muscle. The contents of chapter 3 establish that estradiol utilizes estrogen receptor α (ERα) to elicit its effects on satellite cell number and function. Specifically, these studies determined that the loss of estradiol or the receptor resulted in activation of cell autonomous apoptosis which likely led to the depletion of the satellite cell pool.

Following, the identification of ERα being the primary receptor estradiol utilizes in satellite cells, I began to determine if this held true in skeletal muscle fibers. The contents of chapter 4 investigated the role of ERα in multiple measures of skeletal muscle contractile function. Our lab, along with others, has demonstrated that the loss of estradiol causes a 10-20% deficit in force production (i.e. strength) and treatment with estradiol rescues the force decrement. Recent work from Ribas et al showed ERα was the primary receptor estradiol

utilized for metabolic and mitochondrial functions. In alignment with Ribas et al, muscles lacking ERα had impairments in fatigability and recovery from fatigue due to metabolic defects in the mitochondria. The contents of this chapter establish scientific premise as comprehensive muscle physiology is at the pinnacle of this work. This study thoroughly described a new mouse model (skeletal muscle fiber knockout of ERα) as well as established that ERα is necessary for optimal physiological function. Thereby, establishing that estradiol utilizes ERα to elicits its beneficial effects on skeletal muscle contractility.

Future experiments should further elucidate the estradiol-sensitive apoptosis signaling pathway identified in chapter 3 in order to determine if estradiol directly utilizes this pathway to impact satellite cell maintenance and self-renewal. Additionally, this pathway should be tested in the skeletal muscle fiber knockout model to determine if whole-tissue apoptosis is occurring, which could be a possible mechanism for the decrease in strength. The studies of this dissertation hypothesized that asymmetric division could be affected in satellite cells and the potential mechanism for the impairment in satellite cell maintenance, future studies should directly measure asymmetric division as well as determine if activation, proliferation, and/or differentiation of satellite cells are all equally or preferentially affected by the loss of estradiol. Lastly, since the literature supports the concept that estradiol affects mitochondrial function in muscle fibers, it is possible that satellite cell mitochondria are also impaired with the loss of estradiol and could be the mechanism for satellite cell apoptosis.

Overall, the results of this dissertation show that the loss of estradiol and ERα is detrimental to skeletal muscle and that estradiol treatment is beneficial. Furthermore, it

provides evidence for the study of alternative therapeutic interventions (e.g. selective estrogen receptor modulators) to help mitigate the negative effects of menopause in aged-women.

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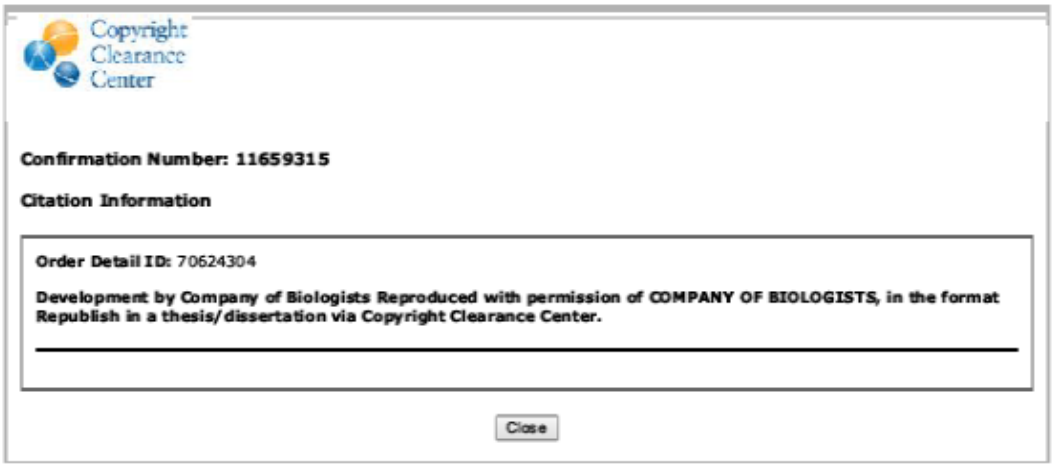
Appendix 1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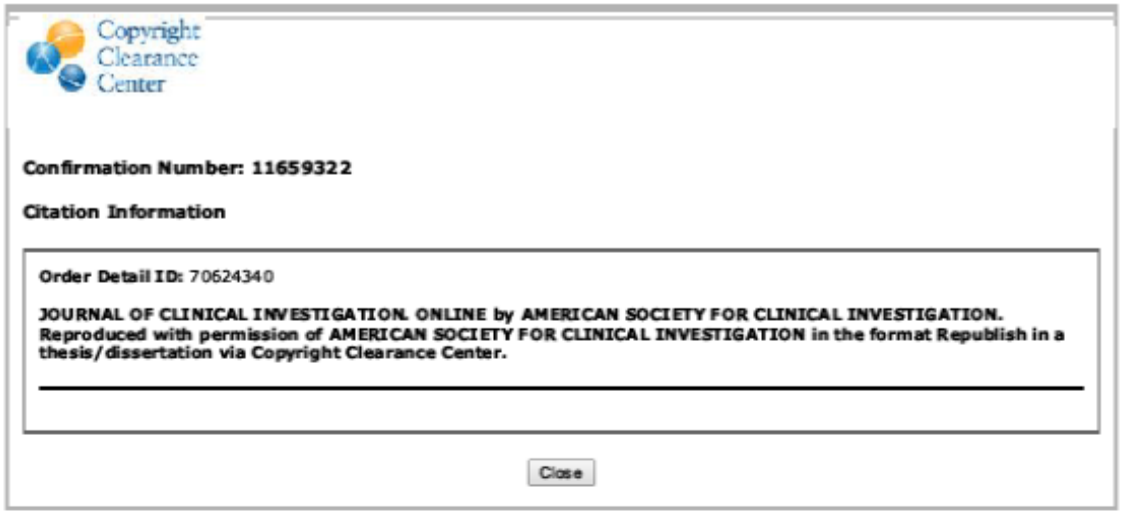

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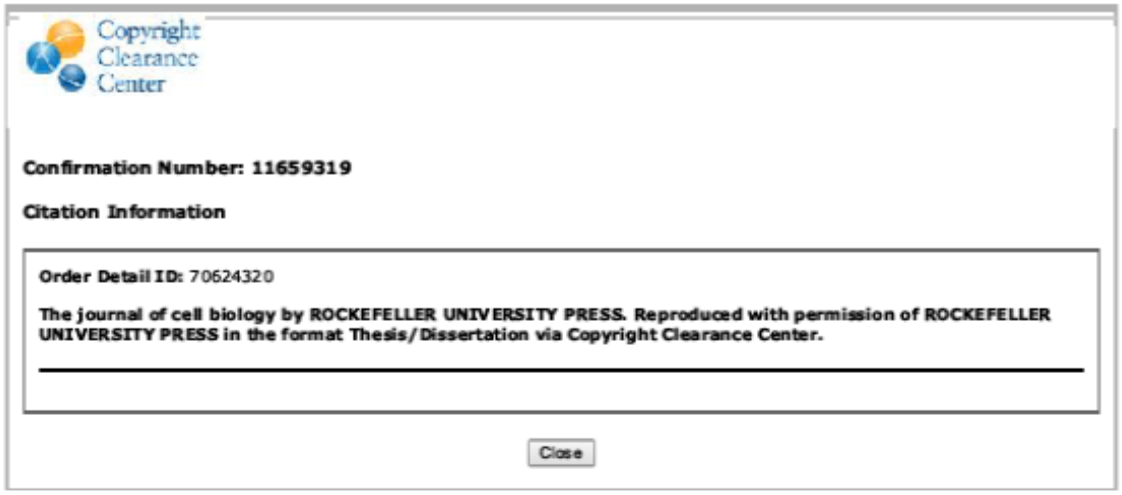

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