Satellite cell maintenance and strength recovery after injury: the impact of estradiol signaling

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Dedication

I dedicate this dissertation to my parents whose wholehearted love and support has raised me up when times are tough. To my mother, for being my daily support system. I can only hope to be as strong and selfless as she is. To my father, who has taught me to be optimistic and live each day to its fullest potential. I can only hope to enjoy life as much as he does. My deepest gratitude to my parents for encouraging me to pursue my dreams. I love them both to the moon and back.
Abstract

My dissertation work established a crucial role for estradiol ($E_2$) in the recovery of muscle strength after injuries and maintenance of the satellite cell population under homeostatic conditions (Chapters 3 & 4). I demonstrated that $E_2$ deficiency impairs the adaptive potential of skeletal muscle after repeated injuries, indicated by blunted muscle mass and strength, and that the reduction in satellite cell number with $E_2$ deficiency likely contributes to this impairment (Chapter 3). With the ovariectomy mouse model and a transgenic female mouse model that specifically ablated ERα in satellite cells, I demonstrated that $E_2$ is the hormone that drives the loss of satellite cells as opposed to any other ovarian hormone, and that the loss of $E_2$ or its receptor for only 14 d impairs satellite cell maintenance (Chapter 4). Mechanistically, I showed that impaired satellite cell maintenance caused by $E_2$ deficiency involves altered satellite cell cycle progression, kinetics, proliferation, and differentiation (Chapter 4). The work of my dissertation highlights a novel mechanism for $E_2$ in maintaining the satellite cell population in female mice through appropriate satellite cell cycle progression. My findings, accompanied by future studies that identify $E_2$-sensitive molecular pathways in satellite cells, are instrumental for developing effective therapies to preserve efficient skeletal muscle regeneration and improve overall skeletal muscle health of post-menopausal women.
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### Abbreviations

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<tbody>
<tr>
<td>4-OHT</td>
<td>4-hydroxytamoxifen</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BaCl₂</td>
<td>barium chloride</td>
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<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2’-deoxyuridine</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinase inhibitors</td>
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<tr>
<td>CS</td>
<td>charcoal-stripped</td>
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<tr>
<td>CSA</td>
<td>cross-sectional area</td>
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<tr>
<td>CTX</td>
<td>cardiotoxin</td>
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<tr>
<td>DAPI</td>
<td>4,6-diamidino-2-phenylindole</td>
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<tr>
<td>DM</td>
<td>differentiation medium</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>E₁</td>
<td>estrone</td>
</tr>
<tr>
<td>E₂</td>
<td>17β-estradiol</td>
</tr>
<tr>
<td>E₃</td>
<td>estriol</td>
</tr>
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<td>EDL</td>
<td>extensor digitorum longus</td>
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<tr>
<td>EdU</td>
<td>5-ethynyl-2’-deoxyuridine</td>
</tr>
<tr>
<td>eMHC</td>
<td>embryonic myosin heavy chain</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
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<tr>
<td>ERE</td>
<td>estrogen response elements</td>
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<td>Abbreviation</td>
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<tr>
<td>ERK</td>
<td>extracellular-signal related kinase</td>
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<tr>
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<td>estrogen receptor-alpha</td>
</tr>
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<td>FACS</td>
<td>fluorescent-activated cell sorting</td>
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<td>fibro-adipogenic progenitor</td>
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<td>fetal bovine serum</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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<td>GPER</td>
<td>G-protein coupled receptor</td>
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<td>HGF</td>
<td>hepatocyte growth factor</td>
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<td>HRT</td>
<td>hormone replacement therapy</td>
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<tr>
<td>HS</td>
<td>horse serum</td>
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<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
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<td>IGFBP</td>
<td>insulin-like growth factor binding protein</td>
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<tr>
<td>Lin</td>
<td>lineage antigens</td>
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<td>MAPK</td>
<td>mitogen-activated protein kinases</td>
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<td>muscle growth medium</td>
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<td>MHC</td>
<td>myosin heavy chain</td>
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<td>mouse myoblast medium</td>
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<td>MRF</td>
<td>myogenic regulatory factor</td>
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<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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<td>myoblast determination protein 1</td>
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<td>MyoG</td>
<td>myogenin</td>
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<tr>
<td>NCAM</td>
<td>neural cell adhesion molecule</td>
</tr>
<tr>
<td>Ovx</td>
<td>ovariectomy</td>
</tr>
<tr>
<td>P13K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>Pax7</td>
<td>paired box transcription factor</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerthrin</td>
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<td>Pen</td>
<td>penicillin solution</td>
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<td>PFA</td>
<td>paraformaldehyde</td>
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<td>PHTPP</td>
<td>2-phenyl-5,7 bis(trifluoromethyl)-pyrazolo[1,5-a] pyrimidin-3-yl)-phenol</td>
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<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PPT</td>
<td>propyl pyrazole triol</td>
</tr>
<tr>
<td>PVC</td>
<td>persistent vaginal cornification</td>
</tr>
<tr>
<td>scERαKO</td>
<td>satellite cell-specific ERα knockout mouse</td>
</tr>
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<td>scERαWT</td>
<td>wild-type littermates</td>
</tr>
<tr>
<td>siERβ</td>
<td>small interfering RNA against ERβ</td>
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<tr>
<td>SM</td>
<td>semimembranosus</td>
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<tr>
<td>Strep</td>
<td>streptomycin solution</td>
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<td>TA</td>
<td>tibialis anterior</td>
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<td>TGF-β</td>
<td>transforming growth factor β</td>
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<td>tyramide signal amplification</td>
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<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
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<td>β-gal</td>
<td>β-galactosidase</td>
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Chapter 1: Introduction

Aging is associated with a loss of muscle mass and strength, known as sarcopenia [1]. Even physically active individuals experience some level of muscle loss with age [2]. Muscle strength declines at 3-5% per decade begin at around 30 years, with an average cumulative decline in muscle strength of 30% over a lifetime [3, 4]. More detrimental declines of up to 50% in muscle mass and strength have been observed at 80 years in inactive individuals [5, 6]. Sarcopenia is a major public health problem that can cause additional health conditions, such as metabolic dysfunction [7] or cardiovascular disease [8], and ultimately is associated with mortality and reduced quality of life [9]. Since the population of individuals over 60 years will nearly double from 12% to 22% by 2050 [10], there is an urgent need to develop therapies to address sarcopenia and the health and social challenges that will arise from this demographic shift.

Sarcopenia has been shown to be regulated by several mechanisms, including dysregulation of protein homeostasis [11], autophagy [12, 13], mitochondrial function [14, 15], increased adipose or fibrotic tissue [16, 17], and impaired maintenance of the muscle stem cell population (i.e., satellite cells) [18]. Because satellite cells have an indispensable role in the regeneration of skeletal muscle [19] and are known decline with age [20], they are an attractive target to explain impaired regenerative capacity that is linked to sarcopenia.

The age-related changes in the satellite cell population have been attributed to cell-autonomous (i.e., intrinsic) and environmental (i.e., extrinsic) changes. Heterochronic parabiosis and transplantation studies have showed that exposing geriatric...
mice to a young systemic environment can restore activation, proliferation, self-renewal and regenerative capacity of aged satellite cells [21, 22]. These studies indicated that systemic factors that change with age are crucial to maintain satellite cell function. Extracellular signaling pathways (e.g., ERK, p38 MAPK, Notch, Wnt) and circulating growth factors (e.g., FGF-2, IGF-1, HGF) have been shown to govern satellite cell fate [23, 24]. Importantly, the sex hormone estradiol (E₂), that is known to decline with age, has been implicated to regulate satellite cell proliferation after injury or exercise [25]. However, the mechanisms whereby satellite cell function is regulated by estradiol is unclear. I propose that estradiol maintains the satellite cell pool through appropriate regulation of the cell cycle and cell fate decisions. My dissertation is focused on investigating the mechanisms whereby estradiol affects satellite cell maintenance under homeostatic conditions and recovery of skeletal muscle strength after injury in females.

The following sections summarize the current understanding of 1) satellite cell biology and skeletal muscle regeneration, 2) satellite cell function with age, and 3) E₂ effects on satellite cells. The first aim of this dissertation is to determine the extent to which E₂ deficiency affects recovery of skeletal muscle strength and satellite cell number when muscle is challenged by multiple injuries. The second aim is to evaluate the role of E₂ in satellite cell maintenance under normal homeostatic conditions (i.e., without injury). This third aim is to investigate the mechanism(s) underlying the decline in satellite cell number with E₂ deficiency. Overall, my dissertation tests the overarching hypothesis that E₂ regulates satellite cell maintenance by augmenting myogenesis, resulting in efficient recovery of strength after injury.
Chapter 2: Literature review

Skeletal muscle overview

Skeletal muscle accounts for 30% and 38% of body weight in adult women and men, respectively, and is necessary for locomotion, oxygen consumption, postural support, temperature regulation, energy storage, and organ protection [26]. The complex architecture of skeletal muscle consists of terminally differentiated, multi-nucleated cylindrical cells called muscle fibers. Each muscle fiber is composed of many myofibrils (approximately 2,000 in an untrained adult), and appears striated under the microscope due to the stacking structure of contractile proteins, actin and myosin, to form sarcomeres [26, 27]. When actin filaments slide along myosin filaments, an action known as the power stroke, the sarcomere shortens and the muscle fiber contracts [26]. Skeletal muscle is highly susceptible to injury due to the intricate structure, mechanical properties, and peripheral location. Though, skeletal muscle is well-equipped to repair damaged muscle fibers because of its resident stem cell population, known as satellite cells.

Skeletal muscle satellite cells

Satellite cells are known to play a crucial role in muscle fiber maintenance, repair, and regeneration. Satellite cells were first observed under an electron microscopic by Alexander Mauro [28] and Bernard Katz [29] as the cells “wedged” between the plasma membrane and basal lamina of frog muscle fibers. Satellite cells have since been observed in close association with muscle fibers across numerous species (e.g., zebrafish, mouse, pig, monkey, human) [30-33]. Along with anatomical location, satellite cells can
be identified by expression of cell-surface markers or cell adhesion molecules, such as: paired box transcription factor (Pax7) [33], vascular cell adhesion protein 1 (VCAM-1) [34], α7 Integrin [35], CD34 [36], M-cadherin [37], caveolin-1 [38], and neural cell adhesion molecule (NCAM) [39]. Cell surface markers can be used to reliably quantify and isolate satellite cells by fluorescence activated cell sorting (FACS) [40, 41]. For satellite cell analysis, hematopoietic and stromal cells are excluded by markers, such as CD45, CD31, and Sca-1, and satellite cells are identified with the previously mentioned cell surface markers, such as α-7 integrin, VCAM, and CD34. However, Pax7 is the most widely used marker for satellite cells and has been deemed the most reliable marker since it marks all functional (quiescent or activated) satellite cells and within muscle is only produced by satellite cells [42-45]. FACS can also be performed using Pax7-ZsGreen transgenic mice which express ZsGreen, an enhanced green fluorescent protein, under the control of the Pax7 promoter [46]. Additionally, the Pax7CreER mice allow for inducible labeling and genetic ablation of satellite cells or a floxed sequence expressed in Pax7+ satellite cells [47].

The size of the satellite cell is approximately 8-10 μm with a small amount of cytoplasm surrounding their nuclei in a quiescent state [28, 48], and an expanded cytoplasm and increase in organelles upon activation [49]. Satellite cells have long projections with filopodia that are heterogeneous and are thought to act as dynamic sensing structures [50]. These projections have also been observed to retract in response to injury or upon isolation of satellite cells [51]. Satellite cells also have few mitochondria and a low metabolic rate under homeostasis, predominantly producing
energy by oxidative phosphorylation or β-oxidation during quiescence and glycolysis during activation and proliferation [52-54]. Satellite cell proliferation is responsible for generating committed myogenic cells, as well as replenishing the satellite cell pool. A satellite cell can generate both a self-renewing daughter cell that returns to quiescence and a committed daughter cell through asymmetric division, or can generate two daughter cells with identical cell fates through symmetric division [55-57]. Since their discovery, satellite cells have been implicated in postnatal muscle growth [58] and regeneration [59] and are established as the *bona fide* myoblast precursor cells capable of self-renewal [56] and differentiation to produce myoblasts that can fuse to existing or create new muscle fibers [60].

The satellite cell population is heterogeneous, indicating that there are subpopulations with different developmental origins and functional properties [Reviewed in 23, 61, 62]. Single-cell analysis studies continue to determine the degree of heterogeneity of the satellite cell population and have recently demonstrated that subpopulations may exhibit altered gene expression [36, 57], cycling dynamics [63], proliferative [64] and self-renewal capacities [65], and temporal and spatial heterogeneity [66]. Heterogeneity is considered to be advantageous to maintain a diverse satellite cell population that can promptly respond to the dynamic demands of growing and regenerating skeletal muscle.

**Satellite cell homeostasis**

In undamaged muscle, adult satellite cells account for 2-7% of sublaminal nuclei on muscle fibers [67] and are predominantly in a non-cycling, quiescent state [68].
However, a small subset of satellite cells will proliferate and contribute new nuclei to differentiated fibers to maintain healthy muscle [69-71]. By measuring the incorporation of thymidine analog 5-bromo-2’-deoxyuridine (BrdU) or 5-ethynyl-2’-deoxyuridine (EdU), studies have shown that 0.2±0.1% of satellite cells proliferate per day [72] or 0.1-2.0% of satellite cells proliferate per week [70, 73]. Satellite cell lineage tracing mouse models (e.g., *Pax7CreERT2/+;R26RtdTomato* or *Pax7CreERT2/+;Rosa26mTmG/+*) allow satellite cell contribution to be determined long-term and have measured much higher satellite cell proliferation than EdU or BrdU incorporation assays. Such studies measure 20% of tdTomato+ satellite cells fused into muscle fibers following a 2-week chase [69] and 8-36% of GFP+ satellite cells fused into muscle fibers following a 12-month chase [71]. Consistent differences in the degree of satellite cell proliferation have been observed under homeostatic conditions between muscles, muscle fiber type, and age [69, 71]. Under homeostatic conditions, satellite cells have also been shown to favor asymmetric division to maintain the satellite cell population throughout life [57, 74, 75]. This is largely because uninjured muscle does not require a large number of satellite cells to generate myogenic precursor cells that can differentiate and fuse to muscle fibers. In contrast, injured muscle does require rapid expansion of the satellite cell pool and achieves this originally through symmetric division and followed by asymmetric division [74, 76, 77].

**Satellite cell function in skeletal muscle regeneration**

Skeletal muscle regeneration includes successive but overlapping degenerative and regenerative phases that include: 1) an inflammatory response, 2) activation,
differentiation, and fusion of satellite cells; and 3) maturation and remodeling of newly formed myofibers [Reviewed in 78]. For an efficient regenerative phase, an intricate balance between satellite cell self-renewal and myogenic commitment are required. Satellite cells are recruited to repair muscle following introduction to natural toxins (cardiotoxin, notexin), chemical reagents (BaCl2), physical injury (freeze, crush, denervation, irradiation), medications (bupivacaine), and eccentric-contraction induced injury [72, 79]. Irrespective to the type of stress, skeletal muscle follows a general regeneration process in mice and humans, including proliferation, differentiation, and fusion to form myofibers [23, 72, 80]. Upon stimulus, quiescent satellite cells that express Pax7 and Myf5 become activated, migrate to the site of injury, and re-enter the cell cycle [81]. The rate at which satellite cells enter the cell cycle and divide (i.e., time to first division) have been shown to substantially influence muscle regeneration with a faster time to first division resulting in enhanced regeneration [53, 82]. Interestingly, the Brack lab [63] recently showed that satellite cells follow a random-walk like process to activation and can exit and re-enter the cell cycle rather than progressing fully through the cell cycle once activated. After cell division, the satellite cell daughter cells expressing high levels of Pax7 and low levels of MyoD (Pax7+MyoD-) return to quiescence, whereas the cells expressing low levels of Pax7 and high levels of MyoD (Pax7-MyoD+) differentiate into myoblasts. These myoblasts then differentiate into MyoG+MRF4+Myogenin+ myocytes, and fuse to form myosin heavy chain (MHC+) myofibers. These myofibers repair the damaged muscle tissues and restore muscle strength. Standard markers of regeneration, including the presence of centralized nuclei,
embryonic myosin heavy chain (eMHC)-positive fibers, and a reduced myofiber cross-sectional area (CSA), can be observed up to a month following the insult [47, 83].

**Satellite cells with age**

Stem cell exhaustion is a hallmark of aging and muscle satellite cells are no exception to this age-related deficiency. The regenerative capacity of muscle is dependent on satellite cell abundance and their ability to respond to intrinsic and extrinsic cues. Numerous groups have observed a decline in satellite cell number in aged rodent [67, 84-91] and human skeletal muscles [92-94] with the rate and extent varying with muscle fiber type and function (e.g., locomotion, respiration, or mastication) [87, 89, 90]. Generally, the size of the satellite cell pool is reported to amount to less the 2% of the total myonuclei with age [95]. Females have also been reported to experience more significant declines in satellite cell number compared to males [89]. The maintenance of the satellite cell pool is known to be crucial throughout life and studies have reported that the inherent myogenic potential of satellite cells does not decline with age, suggesting that the decline in regenerative capacity of muscle is primarily due to a decline in the number of satellite cells [20]. Nonetheless, others have observed a decline in satellite cell function in aged satellite cells [23]. Traditionally studies measuring satellite cell function assess the ability of satellite cells to respond to stress and efficiently regenerate damaged tissue. Inefficient regeneration with age is characterized by a decline in myofiber CSA [77, 84, 96], increased fibrotic or adipose tissue [16, 21, 97], and blunted recovery of strength [98]. Overall, decrements in both satellite cell number and function that result in
impaired regeneration are attributed to intrinsic changes with satellite cells and extrinsic changes in the satellite cell environment that ultimately influence the ability of satellite cells to maintain a balance between quiescence, proliferation, self-renewal, and apoptosis. Changes in the satellite cell environment (i.e., niche) will be further discussed in the next section.

**Satellite cell niche with age**

During aging, changes in local and systemic factors influence satellite cell fate and function resulting in impaired regeneration. Satellite cells reside in close proximity to a variety of cell types, namely fibroblasts, immune cells, the muscle fiber itself, and endothelial cells, so it is no surprise that communication between satellite cells and other cells affect satellite cell function. In fact, 88% of satellite cells are reported to be within ~20 μm of an endothelial cell (i.e., capillary) [99], and this close proximity has been shown to promote satellite cell self-renewal, signifying that cell-cell interactions and circulating factors play crucial roles in regulating satellite cell maintenance [50]. The importance of environmental factors on satellite cell function were first established using heterochronic tissue transplant or heterochronic parabiotic pairing studies. Such studies demonstrated that the success of a tissue transplant is associated with younger age [22, 100, 101], and that exposing an aged animal to the circulation of a young animal can restore satellite cell regenerative properties [16, 21]. Since these studies, several local and systemic signaling molecules and growth factors have been identified to change with age and disrupt satellite cell function.
Satellite cell quiescence is disrupted with age due to a reduction in myofiber secretions of Delta, a Notch ligand [21, 102, 103], and an increase in myofiber secretions of fibroblast growth factor 2 (FGF2) [104-106]. Cytokines produced in the muscle by fibro-adipogenic progenitors (FAPs) or systemically, such as IL-6, have been shown to have an attenuated response following exercise in aged individuals blunting satellite cell activation [107]. In addition, the production of extracellular matrix protein fibronectin is reduced with age impairing proliferation of satellite cells via β1-integrin and FGF2-ERK MAPK signaling [108, 109]. In the aging systemic environment, there is an increase in systemic transforming growth factor β (TGFβ) [110, 111] and Wnt [16, 112], and a 3-fold decrease in systemic oxytocin [113]. These local and systemic changes promote satellite cells to undergo pre-senescence or lose polarity, which impairs satellite cell self-renewal and causes the majority of activated satellite cells to differentiate [114]. Furthermore, age-induced aberrant signals, such as increase canonical Wnt, can trigger satellite cells to switch to a fibrogenic or adipogenic fates or undergo apoptosis further contributing to depletion of the satellite cell pool and functional deficits [16, 74].

Rejuvenation of the aged satellite cell environment has been achieved through targeting several of these extrinsic factors, such as Notch activation, Wnt inhibition, local fibronectin, or systemic administration of oxytocin [113], resulting in maintenance of quiescence, enhancement of satellite cell activation and proliferation via ERK-MAPK, and subsequent restoration of the myogenic potential of aged satellite cells [Reviewed in 114]. Since skeletal muscle regeneration is a tightly controlled process, it is crucial that the key age-specific local and systemic regulators of satellite cells are identified to
combat inefficient regeneration and subsequent deterioration of skeletal muscle mass and strength with age. Our lab proposes that the sex hormone estrogen is a key systemic hormone regulating the satellite cell population [115].

**Estrogen and satellite cells**

**Estrogen and ERs**

Estrogens, or oestrogens, are 18-carbon steroid molecules synthesized primarily by the ovaries (~95%), and to a lesser extent in non-reproductive tissues (~5%), including the adrenal glands, brain, liver, adipose tissue, and male testes [116, 117]. There are three forms of estrogens: estrone (E₁), estradiol (E₂), and estriol (E₃). E₁ is important in postmenopausal women, E₂ is predominant during the reproductive years, and E₃ plays a role during pregnancy when it is produced by the placenta [118]. Of the endogenous estrogens, E₂ is the most biologically active and most commonly studied form. Further discussion of estrogens in this section will focus on E₂.

Serum E₂ concentrations range from approximately 2-50 pg/ml in female mice [119] and 30-600 pg/ml in premenopausal women [117], depending on the phase of the estrous or menstrual cycle, respectively. In mice, the estrous cycle begins at 3 months of age and includes four stages (proestrus, estrus, metestrus, and diestrus) that repeat every 4-5 days [120-122]. Examination of the vaginal epithelium with cytology is used to determine the stage of the cycle in rodents [120]. In women, the menstrual cycle begins at 8.5-13 years at menarche, the day of the first menstrual period, and includes a follicular phase that is generally 14.5 days and a luteal phase of 13.6 days [123, 124]. Nearly all E₂
in circulation is bound to sex hormone binding globulin and albumin, and when the complex reaches its target tissue it dissociates and allows estrogen to enter the cell by passive diffusion [125].

E$_2$ acts via estrogen receptors (ERs) to regulate processes on target tissues, such as uterus, bone, liver, hypothalamus, and skeletal muscle. Once E$_2$ is inside the cell, it binds to ERs in the cytoplasm. First described in the mid-1960s, ER$\alpha$ is the traditional ER, with ER$\beta$ characterized several years later. These ERs are not splice variants, as ER$\alpha$ and ER$\beta$ have been mapped to chromosome 6 and 14, respectively [126]. Canonical signaling occurs when ER$\alpha$ or ER$\beta$ are activated by E$_2$, dimerize, translocate to the nucleus, and bind to estrogen response elements (ERE) to modulate transcription [127]. ER$\alpha$/$\beta$ can also interact with other DNA-bound transcription factors to stabilize the binding of that transcription factor or attract coactivators [128]. For more rapid E$_2$-ER signaling, membrane bound forms of ER$\alpha$, ER$\beta$, and a more recently discovered G-protein estrogen receptor (GPER) can activate protein-kinase cascades (e.g., p38/MAPK or PI3K/AKT) [129, 130], resulting in upregulation or downregulation of genes due to transcription factor phosphorylation [131]. Each of these ERs have been observed in several tissues throughout the body with differences in expression observed between tissues and not sex with exception to the kidneys and gonads [132]. In skeletal muscle and satellite cells, ER$\alpha$ is the most predominant receptor, followed by GPER and then ER$\beta$ [115, 133, 134].
Estradiol and aging

A decline in circulating $E_2$ can occur due to a variety of conditions, such as surgery, chemotherapy, and eating disorders. The most common cause of $E_2$ deficiency is menopause, which is the cessation of the reproductive years [135]. The menopausal transition, characterized by irregular menses, begins 4 years prior to menopause at around 47.5 years [136]. Menopause is clinically diagnosed 12 months following the final menstrual period, which occurs around 51.4 years [137]. During menopause, oocytes are depleted through atresia or ovulation resulting in significant declines in ovarian secretions [135]. Serum $E_2$ levels are reduced from an average peak of 300 pg/ml in reproductive years to 20 pg/ml at 5 years post-menopause [138]. Women experience vasomotor symptoms as a result of $E_2$ withdrawal, including hot flashes, heart palpitations, and night sweats [135]. To prevent these symptoms, as well as increased fat deposition and osteoporosis, hormone replacement therapy (HRT) is considered. The goal of HRT is to balance the female sex hormone levels. The most commonly used forms of HRT are conjugated equine estrogens, micronized $E_2$, and ethinyl $E_2$ [139].

Rodents also experience a cessation of estrous cycling and a decline in ovarian secretions occurring at 19.7 months on average [121]. At this time, rodents experience irregular cycling comparable to women; however, ovarian secretions continue for several months following the loss of estrous cycles and cause persistent vaginal cornification (PVC) [140, 141]. PVC is the predominant anovulatory condition that lasts 2-4 months on average before transitioning into persistent diestrus (i.e., ovarian senescence) [140]. Contrary to women, PVC and ovarian senescence in rodents is attributed to
neuroendocrine impairments that cause a reduction in follicles [141]. Studies have demonstrated that PVC rodents are unable to support estrous cycles after receiving ovarian grafts [142, 143]. Though, some neuroendocrine impairments only occur when the ovaries are present, so if both ovaries are removed by ovariectomy (Ovx) in young rodents they are able to support estrous cycles after receiving young ovarian grafts at an old age [142, 143]. The circulating E2 levels in ovarian senescent and Ovx female mice are reduced to >5 pg/ml and >0.3 pg/ml, respectively [119, 120]. To mimic ovarian senescence (i.e., menopause) in rodents, the Ovx model is often used to study E2 deficiency without the confounding factors associated with aging, and implantation of a time release E2 pellet can be used to mimic HRT following confirmation of ovarian senescence or Ovx.

**Estradiol and skeletal muscle regeneration**

It is well known that skeletal muscle strength declines with age corresponding with the time that E2 declines at menopause and HRT has been shown to mitigate this decrement [144-147]. Our lab is the first to show that recovery of muscle strength is blunted in E2-deficient mice after various types of injury (e.g., eccentric-contraction induced injury, chemical injury, freeze injury) and at several time points after injury (e.g., 3, 4, 8 wk) [79, 148-151] (Fig. 2.1). Others have shown that E2 regulates muscle recovery following hindlimb suspension in female rats with Ovx rats failing to fully recover muscle mass [152]. Furthermore, the Akt-p70s6k signaling pathway has been suggested to be impaired with E2 deficiency, as E2 has been shown to activate
Akt-mTOR in other tissues [153, 154]. Studies by the Tiidus lab have demonstrated that the neutrophil response after injury is attenuated with E₂ supplementation (Ovx+E₂) compared to E₂ deficiency (Ovx) following downhill running [155-157], and suggest that E₂ reduces proinflammatory cytokines and nitric oxide [158]. On the contrary, our lab has shown that a blunted neutrophil response in Ovx mice compared to Ovx+E₂ following freeze injury [159]. The findings from the Tiidus lab and our lab argue that reduced and enhanced neutrophil response with E₂ could be beneficial and lead to a timely recovery, respectively [148, 155-157, 159]. Nonetheless, E₂ has been implicated in regulating the initial degenerative phase of muscle regeneration after injury. Further research in our lab is focused on determining whether E₂ deficiency impairs the infiltration of neutrophils (i.e., chemotaxis).
Estradiol and satellite cells

The role of E$_2$ in satellite cell maintenance and function in skeletal muscle regeneration is unclear with only six labs, including ours, studying E$_2$ actions under specific conditions. A summary of the in vitro experiments that have evaluated the effects of E$_2$ deficiency on satellite cells is shown in Table 1. In general, in vitro studies have indicated that the lack of E$_2$ results in a decline in satellite cell number [160], specifically the differentiating or self-renewing population [161], and that E$_2$ treatment increases bovine satellite cell proliferation, which can be prevented by MAPK or PI3K inhibition [162, 163] (Table 1). The lack of ER signaling via receptor knockout models (scERKO), small interfering RNA against ERs (siER), or receptor antagonists (e.g., PHTPP), also have been shown to reduce satellite cell number and protein synthesis in vitro [160, 164] (Table 1). Furthermore, ER antagonist studies suggest that the effects of E$_2$ on satellite cells is mediated by ER$_\alpha$ and ER$_\beta$ and not GPER [160, 164] (Table 1). While E$_2$ consistently is implicated in regulating satellite cell proliferation, Ogawa et al [165] has revealed that E$_2$ treatment in vitro at supraphysiological concentrations ($10^4$ pM) for a long duration (8 d) results in reduced fusion and increased ubiquitin-specific peptidase 19 (USP 19) via ER$_\alpha$ which is known to be activated in muscle atrophy [166] (Table 1).
Table 2.1. Summary of the effects of estradiol on satellite cell function *in vitro*.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Muscle(s)</th>
<th>Cell model</th>
<th>Hormone treatment</th>
<th>Key Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice (F)</td>
<td>GC; Soleus</td>
<td>Fused satellite cells</td>
<td>10^4 pM E₂ for 8 d</td>
<td>↓ fusion ↑ USP-19 protein</td>
<td>Ogawa et al., 2011</td>
</tr>
<tr>
<td>Mice (F)</td>
<td>EDL</td>
<td>Floating myofiber culture</td>
<td>Mitogen-rich medium for 72 h</td>
<td>Ovx: ↓ differentiating and self-renewing; ↔ activating /proliferating</td>
<td>Kitajima and Ono, 2016</td>
</tr>
<tr>
<td>Mice (M/F)</td>
<td>EDL</td>
<td>scERβKO + 4-OHT</td>
<td>N/A</td>
<td>↓ cell number ↓ proliferation ↓ ccna2 mRNA ↑ p21 mRNA ↑ apoptosis</td>
<td>Seko et al., 2020</td>
</tr>
<tr>
<td>Mice (M/F)</td>
<td>EDL</td>
<td>siERβ</td>
<td>N/A</td>
<td>↓ cell number ↓ proliferation ↑ apoptosis</td>
<td>Seko et al., 2020</td>
</tr>
<tr>
<td>Mice (M/F)</td>
<td>EDL</td>
<td>ERβ antagonist (PHTPP)</td>
<td>N/A</td>
<td>↓ cell number</td>
<td>Seko et al., 2020</td>
</tr>
<tr>
<td>Steer (M)</td>
<td>SM</td>
<td>Proliferating satellite cells</td>
<td>1, 10, 100, 10^3, 10^4 pM E₂</td>
<td>1 pM: ↑ ERα mRNA 10^4 pM: ↑ proliferation</td>
<td>Kamanga-Sollo et al., 2004</td>
</tr>
<tr>
<td>Steer (M)</td>
<td>SM</td>
<td>Proliferating satellite cells</td>
<td>10^4 E₂; 10^4 ICI 182,780; 0, 20, 100, 500 μM PD98059; 0, 100, 500, 1000 nM wortmannin at 48 h</td>
<td>E₂ in FBS: ↑ IGF mRNA E₂ in SS: ↑ proliferation PD98059 and wortmannin: ↔ E₂ induced proliferation</td>
<td>Kamanga-Sollo et al., 2008</td>
</tr>
<tr>
<td>Steer (M)</td>
<td>SM</td>
<td>Fused satellite cells</td>
<td>10, 100, 10^4 pM E₂; 10^4 pM ICI 182,780; 10^3 pM G1 at 48 or 96 h</td>
<td>E₂: ↑ protein synthesis E₂: ↓ protein degradation ICI 182,780: ↔ E₂ effects G1: ↔ no effect</td>
<td>Kamanga-Sollo et al., 2010</td>
</tr>
</tbody>
</table>

F: female; M: male; GC: gastrocnemius; EDL: extensor digitorum longus; SM: semimembranosus; E₂: 17β-estradiol or estradiol valerate; scERβKO: satellite-cell specific estrogen receptor-β knockout; 4-OHT: 4-hydroxytamoxifen; siERβ: small interfering RNA against ERβ; PHTPP: selective antagonist of ERβ, 4-(2-phenyl-5,7 bis(trifluoromethyl)-pyrazolo[1,5-a] pyrimidin-3-yl)-phenol; ICI 182,780: estrogen receptor antagonist; PD98059: MAPK inhibitor; wortmannin: PI3K inhibitor; SS: IGFBP-3-free swine serum; G1: GPR30 agonist.
Results from in vivo studies have used various exercise or injury models and techniques to assess satellite cell fate. A summary of the in vivo experiments that have evaluated the effects of E₂ deficiency on satellite cells is shown in Table 2. The Tiidus lab has demonstrated in five studies that Ovx+E₂ rats compared to Ovx have increased total (Pax7+), activated (MyoD+), and proliferating (BrdU+) satellite cells at 72 h after downhill running [167-170], and similar effects can be observed when Ovx rats receive E₂ or ERβ agonist treatment (e.g., 8β-VE2) after notexin injury [171] (Table 2). The effects of E₂ on satellite cells can be prevented by PI3K inhibition or ER antagonist, further implicating E₂-ER-PI3K signaling in regulation of satellite cell proliferation and survival [169, 170] (Table 2). In contrast, there is no difference in satellite cell number in male female mice following satellite cell specific ERβ (scERβKO); however, female mice have reduced muscle weight and regeneration following BaCl₂ injury [160] (Table 2). These studies suggest that E₂ effects satellite cell fate after exercise or injury; however, these studies did not establish whether the functional recovery of skeletal muscle strength was affected with injury and raise the question whether E₂ or ERs influence satellite cell fate under normal homeostatic conditions (i.e., without injury or exercise).
Table 2.2. Summary of the effects of estradiol on satellite cell function in vivo.

<table>
<thead>
<tr>
<th>Animal model (sex; age)</th>
<th>Muscle(s)</th>
<th>Surgery/Treatment</th>
<th>Exercise/Injury</th>
<th>Key Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sprague-Dawley rat (M; 11 wk)</td>
<td>Soleus; white vastus</td>
<td>0.25 mg/21 d E₂ pellet</td>
<td>72 h post - downhill running</td>
<td>↑ cell number</td>
<td>Tiidus et al., 2005</td>
</tr>
<tr>
<td>Sprague-Dawley rat (F; 11 wk)</td>
<td>Soleus; white vastus</td>
<td>Ovx ± 0.25 mg/21 d E₂ pellet</td>
<td>72 h post - downhill running</td>
<td>Ovx ± E₂ with exercise: ↑ total; activated, and proliferating than exercise alone</td>
<td>Enns and Tiidus, 2008</td>
</tr>
<tr>
<td>Sprague-Dawley rat (F; 11 wk)</td>
<td>Soleus; white vastus</td>
<td>Ovx ± 0.25 mg/21 d E₂ pellet; ICI 182,780</td>
<td>24 and 72 h post - downhill running</td>
<td>Ovx ± E₂ with exercise: ↑ total, activated, and proliferating than exercise alone; ICI 182,780: = E₂ effects</td>
<td>Enns et al., 2008</td>
</tr>
<tr>
<td>Sprague-Dawley rat (F; 11 wk)</td>
<td>Soleus; white vastus</td>
<td>Ovx ± 0.25 mg/21 d E₂ pellet; ERT antagonist PPT</td>
<td>72 h post - downhill running</td>
<td>Ovx ± E₂ or PPT with exercise: ↑ total, activated, and proliferating than exercise alone</td>
<td>Thomas et al., 2010</td>
</tr>
<tr>
<td>Wistar rat (F; 8 wk)</td>
<td>GC</td>
<td>Ovx ± 0.25 µg/kg E₂ pellet; 10 µg/kg 16a-LE; 100 µg/kg 8β-VE2</td>
<td>24 h, 3 d, or 7 d post-notexin</td>
<td>Ovx ± E₂ or 8β-VE2: ↑ activation, proliferation, and eMHC</td>
<td>Velders et al., 2012</td>
</tr>
<tr>
<td>Sprague-Dawley rat (F; 9 wk)</td>
<td>Soleus; white vastus</td>
<td>Ovx ± 0.25 mg/21 d E₂ pellet; 0.7 mg/kg wortmannin</td>
<td>72 h post - downhill running</td>
<td>Ovx ± E₂ with exercise: ↑ total and activated than exercise alone; wortmannin: =↓ exercise and E₂ effects</td>
<td>Mangan et al., 2014</td>
</tr>
<tr>
<td>C56BL/6 mice (F; 6 wk)</td>
<td>EDL</td>
<td>Sham or Ovx ± 0.01 mg/50 µl E₂ pellet for 24 wk</td>
<td>N/A; CTX</td>
<td>Ovx myofibers: ↔ cell number</td>
<td>Kitajima and Ono, 2016</td>
</tr>
<tr>
<td>C56BL/6 or Pax7-ZsGreen mice (F; 3-4 mo)</td>
<td>TA; GC; EDL; Soleus; Diaphragm</td>
<td>Sham or Ovx for 2, 4, and 7 mo; Ovx ± 0.18 mg/50 µl E₂ pellet</td>
<td>N/A; 1.2% BaCl₂</td>
<td>Ovx: ↓ cell number; ↓ self-renewal, ↓ fiber engraftment; ↑ apoptosis than Sham; ↓ recovery of strength after injury than Ovx+ E₂</td>
<td>Collins et al., 2019</td>
</tr>
<tr>
<td>C56BL/6 mice (F; 3-4 mo)</td>
<td>TA</td>
<td>Ovx ± 0.18 mg/50 µl E₂ pellet</td>
<td>1.2% BaCl₂</td>
<td>Ovx: ↓ cell number</td>
<td>Larson et al., 2020</td>
</tr>
<tr>
<td>scERβKO mice (M/F; 6 and 20 wk)</td>
<td>TA; EDL</td>
<td>scERβKO ± Ovx</td>
<td>N/A; Barium chloride</td>
<td>F scERβKO mice: ↓ muscle weight and regeneration after injury than control; ↔ scERβKO ± Ovx than scERβKO alone</td>
<td>Seko et al., 2020</td>
</tr>
<tr>
<td>Human (peri- to post-menopausal)</td>
<td>Vastus lateralis</td>
<td>N/A</td>
<td>N/A</td>
<td>↓ cell number from peri- to post-menopause</td>
<td>Collins et al., 2019</td>
</tr>
</tbody>
</table>

M: Male; F: Female; E₂: 17β-estradiol or estradiol valerate; Ovx: ovariectomy; GC: gastrocnemius; EDL: extensor digitorum longus; TA: tibialis anterior; ICI 182,780: estrogen receptor antagonist; PPT: propyl pyrazole triol; 16α-LE2: ERT antagonist; 8β-VE2: ERβ agonist; eMHC: embryonic myosin heavy chain; wortmannin: PI3K/Akt inhibitor; CTX: cardiotoxin; BaCl₂: barium chloride; scERβKO: satellite cell-specific ERβ knockout (Pax7-CreERβ/Er(beta)F5F10). Note: Lowe lab studies are highlighted in yellow.
Our lab was the first to demonstrate that satellite cell number in muscle is substantially reduced with the loss of $E_2$ or $E_2$-ER$\alpha$ signaling under normal conditions [115] (Fig. 2.2). It is important to note that we focus on $E_2$ rather than other ovarian hormones, such as progesterone, because we have data demonstrating that Ovx mice treated with $E_2$ have restored satellite cell numbers [115] (Fig. 2.3).

**Figure 2.2. Loss of $E_2$ or $E_2$-ER$\alpha$ signaling impairs satellite cell maintenance.** $E_2$ deficiency via loss of the hormone or receptor for 2 months results in lower satellite cell numbers in the tibialis anterior muscles of C57/BL6J and Pax7-ZsGreen female mice quantified by FACS (right) and IHC (left) (n=4-6 per group); *P<0.05, **P<0.005. Adapted from Collins et al., 2019.

**Figure 2.3. $E_2$ is the specific hormone that influences satellite cells.** Representative FACS plot showing % of double positive cells (boxes) identified as satellite cells. Treating ovariectomized (Ovx) mice with $E_2$ prevents the decline in satellite cell number in the tibialis anterior muscle. **P=0.01. Adapted from Collins et al., 2019.
In addition, we have measured that satellite cell number is lower in satellite cell-specific ERα knockout mice (scERαKO) compared to wildtype littermates, regardless if the scERαKO mice are Ovx or Ovx+E₂ (Fig. 2.4 left). We show that the genetic ablation of ERα does not result in the compensation of the other sex hormone receptors in satellite cells (Fig. 2.4 right). Together, these findings indicated that ERα is the main receptor that E₂ utilizes to elicit its effects on satellite cells [115].

![Graph](image_url)

**Figure 2.4. E₂ elicits its effects via ERα.** FACS quantification of TA muscle satellite cells in scERαKO mice with or without Ovx or Ovx+E₂ (left) (n=4-6). TPM of estrogen and progesterone receptors (right). Statistical significance from scERαWT. *P<0.05*, **P<0.005. Adapted from Collins et al., 2019.

To determine if satellite cells are lacking E₂ or E₂-ERα signaling lose their ability to self-renew and thereby cannot maintain the satellite cell pool, we performed several satellite cell transplantation studies. We showed that E₂ deficiency of the recipient results in 75% lower engraftment of the satellite cell compartment compared to control recipients (Fig. 2.5) and that there is a similar decrement in satellite cell engraftment.
when satellite cells lacking ERα are transplanted into a control environment [115]. Mechanistically, we have shown that in the absence of E2 signaling, markers of apoptosis appear within a subset of satellite cells (Fig. 2.6) [115], suggesting that E2 protects against apoptosis which has been observed in C2C12 cells [172-174]. Our recent study identified an important role for E2 in regulating satellite cell number and warranted additional studies investigating the mechanism(s) whereby E2 maintains satellite cell number and the extent by which E2 deficiency impacts skeletal muscle regeneration after repeated injuries. Therefore, this studies in this dissertation aim to shed light on the mechanisms whereby E2 regulates satellite cells under injured (Chapter 3) and uninjured conditions (Chapter 4). All experiments in Chapters 3 and 4 were performed by myself unless otherwise noted in the figure legend.
Chapter 3: Estradiol Affects Skeletal Muscle Mass, Strength and Satellite Cells Following Repeated Injuries

Alexie A. Larson, Cory W. Baumann, Michael Kyba, and Dawn A. Lowe

What is the central question of this study?

Estradiol ($E_2$) plays an important role in regulating skeletal muscle strength in females. Here, we asked to what extent $E_2$ deficiency affects recovery of strength and satellite cell number when muscle is challenged by multiple injuries.

What is the main finding and its importance?

$E_2$ deficiency impairs the adaptive potential of skeletal muscle following repeated injuries, as measured by muscle mass and strength. The impairment is likely multifactorial with our data indicating that one mechanism is reduction in satellite cell number. Our findings have implications for aging, hormone replacement and regenerative medicine in regards to maintaining satellite cell number and ultimately the preservation of skeletal muscle’s adaptive potential.

The contents of this chapter are published in *Experimental Physiology*. 
Abstract

 Estradiol effects on skeletal muscle are multifactorial including the preservation of mass, contractility and regeneration. Here, we aimed to determine the extent to which estradiol deficiency affects strength recovery when muscle is challenged by multiple BaCl$_2$-induced injuries and to assess how satellite cell number is influenced by the combination of estradiol deficiency and repetitive skeletal muscle injuries. A longitudinal study was designed, using an in vivo anesthetized mouse approach to precisely and repeatedly measure maximal isometric torque, coupled with endpoint fluorescent-activated cell sorting to quantify satellite cells. Isometric torque and strength gains were lower in ovariectomized mice at several time points after the injuries compared to those treated with 17β-estradiol. Satellite cell number was 41-43% lower in placebo- than estradiol-treated ovariectomized mice, regardless of injury status or number of injuries. Together, these results indicate that the loss of estradiol blunts adaptive strength gains and that the number of satellite cells likely contributes to the impairment.
Introduction

Estradiol, the primary female sex hormone, is classically known to regulate reproductive organ development and function. It is also recognized that estradiol plays an important role in regulating skeletal muscle strength [147]. For example, muscle strength of women declines at a time corresponding to menopause (i.e., the cessation of estradiol production), and estradiol-based hormone therapy in post-menopausal women has been shown to maintain muscle strength [144, 147]. A common approach to study estradiol in rodent models is the surgical removal of the ovaries (ovariectomy; Ovx) and subsequent treatment with or without 17β-estradiol (E₂), the most biologically active form of estrogen. Using this approach, strength loss has been measured in Ovx mice [147, 175], similar to post-menopausal women [144, 147]. Loss of muscle strength in females due to E₂ deficiency is attributed to inadequate preservation of skeletal muscle mass [176] and reduced quality of the remaining skeletal muscle [146, 175, 177]. Although E₂ likely works through various mechanisms, leading candidates contributing to strength loss are apoptotic-induced reductions in muscle mass [115, 178, 179], modifications to myosin heavy chain function [146, 175] through phosphorylation of the regulatory light chain [177, 180], abnormal inflammation [157], and impaired mitochondrial function [134, 181].

Estradiol also affects recovery of muscle following injury and thus, E₂ deficiency in females would theoretically further exacerbate loss of strength via this mechanism. In support of this theory, muscles from Ovx mice have been shown to be weaker following various injuries (e.g., freeze injury, eccentric contraction-induced injury) compared to
control or Ovx+E2 mice [115, 148, 182, 183]. Estradiol may influence recovery of strength by regulating various pathways of skeletal muscle degeneration and regeneration. These include, but are not limited to, immune cells [148, 157] and muscle stem cells (i.e., satellite cells) [167]. For instance, following a freeze-induced injury, a moderate dose of E2 given to Ovx mice increased neutrophil recruitment and recovery of muscle strength over that of a placebo treatment [148]. While acute inflammation including neutrophils and macrophages dominate the initial degenerative phase following injury [184], the capacity for muscle to regenerate is largely due to the satellite cell population [185, 186]. Satellite cells are small, mitotically quiescent stem cells that reside under the basal lamina of the muscle fiber [28] and express the transcription factor Pax7 [33, 46]. Depleting satellite cells prevents skeletal muscle regeneration following injury [70, 186]. Thus, the regenerative capacity of skeletal muscle requires maintenance of the satellite cell population. We recently reported that loss of E2 or estrogen receptor-α (ERα) in satellite cells reduces satellite cell number in skeletal muscles of female mice through a process involving apoptosis [115]. These data indicate E2 regulates satellite cell maintenance in females under steady-state conditions, and likely muscle regeneration and recovery of strength following a single, isolated injury from an insult such as cardiotxin or barium chloride (BaCl2).

In a physiological setting, skeletal muscle does not typically sustain a single injury, but rather numerous injuries over a lifetime. Repeated injuries have previously been used to assess skeletal muscle stress resistance, resilience, and adaptability in healthy and diseased mouse models, such as muscular dystrophy and malignant
hypothermia [187, 188]. However, is it unclear how repeated injuries to E2 deficient skeletal muscle impacts recovery, satellite cell number, and subsequent strength gains. Therefore, the purpose of this study was to (1) determine the extent to which E2 deficiency affects strength recovery when muscle is challenged by multiple injuries and, (2) assess how satellite cell number is influenced by the combination of E2 deficiency and repetitive skeletal muscle injuries. Considering that skeletal muscle regeneration following injury requires satellite cells and E2 maintains satellite cell number, in this study we hypothesized that recovery of strength following repeated injuries is impaired in mice without E2.

Methods

Ethical approval

All animal procedures were approved by the Institutional Animal Care and Use Committees at the University of Minnesota (A3456). For all in vivo procedures (Ovx surgery and torque measurements), mice were initially anesthetized in an induction chamber using isoflurane, and then maintained by inhalation of isoflurane via a nose cone (1.25%, 125 mL O2 per min). Mice were euthanized with an overdose of sodium pentobarbital (IP injection at 200 mg/kg) at the completion of the study. Investigators understand the ethical principles and ensure that the work complies with the animal ethics checklist of the journal.

Experimental animals and design
Female wildtype (C57Bl/6) mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) at 12 wk of age, and aged to 16 wk prior to the initiation of the study. Mice were housed in groups of 4-5 and had access to phytoestrogen-free rodent chow (Harlan-Teklad #2019; Indianapolis, IN, USA) and water *ad libitum*. The housing room was maintained on a 14:10 h light:dark cycle with controlled temperature and humidity.

Mice were randomly assigned to one of two treatment groups: Placebo (n=24) or E₂ (n=21). Four hours prior to the surgery, mice were given a subcutaneous injection of slow-release buprenorphine. Immediately following the Ovx surgery, mice received a placebo or slow-release E₂ pellet. Three weeks following Ovx surgery, mice were tested for *in vivo* maximal isometric torque of the anterior crural muscles [tibialis anterior (TA), extensor digitorum longus, extensor hallucis muscles] (Pre; Fig. 3.1) and retested weekly four times in a repeated measures design (i.e., pre-injury, and 7, 14 and 21 d post-injury). Immediately after recording *in vivo* maximal isometric torque during the pre-injury and each of the 21-d assessments, TA muscles were injured with intramuscular injections of BaCl₂. Thus, over a 10-wk period TA muscles were injured three times with BaCl₂ and assessed for maximal isometric torque production on 10 separate occasions (Fig. 3.1). Mice were monitored daily for 3 d following all surgical procedure (ovariectomy or BaCl₂ injections). TA muscles that were injured once or three times were given 63 or 21 d to recover, respectively. A subset of mice within each group served as uninjured controls. At the end point, uteri and TA muscles were dissected and weighed. Uterine mass <30 mg was used as an inclusion parameter to indicate successful Ovx surgery [189].
Figure 3.1. **Experimental timeline for treatment, assessment of in vivo isometric torque, injury induction and tissue collection.** Mice were randomly assigned to one of two treatment groups prior to an ovariectomy (Ovx) surgery: Placebo or 17β-estradiol (E2). In vivo isometric torque of the anterior crural muscles was then measured before (Pre), and 7, 14 and 21 d following repeated BaCl2-induced injuries. After recovery from the first or third injuries, tissues were collected and satellite cell number of injured and uninjured tibialis anterior (TA) muscles was measured using FACS.

The total number of satellite cells in TA muscles was measured by FACS, with muscles designated as uninjured, injured once or injured three times. To determine adaptive strength gains, defined as the gain in torque from the initial pre-injury torque (Pre; Fig. 3.1) to 21 d post-injury for each injury, a percent change was calculated as: 

\[
\frac{(21 \text{ d Post-Injury Torque} - \text{Pre-Injury Torque of Injury #1})}{\text{Pre-Injury Torque of Injury #1}} \times 100
\]

Torque data expressed as fractional change following the first and second injuries for a subset of mice were previously reported [115].

**Experimental methodology**

*Ovx surgery and treatment pellets*

Under aseptic conditions, bilateral Ovx was performed through two small dorsal incisions between the iliac crest and the lower ribs [175]. Immediately after Ovx, mice were implanted with pellets containing placebo or 0.18 mg E2 released over a 60-d period.
(Innovative Research of America, Sarasota, FL). This dose of E$_2$ has been shown to mimic physiological levels in female mice [148, 190].

In vivo analysis of muscle torque

As previously described [191], anesthetized mice were placed on a temperature-controlled platform to maintain core body temperature. The left knee was clamped and the left foot was secured to an aluminum “shoe” that is attached to the shaft of a 300B servomotor (Aurora Scientific, ON, Canada). Sterilized platinum needle electrodes were inserted through the skin for stimulation of the left common peroneal nerve. Stimulation voltage and needle electrode placement were optimized with isometric tetanic contractions (200 ms train of 0.1 ms pulses at 200 Hz). Contractile function of the anterior crural muscles was then assessed by measuring isometric torque as a function of stimulation frequency, with the highest recorded torque defined as maximal isometric torque.

$\text{BaCl}_2$-induced injury

To induce skeletal muscle injury several methods have been used previously, including natural toxins (cardiotoxin, notexin), chemical reagents ($\text{BaCl}_2$), physical injury (freeze or crush injury), medications (bupivacaine), and eccentric-contraction induced injury [192]. Here, we use local exposure of $\text{BaCl}_2$ (1.2% in sterile demineralized water; ~57mM) (Ricca Chemical Company, Arlington, TX) to induce muscle injury because, unlike freeze injury, $\text{BaCl}_2$ causes myofiber necrosis through calcium-induced proteolysis, which does not affect the surrounding mononuclear population (i.e., satellite
cells and fibroblasts) [72, 193-197]. BaCl₂ is a widely used method of injury due to its ability to cause consistent, repeatable muscle injury, as well as the absence of regulatory restrictions that accompany the purchase and use of natural toxins. Briefly, to induce injury, a small incision was made to expose the TA muscle, the Hamilton syringe needle was inserted at a 30° angle into the distal end of the TA muscle, pulled parallel to the tibia and 25 µL of BaCl₂ was slowly injected. The syringe was held in place for 30 s following the injection to prevent fluid loss and the skin incision was closed with a single suture. To determine the volume of 1.2% BaCl₂ to completely injure the TA muscle, we performed preliminary experiments with 25 µL or 50 µL injections via insulin or Hamilton syringe. We found that > 90% of the TA myofibers were affected histologically after injecting 25 µL 1.2% BaCl₂ and preferred delivery with Hamilton syringe.

Isolation, staining, and FACS analyses of satellite cells

Isolation of satellite cells from TA muscles was performed as described in detail previously [115]. Briefly, TA muscles were digested with collagenase type II and dispase (17101-015 and 17105-041, respectively; Gibco, Grand Island, NY). Mononuclear cells 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 alpha7 integrin 647 (clone R2F2) from AbLab (Vancouver, B.C., Canada). Samples were incubated with antibody cocktail, washed, and resuspended with FACS staining medium containing propidium iodide for analysis on a FACSARiaII SORP (BD
Biosciences, San Diego, CA). Total satellite cells (lineage negative; VCAM, alpha7 double positive cells) were analyzed while draining the entire sample from each TA muscle sample (Fig. 3.2).

**Figure 3.2.** Representative FACS plots of cells isolated from TA muscles of Ovx mice with and without 17β-estradiol treatment. FACS plots show total satellite cells in TA muscles from Ovx+E2 and Ovx+Placebo mice that were quantified by lineage negative; VCAM, alpha7 integrin double positive cells. All gated events from individual TA muscles are shown.

**Statistical analyses**

To analyze the effect of treatment (Placebo vs. E2) on torque (Pre, 7 d, 14 d, 21 d for Injuries #1-#3) or adaptive strength gains (Pre and 21 d following Injuries #1-#3) across time, a repeated measures two-way analysis of variance (ANOVA) was utilized (with time as the repeated measure). A two-way ANOVA was used to assess the effect of treatment across time (Uninjured, Post-Injury #1 and Post-Injury #3) for satellite cell
number and TA muscle mass. Bonferroni post hoc tests were performed in the event of a significant interaction or main effect of time. T-tests were used to detect difference in body mass and uterine mass between treatment or across time (study start and end times). An α level of <0.05 was used for all analyses. Data are presented as mean±SD. All statistical testing was performed using SigmaPlot version 12.5 (Systat Software, San Jose, CA).

Results

Mouse body and uterine masses

Body mass did not differ between mice designated to Placebo or E2 groups (20.2±0.9 vs. 20.3±1.5 g; p=0.780) prior to the Ovx surgery. Both groups gained body mass 12 weeks following the surgery (p<0.001), however the Ovx+Placebo mice weighed 20% more than the Ovx+E2 mice (30.3±4.4 vs. 25.1±2.3 g; p<0.001). Uterine mass was ~9-fold less in Ovx+Placebo than Ovx+E2 mice (16.1±4.9 vs. 141.3±40.3 mg; p<0.001) with all uteri being < 25 mg in Ovx+Placebo mice.

Maximal isometric torque

To assess recovery of strength after injuries, in vivo isometric torque was measured in Ovx+Placebo and Ovx+E2 mice at several time points. An interaction between treatment and time was observed for maximal isometric torque (p<0.001). Isometric torque did not differ between groups prior to the first injury (Pre; 2.09±0.27 vs. 1.91±0.13 mN·m, p=0.146) and was greater in Ovx+Placebo than Ovx+E2 mice at day 7
following injury #1 (p=0.046; Fig. 3.3, A & B and Table 3.1). In contrast, at 7, 14 and 21
days after the second injury, Ovx+Placebo mice produced 13-22% less isometric torque
than Ovx+E₂ mice (p≤0.021). Isometric torque following the third injury tended to be
lower in Ovx+Placebo mice at day 14 (p=0.070), and was 11% less than Ovx+E₂ mice by
day 21 (2.67±0.23 vs. 3.01±0.40 mN·m, p=0.029; Fig. 3.3, A & B).

Table 3.1. In vivo isometric torque in Ovx+E₂ and Ovx+Placebo mice before (Pre) and after BaCl₂–induced injuries.

<table>
<thead>
<tr>
<th>Sample Size (n)</th>
<th>Ovx+E₂</th>
<th>Ovx+Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre</td>
<td>1.91±0.13</td>
<td>2.09±0.27</td>
</tr>
<tr>
<td>Injury #1, 7 d</td>
<td>1.02±0.29</td>
<td>1.33±0.33*</td>
</tr>
<tr>
<td>Injury #1, 14 d</td>
<td>2.00±0.23</td>
<td>2.22±0.18</td>
</tr>
<tr>
<td>Injury #1, 21 d/Pre</td>
<td>2.40±0.29</td>
<td>2.35±0.15</td>
</tr>
<tr>
<td>Injury #2, 7 d</td>
<td>1.82±0.31</td>
<td>1.42±0.39*</td>
</tr>
<tr>
<td>Injury #2, 14 d</td>
<td>2.61±0.12</td>
<td>2.25±0.20*</td>
</tr>
<tr>
<td>Injury #2, 21 d/Pre</td>
<td>2.99±0.22</td>
<td>2.61±0.22*</td>
</tr>
<tr>
<td>Injury #3, 7 d</td>
<td>1.71±0.37</td>
<td>1.72±0.48</td>
</tr>
<tr>
<td>Injury #3, 14 d</td>
<td>2.71±0.21</td>
<td>2.43±0.38</td>
</tr>
<tr>
<td>Injury #3, 21 d</td>
<td>3.01±0.40</td>
<td>2.67±0.23*</td>
</tr>
</tbody>
</table>

Values are presented as mean±SD. E₂; 17β-estradiol.
*Significantly different from Ovx+E₂ at given time point (p<0.05).

Because in vivo maximal isometric torque appeared to increase above the initial
pre-injury torque during the latter recovery periods (unshaded area, Fig. 3.3B), we
calculated adaptive strength gains at 21 d after each injury. When expressed as a percent
change relative to Pre, both groups experienced an increase in isometric torque by the end
of the study (p=0.005). These adaptive strength gains were not significantly different
between the Ovx+Placebo and Ovx+E₂ mice following first injury (14 vs. 26%; p=0.194).
However, after the second and third injuries, adaptive strength gains at the 21 d post-
injuries were less in Ovx+Placebo mice, with strength only increasing by 26-29% vs. 57-58% in Ovx+E2 mice relative to their respective Pre torque values (p≤0.002).

**Figure 3.3. Mice that lack E2 are weaker following repeated injuries.** (A) Representative tracings of maximal isometric torque by anterior crural muscles before (Pre) and 21 d following one, two or three BaCl2-induced injures. (B) Repeated in vivo isometric torque measurements were made on Ovx+E2 and Ovx+Placebo mice before injury, and at 7-d intervals after one, two and three BaCl2–induced injures. Data points above shaded area represent adaptive strength gains. Ovx; ovariectomized, E2; 17β-estradiol. Sample size per group, n=7 mice. Values are presented as mean±SD. Post hoc results following a significant interaction between treatment and time are shown in B (p<0.05). *Significantly different from Ovx+E2 at given time point. Performed in collaboration with CWB.

**TA muscle mass and satellite cell numbers**

To determine if the differences observed in maximal isometric torque were due to differences in muscle hypertrophy, we assessed mass of the uninjured and recovered TA muscles following the single and triple injury. An interaction between treatment and time was detected for TA muscle mass (p=0.006). Following the third injury, TA muscles of
the Ovx+Placebo mice weighed 20% less than that of the Ovx+E2 mice (p<0.001; Fig. 3.4A). Within the Ovx+E2 group, TA muscle mass was greater following injuries #1 and #3 compared to uninjured muscles indicating hypertrophy at the 21-d recovery times (p≤0.012; Fig. 3.4A).

To quantify satellite cells, we used flow cytometry to count the number of lineage negative; VCAM, alpha7 integrin positive cells in entire TA muscles. The number of satellite cells in TA muscles was approximately 42% lower in Ovx+Placebo than Ovx+E2 mice (treatment effect; p<0.001; Fig. 3.4B). The number of times that the TA muscle was injured did not alter the absolute number of satellite cells (time effect; p=0.230; Fig. 3.4B). No treatment by time interaction was detected (p=0.849).

As with absolute satellite cell number (Fig. 3.4B), satellite cell number per mg of TA muscle mass was lower in Ovx+Placebo than Ovx+E2 mice (treatment effect; p<0.001; Fig. 3.4C). In contrast to absolute numbers, when accounting for hypertrophy as measured by muscle mass, there was a main effect of time (p=0.002). Satellite cell number per muscle mass was 26-40% less after injury #1 and #3 compared to that of uninjured muscles. No treatment by time interaction was detected (p=0.174).
Figure 3.4. E₂ improves the maintenance of satellite cell number before and after injuries. (A) Differences in TA muscle masses due to estradiol treatment depend on the number of injuries (interaction effect; p<0.006). (B) The total number of satellite cells in TA muscles, quantified by FACS as lineage negative;VCAM, alpha7 double-positive cells, is greater in ovariectomized mice treated with estradiol (main effect of treatment; p<0.001). (C) The total number of satellite cells normalized to muscle mass is greater in ovariectomized mice treated with estradiol (main effect of treatment; p<0.001) and lower with injury (main effect of time; p=0.002). Data was derived from the TA muscles of Ovx+E₂ and Ovx+Placebo mice that were not injured, or from TA muscles that had recovered from one or three BaCl₂-induced injuries. Sample size per group, n=7-16 mice. Ovx; ovariectomized, E₂; 17β-estradiol. Values are presented as mean±SD. For A, following the significant interaction pairwise post-hoc results are indicated by: *Significantly different from Ovx+E₂ at given time point; #Significantly different from Uninjured within treatment group. Main effects of two-way ANOVAs are indicated above each set of bars in B and C.

Discussion

The purpose of this study was to determine the extent to which E₂ deficiency affects recovery of skeletal muscle strength and satellite cell number when challenged by multiple injuries. This report is the first to measure recovery of strength at several time points following three injuries in ovariectomized mice with and without estradiol treatment (Ovx+E₂ and Ovx+Placebo, respectively). It is also one of few studies to measure total muscle satellite cell number by FACS using a repetitive injury model. The major findings of this study are: 1) after repeated injuries, muscles from Ovx+Placebo
mice exhibit a blunted ability to adapt as indicated by smaller and weaker muscles compared to those from Ovx+E2 mice, and 2) satellite cell number is lower in Ovx+Placebo mice compared to Ovx+E2 mice regardless of injury status (uninjured or injured) or the number of injuries (1, 2 or 3). Taken together our data support the notion that the loss of E2 blunts adaptive strength gains and that the number of satellite cells likely contributes to the impairment. However, considering that recovery of strength occurred in Ovx mice, we are unable to support the hypothesis that recovery of strength would be impaired in mice without E2.

One of the more salient mechanisms that regulates skeletal muscle regeneration is the homeostatic maintenance of the satellite cell pool [20], with loss of satellite cells being detrimental to regeneration. Decrements in satellite cell number can arise from changes in the muscle environment [198], such as loss of E2-ERα signaling [115]. In the present study, BaCl2-induced injuries were used to evaluate skeletal muscle regeneration with parallel analyses of strength recovery and adaptability. Hardy et al. reported that satellite cell number decreased 53% 18 h after a BaCl2–induced injury followed by an increase in proliferation resulting in a 4-fold increase in satellite cell number [72]. By three months post-injury, satellite cell number returned to baseline [72]. We quantified satellite cell number in TA muscles that were uninjured or recovered following a single or triple injury. The results presented here show that absolute satellite cell number remains low in Ovx+Placebo mice after a single injury or three repeated injuries, while the absolute satellite cell number in Ovx+E2 muscle remains consistently higher (Fig. 3.4B). Since further decrements in absolute satellite cell number with E2 deficiency were

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not observed with repeated injuries, our data suggest that the remaining population of satellite cells in the Ovx+Placebo muscle are resilient to pro-apoptotic signals and maintain their ability to self-renew.

Several studies have investigated factors that influence satellite cell number and muscle regeneration, yet the link between satellite cells and recovery of strength has been minimally studied. Combining satellite cell ablation and hindlimb casting to model muscle contractures, Dayanidhi, Kinney [199] showed that a reduced number of satellite cells impaired muscle’s ability to add sarcomeres in series and recover from the immobilization-induced contracture. That study suggested that there is a relationship between satellite cell number and muscle growth. Using an eccentric contraction-induced injury model and irradiation to eliminate satellite cells, Rathbone, Wenke [185] reported that recovery of in vivo maximal isometric torque 35 d after a single injury was 25% less when satellite cells were eliminated. We are aware of no studies that determined the effect of reduced satellite cell number on strength recovery following multiple injuries. The results presented here show that regardless of BaCl₂ injury number, maximal isometric torque of Ovx+Placebo mice fully recover and increase 21 d post-injury compared to that of their pre-injury torque. However, when E₂ was present and satellite cell number was maintained, Ovx+E₂ mice were able to produce more torque as well as greater strength gains following repeated injures compared to Ovx+Placebo mice (Fig. 3.3). We suggest that satellite cells that survived in the E₂ deficient muscle were resilient and capable of regenerating injured muscle to support recovery of strength, yet were insufficient to induce adaptive strength gains (i.e., hypertrophy) as measured in TA
muscle of Ovx+E₂ mice (Fig. 3.4A). These data are in agreement with Egner et al., [200] who showed that overload hypertrophy was prevented in satellite cell-deficient mice indicating that satellite cells are necessary for effective muscle hypertrophy. Overall, our data suggest that E₂ deficiency impairs the ability of muscle to adapt after repeated injuries and that mechanistically satellite cell number contributes to this functional deficit.

In summary, we report that E₂ deficiency impairs the adaptive potential of skeletal muscle following repeated BaCl₂-induced injuries, as measured by skeletal muscle mass and strength. The blunted ability of E₂ deficient muscle to recover and adapt to repeated injuries is likely multifactorial, however our data suggest one mechanism is reduction in the number of satellite cells. We highlight the role of E₂ in maintaining muscle strength, an important consideration in the context of aging because repeated injuries accumulate over a lifetime and can contribute to age-related strength loss [201]. Thus, our findings have implications for aging, hormone replacement and regenerative medicine in regards to maintaining satellite cell number and ultimately the preservation of skeletal muscle’s adaptive potential.
Chapter 4: Estradiol deficiency reduces the satellite cell pool by impairing cell cycle progression

Alexie A. Larson¹, Ahmed S. Shams², Shawna L. McMillin³, Brian P. Sullivan³, Cha Vue³, Zachery A. Roloff³, Eric Batchelor¹, Michael Kyba², and Dawn A. Lowe³

![Graphical abstract](image)

**Figure 4.** Graphical abstract.

The contents of this chapter are currently under review to be published in the *American Journal of Physiology Cell Physiology.*
Abstract

The size of the satellite cell pool is reduced in estradiol (E₂)-deficient female mice and humans. Here, we use a combination of in vivo and in vitro approaches to identify mechanisms whereby E₂ deficiency impairs satellite cell maintenance. By measuring satellite cell numbers in mice at several early time points post-ovariectomy (Ovx), we determine that satellite cell numbers decline by 33% between 10 and 14 days post-Ovx in tibialis anterior and gastrocnemius muscles. At 14 days post-Ovx, we demonstrate that satellite cells have a reduced propensity to transition from G₀/G₁ to S and G₂/M phases, compared to cells from ovary-intact mice, associated with changes in two key satellite cell cycle regulators, ccna2 and p16INK4a. Further, freshly isolated satellite cells treated with E₂ in vitro have 62% greater cell proliferation and require less time to complete the first division. Using clonal and differentiation assays, we measured 69% larger satellite cell colonies and enhanced satellite cell-derived myoblast differentiation with E₂ treatment compared to vehicle-treated cells. Together, these results identify a novel mechanism for preservation of the satellite cell pool by E₂ via promotion of satellite cell cycling.
Introduction

Skeletal muscle growth and regeneration require a mitotically quiescent stem cell population known as satellite cells [81, 202]. During homeostasis, satellite cells reside on the periphery of terminally differentiated muscle fibers and are marked by expression of paired box transcription factor 7 (Pax7) and several cell surface markers, including α7-integrin [19, 33, 46, 203]. Following a stimulus (e.g., injury or stress), a subset of the satellite cells transition from a quiescent to activated state and enter the G1 phase of the cell cycle [82]. Satellite cells can then undergo asymmetric division where one daughter cell commits to the myogenic lineage, differentiates and fuses to new or existing damaged fibers, and the other daughter cell returns to quiescence to maintain the satellite cell pool, a process known as self-renewal [57]. Satellite cells can also undergo symmetric proliferation followed by stochastic recruitment of proliferated progeny back into the satellite cell pool [204, 205]. In healthy adult muscle, the appropriate balance of intrinsic and extrinsic factors are maintained to coordinate satellite cell fate decisions (i.e., myogenic commitment vs. self-renewal) with the demands of regenerating or growing muscle [198, 206].

The effects of disrupting the complex balance of factors that affect satellite cell fate can be observed in aging skeletal muscle, resulting in markedly compromised muscle regeneration. Major advances towards understanding how changes in the intrinsic and extrinsic factors that influence the satellite cell pool have been made in the last 60 years since the satellite cell was discovered [207]. Numerous groups have observed a decline in satellite cell number in aged rodent [67, 84-91] and human skeletal muscles [92-94] with
the rate and extent varying with muscle fiber type and function (e.g., locomotion, respiration, or mastication) [87, 89, 90]. In addition, several studies have demonstrated that changes in extrinsic factors in the satellite cell microenvironment contribute to impaired regeneration with age [Reviewed in 21, 24, 208-210]. The decline of circulating hormones including insulin-like growth factor-1 (IGF-1) and oxytocin have been identified as such contributors to age-associated impairments of satellite cells [104, 113, 211]. Over the last decade, the relationship between satellite cell function and sex hormones has gained attention to rationalize sex-related differences in skeletal muscle regeneration [212-221]. In particular, evidence is mounting that the major sex hormone in females, estradiol (E$_2$), influences satellite cell function and muscle regeneration.

It is important to understand that E$_2$ levels can decline in females due to a variety of reasons, including 1) natural age-induced menopause [222], 2) menstrual dysfunction experienced with the female athlete triad [223], 3) side effects of hormone therapy to treat cancer [224], 3) congenital conditions (e.g., Turner syndrome) [225, 226], and 5) hysterectomy with or without oophorectomy (i.e., surgical removal of the ovaries; referred to as ovariectomy in animals) [227]. Health issues associated with E$_2$ deficiency traditionally prompted studies focused on osteoporosis [Reviewed in 228] and heart disease [Reviewed in 229] leaving the role of E$_2$ on skeletal muscle and satellite cell biology less clear. Early studies have shown that E$_2$ deficiency blunts satellite cell activation and proliferation induced by injury or exercise [163, 168-171, 229]. Interestingly, E$_2$ treatment has been shown to both impair and enhance satellite cell differentiation [165, 230, 231]. Inconsistencies in the effects of E$_2$ on satellite cells are
presumably due to different experimental conditions including animal model and age, E$_2$ dose and treatment duration, as well as methods of measuring progression of satellite cells through myogenesis. While these studies suggest potential mechanisms of action of E$_2$ on muscle regeneration, well-defined mechanisms whereby E$_2$ regulates satellite cell function are yet to be determined.

Our recent findings show that the size of the satellite cell pool is reduced in ovarian hormone-deficient female mice and humans under normal homeostatic conditions [79, 115]. Using hormone replacement, we demonstrate that E$_2$ is the ovarian hormone responsible for affecting satellite cells [79, 115]. Here we investigate mechanisms whereby the loss of circulating E$_2$ results in the reduced satellite cell number under normal homeostatic conditions, i.e., without any muscle injury. We propose that E$_2$ influences satellite cell maintenance by regulating satellite cell cycle kinetics, progression, proliferation and differentiation. To test this hypothesis, we use ovariectomized (Ovx) female mice to study satellite cell biology in vivo, and thus examine the cell cycle progression of satellite cells with and without circulating E$_2$. In addition, considering that satellite cells are heterogeneous regarding their cell cycle progression, we assess cell cycle kinetics, proliferation, and differentiation in vitro by treating freshly isolated satellite cells with E$_2$. Our results show decrements in satellite cell cycle progression with E$_2$ deficiency suggesting that satellite cell number declines due to the inability of satellite cells to cycle and generate progeny without E$_2$ in the environment. These findings have implications in the preservation of efficient muscle regeneration, including targeting p16-mediated pathways to prevent cell cycle arrest of
satellite cells and subsequent exhaustion of the satellite cell pool. Ultimately, understanding how E₂ regulates satellite cells will help to determine therapies for improving muscle regeneration and recovery of strength that affect quality of life.

Methods

Mice

All procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committees at the University of Minnesota (#A3456-01). All experiments were conducted on female mice when they were young adults (3-6 mo of age). Female wildtype (C57Bl/6) mice were obtained from Jackson Laboratory (000664; Bar Harbor, ME), Pax7-ZsGreen, Pax7\textsuperscript{CreERT2/+}; Esr1\textsuperscript{f/f}; Pax7-ZsGreen (scERαKO) and Pax7\textsuperscript{+/-}; Esr1\textsuperscript{f/f}; Pax7-ZsGreen (scERαWT) female mice were generated in-house [46]. Mice were housed in groups of 4-5 and had access to phytoestrogen-free rodent chow (Harlan-Tekland #2019; Indianapolis, IN, USA) and water \textit{ad libitum}. The housing room was maintained on a 14:10 light:dark cycle with controlled temperature and humidity.

For time-course experiments, female C57Bl/6 mice were assigned to one of two groups: Sham or Ovariectomized (Ovx) and were sacrificed 6, 10, or 14 d post-surgery (n=25 for each group). Female scERαWT and scERαKO mice were treated with tamoxifen (2 mg/kg) for 5 d consecutively [47, 69]. At 14 d after tamoxifen treatment, mice were used for satellite cell harvests (n=6 per group). For the Pax7\textsuperscript{CreERT2/+} effect experiment, female scERαWT and scERαKO mice were used for satellite cell harvests.
(n=6 per group). For the tamoxifen effect experiment, female scERαWT were treated with a vehicle (15% EtOH in sunflower seed oil) or tamoxifen (n=5 per group). For in vivo proliferation experiments, female C57Bl/6 mice that were either Sham or Ovx received 21 d slow-release 5-ethynyl-2’-deoxyuridine (EdU; 25 mg) immediately following surgery (n=7-9). For in vitro experiments, satellite cells were harvested from Pax7-ZsGreen female mice (n=3-6 per group). Mice were euthanized with an i.p. injection of sodium pentobarbital (200 mg/kg) followed with cervical dislocation as secondary euthanasia.

Surgical procedures

Sham and Ovx surgeries were performed as previously described [175]. Briefly, mice were given a subcutaneous injection of slow-release buprenorphine (1 mg/kg) and 2-4 h later were anesthetized by inhalation of isoflurane (1.25%, 125 mL O₂ per min). Bilateral Ovx was performed through two small dorsal incisions between the iliac crest and the lower ribs, and Sham operations consisted of the same procedure as Ovx except that the ovaries were not removed. In a subset of mice, immediately after Ovx, mice were implanted with pellets containing 25 mg EdU released over a 21d period (Innovative Research of America, Sarasota, FL). The daily dose of EdU is equivalent to that given by IP injection daily (50 mg/kg). Mice were monitored daily for 3 d following surgery and incision wounds clips were removed at 10 d post-surgery. The estrous cycle of Sham and Ovx mice was tracked for 3-5 d consecutively via vaginal cytology to confirm normal estrous cycles or persistent diestrus, respectively [232]. At the completion of all
experiments, uteri were dissected and weighed. Uterine mass <30 mg was used as an inclusion parameter to indicate successful Ovx surgery [189].

**Satellite cell isolation**

Isolation of satellite cells from individual muscles (e.g., tibialis anterior [TA] and gastrocnemius [GC]) and bulk muscles (hindlimb muscles) was performed as described in detail previously [87, 115]. Briefly, muscles were dissected, minced in parallel with muscle fibers, and digested with collagenase type II and dispase (17101-015 and 17105-041, respectively; Gibco, Grand Island, NY). Mononuclear cells 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 alpha7 integrin 647 (clone R2F2) from AbLab (Vancouver, B.C., Canada). Samples were incubated with antibody cocktail, washed, and resuspended with FACS staining medium (2% Fetal Bovine Serum [FBS; 16000044; Gibco] in phosphate-buffered saline [PBS]) containing 0.5 µg/mL propidium iodide (PI) for analysis on a FACSAriaII SORP (BD Biosciences, San Diego, CA). Total satellite cells (lineage negative; VCAM, alpha7 double-positive cells) were analyzed from the entire muscle sample. For isolation of satellite cells from Pax7-ZsGreen mice, mononuclear cells were incubated in FACS staining medium containing PI and ZsGreen+ cells were examined as described previously [233]. Absolute satellite cell counts by FACS were confirmed through gating ZsGreen+ cells and counting beads (CountBright absolute counting beads; C36950; Lot #2361079; Invitrogen, Waltham,
MA) according to manufacturer’s instructions. Cell concentration was calculated using the formula: (number of cell events ÷ number of bead events) × (assigned bead count of the lot ÷ volume of sample).

**Pax7 immunostaining**

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, Pax7 and laminin staining was performed on 7 µM cryosections (CM 1850, Leica Microsystems, Buffalo Grove, IL). Sections were fixed in 4% paraformaldehyde (PFA), washed with PBS, and boiled in heat-induced antigen retrieval buffer (1.8 mM Citric Acid and 8.2 mM Sodium Citrate in water) for 30 min using an Instant Pot pressure cooker (Instant™ Appliances). Sections were incubated for 10 min in H₂O₂ to block endogenous peroxidase activity and then blocked for non-specific binding in 0.5% PerkinElmer TNB Blocking Reagent (0.1 M Tris-HCl, pH 7.5; 0.15 M NaCl; 0.5% Tyramide Signal Amplification (TSA) Blocking Reagent, FP1020) for 1 h at room temperature. Following blocking, sections were incubated with anti-pax7 mouse IgG1 primary antibody (PAX7, Developmental Studies Hybridoma Bank, 1:10) and anti-laminin rabbit (L9393; Sigma-Aldrich, 1:250) in TNB blocking buffer overnight at 4°C. After washing with PBS, sections were incubated with goat anti-mouse biotin-conjugated secondary antibody (115-065-205; Jackson Immuno Research Laboratories Inc, West Grove, PA; 1:1000) and Alexa Fluor 488 goat anti-rabbit (A11034; Invitrogen; 1:500) in TNB blocking buffer for 2 h at room temperature. Visualization of the Pax7 primary antibody was achieved by incubating the sections with the Vectastain ABC
reagent (PK-6100; Vector Laboratories, Burlingame, CA) for 3 h and incubation in the dark with TSA Cyanine 3 kit (NEL744; PerkinElmer, Waltham, MA; 1:50) in diluent buffer for 10 min. Finally, the sections were mounted with anti-fade Prolong gold with 4’, 6-diamidino-2-phenylindole (DAPI). All images were processed and analyzed in a blinded manner with samples being de-identified as to group. Mouse muscle 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. Satellite cells were identified by DAPI+ and Pax7+ cells residing along the myofiber border and were quantified using the ROI manager in the ImageJ software package (NIH, Bethesda, MD, USA). For determination of the cross-sectional area of the TA muscle, the freehand and wand selection tools of the ImageJ were used to measure maximum feret’s diameter.

**DNA content analysis**

Pax7-ZsGreen cells were isolated by FACS and fixed by adding cooled 70% EtOH dropwise while vortexing cell suspension. Cells were then washed with PBS and incubated in staining solution containing 0.1% (v/v) Triton-X100 in PBS, 2 mg DNase-free RNase (Sigma) and 1 mg/ml PI for 30 min at 37°C. Samples were analyzed on a FACSARiaII SORP (BD Biosciences, San Diego, CA). Cell cycle distributions for satellite cells in G₁, S, and G₂ phases was performed using FlowJo v.10 univariate modeling with the Watson Pragmatic algorithm.
RT-qPCR

RNA from freshly FACS-isolated satellite cells was isolated using Qiagen RNeasy Plus Universal Mini kit (73404; Hilden, Germany) according to manufacturer’s instructions. cDNA was synthesized from 100 ng RNA according to directions in SUPERVIOLO cDNA Synthesis Kit (11756050; ThermoFisher, Waltham, MA). Relative quantitation of cdkn1b/p27<sup>Kip1</sup> (Mm00438168_m1), cdkn2a/p16<sup>INK4a</sup> (Mm00494449_m1), ccnd1 (Mm00432359_m1), ccna2 (Mm00438063_m1), mapk14/p38 (Mm01301009_m1), and house-keeping gene GAPDH (Mm99999915_g1) were determined using TaqMan fast advanced master mix (4444557; ThermoFisher; Waltham, MA).

In vivo EdU proliferation assay

Sham and Ovx mice received a pellet containing 25 mg EdU. Following 21 d of exposure, flow cytometry analysis was performed as described in the Click-iT EdU Alexa Fluor 488 Flow Cytometry kit (C104020; Invitrogen) combined with the mononuclear antibody mixture as described above in the satellite cell isolation section. A total of 50,000-100,000 events were recorded for the analysis. Proliferating satellite cells (i.e., S-phase satellite cells) were identified as lineage negative; VCAM, alpha7; FITC triple-positive cells (Fig. 4.3.2B). A positive control comprised of a 72 h post-barium chloride injured TA muscle was included to demonstrate robust EdU+ incorporation by satellite cells (Fig. 4.3.2, C and D).
**In vitro EdU proliferation assay**

Pax7-ZsGreen cells were isolated by FACS and plated into 0.1% gelatin-coated 96-well plates (1,000 cells/well) containing MGM with 20% charcoal stripped FBS (CS-FBS; NB036790; Fisher Scientific). The cells received MGM with or without E2 daily (100 pM final concentration; E8875; Sigma-Aldrich). At day 6, Click-it™ EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 594 dye (C10339; Invitrogen) was performed according to manufacturer’s instructions. The cells were then incubated in 4,6-diamidino-2-phenylindole (DAPI; 1:1000 dilution) in PBS for 20 min at room temperature. EdU+ nuclei were identified and imaged at 10X magnification taken on a Zeiss Observer.Z1 inverted microscope equipped with an AxioCam Mrm camera (Thornwood, NY).

**MTT proliferation assay**

Pax7-ZsGreen cells were isolated by FACS and plated into 0.1% gelatin-coated 96-well plate (2,000 cells/well) with Hams/F10 medium (SH30025.01; Hyclone, Logan, UT) supplemented with 20% CS-FBS, 10 ng/mL human basic fibroblast growth factor (bFGF; 100-18C; Peprotech), 1% Pen/Strep (15140122; Gibco), and 1% Glutamax (35050061; Gibco) and were incubated at 37°C and 5% CO₂. Satellite cells were treated with E₂ every 12 or 24 h to establish a final concentration of: 0 pM, 3.125 pM (0.85 pg/mL), 50 pM (13.62 pg/mL), or 100 pM (27.24 pg/mL) E₂. The MTT assay was performed according to manufacturer’s instructions (11465007001; Roche). After 24 or
72 h, the MTT labeling reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide in PBS) was added to each well (final concentration 0.5 mg/ml) and incubated for 4 h. The solubilization solution was then added to each well and incubated overnight. The formazan product was measured in a microplate reader at a 570-nm wavelength.

**ATP cell proliferation luciferase assay**

Pax7-ZsGreen cells were isolated by FACS and plated into 0.1% gelatin-coated 96-well plates (1,000 cells/well) containing MGM with 20% CS-FBS. The cells either received MGM with or without 100 pM E$_2$. At days 4 and 6, CellTiter-Glo Luminescent Cell Viability Assay (G7570; Promega, Madison, WI) was performed. Medium was replaced with CellTitre-Glo reagent (1:3) in 100 µl of PBS. Plates were allowed to equilibrate for 3 min, then read on a Cytation3 plate reader (BioTek, Winooski, VT).

**Time to first division and cell size**

Pax7-ZsGreen cells were isolated by FACS and plated for live-cell imaging into 0.1% gelatin-coated 24-well glass-bottom dishes (NC9988706; Mattek; Thermo Fisher Scientific; Waltham, MA) (12,000 cells/well) containing MGM with 20% CS-FBS. Cells were treated with MGM containing vehicle (0.03% ethanol in PBS) or E$_2$ (final concentration 100 pM) at the time of plating and again 18 h after plating. Time-lapse imaging was performed from 18h to 72h after plating with a Nikon Eclipse Ti-inverted fluorescence microscope equipped with an automated stage (Prior) and a custom chamber to maintain a constant 37°C temperature, high humidity, and 5% CO$_2$. Multiple positions
were analyzed per group with images acquired every 10 min using phase contrast. Images were collected using a 20X CFI Plan Apochromat Lambda (NA=0.75) objective (Nikon). For each condition, at least 100 individual cells were tracked. Following imaging, data were exported as individual TIFFs for each position and time point. ImageJ software package was used to concatenate TIFF images from each location and manually measure time to first division of each cell. Cell size at 18 h after plating was measured following pixel-based classification and cell segmentation with ilastik (version 1.3.3) and CellProfiler (version 4.0.5), respectively.

Colony-forming assay

Pax7-ZsGreen cells were isolated by FACS and single cells were sorted into 0.1% gelatin-coated 96-well plates containing mouse myoblast medium (MMM): Dulbecco’s modified Eagle’s medium (DMEM; SH30284.01; Hyclone) without phenol red containing 4.00 mM L-glutamine, 4,500 mg/L glucose, and sodium pyruvate; 20% CS-FBS; 10% CS-HS (NC9058780; Fisher Scientific); 10 ng/mL human basic fibroblast growth factor (bFGF; 100-18C; Peprotech), 1% Pen/Strep, and 1% Glutamax with or without E_2 (final concentration 100 pM E_2). Cells were allowed to adhere for 24 h and were then supplemented daily with MMM with or without 100 pM E_2. After culturing plates for 8 days at 37°C and 5% CO_2, cells were fixed with 4% PFA for 20 min at room temperature. For immunostaining of colonies, cells were permeabilized with 0.3% Triton-X100 for 20 min at room temperature, washed with PBS, and blocked with 3% BSA in PBS for 1 h at room temperature. Colonies were stained for MF-20 antibody supernatant
(Developmental Studies Hybridoma Bank, University of Iowa; 1:20 dilution) in 3% BSA in PBS overnight at 4°C. After PBS washes, cells were incubated with Alexa Fluor 555 goat anti-mouse secondary antibody (Life Technologies; 1:500 dilution) in the dark for 45 min at room temperature. The cells were then incubated in DAPI (1:1000 dilution) in PBS for 20 min at room temperature. Colonies were imaged at 10X magnification taken on a Zeiss Observer.Z1 inverted microscope equipped with an AxioCam Mrm camera (Thornwood, NY). Intensity thresholding of ImageJ software package was used to measure the number of nuclei and # of colonies. The percentage of clonal efficiency was calculated by dividing the number of colonies in each plate by the number of wells in which a single cell was sorted then multiplying by 100. Colony size was measured using the freehand selection tool.

*Satellite cell-derived myoblast differentiation*

Pax7-ZsGreen cells were isolated by FACS and plated into 0.1% gelatin-coated 48-well plates (20,000 cells/well) containing MGM with 20% CS-FBS. Cells were incubated at 37°C and 5% CO₂ with MGM medium changed every other day. Cells reached 80-100% confluence on day 3 and were induced to differentiate in a low serum medium: DMEM supplemented with 2% normal or CS-HS, 1% Pen/Strep, and 1% Glutamax for 3.5 days with or without 100 pM E₂. Immunofluorescent staining of cells for MF-20 and DAPI was performed as described in clonal ability assay section. Fusion index was calculated as the percentage of nuclei in myotubes.
Statistical analysis

Two-way analysis of variance (ANOVA) was utilized to determine differences among times and groups. Holm-Sidak post hoc tests were performed in the event of a significant interaction or main effect of time. All other data were analyzed with two-tailed unpaired Student t-tests for determining significant differences between two groups or one-way ANOVA with Holm-Sidak post hoc for determining significant differences between three or more groups. An α level of <0.05 was used for all analyses. Data are presented as mean±SEM unless otherwise indicated. Time to first division data are presented as histograms representing individual cells dividing within time-points and as scatter plots with mean±SD. Satellite cell size and colony size data are presented as scatter plots with mean±SD. All statistical testing was performed using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA) or SigmaPlot version 12.5 (Systat Software, San Jose, CA). Sample sizes are reported as the number of independent mice from which the cells were analyzed or isolated. All immunofluorescent images were processed and analyzed in a blinded manner with samples being de-identified as to treatment or group.

Results

Body mass did not differ between Sham and Ovx mice or across 6, 10, and 14 d post-surgery (p≥0.416; Fig. 4.1A). Vaginal cytology confirmed estrous cycling in Sham mice and persistent diestrus in Ovx mice. Further, Ovx surgery was considered successful with uterine mass ~4-fold less in Ovx than Sham mice (p<0.001) and all uteri being <26
mg in Ovx mice (Fig. 4.1B). The duration of E₂ deficiency did not affect the uterine mass (p=0.393). Mass of TA muscles was 6% greater at 14 compared to 6 d post-Ovx (p=0.040) but did not differ between Sham and Ovx (p=0.471; Fig. 4.1C).

Effects of ovarian hormones and ERα signaling on satellite cell number

To identify the best time post-Ovx to study satellite cell cycling, FACS was used to quantify the total number of satellite cells (lineage negative; VCAM alpha7 double-positive cells) in TA and GC muscles (Fig. 4.1, A and B, respectively). An interaction between group and time was observed for satellite cell number in the TA (p=0.049; Fig. 4.1D). Satellite cell number did not differ between Sham and Ovx at 6 or 10 d post-surgery (p≥0.862); however, was 33% lower in Ovx than Sham mice at 14 d (3738±128 vs. 5554±354, respectively; p=0.010; Fig. 4.1D). We evaluated the density of satellite cells, calculated by dividing the absolute cell number by the wet mass of each muscle. Satellite cell density in TA muscles was lower at 14 d compared to 10 d (time effect; p=0.031; Fig. 4.1E). Similarly, satellite cell number and density were 33% lower in GC muscles from Ovx than Sham mice at 14 d post-surgery (group effect; p≤0.001; Fig. 4.1, C, D and E). Compilation of satellite cell number at these early time points of ovarian hormone deficiency with later time points previously reported (i.e., 56, 112, and 196 d post-Ovx; 58), identifies 14 d as the earliest time point analyzed where a decline in satellite cell number is measured (Fig. 4.1, F and G). Pax7-immunostaining of TA muscle cross-sections at 14 d post-Ovx showed 30 and 32% fewer satellite cells per
cross-section and per mm², respectively (p≤0.025; Fig. 4.1, F and G), recapitulating the decline in satellite cell number at 14 d post-Ovx observed with FACS quantification.

Figure 4.1. Effects of ovarian hormone deficiency on organ masses and satellite cell numbers. (A) Body masses, (B) uterine masses, and (C) TA muscle masses of Sham and Ovx female mice. (D) Total number of satellite cells quantified by FACS as lineage negative; VCAM, alpha7 double-positive cells in TA muscles 6, 10, or 14 d following a Sham or Ovx surgery. (E) Density of satellite cells as calculated from the total number of satellite cells normalized to TA muscle masses. (F) Satellite cell number quantified by Pax7 immunohistochemistry in TA muscle cross-sections from Sham and Ovx at 14 d postsurgery. Pax7+ cells are presented normalized to TA cross-section area and as absolute satellite cells per cross-section. Scale bars = 50 µm. Ovx; ovariectomized, TA; tibialis anterior. Values are presented as Mean±SEM. Significant main effects of two-way ANOVAs (p<0.05) are indicated above the bars (B, C, and E) and when significant interactions occurred, Holm-Sidak post hoc results are indicated by *different from Sham at corresponding time (D) and #different from 10 d Ovx (D). *different from Sham by student t-tests (G).
**Figure 4.1.2. Satellite cell FACS gating scheme and the effects of E2 deficiency on satellite cell number.** Gating scheme for lineage negative; VCAM, alpha7 double-positive cells in (A) TA and (B) GC muscles. Skeletal muscle cells were gated based on forward/side scatter (plots 1-3), live cells (propidium iodide negative – plot 4). These cells were then gated for lineage negative cells CD31/CD45 (plot 5) and selected for VCAM, alpha7 double positivity (plot 6). (C) GC muscle masses. (D) Total number of satellite cells in GC muscles of Sham and Ovx mice 6, 10, and 14 d post-surgery (interaction; p=0.016). (E) Total number of satellite cells normalized to GC muscle masses (interaction; p=0.013). (F) Time course line plot of the total number of satellite cells in TA and (G) GC muscles. Muscles from Sham and Ovx mice were harvested and analyzed 6, 10, 14, 56, 112, or 196 d post-surgery. For F and G, square symbols indicate previously reported data from our laboratory in which methods to count satellite cells were identical [115]. Ovx; ovariectomized, TA; tibialis anterior, GC; gastrocnemius. Values are presented as Mean±SEM. Holm-Sidak post hoc results following a significant interaction (p<0.05) are indicated by *different from Sham at corresponding time (D and E).
We recently determined that *Esr1*, the gene encoding estrogen receptor α (ERα), is more highly expressed in satellite cells than the two other estrogen receptors, *Esr2* (ERβ) and *Gper*, and the progesterone receptor, *Pgr* [115]. This led us to develop an inducible satellite cell-specific ERα knockout mouse (scERαKO) to specifically probe E2-ERα signaling in satellite cells [described in 115] by measuring ZsGreen+ cells (Fig. 4.2A). First, we completed control experiments to directly show that the presence of Pax7\textsuperscript{CreERT2/+} and tamoxifen treatment did not influence satellite cell number (Fig. 4.2, B and C). Next, we measured ZsGreen+ cells in TA and GC muscles from scERαKO and control littermates (scERαWT) 14 d after ablation of ERα. Similar to Ovx mice, scERαKO mice have 24-62% fewer satellite cells (p≤0.050; Fig. 4.2D) indicating that E2 deficiency drives the loss of satellite cells with Ovx as opposed to any other ovarian hormone. Accuracy of satellite cell counts by FACS were confirmed by the concurrent analysis of flow cytometry counting beads and ZsGreen+ satellite cells (Fig. 4.2.2, A and B). Together, these results indicate that deficiency of the hormone E2 drives the loss of satellite cells with Ovx as opposed to any other ovarian hormone and that the loss of E2 or its receptor for only 14 d causes a reduction in the number of satellite cells in skeletal muscles of female mice. Importantly, these data identify the most appropriate time point for conducting the following *in vivo* experiments to investigate impaired satellite cell cycling as a mechanism for the decline in satellite cell number with disruption of E2-ERα signaling.
Figure 4.2. Satellite cell number with the loss of E₂-ERα signaling. (A) Gating scheme for ZsGreen+ cells in TA and GC muscles. ZsGreen+ cells were gated based on forward/side scatter (plots 1-3) and live cells (propidium iodide negative – plot 4). These cells were then selected for ZsGreen-positive cells SSC-H X FITC (ZsGreen; absolute). Total number of ZsGreen+ satellite cells normalized to muscle masses from (B) Pax7⁺/⁻ (scERαWT) and Pax7CreERT2/⁻ (scERαKO) female mice, (C) scERαWT female mice treated with vehicle or tamoxifen, and (D) scERαWT and scERαKO female mice treated with tamoxifen. For C and D, TA and GC muscles were harvested and analyzed 14 d after treatment or the loss of E₂-ERα signaling. ERα; estrogen receptor α, TA; tibialis anterior, GC; gastrocnemius. Values are presented as Mean±SEM. *different from scERαWT.

Figure 4.2.2. Verification of satellite cell count by flow cytometry counting beads. (A) Gating scheme for CountBright absolute counting beads and ZsGreen+ cells. Forward scatter was set to less than 50k to include microsphere on the forward scatter vs linear side scatter plot. CountBright beads were gated using forward/side scatter (plot 1) ZsGreen+ cells were gated based on forward/side scatter (plots 1-3) and live cells (propidium iodide negative – plot 4). These cells were then selected for ZsGreen-positive cells SSC-H X FITC (ZsGreen; absolute). ZsGreen+ cell concentration (estimated) was calculated using the formula: (number of cell events ÷ number of bead events) × (assigned bead count of the lot ÷ volume of sample). (B) Total absolute and estimated number of ZsGreen+ satellite cells in TA muscles (p=0.127). TA; tibialis anterior. Values are presented as Mean±SEM.

Effects of E₂ on satellite cell cycle progression
First, we investigated whether the decline in satellite cell number with E2-ERα disruption is due to changes in satellite cell cycle progression. The most common method for evaluating the cell cycle is DNA content; thus, we used isolated satellite cells from Sham and Ovx mice 14 d post-surgery and stained the DNA stoichiometrically with PI (Fig. 4.3A). This analysis revealed significant differences between Sham and Ovx mouse muscle in the distribution of satellite cells in each cell cycle phase (p≤0.048; Fig. 4.3, B-C). To evaluate the percentage of S-phase satellite cells long-term,

**Figure 4.3. Role of E2 in satellite cell cycle progression.** (A) Cell cycle analysis by quantitation of DNA content via flow cytometry. Sorted satellite cells were fixed and gated based on pulse area/width (plot 1), forward/side scatter (plot 2), and propidium iodide histogram plot (plot 3). (B) Overlay histogram of the cell cycle analysis and table of cell cycle distribution of satellite cells from representative Sham (sample 01) and Ovx (sample 10) TA muscles. (C) Cell cycle distributions for satellite cells in G1, S, and G2 phases (n=4 per group). (D) Percentage of EdU+ satellite cells 21 d post-Ovx. (E) RT-qPCR mRNA expression of cell cycle-related genes in ZsGreen+ satellite cells isolated from hindlimb muscles of Sham (n=4) and Ovx mice (n=4). Ovx; ovariectomized. Values are presented as Mean±SEM. *different from Sham.
Sham and Ovx mice received EdU slow-release pellets, implanted on the day of Ovx surgery, for 21 d (Fig. 4.3,2A). Flow cytometry analysis indicated that the percentage of EdU+ satellite cells accumulated over 21 d did not differ between TA muscles from Sham and Ovx mice (p=0.646; Fig. 4.3D). qPCR analysis showed that gene expression of \(p16^{INK4a}\), a negative regulator of the cell cycle, and \(ccna2\), a regulator of both DNA replication and mitotic entry, were upregulated 3-fold in satellite cells from Ovx mice (p≤0.020; Fig. 4.3E).

**Figure 4.3.2. Satellite cell S-phase cell cycle progression with E\(_2\) deficiency.** (A) Schematic of EdU+ proliferation experiment where Sham and Ovx surgeries with EdU pellet implantation were performed and satellite cells subsequently quantified by FACS 21 d post-surgery. (B) Gating scheme for lineage negative; VCAM, alpha7, FITC triple-positive cells in TA muscles. Skeletal muscle cells were gated based on forward/side scatter (plots 1-3), live cells (propidium iodide negative – plot 4). These cells were then gated for lineage negative cells CD31/CD45 (plot 5), selected for VCAM, alpha7 double positivity (plot 6), and then selected for FITC (EdU) positivity (plot 7). (C) Representative FACS plots of EdU+ satellite cells isolated from TA muscles of Sham, Ovx, and an Injured control mouse. (D) Percentage of EdU+ satellite cells in Sham (n=9), Ovx (n=11), and Injured control TA muscles (n=7). Ovx; ovariectomized. Values are presented as Mean±SEM. *different from Sham and #different from Ovx.
Effects of \( E_2 \) on proliferation and cell cycle kinetics of satellite cells in vitro

To further characterize the impaired cycling of the satellite cell pool with \( E_2 \)-ER\( \alpha \) disruption, we isolated satellite cells from female Pax7-ZsGreen mice and assessed cell cycle kinetics in vitro. First, we evaluated satellite cell proliferation at 24 and 72 h post-plating with \( E_2 \) (final concentrations 0, 3.125, 50, and 100 pM). Satellite cell proliferation was 51-67% greater with \( E_2 \)-treatment at 72 h post-plating, regardless of the dose (\( p<0.001; \) Fig. 4.4A). The 100 pM \( E_2 \) concentration was used for all subsequent experiments, as it significantly affected satellite cell proliferation at both 24 and 72 h post-plating and represents physiologically relevant \( E_2 \) levels in mice [232]. Furthermore, satellite cell proliferation measured by ATP luciferase assay was 6- and 7-fold greater with 100 pM \( E_2 \) treatment at 4 and 6 d after plating, respectively (\( p<0.001; \) Fig. 4.4.2), and the percentage of EdU+ nuclei was 2-fold greater with 100 pM \( E_2 \) treatment at 6 d after plating (\( p=0.025; \) Fig. 4.4B).

To measure the rate of satellite cell division, we treated satellite cells with \( E_2 \) at 0 and 18 h after plating and observed cell division by time-lapse imaging from 18-72 h after plating (Fig. 4.4C). We found that \( E_2 \)-treated satellite cells require less time to complete the first division compared to vehicle-treated (37.1\( \pm \)0.6 and 40.2\( \pm \)0.8 h; \( p=0.001; \) Fig. 4.4, D and E). Since cell size is proposed to be indicative of cell growth, we measured satellite cell size at 18 h post-plating; \( E_2 \) treatment did not affect satellite cell size (\( p=0.547; \) Fig. 4.4F).
Figure 4.4. Proliferation and cell cycle kinetics of E2-treated satellite cells. (A) Formazan absorbance expressed as a measure of cell viability from satellite cells treated with physiological doses of E2. (B) Representative images and quantification of the percentage of EdU+ nuclei 6 d post-plating of satellite cells. Scale bars = 50 µm. (C) Schematic of study design for time-lapse microscopy experiment to quantify satellite cell time to first division. (D) Frequency of satellite cell first division (n=3 per group). (E) Mean time of satellite cell first division. (F) Satellite cell size at 18 h after plating and initial E2-treatment. E2; estradiol. Values are presented as Mean±SEM in A and B and Mean±SD for E and F. †different from 0 (vehicle), ‡different from 3.125 pM, ‡different from 50pM at corresponding time points. *different from –E2. (B) Performed in collaboration with ASS.
Figure 4.4.2. Satellite cell proliferation with E2 treatment. Phase contrast micrographs at (A) 4 d or (B) 6 d post-plating with or without E2 treatment and quantification of satellite cell proliferation by luciferase assay. Scale bars = 50 µm. E2; estradiol. Values are presented as Mean±SEM. *different from –E2. Performed in collaboration with ASS.

**Effects of E2 on satellite cell colony-forming ability and differentiation**

To assess clonogenicity of single satellite cells with and without E2, we treated single satellite cells with vehicle or E2 and allowed colonies to form for 8 d (Fig. 4.5A top). The ability of the cells to survive and form colonies in vitro was not affected by E2 treatment (p=0.687; Fig. 4.5B). However, mean colony size and spontaneous differentiation, quantified as nuclei in MHC+ cytoplasm, were approximately 69% and 30% greater with E2 treatment (p<0.001; Fig. 4.5, C and D, respectively). Because we observed greater satellite cell proliferation in vitro with E2 treatment under standard culture conditions (Fig. 4.4, A and B), to evaluate the effects of E2 on satellite cell differentiation alone, we cultured satellite cells under identical conditions until confluent.
and then began vehicle or E₂ treatment after switching to low-serum differentiation medium (Fig. 4.5A bottom). An interaction between serum condition and E₂ treatment was measured for both number of nuclei in MHC⁺ myotubes and fusion index (p<0.001; Fig. 4.5, F-H). Number of nuclei in MHC⁺ myotubes was greater with E₂ treatment regardless of serum condition (p≤0.009; Fig. 4.5G); however, fusion index did not differ between groups when normal HS was used (p=0.250; Fig. 4.5H). In fact, myotubes were almost nonexistent in vehicle-treated wells, supporting the concept that the lack of E₂ impairs satellite cell differentiation (Fig. 4.5F; -E₂ on right).
Figure 4.5. Effects of E2 on satellite cell colony-forming ability and differentiation. (A) Schematic for colony-forming assay (top) where single satellite cells were plated and treated with MMM with vehicle or E2 until day 8 when colony number, size, and spontaneous differentiation are measured (n=3 per group). Schematic for differentiation assay (bottom) where 20,000 satellite cells were plated and treated with vehicle or E2 only after switching to low-serum differentiation medium (n=3-6 per group). (B) Clonal efficiency quantified by counting the number of colonies in each plate and the number of wells in which a single cell was sorted. (C) Colony size. (D) Percentage of nuclei in MHC+ cytoplasm. Representative images of immunofluorescence of MF-20 (MHC) and DAPI (E) after 8 d of MMM with or without E2 or (F) after low-serum medium conditions with normal horse serum (HS; top) and charcoal-stripped horse serum (CS-HS; bottom) for 3.5 d with or without E2. Scale bars = 50 µm. (G) Percentage of MHC+ nuclei (interaction; p<0.001). (H) Quantitative analysis of myotube fusion index (interaction; p<0.001). FACS; fluorescence-activated cell sorting, SC; satellite cell, MMM; mouse myoblast medium, MGM; muscle growth medium, DM; differentiation medium, E2; estradiol, MHC; myosin-heavy chain, HS; horse serum, CS-HS; charcoal-stripped horse serum. Values are presented as Mean±SEM for B, D, and E and Mean±SD for C. *different from −E2 (C and D). †different from HS without E2, #different from HS with E2, and ϕdifferent from CS-HS without E2. (B-E) Performed in collaboration with ASS and (D-H) with BPS.
Discussion

Recent developments in satellite cell biology have highlighted the importance of circulating factors, such as sex hormones, in skeletal muscle growth and regeneration. Here, we expanded upon our previous work demonstrating that there is a substantial decline in the number of satellite cells in muscles from female mice with ovarian hormone deficiency, which can be prevented with E2 treatment [115]. Results of the present study show that deficiency of the hormone E2 drives the loss of satellite cells with Ovx as opposed to any other ovarian hormone and that the loss of E2 or its receptor for only 14 d impairs satellite cell maintenance. We show mechanistically that impaired satellite cell maintenance caused by E2 deficiency includes altered satellite cell cycle progression, kinetics, proliferation, and differentiation.

When satellite cells exit quiescence, the non-cycling G0 phase, they can adopt different cell fates: differentiation, cell death (i.e., apoptosis, necrosis, autophagy), or senescence. These satellite cell fate decisions are carefully regulated by intrinsic and extrinsic cues and significant alterations can lead to exhaustion of the satellite cell pool [84, 234, 235]. In vivo FACS analysis of cell cycle distribution at 14 d post-Ovx indicated that E2-deficient satellite cells have impaired cell cycle progression at both G1 to S and S to G2 transitions. There were 4- to 5-fold fewer satellite cells from Ovx than Sham muscles in S-phase and G2-phase (Fig. 4.3C). In contrast, 93% of the satellite cell population was in G0 phase in Ovx muscles versus only 69% for Sham (Fig. 4.3C). Theoretically, there is no need for elevated cell cycling or proliferation in the ovary-intact mice because the satellite cell pool is maintained. However, given that there are
approximately 33% fewer satellite cells in muscles of Ovx mice compared to ovary-intact mice, we proposed that there would be an increase in proliferating satellite cells to counter the loss of satellite cells caused by E₂ deficiency previously suggested to occur through apoptosis [115]. We found that the cumulative proportion of proliferating satellite cells in vivo over 21 d is the same in muscles of Ovx and ovary-intact mice (Fig. 4.3D), and that the percentage of EdU-labeled satellite cells was similar to that previously reported. Specifically, studies have shown that 1-2% of satellite cells are labeled per week [73] or 0.2±0.1% of satellite cells are labeled per day [72], suggesting that after 21 d approximately 6% of satellite cells will be EdU+, which is supported by our observations in this study. Interestingly, in vitro we observed that E₂-treatment of isolated satellite cells have 97% greater EdU incorporation compared to vehicle-treated satellite cells (Fig. 4.4B). These results are consistent with other reports showing that disruption of E₂-ERα/β signaling impairs proliferation of cultured satellite cells or exercise-induced satellite cell proliferation [160, 162, 168-171].

Consequently, we evaluated genes that are known to regulate satellite cell cycle progression. The progression of satellite cells from G₁ to S phase is promoted by insulin-like growth factor-1 (IGF-1) via downregulation of p27\textsuperscript{Kip1} [236] and ccnd1 via downregulation of transforming growth factor β (TGF-β) signaling [237], whereas accumulation of cyclin-dependent kinase (CDK) inhibitors, such as p16\textsuperscript{INK4a}, prevents satellite cell G₁ to S phase progression resulting in cell cycle arrest and replicative senescence [84]. E₂ deficiency did not affect p27\textsuperscript{Kip1} or ccnd1 mRNA expression (Fig. 4.3E), supporting the notion that p27\textsuperscript{Kip1}, a negative regulator of cell cycling, is not
associated with E$_2$-dependent satellite cell proliferation, and potentially occurs through phosphatidylinositol 3-kinase (PI3K) as previously demonstrated [169]. We instead show a 3-fold upregulation in mRNA expression of $p16^{INK4a}$, a marker of permanent cell cycle arrest, in satellite cells from muscles of Ovx mice (Fig. 4.3E). Sousa-Victor et al. [84] recently showed for the first time that the loss of cell cycle protective mechanisms with age results in senescent $p16^{INK4a}$-expressing satellite cells. That study revealed a 2- and 4-fold upregulation in $p16^{INK4a}$ mRNA expression and 15% and 40% SA-β gal+ satellite cells from 28-32 mo geriatric mice and 75 yr humans compared to young adults, respectively [84]. With $p16^{INK4a}$-induced replicative senescence playing a role in satellite cell maintenance in aged mice and humans, it will be interesting to further investigate whether the inability of satellite cells to efficiently cycle and generate progeny in an environment lacking E$_2$ is due to $p16^{INK4a}$-induced replicative senescence and whether the loss of E$_2$ stimulates a switch to a senescent, non-proliferative state or impairs the maintenance of quiescence.

We also present data that mRNA expression of $ccna2$, an essential regulator of both the onset of the S-phase transition and during the G$_2$-M transition, is upregulated (Fig. 4.3E). This result is in contrast to a previous study that shows a decline in $ccna2$ mRNA expression at 6 d post-inactivation of ERβ signaling in satellite cells [160]. The discrepancy between these studies is presumably due to different analysis time points, hormone vs. receptor signaling, and that the present study did not culture satellite cells prior to qPCR analysis as was done previously (72).
It may seem paradoxical that both $p16^{INK4a}$ and $ccna2$ are upregulated in satellite cells from Ovx muscles, but it is important to note that our study performed a satellite cell population analysis and not an individual satellite cell-based analysis. The satellite cell population analysis suggests that the $p16^{INK4a}$-expressing cells may not be the satellite cells expressing $ccna2$. Functionally heterogeneous subpopulations of satellite cells have been previously identified using techniques such as single cell RNA-sequencing [238-240], single-cell mass cytometry (CYTOF) [241], lineage tracing [57, 82], and label retaining [242, 243]. These cell cycle gene expression data along with our finding that E2 is necessary for transitions between cell cycle phases strongly suggest that reduced cell cycle progression is one of the mechanisms whereby satellite cell number declines with E2 deficiency.

Satellite cell subpopulations are distinguished by differential expression of genes or cell surface markers, or phenotypic changes (e.g., time to first division). For instance, non-cycling satellite cells, in G0 phase, express high levels of Sprouty1 and $p27^{kip1}$ to maintain quiescence [238, 242, 244] and can reversibly transition from G0 to a primed $G_{Alert}$ phase permitting rapid cell cycle entry [82]. We therefore cultured satellite cells in the presence or absence of E2 and observed time to first division. To note, normal serum can contain estrogens from female donors [245], so we utilized serum where the estrogens were removed by charcoal-stripping the serum and then controlled estrogen exposure by adding specific amounts of exogenous E2. We observed that E2-treated satellite cells have reduced time to first division, only taking 33–48 h to undergo first division (Fig. 4.4E). The time to first division was not comparable to that of satellite cells
from muscles 3 d post-injury, which is reported to be less than 20 h [82]. Other time-lapse microscopy studies have shown that 16% of satellite cells do not divide and it takes the remaining satellite cells 36-48 h to undergo cell division [64, 82]. It is worth mentioning that satellite cells grow more slowly in medium containing charcoal-stripped serum than normal serum, so the time to first division observed of vehicle-treated satellite cells in our study may not closely compare to previous satellite cell studies.

Advancements in cell-labeling techniques have allowed analysis of satellite cell division history revealing that satellite cell division can be separated into slow- and fast-dividing subpopulations. Studies have shown that the slow-dividing subpopulation accounts for 10-20% of satellite cells and are the long-term self-renewing population; whereas the fast-dividing subpopulation accounts for 80-90% of satellite cells which generate a great deal of differentiated cells but have limited replication [53, 246-248]. This study showed a faster rate of cell division with E_2 treatment compared to vehicle treatment suggesting that the satellite cells exhausted in an environment without E_2 are from the fast-dividing subpopulation. These results identify impaired cell cycle kinetics as an additional mechanism whereby the absence of E_2 influences satellite cell maintenance.

Our study employed a variety of methods to analyze the satellite cell proliferation in environments with and without E_2, including colorimetric assays that measure metabolic activity (i.e., MTT), fluorescent dyes (i.e., propidium iodide), and incorporation of thymidine analogs (i.e., EdU). The collective data suggest that satellite cell viability and proliferation is enhanced when E_2 is present (Fig. 4.4, A and B; Fig. 4.4.2). Previous rodent studies investigating the effects of E_2 on satellite cell proliferation
did not observe changes in satellite cell proliferation with E2-treatment under normal conditions (i.e., without injury or exercise), when quantifying proliferation using the proliferation marker, proliferating cell nuclear antigen (PCNA) [171] or immunostaining for incorporated thymidine analog BrdU [170]. Kamanga-Sollo et al. used radioactive thymidine (3H-thymidine) in vitro and demonstrated greater proliferation in bovine satellite cells treated with E2 when medium is free of IGF binding protein (IGFBP)-3, a protein previously shown to antagonize IGF-1 actions on myogenic proliferation [249]. The 1.5-fold increase in bovine satellite cell proliferation noted in their study was only observed when bovine satellite cells were cultured with 10^4 pM E2 and not 10^3 pM E2 [162, 163]. Considering that circulating E2 concentrations in rodent models and premenopausal women range from approximately 5 to 200 pM [119, 120], all of the currently published studies evaluating the effects of E2 on cultured satellite cells used supraphysiological E2 doses (ranging from 10^4 to 10^6 pM E2; [162, 163, 165, 173]). Higher doses of E2 were possibly used due to the short half-life of E2 in culture (presumably 3 h), relatively high photo-degradation half-life in solution [250], and adherence of E2 molecules to polypropylene [251]. Here, we use physiologically relevant E2 doses to establish a final concentration of 100 pM E2 in culture and treated either every 24 or 48 h. Our data demonstrated greater proliferative capacity of satellite cells with E2 compared to vehicle at 3.125, 50 and 100 pM (final concentrations) at 72 h post-plating (Fig 3A). These findings suggest that previous studies using supraphysiological E2 doses may have observed opposite effects compared to those observed with physiological ranges, as has been shown in other cells/tissues [252, 253].
Assessment of the colony-forming ability of single satellite cells demonstrated that all satellite cells were able to generate clones, regardless of treatment, but the satellite cells supplemented with E$_2$ had greater colony sizes suggesting enhanced proliferative capacity (Fig. 4.5C). Satellite cell-derived myoblasts treated with E$_2$ had augmented differentiation, indicated by increased MHC$^+$ nuclei and myotube fusion index (Fig. 4.5, G and H). Other studies have yielded similar results using other methodologies. Kitajima et al. cultured myofibers from Ovx in floating conditions for 3 d and observed significantly lower numbers of differentiating satellite cells compared to those from ovary-intact mice [161], and Galluzzo et al. found that E$_2$-treated rat myoblast cells (L6) had augmented expression of differentiation markers, MHC and myogenin [231]. On the contrary, Ogawa et al. previously demonstrate that satellite cells treated with E$_2$ (10$^4$ pM) and cultured for 8 d in differentiation medium displayed inhibited myogenesis and reduced fusion index [165]. We propose this negative regulation by E$_2$ is due to supraphysiological dosing and the prolonged duration of culture in differentiation medium.

The present study showed no noticeable difference in myotube fusion index when satellite cells were cultured in normal HS (Fig. 4.5H). This result emphasizes the importance of using charcoal-stripped serum to deplete E$_2$ and other endogenous non-polar lipid-bound materials (e.g., hormones, growth factors, cytokines), which have demonstrated estrogenic activity and could potentially confound results [254, 255]. Given that the pH indicator phenol-red has also been shown to have estrogenic effects [256-258], this study used phenol-red free DMEM with CS-HS. The absence of myotubes in
vehicle-treated satellite cells cultured in phenol-red free DMEM with CS-HS (Fig. 4.5F) demonstrates that E₂, possibly as well as the other lipid-modified proteins, are crucial for myoblast differentiation. These results suggest that E₂ regulates both satellite cell proliferation and differentiation resulting in enhanced myogenesis.

In summary, we show that mechanisms underlying the E₂ deficiency-induced decline in satellite cell number is multifactorial involving impaired satellite cell cycle progression, kinetics, proliferation, and differentiation. These findings have implications in the preservation of efficient muscle regeneration, including targeting p16-mediated pathways to prevent cell cycle arrest of satellite cells and subsequent exhaustion of the satellite cell pool, and are relevant to all women experiencing a decline in circulating E₂ levels. Ultimately, understanding how E₂ regulates satellite cells will help to determine therapies for improving muscle regeneration and recovery of strength that affect quality of life.
Chapter 5: Summary Statement

The contents of this dissertation add significant findings and mechanisms to the fields of satellite cell biology, E2-ERα signaling, and recovery of skeletal muscle strength. Aged women experience decrements in skeletal muscle strength and regenerative capacity corresponding with the time E2 levels decline at of menopause. Previous studies demonstrated that satellite cells are indispensable for muscle regeneration and that satellite cells rely on cues from their environment, such as circulating hormones, to maintain their quiescent population. Our lab has previously demonstrated that E2 deficiency impairs satellite cell maintenance and self-renewal; however, the mechanisms responsible for these decrements were unknown. The aim of this dissertation was to evaluate the effects of E2 on skeletal muscle recovery of strength after injury and to identify underlying mechanisms whereby E2 influences satellite cell maintenance. Specifically, I investigated the role of E2 on the recovery of muscle strength after repeated BaCl₂ (Chapter 3) and on satellite cell cycle progression, proliferation, and differentiation (Chapter 4).

My first study examined the effects of E2 on recovery of strength at several time points after repeated injury in a longitudinal design. I established that E2-deficient muscles have a blunted ability to adapt and recover muscle strength following repeated injuries. Satellite cell number was also compromised in E2-deficient muscles, as evidenced by 41-43% less satellite cells after one or three injuries in placebo- than E2-treated Ovx. Muscle injury is a complex affair involving several types of cells; therefore, to correlate the blunted recovery of strength with E2 deficiency to the decline in satellite
cell number further investigation is needed. Future work can ablate satellite cells or use the scERαKO mouse model to measure whether E₂-mediated maintenance of satellite cells is necessary for the adaptation of muscle and recovery of strength after injury. Overall, the contents of this chapter establish that E₂ influences strength recovery after injury and suggests that satellite cells are likely involved.

Upon determining that satellite cell maintenance is impaired with E₂ deficiency, my second study investigated the mechanism(s) whereby E₂ regulates satellite cell maintenance. The contents of this chapter thoroughly investigated the effects of E₂ on satellite cells with a combination of in vitro and in vivo approaches. My results showed that the decline in satellite cell number occurs between 10 and 14 d after the loss of E₂ or E₂-ERα signaling, and identified impaired satellite cell cycle progression, kinetics, division, and differentiation as mechanisms. Together, the results of this chapter discovered a novel mechanism for preservation of the satellite cell pool by E₂ through promotion of satellite cell cycling.

Overall, my dissertation work has established that the loss of E₂ or ERα signaling impairs satellite cell maintenance by disrupting proper satellite cell cycle progression, which may play a key role in the recovery of muscle strength after injury. To advance my findings, future studies could investigate the heterogeneity of the satellite cell population with and without E₂ or E₂-ERα signaling. Single cell RNA-seq and CyTOF can be used complementarily to quantify genes or proteins in a cell and identify complex and rare cell populations. Understanding how E₂ deficiency affects satellite cell heterogeneity will help design mechanistic studies to determine whether the failure in cell cycle progression
with E2 deficiency is due to p16INK4a-induced replicative senescence and whether the loss of E2 immediately promotes the switch to a senescent, non-proliferative state or impairs the maintenance of quiescence. We also recognize that the substantial decline in satellite cell numbers of limb muscles (e.g., TA) may not be a typical response to aging, since other muscles, such as the masseter, have been observed to increase with age. Future studies on the satellite cell population will elucidate differences between muscles and fiber types. Furthermore, lineage tracing models under normal homeostatic conditions can be performed to determine if there is a deficit in satellite cell-derived myoblast fusion that results in satellite cell death. Finally, comparative studies of the effects of E2 and other sex hormones (e.g., 17α-estradiol) on female and male satellite cell maintenance can be performed to determine the relevant estrogens in both sexes. Ultimately, once E2-sensitive molecular mechanisms of satellite cell maintenance are identified, potential therapies can be developed to preserve muscle regeneration, strength, and quality of life for aged individuals.

Many hormones are known to affect muscle mass and function. Estrogens are the primary female sex hormones that have both reproductive and non-reproductive functions. Beyond the role of E2 in childbearing, bone health, and maintenance of cholesterol levels, my dissertation demonstrates that E2 is a key hormone in regulating muscle regeneration and strength via satellite cell maintenance. In conclusion, since women spend approximately one-third of their lives post-menopause and E2-based HRT can mitigate detrimental effects of menopause and aging, it is critical that the mechanisms whereby E2 effects skeletal muscle are discovered.
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Estrogen Regulates the Satellite Cell Compartment in Females

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