Induced ablation of skeletal muscle-specific estrogen receptor-alpha in adult female mice increased the susceptibility to develop skeletal muscle inflammation and glucose intolerance under chronic lipid overload

by
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May, 2018

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Abstract

Skeletal muscle-specific ERα appears to play important roles in regulating skeletal muscle glucose and lipid homeostasis. The overall aim of this dissertation was to determine whether skeletal muscle-specific ERα is critical for maintaining metabolic function under conditions of lipid overload. To further advance our understanding of skeletal muscle-specific ERα, this study integrated *in vivo* and *in vitro* loss-of-function approaches by generating a novel inducible skeletal muscle-specific ERα knockout mouse model (ERαKO^{ism}) and by silencing ERα in human myotubes using an adenovirus-driven ERαshRNA. The overarching hypothesis is that induced ablation of skeletal muscle-specific ERα increased the susceptibility to high fat diet (HFD)-induced metabolic dysfunction. ERαKO^{ism} mice exhibited similar adiposity after acute and chronic HFD treatments compared to WT mice, for both females and males. Indirect calorimetry revealed that energy expenditure was similar between female WT and

ERαKO^{ism} mice, even when exposed to acute and chronic HFD treatments. Male ERαKO^{ism} mice exhibited minimally greater energy expenditure after chronic HFD treatment compared to male WT mice, regardless of diet. Spontaneous cage activity was similar between diet-matched WT and ERαKO^{ism} mice for both sexes, even after acute and chronic HFD treatment. Analysis of glucose dynamics revealed that female ERαKO^{ism}-HFD exhibited greater glucose intolerance than WT-HFD after chronic HFD treatment. *Ex vivo* skeletal muscle glucose uptake was similar between female WT and ERαKO^{ism} mice, although GLUT4 protein content was lower in skeletal muscle of female ERαKO^{ism}, regardless of diet. Markers of pro-inflammation were also elevated in female ERαKO^{ism} mice, regardless of diet. Analysis of mitochondrial respiratory capacity, oxidative phosphorylation efficiency, and H_2O_2 emission potential in permeabilized skeletal muscle fibers, revealed that skeletal muscle mitochondrial function was similar between WT and ERαKO^{ism} for both sexes. In human skeletal myotubes sourced from healthy and obese-insulin resistant adult women, ATP production rate was minimally lower in myotubes transduced with ERαshRNA compared to scrambled-shRNA (control) myotubes. Overall, the data suggest that skeletal muscle ERα is critical for maintaining glucose tolerance in females on a chronic HFD and regulating skeletal muscle inflammation.

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A Dissertation

Presented To the Faculty of the Department of Physiology

East Carolina University

In Partial Fulfillment of the Requirements for the Degree

Doctor of Philosophy in Physiology

by

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May, 2018

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Acknowledgements

I would like to express my utmost gratitude to my advisor Dr. Espen Spangenburg, Associate Professor in the Department of Physiology, for this amazing opportunity, for teaching and showing me how to be a successful scientific researcher, and for being an overall great mentor. This work would not have been possible without your guidance and enduring support.

I would like to thank my dissertation committee members: Dr. P. Darrell Neufer, Director of East Carolina Diabetes and Obesity Institute, Dr. Johanna L. Hannan, Assistant Professor of the Department of Physiology, Dr. Joseph M. McClung, Assistant Professor of the Department of Physiology, and Dr. Dawn A. Lowe, Associate Professor of the Department of Rehabilitation Medicine at the University of Minnesota for providing helpful input in this dissertation and for being supportive of my future career.

I would like to thank all our collaborators at East Carolina University: Dr. P. Darrell Neufer and the Neufer Lab, Dr. Carol A. Witczak and the Witczak Lab, Dr. Joseph M. McClung and the McClung Lab, Dr. Kym Gowdy and the Gowdy Lab, and Dr. Katsu Funai and the Funai Lab for the knowledge you have shared and your assistance.

I would like to thank the members of the Spangenburg Lab, the entire ECDOI, and the Physiology Department for your help, support, and friendship.

I would like to thank the people at the Department of Comparative Medicine, who I had the pleasure of working with, for your assistance in caring for the mouse colonies, your friendship, and your support.

Finally, I would like to thank my parents and my siblings for their undying love and support.

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Chapter 1: Introduction and Specific Aims

In most countries worldwide, the prevalence of the Metabolic Syndrome (MetS), continues to rise and is reported to be wide-ranging from ~10% to alarmingly ~50% of all adults within each country (Beltrán-Sánchez, Harhay, Harhay, & McElligott, 2014; J. Moore, Chaudhary, & Akinyemiju, 2017; O'Neill & O'Driscoll, 2015; Ranasinghe, Mathangasinghe, Jayawardena, Hills, & Misra, 2017). MetS comprises of at least 3 of the following conditions: excess adiposity, hypertriglyceridemia, insulin resistance, low high-density lipoprotein-cholesterol (HDL-C), high fasting blood glucose, and hypertension, thus making MetS a precursor to Type 2 Diabetes and other obesity-associated diseases (Beltrán-Sánchez et al., 2014; J. Moore et al., 2017; O'Neill & O'Driscoll, 2015). Large amounts of stored body fat are associated with mechanisms that initiate the development of insulin resistance including lipotoxicity, a reduction in mitochondrial function, alterations in redox homeostasis, inflammation, and endoplasmic reticulum stress (Bosma, Kersten, Hesselink, & Schrauwen, 2012; Lark, Fisher-Wellman, & Neufer, 2012; Muoio, 2014; Muoio & Neufer, 2012).

Epidemiological evidence has documented that alterations in estrogen signaling can increase the risk of developing MetS (Carr, 2003). A significant reduction in estrogen action in humans due to aging (e.g. menopause), surgical outcomes (e.g. oophorectomy) or genetic reasons (e.g. estrogen receptor polymorphisms) are associated with increased risk of MetS (Ahtiainen et al., 2012; Brussaard, Gevers Leuven, Frölich, Kluft, & Krans, 1997; Demir et al., 2008; Dørum et al., 2008; Efstathiadou et al., 2015; Gallagher et al., 2007; Lo, Zhao, Scuteri, Brockwell, & Sowers, 2006; Sites, Brochu, Tchernof, & Poehlman, 2001). Conversely, estrogen

therapy is associated with decreased adiposity and improved insulin sensitivity in postmenopausal women (Ahtiainen et al., 2012; Brussaard et al., 1997; Demir et al., 2008; Sites et al., 2001). Collectively, the data suggest that estrogens protect the body from metabolic disease; however, the protective mechanisms induced by estrogen action remain largely undefined.

Among the estrogen receptors that mediate estrogen signaling in cells, estrogen receptor-alpha (ERα) is thought to be critical in regulating skeletal muscle glucose and lipid homeostasis. Global ablation and conditional skeletal muscle-specific ablation of the gene that encodes for ERα (ESR1) results in significant increases in body fat, impaired whole body insulin sensitivity, skeletal muscle insulin resistance, increased markers of skeletal muscle inflammation, and altered mitochondrial health (Ribas et al., 2010, 2016). Unfortunately, the utilization of global ERα knockout models does not allow the dissection of primary versus secondary effects because ERα is expressed in numerous tissues (Baltgalvis, Greising, Warren, & Lowe, 2010; Couse, Lindzey, Grandien, Gustafsson, & Korach, 1997; Drew et al., 2015; Khalid & Krum, 2016; H.-R. Lee, Kim, & Choi, 2012; Pugach, Blenck, Dragavon, Langer, & Leinwand, 2016; Yuchi et al., 2015). Furthermore, findings from current conditional skeletal muscle-specific ERα knockout models may be confounded by developmental effects.

The current gap in the literature that this dissertation aimed to elucidate was the effect of skeletal muscle-specific ERα deletion in the adult animal on skeletal muscle metabolism and physiological function. This dissertation also sought to determine if estrogen/ERα signaling in adult skeletal muscle has a direct or indirect role in protecting against high fat diet-induced insulin resistance is still unclear. Thus, the overall purpose

of this dissertation was to determine whether skeletal muscle-specific ERα is critical for the regulation of skeletal muscle glucose and lipid homeostasis under acute and chronic lipid overload.

Specific Aim #1:

Determine whether skeletal muscle ERα is critical for maintaining metabolic function under acute exposure to a high fat diet (HFD).

Hypothesis: Induced ablation of skeletal muscle-specific ERα in adult female and male mice increased the susceptibility to develop excess adiposity, impaired whole body metabolism, and glucose intolerance after an acute HFD**.**

This study generated a novel inducible skeletal muscle-specific ERα knockout mouse model (ERαKO^{ism}) using the tamoxifen-inducible Cre-loxP system to avoid confounding developmental mechanisms that may have an impact on metabolism at adulthood. In this study, female and male ERαKO^{ism} mice grew and matured with normal ERα function. ERα was ablated when mice became young adults. To determine whether body composition was affected by the induced ablation of ERα, body mass, fat mass, and lean mass were measured at baseline and after a 1 week HFD. Indirect gas calorimetry was used to assess whether induced ablation of ERα affected whole body oxygen consumption, carbon dioxide production, energy expenditure, and flexibility in fuel selection at baseline and during the acute switch to a HFD. Finally, to assess

peripheral glucose dynamics, a glucose tolerance test was performed after a 1 week HFD.

Specific Aim #2:

Determine whether skeletal muscle ERα is critical for maintaining metabolic function under chronic exposure to a high fat diet (HFD).

Hypothesis: Induced ablation of skeletal muscle-specific ERα in adult female and male mice increased the susceptibility to develop excess adiposity, impaired whole body metabolism, glucose intolerance, skeletal muscle insulin resistance, increased skeletal muscle inflammation, and reduced mitochondrial respiration after chronic HFD.

Long-term exposure to a high fat diet results in the disruption of multiple mechanisms associated with maintaining normal insulin sensitivity. The response of ERαKO^{ism} mice to acute and chronic high fat diets may be different, further providing valuable insight on the importance of skeletal muscle-specific ERα in glucose and lipid metabolism. The aforementioned experiments in Specific Aim #1 were repeated after a 12-week HFD. Additionally, e*x vivo* basal and insulin-stimulated skeletal muscle glucose uptake, markers of inflammation, and indices of mitochondrial function in skeletal muscle were assessed after the 12-week HFD treatment to comprehensively determine whether skeletal muscle ERα regulates mechanisms that maintain skeletal muscle metabolic function.

Specific Aim #3:

Determine whether skeletal muscle ERα critical for maintaining mitochondrial respiration in human myotubes.

Hypothesis: Induced ablation of skeletal muscle-specific ERα in human myotubes altered mitochondrial respiratory capacity.

This study attempted to translate the results found in Specific Aim #2 to human skeletal muscle cells. In this study, primary human skeletal muscle cells, which were isolated from healthy and obese-insulin resistant adult women, were differentiated into mature myotubes. Mitochondrial respiratory capacity was assessed in myotubes transduced with ERαshRNA, which significantly reduced ERα expression.

Chapter 2: Review of Literature

The Metabolic Syndrome (MetS) is a global epidemic (Aguilar, Bhuket, Torres, Liu, & Rj, 2015; Amirkalali et al., 2015; J. Moore et al., 2017; O'Neill & O'Driscoll, 2015; Ranasinghe et al., 2017; Xiao et al., 2016). MetS is defined by the presence of at least 3 of the following risk factors: abdominal obesity (waist circumference of ≥94cm in men, ≥80cm in women), high fasting blood glucose (≥110mg/dl or ≥5.6mmol/L), hypertriglyceridemia (≥150mg/dl or ≥2mmol/L), low high-density lipoprotein-cholesterol (<40mg/dl in men, <50mg/dl in women), and hypertension (≥130/85mmHg) (O'Neill & O'Driscoll, 2015). While the pathogenesis of MetS and the exact mechanisms that link these risk factors are still currently being elucidated, the key components appear to be obesity and insulin resistance (Ervin, 2009; Ferrannini, Haffner, Mitchell, & Stern, 1991). Obesity, a consequence of nutrition overload coupled with a sedentary lifestyle, dramatically increases the susceptibility to insulin resistance and dyslipidemia (Rask-Madsen & Kahn, 2012). Averting and/or treating MetS is critical to prevent more severe conditions linked to MetS such as Type 2 Diabetes Mellitus, cardiovascular disease, and metabolic disease-associated cancers (Esposito, Chiodini, Colao, Lenzi, & Giugliano, 2012; Goldberg & Mather, 2012; Kul et al., 2014; O'Neill & O'Driscoll, 2015; Rask-Madsen & Kahn, 2012; Samson & Garber, 2014).

The prevalence of MetS is high in women and men with significantly reduced estrogen action; however, the etiology of this phenomenon remains poorly defined (Carr, 2003; Gallagher et al., 2007; M. Jones, Chin Boon, Proietto, & Simpson, 2006; Lo et al., 2006; Park et al., 2003; Pu, Tan, Yu, & Wu, 2017). Animal models have provided valuable insight on underlying mechanisms that affect glucose and lipid homeostasis

when estrogen action is impaired. This review summarizes our current understanding of how alterations in estrogen action influences the development of defining features of MetS, obesity and insulin resistance.

Excess Adiposity and Insulin Resistance in Women and Men with Reduced Estrogen Action

Reduced estrogen action occurs with natural menopause, surgical menopause, aromatase deficiency, and estrogen receptor polymorphisms, and these are all associated with obesity and insulin resistance.

Natural Menopause

Analyses of the National Health and Nutrition Examination Survey (NHANES) 1988-1994 data on US adults have revealed that postmenopausal status is a risk factor for MetS, independent from age, ethnicity, body mass index (BMI), smoking, drinking, physical activity level, and household income (Carr, 2003; Park et al., 2003)**.** Natural menopause is characterized by ovarian senescence and the absence of menstruation for ≥12 months. Circulating estrogen levels decline after menopause, which typically occurs when women reach ~51 years of age (Gold, 2011). The most recent NHANES 2007-2012 data implies that the relationship between natural menopause and MetS continues to exist, wherein the prevalence of MetS is ~5-10% in 18-29 year old women, ~35-55% in the 50-69 year age group, and ~45-70% in women over age 70, regardless of race and ethnicity (J. Moore et al., 2017). Cross-sectional studies from different countries (e.g. Korea (Kim, Park, Ryu, & Kim, 2007),

India (Dasgupta et al., 2012), Nepal (Sapkota, Sapkota, Acharya, Raut, & Jha, 2015), Iran (Eshtiaghi, Esteghamati, & Nakhjavani, 2010; Maharlouei, Bellissimo, Ahmadi, & Lankarani, 2013), Brazil (Marchi et al., 2017), China (Chang et al., 2000), French (Trémollières, Pouilles, Cauneille, & Ribot, 1999), Taiwan (W. Y. Lin et al., 2006)) have also similarly documented that MetS is more prevalent in postmenopausal than pre-menopausal women, even after adjusting for age. Longitudinal studies have further supported the notion that the development of MetS accelerates after menopause (Janssen, Powell, Crawford, Lasley, & Sutton-tyrrell, 2008; Lovejoy, Champagne, de Jonge, Xie, & Smith, 2008; Macdonald, New, Campbell, & Reid, 2003). Data from a 4-year longitudinal study on premenopausal women (mean age of 43) have revealed that visceral fat, which is the adipose depot most associated with MetS development, significantly increases when women reach menopause (Lovejoy et al., 2008). Furthermore, these studies have also reported that abdominal obesity (as indicated by high waist circumference or body mass index) and insulin resistance (as indicated by high fasting blood glucose and hyperinsulinemia) significantly increases the risk of MetS development (Chang et al., 2000; Dasgupta et al., 2012; Ervin, 2009; Gaspard, 2009; Lovre & Mauvais-Jarvis, 2015; Park et al., 2003). However, some studies have documented that decreases in physical activity levels, resting energy expenditure, and age confound the association between postmenopausal status and MetS (Lovejoy et al., 2008; Macdonald et al., 2003; Pandey et al., 2010). Nonetheless, the overwhelmingly high prevalence of obesity and insulin resistance in postmenopausal women, has led researchers to postulate that the reduced ovarian production of estrogens triggers the onset of metabolic dysfunction (Clegg, 2012).

Surgically-Induced Menopause

According to a recent meta-analysis, the risk of developing MetS is ~1.5 fold higher with surgical menopause than natural menopause (Pu et al., 2017)**.** Bilateral oophorectomy or the surgical removal of the ovaries abruptly leads to menopause due to the significant reduction in ovarian hormone production (Hendrix, 2005). This procedure is typically performed in cases where surgery is the best option to prevent ovarian and breast cancer, and to alleviate severe endometriosis (Evans et al., 2016; Greene et al., 2016; Hendrix, 2005). Oophorectomy is often performed together with a hysterectomy (Evans et al., 2016; B. J. Moore, Steiner, Davis, Stocks, & Barrett, 2016). Annually, over ~200,000 women in the US have their ovaries removed (B. J. Moore et al., 2016). Oophorectomy reduces ovarian cancer risk by up to 80% and breast cancer risk by up to 50% in premenopausal women with breast cancer type susceptibility gene (BRCA) mutations (Marchetti et al., 2014; Rebbeck, Kauff, & Domchek, 2009). Indeed, cancer is linked to increased mutations that arise from the proliferative and genotoxic effects of estrogens (Mungenast & Thalhammer, 2014; Russo & Russo, 2004; E. Simpson & Santen, 2015). However, oophorectomy has its own consequences. The development of MetS is a major health concern with this procedure, along with osteoporosis and vasomotor symptoms (hot flashes, vaginal dryness, etc.). Indeed, data from 3549 women in the NHANES 1988-1994 study have revealed that women, who have undergone oophorectomy before menopause (<40 years old), display greater waist circumference, skinfold thickness, and percent body fat than age-matched women with intact ovaries (A. M. McCarthy, Menke, & Visvanathan, 2013). A longitudinal study has also shown that hyperglycemia is greater with surgical

menopause compared to natural menopause (Farahmand et al., 2014). Incident diabetes is also higher in women, who have undergone oophorectomy, than women with intact ovaries (Appiah, Winters, & Hornung, 2014). Obesity increases the risk of developing metabolic dysfunction associated with oophorectomy (A. M. McCarthy, Menke, Ouyang, & Visvanathan, 2012). Moreover, the risk of all-cause mortality in obese women is higher when oophorectomy is performed before rather than after the age of 40 (A. M. McCarthy et al., 2012). Collectively, the increased incidence of MetS associated with oophorectomy further supports the concept that the abrupt reduction of estrogen action is strongly associated with the development of obesity and insulin resistance.

Aromatase Deficiency

Case studies on aromatase deficient men and women suggest that aromatase deficiency is also associated with metabolic dysfunction (M. Jones et al., 2006)**.** Aromatase, encoded by the CYP19A1 gene, is the enzyme that converts androgens to estrogens; therefore, genetic mutations of the CYP19A1 gene lead to estrogen deficiency. In contrast to natural or surgical menopause, reduced estrogen action due to aromatase deficiency is rare. However, the incidence of MetS is significantly high in aromatase deficient men (Carani et al., 1997; Z. Chen et al., 2015; Herrmann et al., 2002; M. Jones et al., 2006; Maffei et al., 2004; Morishima, Grumbach, Simpson, Fisher, & Qin, 1995). These case studies have reported increased abdominal adiposity, BMI (25-30 kg/m^2), circulating triglycerides (~305mg/dl), fasting blood glucose (~180mg/dl), and the homeostasis model assessment of insulin resistance (HOMA-IR=3.6, normally <2.4) (Carani et al., 1997; M. Jones et al., 2006). Aromatase deficiency

is also associated with Type 2 Diabetes and hepatic steatosis (Maffei et al., 2004). In addition, the onset of MetS is evident at a young age (mid-20s) in men, who have the CYP19 mutation (Carani et al., 1997; Morishima et al., 1995). Case studies, which have documented MetS in women with aromatase deficiency, are even more uncommon. Due to genital abnormalities and health complications since infancy, these women with the CYP19A1 mutation undergo surgeries and hormone therapy at an early age (as early as 22 months of age) (Morishima et al., 1995). These are performed to restore reproductive function and prevent the recurrence of ovarian cysts (Morishima et al., 1995). Thus, early treatments to normalize hormone levels may have protected these women from MetS. However, a few case studies have documented that women diagnosed with aromatase deficiency and treated at a later age exhibit MetS (Gagliardi, Scott, Feng, & Torpy, 2014; Guercio et al., 2009). For example, a CYP19A1 mutation has been found in a 25yr old woman with central obesity (waist circumference of 131cm), high fasting blood glucose (6.1mmol/L), high fasting insulin (57mU/L, normally <12mU/L), and impaired glucose tolerance (Gagliardi et al., 2014). Collectively, the high incidence of increased adiposity and insulin resistance in men and women with aromatase deficiency suggests that estrogens may have important metabolic functions in both sexes.

Estrogen Receptor Mutations and Polymorphisms

Men and women with reduced estrogen action due to certain estrogen receptor (ER) mutations and polymorphisms also have an increased risk of excess adiposity and insulin resistance. Estrogens exert their functions by initially binding to estrogen receptors. A case study in 1994 on a 28-year old man with a single

base pair mutation (cytosine-to-thymine transition) in the second exon (that results in a protein with no DNA- and ligand-binding domains) is the first to document an association between disrupted ER function and insulin resistance in a human (Smith et al., 1994). This man presented with moderately high fasting blood glucose levels (135mg/dl) and impaired glucose tolerance, despite having high insulin levels and high endogenous estradiol concentrations (Smith et al., 1994). By 2006, single nucleotide polymorphisms (SNPs) of the estrogen receptor-1 (ESR1) and -2 (ESR2) genes have been linked to MetS (Lo et al., 2006). For instance, *ESR1 rs3798577 TT* is associated with low insulin sensitivity in US-based Chinese women, and *ESR2 rs1255998 CC* and *ESR2 rs1256030 CC* are associated with MetS in US-based Chinese and Japanese women, respectively (Lo et al., 2006). These specific SNPs are not associated with MetS in Caucasian and African American women (Lo et al., 2006). However, many other SNPs within the intron 1-intron 2 regions of ESR1 are associated with Type 2 Diabetes, MetS, and each of the components of MetS in African American men and women (Gallagher et al., 2007). Another study has shown that presenting with 3 or more polymorphic alleles is associated with lower human chorionic gonadotropin-induced estradiol production (Anagnostou et al., 2013). One of the most studied ESR1 SNPs located in intron 1 is the *c.454-351 A>G (rs9340799)*, and is associated with MetS in African Americans (Gallagher et al., 2007). Moreover, the G allele of *ESR1 rs9340799* is associated with the presence of all components of MetS in premenopausal Egyptian women (Ghattas, Mehanna, Mesbah, & Abo-Elmatty, 2013) as well as total lipids and triglycerides in postmenopausal Brazilian women (Gomes-Rochette et al., 2017). The minor C allele of another well-characterized ESR1 SNP in intron 1, *c.454-*

397 T>C (rs2234693), is also associated with components of MetS in Egyptian women, except for waist circumference (Ghattas et al., 2013). Moreover, the C allele of *rs2234693* is associated with high BMI in postmenopausal Caucasian women (Deng et al., 2000). Other pathologic conditions that are associated with *rs9340799* and *rs2234693* polymorphisms of ESR1 include infertility (Ayvaz, Ekmekçi, Baltacı, Önen, & Ünsal, 2009), breast cancer (Zhang et al., 2015), and cardiovascular disorders (Schuit et al., 2004; Shearman et al., 2003). Notably, these studies suggest that the relationship between particular ER polymorphisms and cellular dysfunction may be specific to one's sex and/or ethnicity.

Mechanisms that Link Obesity and Skeletal Muscle Insulin Resistance

Insulin is a hormone secreted by the β cells of pancreas in response to changes in blood glucose levels. The major metabolic functions of insulin include the uptake of glucose in skeletal muscle, suppression of glucose production in the liver, and inhibition of adipose tissue lipolysis. In skeletal muscle, insulin binds to the insulin receptor on the sarcolemma, which triggers the phosphorylation of tyrosine residues on the β-subunit of the insulin receptor. The insulin receptor substrate-1 (IRS-1) subsequently moves to the sarcolemma, binding to the insulin receptor, which leads to the activation of the p85 regulatory subunit and p110 catalytic subunit of phosphatidylinositol-3 (PI-3) kinase, and the increase in phosphatidylinositol-3,4,5 triphosphate. Subsequent docking of phosphoinositide-dependent protein kinase-1 (PDPK1) and protein kinase B (Akt) on the sarcolemma occurs. PDPK1 phosphorylates Akt, which then inactivates the Akt

substrate of 160kDa (AS160). In turn, this inactivates other GTPases involved in retaining glucose transporter-4 (GLUT4) in the cytosol. Once GLUT4 docks on the cell membrane, glucose enters the cell by facilitated diffusion. Normally, approximately 80-90% of insulin-stimulated glucose uptake takes place in skeletal muscle while 5-10% occurs in adipose tissue (Thiebaud et al., 1982).

Insulin resistance is defined as a condition wherein target tissues do not respond normally to insulin. As a result, blood glucose levels remain elevated in women and men with normal pancreatic beta cell function. Skeletal muscle insulin resistance, characterized by reductions in muscle glucose uptake in the presence of insulin, primarily affects the progression to Type 2 Diabetes Mellitus (DeFronzo & Tripathy, 2009). Although insulin resistance develops with age (Halter et al., 2014), obesity due to excess caloric intake and physical inactivity significantly increases one's susceptibility to impaired insulin sensitivity (O'Neill & O'Driscoll, 2015).

This section will review mechanisms that have shed light to the link between excess adiposity and skeletal muscle insulin resistance. Three different mechanisms have been proposed: lipotoxicity, inflammation, and alterations in mitochondrial respiration and redox homeostasis (de Luca & Olefsky, 2008; Fisher-Wellman & Neufer, 2012; Glass & Olefsky, 2012; Kitessa & Abeywardena, 2016; Muoio, 2014; Samuel, Petersen, & Shulman, 2010; Samuel & Shulman, 2016).

Lipotoxicity

Excess adiposity, which increases intramuscular lipid accumulation, is associated with impaired insulin signaling. The skeletal muscle can store a substantial amount of lipids, which functions as a fuel reserve (Schrauwen-Hinderling et

al., 2003). Intramyocellular lipids (IMCLs) are mostly composed of triacylglycerol (TAG), but also contain diacylglycerol (DAG), sphingolipids (e.g. ceramides), cholesterol esters, and free cholesterol. Energetic demand causes IMCLs to be oxidized in the mitochondria to produce ATP, the primary fuel for muscle contraction. IMCLs are more abundant in oxidative (Type 1) fibers than glycolytic (Type 2x/2b) fibers, and are attributed to the oxidative fiber's greater capacity to oxidize and preferentially utilize fat as the substrate for ATP generation (Schrauwen-Hinderling, Hesselink, Schrauwen, & Kooi, 2006). Accordingly, increased IMCL accumulation is beneficial to the sport performance of healthy, insulin sensitive endurance athletes (Coen & Goodpaster, 2012). However, excess IMCL deposition also occurs in non-athletic, overweight/ obese individuals and is associated with the development of insulin resistance (Amati, 2012). IMCL accumulation is thought to occur due to increased free fatty acid mobilization from adipose tissue to skeletal muscle in the absence of increased fat utilization (Blaak, 2003). Studies on lipid-induced insulin resistance have documented that fatty acid re-esterification into bioactive lipid intermediates – diacylglycerol (DAG) and ceramide – activate inflammatory or stress signaling complexes that decrease the activity of the insulin signaling pathway in skeletal muscle, consequently decreasing glucose uptake (Kitessa & Abeywardena, 2016). For example, previous studies have observed that DAG activates different isoforms of protein kinase C-θ, βII, δ (Itani, Ruderman, Schmieder, & Boden, 2002; M. Li, Vienberg, Bezy, O'Neill, & Kahn, 2015), which activates c-Jun N-terminal kinase (JNK). JNK suppresses insulin signaling by phosphorylating IRS-1 at the serine site (Ser307), and results in impaired GLUT4 translocation towards the cell membrane and facilitation of glucose entry into the cell

(Aguirre, Uchida, Yenush, Davis, & White, 2000). Thus, glucose uptake is impaired in the presence of accumulated DAGs in skeletal muscle. Specific DAG species such as C16:0, C18:0, C18:1, C18:2, C20:4 DAGs are found to more strongly associate with insulin resistance (Szendroedi et al., 2014). Greater ceramide accumulation is also observed in C2C12 myotubes pre-treated with palmitate than untreated myotubes, which coincides with lower insulin-stimulated phosphorylation of Akt, lower basal and insulin-stimulated glucose uptake, lower phosphorylation of glycogen synthase kinase-3, and lower glycogen synthesis (Schmitz-Peiffer, Craig, & Biden, 1999). One study has attributed the ceramide-associated reduction of phosphorylated Akt to the increased activity of a stress signaling kinase, protein kinase C-ζ (Hajduch et al., 2008). Dexamethasone treatment, which is known to increase ceramide accumulation, also leads to reductions in muscle glucose uptake (Holland et al., 2007). Conversely, myriocin treatment, a known inhibitor of ceramide synthesis, protects mice and rats from developing skeletal muscle insulin resistance (Holland et al., 2007; Kurek et al., 2015). Similar to DAG, measuring total ceramide does not always associate with insulin resistance. Specific long chain ceramide species such as C18:0 (Bergman et al., 2016), C20:1 and C22:0 ceramides (de la Maza et al., 2015) are reported to more strongly associate with a decline in insulin sensitivity. More studies are needed to define how DAG and ceramide species directly induce insulin resistance.

Inflammation

Lipids induce skeletal muscle inflammation and is associated with insulin resistance. Myocytes isolated from obese insulin resistant individuals exhibit increased tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1, also known as chemokine (C-C motif) ligand 2, CCL2) expression (Saghizadeh, Ong, Garvey, Henry, & Kern, 1996; Wu & Ballantyne, 2017). In turn, TNF-α and MCP-1 are thought to induce increased migration of immune cells that originate from lipid depots surrounding skeletal muscle, termed intermuscular and perimuscular adipose tissues (Wu & Ballantyne, 2017). These depots are reported to contain greater concentrations of pro-inflammatory factors compared to skeletal muscle (Khan et al., 2015; Wu & Ballantyne, 2017). Specifically, macrophage and T-cell infiltration in skeletal muscle are increased in non-diabetic obese insulin resistant and Type 2 diabetic individuals (Khan et al., 2015; Wu & Ballantyne, 2017). M1-macrophages are present in skeletal muscle, as indicated by the increase in interferon gamma (IFN-γ) expressing T-helper-1 (Th1) cells that have previously been shown to promote the M1-like phenotype (Khan et al., 2015; Wu & Ballantyne, 2017). Increased M1-macrophages are consistently documented in adipose tissue during obesity and secrete pro-inflammatory cytokines such as TNF-α (Nguyen et al., 2007). Indeed, previous studies have demonstrated that overexpression of MCP-1 as well as incubation of myocytes with long chain saturated fatty acids increases M1-macrophages in skeletal muscle (Nguyen et al., 2007; Patsouris et al., 2014). Several studies have also shown that in myocytes, TNF-α activates the inhibitor of nuclear factor kappa-B kinase (IKK) complex, which leads to the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB),

and in turn, the development of insulin resistance (De Alvaro, Teruel, Hernandez, & Lorenzo, 2004; Khan et al., 2015; Nguyen et al., 2007; Saghizadeh et al., 1996). Skeletal muscle inflammation coincides with increased serine phosphorylation of IRS-1 (Rui et al., 2001), and reduced muscle glucose uptake (Wu & Ballantyne, 2017). Other studies have documented that the lipid-induced increase in TNF-α also activates JNK in skeletal muscle (Wu & Ballantyne, 2017). As eluded to earlier, previous studies have observed that JNK activity is elevated in skeletal muscle of obese individuals (Pal, Febbraio, & Lancaster, 2016) and in myocytes incubated with palmitate (Senn, 2006). JNK activity is associated with lipid-insulin resistance for JNKs impair insulin signaling by phosphorylating serine and threonine residues of IRS-1, resulting in reduced insulin action (Aguirre et al., 2000).

Alterations in Skeletal Muscle Mitochondrial Respiration and Redox Homeostasis

Alterations in skeletal muscle mitochondrial function are also linked to lipid-induced insulin resistance. Some studies have observed that mitochondrial respiratory capacity, mitochondrial content, and expression of genes necessary for oxidative metabolism are lower in skeletal muscle of obese insulin resistant compared to healthy individuals (Lowell & Shulman, 2005; Mogensen et al., 2007)**.** Others have found that skeletal muscle mitochondria of young adult, obese insulin resistant men exhibit increased H_2O_2 emission potential (E. J. Anderson et al., 2009; Fisher-Wellman et al., 2014). Whether insulin resistance develops due to inherent mitochondrial dysfunction that leads to IMCL accumulation or due to chronic overnutrition that leads to increased H_2O_2 and altered redox homeostasis is still under debate.

Researchers have hypothesized that the onset of lipid-induced insulin resistance occurs with the inability of the mitochondria to cope with the presence of excess lipids, thus exhibiting an inflexibility to completely oxidize fat (Lowell & Shulman, 2005)**.** Mitochondrial content and activity of key mitochondrial proteins are thought to be altered, reducing mitochondrial function (Lowell & Shulman, 2005; Morino, Petersen, & Shulman, 2006). Specifically, a reduction in mitochondrial respiratory capacity may cause the accumulation of lipid intermediates, which activates the cascade of events leading to impaired skeletal muscle glucose uptake (Morino et al., 2006). For example, transport of mobilized free fatty acid from adipose tissue to skeletal muscle results in IMCL build up that is associated with reduced mitochondrial fat oxidation (Boyle, Zheng, Anderson, Neufer, & Houmard, 2012). In human permeabilized myotubes, state 3-supported mitochondrial respiration in the presence of a lipid substrate is significantly lower in the obese group compared to the lean group (Boyle et al., 2012). However, while there is substantial evidence that excess IMCL is associated to the development of insulin resistance, this is not always accompanied by reductions in the capacity of mitochondria to oxidize fat. Previous works have demonstrated that permeabilized skeletal muscle fibers of obese insulin resistant adults and high fat diet-fed rodents with insulin resistance exhibit normal mitochondrial respiratory capacity (E. J. Anderson et al., 2009; Fisher-Wellman et al., 2014). In addition, certain mouse models, which can have reduced mitochondrial respiration, can exhibit normal glucose tolerance (Jackson et al., 2018).

Alternatively, excess nutritional load can induce chronic elevations in skeletal muscle mitochondrial H_2O_2 production, thus altering the redox

environment and initiating the onset of insulin resistance (Fisher-Wellman & Neufer, 2012)**.** Here, the electron transport system of the mitochondria receives excess hydrogen from reducing equivalents NADH and $FADH_2$. As more hydrogen protons build-up in the intermembrane space, creating back pressure, the mitochondria relieves this pressure by generating superoxide (Fisher-Wellman & Neufer, 2012). Superoxide dismutases rapidly convert superoxide into H_2O_2 (Schieber & Chandel, 2014). Excess H_2O_2 can alter redox biology by oxidizing thiolate anions of cysteine (Schieber & Chandel, 2014). In turn, allosteric changes in proteins occur, modifying function or damaging proteins. The glutathione and thioredoxin redox buffering systems that scavenge H_2O_2 are also affected, which further contributes to the increasingly oxidized cellular environment (Fisher-Wellman & Neufer, 2012). Recent studies have demonstrated that skeletal muscle mitochondria of mice, which exhibit insulin resistance as a result of high fat diet exposure, display a lower reduced/oxidized glutathione ratio (Fisher-Wellman et al., 2016). The lower ratio indicates a reduction in the buffering capacity, which promotes prolonged exposure to elevated intracellular H_2O_2 (E. J. Anderson et al., 2009). Importantly, H_2O_2 emission potential is higher in these mice, but mitochondrial respiratory capacity is normal (E. J. Anderson et al., 2009; Fisher-Wellman et al., 2014). The importance of H_2O_2 is confirmed when overexpressing catalase (the enzyme responsible for converting H_2O_2 to H_2O) targeted to the mitochondria protects mice (MCAT mice) from high fat diet-induced insulin resistance (E. J. Anderson et al., 2009; Ryan et al., 2016). Previous works have demonstrated that elevations in H_2O_2 oxidize phosphatases, inactivating phosphatase function in inhibiting kinase activity (Fisher-Wellman & Neufer, 2012; Schieber & Chandel, 2014).

Subsequently, stress sensitive kinases are activated, which phosphorylate insulin signaling molecules, like IRS-1, at the serine site, reducing the activity of the insulin signaling cascade (Fisher-Wellman & Neufer, 2012). These data support the model where mitochondrial H_2O_2 links excess adiposity to insulin resistance by impairing insulin signaling. However, in contrast to this model, a recent study has demonstrated that MCAT mice are not protected from skeletal muscle insulin resistance induced by an acute lipid infusion (H. Y. Lee et al., 2017). The authors of the study have concluded that in some circumstances, insulin resistance occurs in the absence of elevated H_2O_2.

Collectively, the etiology of lipid-induced insulin resistance is complex such that the underlying mechanisms: lipotoxicity, inflammation, and alterations in mitochondrial function and redox biology appear to be intertwined. Nonetheless, estrogens interestingly influence each of these mechanisms. The remaining sections of this review will discuss how estrogens have critical roles in regulating glucose and lipid homeostasis as well as mitochondrial function.

Estrogens' Protective Role against Obesity and Insulin Resistance

Historically, ovarian extracts were used as early as the 1900s to alleviate menopausal symptoms due to the association between such vasomotor symptoms and bilateral removal of the ovaries (E. Simpson & Santen, 2015). Shortly after the discovery and isolation of estradiol in 1940 (E. Simpson & Santen, 2015), a pharmaceutical company, Wyeth, synthesized a complex of conjugated estrogens (CEE), named

Premarin, which was isolated from urine of pregnant mares (E. Simpson & Santen, 2015; Vance, 2007). Premarin, which mostly constituted estrone sulfate and 10 other weak estrogen agonists (Pinkerton et al., 2017), became the main hormone therapy (HT) used to treat menopausal symptoms for many years in Canada and US until the 1970s (Vance, 2007). Later on, CEE was also prepared in combination with synthetic forms of progesterone (e.g. medroxyprogesterone acetate) (CEE + P) (Pinkerton et al., 2017). CEE and estradiol alleviated vasomotor symptoms to a similar extent (Pinkerton, Abraham, Bushmakin, Cappelleri, & Komm, 2016).

The role of estrogens in maintaining metabolic homeostasis is documented in numerous reports that associate HTs with decreased adiposity (Ahtiainen et al., 2012; Macdonald et al., 2003; Munoz, Derstine, & Gower, 2002; Pu et al., 2017; Salpeter et al., 2006; Sites et al., 2001) **and increased insulin sensitivity** (Ahtiainen et al., 2012; Brussaard et al., 1997; Demir et al., 2008; Munoz et al., 2002)**.** A meta-analysis of 107 trials in 2006 has revealed that HT lowers body fat percentage and HOMA-IR in postmenopausal women (Salpeter et al., 2006). HT also lowers HOMA-IR and improves the serum lipid profile in postmenopausal women with diabetes (Salpeter et al., 2006). More recently, a meta-analysis in 2017 has also revealed that 17β-estradiol (E2) treatment lasting ≥3 months lowers total triglyceride levels, and CEE is beneficial in improving circulating lipoprotein profiles in postmenopausal women (Pu et al., 2017). The following randomized control trials provide strong evidence for the beneficial effects of HT on glucose and lipid metabolism. First, results of the 10-year follow-up on over ~48000 postmenopausal women in the 1976 Nurses' Health Study show HT users to more likely have undergone oophorectomy, be leaner, and less likely

to be diabetic compared to non-users (Stampfer et al., 1991). Second, data from the 1987 Postmenopausal Estrogen/Progestin Intervention (PEPI) Trial on 875 healthy postmenopausal women (45-64 years old) have also revealed that CEE and CEE + P improve fasting glucose levels (Miller et al., 1995). Third, the Women's Health Initiative (WHI), the largest randomized control trial on HT (n= ~160,000 postmenopausal women), has documented that HT (CEE and CEE + P) lowers the incidence of Type 2 Diabetes (Manson et al., 2013). Moreover, a 7-year longitudinal study on 907 women transitioning to their postmenopausal years has shown that HT (CEE and CEE + P) users exhibit lower BMI and body weight than non-users (Macdonald et al., 2003). The only study on monozygotic twins discordant for estrogen therapy has also observed that long term estrogen therapy (CEE and CEE + P) users (~7.5 years, range 2-16 years) display lower adiposity, lower fasting serum glucose levels, and lower glycated hemoglobin (HbA1c) levels than their identical co-twin non-users (Ahtiainen et al., 2012). Estrogen pills also lower body fat in women who have undergone oophorectomy (A. M. McCarthy et al., 2013), and transdermal estradiol lowers triglyceride levels, fat accumulation in the liver, fasting glucose and insulin levels in aromatase deficient men (Carani et al., 1997; Maffei et al., 2004). Overall, these studies provide evidence for the notion that raising estrogens to physiological levels can ameliorate the detrimental effects of natural and surgical menopause as well as aromatase deficiency on adiposity and insulin sensitivity.

Risks of Hormone Therapy

Previous observational and randomized control trial studies have revealed that HT increases cancer and cardiovascular disease risk, making HT a controversial form of treatment. Studies in the 1970s have reported that Premarin (CEE) increases the risk of endometrial cancer, especially with long term use (≥7 years) (Ziel & Finkle, 1975). Low and high doses of unopposed CEE also increase the risk of simple or complex endometrial hyperplasia (Furness et al., 2012; Herrington et al., 2000), which may in turn, lead to endometrial cancer. CEE + P (e.g. Prempro) is currently regarded as an equally effective but safer drug in alleviating vasomotor symptoms, as it diminishes the risk of endometrial hyperplasia and cancer (Furness et al., 2012; Vance, 2007). Data from the 10-year follow-up on the 1976 Nurses' Health Study have shown that very high doses of HT (i.e. 1.25mg/day) increase the risk of coronary heart disease (Stampfer et al., 1991). Notably, ~71% of ~48,000 women in the Nurses' Health Study used unopposed CEE, only ~3% were on CEE + P, and only ~4% of the cohort were prescribed a dosage of ≥1.25mg/day (Stampfer et al., 1991). The study that convinced the United States of America to significantly decrease the use of HT is the only large-scale randomized control trial, called the Women's Health Initiative (WHI) (Vance, 2007). The WHI has revealed that unopposed CEE is associated with a higher incidence of stroke (HR, 1.39; 95% CI, 1.10-1.77) and total cardiovascular disease (arterial and venous) (HR, 1.12; 95% CI 1.01-1.24) in women who have undergone a hysterectomy (G. Anderson et al., 2004). Moreover, CEE + P is associated with the increased incidence of coronary heart disease (HR, 1.29; 95% CI, 1.02-1.63),

stroke (HR, 1.41; 95% CI, 1.07-1.85), pulmonary embolism (HR, 2.13; 95% CI, 1.39-3.25), and breast cancer (HR, 1.26; 95% CI, 1.00-1.59) in postmenopausal women with an intact uterus (Rossouw et al., 2002). These findings have led to the untimely end of the WHI for the risks outweighed the beneficial reduction in the incidence of hip fractures and Type 2 Diabetes (G. Anderson et al., 2004; Manson et al., 2013; Rossouw et al., 2002). Since then, the deliberation on whether or not HT should still be prescribed continues to this day. Substantial benefits of HT on adiposity and insulin sensitivity may outweigh the risks, but this depends on the individual's age, number of years past the onset of menopause, and the presence of other health contraindications (Pinkerton et al., 2017). More recent studies, which re-analyzed the WHI data, have reported that healthy women within 10 years of menopause and younger than 60 years of age can safely use HT (Pinkerton et al., 2017). The data suggest that the incidence of myocardial infarction, total cancer (all types), and all-cause mortality is lower in the CEE and CEE + P groups than the placebo group in women who are 50-59 years of age (Manson et al., 2013). The absolute risk of adverse events with CEE and CEE + P is also lower in 50-59 year old women than 70-79 year old women (Manson et al., 2013). Finally, the risk of coronary heart disease is not significantly different between CEE or CEE + P and placebo users in 50-59 year old women whereas the excess risk is high in 70-79 year old women with moderate to severe vasomotor symptoms (CEE HR, 4.34; 95% CI, 1.43-13.14 and CEE + P HR, 5.79; 95% CI, 1.29-25.97) (Manson et al., 2013). The most recent Position Statement of the North American Menopause Society in 2017 highlight that HT prescription must be individualized (Pinkerton et al., 2017). Overall, HT provides beneficial effects on metabolic health. However, the type, dose, and duration

of HT in conjunction with age, postmenopausal status, and medical history must all be considered in order to minimize the risks associated with HT (Pinkerton et al., 2017).

The challenge today is to develop safer therapies that target specific tissues involved in the regulation of adiposity and insulin sensitivity in men and women with reduced estrogen action. The goal is to prevent accelerated adiposity and insulin resistance without increasing the risk for breast cancer, endometrial cancer, and cardiovascular disease. Thus, deciphering estrogens' mechanisms of action in regulating glucose and lipid metabolism in insulin sensitive tissues is critical in advancing the field.

Estrogens' Mechanism of Action

Estrogens are a class of steroid hormones. The 3 main forms of endogenous estrogens in humans are estrone (E1), 17β-estradiol (E2), and estriol (E3). Two scientists, Edward Doisy and Adolf Butenandt, independently but simultaneously, purified estrone from urine of pregnant women in 1929 (E. Simpson & Santen, 2015). Later on, Doisy purified estriol in 1931 and estradiol in 1940 (E. Simpson & Santen, 2015). The Nobel Prize was given to Butenandt in 1939 for his discovery of estrone along with testosterone while Doisy was awarded his Nobel Prize in 1943, but for the purification of vitamin K (E. Simpson & Santen, 2015). E2 is the predominant and most potent form of endogenous estrogen (Kuhl, 2005). Estrogens, from both gonadal and extragonadal sites, exert their effects through genomic and non-genomic signaling (Cui, Shen, & Li, 2013).

Estrogens are primarily synthesized in the ovaries, and their production periodically rises and falls as part of the menstrual cycle. Menstrual cyclicity begins at ~12.4 years old (Chumlea et al., 2003) and ends at the age of ~51 years old (Greendale, Lee, & Arriola, 1999). The menstrual cycle is typically 28 days long and is divided into menstrual phases (Reed & Carr, 2015). Granulosa cells in the ovary synthesize estrogens by aromatase cytochrome P450 action, which converts circulating C19 androgens, testosterone and androstenedione, to estrogens (N. G. Anderson & Lieberman, 1980; Labrie, 2015; E. R. Simpson, 2003). Estrogens released into circulation are low during the early follicular phase (days 1-7) and progressively increase during the late follicular phase as the size of the follicle increases (days 9-14) (Reed & Carr, 2015). Ovulation typically occurs on day 14 and the succeeding days after ovulation are part of the luteal phase. During the mid-luteal phase (days 20-25), circulating estrogen levels remain higher than their concentrations during the early follicular phase and then eventually decline on days 26-28 when the egg is not fertilized (Reed & Carr, 2015). This menstrual cycle keeps occurring until the last menstrual period (often referred to as menopause). This postmenopausal stage in a woman's life occurs naturally and is characterized by senescent ovaries that are no longer able to produce estrogens (E. R. Simpson, 2003). In contrast to humans, female rodents experience the estrous cycle beginning at ~4-6 weeks of age, which lasts 4-5 days (Nelson, Felicio, Randall, Sims, & Finch, 1982). In a typical estrous cycle, circulating estrogen levels peak during proestrus, declines during estrus, remains low during metestrus, and once again increases during diestrus (Walmer, Wrona, Hughes, &

Nelson, 1992). Cyclicity ends at ~18 months of age; however, ovary secretions cease in mice around ~24 months of age (Mobbs, Gee, & Finch, 1984).

Men and postmenopausal women produce estrogens in extragonadal tissues. Estrogens are also synthesized in the mesenchymal cells of adipose tissue, breast, osteoblasts and chondrocytes of bone, the vascular endothelium, and aortic smooth muscle cells, brain, liver, adrenal glands, and skeletal muscle (Barakat, Oakley, Kim, Jin, & Ko, 2016; Longcope, Pratt, Schneider, & Fineberg, 1978; E. R. Simpson, 2003). The estrogen concentrations produced from these tissues and cells are considerably lower compared to ovarian production of estrogens; however, studies have demonstrated that extragonadal estrogens exert important paracrine and intracrine effects (E. R. Simpson, 2003). For instance, some suggest that the development of postmenopausal breast cancer is more tightly associated with the estradiol concentrations in breast tissue than the low levels of circulating estrogens (E. R. Simpson, 2003).

In genomic signaling, estrogens regulate the expression of target genes by binding to estrogen receptors-alpha (ERα) and beta (ERβ), which subsequently enable the receptors to bind to the target DNA. ERα and ERβ are two different receptors. The gene for ERα is ESR1, and is located on chromosome 6q25.1 in humans (Menasce, White, Harrison, & Boyle, 1993) and chromosome 10 in mice (Sluyser, Rijkers, de Goeij, Parker, & Hilkens, 1988). On the other hand, the gene for ERβ is ESR2 and is located on chromosome 14q22-24 in humans (Enmark et al., 1997) and chromosome 12 in mice (Tremblay et al., 1997). They are primarily known as nuclear receptors that act as transcription factors and signal transducers (Murphy, 2011). These

receptors contain 6 functional domains (A-F), similar to other members of the superfamily of nuclear receptors (Ruff, Gangloff, Marie Wurtz, & Moras, 2000). ERs have an A/B domain at the N-terminal region that has an activation function-1 (Ruff et al., 2000). ERα and ERβ have relatively different A/B domains (only 17% homology) (Enmark et al., 1997). The C domain contains the DNA-binding region, which is highly conserved between ERα and ERβ (97% homology) (Enmark et al., 1997). The D domain links the C to the E domain, which is the ligand-binding domain and contains an activation function-2 (Ruff et al., 2000). The ligand-binding domains of ERα and ERβ are ~59% homologous (Enmark et al., 1997). Lastly, the F domain is located at the C-terminal region and serves as an extension of the ligand binding domain (Ruff et al., 2000). Estrogens are bound to carriers in circulation (sex hormone binding globulin and albumin) and are lipophilic, allowing the spontaneous dissociation from the carriers and passive diffusion into the cell. Genomic signaling initially begins with the dissociation of chaperone proteins (heat shock protein-90, heat shock protein-70, and other proteins) from the ERs in the cytosol when estrogens enter the cell (Oren, Fleishman, Kessel, & Ben-Tal, 2004). Estrogens bind to the ligand-binding domain of ERs, which trigger the translocation of these estrogen-ER complexes to the nucleus (Raam et al., 1983). Once in the nucleus, the DNA-binding domain of ER binds to the estrogen response element (ERE) within the promoter region of the target gene, modulating gene expression (Driscoll et al., 1998; Klinge, 2001). Indeed, ERs mainly localize in the nucleus (Pugach et al., 2016; Yuchi et al., 2015). ERα is highly expressed in the uterus and ovaries, but are also found in the prostate stroma, testes, epididymis, breast, skeletal muscle, hypothalamus, pituitary, liver, pancreas, adipose, heart, bone, lungs, and blood vessels

(Baltgalvis et al., 2010; Couse et al., 1997; Drew et al., 2015; Khalid & Krum, 2016; H.-R. Lee et al., 2012; Pugach et al., 2016; Yuchi et al., 2015). ERβ is highly expressed in ovaries, but are also found in the prostate epithelium, testes, epididymis, bone marrow, hypothalamus, adipose, lungs, bone, and blood vessels (Couse et al., 1997; Dieudonné, Leneveu, Giudicelli, & Pecquery, 2004; Enmark et al., 1997; Khalid & Krum, 2016; H.-R. Lee et al., 2012). Furthermore, estrogens can also exert their effects on genes that do not have ERE. About 1/3 of genes that are influenced by estrogens do not have ERE (O'Lone, Frith, Karlsson, & Hansen, 2004). Here, the ER binds to transcription factors such as activator protein-1 (AP-1) and specificity protein-1 (SP-1), acting as cofactors (Safe & Kim, 2008). For example, ER/SP-1 plays a role in the differentiation, proliferation, and survival of breast cancer cells by regulating estrogen-dependent genes like insulin-like growth factor-binding protein-4 and Cathepsin D (Safe & Kim, 2004). ER/AP-1 also plays a role in breast cancer cell growth by regulating E2F transcription factor-1 and cAMP-dependent protein kinase inhibitor β (Dahlman-Wright et al., 2012; Safe & Kim, 2008). Other estrogen responsive genes that are regulated by ER through this indirect genomic signaling pathway include insulin like growth factor-1, Cyclin D1, low density lipoprotein receptor, and progesterone receptor (O'Lone et al., 2004).

ERs can also localize on the plasma membrane and exert the effects of estrogens through non-genomic signaling pathways, albeit there are less membrane ERs than nuclear ERs (Levin, 2009b; Razandi, Pedram, Greene, & Levin, 1999). These membrane ERs have been initially identified in the 1970s (Pietras & Szego, 1977; E. Simpson & Santen, 2015), but have been more recently confirmed by

experiments with estrogen-linked dendrimer conjugates (EDCs), which cannot enter the nucleus (Harrington et al., 2006), and mutations inhibiting palmitolyation (Acconcia, Ascenzi, Fabozzi, Visca, & Marino, 2004; Adlanmerini et al., 2014). For example, one study in mice has shown that reendothelialization after carotid injury is similar between EDC and estradiol treatment (Adlanmerini et al., 2014). Previous studies have also demonstrated that palmitoylation is required for ER localization on the membrane (Acconcia et al., 2004; Adlanmerini et al., 2014). Upon estrogen stimulation, ERs can activate rapid signaling pathways, such as mitogen-activated protein kinase (MAPK), phosphoinositide-3 kinase/protein kinase B (PI3-K/Akt), and Gαi coupled protein signaling (E. Simpson & Santen, 2015). For instance, in breast cancer cells, estrogen binding to membrane-bound ERα can trigger the subsequent binding of ERα to MAPK (Song et al., 2002). Upon MAPK activation, ERα-MAPK translocates to the nucleus, where they can bind to either ERE or activate other transcription factors like nuclear receptor coactivator-3, nuclear receptor interacting protein-1, E1A binding protein p300, and cAMP responsive element binding protein-1 (E. Simpson & Santen, 2015). Notably, a key characteristic of non-genomic estrogen signaling is its rapid (within minutes) effects compared to genomic signaling (may take several hours) (Rainville, Pollard, & Vasudevan, 2015). Apart from full-length membrane-bound ERs (ERα-66kDa and ERβ-56kDa) (Razandi et al., 1999), alternative splicing of ERα appears to promote palmitoylation, resulting in membrane localization and non-genomic signaling (L. Li, Haynes, & Bender, 2003; A. H. Y. Lin et al., 2013). Immunohistochemistry experiments of the 36-kDa ERα isoform (ERα36) have revealed that this isoform is primarily localized in the cytoplasm and plasma membrane in human breast cancer tissue (L. M. J. Lee et

al., 2008). A previous study has demonstrated that membrane-bound ERα36 induces calcium mobilization in breast cancer cells (Kang et al., 2010). In endothelial cells, 46-kDa ERα (ERα46) localization has been observed in the nucleus, cytosol, and plasma membrane (L. Li et al., 2003). Membrane-bound ERα46 in endothelial cells activates NOS through the c-Src-PI3-K/Akt pathway (L. Li et al., 2007, 2003). Furthermore, a G-protein coupled estrogen receptor (GPR30, now called GPER) is argued to be an estrogen receptor as well. GPER is a plasma membrane-bound receptor that is thought to mediate estrogen-driven non-genomic function through rapid kinase signaling (Prossnitz & Hathaway, 2015). For example, GPER mediates breast cancer cell growth via MAPK and PI3-K/Akt pathways (Prossnitz & Hathaway, 2015). However, whether GPER is truly an estrogen receptor is still under debate. Some studies have demonstrated that GPER activates membrane ERs, and that these membrane ERs are the receptors that activate downstream non-genomic signaling (Kang et al., 2010; Levin, 2009a).

Regulation of Glucose and Lipid Homeostasis by Estrogens: Effects on Adipose, Liver, and Skeletal Muscle Based on Animal Models of Reduced Estrogen Action

The following animal models of reduced estrogen action: aromatase knockout, ovariectomized model, and global ER knockout models provide evidence for the importance of estrogens in maintaining glucose and lipid homeostasis.

Aromatase Knockout

Global genetic ablation of the aromatase enzyme (ARKO) leads to increased body fat and insulin resistance in both females and males. Aromatase is the enzyme responsible for converting testosterone to estradiol. Thus, the global loss of aromatase function leads to estrogen deficiency. Previous studies have found that female and male ARKO mice exhibit greater percent body fat and gonadal fat mass than wild-type (WT) mice by ~ 50-60% at 3 months of age, and progressive fat mass gain continues as these mice age (M. E. Jones et al., 2000; Takeda et al., 2003). Not surprisingly, adipocyte volume and adipocyte size are higher in both female and male ARKO mice by ≥50% at 4 months of age, and serum triglyceride levels are higher at 1 year of age than WT mice (M. Jones et al., 2006; M. E. Jones et al., 2000). ARKO mice also exhibit severe hepatic steatosis at 2-3 months of age, which is up to 7-fold greater than in WT mice (Amano et al., 2017; M. Jones et al., 2006; M. E. Jones et al., 2000; Takeda et al., 2003). Indeed, lipogenic enzymes such as fatty acid synthase, acetyl coenzyme A carboxylase α, fatty acid transporter adipocyte differentiation-related protein are higher in ARKO than WT mice (M. Jones et al., 2006). Another study has demonstrated that fatty acid beta-oxidation activity is impaired in liver of male ARKO mice, which is accompanied by a reduction in liver very long chain fatty acyl-CoA synthetase, peroxisomal acyl-CoA oxidase, and medium-chain acyl-CoA dehydrogenase (Egawa, Toda, Nemoto, & Ono, 2003). Male ARKO mice also develop insulin resistance, as indicated by hyperinsulinemia and marked insulin intolerance at 4-5 months of age (Takeda et al., 2003). Glucose tolerance is normal in male ARKO mice at 3 months of age, but is impaired by the 6th month of age (Takeda et al., 2003).

Furthermore, insulin signaling, as indicated by p-Akt protein content, is impaired in the liver, but not in adipose or skeletal muscle of ARKO mice (Van Sinderen et al., 2015). Three-week and 12-week estradiol treatments prevent excess fat mass gain (M. E. Jones et al., 2000), and improve glucose and insulin tolerance in male adult ARKO mice (Takeda et al., 2003). Similarly, female ARKO mice treated with estradiol for 6 weeks exhibit lower gonadal and omental fat mass, liver triglycerides, lower fasting insulin, and higher liver insulin signaling than untreated ARKO mice, all of which are improved to levels that are similar to WT (Van Sinderen et al., 2015). Collectively, estradiol treatment ameliorates excess fat mass gain and insulin resistance in female and male ARKO mice.

Ovariectomized Rodents

Ovariectomized rodents consistently develop obesity and insulin resistance. The surgical removal of the ovaries (OVX), which is the primary organ that produces estrogens, leads to increased adiposity and insulin resistance (Camporez et al., 2013; Jackson et al., 2013; Wohlers & Spangenburg, 2010). The OVX model is most commonly used to study the effects of the reduction in estrogens in women, who have undergone natural or surgical menopause. Our laboratory has shown that at eight weeks post-OVX, OVX mice exhibit greater body weight, visceral and subcutaneous fat mass, intramuscular lipid content, and monounsaturated fatty acid accumulation in the liver (Jackson et al., 2011, 2013; Wohlers, Powers, Chin, & Spangenburg, 2013; Wohlers & Spangenburg, 2010). OVX mice consume similar amounts of food compared to sham-operated mice (SHAM); however, spontaneous cage activity and energy expenditure are lower in OVX than SHAM mice, which partially explain the greater fat

mass accumulation in OVX mice (Witte, Resuehr, Chandler, Mehle, & Overton, 2010). We have also shown that triglyceride and diglyceride concentrations in the liver are similar between SHAM and OVX mice at 8 weeks post-surgery (Valencia et al., 2016). However, one study has documented that OVX mice exhibit hepatic steatosis at 3 months post-surgery (Rogers, Perfield, Strissel, Obin, & Greenberg, 2009). Our previous data have also indicated that OVX mice exhibit impaired insulin responsiveness in skeletal muscle fibers compared to sham-operated mice (Wohlers et al., 2013). Another study has shown that over a longer period of time (i.e. 26 weeks after ovariectomy), OVX mice remain insulin resistant (Vieira Potter et al., 2012). High fat diet treatment (HFD) exacerbates this obese and insulin resistant metabolic phenotype of OVX mice (Camporez et al., 2013). Although the ovary secretes other proteins and hormones, estrogen treatment (E2) lowers fat mass and improves glucose tolerance, insulin signaling in skeletal muscle, and fasting insulin levels (Riant et al., 2009). Notably, results from a hyperinsulinemic-euglycemic clamp study has indicated that whole body insulin sensitivity is reduced in OVX mice while estradiol-treated OVX mice exhibit markedly improved insulin sensitivity that is similar to SHAM mice (Camporez et al., 2013). Furthermore, systemic insulin resistance is attributed to a decline in skeletal muscle insulin sensitivity (Camporez et al., 2013). OVX rats also display a similar obese and insulin resistant phenotype and respond similarly to estradiol treatment (Abbas & Elsamanoudy, 2011; Ainslie et al., 2001). However, OVX rats exhibit hyperphagia (Witte et al., 2010), which does not closely translate to the eating habits of postmenopausal women. Nonetheless, the data clearly show that significantly reduced levels of estrogens by ovariectomy leads to metabolic dysfunction.

Global ERα Knockout

The global ERα knockout mouse model (ERαKO) recapitulates the obese and insulin resistant phenotype, which suggests that ERα is critical for the regulation of lipid and glucose metabolism. Previous studies have documented that adult female and male ERαKO mice exhibit ~20% greater body weight and ~50-60% greater fat mass than age-matched WT mice under both standard chow and long term high fat diet treatments, even though both groups consume similar amounts of food (Bryzgalova et al., 2006; Heine, Taylor, Iwamoto, Lubahn, & Cooke, 2000; Ohlsson et al., 2000; Ribas et al., 2010). Consistent with the increase in overall adiposity, ERαKO mice display greater amounts of skeletal muscle triglycerides, diglycerides, and ceramides than WT mice (Ribas et al., 2010). Energy expenditure and spontaneous cage activity are also lower in ERαKO compared to WT mice (Ribas et al., 2010). Additionally, ERαKO mice exhibit lower mRNA expression of fatty acid oxidation markers (peroxisome proliferator activated receptor-α, peroxisome proliferator activated receptor-δ, and uncoupling protein-2) compared to WT mice, which likely implies a reduction in skeletal muscle oxidative metabolism (Ribas et al., 2010). Pro-inflammatory signaling markers such as TNFα and p-JNK are also elevated in skeletal muscle and adipose tissue of ERαKO mice (Ribas et al., 2010). Excessive ectopic lipid accumulation in skeletal muscle is associated with cellular stress and activation of inflammatory signaling pathways, which are thought to impair insulin signaling, glucose uptake, and insulin sensitivity (Erion & Shulman, 2010; Holland et al., 2007; Schmitz-Peiffer et al., 1999). Indeed, one study has documented that ERα ablation results in the development of skeletal muscle insulin resistance, with minor but detectable onset of

hepatic insulin resistance (Ribas et al., 2010). Data from the hyperinsulinemic-euglycemic clamp have indicated that the insulin-stimulated glucose disposal rate, a marker for skeletal muscle insulin sensitivity, is 40% lower in ERαKO than WT mice (Ribas et al., 2010). This is supported by the ~50-65% reduction in insulin-induced activation of signaling molecules (pIRS-1^{tyr}, p85 subunit of IRS-1-associated PI3-kinase, and $pAkt^{ser473}$) in skeletal muscle (Ribas et al., 2010). Furthermore, skeletal muscle glucose uptake *ex vivo* is lower in ERαKO compared to WT mice (Bryzgalova et al., 2006). Reports on skeletal muscle glucose transporter-4 (GLUT4), the main glucose transporter for glucose uptake in skeletal muscle, have been mixed. Some studies have shown lower GLUT4 protein content in ERαKO mice (R. Barros, Gabbi, Morani, Warner, & Gustafsson, 2009; R. Barros, Machado, Warner, & Gustafsson, 2006) while another study has reported similar GLUT4 protein content between ERαKO and WT mice (Ribas et al., 2010). In contrast, another hyperinsulinemic-euglycemic clamp study has provided evidence for marked hepatic insulin resistance in female and male ERαKO mice, such that the endogenous glucose output is ~50% higher than in WT mice despite high basal insulin levels (Bryzgalova et al., 2006). Moreover, these ERαKO mice only display modest skeletal muscle insulin resistance (Bryzgalova et al., 2006). A recent study has demonstrated the importance of considering the molecular domains of ERα (Handgraaf et al., 2013). ERα has 2 separate activation functions, AF-1 (A/B domain) and AF-2 (E domain), that can act synergistically or independently in order to mediate the transcription of target genes (Tora et al., 1989). When mice lacking whole body AF-2 (ERαAF-2KO) are compared to ERαAF-1KO mice, the results suggest that ERαAF-2 is necessary for normal lipid and glucose metabolism while ERαAF-1 is not required

(Handgraaf et al., 2013). ERαAF-2KO female and male mice exhibit increased adiposity and insulin resistance while ERαAF-1KO mice display no metabolic phenotype (Handgraaf et al., 2013). Moreover, ovariectomized ERαAF-2KO mice treated with estrogen are not protected from high fat diet-induced insulin resistance, unlike ERαAF-1KO mice (Handgraaf et al., 2013). Additionally, global membrane-only ERα (MOER) and nuclear-only ERα (NOER) mouse studies have revealed that both pools of ERα are required to prevent excess adiposity (Pedram, Razandi, Blumberg, & Levin, 2016). ERα of MOER mice contains a ligand binding domain that is specifically targeted to the plasma membrane. As a result, MOER mice do not express ERα in the nucleus (Pedram et al., 2016). On the other hand, NOER mice have a point mutation C451A that does not permit palmitoylation; therefore, the mice only express ERα in the nucleus. Interestingly, both mice exhibit greater body weight and visceral fat than WT mice (Pedram et al., 2016). Moreover, estradiol treatment on isolated bone marrow stem cells (BMSCs) from WT mice inhibits cell differentiation into adipocytes (Pedram et al., 2016). This inhibitory effect is lost in BMSCs from NOER and MOER, which suggest that partial loss of ERα increases the susceptibility to form new adipocytes (Pedram et al., 2016). Membrane ERα also inhibits carbohydrate response element-binding protein-α and -β in differentiated 3T3-L1 (murine adipocyte-like cells) via AMPK and PKA, and in turn, inhibits the lipogenic enzymes, acetyl-CoA carboxylase and fatty acid synthase (Pedram et al., 2016). Overall, the loss of ERα significantly affects metabolic homeostasis. The disparity for the primary source of insulin resistance between studies calls for more tissue-specific estrogen receptor knockout models in order to advance our understanding of ERα function.

Global ERβ Knockout

Global ERβ knockout mice (ERβKO) do not exhibit increased adiposity or impaired peripheral insulin sensitivity. A previous study has shown that body weight and fat mass are similar between adult ERβKO and WT mice (Bryzgalova et al., 2006). ERβKO mice are also protected from high fat diet-induced insulin resistance and ectopic lipid deposition, even though ERβKO mice exhibit greater body weight and adiposity than WT mice after 12 weeks of high fat diet treatment (Foryst-Ludwig et al., 2008). Furthermore, double ERα and ERβ knockout (ERα/βKO) mice display significantly increased visceral fat mass that is similar to age-matched ERαKO mice (Ohlsson et al., 2000). Thus, it appears that ERβ is not necessary for preventing metabolic dysfunction.

Global GPER Knockout

The metabolic phenotype of the GPERKO mouse is currently unclear. Some investigators have reported that genetic ablation of GPER (GPERKO) in mice leads to obesity (Haas et al., 2009; Sharma et al., 2013) while others have documented no significant differences in adiposity between GPERKO and WT mice. (Isensee et al., 2009; Martensson et al., 2009). Some studies have shown that adult GPERKO mice also display impaired glucose tolerance due to reduced pancreatic secretion of insulin in (Martensson et al., 2009), while others have observed that GPERKO mice exhibit normal glucose tolerance, similar to WT mice (Isensee et al., 2009). Indeed, GPER function in metabolism remains elusive since the reported effects of GPERKO depend on the genetic strain background of the animal models. Other investigators have even contested that GPER is not an estrogen receptor since the binding affinity of estrogen to GPER is low, classic ER antagonists (e.g. ICI182780) act as a GPER agonist, and

finally, all established GPERKO mouse models have normal reproductive function, in contrast to global ERαKO and ERβKO models (Levin, 2009a; Otto et al., 2008).

Regulation of Glucose and Lipid Homeostasis by Adipose-, Liver-, and Skeletal Muscle-Specific ERα: Evidence from Animal Models

Effects of the significant reduction in estrogens or the global reduction of ERα function in both females and males support the notion that estrogens play an important role in protecting both sexes from the development of obesity and insulin resistance. However, global ERα knockout studies are not able to fully determine how ERα mediates the regulation of lipid and glucose metabolism. These studies have yielded critical information, but have been unable to determine tissue-specific effects since ERα is expressed at various levels across most tissues in the body. Specifically, ERα ablation alters multiple organ systems confounding the ability to determine the mechanistic function of tissue-specific ERα. These mouse models exhibit numerous alterations in peripheral tissue function, making it challenging to differentiate primary versus secondary effects. Research on tissue-specific functions of ERα are currently underway. Generation of tissue-specific ERα knockout models have allowed the possibility of determining which metabolic tissue is primarily affected by the loss ERα. Thus far, only conventional tissue-specific models of *in vivo* ERα ablation have been developed. The following section will discuss our current understanding of the role of ERα in insulin sensitive tissues: liver, adipose tissue, and skeletal muscle.

Adipose-specific ERα Knockout

Adipose tissue-specific ablation of ERα increases adiposity, adipose tissue inflammation, and fibrosis. Researchers have investigated the role of adipose tissue ERα in metabolism using 2 conventional adipose tissue-specific ERαKO mouse models that utilize either the adiponectin promoter driven-Cre recombinase (AdipoERαKO) (Davis et al., 2013) or the aP2-Cre recombinase (FERKO) (Drew et al., 2015). Four and a half month old AdipoERαKO mice exhibit ~60% less ERα expression than the WT while adult FERKO mice display a ~90% reduction in ERα. Gonadal fat mass and adipocyte size are greater in both adipose tissue-specific ERαKO mouse models. Glucose tolerance is not affected in female AdipoERαKO, but male AdipoERαKO are glucose intolerant. This finding suggests that adipose-specific ERα does not modulate glucose homeostasis in female mice. Fasting glucose and insulin levels are also not affected in female and male FERKO mice. Adipose tissue inflammation and fibrosis also manifest in a sex-specific manner. Macrophage infiltration marker F4/08 and pro-inflammatory markers TNFα, toll-like receptor-4, and serum amyloid A-3 are ~80-90% greater in adipose tissue of male AdipoERαKO than their WT counterpart, but are not elevated in female AdipoERαKO mice. In male AdipoERαKO, inflammation is accompanied by increased lysyl oxidase and collagen type-VI expression, which are markers of adipose tissue fibrosis. Additionally, data from the offspring of the AdipoERαKO-global ERβKO cross-breed have revealed that ERβ has an important synergistic role with adipose ERα in the regulation of inflammation and fibrosis (Davis et al., 2013). The loss of ERβ impairs glucose tolerance in female AdipoERα/ERβKO mice, but not in male AdipoERα/ERβKO mice. ERβ loss also additively exacerbates adipose

tissue inflammation in male AdipoERα/ERβKO. Female AdipoERα/ERβKO mice also display greater inflammation and fibrosis in adipose tissue.

Liver-specific ERα Knockout

Deletion of liver-specific ERα by conventional conditional knockout yields different results from temporal induced knockdown in adult mice. Liver-specific ERαKO (LERαKO) studies have examined whether liver ERα plays a role in liver lipid deposition and insulin-stimulated suppression of endogenous glucose production, both of which are negatively affected in models of reduced estrogen action (OVX, ARKO, and global ERαKO) (Bryzgalova et al., 2006; Camporez et al., 2013; Egawa et al., 2003; Rogers et al., 2009). Current LERαKO mice are generated using the Cre-loxP approach under the liver-specific albumin promoter (Han et al., 2014; Matic et al., 2013). LERαKO mice exhibit little to no liver-specific ERα since birth, with about a ~90% and 99% reduction in liver ERα mRNA (Esr1) and protein content, respectively (Matic et al., 2013). Adult female and male LERαKO mice are similar to age-matched WT mice in body weight, whole body and liver adiposity, glucose tolerance, and insulin signaling in the liver (Matic et al., 2013). Expression of known hepatic regulators of metabolism that are influenced by estrogen: sterol regulatory element binding transcription factor-1, stearoyl-coenzyme A desaturase-1, and glucose-6-phosphatase (G6Pase) (Han et al., 2014; Jackson et al., 2011) are not affected by the loss of liver ERα (Matic et al., 2013). Furthermore, a 5-month HFD treatment in LERαKO mice does not exacerbate the glucose intolerance or elevated fasting insulin levels typically observed in WT-HFD mice (Matic et al., 2013). These effects are not a result of compensatory increases in ERβ and GPER expression. The authors have concluded that the metabolic phenotype of

mouse models with reduced estrogen action is not likely due to the loss of liver ERα. However, a recent study has demonstrated that liver-specific ERα knockdown by injection of adeno-associated virus-Cre (driven by the liver-specific thyroid binding globulin promoter) in 2-3 month old male ERα floxed mice (AAV-LERαKO), results in hepatic steatosis and elevated fasting glucose after 1 month post-injection of AAV (Qiu et al., 2017). Although body weight is not affected, the liver of AAV-LERαKO mice exhibit ~50% greater triglyceride deposition than WT mice, which is attributed to greater expression of *de novo* lipogenic markers, fatty acid synthase and acetyl-coa carboxylase-1. AAV-LERαKO mice also display high fasting glucose, which is in part, due to ~50% higher expression of gluconeogenic enzymes, G6Pase and phosphoenolpyruvate carboxykinase-1 (Pck1), than WT mice. This finding indicates that glucose production is less suppressed in AAV-LERαKO mice. In primary hepatocytes sourced from WT mice, estradiol-ERα has been demonstrated to directly bind to G6Pase and Pck1, both of which contain estrogen response element half-sites in their promoter region, and thus, inhibit G6Pase and Pck1 expression (Qiu et al., 2017). Indeed, estradiol treatment does not modulate G6Pase and Pck1 in primary hepatocytes from AAV-LERαKO mice (Qiu et al., 2017). No studies on liver-specific ERα in adult female mice have been conducted. Collectively, the data intriguingly suggest that liver ERα is critical in maintaining glucose and lipid homeostasis in male adult mice, but is not essential during development. More studies are needed to investigate the similarities and differences between embryonic and induced knockout of tissue-specific ERα function, its underlying mechanisms, and whether these functions are sexually dimorphic.

Skeletal Muscle-specific ERα Knockout

Ablation of skeletal muscle-specific ERα results in excess adiposity and significant skeletal insulin resistance. The skeletal muscle is the primary site for postprandial insulin-stimulated glucose uptake, lipid and glucose utilization, as well as a site for lipid storage (Thiebaud et al., 1982). Skeletal muscle ERα is, therefore, a potential potent regulator of glucose and lipid homeostasis. Thus far, only one study has utilized a skeletal muscle-specific ERαKO mouse model (MERKO), which is generated using the Cre-loxP approach, driven by the muscle creatine kinase promoter (Ribas et al., 2016). Adult female MERKO mice exhibit greater body mass due to 2.75-fold greater gonadal fat mass than WT mice. Skeletal muscle lipid deposition is also evident, wherein triglyceride, diglyceride, and ceramide levels are ~40-50% greater than WT mice. Markers of inflammation, p-JNK and p-IKKβ are also higher in MERKO than WT mice. As mentioned earlier, both ectopic deposition of lipid intermediates and skeletal muscle inflammation are implicated in the etiology of lipid-induced insulin resistance (Kitessa & Abeywardena, 2016; Wu & Ballantyne, 2017). Consistent with this notion, MERKO mice exhibit reduced whole body insulin sensitivity. Data from the hyperinsulinemic-euglycemic clamp have revealed that insulin-stimulated glucose disposal rate is reduced by 45% in MERKO mice, indicating significant skeletal muscle insulin resistance. *Ex vivo* insulin-stimulated skeletal muscle glucose uptake is also lower by 55% in MERKO mice, along with lower protein content of skeletal muscle insulin signaling molecules, p85 subunit of IRS-1 and p-Akt, than WT mice. Currently, no studies on skeletal muscle-specific ERα in male mice have been conducted;

therefore, whether ERα function in skeletal muscle is sexually dimorphic remains to be determined.

Overall, these studies suggest that from a metabolic perspective, the ERα site of action for maintaining both glucose and lipid homeostasis is in the skeletal muscle. However, compensatory mechanisms that occur during development may have substantially contributed to the phenotype of adult skeletal muscle-specific ERαKO mice, and as a result, primary and secondary effects of the loss of skeletal muscle ERα cannot be differentiated. Finally, considering that most women are born with normal estrogen function, the existing global and skeletal muscle-specific ERαKO mouse models do not truly represent women since these ERαKO mice lack the estrogen-ERα interaction since development. Therefore, our current understanding of the importance of ERα to skeletal muscle is limited by the current state of the animal models.

Regulation of Mitochondrial Function by Estrogens

The following section will discuss how estrogens are thought to influence aspects of mitochondrial function, including mitochondrial respiration, reactive oxygen species (ROS) emission, and mitochondrial fusion-fission dynamics, by direct and indirect regulation of mitochondrial-encoded gene transcription.

Estrogens indirectly regulate mitochondrial-encoded gene transcription (Jin-qiang Qiang Chen, Brown, & Russo, 2009; Klinge, 2017)**.** For example, COX-7RP is a nuclear-encoded mitochondrial gene that contains an ERE, which has been

documented to increase with estradiol treatment (Watanabe et al., 1998). Studies in MCF-7 breast cancer cells and H1793 lung adenocarcinoma cells have also shown that ERα mediates the estradiol-induced increase in the nuclear-encoded nuclear respiratory factor-1 (NRF-1), which plays a major role in mitochondrial biogenesis (Mattingly et al., 2008). ChIP assays in MCF-7 cells have demonstrated the interaction between ERα and NRF-1, which contains an imperfect ERE (Mattingly et al., 2008). Furthermore, a previous study has shown that silencing ERα by siRNA inhibits the estradiol-induced increase in NRF-1, but silencing ERβ has no effect (Mattingly et al., 2008). NRF-1 activation promotes mitochondrial biogenesis by activating Transcription Factor A, Mitochondrial (TFAM), which is a major inducer of mtDNA gene transcription (Scarpulla, 2012). Indeed, the estradiol-induced upregulation of NRF-1 increases TFAM, and concurrently increases the expression of mitochondrial-encoded cytochrome c oxidase subunit-1 (COX-1) and NADH dehydrogenase subunit-1 in MCF-7 cells (Mattingly et al., 2008). Other studies have also demonstrated that estradiol, via NRF-1, regulates the following nuclear DNA-encoded genes: Complex I subunit NDUSF-8, succinate dehydrogenase complex subunit B, cytochrome C, COX-4, COX-5a, COX-5b, COX-6b, COX-6c, COX-7b, COX-7c, COX-8, COX17p, F0 ATP synthase subunits d-g, and F1 ATP synthase γ (J. Chen, Cammarata, Baines, & Yager, 2009). NRF-1 and TFAM expression are also increased in skeletal muscle of OVX rats (Capllonch-Amer et al., 2014), and in hearts, mammary gland, and uterus of OVX mice (Y. Chen et al., 2015; Ivanova, Radde, Son, Mehta, & Klinge, 2013) after short (e.g. 4 weeks) and long term (e.g. 3 months) estradiol treatment.

Estrogen-ER signaling also appears to regulate mitochondrial function by direct mtDNA-encoded gene transcription of certain Complex I-V subunits in electron transport system (ETS) (Jin-qiang Qiang Chen et al., 2009; Jin Qiang Chen, Yager, & Russo, 2005)**.** MtDNA has been previously shown to contain ERE-like sequences, which coincides with an estradiol-induced increase in ETS activity (J. Chen et al., 2009). Furthermore, electrophoresis mobility shift and surface plasmon resonance assays demonstrate that ERα and ERβ can bind to mtDNA ERE-like sequences in MCF-7 cells, and that their binding affinity increases with increasing dosage and longer estrogen exposure (Jin Q. Chen, Eshete, Alworth, & Yager, 2004). Some studies have also reported that ERα and ERβ localize in the mitochondria of different tissues and cell types such as human heart, human fetal brown adipose tissue, rat hippocampal neurons, MCF-7 cells, HepG2 cells, providing added support for the direct regulation of mtDNA-encoded genes (Klinge, 2017; Pedram, Razandi, Wallace, & Levin, 2006; Yager & Chen, 2007). Notably, the concept that ERs localize in the mitochondria remains to be controversial or appears to be tissue-dependent. Since most studies have used immunoblot and immunohistochemistry techniques (Klinge, 2017), some have argued that such data are not reliable since ER antibodies have been previously shown to yield non-specific results [141]. Data from mass spectrometry studies are also mixed (Klinge, 2017). Only one study has detected ERβ in human heart mitochondria by MALDI-TOF (Yang et al., 2004). However, other mass spectrometry studies on mitochondria in over 14 different mouse tissues have documented that ERs do not localize in the mitochondria (Klinge, 2017). Furthermore, a recent study on OVX and skeletal muscle-specific ERαKO mice has demonstrated that estradiol directly incorporates into the

mitochondrial membrane, in the absence of ERα, which enhances the fluidity of the membrane and in turn, improves Complex I- and Complex III-specific activities and reduces H_2O_2 emission potential (Torres et al., 2018).

Direct and indirect regulation of mitochondrial proteins by estradiol can affect mitochondrial respiratory capacity. Decreased circulating estrogens by ovariectomy results in reduced mitochondrial respiratory capacity, and estrogen treatment improves it. For instance, isolated heart mitochondria of young adult OVX rats display lower protein content and enzyme activities of Complex-I, Complex-IV, pyruvate dehydrogenase, and 2-oxoglutarate dehydrogenase at 2 months post-surgery (Pavón et al., 2017). At 3 months post-surgery, OVX rats exhibit lower maximal respiratory capacity in the presence of glutamate/malate compared to controls (Pavón et al., 2017). Another study has also demonstrated that estradiol treatment for 4 weeks results in higher activity of ETS enzymes important for ATP production, citrate synthase, and COX-4, in cerebral blood vessels of young adult OVX rats (Stirone, Duckles, Krause, & Procaccio, 2005). In skeletal muscle, respiratory capacity of permeabilized muscle fibers at 8 weeks post-ovariectomy is similar between young adult OVX rats and age-matched sham-operated mice when normalized to citrate synthase activity, but is lower when normalized to fiber weight (Cavalcanti-de-Albuquerque et al., 2014). The authors of this study have suggested that muscle fibers of these OVX rats have lower mitochondrial content (Cavalcanti-de-Albuquerque et al., 2014). In mice, ovariectomy leads to a reduction in muscle fiber respiratory capacity by ~25% in the presence of glutamate/malate and ~45% in the presence of fatty acids in as early as 2 weeks post-surgery (OVX-2w), which decreases even more at 4 weeks post-ovariectomy (Torres et

al., 2018). Conversely, respiratory capacity (in the presence of pyruvate/malate and glutamate/malate) of OVX-2w mice treated with estradiol for 2 weeks is similar to the rates observed in ovary-intact mice, which is accordingly higher than untreated OVX mice (Torres et al., 2018). The role of estradiol in maintaining respiratory capacity appears to be mediated by ERα in skeletal muscle. This has been demonstrated in a study on C2C12 myotubes transduced with lentiviral ERαshRNA, which exhibit a 40-50% decrease in basal and maximal respiratory capacity, a ~50% decrease in ATP synthesis, and a ~50% lower palmitate oxidation rate (Ribas et al., 2016). However, no direct measures have been made in mature skeletal muscle with intact cellular and mitochondrial architecture. Furthermore, as mentioned earlier, estradiol can localize to the mitochondrial membrane and improve membrane fluidity, which in turn increases respiratory capacity of skeletal muscle mitochondria, independent of ERα (Torres et al., 2018). Thus, whether skeletal muscle-specific ERα mediates the reductions in respiratory capacity *ex vivo* or *in vivo* remains to be determined.

Estradiol also regulates mitochondrial reactive oxygen species (ROS) exposure. In models of reduced estrogen action, the cellular antioxidant responses are altered, resulting in increased emitting potential of hydrogen peroxide (H_2O_2), a major form of ROS in the mitochondria (Wong, Dighe, Mezera, Monternier, & Brand, 2017). For example, our lab has shown that H_2O_2 emission potential is higher in isolated liver mitochondria of OVX mice than sham-operated mice, which is attributed to a reduction in protein content of glutathione peroxidase-1, a major antioxidant enzyme in liver mitochondria of OVX mice (Valencia et al., 2016). Another study has also demonstrated that cerebral blood vessels of OVX rats treated with estradiol for 4 weeks exhibit lower

H_2O_2 emission potential and concomitantly, higher MnSOD protein content compared to untreated OVX rats (Stirone et al., 2005). Young adult OVX rats also display greater hydrogen peroxide (H_2O_2) emission potential in skeletal muscle fibers than sham-operated rats at 4 weeks post-ovariectomy (Capllonch-Amer et al., 2014). Similarly, a recent study has reported that permeabilized skeletal muscle fibers of OVX mice exhibit higher H_2O_2 emission potential than SHAM mice in as early as 2 weeks post-surgery, accompanied by a reduction in the capacity of glutathione to reduce ROS levels, as indicated by a lower reduced-to-oxidized glutathione (GSH/GSSG) ratio (Pavón et al., 2017). Conversely, permeabilized muscle fibers of OVX mice treated with estradiol for 2 weeks display lower H_2O_2 emission potential (in the presence of pyruvate) than untreated OVX mice (Pavón et al., 2017). Overall, these studies support the notion that estradiol plays a role in regulating redox homeostasis. One study has reported that ERα function is critical for the regulation of ROS levels in skeletal muscle, such that muscle-specific ERαKO (MERKO) mice exhibit greater protein carbonylation in skeletal muscle (a marker of elevated mitochondrial and non-mitochondrial ROS) and lower glutathione peroxidase-3 (also a member of the glutathione antioxidant system) (Ribas et al., 2016). Increased ROS levels are similarly observed in C2C12 myocytes transduced with lentiviral ERαshRNA (Ribas et al., 2016). However, no study has directly measured whether ERα functions to regulate mitochondrial H_2O_2 emission in mature skeletal muscle with intact mitochondrial architecture.

Mitochondrial function is also influenced by estradiol's regulation of mitochondrial fusion and fission via both ER-dependent and independent mechanisms. A previous study has demonstrated that estradiol increases the

expression of fusion proteins mitofusin-1 and -2 (MFN-1 and MFN-2) in MCF-7, MDA-MB-231, and T47D cancer cells (Sastre-Serra, Nadal-Serrano, Pons, Roca, & Oliver, 2013). Fusion protein optic atrophy-1 homolog (OPA-1) expression is higher in estradiol-treated MCF-7 (ER+) cells, but not in MDA-MB-231 (ER-) cells, indicating that estradiol modulates the expression of OPA-1 in an ER-dependent manner (Sastre-Serra et al., 2013). On the other hand, dynamin-related protein-1 (DRP-1) is higher in estradiol-treated MDA-MB-231 cells, but not in MCF-7 cells, indicating the involvement of an ER-independent mechanism (Sastre-Serra et al., 2013). Estradiol also reduces fission-1 homolog (FIS-1) expression in MCF-7 cells, but not MDA-MB-231 (Sastre-Serra et al., 2013). Alterations in mitochondrial fusion and fission dynamics may alter mitochondrial function. For instance, a previous study has reported that young adult, OVX rats exhibit lower MFN1, MFN2, and DRP1 protein content in skeletal muscle than SHAM rats, which coincide with lower mitochondrial respiratory capacity compared to control rats (Capllonch-Amer et al., 2014). However, skeletal muscle-specific ERα loss may not be the mediator of altered mitochondrial fusion that occurs with ovariectomy. A recent study has observed that conventional skeletal muscle-specific ERαKO mice (MERKO) exhibit impaired mitochondrial fission, as indicated by hyper-fused intermyofibrillar mitochondria, less dense subsarcolemmal mitochondria, ~50% lower FIS1, and ~50-60% greater MFN-1, MFN-2, and OPA-1 than WT mice (Ribas et al., 2016).

The current state of the literature points to skeletal muscle-specific ERα action as a critical role in regulator of glucose dynamics, lipid homeostasis, and mitochondrial function in skeletal muscle. However, the available data on muscle-specific ERα function is limited, and is likely confounded by developmental effects due to losing ERα at conception. The inducible tissue-specific conditional knockout overcomes the aforementioned limitations, and is one of the most powerful and innovative techniques to date (J. J. McCarthy, Srikuea, Kirby, Peterson, & Esser, 2012). This tool allows mice to mature with normal estrogen-ERα function until genetic ablation of ERα is desired. The current study aimed to investigate the role of skeletal muscle ERα in preventing metabolic dysfunction by utilizing an inducible skeletal muscle-specific ERα knockout mouse model so that the function of ERα can be precisely defined without the confounding factors of development.

Chapter 3: Induced ablation of skeletal muscle-specific estrogen receptor alpha in adult female mice increased the susceptibility to develop skeletal muscle inflammation and glucose intolerance under chronic lipid overload

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Abstract

Estrogen receptor-α (ERα) belongs to the nuclear receptor family and is thought to play a significant role in the regulation of skeletal muscle glucose and lipid homeostasis. However, current skeletal muscle-specific ERα loss-of-function approaches have likely affected developmental mechanisms, making it difficult to assess the effect of ERα loss in adult mice. Here, we determined whether skeletal muscle ERα is critical for maintaining metabolic function in adult mice by utilizing a novel inducible skeletal muscle-specific ERα knockout mouse model (ERαKO^{ism}). We hypothesized that the loss of ERα function in adult skeletal muscle increases the susceptibility to high fat diet-induced metabolic dysfunction. At 20-22 weeks of age, female and male wild-type (WT) and ERαKO^{ism} mice were given either a control (low fat) diet (CD, 10% kcal fat) or high fat diet (HFD, 45% kcal fat) for 12 weeks. Body composition and glucose tolerance were assessed after acute (1 week) and chronic (11 weeks) HFD treatment. Whole body metabolism was measured at baseline, during the switch to a HFD, and on the 11th week of HFD treatment by indirect calorimetry. *Ex vivo* skeletal muscle glucose uptake, indices of mitochondrial function, and markers of inflammation in skeletal muscle were assessed after the 12-week HFD treatment. At baseline, body mass, fat mass, and lean mass were similar among the groups, for both females and males. The change in body mass and fat mass over the course of the study were similar between WT-CD and ERαKO^{ism}-CD mice, for both females and males. Chronic HFD treatment increased total body fat mass with no significant differences between genotype, for both females and males. After chronic HFD treatment, energy expenditure of male ERαKO^{ism} mice was minimally higher than WT

mice, regardless of diet, despite no significant differences in spontaneous cage activity. Analysis of glucose dynamics revealed that female ERαKO^{ism}-HFD mice were more glucose intolerant than WT-HFD mice. *Ex vivo* basal and insulin-stimulated skeletal muscle glucose uptake was similar between WT and ERαKO^{ism} mice, and chronic HFD treatment led to a reduction in glucose uptake, regardless of genotype. Interestingly, female ERαKO^{ism} have lower skeletal muscle GLUT4 protein content and increased expression of pro-inflammatory markers in skeletal muscle compared to WT mice, regardless of diet. Finally, permeabilized muscle fibers of WT and ERαKO^{ism} mice, regardless of diet, exhibited similar mitochondrial respiratory capacity, OXPHOS efficiency, and H_2O_2 emission potential for both females and males. Chronic HFD treatment did not negatively affect skeletal muscle mitochondrial function of female and male ERαKO^{ism} mice. To determine whether our findings on mitochondrial function similarly occur in human skeletal muscle, we silenced ERα in cultured human myotubes using an adenovirus-driven ERαshRNA (ERαKD). ERαKD myotubes sourced from healthy women displayed a minimal reduction in ATP production rate. ERαKD myotubes from obese-insulin resistant women exhibited slightly reduced basal O_2 consumption and ATP production rates. Overall, our data suggest that skeletal muscle ERα is critical for preventing glucose intolerance in females on a chronic HFD and regulating skeletal muscle inflammation. Furthermore, induced loss of ERα in adult skeletal muscle does not result in excess adiposity and does not deleteriously affect mitochondrial function.

Introduction

Ovarian senescence (Gold, 2011), bilateral oophorectomy (Hendrix, 2005), aromatase deficiency (M. Jones et al., 2006), and estrogen receptor polymorphisms (Smith et al., 1994) lead to a significant reduction in estrogen action, and all these conditions are associated with the increased risk of developing obesity and peripheral insulin resistance, which can progress to overt Type 2 Diabetes (Carr, 2003; Lo et al., 2006; Park et al., 2003; Pu et al., 2017). Conversely, administering pharmacological levels of estrogen is an effective approach for decreasing adiposity and improving peripheral insulin sensitivity (Ahtiainen et al., 2012; A. M. McCarthy et al., 2013; Salpeter et al., 2006). However, observational studies in the 1970s have documented that the use of conjugated equine estrogen (CEE), the most popular form estrogen therapy at that time, increases the risk of endometrial cancer (Ziel & Finkle, 1975). Moreover, the largest randomized control trial study on postmenopausal hormone therapy, called the Women's Health Initiative, has found statistical associations between estrogen therapies (CEE and CEE + medroxyprogesterone acetate) and increased incidence of breast cancer, coronary heart disease, stroke, and pulmonary embolism (G. Anderson et al., 2004; Rossouw et al., 2002). Since then, the urgency to improve our therapeutic strategies has required a better understanding of estrogen-sensitive mechanisms across all tissues.

Estrogen action at each individual tissue is predominantly mediated by tissue expression of each specific receptor (Couse et al., 1997) and estrogens' affinity for the expressed receptor (Kuhl, 2005). Numerous studies on estrogen receptors may have revealed a potential new target for modulating metabolism (R. P. a Barros &

Gustafsson, 2011; Hevener, Clegg, & Mauvais-Jarvis, 2015). Among the estrogen receptors that mediate estrogen signaling in cells, estrogen receptor-alpha (ERα) appears to play a key role in the regulation of glucose and lipid homeostasis. Global ERα knockout mice (ERαKO) exhibit excess visceral adiposity and insulin resistance (Bryzgalova et al., 2006; Heine et al., 2000; Ohlsson et al., 2000; Ribas et al., 2010), whereas global ERβKO mice have normal body mass, fat mass, and insulin sensitivity (Bryzgalova et al., 2006; Ohlsson et al., 2000). Additionally, the consensus on whether the G-protein coupled estrogen receptor is a true estrogen receptor remains to be determined (Levin, 2009a).

Tissue-specific ERα loss-of-function models have allowed the dissection of primary versus secondary effects and the functions of ERα in regulating glucose and lipid metabolism at each specific tissue. For instance, induced knockdown of liver ERα by injection of adeno-associated virus-Cre (driven by the liver-specific thyroid binding globulin promoter) in 2-3 month old male ERα-floxed mice results in hepatic steatosis, along with a modest increase in fasting glucose that is attributed to decreased inhibition of gluconeogenic enzymes in the liver, glucose-6-phosphatase and phosphoenolpyruvate carboxykinase-1 (Qiu et al., 2017). However, conventional liver-specific ERαKO mice with no liver ERα function since embryonic development display normal body mass, fat mass, glucose tolerance, and even after a 5-month high fat diet (Matic et al., 2013). The disparity between these findings from 2 different loss-of-function approaches emphasize the importance of conducting more studies on ERα function without the confounding factors of development.

Skeletal muscle-specific ERα appears to play key roles in preventing metabolic dysfunction. Global ERαKO female mice exhibit ectopic deposition of triglycerides, diglycerides, and ceramides, reduced expression of fatty acid oxidation markers, as well as elevated expression of pro-inflammatory markers in skeletal muscle (Ribas et al., 2010), all of which have been associated with the development insulin resistance (Befroy et al., 2007; Holland et al., 2007; Szendroedi et al., 2014; Wu & Ballantyne, 2017). Indeed, a hyperinsulinemic-euglycemic clamp study has demonstrated that global ERαKO female mice display significantly decreased glucose disposal rate, indicating substantial skeletal muscle insulin resistance [207]. This is accompanied by impaired insulin signaling; although, GLUT4, the main glucose transporter in skeletal muscle, is not affected by the loss of ERα (Ribas et al., 2010). Other studies, however, have reported reduced GLUT4 protein content in skeletal muscle of global ERαKO mice (R. Barros et al., 2009, 2006). Thus far, only one study has investigated the role of skeletal muscle-specific ERα in the regulation of glucose and lipid homeostasis by utilizing conditional tissue-specific knockout mice, which have lost skeletal muscle-specific ERα function during the embryonic stage of development (MERKO mice) (Ribas et al., 2016). Female MERKO mice exhibit increased visceral adiposity and reduced insulin sensitivity at 7 months of age (Ribas et al., 2016). Skeletal muscle of female MERKO mice display significant accumulation of lipid intermediates, inflammation, and insulin resistance, similar to the metabolic phenotype to global ERαKO mice (Ribas et al., 2016). These findings suggest that skeletal muscle-specific ERα may be critical for preventing significant increases in adiposity and inflammation as well as maintaining insulin sensitivity in adult mice. However, no studies have investigated the *in vivo* role of

skeletal muscle ERα in regulating metabolic homeostasis, without the confounding factors associated with development.

Estrogens also regulate mitochondrial function, which appears to be mediated by ERα. Indeed, our lab (Valencia et al., 2016) and others (Cavalcanti-de-Albuquerque et al., 2014; Pavón et al., 2017; Stirone et al., 2005; Torres et al., 2018) have previously demonstrated that reduced estrogen action by ovariectomy results in increased mitochondrial reactive oxygen species (ROS), particularly H_2O_2 emission potential and/or reduced mitochondrial respiratory capacity in multiple tissues, including skeletal muscle (Torres et al., 2018). 17β-estradiol treatment has also been recently reported to improve these aspects of mitochondrial function in skeletal muscle fibers of ovariectomized mice (Torres et al., 2018). Recent evidence suggest that ERα is important for maintaining mitochondrial health (Ribas et al., 2016). Female MERKO mice exhibit mitochondrial morphological abnormalities such as hyper-fused intermyofibrillar mitochondria and a reduction in the density of subsarcolemmal mitochondria (Ribas et al., 2016). Additionally, mitochondrial DNA (mtDNA) turnover is reduced, and protein carbonylation levels, a marker of increased ROS levels (mitochondrial and non-mitochondrial ROS), are significantly elevated in skeletal muscle of MERKO mice. In C2C12 myotubes, ERα knockdown by lentiviral-ERαshRNA is reported to drastically lower basal and ADP-supported maximal O_2 consumption, ATP production, and fatty acid oxidation rates by approximately 40-50% (Ribas et al., 2016). ERα has also been demonstrated to regulate mitochondrial DNA- and nuclear DNA-encoded gene transcription of certain subunits of the electron transport system via direct and indirect mechanisms in other cell types such as breast cancer (MCF-7) cells

(Klinge, 2017). However, at this point, no direct functional measures have been made in adult skeletal muscle with intact mitochondrial architecture to assess whether skeletal muscle-specific ERα regulates mitochondrial respiratory capacity, oxidative phosphorylation efficiency, and mitochondrial-specific ROS emission. Since lipid-induced insulin resistance has been linked to elevated mitochondrial H_2O_2 emission potential and reduced mitochondrial function that occur with chronic overnutrition (E. J. Anderson et al., 2009; Fisher-Wellman & Neufer, 2012), the role of skeletal muscle ERα in maintaining mitochondrial function may be critical for the regulation of glucose and lipid homeostasis.

We sought to advance our understanding of skeletal muscle ERα function by generating a novel inducible skeletal muscle-specific ERα knockout ($ERαKO^{ism}$) mouse model, using the tamoxifen-inducible Cre-loxP system. In this study, skeletal muscle ERα function was ablated in adult mice after Cre activation at 10-12 weeks of age, which eliminated the confounding factors associated with ERα loss during development. The purpose of the study was to determine whether skeletal muscle-specific ERα is critical for maintaining metabolic homeostasis under acute and chronic conditions of nutrition overload. We hypothesized that induced ablation of skeletal muscle-specific ERα in female and male adult mice increases the susceptibility to high fat diet-induced metabolic dysfunction. The major finding of this study was that induced ablation of skeletal muscle ERα in female adult mice led to glucose intolerance after chronic HFD treatment, which may, in part, be due to increased expression of pro-inflammatory markers and reduced GLUT4 protein content in skeletal muscle. These findings were not due to alterations in adiposity or mitochondrial function. Altogether, our data suggest

that skeletal muscle ERα mediates the protective role of estrogens against HFD-induced glucose intolerance in females by regulating skeletal muscle inflammation and preserving skeletal muscle GLUT4 levels.

Methods

Generation of Inducible Skeletal Muscle-Specific Estrogen Receptor-Alpha Knockout Mice

To assess the *in vivo* role of ERα, specifically in skeletal muscle, we generated an inducible skeletal muscle-specific ERα knockout (ERαKO^{ism}) mouse model. ERα$^{flox/flox}$ mice, which carry ESR1 with exon 3 flanked by loxP sites (a kind gift from Dr. K. Korach, National Institute of Environmental Health Sciences), were crossed with the tamoxifen-inducible Cre transgenic line driven by the human α-skeletal actin promoter (HSA-MCM) (a kind gift from Dr. K. Esser, University of Kentucky College of Medicine). Mice were backcrossed 8-9 generations, with SNP analysis demonstrating >99% of genome from C57Bl/6N. To determine whether the offspring possessed ERα$^{flox/flox}$ and HSA-MCM, mice were genotyped by polymerase chain reaction (PCR) using the following primer sets: 1) ERα$^{flox/flox}$ F: 5'-GACTCGCTACTGTGCCGTGTGC-3' and R: 5'-CTTCCCTGGCATTACCACTTCTCCT-3', and 2) HSA-MCM F: 5'-GGCATGGTGGAGATCTTTGA-3' and R: 5'-CGACCGGCAAACGGACAGAAGC-3'. In this model, Cre recombinase is only activated when tamoxifen binds to Mer (mutated estrogen receptor), and is not activated by endogenous estrogens. Adult female and male mice (10-12 wks of age) with ERα$^{flox/flox}$ x HSA-MCM were injected intraperitoneally with 2mg of tamoxifen (Sigma #T5648), once a day for 5 days, as previously described (Jackson et al., 2018), to induce recombination of flox sites on

exon 3. In parallel, genetically-identical mice (age- and gender-matched) were injected with the vehicle and served as the control or wild-type (WT) mice in this study. Mice were further aged for 10 weeks before any experiments and diet treatments were performed to ensure the washout of tamoxifen and that ERα protein was no longer detected in skeletal muscle. Successful recombination of ERα in skeletal muscle was assessed through PCR. DNA was extracted using the Puregene Tissue Kit (Qiagen). PCR was performed using the HotStarTaq Plus Master Mix Kit (Qiagen) along with the following primers: primer 1, 5'-TTGCCCGATAACAATAACAT-3' and primer 2, 5'-GGCATTACCACTTCTCCTGGGAGTCT-3'. Finally, in previous experiments, we found no evidence of Cre-toxicity. We also injected HSA-MCM with tamoxifen per the above protocol and found no effect on glucose dynamics or mitochondrial respiration measures with this approach (Jackson et al., 2018).

Research Experimental Design

For both females and males, mice were divided into 4 groups based on genotype and diet: 1) wild-type on control low fat diet (WT-CD), 2) wild-type on high fat diet (WT-HFD), 3) ERαKO^{ism} on control (low fat) diet (ERαKO^{ism}-CD), and 4) ERαKO^{ism} on high fat diet (ERαKO^{ism}-HFD). All mice were given water and food ad libitum in a room on a 12h light/dark cycle. WT and ERαKO^{ism} mice were first fed a CD for 1 week prior to the HFD. Mice were subsequently fed either a CD or HFD for 12 weeks. CD contained 10% fat, 70% carbohydrates, and 20% protein while HFD contained 45% fat, 35% carbohydrates, and 20% protein. All rodent diets were obtained from *Research Diets, Inc., New Brunswick, NJ* (CD: D12450Ki, HFD: D12451i). Whole body and skeletal muscle metabolic function were assessed after acute (1-week) and chronic (12-week)

exposure to the HFD. Mice were sacrificed at the end of the 12-week HFD treatment. All experiments were approved by the Institutional Review Committee of East Carolina University.

Body Composition and Food Intake

Body mass and food intake were recorded weekly, and data from baseline until the 11th week of HFD treatment were analyzed. Fat mass and lean mass were measured using the EchoMRI^{TM}-500 Body Composition Analyzer (EchoMRI, Houston, Tx) according to the manufacturer's protocol.

Indirect Gas Calorimetry

O_2 consumption (VO_2), CO_2 production (VCO_2), energy expenditure (H), and spontaneous cage activity levels of mice were measured using the TSE LabMaster System (TSE Systems, Chesterfield, MO) according to the manufacturer's protocol. Calorimetry data were normalized to lean body mass. Activity levels were based on the absolute number of times infrared sensors in the x, y, and z planes were disturbed. All data were presented as the average of at least two 12hr light and dark cycles. Measurements were conducted at baseline, during the acute switch from control to high fat diet, and at the 11th week of HFD treatment.

Glucose Tolerance Test

Glucose tolerance was measured at the end of the 1st week and end of the 11th week of HFD treatment. Mice were fasted for 12 hours. A glucose bolus of 2mg/kg of body mass was injected in the intraperitoneal (IP) region. A glucometer was used to

measure glucose from blood drawn at the tail vein at the 0, 30th, 60th, and 120th minute.

Ex Vivo Skeletal Muscle Glucose Uptake

Extensor digitorum longus and soleus muscles were dissected from 12hr-fasted mice and used to measure glucose uptake as previously described (McMillin, Schmidt, Kahn, & Witczak, 2017). Briefly, muscles were incubated in 37°C gassed (95%O_2, 5% CO_2) Krebs Ringer Bicarbonate (KRB) buffer supplemented with 2mM pyruvate for 60 min, and stimulated in the absence or presence of insulin (600µU/ml) for 20 min. Muscles were transferred to vials containing 30°C KRB buffer supplemented with 1.5µCi/ml [^{3}H]-2-deoxyglucose, 1mM 2-deoxyglucose, 0.45µCi/ml [^{14}C]-mannitol, 7mM mannitol, with and without insulin for 10 min, and frozen in liquid nitrogen. Muscles were solubilized in 1N NaOH at 80℃ for 15 min, and neutralized with 1N HCl. Samples were vortexed and centrifuged at 10,000xg for 1 min, and aliquots were removed for scintillation counting of the [^{3}H] and [^{14}C] labels.

Mitochondrial Respiration using High-Resolution Respirometry

Permeabilized skeletal muscle fibers from red gastrocnemius were used to measure mitochondrial respiratory capacity as previously described (Fisher-Wellman et al., 2015; Lark et al., 2016). Briefly, muscles were placed in buffer X (7.23mM K_2EGTA, 2.77mM CaK2EGTA, 20mM imidazole, 20mM taurine, 5.7mM ATP, 14.3mM phosphocreatine, 6.56mM MgCl2, and 50mM MES (pH 7.1), and fibers were separated with the aid of a dissecting microscope. For permeabilization, fiber bundles were incubated in buffer X, supplemented with 40µg/ml saponin, for 30 min. Permeabilized

muscle fibers were washed in buffer Z (110mM K-MES, 35mM KCl, 1mM EGTA, 5mM K_2HPO_4, 3mM $MgCl_26H_2O$, 5mg/ml bovine serum albumin, pH 7.4) at 4℃ for 15 min. Permeabilized fibers were placed in the OROBOROS O2K Oxygraph chambers (Oroboros Instruments) filled with buffer Z that was supplemented with creatine monohydrate (20mmol/L) and 12.5μM blebbistatin. To determine respiratory capacity using carbohydrate-derived substrates, O_2 consumption rate (OCR) was measured after each sequential addition of the following: 4mM pyruvate/ 0.5mM malate/ 5mM glutamate, 2.5mM ADP, 5mM Succinate, 5μM cytochrome c, 10mM rotenone, 5μM antimycin A/ 2mM ascorbate, 0.5mM TMPD. To determine respiratory capacity using fatty acid substrates, OCR was measured after each sequential addition of the following: 18μM palmitoyl-carnitine/20μM palmitoyl-CoA/5mM l-carnitine, 0.5mM malate, 1mM ADP, 500nM FCCP. Data were normalized to fiber dry weight.

Direct Measurement of Mitochondrial Oxidative Phosphorylation (OXPHOS) Efficiency

OXPHOS efficiency was measured in permeabilized muscle fibers via the simultaneous quantification of ATP production and O_2 consumption (OCR) rates as previously described (Lark et al., 2016), with minor modification. Permeabilized fibers were placed in the OROBOROS O2K Oxygraph (Oroboros Instruments) filled with buffer Z that was supplemented with 0.01mM blebbistatin and 0.2mM Ap5A. OCR was simultaneously measured with ATP production rate by quantification of NADPH fluorescence (Excitation/Emission: 340/460nm; FluoroMax-3, Horiba Jobin Yvon, Edison, NJ) at 37℃. In this assay, ATP production was determined using an enzyme-linked assay, where the ATP hydrolyzed in a hexokinase reaction and subsequent

NADPH production through the glucose-6-phosphate dehydrogenase reaction results in a 1:1 stoichiometry. OXPHOS flux in permeabilized fibers was measured in the presence of 0.5mM malate/5mM pyruvate/ glutamate /5mM succinate followed by ADP titration (20μM, 200μM, and 2000μM). Data were normalized to fiber dry weight. OXPHOS efficiency (ATP/O ratio) was calculated by dividing the ATP production rate by the rate of atomic oxygen consumed. Permeabilized muscle fibers were prepared as mentioned above.

Mitochondrial H_2O_2 Emission Potential

Mitochondrial H_2O_2 emission potential was measured fluorometrically (FluoroLog, Horiba Jobin Yvon, Edison, NJ) in permeabilized muscle fibers using the Amplex Ultra Red/horseradish peroxidase detection system as previously described (Fisher-Wellman et al., 2015). Briefly, fibers were placed in a cuvette containing 10μM Amplex Ultra Red, 1mM EGTA, supplemented with 25U/ml superoxide dismutase to convert superoxide into H_2O_2 and 25μM blebbistatin to prevent contraction. H_2O_2 emission rate was measured under 3 substrate conditions: 1) 10mM succinate, 2) 37.5μM palmitoyl-carnitine/20μM palmitoyl-coA/5mM l-carnitine, and 3) 1mM pyruvate/ 5mM l-carnitine. For each assay, H_2O_2 emission rate was also measured in the presence of 1μM auranofin (AF), a thioredoxin reductase inhibitor, and 100μM bis-chloroethylnitrosourea (BCNU), a glutathione reductase inhibitor. Data were normalized to fiber dry weight.

Muscle Contractile Force Measurements

Extensor digitorum longus muscle (EDL) was dissected and used to assess parameters of contractile function using a force transducer (Aurora Scientific Inc.) as previously described (Spangenburg, Le Roith, Ward, & Bodine, 2008).

Silencing ERα in Human Myotubes

Primary human skeletal muscle cells (HSKMCs) were provided as a kind gift by Dr. K. Funai (East Carolina University, Department of Kinesiology). HSKMCs were from vastus lateralis muscle biopsies of healthy and obese-insulin resistant young adult women (18-24 yrs of age). HSKMCs were differentiated into myotubes using standard methods. Briefly, HSKMCs were grown in DMEM with 5mM glucose (#11885 Gibco), supplemented with 10% FBS, Lonza SkGM SingleQuot (#CC-4139, Lonza) without gentamicin and insulin, and 100mg/ml penicillin-streptomycin (Gibco). At 90% confluency, differentiation was induced using DMEM with 5mM glucose (#11885 Gibco), supplemented with 2% heat inactivated horse serum (#26050, Gibco), 0.3% bovine serum albumin (#A-9025, Sigma Aldrich), 0.05% fetuin (#F-3385, Sigma Aldrich) and 100mg/ml penicillin-streptomycin (Gibco). On the first day of differentiation, human myotubes were transduced with either scrambled-shRNA (Ad-U6-shRNA-RFP) or RFP-tagged ESR1shRNA adenovirus (Ad-RFP-U6-h-ESR1-shRNA) that targets the coding region of ERα, both purchased from Vector Biolabs. Polybrene (SCBT #SC-134220) was used to increase transduction efficiency. Adenovirus infection was confirmed by imaging myotubes using a fluorescence microscope (EVOS FL Auto Cell Imaging System). No differences in differentiation potential were observed (data not shown). In all experiments, cells at passage 4-5 were used.

Mitochondrial Respiration Measurement in Human Myotubes

On the 5th day of differentiation, O_2 consumption rate (OCR) was measured using the XF24 Extracellular Analyzer and XFe96 Extracellular Analyzers according to the manufacturer's protocols. Briefly, 50,000 HSKMCs and 20,000 HSKMCs were seeded in 24- and 96-well plates, respectively. Wells were coated with 20µg/ml ECL cell attachment matrix (#08-110, EMD Millipore). Myotubes were incubated in XF Base Medium with 1mM pyruvate, 2mM glutamine, and 10mM glucose (pH 7.4) for 1 hr at 37℃ in a non-CO_2 incubator. Then, cells were placed into the XF Analyzer at 37℃, and OCR was measured after each sequential addition of the following: 1µM oligomycin, 1µM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), and 0.5µM rotenone/ antimycin A. Results were normalized to the amount of protein in each well, which was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

Expression Analysis by Real-Time Quantitative PCR (QT-PCR)

Total mRNA was extracted from gastrocnemius muscle of mice using TRIzol (Ambion) and from human myotubes using RNeasy Mini Kit (Qiagen). Complementary DNA was synthesized from mRNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. Using Quant Studio 3 (Applied Biosystems), the following Taqman Gene Expression Assays (Thermo Fisher Scientific) were used to quantify mRNA expression: human Esr1 (Hs00174860_m1), murine Esr1 (Mm00433149_m1), murine Esr2 (Mm00599821_m1), murine Tnf (Mm03928990_m1), murine Il6 (Mm00446190_m1), murine Il1b (Mm00434228_m1), murine Cxcl2 (Mm 00436450_m1), murine Cxcl1

(Mm04207460_m1), murine Ccl2 (Mm 00441242_m1), murine Ccl3 (Mm00441259_g1), human 18S (Hs99999901_s1), murine 18S (Mm03928990_g1).

Immunoblotting

Western blotting was performed using standard methods. Proteins were extracted from tibialis anterior muscles and human myotubes with lysis buffer (.05% IGEPAL CA-630, 20mM Tris-HCl, 150mM NaCl, 1mM EDTA) supplemented with a protease inhibitor cocktail (Pierce Protease Inhibitor Mini Tablets – Thermo Fisher Scientific). Lysates were centrifuged at 800g for 15 min at 4℃. Proteins (20-80µg) were separated by SDS-PAGE then transferred to PVDF membranes. Blots were probed with the following primary antibodies: ERα (SCBT #MC-20, rabbit, 1:800), total OXPHOS Human WB Antibody Cocktail (Abcam #ab110411, mouse, 1:250), GLUT4 (Millipore Corp #07-1404, rabbit, 1:2000), hexokinase II (SCBT #SC-6521, rabbit, 1:1000), and glycogen synthase (Cell Signaling #3886, goat, 1:2000). Then, blots were incubated with the appropriate secondary antibodies: donkey anti-rabbit IgG-HRP (SCBT #SC-2313, 1:2000), goat anti-rabbit IgG (H+L) HRP (Thermo Fisher Scientific #P131460, 1:2000), donkey anti-goat IgG (H+L) HRP (Thermo Fisher Scientific #PA128664, 1:5000), or goat anti-mouse IgG (H+L) HRP (Thermo Fisher Scientific, #62-6520, 1:2000). Blots were developed using chemiluminescent substrate (Pierce™ ECL Western Blotting Substrate, Thermo Fisher Scientific and Western™ Lighting Plus Substrate Perkin Elmer).

Statistical Analysis

All data are presented as Mean ± SEM. Statistical analyses were performed using *PASW Statistics 18, Chicago, IL* statistical software. The 2-way ANOVA was used for comparisons between mice that were grouped based on genotype and diet. The 3-way mixed ANOVA was used to determine group differences (genotype and diet) over time. Two-tailed Independent and Dependent Student's t-tests were also used when appropriate. For all statistical tests, $p < .05$ was considered significant. In cases where simple 2-way interactions were determined, the Bonferroni adjustment method was used, thus the statistically significant *p*-value was adjusted for the family of comparisons, dividing $p < .05$ by the number of comparisons made (i.e. two simple 2-way interactions: $.05 \div 2 = .025$).

Results

Generation of Inducible Skeletal Muscle-Specific Estrogen Receptor-Alpha Knockout Mice

Exon 3 of the gene that codes for ERα (ESR1) was genetically deleted in the skeletal muscle of adult (10-12 week old) mice using a tissue-specific tamoxifen inducible Cre-LoxP-based approach; thereby, ablating ERα function only in skeletal muscle (Fig. 1A). Recombination of ESR1 was not detected in skeletal muscle of vehicle-treated $ERα^{flox/flox}$ x HSA-MCM (WT) mice (Fig. 1B-C). Recombination of ESR1 was detected in skeletal muscle of tamoxifen-treated $ERα^{flox/flox}$ x HSA-MCM ($ERαKO^{ism}$) mice, which was not detected in other organs (Fig. 1C). QT-PCR and western blot analyses confirmed that Esr1 mRNA and ERα protein were significantly reduced in skeletal muscle of tamoxifen-treated, but not in vehicle-treated female and male

ERα$^{flox/flox}$ x HSA-MCM mice (Fig. 1D-F). Chronic high fat diet (HFD)-fed female ERαKO^{ism} mice exhibited greater Esr1 mRNA expression than female ERαKO^{ism} mice on control (low fat) diet (CD) (Fig. 1D). In males, WT-HFD mice displayed lower Esr1 expression than WT-CD mice (Fig. 1E). Although estrogen receptor beta (Esr2) mRNA expression was greater in female ERαKO^{ism} than WT mice, regardless of diet (Fig. 1D), Esr2 expression was several thousand fold lower than Esr1 expression in gastrocnemius of mice (Fig. 1D-E). Esr2 expression was lower in gastrocnemius of male WT-HFD, ERαKO^{ism}-CD, ERαKO^{ism}-HFD than WT-CD mice (Fig. 1E). Esr1 expression was also detected across multiple skeletal muscle tissues and other organs of WT mice, was highest in murine uterus, and was several thousand lower in C2C12 myotubes compared to murine skeletal muscle (Supplementary Fig. 1). Finally, we found that the three major isoforms of ERα are localized to the nucleus in skeletal muscle (Supplementary Fig. 2).

Body Composition of ERαKO^{ism} Mice after Acute and Chronic HFD

At baseline, all groups exhibited similar body mass, fat mass, and lean mass for both females (Fig. 2A, C, E) and males (Fig. 2I, K, M). The change in body composition over time was greater in HFD-fed than CD-fed mice, but no significant differences between genotype were observed, for both females (Fig. 2B, D, F) and males (Fig. 2J, L, N). In female mice, body mass and fat mass were similar among groups after acute (1-week) exposure to HFD, and greater in HFD fed mice, regardless of genotype, after chronic (11-week) exposure to HFD (Fig. 2A, C). Lean mass of female ERαKO^{ism} mice, regardless of diet, was statistically lower than WT mice after acute HFD treatment, but was not significantly different among groups after chronic HFD treatment (Fig. 2E). In

male mice, body mass, fat mass, and lean mass were similar among the groups after acute HFD, and were greater in HFD-fed male mice after chronic HFD, regardless of genotype (Fig. 2I, K, M). Food intake was similar among the groups for both sexes over the course of the study (Fig. 2H, P). Liver and heart mass were not affected by diet or genotype (Table 1). In females, gonadal fat mass was greater in HFD-fed mice, regardless of genotype (Table 1). On the other hand, male ERαKO^{ism}-HFD mice exhibited lower gonadal fat mass than WT-HFD mice (Table 1).

Whole Body Metabolic Assessment during Acute and Chronic HFD

Indirect gas calorimetry revealed that female ERαKO^{ism} was similar to WT, regardless of diet. O_2 consumption (VO_2), CO_2 production (VCO_2), respiratory exchange ratio (RER), energy expenditure (H), and activity counts were not significantly different among groups at baseline (Fig. 3A, 4A). The acute switch from CD to HFD lowered VCO_2 during the dark cycle in female WT- and ERαKO^{ism}-HFD mice, which resulted in decreased RER (Fig. 3B). There were no significant differences in energy expenditure and activity counts among the groups during the switch from CD to HFD (Fig. 4B). Female HFD-fed mice, regardless of genotype, exhibited lower RER during the light and dark cycles after chronic HFD treatment, than CD-fed mice (Fig. 3C). Female HFD-fed mice, regardless of genotype, also exhibited greater energy expenditure and lower spontaneous cage activity after chronic HFD treatment than CD-fed mice, especially during the dark cycle (Fig. 4C).

Male ERαKO^{ism} mice, regardless of diet, exhibited higher baseline RER than WT mice during the dark cycle (Fig. 5A). There were no significant differences in baseline energy expenditure and activity counts among the male groups (Fig. 6A). The switch

from CD to HFD lowered RER in male HFD-fed mice, regardless of genotype (Fig. 5B). Notably, RER was similar between male WT-CD and ERαKO^{ism}-CD mice during the diet switch, suggesting our finding at baseline was only a transient effect (Fig. 5B). Energy expenditure and activity counts were not affected in male mice during the diet switch (Fig. 6B). Chronic HFD feeding reduced RER of male HFD-fed mice, regardless of genotype (Fig. 5C). Male ERαKO^{ism} mice, regardless of diet, exhibited minimally higher VO_2, VCO_2, and energy expenditure than the WT, especially during the dark cycle, after chronic HFD treatment (Fig. 5C, 6C). However, there were no statistical differences in spontaneous cage activity among the male groups (Fig. 6C).

Glucose Dynamics after Acute and Chronic HFD

The impact of skeletal muscle ERα ablation on glucose tolerance was assessed. Glucose tolerance was significantly different between CD- and HFD-fed female mice after acute exposure to a HFD, but no significant differences between genotype were observed (Fig. 7A). Interestingly, female ERαKO^{ism}-HFD exhibited greater glucose intolerance than WT-HFD after chronic exposure to a HFD (Fig. 7B). In male mice, the acute and chronic HFD treatments resulted in glucose intolerance in WT-HFD and ERαKO^{ism}-HFD, but there were no significant differences between genotype (Fig. 7A-B).

Skeletal muscle glucose uptake was assessed *ex vivo*, using [3H]-2-deoxy-D-glucose, after chronic HFD treatment. Basal and insulin-stimulated glucose uptake in both extensor digitorum longus (EDL) and soleus muscles were not impaired in female ERαKO^{ism}-CD and ERαKO^{ism}-HFD mice; although, muscle glucose uptake was lower in HFD-fed mice, regardless of genotype (Fig. 7C-D). After chronic HFD treatment, skeletal muscle of female ERαKO^{ism} exhibited lower GLUT4 protein content compared

to WT mice, regardless of diet (Fig. 7E). There were no significant differences in GLUT4 protein content between male WT and ERαKO^{ism} (Fig. 7E). Hexokinase and glycogen synthase protein content were similar among groups, for both females and males (Fig. 7F-G).

Skeletal Muscle Inflammation after Chronic HFD

Skeletal muscle mRNA expression of majority of the pro-inflammatory cytokines and chemokines that we measured were higher in female ERαKO^{ism} than WT mice, regardless of diet (Fig. 8). Specifically, tumor necrosis factor (TNF, also known as TNFα), interleukin-6 (IL6), chemokine (C-X-C motif) ligand-1 (CXCL1, also known as growth-regulated oncogene α, GROα), chemokine (C-C motif) ligand-3 (CCL3, also known as macrophage inflammatory protein-1-alpha, MIP-1-alpha), and chemokine (C-C motif) ligand-2 (CCL2, also known as monocyte chemoattractant protein-1, MCP-1) were significantly greater in female ERαKO^{ism} than WT mice, regardless of diet (Figure 8). Interleukin-1β (IL1B) expression was higher in female HFD-fed mice, regardless of genotype. Chemokine (C-X-C motif) ligand 2 (CXCL2, also called macrophage inflammatory protein 2-alpha, MIP2-alpha) expression was not significantly different among the groups.

Functional Assessment of Skeletal Muscle Mitochondria *In Situ* after Chronic HFD

Given that ERα is thought to play a prominent role in regulating mitochondrial function, we tested whether mitochondrial respiration in permeabilized skeletal muscle fibers was also affected by the induced *in vivo* ablation of skeletal muscle ERα. Mitochondrial respiratory capacity in the presence of pyruvate/malate/glutamate was not

reduced in permeabilized fibers of female and male ERαKO^{ism} mice, regardless of diet (Fig. 9A,C). In female mice, an increase in complex IV-supported respiration was observed in fibers of WT-HFD compared to WT-CD mice (Fig. 9A). In male mice, complex I-supported state 4 respiration was higher in HFD-fed than CD-fed mice, but there were no significant differences between genotype (Fig. 9C). Fatty acid-supported respiratory capacity (in the presence of palmitoyl-carnitine/palmitoyl-coA) was similar among the female groups (Fig. 9B). In male mice, fatty acid-supported state 4 respiration was statistically greater in the HFD-fed than CD-fed groups, but there were no significant differences between genotype (Fig. 9D). Maximal ADP-supported respiration in the presence of fatty acid substrates was also higher in fibers of WT-HFD than WT-CD male mice (Fig. 9D). Nonetheless, the data suggest that mitochondrial respiratory capacity is not affected by the induced ablation of skeletal muscle ERα nor was it decreased after 12 weeks of high fat diet treatment. The rates of ATP production (*J*ATP) and O_2 consumption (JO_2) were simultaneously measured to determine OXPHOS efficiency (defined as the molar amount of ATP produced per mole of atomic oxygen consumed, thus the ATP/O ratio) in skeletal muscle of ERαKO^{ism} mice. *J*ATP, JO_2, as well as ATP/O ratio in permeabilized fibers were not significantly different among the female groups (Fig. 10A-C). Permeabilized fibers of male HFD-fed mice, regardless of genotype, exhibited higher *J*ATP in the presence of 200μM ADP as well as higher JO_2 in the presence of 20μM ADP and 200μM ADP than CD-fed mice (Fig. 10D-E). ATP/O ratio was not significantly different between WT and ERαKO^{ism} male mice, regardless of diet (Fig. 10F). In line with our findings on mitochondrial ATP production and OXPHOS efficiency *in situ*, contractile force and fatigue susceptibility

were not affected in female and male ERαKO^{ism} mice, regardless of diet (Supplementary Fig. 3). Furthermore, permeabilized fibers of female and male ERαKO^{ism} mice did not exhibit any significant differences in H_2O_2 emitting potential in the presence of succinate (Fig. 11A,D), palmitoyl-carnitine/palmitoyl-coA (Fig. 11B,E), or pyruvate/carnitine (Fig. 11C,F) compared to their WT counterparts, regardless of diet. For both sexes, JH_2O_2 increased in the presence of AF/BCNU in all substrate conditions; however, there were no significant differences in AF/BCNU response among the groups (Fig. 11A-F).

Mitochondrial Respiration in Human Myotubes with Reduced ERα Function

In an attempt to reconcile our investigation on the role of skeletal muscle ERα in mitochondrial function with a previous publication (Ribas et al., 2016), we measured mitochondrial respiration in human myotubes with significantly reduced ERα. Myotubes were cultured from primary human skeletal muscle cells isolated from muscle biopsies of female healthy and obese-insulin resistant (OIR) young adult women. Healthy women exhibited lower body weight, body mass index, fasting insulin and glucose levels, HOMA-IR, and higher maximal O_2 consumption (VO_2max) compared to OIR women (Table 2). Myotubes were transduced with an adenovirus expressing RFP-tagged ERαshRNA (ERαKD). Control myotubes expressed scrambled shRNA (Scrmbl). Transduction efficiency was similar between Scrmbl and ERαshRNA myotubes (Fig. 12A). QT-PCR revealed that Esr1 (ERα) mRNA expression was significantly reduced in ERαKD myotubes compared to the Scrmbl condition (Fig. 12B). Mitochondrial respiration was not significantly different between the healthy- and OIR-Scrmbl myotubes (Fig. 12C). In myotubes from healthy subjects, ATP production rate was

minimally reduced by 16% in the ERαKD group compared to the Scrmbl group (Fig. 12D). ERαKD myotubes from OIR subjects also exhibited statistically lower ATP production rate (~18%) in addition to the slightly lower basal O_2 consumption rate (~16%) compared to the Scrmbl condition (Fig. 12E). These results were not due to alterations in protein content of the electron transport system complexes (Fig. 12F). No significant differences in uncoupled oxygen consumption rate were found between control and ERαKD myotubes from either healthy or obese groups (Fig. 12D-E).

Discussion

ERα is expressed in multiple tissues, and emerging evidence suggest that skeletal muscle-specific ERα has important roles in maintaining metabolic homeostasis (Ribas et al., 2010, 2016). However, available data from global (R. Barros et al., 2009, 2006; Ribas et al., 2010) and conventional skeletal muscle-specific ERα knockout models (Ribas et al., 2016) may be confounded by factors associated with the loss of ERα during development. To overcome this limitation, we generated an inducible skeletal muscle-specific ERα knockout mouse. Our results confirmed that induced ablation of skeletal muscle-specific ERα increased the susceptibility to develop glucose intolerance in adult female mice on chronic HFD treatment. Skeletal muscle GLUT4 protein content, the major glucose transporter in skeletal muscle, was lower in female ERαKO^{ism} than WT mice, regardless of diet. We observed sex differences in glucose tolerance and skeletal muscle GLUT4 protein expression as well. Glucose tolerance and GLUT4 protein content were similar between diet-matched ERαKO^{ism} and WT male mice, suggesting that skeletal muscle ERα function in the regulation of glucose homeostasis is critical in females, but not in males. Furthermore, we found that total

body fat, gonadal fat, and gain in fat mass over time were not greater in diet-matched ERαKO^{ism} than WT mice, yet skeletal muscle of female ERαKO^{ism} mice, regardless of diet, exhibited greater expression of key pro-inflammatory cytokines, TNF, IL6, and chemokines CCL2, CCL3 compared to WT mice. Collectively, our data suggest that induced ablation of skeletal muscle-specific ERα in adult female mice increased the susceptibility to develop skeletal muscle inflammation and reduced GLUT4 protein levels, independent of adiposity, which may have contributed to the glucose intolerance under chronic lipid overload.

Our findings support the notion that estrogens play a role in preventing local inflammation in the skeletal muscle. Similar ideas have been seen in literature using other models and other tissues. For instance, exogenous estradiol treatment for 1 month has been shown to lower the expression of circulating serum pro-inflammatory cytokines IL1B, IL8, and IL12 in young regularly menstruating obese women (Al-Safi et al., 2015). Conversely, reduced circulating estrogens by ovariectomy in mice results in greater expression of intraabdominal adipose pro-inflammatory markers CCL2, F4/80, CD11C, IL12p40, and IFNγ than sham-operated mice at 26 weeks post-surgery, which is associated with lower insulin sensitivity but is not due to excess adiposity (Vieira Potter et al., 2012). ERα has been shown to mediate estrogen-induced reduction of local inflammation in lung tissue, as indicated by the greater expression of ICAM-1 and TNF in global ERαKO mice compared to WT mice (Vegeto et al., 2010). Our results are also similar with previous studies, which have shown that global ERαKO and conventional skeletal muscle-specific ERαKO (MERKO) mice exhibit increased expression of pro-inflammatory markers, TNF and p-JNK in skeletal muscle; although,

global ERαKO and MERKO mice also display greater visceral adiposity than WT mice (Ribas et al., 2010, 2016). Indeed, elevated levels of TNF, IL6, and CCL2 are associated with lipid-induced insulin resistance and are consistently observed in skeletal muscle of non-diabetic obese insulin resistant and Type 2 diabetic individuals (Khan et al., 2015; Wu & Ballantyne, 2017). Moreover, studies in myocytes have demonstrated that elevated TNF and CCL2 expression further increases skeletal muscle infiltration of immune cells, which originates from intermuscular and perimuscular adipose depots (Wu & Ballantyne, 2017). These local skeletal muscle pro-inflammatory factors are known to exert paracrine effects, which results in decreased insulin signaling and glucose uptake (Wu & Ballantyne, 2017). Taking this into consideration, our data, which show that *ex vivo* skeletal muscle glucose uptake is similar between ERαKO^{ism} mice and WT mice, may be attributed to the loss of paracrine effects due to the nature of the experiment.

Our data also indicate that CXCL1 expression is higher in skeletal muscle of female ERαKO^{ism} than WT mice, and this upregulation of CXCL1 may have contributed to the regulation of adiposity. A previous study has demonstrated that visceral and subcutaneous fat mass as well as body fat percentage are lower in mice with overexpressed skeletal muscle-specific CXCL1 compared to WT mice (Pedersen, Olsen, Pedersen, & Hojman, 2012). Indeed, our findings suggest that induced ablation of skeletal muscle-specific ERα in adult female mice does not exacerbate body mass and fat mass gain over the 12-week HFD treatment period. However, ERα appears to modulate CXCL1 expression in a cell-specific manner. In contrast to our findings in skeletal muscle, ERα has been documented to regulate CXCL1 in uterine epithelial

cells, such that inhibition of ERα decreases CXCL1 expression (Hickey, Fahey, & Wira, 2013). Thus, future studies need to consider the possible regulation of CXCL1 expression by estrogens in skeletal muscle.

Previous studies have suggested that skeletal muscle mitochondrial function is important for maintaining insulin sensitivity (E. J. Anderson et al., 2009; Fisher-Wellman & Neufer, 2012) and that skeletal muscle ERα may be critical for maintaining mitochondrial function (Ribas et al., 2016). Specifically, insulin resistance can develop as a result of elevations in mitochondrial H_2O_2 levels, a major form of reactive oxygen species (ROS) in mitochondria, and a reduction in mitochondrial respiratory capacity, which can occur with chronic overnutrition (E. J. Anderson et al., 2009; Fisher-Wellman & Neufer, 2012). ERα is thought to regulate mitochondrial function by direct and indirect mitochondrial DNA-encoded gene transcription of several Complex I-V subunits in the electron transport system (Klinge, 2017). For instance, MCF-7 (ERα positive) breast cancer cells have mtDNA containing estrogen response element-like sequences, which ERα binds to in the presence of estradiol, with a dose- and time-dependent increase in binding affinity (J. Chen et al., 2009; Jin Q. Chen et al., 2004). ERα has also been reported to induce the transcription of certain mtDNA-encoded Complex I-V subunits in the electron transport system (ETS) (J. Chen et al., 2009; Jin Qiang Chen et al., 2005). Studies in MCF-7 breast cancer cells and H1793 lung adenocarcinoma cells have also demonstrated that ERα interacts with the estrogen response element-like sequence on nuclear respiratory factor-1 (NRF-1) (Mattingly et al., 2008), a nuclear transcription factor known to directly increase the expression of key nuclear DNA-encoded subunits in ETS (Scarpulla, 2012). NRF-1 also indirectly upregulates the expression of several

mtDNA-encoded subunits in ETS via activation of Transcription Factor A, Mitochondrial (TFAM) (Scarpulla, 2012). A recent study has also demonstrated that ablation of skeletal muscle ERα (MERKO mice), where ERα function is lost during embryonic development, leads to a reduction insulin sensitivity and impairment in aspects of mitochondrial function, specifically mitochondrial fission and fusion dynamics, mitochondrial calcium retention capacity, and total ROS (mitochondrial and non-mitochondrial) regulation by 7 months of age (Ribas et al., 2016). Furthermore, basal and maximal respiratory capacity, ATP synthesis, and palmitate oxidation rate are drastically (~50%) lower in C2C12 myotubes transduced with ERαshRNA compared to scramble myotubes (Ribas et al., 2016). In contrast, our data indicate that induced ablation of skeletal muscle ERα in adult female and male mice does not affect mitochondrial function. Mitochondrial respiratory capacity, H_2O_2 emission potential, ATP production rate, and OXPHOS efficiency in the presence of carbohydrate and fatty acid substrates in permeabilized fibers of female and male ERαKO^{ism} mice are not significantly different from diet-matched WT mice, even after chronic HFD. Our data suggest that fat oxidation is not reduced in ERαKO^{ism} mice, which may also explain why adiposity is not negatively affected in female and male ERαKO^{ism} mice. Energy expenditure is also unaltered in female ERαKO^{ism} mice and is only slightly increased in male ERαKO^{ism} mice at ~8 months of age. In line with our inducible ERα knockout mouse model, ERα knockdown in human myotubes isolated from adult healthy and obese-insulin resistant women only minimally reduces ATP production rates. Overall, our findings highlight that skeletal muscle ERα action in adult mice is not critical for maintaining adiposity and skeletal muscle mitochondrial function. We speculate that the

loss of skeletal muscle ERα function is not the primary mechanism, which underlies the increased susceptibility for excess adiposity associated with natural and surgical menopause.

The results of the present study are not likely due to a meaningful compensatory increase in ERβ expression. Although, ERβ expression is higher in skeletal muscle of female ERαKO^{ism} than WT mice, ERα expression is several thousand-fold higher than ERβ. At present, whether GPER is a true estrogen receptor is still unclear (Levin, 2009a), thus we did not assess GPER expression in our mice.

Our findings are largely in contrast to previous studies, which have documented that global ERαKO and conventional skeletal muscle-specific ERαKO (MERKO) mice on control (low fat) diet display greater gonadal adiposity, systemic and local skeletal muscle insulin resistance, and altered mitochondrial health (R. Barros et al., 2009, 2006, Ribas et al., 2010, 2016). Given that global ERαKO and MERKO mice (which are built using the muscle creatine kinase promoter to drive Cre expression) are generated in a manner where ERα function is ablated during the embryonic stage of development, the disparity between our data and previous findings suggest that ERα is essential for development, but not nearly as critical for maintaining metabolic function post-development. Several lines of evidence support this observation. First, estrogens are known to play major roles in cell proliferation and organ differentiation during fetal development (Bondesson, Hao, Lin, Williams, & Gustafsson, 2015; Kaludjerovic & Ward, 2012), including skeletal muscle (Byrne et al., 2010). One study on ovine skeletal muscle development has shown that during the transition from late fetal period to 1-3 days postpartum, muscle oxidative capacity increases in conjunction with the

upregulation of a multitude of genes, including those that contained ERα cis-regulatory motifs (Byrne et al., 2010). Second, reduced estrogen action alters fetal developmental programming (Abbott, Padmanabhan, & Dumesic, 2006) and affects metabolic homeostasis that manifests later in life. A recent study has documented that 3-year old offspring of pregnant baboons injected with the aromatase inhibitor, letrozole, exhibit insulin resistance, as reflected by greater fasting glucose, fasting insulin, glucose intolerance, and HOMA-IR than both untreated offspring and offspring of baboons injected with letrozole plus estradiol (Maniu et al., 2016). Insulin resistance persists in offspring of baboons injected with letrozole through 6 years of age (Pepe et al., 2016). We speculate that skeletal muscle ERα is critical for mediating estrogen function during fetal developmental programming given that ERα ablation in global ERαKO and MERKO mice also results in marked insulin resistance (Ribas et al., 2010, 2016). Third, adaptive/redundant mechanisms can maintain homeostasis. Researchers have previously proposed that such mechanisms may explain disparities between loss-of-function since birth and loss-of-function in adult mice (Luquet, Perez, Hnasko, & Palmiter, 2005; Qiu et al., 2017). For example, *in vivo* neonatal ablation of neuropeptide Y (NPY)/agouti-related protein (AgrP) in neurons of mice results in slightly lower food intake than the age-matched WT, whereas induced *in vivo* NPY/AgrP ablation in neurons of adult (12-week old) mice leads to a major reduction in food consumption and an immediate ~20% drop in body mass in 2 days (Luquet et al., 2005). The authors have suggested that a network-based compensatory mechanism may be present in neonates; albeit, the nature of adaptive changes are still unknown (Luquet et al., 2005). In the present study, we speculate that estrogen receptor-independent mechanisms

may have played a role in maintaining adiposity and skeletal muscle mitochondrial function in adult ERαKO^{ism} mice. Indeed, a recent study has demonstrated that ovariectomy reduces skeletal muscle mitochondrial respiratory capacity and increases mitochondrial H_2O_2 emission potential, whereas 17β-estradiol can localize in mitochondrial membranes in the absence of ERα and functions to enhance membrane fluidity, which in turn, optimizes mitochondrial function and redox homeostasis (Torres et al., 2018). Future studies are needed to directly define adaptive or redundant mechanisms that occur with the loss of skeletal muscle ERα function in adults.

Collectively, our data suggest that skeletal muscle ERα in adult female mice is critical for preventing excessive elevations in skeletal muscle inflammation and preserving GLUT4 levels, which in part, contribute to the maintenance of glucose tolerance under chronic lipid overload. Furthermore, induced ablation of ERα in adult skeletal muscle does not exacerbate fat mass gain nor detrimentally affect mitochondrial function. These findings suggest that adaptive or redundant mechanisms are likely established by the time ERα function is lost in adult skeletal muscle. Indeed, our work also brings light to the importance of investigating the function of genes without the confounding factors of development.

Table 1. *Tissue Mass of WT and ERαKO^{ism} Mice after Chronic (12-week) HFD*

	Females				Males			
	WT-CD	WT-HFD	KO-CD	KO-HFD	WT-CD	WT-HFD	KO-CD	KO-HFD
Heart (mg)	118 ± 4	122 ± 3	114 ± 4	119 ± 7	143 ± 6	143 ± 8	139 ± 6	150 ± 15
Liver (mg)	1186 ± 66	1257 ± 71	1239 ± 75	1138 ± 96	1557 ± 179	2506 ± 455	1824 ± 221	1717 ± 254
Gonadal fat (mg)	1037 ± 115	2908 ± 345*	1317 ± 209	2960 ± 458*	1553 ± 87	3460 ± 250*	1898 ± 190	2829 ± 100*†

Data are means ± SEM. *Main effect of diet, $p<.05$ within each sex. †Significant difference between male WT-HFD and ERαKO^{ism}-HFD mice, $p<.05$. Females: n=6-8/group, Males: n=4-6/group.

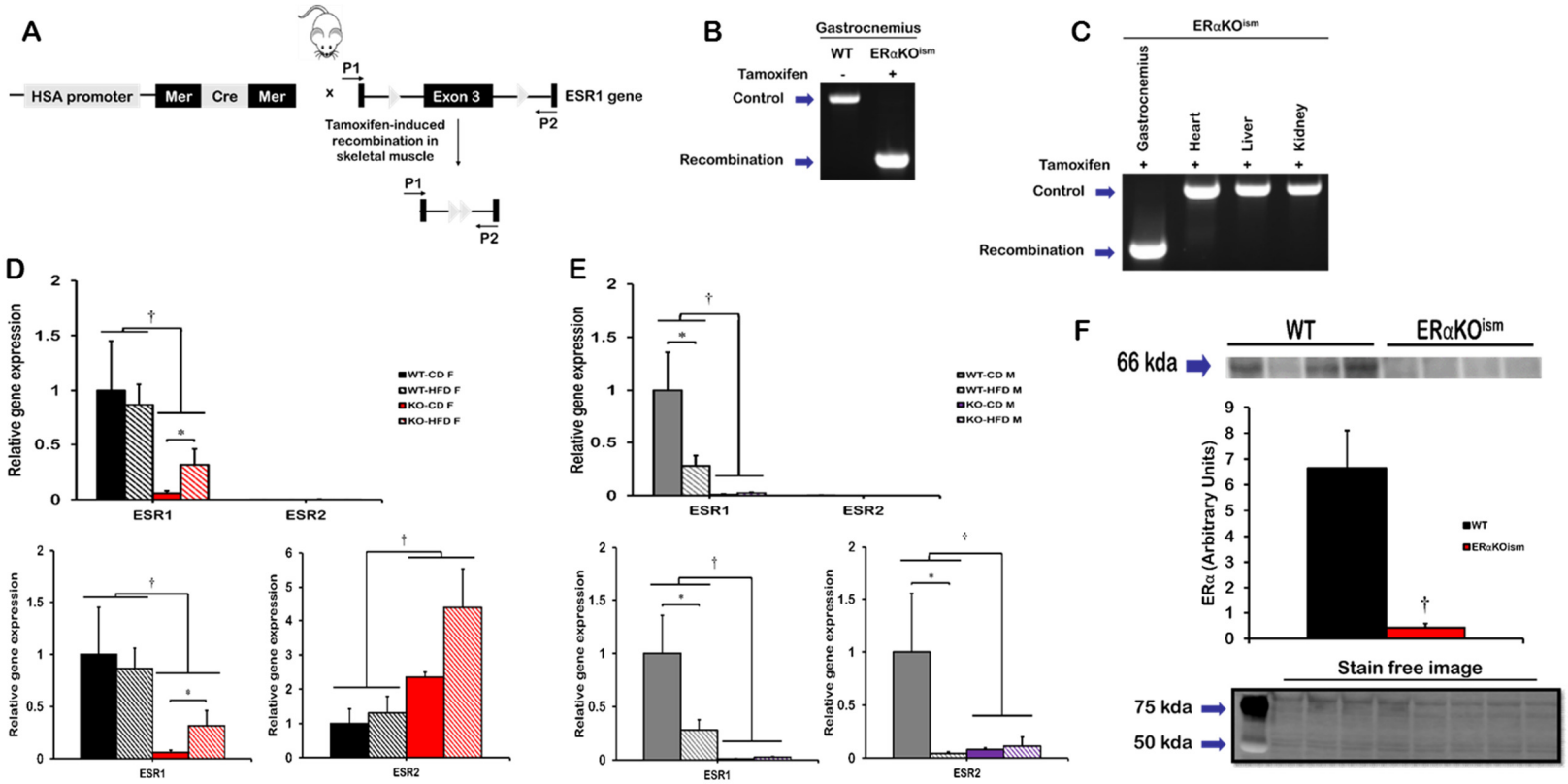


Figure 1. Characterization of a novel inducible skeletal muscle-specific ERαKO mouse
(A) Mice that possessed ERα$^{flox/flox}$ x HSA-MCM were used in this study. Exon 3 of ESR1 was deleted upon Cre activation, subsequently inactivating ERα function. (B-C) Recombination of ESR1 in skeletal muscle of vehicle-injected (WT) and tamoxifen-injected ERα$^{flox/flox}$ x HSA-MCM (ERαKO^{ism}) mice. (D) Esr1 and Esr2 mRNA expression normalized to 18s rRNA in gastrocnemius of female WT and ERαKO^{ism} mice. (E) Esr1 and Esr2 mRNA expression normalized to 18s rRNA in gastrocnemius of male WT and ERαKO^{ism} mice. (F) ERα protein content in tibialis anterior of WT and ERαKO^{ism} mice. Data are means ± SEM. *Simple main effect of diet, p<.05. †Main effect of genotype, p<.05. Females: n= 6/group, Males: n= 5-6/group.

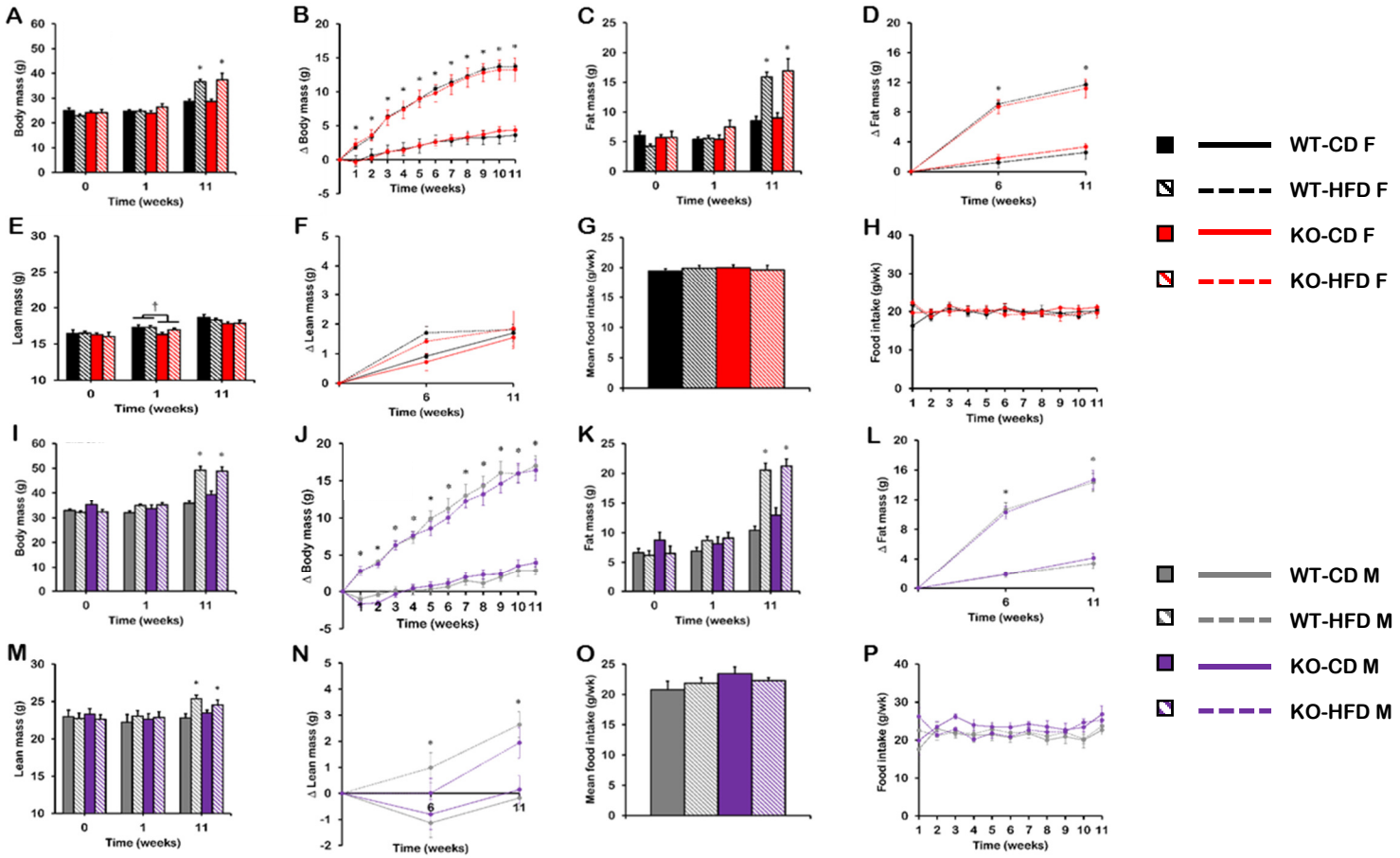


Figure 2. Induced ablation of skeletal muscle ERα in adult mice had no effect on adiposity
(A,I) Body mass, (B,J) Change in body mass over time, (C,K) Total fat mass, (D,L) Change in total fat mass over time, (E,M) Total lean mass, (F,N) Change in total lean mass over time, (G,O) Average food intake over the course of the study, and (H,P) Average food intake per week of female (A-H) and male (I-P) WT and ERαKO^{ism} mice fed a control (low fat) diet (CD) or high fat diet (HFD). Data are means ± SEM. *Main effect of diet, p<.05. †Main effect of genotype, p<.05. Females: n=8/group, Males: n=6-8/group.

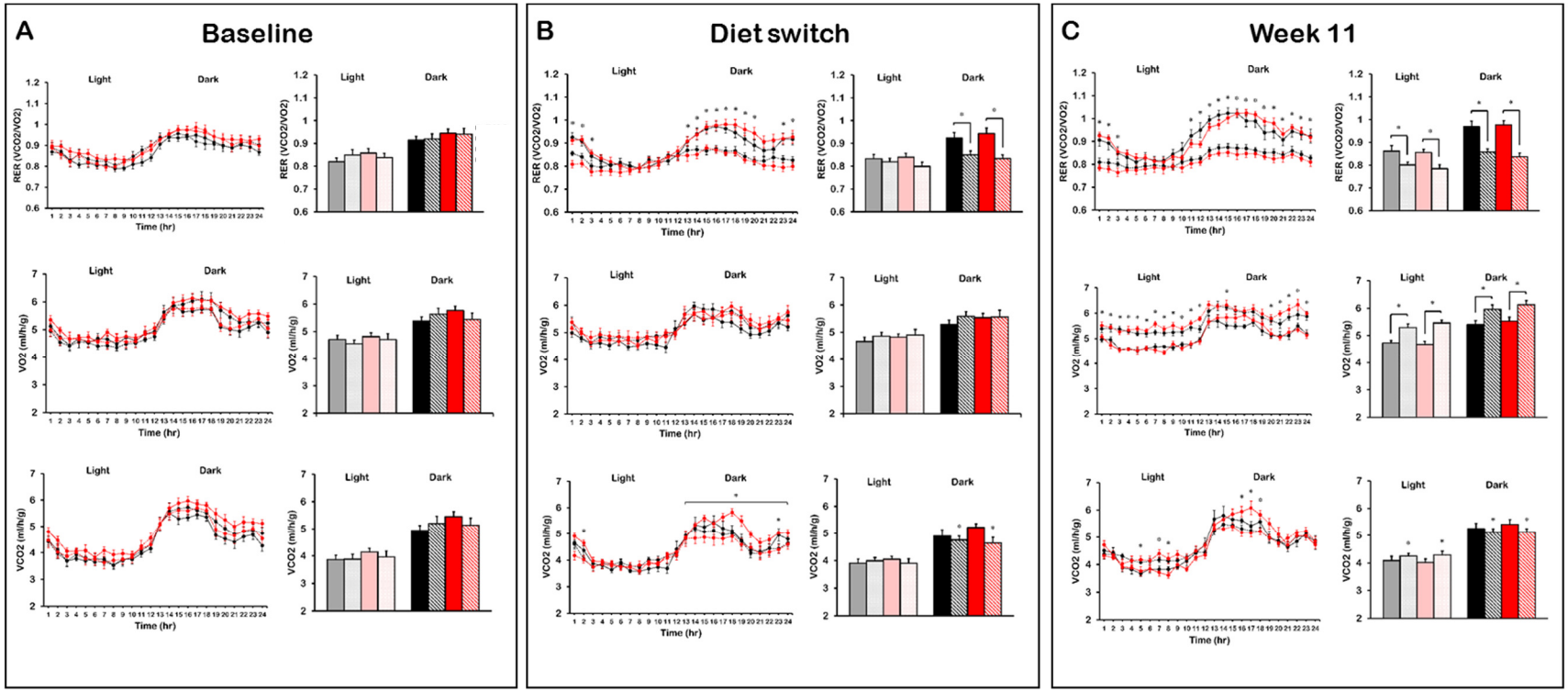


Figure 3. Induced ablation of skeletal muscle ERα in adult female mice had no effect on respiratory exchange ratio, whole body O_2 consumption, and CO_2 production

Whole body calorimeter measurements of respiratory exchange ratio (RER), whole body O_2 consumption (VO_2), and CO_2 production (VCO_2) at (A) baseline, (B) during the diet switch (first 2-3 days of HFD), and (C) after chronic HFD. Data are means ± SEM. *Main effect and simple main effects of diet, p<.05. Females: n=7-10/group.

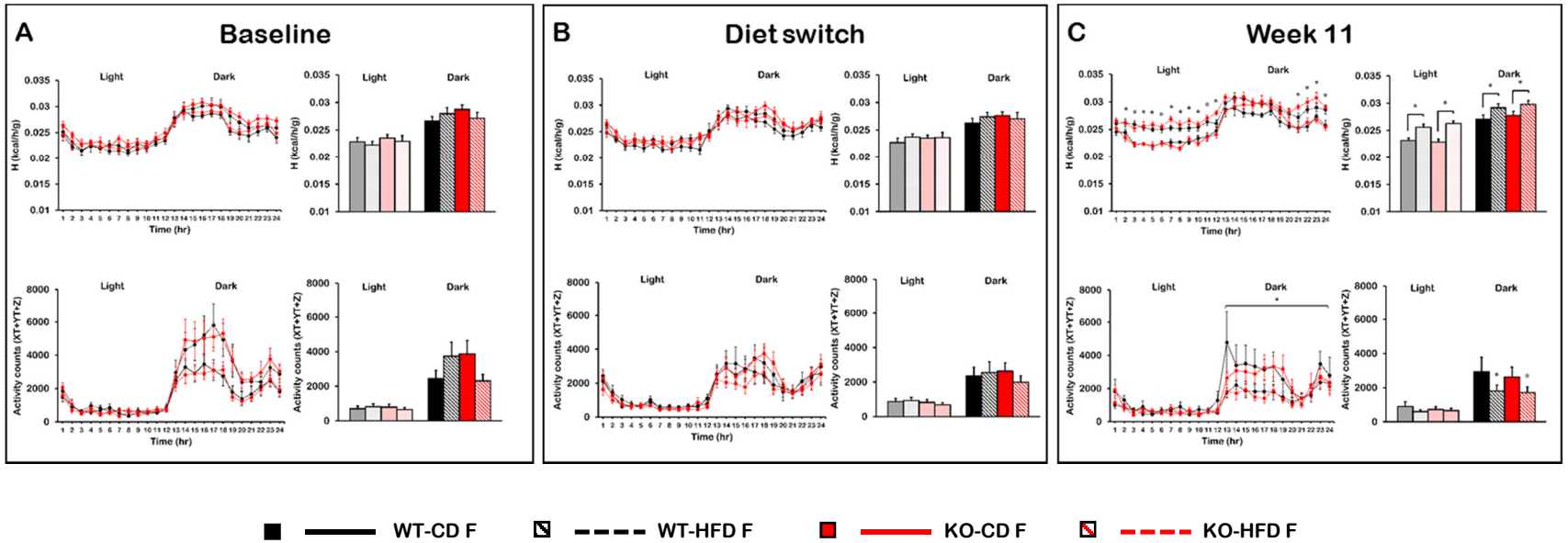


Figure 4. Induced ablation of skeletal muscle ERα in adult female mice had no effect on energy expenditure and spontaneous cage activity

Whole body calorimeter measurements of energy expenditure (H) and activity counts at (A) baseline, (B) during the diet switch (first 2-3 days of HFD), and (C) after chronic HFD. Data are means ± SEM. *Main effect of diet, p<.05. Females: n=7-10/group.

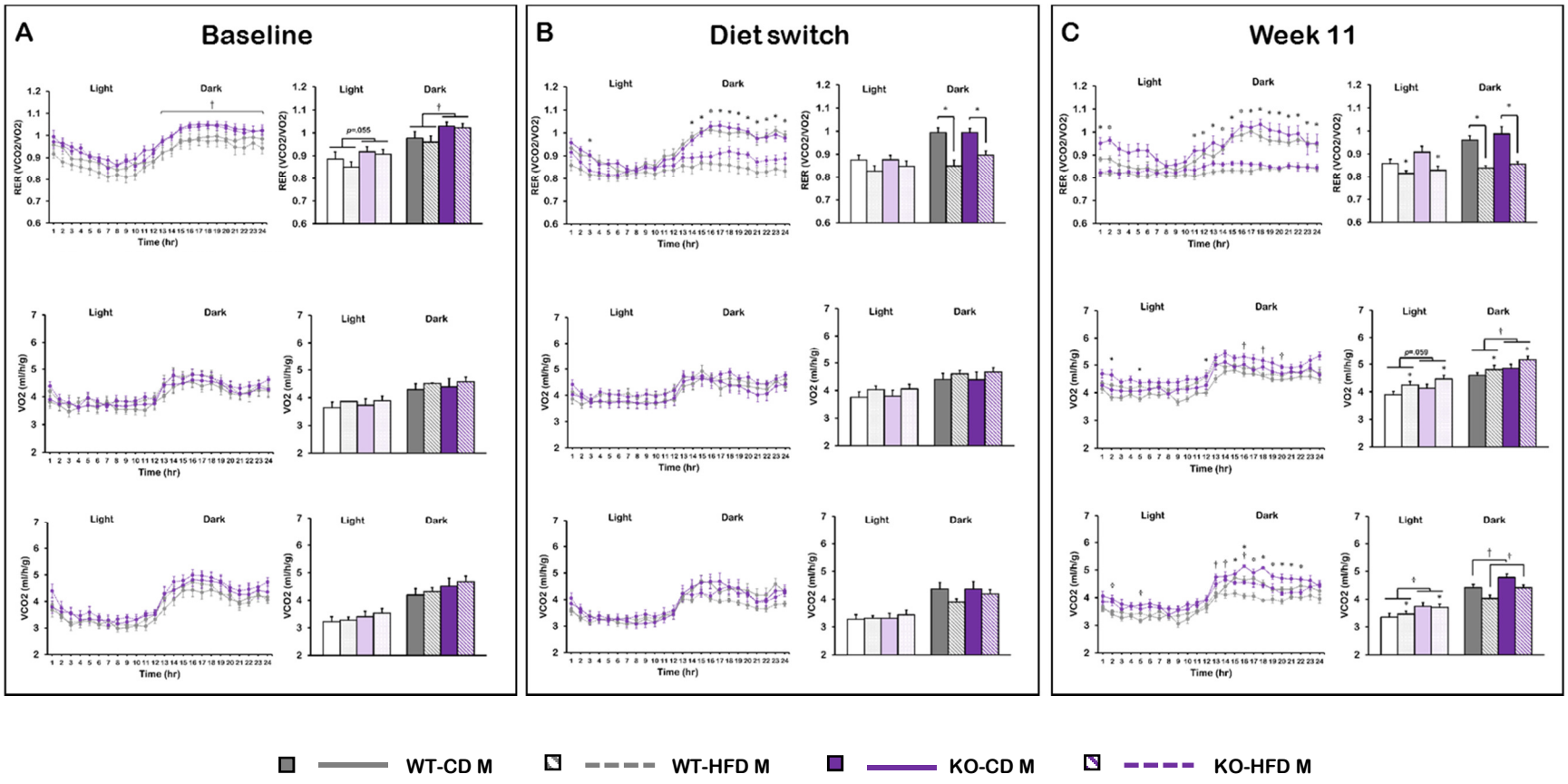


Figure 5. Induced ablation of skeletal muscle ERα in adult male mice minimally altered whole body O_2 consumption and CO_2 production after chronic HFD

Whole body calorimeter measurements of respiratory exchange ratio (RER), whole body O_2 consumption (VO_2), and CO_2 production (VCO_2) at (A) baseline, (B) during the diet switch (first 2-3 days of HFD), and (C) after chronic HFD. Data are means ± SEM. *Main effect of diet, $p<.05$. †Main effect and simple main effects of genotype, $p<.05$. Males: n=5-8/group.

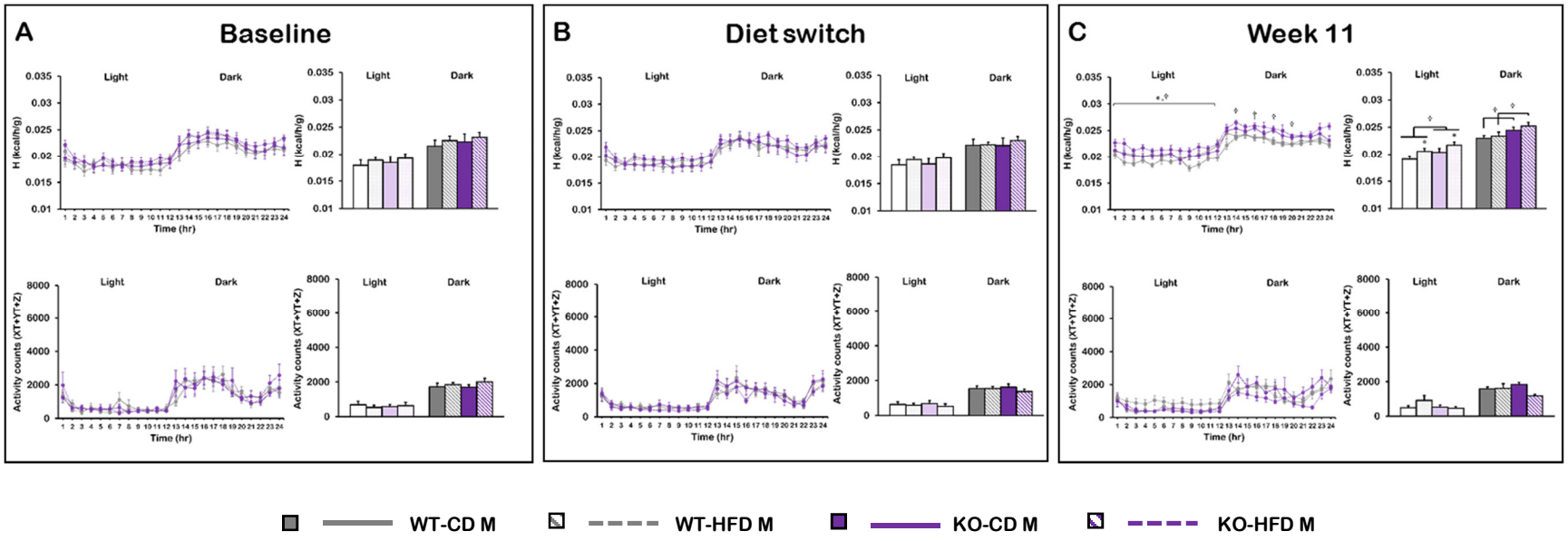


Figure 6. Induced ablation of skeletal muscle ERα in adult male mice minimally altered energy expenditure after chronic HFD

Whole body calorimeter measurements of energy expenditure (H) and activity counts at (A) baseline, (B) during the diet switch (first 2-3 days of HFD), and (C) after chronic HFD. Data are means ± SEM. *Main effect of diet, $p<.05$. †Main effect and simple main effects of genotype, $p<.05$. Males: n=5-8/group.

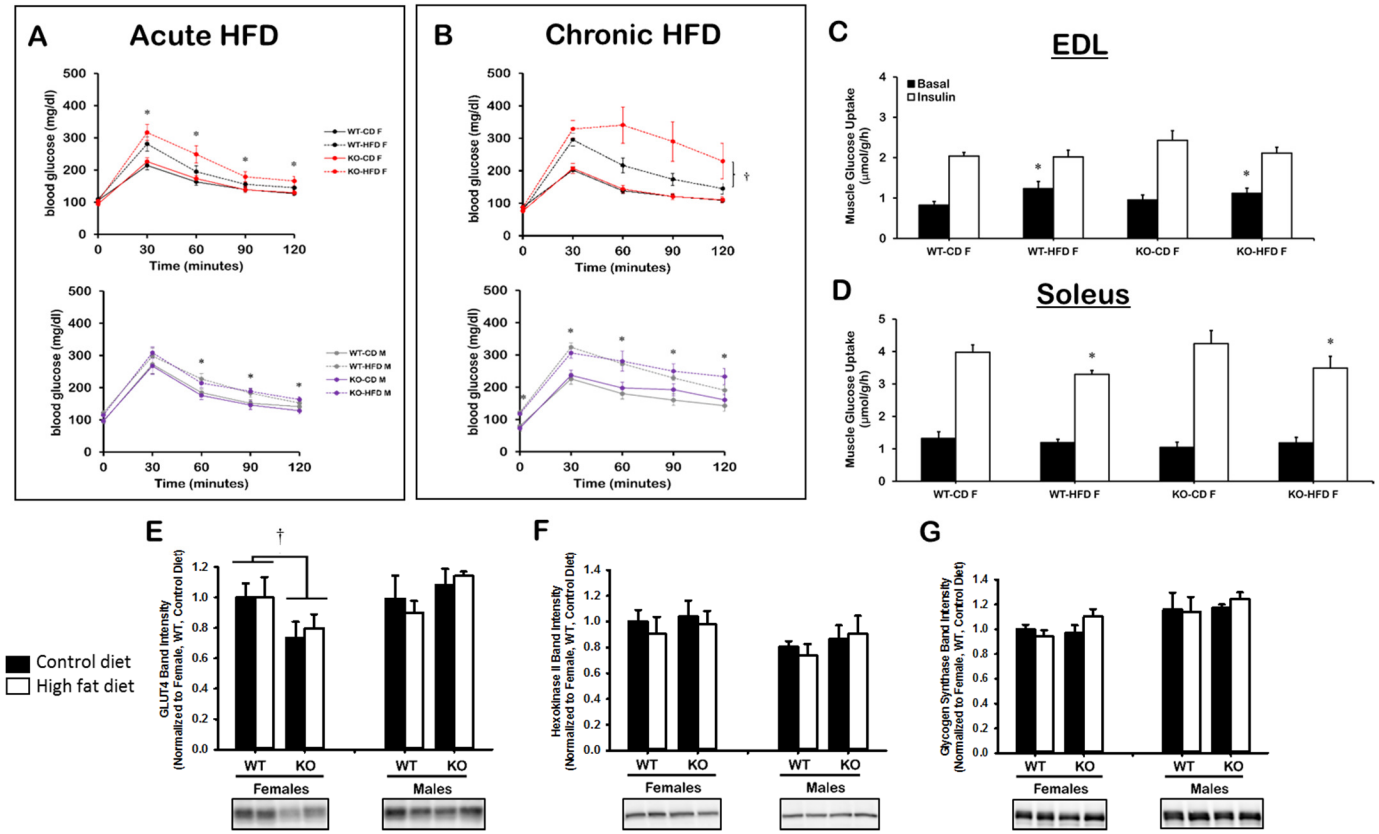


Figure 7. Induced ablation of skeletal muscle ERα in adult female mice increased the susceptibility to develop glucose intolerance after chronic HFD treatment that is associated with reduced skeletal muscle GLUT4 protein content

Glucose tolerance of female and male WT and ERαKO^{ism} after (A) acute HFD (1 week) and (B) chronic HFD (11 weeks). Females: n=6-9/group, Males: n=5-7/group. *Ex vivo* basal and insulin-stimulated skeletal muscle glucose uptake in (C) extensor digitorum longus (EDL) and (D) soleus muscles of female WT and ERαKO^{ism} mice after chronic HFD. Females: n=6-8/group. Western blots for tibialis anterior muscle protein content of (E) glucose transporter-4 (GLUT4), (F) hexokinase II, and (G) glycogen synthase. Females: n=5/group, Males: n=4-/group. Data are means ± SEM. *Main effect of diet, p<.05. †Main effect of genotype, p<.05.

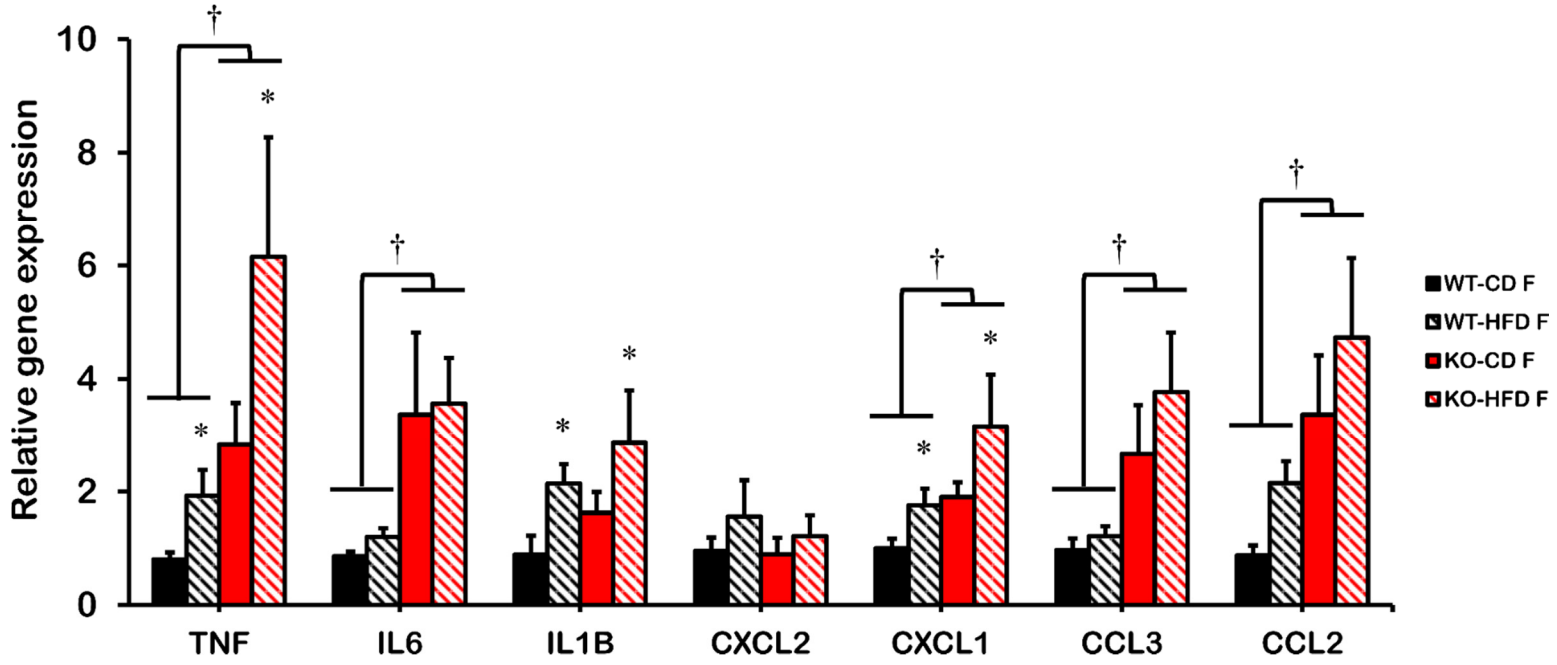


Figure 8. Induced ablation of skeletal muscle ERα in adult female mice alters the gene expression of inflammatory markers in skeletal muscle

Key pro-inflammatory cytokines and chemokines in gastrocnemius of female WT and ERαKO^{ism} after chronic HFD. mRNA expression was measured by real-time PCR and normalized to 18s rRNA. TNF = tumor necrosis factor; IL6 = interleukin-6; IL1B = interleukin-1β; CXCL2 = chemokine (C-X-C motif) ligand 2; CXCL1 = chemokine (C-X-C motif) ligand 1; CCL3 = Chemokine (C-C motif) ligand 3; Chemokine (C-C motif) ligand 2. Data are means ± SEM. *Main effect of diet, p<.05. †Main effect of genotype, p<.05. Females: n=7-9/group.

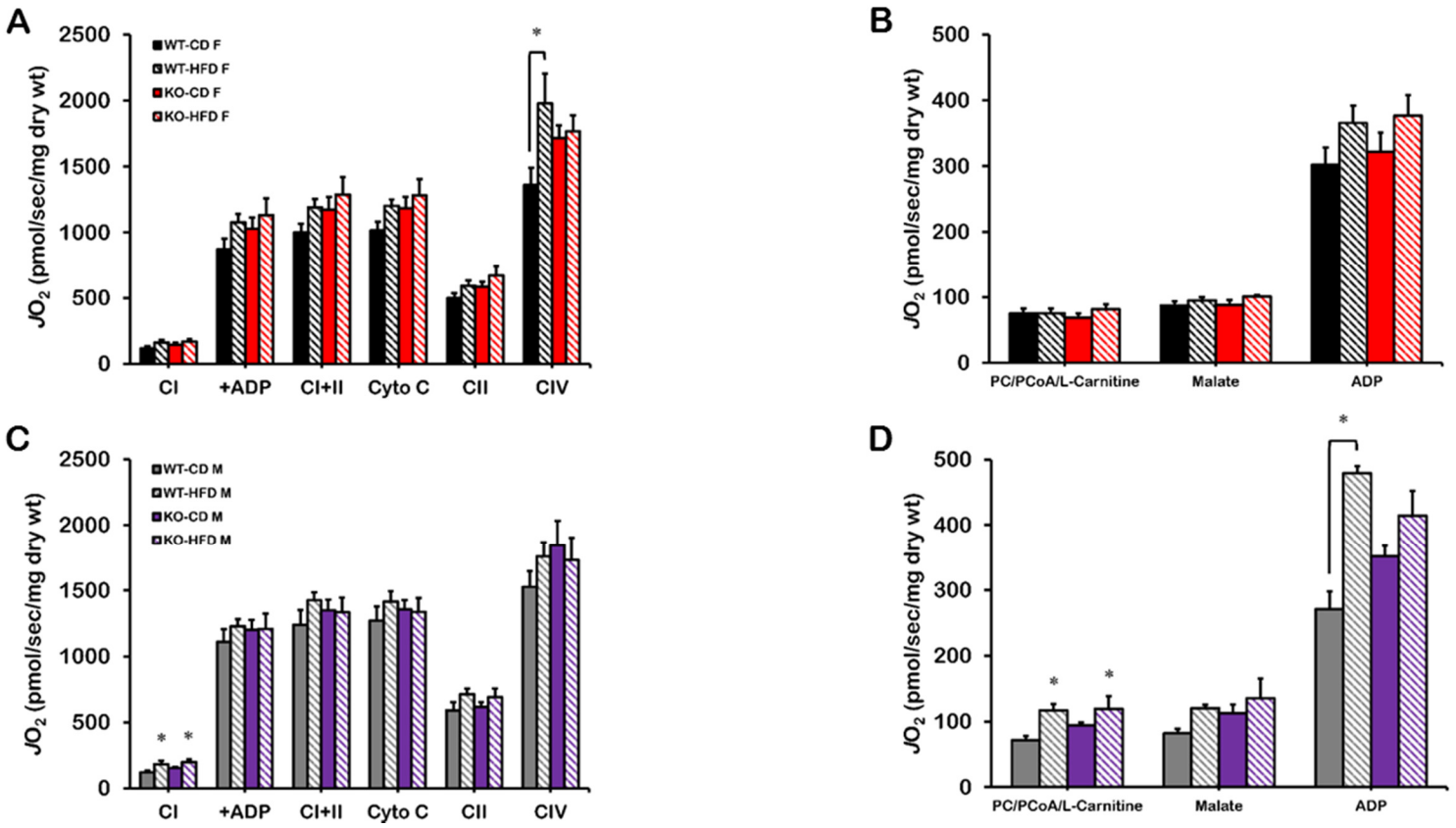


Figure 9. Induced ablation of skeletal muscle ERα in adult mice did not alter mitochondrial respiratory capacity even after chronic HFD

Oxygen consumption rate (JO_2) in permeabilized muscle fibers of WT and ERαKO^{ism} CD- and HFD-fed female (A-B) and male mice (C-D). Carbohydrate (pyruvate/malate/glutamate)-supported JO_2 (A,C). Fatty acid (palmitoyl-carnitine/palmitoyl-coA/l-carnitine)-supported JO_2 (B,D). CI = Complex I; CII = Complex II; CI+II = Complex I + Complex II; CIV = Complex IV; Cyto c = cytochrome c; wt = weight. Data are mean ± SEM. *Main effect and simple main effects of diet, $p<.05$. Females: n=6-8/group. Males: n=4-6/group.

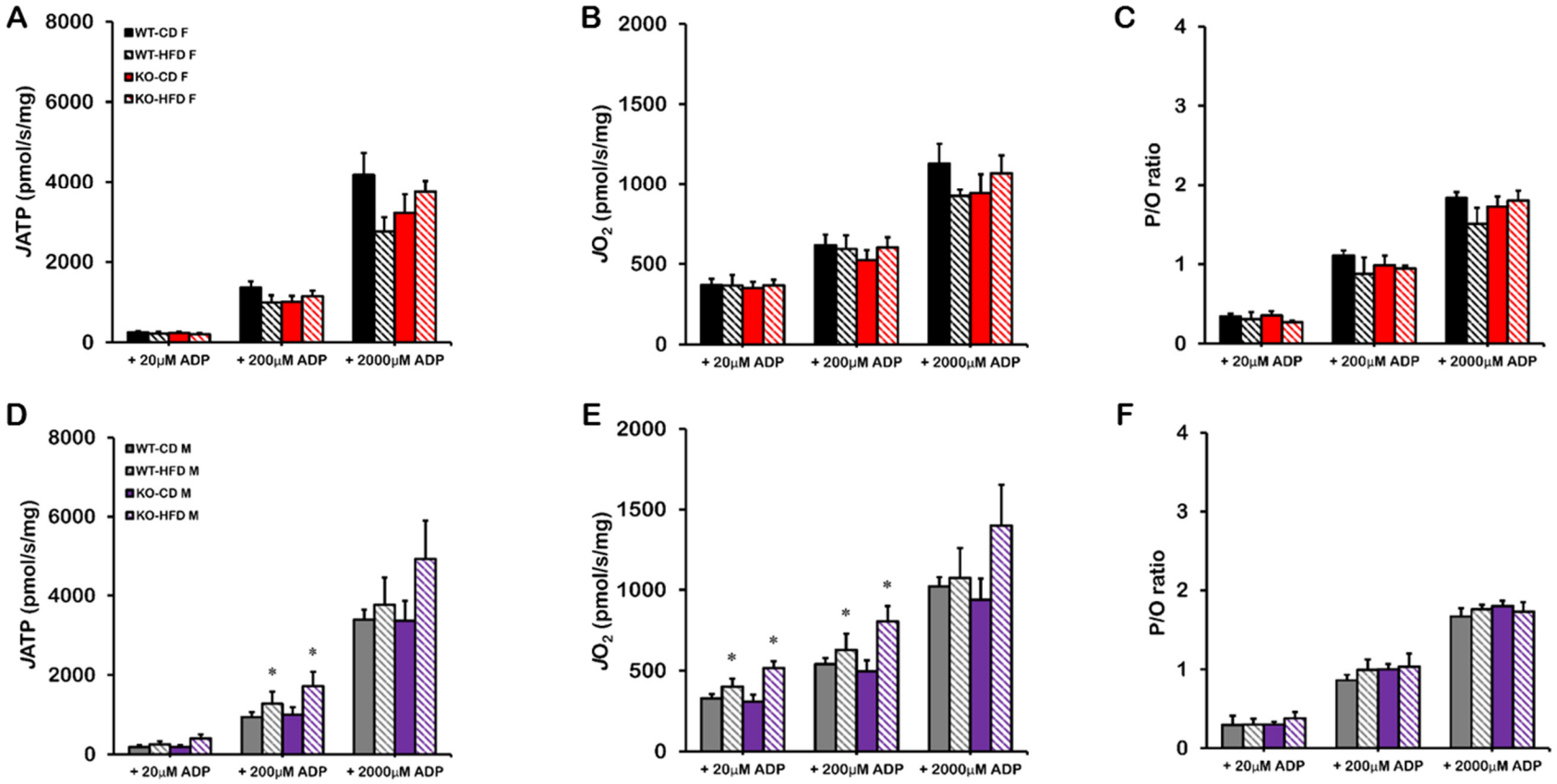


Figure 10. Induced loss of skeletal muscle ERα in adult mice did not affect ATP production rate and OXPHOS efficiency

ATP/O ratio in permeabilized muscle fibers of WT and ERαKO^{ism} CD- and HFD-fed female (A-C) and male mice (D-F) mice. ATP production rate (A,D), O_2 consumption rate (B,E), and ATP/O ratio (C,F). Data are mean ± SEM

*Main effect of diet, $p<.05$. wt, weight.

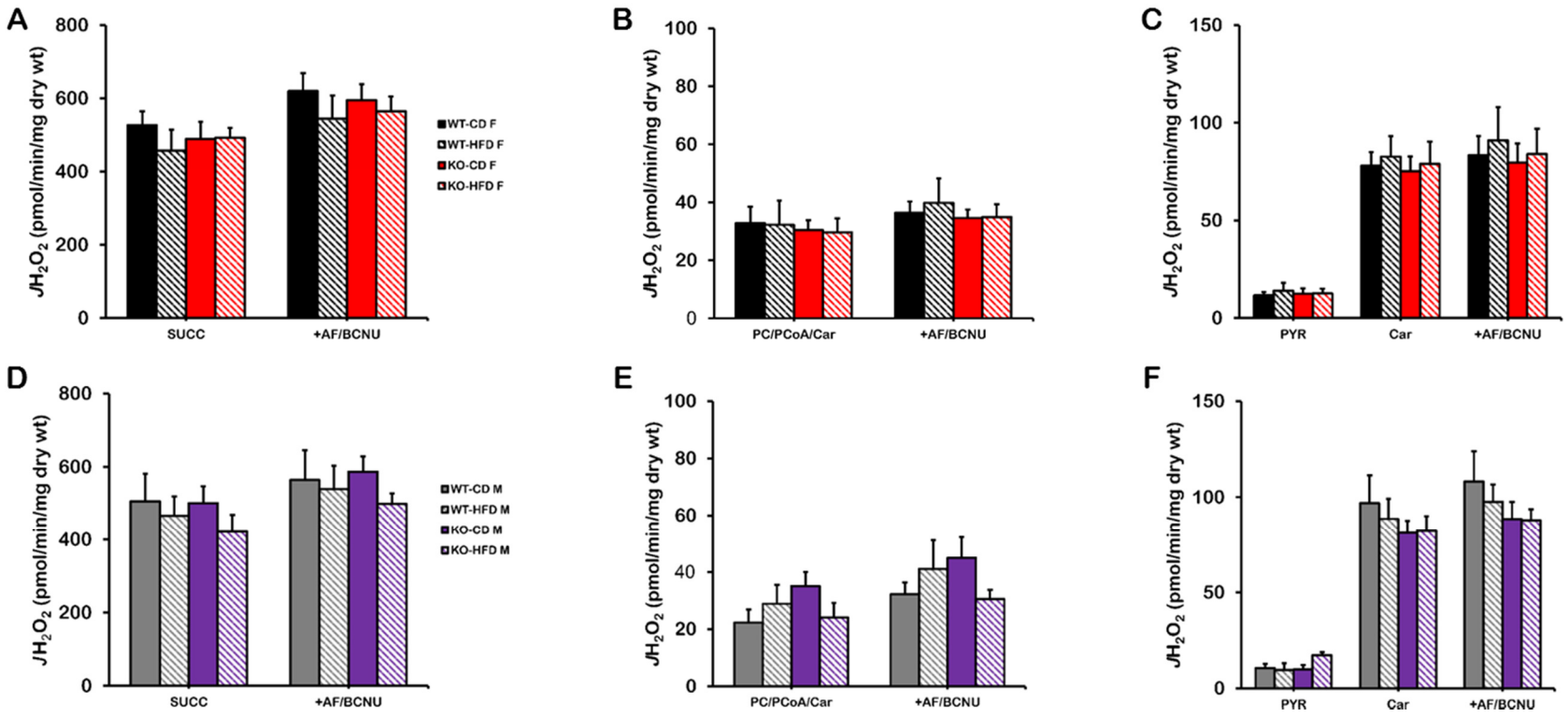


Figure 11. Induced loss of skeletal muscle ERα in adult mice did not affect H_2O_2 emitting potential even after a chronic HFD

H_2O_2 emission rate (JH_2O_2) in permeabilized muscle fibers of WT and ERαKO^{ism} CD- and HFD-fed female (A-C) and male mice (D-F). Succinate-supported H_2O_2 emission rate (A,D), fatty acid (palmitoyl-carnitine/palmitoyl-coA/l-carnitine)-supported H_2O_2 emission rate (B,E) , and pyruvate (pyr)/carnitine (car)-supported H_2O_2 emission rate (C,F). Data are mean ± SEM. Females: n=6-7/group. Males: n=4-6/group. wt, weight.

Table 2. *Subject Characteristics*

	Healthy	Obese-Insulin Resistant
Sample size (N)	9	5
Age (yrs)	24±1.6	41±3.6*
Weight (kg)	62.1±2.0	112.9±5.3*
BMI (kg/m^2)	22.9±0.8	41.0±1.8*
Insulin (mU/L)	5.4±0.6	15.3±1.5*
Glucose (mg/dl)	86±7.8	96.4±4.34*
HOMA IR	1.2±0.5	3.7±0.5*
VO2max (ml/kg/min)	44.9±1.6	23.0±1.8*

Data are means ± SEM. *Significant difference ($p \leq .05$)

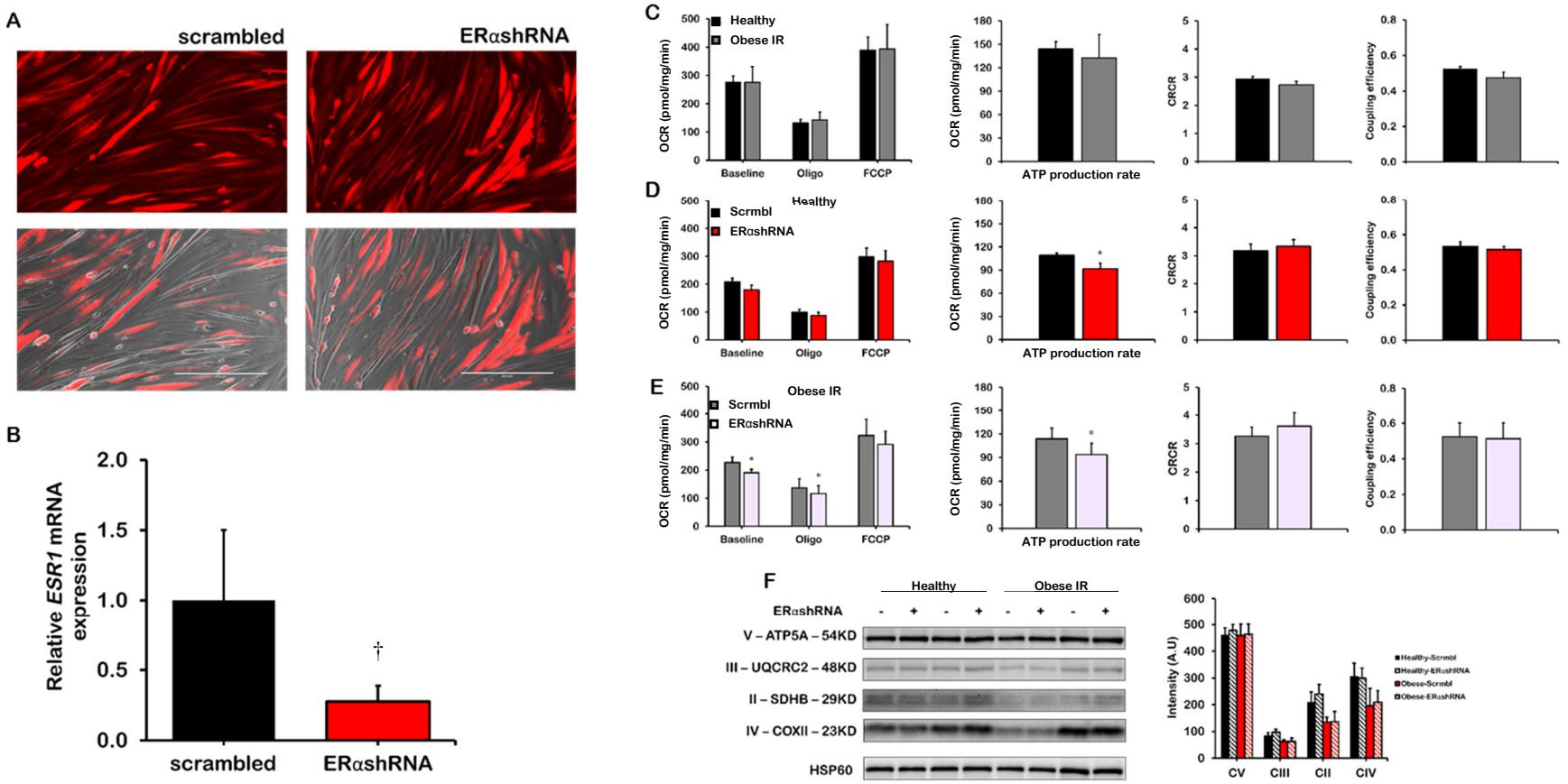
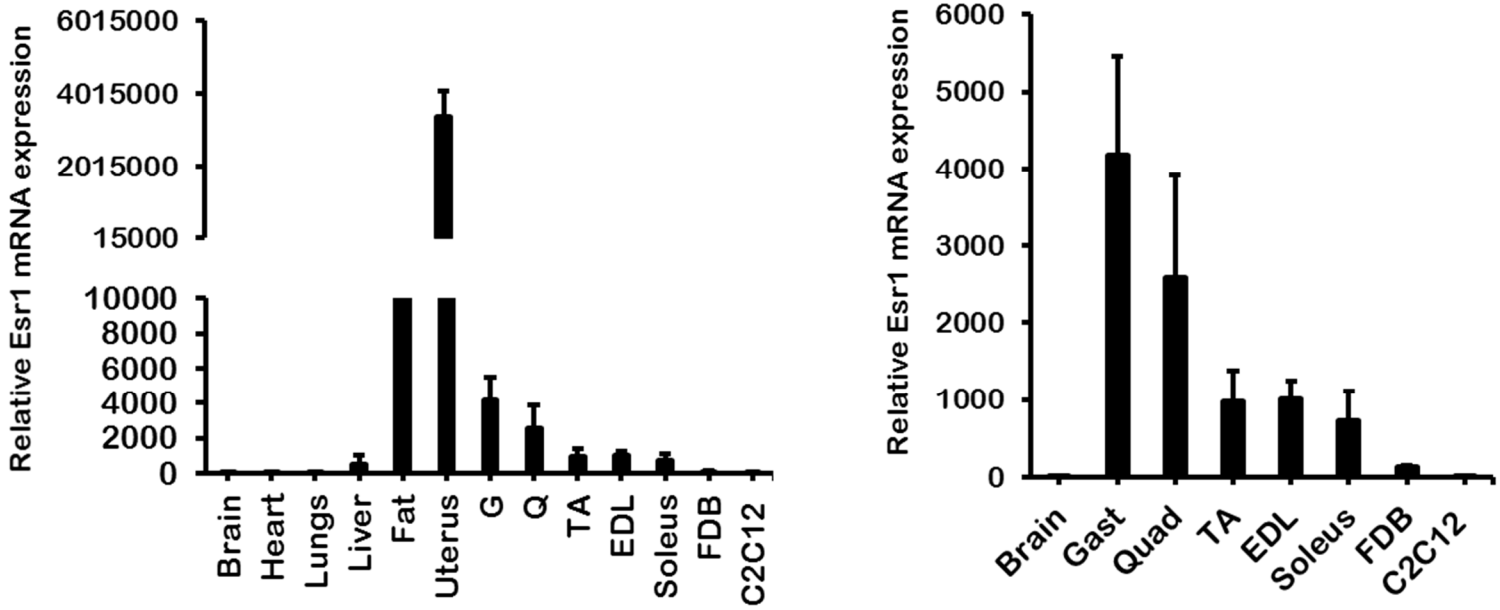
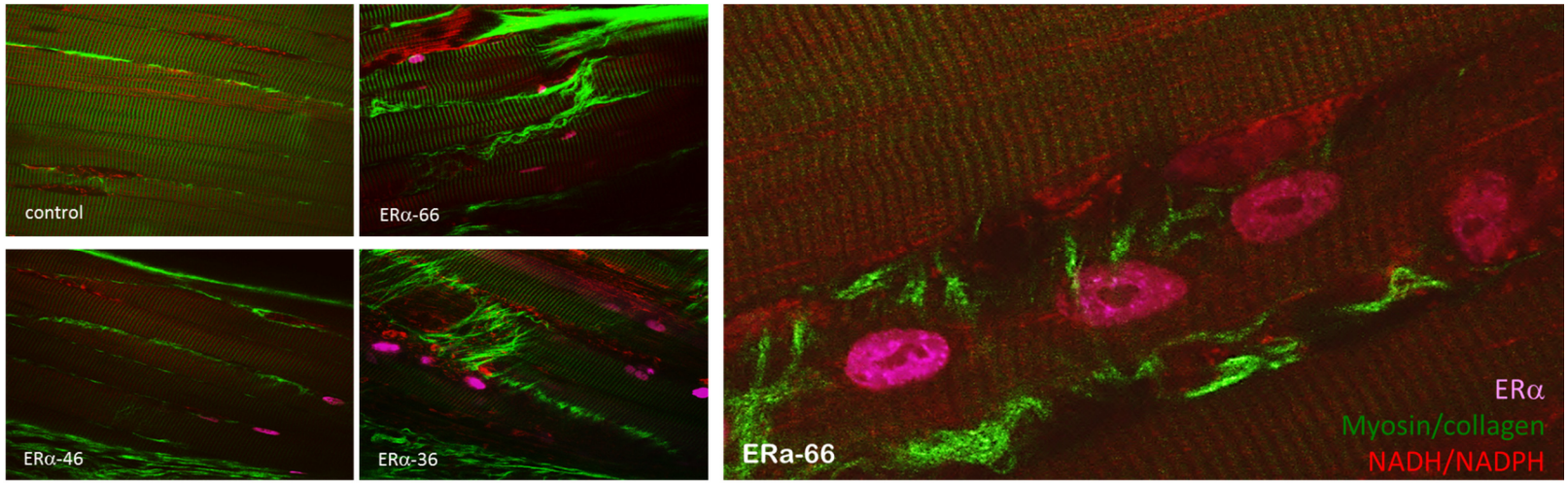


Figure 12. Knockdown of ERα in human myotubes from healthy and obese insulin-resistant subjects minimally affects basal O_2 consumption and ATP production rates

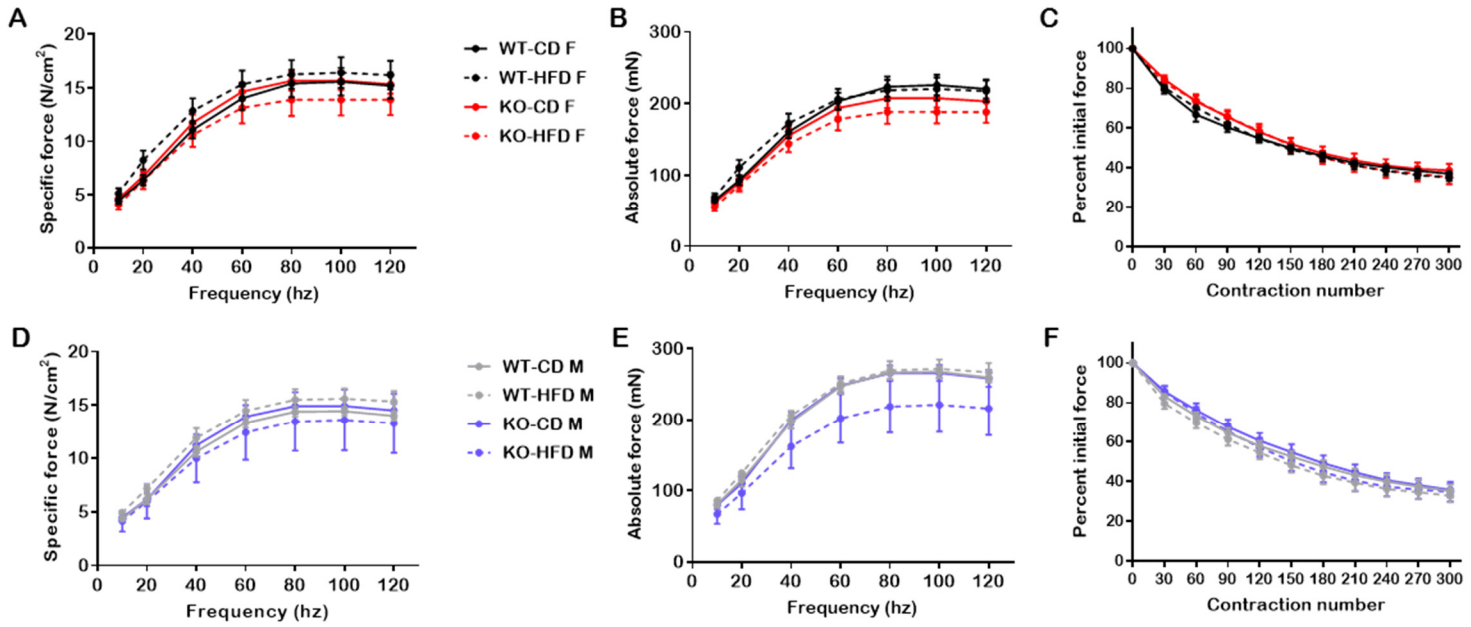
OCR = oxygen consumption rate; Oligo = oligomycin; FCCP = carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; CRCR = cell respiratory control ratio; CII = Complex II; CIII = Complex III; CIV = Complex IV; CV = Complex V. Data are means ± SEM. †Significant difference between scrambled-shRNA and ERαshRNA, $p<.05$.



Supplementary Figure 1. ERα (Esr1) mRNA expression across multiple tissues in WT mice and C2C12 myotubes G = gastrocnemius muscle; Q = quadriceps muscle; TA = tibialis anterior muscle; EDL = extensor digitorum longus; FDB = flexor digitorum brevis muscle.



Supplementary Figure 2. ERα (ESR1) localizes in the nuclei
Sample images of two-photon-second harmonic imaging of GFP-tagged ESR1-66kDa, ESR1-46kDa, and ESR1-36kDa electroporated into skeletal muscle fibers of the flexor digitorum brevis muscle.



Supplementary Figure 3. Induced ablation of skeletal muscle ERα in adult mice did not affect muscle contractility even after a chronic HFD

Specific force (A,D), absolute force (B,E), and fatigue development (C,F) of female WT and ERαKO^{ism} mice (A-D) and male WT and ERαKO^{ism} mice (E-H). Data are mean ± SEM.

Chapter 4: Summary, Clinical Significance, and Future Directions

Summary

The purpose of this dissertation was to investigate whether skeletal muscle ERα is critical for maintaining metabolic function under conditions of lipid overload. The approach in this study involved the generation of a novel inducible skeletal muscle-specific ERα knockout mouse model. The model allowed us to determine the functions of skeletal muscle ERα in the regulation of glucose and lipid homeostasis without the confounding factors associated with development. The results indicated that induced ablation of skeletal muscle ERα did not affect adiposity, whole body oxygen consumption, energy expenditure, spontaneous cage activity, and glucose tolerance in female and male mice under low fat and acute high fat diet (HFD) treatments. Interestingly, skeletal muscle ERα ablation led to elevated expression of pro-inflammatory cytokines and chemokines that was associated with reduced GLUT4 protein content in skeletal muscle of female mice, which may have contributed to the glucose intolerance after chronic HFD treatment. In male mice, induced ablation of skeletal muscle ERα led to a subtle increase in energy expenditure after chronic HFD, despite no significant alterations in spontaneous cage activity. Finally, adiposity and indices of mitochondrial function, particularly respiratory capacity, oxidative phosphorylation efficiency, and H_2O_2 emission potential, in the presence of carbohydrate-derived and fatty acid substrates, were not affected by the loss of skeletal muscle ERα function in both female and male mice after chronic HFD. The function of ERα in modulating mitochondrial respiration of human skeletal muscle was also

assessed in an attempt to translate the findings from ERαKO^{ism} mice. Primary human skeletal muscle cells from healthy and obese-insulin resistant adult women were transduced with an adenovirus-driven ERαshRNA and differentiated into mature myotubes. The data indicated that ATP production rate was only minimally affected in ERα knockdown human myotubes from both healthy and obese-insulin resistant groups. Collectively, the results of this dissertation bring light to the functions of skeletal muscle ERα in the maintenance of glucose homeostasis and skeletal muscle inflammation. Importantly, these key findings were observed after ERα ablation in adult skeletal muscle and thus, were not confounded by developmental effects that are likely present in conventional embryonic skeletal muscle-specific ERα deletion strategies.

Clinical Significance

Women and men with reduced estrogen action are at increased risk of gaining excess adiposity and developing insulin resistance; however, underlying mechanisms remain poorly defined, requiring a better understanding of estrogen-sensitive processes to either improve preventive approaches or develop appropriate treatment strategies. This dissertation showed that ERα in adult skeletal muscle mediates the protective role of estrogens against HFD-induced glucose intolerance in females by regulating skeletal muscle inflammation and preserving skeletal muscle GLUT4 levels. Future studies should be directed towards defining how skeletal muscle ERα influences mechanisms that regulate the expression of genes associated with inflammatory signaling.

Interestingly, the results of this study also indicated that skeletal muscle ERα does not play a role in the regulation of skeletal muscle mitochondrial function. This

finding contradicted previous results using global ERαKO and conventional skeletal muscle-specific ERαKO mouse models, which documented that ERα ablation during the embryonic stage of development led to increased visceral adiposity and alterations in skeletal muscle mitochondrial health (Ribas et al., 2010, 2016). Similar results were found in fetal estrogen deficiency that resulted in altered developmental programming of mechanisms that regulate metabolic homeostasis during adulthood (Maniu et al., 2016; Pepe et al., 2016). ERα in adult skeletal muscle may not be as necessary for maintaining lipid homeostasis and mitochondrial function because of compensatory or redundant mechanisms that are likely established in adults. For example, a recent study in mice demonstrated that estradiol directly incorporates in the mitochondrial membrane, independent of ERα, decreasing membrane microviscosity, which in turn, optimizes mitochondrial function (Torres et al., 2018). The nature of these ERα-independent mechanisms remain to be clearly elucidated and may serve as a foundation for future potential strategies that can decrease or eliminate the risk of excess adiposity and insulin resistance associated with reduced estrogen action by natural or surgical menopause.

Future Directions and Limitations

Female ERαKO^{ism} mice exhibited increased expression of pro-inflammatory markers in skeletal muscle, reduced skeletal muscle GLUT4 protein content, and glucose intolerance after chronic HFD, implying loss of skeletal muscle ERα function may have increased the susceptibility to develop skeletal muscle insulin resistance in adult female mice exposed to a long-term HFD. However, *ex vivo* basal and insulin-stimulated glucose uptake in the extensor digitorum longus and soleus muscles were similar between WT and ERαKO^{ism} mice, suggesting that detecting the role of skeletal muscle ERα in modulating *in vivo* glucose uptake may have been lost due to the nature of the *ex vivo* glucose uptake experiment. Future studies must consider measuring skeletal muscle insulin sensitivity in ERαKO^{ism} mice utilizing *in vivo* methods such as the hyperinsulinemic-euglycemic clamp or *in vivo* glucose uptake experiments.

The elevated mRNA expression of several known pro-inflammatory cytokines and chemokines in skeletal muscle of female ERαKO^{ism} mice also strongly suggested that induced ablation of skeletal muscle ERα increased the susceptibility to exhibit heightened skeletal muscle inflammation, independent of excess adiposity, in adult female mice. This is an important finding given that inflammation is often associated with increased adipose tissue and intramuscular fat accumulation (Wu & Ballantyne, 2017), which was indeed observed in previous global ERαKO and conventional skeletal muscle-specific ERαKO mice (Ribas et al., 2010, 2016). Thus, further studies are required to confirm the mRNA expression data and comprehensively determine whether skeletal muscle inflammation is a primary consequence of the loss of skeletal muscle ERα in females. A multiplex protein assay may be considered in order to assess

whether gene expression accurately parallels protein levels. Flow cytometry (Fink et al., 2014), immunohistochemistry, or in situ hybridization experiments (Amsen, Visser, & Town, 2009) may reveal the type of immune cells that are excessively producing cytokines in skeletal muscle of female ERαKO^{ism} mice. Furthermore, future experiments are required to identify specific targets that are involved in the regulation of skeletal muscle inflammation, which have ESR1 binding sites in their promoter regions. Finally, determining whether systemic inflammation is affected in female ERαKO^{ism} mice can be addressed by flow cytometry experiments.

Adiposity and mitochondrial function were not affected in ERαKO^{ism} mice in the present study whereas previously studied conventional skeletal muscle-specific ERαKO mice exhibited greater body fat, intramuscular fat accumulation, and altered mitochondrial health compared to WT mice (Ribas et al., 2016). ERαKO^{ism} mice were generated by crossing ERα-floxed mice with human skeletal actin-MerCreMer mice, which allowed the temporal ablation of ERα. On the other hand, the previous conditional skeletal muscle-specific ERαKO mice used to determine the role of skeletal muscle-specific ERα in maintaining metabolic homeostasis were generated by crossing ERα-floxed mice with muscle creatine kinase-Cre mice, which resulted in the loss of skeletal muscle ERα function during the embryonic stage of development. This inherent difference between mouse models, along with their divergent metabolic phenotypes at adulthood have led to the speculation that skeletal muscle ERα is essential for fetal programming, but not nearly as critical post-development for the regulation of lipid homeostasis and mitochondrial function. This hypothesis will need to be addressed either by determining the metabolic phenotype of offspring of ERα-floxed mice crossed

with human skeletal actin-Cre mice (Collins et al., 2018) or by identifying compensatory/redundant mechanisms that are likely established in adult ERαKO^{ism} mice.

Finally, the novel ERαKO^{ism} mouse model is a valuable tool; however, future studies must be conducted to determine the functions of skeletal muscle-specific ERα in humans. Aside from the phylogenetic differences between mice and humans, inbred mice in this study also lack the genetic variations of outbred mice and humans. Therefore, the mechanisms involved in disease susceptibility may be different between the two species. For instance, this dissertation showed that ERαKO^{ism} mice exhibited normal skeletal muscle mitochondrial respiratory capacity, ATP production rate, and OXPHOS efficiency; however, silencing ERα in human myotubes that were isolated from healthy and obese-insulin resistant women resulted in a subtle reduction in ATP production rate. Although both findings support the notion that induced ablation of skeletal muscle ERα does not lead to drastic impairments in mitochondrial respiration, future experiments can be directed towards verifying whether skeletal muscle ERα is important for mitochondrial respiration in human skeletal muscle derived from biopsies. To avoid the confounding effects of age, differences in respiratory capacity can be compared between skeletal muscle biopsies obtained from women treated with leuprolide acetate (a gonadotropin releasing hormone agonist that suppresses ovarian hormones) (Melanson et al., 2015; Shea et al., 2015), women treated with leuprolide acetate + estradiol, and women treated with leuprolide acetate + a selective estrogen receptor modulator (e.g. bazedoxifene or a future novel selective estrogen receptor modulator that specifically targets skeletal muscle ERα) (Xu, Lovre, & Mauvais-Jarvis,

2017). Additionally, ERαKO^{ism} mice exhibited elevated skeletal muscle inflammation under low fat and chronic HFD conditions. Given that estradiol treatment has anti-inflammatory effects in obese women (Al-Safi et al., 2015) and menopause is associated with increased inflammation in certain tissues (Iyengar et al., 2015), ERα is likely a key regulator of skeletal muscle inflammation in women, but this remains to be tested.

Collectively, these aforementioned studies can uncover direct targets that are regulated by ERα, which may lead to the discovery of safer and more effective strategies for preventing or treating excess adiposity and insulin resistance associated with reduced estrogen action.

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Appendix

EAST CAROLINA UNIVERSITY
ANIMAL USE PROTOCOL (AUP) FORM
LATEST REVISION NOVEMBER, 2013

Project Title:

ER-alpha protects skeletal muscle from lipid based insults.

	Principal Investigator	Secondary Contact
Name	Espen Spangenburg	Click here to enter text.
Dept.	Physiology/ECDOI	Click here to enter text.
Office Ph #	252-737-5035	Click here to enter text.
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For IACUC Use Only

AUP # Q333			
New/Renewal New 7/22/15			
Full Review/Date	DR/Date		
Approval Date 7/23/15			
Study Type Skeletal muscle			
Pain/Distress Category D			
Surgery ✓	Survival ✓	Multiple	EchT 43B
Prolonged Restraint			foot injections
Food/Fluid Regulation			
Other			
Hazard Approval/Dates	Rad	IBC 7/23/15	EHS tamoxifen,
OHP Enrollment		transgenic	ethanol, Meloxicam
Mandatory Training			
Amendments Approved			

Single housing

I. Personnel

A. Principal Investigator(s):

Espen E. Spangenburg, PhD

B. Department(s):

Physiology/ECDOI

C. List all personnel (PI's, co-investigators, technicians, students) that will be working with live animals and describe their qualifications and experience with these specific procedures. If people are to be trained, indicate by whom:

Name/Degree/Certification	Position/Role(s)/ Responsibilities in this Project	Required Online IACUC Training (Yes/No)	Relevant Animal Experience/Training (include species, procedures, number of years, etc.)
Espen Spangenburg	PI/oversight/will help with basic procedures when necessary	yes	Completed numerous animal training courses/rodent (mouse/rat), aseptic technique and tissue removal/20+ years of experience
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II. Regulatory Compliance

A. Non-Technical Summary

Using language a non-scientist would understand, please provide a clear, concise, and sequential description of animal use. Additionally, explain the overall study objectives and benefits of proposed research or teaching activity to the advancement of knowledge, human or animal health, or good of society. (More detailed procedures are requested later in the AUP.)
Do not cut and paste the grant abstract.

Loss of ovarian function in females is associated with the increased risk for the development of type 2 diabetes. Currently, we have a poor understanding of how ovarian hormones affects metabolic function of skeletal muscle. In these experiments we will seek to determine if estrogens have a protective role in skeletal muscle. The primary goal of this study is therefore, to determine if endogenous estrogen receptor alpha (ERa) expression is necessary for regulating metabolic and physiological mechanisms of skeletal muscle in mice. To address this question, we will use a previously developed mouse model that allows us to induce a skeletal muscle specific loss of ERa expression. To determine the function of ERa in skeletal muscle, we will make a number of metabolic and physiological measures in these mice. ERa will be ablated from skeletal muscle using a tamoxifen-induced human skeletal alpha actin driven Cre model crossed with a ERa -floxed mouse model. Mice will be injected with tamoxifen or vehicle for 5 days. Tamoxifen injections induce the loss of ERa function, which will only occur in skeletal muscle. Then the animals will be placed into groups and exposed to either a high fat diet or a control diet. Subset of animals will be exposed to voluntary running wheels and/or placed in metabolic cages. At the appropriate time points, we will remove skeletal muscle from the animals to measure muscle force production or to measure metabolic function through biochemical assays. Finally we assess the impact of known genetic mutations in ERa on skeletal muscle function using our model by cDNA electroporation procedures in the flexor digitorum brevis muscle (FDB). After, two weeks the FDB muscle will be removed from the animal for biochemical assays. It is expected that data obtained from these studies will improve our understanding of ERa and demonstrate that ERa has an important role outside of breast tissue.

B. Ethics and Animal Use

B.1. Duplication

Does this study duplicate existing research? No
If yes, why is it necessary? (note: teaching by definition is duplicative)
Click here to enter text.

B.2. Alternatives to the Use of Live Animals

Are there less invasive procedures, other less sentient species, isolated organ preparation, cell or tissue culture, or computer simulation that can be used in place of the live vertebrate species proposed here? No

If yes, please explain why you cannot use these alternatives.
Click here to enter text.

B.3. Consideration of Alternatives to Painful/Distressful Procedures

a. Include a literature search to ensure that alternatives to all procedures that may cause more than momentary or slight pain or distress to the animals have been considered.

1. Please list all of the potentially painful or distressful procedures in the protocol:

Foot muscle injections and electroporation, non-survival surgery animal tissue removal

2. For the procedures listed above, provide the following information (please do not submit search results but retain them for your records):

Date Search was performed:	06/14/2015
Database(s) searched:	Pubmed, Agricola,
Time period covered by the search (i.e. 1975-2013):	1980-2015
Search strategy (including scientifically relevant terminology):	Mouse, pain, tissue removal, under anesthetic, analgesia, injection, cDNA muscle electroporation, muscle stimulation
Other sources consulted:	google

3. In a few sentences, please provide a brief narrative indicating the results of the search(es) to determine the availability of alternatives and explain why these alternatives were not chosen. Also, please address the 3 Rs of refinement, reduction, and replacement in your response. Refinement refers to modification of husbandry or experimental procedures to enhance animal well-being and minimize or eliminate pain and distress. Replacement refers to absolute (i.e. replacing animals with an inanimate system) or relative (i.e. using less sentient species) replacement. Reduction involves strategies such as experimental design analysis, application of newer technologies, use of appropriate statistical methods, etc., to use the fewest animals or maximize information without increasing animal pain or distress.

The experimental approaches in this protocol will expose the animals to minimal distress or pain. In addition, we will provide analgesia or the animal will be brought to a surgical depth with anesthetic to relieve any discomfort the animal might experience.

Refinement: We only have minor survival procedures in which we will inject the animals. If the animal experiences discomfort we will give them analgesia through treatment with meloxicam as an analgesic. In addition, if the animal shows signs of discomfort (e.g. weight loss, excessive grooming, huddling in corner) we will seek the advice of the campus veterinarian.

Replacement: For these experiments we are using the lowest sentient species for our hypothesis that accurately replicates human physiology. At this time, it is not possible to test this hypothesis in an inanimate system. Mice are an appropriate species because this is a genetic mouse model, in which ERa function was disrupted by genetic removal of the coding sequence of ERa. This allows us to evaluate the role of ERa in skeletal muscle using in vivo and ex vivo-based approaches. This is not possible with other animal or culture based models.

Reduction: For these experiments, we have applied a priori statistical analysis to use the lowest number of animals possible while maintaining sufficient statistical power. In addition, where possible we are using multiple different tissues from the same animal to reduce animal number.

C. Hazardous Agents

1. Protocol related hazards (chemical, biological, or radiological): Please indicate if any of the following are used in animals and the status of review/approval by the referenced committees:

HAZARDS	Oversight Committee	Status (Approved, Pending, Submitted)/Date	AUP Appendix I Completed?
Radioisotopes	**Radiation**	Click here to enter text.	Choose an item.
Ionizing radiation	**Radiation**	Click here to enter text.	Choose an item.
Infectious agents (bacteria, viruses, rickettsia, prions, etc.)	**IBC**	Click here to enter text.	Choose an item.
Toxins of biological origins (venoms, plant toxins, etc.)	**IBC**	Click here to enter text.	Choose an item.
Transgenic, Knock In, Knock Out Animals---breeding, cross breeding or any use of live animals or tissues	**IBC**	Yes, we are using transgenic animal models. Protocol pending.	Yes
Human tissues, cells, body fluids, cell lines	**IBC**	Click here to enter text.	Choose an item.
Viral/Plasmid Vectors/Recombinant DNA or recombinant techniques	**IBC**	Yes, we are using plasmid cDNA in these experiments. None of the plasmids	Yes

		contain viral backbone. Protocol pending.	
Oncogenic/toxic/mutagenic chemical agents	**EH&S**	Tamoxifen, protocol pending.	yes
Nanoparticles	**EH&S**	Click here to enter text.	Choose an item.
Cell lines, tissues or other biological products injected or implanted in animals	**DCM**	Click here to enter text.	Choose an item.
Other agents		Click here to enter text.	Choose an item.

2. Incidental hazards

Will personnel be exposed to any incidental zoonotic diseases or hazards during the study (field studies, primate work, etc)? If so, please identify each and explain steps taken to mitigate risk:

No

III. Animals and Housing

A. Species and strains:

Mouse and C57BL/6 X C3H (B6C3F1)

Wildtype (WT) = non-injected B6.Cg-Tg(ACTA1-cre/Esr1* $ESR1^{tm2Cxd}$)1EES/J

ERalpha knockout (KO) = injected B6.Cg-Tg(ACTA1-cre/Esr1* $Esr1^{tm2Cxd}$)1EES/J

B. Weight, sex and/or age:

Weight will vary depending on sex (~18-35g), both males/females, 10-45 weeks old

C. Animal numbers:

1. Please complete the following table:

Total number of animals in treatment and control groups	**Additional animals (Breeders, substitute animals)**	**Total number of animals used for this project**
Total number for duration of protocol including breeders	Total amount	=1180
Breeding group	breeders-Non-transgenic offspring	=560

	that will be culled Breeders (30 prs)	 =60
Wild type control diet (140)	Exps 1, 2, 3 Exp 1 only Exp 2 only Exp 3 only	total=140 exp 1 = 40 exp 2 = 40 exp 3 = 60
Wild type – high fat diet (140)	Exps 1, 2, 3 Exp 1 only Exp 2 only Exp 3 only	total =140 exp 1 = 40 exp 2 = 40 exp 3 = 60
Knock out – control diet	Exps 1, 2, 3 Exp 1 only Exp 2 only Exp 3 only	total=140 exp 1 = 40 exp 2 = 40 exp 3 = 60
Knock out – high fat diet	Exps 1, 2, 3 Exp 1 only Exp 2 only Exp 3 only	total=140 exp 1 = 40 exp 2 = 40 exp 3 = 60

2. Justify the species and number (use statistical justification when possible) of animals requested:

In this section, we provide the total number of animals (n=1120) needed to establish the experimental groups and complete the experiments. This total number of animals includes breeders, born pups, and the total number of animals that will be used in each experiment. So, we will not actually use all of these animals in our specific experiments. Please note that we will never have this many animals in the facility at once, but these estimations are based on mathematical calculations of what it will require to maintain our experiments over a three year period.

Experimental Numbers: Using a priori power measure we have determined that in order to maintain statistical power (0.80), the minimum number of animals needed per group for each experiment is 20 (10 males and 10 females). For more details on each experimental group you may refer to Table 1 in the experimental design section.

We have broken down the whole proposal into three independent experiments. In experiment 1, we seek to determine the effect of ERa ablation on skeletal muscle function, and also determine the effects of exercise across genotype and sex. In experiment 2, we seek to determine the effect of ERa ablation in skeletal muscle on cellular and molecular mechanisms that regulate metabolic function across genotype and sex. In experiment 3, we seek to determine the effect of ERa genetic mutation on mitochondrial function in skeletal muscle by in vivo gene delivery approaches. In order to determine the mechanistic function of the targets identified in experiments 1-3 on metabolic function *ex vivo*, we will isolate whole muscle or primary muscle cells from each group.

In these experiments, we will use an inducible skeletal muscle specific ERa knock-out (KO) mouse that we developed at University of Maryland (UMD). We have been previously approved to perform very similar experiments at UMD and we are now proposing to continue this highly promising line of research at ECU. The induction of ERa gene ablation is accomplished through daily intraperitoneal injections of tamoxifen (12.75 mg/ml) for 5 consecutive days in ten week-old mice; whereas the control group (WT) receives a vehicle injection in the same pattern. We then allow the animals to remain in their cages with ad libitum food and water for a 14-day wash-out period before assigning them to control food diet group (CD; 10%kcal fat, D1245K) or high-fat diet group (HFD; 45%kcal fat, D12451) (Research Diets Inc. New Brunswick, NJ). The animals remain in diet for 10 weeks prior to use. Since it is critical to maintain a consistent and controlled diet, no animals will receive food-based enrichment in their cages.

Three different overall experiments will be conducted to determine the importance of ERa signaling on skeletal muscle function (see table above). We have divided the total number of animals into three distinct experiments, each which requires us to process in a completely different fashion.

3. Justify the number and use of any additional animals needed for this study:

Working with genetic models results in a degree of unpredictability because we have no way of knowing how many of the litter will express the needed genotype until after birth. Thus, any extra animals will be needed to ensure that we achieve the required animals described in our experimental design (see above).

a. For unforeseen outcomes/complications:

Click here to enter text.

b. For refining techniques:

Click here to enter text.

c. For breeding situations, briefly justify breeding configurations and offspring expected:

Breeding numbers: We are crossing two different genotypes of mice to establish our animal model. With this crossing, approximately 50% of each litter are the correct genotype. In addition, because we plan to assess sex-differences we will need males and females for the groups. Thus, the large animal numbers are largely the result of the necessary breeding paradigm to establish our desired experimental design. All animals that are not appropriate genotype will be euthanized or provided to other ECU investigators who may be able to use them. Since, we need 560 total animals it is predicted that over the course of three years, we will need to breed approximately 1120 animals to achieve the correct genotypes and genders needed for our experimental design. We are predicting that each breeder pair will provide us 6-10 animals, thus we conservatively assume four viable litters per breeder pair then we will need approximately 30 breeder pairs. Thus, we are anticipating a total of 1180 animals for these experiments when breeding numbers are included in the total animal number.

d. Indicate if following IACUC tail snip guidelines: we are following IACUC tail snip guidelines

(if no, describe and justify)
Click here to enter text.

4. Will the phenotype of mutant, transgenic or knockout animals predispose them to any health, behavioral, physical abnormalities, or cause debilitating effects in experimental manipulations? NO **(if yes, describe)**
Click here to enter text.

5. Are there any deviations from standard husbandry practices?
yes **If yes, then describe conditions and justify the exceptions to standard housing (temperature, light cycles, sterile cages, special feed, prolonged weaning times, wire-bottom cages, etc.):**
For a part of the study the animals will be placed on a specialty chow diet or high fat diet. The Spangenburg lab will assume responsibility for the food during this period.

6. The default housing method for social species is pair or group housing (including mice, rats, guinea pigs, rabbits, dogs, pigs, monkeys). Is it necessary for animals to be singly housed at any time during the study?
yes **(If yes, describe housing and justify the need to singly house social species):**
For a short time, a subset of animals will be placed in the metabolic cages housed in Brody School of Medicine. In order, to gather the metabolic data appropriately the animals need to be housed in the cages individually for 7 days. The animals will be returned to group housing after the 7 days is complete.

7. Are there experimental or scientific reasons why routine environmental enrichment should not be provided? yes
(If yes, describe and justify the need to withhold enrichment)
No food based enrichment can be provided to the animals, any other form of enrichment is acceptable (i.e. nestlets, plastic domes, etc...)

8. If wild animals will be captured or used, provide permissions (collection permit # or other required information):
n/a

9. List all laboratories or locations outside the animal facility where animals will be used. Note that animals may not stay in areas outside the animal facilities for more than 12 hours without prior IACUC approval. For field studies, list location of work/study site.
Click here to enter text.

IV. Animal Procedures

A. Outline the Experimental Design including all treatment and control groups and the number of animals in each. Tables or flow charts are particularly useful to communicate your design. Briefly state surgical plans in this section. Surgical procedures can be described in detail in IV.S.

We have broken down the whole proposal into three independent experiments. In experiment 1, we seek to determine the effect of ERa ablation on skeletal muscle function, and also determine the effects of exercise across genotype and sex. In experiment 2, we seek to determine the effect of ERa ablation in skeletal muscle on cellular and molecular mechanisms that regulate metabolic function across genotype and sex. In experiment 3, we seek to determine the effect of ERa genetic mutations on mitochondrial function in skeletal muscle by in vivo gene delivery approaches. In order to determine the mechanistic function of the targets identified in experiments 1-3 on metabolic function *ex vivo*, we will isolate whole muscle or primary muscle cells from each group.

In these experiments, we will use an inducible skeletal muscle specific ERa knock-out (KO) mouse that we developed at University of Maryland (UMD). We have been previously approved to perform very similar experiments at UMD and we are now proposing to continue this highly promising line of research at ECU. The induction of ERa gene ablation is accomplished through daily intraperitoneal injections of tamoxifen (12.75 mg/ml) for 5 consecutive days in ten week-old mice; whereas the control group (WT) receives a vehicle injection in the same pattern. We then allow the animals to remain in their cages with ad libitum food and water for a 14-day wash-out period before assigning them to control food diet group (CD; 10%kcal fat, D1245K) or high-fat diet group (HFD; 45%kcal fat, D12451) (Research Diets Inc. New Brunswick, NJ). The animals remain in diet for 10 weeks prior to use. Since it is critical to maintain a consistent and controlled diet, no animals will receive food-based enrichment in their cages.

Three different overall experiments will be conducted to determine the importance of ERa signaling on skeletal muscle function (see table 1 below). We have divided the total number of animals into three distinct experiments, each which requires us to process in a completely different fashion.

In experiment 1, the focus will be on skeletal muscle function that will be assessed by performing in vitro and ex vivo based experiments. Prior to collecting tissues for physiological based measures the animals will be anesthetized and euthanized after the tissue collection. Additionally, a subset of these animals will be exposed to metabolic cages, wheel running and/or a high fat diet for 7 days prior to tissue collection (see below). After the tissue is collected, the animals will be euthanized as well. The animal numbers are as follows: WT Normal Chow sedentary: Males =10; Females=10; WT High fat diet sedentary: Males =10; Females=10; WT Normal Chow Wheel Running: Males =10; Females=10; WT High fat diet Wheel Running: Males =10; Females=10; ERa KO Normal Chow sedentary: Males =10; Females=10; ERa KO High fat diet sedentary: Males =10; Females=10; ERa KO Normal Chow Wheel Running: Males =10; Females=10; ERa KO High fat diet Wheel Running: Males =10; Females=10.

In experiment 2, the focus will be on mitochondrial function. We will collect the necessary tissue for measures of mitochondrial function and snap freeze tissue for subsequent biochemical measures. In a second set of animals, we longitudinally assess the whole body oxygen consumption kinetics of the animals. The animal numbers are as follows: WT Normal Chow: Males =20; Females=20; WT High fat diet: Males =20; Females=20; ERa KO Normal Chow: Males =20; Females=20; ERa KO High fat diet: Males =20; Females=20. Note: there are two sets of animals in this animal number total.

Finally, in experiment 3 we will remove tissue from each group and isolate primary cell lines from the skeletal muscle tissue. In these experiments, we will perform a cDNA electroporation procedure in both flexor digitorium brevis (FDB) muscles. The FDB muscle is located on the bottom of the foot and is chosen because the muscle is amenable to primary cell isolation. cDNA electroporation is a simple procedure in which a mild collagenase mixture is injected into the FDB muscle with the cDNA of interest. The muscle then receives a mild electrical stimulation (1Hz) via needle electrodes (33 gauge). The animal will be given meloxicam at 5-10 mg/kg for pain relief and returned to their cage for 10 days with ad libitum water and food. Based on the targets identified in experiment 3, we will use these cultured cells to determine the critical nature of each target for the regulation of physiological function. Due to limited amount of tissue in the FDB, we will be required to repeat portions of the study three different times. WT Normal Chow: Males =30; Females=30; WT High fat diet: Males =30; Females=30; ERa KO Normal Chow: Males =30; Females=30; ERa KO High fat diet: Males =30; Females=30. Note: there are three sets of animals in this animal number total.

In sections IV.B-IV.S below, please respond to all items relating to your proposed animal procedures. If a section does not apply to your experimental plans, please leave it blank.

Please refer to DCM and IACUC websites for relevant guidelines and SOPs.

B. Anesthesia/Analgesia/Tranquilization/Pain/Distress Management For Procedures Other than Surgery:

Adequate records describing anesthetic monitoring and recovery must be maintained for all species.

If anesthesia/analgesia must be withheld for scientific reasons, please provide compelling scientific justification as to why this is necessary:

We will not withhold anesthesia/analgesia for any reason.

1. **Describe the pre-procedural preparation of the animals:**
   a. **Food restricted for** Click here to enter text. **hours**
   b. **Food restriction is not recommended for rodents and rabbits and must be justified:**

Click here to enter text.

c. Water restricted for Click here to enter text. **hours**

d. Water restriction is not recommended in any species for routine pre-op prep and must be justified:

Click here to enter text.

2. Anesthesia/Analgesia *for Procedures Other than Surgery*

	Agent	Concentration	Dose (mg/kg)	Max Volume	Route	Frequency	Number of days administered
Pre-procedure analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Pre-anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Anesthetic	Click here to enter text.		Click here to enter text.	Click here to enter text.			
Post procedure analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

3. Reason for administering agent(s):

Click here to enter text.

4. For which procedure(s):

Click here to enter text.

5. Methods for monitoring anesthetic depth:

Click here to enter text.

6. Methods of physiologic support during anesthesia and recovery:
Click here to enter text.

7. Duration of recovery:
Click here to enter text.

8. Frequency of recovering monitoring:
Click here to enter text.

9. Specifically what will be monitored?
Click here to enter text.

10. When will animals be returned to their home environment?
Click here to enter text.

11. Describe any behavioral or husbandry manipulations that will be used to alleviate pain, distress, and/or discomfort:
Click here to enter text.

C. Use of Paralytics

1. Will paralyzing drugs be used? NO

2. For what purpose:
Click here to enter text.

3. Please provide scientific justification for paralytic use:
Click here to enter text.

4. Paralytic drug:
Click here to enter text.

5. Dose:
Click here to enter text.

6. Method of ensuring appropriate analgesia during paralysis:
Click here to enter text.

D. Blood or Body Fluid Collection

1. Please fill out appropriate sections of the chart below:

	Location on animal	Needle/catheter size	Volume collected	Frequency of procedure	Time interval between collections
Blood Collection	Click here to	Click here to	Click here to	Click here to enter	Click here to enter

	enter text.	enter text.	enter text.	text.	text.
Body Fluid Collection	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

E. Injections, Gavage, & Other Substance Administration

1. Please fill out appropriate sections of the chart below:

	Compound	Location & Route of admin	Needle/catheter/gavage size	Max volume admin	Freq of admin (ie two times per day)	Number of days admin (ie for 5 days)	Max dosages (mg/kg)
Injection/ Infusion	tamoxifen	IP-injection	27 gauge	150uL	1/day	5 days	2mg/mouse
Injection	Vehicle (15:85 ethanol:sunflower (control for tamoxifen injections))	IP-injection	27 gauge	150 uL	1/day	5 days	n/a
Injection	Hyaluronidase	Foot pad injection	33 gauge	20 uL	1x total	1	0.36 mg/mL
Injection	cDNA	Foot pad injection	33 gauge	20 uL	1x total	1	50ug/ mouse

3. Pharmaceutical grade drugs, biologics, reagents, and compounds are defined as agents approved by the Food and Drug Administration (FDA) or for which a chemical purity standard has been written/established by any recognized pharmacopeia such as USP, NF, BP, etc. These standards are used by manufacturers to help ensure that the products are of the appropriate chemical purity and quality, in the appropriate solution or compound, to ensure stability, safety, and efficacy. For all injections and infusions for CLINICAL USE, PHARMACEUTICAL GRADE compounds must be used whenever possible. Pharmaceutical grade injections and infusions for research test articles are preferred when available. If pharmaceutical grade compounds are not available and non-pharmaceutical grade agents must be used, then the following information is necessary:

a. **Please provide a scientific justification for the use of ALL non-pharmaceutical grade compounds. This may include pharmaceutical-grade compound(s) that are not available in the appropriate concentration or formulation, or the appropriate vehicle control is unavailable.**
b. **Indicate the method of preparation, addressing items such as purity, sterility, pH, osmolality, pyrogenicity, adverse reactions, etc. (please refer to ECU IACUC guidelines for non-pharmaceutical grade compound use), labeling (i.e. preparation and use-by dates), administration and storage of each formulation that maintains stability and quality/sterility of the compound(s).**

The compound is not available at the appropriate concentrations in a pharmaceutical grade. Thus, we prepare the solution by solubilize the compound (Sigma T5648) in a sterile sunflower oil/ethanol (15% EtOH) solution. The solution is aliquoted into sterile tubes and stored at -20C until use. We discard any stored material after 4 weeks. We have been using this approach for more than two years and we have never seen any adverse reactions in our animals.

F. Prolonged restraint with mechanical devices

Prolonged restraint in this context means *beyond routine care and use procedures* for rodent and rabbit restrainers, and large animal stocks. Prolonged restraint also includes *any* use of slings, tethers, metabolic crates, inhalation chambers, primate chairs and radiation exposure restraint devices.

1. For what procedure(s):
Click here to enter text.
2. Explain why non-restraint alternatives cannot be utilized:
Click here to enter text.
3. Restraint device(s):
Click here to enter text.
4. Duration of restraint:
Click here to enter text.
5. Frequency of observations during restraint/person responsible:
Click here to enter text.
6. Frequency and total number of restraints:
Click here to enter text.
7. Conditioning procedures:
Click here to enter text.
8. Steps to assure comfort and well-being:

Click here to enter text.

9. Describe potential adverse effects of prolonged restraint and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

G. Tumor Studies, Disease Models, Toxicity Testing, Vaccine Studies, Trauma Studies, Pain Studies, Organ or System Failure Studies, Shock Models, etc.

1. Describe methodology:

Click here to enter text.

2. Expected model and/or clinical/pathological manifestations:

Click here to enter text.

3. Signs of pain/discomfort:

Click here to enter text.

4. Frequency of observations:

Click here to enter text.

5. Describe potential adverse side effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

H. Treadmills/Swimming/Forced Exercise

1. Describe aversive stimulus (if used):

Click here to enter text.

2. Conditioning:

Click here to enter text.

3. Safeguards to protect animal:

Click here to enter text.

4. Duration:

Click here to enter text.

5. Frequency:

Click here to enter text.

6. Total number of sessions:

Click here to enter text.

7. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

I. Projects Involving Food and Water Regulation or Dietary Manipulation

(Routine pre-surgical fasting not relevant for this section)

1. Food Regulation

a. Amount regulated and rationale:
N/A

b. Frequency and duration of regulation (hours for short term/weeks or months for long term):
Click here to enter text.

c. Frequency of observation/parameters documented (i.e. recording body weight, body condition, etc.):
Click here to enter text.

d. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

2. Fluid Regulation

a. Amount regulated and rationale:
N?A

b. Frequency and duration of regulation (hours for short term/weeks or months for long term):
Click here to enter text.

c. Frequency of observation/parameters documented (body weight, hydration status, etc.):
Click here to enter text.

d. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

3. Dietary Manipulations

a. **Compound supplemented/deleted and amount:**
A subset of animals will receive a diet supplemented with extra fat.

b. **Frequency and duration (hours for short term/week or month for long term):**

For 12 weeks animals will receive this diet and will have ad libitum access to the diet.

c. **Frequency of observation/parameters documented:**
Daily observations will be performed by animal care staff

d. **Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**
The animals are expected to develop peripheral insulin resistance, but this diet is not expected to induce any effects that would require induction of euthanasia. If we encounter adverse side effects we will contact the campus veterinarian.

J. Endoscopy, Fluoroscopy, X-Ray, Ultrasound, MRI, CT, PET, Other Imaging

1. Describe animal methodology:
Click here to enter text.

2. Duration of procedure:
Click here to enter text.

3. Frequency of observations during procedure:
Click here to enter text.

4. Frequency/total number of procedures:
Click here to enter text.

5. Method of transport to/from procedure area:
Click here to enter text.

6. Describe potential adverse side effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

7. Please provide or attach appropriate permissions/procedures for animal use on human equipment:
Click here to enter text.

K. Polyclonal Antibody Production

1. **Antigen/adjuvant used and justification for adjuvant choice:**
   Click here to enter text.
2. **Needle size:**
   Click here to enter text.
3. **Route of injection:**
   Click here to enter text.
4. **Site of injection:**
   Click here to enter text.
5. **Volume of injection:**
   Click here to enter text.
6. **Total number of injection sites:**
   Click here to enter text.
7. **Frequency and total number of boosts:**
   Click here to enter text.
8. **What will be done to minimize pain/distress:**
   Click here to enter text.
9. **Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**
   Click here to enter text.

L. Monoclonal Antibody Production

1. Describe methodology:
Click here to enter text.

2. Is pristane used: Choose an item.

Volume of pristane:
Click here to enter text.

3. Will ascites be generated: Choose an item.

i. **Criteria/signs that will dictate ascites harvest:**
   Click here to enter text.
ii. **Size of needle for taps:**
   Click here to enter text.
iii. **Total number of taps:**
   Click here to enter text.
iv. **How will animals be monitored/cared for following taps:**

Click here to enter text.

4. What will be done to minimize pain/distress:

Click here to enter text.

5. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

M. Temperature/Light/Environmental Manipulations

1. Describe manipulation(s):

Animals will placed in the metabolic cages housed in the Brody building. The animals will retain ad libitum food and water access. In addition, the animals will receive unrestricted access to the enrichment (including wheel running) with the exception of food enrichment.
We will follow the ECDOI previously established SOP.

2. Duration:

7 days

3. Intensity:

n/a

4. Frequency:

once

5. Frequency of observations/parameters documented:

Oxygen utilization and CO2 production will be measured. In addition, total wheel activity will be measured. We will check the cages 2X per day.

6. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

If the animal appears to develop indicators of chronic discomfort that do not resolve, we will seek the advice of the campus veterinarian. If no solution is available, the animal will be removed from the study and humanely euthanized.

N. Behavioral Studies

1. Describe methodology/test(s) used:

Click here to enter text.

2. Will conditioning occur? If so, describe:

Click here to enter text.

3. If aversive stimulus used, frequency, intensity and duration:

Click here to enter text.

4. Length of time in test apparatus/test situation: ***(i.e., each test is ~10 mins)***

Click here to enter text.

5. Frequency of testing and duration of study: ***(i.e., 5 tests/week for 6 months)***

Click here to enter text.

6. Frequency of observation/monitoring during test:

Click here to enter text.

7. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

O. Capture with Mechanical Devices/Traps/Nets

1. Description of capture device/method:

Click here to enter text.

2. Maximum time animal will be in capture device:

Click here to enter text.

3. Frequency of checking capture device:

Click here to enter text.

4. Methods to ensure well-being of animals in capture device:

Click here to enter text.

5. Methods to avoid non-target species capture:

Click here to enter text.

6. Method of transport to laboratory/field station/processing site and duration of transport:

Click here to enter text.

7. Methods to ensure animal well-being during transport:

Click here to enter text.

8. Expected mortality rates:

Click here to enter text.

9. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

P. Manipulation of Wild-Caught Animals in the Field or Laboratory

1. Parameters to be measured/collected:

Click here to enter text.

2. Approximate time required for data collection per animal:
Click here to enter text.

3. Method of restraint for data collection:
Click here to enter text.

4. Methods to ensure animal well-being during processing:
Click here to enter text.

5. Disposition of animals post-processing:
Click here to enter text.

6. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

Q. Wildlife Telemetry/Other Marking Methods

1. Describe methodology (including description of device):
Click here to enter text.

2. Will telemetry device/tags/etc. be removed? Choose an item. **If so, describe:**
Click here to enter text.

3. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

R. Other Animal Manipulations

1. Describe methodology:
Click here to enter text.

2. Describe methods to ensure animal comfort and well-being:
Click here to enter text.

3. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

S. Surgical Procedures

All survival surgical procedures must be done aseptically, regardless of species or location of surgery. Adequate records describing surgical procedures, anesthetic monitoring and postoperative care must be maintained for all species.

1. Location of Surgery (Building & Room #):
ECDOI, room 4313F

2. Type of Surgery (check all that are appropriate):
Two different surgeries will be performed. Only one is a survival surgery and is specific to experiment 3. Animals in experiments 1, 2, 3 will undergo non-survival surgery to allow for necessary tissue collection.

☒ **Non-survival surgery (animals euthanized without regaining consciousness)**
☐ **Major survival surgery (major surgery penetrates and exposes a body cavity or produces substantial impairment of physical or physiologic function)**
☒ **Minor survival surgery**
☐ **Multiple survival surgery**
If yes, provide scientific justification for multiple survival surgical procedures:
Click here to enter text.

3. Describe the pre-op preparation of the animals:

a. **Food restricted for** 4 **hours**
b. **Food restricted is not recommended for rodents and rabbits and must be justified:**
It is important that the tissue we remove from the animal to not have been exposed to post-prandial hyperglycemic conditions. High glucose exposure disrupts our downstream measures in these tissues. Thus, food is removed for 4 hr prior to euthanasia only.
c. **Water restricted for** 0 **hours**
d. **Water restriction is not recommended in any species for routine pre-op prep and be justified:**
Click here to enter text.

4. Minimal sterile techniques will include (check all that apply):

Please refer to DCM Guidelines for Aseptic Surgery for specific information on what is required for each species and type of surgery (survival vs. non-survival).

☒ **Sterile instruments**

How will instruments be sterilized?

Autoclave

If serial surgeries are done, how will instruments be sterilized between surgeries:

dry bead sterilizer

☒ **Sterile gloves**
☐ **Mask**
☐ **Cap**
☐ **Sterile gown**
☐ **Sanitized operating area**
☐ **Clipping or plucking of hair or feathers**
☐ **Skin preparation with a sterilant such as betadine**
☐ **Practices to maintain sterility of instruments during surgery**
☒ **Non-survival (clean gloves, clean instruments, etc.)**

5. Describe all surgical procedures:

a. Skin incision size and site on the animal:

No incision will be made, but injections on pad of each foot on both rear legs.

b. Describe surgery in detail (include size of implant if applicable):

1. In experiments 1 and 2, at the appropriate time point the animals will be anesthetized and the necessary tissue will be collected.
2. However, in experiment 3 we will deliver specific cDNA plasmids to the FDB muscles. These are standard DNA plasmids and have no viral packing contents to them (i.e. lenti or adenovirus). The animal will be anesthetized (4% isoflurane) to a necessary surgical plane (determined by toe pinch) and then placed on a heating pad (37 °C) and maintaining the anesthesia using a rodent face mask. Anesthetic depth will be monitored by toe pinch reflex.
3. Both FDBs will be injected 10 µL of a sterile hyaluronidase solution under the footpads of the mouse using a 1" long 33 gauge sterile needle. This will be done by penetrate the skin at a point close to the heel of the foot and advance the needle subcutaneously towards the base of the toes for ~1/4".
4. We will repeat the procedure with the other foot.
5. We will then place the mouse in a cage and allow it to fully recover from anesthesia using a heating pad.

6. After one hour, anesthetize the animal for a second time and place it on the heating pad. Following the same procedure described for the hyaluronidase solution, we inject a total of 20-50 µg of the plasmid DNA (depending on the size of the cDNA plasmid construct). The total injection volume will be less than 20 µL/foot. Previous experience with this procedure has shown us that as long as very low volumes are used the needle entry points do not affect the foot. However, if we find issues with the needle entry point we will close them with approved tissue-glue. We will consult with DCM vets to determine the best kind of tissue glue.
7. After both feet have been injected with the cDNA plasmid, we will place a sterile platinum-plated stimulation needle under the skin at heel, and a second one at the base of the toes. The electrodes will be oriented parallel to each other and perpendicular to the long axis of the foot.
8. We will connect the head of the needle electrodes to the electrical stimulator using micro-clip connectors. We will electroporate the muscles by applying 20 pulses, 20 ms in-duration/each, at 1Hz yielding an electric field of ~100 V/cm. *Note: This is a very minor stimulus resulting in no visible contractions of the FDB muscle.* We will repeat the above procedures in the contralateral foot of the animal.
9. We will then return the animal to its cage and once fully recovered from anesthesia maintain it under observation. Note: if the procedure went normally, the animal should regain full mobility within 30 minutes and afterwards is ready to be sent back to the animal room at the vivarium.
10. In our past experience, the injections of hyaluronidase and cDNA in the footpads do not have noticeable adverse effects on the animals. Once recovered from anesthesia, mice are able to amble normally around the cage. As an additional precaution, mice will be treated with meloxicam at 5-10 mg/kg PO once a day for 3 days as an analgesic. Treatment will begin prior to the procedure and continue for 2 more days.

If we see unusual behavior from the animal after injection, we will consult with DCM on how to proceed. Unusual behavior could include but is not limited to: reduced mobility, failure to groom, reduced eating or water consumption

c. Method of wound closure:

No incision is made, so in our experience wound closure is not necessary. However, if open wounds appear at the injection site sterile wound glue will be applied.

i. Number of layers

Click here to enter text.

ii. Type of wound closure and suture pattern:

Click here to enter text.

iii. Suture type/size/wound clips/tissue glue:

Click here to enter text.

iv. Plan for removing of skin sutures/wound clip/etc:

Click here to enter text.

6. Anesthetic Protocol:

a. If anesthesia/analgesia must be withheld for scientific reasons, please provide compelling scientific justification as to why this is necessary:

We will not withhold anesthesia/analgesia for any reason.

b. Anesthesia/Analgesia For Surgical Procedures

	Agent	Dose (mg/kg or %)	Volume	Route	Frequency	Number of days administered
Pre-operative analgesic	meloxicam	5-10 mg/kg	50 uL	PO	Once a day	1
Pre-anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Anesthetic	Isoflurane	4%	4mL flow rate	inhalation	constant	Acute only during surgery
Post-operative Analgesic	meloxicam	5-10 mg/kg	50 uL	PO	Once a day	2 more days after initial dose
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

c. Methods that will be used to monitor anesthetic depth (include extra measures employed when paralyzing agents are used):

toe pinch and respiration rate

d. Methods of physiologic support during anesthesia and immediate post-op period (fluids, warming, etc.):

warming pad and fluids

e. List what parameters are monitored during immediate post-op period. Provide the frequency and duration:

animals weight, food consumption and behavior are monitored for 72 hrs

f. Describe any other manipulations that will be used to alleviate pain, distress, and/or discomfort during the immediate post-op period (soft bedding, long sipper tubes, food on floor, dough diet, etc.):

None are necessary. If we encounter issues we will locate the campus veterinarian and seek advice.

g. List criteria used to determine when animals are adequately recovered from anesthesia and when the animals can be returned to their home environment:

normal cage mobility, normal eating/drinking

no shivering, no huddling in corner of cage, no signs of excessive licking and/or self-mutilation,

7. Recovery from Surgical Manipulations (after animal regains consciousness and is returned to its home environment)

Click here to enter text.

a. What parameters (behavior, appetite, mobility, wound healing, etc.) will be monitored:

We will monitor all appetite, mobility, wound healing

b. How frequently (times per day) will animals be monitored:

2x per day in first 24 hrs and 1x/day in next 48hrs

c. How long post-operatively (days) will animals be monitored:

3 days total

8. **Surgical Manipulations Affecting Animals**

a. Describe any signs of pain/discomfort/functional deficits resulting from the surgical procedure:

typically reduced mobility or excessive licking of foot

b. What will be done to manage any signs of pain or discomfort (include pharmacologic and non-pharmacologic interventions):

Meloxicam will be given if the animal shows signs of pain.

c. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

If the animal appears to develop indicators of chronic discomfort that do not resolve, we will seek the advice of the campus veterinarian. If no solution is available, the animal will be removed from the study and humanely euthanized.

V. Euthanasia

Please refer to the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition and DCM Guidelines to determine appropriate euthanasia methods.

A. **Euthanasia Procedure.** ***All investigators, even those conducting non-terminal studies, must complete this section in case euthanasia is required for humane reasons.***

1. Physical Method- If a physical method is used, the animal should be first sedated/anesthetized with CO_2 or other anesthetic agent. If prior sedation is not possible, a scientific justification must be provided:

Click here to enter text.

2. Inhalant Method- isoflurane

(if other, describe the agent and delivery method)

Animal will be anesthetized to an appropriate surgical plane. Surgical depth will be monitored through toe pinch and visual examination of respiration rate. Upon reaching surgical depth, we will remove the skeletal muscles from the rear hind quarters. Upon removal of tissue from both hindlimbs, we will then remove the diaphragm and heart from the animal.

3. Non-Inhalant Pharmaceutical Method (injectables, MS-222, etc.)- Please provide the following:

a. Agent:

Click here to enter text.

b. Dose or concentration:

Click here to enter text.

c. Route:

Click here to enter text.

B. Method of ensuring death (can be physical method, such as pneumothorax or decapitation for small species and assessment method such as auscultation for large animals):

Pneumothorax

C. Describe disposition of carcass following euthanasia:

Carcasses will be bagged and disposed of in either ECDOI freezer or Brody/LSB coolers

I acknowledge that humane care and use of animals in research, teaching and testing is of paramount importance, and agree to conduct animal studies with professionalism, using ethical principles of sound animal stewardship. I further acknowledge that I will perform only those procedures that are described in this AUP and that my use of animals must conform to the standards described in the Animal Welfare Act, the Public Health Service Policy, The Guide For the Care and Use of Laboratory Animals, the Association for the Assessment and Accreditation of Laboratory Animal Care, and East Carolina University.

Please submit the completed animal use protocol form via e-mail attachment to iacuc@ecu.edu. You must also carbon copy your Department Chair.

PI Signature: e-mail 7/22/15 ~~06-17-15~~ Date: _____

Veterinarian: Karen A. Dypalt Date: 7/22/15

IACUC Chair: [signature] Date: 7/23/15

Pending IBC approval
Kad 7/22/15
DeC'd 7/23/15

APPENDIX 1-HAZARDOUS AGENTS			
Principal Investigator: Spangenburg, espen	Campus Phone: 252-737-5041		Home Phone: 530-574-4825
IACUC Protocol Number:	Department: Physiology/ECDOI		E-Mail: Spangenburge14@ecu.edu
Secondary Contact: Department: Robert Lust	Campus Phone: 252-744-2762	Home Phone:	E-Mail: lustr@ecu.edu
Chemical Agents used: tamoxifen		Radioisotopes used:n/a	
Biohazardous Agents used: cDNA plasmid	Animal Biosafety Level: 1		Infectious to humans? NO
PERSONAL PROTECTIVE EQUIPMENT REQUIRED:			
Route of Excretion: none			
Precautions for Handling Live or Dead Animals: Gloves, lab coats, and safety glasses will be worn for live animals. Dead animals will placed in sealed bags			
Animal Disposal: All dead animals will be placed in sealed bag and placed in the -20 C freezer located at 4300J in the ECDOI.			
Bedding/Waste Disposal: No special requirements needed.			
Cage Decontamination: No special requirements needed.			
Additional Precautions to Protect Personnel, Adjacent Research Projects including Animals and the Environment: none required			
Initial Approval Safety/Subject Matter Expert Signature & Date _____			

Davenport, Janine

From:	Bagley, Alana
Sent:	Friday, July 17, 2015 4:32 PM
To:	Davenport, Janine
Cc:	Spangenburg, Espen Eric
Subject:	AUP Q332-333
Attachments:	Spangenburg approval Q332-333.pdf

Attached are the approved documents for AUP Q332 and Q333. If you have any questions or concerns do not hesitate to contact me.

Thanks,

Alana E. Bagley, AOEE
EH&S Specialist
bagleya@ecu.edu (primary)
bagleya00@students.ecu.edu
252-328-6166 (office)
252-737-1458 (fax)
www.ecu.edu/oehs

7.17.15

Laboratory Safety Plan for Using Tamoxifen

(Required fields are noted with a *. This document may be saved in Microsoft Word and 0.changed using the table commands.)

***Process**	Treatment of Mice with Tamoxifen
***Hazardous Chemical/ Chemical Class**	Tamoxifen Target Organs: Eyes, Liver, Kidneys, Blood,
***Hazardous Equipment**	syringes
***Potential Hazards**	Harmful if inhalation, ingestion, absorption through the skin and eyes occurs. Substance may cause cancer, may be a reproductive toxin (harmful to fertility and unborn children) and teratogen.
***Personal Protective Equipment**	Safety glasses/goggles, nitrile or other chemical compatible gloves (no latex), closed front/back lab coat, long pant and closed-toed shoes.
***Engineering and Ventilation Controls**	All handling, preparation and injections will be done in a chemical fume hood or certified biological safety cabinet. The floor of the hood will be covered with a plastic backed paper liner. All extraneous equipment will be removed from the hood before work begins. All equipment required for the injections will be placed in the hood prior to beginning work. Tubes and vials containing these materials can only be removed from the fume hood if tightly capped and the exterior is wet wiped.
Designated Use Area for Carcinogens, Reproductive Toxins or Acute Toxins	The reagent will be prepared and handled in the fume hood. Vials should be kept in a separate container out of contact with other substances. The fume hood must be used when handling the chemical. The storage site in the lab will be secured at all times except when it necessitates the chemical's use.
Special Use Procedures	Drug delivery will be accomplished in a closed loop system. Syringes used for delivery will be sized large enough so that they are not full when the entire drug dose is present. Never re-cap syringes. Syringes will be disposed at the end of administration in a sharps disposal container labeled with the cancer hazard symbol. If moving the chemical ensure the lid is secure, placed in secondary containment with absorbent paper and lid. Use freight elevator or stairs to transport.
Special Handling and Storage Requirements	Stock and prepared dilutions will be securely stored. Containers will be marked with a cancer hazard symbol and segregated from other chemicals by secondary containment lined with a plastic backed absorbent paper. It will be labeled in compliance with OSHA/ECU EH&S mandates. Avoid prolonged or repeated exposure. Keep tightly closed. Store at required temperatures.
***Spill and Accident Procedures**	If a spill occurs under the fume hood, the substance will be wiped up with absorbent gauze pads and the sill area cleaned 3 times with soap and water by trained personnel. Any broken glass fragments will be collected with a scoop and brush and placed in the sharps container. Contaminated items and clean-up material will be double bagged and placed in a red bag for incineration. Red bags must be marked with the cancer hazard symbol. If a spill takes place outside of a fume, trained staff will call EH&S at 328-6166 and the area of spill will be isolated from lab traffic and activities and/or the lab personnel will be instructed to evacuate the area.
***Waste Minimization Plan**	Smallest quantity required will be purchased.
***Hazardous Waste Disposal**	All chemical waste material will be discarded through the ECU hazardous waste management system.
Decontamination Procedures	The fume hood will be decontaminated if there is an incident that would result in the hood being contaminated. This will be accomplished by removing the paper liner and wiping the hood interior surfaces with 1:5 bleach from the top to bottom and back and forth.
Animal Care Precautions	Animal care staff will wear nitrile gloves, isolation gowns, closed toed shoes while handling bedding and other animal wastes.
***Chemical Procurement**	Authorized lab personnel will obtain the minimum quantities needed. The reagent will be procured using a needle and syringe. The minimal amount necessary for injections will be procured at each injection.
***Revision Date**	5-13-2015

APPENDIX 1 - HAZARDOUS AGENTS			
Principal Investigator: Espen Spangenburg	Campus Phone: 252-737-5041		Home Phone: 530-574-4825
IACUC Protocol Number: Q332	Department: Physiology		E-Mail: spangenberge14@ecu.edu
Secondary Contact: Bob Lust Department: Physiology	Campus Phone: 252-744-2762	Home Phone:	E-Mail: lustr@ecu.edu
Chemical Agents Used: Tamoxifen		Radioisotopes Used:	
Biohazardous Agents Used:		Animal Biosafety Level:	Infectious to humans? No
PERSONAL PROTECTIVE EQUIPMENT REQUIRED: STANDARD PERSONAL PROTECTIVE EQUIPMENT FOR DCM ANIMAL LAB TECHNICIANS			
Route of Excretion: mostly feces, much less in urine (some unchanged).			
Precautions for Handling Live or Dead Animals: All personnel should wear personal protective equipment when handling live or dead animals.			
Animal Disposal: All materials will be discarded in biohazard bags with a cancer hazard sticker and incinerated through ECU Hazardous Waste Management.			
Bedding / Waste Disposal: When changing or dumping animal bedding and waste ensure it is completed under a changing stations. All materials will be discarded in biohazard bags with a cancer hazard sticker and incinerated through ECU Hazardous Waste Management.			
Cage Decontamination: Normal cage washing will be sufficient.			
Additional Precautions to Protect Personnel, Adjacent Research Projects including Animals and the Environment: **PI will notify Dale Aycock or covering supervisor when the injection schedule will begin.** Standard PPE for DCM personnel including mask, chemical compatible gloves, safety glasses/goggles, and isolation gowns, long pants and closed toed shoes.			
Initial Approval Safety/Subject Matter Expert Signature & Date 7·17·15			

Veterinarian:_____ Date:_____

IACUC Chair: _____Date:_____

Laboratory Safety Plan for 14% Ethanol with Sunflower oil injection

***Process**	15 % Ethanol with sunflower oil injection
***Hazardous Chemical/ Chemical Class**	Flammable Liquid/Vapor Class 3 Target Organs: Kidneys, Liver and Central Nervous System (CNS)
***Hazardous Equipment**	Syringe
***Potential Hazards**	This substance is considered harmful if swallowed, or inhaled. Contact with skin and eyes will cause severe irritation, possible painful sensitivity to light. If ingested could cause blindness. Inhaling this material may cause CNS depression, dizziness and possible respiratory failure.
***Personal Protective Equipment**	Safety glasses/goggles, full-buttoned lab coat, long pants, closed toe shoes, and nitrile or neoprene gloves
***Engineering and Ventilation Controls**	Preparation and handling of ethanol preservation containers in a certified fume hood. The floor of the hood will be covered with plastic backed paper liner. All extraneous equipment will be removed from the hood before work begins. All equipment required for preparing the solutions should be in place before continuing with chemical. Containers with this material can only be removed from fume hood if tightly capped and the exterior wet wiped.
Designated Use Area for Carcinogens, Reproductive Toxins or Acute Toxins	n/a
Special Use Procedures	When transporting containers, ensure they are in an absorbent lined secondary containment large enough to contain a spill from the largest chemical container.
Special Handling and Storage Requirements	Store in a cool, well-ventilated area, preferably in a flammable cabinet. Keep away from flames, sparks and ignition sources. Do not store near oxidizing agents, perchlorates, nitric acid, chromic acid or peroxides. Ensure the container lid is secure screw top, check security of lid before and after each use and before storage. And use only required amount for each experiment.
***Spill and Accident Procedures**	Clean spills only if proper materials are available and if researcher is properly trained to do so. For minor spills ventilate area. All other spills should be reported to EH&S for clean-up
***Waste Minimization Plan**	Smallest amount required will be purchased. Use minimum amount of chemicals for each experiment.
***Hazardous Waste Disposal**	Disposal of hazardous waste shall be completed by EH&S Hazardous Waste Management.
Decontamination Procedures	If contamination occurs wipe fume hood with soap and water from back to front. Wash hands immediately after use and before leaving the laboratory.
Animal Care Precautions	n/a
***Chemical Procurement**	Smallest quantity required will be purchased.
***Revision Date**	6-9-2015

7-17-15

Laboratory Safety Plan for Meloxicam
(Required fields are noted with a *. This document may be saved in Microsoft Word and changed using the table commands.)

***Process**	Meloxicam (anesthetic/analgesic)
***Hazardous Chemical/ Chemical Class**	Meloxicam- Flammable Class 3 (Flammable Liquid)
***Hazardous Equipment**	N/A
***Potential Hazards**	If absorbed through eyes chemical may cause serious damage. Harmful if ingested, inhaled or absorbed through skin.
***Personal Protective Equipment**	Safety glasses/goggles; nitrile gloves (resistant to these materials); chemical resistant lab coats; closed toed shoes; long pants.
***Engineering and Ventilation Controls**	All handling, preparation, dilutions of this chemical must be done in a certified chemical fume hood, unless authorization is given by DCM to use outside of fume hood. The floor of the hood will be covered with plastic backed paper liner. All equipment required for dilutions will be placed in the hood prior to beginning work.
Special Use Procedures	N/A
Special Handling and Storage Requirements	Keep container tightly closed. Store in cool, dry, well-ventilated space, out of direct sun light. Do not create/breathe dust or aerosols. Store with like chemicals.
***Spill and Accident Procedures**	Clean spills only if proper materials are available and if researcher is properly trained to do so; all other spills should be reported to EH&S for clean-up. For minor spills: ventilate area; if spill occurs outside hood, cover liquid with absorbent material; slowly brush into dust pan and place in plastic bag; do not breath the dust from absorbent. Contact EH&S for removal of sealed waste clean-up material. Do not place clean up materials in regular waste.
***Waste Minimization Plan**	Smallest quantity required will be purchased.
***Hazardous Waste Disposal**	All waste material, including liquid waste, will be discarded through the ECU hazardous waste management system. Any biological waste should be placed in a red bio-hazard bag and request a pick up through Prospective Health.
Decontamination Procedures	If hood is contaminated, then at the end of the work session decontaminate by wiping down the hood interior with soap and water. (PPE as listed above must be worn during decontamination.)
Animal Care Precautions	Animal care workers should wear standard PPE required.
***Chemical Procurement**	Meloxicam will be procured in the minimum quantity necessary.
***Revision Date**	February 25, 2015

Davenport, Janine

From:	Taylor, Yvonne
Sent:	Thursday, July 23, 2015 10:50 AM
To:	Spangenburg, Espen Eric
Cc:	Johnson, Edward Harvey; Spruill, Chad; Smith, Charles Jeffrey; Lust, Bob; Davenport, Janine; McRae, Susan; Aycock, Dale
Subject:	Biosafety
Attachments:	Scan0001.pdf

Dr. Spangenburg,
Please see the attachment regarding your Biosafety registration.
Thank you,

Yvonne B. Taylor
ECU Brody School of Medicine
Office of Prospective Health
640 Life Sciences Bldg. 188
Greenville, NC 27834
252-744-2070 252-744-2417 (Fax)

The Brody School of Medicine
Office of Prospective Health
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188 Warren Life Sciences Building • Greenville, NC 27834
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EAST CAROLINA UNIVERSITY

Occupational Medicine
Employee Health
Radiation Safety
Infection Control
Biological Safety

TO: Dr. Espen E. Spangenburg
Physiology

FROM: Eddie Johnson EMJ
Chad Spruill CS
Biological Safety Officers

RE: Registration Approval

Date: July 23, 2015

Your Biological Safety Protocol, Spangenburg, 15-01, **Gene regulation of skeletal muscle function** has received **approval** to be conducted at Biosafety Level 1 and Animal Biosafety Level 1 in ECDOI 4th floor Heart Institute/4101 based on your registration/revisions submitted,

using: A. Biohazards

- ☐ Infectious Agent(s)
- ☐ Biotoxin(s)
- ☐ Allergen(s)
- ☐ Prion(s)
- ☐ Human blood, fluid, cells, tissue or cell cultures
- ☐ Transformed cells
- ☒ Other – Recombinant DNA and Transgenic animals

and/or B. ☒ NIH Use of Recombinant DNA (or RNA) molecules, microorganisms use or breeding transgenic or techniques (plasmids, viral vectors, transfection); of transgenic animals or plants at NIH Category III-F.

This work will receive final approval pending laboratory inspection and Blood Borne Pathogen training. We do not foresee any issues arising with the Biosafety registration or the lab in preventing final approval.

This approval is effective for a period of 3 years and may be renewed with an updated registration if needed at that time. Your laboratory will be inspected periodically (every 1-3 years) depending upon the materials/techniques used.

Please notify the Animal Care staff before beginning work with Biohazard agents in animals. Also please keep in mind all individuals who will be exposed to or handle human-derived biohazardous agents will be due for Blood Borne Pathogens refresher training annually.

Please do not hesitate to contact Biological Safety at 744-2070 if you have any questions, concerns, or need any additional information. Best wishes on your research.

cc: Dr. Jeff Smith, Chair, Biosafety Committee
Dr. Robert Lust, Chair
Janine Davenport, IACUC
Dr. Susan McRae, IACUC
Dale Aycock, Comparative Medicine