



# The impact of ER $\alpha$ action on muscle metabolism and insulin sensitivity — Strong enough for a man, made for a woman

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## ABSTRACT

**Background:** The incidence of chronic disease is elevated in women after menopause. Natural variation in muscle expression of the estrogen receptor (ER) $\alpha$  is inversely associated with plasma insulin and adiposity. Moreover, reduced muscle ER $\alpha$  expression levels are observed in women and animals presenting clinical features of the metabolic syndrome (MetSyn). Considering that metabolic dysfunction impacts nearly a quarter of the U.S. adult population and elevates chronic disease risk including type 2 diabetes, heart disease, and certain cancers, treatment strategies to combat metabolic dysfunction and associated pathologies are desperately needed.

**Scope of the review:** This review will provide evidence supporting a critical and protective role for skeletal muscle ER $\alpha$  in the regulation of metabolic homeostasis and insulin sensitivity, and propose novel ER $\alpha$  targets involved in the maintenance of metabolic health.

**Major conclusions:** Studies identifying ER $\alpha$ -regulated pathways essential for disease prevention will lay the important foundation for the rational design of novel therapeutics to improve the metabolic health of women while limiting secondary complications that have plagued traditional hormone replacement interventions.

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**Keywords** Estrogen action; Estrogen receptors; Insulin sensitivity; Metabolic homeostasis

## 1. INTRODUCTION

For over two decades researchers have shown strong relationships between estrogen action and metabolic health in women. Moreover, epidemiological reports indicate that chronic disease incidence increases in women following menopause. Considering that menopause occurs on average by age 51 (National Institutes of Health, NIA [www.nia.nih.gov](http://www.nia.nih.gov)), and that life expectancy has increased for white females to ~80.6 years (The National Vital Statistics Report, 2012), women in the modern era are challenged with heightened disease risk associated with increasing adiposity and metabolic dysfunction for up to three decades of life. Although many researchers and clinicians have focused on the impact of replacement estrogens to ameliorate clinical symptoms and provide protective health benefit, an incomplete understanding of hormone action as well as estrogen receptor distribution and function has contributed to our continued confusion and failure to advance therapeutic strategies to combat chronic disease-associated pathologies for women.

Regarding the benefits of exogenous hormone replacement therapy (HRT) on diabetes risk after menopause, large randomized clinical trials of postmenopausal estrogen-based HRT compared with placebo as well as prospective cohort studies, have shown reductions in fasting glucose, insulin, and incidence of new onset T2D [1–6]. Meta-analyses indicate a 30% lower relative risk [RR 0.7 (CI, 0.6–0.9)] of

new-onset T2DM in postmenopausal women following HRT compared with placebo [7]. The mechanism by which HRT reduces T2D incidence in postmenopausal women is not yet known however molecular studies in rodents indicate that this protective effect may be achieved in part as a consequence of estrogen-induced insulin-sensitization. Considering that 75–85% of insulin-stimulated glucose disposal is into skeletal muscle and since skeletal muscle typically represents 30–40% of total body mass, we have focused our efforts in understanding the effects of estradiol/ER $\alpha$  action in this tissue.

In this review, we will present studies related to the biological actions of estrogen receptors in skeletal muscle in controlling glucose homeostasis and insulin sensitivity, as insulin resistance and metabolic dysfunction are identified as major underpinnings involved in the pathobiology of chronic diseases that plague our society today. We will present basic research suggesting that the estrogen receptor (ER)  $\alpha$  form ER $\alpha$  (encoded by the gene *ESR1*) is an important target to combat metabolic dysfunction.

## 2. ER LIGAND-MEDIATED EFFECTS ON METABOLISM AND INSULIN ACTION

Identification and phylogenetic analysis of steroid receptors in basal vertebrates and reconstruction of the sequences and functional attributes of ancestral proteins led to the conclusion that the first steroid

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receptor was an estrogen receptor [8]. Early studies in reproductive tissues investigating the actions of estradiol led to the paradigm of classical nuclear ERs as ligand-activated transcription factors [9]. Although ERs exist in two main forms,  $\alpha$  and  $\beta$ , which have multiple splice variants of unknown function, ERs exhibit tissue specificity in expression and function [10]. The classical, or genomic mechanism of ER action, describes a scenario whereby the ligand-activated ER dissociates from its chaperone and binds as a dimer either directly to estrogen response elements (ERE) in target genes promoters or indirectly to AP-1 or SP-1 response elements through protein tethering association with other DNA-bound transcription factors [11,12] (Figure 1). Following DNA binding, ER dimers interact with basal transcription factors leading to activation or repression of target gene expression. Overlap in binding sites for  $E_2$ -liganded ER $\alpha$  and ER $\beta$  is observed when receptors are expressed individually; however, when both ERs are present, few sites are shared. Each ER restricts the binding site occupancy of the other, with ER $\alpha$  typically dominating [13]. Moreover, ligand-activated ERs promote transcription in a cyclic fashion. The repeated cycling of the receptor complex on and off target promoters in the presence of continuous  $E_2$  stimulation may represent a mechanism of continuous sensing and adaptation to the external hormonal milieu to yield the appropriate transcriptional response [14].

In addition to classical signaling,  $E_2$ -ER $\alpha$  can act within seconds to minutes via extranuclear and membrane-associated forms of the receptor [15] (Figure 1). Membrane associated receptors localize to caveolae where they congregates with other signaling molecules, including G proteins, growth factor receptors, tyrosine kinases (Src), linker proteins (MNAR), and orphan G-protein coupled receptors (GPCRs) [16]. In a variety of cell types, membrane and extranuclear pools of ERs activate protein kinases that phosphorylate transcription factors to promote their nuclear translocation and transcriptional action [15,17]. The G protein-coupled estrogen receptor (GPER), or GPR30, has been reported to respond to  $E_2$ ; however, its role as an ER is still controversial. Although emerging evidence in murine muscle cells shows diverse distribution of GPR30 in the nucleus, mitochondria, and cytoplasm [18], functional aspects of this receptor *in vivo* remain unclear; thus, GPR30 will not be discussed in this review.

Although ERs are believed to function almost exclusively as classical nuclear ligand-activated transcription factors with respect to their reproductive actions, the role of nuclear vs. extranuclear actions of ERs in the regulation of metabolism and insulin action remains controversial and inadequately interrogated [19]. More recently, an emerging theme in the field is that for many targets, nuclear and non-nuclear signaling must collaborate to achieve the full biological action of estradiol [20]. Although non-genomic signaling is supported for specific cell types under defined conditions, scientific dissection of these pathways remains challenging, thus a central question in the field pertaining to the tissue-specific sites of action and the molecular mechanisms by which ER $\alpha$  selectively activates or represses target genes remains.

Reduced whole body ER $\alpha$  expression or impaired ER $\alpha$  function due to genetic alteration (including genetic variants) has been linked with increased prevalence of specific features of the metabolic syndrome including insulin resistance and obesity in both male and female human subjects and rodents [21–28]. Since obesity is a prominent phenotype observed in estrogen or ER $\alpha$  deficient rodent models, the specific role of ER $\alpha$  in adipocytes and the phenotypic outcomes of obesity as a consequence of adipose-specific ER $\alpha$  deletion in mice is currently under investigation by several laboratories around the world. Whether the obesity phenotype observed in whole body *Esr1*<sup>-/-</sup> mice or women harboring an *ESR1* polymorphisms is explained by impaired ER $\alpha$  action in adipose tissue specifically, or as a secondary phenotype of ER $\alpha$  impairment in other metabolic tissues requires resolution.

Insulin resistance is a central disorder in the pathogenesis of obesity and type 2 diabetes and is a defining feature of the Metabolic Syndrome, a clustering of metabolic abnormalities including obesity, hypertension, glucose intolerance, and dyslipidemia [29,30]. Metabolic dysfunction and a clustering of these abnormalities is worrisome as this clinical distinction is now thought to impact nearly a quarter of the US population and drive increased risk of numerous chronic disease states including diabetes, cardiovascular disease, neurodegeneration, and certain forms of cancer [31,32]. Normally cycling pre-menopausal women show enhanced insulin sensitivity compared to men when sensitivity is normalized to lean mass (since women have a reduced

## Estrogen Receptor Action

### Target Gene Regulation

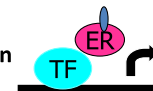
- **Classical Mechanism**

$E_2$ -ER complex binds directly to estrogen response elements (EREs) in target gene promoters.



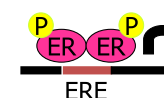
- **Indirect DNA Binding Mechanism—ERE independent Genomic Action**

Protein-protein interactions with other transcription factors (e.g. NF $\kappa$ B, AP1, Runx).



- **Ligand-Independent Genomic Action**

Growth Factors activate Protein Kinase Cascades leading to phosphorylation (P) of ER at EREs.



- **Non-Genomic Mechanism**

Membrane-associated ERs mediate estrogen actions (e.g. G-protein coupled receptors).



**Figure 1: Molecular actions of ER $\alpha$  to activate or repress target genes by classical DNA binding, non ERE genomic action, or non-genomic actions.** ERE, Estrogen response element in target gene promoters; P, phosphorylation; TF, transcription factor.

lean body mass compared to men) [33]. Improved insulin sensitivity and protection against factors promoting insulin resistance are likely underpinnings of reduced type 2 diabetes incidence observed for premenopausal women compared with men [33,34]. Case-in-point, although a 40–50% reduction in insulin-mediated glucose disposal is consistently observed in males following high fat feeding [35,36], E<sub>2</sub>-replete females, humans and rodents, are typically protected against a high fat diet and acute fatty acid-induced insulin resistance [37,38]. In contrast to the metabolic protection seen in normally cycling premenopausal women, following menopause (biological or surgically-induced), a precipitous decline in insulin sensitivity coincides with a dramatic increase in fat mass, and elevated circulating inflammatory markers, LDL, triglycerides, and fatty acids [176]. Similar to humans, ovariectomy (OVX) mice and rats become insulin resistant, show impaired exercise-stimulated glucose disposal into muscle [39], and are more susceptible to the deleterious effects of high fat diet or lipid oversupply. The physiological consequences of OVX are prevented by treatment with an ER $\alpha$ -specific agonist or by restoration of circulating estradiol within a physiological concentration [40–42].

Although chronic administration of E<sub>2</sub> is shown to improve insulin sensitivity in rodents, the acute action of E<sub>2</sub> to promote insulin-stimulated glucose uptake into muscle remains disputed; this despite consistent observations of E<sub>2</sub>-induced activation of Akt and AMP-activated Protein Kinase (AMPK) [42,43]. Furthermore, although administration of intravenous conjugated estrogens and E<sub>2</sub> to postmenopausal women or OVX rats, respectively, elicited a significant increase in glucose disposal during hyperinsulinemic-euglycemic clamp studies [44,45], ex vivo treatment of skeletal muscle with E<sub>2</sub> failed to recapitulate the same increase in insulin-stimulated glucose disposal [43]. It could be that methodological issues have clouded this relationship since only super-physiological insulin concentrations have been tested thus far, potentially masking the true effects of estradiol on insulin action at physiological insulin doses. This ex vivo observation by Rogers et al. [43] is also in contrast to short-term estradiol effects on insulin action in myotubes from postmenopausal women and age-matched men [46]. Additionally, recent research by Park et al. shows that the timing of E<sub>2</sub> administration following menopause may also be of importance given the reduced nuclear expression of ER $\alpha$  found in muscle from women >10 years from final menstrual period (FMP) late postmenopausal (LPM), versus early postmenopausal (EPM) women <6 years from FMP (EPM). Moreover, in parallel with a lack of E<sub>2</sub> effect to improve insulin sensitivity in LPM, E<sub>2</sub> failed to increase the phosphorylation of AMPK and induce Pgc1 $\alpha$  expression unlike EPM [44,47]. Collectively, these data suggest that there could be a critical window of therapeutic opportunity in which E<sub>2</sub> administration could be utilized for improving metabolic health and that the expression and functionality of ER $\alpha$  may be a key determinant of therapeutic efficacy [44,47].

Similar to findings for ovarian failure in women and rodents, a reduction in circulating estrogens resulting from rare inactivating mutations of the Cyp19 (aromatase) or experimental deletion in Cyp19 in mice confers an obesity-insulin resistance phenotype [21,48–55]. The physiological and genetic evidence argues that E<sub>2</sub> and ER favor insulin sensitivity in rodents and humans of both sexes when E<sub>2</sub> is maintained within a tight physiological concentration. Indeed, replacement or augmentation of E<sub>2</sub> to supraphysiological levels or over-stimulation of ERs is thought to induce insulin resistance secondary to hyperinsulinemia and/or a reduction in total GLUT4 expression in muscle [56,57]. In fact, two studies have reported that in postmenopausal women, higher plasma levels of E<sub>2</sub> were prospectively associated with increased risk of developing T2D [58,59].

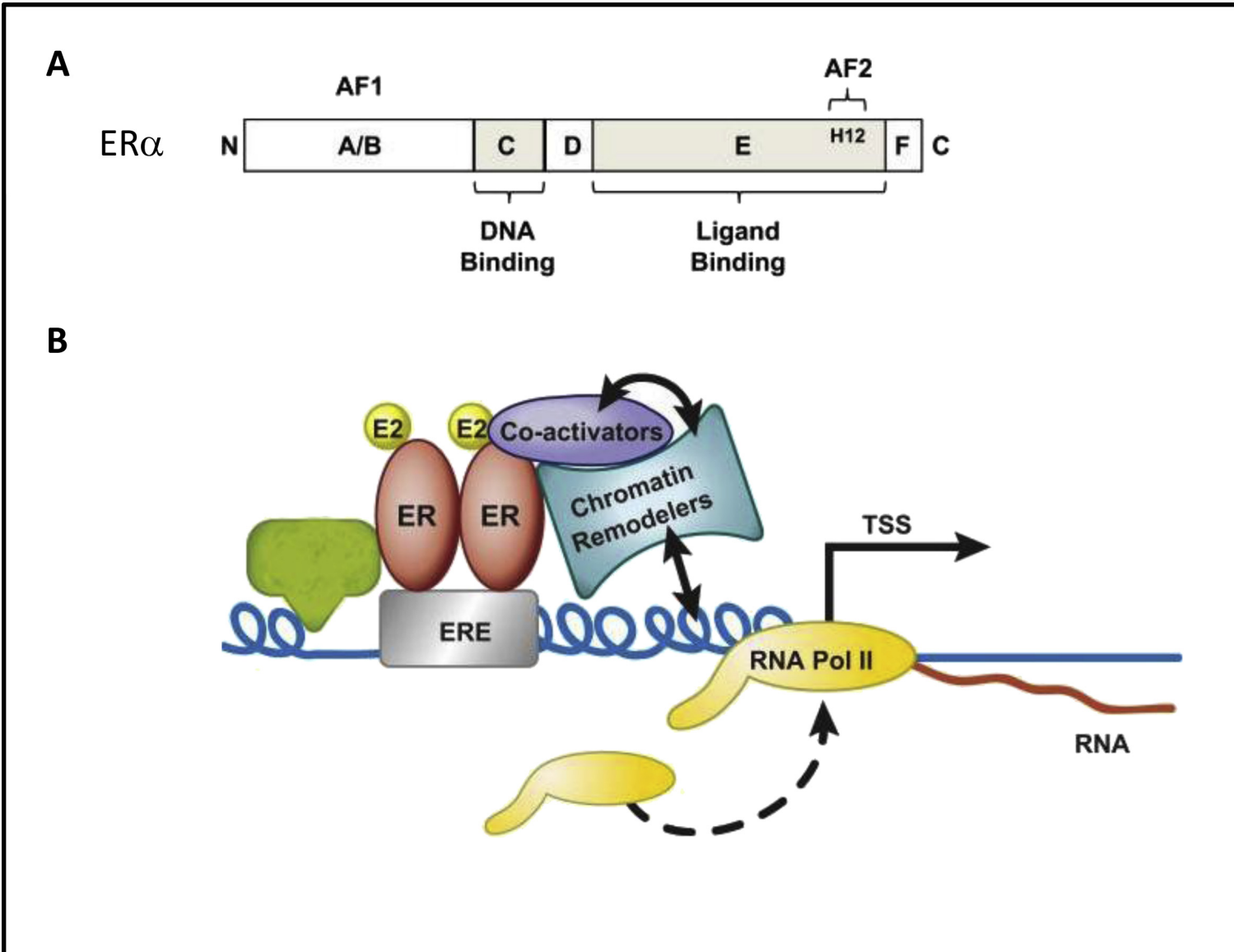
Clearly, additional studies in rodents and humans using a dose response strategy are necessary to better understand the interplay of steroid hormones including E<sub>2</sub>, testosterone and progesterone on the regulation of metabolism and insulin action in glucoregulatory tissues. Thus, many questions remain: does E<sub>2</sub> enhance skeletal muscle insulin sensitivity and at what minimal pharmacological dose, and what are the critical tissues of E<sub>2</sub> action that confer protection against nutrient-induced insulin resistance?

### 3. ESTROGEN RECEPTOR $\alpha$ — STRUCTURE AND GENOMIC ACTION

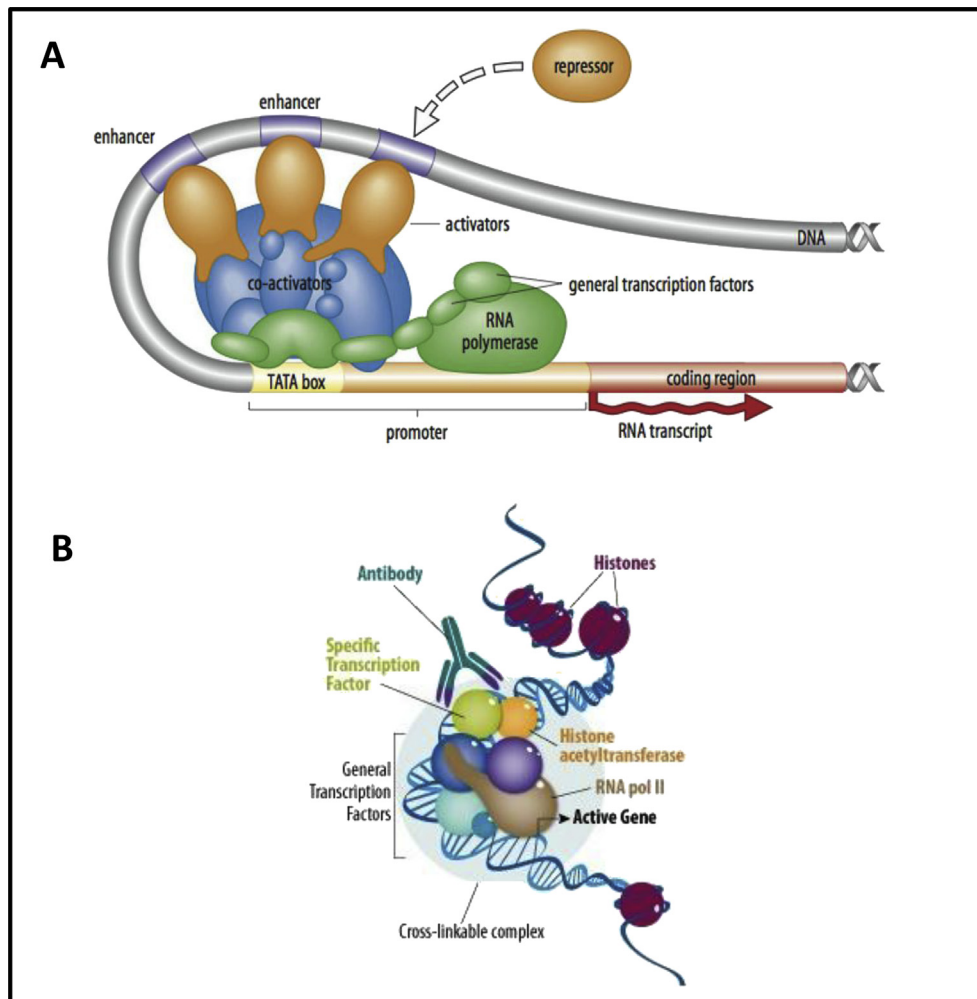
The cellular effects of estrogens are mediated by two ERs: ESR1 (the gene that encodes ER $\alpha$ ) and ESR2 (ER $\beta$ ). ESR1 was identified in 1958 [60], and ESR2 was first identified in the rat prostate and ovary in 1996 [61]. Different splice variants of each receptor have been identified and each exhibit distinct tissue expression patterns and functions [10,62]. With the exception of the ER $\alpha$ D3 isoform, all other ERs are composed of six functional domains, from A to F, containing an NH<sub>2</sub>-terminal domain (NTD), DNA-binding domain (DBD), and COOH-terminal ligand-binding domain (LBD) (Figure 2). Two regions, named activation function (AF) have been identified as crucial for the transcriptional response of the ERs; the first is localized at the NTD, and the second is in the LBD [63]. ESR1 and ESR2 are structurally different in the ligand binding pockets, and this knowledge was responsible for driving the development of receptor-specific selective ligands [62], since ERs primarily act as ligand-mediated transcriptional factors [9,62,64,65]. Although ERs were the first of the nuclear superfamily to be cloned over two decades ago [66], the tissue-specific gene targets and mechanisms of action, including activation and repression of genes involved in the integrative regulation of metabolic health, remains an area of intense investigation.

Since ER $\alpha$  is a ligand-dependent transcription factor that regulates a large number of genes in diverse target tissues to achieve selective action, the question arises as to how ER $\alpha$  exerts such specific and exacting control over so many different processes. The interplay between ligand, receptor, DNA sequence, cofactors, chromatin context, and post-translational modifications collectively govern transcriptional regulation by ER $\alpha$ . As stated above, ER $\alpha$  can bind directly to DNA (classical pathway) or can impact gene transcription indirectly via protein–protein tethering. In the classical sense, ER $\alpha$  homodimers are thought to bind specific sequence motifs called estrogen response elements (ERE) (Figure 2). The ERE is a 15bp palindrome consisting of two PuGGTA half site separated by a 3bp spacer [67–69] and was first identified in 1986 in the promoter of the *Xenopus vitellogenin* gene. Interestingly, there are over 70,000 ERAs present in the human genome and 9944 ERAs in the mouse genome. In total, 660 evolutionarily conserved elements have been identified [70]. However, in silico studies suggest that ER $\alpha$  binding is dependent on more factors than just DNA sequence.

The chromatin structure and binding accessibility of ER $\alpha$  target sites remains inadequately understood, but is currently under intense investigation (Figure 3). It is estimated that only 23% of E<sub>2</sub> responsive genes are direct ER $\alpha$  targets [69]. Using ChIP-PET analysis in MCF-7 cells, Lin et al. identified 1,234 high confidence binding clusters of which 94% were projected to be bona fide ER $\alpha$  binding regions [69]. Of importance, only 5% of the mapped estrogen receptor binding sites were located within 5 kb upstream of the transcriptional start sites of adjacent genes (regions containing the proximal promoters); therefore, the vast majority of ER $\alpha$  binding sites mapped to intronic or distal locations (>5 kb from 5' and 3' ends of adjacent transcript),



**Figure 2:** ER $\alpha$  structure (A) and DNA binding at an estrogen response element (ERE) (B) (adapted from [170]).



**Figure 3:** Schematic showing potential mechanism(s) by which enhancer elements cooperate to regulate putative ER $\alpha$  target genes (A), and a cartoon showing a crosslinked chromatin complex immunoprecipitated by a specific transcription factor of interest, herein ER $\alpha$ . (adapted from [78,171,172]).

suggesting transcriptional regulatory mechanisms act over significant physical distances [69,71,72]. Of all the identified sites, 71% harbored putative full estrogen response elements (EREs), 25% ERE half sites, and 4% had no recognizable ERE sequences [69]. Of important consideration, Lin et al. found that only ~20% of the bona fide human ER $\alpha$  binding sites overlapped with whole genome vertebrate alignments suggesting limited conservation of functional ER $\alpha$  binding sites between species [69]. Thus, since most studies interrogating ER $\alpha$  binding on a genome-wide scale are performed in breast cancer cell lines, species, sex, and cell-type specific dynamics of estrogen action should be deliberated.

Classical genetics approaches provide evidence of redundant, additive, and synergistic enhancer relationships over a variety of loci. More recent studies, using a multiplex interference approach (in Isikawa and T-47D cells), have revealed strong collaboration between predominant and supportive ER $\alpha$  binding sites exposing a complex functional hierarchy of enhancers regulating the expression of ER $\alpha$  target genes [72]. Current thinking is that chromosomal looping allows for the collaborative action of these distal sites and that distance to the target gene and strength of the ERE motif predict the ER $\alpha$  binding site importance [72]. At least in liver, ERE sites, ERE half sites, AP1, bHLH, ETS, and Forkhead-binding motifs were enriched DNA sequences in

ER $\alpha$  binding regions [73]. Considering that most of what we know about ER $\alpha$  action is gleaned from MCF7 cells, an important question is whether the genomics of ER $\alpha$  in breast cancer can be translated to muscle and other metabolic tissues [74]. Now that we are moving beyond whole genome binding site cartography, putative ER $\alpha$  binding sites will require validation by functional interrogation using mutagenesis and chromatin immunoprecipitation approaches in a cell specific context [71,72,75–78].

#### 4. THE ROLE OF ER $\alpha$ IN REGULATING INSULIN ACTION AND GLUCOSE DISPOSAL

*ESR1* is broadly expressed in the central nervous system and in peripheral tissues including adipose, skeletal muscle, liver, and immune cells [79]. Women and men as well as male and female mice carrying specific *ESR1* variants develop features of the metabolic syndrome including obesity, glucose intolerance, and insulin resistance. Clinical evidence is undeniable; the clustering of these metabolic abnormalities increases disease risk (heart disease, type 2 diabetes, and certain forms of cancer) [21,22,80,81]. Of translational relevance, whole body ER $\alpha$  knockout mice (ER $\alpha$ KO) recapitulate the metabolic dysfunction observed in a male human subject with a rare inactivating receptor

mutation, as well as aspects of the phenotypes observed in subjects with genetic polymorphisms in the receptor [21,22,80]. Not only do ER $\alpha$ KO mice have increased adiposity caused by reductions in energy expenditure, but they also exhibit glucose intolerance and insulin resistance, thus demonstrating the critical role for *ESR1* in regulating energy and metabolic homeostasis [80–82]. The integration of central and peripheral *ESR1* action as well as the interaction of ER $\alpha$  and sex chromosome action remains to be defined; however, the tissue dissection approach to studying ER $\alpha$  using mice with conditional deletion alleles has allowed the research community the opportunity to delineate unique aspects of ER $\alpha$  biology in a tissue and sex-specific context.

Observational findings indicate that *ESR1* expression levels are reduced in muscle from women with the metabolic syndrome, and that natural variation in muscle *ESR1* expression in women is inversely correlated with adiposity and fasting insulin, markers of metabolic health (i.e. low muscle *ESR1* expression levels are associated with metabolic dysfunction and increased adiposity) [83]. Remarkably similar findings were observed across numerous strains of inbred female mice, as well as in genetically obese animals, thereby illustrating the strong relationship between muscle ER $\alpha$  expression and metabolic health. Collectively, these data suggest that maintenance of ER $\alpha$  expression or activation of muscle *ESR1* could serve as an effective means to combat diseases associated with metabolic dysfunction [83]. Although these strong correlative findings suggest a relationship between muscle ER $\alpha$  expression levels and metabolic health, few studies have directly tested a causal relationship. Does a loss of ER $\alpha$  specifically from myocytes drive skeletal muscle insulin resistance, or does the insulin resistance phenotype observed in the ER $\alpha$ KO model arise from increased adiposity/altered adipokine/

cytokine secretion and impaired central drive of feeding and ambulatory movement?

Although two forms of the receptor are expressed in many of the glucoregulatory tissues, ER $\alpha$  is expressed at much higher abundance than ER $\beta$  or GPR30, as these transcripts are nearly undetectable in muscle from humans and rodents [46,83–85]. Consistent with these observations, homozygous deletion of ER $\beta$  failed to produce insulin resistance [86] in contrast to the marked skeletal muscle insulin resistance observed in ER $\alpha$ KO animals (Figure 4) [81,87]. The underlying mechanism contributing to impaired insulin action in muscle of ER $\alpha$ KO animals remains disputed. Findings reported by Bryzgalova et al. [82] suggest reduced total GLUT4 levels in muscle as an underlying cause for the ER $\alpha$ KO insulin resistance phenotype; however, these findings were not supported by Ribas et al. [81]. Furthermore, despite maintenance of GLUT4 mRNA and protein, Ribas et al. reported more dramatic skeletal muscle insulin resistance in ER $\alpha$ KO mice than Bryzgalova et al. Hevener and colleagues suggest that the skeletal muscle insulin resistance observed in ER $\alpha$ KO mice is predominantly a consequence of direct ER $\alpha$  deletion effects on insulin action with a secondary impact of inflammation on proximal insulin signaling. This hypothesis was later confirmed using muscle-conditional deletion alleles studied *in vivo*, *ex vivo*, and *in vitro*.

Indeed, in female muscle-specific ER $\alpha$  knockout mice and myotubes with ER $\alpha$  knockdown, no alteration in GLUT4 mRNA or protein in skeletal muscle was observed despite reduced insulin-stimulated glucose disposal into muscle during clamp studies. Findings in the muscle-specific ER $\alpha$  mouse are consistent with those of whole body ER $\alpha$  mice [88]. Furthermore, additional studies by Barros et al. [57,89] assessing GLUT4 expression in response to ovariectomy with/without E<sub>2</sub> supplementation are in conflict with other studies of similar design

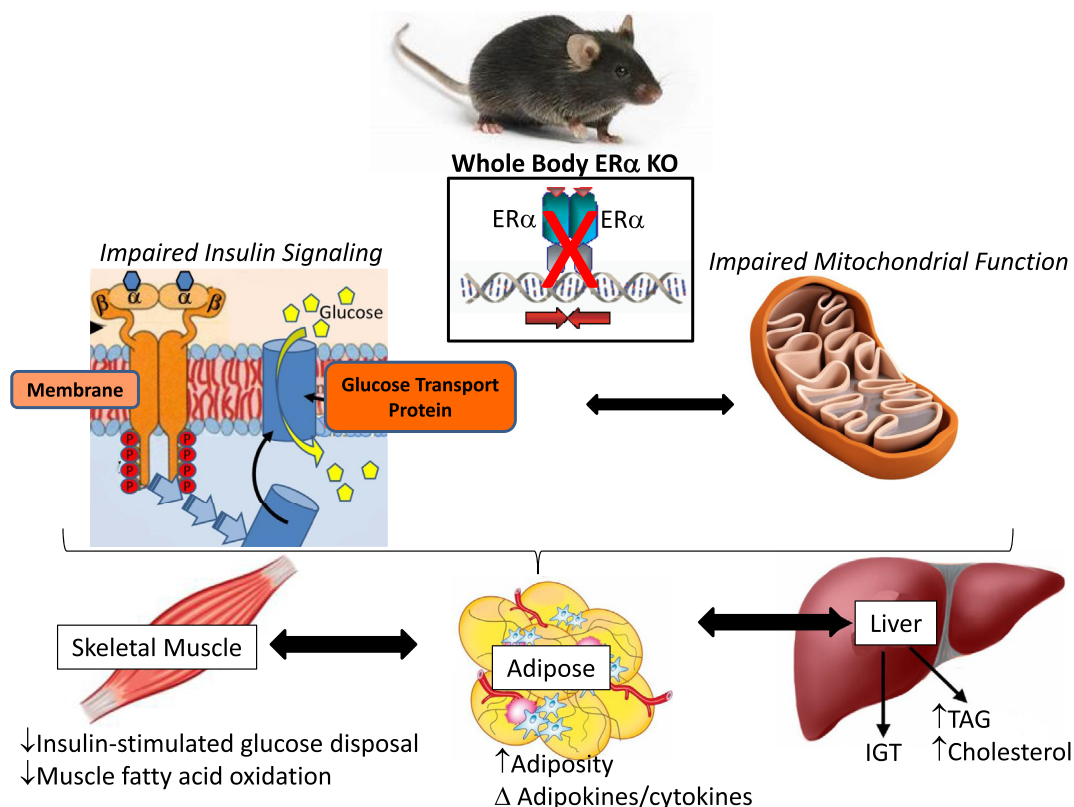


Figure 4: The impact of whole body ER $\alpha$  deletion on metabolism and insulin action [80–82,173–175].

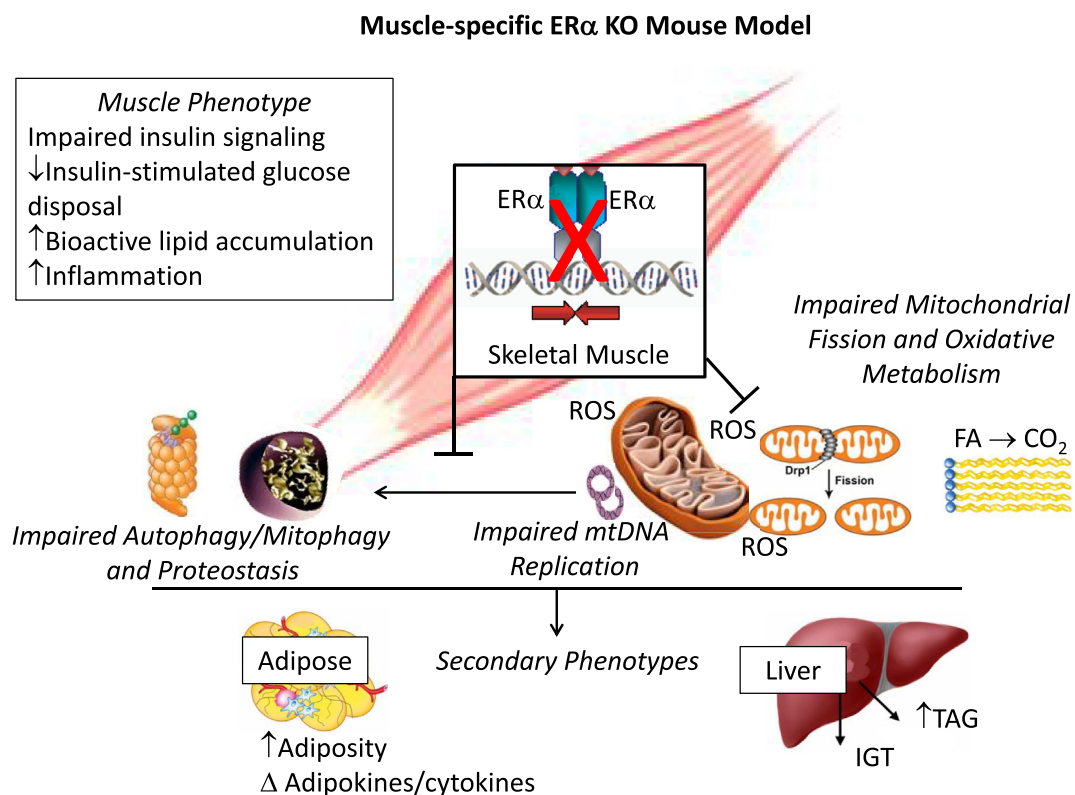
[46,85,90–92]. Given the lack of consensus ERE in the GLUT4 promoter [93] and absence of confirmatory findings in cellular reporter and chromatin immunoprecipitation assays, the regulation of GLUT4 expression by ER $\alpha$  requires further investigation. GLUT4 is regulated by several redundant transcriptional pathways [94,95]. Considering that total GLUT 4 transcript and protein levels are not reduced in humans or rodents in the context of insulin resistance, obesity, and type 2 diabetes, or between men and women [96,97], it is likely that in the absence of ER $\alpha$ , other transcription factors compensate to maintain GLUT4 levels [98–103]. This is not to say that ER $\alpha$  is not involved in the exercise-stimulated increase in GLUT4 observed following training [96,104,105], given the concomitant increase in ER $\alpha$  expression observed in muscle of exercise-trained humans and mice [84,106,107].

Myocyte enhancer factor 2 (MEF2) expression and a functional MEF2 element in the GLUT4 promoter are critical for GLUT4 gene expression [108]. Furthermore, reciprocal regulation between ER $\alpha$  and MEF2 is observed in cardiomyocytes via ER $\alpha$  interaction with class II HDAC in female mice only [109]. Despite complex transcriptional signal integration in the regulation of GLUT4 expression [94,95,110–113], it is conceivable that elevated ER $\alpha$  action could promote increased GLUT4 transcription via heightened protein tethering with MEF2 on the GLUT4 promoter or by indirect action via AMPK [43,114]. It is important to note that transcriptional activity of the GLUT4 promoter is quite low under basal conditions, and other ovarian hormones (e.g. progesterone) are

shown to play an antagonistic role in the regulation of GLUT4 expression [39]. These issues as well as the dose of E<sub>2</sub> administration during interventional studies (presumably off-target effects of super-physiological doses of E<sub>2</sub> are deleterious to metabolism), the age and hormone status of the human subjects and rodents (species and strain) used are important considerations when interpreting the literature.

Considering the varying roles that muscle and adipose tissue play in controlling whole body metabolic homeostasis, it is likely that the interplay of transcriptional regulators of GLUT4 vary markedly between tissues. Taken together, these data would suggest a potential role for ER $\alpha$  as an enhancer of GLUT4 transcription in muscle under certain conditions, but not necessarily obligatory in the direct regulation of GLUT4 expression under basal conditions.

Collectively, work by Ribas et al. suggests that the skeletal muscle insulin resistance observed in whole body ER $\alpha$ KO mice and animals with a muscle-specific deletion of ER $\alpha$  is predominantly the result of impaired insulin signal transduction (Figure 5) [88]. A role for ER $\alpha$  in the regulation of proximal insulin signal transduction has been suggested previously as E<sub>2</sub> administration to insulin resistant rodents increases insulin receptor substrate (IRS)-1 abundance and insulin-stimulated tyrosine phosphorylation and as well as phosphorylation of Akt at activation site Ser473 [87,115]. Akt serves many functions in myocytes including ER $\alpha$ -induced regulation of myogenic differentiation [116], suppression of muscle-atrophy ubiquitin ligases via FOXO1 inhibition [117], and induction of genes associated with myocellular



**Figure 5: The impact of skeletal muscle-specific ER $\alpha$  deletion on metabolism and insulin sensitivity.** Skeletal muscle-specific ER $\alpha$  deletion reduced mitochondrial DNA replication and impaired muscle oxidative metabolism, despite maintenance of mtDNA copy number. Increased PKA and reduced calcineurin activity promoted elongated, hyperfused mitochondria in MERKO muscle. The morphological changes coupled with an imbalanced PKA-calcineurin axis blunted mitochondrial fission signaling through DRP1 and impaired macroautophagy, both processes critical for mitochondrial turnover by mitophagy. Collectively, the retention of damaged mitochondria to the network was paralleled by increased ROS production, inflammation, and insulin resistance in skeletal muscle of MERKO mice. Findings implicate a critical role for ER $\alpha$  in the maintenance of muscle mitochondrial and metabolic health [83].

proliferation [116,118–121]. In breast cancer cell lines, endothelial cells, and cortical neurons, ER $\alpha$ -specific binding and activation of PI3kinase as well as suppression of the tumor suppressor and PI3kinase inhibitory protein, PTEN, is well-established [122–126]; however, studies on this direct interaction are limited in skeletal muscle. Additionally, E<sub>2</sub> acting via ER $\alpha$  is also shown to promote phosphorylation of p38 MAPK [127,128], and transduction of a signaling cascade shown to enhance GLUT4 intrinsic activity and glucose uptake [129–131]. Furthermore, ER $\alpha$  activation of Akt and MAPK pathways is thought to underlie E<sub>2</sub>-mediated protection of muscle against age-induced sarcopenia [132–138], exercise-induced muscle damage [120,134,139,140], and myocyte apoptosis in the face of a variety of cellular perturbations [141–144]. Thus, ER $\alpha$  stimulation of muscle growth and insulin sensitivity via these pathways is reasonable to posit.

## 5. ER $\alpha$ AND SKELETAL MUSCLE FATTY ACID METABOLISM AND INFLAMMATION

Normally cycling pre-menopausal women are protected against acute lipid-induced insulin resistance compared with estrogen-deficient women and men [38,145]. Furthermore, muscle from premenopausal women shows enhanced insulin sensitivity despite 47% higher triglyceride content compared with age-matched men [97]. This observation in women, is consistent with a reduced respiratory quotient and greater reliance on fatty acids as a fuel source [146]. These data indicate interesting similarities between E<sub>2</sub> replete women and exercise trained subjects including elevated muscle ER $\alpha$  expression [84,106,107], heightened insulin sensitivity [101], elevated muscle lipid tolerance [147], and enhanced oxidative capacity [148,149]. Consistent with the reported effects of E<sub>2</sub> on metabolism, estrogen supplementation is shown to enhance lipid oxidation *in vivo* in men during acute endurance exercise [150], and palmitate oxidation in myotubes from male subjects *ex vivo* [46]. The effect of E<sub>2</sub> to increase the expression of fatty acid transport protein FAT/CD36 and FABP as well as transcription factors and key enzymes that regulate oxidative metabolism [90,96,151] likely underlie these observations in male subjects. Moreover, E<sub>2</sub> treatment reduced HFD-induced insulin resistance in skeletal muscle by 50% (assessed by hyperinsulinemic-euglycemic clamp) in an ER $\alpha$ -dependent manner [87]. In addition, similar to exercise, E<sub>2</sub> is shown to rapidly stimulate AMPK phosphorylation in both muscle and myotubes in culture [43,152]. AMPK is considered a central regulator of many cellular processes including growth, mitochondrial biogenesis, and oxidative metabolism [153,154]. Comparable to the effects of E<sub>2</sub>, the ER $\alpha$ -selective agonist PPT stimulates AMPK phosphorylation in muscle of ovariectomized female rats [42] while OVX or whole body ER $\alpha$  deletion is associated with reduced skeletal muscle levels of phosphorylated AMPK [81,155]. Recent evidence from Lipovka et al. shows that ER $\alpha$  but not  $\beta$  directly binds the  $\beta\gamma$ -subunit domain of AMPK  $\alpha$  [156]. Muscle PPAR $\alpha$ , PPAR $\delta$ , and UCP2 expression are also reduced in whole body ER $\alpha$ KO mice suggesting that E<sub>2</sub> acting via ER $\alpha$  is essential in the regulation of a coordinated program regulating oxidative metabolism. Interestingly, although the phenotype of impaired muscle fatty oxidation was recapitulated in the muscle-specific ER $\alpha$ KO mice (MERKO), no alteration in basal p-AMPK, PPAR $\alpha$ , PPAR $\delta$ , or UCP2 was observed [88], suggesting that these specific alterations in muscle gene expression are secondary to the loss of ER $\alpha$  in other metabolic tissues (e.g. CNS, adipose tissue, or liver).

The mechanistic link between the accumulation of lipid intermediates, activation of inflammatory signaling cascades, and impaired insulin

action is shown in myocytes and rodent muscle, and these factors are observed concurrently in obese, type 2 diabetic subjects [157–160] as well as in muscle from whole body and muscle-specific ER $\alpha$ KO mice [81]. Bioactive lipid intermediates including diacylglycerol, DAG, and ceramides are believed to activate stress kinases including IKK $\beta$ , c-Jun-N-terminal kinase (JNK), and certain nPKCs [158,161–163]. Indeed, muscle from normal chow-fed whole body ER $\alpha$ KO mice showed heightened inflammatory signaling as reflected by markedly increased JNK phosphorylation and TNF $\alpha$  transcript [81]. A similar inflammatory profile was observed in muscle from female MERKO mice [83]. Collectively, these data illustrate the essential role of ER $\alpha$  in regulating fatty acid oxidation (FAO) and inflammation in muscle and highlight the primary nature of ER $\alpha$  in the direct control of these processes. The impact of pathway crosstalk and other cellular signaling mechanisms engaged in altering FAO and inflammation in the context of ER $\alpha$  deficiency, are not well fleshed out. In addition to the action of ER $\alpha$  on enzymes involved in fatty acid transport and processing, new evidence is emerging regarding the direct role of ER $\alpha$  on oxidative metabolism via control over specific mitochondrial processes (discussed in greater detail below) [83]. With respect to inflammatory signaling activation, beyond ER $\alpha$  de-repression of selective inflammatory targets within the nucleus, it is likely that mitochondrial ROS production, sarcoplasmic reticulum stress, and proinflammatory lipid species are additional mediators of heightened inflammation in muscle.

## 6. THE ROLE OF MUSCLE ER $\alpha$ IN THE REGULATION OF MITOCHONDRIAL FUNCTION

Despite model differences in gene and protein expression, skeletal muscle insulin resistance and bioactive lipid accumulation was surprisingly similar between ER $\alpha$ KO and MERKO animals [81,83]. Triacylglycerol, diacylglycerol, and ceramides were all elevated significantly in muscle from female mice lacking ER $\alpha$  globally or specifically in muscle [81,83]. Consistent with these observations, oxygen consumption rates in C2C12 myotubes with ER $\alpha$  knockdown (KD) were reduced significantly [83]. In addition, mitochondria from muscle cells depleted of ER $\alpha$  produced high levels of reactive oxygen species (ROS) thus promoting cellular oxidative stress. Analysis of mitochondrial function confirmed a defect in respiratory complex 1 activity in MERKO muscle [83]. Moreover, mitochondria from MERKO mice produced increased levels of H<sub>2</sub>O<sub>2</sub> and superoxide, thus recapitulating findings in C2C12 myotubes with Esr1-KD.

This defect in mitochondrial function was paralleled by a reduction in expression of the only mammalian mitochondrial (mt) DNA polymerase, *Polg1*, in both MERKO muscle as well as murine myotubes with *Esr1* knockdown (Figure 5). Additionally, heavy water labeling of newly synthesized mtDNA showed a reduction in the rate of mtDNA replication, functionally supporting an impact of the reduction in *Polg1* expression in MERKO mouse muscle [83]. Additional mechanistic studies showed that estradiol and ER $\alpha$ -selective ligand treatment induced *Polg1* expression in muscle cells; however, the ligand was ineffective to induce gene expression when the receptor was absent. Considering the presence of a consensus ERE in the *Polg1* promoter, ongoing studies in the Hevener laboratory will delineate the mechanism(s) by which ER $\alpha$  regulates mtDNA replication via this polymerase. mtDNA replication is intimately linked with mitochondrial remodeling by a process known as fission [164]. The Hevener laboratory has shown that treatment of murine myotubes with ER $\alpha$  agonists also promotes this mitochondrial morphological alteration inducing the severing of a mitochondrion into two daughter organelles achieved



by high order dynamin related protein (Drp) 1 oligomers promoting scission. Interestingly, although ER $\alpha$  activation drives mitochondrial fission, it appears to achieve this shift in mitochondrial architecture by a coordinated enzymatic regulation of the fission activator calcineurin and the calcineurin inhibitor Rcan1 [83]. Supporting the role of ER $\alpha$  in the regulation of mitochondrial morphology, we observed that mitochondria from both female and male MERKO mouse muscle were enlarged, elongated, and hyperfused, suggesting a reduction in fission:fusion dynamics.

Internally consistent with the morphological data obtained by transmission electron microscopy, analysis of mitochondrial dynamics signaling showed reduced fission signaling by Drp1 (including increased phosphorylation at the inhibitory Ser<sup>637</sup> site and reduced total Drp1 protein on the outer mitochondrial membrane), as well as increased abundance of the inner and outer mitochondrial membrane fusion proteins OPA1 and Mfn2 [83] (Figure 5). Ribas et al. observed a marked increase in expression of the mitochondrial fission inhibitor Rcan1 in myotubes with Esr1-KD, female MERKO muscle, and muscle from women displaying clinical features of the metabolic syndrome. Ribas et al. overexpressed Rcan1 in myotubes using lentivirus and showed that *Rcan1* expression elevated to levels seen in MERKO mouse muscle impaired insulin action [83]. Moreover, impairment in muscle mitochondrial fission promoted dysfunction in mitochondrial respiration and insulin action in primary myotubes from female mice with *Dnm1L* deletion and in C2C12 myotubes with lentiviral-mediated *Dnm1L* knockdown [83]. Therefore, we hypothesize that both a reduction in the direct effects of ER $\alpha$  on insulin signaling as well as indirect effects of ER $\alpha$  on insulin action mediated by mitochondrial dysfunction contribute to the development of global disturbances in metabolic health and insulin sensitivity (Figure 5).

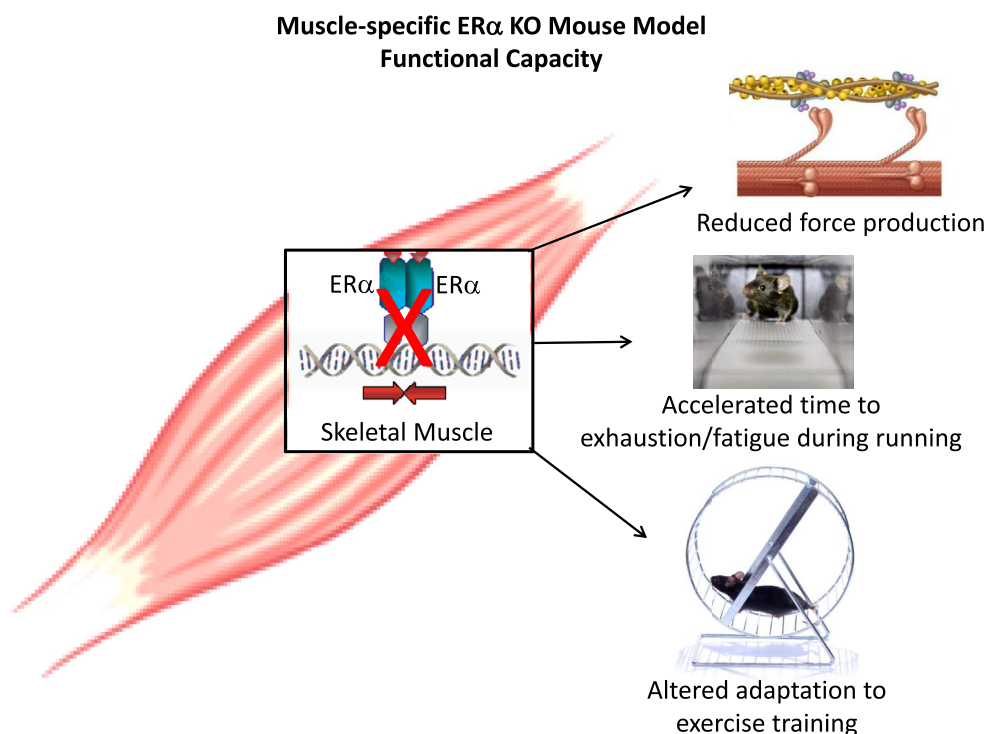
In light of the observation that Rcan1 was only induced in female MERKO muscle (not in males) despite a similar impairment in fission

signaling in both sexes of MERKO mice, the Hevener laboratory has initiated additional studies to flesh out the sex-specific mechanisms that underlie altered mitochondrial dynamics and function in the absence of ER $\alpha$ . These studies are viewed to be of translational importance since it is well known that sex is an important biological variable contributing to differences in disease incidence and mechanisms of pathobiology.

Follow-up studies in the Hevener laboratory in MERKO mice, as well as new studies in muscle-specific *Polg1*, Parkin (*Park2*), and Drp1 (*Dnm1L*) KOs, and mice with muscle-specific ER $\alpha$  overexpression, will allow for the determination of whether the stall in mtDNA replication is a primary defect driving mitochondrial dysfunction and insulin resistance. Secondly, we will learn whether the impairment in mitochondrial quality control and turnover seen in MERKO muscle is a consequence or causal of the stall in mtDNA replication, and contributory or resultant of insulin resistance. The use of broad transcriptomic, proteomic and metabolomic approaches in rodents harboring conditional ER $\alpha$  deletion alleles coupled with more targeted chromatin immunoprecipitation analyses in ER $\alpha$  ligand treated animals will allow for the identification of novel ER $\alpha$  target genes as well as reveal new mechanism(s) controlling metabolic signaling nodes in insulin responsive tissues.

## 7. THE ROLE OF ER $\alpha$ IN THE REGULATION OF MUSCLE PERFORMANCE AND EXERCISE TRAINING

In addition to the skeletal muscle-induced insulin resistance, MERKO mice also fail to adapt to endurance training (Figure 6). Whether defects in exercise training are linked with impaired mitochondrial function and disruption of calcium homeostasis is unclear. In addition to diminished muscular endurance in MERKO, Lai and Collins showed that ER $\alpha$  deficient muscles are also fast fatiguing and have reduced performance capability [165,166] (Figure 6). Studies from this group



**Figure 6:** Skeletal muscle-specific deletion of ER $\alpha$  reduces running performance, isometric force contraction, and impairs specific molecular adaptations to endurance exercise training — adapted from [83,166].

show that  $E_2$ , in a concentration-dependent manner, elevates phosphorylation of the myosin regulatory light chain (pRLC) in C2C12 myotubes in culture and in muscles from C57BL/6J mice [166]. Female mice with a skeletal muscle-specific KO of ER $\alpha$  produced 16% less eccentric and 16–26% less submaximal and maximal isometric force with impaired force recovery relative to WT mice. Maximal torque production by plantar- and dorsi-flexors were 16% and 12% lower in female KO than WT mice, and produced 21–32% less power, and submaximal isometric and peak concentric torques [166]. Data support the hypothesis that ablation of ER $\alpha$  in skeletal muscle underlies muscle weakness, suggesting that the beneficial effects of estradiol on muscle strength are receptor mediated via ER $\alpha$ . Since metabolic function is clearly linked with muscle quality and mass, and these traits are most highly associated with morbidity and mortality in aging, strategies aimed at maintaining muscle metabolism appear warranted especially in women during the menopausal transition.

## 8. CONCLUSIONS AND PERSPECTIVES

In recent years, novel molecular targets have emerged offering the prospect of pharmacological intervention to restore metabolic homeostasis and insulin action, as well as ameliorate complications associated with type 2 diabetes and obesity. The inherent beauty of targeting ER $\alpha$  therapeutically is underscored by decades of research and in depth knowledge related to biological/clinical efficacy and toxicity profiles obtained for estradiol/SERMs during preclinical studies in animal models and clinical trials in human subjects. Although estrogen treatment (primarily 17 $\beta$ -estradiol) is shown to promote energy homeostasis, improve body fat distribution, and diminish insulin resistance,  $\beta$ -cell dysfunction and inflammation, the challenge with chronic hormone administration is the narrow therapeutic index. Thus, the translation of the basic advances in diabetes and obesity treatment described in this review, although successful in rodents, is problematic when extending to clinical practice. However, ten years after the WHI concluded that the risks of hormone therapy outweighed its benefits, reevaluation of the WHI findings and determination that the risks of breast cancer, coronary heart disease, stroke, and pulmonary embolism with estrogen-progestin treatment were overstated, prompted a position statement by the North American Menopause Society stating that HRT has a role in short-term treatment of menopausal symptoms [167]. Thus, considering the new and more positive light estradiol is receiving by clinical experts, it will be important to determine whether short-term treatment with a well-designed ER $\alpha$  agonist during early menopause offers protection against metabolic dysfunction and insulin resistance.

Additionally, it is imperative that we determine how to modulate the specific ER-controlled pathways involved in energy balance and glucose homeostasis and develop estradiol mimetics that initiate specific cellular events promoting metabolic benefit without unwanted side effects. This could possibly be achieved by fusion peptides [168,169] or through the development of novel SERMs that retain the beneficial metabolic effects of  $E_2$  in desired tissues including skeletal muscle, while exerting antagonist action in breast and uterus. With regard to whole body metabolism, obesity, and insulin sensitivity, future studies should focus on identifying the critical nodes of ER $\alpha$ -mediated metabolic crosstalk between all glucoregulatory tissues as these integrative networks may reveal new pharmacological targets for therapeutic exploitation.

Although this review focused on the role of ER $\alpha$  in controlling metabolism and insulin action in skeletal muscle, clearly the expression of ER $\alpha$  in other metabolic tissues contributes to specific aspects of global

metabolism and metabolic health. Once the general phenotype of each of the ER $\alpha$  deletion models is published, it will be important to integrate the findings so that the field has a better perspective on the tissue selective contribution of ER $\alpha$  to a phenotypic trait. Determination of the overlapping and distinctive phenotypic traits of conditional ER $\alpha$  deletion models will allow the field to better target ER $\alpha$  for global impact. Since insulin resistance underlies most chronic diseases, the Hevener laboratory has focused its efforts on understanding pathways known to impair insulin action including inflammation, and organelle, metabolic, and oxidative stress. Although collective evidence suggests that ER $\alpha$  regulates a vast number of metabolic processes, emerging findings suggest that ER $\alpha$  exerts a primary regulatory role on metabolism by controlling specific aspects of mitochondrial function. Now that novel technologies allow us to study this complex organelle in a more precise and comprehensive way, a new era of mitochondrial biology has emerged. A major area of focus for diabetes researchers is to understand the genes that regulate key aspects of mitochondrial function and determine how this organelle controls other pathways including insulin action, inflammation, adiposity and muscle mass. Lastly, a major limitation in our understanding and interpretation of  $E_2$ -ER action is the lack of information regarding the contribution of extranuclear vs. nuclear ER actions, as well as ligand vs. non-ligand-mediated functions of ERs in controlling key metabolic nodes in insulin responsive tissues. Delineation of these pathways will be critical for moving the field forward and advancing therapeutic strategies to improve women's health.

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## CONFLICT OF INTEREST

None declared.

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