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Estrogen Hormone Biology

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Abstract

The hormone estrogen is involved in both female and male reproduction, as well as numerous other biological systems including the neuroendocrine, vascular, skeletal, and immune systems. Therefore, it is also implicated in many different diseases and conditions such as infertility, obesity, osteoporosis, endometriosis, and a variety of cancers. Estrogen works through its two distinct nuclear receptors, Estrogen Receptor alpha (ERα) and Estrogen Receptor beta (ERβ). Various transcriptional regulation mechanisms have been identified as the mode of action for estrogen, mainly the classical mechanism with direct DNA binding but also a non-genomic mode of action and one using tethered or indirect binding. The expression profiles of ERα and ERβ are unique with the primary sites of ERα expression being the uterus and pituitary gland and the main site of ERβ expression being the granulosa cells of the ovary. Mouse models with knockout or mutation of Esr1 and Esr2 have furthered our understanding of the role each individual receptor plays in physiology. From these studies, it is known that the primary roles for ERα are in the uterus and neuroendocrine system, as female mice lacking ERα are infertile due to impaired ovarian and uterine function, whereas female mice lacking ERβ are subfertile due to ovarian defects. The development of effective therapies for estrogen-related diseases has relied on an understanding of the physiological roles and mechanistic functionalities of ERα and ERβ in various human health and disease.

Keywords

Estrogen; estrogen receptor; ER; ERα; ERβ

Introduction

Our understanding of mechanisms by which hormones act has evolved since the first description over 100 years ago; especially, regarding the role of nuclear receptors and receptor-mediated signaling first proposed by Jensen over 50 years ago (Jensen, 1962; Jensen and Jacobson, 1960, 1962). Our knowledge of cellular mechanisms from the initial concepts of ligand receptor binding, activation, direct DNA binding and resulting gene regulation now includes non-DNA binding or tethering, cellular non-genomic signaling and receptor mediated non-ligand hormone activities (Hewitt et al., 2016). Estrogen, one of the first hormone substances identified, was thought to have only female-selective activities important in female reproduction. We now know, however, that estrogen is also involved in male reproduction and in numerous other systems including the neuroendocrine, vascular, skeletal, and immune systems of both males and females. Estrogen influences many

physiological processes, as it is also implicated in many different diseases including obesity, metabolic disorder, a variety of cancers, osteoporosis, lupus, endometriosis, and uterine fibroids (Burns and Korach, 2012; Deroo and Korach, 2006).

Cell Mechanisms

It is now accepted that the predominant mechanism of estrogen action is through nuclear estrogen receptor (ER) expression in estrogen target organs (Mangelsdorf et al., 1995). For many years it was thought there was only a single estrogen receptor (ERα), but a second form of estrogen receptor (ERβ) was later discovered (Kuiper et al., 1996). The biological effects of estrogen, described later in this chapter, are mediated through these two distinct ER proteins, ERα and ERβ. Separate genes on non-homologous chromosomes encode these receptors; the expression profiles of which are quite different across tissues and cell types. The predominant expression tissues for ERα include: uterus and pituitary gland with the highest levels, liver, hypothalamus, bone, mammary gland, cervix and vagina. ERβ expression on the other hand is expressed in fewer tissues by most analyses, but tissues with predominant levels include ovary, lung, and prostate (Couse et al., 1997). ERβ expression is especially high in the ovary and is found exclusively in the granulosa cells. Many therapeutic interventions to estrogen related diseases target the functions of ERα and ERβ. Such therapeutic approaches highlight the importance of understanding the physiological role of ERα and ERβ in tissues and their *in vivo* mechanistic functionality to identify effective treatments and minimize side effects.

The estrogen receptors are members of the nuclear receptor superfamily of hormone receptors, and are composed of several main structural features that are consistent in these proteins (Aagaard et al., 2011). All members of this superfamily are comprised of four structural and functional domains: an amino-terminal domain (A/B-domain), a DNA binding domain (DBD; C-domain), a hinge region (D-domain), and a ligand-binding domain (LBD; E-domain). The ERs have an additional fifth domain: the carboxyl-terminal domain (F-domain) whose function is still unknown (Mangelsdorf et al., 1995). In the case of ERα and ERβ the C and E domains carry a high-degree of homology between the two forms, however the A/B, D and F domains are divergent (Germain et al., 2006; Mangelsdorf et al., 1995). The A/B-domain contains the transcription activation function 1 (AF-1) which is reported to be important for ligand-independent transactivation (Bourguet et al., 2000). The LBD or E-domain of ERs contains the transcription activation function 2 (AF-2) that is important for ligand-dependent transcriptional regulation (Bourguet et al., 2000). Helix 12 is a highly conserved region within the LBD and is the core of the AF-2 functionality. The structural configuration of helix 12 is changed by ligand binding resulting in either an active (agonist bound) or inactive (antagonist bound) form for the transcription regulation (Green and Carroll, 2007; Klinge, 2000).

Estrogen works through several possible cellular mechanisms to mediate its biological responses as shown in Figure 1. These include two major cellular actions involving the receptors: rapid non-genomic effects and genomic activities (Hewitt et al., 2016). Several studies, primarily in cell culture, have shown these rapid actions occur within minutes of hormone treatment and can be silenced by inhibition of either the MAPK/ERK or AKT

signaling pathways (Clark et al., 2014; Kelly and Levin, 2001). Activation of these intracellular signaling pathways has been shown to involve a plasma membrane-associated process that is mediated by either a G-protein coupled receptor, GPER1 (originally designated GPR30), or a caveolin-associated form of ERα (Levin, 2015; Prossnitz and Hathaway, 2015). Estrogen signaling at the membrane solely involves ERα and is shown to require palmitoylation at cysteine 447 (human) or cysteine 451 (mouse) (Levin, 2015). Mutation of this cysteine in mouse models results in differing phenotypes, effects, and mechanistic interpretations (Adlanmerini et al., 2014; Pedram et al., 2014). How significantly non-genomic signaling contributes to genomic actions of ERα and the biology of hormone responsiveness is still not totally resolved and requires continued study.

Three major genomic ER-mediated transcriptional regulation mechanisms have been characterized. These include the direct binding to regulatory elements of DNA (classical), indirect binding to other existing transcription factors which are bound to DNA (tethering), and ligand-independent receptor activation, which is proposed to involve altered phosphorylation of sites on the receptor protein. Examples of each of these genomic modes of action for estrogen and ER have been published, although most studies have investigated the activity primarily involving ERα. In the first example, hormone-ER binding causes a conformational change in the LBD, allowing helix 12 to accept coactivator interactions. Coactivator binding is required for the resulting genomic response, and is directly proportional to the amplitude of this response. In the absence of hormone, ERα is bound to DNA in an inactive state, as shown in both cell culture and *in vivo* mouse studies by ChIP-Seq. (Carroll et al., 2005; Hewitt et al., 2012). Hormone binding increases the number of binding peaks in the genome. Mouse models in which the DBD of ERα is mutated indicate that direct DNA binding is required to elicit hormone responses and biological activity. Further research will determine whether it is the only activity that is required or if complementary actions of other signaling mechanisms (Ahlbory-Dieker et al., 2009; Hewitt et al., 2014). As shown in Figure 1, other nuclear factors influence direct binding such as a pioneering factor FoxA1, which is bound at sites to allow the recruitment of chromatin remodeling proteins, opening the chromatin to give ER accessibility to its regulatory DNA sites. Following the assembly of the ER transcription complex, composed of a multitude of components (Carroll and Brown, 2006) gene transcription is initiated by recruitment of polymerase II. A second method, (shown in figure 1 Box) primarily described in cell culture is the indirect or tethered mechanism of hormone receptor action in which the hormone receptor modulates gene expression by protein-protein interactions with existing transcription factors (eg. *Fos/jun)*, which bind directly to their respective response elements (AP1) (Jakacka et al., 2001). Other examples of regulatory elements have included binding to factors in Sp1 sites in GC rich regions of DNA (Kushner et al., 2000). Lastly, ER can have regulatory activities and drive hormone responses in the absence of hormone through ligand-independent activation by growth factors or other intracellular signaling pathways, thought to involve phosphorylation of certain serine residues on the receptor (Smith, 1998). Such a coupling of non-genomic and genomic signaling may be an explanation for the complementation of different cellular signaling pathways, which elicit the broad spectrum of hormone responses of estrogen action.

Uterine Estrogen Response

The ovariectomized (ovexed) mouse uterus is an estrogen responsive organ and therefore a valuable model to study ER-mediated responses. The rodent uterus is a bicornuate tube made up of outer muscle cell layers (myometria), an inner lumen lined with luminal epithelial cells, and a layer of stroma cells between the lumen and myometrium. The uterus also contains glandular structures that are lined with epithelial cells. The endogenous stimulation of the uterus occurs during the transient proestrus surge of the reproductive cycle. Experimentally, a single injection of estrogen can mimic this stimulation by being administered to an ovexed animal, and the uterus will then undergo a series of ordered, well-characterized events that can be divided into an initial (early) phase and subsequent (late) responses culminating specifically in waves of mitosis restricted to only uterine epithelial cells. These responses are mediated by ERα, which is expressed in all uterine cells (luminal and glandular epithelial, stromal and myometrial cells).

Studies using the ovexed mouse uterine model have examined the regulation of endogenous uterine genes over a 24-hour stimulatory time course, and shown that the gene regulation pattern follows the progression of early (within 2 hours after estrogen was administered) or late (occurring 12–24 hours after estrogen was administered) events. Some gene regulation was seen at intervening time points, but most fall within either the early or late clusters (Hewitt et al., 2003). In experimental analysis of estrogen responsive uterine genes, it is apparent that samplings at 2 or 24-hour time points will represent most of the observed gene responses correlating with tissue physiological actions

Interaction of ERα and RNA polymerase II (PolII) were analyzed using ChIP-seq in uterine tissue (Hewitt et al., 2012) to understand ERα DNA binding within an *in vivo* system. In vehicle-treated unstimulated samples, more than 5000 peaks were mapped indicating ERα was already bound to the chromatin in the absence of hormone (Hewitt et al., 2012). Estrogen treatment increased the amount of ERα binding at these sites, and also led to ERα binding to additional regions (Hewitt et al., 2012) with a total of more than 17000 sites. The number of active annotated genes (with PolII at the transcription start site (TSS)) within 100 KB of ERα peaks increased from 4672 (1.1 ERα peaks/active gene) to 6519 (2.6 ERα peaks per active gene) thereby showing that in the absence of hormone, ERα is bound to DNA sites, and that hormone treatment increased the number 2.4-fold. Analysis of the ER binding sites for transcription factor binding motifs revealed that ERE motifs were present in 35% of the vehicle sites and were more abundant (59%) in the estrogen-treated sites (Hewitt et al., 2012). Thus, ERE motifs are important for estrogen-dependent ER recruitment. The computed consensus motif for the sequences bound to ERα in uterine chromatin matched the experimentally derived ERE (GGTCAnnnTGACC) (Hewitt et al., 2012), indicating preference for this motif in a biological system. Interestingly, at the sites that were not enriched for ERE motifs, numerous other motifs were seen; notably homeobox (Hox) motifs were highly enriched (Hewitt et al., 2012). Many Hox family members are expressed in the uterus (Hewitt et al., 2012), and Hoxa10 and Hoxa11 have been demonstrated to play key roles in uterine function (Eun Kwon and Taylor, 2004). ERα binding in the uterine tissue was primarily distal from promoters (Hewitt et al., 2012), which has similarly been observed in ERα ChIP-seq in MCF-7 cells. When comparing ChIP-seq data to microarray profiles,

up-regulated transcripts at early time points (2h, 6h) were significantly more likely to have ERα binding at their promoters (0 to 10 kb 5') than down-regulated genes (Hewitt et al., 2012).

Genetic Control of Estrogen Responses

Differences in uterine estrogen sensitivity of two mouse strains, C57Bl6 (more responsive uterus) and C3H (less sensitive uterus) have been mapped to associated quantitative trait loci (QTL) (Roper et al., 1999). Uterine transcriptional profiles of C57Bl6 and C3H mice (basal or 2 or 24 hours after estrogen treatment) include response differences correlated with the QTL on chromosomes 4, 5, 11 and 16 (Wall et al., 2013). For example, *Ngfr* is in the chromosome 11 QT locus, and its transcript is expressed at a 3-fold higher level in ovexed untreated C57Bl6 than in ovexed untreated C3H uterine samples. Uterine NGF signaling has been shown to impact pregnancy (Hah and Kraus, 2014). *Runx1*, which was within the chromosome 16 QT locus, and can enhance estrogen responses (Chimge and Frenkel, 2013), was shown to be present at higher levels in C57Bl6 than C3H uterine epithelial cells (Wall et al., 2013). Transcripts that showed strain-selective differences indicated C3H-selective enrichment of apoptosis, consistent with increase in the apoptosis indicator *Casp3*, and decrease in the apoptosis inhibitor *Naip1* (*Birc1a*) in C3H vs. C57Bl6 following treatment with estrogen (Wall et al., 2013). Mammary gland response differences were also examined (Wall et al., 2014), where an opposite strain sensitivity observation was reported (C3H more sensitive than C57Bl6). Strain-selective transcripts were identified in the mammary samples as well. Most interesting was the opposite pattern of *Runx1* expression, with higher levels in C3H than C57Bl6 mammary epithelia, a pattern consistent with higher estrogen sensitivity of C3H mammary glands (Wall et al., 2014). Understanding differences in sensitivity to estrogen is important in understanding genetic contributions to the impact of xenoestrogens on populations of exposed humans and wildlife.

ERα Mutations Demonstrate Uterine Mechanisms

Since ERα is detected in all uterine cells, deletion or mutation of the receptor is expected to have a profound impact on 17β-estradiol (E2) -mediated responses. Mice with ERα deletion or mutation are therefore an optimal biological model in which to dissect details of ERα mechanisms (Table 1). Mice that lack ERα (*Esr1*$^{-/-}$; aka αERKO) develop a hypoplastic uterus that includes all uterine cell types and structures, however there is no uterine maturation or growth at puberty, and no response to E2 (Couse and Korach, 1999). Since the female reproductive tract is composed of several tissue types, all expressing ERα, identifying the cell type selectivity of ERα activity related to biological responses is critical. This cell specificity becomes of particular significance when comparing the varying physiological responses of the uterus to its inherent functions involving proliferative to luteal secretory responses and implantation (Wang and Dey, 2006) and the development of diseases, including endometriosis, cystic endometrial hyperplasia, fibroids and endometrial cancer. More specifically, in contrast to adults, neonates and prepubertal experimental animals exhibit both stromal and epithelial tissue proliferation under E2 stimulation, while in adults, E2 selectively stimulates growth only in epithelial cells with the stroma remaining quiescent (Quarmby and Korach, 1984). Two major concepts have been published to explain

the mitogenic mechanisms for the E2 activity; one involves direct estrogen action through ER in the epithelial cell, and the second regards a paracrine mechanism of direct stimulation of E2/ER in stromal cells to induce a mitogenic signal (eg. growth factor) on the epithelium. To identify the specific epithelial responses to E2, uterine epithelial-specific ERα knockout mice (*Wnt7a*$^{Cre/+}$;*Esr1*$^{f/f}$ or referred to as "epithelial ER cKO") were generated by crossing *Esr1*-floxed mice (Hewitt et al., 2010a) with *Wnt7a*$^{Cre/+}$ mice (Huang et al., 2012). Using the epithelial ER cKO mouse model, it has been shown that ERα in uterine epithelium is dispensable for epithelial growth response to E2. This finding supports mediation by stromal mitogenic paracrine factors, such as IGF-1. However, epithelial ERα is required for a full growth response of endometrial hyperplasia by actively inhibiting epithelial apoptosis in the uterus (Winuthayanon et al., 2010). To dissect the mediators of epithelial ERα response during uterine transcription, microarray analysis was performed to evaluate the differentially expressed genes in the presence (WT control littermates, referred to as WT) or absence (epithelial ER cKO) of epithelial ERα after E2 treatment for 2 h or 24 h in ovexed adult females (Winuthayanon et al., 2014). RNA microarray analysis revealed approximately 20% of the genes differentially expressed at 2 h were epithelial ERα-independent, as they were preserved in the epithelial ER cKO uteri. This indicates that regulation of the early uterine transcripts mediated by stromal ERα is sufficient to promote initial proliferative responses. However, more than 80% of the differentially expressed transcripts at 24 h were not regulated in the epithelial ER cKO uteri, indicating most late transcriptional regulation required epithelial ERα, especially those involved in mitosis. This shows that loss of regulation of these later transcripts results in the blunted subsequent uterine growth after 3 days of E2 treatment. These transcriptional profiles at 2 and 24 h of E2 treatment correlate with previously observed biological responses, in which the initial proliferative response (at 24 h E2 treatment) is independent of epithelial ERα and thus dependent on stromal ERα, yet epithelial ERα is essential for subsequent maintenance of tissue responsiveness during 3 days of E2 treatment.

In addition to uterine response to E2, epithelial ER cKO females were infertile partly due to an implantation defect (Winuthayanon et al., 2010). In addition, they fail to decidualize (Pawar et al., 2015). The role of epithelial ERα during implantation was examined using a uterine receptivity model that has been previously published (Tong and Pollard, 1999) by treating the mice with a series of E2 and P4 injections to mimic the hormonal profile during implantation. E+Pe treatment significantly increases uterine weight in wildtytpe (WT) females, as well as proliferation of stromal cells, but not epithelial cells. In epithelial ER cKO uteri, treatment with E+Pe showed a dampened uterine weight increase when compared to the WT treated group (Winuthayanon et al., 2014), and a slight decrease in stromal cell proliferation. However, epithelial cell proliferation was significantly higher in epithelial ER cKO compared to WT uteri. This suggests that lack of uterine epithelial ERα does not affect stromal cell proliferation but leads to an inability to appropriately arrest epithelial cell proliferation, a key requirement for embryo attachment and implantation. Additionally, leukemia inhibitory factor (*Lif*) (Stewart et al., 1992) and indian hedgehog (*Ihh*) (Lee et al., 2006), both required for uterine receptivity and induced in WT uteri, were not induced in the epithelial ER cKO samples, confirming induction of these factors requires epithelial ERα. Comparable expression of PR is seen in WT and epithelial ER cKO uteri using

immunohistochemical analysis. Moreover, expression of HAND2, a PR-regulated transcription factor expressed in uterine stromal cells during implantation (Li et al., 2011), showed a similar pattern in the epithelial ER cKO uteri and WT. This indicates that the expression of PR and its downstream effector (HAND2) in the stromal cells were not disrupted by a lack of epithelial ERα. In summary, loss of epithelial ERα disrupted progesterone's ability to inhibit E_2-induced epithelial cell proliferation, but did not affect uterine stromal cell proliferation. Understanding the ERα epithelial cell-specific mechanisms and gene responses for controlling cell growth in the uterus is informative towards understanding a basis for uterine diseases such as endometriosis and endometrial cancer.

Tethered pathway analysis using DNA binding deficient ERα mutants

Studies using *in vitro* cell culture based models have indicated that estrogen responsive genes that lack the canonical ERE sequence can interact with estrogen receptors via a tethering mechanism whereby ER is recruited by AP1 or SP1 bound to their respective response elements. (Jakacka et al., 2001; Kushner et al., 2000; O'Lone et al., 2004; Safe, 2001) (Figure 1). To study the relative biological roles for tethered and ERE DNA binding mechanisms *in vivo*, two different ERα "knock in" mouse models have been created that have mutations of the first zinc finger of the ERα DNA binding domain. Both DNA binding mutations were designed to prevent ER-ERE binding, while retaining the ability to regulate genes via the tethered pathway (Ahlbory-Dieker et al., 2009; Jakacka et al., 2001). The first mouse model was referred to as the "nonclassical ER knock in" (Nerki) (Jakacka et al., 2002a). Female mice heterozygous for this mutation are infertile due to ovarian and uterine pathologies (Jakacka et al., 2002a); however, by intercrossing with the $Esr1^{-/-}$ global knockout line, a mouse possessing one copy each of the Nerki ERα allele and one copy of the null ERα allele has been generated (O'Brien et al., 2006) thereby expressing the Nerki mutant as its only ERα protein. The Nerki/αERKO ($Esr1^{AA/-}$; aka KIKO) uterus is not hypoplastic, however it resembles the $Esr1^{-/-}$ in that estrogen fails to elicit uterine weight increase or cell proliferation (Hewitt et al., 2010b; O'Brien et al., 2006). Microarray comparison of transcripts after estrogen treatment indicated the KIKO uterus retains some of the gene regulation (~24%) also seen in the WT uterus (Hewitt et al., 2009). WT vs. KIKO differentially regulated genes in this microarray profile were enriched for components of the Wnt/Ctnnb signaling pathway as transcripts for Wnt ligands, receptors, transducers and targets were misregulated by E2 in KIKO vs. WT uteri (Hewitt et al., 2009).

Microarray and later ChIP-seq analysis (Hewitt et al., 2014) also showed unexpected results, which were the appearance of numerous estrogen regulated responses in the KIKO that were not observed in normal WT uteri. Evaluation of the KIKO uterine cistrome by ERα ChIP-seq revealed that these transcripts result from an unanticipated "gain of function" of the KIKO DNA binding mutation. Analyses of the sequences bound to KIKO ERα revealed enrichment of hormone response element (HRE) DNA motifs, which typically bind androgen, progesterone and glucocorticoid receptors (AR, PR, GR). Further *in vivo* and *in vitro* analyses have shown that the KIKO ERα binds HRE DNA and regulates uterine genes that are normally PR targets (Hewitt et al., 2014), indicating this particular ERα mutation has an aberrant binding activity with loss of ERE binding but a gain of HRE binding and

gene regulation (Hewitt et al., 2014). The KIKO ERα was created by introducing two point mutations (E207A G207A) at the base of the first zinc dinger of the DBD. These positions in the PGR, AR and GR are occupied by GS. However, modeling the interaction between ERα and ERE based on structural studies revealed the critical importance of E207 in forming a hydrogen bond with the G/C nucleotides in an ERE at position 2/12 (G**G**TCAnnnTGA**C**C). Additionally, attempting to interact with the T/A found at the equivalent position of an HRE (G**A**ACAnnnTGT**T**C) results in significant steric clash, thus excluding ERα/HRE binding. However, by replacing the critical E207 residue with A, the hydrogen binding with ERE and the exclusion of HRE are lost, leading to an ability to interact with HRE (Hewitt et al., 2014)

The second DNA binding mutant ERα mouse model (EAAE), has an ERα that does not bind to HRE or ERE motifs either *in vitro* or *in vivo* (Hewitt et al., 2014), but retains AP1 mediated gene induction (Hewitt et al., 2014). It was created by introducing 4 point mutations (Y201A, K210A K214A, R215E) in the first zinc finger and between the first and second zinc finger. Since EAAE ERα maintains the critical E207 residue, it lacks aberrant HRE binding seen with the KIKO ERα. The EAAE mouse uterus is hypoplastic and refractory to estrogen responses, like the αERKO, indicating that the tethering HRE mechanism is not a major physiological regulatory response in the uterus. Microarray profiling of uterine RNA indicated a lack of estrogen responsive transcripts (Hewitt et al., 2014). Altogether, this shows that DNA binding activity of the ERα is critical for uterine function and estrogen response, and DNA binding-independent activity appears to have little role on its own, but may complement the direct DNA binding activity in eliciting the full uterine hormone response.

Induction of IGF1 signaling by E2 is known to be a major mediator of uterine growth in a paracrine manner, whereby uterine stromal cells secrete IGF1, which then stimulates epithelial cell growth (Adesanya et al., 1999; Cunha et al., 2004; Zhu and Pollard, 2007). One major surprising observation in the KIKO and EAAE models was the inability of estrogen to increase the transcript of insulin-like growth factor 1 (*Igf1*), which had been reported to be regulated via interaction between ERα to AP1 motifs in the Igf1 promoter via tethering. Analysis of *Igf1* genomic sequences indicated that the AP1 motif previously identified using the chicken Igf1 gene is absent in mammals. Several potential ERE sequences were identified and tested for ERα binding by ChIP and gel shift (Hewitt et al., 2012; Hewitt et al., 2010b). ChIP-PCR analysis confirmed ER bound to specific ERE sequences of the *Igf1* in WT but not KIKO uteri. Interestingly, exogenous treatment with IGF-1 did not restore KIKO uterine growth, indicating additional ERE mediated responses are needed to modulate the stimulatory action of IGF-1 in the full uterine response. Additionally, analysis of our uterine ERα ChIP-seq data revealed an enhancer 50 kb 5’ of the *Igf1* promoter that has more estrogen dependent ERα enrichment than the previously tested EREs (Hewitt et al., 2012).

Analysis of AF-1 and AF-2 mediated responses

As described in the mechanism section, ERα activity requires interaction with co-regulators through AF-1 and AF-2. To understand how these impact uterine responses, mice with mutations in AF-1 (ERαAF-1^0) or AF-2 (ERαAF-2^0 and AF2ER) have been created.

ERαAF-1^0 mice were made by deleting amino acids 2–128, which includes the AF-1. The uterus develops normally, but exhibits a blunted response to E2 (Abot et al., 2013; Billon-Gales et al., 2009). In contrast, mice lacking AF-2 exhibit more severe uterine phonotypes, with development of a hypoplastic uterus and complete insensitivity to E2 treatments (Arao et al., 2011a; Billon-Gales et al., 2011). ERαAF-2^0 mice were made by deleting amino acids 543–549, whereas AF2ER mice were made using 2 point mutations in the LBD. Both experimental approaches resulted in inactivation of the AF-2 function and lack of response to E2. One advantage of the AF2ER model is that ER antagonists such as tamoxifen and fulvestrant (ICI182780) exhibit agonist activity, resulting in an antagonist-agonist reversal, thus allowing re-activation of some ERα-mediated responses, including uterine epithelial cell proliferation, presumably through the AF-1 function of the mutant ERα. Overall, studies with AF-1 and AF-2 mutant mice demonstrate that AF-2 activity is critical for uterine E2 response, but that AF-1 activity can promote uterine response. Further characterization of these mice has also uncovered the tissue selectivity of ER actions through either AF-1 or AF-2. AF-1 activity is sufficient in promoting uterine and male efferent duct responses; in contrast, AF-2 activity is necessary for pituitary and mammary responses (Arao et al., 2013; Arao et al., 2012; Arao et al., 2011a).

A mouse with G525L mutation in the LBD called the estrogen-nonresponsive ER knock-in (ENERKI), shows lack of response to E2 (Sinkevicius et al., 2008). Like the AF2ER mice, high doses of the synthetic ERα selective agonist propyl pyrazole triol (PPT) and the ER agonist diethylstilbestrol (DES) are able to induce uterine growth (Sinkevicius et al., 2008). These observations support the findings from mice with mutations in AF-1 or AF-2 activities, that full ER function is needed for optimal uterine response.

Analysis of biological impact of membrane initiated signaling

To address the impact of cell membrane-associated signals, two mouse models with an identical mutation of the palmitoylation site (C451A) have been made which prevent membrane localization of ERα; Nuclear-only ER (NOER) (Pedram et al., 2014) and C451A-ERα (Adlanmerini et al., 2014). The two models differed in their uterine phenotypes, the NOER having a hypoplastic uterus that lacks E2 responses, and the C451A-ERα having normal uterine development and E2 responses. The C451A-ERα model only had ~55% reduction in membrane ERα (measured only in hepatocytes) which perhaps explains the different phenotypes {Pedram, 2014 #9}Conversely, another mouse model was created that prevented nuclear localization (Membrane-only ER, MOER) (Pedram et al., 2009) by expressing the LBD fused with multiple palmitoylation sites from the neuromodulin protein, resulting in a hypoplastic, E2-insensitive uterus (Pedram et al., 2009). Clearly, nuclear ERα is critical to uterine function, however the role of membrane-localized ERα is uncertain and the focus of ongoing investigations is to identify the intracellular signaling pathways involved.

ERβ does not impact uterine responses

Observation from ERβ-null females indicates their subfertility is due to diminished ovarian responses, with normal uterine development, function, and responses to E2 (Hewitt et al.,

2003; Krege et al., 1998b). Although females with deletion of both ERα and β have more severe ovarian defects, the uterine phenotypes are similar to that observed in ERα-null mice (Couse, 1999).

Importance of ERα to uterine function informs mechanisms of disease

Owing to the biological and molecular events that require ERα, the mouse uterus has enabled advancement of our understanding of the details underlying estrogen-initiated responses. Studies in the many mouse models developed with deletion or mutation of ERα have highlighted the essential role of DNA binding and AF-1 and 2 functions in achieving optimal development and response. Additionally, ERα has cell type dependent roles. Our increased understanding of these molecular details and their roles in normal uterine function are critical to understanding perturbation that leads to impaired embryo implantation or endometrial diseases including endometriosis, endometrial cancer and leiomyoma.

Estrogen Receptor in the Ovary

Both known forms of nuclear estrogen receptor, ERα and ERβ, are expressed in mammalian ovaries but localized to distinct functional compartments. ERβ is highly expressed but limited to the granulosa cells of growing follicles, while ERα is generally localized to the interstitium and theca cells. This expression pattern is highly conserved among several mammalian species. Adult estrogen receptor alpha null (αERKO) females are anovulatory, possessing pre- and small antral follicles but lacking corpora lutea, resulting in infertility. By 50 days of age, αERKO mice are infertile and have ovaries that exhibit multiple enlarged, hemorrhagic, and cystic follicles, with increased gonadotropin and gonadotropin receptor levels, elevated steroid synthesis, and hypertrophied theca cells (Couse and Korach, 1999, 2001; Schomberg et al., 1999). Adult estrogen receptor beta null (βERKO) females are sub-fertile, as evidenced by reduced litter number and size (Couse et al., 2005). Despite speculated roles of ER in granulosa cells, βERKO ovaries appear relatively normal and possess follicles at all stages of growth and are not overtly impaired by losing ERβ (Couse et al., 2005; Krege et al., 1998b). Consistent with the subfertility, superovulatory treatments in βERKO females result in significantly fewer ovulations and observation of trapped oocyte follicles (Couse et al., 2005; Krege et al., 1998b). In addition, reduced expression of PR and Cox2, increased rates of follicle atresia, and a paucity of corpora lutea in βERKO ovaries indicate that the subfertility is likely due to a reduced ovulatory frequency (Emmen et al., 2005; Krege et al., 1998b).

Ovarian Phenotypes of ERα Mutant Mice

Although αERKO females are anovulatory, it is generally thought to be due to the cystic ovarian phenotype seen in these mice (Couse and Korach, 1999, 2001; Schomberg et al., 1999). Accompanying the pathology is a severe disruption in steroid hormone levels; mainly, αERKO mice have chronically elevated luteinizing hormone (LH), E2 and testosterone (T) due to a disruption of negative feedback (Couse et al., 1999a; Couse et al., 2003). Treatment of αERKO females with a gonadotropin-releasing hormone (GnRH)- antagonist (antide) corrected the cystic follicle and elevated steroidogenesis levels and is therefore thought to be

the primary cause of the ovarian phenotypes seen in αERKO females (Couse et al., 1999a; Couse et al., 2005). One distinct phenotype of the ERα null females is the aberrant expression and extremely high levels of the enzymes involved in androgen biosynthesis (Couse et al., 2003). Aberrant expression of *Hsd17b3*, a testis specific gene, is observed in the αERKO female ovary. HSD17B3 catalyzes the conversion of androstendione to testosterone (T), is expressed in theca cells, and contributes to the high serum T in the αERKO female. T produced by HSD17B3 in theca cells is not converted to E2 by granulosa Cyp19 (aromatase), even though higher expression of Cyp19 was observed in αERKO female ovary. The expression of *Hsd17b3* in the αERKO ovary is regulated by LH and treatment with a GnRH-antagonist can normalize T levels in the αERKO female (Couse et al., 2003). *Cyp17*, the enzyme necessary for androstendione synthesis, is increased 3-fold in αERKO females when compared to WT (Couse et al., 2003), resulting in increased serum levels of E2 and T. *Cyp17* is found in the theca cells, where ERα is also localized, and therefore it is speculated that ERα mediates thecal cell steroidogenesis. Additionally, our data shows a very modest increase in *Cyp17* in WT females with chronic LH expression (Couse et al., 2003). ERα null follicles grown in culture produce more androgens relative to wild type follicles under a controlled gonadotropin environment (Emmen et al., 2005), further supporting a role for ovarian ERα in T production. Taken together, these data suggest that the role of ERα in ovulation is through regulation of androgen biosynthesis by way of a short loop feedback mechanism in theca cells within the ovary.

It is also important to note the ovarian phenotypes of various ERα mutants that have been described (Table 2). As discussed earlier, there are a group of mutants that were developed to limit the genomic activity of ERα and their mutations only allow estrogen signaling through the non-classical mechanism. One such model is the Nerki. Female mice that are heterozygous for this mutation are infertile and the ovaries contain follicles of all stages but no corpora lutea and lipid-filled cells in the ovarian stroma (Jakacka et al., 2002a). When superovulated, Nerki heterozygous females develop large hemorrhagic cysts like those seen in the $Esr1^{-/-}$ and ovulation does not reach the level seen in WT mice; however, they do not display the altered steroidogenic enzyme or hormone profile (Jakacka et al., 2002a). Jackacka *et al.* speculated that the ovarian phenotypes result from the Nerki ERα acting in a dominant-negative matter (Jakacka et al., 2002a). As described earlier, more recent research revealed that Nerki ERα has aberrant DNA binding activity and binds to steroid hormone responsive elements (HRE) sequences including progesterone receptor responsive element (PRE) (Hewitt et al., 2014). Unexpected PRE-mediated gene regulation together with the ERE-mediated gene regulation by WT and heterozygote mice may be a cause of the disrupted phenotype described in the Nerki ovary.

As described in the uterine section of this chapter, the $ERα^{AA/-}$ (KIKO) and $ERα^{EAAE/EAAE}$ (EAAE) mouse models were developed to further understand the role of ERα in non-classical estrogen signaling (Ahlbory-Dieker et al., 2009; O'Brien et al., 2006; Sinkevicius et al., 2008). These models both express ERα that is unable to bind to ERE sequences. The ovaries of KIKO mice have follicles with most stages of development, but lack corpora lutea (O'Brien et al., 2006). EAAE mice have hemorrhagic and cystic ovaries and are infertile, similar to the $Esr1^{-/-}$ mice (Ahlbory-Dieker et al., 2009). Taken together, these data suggest

that direct ERE binding by ERα is critical for ovarian ERα functionality and regulation required for proper ovulation and therefore fertility.

There are also two notable ERα mouse lines that have mutations in the LBD of ERα. In one model, the ENERKI, a single point mutation was created by switching a glycine to a leucine at residue 525 creating an altered ligand binding pocket which prevents ligand binding (Sinkevicius et al., 2008). ENERKI has hemorrhagic and cystic ovaries and does not ovulate based on the lack of corpora lutea found in ovarian sections (Sinkevicius et al., 2008). The ovarian defects and lack of ovulation in the ENERKI reveal the importance of estrogen hormone binding for normal ovarian function and neuroendocrine negative feedback (Sinkevicius et al., 2008). A second line, the AF2ER, has two point mutations in the AF-2 region of ERα and, while E2 can be bound by this mutant, it is not able to engage transcriptional machinery due to the inability to interact with coactivators (Arao et al., 2011a). The ovarian phenotype of AF2ER females looks very similar to the ERα null mice, as they have hemorrhagic and cystic ovaries and ovarian sections have no corpora lutea (Arao et al., 2011a). AF2ER female mice also have a disruption in negative feedback and have elevated E, T, and LH (Arao et al., 2011a). This suggests that the AF-2 region of ERα is critical for regulating ovarian function, neuroendocrine control and ovulation.

Ovary-Specific ERα knockouts

The global ERα knockout mouse has shown that loss of estrogen is detrimental to ovarian function, as evidenced by large hemorrhagic cysts and infertility. However, this approach does not allow us to dissect the role of ERα in the ovary due to confounding factors, such as the high persistent steroid and gonadotropin serum levels following disruption of negative feedback. However, theca cell-specific ERα knock-out mice (thEsr1KO) were generated by crossing the floxed ERα strain with *Cyp17* cre mice (Bridges et al., 2008). At 2 months of age, thEsr1KO mice had comparable fertility to WT mice and displayed a normal estrus cycle despite a reduction of serum LH. After superovulation, the same numbers of oocytes were found in the oviduct of the cKO and WT suggesting there was no defect in ovulation (Lee et al., 2009). However, by 6 months of age the thEsr1KO ovaries displayed hemorrhagic cysts and superovulation resulted in significantly fewer oocytes (Lee et al., 2009). Females had an even further reduction of serum LH, suggesting that ERα expression in the theca cells of the ovary is important for feedback regulation and control of LH expression. At both time points, testosterone was elevated, confirming the original role of ovarian ERα regulation of androgen production in the ovary as proposed in the $Esr1^{-/-}$ mice (Couse et al., 2006) (Lee et al., 2009). These data suggest that thecal cell ERα is associated with an age related reduction in ovarian function, but is not required for ovulation and normal ovarian function in young mice.

Ovarian Phenotypes of ERβ Mutant Mice

The role of ERβ in the maintenance of normal reproduction and fertility has not been fully elucidated. It has been reported that female βERKO mice are subfertile compared to WT, as characterized by fewer pregnancies, fewer litters, and smaller litter sizes (Dupont et al., 2000; Krege et al., 1998b). Ovaries of adult βERKO mice contain a reduced number of

corpora lutea, indicating fewer ovulations, and ovulation rates cannot be rescued by exogenous gonadotropins (Couse et al., 2005; Emmen et al., 2005; Krege et al., 1998b). ERβ deletion results in impaired follicular maturation and a reduced number of follicles responsive to LH, which could explain why βERKO mice are poor responders to ovulatory stimulation and have smaller litters. The fact that βERKO mice have fewer pregnancies and produce fewer litters may also be due to fewer adequate ovulatory signals (i.e., LH surges). βERKO ovaries and granulosa cells isolated from βERKO mice have an attenuated cAMP accumulation in response to FSH and altered expression of several genes including *Lhcgr* (LH receptor) and *Cyp19a1* (aromatase) (Couse et al., 2005; Emmen et al., 2005). Cultured follicles from βERKO follicles produce less estrogen than WT follicles (Emmen et al., 2005; Rodriguez et al., 2010) and may fail to provide a sufficient stimulus required to trigger physiologically relevant LH surges. The amplitude and timing of the naturally occurring LH surge was measured in individual intact βERKO and WT mice (Jayes et al., 2014) and it was determined that while the pituitary levels of LH revealed no differences, the amplitude of the LH surge was severely blunted in βERKO mice compared to WT. The βERKO mice did not produce an adequate preovulatory E2 surge. To determine if the smaller LH surges and the reduced number of litters in βERKO were due to the lack of ERβ in the hypothalamic-pituitary axis or ovary, ovaries were transplanted from WT into βERKO mice and vice versa. The size of the LH surge was reduced only in mice lacking ERβ within the ovary, and these mice had fewer litters. Fertility and size of the LH surge were rescued in βERKO mice receiving a WT ovary. These data provided the first experimental evidence that the LH surge is impaired in βERKO females and may be another aspect of their overall reduced fertility. This study shows that ERβ is not necessary within the pituitary and hypothalamus for the generation of a normal LH surge and for normal fertility, but ERβ is essential within the ovary to provide proper hormonal signals for the ovulatory cycle.

Role of ERβ signaling in Granulosa Cells

In the ovary, there is a heterogeneous cell population, and ERβ is specifically expressed in granulosa cells, while ERα is predominately expressed in the theca cells (Binder et al., 2013; Krege et al., 1998b). To isolate pure populations of granulosa cells, Laser Capture Microdissection (LCM) was performed using ovaries from WT and βERKO mice after stimulation with FSH alone or FSH in combination with LH. Use of LCM allowed for targeted isolation of granulosa cells from large antral follicles (FSH) or pre-ovulatory follicles (FSH+LH) in both WT and βERKO mice so that cells at the similar stages of development could be compared. Microarray analysis demonstrated that granulosa cells isolated at similar stages of follicular maturation have altered gene expression in βERKO mice compared to WT mice. While a subset of follicles can grow and respond to FSH and LH in βERKO mice, the transcriptional profile differs in these cells from that observed in WT cells from similar sized follicles, suggesting ERβ-null granulosa cells are not properly differentiated to respond to hormonal stimulation (Binder et al., 2013).

Examination of the genes from large antral follicles after FSH stimulation revealed 414 genes differentially expressed in βERKO granulosa cells compared to WT. These genes included several implicated in E2 biosynthesis, including *Adcyap1* and *Runx2*, which correlates with reduced E2 concentrations in βERKO follicles (Emmen et al., 2005;

Rodriguez et al., 2010). While a subset of granulosa cells were able to respond to LH and differentiate into preovulatory granulosa cells in βERKO mice, these cells showed 1,258 genes differentially expressed compared to WT preovulatory granulosa cells. These genes included members of several signaling pathways, including *Akap12* and other members of the cAMP/PKA signaling pathway.

These findings indicate that ERβ is necessary for proper differentiation of ovarian granulosa cells in response to gonadotropins during folliculogenesis and provides a novel list of ERβ-dependent estrogen-regulated genes that may contribute to proper follicle maturation and ovulation *in vivo*.

Ovarian Phenotypes of ERα and ERβ Compound Mutant Mice

Mice that possess neither estrogen receptors alpha nor beta (αβERKO) are anovulatory and infertile similar to the αERKO (Couse et al., 1999b; Dupont et al., 2000). In ovarian sections, there are normal follicular stages found but upon aging to 6–12 months the antrum is underdeveloped, granulosa cell number is small, and the theca is thin and disorganized. Further, there are cystic follicles present and the mice have the same hormonal disruption as seen in the αERKO mice, with even higher LH levels (Couse et al., 1999b; Dupont et al., 2000). The main difference seen in the αβERKO when compared to the single ER KOs is the presence of seminiferous tubule-like structures in the post-pubertal ovary that appear to arise from atretic follicles and have cells with characteristics of Sertoli-like cells found in the male testis (Couse et al., 1999b; Dupont et al., 2000). This phenotype of transdifferentiation in the αβERKO, while absent in each individual KO (αERKO or βERKO), suggests that estrogen signaling involving both ERα and ERβ is necessary for proper ovarian formation and function. There is possible compensation by one or the other ER present in the individual knockout lines or an even more overt effect of the even higher levels of LH in combination with the improper cellular tissue differentiation.

Ovarian Phenotypes in Mice Lacking Estradiol Synthesis

When considering the role of E2 in various body functions, it is helpful to not only look at the estrogen receptor mutants but also mice that lack the ability to synthesize E2. Taking this approach, it is possible to dissect hormone ligand dependent responses from non-ligand dependent. Cyp19-null mice (ArKO) were developed for this purpose. Initially these mice had no noticeable phenotype; however, transition to a soy-free diet resulted in noticeable differences between ArKO and WT mice (Fisher et al., 1998). The discrepancy appears to be due to hormonally active components of the feed. The ovaries of ArKO mice have follicles of all stages of development, but no corpora lutea (Britt et al., 2000; Fisher et al., 1998; Toda et al., 2001a). Additionally, ArKO mice develop hemorrhagic and cystic follicles with age due, in part, to disrupted negative feedback that can be corrected with E2 treatment (Toda et al., 2001a). With carefully timed exogenous hormone treatment, ovulation can be partially rescued, showing that the ovulation defect is estrogen-dependent (Toda et al., 2012). When fed a soy-free diet, ArKO ovaries develop the transdifferentiation phenotype seen in the αβERKO ovaries (Britt et al., 2001). The steroid hormone serum composition of the ArKO female is disrupted (E is undetectable, androgens are elevated) and the mice have elevated

serum LH (Britt et al., 2000; Fisher et al., 1998). When data from the ArKO mice (lacking ligand) is compiled with mice lacking ERα or ERβ (lack of receptors), it is clear that estrogen plays a major role in ovarian physiology. This is a compound issue that can be attributed to estrogen signaling not only in the ovary, but also in the hypothalamus and pituitary gland, which are involved in negative feedback controlling the trophic hormone levels.

Estrogen receptor in metabolism

Estrogen regulates multiple physiological functions, including reproduction, bone density and metabolic regulations. As a consequence of pleiotropic effects of estrogen, the decline of endogenous estrogen production by the ovaries at menopause often leads to functional disorders including dyslipidemia, impaired glucose tolerance (IGT) and type 2 diabetes mellitus, which increase cardiovascular disease risk in postmenopausal women and directly affect quality of life (Munoz et al., 2002). Several animal models have been developed to further explore the clinical findings of estrogen-dependent metabolic regulation. Indeed, ovexed mice lacking intact estrogen signaling display obesity and IGT; these effects are reversible with the reintroduction of estrogen (E2) (Zhu et al., 2013). Similar results have been seen in *Cyp19* (aromatase) knockout (KO) mice, which unable to synthesize E2 from testosterone. Treatment of *Cyp19*KO mice with exogenous E2 restores the E2 protective effect against the development of metabolic syndrome in both male and female mice (Hewitt et al., 2004; Jones et al., 2000). Studies using the estrogen receptor (ERα and ERβ) knockout mice have demonstrated that ERα plays the essential role in estrogen-mediated metabolic regulation, whereas ERβ does not (Bryzgalova et al., 2006).

Metabolic Phenotype of ERα knockout mice

Metabolic phenotypes of αERKO have been described previously. αERKO females present with obesity, IGT and insulin resistance (Heine et al., 2000). Fat deposition of parametrial and inguinal white adipose tissues (WAT) were higher in regular diet fed 3-month-old αERKO females than in wild-type (WT) littermates. No difference in WT vs. αERKO perirenal WAT or brown adipose tissue (BAT) was observed. Increased adipocyte volume in parametrial and inguinal WAT was accompanied by increased adipocyte number (Heine et al., 2000). These observations suggested that ERα is involved in the adipogenesis; however, the mechanisms responsible for ERα-dependent regulation of adipogenesis remain unclear. Energy intake of WT and αERKO was equal, indicating that obesity was not induced by hyperphagia. In contrast, energy expenditure was reduced in αERKO compared with WT, indicating that altered energy expenditure may contribute to the observed obesity (Heine et al., 2000). Recent reports suggest that decreased locomotion is a cause of reduction of energy expenditure in αERKO mice (Park et al., 2011; Xu et al., 2011).

Physiological role of ERα transactivation domains in metabolism

As described previously, ERα has two transcription activation domains, named AF-1 and AF-2. Physiological roles of ERα AF-1 and AF-2 have been reported using the mouse models, which deleted ERα AF-1 (ERaAF-1°) or ERα AF-2 (ERaAF-2°) (Handgraaf et al.,

2013). ERaAF-2° females present with obesity, IGT and insulin resistance, that mimics that seen in αERKO females. In striking contrast, metabolic phenotypes were lacking in ERaAF-1° mice being identical to WT. HFD-induced metabolic disturbances in ovexed ERaAF-1° and WT mice were prevented by E2 administration, whereas an E2-mediated protective effect was totally abrogated in ERaAF-2° and αERKO mice. Thus, the report concluded that the protective effect of E2 towards obesity and insulin resistance is ERα AF-2 dependent but does not require AF-1 (Handgraaf et al., 2013). The molecular mechanism of AF-1 or AF-2 activation or cooperative regulation of ERα AF-1 by AF-2 is still unresolved (Arao et al., 2015). Although the ERα AF-2 mutations (ERaAF-2° and AF2ER) disrupt E2-mediated physiological responses, antagonistic ligands such as fulvestrant and tamoxifen activate AF-1 mediated physiological functions in these mutant mice (Arao et al., 2012; Arao et al., 2011a; Moverare-Skrtic et al., 2014). AF2ER females present with disrupted metabolic phenotypes similar to ERaAF-2° and αERKO mice. Treatment with Tamoxifen to AF2ER females rescued the metabolic phenotypes (Arao A, 2016). This result suggested that ERα AF-1 is able to modulate metabolic regulation, even though it is in contrast to the previous report using a different model system (ERaAF-1°) (Handgraaf et al., 2013). Understanding the mechanism of ligand dependent ERα AF-1 and AF-2 cooperative regulation will be necessary to delineate new therapeutic options for selective modulation of ERα mediated metabolic regulation.

Phenotype of ERα DNA binding domain mutant mice in metabolism

ERα DNA binding domain mutant mice (KIKO) were analyzed to characterize the role for non-genomic and indirect DNA binding transcription (nonclassical ERα signaling) towards mediating metabolic regulation (Park et al., 2011). KIKO mice restored metabolic parameters dysregulated in αERKO mice to normal values, suggesting that the nonclassical ERα signaling rescues body weight and metabolic function. The normalization of energy expenditure, including voluntary locomotor activity leads to nonclassical ERα signaling-mediated normalization of metabolic regulation (Park et al., 2011). The phenotype of KIKO mice suggested that the nonclassical ERα signaling is a potential target for selective modulation of ERα-mediated metabolic regulation. Based on the aberrant DNA binding activity of the KIKO mouse model, further consideration of the metabolic phenotype of the EAAE mouse model with no DNA binding activity will provide a more accurate assessment of the signaling mechanisms involved in metabolic regulation. Additionally, development of other knock-in mutation mouse models will facilitate further evaluation of non-genomic extra-nuclear ERα action. The H2NES ERα mutation which is a cytosol-only form of ERα mutant, even in the presence of hormone, might be useful for such purposes (Burns et al., 2014). We have currently developed such a mouse model and are characterizing the phenotypes to assess the role of non-genomic ERα signaling.

As described above, various functional domains of ERα contribute to differential estrogen mediated metabolic regulations. Development of ligands that selectively regulate specific ERα functional domains and ER cellular signaling mechanisms may be useful for developing more effective targeted therapies for postmenopausal women without undesirable side effects.

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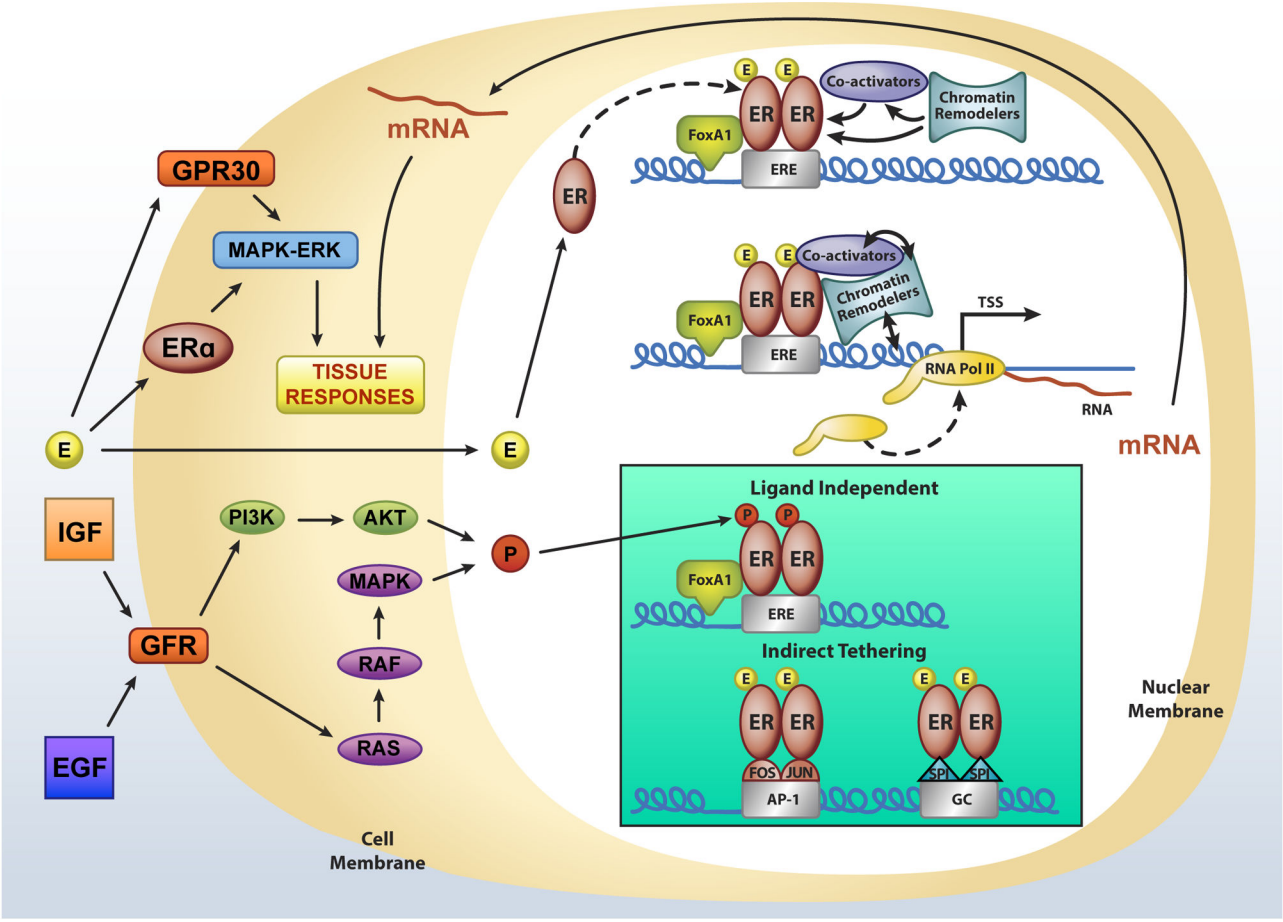

Figure 1. Cellular Mechanisms of Estrogen Action

Model of nuclear and non-nuclear estrogen receptor action. Estrogen (E circles) and estrogen receptor (ER) complex binds directly to the regulatory DNA elements (estrogen responsive element (ERE) recruiting additional factors involved in transcriptional regulation. ER can also bind indirectly through a tethering mechanism to AP1 or Sp1 binding sites (GC) to regulate transcription. Growth factors (IGF, EGF) can phosphorylate ER through membrane growth factor receptor (GFR) mediated intracellular signaling pathways (P circles) to regulate gene expression in the absence of ligand (nuclear action). Estrogen also binds and activates membrane ERα or GPR30, inducing the intracellular signaling pathway (non-nuclear action) that is rapid.

Table 1.

Uterine Phenotypes of Estrogen Receptor Mutants

Gene	Mutation	Nick-names	Uterine phenotypes	References
Esr1	Homozygous null for ERα	αERKO, Ex3αERKO	Normal uterine development but exhibits hypoplastic uteri. Insensitive to the proliferative and differentiating effects of endogenous E2, growth factors and exogenous E2. Implantation defect. *lack decidualization. Infertile.	(Antonson et al., 2012b; Curtis Hewitt et al., 2002; Curtis and Korach, 1999; Dupont et al., 2000; Hewitt et al., 2010a; Lubahn et al., 1993b)
Esr1	One mutated allele of two-point mutation in ERα DBD (E207A, G208A) and one WT allele	*NERKI*$^{+/-}$ ER$^{AA/+}$	Normal uterine development but exhibits hyperplastic uteri. Hypersensitive to estrogen. Infertile.	(Jakacka et al., 2002a)
Esr1	One mutated allele of two-point mutation in DNA binding domain of ERα (E207A, G208A) and one ERα null allele	*ERα KIKO, ER*$^{AA/-}$	Normal uterine development. Insensitive to the proliferative effects of exogenous E2 treatment. Infertile.	(Hewitt et al., 2010b; O'Brien et al., 2006)
Esr1	4-point mutation of DBD ERα (Y201E, K210S, K214A, and R215A)	*ERα*$^{EAAE/EAAE}$	Normal uterine development but exhibits hypoplastic uteri. Loss of E2-induced uterine transcripts. Infertile.	(Ahlbory-Dieker et al., 2009)
Esr1	Deletion of amino acids 2-128 including AF2 domain of ERα	*ERαAF-1*^{0}	Normal uterine development and architecture. Blunted E2 response. Infertile.	(Abot et al., 2013; Billon-Gales et al., 2009)
Esr1	Deletion of amino acids 543-549 in LBD/AF-2 of ERα	*ERαAF-2*^{0}	Normal uterine development but exhibits hypoplastic uteri. Insensitive to E2 treatment. Infertile.	(Billon-Gales et al., 2011)
Esr1	Two-point mutation in LBD/AF-2 of ERα (L543A, L544A)	*AF2ER*$^{KI/KI}$	Normal uterine development but exhibits hypoplastic uteri. Insensitive to E2 treatment. ER antagonists and partial agonist (ICI 182,780 and TAM) induced uterine epithelial proliferation. Growth factor did not induce the uterine epithelial cell proliferation. Infertile.	(Arao et al., 2011a)
Esr1	Point mutation in LBD of ERα (G525L)	*ENERKI ERα*^{G525L}	Normal uterine development but exhibits hypoplastic uteri. Insensitive to E2 treatment. Synthetic estrogens PPT and DES induce uterine growth. IGF-1 induced patchy uterine epithelial growth. Infertile.	(Sinkevicius et al., 2008)
Esr1	Female reproductive tract epithelial cell specific deletion of ERα	*Wnt7a*$^{Cre+}$;*Esr1*$^{f/f}$ Epi ERα cKO WE$^{d/d}$	Normal uterine development. Sensitive to E2- and growth factors-induced epithelial cell proliferation. Selective loss of E2-target gene response. Implantation defect. Decidualization defect. Infertile.	(Pawar et al., 2015; Winuthayanon et al., 2014; Winuthayanon et al., 2010)
Esr1	Uterine specific deletion of ERα	*PgrCre*$^{Cre+}$;*Esr1*$^{f/f}$ERα Ut cKO Esr$^{d/d}$	Normal Uterine development. Insensitive to E2. Decidualization defect. Infertile.	(Pawar et al., 2015)

Gene	Mutation	Nick-names	Uterine phenotypes	References
Esr1	Point mutation of ERα palmitoylation site (C541A)	C451A-ERα, NOER (nuclear-only ERa)	**C451A-ERα:** normal uterine development, E2 growth response **NOER:** hypoplastic ERα-null like uterus	(Adlanmerini et al., 2014; Pedram et al., 2014)
Esr1	LBD of ERα fused with multiple palmitoylation sites from the neuromodulin protein	MOER	Normal uterine development but exhibits hypoplastic uteri.	
Esr2$^{-/-}$	Homozygous null for ERβ	*Esr2*$^{-/-}$ (βERKO, Ex3βERKO, **ERβ$_{ST}^{L-/L-}$	Exhibit grossly normal uterine development and function. Sensitive to E2 treatment. Some *Esr2*$^{-/-}$ lines reported elevated uterine epithelial proliferation after E treatment.	(Antal et al., 2008a; Dupont et al., 2000; Krege et al., 1998b; Wada-Hiraike et al., 2006a) (Binder et al., 2013)
Esr1 and *Esr2*	Homozygous null for both ERα and ERβ	αβERKO	Normal uterine development but exhibit hypoplastic uteri, similar αERKO. Insensitive to E2, infertile	(Couse et al., 1999b; Dupont et al., 2000)

Table 2.

Ovarian Phenotypes of Estrogen Receptor Mutants

Gene	Mutation	Nickname	ovarian phenotypes	Hormone Levels	References
Esr1	Homozygous null for ERα	αERKO or Ex3αERKO	-Anovulatory and Infertile Hemorrhagic and cystic ovaries with no CLs present in histological sections. -Increased expression of steroidogenic enzymes Lack of response to superovulation.	Elevated T, E2 and LH Normal FSH & P	(Antonson et al., 2012a; Curtis Hewitt et al., 2002; Curtis et al., 1999; Dupont et al., 2000; Hewitt et al., 2010a; Lubahn et al., 1993a)
Esr1	One mutated allele of two-point mutation in ERα DBD and one WT allele	*NERKI*$^{+/-}$	-Anovulatory and Infertile -Lack of plugs in NERKI females after superovulation and natural mating -Superovulation partially restored ovulation while increasing cyst presence	Normal LH, FSH and E2 Reduced P	(Jakacka et al., 2002b)
Esr1	One mutated allele of two-point mutation in DNA binding domain of ERα and one ERαKO allele	*KIKO (ER*$^{AA/-}$)	-Anovulatory and Infertile -No CLs present in histological sections	Normal E2 and P	(Hewitt et al., 2010b; O'Brien et al., 2006)
Esr1	Homozygous animal of 4-point mutation of DBD ERα	*ERα*$^{EAAE/EAAE}$	-Infertile -Hemorrhagic ovaries	Not reported	(Ahlbory-Dieker et al., 2009)
Esr1	Homozygous animal of one point mutation in LBD of ERα	*ENERKI (ERα*^{G525L}	-Anovulatory -Hemorrhagic and cystic ovaries with increased atretic antral follicles and No CLs in histological sections. -Hyperplastic theca cells in response to LH (data not shown)	Elevated serum E2, T and LH Normal FSH	(Sinkevicius et al., 2008)
Esr1	Homozygous knock-in of two-point mutation in LBD of ERα	*AF2ER*$^{KI/KI}$	-Anovulatory and Infertile -Hemorrhagic and cystic ovaries with no CLs present in histological sections. -Lack of reseponse to superovulation.	Elevated serum LH and E2	(Arao et al., 2011b)
Esr2	Homozygous null alleles for ERβ	*Esr2*$^{-/-}$ βERKO, Ex3βERKO, and **ERβ$_{ST}^{L-/L}$	-Subfertile – Infertile (lines vary) -Reduction or failure to respond to superovulation -Lack of COC expansion	Normal LH and FSH	(Antal et al., 2008b; Dupont et al., 2000; Krege et al., 1998a; Wada-Hiraike et al., 2006b) (Binder et al., In Reivew)
Esr1 and Esr2	Homozygous null for both ERα and ERβ	αβERKO	-Anovulatory and infertile -No CLs and few large follicles	Elevated LH and T, Normal FSH and P	(Couse et al., 1999b; Dupont et al., 2000)

Gene	Mutation	Nickname	ovarian phenotypes	Hormone Levels	References
			-Ovarian transdifferentiation to Sertoli-like cells -Altered expression of steroidogenic enzymes		
Cyp19a1	Homozygous null aromatase: ArKO Unable to synthesize endogenous E2.	*Cyp19a1*$^{-/-}$	-Anovulatory and infertile -Hemorrhagic and cystic ovaries with no CLs present in histological sections. -Failure to respond to superovulation with partial rescue with E2 treatment. Ovarian transdifferentiation to Sertoli-like cells that express *Sox9*	No E2 Elevated LH, FSH and T	(Fisher et al., 1998; Honda et al., 1998; Toda et al., 2001a, b) (Britt et al., 2000; Britt et al., 2002; Fisher et al., 1998; Honda et al., 1998; Toda et al., 2012; Toda et al., 2001a)
Esr1	Theca cell specific ERα knockout	*Cyp17cre;ERα*$^{flox/flox}$	Fertility normal in young mice, but 6 month old animals have reduced fertility and longer estrous cycle	Elevated T at both 2 & 6 mo. Normal FSH Decreased LH at 2 mo. with further decrease at 6 mo.	(Bridges et al., 2008; Lee et al., 2009)
Esr1	palmitoylation deficient mutants	Esr1 C541A	Cystic ovaries Cystic ovaries	LH elevated, E2 normal LH and E2 elevated	(Adlanmerini et al., 2014) (Pedram et al., 2014)