What's new in estrogen receptor action in the female reproductive tract

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Abstract

Estrogen receptor alpha (ERα) is a critical player in development and function of the female reproductive system. Perturbations in ERα response can affect wide-ranging aspects of health in humans as well as in livestock and wildlife. Because of its long-known and broad impact, ERα mechanisms of action continue to be the focus on cutting-edge research efforts. Consequently, novel insights have greatly advanced understanding of every aspect of estrogen signaling. In this review, we attempt to briefly outline the current understanding of ERα mediated mechanisms in the context of the female reproductive system.

Keywords
- estrogen receptors
- gene expression
- gene regulation
- hormone action
- uterus/endometrium

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Estrogen receptor

The vast majority of estrogen's activities are mediated by the estrogen receptor (ER), a member of the nuclear receptor family of hormone activated transcription factors. Our understanding of the physiological role of estrogen action has been greatly advanced by the generation of experimental mouse and rat models with knockout of receptors or coactivators either globally or in specific tissues and cells, or with knock-in expression of mutated forms of these molecules. These models, used in combination with microarray, RNA next generation sequencing (RNA-seq), and chromatin immunoprecipitation next generation sequencing (ChIP-seq) methods, allow comprehensive mapping of interaction of ERs with the chromatin landscape to impact genomic response. Together, these models and techniques have led to better understanding of the molecular details of ER roles in biological processes.

Estrogen receptor alpha (ERα) cDNA was the first described and cloned estrogen receptor (termed *ESR1* (ERα)) (Walter *et al.* 1985). A second ER gene, termed *ESR2* (ERβ), was discovered in 1996 (Kuiper *et al.* 1996). ERα and ERβ are not isoforms but rather distinct receptors encoded by two separate genes on different chromosomes. ERα is found on chromosome 6 in humans and chromosome 10 in mice. ERβ is found on chromosome 14 in humans and chromosome 12 in mice. The ERα proteins are 595 and 599 amino acids in length in humans and mice respectively with an approximate molecular weight of 66 kDa (Fig. 1) (Heldring *et al.* 2007, Le Romancer *et al.* 2011, Gibson & Saunders 2012).

The *ESR2* encodes a receptor of 549 amino acids in rodents and 530 amino acids in humans, each with an approximate molecular weight of 60–63 kDa (Fig. 1) (Gibson & Saunders 2012). Therefore, ERβ is slightly smaller than ERα, and most of these differences lie within the smaller N-terminus.

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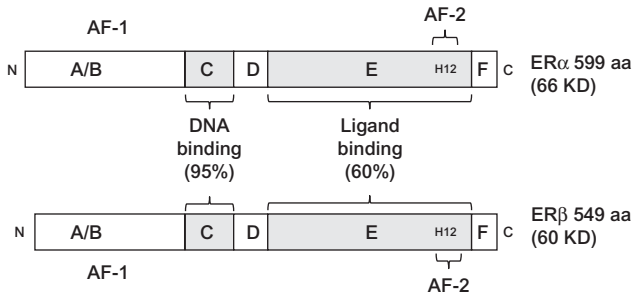

Figure 1
Structures of ERα and ERβ protein with functional domains. Estrogen receptors ERα and ERβ share a conserved domain structure. The A/B domain, at the amino terminus (N) of the protein contains AF-1. The C domain binds to DNA motifs called EREs. The D domain is called the hinge region, and contributes to DNA binding specificity and nuclear localization of the ERs. The E domain is called the ligand binding domain because it interacts with estrogen, through an arrangement of 11 α helices (H1, and H3 through H12). H12 in this region of the receptor is critical to mediating transcriptional activation via AF-2. At the carboxy terminus (C) is the F domain. The % homology shared between ERα and ERβ in the C and E domains is shown.

Receptor structure

The estrogen receptors are composed of five functional domains (Fig. 1), an N-terminal domain (NTD) or A/B domain, the DNA-binding (DBD or C) domain, a hinge (D) region, LBD (LBD or E), and a C-terminal F domain (Laudet & Gronemeyer 2001, Aagaard *et al*. 2011, Hilser & Thompson 2011, Brelivet *et al*. 2012, Helsen *et al*. 2012).

NTD or A/B domain

Crystallography of the ER NTD or A/B domain has been largely unsuccessful because this portion of the receptor is unstructured and fluctuates in aqueous solutions. However, evidence suggests that intramolecular interactions between the A/B and other receptor domains are likely to induce a more structured NTD (McEwan 2004, Aagaard *et al*. 2011, Hilser & Thompson 2011), as evidenced from recent cryogenic Electron Microscopy (cryo-EM) studies (Yi *et al*. 2015). Current models of ER signaling incorporate the flexibility of intrinsically disordered (ID) regions of the receptor, including the NTD, into a mechanism of allosteric interaction and coordination of ligand, DNA motif and ER domain functions (Aagaard *et al*. 2011, Hilser & Thompson 2011). The NTD contains the transcriptional activation function-1 (AF-1) domain and provides for cell- and promoter-specific activity of the receptor as well as a site for interaction with coreceptor proteins (Table 1). More recent description of full-length ERα structure derived using cryo-EM indicates A/B domain is positioned near the LBD and facilitates recruitment of the steroid receptor transcriptional coactivator, SRC-3 (Yi *et al*. 2015). Post-translational modifications, such as phosphorylation, of the A/B domain can dramatically affect the overall behavior of the receptor and are thought to be an important mechanism for the modulation of AF-1 functions (Le Romancer *et al*. 2011).

DNA-binding or C domain

The C domain of the ER recognizes and binds to the cis-acting enhancer sequences, called estrogen responsive elements (EREs) (Helsen *et al*. 2012). The C domain contains two zinc fingers, each composed of four cysteine residues that chelate a single Zn^2 ion. Crystallography studies indicate a highly conserved structure consisting of dual α-helices positioned perpendicular to each other (Aagaard *et al*. 2011, Hilser & Thompson 2011, Helsen *et al*. 2012). Amino acids in the C-terminal 'knuckle' of the first zinc finger form the proximal box ('P-box') of the DNA binding domain and confer DNA sequence recognition specificity to the receptor for binding DNA sequences; hence, the proximal zinc finger is often referred as forming the 'recognition helix. ' Amino acids at the N-terminal 'knuckle' of the second zinc finger form the distal box ('D-box') and are more specifically involved in differentiating the 'spacer' sequence within the ERE as well as providing a secondary interface for receptor dimerization.

The consensus motif (ERE) that ER binds is composed of a six-base pair (bp) palindromic sequence arranged as an inverted repeat and separated by a three-bp spacer, GGTCAnnnTGACC. The inverted-repeat arrangement of the ERE dictates that the ER homodimerizes in a 'head-to-head' position when bound to DNA. Structural analysis has revealed the importance of the 10–30 amino acid carboxy terminal extension (CTE) of the DBD in DNA interaction (Aagaard *et al*. 2011, Hilser & Thompson 2011, Helsen *et al*. 2012). Although this CTE region is variable between steroid receptors, it is crucial for DNA binding, particularly for sequence selectivity of DNA binding, by extending the interaction surfaces between the receptor and the DNA.

Hinge region or D domain

The above described CTE extends into the hinge region, which also contains a nuclear localization signal, and influences cellular compartmentalization of ER, as well as

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Table 1 ER coregulator complexes. Adapted, with permission, from Binder AK, Winuthayanon W, Hewitt SC, Couse JF & Korach KS (2015) Steroid receptors in the uterus and ovary. In *Knobil and Neill's Physiology of Reproduction*, 4th Edn, pp 1099–1193. Eds TM Plant & AJ Zeleznik. Elsevier

Complex	Functions	Comments	References
Src1, Src2, Src3	Interact with Helix12 of agonist bound ER, interact with SWI/SNF, histone modifiers		Hsia *et al*. (2010) and Johnson & O'Malley (2012)
Mediator	'Bridges' ER and transcriptional 'machinery' (RNA Pol II) to control transcription	Made up of >20 subunits, MED 1–31, arranged in three modules (head, middle, tail)	Malik & Roeder (2010) and Conaway & Conaway (2011)
SWI/SNF	Regulate access to enhancer sequences via chromatin remodeling, ATPase activity	Made up of 9+ subunits, examples include BRG1, BRM, BAF subunits	Roberts & Orkin (2004)
Histone modifiers	Modify histones to increase or decrease transcription	Acetyl transferase (HAT; e.g., p300/CBP), deacetyase (HDAC; e.g., NCoR), methyl transferase (e.g., PMRT/CARM), de-methylase	Barnes *et al*. (2005) and Wu & Zhang (2009)
26S proteasome	'Clears' transcriptional modulatory proteins to facilitate subsequent transcription, transcriptional termination	Structure made up of 20S catalytic core particles (CP), 19S regulatory particles (RP)	Keppler *et al*. (2011) and Kim *et al*. (2011*a*,*b*)

sites of post-translation modifications (Kim *et al*. 2006). Current mechanisms suggest this non-conserved and ID domain is important for intra-molecular allosteric interactions involving the N-terminal and LBD. This type of flexible structural interaction works to allow rapid response to diverse modulators governing changes in biological environments (Kumar & McEwan 2012).

LBD or E domain

The LBD or E domain of the ER is a highly structured multifunctional region that primarily serves to specifically bind estrogen and provide for hormone-dependent transcriptional activity through an activation function 2 (AF-2) domain located close to the C-terminus of the E domain. A strong receptor dimerization interface, sites for interaction with heat shock proteins, and nuclear localization signals are also within the E domain (Laudet & Gronemeyer 2001, Kumar & McEwan 2012). Structural studies indicate that the LBD is composed of 11 α-helices (H1, and H3 through H12) arranged in a three-layer α-helical sandwich to create a hydrophobic ligand-binding pocket near the C-terminus of the receptor (Huang *et al*. 2010). Receptor binding to an estrogen agonist leads to rearrangement of the LBD such that H11 is repositioned and H12 rotates back toward the core of the domain to form a 'lid' over the binding pocket. This agonist-induced repositioning of H12 leads to the formation of a hydrophobic cleft, or 'NR box,' by helices 3, 4, and 5 on the receptor surface, constituting the AF-2, which serves to recruit coactivators (Table 1) to the receptor complex. In contrast, estrogen antagonists are unable to induce a similar repositioning of H12, leading to a receptor formation that is incompatible with coactivator recruitment and is therefore less likely to activate transcription. The LBDs of ERα and ERβ exhibit ~60% homology (Fig. 1) but bind the endogenous estrogen, estradiol (E_2), with similar affinity (ERα, 0.1 nM; ERβ, 0.4 nM) (Le Romancer *et al*. 2011, Gibson & Saunders 2012) indicating only a small portion of the LBD sequence governs the specificity of ligand binding. However, given the divergence in homology, it is not surprising that ERα and ERβ exhibit measurable differences in their affinity for other endogenous steroids and xenoestrogens (Le Romancer *et al*. 2011, Gibson & Saunders 2012). Natural and synthetic steroidal and non-steroidal ER agonists and antagonists have been described, some of which show specificity or preference for one or the other ER subtype, illustrating differences between the LBDs of ERα and ERβ and provide for conceptual pharmacological tools to discern the overall function of each ER. The most widely used ER sub-type selective ligands currently in use are propylpyrazole (PPT), an ERα selective agonist, and diarylpropionitrate (DPN), an agonist showing preference, but not exclusive selectivity, towards ERβ (Stauffer *et al*. 2000, Meyers *et al*. 2001).

F domain

Among the sex steroid receptors, only ERs possess a well-defined F domain (Fig. 1). This region is relatively unstructured with little known function, although some data indicate a role in coactivator recruitment,

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dimerization and receptor stability (Katzenellenbogen *et al*. 2000, Koide *et al*. 2007, Yang *et al*. 2008, Kumar *et al*. 2011, Arao *et al*. 2013).

Coregulatory complexes

All steroid receptors interact with coregulatory molecules, coactivators, and corepressors (Hsia *et al*. 2010, George *et al*. 2011). The primary coactivator interaction for steroid receptors is with a family of p160/SRC (steroid receptor coactivator) 1, 2, and 3 coactivators (Lonard & O'Malley 2005, Bulynko & O'Malley 2011, Johnson & O'Malley 2012). SRC1 (NCOA1), SRC2 (GRIP1 and TIF2), and SRC3 (pCIP, RAC3, ACTR, TRAM, and A1B1) interact with helix 12 of ERs via 'LXXLL' motifs in their nuclear receptor interacting domains, which are leucine rich regions with 'X' designating any amino acid (Johnson & O'Malley 2012). SRCs also contain activation domains that recruit secondary molecules such as p300, and a bHLH-PAS motif within the N-terminal region, which can interact with other transcription factors (Johnson & O'Malley 2012). ERs and SRCs function as a nexus interacting with massive multimeric complexes, including the SWI/SNF chromatin remodeler, mediator complex, or proteasomes (Table 1) (Bulynko & O'Malley 2011). These interactions coordinate the specific functions necessary to allow appropriate gene and cell selective access to chromatin, via modifications of histones or members of coregulatory complexes (O'Malley *et al*. 2012). In this way, coactivators dynamically mediate and coordinate processes necessary to accomplish transcription, including initiation, elongation, termination, and clearing or turnover of the transcriptional modulators.

Mechanisms of estrogen response

Our understanding of the mechanisms by which estrogens influence cell function and behavior has expanded profoundly since initial models of ligand-dependent activation, which is now referred to as the 'classical' or ligand dependent direct DNA binding model of receptor function (Fig. 2). In the years since, numerous discoveries primarily in cell-based systems have been made that illuminate the complexity of ER signaling in cells and tissues. The entrée into the 'omics' era has facilitated massive expansion for the study of transcriptional regulation and chromatin remodeling. In addition, several alternative receptor signaling mechanisms that diverge from the classic model have become apparent, including 'tethering' of the ER to heterologous DNA-bound transcription factors to provide for regulation of genes that lack ERE sequences (Fig. 2); plasma membrane estrogen signaling, often referred to as 'nongenomic' steroid actions and ligand-independent 'cross-talk' with intracellular and second messenger systems that provide for ER activation in the absence of the cognate steroid ligand (Fig. 2). These modes of ER responses as currently understood are discussed below.

Ligand-dependent actions: direct or classical

In the classic model of estrogen response (Figs 2 and 3) estrogen ligands diffuse across the plasma and nuclear membranes to bind ER, primarily localized to the nucleus, resulting in a conformational change in the receptor, transforming it to an 'activated' state that interacts with chromatin via ERE motifs and transcriptional mediators. ERs seem to be preferentially recruited to open regions of chromatin (Biddie *et al*. 2010). Studies using MCF7 breast cancer cells indicate that FoxA1 acts as a pioneering factor, providing accessible regions in the chromatin that recruit ERα (Fig. 3) (Carroll *et al*. 2005, Carroll & Brown 2006, Fu *et al*. 2011, Zaret & Carroll 2011). The ligand–ERE-bound receptor complex then engages coactivator molecules as described above (Johnson & O'Malley 2012) leading to modulation of transcription rates of responding genes. This classic steroid receptor mechanism is dependent on the functions of both AF-1 and AF-2 domains of the receptor, which synergize via the recruitment of coactivator proteins, most notably the p160 family members (Johnson & O'Malley 2012). Depending on the cell and target gene promoter context, the DNA-bound receptor complex may positively or negatively affect expression of the downstream target gene. Initially, study of ER mediated gene regulation was carried out on a gene-by gene basis using a handful of known hormone regulated transcripts. Now, after numerous comprehensive analyses of hormonally regulated transcriptional profiles, using microarray and more recently RNA-seq, thousands of ER targets have been found in various cell lines and tissues.

Indirect/tethered actions (ERE independent)

In *in vitro* reporter gene systems, ligand-activated ER can modulate the expression of genes that lack a conspicuous ERE within their promoter (Kushner *et al*. 2000, Safe & Kim 2004, 2008). This mechanism of ERE-independent steroid receptor activation is postulated to involve a 'tethering' of the ligand-activated receptor to transcription factors that are directly bound to DNA via their respective response

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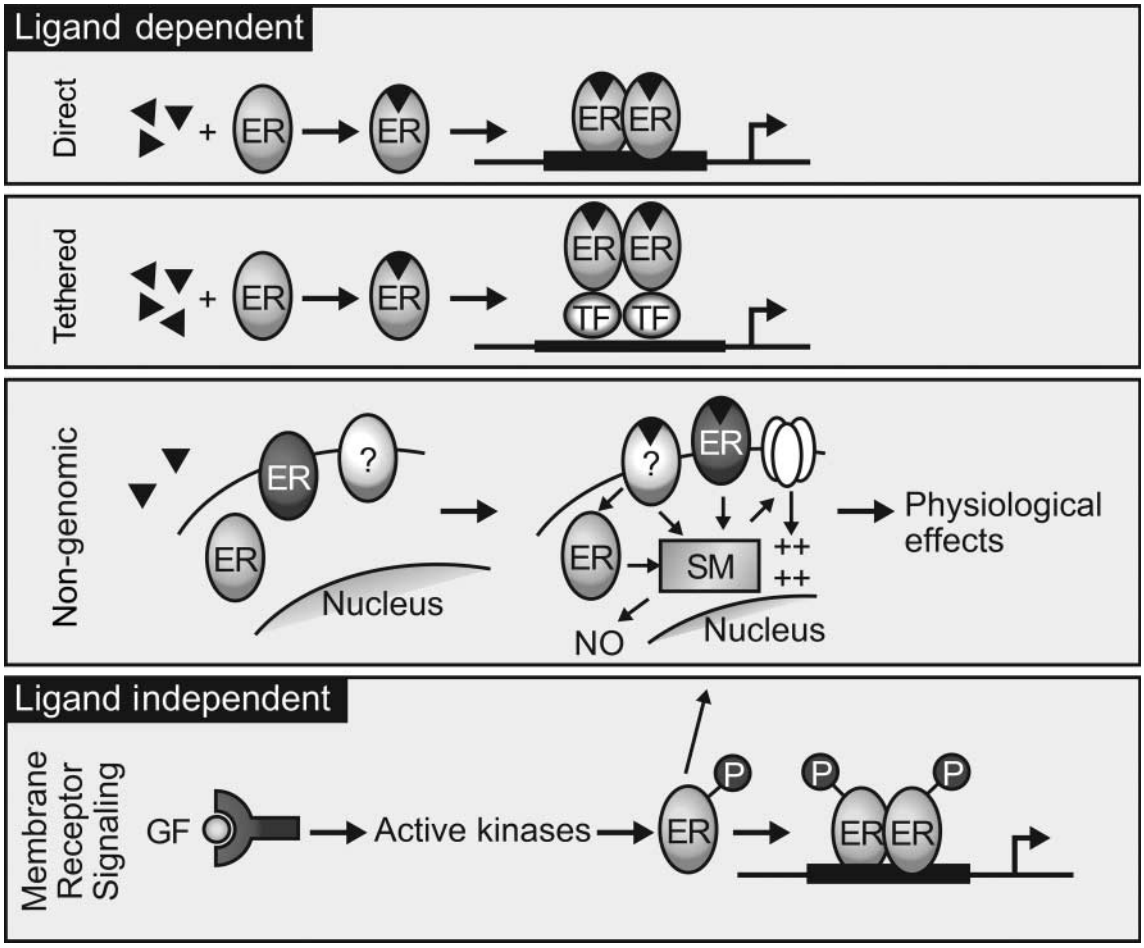

Figure 2

Ligand-dependent and ligand-independent nuclear receptor mechanisms. The direct 'classic' model of ER action involves direct interaction between ER bound to estrogen (triangles) and ERE; the tethered pathway utilizes indirect 'tethering' of ER to genes via interactions with other transcription factors (TF). 'Nongenomic' signaling is initiated by membrane-localized receptors modulating extranuclear second messenger (SM) signaling pathways. Ligand-independent responses occur as a result of transduction of membrane receptor signaling, such as growth factors (GF), to nuclear ER. Adapted, with permission, from Heldring N, Pike A, Andersson S, Matthews J, Cheng G, Hartman J, Tujague M, Ström A, Treuter E, Warner M & Gustafsson JA (2007) Estrogen receptors: how do they signal and what are their targets. *Physiological Reviews* **87** 905–931. Copyright 2007 The American Physiological Society (APS). All rights reserved.

elements (Fig. 2). However, the ER$\alpha^{EAAE/EAAE}$ mouse, which is mutated in the ERα DBD and lacks ERE binding, does not exhibit estrogen response *in vivo*, indicating the tethering mechanism, at least on its own, is unable to mediate hormonal responses (Ahlbory-Dieker *et al*. 2009, Hewitt *et al*. 2014) and is likely complimentary to the direct DNA stimulated responses.

Non-genomic actions

Rapid effects of E_2 have been described, including a rapid activation of endothelial nitric oxide synthase in endothelial cells (Levin 2011) and potentiation of nerve conductance (Takeo & Sakuma 1995, Kim *et al*. 2011*a*,*b*). Because these estrogen effects occur within minutes, they have been thought not to involve direct ER activation of gene transcription, they are often collectively referred to as representing 'non-genomic' pathways of estrogen action. Questions remain concerning whether the membrane-associated receptors mediating these events are identical or variant forms of the ER or instead distinct receptors altogether.

One potential mediator of rapid membrane localized hormone response is the G protein coupled ER (GPER, originally referred to as GPR30), which is activated by E_2 (Prossnitz & Barton 2011). *Gper* null mice lack reproductive phenotypes (Langer *et al*. 2010), although effects on the degrees of uterine responses elicited by E_2 have been

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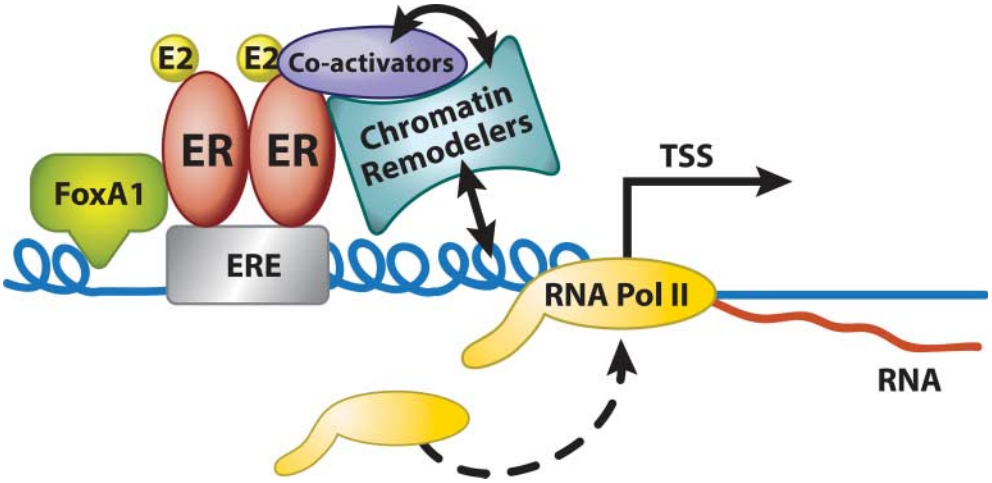

Figure 3
Model of chromatin dynamics in ER mediated transcription. FoxA1 interacts with chromatin, providing access for ER to nearby EREs. ER then interacts with transcriptional coactivators and chromatin modifying enzymes to open up transcription start sites (TSS) for RNA polymerase II (PolII), allowing initiation of transcription. Adapted, with permission, from Wall EH, Hewitt SC, Case LK, Lin CY, Korach KS & Teuscher C (2014) The role of genetics in estrogen responses: a critical piece of an intricate puzzle. *FASEB Journal* **28** 5042–5054.

observed with G15, a GPER selective antagonist, suggesting a potential role for GPER in modulating ERα mediated responsiveness (Gao *et al*. 2011).

Ligand independent actions: membrane receptor cross-talk

Peptide growth factors are able to activate ERα-mediated gene expression via mitogen-activated protein kinase activation of ERα in the absence of E_2 (Fig. 2). Likewise, growth factors are able to mimic the effects of E_2 in the rodent uterus via E_2 independent activation of ERα (Curtis & Korach 1999, Fox *et al*. 2009). In some cases, the MAP kinase protein ERK is corecruited to chromatin with ERα (Madak-Erdogan *et al*. 2011). Ligand-independent activation of estrogen receptors is believed to rely largely on cellular kinase pathways that alter the phosphorylation state of the receptor and/or its associated proteins (e.g., coactivators, heat shock proteins) (Fig. 2).

Uterine response to E_2

Utilizing animal models to follow and manipulate estrogen responsiveness is one way to understand and describe mechanisms of estrogen responses. The reproductive function of the mouse has been especially well studied and characterized in this manner.

Treatment of ovariectomized mice with estrogens (e.g., E_2 or diethylstilbestrol – DES) has long served as an experimental model to mimic the uterine events that occur during the estrous phase of the rodent cycle or immediately after the preovulatory E_2 surge. Morphological and biochemical changes occur in the rodent uterus after estrogen stimulation following an established biphasic temporal pattern (Hewitt *et al*. 2003). Estrogen-stimulated changes in the rodent uterus that occur early, within the first 6 h after treatment, include increases in nuclear ER occupancy, water imbibition, vascular permeability and hyperemia, prostaglandin release, glucose metabolism, eosinophil infiltration, gene expression (e.g., c-*fos*), lipid and protein synthesis. ERα ChIP-Seq profiles from *in vivo* studies of uterine tissues show that in the unstimulated state the receptor pre-occupies chromatin sites in the absence of hormone and that E_2 treatment increases ERα recruitment (Hewitt *et al*. 2012). The above processes are followed by responses that peak after 24–72 h and include dramatic increases in RNA and DNA synthesis, epithelial proliferation, and differentiation of epithelial cells toward a more columnar secretory phenotype, dramatic increases in uterine weight, and continued gene expression.

Changes in uterine gene expression

The dramatic physiological changes that occur in the uterus in response to steroid hormones are presumably the ultimate effects of equally dramatic changes in gene expression among the uterine cells. It is unlikely that the

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E_2–ER complex is directly involved in mediating the whole genomic response in the uterus but more plausibly serves to stimulate a cascade of downstream signaling pathways that act to amplify the estrogen action. However, early investigations of the genomic response to estrogens in the rodent uterus discovered a handful of genes that are directly regulated via the classic ER mode of action, including progesterone receptor (*Pgr*) and lactoferrin or lactotransferrin (*Ltf*). Microarray analysis has significantly advanced understanding of genomic response of the rodent uterus to E_2. Numerous studies have used microarray techniques to map the global gene expression patterns after estrogen exposure in the uterus and largely demonstrate that the biphasic uterine response to estrogens, so well characterized by physiological indicators above, is mirrored by the global changes in gene expression (Andrade *et al*. 2002, Fertuck *et al*. 2003, Hewitt *et al*. 2003, Watanabe *et al*. 2003, Ho Hong *et al*. 2004, Moggs *et al*. 2004, Hewitt *et al*. 2005, Hong *et al*. 2006). The clearly defined patterns of early and late response genes found in mouse uterine tissues are completely lacking in ERα–null (αERKO, Ex3αERKO) uteri (Hewitt *et al*. 2003, 2010*a*,*b*). The identified genes fall into functional groups, including signal transduction, gene transcription, metabolism, protein synthesis and processing, immune function, and cell cycle. The expression levels of a striking number of genes are actively repressed by estrogen in the mouse uterus, and these effects were absent in ERα-null uteri or are relieved by cotreatment with ER antagonists in the presence of ERα, indicating that ERα is also actively involved in transcriptional repression as part of mediating the physiological responses (Hewitt *et al*. 2003, 2010*a*,*b*).

Whole transcriptome analyses are now routinely incorporated into studies of disruptions in signaling pathways underlying uterine phenotypes of mouse models such as those described in Table 2. Thus, microarray comparisons have now become just one of many tools employed for investigation of uterine functions.

Chip-seq

Evaluation of sites of transcription factor interaction with chromatin, by enriching a DNA binding protein, such as ERα, that has been crosslinked *in situ* to chromatin, with immunoprecipitation (ChIP), followed by hybridizing the associated DNA to a chip tiled with promoter region sequences (ChIP–Chip) or by 'next generation' massively parallel sequencing (ChIP-seq), have been developed and widely utilized to study sites of ER interaction (Farnham 2009, Park 2009, Biddie *et al*. 2010, Green & Han 2011, Martens *et al*. 2011, Meyer *et al*. 2012). Initial studies focused on ERα binding in MCF7 breast cancer cells, and several similar studies followed, which are summarized and compared in several review articles (Deblois & Giguere 2008, Cheung & Kraus 2010, Gao & Dahlman-Wright 2011, Tang *et al*. 2011, Gilfillan *et al*. 2012). These articles reported that most sites were distal from transcriptional start sites (TSS), or were in intronic regions, rather than adjacent to TSS, as models of ER regulation of target transcripts had hypothesized. These comprehensive maps of cis-acting transcriptional regulators have been dubbed 'cistromes.' The initial ERα cistrome-associated sequences were evaluated for enrichment of transcription factor motifs and confirmed binding to the experimentally defined 'ERE' sequence. In the case of the MCF7 tumor cells, enrichment of motifs for forkhead binding factors (Fox) was apparent as mentioned in the earlier section. Owing to the abundant expression of the FoxA1 member of the Fox family, a potential role for FoxA1 in estrogen response was pursued with an arsenal of bioinformatic, Next Gen sequencing and biological studies that demonstrated FoxA1's role as 'pioneer,' creating accessible regions of the chromatin that were subsequently targeted by ERα (Lupien *et al*. 2009, Zaret & Carroll 2011).

ChIP-seq analysis is examining the ERα binding sites in mouse uterine tissue indicated that, much like the MCF7 breast cancer study, most ERα sites were not proximal to TSS (Hewitt *et al*. 2012). ERs bind to thousands of sites within the cellular chromatin, and not all potential EREs in every cell bind ER. Rather, it is apparent that chromatin exhibits 'pre-opened' regions destined to recruit ER (Grontved & Hager 2012). For ER in MCF7 and FoxA1 can establish ER accessible regions. The accessible chromatin regions are colocalized within nuclear 'hubs,' which seem to optimize frequency of interaction with ER (Grontved & Hager 2012). ChIP-seq is also used to locate other molecules involved in chromatin remodeling and transcriptional regulation, and to examine activating or repressive histone modifications or 'marks.' These maps of relative locations and dynamics of ER and chromatin components greatly enhance our understanding of hormone response mechanisms (Deblois & Giguere 2008, Green & Han 2011, Martens *et al*. 2011, Gilfillan *et al*. 2012, Meyer *et al*. 2012).

Uterine phenotypes in mouse models of disrupted estrogen signaling

Mouse models of disrupted ER signaling have proven invaluable to experimental investigation of estrogen

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Table 2 Uterine phenotypes in mice null or mutated for estrogen receptors or estrogen signaling. Adapted, with permission, from Binder AK, Winuthayanon W, Hewitt SC, Couse JF & Korach KS (2015) Steroid receptors in the uterus and ovary. In *Knobil and Neill's Physiology of Reproduction*, 4th Edn, pp 1099–1193. Eds TM Plant & AJ Zeleznik. Elsevier

Mutated or null for sex steroid receptors and signaling	Uterine phenotypes	References
Esr1$^{-/-}$ (homozygous null alleles for ERα: αERKO and Ex3αERKO)	Normal uterine development but exhibits hypoplastic uteri Insensitive to the proliferative and differentiating effects of endogenous, growth factors and exogenous E_2 Implantation defect [a]Lack decidualization Infertile	Lubahn *et al*. (1993), Curtis *et al*. (1999), Dupont *et al*. (2000), Curtis Hewitt *et al*. (2002), Hewitt *et al*. (2010*a*,*b*), Antonson *et al*. (2012)
NERKI$^{+/-}$ (one mutated allele of two-point mutation in ERα DBD and one WT allele)	Normal uterine development but exhibits hyperplastic uteri Hypersensitive to estrogen Infertile	Jakacka *et al*. (2002)
KIKO (*ER*$^{AA/-}$) (one mutated allele of two-point mutation in DNA binding domain of ERα and one ERαKO allele)	Normal uterine development Insensitive to the proliferative effects of exogenous E_2 treatment ER^{AA} binds HRE and induces genes that are normally progesterone responsive Infertile	O'Brien *et al*. (2006) and Hewitt *et al*. (2010*a*,*b*)
ERα$^{EAAE/EAAE}$ (homozygous animal of four-point mutation of DBD ERα)	Normal uterine development but exhibits hypoplastic uteri Loss of E_2-induced uterine transcripts Infertile	Ahlbory-Dieker *et al*. (2009)
ERαAF-1° (deletion of amino acids 2–128 on ERα)	Normal uterine development and architecture Blunted E_2 response Infertile	Billon-Gales *et al*. (2009) and Abot *et al*. (2013)
ERαAF-2° (deletion of amino acids 543–549 on ERα)	Normal uterine development but exhibits hypoplastic uteri Insensitive to E_2 treatment Infertile	Billon-Gales *et al*. (2011)
ENERKI (*ERα*^{G525L}) (homozygous animal of one point mutation in LBD of ERα)	Normal uterine development but exhibits hypoplastic uteri Insensitive to E_2 treatment IGF1 induced slight uterine epithelial proliferation compared to control littermates (non-homogenous pattern) Infertile	Sinkevicius *et al*. (2008)
AF2ER$^{KI/KI}$ (homozygous knock-in of two-point mutation in LBD of ERα)	Normal uterine development but exhibits hypoplastic uteri Insensitive to E_2 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*. (2011)
ERα Epi-cKO (epithelial cell specific deletion of ERα using *Wnt7a*$^{Cre+}$; *Esr1*$^{f/f}$ mouse model)	Normal uterine development Sensitive to E_2- and growth factor-induced epithelial cell proliferation Lack full uterine growth response to E_2 Selective loss of E_2-target gene response Implantation and decidualization defects Infertile	Winuthayanon *et al*. (2010 2014) and Pawar *et al*. (2015)
Esr1$^{d/d}$ (uterine deletion of ERα using *Pgr*$^{Cre+}$; *Esr1*$^{f/f}$ mouse model)	Normal uterine development Hypoplastic uteri Defective decidual response	Pawar *et al*. (2015)
Esr2$^{-/-}$ (homozygous null alleles for ERβ: βERKO, Ex3βERKO, and [b]ERβ$^{L-/L-}_{ST}$)	Exhibit grossly normal uterine development and function Sensitive to E_2 treatment Some *Esr2*$^{-/-}$ lines reported elevated uterine epithelial proliferation after E treatment compared with WT Some are complete sterile (due to ovarian phenotype)	Krege *et al*. (1998), Dupont *et al*. (2000), Wada-Hiraike *et al*. (2006) and Antal *et al*. (2008)
αβERKO (homozygous null for both ERα and Erβ)	Normal uterine development but exhibit hypoplastic uteri, similar to those of Esr1$^{-/-}$. Insensitive to E_2, infertile	Couse *et al*. (1999) and Dupont *et al*. (2000)
Cyp19a1$^{-/-}$ (homozygous null aromatase: ArKO)	Normal uterine development but exhibits hypoplastic uteri Sensitive to E_2-induced epithelial cell proliferation Infertile	Fisher *et al*. (1998) and Toda *et al*. (2001)
*Esr1*C541A palmitoylation deficient mutants	C451A-ERα normal uterine development, E_2 growth response Nuclear-only ERα [NOER] hypoplastic ERα-null like uterus	Adlanmerini *et al*. (2014) and Pedram *et al*. (2014)

[a]αERKO females have a similar uterine phenotype to the newer Ex3αERKO except for maintaining decidualization response, which may due to the splice variants in the original αERKO that retains ER activities.
[b]ERβ$^{L-/L-}_{ST}$ females are the only line of ERβ knockout animals that reported to be completely sterile.

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actions and the contribution of each ER form to these functions (Table 2). In addition to the ER-null models are lines of mice that lack the capacity to synthesize E_2 due to disruption of the *Cyp19* gene (Fisher *et al*. 1998, Toda *et al*. 2001). Below we will describe how these different mouse models have helped to delineate the biological role of ER mechanisms in estrogen hormone action.

ERα null patients and mice

Only one male patient and one female patient with ERα mutation have been described (Smith *et al*. 1994, Quaynor *et al*. 2013). The male patient's mutation is a true null since no ERα protein is expressed due to the mutation generating a premature stop codon in the A/B domain. The female patient has a single point mutation in her ERα LBD that results in decreased activity by reducing the receptors affinity for coactivator proteins more than 200-fold.

There are currently numerous reported lines of ERα-null mice and additional lines of mice with mutations in functional domains of ERα. Three separate lines of ERα-null mice were generated: the αERKO, first described by Lubahn *et al*. (1993), the ERαKO (or Ex3αERKO), described by Dupont *et al*. (2000) and by Hewitt *et al*. (2010*a*,*b*), and ERα$^{-/-}$ described by Antonson *et al*. (2012). Homologous recombination was employed to disrupt ERα (αERKO), or cre-mediated recombination was used to completely excise exon 3, which encodes the ER DNA binding domain (Dupont *et al*. 2000, Hewitt *et al*. 2010*a*,*b*, Antonson *et al*. 2012) of the murine *Esr1* (ERα) gene (ERαKO, Ex3αERKO, and ERα$^{-/-}$). The uterine estrogenic response in αERKO females differs from the latter two lines, but the overall spectrum of phenotypes are the same, as αERKO animals have minimal level of truncated ERα protein produced from a splice variant, which preserves some residual biological functions (Couse *et al*. 1995), but all ERα null female mice are infertile. Recently, an ERα null rat has been derived using zinc finger nuclease (ZFN) genome editing. All phenotypes in the ERα null rats examined thus far were previously seen in the ERα null mice, including infertility due to hypoplastic uteri, polycystic ovaries, and ovulation defects (Rumi *et al*. 2014). The female patient with homozygous ERα mutation also has cystic ovaries and a small uterus despite elevated circulating serum E_2 (Quaynor *et al*. 2013).

The essential role of ERα in uterine response to estrogen is indicated by the loss of early phase effects of water imbibition and hyperemia as well as the late-phase effects of increased DNA synthesis and epithelial proliferation in ERα-null uteri (Couse *et al*. 1995, Korach *et al*. 1996, Hewitt *et al*. 2010*a*,*b*). The αERKO model was the first test of a prevailing hypothesis that early uterine effects were non-receptor mediated (Lubahn *et al*. 1993). Lack of these early responses of water imbibition, hyperemia, and eosinophil infiltration in αERKO indicated that ERα was involved in some manner and these responses clearly require the estrogen receptor. Additionally, ovariectomized mice normally exhibit a three- to four-fold increase in uterine weight after three daily treatments with E_2 or DES, whereas no such response is observed in the uteri of ERα-null females (Lubahn *et al*. 1993, Korach 1994, Hewitt *et al*. 2010*a*,*b*). Uteri of mice that lack ERα just in uterine epithelial cells (*Wnt7a*$^{Cre+}$; *Esr1*$^{f/f}$, called *ERα Epi-cKO*) have an initial proliferative response to estrogen, but full uterine response is impaired, as the growth after 3 days of estrogen treatment is significantly less than expected (Winuthayanon *et al*. 2010). The total lack of response to estrogens in ERα-null uteri as well as a lack of late biological response in epithelial ERα knockout uteri provide strong evidence that ERα is required to mediate the full biochemical and biological uterine response to estrogens (Hewitt *et al*. 2010*a*,*b*, Winuthayanon *et al*. 2010, 2014).

Numerous studies have demonstrated some of the molecular mechanisms of E_2-induced uterine epithelial cell proliferative responses in animal models. The transcription factor CCAAT enhancer binding protein beta (C/EBPβ) is involved in hormone-induced uterine proliferation (Mantena *et al*. 2006). Maximum uterine expression of C/EBPβ is induced 1 h after E_2 treatment in both epithelial and stromal cells (Mantena *et al*. 2006, Ramathal *et al*. 2010). ICI 182,786 (ER antagonist) strongly inhibited E_2-induced *Cebpb* transcript in the uterus suggesting an ER-dependent expression of C/EBPβ (Bagchi *et al*. 2006). In addition, loss of epithelial ERα in the uterus did not alter E_2-induced *Cebpb* expression, indicating that *Cebpb* expression is independent of epithelial ER (Winuthayanon *et al*. 2010), and suggesting the stimulation was through a paracrine mechanism via stromal ERα. This points to the action of estrogen through ERα as the major mediator of C/EBPβ expression in the uterus. Indeed, the deletion of C/EBPβ (C/EBPβ$^{-/-}$) leads to a lack of the E-induced uterine proliferative response (Mantena *et al*. 2006) as reflected by the absence of mitotic activity, S-phase activity and an increase in apoptotic activity in the uterine epithelial cells (Ramathal *et al*. 2010). In addition to a blunted uterine growth response to hormones, the C/EBPβ$^{-/-}$ females also exhibit complete infertility (Bagchi *et al*. 2006), due to implantation and decidualization defects (Mantena *et al*. 2006).

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Pan *et al.* (2006) demonstrated that the uterine expression of minichromosome maintenance proteins (MCMs), a complex required for DNA synthesis initiation, is induced after E_2 treatment, specifically MCM2 and MCM3. MCM2 activity is crucial and required for DNA synthesis in uterine epithelial cells (Ray & Pollard 2012). Further study demonstrated E_2-mediated induction of the transcription factor KLF4, which then targets the *Mcm2* promoter (Ray & Pollard 2012).

Mice lacking ERβ

ERβ-null mice have provided insight into the importance of ERβ to female fertility and studies to date indicate ERβ plays a particularly important role in ovarian function. Four different lines of ERβ-null mice have been described. The βERKO mouse, made using homologous recombination was first described by Krege *et al.* (1998), and the ERβKO or Ex3βERKO, was described by Dupont *et al.* (2000), and by Binder *et al.* (2013). Cre-mediated recombination was employed in both lines to disrupt exon 3 (Dupont *et al.* 2000, Binder *et al.* 2013) of the murine *Esr2* (ERβ) gene. As described to date, the reproductive, endocrine, and ovarian phenotypes of both lines are indistinguishable, with both exhibiting female subfertility. Shughrue *et al.* (2002) reported the third line of ERβKO animals, however, no uterine or ovarian phenotypes were reported (Shughrue *et al.* 2002). Recently, $ERβKO^{L-/L-}_{ST}$ animals, which contain *LoxP* sites flanking exon 3 of *Esr2*, were generated using the *Cre/loxP* recombination system (Antal *et al.* 2008). Interestingly, female mice from this recently described $ERβKO^{L-/L-}_{ST}$ colony were reported to be sterile due to an ovarian defect while Ex3βERKO (Binder *et al.* 2013) are subfertile, due to ovulatory defects.

Mice lacking ER α and β

The two reported lines of compound ER-null mice are the αβERKO, described by Couse *et al.* (1999), and the ERαβKO, described by Dupont *et al.* (2000). Both were generated by cross breeding animals heterozygous for the respective individual ER-null mice and as described to date, exhibit comparable reproductive, endocrine, and ovarian phenotypes. The most striking phenotype is the unique trans-differentiation of the ovarian granulosa cells to sertoli-like cells in follicles of αβERKO females which is age dependent. To date, no manipulation of the individual αERKO or βERKO mouse lines can reproduce this novel phenotype. This model clearly uncovered that both ER signaling systems are required to maintain the proper differentiation state of the adult granulosa cells.

Mice lacking Cyp19

Estrogens are produced by aromatase cytochrome P450, the product of *Cyp19* gene. Female mice with disruption of circulating estrogen production exhibit altered reproduction (Fisher *et al.* 1998, Honda *et al.* 1998, Toda *et al.* 2001). There are three animal models of Cyp19-null mice (called ArKO). Fisher *et al.* (1998) reported the first mouse line in 1998, which disrupted exon 9 of *Cyp19* gene, as the region is highly conserved. Later, Honda *et al.* (1998) reported a mouse line with targeted disruption of exons 1 and 2 of the *Cyp19* gene. Subsequently, Toda *et al.* (2001) generated the most recent mouse line of Cyp19-null in 2001 with a targeted disruption of exon 9 of the *Cyp19* gene. These ArKO female phenotypes are indistinguishable (Fisher *et al.* 1998, Honda *et al.* 1998, Toda *et al.* 2001), with similarity to the αβERKO mice with a clear metabolic syndrome (Couse *et al.* 1999) and infertility due to ovarian dysfunction marked by cystic follicles and a failure to respond to exogenous gonadotropins. Interestingly, the phenotype of the original ArKO mice (Fisher *et al.* 1998) were also shown to exhibit the same age related ovarian phenotype (Britt *et al.* 2002) as the αβERKO mice, indicating that hormone mediated ER action is required.

Female reproductive phenotypes in mice with disrupted estrogen signaling

Females within each respective model exhibit a similar phenotypic syndrome. Female mice lacking ERα or aromatase are infertile due to dysfunction of numerous physiological systems, including the ovary and uterus, whereas ERβ-null females exhibit reduction or loss of fecundity that is largely attributable to ovarian dysfunction. A level of caution is warranted when making phenotypic comparisons between the ER-null and Cyp19-null models because sensitivity to maternally derived estrogens may provide a more normal developmental environment during gestation in Cyp19-null mice and sensitivity to dietary estrogens during adulthood is able to abate several phenotypes in Cyp19-null mice (Britt *et al.* 2002).

The reported uterine phenotypes of these models are summarized in Table 2. All lines of ER-null females exhibit uteri that possess the expected tissue compartments, myometrium, endometrial stroma, and epithelium

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(Couse & Korach 1999, Hewitt *et al*. 2010*a*,*b*). However, in females lacking functional ERα or Cyp19, uteri are overtly hypoplastic and exhibit severely reduced weights relative to wild-type littermates (Fisher *et al*. 1998, Couse & Korach 1999, Britt *et al*. 2001, Toda *et al*. 2001), whereas ERβ-null uteri are grossly normal and normally responsive to ovarian-derived steroids (Couse & Korach 1999). The uterus of ERα-null females is severely hypotrophic, poorly organized, and possesses a paucity of glandular structures (Korach *et al*. 1996, Hewitt *et al*. 2010*a*,*b*). The luminal and glandular epithelial cells in ERα-null uteri are severely immature with fewer glands present in the adults (Nanjappa *et al*. 2015) and consistently exhibit a cuboidal morphology, vs the tall columnar morphology and basal location of the nucleus of an 'estrogenized' epithelium in WT uteri. Therefore, fetal, neonatal, and perinatal development of the female reproductive tract in mice is largely independent of ERα- and ERβ-mediated actions, but estrogen responsiveness and sexual maturation of the adult uterus are ablated after the loss of functional ERα. The totality of the ERα-null phenotype and lack of any overt uterine abnormalities in ERβ-null females suggest that ERβ has little meaningful function in mediating estrogen actions in the uterus. Moreover, ERαβ-null also demonstrated a similar uterine phenotype as ERα-null (Walker & Korach 2004). Weihua *et al*. (2000) reported that ERβ-null females exhibited a slightly aberrant uterine growth response after estrogen replacement; however, the uterine bioassay was conducted in immature intact, not ovariectomized adult, animals. In addition, Wada-Hiraike *et al*. (2006) showed that in immature females, loss of ERβ leads to increased uterine epithelial proliferation induced by E_2 compared with WT uteri. Although ERβ-null females are subfertile, when pregnancies are established they are sustained to term (Krege *et al*. 1998), indicating uterine competence. More recent findings suggest that loss of ERβ leads to complete sterility due to a defect in ovarian function (Dupont *et al*. 2000, Antal *et al*. 2008).

Mice with uterine specific deletion of ERα

Selectively deleting ERα in the uterus postpubertally, using the *Cre*/*Lox*P recombination system, by crossing Pgr^{Cre+} with $Esr1^{f/f}$ animals ($Esr1^{d/d}$), leads to a hypoplastic uterus that lacks a decidual response (Pawar *et al*. 2015). Our laboratory has described uterine epithelial cell selective deletion of ERα, using the *Cre*/*Lox*P recombination system, by crossing $Wnt7a^{Cre+}$ (Huang *et al*. 2012) with $Esr1^{f/f}$ animals (Hewitt *et al*. 2010*a*,*b*) (*ERα Epi-cKO*). The expression of ERα in the uterine luminal and glandular epithelium of these animals was ablated, while the ERα expression in the stromal cells and other uterine cells remains intact (Winuthayanon *et al*. 2010). The epithelial ERα was ablated not only in the uterus in this mouse line (Winuthayanon *et al*. 2010), but also in the oviduct (Winuthayanon *et al*. 2015). As expected, based on findings in the global ERα knockouts, loss of uterine epithelial ERα has no effect on female reproductive tract development. Uterine histological analysis showed a similar uterine morphology as WT control (Winuthayanon *et al*. 2010). The *ERα Epi-cKO* uteri are sensitive to 24 h treatment of E_2, as the uterine epithelial proliferation is preserved. However, *ERα Epi-cKO* uteri lack a complete uterine response to E_2, following a 3-day uterine bioassay, which demonstrated a blunted growth response and increased apoptotic activity in *ERα Epi-cKO* compared with the control uteri. Additionally, a lack of ERα expression in the uterine epithelial cells contributes to complete infertility, due to oviduct, and uterine implantation and decidulaization defects (Winuthayanon *et al*. 2010, Pawar *et al*. 2015, Winuthayanon *et al*. 2015). This suggests that uterine epithelial ERα is dispensable for early uterine proliferative responses but crucial for a complete adult biological response induced by E_2, as well as for establishing pregnancy.

Mice with mutated DNA binding domains of ERα

To date, there are two mouse lines with mutations that are designed to disrupt the DNA binding function of the ERα that have been 'knocked-in' (KI) at the ERα gene locus. The first line was generated by replacing critical P-box amino acids E207 and G208 with alanines ($ERα^{AA}$). This line was named 'non-genomic ER knock-in' (NERKI), as these mutations were intended to restrict ERα signaling to the non-genomic and tethered mechanisms. Female $NERKI^{+/-}$ animals that have one mutated allele and one WT allele (Jakacka *et al*. 2002) were infertile, exhibiting a highly novel hyperplastic uterine phenotype, so $NERKI^{+/-}$ males were crossed with ERα null heterozygous (WT/KO) females to produce mice with one NERKI mutated allele and one deleted *Esr1* allele, called ERα KIKO or $ERα^{AA/-}$ as described by O'Brien *et al*. (2006). The second line of DNA-binding domain knock-in animals were created through mutation of four amino acids in the first zinc finger of the *Esr1* gene, substituting Y at position 201 with E, and in the critical P box, K at position 210 with A, K at position 214 with A, and R at position 215 with E as described by Ahlbory-Dieker *et al*. (2009; called $ERα^{EAAE/EAAE}$).

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The NERKI$^{+/-}$ females have normal uterine development but exhibit hyperplastic uteri, and are hypersensitive to estrogen (Jakacka *et al*. 2002). These NERKI$^{+/-}$ are infertile and exhibit a uterine abnormality of enlarged hyperplastic endometrial glands despite possessing normal levels of circulating sex steroids.

ERα$^{AA/-}$ females have normal uterine development. Initially, O'Brien *et al*. (2006) reported that ERα$^{AA/-}$ females, with mutation of the DNA binding domain, maintained proliferative responses induced by E_2. However, in subsequent studies, no uterine proliferation was observed (Hewitt *et al*. 2009, 2010). Ahlbory-Dieker *et al*. (2009) showed that, unlike the NERKI$^{+/-}$, females heterozygous for the ERα^{EAAE} mutation are fertile. The homozygous ERα$^{EAAE/EAAE}$ females have normal reproductive tract development but uteri are severely hypoplastic, similar to global ERα-null uteri. Additionally, ERα$^{EAAE/EAAE}$ uteri do not respond to E treatment, as normally estrogen-responsive uterine and liver genes are not regulated in ERα$^{EAAE/EAAE}$ (Ahlbory-Dieker *et al*. 2009, Hewitt *et al*. 2014). The females from these two mouse lines with point mutations in the DNA binding domain of ERα are infertile. Thus the physiological function of the DNA binding domain of ERα is crucial for female reproduction. ERα ChIP-seq analysis of the ERα$^{AA/-}$ uterus revealed that the DBD mutation, rather than completely disrupting DNA binding instead altered the motif specificity, so that ERα^{AA} could bind HRE motifs normally occupied by progesterone receptor (Pgr or PR). Additionally, this HRE binding lead to E_2 regulation of uterine transcripts that are normally progesterone responsive (Hewitt *et al*. 2014). This novel ERα^{AA} binding activity may also explain the hyperplastic phenotype of the heterozygous ERα$^{AA/+}$ females where the normally activated uterine HRE sites are occupied by the mutant ERα^{AA} and thus blocking the dampening activity of uterine PR at those sites. Adding to this abnormal regulation is the expression of ERα^{AA} in all uterine cells at all times, whereas, the PR is restricted at times, to epithelial cells and is dynamically induced in the stromal cells during the estrous cycle. Additionally, the phenotype also indicates the specificity of the action at the HRE requires the proper activity of the PR to elicit the dampening action.

Mice with mutated AF-1 or AF-2 domains of ERα

As discussed in the Receptor structure section, AF-1 and AF-2 are important for ER transcriptional activity (Fig. 1). Amino acids 2–128 were deleted from exon 1 of *Esr1*, which removes the AF-1 domain, and knocked into a mouse line (called ERαAF-1°) (Billon-Gales *et al*. 2009). There are three reported mouse lines with mutation in the AF-2 domain of ERα. One with a single point mutation in ERα of G at position 525 to L in the ligand binding domain (LBD), called 'estrogen-nonresponsive ERα knock-in or ENERKI' (ERα^{G525L}) (Sinkevicius *et al*. 2008). Amino acids 543–549 were deleted from the LBD of ERα, removing helix 12 and thus AF-2 functionality, to create a second mouse line (called ERαAF-2°) (Billon-Gales *et al*. 2011). Two point mutations in the AF-2 of the LBD of ERα were knocked into a mouse (L543A and L544A, called AF2ER$^{KI/KI}$ animals) (Arao *et al*. 2011). ERαAF-1°, ERα^{G525L}, ERαAF-2°, and AF2ER$^{KI/KI}$ females are all sterile (Sinkevicius *et al*. 2008, Billon-Gales *et al*. 2009, Arao *et al*. 2011, Billon-Gales *et al*. 2011).

ERαAF-1° females exhibited minimal uterine wet weight gain compared with ER$^{+/+}$ uteri after treatment with E_2 pellets for two consecutive weeks, while ERαAF-2° females did not respond (Billon-Gales *et al*. 2009, 2011, Abot *et al*. 2013). This indicates that the ERα AF-2 functional domain contributes to minimal uterine weight increase induced by E_2 in the absence of AF-1. Both lines of AF-2 mutated animals (ERα^{G525L} and AF2ER$^{KI/KI}$) display severely hypoplastic uteri, and lack uterine growth response to E_2 treatment (Sinkevicius *et al*. 2008, Arao *et al*. 2011, Billon-Gales *et al*. 2011). Interestingly, uterine wet weight can be increased by using the synthetic ERα agonist PPT in ERα^{G525L} or by using the ER antagonists ICI 182,780 or tamoxifen in AF2ER$^{KI/KI}$ females (Sinkevicius *et al*. 2008, Arao *et al*. 2011). The ability of the antagonists to mediate responses seems to be due to a unique conformation of the LBD of the AF2ER that leads to AF-1-dependent transcriptional activity (Arao *et al*. 2011, 2013). Arao *et al*. (2011) also demonstrated that the uterine response to ICI or tamoxifen includes increased DNA synthesis in the uterine epithelial cells of AF2ER$^{KI/KI}$. The growth factor IGF-1 induced minimal uterine epithelial proliferation in ERα^{G525L}, and was not seen in AF2ER$^{KI/KI}$ uteri (Sinkevicius *et al*. 2008, Arao *et al*. 2011). Together, these findings indicated that both AF-1 and AF-2 activation domains of ERα contribute to a normal regulation of the complete biological response of uterine growth and reproductive functions. As the AF domains mediate ER-coregulator interaction (Table 1), this emphasizes the importance of effective ERα coactivator protein recruitment for successful uterine E_2 response. Similarly, mice lacking sufficient SRC-1 coactivator (SRC1$^{-/-}$), exhibit measurably diminished uterine response to E_2 (Xu *et al*. 1998).

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Mice with altered localization of ERα

A mutated mouse ERα that remains sequestered outside the nucleus (ERαH2NES), is unable to mediate transcriptional responses in a cell based assay, but maintains estrogen induced MAPK phosphorylation (Burns *et al.* 2011). Targeting steroid receptors to the membrane involves palmitoylation, which is facilitated by HSP27 (Levin 2011). The palmitoylation promotes interaction with caveolin-1, which then results in localization of the receptor in membrane caveolin rafts. Two laboratories have mutated the palmitoylation site of the mouse ERα, and created knock in mouse models to study the effect of disabling this mechanism *in vivo* (Adlanmerini *et al.* 2014, Pedram *et al.* 2014). Both mouse lines have ovarian defects, but differ in several aspects (Table 2). Both involved knocking in an ERα with the same mutation of cysteine 451 to alanine. The first, C451A-ERα, exhibits normal uterine development and E_2 induced growth response (Adlanmerini *et al.* 2014), whereas the nuclear-only ERα (NOER) has a hypoplastic ERα-null like uterus that fails to respond to E_2 (Pedram *et al.* 2014). Both models have elevation in LH, but only the NOER has elevated E_2. These mixed results remain to be reconciled to definitively illustrate the role of membrane associated ERα in these physiological systems.

Conclusion

Female reproduction is a complex staged series of physiological responses occurring in multiple organ systems activated by estrogen and estrogen receptors. Cell based studies have uncovered that cellular signaling mechanisms for ER are multifaceted regarding gene regulation. Because of the complexity with what is known about female reproduction and fertility, the mechanisms and activities cannot be clearly studied or tested in cell based systems. The development of gene targeting has allowed the evaluation of the physiological roles of estrogen action and ER functionality under natural biological conditions. It is now apparent from the experimental and clinical reports outlined in this review that the primary mediator of female reproduction is ERα. What functional aspects of the ERα action are required will be forthcoming with the continued use of new technologies and experimental approaches, which will lead to a better understanding for the potential origins of infertility, reproductive tract disease and development of reproductive therapeutics.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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