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Estrogen hormone physiology: Reproductive findings from estrogen receptor mutant mice

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Abstract

Estrogen receptors (ERs) play a crucial role in reproduction and normal physiology. The two subtypes of ER (ER α and β) are expressed in various levels in different tissues and selective cell types. Gene targeting technology allowed us to produce lines of mice with disrupted ER α (α ERKO) and ER β genes (β ERKO) as well as a compound $\alpha\beta$ ERKO in the whole body. Male and female α ERKO mice are infertile. Estrogen, EGF and IGF-1 treatments failed to induce uterine growth and DNA synthesis in α ERKO uteri. α ERKO females are infertile due to hypoplastic uteri and hyperemic ovaries with no corpora lutea due to persistent LH stimulation from loss of negative feedback. α ERKO males are infertile, with testicular atrophy and seminiferous tubule dysmorphogenesis producing decreased spermatogenesis and inactive sperm. β ERKO females show arrested folliculogenesis and subfertility. Ovarian analyses indicate differential gene expression related to ovulatory stimulation deficits including lack of LH, PR, Cyp19 and Cox2 expression. A unique ovarian phenotype is found only in $\alpha\beta$ ERKO females showing transdifferentiation of granulosa cells to Sertoli cells.

We describe here several novel mouse models which possess ER α gene modification. To understand ER α function in uterine endometrial epithelial cells, we generated a tissue selective ER α gene disrupted mouse model, the uterine epithelial-specific ER α knockout (UtE-pi α ERKO). To understand the physiological role of ER α functional domains, we generated a mouse model with a mutation in the ligand dependent transcription activation domain of ER α (AF2ERKI). Findings from the ER α mutant mice suggest that the absence of functional ER α is not lethal and results in significant endocrine effects and altered physiological processes.

Keywords

Estrogen receptor; Transactivation function; Genetically modified mouse; Uterus; Ovary; Fertility

1. Introduction

Estrogen is a well-known female steroid hormone synthesized from the ovary that controls the estrous or menstrual cycle in females, therefore estrogen is imperative for female reproduction. Estrogen is not only important in female reproduction but also in male

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reproduction and in numerous other systems including the neuroendocrine, skeletal and immune systems in males and females. Along with the influence of estrogen on many physiological processes, it is also implicated in many different diseases including obesity, metabolic disorder, cancer, osteoporosis, endometriosis and fibroids [1,2]. The predominant mechanism of estrogen action is through nuclear estrogen receptor (ER) expression in estrogen target organs [3]. The biological effects of estrogen are mediated through two distinct ER proteins, ER α and ER β (ERs). These receptors are encoded from separate genes on different chromosomes and the expression profiles in the tissues are different. The predominant expression tissues for ER α include: liver, uterus, mammary gland, pituitary, hypothalamus, cervix, and vagina. ER β expression is more limited and predominant expression tissues include: ovary, lung, and prostate [4]. Controlling the functions of ER α and ER β is the basis for many therapeutic interventions to estrogen related diseases. Thus it is quite important to reveal the physiological role of ER α and ER β in the tissues and the *in vivo* functionality of ER proteins.

2. Estrogen receptors – structure and functions

The ERs are comprised of six structural domains: an amino-terminal domain (A/B-domain), a DNA binding domain (DBD; C-domain), a hinge region (D-domain), a ligand-binding domain (LBD; E-domain), and a carboxyl-terminal domain (F-domain) [3]. The C and E domains carry a high-degree of homology between ER α and ER β ; however the A/B, D and F domains are divergent [5,6]. The A/B-domain contains the transcription activation function 1 (AF-1) which is reported to be important for ligand-independent transactivation [7]. The LBD or E-domain of ERs contains the transcription activation function 2 (AF-2) that is important in ligand-dependent transcriptional regulation [7]. The helix 12 in the LBD is the core of AF-2 and the configuration of helix 12 is changed by ligands to either active (agonist bound) or inactive (antagonist bound) forms for transcription regulation [8,9].

Multiple ER-mediated transcription regulation mechanisms have been characterized. In the “classical” mechanism of estrogen action, hormone bound ERs directly bind to a specific DNA sequence called the estrogen responsive element (ERE) through the DBD or C-domain of ER [10]. Estrogen can modulate other transcription factor functions through tethered ER with other transcription factors such as c-Jun and Sp1. In the “tethered” mechanism, ER does not bind to a DNA element directly but forms a transcription activation complex on the AP-1 and Sp1 responsive elements [11,12]. It is also known that growth factors can activate estrogen-independent ER α mediated transcription and the AF-1 has an important role in the growth factor mediated ER α activation [13]. Furthermore, while ER α is a well-characterized nuclear transcription regulator, it is believed that the ER α protein is also involved in extranuclear non-genomic signal transduction [14,15].

3. Estrogen receptor knock-out (ERKO) mouse models (whole tissue KO)

The development of multiple genetic models has led to an increase in understanding and knowledge of the physiological roles of estrogen receptors. These models include mice lacking functional ER α (α ERKO), ER β (β ERKO) or both estrogen receptors ($\alpha\beta$ ERKO). Several methods have been utilized to generate the ERKO mice. Currently, the cre-loxP

system is the most widely used method to create whole tissue KO or tissue selective KO mice. The first α ERKO mouse (*Esr1^{tm1KSK}*) was generated by an insertion method to disrupt ER α expression. Namely, a neo expression cassette was inserted into exon 2 to create the frame shift mutation, resulting in the disruption of functional ER α protein expression [16]. Unexpectedly, the *Esr1^{tm1KSK}* mouse expresses a trace level of N-terminal truncated mutant ER α (E1-ER α) which is generated by non-controlled alternative splicing [17]. We have reevaluated the ER α function using the Ex3 α ERKO (*Esr1^{tm4.2KSK}*) mice, in which exon 3 is excluded by the cre-loxP system to disrupt the functional ER α expression and the major phenotypes of both ER α null strains are nearly identical [18,19]. In our preliminary results, there are some differences between the two α ERKO mouse lines, suggesting that the E1-ER α variant might have affected some physiological responses in the first α ERKO mouse (*Esr1^{tm1KSK}*) line [18,19].

3.1. Phenotypes of the reproductive tissues of the ER α knock-out (α ERKO) mouse

Estrogens stimulate the process of uterine epithelial proliferation and differentiation that is necessary for establishment and maintenance of pregnancy. Progesterone and many growth factors are also important for these processes. Establishment of a model with a null ER α (α ERKO) has given us a model to determine and verify the physiological roles of ER α . Because ER α is the predominant factor for estrogen action in the adult mouse uterus, adult α ERKO mice are infertile and have hypoplastic uteri [4]. A well-established model to examine uterine estrogenic response is to treat the ovariectomized (OVX) mouse with an agonist (i.e. estradiol or DES). In response to agonist treatment in a 3-day bioassay, uterine wet weight and the expression of estrogen responsive genes are induced by agonists in the wild type (WT) mouse. In contrast, α ERKO uteri show neither uterine stimulation nor increased expression of estrogen-responsive genes [17]. α ERKO uteri express residual progesterone receptor (PR) levels and respond to progesterone mediated gene stimulation but PR is not inducible and does not support embryo implantation [20]. α ERKO mice have hemorrhagic cystic ovaries which are due to constitutively high serum luteinizing hormone (LH) levels causing the disruption of negative feedback regulation in the hypothalamic–pituitary–gonadal (HPG) axis. The treatment of an antagonist (antide) for gonadotropin releasing hormone reduces serum LH levels and prevents cyst formation in the α ERKO ovaries [21]. The expression levels of several steroidogenic enzymes, including *Cyp17a1*, *Cyp19*, and *HSD17 β 3* are elevated in the α ERKO ovary. *HSD17 β 3* is a testicular steroidogenic enzyme not observed in the WT ovary but is aberrantly expressed in the α ERKO ovary [21]. α ERKO females have elevated estradiol and androstendione levels in their serum and also have an elevated testosterone level [21]. Mammary gland development in the α ERKO female is impaired and remains rudimentary after puberty when normal WT mammary gland development expands [18].

Adult male mice lacking ER α are infertile with lower sperm count and motility than WT mice and exhibit dilated seminiferous tubules [19]. The dilation is presumably due to loss of expression of efferent ductal testicular fluid reabsorption related proteins such as sodium–hydrogen exchanger 3 (NHE3/Slc9a3) and carbonic anhydrase 2 (Car2) [22,23]. ER α is not required by the male germ cells; demonstrated by transplantation experiments where male germ cells were put into germ cell-depleted WT testes and the recipients were able to sire

offspring from the transplanted α ERKO germ cells [24,25]. The serum hormone levels in male α ERKO mice are also disrupted with elevated testosterone and LH levels [19].

3.2. Phenotypes of the ER β knock-out (β ERKO) mouse

Female β ERKO mice are subfertile in continuous mating studies and have reduced litter sizes when compared to WT mice [26]. Mouse uteri have very low expression of ER β and the uteri of β ERKO mice are indistinguishable from WT with normal organization and development [4,27]. β ERKO uteri show a comparable response to WT when challenged with estradiol in a 3-day bioassay [27]. ER β is predominantly expressed in the granulosa cells of the ovarian follicles and β ERKO mice have defects resulting in an inefficient and infrequent ovulatory response [27,28]. This deficiency is highlighted by the appearance of unruptured follicles following superovulation and a lower number of recovered oocytes after superovulation when compared to WT mice [26]. Granulosa cells of the β ERKO mice did not differentiate fully after PMSG treatment and had reduced LH receptor expression, aromatase activity and estradiol synthesis [29]. These factors result in a deficient ovulatory response to gonadotropin treatment and failure of the cumulus–oocyte complex to expand fully [29]. *In vitro* studies of β ERKO granulosa cells showed that ER β is involved in induction of the cAMP pathway and this disruption may cause ovulation and fertility defects [29]. Male β ERKO mice are fertile and overall exhibit a phenotype widely similar to WT littermates [27,28].

3.3. Phenotypes of the ER α and ER β double knock-out ($\alpha\beta$ ERKO) mouse

Mice lacking either ER α or ER β have been instrumental in the study of estrogen action, however using these models does not account for the possible compensatory mechanisms provided by the opposite ER in each ERKO model. Therefore, mice lacking both ERs ($\alpha\beta$ ERKO) were generated to study the potential compensatory and cooperative actions of ER α and ER β . Mice lacking both ER α and ER β display a phenotype similar to the α ERKO mice. Females are infertile, have an altered endocrine environment, and exhibit deficient ovulation and male mice have dilated seminiferous tubules and deficient sperm [26,28,30]. The similar phenotype between the α ERKO and $\alpha\beta$ ERKO mice suggests that ER β is not able to compensate for ER α functions in the single ER α knockout. However, the compensational function of ER α in the single ER β knockout is still debatable. A very unique feature of the compound ER knockout adult female is the presence of seminiferous-like tubules in the ovary which develop post-pubertally and are not present in the ovary of either single ER knockout [28,30]. The cells found in these structures have features highly similar to Sertoli cells which are normally found only in the testes [28,30]. Along with physical commonality between the structures, the Sertoli-like cells found in the $\alpha\beta$ ERKO aberrantly express genes known to be involved in Sertoli cell differentiation such as Sry-related transcription factor (Sox9) [30,31]. Sox9 is required for normal Sertoli cell differentiation in the development of the testis in both humans and rodents [32,33]. These unique features suggest that the double knockout granulosa cells undergo trans-differentiation and the structures change from ovarian features into more male-like structures [30,31]. This phenomenon has never been observed in α ERKO mouse suggesting that granulosa cell ER β might be involved in the prevention of granulosa cell trans-differentiation. While sex

reversal of the ovarian structure has been shown in other strains/species, this model is noteworthy in that the differentiation occurs postnatally in $\alpha\beta$ ERKO mice [30].

4. Tissue specific ERKO mouse models

Whole animal knockout models have allowed tremendous insight into the physiological functions of the ERs. However, in the global knockout models it is not possible to look at the role of the ERs in specific tissues. With the advancement of the cre-loxP technology, the floxed ER α line can be crossed with other cre lines to produce mice lacking functional ER in specific cell types or tissues. Multiple ER α mouse models using this technique have been developed and studied [34–39]. In this review, we will discuss mice lacking ER α in uterine epithelial cells, referred to as the UtEpi α ERKO mouse.

4.1. Phenotypes of the UtEpi α ERKO mouse

Because the uterus is a primary target tissue for estrogen and is composed of different cell types, tissue or cell-type specific knockouts of this organ are instrumental in deciphering the role of ER α in uterine biology. In order to look at the individual roles of ER α in the stromal cells versus epithelium of the mouse uterus, a uterine epithelial-specific ER α knockout (UtEpi α ERKO) mouse line was generated by crossing the floxed *Esr1* mice (*Esr1^{tm4.1KSK}*) with *Wnt7a*-Cre mice [34]. The UtEpi α ERKO female mice are infertile however they exhibit regular estrous cycles and ovaries possess all stages of follicular development including corpus lutea, indicating ovulation. Implantation was not observed in UtEpi α ERKO uteri after either natural mating or embryo transfer suggesting ER α in the uterine epithelium is needed for embryo receptivity. Three days of estradiol treatment to the OVX UtEpi α ERKO mice induced uterine wet weight and epithelium proliferation suggesting that ER α in the uterine stromal cells, but not the epithelium, regulates uterine epithelial cell proliferation. While estrogen induced uterine epithelium proliferation in UtEpi α ERKO mice, apoptosis in the epithelial cells was significantly increased when compared to WT. Taken together these observations suggest that epithelial ER α is needed for proper endometrial epithelial cell functions.

5. ER domain mutated mouse models (estrogen receptor knock-in mouse)

It is impossible to examine the physiological role of ER functional domains using the ERKO mice since no protein is made. Therefore, mouse models have been made with genetically modified estrogen receptor domains to allow dissection of the physiological function of the ER domains. These include models with altered DNA binding domains (NERKI and EAAE) which disrupt binding to the estrogen responsive DNA element, as well as one model with a point mutation in the ligand binding domain (ENERKI) which disrupts estradiol mediated transcription activation by inhibiting estradiol binding [40–42]. Furthermore, mouse models have been made with the deletion of AF-1 or AF-2 (AF-1⁰ and AF-2⁰) and point mutations in the core AF-2 (AF2ERKI) [43,44].

5.1. Phenotypes of the AF2ERKI mouse

In order to examine the physiological function of AF-1 and AF-2 of ER α , our laboratory established a knock-in mouse model (AF2ERKI, *Esr1^{tm3.1KSK}*) that contains two point

mutations in the AF-2 region, disrupting the AF-2 mediated transactivation [45]. Even though the AF2ER mutant ER α protein is expressed in AF2ERKI mice, the phenotypes of the AF2ERKI male and female are indistinguishable from α ERKO mice, suggesting that the AF-2 is indispensable for ER α -mediated physiological responses. Growth factors, such as insulin like growth factor 1 (Igf1) and EGF are able to stimulate uterine cell proliferation without estrogen in OVX WT mice but not in the α ERKO uteri [18,46,47]. This observation suggests that the growth factor dependent phosphorylation signals activate “ligand-independent” transcription through ER α *in vivo* [13,47]. However, Igf1 and EGF did not induce the uterine cell proliferation in the AF2ERKI [45], suggesting that the AF-1 activity of ER α is regulated by the “ligand-dependent” functional domain, AF-2. One unique characteristic of the AF2ERKI mouse model is that traditional antagonists such as fulvestrant/ICI182780 (ICI) and tamoxifen (Tam) act as agonists through the AF2ER mutant ER α [45]. Our *in vitro* experiments suggest that the AF-1 activity is necessary for the antagonist dependent AF2ER activation [45]. In a 3-day bioassay, ICI and Tam stimulated uterine growth and gene expression in a comparable way to WT treated with E2 [45]. In contrast, E2 induced prolactin (*Prl*) gene expression in the OVX WT mouse pituitary, however, ICI and Tam treatment were not able to stimulate *Prl* gene expression in the AF2ERKI pituitary [45]. These results indicate that the AF-1 functionality may be different between the uterine tissue and the pituitary. Furthermore, we were able to show that ER activation by Tam treatment to the male AF2ERKI was sufficient to partially rescue male fertility [48]. Thus the AF2ERKI mouse is a useful tool in determining the differential roles of AF-1 and AF-2 of ER α in the estrogen target tissues and hormone responsiveness.

6. Conclusions

Multiple mouse models with different mutations to the ERs have allowed great advances in deciphering the role of estrogen receptor in hormonal physiology as summarized in Table 1 (female) and Table 2 (male). Generation of ERKO mice demonstrated that loss of function through ER gene mutations is not lethal and phenotypes of these lines have given insight into the roles of the individual estrogen receptors. In particular, we now have advanced knowledge of the role and function of each estrogen receptor in female reproduction and other physiological processes which has led to therapeutic advances in estrogen receptor related diseases.

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Table 1

Phenotype summary of female estrogen receptor mutant mice.

	αERKO	AF2ER	βERKO	αβERKO	Ute^{ptc}αERKO
Reproductive tract development	Normal	Normal	Normal	Normal	Normal
Fertility	Infertile	Infertile	Subfertile	Infertile	Infertile
Uterine morphology	Hypoplastic	Hypoplastic	Normal	Hypoplastic	Normal
Uterine estrogen responsiveness	Absent	Absent	Present	Absent	Partially absent
Ovarian steroidogenesis	Elevated	Elevated	Reduced	Elevated	Normal
Ovarian folliculogenesis	Anovulatory	Anovulatory	Partially disrupted	Anovulatory	Normal
Mammary gland	Disrupted	Disrupted	Normal	Disrupted	Normal
Neuroendocrine	Disrupted	Disrupted	Normal	Disrupted	Normal

Table 2

Phenotype summary of male estrogen receptor mutant mice.

	αERKO	AF2ER	βERKO	αβERKO	UteptidαERKO
Reproductive tract development	Normal	Normal	Normal	Normal	Normal
Fertility	Infertile	Infertile	Fertile	Infertile	Fertile
Testicular morphology	Dysmorphogenic	Dysmorphogenic	Normal	Dysmorphogenic	Normal
Sperm motility	Reduced	Reduced	(Fertile)	Reduced	(Fertile)