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Membrane estrogen receptor 1 is required for normal reproduction in male and female mice

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Abstract

Steroid hormones, acting through their cognate nuclear receptors, are critical for many reproductive and non-reproductive functions. Over the past two decades, it has become increasingly clear that in addition to cytoplasmic/nuclear steroid receptors that alter gene transcription when liganded, a small fraction of cellular steroid receptors are localized to the cell membranes, where they mediate rapid steroid hormone effects. 17β-Estradiol (E2), a key steroid hormone for both male and female reproduction, acts predominately through its main receptor, estrogen receptor 1 (ESR1). Most ESR1 is nuclear; however, 5-10% of ESR1 is localized to the cell membrane after being palmitoylated at cysteine 451 in mice. This review discusses reproductive phenotypes of a newly-developed mouse model with a C451A point mutation that precludes membrane targeting of ESR1. This transgenic mouse, termed the nuclear-only ESR1 (NOER) mouse, shows extensive male and female reproductive abnormalities and infertility despite normally functional nuclear ESR1 (nESR1). These results provide the first *in vivo* evidence that membrane-initiated E2/ESR1 signaling is required for normal male and female reproductive functions and fertility. Signaling mechanisms for membrane ESR1 (mESR1), as well as how mESR1 works with nESR1 to mediate estrogen effects, are still being established. We discuss some possible mechanisms by which mESR1 might facilitate nESR1 signaling, as well as the emerging evidence that mESR1 might be a major mediator of epigenetic effects of estrogens, which are potentially linked to various adult-onset pathologies.

Keywords: Efferent Ductules, 17β-Estradiol, Spermatogenesis, Testis, Uterus

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Abbreviations

DES, diethylstilbestrol; **EGF**, epidermal growth factor; **ER**, estrogen receptor; **ESR1**, estrogen receptor 1; **ESR2,** estrogen receptor 2; **E2**, 17β-estradiol; ***Esr*1KO**, estrogen receptor 1 knockout; **ERK**, extracellular signal-regulated kinase; **GPER**, G protein-coupled estrogen receptor; **IGF-1**, insulin-like growth factor 1; **mESR1**, membrane ESR1; **MAPK**, mitogen-activated protein kinase; **nESR1**, nuclear ESR1; **NOER**, nuclear-only estrogen receptor 1; **PI3K**, phosphoinositide 3-kinase; **T**, testosterone; **WT**, wild-type

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1. Introduction

Estrogen plays critical roles in male and female reproductive and non-reproductive tissues. This review describes general steroid hormone signaling, and the receptors mediating their effects. Development of various transgenic mouse models has advanced our understanding of signaling by estrogen and other steroid hormones. Here we focus on a new and unique transgenic mouse that for the first time allows us to determine *in vivo* the effects of the loss of the membrane form of the main estrogen receptor, estrogen receptor 1 (ESR1). Insights that this new model system provides into estrogen signaling in reproduction are discussed here, along with potential mechanisms through which these receptors may act.

2. Steroid Hormone Receptor Signaling

The large family of steroid hormone receptors (SHRs) is believed to have arisen from multiple duplications of a common ancestral gene[1] and is evolutionarily conserved in all mammals. It includes receptors for androgens, estrogens, progestins, glucocorticoids, mineralocorticoids, thyroid hormones, vitamin D, and retinoic acid (Table 1). A number of mechanisms of action for SHRs have been described, of which the best understood is nuclear SHR signaling, where hormone-receptor complexes function as nuclear transcription factors[[2]; reviewed in[3]].

Two other mechanisms are extra-nuclear and are commonly referred to as non-genomic or membrane-initiated pathways [reviewed in[4, 5]]. One mechanism involves membrane signaling by the same molecule that functions as a nuclear receptor inside the cell, but is now bound to the cell membrane. This mode of action will be a major focus of this review. The other mechanism utilizes a membrane-associated receptor that is totally unrelated to classic nuclear receptors to transmit membrane-initiated hormone signaling. An example of this final type of receptor is G protein-coupled estrogen receptor 1 (GPER), which is exclusively in the membrane, binds estrogens but also other steroids, and signals through G proteins following ligand binding. The promiscuity of GPER, as well as the lack of male or female reproductive phenotypes in four strains of GPER knockout mice that have been developed [reviewed in[6]], have hindered a definitive elucidation of its role in modulating signaling by estrogens and other steroids. Finally, in addition to genomic and non-genomic pathways through membrane receptors, estrogens and other steroids signal through SHRs present in mitochondria. Understanding of the last three modes of steroid hormone action remains limited compared to nuclear steroid hormone signaling but is an area of active investigation [reviewed in[7-9]].

Since the time of their discovery, it has been proposed that rapid effects produced by steroid hormones could best be explained by extra-nuclear actions, and indeed literature describing effects of steroid hormones too rapid to be mediated by the classical nuclear pathway dates back more than 70 years[10, 11]. However, once SHRs were isolated, the genomic mechanism of signaling quickly came to dominate both the experimentation and thinking in the field. Rapid effects of steroid hormones were often discounted, due in part to the initially slow progress in identifying receptor molecules responsible for this mode of action as well as the ongoing focus by much of the endocrinology community on nuclear actions. Nonetheless, evidence for rapid actions by several steroids outside of the nucleus continued to accumulate, suggesting that membrane receptors could play important roles in overall steroid hormone responses[12].

Non-genomic effects of membrane receptors include protein-protein interactions at the level of the plasma membrane that include partners like ion channels and G-protein-coupled receptors [reviewed in[13, 14]]. Non-genomic SHR action is often defined as any action that does not change gene expression directly, but instead triggers rapid effects (e.g., activation of signal transduction pathways)[15]. It stands to reason that these effects should be resistant to inhibitors of transcription or protein synthesis, occur in cells lacking nuclei (e.g., erythrocytes) and be inducible by conjugated steroid analogs that cannot permeate the cell membrane[16].

2.1 Classical Genomic Signaling by Steroid Hormone Receptors

The SHRs are clearly the best understood system for ligand-induced transcriptional gene regulation, and this system displays remarkable precision as well as plasticity. Activation of SHRs is initiated by binding of the endogenous steroid molecule, but drugs or environmental chemicals can induce signaling through these receptors,

especially ESR1. The non-liganded latent state of SHRs is maintained through the binding of heat-shock chaperone proteins that preserve the high-affinity conformation of the receptor for hormone interaction while inhibiting nuclear localization and DNA binding. According to the classical model, the signaling cascade is initiated by influx of steroid hormones through the plasma membrane, and formation of complexes with their cognate receptors. Some SHRs (e.g., androgen and glucocorticoid receptors) are located in the cytoplasm, and translocate into the nucleus upon hormone binding. Other SHRs such as ESR1 and estrogen receptor 2 (ESR2) are typically located in the nucleus and bind hormone there. The hormone-receptor complex recognizes and binds conserved DNA hormone response elements in promoter regions of target genes, leading to altered expression of hormone-responsive target genes. Additional mechanisms for SHR regulation of transcription have also been described, and these have been recently reviewed[3].

Nuclear SHRs are essential for typical steroid responses, but many observations gathered in parallel with and even before the nuclear function of SHRs was established demonstrated that some hormone effects were too rapid (sometimes measured in seconds) to be explained by transcriptional effects. These rapid responses also suggested that the hormone is likely binding a protein located in the membrane. Over time, it became apparent that in some cases the unknown protein that a steroid ligand engages in the membrane is the same classical SHR that mediates nuclear signaling. For example, antibodies to classical ESR1 also recognize membrane-bound ESR1 and binding of estrogens to a membrane receptor could be reduced by targeting a nuclear receptor with siRNA (Table 2). Importantly, a DNA vector encoding ESR1 reintroduced into ESR1-null cells restored both mESR1 and nESR1 expression and the rapid responses they mediate [[17]; reviewed in[18]; Table 2]. Many SHRs are now known to be associated with the plasma membrane, and their specific functions are now being defined in animal models. Interestingly, the existence of the membrane-initiated steroid signaling in plants (Table 1) suggests that this mode of SHR function likely predates genomic effects[19].

Membrane SHRs signal through several mechanisms. The hallmarks of their specialized function are rapid independent responses that do not require nuclear localization/action and directly engage membrane-associated proteins (mostly G-protein based) or ion channels. It is conceivable that rapid signaling mediated exclusively by the membrane receptor pool could be sufficient to cause some physiological effects. For example, a syn-

Table 1. Steroid hormones and their classic receptors implicated in non-genomic signaling in commonly studied model organisms

Model organism (eukaryotes)	Steroid hormone	Receptor	Rapid non-genomic effect (References)
Yeast	Not found	Not found	Reviewed in[83]
C. elegans	dafachronic acids (DAs, e.g., 25(*S*),26-3-keto-4-cholestenoic acid)	DAF-12	Not found. Reviewed in[84]
Drosophila	Ecdysteroids (e.g., ecdysone)	EcR, ecdysteroid (ecdysone) receptor	Reviewed in[85]
Plants	Brassinosteroids (e.g., brassinolide)	Receptor like kinase (RLK) BRI1 (BRASSINOSTEROID INSENSITIVE 1)	Reviewed in [86, 87]
Rodents and Humans	Classical steroids: mineralocorticoids (aldosterone), glucocorticoids (cortisol), androgens (testosterone), estrogens (E2), and progestins (progesterone)	Mineralocorticoid, glucocorticoid, progesterone and androgen receptor; estrogen receptor 1 and 2	Reviewed in[5]
	Thyroid hormones (thyroxine)	Thyroid hormone receptor b1, truncated forms of a1	Reviewed in[5]
	Secosteroids (e.g., 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$]	Vitamin D receptor	Reviewed in[5, 88]

thetic estrogen that binds and signals through mESR1, but does not activate transcription through nESR1, has been developed[20]. This compound has been reported to induce some cellular responses, although it does not induce the classical responses associated with estrogen exposure. Available data indicates that one key role of membrane SHRs is to facilitate the actions of nuclear estrogen receptors, and there are notable instances where membrane-initiated signaling cooperates with the classic nuclear receptors to regulate gene expression[21]. In contrast to classic receptors that frequently act as homodimers, membrane-initiated signaling appears to be more promiscuous and often employs heterodimers composed of different steroid receptors[22,23]. Mouse models specifically developed to examine the membrane functions of steroid hormone receptors have been informative and are described in detail later in this review.

3. Mitochondrial Effects of Steroid Hormones

Increasing evidence over the past few years indicates that steroid hormones such as estrogens may induce effects mediated through estrogen receptors in mitochondria. Extensive work has documented the expression of both ESR1 and ESR2 in mitochondria from both reproductive and non-reproductive organs[9, 24]. Estrogen signaling through ESR1 regulates mitochondrial structure, promotes mitochondrial electron transport chain efficiency and preserves mitochondrial integrity in various tissues[9, 24]. These effects presumably involve direct actions of E2 on mitochondrial ESR1. However, E2 also acts through the classical genomic pathway to stimulate transcription and protein expression of nuclear respiratory factor-1 (NRF-1), a nuclear transcription factor that upregulates mitochondrial transcription factor A (TFAM). The TFAM then acts in mitochondria to increase transcription of mtDNA-encoded genes, mitochondrial biogenesis and oxidative phosphorylation[25, 26]. Furthermore, overexpression of NRF-1 inhibits apoptosis, an event in which mitochondria play a crucial part[27]. Although overall estrogen effects on mitochondrial function appear to involve classical genomic effects through the NRF-1/TFAM pathway, direct mitochondrial estrogen actions are a pathway distinct from either genomic or non-genomic estrogen signaling.

The overall importance of estrogen signaling in mitochondrial activity has been difficult to establish, but new results obtained with a conditional knockout system suggest that it may be required for normal mitochondrial function. Ribas et al.[28] recently developed a conditional knockout mouse lacking ESR1 in skeletal muscle. Female mice lacking only muscle ESR1 had a constellation of structural abnormalities in their skeletal muscle mitochondria, as well as striking metabolic abnormalities. These mice had diminished muscle oxidative metabolism, altered mitochondria morphology, increased production of reactive oxygen species and altered mitochondrial turnover. Furthermore, the conditional knockout females showed increased adiposity and impaired glucose homeostasis characteristic of female or male mice with a global ESR1 knockout[29]. These striking results emphasize the importance of ESR1 for mitochondrial activity and will almost certainly stimulate further studies to better understand the role of direct and indirect actions of estrogen in mitochondrial function.

4. Structure of Steroid Hormones

All steroid ligands have a common chemical structure involving three 6-carbon fused cyclohexane rings and one 5-carbon cyclopentane ring. This basic structure is shared by all hormonally active steroids in higher vertebrates, but these structures also occur widely in species ranging all the way to insects and plants (e.g., ecdysone and the brassinosteroids)[30]. Therefore, it is not entirely surprising that their receptors developed a conserved structure that is commonly divided into distinctive domains.

The most variable part of the SHR is its amino terminus that serves as a binding site for recruited transcriptional co-regulators. The centrally located zinc finger DNA binding domain contains two highly conserved structures, with four cysteine residues coordinating zinc atoms. The hinge region that contains the nuclear localization signal is followed by the carboxy terminus that harbors the hormone-binding domain and controls dimerization properties[3]. As mentioned earlier, for some hormones the same receptor molecule can mediate nuclear and membrane signaling. Unique membrane isoforms (e.g., formed by alternative splicing, promoter use

or translational site) have been reported, especially for ESR1, where ESR1 variants may have important roles in transformed cancer cells but also occur in normal reproductive tissue. While these alternatively spliced products could contribute to non-genomic signaling, their roles remain enigmatic[31-33].

5. Membrane Localization of Steroid and Thyroid Hormone Receptors

One of the key advances in the membrane SHR field was establishment of how SHRs are directed to the cell membrane. Receptors for sex steroids, vitamin D receptor and truncated thyroid hormone receptor are guided to the membrane through post-translational modification of SHRs by palmitoylation, a covalent attachment of a hydrophobic palmitate group onto a cysteine (Cys) moiety in a highly conserved, nine-amino-acid palmitoylation motif (Table 2). A small fraction (approximately 5-10%) of the ESR1 protein is post-translationally modified by addition of palmitic acid to cysteine 451 in mice or cysteine 447 in humans[34].

Receptor palmitoylation increases its hydrophobicity and membrane affinity, while receptor dimerization promoted by ligand binding is required for both nuclear and membrane receptor activity. It appears that this process is important in creating uneven size pools of separate receptor compartments. Upon ligand binding, receptors rapidly undergo dimerization that limits the number of molecules available for palmitoylation and membrane allocation. This assures that most of the receptors are destined to reach the nucleus to function in classical genomic signaling[34].

The mechanism of the palmitoylation reaction has been studied in detail for SHRs. It has been demonstrated that oligomerized heat shock protein 27 (HSP27) associates with the cytoplasmic receptor, and maintains the structure of the palmitoylation motif in an open position. The cysteine residue in the core motif of the receptor becomes palmitoylated by palmitoyl acyltransferases (PATs) such as zinc-finger DHHC domain-containing 7 and 21[35]. Palmitoylated receptor associates with caveolin-1, a main component of caveolae that concentrate steroid hormones that enter the cells[36]. This spatial proximity potentiates interactions between hormones and receptor pools. Mutations in the membrane-localization signal of caveolin-1 inhibit membrane trafficking of SHRs, underscoring a key role for caveolin-1 in mediating this interaction[36].

5.1 Membrane Signaling by Steroid Hormones

Membrane-initiated signaling relies on steroid interactions with membrane-associated receptors to promote rapid signaling and does not require nuclear receptor. Within seconds, the steroid ligands activate a rapid response through generation of secondary messengers such as cAMP and cGMP and calcium influx into the cells. This rapid signaling normally involves interactions between the liganded membrane SHR and the signalosome, a multi-protein complex that incorporates a G-protein and receptor kinases such as SRC, PIK3 or AKT. In contrast, the intermediate duration signaling that is often found following steroid hormone signaling involves growth factor receptors and their ligands, most notably insulin-like growth factor 1 (IGF1) and epidermal growth factor (EGF) and their respective receptors [reviewed in[37];[38]]. Proliferative and metastatic effects of steroid hormone signaling in cancer are thought to require cooperation between growth factor receptors and membrane SHRs to activate extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K)–AKT signaling[39].

5.2 Loss of Membrane ESR1 Impairs Female Reproduction

Despite decades of literature documenting membrane receptors and their actions, it was unclear what role these receptors play in overall steroid signaling or if their absence would lead to significant or even demonstrable changes in target organs. To investigate mESR1 function *in* vivo, two independent laboratories developed knock-in mice where cysteine 451 was replaced by alanine in the mouse ESR1 protein (Table 2). Since alanine cannot be palmitoylated, this prevents membrane translocation of ESR1 and results in a mouse that has functional nESR1 but lacks mESR1[40, 41]. Pedram et al.[40] described a complete loss of mESR1 expression in hepatocytes and uterine and mammary epithelium in homozygous NOER mice. In contrast, using the same cysteine-alanine substitution at position 451, Adlanmerini et al.[41] reported a reduction of

approximately 55% in mESR1 in hepatocytes but did not quantitate mESR1 in uterine or mammary epithelium.

Although both studies reported a crucial role of mESR1 in E2/ESR1 signaling, there were differences in observed phenotypes of these two knock-in mice. Uteri from intact homozygous NOER mice developed by Pedram et al.[40] were hypoplastic, and had a thinner luminal epithelium compared to wild-type(WT) and heterozygous NOER females. This reduced epithelial thickness was not increased by E2 treatment of ovariectomized NOER mice, indicating that impaired uterine E2 responses resulted from loss of mESR1. In contrast, Adlanmerini et al.[41] reported normal uterine E2 responses in their mice, possibly resulting from residual mESR1 expression in uterine cells of these transgenic mice.

Both groups, however, reported that homozygous NOER female mice were infertile, despite normal fertility in heterozygous NOER females. Furthermore, NOER females were acyclic as revealed in vaginal smears and failed to ovulate as indicated by an absence of corpora lutea and hemorrhagic cysts. In contrast, heterozygous female NOER mice exhibited follicular development comparable to WT mice, consistent with their normal fertility[40].

Some reproductive hormone concentrations were also altered in NOER mice and thus appear to be regulated by mESR1. Serum E2 concentrations were increased in homozygous NOER females due to alterations in the hypothalamic-pituitary-gonadal axis, and high endogenous E2 concentrations failed to suppress LH secretion, similar to E2-treated ovariectomized NOER females. However, concentrations of other hormones, such as FSH and IGF-1, were comparable in NOER and WT mice. Mammary gland development was also impaired in homozygous NOER females, as shown by reduced mammary ductal branching and blunted duct termini. However, the mammary phenotype of heterozygous NOER mice was unaffected[40].

5.3 Loss of Membrane ESR1 Leads to Male Reproductive/Fertility Abnormalities

The infertility and reproductive abnormalities in NOER females raised the possibility that loss of mESR1 would lead to reproductive and other effects in males as well. Recently, our group reported that loss of mESR1 caused extensive abnormalities in the male reproductive tract that were similar to those in *Esr1*KO males, which have complete loss of ESR1[42]. In *Esr1*KO males, efferent duct epithelial dysfunction leads to fluid accumulation in the rete testes and seminiferous tubules, which causes backpressure atrophy and degeneration of seminiferous tubular epithelium and infertility[43]. Efferent ducts from 4-month-old NOER mice showed similar signs of epithelial dysfunction as indicated by luminal dilation and epithelial atrophy due to fluid accumulation[42].

The NOER males showed age-related progressive changes in the testes that were also similar to Esr1KO mice. At 4 months of age, NOER testes had an enlarged rete testis and a high number of abnormal seminiferous tubules with atrophic and degenerative epithelium; some of the tubules showed increased luminal and tubular diameter. By 8 months of age, these changes were more severe than in 4-month-old mice. The morphological abnormalities were accompanied by functional deficits as

Table 2. **Key milestones in establishment of the critical effects of mESR1 in reproductive function**

Year	Description	References
1942	Rapid effects of glucocorticoids demonstrated	10
1960's, 1970's	Rapid effects of estrogen and estrogen binding in the cell membrane reported	12, 58, 59
1999	Antibody against ESR1 shows cell membrane staining, and transfection of plasmid for *Esr1* into cells lacking ESR1 induces expression of both mESR1 and nESR1, indicating that these are the same molecule	17
2007	Blocking palmitoylation of ESR1 prevents its membrane localization in a cell line, allowing *in vitro* studies of mESR1 actions and emphasizing the importance of palmitoylation for mESR1 function	34
2014	Development of transgenic knock-in NOER mice lacking membrane ESR1 shows that mESR1 is essential for female fertility and normal estrogen responses	40, 41
2016	Male NOER mice lacking mESR1 show progressive infertility and reproductive tract abnormalities, indicating that mESR1 is essential for male reproductive tract function	42

well, and daily sperm production was decreased by 62% in NOER males compared to WT controls[42].

Global loss of ESR1 in male mice results in hypo-osmolar alkaline conditions in the lumen of the epididymis, which contributes to abnormal sperm maturation, as indicated by increased morphological abnormalities and loss of sperm motility[44, 45]. Critically, germ cells from *Esr1*KO male mice are capable of forming normal fertilization-competent sperm when transplanted into testes of WT mice[46]. This suggests that infertility and pathological changes in *Esr1*KO mice are due to dysfunction of somatic cells of either the testes or male excurrent ducts[46]. Similar to *Esr1*KO mice, NOER male sperm from the caudal epididymis had a high incidence (>95%) of morphological defects[45]. These abnormalities were not present at spermiation, but were seen when sperm reached the rete testes[6]. Thus, these abnormalities arise when in lumen of seminiferous tubules and excurrent ducts and may be due to an abnormal luminal milieu, as seen in *Esr1*KO mice[45]. In addition to their structural defects, NOER sperm had decreased motility, again consistent with changes in *Esr1*KO mice reported two decades ago[47]. Young post-pubertal NOER males around 2 months of age were fertile, although the size of the litters they sired was reduced. However, by 3.5 to 4 months of age, all NOER males became completely infertile[42].

Efferent ductules and epididymis express ESR1 and are believed to be the main targets of E2/ESR1 signaling in male reproductive system[47-49]. However, it is still unclear how lack of E2/ESR1 signaling affects male reproduction[47]. Aromatase knockout male mice[47, 50] lacking endogenous estrogens do not exhibit the severe phenotype seen in *Esr1*KO mice. In addition, anti-estrogen treatments of WT male mice[47, 51] do not fully replicate the *Esr1*KO phenotype. Loss of ESR1 results in down-regulation of proteins involved in water absorption [e.g., aquaporin 1 and 9 (AQP1 and AQP9)] and acid-base balance [e.g., sodium/hydrogen exchanger 3 (NHE3; SLC9A3) and carbonic anhydrase II (CAR2)] in efferent ducts and epididymis[44, 45, 52]. Similarly, NOER males also show decreases in mRNA expression for *Slc9a3 and Aqp1* in the epididymis[53]. These results indicate that similar molecular mechanisms may account for the reproductive abnormalities in *Esr1*KO and NOER males.

Previous results have shown that *Esr1*KO mice have seminal vesicle hypertrophy[42] and increased serum testosterone (T) due to enhanced Leydig cell T production capacity[54]. Similarly, NOER mice showed increased seminal vesicle weight and higher serum T levels[42], again emphasizing the similar reproductive phenotype of *Esr1*KO and NOER mice.

These results indicating that loss of mESR1 in the NOER mouse caused progressive infertility, histopathological changes in testes, efferent ducts and epididymis, and defective sperm maturation[42] are all similar to *Esr1*KO mice[44, 45]. Thus, mESR1 is essential for normal male reproductive tract development and function, and fertility[42].

6. Role of mESR1 in Facilitating Normal Estrogen Signaling Through Nuclear ESR1

Over the past 60 years, mechanistic research in E2/ESR1 signaling emphasized genomic E2 signaling through the canonical nuclear estrogen receptor pathway, which is essential for major reproductive effects of E2[55-57]. This involves E2 binding to nuclear ESR1, which is then phosphorylated and dimerizes, leading to translocation of ligand-receptor complexes to DNA, where they bind estrogen-response elements (EREs) in target genes. This causes co-activator recruitment and ultimately transcription of target genes, resulting in cell proliferation, secretory protein production and other processes.

Estrogen signaling through mESR1 activates PI3K/AKT and mitogen-activated protein kinase/ERK (MAPK/ERK) pathways (Figure 1) which induces release of EGF receptor ligands, and increases intracellular cAMP and Ca^{++}[12, 40, 41, 58-60]. Previous literature indicates that mESR1 signaling through these pathways may facilitate nESR1 signaling (Figure 1), but the exact mechanism of this effect is unknown. Phosphorylation of nESR1 is critical for early E2 responses[57, 61, 62]. The main amino acid phosphorylated is Ser^{118} in the A/B region of ESR1; this and surrounding amino acids are highly conserved among various species[63]. Most importantly, if another amino acid is substituted for Ser^{118}, ESR1 cannot be normally phosphorylated, which severely reduces transcriptional activity of liganded ESR1[63]. It is likely that the mESR1-mediated MAPK/AKT activation following estrogen treatment that phosphorylates and acti-

vates nESR1 (Figure 1) may be a critical aspect of how mESR1 facilitates full E2 responses.

Transcriptional activation by liganded nESR1 depends on various co-activator molecules. The most important are the p160 family, which includes SRC-1, SRC-2 and SRC-3[64, 65]. For both SRC-2[64, 65] and SRC-3[66], a crucial step in their recruitment and activation is phosphorylation, which allows them to bind to the liganded nESR1/ERE complex in target genes and facilitate transcription. This phosphorylation appears to be MAPK-dependent and, again, may be induced through mESR1 (Figure 1). In the absence of mESR1 in NOER mice, altered/deficient co-activator recruitment may impair normal interaction of liganded nESR1 with EREs in target genes.

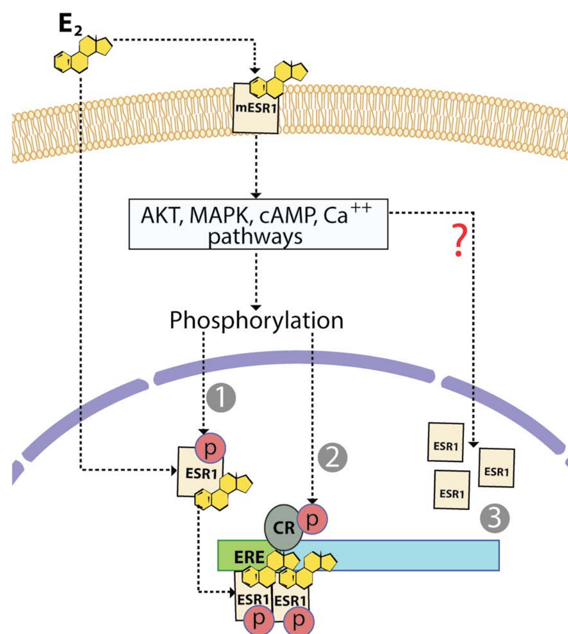

Figure 1. Potential mechanisms for interactions between membrane-initiated and nuclear ESR1 signaling. Binding of E2 or other estrogens to mESR1 activates protein kinases [protein kinase B (AKT) and mitogen-activated protein kinase (MAPK)] and second messengers [cyclic adenosine monophosphate (cAMP) and calcium (Ca^{++})] shortly after E2 stimulation. 1. Activated protein kinases (AKT and/or MAPK) may phosphorylate nESR1, one of the critical early E2 responses which is required for efficient nESR1 transcriptional activity. 2. Protein kinases also activate co-regulators by phosphorylation. Phosphorylated co-activators bind to E2/ESR1 transcription initiation complexes and regulate ESR1 transcription activity. 3. Lack of palmitoylation of ESR1 prevents membrane-initiated E2/ESR1 signaling, and this has been shown to decrease nESR1 protein abundance after E2 stimulation and potentially affect the overall E2 response, although the mechanism of this effect is currently unknown.

One classic E2 effect on cells after stimulation is a decrease in ESR1 protein, which results from increased degradation of nESR1 in response to E2 binding rather than down-regulation of ESR1 synthesis[67]. Critically, in MCF-7 cells *in vitro*, pharmacological inhibition of localization of mESR1 to the membrane resulted in increased degradation of ESR1 following E2 stimulation, although it did not affect basal ESR1 levels[67]. The lack of mESR1 resulted in 70% decreases in nESR1 in cells lacking mESR1 compared to normal cells following E2 treatment. This nESR1 reduction could attenuate E2 responses in NOER mouse. Thus, both membrane and nuclear fractions of ESR1 are critical for full E2/ESR1 functions *in vivo*, but the details of how these receptors interact remain to be determined.

6.1 Role of mESR1 in Mediating Epigenetic Effects Induced by Estrogens

In recent decades, there has been intense interest in the role that epigenetic changes induced by perinatal exposures to estrogens may play in disease susceptibility in adults. Epigenetic changes heritably alter gene functions without causing any changes in nucleotide sequences of the genomic DNA. Although epigenetic effects induced by the potent synthetic estrogen diethylstilbestrol (DES) were first reported 20 years ago[68], the mechanisms of these epigenetic effects are still not completely understood. Early DES exposure has a wide variety of effects on targets such as uterus[69], where it stimulates target gene activity, producing short-term changes in uterine epithelial proliferation, secretory protein production and other effects. In addition, early exposure to DES or other environmental estrogens induces changes in methylation of target genes and histone methylation marks in target genes[70], both of which can result in long-term alterations of target gene expression that increase susceptibility to or exacerbate adult pathologies.

Extensive evidence indicates mESR1 may be involved in epigenetic changes arising from early estrogen exposure, but the exact role of mESR1 in mediating epigenetic

effects is unclear (Figure 2). Membrane estrogen receptors activate PI3K and MAPK pathways, and also increase intracellular cAMP and Ca^{++}[12, 40, 41, 58, 59]. In the presence of E2, mESR1 interacts with the p85α regulatory subunit of PI3K, leading to activation of AKT[71]. DES or other estrogens can act through mESR1 to increase signaling through the PI3K/AKT pathway in neonatal rodent uteri[39, 72, 73]. Increased PI3K/AKT signaling then alters histone methylation by regulating the activity of various histone methyltransferases such as polycomb repressive complex 2 (PRC2) enzyme complex and the MLL/complex of proteins associated with Set I (COMPASS) complexes (Figure 2). The PRC2 has major effects on gene function by silencing gene activity through the addition of up to three methyl groups at lysine-27 of histone H3 (H3K27) to form trimethylated histone H3 (H3K27me3), a well-known repressive histone mark.

Activity of PRC2 is regulated primarily by expression of enhancer of Zeste homolog 2 (EZH2), the catalytic subunit of the PRC2 complex that provides methyltransferase activity (Figure 2). Estrogen effects mediated through EZH2/H3K27me3 appear to be the main mechanism of its epigenetic effects[39, 72, 73]. In addition, EZH2 regulation of H3K27me3 is involved in breast, prostate and endometrial cancer, as well as non-reproductive cancers and Weaver syndrome[74-77]. In response to mESR1 signaling, activated AKT phosphorylates EZH2 and inactivates it, causing reduced H3K27me3 levels (Figure 2). Since H3K27me3 is a repressive mark, this reduction may lead to hyper-responsiveness to estrogen in adulthood[72], resulting in increased tumorigenesis and other reproductive diseases such as leiomyoma in adult rodents, and potentially in women, after early estrogen exposure[75-77].

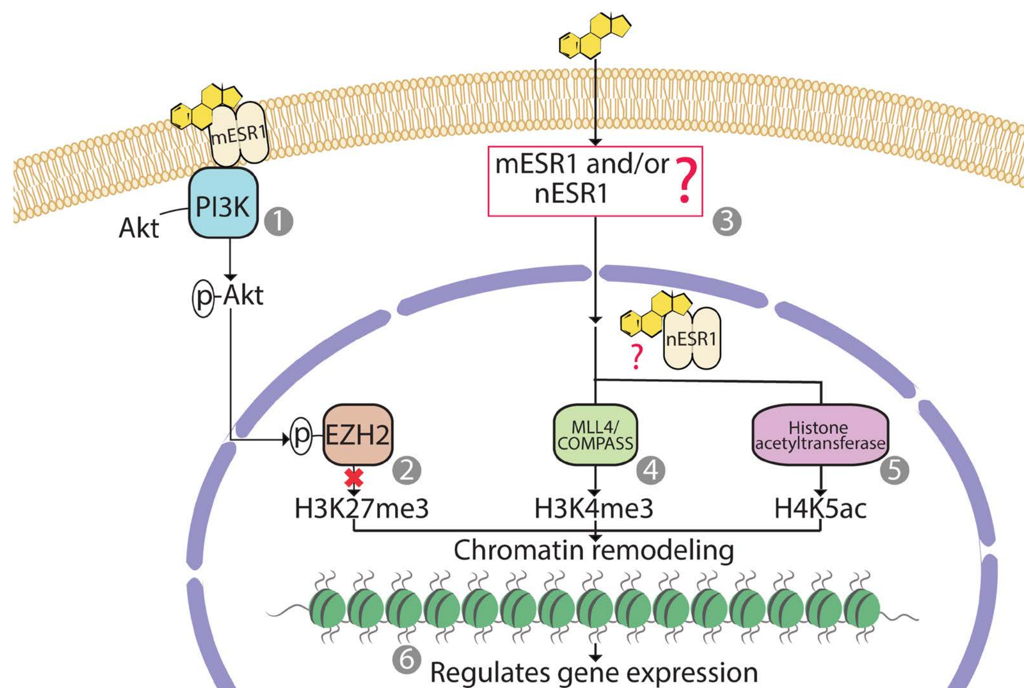

Figure 2. Potential mechanisms by which estrogens induce epigenetic effects. 1. Estrogen signaling through mESR1 activates PI3K, which phosphorylates (activates) AKT. 2. This leads to EZH2 phosphorylation (inactivation), which reduces H3K27me3, a repressive histone mark. 3. Early estrogen exposure and mESR1 signaling also leads to activation of the MLL4/COMPASS methyltransferase, which trimethlylates histone 3 at lysine 4 (H3k4me3) to activate transcription. 4, 5. Estrogen induces acetylation of histone 4 (H4K5ac) through unknown mechanisms that may also involve mESR1 as well as nESR1. 6. Although estrogen has differing effects on various histone marks, changes in all these lead to altered gene expression associated with pathological changes.

The mixed-lineage leukemia (MLL) protein is a subunit of the COMPASS complexes that trimethylate histone 3 at the lysine 4 residue (H3K4me3). This H3K4me3 mark is considered an active histone mark (Figure 2). Thus, H3K4me3 mark is a positive regulator of gene transcription, in contrast to the repressive H3K27me3 mark. Recent evidence suggests that estrogens can regulate MLL and alter the H3K27me3 mark in prostate and cancer cell lines[78]. Rearrangements in *Mll* are associated with leukemias, but MLL is also expressed in uterus. Previous work has shown that estrogen alters MLL4 expression[79], and plays critical roles in estrogen-mediated regulation of HOX genes in breast cancer cells[80-82].

Histone acetylation is another epigenetic modification that, along with histone methylation, is critically involved in gene regulation (Figure 2). A recent study has shown that neonatal DES administration alters uterine H4K5ac levels at various uterine target genes[79]. In general, H4K5ac is associated with active chromatin and transcription. These results indicate that by altering various histone methytransferases and acetylases activity during critical windows of development estrogens cause aberrant long-term expression in various estrogen target genes by epigenetic modifications. These epigenetic changes can predispose individuals to a variety of adult-onset pathologies, and thus early estrogen-induced epigenetic effects have significant clinical importance. Current evidence strongly suggests that mESR1 may have a critical role in epigenetic effects of estrogens, and determining its role in this effect will be a critical focus of future work in this area.

7. Summary and Conclusions

In summary, steroid hormones are ancient and critical regulators of homeostasis and reproduction. Work during the past half-a-century has elucidated many aspects of nuclear steroid signaling, but also has suggested that steroid signaling through membrane receptors could be important. Recent development of NOER mice provides the first experimental system in which the membrane fraction of SHR can be eliminated, and presents unique opportunities to establish roles of membrane SHRs in general and mESR1 in particular. Work with NOER mouse has indicated that mESR1 plays critical roles in male and female reproduction, but our understanding of the role of mERS1 in estrogen signaling remains rudimentary. Future work in this area should elucidate more details of mESR1 signaling, and establish how mESR1 and nESR1 work together to mediate estrogen actions. This promises to be an active area of investigation for several years to come.

8. References

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