

Membrane-Localized Estrogen Receptor α Is Required for Normal Organ Development and Function

Ali Pedram,¹ Mahnaz Razandi,² Michael Lewis,³ Stephen Hammes,⁴ and Ellis R. Levin^{1,2,*}

¹Division of Endocrinology, Department of Medicine, University of California, Irvine, Irvine, CA 92717, USA

²Department of Veterans Affairs Medical Center, Long Beach, Long Beach, CA 90822, USA

³Department of Cellular Biology, Baylor College of Medicine, Houston, TX 77030, USA

⁴Department of Medicine, University of Rochester, Rochester, NY 14642, USA

*Correspondence: ellis.levin@va.gov

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SUMMARY

Steroid receptors are found in discrete cellular locations, but it is unknown whether extranuclear pools are necessary for normal organ development. To assess this, we developed a point mutant estrogen receptor α (ER α) knockin mouse (C451A) that precludes palmitoylation and membrane trafficking of the steroid receptor in all organs. Homozygous knockin female mice (nuclear-only ERα [NOER]) show loss of rapid signaling that occurs from membrane ERα in wild-type mice. Multiple developmental abnormalities were found, including infertility, relatively hypoplastic uteri, abnormal ovaries, stunted mammary gland ductal development, and abnormal pituitary hormone regulation in NOER mice. These abnormalities were rescued in heterozygous NOER mice that were comparable to wild-type mice. mRNAs implicated in organ development were often poorly stimulated by estrogen only in homozygous NOER mice. We conclude that many organs require membrane ERa and resulting signal transduction to collaborate with nuclear ERa for normal development and function.

INTRODUCTION

Steroid receptors are found in discrete cellular locations in most organs. In female mammals, genetic deletion of estrogen receptor α (ER α) produces phenotypes of abnormal development in multiple organs (Lubahn et al., 1993). Deletion of only the nuclear ER α pool simulates the total ER α knockout (KO) mouse (Pedram et al., 2009), indicating the importance of nuclear ER α for normal organ development and function. However, it is unknown as to whether extranuclear receptor pools are also required. Palmitoylation of ER α at cysteine 447 (human)/451 (mouse) is required for trafficking of the endogenous receptor to the plasma membrane (Acconcia et al., 2005; Pedram et al., 2007), and acylation is also required for membrane localization of progesterone and androgen receptors (Pedram et al., 2007, 2012). At the membrane, ER α acts as a G protein-coupled receptor, enacting multiple signal transduction pathways that impact genomic and nongenomic functions (Kumar et al., 2007). To assess importance, we developed a point mutant ER α knockin (KI) mouse (C451A) that precludes steroid receptor palmitoylation and membrane trafficking in all organs. Homozygous KI female mice (nuclear-only ER α [NOER]) show loss of rapid signaling that occurs from membrane ER α in wild-type (WT) mice. Multiple developmental abnormalities were seen in homozygous NOER mice, and these abnormalities were rescued in heterozygous NOER mice that were comparable to WT mice. Multiple mRNAs implicated in organ development were poorly stimulated by estrogen (E2) only in homozygous NOER mice. We conclude that many organs require membrane ER α and resulting signal transduction to collaborate with nuclear ER α for normal development and function.

RESULTS

Loss of Membrane ER Signaling Impacts Transcription

NOER KI mice that lack ERa palmitoylation were generated by inserting a cysteine 451-to-alanine mutation into the esr1 locus in embryonic stem (ES) cells by homologous recombination; the cells were then used to generate mice. Expression of the point mutant ERa transgene was found in all organs assessed (Figure S1A available online). Mammary gland epithelial cells and hepatocytes from intact WT and heterozygous KI mice showed comparable nuclear ERa expression and presence of membrane-localized steroid receptors (Figures 1A and 1B). However, homozygous KI cells showed complete absence of membrane-localized ERa but strong nuclear receptor expression that was comparable to WT mouse cells. The nuclear ER α pool does not undergo palmitoylation (Pedram et al., 2009), and hence, localization was not affected by the mutation (Figure 1B). Because \sim 90% of total ER α protein is in the nucleus, ER protein was similar in cells from all mice (Figure S1B).

Membrane ERs are solely responsible for rapid signal transduction stimulated by 17- β -estradiol (E2) (Pedram et al., 2006, 2013). WT and heterozygous NOER hepatocytes responded to E2 with rapid activation of AKT and extracellular mitogenresponsive protein kinase (ERK) (Figure 1C). In contrast, there was no significant signaling by E2 in homozygous NOER cells, consistent with lack of membrane ER α . We determined pS2 mRNA expression that is regulated by nuclear ER α binding estrogen response elements (EREs) (Won Jeong et al., 2012) and



by membrane ER signaling through ERK and phosphatidylinositol 3-kinase (PI3K) (Björnström and Sjöberg, 2005; Madak-Erdogan et al., 2008; La Rosa et al., 2012). E2 stimulated comparably increased pS2 mRNA in WT and heterozygous NOER hepatocytes that depended upon ERK and AKT (Figure 1D). However, pS2 mRNA response to E2 was substantially less in homozygous NOER mouse hepatocytes, as was activation of an expressed ERE×3/luciferase reporter (Figure 1E). After cell exposure to E2, nuclear ER α occupancy at an ERE within the pS2 gene promoter was robust in WT and heterozygous NOER hepatocytes but significantly less so in homozygous NOER cells (Figure 1F).

To determine whether decreased nuclear ERa recruitment to the pS2 promoter resulted from loss of signaling by membrane ER α , we expressed the E domain of ER α exclusively targeted to the plasma membrane in homozygous NOER hepatocytes. This portion of ERa is sufficient for numerous signaling pathways (Pedram et al., 2009), and we used this construct to derive the membrane ERa-only (MOER) mouse (Pedram et al., 2009, 2013). pEmem (E domain of ER α targeted exclusively to the plasma membrane) enhanced cyan fluorescent protein (ECFP)transfected homozygous NOER hepatocytes showed membrane localization from the expressed ERa E domain, E2 activation of ERK and PI3K-AKT, and restored E2 stimulation of pS2 mRNA that was ERK dependent (Figures S1C-S1E). Importantly, restoring E2 signaling from the membrane resulted in recruitment of nuclear ER α to the pS2 promoter ERE (Figure 1G). Thus, the C451A homozygous mutation prevents the collaboration between membrane and nuclear receptor pools from kinase activation by the membrane receptor. From Scatchard analysis of competition binding (Figure 1H), association, and dissociation studies (Figure S1F), labeled E2 binding to nuclear ER in WT and homozygous NOER hepatocytes was very similar.

Reproductive Tract Phenotypes in the Homozygous NOER Mice

The mating of proven breeder WT males with homozygous NOER female mice produced no pregnancies (Table S1). Breeding homozygous NOER male and female NOER mice was similarly not fruitful. This contrasted to matings between heterozygous male and female NOER mice (WT/KI) that were comparably successful to matings between WT male and female mice (12–18 weeks). Thus, heterozygous expression of membrane ER α rescues the infertility phenotype of homozygous female NOER mice.

From vaginal smears, homozygous NOER female mice fail to ovulate, whereas heterozygous mice show epithelial cell cornification, indicating estrous cycling. To understand the basis for infertility, we determined that uteri from intact homozygous NOER mice were relatively hypoplastic compared to WT and heterozygous NOER mouse uteri that were comparable (n = 5 each) (Figure 2A). Representative uterine tissue sections from the three types of mice showed that the luminal epithelium in particular was significantly thinner in the intact homozygous NOER females compared to WT and heterozygous NOER mice that were comparable (Figure 2B). This correlated to the complete loss of membrane ER α in isolated endometrial epithelial cells only from the homozygous NOER mice were ovariectomized, and the responses to E2 for 21 days were determined (Figure 2C).

E2 replacement was from E2 pellets inserted under the skin, producing physiological levels of this sex steroid in mouse serum (Pedram et al., 2008). Homozygous NOER KI mice uterine epithelium showed little effects from ovariectomy and insignificant epithelial response to E2 (Figure 2C), despite abundant nuclear ER α (Figure 1B). In contrast, ovariectomy caused a marked loss of epithelial thickness in both WT and heterozygous NOER mice that was restored from E2 exposure (Figure 2C).

Ovariectomized WT and homozygous NOER mice treated by E2 pellet for 12 days were further compared. Three representative homozygous KI mice uteri are shown to have little proliferative response to E2 and no significant change in uterine weight (Figure S2B). This is in contrast to the significant responses seen in WT mice. We also found decreased Ki67 staining in luminal epithelium of the homozygous NOER mice at 12 days' exposure to the steroid, compared to WT mice (Figure S2C). These results indicate that nuclear ER α alone is not sufficient to maintain normal uterine epithelial proliferation and development.

We assessed mRNAs recognized as both important for uterine development and that are regulated by ERa (Grümmer et al., 1999; Fleming et al., 2006; Teng, 1999). Expression of connexin 43, oxytocin receptor, lactoferrin, and insulin-like growth factor-1 (IGF-1) mRNAs was comparable in uteri from intact WT and heterozygous NOER mice, but all except IGF-1 were significantly reduced in intact homozygous NOER mice (Figure 2D) despite high-serum E2 levels (Table 1). The IGF-1 results from intact mice suggest that nuclear ERa is sufficient for regulation of this mRNA in the endometrium. Ovariectomized WT mice responded to E2 pellets for 12 days with increases of all uterine mRNAs (Figure S2D). However, in homozygous NOER mice, E2 failed to significantly stimulate these mRNAs except for connexin 43. Thus, loss of membrane ERα compromises E2-induced expression of some important developmental mRNA(s) in the uterus. The reduced but present epithelial development in homozygous NOER mice probably reflects signaling by the IGF-1 receptor to transactivate nuclear ERa in an E2-independent fashion (Winuthayanon et al., 2010). Nevertheless, growth receptor crosstalk to nuclear ERa is inadequate to fully compensate for the loss of membrane ERa function.

Abnormalities of the ovary in homozygous NOER mice included large hemorrhagic cystic (HC) areas and small, malformed ovaries (Figure 2E). The ovaries lack corpora lutea (CLs) that indicate anovulation, consistent with the vaginal smears and a basis for infertility. In contrast, heterozygote NOER and WT mice ovaries show normal development, consistent with their comparable fertility. Expression of the progesterone receptor (PR), transforming growth factor β , and steroidogenic acute regulatory protein mRNAs was determined from pooled ovaries and individual mice (n = 5; Figure S3A, bar graph). For each, intact homozygous NOER mice showed significantly diminished mRNAs compared to WT and heterozygous NOER mice.

Hormone Regulation Is Abnormal in Homozygous NOER Mice

We then determined sex steroid concentrations and impact on the hypothalamic-pituitary axis. Serum progesterone was measured without regard to estrous cycle phase because homozygous NOER mice do not cycle. Progesterone in intact WT and



Figure 1. NOER Mouse Characterization

(A) Cellular ERα location by immunofluorescent microscopy of representative WT, heterozygous, and homozygous NOER mice cells. Arrows identify membrane receptors.

(B) ER α protein blots in nuclear and membrane cell fractions. Graph is mean ± SEM from five mice each. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. (C) Representative kinase activity as AKT Ser473 phosphorylation (left) and ERK tyrosine 202/204 phosphorylation (right). Kinase total proteins are controls; graph data are from two individual mice per genotype in each of three experiments combined. *p < 0.05 by ANOVA plus Scheffé's test for control versus E2. heterozygous NOER mice was comparable and ~200% higher than homozygous NOER mice (Table 1). Very low progesterone in the latter reflects lack of ovarian CLs, the source of postovulatory, circulating progesterone (Carr et al., 1981). Serum E2 was ~100% higher in homozygous NOER mice, compared to WT and heterozygous NOER mice that were comparable. Despite significantly higher E2 levels, serum luteinizing hormone (LH) was not suppressed in homozygous NOER mice. This is consistent with impaired negative feedback regulation of the hypothalamic-pituitary-ovarian axis, as reported in ERa KO mice (Couse and Korach, 1999), and indicates a role for membrane ERa action. The higher E2 levels did not stimulate LH secretion because, upon ovariectomy, serum LH was not diminished in the homozygous NOER mice and increased in WT and heterozygous NOER mice. IGF-1, growth hormone (GH), and follicle-stimulating hormone (FSH) concentrations were not significantly different between the mice types, but prolactin was significantly reduced in the intact homozygous KI mice. Upon ovariectomy, serum prolactin significantly decreased only in WT and heterozygous NOER mice. In vitro, E2 signals to ERK activation from undetermined ERs, and ERK stimulates prolactin mRNA levels in cultured rodent pituitary cells (Watters et al., 2000). Our in vivo findings are consistent with a role for membrane ERa signaling to increased prolactin production through ERK.

Restricted Development of the Mammary Gland in Homozygous NOER Mice

Mammary gland development is rudimentary in ERa KO mice, mainly from loss of postpubertal E2 actions in the epithelium (Bocchinfuso and Korach, 1997). Whether membrane ERa signaling is required for glandular development is unknown. The mammary glands of pubertal, virgin WT, and heterozygous NOER mice (~12 weeks old) were fully developed. In contrast, homozygous NOER female mice showed profoundly diminished ductal side branching and formation of blunted duct termini (Figure 3A and insets). Progressive development of postpubertal mammary gland ducts results initially from E2 action, followed by progesterone-induced ductal side branching through its epithelial receptor, PR-B (Lydon et al., 1995; Brisken and O'Malley, 2010). The low-serum progesterone in homozygous NOER mice therefore likely contributes to the abnormal mammary phenotype. ER stimulates PR transcription including in the mammary gland (Kastner et al., 1990; Chauchereau et al., 1992), and membrane ERa signaling is important for PR expression in breast cancer epithelial cells (Pedram et al., 2012). WT and heterozygote NOER mice showed comparable mammary gland PR-B protein (left) and mRNA (right) expression (Figure 3B), markedly diminished in homozygous NOER glands.

How might membrane ER α collaborate with nuclear ER α to upregulate PR expression? Several possibilities may be relevant (Björnström and Sjöberg, 2005), but others and we have recently shown that membrane ER α rapidly signals to the epigenetic regulation of genes in breast cancer cells (Bredfeldt et al., 2010; Pedram et al., 2012). In WT and heterozygous NOER mammary epithelial cells, E2 stimulated AKT-dependent phosphorylation of the histone methylase, EZH2, at an activity inhibitory site, serine 21 (Figure 3C). As a result, the H3K27me3-repressive mark at the PR promoter was significantly reduced. This correlated to E2enhanced PR mRNA expression that was substantially reduced upon inhibition of PI3K-AKT (LY294002), further supporting the importance of rapid signaling from membrane ER α (Figure 3D). In contrast, H3K27 trimethylation was strong, and PR mRNA was not stimulated by E2 in homozygous NOER epithelial cells.

Amphiregulin is an important ligand for the epidermal growth factor receptor (EGFR) to stimulate mammary gland duct and terminal end bud development (Brisken and O'Malley, 2010; Ciarloni et al., 2007). mRNA for this EGFR ligand is induced by ERa, and the protein has been implicated in E2-stimulated mammary epithelial proliferation and ductal elongation (Ciarloni et al., 2007). Amphiregulin, EGFR, and ErbB2 mRNAs in mammary glands from intact, homozygous NOER mice were significantly reduced compared to WT and heterozygous NOER glands (Figure S3A). Additionally, E2 or propyl-pyrazole-triol (PPT; ERa agonist) injection into ovariectomized mice resulted in stimulation of these and PR mRNAs, only in WT and heterozygous NOER mammary glands (Figure S3B). Thus, membrane ERa signaling is required for amphiregulin, EGFR, and ErbB2 mRNA expression, progesterone production, and mammary gland PR mRNA expression, thereby contributing to mammary gland ductal development. We also identify a potentially important mechanism where membrane ER signaling to the epigenetic regulation of PR mRNA impacts organ development.

Homozygous estrogen receptor α knockout (ERKO) and MOER mice have comparable, rudimentary mammary development (Pedram et al., 2009). Comparing 10-week-old, homozygous ERKO, MOER, and NOER mice, the phenotype in NOER mice is different. Homozygous NOER mice show greater extension of large ducts and filling in the mammary gland fat pad, compared to ERKO and MOER mice (Figure S3C). However, all three mice show the loss of ductal side branching and blunt end budding. We conclude that primary ductal extension through the mammary fat pad requires nuclear ER α action that is missing in ERKO and MOER mice, and is not rescued by only the membrane localization of expressed E domain of ER α (MOER). However, completely normal ductal development after puberty as seen in WT or heterozygous NOER mice requires both functional membrane and nuclear ER α pools.

⁽D) pS2 mRNA in hepatocytes and regulation from ERK and PI3K signaling in response to 10 nM E2. RT-PCR results are from three experiments; GAPDH served as control. *p < 0.05 versus control; +p < 0.05 E2 versus E2 plus PD or LY.

⁽E) ERE-luciferase activity in hepatocytes from five mice each, in three separate experiments. *p < 0.05 versus E2.

⁽F) Nuclear ERa occupancy of the pS2 promoter ERE. Graph is from cells of five mice each, in three experiments. *p < 0.05 versus control.

⁽G) Expression of the E domain of ER α targeted exclusively to the membrane promotes E2 recruitment of nuclear ER α to the pS2 promoter ERE. Graph is from cells of five mice for three experiments. *p < 0.05 versus control in (F) and (G).

⁽H) Competition binding of increasing amounts of unlabeled E2 and H^3 -E2 to WT and homozygous NOER hepatocytes. Scatchard analysis of a representative experiment provides K_D and B_{max} from cells from five livers per mouse type; the study was done three times. See Figure S1 for additional results.



(A) Representative reproductive tract development.
(B) Cross-sections of uterine layers from WT, heterozygous, and homozygous NOER mice.

Table 1. Adult Female Mouse Serum Hormone Levels							
Mice	LH (ng/ml)	FSH (ng/ml)	E2 (g/ml)	Prolactin (ng/ml)	IGF-1 (ng/ml)	GH (ng/ml)	Progesterone (ng/ml)
WT	0.7 ± 0.2^{a}	6.7 ± 0.5	32.2 ± 4.8	49.1 ± 4.6 ^a	115 ± 8.1	61 ± 6	3.4 ± 0.8^{a}
WT (ovx)	5.3 ± 0.5	30.1 ± 2.4	0.4 ± 0.1	17 ± 2.6	112 ± 4.1	59 ± 2.8	1.1 ± 0.5
Hetero-NOER KI	1.2 ± 0.3	6.1 ± 0.6	37 ± 3.9	52.3 ± 4.7^{a}	120 ± 9.3	59 ± 3.9	3.0 ± 0.5^{a}
Hetero-NOER KI (ovx)	4.8 ± 1.0 ^a	29 ± 2.1	0.8 ± 0.2	21 ± 2.6	109 ± 5.1	52 ± 2.8	0.8 ± 0.3
Homo-NOER KI	2.3 ± 0.5^{b}	5.3 ± 0.3	69 ± 5.8 ^b	32.6 ± 1.9 ^b	110 ± 7.6	49 ± 6.1	0.9 ± 0.4^{b}
Homo-NOER KI (ovx)	2.7 ± 0.8 ^b	24 ± 1.5 ^b	2.6 ± 1.3	28 ± 3.4	99 ± 4.6	50 ± 3.7	0.8 ± 0.2

Data are from six mice per group per condition.

^ap < 0.05 by ANOVA plus Scheffé's test for intact WT or Hetero-NOER KI versus same mice under ovariectomized conditions.

^bp < 0.05 for intact Homo-NOER KI versus intact WT or Hetero-NOER KI or same comparisons under ovariectomized conditions.

DISCUSSION

Important functional roles for extranuclear pools of ER α have increasingly been shown, particularly for membrane-localized receptors. In vivo, membrane ER α signaling through AMP kinase suppresses key mRNAs and production of all lipids in liver, independently of nuclear ER α (Pedram et al., 2013). Administration of an estrogenic compound to mice that only binds membrane ER α prevents several forms of arterial injury in vivo (Chambliss et al., 2010).

Here, we provide evidence that membrane $ER\alpha$ is necessary for organ development and function. The ERa palmitoylation site mutant KI mouse shows nuclear ERa localization comparable to WT mice, but E2-stimulated rapid signal transduction is markedly deficient, consistent with absence of membrane ERa. Importantly, loss of membrane ERa results in the markedly reduced expression of multiple mRNAs in response to E2 in several organs. Signaling from membrane ERa impacts transcription in several ways (Björnström and Sjöberg, 2005), but our results indicate that membrane ER signaling through mitogen-activated protein kinase kinase-ERK is important to recruit the nuclear receptor pool to the prototype pS2 promoter that is regulated from a recognized ERE. We also find that epigenetic regulation of PR-B mRNA in WT and heterozygous NOER mammary epithelial cells results in part from membrane ERa activation of AKT, causing an inhibitory phosphorylation of EZH2 (Cha et al., 2005). Recently described, a mouse expressing inducible small hairpin RNA to EZH2 in the mammary epithelium showed diminished ductal elongation and impaired duct termini formation during puberty (Michalak et al., 2013), similar to the phenotype in homozygous NOER mice. Both mechanisms we describe provide a further understanding of how membrane and nuclear ERs integrate their functions, but extensive investigation should uncover additional aspects. Abnormal development, mechanisms, and mRNA targets in homozygous NOER endometrium, ovary, and mammary glands are rescued in heterozygote KI mice that are comparable to WT mice.

Despite high E2 levels in serum, NOER female mice fail to show suppressed serum LH, indicating a necessary role of mem-

brane ER α to participate in negative feedback regulation of LH by E2. Signaling by E2 and ER α to kisspeptin/neurokinin B/dynorphin-expressing neurons inhibits gonadotropin-releasing hormone production in the hypothalamus and suppresses serum LH (Rance, 2009; Navarro et al., 2009; Gottsch et al., 2009). However, our female MOER mice that lack nuclear ER α show nonsuppressed serum LH despite high-serum estradiol levels (Pedram et al., 2009). We therefore conclude that both receptor pools are involved.

While this paper was under revision, Adlanmerini et al. characterized a newly developed mouse that also substituted alanine for cysteine at position 451 of ERα (Adlanmerini et al., 2014). These investigators described infertility and ovarian phenotypes and loss of negative feedback regulation of LH that were very similar to our findings. However, in contrast to our studies, these investigators described similar uterine endometrial responses to E2 in homozygous WT and C451A mutant mice and an 82% overlap of expressed genes by DNA microarray. Importantly, these investigators found that membrane ER α was only ~55% reduced in hepatocytes from their homozygous C451A mice and did not characterize membrane ERa abundance in the endometrium. In contrast, we find the expected complete loss of membrane ERa in luminal epithelial cells from the endometrium, as well as mammary gland epithelium and hepatocytes of homozygous NOER mice. Also, we find the rescue of the endometrial and all phenotypes in heterozygous NOER mice. We thus propose that the presumed persistence of membrane $\mathsf{ER}\alpha$ in the endometrium of the C451A homozygous mice (Adlanmerini et al., 2014) may account for the differences described.

Recently, arcuate nucleus kisspeptin/neurokinin B/dynorphin neurons were implicated to contribute to E2-suppressing gonadotropin secretion and abdominal visceral obesity (Mittelman-Smith et al., 2012). In the latter regard, we found that intact homozygous NOER female mice fed normal chow have extensive visceral fat deposition at 10 weeks of age, compared to WT and heterozygote NOER female mice (Figure S4B). Suppression of fat by E2 is very complicated, involving food intake and energy metabolism differentially mediated through ER α in discrete hypothalamic nuclei (Xu et al., 2011) and direct or

⁽C) Epithelial uterine thickness in intact or ovariectomized (Ovx) mice with or without E2 pellets (21 days). *p < 0.05 versus intact, +p < 0.05 versus Ovx (n = 5). (D) Expression of key E2-responsive mRNAs in uteri samples from intact WT, hetero-, and homozygous NOER mice (bar graphs, five mice each). *p < 0.05 versus WT or heterozygous NOER organs.

⁽E) Ovarian sections from WT and heterozygous NOER mice and three homozygous NOER mice (n = 5). HC, hemorrhagic cysts. See also Figures S2 and S3.



Figure 3. Mammary Gland Development

(A) Representative sections (n = 5 mice per group) stained with carmine also show magnified insets. Terminal end bud formation is shown below.

(B) PR-B proteins (left) and mRNAs (right) from mammary glands. Bar graphs are five mice each. *p < 0.05 WT or heterozygous NOER versus homozygous NOER. (C) AKT activation by E2 in mammary epithelial cells results in EZH2 serine 21 phosphorylation and diminished H3K27 methylation at the PR-B promoter, correlating to increased PR-B mRNA expression (bottom). Bar graphs are individual mouse data combined (n = 5). *p < 0.05 versus control; +p < 0.05 versus E2.

(D) PR-B mRNA expression in dissociated mammary epithelial cells exposed in culture to medium alone (control), E2, or E2 with or without LY294002. *p < 0.05 versus control; +p < 0.05 versus E2. Combined data are from individual mouse cultures (n = 5). See also Figure S3.

indirect actions on adipocyte lipogenesis and differentiation (Pedersen et al., 1992; Heine et al., 2000; Homma et al., 2000). Our findings indicate that membrane ER α plays an important role(s). In summary, we provide clear evidence that the membrane ER α pool is required for normal organ development and function in female mice, mainly acting in conjunction with the nuclear sex steroid receptor pool (Pedram et al., 2009).

EXPERIMENTAL PROCEDURES

Mouse Construction

A bacterial artificial chromosome with a cysteine 451-to-alanine mutation in ER α was created to insert into the *esr1* locus in ES cells. ES cells were then injected into blastocysts from C57BL/6NTac mice, the chimeric male mice positively selected by *neo* gene expression and chimeric males crossed with Flpe-expressing females to delete the neo cassette. Detailed construction methods are described in Supplemental Experimental Procedures. All mouse experiments were approved by the Animal Research and Research and Development Committees at the Department of Veterans Affairs Medical Center, Long Beach.

ER_α Localization

Hepatocytes and mammary epithelial cells from 8- to 10-week-old WT, hetero-, and homozygous NOER female mice were acutely cultured for immunofluorescence microscopy and western blot. First antibody to ER α from Santa Cruz Biotechnology (C terminus, MC-20) and fluorescein isothiocyanate (FITC)-conjugated second antibody (Vector Laboratories) were used.

Kinase Activities

Cell ERK activity was determined at 15 min as phosphorylation at the active site, tyrosine 202/204 with phospho-antibodies to tyrosine 202/204 (Santa Cruz). AKT activity at 15 min was determined as Ser473 phosphorylation using phospho (and total) antibodies from Cell Signaling Technology.

Measurement of Plasma Hormone Levels

Blood samples were collected by cardiac puncture and centrifuged. Serum was stored at -80° C until assayed using ELISA or RIA kits (Diagnostic Systems Laboratories; Cayman Chemical).

mRNA, Epigenetic, and ChIP Assays

mRNA expression by RT-PCR, epigenetic studies, and chromatin immunoprecipitation (ChIP) assays are described in Supplemental Experimental Procedures.

Fertility Studies

The 12- to 18-week-old male and female WT, heterozygous, and homozygous NOER mice were used for multiple matings, and success rates and periods were determined.

Histology, Histochemistry, and Serum Hormone Measurements

Whole organs were obtained under anesthesia/euthanasia for mounts and thin-tissue sections (Pedram et al., 2009). Hematoxylin and eosin staining (reproductive tract tissues) and carmine alum staining for mammary glands were done. Ki67 staining of mouse uteri served as a proliferation marker using ab15580 antibody (Abcam) with formaldehyde-fixed, paraffin-embedded tissue sections. Sections were deparaffinized, rehydrated, then blocked with 4% serum (30 min, 25°C); antigen retrieval by heat mediation was done in citrate buffer (pH 6.0). Incubation with primary antibody (5 μ g/ml in blocking buffer, 16 hr at 4°C) was followed by FITC-conjugated, goat/anti-rabbit IgG polyclonal (1/100), secondary antibody (Vector Labs) incubation overright. Fluorescent microscopy ensued.

Uterine Response to E2

The 10- to 12-week-old female mice were ovariectomized and recovered for 1 week, and E2 pellets were then inserted under the skin for 12 or 21 days, producing physiological serum E2 levels (Pedram et al., 2008). Uterine epithelial thickness was measured. For mRNA, mice were exposed to E2 from pellets for 12 days; the uteri were removed and processed for RT-PCR. Uterine weight studies are described in Supplemental Experimental Procedures.

E-mem-ECFP Expression Studies

Hepatocytes from homozygous NOER mice were transfected with a plasmid expressing only the E domain (ligand binding domain) of ER α , targeted exclusively to the membrane by paired myristylation sequences (E-mem ECFP) (Pedram et al., 2009). As control, some cells were transfected with empty vector (ECFP), and studies were performed.

Epigenetic Modulation Studies

Mammary gland epithelial cells from the three mice types were isolated and cultured. Precipitated histone proteins were collected and resolved by SDS-PAGE on a 10%–20% Tris-tricine gel (Bio-Rad), and changes in trimethylation of histone 3 at lysine 27 relative to histone 3 at the PR promoter were investigated (antibodies from Abcam). Total EZH2 and histone 3 proteins were loading controls. PR-B expression was determined by RT-PCR at 24 hr of E2 treatment.

Statistical Analysis

Mean \pm SEM was calculated for ANOVA plus Scheffé's test. p < 0.05 was considered significantly different.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.04.016.

AUTHOR CONTRIBUTIONS

A.P. and M.R. performed the research. M.L., S.H., and E.R.L. designed the research and analyzed the data. E.R.L. wrote the paper.

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