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Developmental Phenotype of a Membrane Only Estrogen Receptor α (MOER) Mouse^{*S}

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Estrogen receptors (ERs) α and β exist as nuclear, cytoplasmic, and membrane cellular pools in a wide variety of organs. The relative contributions of each ER α pool to *in vivo* phenotypes resulting from estrogen signaling have not been determined. To address this, we generated a transgenic mouse expressing only a functional E domain of ER α at the plasma membrane (MOER). Cells isolated from many organs showed membrane only localized E domain of ER α and no other receptor pools. Liver cells from MOER and wild type mice responded to 17- β -estradiol (E2) with comparable activation of ERK and phosphatidylinositol 3-kinase, not seen in cells from ER α KO mice. Mating the MOER female mice with proven male wild type breeders produced no pregnancies because the uterus and vagina of the MOER female mice were extremely atrophic. Ovaries of MOER and homozygous Strasbourg ER α KO mice showed multiple hemorrhagic cysts and no corpus luteum, and the mammary gland development in both MOER and ER α KO mice was rudimentary. Despite elevated serum E2 levels, serum LH was not suppressed, and prolactin levels were low in MOER mice. MOER and Strasbourg female mice showed plentiful abdominal visceral and other depots of fat and increased body weight compared to wild type mice despite comparable food consumption. These results provide strong evidence that the normal development and adult functions of important organs in female mice requires nuclear ER α and is not rescued by membrane ER α domain expression alone.

Estrogen receptor (ER)³ α exists in many cellular locations, each potentially contributing to sex steroid action (1). Genetic

deletion of ER α in mice established important roles of this receptor for normal adult female mammary gland and reproductive tract development and function (2–4). In these regards, adult female ER α knock-out (KO) mice show atrophy of the uterus and vagina, abnormal ovarian histology, and rudimentary mammary gland development. As a result, the normal adult functions of these organs were markedly compromised, and many of these abnormalities were phenocopied by aromatase knock-out mice (5). Thus, estrogen or its metabolites acting at ER α is necessary for these normal developmental functions.

Since the original descriptions of both the Chapel Hill (2) and Strasbourg (4) ER α KO mice, it has become appreciated that these mice represent depletion of all ER α cellular pools. For instance, endothelial cells derived from homozygous ER α /ER β combined deletion mice show no evidence of any cellular ER (6). Furthermore, E2 cannot rapidly signal nor stimulate proliferation and survival in these cells. Thus, in ER α KO mice, it cannot be determined where estrogen acts in the cell to effect normal development and function. This limits understanding of what specific actions occur through discrete ER α pools, contributing to the overall effects of this receptor *in vivo*.

To begin to address this issue we generated a mouse that expresses a functional E domain of ER α only at the plasma membrane of cells from multiple organs. No cytoplasmic or nuclear ER α were found in any cells from this membrane only E domain of ER α (MOER) mouse. This allowed us to investigate possible roles of membrane ER α in normal female reproductive tract and mammary gland development and function in this model in mice lacking nuclear ER α .

EXPERIMENTAL PROCEDURES

Materials—All of the antibodies were from Santa Cruz except FITC-conjugated antibodies (Vector Laboratories) and the C-terminal ER α antibody (Abcam). Enzyme-linked immunosorbent assay kits for leuteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin, growth hormone, insulin-like growth factor 1, and E2 were from Diagnostic Systems Laboratories, Inc. and R & D Systems.

Generation of MOER Mice—The E domain of ER α was amplified by PCR from a full-length human ER α -containing plasmid (pcDNA3-hER α) and cloned into the multiple cloning site of the pECFP-mem plasmid (Clontech), with slight changes from previously described methods (7). This plasmid has extensively been expressed in cells, and the E domain of ER α localizes only to the plasma membrane because of multiple palmitoyla-

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^S The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1 and S2.

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³ The abbreviations used are: ER, estrogen receptor; WT, wild type; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; KO, knock-out; FITC, fluorescein isothiocyanate; LH, leuteinizing hormone; FSH, follicle-stimulating hormone; RT, reverse transcription; ERE, estrogen response element.

tion sites from the neuromodulin protein cloned into the parent plasmid (7, 14). The construct was injected into fertilized CB6F1 mouse oocytes and implanted into pseudopregnant female mice. Founder transgenic mice were first identified by PCR of tail snip DNA, confirmed by PCR of DNA extracted from multiple organs, using the primers 5'-CGTGTACGGTGGGAGGTCTA-3' (forward) and 5'-ATGTGAACCAGCTCCTGTGTC-3' (reverse). The PCR amplification conditions were 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, followed by 72 °C for 5 min. This produced a 226-bp product composed of both plasmid and E domain sequences and was confirmed by sequencing. The transgenic mice were then bred to Strasbourg ER α KO heterozygous mice (kindly obtained from Dr. Pierre Chambon, INSERM, France, and through Dr. Michael Mendelsohn, Tufts University), and the resulting compound mice were bred to each other, creating MOER mice (see Fig. 1A). Deletion of full-length ER α was confirmed by PCR of tail DNA, as described (4). Briefly, two primer sets were used to yield different product patterns, identifying either homozygous or heterozygous deletion of ER α . Homozygous deletion (ER $\alpha^{-/-}$) is indicated by the absence of a PCR product with primers P1 and P2 (5'-TTGCCCGATAACAATAACAT-3' and 5'-ATTGTCTCTTTCTGACAC-3', respectively) and a 255-bp product with primers P3 and P4 (5'-GGCATTACCACTTCTCCTGGGAGTCT-3' and 5'-TCGCTTTCCTGAAGACCTTTCATAT-3', respectively). Tail DNA samples from heterozygous ER α (ER $\alpha^{+/-}$) mice produce a 387-bp band with P1 and P2 and a 255-bp band with P3 and P4. Wild type mice (ER $\alpha^{+/+}$) DNA yield a 387-bp band with P1 and P2, and an 815-bp band with P3 and P4 as primers. Establishing transgene expression in the organs of MOER mice was done by RT-PCR. Total RNA was isolated from various organs and used for cDNA synthesis, followed by PCR with the primers described for genotyping. To quantify the transgene expression, RNA was extracted from the various organs, and cDNA synthesis was performed. Real time PCR was carried out using the cDNA with SYBR[®] GreenER[™] qPCR SuperMix for the iCycler (Invitrogen) according to the manufacturer's manual. The melting curve analysis was performed following real time PCR to ensure specificity of the reaction and identify the presence of primer dimers. The forward and reverse primer sequence to detect the E domain were: 5'-AGCACCCCTGAAGTCTCTGGA-3' and 5-GATGTGGGAGAGGATGAGGA-3', respectively. The change in gene expression was calculated using the $\Delta\Delta C_t$ method with glyceraldehyde-3-phosphate dehydrogenase as the internal control.

Cell Culture—Mouse hepatocytes were isolated from 6–12-week-old female C57 Black/J6 (WT), MOER, and ER α KO mice by enzymatic and mechanical methods and established as primary cultures for experiments. In addition, thin tissue sections of mammary gland, uterus, vagina, and ovary were prepared for immunohistochemistry.

Microscopy—Tissues from the various mice or hepatocytes cultured on glass slides were incubated with antibodies to the E/F or A/B domains of ER α (Santa Cruz Biotechnology) overnight at 4 °C. This was followed by second antibody conjugated to FITC (Vector Laboratories) for imaging by immunofluores-

cent confocal microscopy. Cytokeratin 8 antibody (epithelial cell marker) was from Santa Cruz Biotechnology.

Kinase and pS2 Gene Expression Assays—ERK and PI3K assays in primary cultures of liver cells from all mice were carried out as previously described (6, 8). Immunoblots for total ERK or AKT serve as loading controls. RT-PCR for pS2 gene expression was carried out from the DNA of cells not exposed (control) or exposed to 10 nM E2 for 24 h. Primers used were pS2, 5'-ATACCATCGACGTCCCTCCA and AAGCGTGCTTGAGGTGTCCG and for control glyceraldehyde-3-phosphate dehydrogenase expression, 5'-AGCCACATCGCTCAGAACAC and GAGGCATTGCTGATGATCTTG.

Tissue and Blood Collection and Histology—Prior to ovariectomy, mice underwent daily vaginal lavage for cell cytology, sequentially over several weeks. Organs and fat were obtained under anesthesia and just before euthanasia of the mice, in compliance with studies approved by the Animal Care and Research and Development Committees at the Long Beach Veterans Affairs Medical Center. Dissected mammary glands, ovaries, and uteri/vaginal tissues were embedded in paraffin before tissue sectioning, and the sections were stained with hematoxylin and eosin or antibodies for immunofluorescent microscopy. The blood samples (400 μ l) for hormone assay were obtained by cardiac puncture during diestrus (WT), followed by euthanasia of the mice.

Vaginal Response to E2—14-week-old female mice were ovariectomized, recovered for 1 week and then given 0.1 mg of E2 in oil or vehicle alone (control) by intraperitoneal injection daily for 3 days. Twenty-four hours after the final E2 injection, the vagina and attached uterus from each anesthetized mouse was removed, fixed, paraffin-embedded, and sectioned. The tissue sections were deparaffinized for hematoxylin and eosin or cytokeratin 10 staining. For cytokeratin 10 staining slides were immersed in antigen retrieval solution (Vector Laboratories) and microwaved for 10 min. The sections were incubated overnight at 4 °C with the primary monoclonal antibody, diluted 1/50 in SuperBlock (Pierce) or a control nonspecific IgG (DAKO), and rinsed in phosphate-buffered saline (Dulbecco's phosphate-buffered saline; Sigma). The sections were then stained with secondary antibody (FITC-conjugated) for 2 h at room temperature and observed under fluorescent microscopy. Nonspecific binding was blocked using Super Block (Pierce). Cytokeratin 10 is a marker of E2-induced differentiation in vaginal epithelia. Epithelial layer thickness was also quantified.

Body Composition Determinations—Four mice from each group (MOER, ER α KO, and WT mice) were anesthetized then imaged by Magnetic Resonance, using a 4.0 Tesla magnet and Surrey Medical Imaging Systems console. The data consists of three sets of images for each mouse, combined to constitute Dixon imaging with a few modifications, generating fat and water images. There were 54 slices taken of each animal, and each slice was 2 mm thick. For processing, the images were assigned a threshold to remove artifacts around the animal that may contaminate the calculation of fat composition. This value was chosen for each animal individually, but for most animals was ~10% of the maximum voxel value. Each image was normalized by the maximum fat and water voxel values, respectively, to compensate for the differences in relative intensities

Membrane Only ER α Mouse

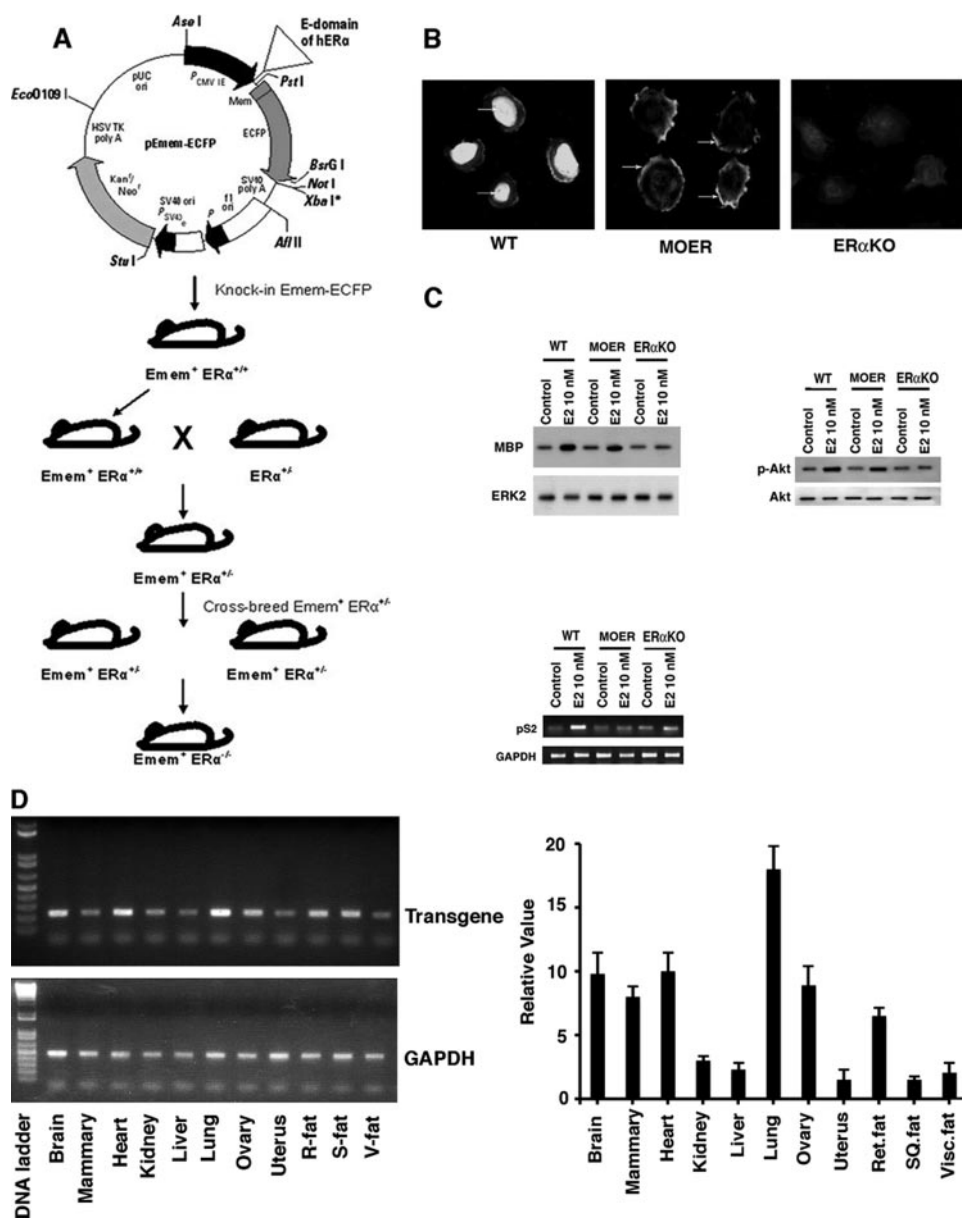


FIGURE 1. Validation of MOER mice. *A*, construction of the MOER mouse. *B*, primary cultures of hepatocytes from WT, MOER, and homozygous ER α KO mice. Immunofluorescent confocal microscopy utilized antibody to the E domain of ER α and FITC-conjugated second antibody. Arrows depict nuclear localization of ER α (in WT) or the membrane-localized E domain (in MOER). *C*, *top panels*, ERK and PI3K activation by E2. *In vitro* kinase activity assays were determined from cultured mouse hepatocytes derived from four mice each, and the cells were exposed to E2 for 10 min. Activities of ERK (substrate protein is myelin basic protein, MBP) and PI3K (reflecting phosphorylation of serine 473 of AKT) are shown. *Bottom panels*, activation by E2 of the endogenous pS2 gene. RT-PCR was carried out from RNA extracted from cultured hepatocytes as described, and glyceraldehyde-3-phosphate dehydrogenase serves as control gene expression. All of the studies were repeated twice. *D*, *left panel*, transgene expression in organs from female MOER mice determined by RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase expression serves as RNA and loading control. Distribution from a representative mouse is shown, and the experiment was repeated. *R* is retro-peritoneal, *S* is subcutaneous, and *V* is abdominal visceral. *Right panel*, real time relative PCR values of transgene expression in multiple organs, from MOER mice. The bar graphs are the mean \pm S.D. from two experiments combined.

between the fat and water images. The values were summed over the entire data set for each animal to give a total amount of fat and water. These two numbers were then divided by the sum of the two to give a fat percentage, and the water percentage is one minus the fat percentage.

Body fat distribution in abdominal, retroperitoneal, and subcutaneous depots were also examined for clear differences.

Abdominal fat was cut out and weighed for comparison between the three mice groups. Consumption of food pellets in grams and weight change over a 4-week period was measured in four mice from each group, and the means \pm S.D. for each group of mice were compared by analysis of variance plus Scheffe's test, where $p < 0.05$ was significant between groups.

RESULTS

Construction and Validation of the MOER Mouse—The E domain of human ER α was cloned into a plasma membrane targeting plasmid that also contains a cytomegalovirus promoter (Fig. 1A). Transfecting the plasmid in ER null cells resulted in strictly plasma membrane localization of the E domain of ER α and E2-induced rapid kinase signaling and cell biological effects (7, 8). Additionally, signaling in these cells was comparable with the rapid effects of E2 engaging endogenous, full-length ER α in breast cancer cells (8).

The construct was injected into fertilized CB6F1 mouse oocytes and implanted into pseudopregnant female mice. Expression of the E domain of ER α transgene in founder transgenic mice was confirmed in many organs by PCR of extracted DNA (supplemental Fig. S1A). Founder transgenic mice were then bred to Strasbourg ER α KO heterozygous mice (4), and the resulting compound mice were bred to each other. This created a mouse that expresses the E domain of ER α at the plasma membrane on the background of homozygous ER α deletion (MOER) (Fig. 1A). Deletion of full-length ER α and expression of the E domain of ER α transgene in MOER mice was initially confirmed by PCR using tail DNA, as previously described (Ref. 4 and "Experimental Procedures") (supplemental Fig. S1A).

To validate the mouse model, the liver was removed, hepatocytes were isolated from MOER, Strasbourg ER α KO, or WT female mice, and primary cultures were established. This was possible because this organ was intact in all three mice. Hepatocytes of MOER mice revealed only membrane-localized E domain of ER α protein, and no intracellular ER α protein

expression (Fig. 1B). In contrast, Strasbourg ER α KO mice hepatocytes produced no ER α anywhere in the cell, whereas WT liver cells showed abundant ER α in the nucleus but also localized at the plasma membrane. ER β expression was comparable in all mouse cells (data not shown).

Functionally, we found that rapid ERK and PI3 kinase activation occurred in response to E2 in MOER or WT mouse liver cells but not in Strasbourg ER α KO mouse cells (Fig. 1C, *top panels*). These are the first *in vivo* data to establish that membrane-localized ER α (and specifically the E domain) alone supports rapid kinase activation by E2. The endogenous pS2 gene is regulated through an estrogen response element in the promoter. Only the WT cells responded to E2 with significantly increased expression (Fig. 1C). Because nuclear ER α is required for pS2 activation, these results validate that MOER and ER α KO mice lack this receptor pool (Fig. 1C, *bottom panels*). We also assayed many target organs for E domain of ER α transgene expression in the MOER mice. Although there was expected variability, transgene expression was widespread in all organs assessed (Fig. 1D, *left panels*). This included mammary gland, ovary, uterus, brain, and various fat depots. Transgene expression was also quantified by real time PCR (Fig. 1D, *right panel*). Strong expression was seen in lung, brain, heart, mammary gland, ovary, and retroperitoneal fat. It must be appreciated, however, that although the transgene expression in liver was relatively low, this was sufficient to produce functional protein expression (Fig. 1C).

Fertility and Female Reproductive Tract Development and Function—Fourteen-week-old female MOER mice ($n = 6$) were mated with WT C57 Black/J6 male proven breeders. Despite multiple matings, no pregnancies resulted. The reason for infertility became apparent when the reproductive tracts of the MOER female mice were examined. The uteri and vaginas of all of the female MOER mice were extremely atrophic and were comparable with these organs from homozygous Strasbourg ER α KO female mice (Fig. 2A). Homozygous female Strasbourg ER α KO mice are known to be infertile, in part because of the extremely atrophic uterus and vagina (4). Atrophy in MOER mice was seen despite expression of the E domain of ER α at the membrane of cells from these organs (Fig. 2C). In contrast, WT female mice had the expected normal reproductive tract development (Fig. 2A).

We then examined the ovaries of the mice. The E domain (but not N terminus) of ER α was only identified at the membrane of cells in MOER ovarian tissue; WT ovaries showed mainly nuclear ER α , and Strasbourg ER α KO mice demonstrated no cellular ER α (Fig. 2B). We also determined whether the transgene was expressed in epithelial cells of these organs that are important targets for E2 action. As seen in Fig. 2C, ER α was found predominantly in the nucleus of ovarian and uterine epithelial cells from WT female mice, identified by surface staining of these cells with the cytokeratin 8 marker. In contrast, E domain of ER α protein expression in MOER mice was found exclusively at the membrane in epithelial cells (overlap with cytokeratin 8). Some expression in the adjacent stroma was also present.

Both female MOER and Strasbourg ER α KO mice comparably showed multiple hemorrhagic cysts in the ovary (Fig. 2D).

Furthermore, there was no evidence for corpus luteum development. This suggested that the mice did not ovulate and did not undergo estrous cycling, and we confirmed this by sequential vaginal smears from MOER mice (supplemental Fig. S1B). After ovariectomy, neither the MOER nor the Strasbourg mice showed a vaginal response to estrogen injection (Fig. 2E). In contrast, ovariectomized WT female mice responded to E2 injections with robust epithelial cell proliferation and cytokeratin staining (Fig. 2E and supplemental Fig. S1C).

Feedback of E2 on LH and Prolactin Secretion Is Compromised—WT female mice showed appropriate suppression of LH and FSH by physiological E2 levels in blood or by inhibin production from the ovary (Table 1). The expected increase in serum gonadotropins resulted from ovariectomy. However, nonovariectomized MOER and ER α KO mice produced markedly elevated serum estradiol that failed to suppress LH. This indicates that for LH, the negative feedback effects of E2 on the hypothalamic-pituitary gonadotrope axis are impaired in the absence of nuclear ER α . After ovariectomy, LH values did not change in MOER or in ER α KO mice but FSH markedly increased, consistent with a loss of inhibin and its negative feedback on FSH production.

Insulin-like growth factor 1 and growth hormone levels were not statistically different between the three groups of mice and were unaffected by ovariectomy. In contrast, E2 is known to stimulate pituitary-derived prolactin in rodents, and this occurs through ER α (9). In our WT mice, ovariectomy resulted in a 50% decrease in serum prolactin. Basal prolactin secretion was decreased in both ER α KO or MOER mice, but no further effect of ovariectomy was seen. This suggests that nuclear ER α is important to the up-regulation of prolactin by E2 in female mice. Furthermore, maldevelopment of the mammary glands in the two genetic mice may reflect the loss of E2 action at the nucleus and lower prolactin levels, a hormone that collaborates with E2 in this respect.

Rudimentary Mammary Gland Development in MOER Mice—ER α KO mice have been reported to show little mammary gland development in the adult (3). To determine whether membrane localization of the E domain of ER α can rescue this phenotype, whole mounts of the mammary glands of 12-week-old female MOER mice were compared with those from Strasbourg or WT female mice. The expected membrane only distribution of the E domain of ER α was prominent in MOER mice mammary epithelial cells seen in tissue sections that showed overlapping with cytokeratin 8 expression (supplemental Fig. S1D). Endogenous nuclear ER α expression in epithelial cells was seen in tissue sections from WT mice (supplemental Fig. S1D), whereas ER α KO mice showed no ER α in any cell type within the tissue sections (data not shown). Both genetically modified mice showed comparable rudimentary mammary gland development (Fig. 3). Mammary ductal elongation was severely retarded in both MOER and Strasbourg mice, and terminal end buds were small with scant lobuloalveolar development. ER α in mammary epithelium is required for ductal elongation and side branching in pubertal, virgin female mice, and conditional deletion of this receptor significantly impairs normal mammary gland development and function during and after pregnancy (10). Our results indicate that the presence of nuclear ER α is

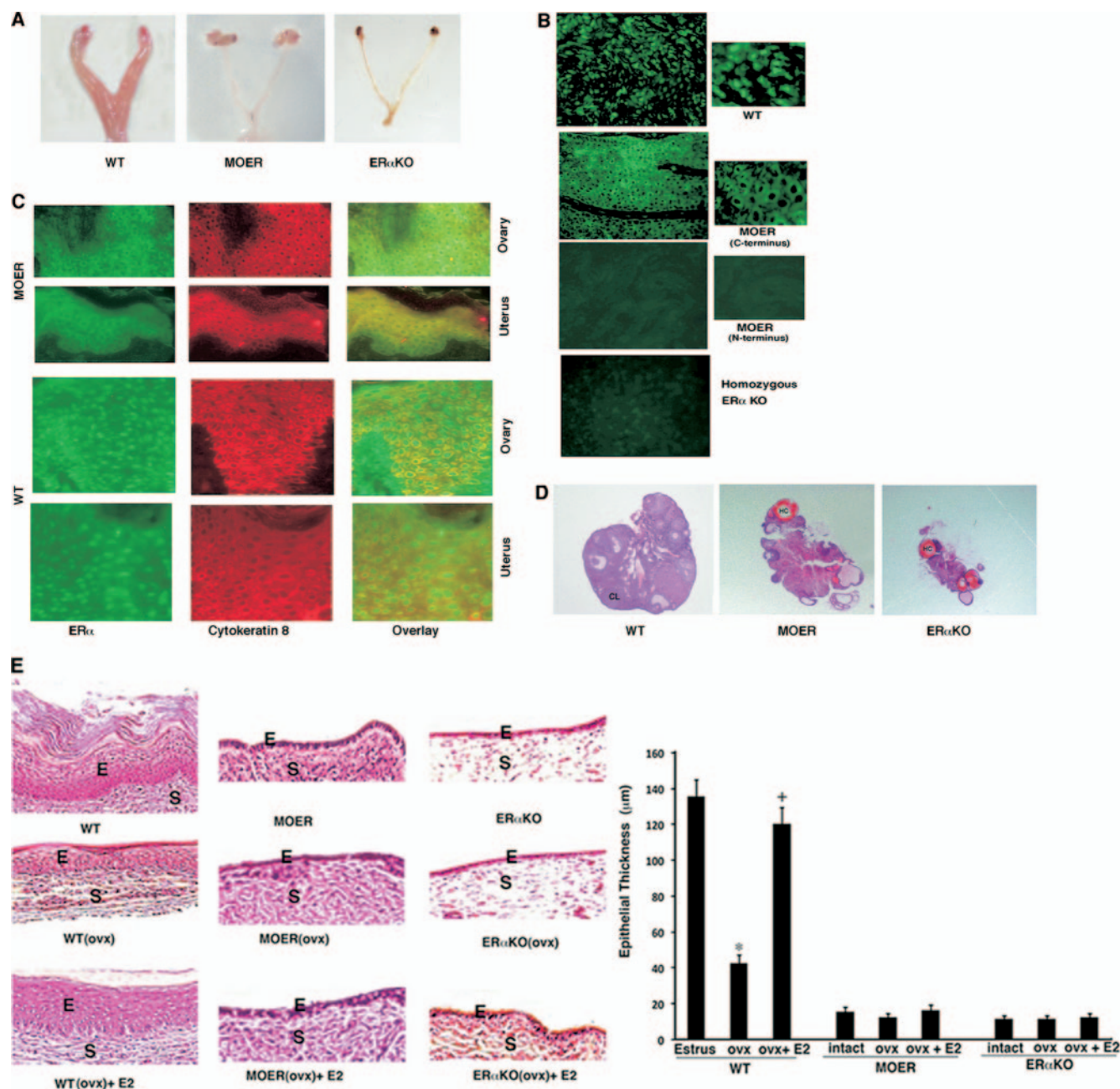


FIGURE 2. Reproductive tract phenotype of MOER mice. *A*, representative ovary, uterus, and vagina of female ER α KO, WT, and MOER mice, aged 16 weeks. *B*, ovarian tissue immunofluorescent histochemistry for ER α expression. Antibodies to the ER α E domain (all mouse ovarian sections) or the N terminus (MOER only) were used with deparaffinized tissue sections, followed by second antibody staining (FITC-conjugated) for confocal microscopy. The insets are magnified views of the tissues. *C*, immunofluorescent histochemistry of tissue sections from MOER and WT mice, using antibodies to the E domain of ER α or cytokeratin 8 (epithelial cell marker). *D*, hematoxylin and eosin staining of representative ovarian sections from the mice. CL, corpus luteum; HC, hemorrhagic cyst. *E*, vaginal epithelium response to E2 injection. Ovariectomized (ovx) mice were consecutively injected in the abdomen for 3 days with 0.1 mg 17- β -estradiol (E2) in oil or vehicle alone (control). *E*, epithelium; S, stroma. The bar graph is the mean \pm S.E. response to E2 injection from each group of mice ($n = 5$). *, $p < 0.05$ by analysis of variance plus Scheffe's test for WT estrous versus WT ovariectomized; +, $p = 0.05$ for WT ovariectomized versus WT ovariectomized plus E2 injection.

required for the normal development and differentiation of the mammary gland.

MOER Mice Develop Extensive Fat Deposition—Women after the menopause often develop abdominal obesity that is reversed by estrogen administration (11), and both ER α KO and aromatase gene-deleted female mice develop truncal obesity (12, 13). We therefore first compared the abdominal fat content of MOER, Strasbourg ER α KO, and WT female mice. In both genetic models, extensive visceral fat deposition was seen and

was 8-fold greater than WT mice (Fig. 4). Furthermore, body weight was increased by almost 60% in MOER and Strasbourg mice, compared with WT mice. Retroperitoneal fat (supplemental Fig. S2) and subcutaneous fat were also much more abundant in both MOER and ER α KO mice compared with WT mice, and the two genetic mice were comparable. Body composition studies were determined by magnetic resonance imaging (Table 2). MOER and ER α KO mice comparably had significantly more overall body fat and less water content compared

TABLE 1
Adult female mouse serum hormone levels

The data are the means \pm S.E. from five or six mice/condition. ovx, ovariectomized. ND, not determined.

Mice	LH	FSH	E2	Prolactin	Insulin-like growth factor 1	Growth hormone
	ng/ml	ng/ml	pg/ml	ng/ml	ng/ml	ng/ml
WT	0.8 \pm 0.1	6.8 \pm 0.5	36.2 \pm 1.9	53 \pm 4.3	119 \pm 6.8	67 \pm 2.5
WT (ovx)	5.8 \pm 1.0 ^a	33.4 \pm 2.5	0.2 \pm 0.1	19 \pm 2.6 ^b	111 \pm 3.6	65 \pm 3.2
ER α KO	4.4 \pm 0.7 ^a	5.4 \pm 0.9	297 \pm 12 ^c	23 \pm 2.9	105 \pm 7.0	52 \pm 3.6
ER α KO (ovx)	5.2 \pm 0.6	18.7 \pm 1.9	ND	21 \pm 2.1	99 \pm 4.8	48 \pm 3.6
MOER	5.0 \pm 0.8 ^a	6.1 \pm 1.0	178 \pm 3.5 ^c	25 \pm 2.8	113 \pm 7.8	52 \pm 2.8
MOER (ovx)	4.7 \pm 0.6	16.3 \pm 2.1	ND	20 \pm 3.2	101 \pm 2	51 \pm 3.1

^a p < 0.05 for WT LH versus ER α KO or MOER.

^b p < 0.05 for WT prolactin versus WT ovx.

^c p < 0.05 for WT E2 versus ER α KO or MOER by analysis of variance plus Scheffe's test.

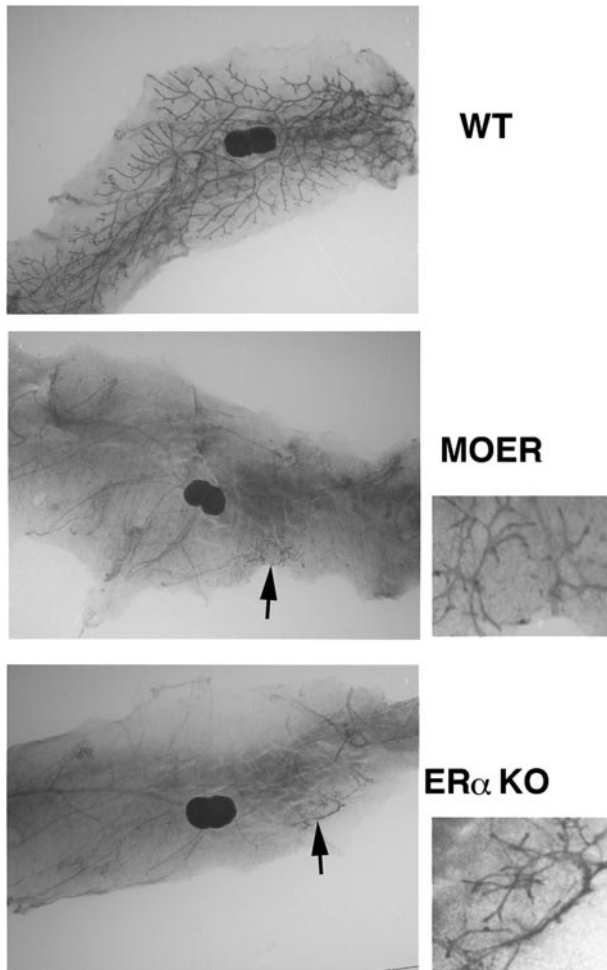


FIGURE 3. Mammary gland phenotype of MOER mice. Whole mounts of removed inguinal mammary glands were sectioned and then stained with hematoxylin and eosin. The arrows point to enlarged areas (insets) in MOER and ER α KO glands. The central lymph node is present in each gland. The studies shown are each representative of five mice.

with WT female mice. We also compared food intake in the mice over a 4-week period ($n = 4$ mice per group). MOER, ER α KO, and WT mice consumed 50 \pm 4 (mean \pm S.D.), 55 \pm 2, and 51 \pm 5 grams of chow pellets/mouse, respectively. Despite these similar intakes, the MOER and ER α KO mice each gained 6.5 \pm 0.9, and 7.8 \pm 1 grams of body weight, compared with 1.5 \pm 0.3 grams gained by the WT mice (*, p < 0.05 for MOER or ER α KO versus WT by analysis of variance and Scheffe's test). These results suggest that nuclear ER α is important to prevent excessive fat deposition throughout the body, and the absence

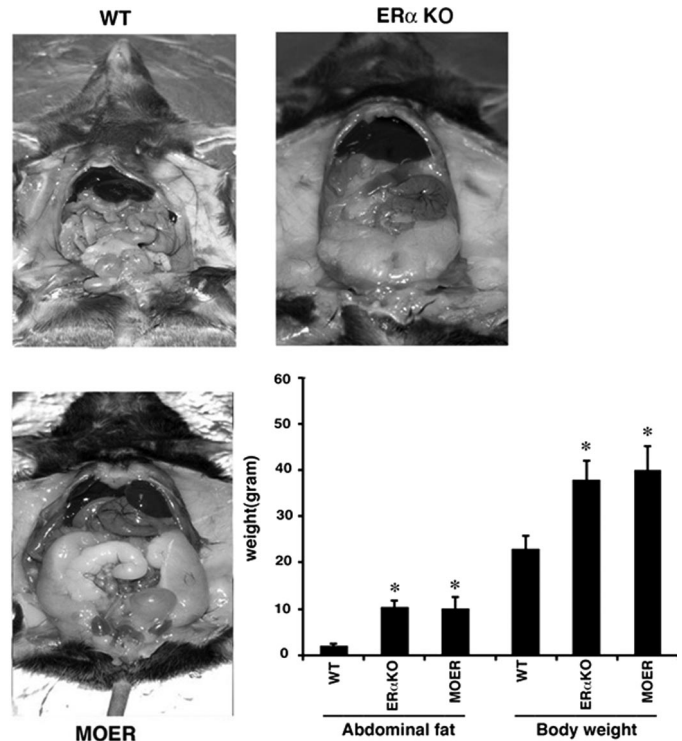


FIGURE 4. Abdominal fat deposition in MOER mice. Representative visceral abdominal fat is shown from each type mouse. Bar graphs show the means \pm S.E. abdominal visceral fat removed and weighed or body weight ($n = 5$). *, p < 0.05 for WT versus MOER or ER α KO mice in both abdominal fat weight and body weight measurements.

TABLE 2
Total body composition studies of fat and water content in 16-week-old female mice

The data are the means \pm S.E. from four mice/group. The results were analyzed by analysis of variance plus Scheffe's test.

Mice	Fat	Water	p value
	%	%	
MOER	45.4 \pm 2.3 ^a	54.6 \pm 3.4 ^b	p < 0.05
ER α KO	47.1 \pm 3.3 ^a	52.9 \pm 4.1 ⁺	p < 0.05
WT	28.3 \pm 1.4	71.7 \pm 4.6	

^a p < 0.05 for MOER versus WT mouse.

^b p < 0.05 for ER α KO versus WT mouse.

of this receptor pool contributes to weight gain. Excessive abdominal visceral fat in post-menopausal women contributes to increased morbidity from cardiovascular disease and cancer (14).

DISCUSSION

Strasbourg and Chappel Hill ER α KO female mice (2, 4) and aromatase KO mice (5) have extensive abnormalities of repro-

Membrane Only ER α Mouse

ductive tract and mammary gland development and function. ER α KO mice also show the loss of negative feedback regulation of LH, and extensive body fat deposition. However, these models represent total ER α depletion. Thus, the specific contributions from discrete cellular pools of ER α at the membrane or nucleus to normal development are unknown. Our results provide the clearest evidence to date that nuclear ER α is required for the normal female phenotype investigated here and that membrane-localized ER α alone is insufficient in this regard. We confirmed that the transgene was expressed in the target organs of female MOER mice investigated here. For example, transgene expression by real time PCR was strong in mammary gland, ovary, and retroperitoneal fat, where both MOER and ER α KO mice showed comparable abnormalities. Furthermore, transgenic protein expression was very evident in tissues that showed moderate transgene expression. Thus, membrane ER α E domain expression failed to rescue the ER α KO phenotype in these and other organs. It may be, however, that integration of membrane and nuclear ER function is important and that loss of either pool results in an abnormal phenotype.

The question arises of whether the E domain localized at the membrane in our model recapitulates the signaling by endogenous membrane-localized ER α . First, published cell models of membrane-localized E domain of ER α , cells that express endogenous 66kDa ER α , or cells transfected to express the full-length ER α at the membrane respond to E2 with similar signal transduction (7, 8). Further, we previously reported that expression of ER α lacking either A/B, C, or D domains localized to the membrane and activated ERK comparably to full-length ER α expression: In contrast, deletion of just the E domain prevented this (15). Second, we find that important kinase cascades are rapidly and comparably activated by E2 in cells derived from MOER and wild type mice. In contrast, no signal transduction was stimulated in cells from ER α KO mice. These results address the important issue of which ER pool is responsible for signal transduction. Although rapid signaling occurs from steroid hormone action at the membrane (1), chronic regulation of signal transduction may involve nuclear ER α transcription of the genes coding for kinases, phosphatases, and modulating proteins. Our *in vivo* and chronic model suggests the novel finding that membrane-localized ER α and specifically the E domain is required and sufficient for signal transduction by E2.

A recently described transgenic mouse model (NERKI) began to address the mechanism of nuclear ER α action in mammary gland and reproductive tract development. Knocking in a mutation in the DNA-binding domain of the mouse ER α resulted in a rodent that lacked classical, ERE-mediated transcription for one allele, yet preserved nonclassical (tethering) mediated transcription (16). NERKI female mice have hyperplastic uteri, different from the hypoplastic uteri of ER α KO or MOER mice. This indicates that a complex interaction occurs between receptor pools and the various mechanisms of gene transcription that leads to normal development of this organ.

Heterozygous NERKI female mice also show decreased differentiation of structures in the breast after puberty, compared with WT mice, and their ovaries demonstrate large cysts and lack corpus luteum. It is not clear why a 50% loss of ERE-mediated

transcription results in such a phenotype, considering that heterozygous ER α KO mice are fertile and have normal mammary gland development. In contrast, uterine luminal epithelial proliferation in response to E2 administration is present in NERKI or combined NERKI heterozygote/ER α KO heterozygote female mice (17). These findings indicate that some uterine responses to E₂ occur independently of ERE-mediated transcription. One possible explanation is that nongenomic signaling from membrane ER in the NERKI mouse is important for these responses to steroid hormone.

Regarding membrane-initiated steroid signaling, it has been proposed that membrane ER α collaborates with nuclear ER α to modulate transcription (18). Thus, the loss of nuclear ER α function at ERE promoters may compromise a physiological and integrated transcriptional action involving membrane ER α . This may include membrane ER α signaling to the phosphorylation and recruitment of co-activators or phosphorylation of nuclear ER α , enhancing its transcription at ERE-regulated genes (19). Possibly, membrane ER α signaling contributes to transcription resulting from nuclear ER α tethering. We therefore expressed the ER α E207A/G208A NERKI plasmid into ER null cells and found that ERK and PI3K kinase activation by E₂ was comparable with E₂ action in native MCF-7 cells (data not shown). Furthermore, Kousteni *et al.* (20) recently reported that E2-induced ERK was present in the vertebrae of combined NERKI heterozygote/ER α KO heterozygote female mice. Thus, kinase activation by membrane ER α appears to be preserved from the NERKI mutation, and it is plausible that membrane ER α contributes to tethering-mediated transcription. Alternatively, membrane ER α -initiated signaling alone could mediate some aspects of target organ response to E2. Our model begins to sort this out in that membrane-initiated signaling by E2 is evident but is not sufficient by itself for the normal organ development investigated here. A requirement of E2 binding to ER α for normal reproductive tract and mammary gland development and function in female mice has also recently been established (21).

In summary, expressing a functional E domain of ER α at the plasma membrane does not rescue the abnormal reproductive tract, mammary gland, pituitary hormone secretion, or increased body fat phenotypes of the total cell ER α KO female mouse. No *in vivo* model of selective nuclear ER α deletion has previously been described. From our results, we conclude that nuclear ER α is critical for the normal development, differentiation, and function of these organs.

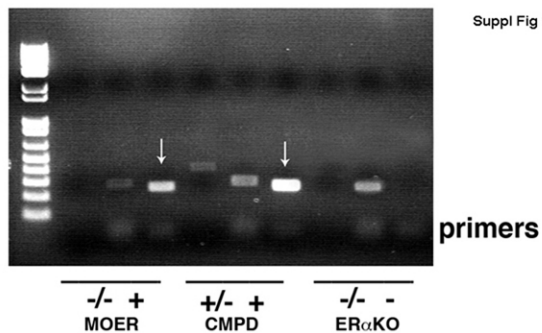
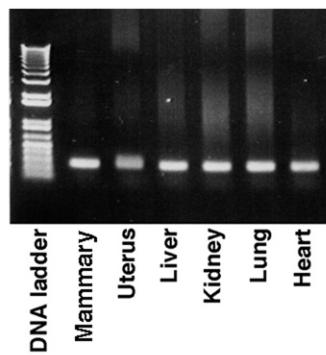
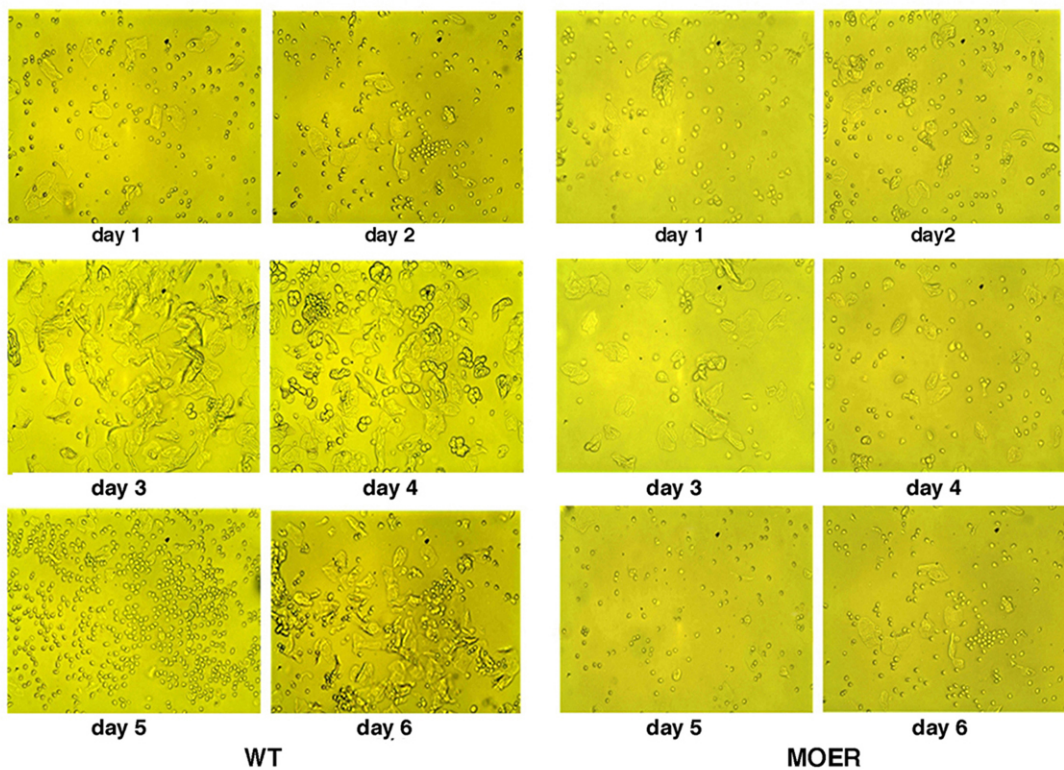
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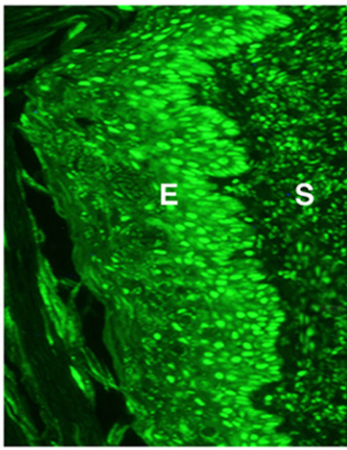
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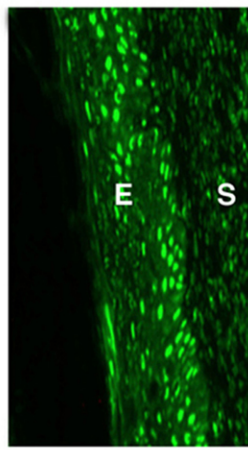
Supplementary Figure 1-(A) Genotype of MOER mice. *Left*, PCR identification of the E domain of ER ζ expressed in various organs from founder mice. Identification from extracted DNA used primers that span the vector and E domain insert. *Right*, PCR identification of the E domain transgene and ER ζ KO pattern (hemi or homozygous) as described in Methods. CMPD is compound mouse. (B) Six day consecutive vaginal smears from representative wild type (WT) and MOER mice (n=5 each). (C) Cytokeratin 10 immuno-fluorescent staining in vaginal sections from WT, MOER and ER ζ KO mice. Mice were under intact and ovxed conditions, or after E2 administration for 3 days. Representative mice are shown (n=5 each). (D) Mammary gland tissue sections stained for ER ζ expression. Antibody against the E domain of ER ζ or cytokeratin 8 was followed by FITC-conjugated second antibody for immunofluorescent microscopic visualization. Overlay of cytokeratin 8 and ER ζ (E domain) was seen in the MOER mice.

Supplementary Figure 2- Retro-peritoneal fat deposition in WT, MOER and ER ζ KO mice. Arrows point to the fat deposited in the kidney/adrenal region of the retro-peritoneum in representative mice. Fat mass at this site in the genetic mice comparably and significantly exceeded the wild type mice.

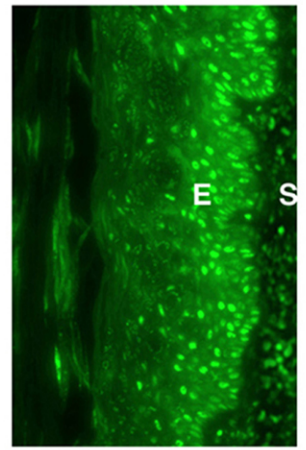
A**B**

C

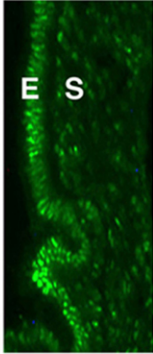
WT



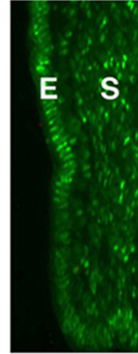
WT(ovx)



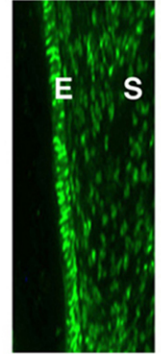
WT(ovx) + E2



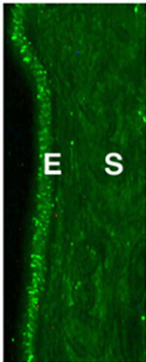
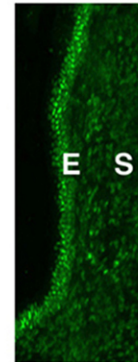
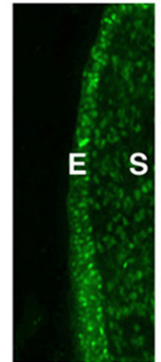
MOER



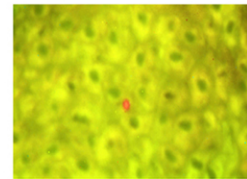
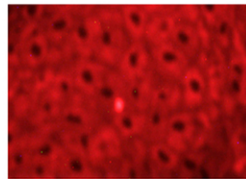
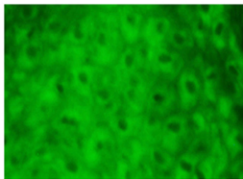
MOER(ovx)



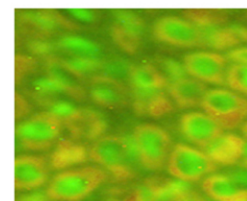
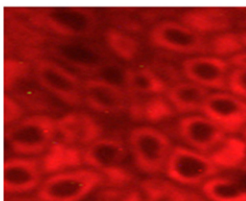
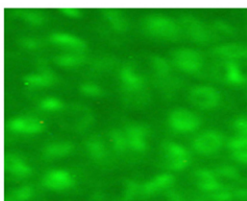
MOER(ovx) + E2

ER α KOER α KO(ovx)ER α KO(ovx) + E2**D**

Mammary



MOER

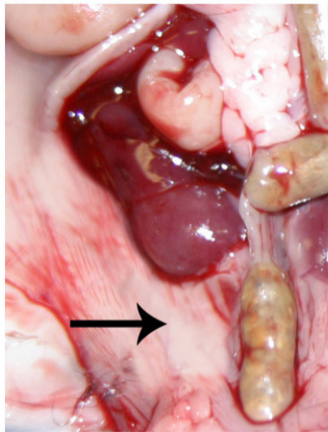


WT

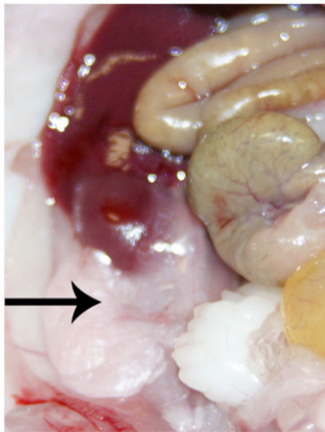
ER α

Cytokeratin 8

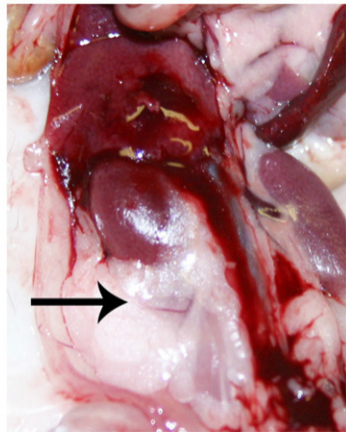
Overlay



WT



MOER



ER α KO

Developmental Phenotype of a Membrane Only Estrogen Receptor α (MOER) Mouse

Ali Pedram, Mahnaz Razandi, Jin K Kim, Fiona O'Mahony, Eva YHP Lee, Ulrike Luderer and Ellis R. Levin

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