Estrogen receptor α AF-2 mutation results in antagonist reversal and reveals tissue selective function of estrogen receptor modulators

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The estrogen receptor (ER) is a ligand-dependent transcription factor containing two transcriptional activation domains. AF-1 is in the N terminus of the receptor protein and AF-2 activity is dependent on helix 12 of the C-terminal ligand-binding domain. Two point mutations of leucines 543 and 544 to alanines (L543A, L544A) in helix 12 minimized estrogen-dependent transcriptional activation and reversed the activity of the estrogen antagonists ICI182780 (ICI) and tamoxifen (TAM) into agonists in a similar manner that TAM activated WT ER α through AF-1 activation. To evaluate the physiological role of AF-1 and AF-2 for the tissue-selective function of TAM, we generated an AF-2-mutated ER α knock-in (AF2ERKI) mouse model. AF2ERKI homozygote female mice have hypoplastic uterine tissue and rudimentary mammary glands similar to ER α -KO mice. Female mice were infertile as a result of anovulation from hemorrhagic cystic ovaries and elevated serum LH and E2 levels, although the mutant ER α protein is expressed in the AF2ERKI model. The AF2ERKI phenotype suggests that AF-1 is not activated independently, even with high serum E2 levels. ICI and TAM induced uterotrophic and ER-mediated gene responses in ovariectomized AF2ERKI female mice in the same manner as in TAM- and E2-treated WT mice. In contrast, ICI and TAM did not act as agonists to regulate negative feedback of serum LH or stimulate pituitary prolactin gene expression in a different manner than TAM- or E2-treated WT mice. The functionality of the mutant ER α in the pituitary appears to be different from that in the uterus, indicating that ER α uses AF-1 differently in the uterus and the pituitary for TAM action.

Estrogen regulates gene transcription via the estrogen receptors (ERs) α and β , which are ligand-dependent transcription factors. Transcriptional activation is mediated by AF-1 in the N-terminal domain and AF-2 in the C-terminal ligand-binding domain (LBD). ER ligands bind to the LBD and induce a conformational change of this LBD domain to modulate transcriptional activation. A portion of the AF-2 domain resides in helix 12 and plays a crucial role in determining interactions with coactivators and corepressors for transcriptional regulation influencing respective agonist or antagonist effects of the ligand (1, 2). Helix 12 has conserved hydrophobic amino acids between species. The mouse ER α residues L543 and L544 are correlated to the L539 and L540 residues of human ER α helix 12. The mutation of these residues in mouse and human ER α has been reported to have similar properties (3–9). Despite these amino acid mutations, binding to estrogen-responsive DNA sequences and estradiol (E2) is unaffected (3, 6); however, transcription activity is markedly lower in the presence of E2 compared with WT ER α because of the failure to recruit the p160 transcriptional coactivators (4). The mutation of these residues has been shown to convert the antiestrogens, including ICI164384, RU54876, and tamoxifen (TAM), into agonists (5, 9). The AF-1 region is required for a transcriptionally active configuration of this mutant with antagonists (5, 9). TAM is a well known selective ER modulator (SERM) that is a partial antagonist/weak agonist for ER α WT (10, 11). Several reports proposed that the N-terminal AF-1 of ER α WT is required for TAM-mediated partial activity

and that may be related to the cell type specific functionality of TAM (12). However, it is still not entirely clear how TAM manifests agonist activities through ER α WT in different tissues. We focused on the L543A and L544A mutations in the ER α AF-2 domain (AF2ER) to evaluate the ER α AF-1 and AF-2 functions in vivo and the SERM functionality in the tissues.

The ER α -KO (α ERKO) mouse is an established model for evaluating ER α function in vivo. The α ERKO does not express functional ER α protein as a result of a genetic modification of *Esr1* (13, 14). The α ERKO mouse model has revealed various physiological functions involving ER α (15). However, this model cannot discern the selective functionality of the ER α AF-1 or AF-2 in ER α -mediated physiological responses in vivo because no receptor protein is expressed. The aim of this study was to evaluate the physiological function of the ER α AF-1 and AF-2 in vivo. We developed a knock-in mouse model with the AF2ER mutations (AF2ERKI). In the AF2ERKI mice, we can definitively determine that the mutation of helix 12 inactivates the ER α -mediated response to endogenous estrogens, making the AF2ERKI mice comparable to the α ERKO mice. Our present studies confirmed that estrogen-induced AF-2 activation is critical for regulating female reproductive tissue hormone responses and AF-1 is not activated independently without AF-2. In addition, this report shows that the antiestrogens ICI182780 (ICI) or TAM can be shown to act as agonists in vivo involving AF2ER mutations. Our in vitro studies indicated the AF-1 activity of ER α is involved in ICI-mediated AF2ER activation and that activation is similar to the condition of TAM-mediated ER α WT transactivation. Therefore, the in vivo observation of ICI-mediated regulation suggests the ER α AF-1 mediates physiological functions in certain tissues and may represent tissue specific SERM functions.

Results

Properties of the L543A, L544A Mutant ER α . We demonstrated the differential functionality of the AF2ER (L543A, L544A mutated ER α) on the ER α -mediated transcription regulation with in vitro transient transfection assays using two different ER α -negative cell lines (HepG2 and HeLa cells; Fig. 1 and Fig. S1). First, we assessed estrogen response element (ERE)-mediated transcription activity. As expected, E2 produced strong activation of the ERE reporter with WT ER α . In contrast, the antiestrogen ICI was inactive with WT ER α but activated the ERE-mediated transcription of AF2ER in both cell lines. ICI-mediated AF2ER activation was more potent than E2 activity. This activation was not observed in the N-terminal truncated AF2ER (121-AF2ER)-transfected HepG2 cells, but very weak activity was observed in

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Fig. 1. Transcription function of AF2ER (L543A, L544A mutated ERα). (A) Schematic illustration of the ERα mutants. (B) HepG2 or HeLa cells were transfected with the reporter gene (3xERE-TATA-luc) and expression vectors for WT or mutated receptors were maintained with or without ligands. (C) HepG2 or HeLa cells were transfected with the reporter gene (7xAP1-TATA-luc) and expression vectors for WT or AF2ER. The cells were maintained with or without ligands. The luciferase activities for the each treatment were represented as fold change for the empty expression vector, pcDNA3 (no ERα). (D) Transrepression function of WT ERα and AF2ER. HeLa cells were transfected with the NF-κB reporter gene (3xMHC-luc), and expression vectors for p65/RelA and WT ERα or AF2ER were maintained with or without ligands. The luciferase activities were expressed relative to p65 activity in the absence of ligand (100%). (E) The effect of cofactors on ICI- or OHT-dependent AF2ER activation. HeLa cells were transfected with the 3xERE reporter gene and expression vectors for cofactors and AF2ER. The cells were maintained with or without ligands (100 nM E2, ICI, or OHT were used for treatments). Luciferase activity is represented as the mean $\pm$ SD of three independent experiments.

HeLa cells (Fig. 1B), indicating that the AF2ER mutant has minimized the AF-2 function and AF-1 is necessary for ICI-mediated AF2ER activation. Furthermore, to confirm the level of ERα AF-1 activity in these cells, C-terminal truncated ERα (ERα339), which activates ERE-mediated transcription irrespective of the presence or absence of ligand, was cotransfected with the ERE reporter. Coincidentally, the level of ERα339-mediated activation in these cells is similar to the level of ICI-mediated AF2ER activity (Fig. 1B), which suggests that the ICI-dependent AF2ER activity is derived from AF-1. Second, to assess AF2ER-mediated DNA-tethered transactivation, we tested AF2ER activity with an AP-1 reporter. ICI activated the AP-1-mediated transcription through WT ERα as previously reported (16). However, AF2ER did not activate the AP-1-mediated transcription with any ligands [E2, ICI, and 4-hydroxy-TAM (OHT); Fig. 1C]. Furthermore, we assessed the transrepression activity of AF2ER in NF-κB-dependent gene activation. AF2ER did not repress the NF-κB-mediated transcription (Fig. 1D). These results suggest that AF2ER is likely to predominantly regulate the ERE-mediated transcription. OHT, a known ERα AF-1-activating partial antagonist, weakly activated WT ERα and activated AF2ER, but to a lesser extent than ICI (Fig. 1B). Therefore, we used ICI for the further experiments. To determine if coregulators for WT ERα were involved in the ICI agonist activity of the AF2ER, expression vectors for coregulators were cotransfected, and p300, CREB binding protein (CBP), and SRC1a were found to increase the ICI-mediated AF2ER activity in HeLa and HepG2 cells (Fig. 1E and Fig. S1A, Top). We also determined the effect of coregulators on the OHT agonistic activity of WT ERα. Surprisingly, the profile of coregulators' effect on OHT-bound WT ERα shows an almost identical pattern to ICI-liganded AF2ER (Fig. S1A, Middle, and Fig. S1B, Top). In this condition, however, the profile of coregulators effect on E2-bound WT ERα is different from OHT-bound WT ERα (Fig. S1A, Bottom, and Fig. S1B, Bottom). The p300/CBP coactivators also increased ERα339-mediated ERE activation regardless of the presence or absence of ICI, but did not enhance 121-AF2ER-mediated activity (Fig. S1C and D). SRC1a slightly enhanced 121-AF2ER activation but did not alter ERα339 transactivation (Fig. S1C and D). Furthermore, we found an additive effect of p300/CBP together with SRC1a on the ICI-dependent AF2ER activation

that was observed in ERα339- but not 121-AF2ER-mediated activation. A similar activation profile was observed for OHT-mediated WT ERα activation (Fig. S1E and F). These results suggest that ICI liganded AF2ER is likely to mimic the OHT-mediated WT ERα activation.

Generation of AF2ERKI Mice. To assess the effect of loss of AF-2 function in vivo, we generated AF2ERKI mice through homologous recombination in mouse ES cells. The construct targeted the ninth exon of mouse *Esr1*, which contains helix 12, possessing the AF-2 region of the LBD as well as the stop codon. Leucines 543 and 544 were mutated to alanines, and a 6xHis-tag was added to the C-terminal end of the mouse ERα protein (Fig. 2A, *SI Materials and Methods*, and Fig. S2). The expression of AF2ER mutant protein in the AF2ERKI mice was evaluated by Western blot analysis of uterine tissues by using the anti-mouse ERα antibody and anti-His-tag antibody. The uterine tissue was collected from AF2ERKI homozygote (AF2ER^{KI/KI}), heterozygote (AF2ER^{KI/+}), and WT female mice. As shown in Fig. 2B, we detected a 66-kDa signal using the anti ERα antibody. After stripping, the membrane was probed with the anti-His-tag antibody. A 66-kDa signal was detected in the AF2ER^{KI/KI} but not in WT, and a faint signal was detected in the AF2ER^{KI/+}. Furthermore, we performed immunohistochemistry using the AF2ER^{KI/KI} and WT female uteri. As shown in Fig. 2C, anti-ERα antibody-derived signal was detected in all the tissue compartments of the uterus, including the endometrial epithelial cells and stromal cells of both genotypes. Taken together, these results indicate that the mutant AF2ER protein is being expressed in the mice.

Phenotype of AF2ERKI Female Is Similar to That of αERKO Female. Continuous breeding studies indicated that AF2ER^{KI/KI} female mice were infertile. We evaluated the estrous cycle of WT, AF2ER^{KI/+}, and AF2ER^{KI/KI} female mice by daily vaginal lavage for nine consecutive days. WT and AF2ER^{KI/+} female mice showed a normal estrous cycle. On the contrary, the AF2ER^{KI/KI} female mice showed no estrous cycle (Fig. S3). Another endpoint linked to reproductive cyclicity and responsiveness is serum luteinizing hormone (LH) levels. The serum LH level of AF2ER^{KI/KI} (5.39 $\pm$ 2.33 ng/mL) was elevated fourfold vs. WT (1.29 $\pm$ 2.12 ng/mL), and the serum E2 level of AF2ER^{KI/KI} (96.8 $\pm$ 10.1 pg/mL)

Fig. 2. Targeting strategy and confirmation of L543A, L544A ERα knock-in mutation. (A) Schematic illustration of the targeting strategy used to introduce mutation. Diagrams show the WT ERα locus, targeting construct, targeted mutant allele in the ES cells/chimera mice, and F1 mutant allele after ACN cassette self-excision. The targeting construct contained ERα exon 9 (light gray boxes show coding sequence and dark gray boxes shows 3' UTR), the L543A, L544A mutations ("mutation"), an extra XbaI site (Xb), and a 6xHis-tag epitope (6xHis). The ACN cassette was flanked at the 5' and 3' ends by loxP sites (closed arrowheads). Open box suggests the position of 5' external probe for Southern blot (5'Ex), and pairs of open arrowheads suggest PCR primer sets for 3' external PCR (3'Ex PCR), His-tag PCR, and PCR genotyping. D, DrrdI; Xm, XmnI; B, BamHI; Xh, XhoI; H, HindIII. (B) Representative results of Western blot probed for the ERα, His-tagged ERα (His-Tag), and β-tubulin in the 8-wk-old individual mouse uterus are shown. β-Tubulin was used as a loading control. +/+, WT; KI/+, heterozygote; KI/KI, homozygote. (C) Uterine ERα immunohistochemistry of 8-wk-old representative mice. (D) Morphology of AF2ERKI female reproductive organs in the 8-wk-old representative mice. (E) Histology of 8-wk-old representative AF2ERKI female mice. Uterine (Top) and ovarian (Middle) tissue H&E staining from WT (Left) and AF2ERKI homozygote (Right) mice. (Scale bar: 100 μm.) Mammary gland (Bottom) whole-mount Carmine alum staining from 8-wk-old representative mice. (Scale bar: 1 cm.) (F) The mRNA level of *Foxa2* was quantified by real-time PCR. The mRNA levels were represented as fold change for the WT. Values are presented as mean ± SD.

was twofold higher than WT (44.2 ± 9.43 pg/mL). These hormone levels were similar to those from a pool of αERKO serum from several female mice (LH, 5.78 ng/mL; E2, 87.6 pg/mL). Such elevated LH levels suggested that the negative feedback of the hypothalamic–pituitary–gonadal axis in AF2ER^{KI/KI} is disrupted, as has been reported in the αERKO animals (17), consistent with the infertility. Female reproductive tract organs from WT and AF2ER^{KI/KI} mice were analyzed for morphological and histological parameters. AF2ER^{KI/KI} uteri were significantly smaller and more hypoplastic than those of their WT littermates (Fig. 2D). AF2ER^{KI/KI} uterine tissues possess luminal epithelium, but fewer glandular structures were evident compared with WT uteri (Fig. 2E, Top). We evaluated the expression level of uterine *Foxa2*, a gene that is implicated in uterine gland development (18). The level of uterine *Foxa2* mRNA was significantly lower in AF2ER^{KI/KI} than WT (Fig. 2F), which is consistent with less glandular morphology. The ovaries from the AF2ER^{KI/KI} female mice show cystic and hemorrhagic follicles, reminiscent of the αERKO ovarian phenotype. A few primary follicles can be seen, but no corpora lutea were observed in AF2ER^{KI/KI} ovaries (Fig. 2E, Middle). Mammary gland whole-mounts were analyzed for evidence of ductal proliferation and differentiation. In 8-wk-old WT mice, ductal trees extended past the lymph node and had enlarged terminal end buds. AF2ER^{KI/KI} mammary glands never developed beyond a rudimentary epithelial ductal tree (Fig. 2E, Bottom), similar to αERKO females. Taken together, these results clearly suggest that estrogen-dependent AF-2-mediated transactivation of ERα is essential for developing and maintaining female reproductive functions.

Antiestrogens ICI and TAM Induce Uterotropic Responses in AF2ERKI Homozygote. Based on our in vitro studies, to assess the functionality of AF2ER in vivo, ovariectomized (OVX) AF2ER^{KI/KI} female mice were injected in a 3-d bioassay with vehicle, ICI, TAM, or E2, and uteri were collected 24 h after the last treatment. E2 and TAM significantly induced the uterine growth of WT mice, and ICI treatment was ineffective. In contrast, ICI and

TAM treatment increased the uterine wet weight of AF2ER^{KI/KI} mice (Fig. 3A and B and Fig. S4). We detected the incorporation of 5-ethynyl-2'-deoxyuridine (EdU) into newly synthesized DNA in the ICI- and TAM-treated AF2ER^{KI/KI} uterine endometrial epithelial cells (Fig. 3C), suggesting that ICI or TAM induces the proliferation of AF2ER^{KI/KI} endometrial epithelial cells. Furthermore, we evaluated the regulation of some uterine estrogen-responsive genes—lactotransferrin (*Ltf*) (19), insulin-like growth factor 1 (*Igf1*) (20), and cytochrome c oxidase subunit VIIa polypeptide 2-like (*Cox7a2l*) (21)—by quantitative PCR. As shown in Fig. 3D, the expression of these genes was regulated by E2 and TAM in the WT uterus and not by ICI treatment. In contrast, the expression of those genes was regulated by ICI and TAM in the AF2ER^{KI/KI} uterus but not by E2. These results indicate that the functionality of the AF2ER mutant in the in vivo AF2ERKI mouse model is similar to the in vitro findings. We also assessed the AF2ER function in the receptive uterus model. In the receptive uterus, estrogen induces stromal cell but not epithelial cell proliferation (22). We detected that ICI increased proliferation of stromal cells but not epithelial cells in the AF2ER^{KI/KI} uterus, as did E2 in WT (Fig. S5).

One of the major antagonistic effects of ICI has been shown to result from loss of ERα protein in vivo and in vitro, which involves a proteasome-mediated proteolysis of ERα (23, 24). As ICI was an agonist in AF2ER^{KI/KI}, we examined the effect of ICI on the level of AF2ER protein by Western blot. The level of AF2ER protein was not reduced, whereas the level of WT ERα was markedly reduced, after ICI treatment (Fig. 3E). It appears that, in contrast to WT, the pure antiestrogen does not accelerate the turnover and loss of the AF2ER protein.

ICI and TAM Do Not Regulate Pituitary Gene Expression in AF2ERKI Homozygote. It is known that ovariectomy releases negative feedback, resulting in increased serum LH level. Additionally, E2 replacement down-regulates serum LH level. As expected, serum LH levels were high in the vehicle-treated OVX WT female mice and were decreased by E2 treatment to 25% of the vehicle level.

Fig. 4. Functional difference of AF2ER in the AF2ERKI mouse pituitary. (A) Serum LH levels were determined after vehicle (Veh), ICI (2 mg/kg), TAM (2 mg/kg), or E2 (10 μ g/kg) treatments for three consecutive days. Values are presented as mean $\pm$ SEM; * P < 0.05 vs. vehicle in each genotype. The pooled serum from the non-OVX WT and α ERKO female mice was used as reference. (B) The mRNA level of *Lhb* was quantified by real-time PCR. (C) The mRNA level of *Prl* was quantified by real-time PCR. The mRNA levels are presented as fold change for vehicle of WT. Values are presented as mean $\pm$ SD; * P < 0.05 vs. vehicle in each genotype. (D) The mRNA level of *Pit1* was quantified by real-time PCR. The mRNA levels are presented as fold change vs. WT. Values are presented as mean $\pm$ SD. (E) Representative results of Western blots probed for the ER α , His-tagged ER α , and β -tubulin from WT and AF2ER homozygote individual mouse uteri and pituitaries are shown.

increased AF-1 activity but not AF-2 activity. On the contrary, SRC1a increased ICI-dependent AF-2 activity modestly, but not that of AF-1. These results may suggest that the ICI-liganded AF2ER induces modest activation of AF-2 and may involve enhancing the AF-1 activity in such cells or tissues. It may explain the differential tissue response of AF2ER with ICI. We also tested the OHT-mediated WT ER α activation to compare ICI-mediated AF2ER activation because the OHT is known as a WT ER α AF-1-activating SERM (27). In our experimental condition, the activation functions of coregulators for E2 or OHT bound WT ER α were different. This result may suggest that OHT-bound WT ER α makes a different coactivator interface for E2-bound WT ER α (28). Interestingly, the profile of coactivators' effect on ICI-mediated AF2ER activation is almost identical to that of OHT-mediated WT ER α activation. These results suggest that the ICI-liganded AF2ER is mimicking the OHT-bound WT receptor. To analyze the involvement of co-regulators in the ICI-dependent AF2ER activation of various tissues, it will be helpful to understand AF-1-dependent tissue-selective functionality of SERMs.

We confirmed that the AF2ERKI mouse expresses the AF2ER mutant ER α protein in the uterus (Fig. 2) and pituitary (Fig. 4) by identifying the His-tag incorporated into the knock-in allele, using an anti-His-tag antibody. Even though the in vitro analysis indicated weak activation of AF2ER by E2, the phenotype described herein of the AF2ERKI female is similar to that of the α ERKO female (15), indicating that the mutation of AF-2 is unable to mediate physiological responses to E2. We also confirmed that treatment with a pharmacological level of E2 (2 mg/kg) does not induce the uterotrophic growth in the AF2ER^{KI/KI} female (Fig. 3 and Fig. S4). These results clearly suggested that the AF-1 function is not activated by E2 independently. Our preliminary results suggest that growth factors (IGF-1 or EGF) are unable to activate uterine proliferation, specifically DNA synthesis, as reflected by lack of incorporation of the deoxyuridine analogue EdU in the AF2ER^{KI/KI} female (Fig. S6A). This result is different from those in a similar mouse model in which the ER α AF-2 is mutated: the ENERKI mouse. The ER α in the ENERKI mouse is mutated in the ligand-binding pocket (G525L), is unable to bind E2, and exhibits reduced E2-dependent transcription activation while maintaining helix 12 structure (29). In the ENERKI homozygous female, IGF-1 induced the proliferation of uterine endometrial epithelial cells without E2, as reflected by an increase in the general cell cycle marker Ki67. However, progression to S-phase as reflected by

DNA synthesis was not evaluated in the ENERKI mouse. These differences between AF2ERKI and ENERKI may suggest that helix 12 also contributes toward regulating the growth factor-mediated ER α activation.

It has been suggested from in vitro cell studies that the differential functionality of AF-1 in different cell types and on different gene promoters is an explanation for the mechanism of SERM action (12, 30). We demonstrated here that TAM regulated uterine responses (Fig. 3) and serum LH level but not pituitary *Prl* gene expression in the OVX WT female (Fig. 4). On the contrary, ICI or TAM regulated uterine responses in the OVX AF2ERKI female, like the response seen in TAM-treated WT female mice (Fig. 3); however, it had no response in the pituitary (Fig. 4). Differential tissue actions of ER α have been proposed as being dependent on the ER α isoforms ER α 66 and ER α 46 (31). ER α 66 is the full-length ER α (harboring AF-1 and AF-2) and ER α 46 is the shorter AF-1-deficient isoform. In this report, we confirmed that the full-length ER α is expressed in the uterus and pituitary by using the anti-ER α antibody and anti-His-tag antibody (Fig. 4). This result clearly suggests that the differences in the TAM-mediated transcriptional activity of the ER α in the uterus and pituitary are results of the differential AF-1 functionality, not differential isoform action.

The TAM responses in WT female pituitary suggest that TAM affects the pituitary functions differently than E2. A study that used the ER α ^{-/-} (NERKI) mouse, which lacks ERE-dependent transactivation but retains ERE-independent activity (32), identified that the negative feedback action of estrogen on LH is mediated by ERE-independent mechanisms and that *Prl* gene regulation is mediated by ERE-dependent mechanisms (33). Taken together, these findings suggested that TAM affects ERE-independent responses (serum LH regulation) but not ERE-dependent actions (*Prl* gene regulation) in the pituitary. This result implies that the ER α AF-1 is not functioning to regulate *Prl* gene expression. Neither ICI nor TAM were able to regulate any responses in AF2ERKI female pituitary (Fig. 4). Our in vitro results indicated that the AF2ER mutant regulates ERE-mediated but not non-ERE-mediated transcription (Fig. 1). These findings suggest that our results are consistent with previous reports and the AF2ERKI mouse model is able to distinguish the differential TAM functionality in tissues in vivo.

Recently, Börjesson et al. reported generation of AF-1 truncated mouse (ER α AF-1⁰) and helix 12 (AF-2) truncated mouse (ER α AF-2⁰) models to evaluate the in vivo functionality of ER α AF-1 and AF-2 (34). The report used a loss-of-function approach of truncated

mutants for comparison with WT ER α and observed varied E2 responses in different tissues of the ER α AF-1⁰, but no responses to E2 in the ER α AF-2⁰ tissues, similar to α ERKO mice lacking the receptor protein (34), indicating that ER α AF-1 function is tissue dependent and AF-2 is required for the estrogenic responses. Our AF2ERKI phenotypes and differential stimulated tissue activities with ICI or TAM would be consistent with those observations and allow assignment of a differential activity of female neuroendocrine and reproductive tract tissues. Our preliminary results suggested that raloxifene, a new-generation SERM, does not activate AF2ER function in vitro (Fig. S6B) and in vivo uterine response (Fig. S6C). Our results so far suggest that AF2ERKI mice can experimentally demonstrate tissue selective function and response of ER α AF-1 in estrogen target tissues for evaluating tissue-selective SERM functions and identifying the beneficial actions of SERMs as potential hormonal therapeutic agents.

Materials and Methods

Cell Culture and Luciferase Assay. Cells were cultured in phenol red-free medium supplemented with 10% charcoal-stripped FBS for transient transfection. The cells were transfected for 6 h, then changed to fresh media supplemented with 100 nM of E2, ICI, or OHT. Luciferase assay was performed 18 h after treatments. Details are described in *SI Materials and Methods*.

Generation of Knock-In Animals. A targeting vector containing mouse ER α exon 9 with an additive XbaI site in exon 9, an 18-bp 6xHis-tag epitope sequence, the L543A and L544A mutations, and the ACN cassette was used for the selection of ES cells. The targeted ES clones were injected into C57BL/6 blastocysts to generate chimeric mice. Male chimeras were bred to C57BL/6 female mice to establish germline transmission, and the resulting heterozygous mice were interbred. Further details are described in *SI Materials and Methods*.

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ER Ligand Treatments. OVX WT or AF2ERKI female mice ($n = 6$ per group) were injected s.c. daily for 3 d with 10 μ g/kg or 2 mg/kg E2 dissolved in sesame oil, intraperitoneally with 2 mg/kg ICI, or 2 mg/kg TAM dissolved in DMSO. DMSO (50 μ L) was used as vehicle in all experiments. The tissues and serum were collected 24 h after the last injection. All procedures involving animals were conducted in accordance with National Institutes of Health guidelines and were in compliance with a National Institute of Environmental Health Sciences-approved animal protocol.

Quantitative PCR. For quantitative PCR, tissues were homogenized and total RNA was extracted. The RNA was reverse-transcribed, and then real-time PCR was performed. The primer sets are described in Table S1. Samples were analyzed in triplicate, and the 18s rRNA was used as an internal control for all experiments.

Statistical Analysis. Statistical analysis was performed with two-way ANOVA with GraphPad Prism software (GraphPad), and a P value lower than 0.05 was considered statistically significant. Other methods are described in *SI Materials and Methods*.

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