Mutation of the palmitoylation site of estrogen receptor α in vivo reveals tissue-specific roles for membrane versus nuclear actions

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Estrogen receptor alpha (ERα) activation functions AF-1 and AF-2 classically mediate gene transcription in response to estradiol (E2). A fraction of ERα is targeted to plasma membrane and elicits membrane-initiated steroid signaling (MISS), but the physiological roles of MISS in vivo are poorly understood. We therefore generated a mouse with a point mutation of the palmitoylation site of ERα (C451A-ERα) to obtain membrane-specific loss of function of ERα. The abrogation of membrane localization of ERα in vivo was confirmed in primary hepatocytes, and it resulted in female infertility with abnormal ovaries lacking corpora lutea and increase in luteinizing hormone levels. In contrast, E2 action in the uterus was preserved in C451A-ERα mice and endometrial epithelial proliferation was similar to wild type. However, E2 vascular actions such as rapid dilatation, acceleration of endothelial repair, and endothelial NO synthase phosphorylation were abrogated in C451A-ERα mice. A complementary mutant mouse lacking the transactivation function AF-2 of ERα (ERα-AF2) provided selective loss of function of nuclear ERα actions. In ERα-AF2, the acceleration of endothelial repair in response to estrogen–dendrimer conjugate, which is a membrane-selective ER ligand, was unaltered, demonstrating integrity of MISS actions. In genome-wide analysis of uterine gene expression, the vast majority of E2-dependent gene regulation was abrogated in ERα-AF2, whereas in C451A-ERα it was nearly fully preserved, indicating that membrane-to-nuclear receptor cross-talk in vivo is modest in the uterus. Thus, this work genetically segregated membrane versus nuclear actions of a steroid hormone receptor and demonstrated their in vivo tissue-specific roles.

Although estrogens classically serve as reproductive hormones, they induce cellular responses in almost all tissues in mammalian species. The biological effects of estrogens, and particularly of 17β-estradiol (E2), are initiated by their binding to intracellular estrogen receptors (ERs), ERα and ERβ, which classically serve as nuclear transcription factors (1, 2). The ERs regulate the transcription of hundreds of genes in a cell- and tissue-specific manner through their two activation functions (AFs), AF-1 and AF-2. The roles of the activation functions of ERα have been studied in vivo using mice deleted for ERα AF-1 or ERα AF-2 (3–5). These results, in particular the two models of ERαAF-2 inactivation (4, 6), suggested that many physiological functions strongly rely on nuclear ERα and gene transcription regulation. However, in addition to the nuclear, termed “genomic actions of ER,” the receptors stimulate rapid (from seconds to minutes), nonnuclear signal transduction, usually termed “nongenomic” or “extranuclear” effects. The rapid mobilization of intracellular calcium and the generation of cAMP by E2 were demonstrated several decades ago (7, 8). More recently, the modulation of potassium currents, phospholipase C activation, the increase in endothelial nitric oxide production, and the stimulation of protein kinase pathways (PI3K/Akt, Erk) have been described (9–13). These rapid effects have been attributed to cell membrane-initiated steroid signaling (MISS) by a subpopulation of receptors associated with the plasma membrane.

In the plasma membrane, ERα has been localized to caveolae/lipid rafts by direct binding to caveolin-1 through Ser-522 or indirectly via the scaffold protein striatin, forming complexes with G proteins (Gq and Gs) (14–17). In addition, Cys-447 of human ERα is a site of palmitoylation that promotes plasma membrane

Significance

The in vivo roles of plasma membrane-associated estrogen receptor (ER)α, including cross-talk with nuclear ERα, are poorly understood. We created a mouse with a point mutation of the palmitoylation site of ERα (C451A-ERα) to obtain membrane-specific loss of function. A complementary mouse lacking the ERα activation function AF-2 (ERα-AF2) provided selective loss of function of nuclear ERα actions. Physiologic studies revealed critical requirements for membrane receptors in ovarian function and thereby in fertility, and in vascular physiology. In contrast, nuclear ERα actions mediate uterine responses to estrogen and genome-wide analysis indicates that membrane-to-nuclear receptor cross-talk in vivo is quite modest in uterus. These findings demonstrate for the first time critical tissue-specific roles for membrane versus nuclear actions of a steroid hormone receptor in vivo.
association of the receptor (18). A nonpalmitoylatable Cys447Ala mutant form of ERα, or its C451A mutant mouse counterpart, expressed in cultured cells lacks interaction with caveolin-1 and downstream activation of signaling pathways and cell proliferation (18, 19). Numerous cellular culture experiments further suggest potentially important kinase-mediated cross-talk between membrane and nuclear ERα that modifies genomic responses to E2 (20, 21), including recent studies revealing that MAPK dependent on receptor palmitoylation influences receptor nuclear actions (22). Our current understanding of these processes has relied primarily on experimentation in cell culture. As a result, the in vivo roles of membrane-associated ERα in numerous physiologic processes are poorly understood.

Using a pharmacological approach in cell-based assays, Harrington et al. (23) synthesized estrogen–macromolecule conjugates (EDCs) to provide a gain-of-function strategy. EDC consists of estrogen attached to a large, positively charged non-degradable poly(amide)amine dendrimer via hydrolytically stable linkages. Studies in breast cancer cells clearly showed that EDC was highly effective in stimulating nonnuclear signaling but inefficient in stimulating nuclear ER target gene expression because EDC does not enter the nucleus (23). Importantly, in vivo administration of EDC fully stimulated carotid artery reendothelialization but not uterine proliferation, suggesting for the first time that activating nonnuclear ERα signaling was sufficient to promote beneficial vascular effects of estrogen (24). Membrane only ERα mRNA expressing the ERα E-domain under the CytoMegaIsoVirus promoter in an ERα/− background were also generated, but the vascular phenotype, such as NO production or reendothelialization, was not assessed in this model (25).

To investigate the physiologic importance of membrane-initiated ERα actions in vivo, we created a knock-in mouse model with a selective loss of function of membrane ERα action by mutating the palmitoylation site at mouse ERα Cys451 to Ala (designated C451A-ERα). A complementary mutant mouse model lacking the activation function AF-2 of ERα (designated ERα-AF2(6)) (4, 6) provided a selective loss of function of nuclear ERα actions. Physiological studies in these two models revealed critical requirements for membrane receptors in ovarian function and thereby in fertility, and in vascular physiology. In contrast, genome-wide analysis of uterine gene expression demonstrated that nuclear ERα actions mediate uterine responses to estrogen, and also that membrane-to-nuclear receptor cross-talk in vivo is modest in this tissue. These findings demonstrate critical tissue-specific roles for membrane versus genomic actions of a steroid hormone receptor in vivo.

**Results**

The C451A Mutation of ERα In Vivo Alters the Membrane Localization of ERα. Compared with the three other cysteines present in the ligand-binding domain of ERα, C447/451 is the least reactive to iodoacetic acid, suggesting a nonexposed position of this amino acid (26), and consequently the least likely candidate for lipid conjugates (EDCs) to provide a gain-of-function strategy. EDC consists of estrogen attached to a large, positively charged non-degradable poly(amide)amine dendrimer via hydrolytically stable linkages. Studies in breast cancer cells clearly showed that EDC was highly effective in stimulating nonnuclear signaling but inefficient in stimulating nuclear ER target gene expression because EDC does not enter the nucleus (23). Importantly, in vivo administration of EDC fully stimulated carotid artery reendothelialization but not uterine proliferation, suggesting for the first time that activating nonnuclear ERα signaling was sufficient to promote beneficial vascular effects of estrogen (24). Membrane only ERα mRNA expressing the ERα E-domain under the CytoMegaIsoVirus promoter in an ERα/− background were also generated, but the vascular phenotype, such as NO production or reendothelialization, was not assessed in this model (25).

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**As an initial characterization of this mouse model, we evaluated the expression of ERα protein in various organs from littermate wild-type (WT-C451) and mutant C451A-ERα mice (Fig. 1 A–C). ERα protein abundance in the uterus, aorta, and hepatocytes was similar in the two groups. Plasma membranes were then prepared from primary cultures of hepatocytes that can be obtained in sufficient quantities to allow sucrose gradient isolation of plasma membranes. Whereas ERα protein was abundant in plasma membrane fractions (2–4) prepared from WT-C451 hepatocytes, a 60% decrease was observed in fraction 4 of the mutant C451A-ERα hepatocytes (Fig. 1D). Functionally, we also found that phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) was increased twofold in response to E2 in WT-C451 mice but unchanged in C451A-ERα (Fig. 1E). These data reveal that the C451A mutation effectively alters the plasma membrane localization of ERα in vivo, which further abrogates the signaling pathway.**

**Membrane-Associated ERα Is Required for Ovarian Function.** When eight C451A-ERα female mice were mated with WT-C451 males, no litters were observed despite continuous mating over a 5-mo period. To investigate the basis of this infertility, histomorphologic analysis was performed on ovaries from 10- to 12-wk-old female mice. There was an excess of large, hemorrhagic, and/or cystic follicles originating from antral follicles in C451A-ERα ovaries, along with an almost total absence of typical mature corpora lutea (Fig. 2 A and B). Counting of the number of follicles at different stages revealed that ovaries from C451A-ERα mice displayed reduced frequency of primordial follicles but normal percentages of primary, secondary, and tertiary follicles (Fig. S2A). Wall composition of the tertiary follicle was also altered, with a decrease in the percentage of the follicle area composed of granulosa cells, whereas the percentage of the thecal cell area was conserved (Fig. S2B). Additionally, there was a marked reduction in the density of glandular interstitial cells when WT-C451 were compared with C451A-ERα ovaries, which appeared loosely arranged and associated with areas of hemorrhage.

**Fig. 1.** Validation of the C451A-ERα mouse model. (A) ERα protein levels in uterus, aorta, and hepatocytes homogenates from ovariectomized WT-C451 and C451A-ERα mice. (B) Representative Western blot of plasma membrane-associated ERα isolated from WT-C451 or C451A-ERα mice. (C) When ERα C447/451 occurs before ligand binding, when the ligand-binding domain is less tightly folded (27). **Fig. S1A** displays a model of palmitoylation of C447/451 showing that despite the buried nature of this residue a considerable portion of the attached palmitoyl chain extends beyond the surface of the ERα ligand-binding domain, and therefore could function as a membrane tether. To study the role of MIISS actions of ERα, a knock-in strategy was used in which the palmitoylation site Cys451 was mutated to alanine using a targeting construct containing two base pair changes in exon 7 (Fig. S1B). The C451A mutation was confirmed by PCR using tail DNA (Fig. S1C).
We also measured steroid sex hormone levels in intact 10- to 12-wk-old female mice (Fig. 2C). C451A-ERα mice exhibited serum E2 levels similar to those of WT-C451 mice, whereas progesterone levels were markedly decreased in mutant mice. The level of FSH tended to be higher (P = 0.067) and luteinizing hormone (LH) was markedly increased (P = 0.0005) in C451A-ERα mice in comparison with WT-C451 mice. Collectively, these findings indicate that membrane ERα is required for normal ovarian morphology and function.

Membrane Actions of ERα Are dispensable for Uterine Responses to Estrogen. The uteri of intact WT-C451 and C451A-ERα mice were found to be normal in gross appearance (Fig. 3A). The uterotrophic response to E2 was also similar in the two genotypes when the uterine wet weights were compared in ovariec-tomized female mice treated or not for 28 d by E2 (8 μg/kg per day), n = 4-6. Serum E2, progesterone (PG), FSH, and LH concentrations in 3-mo-old WT-C451 and C451A-ERα mice, n = 9.

Gene Expression Profiling Analysis Reveals Differential Roles of Membrane Versus Nuclear ERα in Gene Regulation. In contrast to the C451A mutation, previous studies in mice lacking the ERα activation function AF-2 (ERα-AF20) revealed that the uterotrophic response to E2 is entirely dependent on AF-2 (4, 6). We therefore compared the gene expression profiles in the uteri of C451A-ERα and ERα-AF20 mice (4), including littermate controls, 6 h after administration of the vehicle versus E2 (8 μg/kg) using pangenomic microarrays.

The interaction between genotype and treatment effect is shown in the heat map in Fig. 4A (P < 0.05). Cluster analysis of all of the conditions tested identified two major patterns of gene expression. The first cluster contained genes regulated by E2 in both WT-C451 and C451A-ERα mice. The patterns of genes expressed in all of the vehicle-treated animals were very similar, and they closely resembled the gene pattern observed in E2-treated ERα-AF20 mice, demonstrating an absence of transcriptional response to E2 in ERα-AF20 mice, at least in this tissue. Accordingly, E2 affected a very high number of genes in the two sets of wild-type mice: 17,248 genes in WT-AF2 and 18,112 genes in WT-C451 (Fig. S4A). The slight differences may be due to the fact that the wild-type mice are, respectively, on C57BL/6N and C57BL/6J background. More importantly, 16,659 genes were altered by E2 treatment in C451A-ERα
mice, with 14,907 commonly regulated in WT-C451, which represents 82% of the genes targeted by E2 in WT-C451 mice. In contrast, only 800 genes were regulated by E2 in ERα-AF2A mouse, with 640 genes in common to those altered by E2 in WT-AF2-α mouse (3.7%).

A Venn diagram was generated to assess the overlap of up- and down-regulated genes found in the genotype and treatment groups. The vast majority of genes (7,551 and 7,201 genes in groups C and D) were respectively up-regulated or down-regulated in a specific ERα-AF2-dependent manner (Fig. 4C). In contrast, a small number of genes were regulated specifically in an ERα-C451 palmitoylation site-dependent manner (35 up-regulated and 67 down-regulated, groups A and B). Finally, a very limited number of genes was either repressed or activated by E2 via both AF2- or C451-dependent processes in the same or opposite manner (groups E–H). Therefore, there is modest, if any, cross-talk between the two receptor populations in uterus.

Gene ontology analysis (Fig. S4B and Dataset S1) indicated that the limited number of palmitoylation-dependent genes (groups A and B) are involved in membrane-, transmembrane-, or extracellularly related processes or lipid transport pathways. The large number of AF2-dependent genes (groups C and D) are primarily involved in nuclear, phosphoprotein-related, or splicing events.

Altogether, the genome-wide analysis of the uterus response to E2 demonstrates that most of gene regulation by ERα is related to AF2-dependent, transcripational nuclear processes, whereas membrane ERα signaling has a minor impact, affecting only a very limited number of genes in this tissue.

Membrane-Initiated Vascular Actions of ERα Are Abrogated in C451A-ERα Mice. The membrane-initiated actions of E2 have been particularly explored in the vasculature, where estrogen can acutely induce vasodilation in humans (28), at least in part through the rapid stimulation of endothelial nitric oxide synthase (eNOS) activity in an ERα-dependent manner (29, 30). Studies using EDC, which selectively activates nonnuclear ERα, have also previously suggested that nonnuclear ERα signaling is important for the endothelial effects of E2 in vivo (24). We then evaluated the vasodilatory effects of both E2 and EDC in WT-ERα and C451A-ERα mice by measuring dilatory responses of isolated phenylephrine-preconstricted mesenteric arteries (Fig. S5A). Within minutes, E2 (1 μM) increased the diameter of arteries from wild-type mice by 32%, whereas there was no significant vasodilatory response in arteries from ERα−/− or C451A-ERα mice (Fig. S5A). Therefore, there is modest, if any, cross-talk between the two receptor populations in uterus.

To directly evaluate how ERα C451 palmitoylation and the resulting plasma membrane targeting of the receptor does affect a functional response of endothelial cells to E2, endothelial cell migration was tested in scratch assays using primary cells isolated from the aorta of WT-C451 and C451A-ERα mice. Indeed, activation of endothelial cell migration by E2 is mediated by plasma membrane-associated ERα coupled to eNOS via Gαd (24). E2 stimulated migration of WT-C451 endothelial cells, but not of C451A-ERα endothelial cells (Fig. 5C). The role of membrane-associated ERα in the vascular actions of E2 was then investigated in vivo by evaluating reendothelialization of the carotid artery (Fig. 5D). E2 treatment caused an increase in endothelial repair in control WT-C451mice, whereas it had no effect in C451A-ERα mice (Fig. 5D).

Previous cell culture studies indicated that disrupting the palmitoylation of ERα resulted in a receptor that is more sensitive to E2-dependent degradation (22). We therefore evaluated possible alterations in ER abundance, in presence of E2 treatment, in the arteries of C451A-ERα mice. Indeed, ERα and ERβ mRNA expression was assessed in aorta from ovariectomized C451A-ERα and WT-C451 mice treated with vehicle versus E2 (8 μg/kg) for 6 h or 4 wk (Fig. S5B). Aortas from both vehicle and E2-treated C451A-ERα mice displayed ERα and ERβ mRNA levels similar to those of WT mice. ERα protein abundance was also equivalent in aorta from untreated ovariectomized C451A-ERα and WT-C451 mice treated with vehicle versus E2 (8 μg/kg) for 6 h or 4 wk (Fig. S5B and C). Aortas from both vehicle and E2-treated C451A-ERα mice displayed ERα and ERβ mRNA levels similar to those of WT mice. ERα protein abundance was also equivalent in aorta from untreated ovariectomized C451A-ERα and WT-C451 mice, and a similar decrease in the amount of the receptor was observed after E2 treatment (80 μg·kg−1·d−1 for 2 wk) in the two genotypes (Fig. S5D). Thus, although previous in vitro experiments suggested that ERα palmitoylation influences the abundance of the receptor protein (22), this was not detected in vivo in arteries and uterus.

Altogether, these findings indicate that preventing membrane-initiated ERα signaling by targeting the palmitoylation site of ERα abrogates major vascular actions of E2, including rapid artery dilation and the promotion of endothelial repair, despite the presence of a global similar ERα arterial abundance.
Membrane-initiated Vascular Actions of ERα Are Conserved in ERα-AF20 Mice. The marked contrast between ERα function in the uterus of C451-ERα and ERα-AF20 mice suggests that parallel studies in the two models may reveal potential tissue-specific roles for nuclear versus nonnuclear receptors. We have previously shown that the activation function AF2 is dispensable for the accelerating effect of E2 on endothelial healing (4). Here, we further directly tested whether membrane-initiated endothelial effects of E2 are abrogated in C451A-ERα mice treated or not with E2 (10−8 M, 30 min), n = 6. Representative Western blot is shown. AU, arbitrary units. (C) Percentage of migration of primary endothelial cells treated or not with E2 (10−8 M, 16 h), n = 3–4. (D) Carotid artery reendothelialization in ovariectomized WT-C451 and C451A-ERα mice treated or not with E2 (80 μg kg−1 d−1, 2 wk), n = 8–10. For C and D, statistical results of two-way ANOVA are shown, reporting the statistical interaction between treatment and genotype.

Discussion

In the present study we investigated the physiological roles of a pool of ERα localized at the plasma membrane by mutating in mice the ERα C451 recognized as a key palmitoylation site in vitro. This genetic loss-of-function model revealed that ERα is absolutely required for plasma membrane location and signals that are critical for arterial effects of E2, including the stimulation of vasodilation and the promotion of endothelial repair, but also female fertility. In contrast, the uterine proliferation in response to E2 occurs independently of membrane ERα. Complementary studies in ERα-AF20 mice further demonstrated a preservation of membrane ERα action and vascular function in this model. Moreover, genome-wide analysis of uterine gene expression revealed that membrane ERα signaling has only a minor impact on gene expression in this sex target tissue. These observations reveal tissue-specific roles for membrane versus nuclear ERα in vivo. In that broader context, this work genetically segregated nonnuclear and nuclear actions of a steroid hormone receptor and demonstrated their tissue specificity.

We first determined whether mutation of the palmitoylation site of mouse ERα alters the expression level of the protein in vivo, because in vitro studies (22) had shown that such a mutated human ERα (C447A) was more sensitive to E2-dependent degradation. The mutated C451A-ERα protein was normal in abundance in the different murine tissues analyzed. With respect to the abrogation of vascular E2 effects in the C451A-ERα mouse, it was also important to demonstrate that the abundance of the C451A-ERα protein in the vasculature in vivo is normal under basal and E2-stimulated conditions. Furthermore, the anticipated decrease in plasma membrane association of the mutant protein in vivo mirrors in vitro observations (18, 19), and it validates the C451A-ERα mouse as a selective loss-of-function model attenuating membrane receptor localization.

Our study on the reproduction of the C451A-ERα mice demonstrates the crucial role of membrane ERα in female fertility. This is at least in part due to alterations in ovarian function, with the ovaries of adult C451A-ERα mice being almost completely devoid of corpora lutea and displaying a high number...
of hemorrhagic and/or cystic follicles, indicating that the maturation of follicles into corpora lutea (luteinization, which allows the transition to the next estrous cycle) is abnormal. The capacity for ovulation per se has not yet been directly evaluated in C451A-ERα mice. The absence of corpora lutea, which produce progesterone, likely underlies the low serum progesterone found in C451-ERα mice. Furthermore, the level of LH in intact mice was increased, suggesting that the control of LH production by the hypothalamic–pituitary axis is adversely affected in C451-ERα mice. Female mice deficient in either ERαAF1 or -2 (named ERα-AF1 and ERα-AF2, respectively) are also sterile, primarily owing to absence of uterine hypertrophy (3, 4, 6). Thus, both plasma membrane and nuclear actions of ERα are absolutely required for female fertility.

We then explored to what extent membrane ERα is important for estrogen vascular effects. We found that both E2 and EDC elicit a rapid vasorelaxant effect in isolated mesenteric arteries from wild-type mice, whereas these effects are lost in ERα−/− and C451A-ERα mice. eNOS1177 phosphorylation in response to E2 was also abolished in arteries from C451A-ERα mice, as was the accelerative action of E2 on endothelial healing both in vivo and in vitro. In the past, the selective membrane ER activator EDC was shown to be sufficient to stimulate endothelial cell and in vitro. In the past, the selective membrane ER activator EDC was shown to be sufficient to stimulate endothelial cell migration and the acceleration of reendothelialization (24). Furthermore, the accelerative action of endothelial repair by E2 or EDC in ERα-AF2 mice was preserved, demonstrating that the MISS effects are fully activable in this model. Altogether, the current findings indicate that the activation of membrane ERα is both necessary and sufficient to promote endothelial repair and contribute to the integrity of the endothelial monolayer.

In contrast, C451A-ERα mice also revealed that receptor palmitoylation and membrane targeting are dispensable for uterine growth responses to E2. Previous work showed that the selective activation of membrane ERα by EDC does not induce uterine hypertrophy (24), suggesting that MISS activation alone is not sufficient to promote a proliferative response in uterine epithelium. Using a loss-of-function strategy, we now show that MISS effects involving ERα palmitoylation are also not necessary. In contrast, previous studies have demonstrated the importance of genomic (i.e., transcriptional) activity of ERs in this process, because E2 does not induce a uterine proliferative response in C451A-ERα mice (5, 6).

We further explored the gene expression profiles of ERα-AF2 and C451A-ERα mice in the uterus, a tissue that is highly responsive to E2. Genome-wide gene array studies revealed that, in contrast to nuclear ERα-mediated processes, membrane ERα-mediated signaling has only a minor impact on gene expression. A small set of genes was found to be regulated in the uterus by both membrane and nuclear ERα. Altogether, we can propose that C451A-ERα and ERα-AF2 mice can be considered as mouse models of membrane ERα and nuclear ERα loss of function, respectively, but with preservation of nuclear ERα and membrane ERα, respectively.

Cell culture studies (18, 19, 22, 32) previously suggested that rapid E2-dependent signaling may modify ERα transcriptional activity and thereby modulate final receptor-dependent nuclear actions, representing cross-talk between nonnuclear and nuclear receptor subpopulations. The function of many transcription factors is regulated through protein kinase-mediated phosphorylation, and these transcription factors may be targets for extranuclear actions of estrogen (20, 33). An important role for ERα MISS was reported in cancer cell proliferative responses to E2 (34–36), and ERK 2, a downstream effector of the MAPK pathway, cooperates in regulating gene transcription (21). Similarly, a cross-talk between membrane-initiated signaling of steroid receptors, such as progesterone or androgen receptor, and gene regulation and cell proliferation was also reported in various culture models of cancer cells (37). However, in contrast to the conclusions raised from these models of cultured cancer cells, our large-scale gene analysis reveals that although MISS via ERα can influence the regulation of a small subset of genes in the uterus, the impact of nonnuclear ERα on gene regulation is quite modest in this organ, paradigmatic of the proliferative action of E2.

This context-specific role for membrane-initiated signals fits along the complexity of the palmitoylation process (38), emphasizing the regulation and the spatiotemporal dynamics of protein palmitoylation. These levels of complexity should be addressed in future studies, even if such in vivo studies seem particularly difficult.

The discoveries that were rendered possible via the generation of the C451A-ERα mouse model indicate that membrane and nuclear actions of ERα in vivo are highly tissue-specific in some key targets and functions. The C451A-ERα mouse now provides a valuable loss-of-function model for the study of the role of plasma membrane-associated ERα in numerous additional physiological and pathophysiological processes affected by the receptor, as well as a means to conceive new selective ERα modulators with an optimized medical profile.

Materials and Methods

**Mice.** All procedures involving experimental animals were performed in accordance with the principles established by the International Union for the Scientific Study of Animal Research (2008). C451A-ERα mice were generated on a 129SvJOut genetics background, and were approved by the local Ethical Committee of Animal Care. The C451A-ERα knock-in mouse line was generated on a C57B1/6J background through the strategy outlined in Fig. S1A at the Mouse Clinical Institute (Liévin, France). ERα−/−, ERα-AF2 mouse have been previously described (4, 39). C451A-ERα and their corresponding wild-type littermates (WT-C451) were ovariec- tomized at 4 wk of age. For acute E2 treatment, they were injected s.c. with vehicle (castor oil) or 17β-estradiol (E2, 8 μg/kg) 3 wk after ovariectomy. For chronic E2 treatment, ovariec- tomized mice were implanted with s.c. pellets releasing either vehicle or E2 (or 80 μg kg−1·d−1) as indicated, 60-d release; Innovative Research of America)

**Isolation of Plasma Membranes.** Primary hepatocyte cultures were isolated from the livers of 8- to 10-wk-old mice by a modification of the collagenase method (40). Briefly, livers from WT-C451 and C451A-ERα mice were perfused using the portal vein with HBSS before collagenase perfusion (C513B; Sigma). Hepatocytes were then put in cultures in DMEM. Plasma membranes were isolated by a discontinuous sucrose step gradient, from which 12 fractions were recovered (41). The first fractions (1–4) contained the plasma membrane proteins, which were identified after protein precipitation by Western blot using polyclonal antibodies against either ERα or annexin II.

**Mouse Carotid Artery Injury.** Perivascular carotid artery injury was performed as previously described (3) in ovariectomized mice treated or not for 2 wk before surgery with E2 (80 μg kg−1·d−1) in 60-d-release pellets) or EDC (ethinyl-estradiol dimer conjugated, 240 μg kg−1·d−1). Briefly, the left carotid artery was exposed via an anterior cervicotomy. The electric injury was applied to the distal part (3 mm precisely) of the common carotid artery with a bipolar microregulator. The percentage of reendothe- lialization was calculated relative to the initial deendothelialized area by assessing Evans blue dye uptake 3 d after injury. Images were acquired under a DMR 300 Leica microscope, using Leica Application Suite V3.8 and ImageJ softwares.

**Vascular Reactivity of Isolated Mesenteric Arteries.** As previously described (42), 5-mm-long segments of second-order mesenteric arteries were dissected and mounted between two glass cannulae and bathed in a physio- logical salt solution (PH 7.4, PO2 120 mm Hg, and PCO2 37 mm Hg). Pressure was controlled by a servo-perfusion system, and diameter changes were measured continuously using a video-monitored system (Living Systems Instruments). Artery viability was assessed using KCl (80 mM) and endothelium integrity with acetylcholine (1 μM). EDC (1 μM) and E2 (0.01-1 μM) dependent dilation was then assessed after precontraction with phenyl- ephrine to 70% of the maximal contractile response obtained with KCl (80 mM).

**Mouse Endothelial Cell Migration Assay.** Endothelial cells were isolated from the aortae of wild-type and C451A-ERα mice, and cell migration was assessed using methods modified from those previously described (43). At first pass-
sage, the cells were transferred into six-well plates, and following growth to confluence in EGM-2 medium (Lonza) containing 10% FBS, a defined region of the cells was removed using a pipette tip. The initial area devoid of cells was marked and quantified on images obtained at baseline, and treatments with vehicle or E2 (10−9 M) were initiated in phenol red-free DMEM with 5% charcoal-stripped FBS. Sixteen hours later the cells were fixed and stained with Coomassie blue, and repeat images were obtained to quantify the remaining area devoid of cells. Cells were imaged using an inverted phase-contrast microscope (Nikon Eclipse TS100; Nikon Corporation) and a digital camera (Infinity 1). The area deficient of cells was quantified using Adobe ImageReady CS2 software. The degree of migration was calculated as the percentage of the initial area devoid of cells that was occupied by cells following 16-h treatment.

**Hormone Assays.** Serum levels of LH and FSH were determined using the Multiplex Immunoassay Technology Xmap (MILLIPLEX; Millipore). Progesterone and 17-estradiol were measured by gas chromatography-mass spectrometry (44), with minor modifications. These assays were performed on intact 10- to 12-wk-old female mice.

**Immunohistochemistry.** Paraffin-embedded transverse sections (4 µm) from formalin-fixed uterine or ovary specimens were stored as previously described (5) with anti-Ki-67 antigen (RM-9106, Thermo-scientific). Sections were treated with hydrogen peroxide to inhibit endogenous peroxidase activity and immunostained using an automated Immunostainer (Dako Autostainer Plus). The number of follicles in each section was expressed as a percentage of the total area of follicle.

**Gene Expression Analysis.** Total RNA was extracted using TriPure reagent (Roche) and reverse-transcribed (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems). Microarray data were obtained from 200 ng of total RNA labeled with Cy3 using Low Input Quickamp Labeling Kit (Agilent) and hybridized to a SurArray mouse M44E (8 x 60K) microarrays following the manufacturer’s instructions. All experimental details are available in the Gene Expression Omnibus (GEO) database under accession no. GSE2327. Data were analyzed under R (R 2.13; www.R-project.org). Hierarchical clustering was applied to the samples and the probes using 1-Pearson correlation coefficients as distance and Ward’s criterion for agglomeration. Functional analysis was carried out using DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov), and comparisons are realized with the Venn Diagrams plug-in based upon the VENNY tool developed by J. C. Oliveros.

**Statistical Analyses.** Results are expressed as the mean ± SEM. To test the effect of treatments or genotypes, t test or Mann–Whitney test was performed. To test the interaction between treatments and genotypes, a two-way ANOVA was carried out. When an interaction was observed between two variables, the effect of treatment was studied in each genotype using the Bonferroni post hoc test. A value of P < 0.05 was considered statistically significant (*P < 0.05; **P < 0.01; ***P < 0.001).

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**Supporting Information**

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**Fig. S1.** Generation of estrogen receptor (ER)α mice mutated on C451A. (A) Computer-generated model of the surface exposure of a palmitoyl group attached to ERα C451 (discussed in main text). The C451 residue is colored red, and the palmitoyl chain atoms, shown explicitly, are colored gray. (B) The targeting vector was constructed as follows: A 809-bp fragment encompassing ERα exon 7 and part of intron 6 and 7 was PCR-amplified on RP24-73H17 C57BL/6J BAC in two steps to allow the introduction of the point mutation of the base G into C (giving C451A) in exon 7. This fragment was subcloned into an ICS proprietary vector that contains a floxed neomycin resistance cassette (NeoR). The 5′ homology arm (4.3 kb) and 3′ homology arm (3.5 kb) were then sequentially subcloned into the same vector. The linearized construct was electroporated in C57BL/6NTac mouse embryonic stem (BD10 ES) cells. After selection, targeted clones were identified by PCR using 5′ and 3′ external primers; the insertion of the point mutation was confirmed by sequencing (C) and further confirmed by Southern blot with a Neo (5′ digest AvrII and 3′ digest BclI) and a 5′ external probes (BamHI and Scal digests). The targeted ES clone was microinjected into BALB/cN blastocysts. Resulting male chimaeras were bred with a Cre deleter line (to excise the NeoR floxed cassette) and germ-line transmission was achieved.

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<table>
<thead>
<tr>
<th>Follicles</th>
<th>WT-C451</th>
<th>C451A-ERα</th>
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<tr>
<td>Primordial</td>
<td>14.7 ± 1.8</td>
<td>5.9 ± 1.5</td>
<td>*</td>
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<tr>
<td>Primary</td>
<td>20.7 ± 5.3</td>
<td>17.8 ± 3.7</td>
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<td>Secondary</td>
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<tr>
<td>Atretic</td>
<td>24.3 ± 5.3</td>
<td>30.9 ± 5.1</td>
<td>ns</td>
</tr>
</tbody>
</table>

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**Fig. S2.** Adult ovarian phenotype in the C451A-ERα mice. (A) Percentages of primordial follicles, primary follicles, secondary follicles, tertiary follicles, and atretic follicles were determined in ovaries from 3-mo-old WT-C451 and C451A-ERα mice, n = 4. (B) Representative images (×100) of granulosa cell layer (GCL) and thecal cell layer (TCL) of tertiary follicles in WT-C451 and C451A-ERα mice are shown. The magnification (×200) shows the stromal glandular interstitial cells. The percentages of the GCL and of the TCL were analyzed in tertiary follicles from WT-C451A and C451A-ERα mice, n = 4-6.
Fig. S3. Protein expression and mRNA level of ERα, ERβ, and GPR30 in C451A-ERα uterus. (A) ERα protein level in uterus after 24 h of 8 μg/kg 17β-estradiol (E2) treatment in WT-C451 and C451A-ERα mice by Western blot analysis. Statistical results of two-way ANOVA are shown, explaining the effect of treatment, genotype, and the interaction between these two variables, n = 2.

ERα, ERβ, and GPR30 mRNA levels after (B) acute (6 h) or (C) chronic (4 wk) treatment with 8 μg·kg⁻¹·d⁻¹ of E2 in uterine homogenates from ovariectomized WT-C451 and C451A-ERα mice. Statistical results of two-way ANOVA are shown, explaining the effect of treatment (Treat.), genotype (Geno.), and the interaction between these two variables, n = 3.

Fig. S4. Difference between genetic backgrounds in the transcriptional response to E2. (A) The Venn diagram illustrates the overlaps between genes significantly (P < 0.05) up- or down-regulated in the uterus following E2 treatment in WT-AF2 or WT-C451. (B) The first three biological pathways of most significantly enriched genes are indicated for each group of Venn diagram comparison of Fig. 4C.
Fig. S5. Membrane-initiated vascular effects of E2 in C451A-ERα mice. (A) Response of phenylephrine-precontracted mesenteric arteries from WT-C451 and C451A-ERα mice to stepwise concentrations of E2. Comparison of mRNA levels of ERα, ERβ in aorta from ovariectomized (OVX) WT-C451 and C451A-ERα mice treated or not with E2 (8 μg/kg) (B) acutely (6 h) or (C) chronically (4 wk), n = 3–4. (D) ERα protein expression detected by Western blot analysis in aorta from OVX WT-C451 and C451A-ERα mice treated or not with E2 (80 μg·kg⁻¹·d⁻¹, 2 wk). AU, arbitrary units.

Dataset S1. List of genes significantly regulated in interaction with treatment and genotype in the different groups identified in Fig. 6C

Dataset S1

Genes significantly regulated in interaction with treatment and genotype in C451A-ERα or ERα-AF2 mice. Genes are clustering according to the Venn diagram overlapping in Fig. 4C. FC, fold change.