The AF-1 Activation Function of Estrogen Receptor α Is Necessary and Sufficient for Uterine Epithelial Cell Proliferation In Vivo

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Estrogen receptor-α (ERα) regulates gene transcription through the 2 activation functions (AFs) AF-1 and AF-2. The crucial role of ERαAF-2 was previously demonstrated for endometrial proliferative action of 17β-estradiol (E2). Here, we investigated the role of ERαAF-1 in the regulation of gene transcription and cell proliferation in the uterus. We show that acute treatment with E2 or tamoxifen, which selectively activates ERαAF-1, similarly regulate the expression of a uterine set of estrogen-dependent genes as well as epithelial cell proliferation in the uterus of wild-type mice. These effects were abrogated in mice lacking ERαAF-1 (*ERαAF-1^0*). Four weeks of E2 treatment led to uterine hypertrophy and sustained luminal epithelial and stromal cell proliferation in wild-type mice, but not in *ERαAF-1^0* mice. However, *ERαAF-1^0* mice still presented a moderate uterine hypertrophy essentially due to a stromal edema, potentially due to the persistence of *Vegf-a* induction. Epithelial apoptosis is largely decreased in these *ERαAF-1^0* uteri, and response to progesterone is also altered. Finally, E2-induced proliferation of an ERα-positive epithelial cancer cell line was also inhibited by overexpression of an inducible ERα isoform lacking AF-1. Altogether, these data highlight the crucial role of ERαAF-1 in the E2-induced proliferative response in vitro and in vivo. Because ERαAF-1 was previously reported to be dispensable for several E2 extrareproductive protective effects, an optimal ERα modulation could be obtained using molecules activating ERα with a minimal ERαAF-1 action. **(*Endocrinology* 154: 2222–2233, 2013)**

Estrogens, particularly 17β-estradiol (E2), play a pivotal role in sexual development and reproduction but are also implicated in other physiologic processes in mammals. The uterus is a major estrogen target, and waves of steroid hormone-induced cell proliferation and differentiation dictate the cyclical changes that occur in the uterine epithelium during the reproductive cycle (1). In addition, estrogens induce a rapid increase in endometrial microvascular permeability, leading to stromal edema and marked uterine weight increase. Stromal edema is believed to create an optimal environment for the growth and remodeling of the endometrium in preparation for embryo implantation and pregnancy (2). Estrogens and progesterone (P4) actions, mediated by their respective receptors, cause molecular and cellular events during uterine receptivity, and the balance between them is important for the

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Abbreviations: AF, activation function; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen-responsive element; 16α-LE2, cpd1471; LEH, luminal epithelial height; P4, progesterone; PR, progesterone receptor; SERM, selective ER modulator; VEGF, vascular endothelial growth factor.

endometrium functions (3). At menopause, after cessation of ovarian function, estrogen replacement is used to relieve climacteric symptoms, in order to prevent osteoporosis but also coronary disease when given early after menopause (4, 5). To minimize the proliferative effects of estrogens on the uterus and reduce the risk of endometrial cancer, progestins are routinely administered together with estrogens in hormone replacement therapy. However, the Women's Health Initiative trial highlighted that the estrogen-medroxyprogesterone acetate association might increase the risk of breast cancer and cardiovascular diseases, whereas administration of estrogens alone in hysterectomized women have protective (breast cancer) and neutral (coronary heart disease) effects (6, 7).

The different physiologic responses to estrogens are initiated by their binding to the estrogen receptors (ERs) ERα and ERβ, which belong to the nuclear receptor superfamily and are structurally organized into 6 functional domains (A to F). The E domain allows hormone binding, an event that induces specific conformational changes that are required for ER transcriptional activity through the modulation of 2 activation functions (AFs), AF-1 and AF-2, located in the A/B and E domains, respectively (8). ER-mediated transcriptional regulation involves either direct interaction of ER with specific estrogen-responsive elements (EREs) in or near the promoter region of target genes, or an indirect mechanism via protein/protein interactions with other transcriptional factors (9). ER-mediated transactivation is then achieved via an ordered sequence of interactions between the AFs and various coactivators, such as members of the p160 subfamily or the cAMP response element binding protein/p300 (10–12).

In the rodent uterus, E2 generates a robust and rapid transcriptional response with a biphasic temporal effect. Early response includes RNA transcription, hyperemia, and water imbibition, whereas later responses comprise cycles of DNA synthesis and mitosis in epithelial cells (13–15). The strong expression of ERα in the uterus is powerful evidence for its importance in the response to E2. In fact, ERα ligand-induced signaling is critical for the normal development of uterine tissue (13, 16, 17). Recently, an ERαAF-2 mutant mouse line allowed us to show that this AF-2 is required for regulating some uterine gene expression and epithelial cell proliferation in response to E2 (18). Although weak E2 action on uterine weight in *ERαAF-1^0* mice has been previously reported (19), the precise role of ERαAF-1 in uterine function has not been precisely studied. Therefore, to explore the role of AF-1 in the molecular mechanisms of uterine growth, we used a combination of pharmacologic and genetic approaches to compare the effects of acute and chronic administration of E2 on uterine gene expression and epithelial cell proliferation in wild-type mice and in mice lacking ERαAF-1 (*ERαAF1^0*) (19). We proved that ERαAF-1 is required for E2-induced uterine epithelial cell proliferation, whereas it is partially dispensable for the induction of edema after chronic E2 stimulation.

Materials and Methods

Mice

All procedures involving experimental animals were performed in accordance with the principles and guidelines established by the National Institute of Medical Research (INSERM) and were approved by the local Animal Care and Use Committee. *ER$\alpha^{+/-}$* and *ERαAF-1$^{+/0}$* mice were generated as previously described (19, 20). C57Bl/6J as well as *ER$\alpha^{-/-}$*, *ERαAF-1^0*, and their corresponding wild-type littermates (all backcrossed at least 10 times on a C57Bl/6J genetic background) were ovariectomized at 4 weeks of age, and 3 weeks after were sc injected with vehicle (castor oil), 17β-estradiol (E2, 8 μg/kg), the ERα agonist 16α-LE2 (also termed cpd1471) (28.8 μg/kg), tamoxifen (4 mg/kg) or P4 (1 mg). Mice were humanely destroyed 2, 6, 12, and 24 hours after unique or daily repeated treatment (during 3 d). For chronic E2 treatment, ovariectomized mice were implanted with sc pellets that release either placebo or E2 (0.01 mg 17β-estradiol, 60-d release, ie, 8 μg/kg/d; Innovative Research of America, Sarasota, Florida). Because no significant statistical difference was observed in the parameters of *ER$\alpha^{+/+}$* and *ERαAF-1$^{+/+}$* mice, these two control groups were pooled and indicated as WT in Figures 4 and 6 and Supplemental Figure 10.

Analysis of mRNA levels by quantitative RT-PCR

Dissected uteri were homogenized using a Precellys tissue homogenizer (Bertin Technology, Cedex, France) and total RNA from tissues was prepared using TRIzol (Invitrogen, Carlsbad, California). One microgram of RNA was reverse transcribed at 25°C for 10 minutes and then at 37°C for 2h in 20 μL final volume using the High Capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster City, California). The 96.96 Dynamic Arrays for the microfluidic BioMark system (Fluidigm Corp., South San Francisco, California) were used to study by high throughput quantitative PCR the gene expression profile in 6.5 ng cDNA from each mouse uterus, as described previously (21, 22). Primers (Supplemental Figure 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org) were validated by testing the PCR efficiency using standard curves (95% $\leq$ efficiency $\leq$ 105%). Gene expression was quantified using the comparative C_t (threshold cycle) method, and *HPRT1* expression is used as reference. Each probe was indeed normalized to each respective genotype placebo in order to better visualize the impact of E2 or tamoxifen on gene expression changes because no significant difference was detected between wild-type and *ERaAF-1^0* untreated ovariectomized mice.

Immunohistochemistry

Paraffin-embedded transverse sections (4-μm) from formalin-fixed uterine specimens were dewaxed in toluene and rehydrated through acetone bath to deionized water. Antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) for 30 minutes in a water bath at 95°C. Cooled sections were then incubated in peroxidase blocking solution (DAKO Corp., Carpinteria, California) to quench endogenous peroxidase activity. To block nonspecific binding, sections were incubated in normal goat serum (DAKO) for 20 minutes at room temperature. Primary antibodies were all rabbit polyclonal antibodies: anti-Ki-67 antigen (Monosan; Sanbio B.V., Uden, The Netherlands), anti-ERα (Santa Cruz Biotechnology, Santa Cruz, California), anti-progesterone receptor (PR) (DAKO) and antiactive caspase-3 (R&D Systems, Minneapolis, Minnesota). Sections were incubated 50 minutes at room temperature with primary antibodies. The secondary antibody, biotinylated goat antirabbit Igs (Thermo-Scientific, Rockford, Illinois), was applied for 25 minutes at room temperature followed by an horseradish peroxidase-streptavidin solution (DAKO) for 25 minutes. Peroxidase activity was revealed by 3,3′-diaminobenzidine tetrahydrochloride substrate (DAKO). Finally, sections were counterstained with Harris hematoxylin, dehydrated, and coverslipped. The luminal epithelial height was measured from the basal membrane to the apical surface. The values are the mean of 10 measurements in each transverse uterus section.

Determination of apoptotic index

The number of apoptotic cells present in a section is expressed as a fraction of the total number of epithelial cells, so called activated caspase-3 labeling apoptotic index.

Statistical analyses

Results are expressed as the mean ± SEM. To test the effect of treatments, 1-way ANOVA was performed. To test the interaction between treatments and genotypes a 2-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 $P < .05$ was considered as statistically significant.

Results

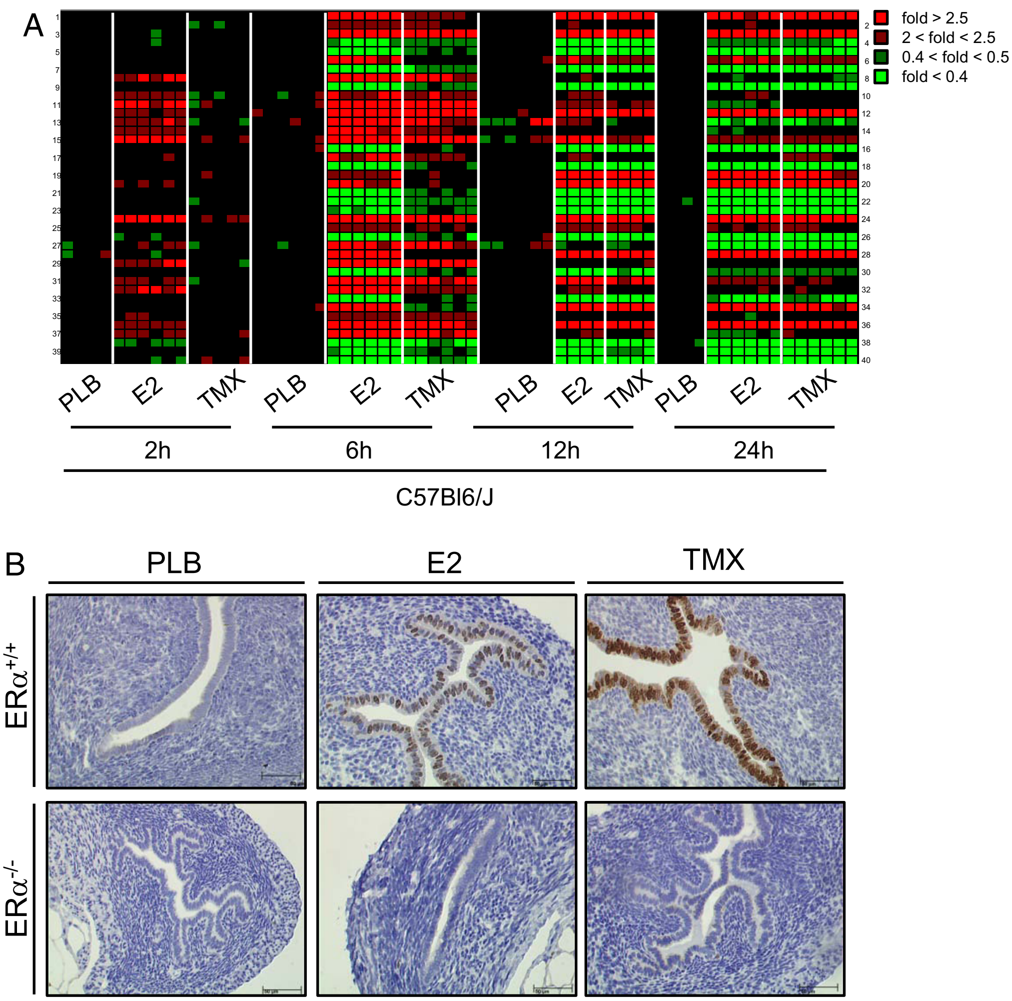
ERαAF-1 is sufficient to mediate ERα-dependent uterine response to estrogens in vivo

We first confirmed the crucial role of ERα in the uterine response to E2 (ie, gene transcription and cell proliferation). To this aim, we selected a set of genes known to be regulated by E2 in the uterus (23–27) (Supplemental Figure 1) and evaluated their expression profile in ovariectomized C57Bl/6J mice after a unique acute administration of 8 μg/kg E2 or placebo. We first observed a similar transcriptional regulation of this set of genes upon acute administration of E2 or using the selective ERα agonist 16α-LE2 (Supplemental Figures 2A and 3). Conversely, acute administration of E2 in ovariectomized $ER\alpha^{-/-}$ mice did not have any effect on the expression of these genes. In addition, 24 hours after E2 or 16α-LE2 treatment, both luminal epithelial height (LEH) and uterine weight were significantly increased in ovariectomized C57Bl/6J mice (Supplemental Figure 2, B and C). Nuclear expression of Ki-67 (a proliferation marker) was observed in 87% (E2) and 93% (16α-LE2) of uterine luminal epithelial cells, whereas no Ki-67-positive cells were detected in placebo-treated animals and $ER\alpha^{-/-}$ mice (Supplemental Figure 2, B and C).

Then, to determine the role of ERαAF-1 in the uterine response to E2, we compared uterine gene transcription and cell proliferation in ovariectomized wild-type mice given either 8 μg/kg E2 or 4 mg/kg tamoxifen, a selective ERαAF-1 agonist and ERαAF-2 antagonist (28–30) (Figure 1 and Supplemental Figure 4). The set of genes regulated by E2 was very similarly regulated by tamoxifen after 12 or 24 hours, although the earlier response (at 2 and 6 h) to tamoxifen was somewhat delayed compared with E2 (Figure 1A and Supplemental Figure 4), in agreement with a previous report (31). The effects of E2 and tamoxifen on uterine weight, LEH, and Ki-67 expression were also quite similar in wild-type mice, and abrogated in $ER\alpha^{-/-}$ mice (Figure 1, B and C). These findings suggest that ERαAF-1 activation is sufficient to mediate the ERα-dependent uterine response.

Uterine gene expression in response to acute E2 or tamoxifen administration requires ERαAF-1

To further evaluate the role of ERαAF-1 in the E2 or tamoxifen-induced uterine transcriptional response in vivo, we used transgenic mice, in which the sequence coding for the main part of the A/B domain of ERα including AF-1 was deleted ($ER\alpha AF\text{-}1^0$ mice) (19). Regulation of most of the genes regulated by acute E2 treatment in $ER\alpha AF\text{-}1^{+/+}$ mice was lost in $ER\alpha AF\text{-}1^0$ mice (Figure 2A and Supplemental Figure 5). Only 2 genes, encoding the family with sequence similarity 65 member B (*Fam65b*) and the progesterone receptor (*Pr*) were still significantly up-regulated by E2, although in a lesser extent than in wild-type mice (Figure 2B and Supplemental Figure 5). The transcriptional regulation of the classical ERE-responsive genes by E2, such as insulin-like growth factor (*Igf1*) and cyclin-dependent kinase inhibitor 1A (*P21*), was strictly ERαAF-1 dependent. Interestingly, *Vegf-a*, which is implicated in vascular permeability and water imbibition (32), was up-regulated in response to an acute treatment with E2 in wild-type but not in $ER\alpha AF\text{-}1^0$ mice (Figure 2B). In the same way, E2 strongly induced the activity of the C3 (ERE) promoter and of the AP-1 site in human endometrial adenocarcinoma Ishikawa cells transfected with the wild-type ERα, but not with the $ER\alpha AF\text{-}1^0$ in luciferase reporter assays (Supplemental Figure 6). The transcriptional effect of tamoxifen was totally abrogated using the $ER\alpha AF\text{-}1^0$ mice (Figure 2 and Supplemental Figure 5).



C

	ERα+/+			ERα-/-		
	PLB	E2	TMX	PLB	E2	TMX
Uterine weight (mg)	6.0 ± 0.8	16.2 ± 1.5***	17.0 ± 1.0***	3.5 ± 0.5	3.7 ± 0.3	3.7 ± 0.7
LEH (μm)	11.4 ± 0.4	22.8 ± 2.9**	23.8 ± 0.1**	11.7 ± 1.8	12.5 ± 2.4	11.1 ± 1.3
Ki67 epith (%)	< 1	67 ± 6***	64 ± 4***	< 1	< 1	< 1

Figure 1. ERαAF-1 Is Sufficient to Induce Uterine Epithelial Proliferation in Response to Acute E2 or Tamoxifen Treatment in Vivo. Ovariectomized C57Bl/6J, *ERα*$^{+/+}$ and *ERα*$^{-/-}$mice (7 wk of age) were injected sc with placebo (PLB, castor oil), 17β-estradiol (E2, 8 μg/kg). or tamoxifen (TMX, 4 mg/kg), and were euthanized at different time points (A) or 24 hours after treatment (B and C). A, Data obtained from 96.96 Dynamic Arrays were used to generate a cluster diagram describing the significant changes in the expression of 40 E2-regulated genes labeled 1-40. Each horizontal line represents a single gene, and each vertical line represents an individual sample. Genes that were up-regulated at least 2-fold after E2 administration relative to placebo are in red, whereas down-regulated genes are in green. The color intensity indicates the degree of variation in expression. B, Ki-67 detection in transverse uterus sections (scale bar, 50 μm). C, Uterine weight, LEH, and percentage of Ki-67-positive epithelial cells. Results are expressed as mean ± SEM. To test the respective roles of treatment and genotype, a 2-way ANOVA was performed. When an interaction was observed between the 2 factors, effect of treatment was studied in each genotype using Bonferroni post test (**, $P < .01$; and ***, $P < .001$; n = 4 to 6 mice per group).

ERαAF-1 is required for cell proliferation in response to acute E2 or tamoxifen treatment

Next we investigated the role of ERαAF-1 in the induction of uterine epithelial cell proliferation after acute administration of E2 or tamoxifen. First, we verified that ERα was similarly expressed in the epithelium and in the stroma of both wild-type and *ERαAF-1*^{0} mice using immunochemistry (Figure 3A). Uterine weight, LEH, and stromal height were significantly increased in wild-type mice, but not in *ERαAF-1*^{0} mice 24 hours after acute ad-

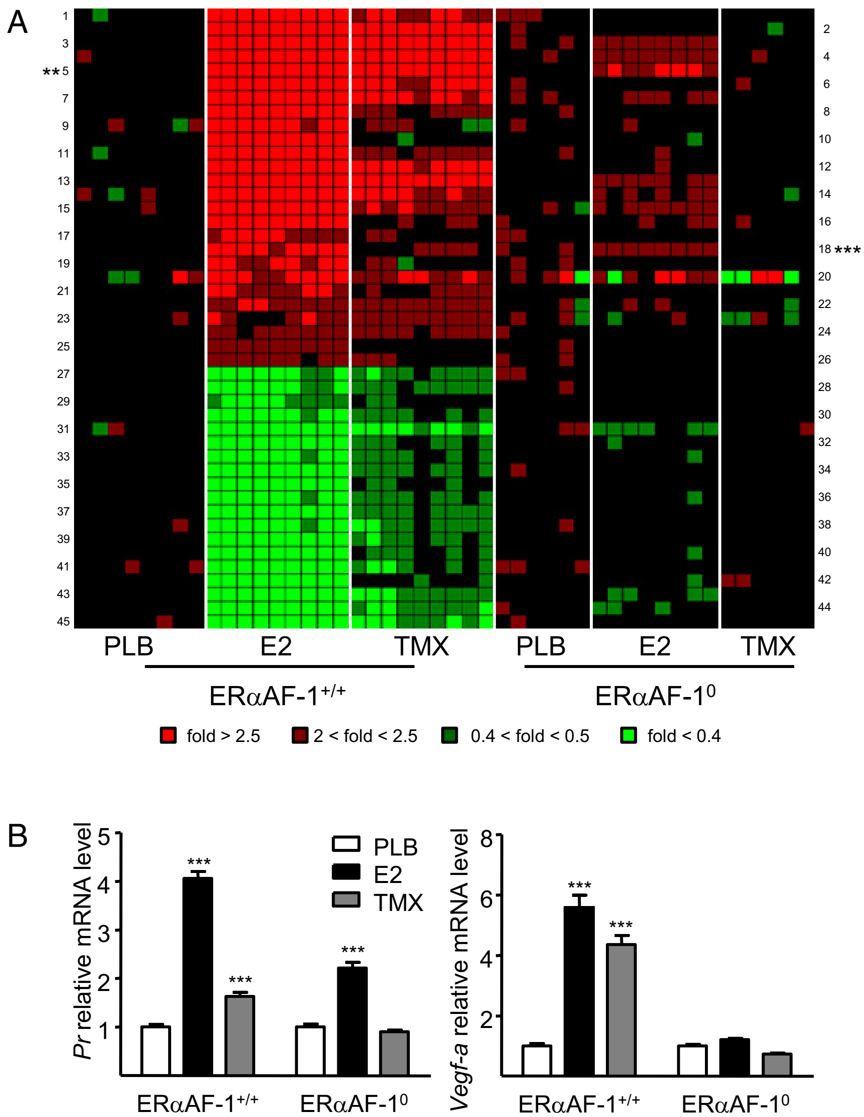


Figure 2. ERαAF-1 Is Necessary for the Uterine Transcriptional Response to E2 or Tamoxifen (TMX) Acute Exposure in Vivo. Ovariectomized *ERαAF-1*$^{+/+}$ and *ERαAF-1*^{0} mice (7 wk of age) were injected sc with placebo (PLB, castor oil), 17β-estradiol (E2, 8 μg/kg), or TMX (4 mg/kg) and euthanized 6 hours later. A and B, Data obtained from 96.96 Dynamic Arrays were used to generate a cluster diagram of the significant changes in the expression of the E2-regulated genes. Each horizontal line represents a single gene, and each vertical line an individual sample. Genes that were up-regulated at least 2-fold after E2 administration relative to placebo are in red, whereas down-regulated genes are in green. The color intensity indicates the degree of variation in expression. C, Quantification of the relative mRNA level of *PR* and *Vegf-a* by quantitative PCR. Data were normalized to *HPRT1* expression. Results are expressed as mean ± SEM. To test the respective roles of treatment and genotype, a 2-way ANOVA was performed. When an interaction was observed between the two factors, the effect of treatment was studied in each genotype using Bonferroni post test (***, $P < .001$; n = 6 to 9 mice per group).

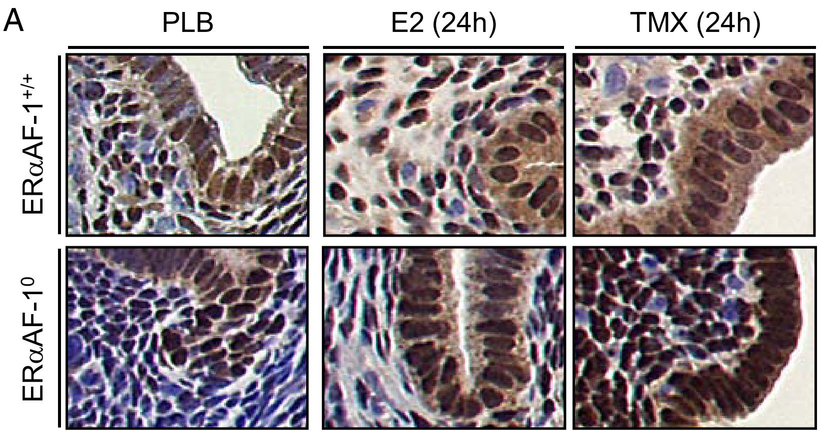
ministration of E2 (8 μg/kg) or tamoxifen (4 mg/kg) (Figure 3B). A strong induction of epithelial proliferation, as indicated by Ki-67 nuclear expression, was observed in wild-type, but not in *ERαAF-1*^{0} mice after E2 or tamoxifen exposure (Figure 3, C and D). The absence of uterine hypertrophy after E2 or tamoxifen treatment in *ERαAF-1*^{0} mice was associated with the absence of regulation at 24 hours of most of the studied genes (Supplemental Figure 7).

To assess the role of the other AF of ERα, ie, AF-2, we used *ERαAF-2*^{0} mice in which the sequence encoding 7 amino acids in helix 12 that are crucial for AF-2 activity has been deleted (33). No increase in uterine weight, LEH, and epithelial proliferation were observed in these *ERαAF-2*^{0} mice after acute E2 or tamoxifen (Supplemental Figure 8). Thus, we confirmed the crucial role of ERαAF-2 for uterine response to E2 previously demonstrated with another mouse model of invalidation of this AF (18).

Interestingly, overexpression of an AF-1-deficient ERα isoform inhibited the cell proliferation in response to E2 of the MCF7 ERα-positive breast cancer cell line (Supplemental Figure 9). Altogether, these results show the crucial role of ERαAF-1 in the proliferative response to acute E2 treatment both in vitro and in vivo.

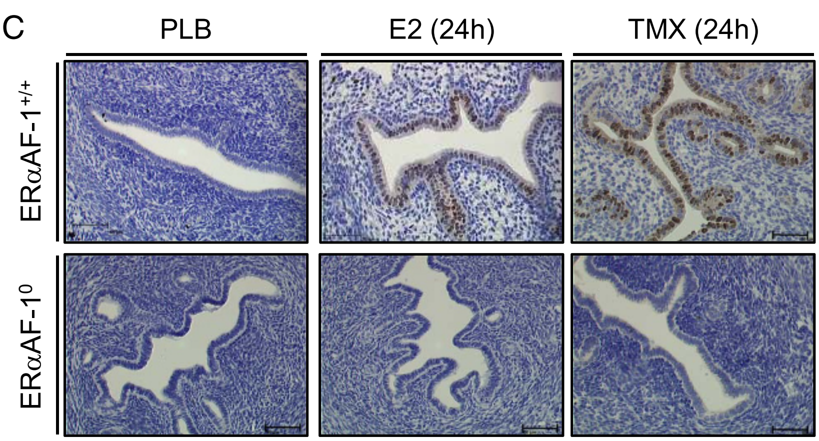
Luminal epithelial cell proliferation is inhibited in *ERαAF-1*^{0} mice after chronic exposure to E2

We then evaluated the role of ERαAF-1 in uterus-mediated E2 signaling after chronic exposure to E2 (8 μg/kg/d for 4 wk) (Figure 4). In wild-type animals, chronic E2 treatment strongly increased uterine weight in comparison to placebo (Figure 4A). The luminal epithelium from wild-type E2-treated mice was characterized by the presence of large columnar cells with eosinophilic cytoplasm, apoptotic bodies, and neutrophilic infiltration; 19% of luminal epithelial cells were Ki-67 positive (Figure 4B). The uterine stroma presented significant decrease of cell density and presented loosely arranged oval fibroblasts, glands with cystic dilatation and neutrophilic infiltration. In parallel, stromal cell proliferation was increased by E2 in comparison with placebo-treated controls (Figure 4B). Conversely, chronic E2 treatment did not have any effect in *ERα*$^{-/-}$ animals. Indeed, in these mice, uterine stroma was compact and dense without mitotic activity, and the luminal epithelium was composed of



B

	ERαAF-1+/+			ERαAF-10		
	PLB	E2 (24h)	TMX (24h)	PLB	E2 (24h)	TMX (24h)
Uterine weight (mg)	4.3 ± 0.3	20.5 ± 1.5***	18.7 ± 0.9***	3.7 ± 0.3	4.7 ± 0.7	4.3 ± 0.3
LEH (μm)	12.0 ± 0.9	19.4 ± 1.2***	16.2 ± 0.5*	11.1 ± 0.5	11.7 ± 0.3	10.4 ± 1.0
SH (μm)	95.3 ± 9.3	171.7 ± 11.7***	174.3 ± 4.8***	81.0 ± 1.8	75.5 ± 1.7	72.9 ± 4.3



D

		ERαAF-1+/+			ERαAF-10		
		PLB	E2 (24h)	TMX (24h)	PLB	E2 (24h)	TMX (24h)
Ki67 (%)	**Epith**	< 1	89 ± 3***	80 ± 6***	< 1	< 1	< 1
	Stroma	< 1	< 1	< 1	< 1	< 1	< 1

Figure 3. ERαAF-1 Is Required to Induce Uterine Epithelial Proliferation in Response to Acute E2 or Tamoxifen (TMX) Treatment in Vivo. Ovariectomized *ERαAF-1*$^{+/+}$ and *ERαAF-1*^{0} mice (7 wk of age) were injected sc with placebo (PLB, castor oil), 17β-estradiol (E2, 8 μg/kg), or TMX (4 mg/kg) and euthanized 24 hours later. A, ERα immunodetection in stromal and epithelial compartments from transverse uterus sections. B, Uterine weight, LEH, and stromal height (SH) were calculated. C, Ki-67 immunodetection in transverse uterus sections (scale bar, 50 μm). D, Percentage of Ki-67-positive cells in luminal epithelial (Epith) and stroma were calculated. Results are expressed as mean ± SEM. To test the respective roles of treatment and genotype, a 2-way ANOVA was performed. When an interaction was observed between the two factors, the effect of treatment was studied in each genotype using Bonferroni post test (*, $P < .05$; ***, $P < 0.001$; n = 4 to 5 mice per group).

cuboidal, nonproliferating cells with a high nuclear-cytoplasmic ratio (Figure 4B). On the other hand, in *ERαAF-1*^{0} mice, chronic E2 administration had a moderated, but significant, effect on uterine weight, luminal epithelial height (LEH), stromal height, and stromal cell density (Figure 4A). The uterine stroma cell density of *ERαAF-1*^{0} mice was modest, but significantly reduced in response to chronic E2 treatment in comparison with wild-type mice, highlighting the persistent induction of stromal edema. Moreover, in *ERαAF-1*^{0} mice, glandular epithelial cells showed mitotic activity, whereas no proliferation was observed in the luminal epithelium and in the stromal compartment. No apoptotic events were observed in the epithelium from all 3 genotypes in ovariectomized untreated mice. The increase in active caspase-3 in wild-type mice (~11%), which indicates a normal regulation between growth and apoptosis after E2-induced epithelial proliferation, was abrogated in *ERα*$^{-/-}$ mice and largely attenuated in *ERαAF-1*^{0} mice (~2%) (Figure 4C). Detection of ERα by immunochemistry showed same protein expression levels in epithelium and stroma of wild-type and mutant mice (Figure 4D). Whereas acute E2 treatment did not up-regulate the expression of vascular endothelial growth factor (VEGF) A (*Vegf-a*) in *ERαAF-1*^{0} mice (Figure 2B), this gene became quite similarly regulated after chronic E2 treatment in wild-type and *ERαAF-1*^{0} mice (Figure 4E), probably explaining the delayed decrease in uterine stroma cell density and revealing another impact on edema.

PR protein is expressed exclusively in the epithelium from ovariectomized untreated mice independent of the genotype, including *ERα*$^{-/-}$ mice (Figure 4F). In wild-type mice, E2 caused a redistribution in which the expression of epithelial PR was repressed whereas its expression in the stromal compartment was induced (34–36) (Figure 4F). In *ERαAF-1*^{0} mice, the PR labeling was still observed on the epithelium whereas a significant labeling

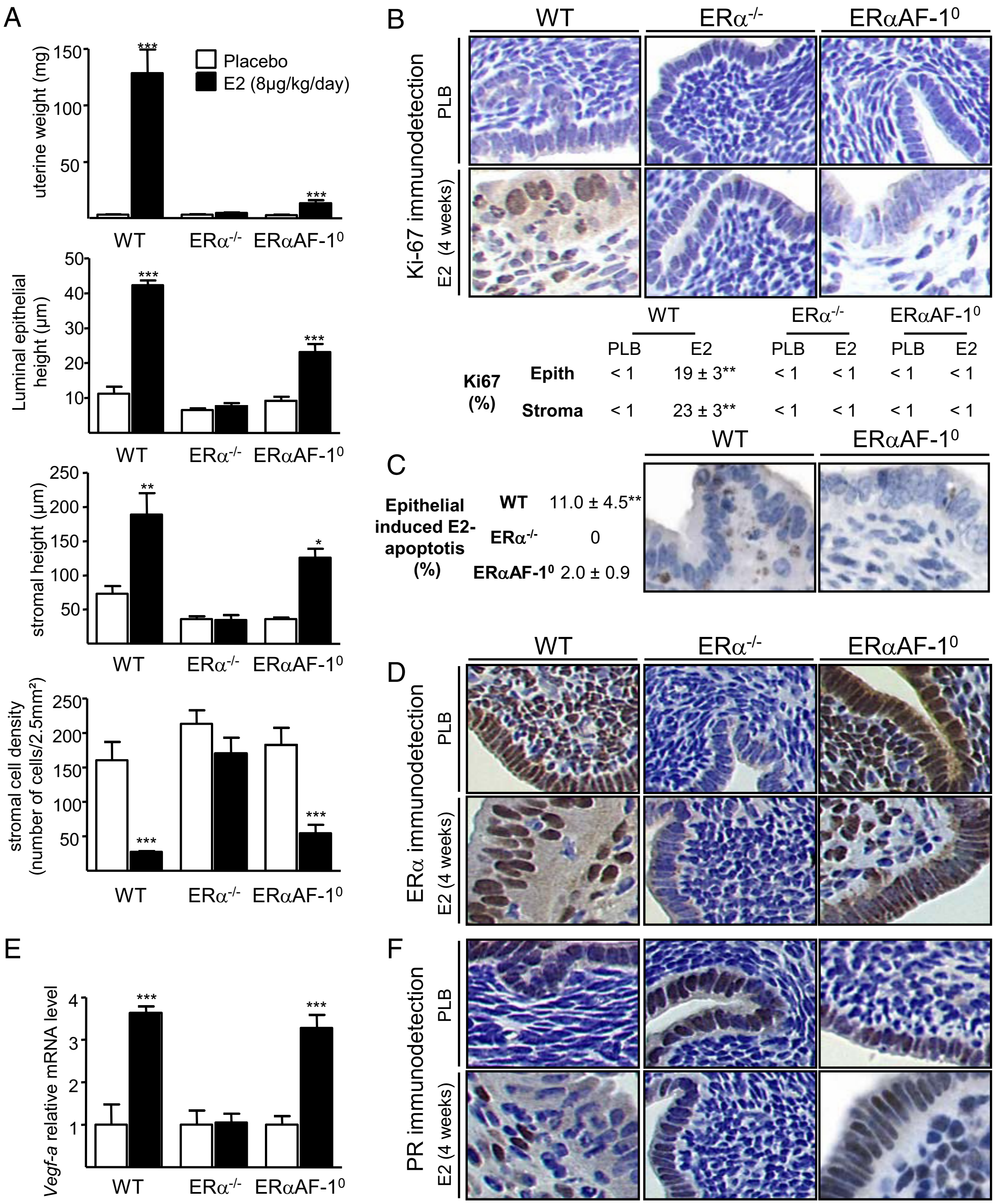


Figure 4. ERαAF-1 Is Necessary to Induce Uterine Endometrial Proliferation in Response to Chronic and Physiologic E2 treatment but Is Partially Dispensable for Water Imbibition Ovariectomized wild-type (WT), *ERα*$^{-/-}$ and *ERαAF-1*^{0} mice (7 wk of age) were given placebo (PLB) or 17β-estradiol (E2, 8 μg/kg/d) for 4 weeks. A, Uterine weight, LEH, stromal height (SH), and stromal cell density (number of cells/2.5 mm^2) were calculated. B, Ki-67 immunodetection in transverse uterus sections. Percentage of Ki-67-positive cells in uterine stroma (strom) and epithelium (epith). Results are expressed as mean ± SEM. To test the respective roles of treatment and genotype, a 2-way ANOVA was performed. When an interaction was observed between the two factors, the effect of treatment was studied in each genotype using Bonferroni post test (*, $P < .05$; **, $P < .01$; ***, $P < .001$; n = 4 to 6 mice per group). C, Active caspase-3 immunodetection in epithelial compartment of wild-type and *ERαAF-1*^{0} mice. D, ERα immunodetection in stromal and epithelial compartment. E, mRNA levels of *Vegf-a* in wild-type littermates, *ERα*$^{-/-}$ and *ERαAF-1*^{0} mice were measured by quantitative PCR and normalized to *HPRT1* expression. Results are expressed as mean ±SEM. To test the respective roles of treatment and genotype, a 2-way ANOVA was performed. When an interaction was observed between the two factors, the effect of treatment was studied in each genotype using Bonferroni post test (***, $P < .001$). F, PR immunodetection in stromal and epithelial compartment.

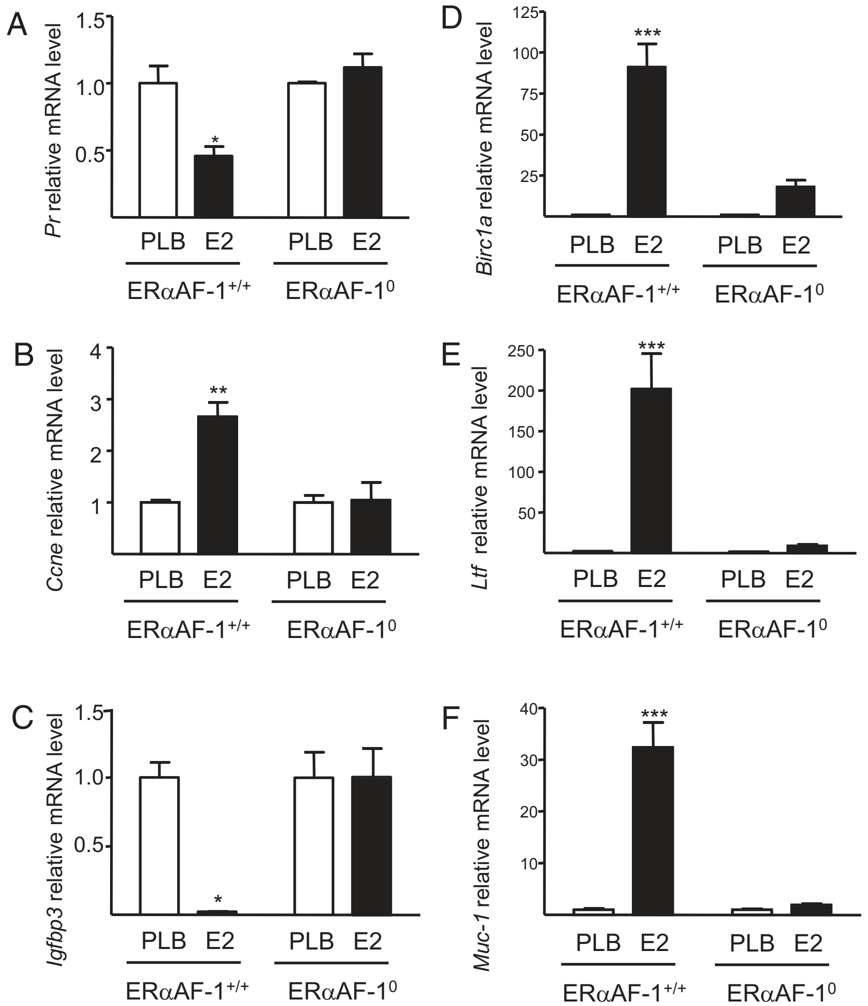


Figure 5. ERαAF-1 Is Necessary for the Uterine Transcriptional Proliferative Response to E2 Chronic Exposure in Vivo but Is Dispensable for Water Imbibition. Ovariectomized *ERαAF-1*$^{+/+}$ and *ERαAF-1*^{0} mice (7 wk of age)were given placebo (PLB) or 17β-estradiol (E2, 8 μg/kg/d) for 4 weeks. mRNA levels of *PR* (A) *CCNE* (B), *IGFBP3* (C), *BIRC1A* (D), *LTF* (E), *MUC1* (F) in wild-type littermates and in *ERαAF-1*^{0} mice were measured by quantitative PCR and normalized to *HPRT1* expression. Results are expressed as mean ± SEM. To test the respective roles of treatment and genotype, a 2-way ANOVA was performed. When an interaction was observed between the two factors, effect of treatment was studied in each genotype using Bonferroni post test (*, $P < .05$; **, $P < .01$; ***, $P < 0.001$; n = 4 to 6 mice per group).

appeared in the stroma as compared with ERα$^{-/-}$ mice. E2 thus caused a partial redistribution, PR being found in both epithelial and stromal compartments (Figure 4F), but *Pr* mRNA level was not regulated by E2 in whole uterus of deficient mice (Figure 5A). As expected from the absence of epithelial proliferation, the regulation of genes implicated in uterine proliferation, such as cyclin E (*Ccne*) (Figure 5B) and IGF-binding protein 3 (*Igfbp3*) (Figure 5C) (13), by chronic E2 treatment was abrogated in *ERαAF-1*^{0} mice. Interestingly, the regulation of 3 other genes known to be regulated by epithelial ERα (37), baculoviral inhibitors of apoptosis repeat-containing 1 (*Birc1a*) (Figure 5D), lactotransferrin (*Ltf*) (Figure 5E), and mucin-1 (*Muc-1*) (Figure 5F), was abrogated by E2 in *ERαAF-1*^{0} mice.

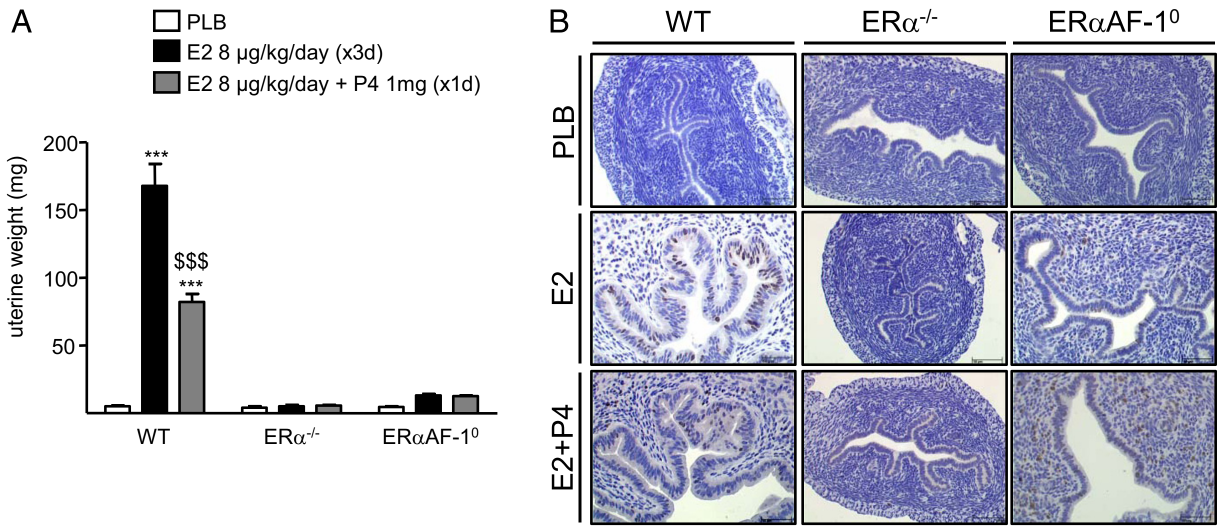
Taken together, these results demonstrate that ERαAF-1 is necessary for the proliferation of luminal epithelial cells in the uterus but partially dispensable for the induction of stromal edema.

P4 fails to inhibit the residual uterine E2 effect in the absence of ERαAF-1

The role of ERαAF-1 in the inhibition of E2-induced uterine proliferation by P4 in uterus was studied using ovariectomized mice injected sc with placebo, E2 alone (3 d), or E2 (3 d) together with P4. As expected, after 3 days of treatment, E2 uterine effects were attenuated by a single injection of P4 in wild-type mice (38) (Figure 6). The P4 inhibitory effect on uterine weight growth in response to E2 treatment was not observed in *ERαAF-1*^{0} mice (Figure 6A), and P4 injection had no significant effect on epithelial proliferation in E2-treated *ERαAF-1*^{0} mice (Figure 6, B and C). Moreover, we observed a significant increase in stromal proliferation under E2 and P4 cotreatment in *ERαAF-1*^{0} mice compared with wild-type mice (Figure 6, B and C) but without impact on the stromal height (Figure 6D). Detection of ERα by immunochemistry showed similar protein expression level in epithelium and stroma of wild-type and mutant mice (Supplemental Figure 10).

Discussion

In this work using pharmacologic and genetic approaches, we confirmed that ERα and its AF-2 function are absolutely required for the uterine response to E2 stimulation and demonstrated, for the first time, the crucial role of AF-1 in uterine luminal epithelial proliferation. However, a moderate uterine hypertrophy was still observed in *ERαAF-1*^{0} mice after chronic administration of E2, essentially due to stromal edema in the absence of epithelial and stromal proliferation.



C

		WT			ERα-/-			ERαAF-1⁰		
		PLB	E2	E2+P4	PLB	E2	E2+P4	PLB	E2	E2+P4
Ki67 (%)	Epith	< 1	19 ± 2*** $$$	5 ± 1*	< 1	< 1	< 1	< 1	< 1	3 ± 1 ns
	Stroma	< 1	5 ± 1***	5 ± 1***	< 1	< 1	< 1	< 1	< 1	10 ± 1*** $$$

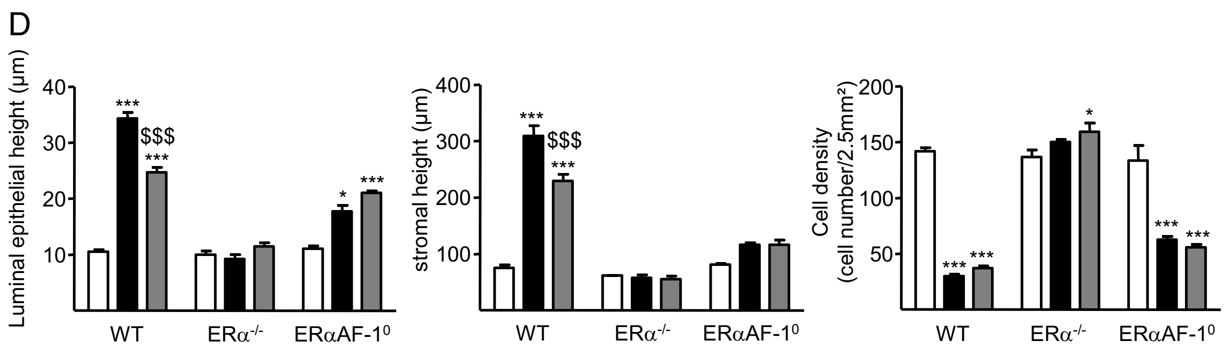


Figure 6. P4 Fails to Inhibit the Residual Uterine E2 Effect in the Absence of ERαAF-1 Ovariectomized wild-type (WT), *ERα*$^{-/-}$ and *ERαAF-1*^{0} mice (7 wk of age) were given placebo (PLB), 17β-estradiol (E2, 8 µg/kg/d) for 3 days or 17β-estradiol (E2, 8 µg/kg/d) for 3 days followed by a single injection of P4 (1 mg). A, Uterine weight. B, Ki-67 immunodetection in transverse uterus sections (scale bar, 50 µm). C, Percentage of Ki-67 positive cells in uterine stroma and epithelium (epith) were calculated. D, LEH, stromal height (SH), and stromal cell density (number of cells/2.5 mm^2) were calculated. Results are expressed as mean ± SEM. To test the respective roles of treatment and genotype, a 2-way ANOVA was performed. When an interaction was observed between the two factors, the effect of treatment was studied in each genotype using Bonferroni post test (*, $P < .05$ vs PLB, ***, $P < .001$ vs PLB; $$$, $P < .001$ vs E2; n = 4 to 6 mice per group).

So far, the specific roles of ERαAF-1 and ERαAF-2 have been explored mainly in vitro in cultured overexpressed cell lines. These studies have shown that the relative involvement of ERαAF-1 and ERαAF-2 in ERα activity depends on the type and the differentiation stage of the cells and requires specific cofactors and posttranslational modifications (12, 39). ERαAF-1 and ERαAF-2 can synergize for the recruitment of different cofactors (40–43), and this functional synergism depends strongly on the promoter context (44). In addition, an interaction between the A and E domains has been identified (45), suggesting that "repositioning" of helix 12 in response to E2 binding to the ligand pocket not only unmasks ERαAF-2 activation, but also allows the activation of ERαAF-1 through the release of the A domain.

An important role of the nuclear, "genomic," transcriptional action of ERα was previously demonstrated in vivo. Indeed, the EAAE mice, which harbor mutations in 4 amino acids crucial for ERα DNA binding activity are characterized by full abrogation of E2-dependent uterine gene expression and growth (46). Moreover, in the mouse model where 2-point mutations of leucine 543 and 544 to alanine (L543A, L544A) have been introduced in helix 12 (also named AF2ERKI mouse), Korach and coworkers (18) previously demonstrated that ERαAF-2 mutation results in an abrogation of the E2 action in the uterus. However, in this model, tamoxifen still induced endometrial proliferation and ER-mediated gene responses. In another model of ERαAF2 in which 7 amino acids, 543-549 in the helix 12, were deleted (33), we report here that the proliferative effect of both E2 and tamoxifen on the endometrial epithelium are abrogated. Thus, whereas 2-point mutations (L543A, L544A) in ERα allow to tamoxifen to act as an agonist, a more extensive alteration of helix 12 (deletion of 7 amino acids in the helix 12) abrogates the effect of tamoxifen. These discrepancies may be a consequence of an altered synergism between ERαAF-1 and ERαAF-2, at least on endometrial proliferation. On the other hand, E2 can also induce a rapid nongenomic response through the activation of a pool of ERs localized at the plasma membrane (47). However, the selective activation of this response using an estrogen-dendrimer conjugate has no effect on uterine growth (48), demonstrating that the "nongenomic"/membrane-initiated activation of ERα is not sufficient to elicit uterine growth or cell proliferation. Altogether, these findings demonstrate that endometrial proliferation induced by E2 is highly dependent on the ERα genomic actions and requires the recruitment of ERαAF-1. We demonstrate here that ERαAF-1 is required for the uterine transcriptional and proliferative responses to E2. Indeed, although the expression of 2 genes remained significantly regulated in early response to E2 in *ERαAF-1⁰* mice, the magnitude of their induction or repression was strongly attenuated compared with wild-type littermates. Similarly, we show that ERαAF-1 is required for E2 induction of C3 (ERE)- and AP-1-dependent luciferase activity in a human endometrial adenocarcinoma cell line (Ishikawa cells), suggesting a crucial role of ERαAF-1 in this cell type. We then provide the first evidence that ERαAF-1 is necessary for luminal epithelial cell proliferation in response to both acute and chronic E2 treatment in vivo. Tamoxifen, known as a selective activator of AF-1, further emphasizes the crucial role of AF-1 because the specific induced endometrial proliferation by tamoxifen is lost in *ERαAF-1⁰* mice.

It was previously demonstrated that activation of ERα in the uterine stromal cells elicits the release of paracrine factors that are required to induce epithelial cells proliferation (49). However, ERα of the uterine epithelial cells is dispensable for their proliferative response (37). Similarly, tissue recombination studies (50) and genetic approach with epithelial ERα-deficient mice (37) indicated that down-regulation of epithelial PR by E2 requires stromal, but not epithelial, ERα. Here, we report that the complete redistribution of PR expression from epithelium to stroma under E2 treatment is lost in $ER\alpha^{-/-}$ mice, but partially preserved in the *ERαAF-1⁰* mice. Whereas epithelial proliferation is completely abrogated in *ERαAF-1⁰* mice, these data suggest that stromal ERαAF-1 activation could play a crucial role for endometrial epithelial proliferation and a significant role in PR redistribution. During the female reproductive cycle, a balance between proliferation and subsequent elimination of proliferative cells by apoptosis is regulated, in particular, by E2. In wild-type mice, an increase in active caspase-3 is a key actor of this balance. Epithelial ERα contributes to prevent uterine epithelial apoptosis after E2 stimulation and, at its targeted deletion, doubles the level of apoptosis without directly altering the proliferative response (37). Here, we report that the level of epithelial apoptosis is strongly attenuated in *ERαAF-1⁰* mice compared with wild-type controls, and the precise role of ERαAF-1 in the proliferation-apoptosis balance should be delineated in future studies.

In contrast to the crucial role in endometrial proliferation, we found that ERαAF-1 was partially dispensable to the E2 effect on the uterine vascular permeability. Indeed, E2 was still able to increase uterine weight in *ERαAF-1⁰* mice, a response essentially due to an interstitial tissue infiltration. The persistence of the normal regulation of some E2-dependent gene at 4 weeks, in particular *Vegf-a* known vascular permeability factor, probably plays a significant role in this residual vascular action. Importantly, this dissociation between endometrial proliferation and uterine weight highlights the limitation of this later parameter as a marker of the pathophysiologic impact of E2 on the uterus.

We and others previously showed that the beneficial effects of estrogens on cortical bone (51) and atheroma (19, 33) are ERαAF-1 independent and ERαAF-2 dependent. We provide evidence here that ERαAF-1 plays a crucial role in uterine cell proliferation and could thereby contribute to the physiopathology of endometrial cancer, in line with the harmful action of tamoxifen on this target. Finally, we confirm here that full ERαAF-1 activity is required for E2-dependent proliferation of cultured MCF-7 breast cancer cells (52–54).

Prevention of breast cancer, type 2 diabetes, osteoporosis, and cardiovascular diseases by novel selective ER modulators (SERMs) represents the major challenge for the future treatment of menopause (55). We hypothesize that a SERM that preferentially stimulates ERαAF-2 and has a minimal effect on ERαAF-1 would retain many of the E2-protective responses, but would not elicit uterine and breast cell proliferation. This SERM would not require the addition of a progestin to prevent uterine proliferation, thereby offering an optimized therapeutic profile for menopausal women. Alternatively, strategies aiming at blocking ERαAF-1 in the presence of E2 would confer a similar benefit, as already reported for inhibitors of the androgen receptor AF-1 (56).

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References

1. **Das RM.** The effects of oestrogen on the cell cycle in epithelial and connective tissues of the mouse uterus. *J Endocrinol*. 1972;55:21–30.
2. **Rockwell LC, Pillai S, Olson CE, Koos RD.** Inhibition of vascular endothelial growth factor/vascular permeability factor action blocks estrogen-induced uterine edema and implantation in rodents. *Biol Reprod*. 2002;67:1804–1810.
3. **Large MJ, DeMayo FJ.** The regulation of embryo implantation and endometrial decidualization by progesterone receptor signaling. *Mol Cell Endocrinol*. 2012;358:155–165.
4. **Rossouw JE, Prentice RL, Manson JE, et al.** Postmenopausal hormone therapy and risk of cardiovascular disease by age and years since menopause. *JAMA*. 2007;297:1465–1477.
5. **Lenfant F, Trémollières F, Gourdy P, Arnal JF.** Timing of the vascular actions of estrogens in experimental and human studies: why protective early, and not when delayed? *Maturitas*. 2011;68:165–173.
6. **Rossouw JE, Anderson GL, Prentice RL, et al.** Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. *JAMA*. 2002;288:321–333.
7. **Anderson GL, Limacher M, Assaf AR, et al.** Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. *JAMA*. 2004;291:1701–1712.
8. **Tora L, White J, Brou C, et al.** The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell*. 1989;59:477–487.
9. **Marino M, Galluzzo P, Ascenzi P.** Estrogen signaling multiple pathways to impact gene transcription. *Curr Genomics*. 2006;7:497–508.
10. **McKenna NJ, O'Malley BW.** Nuclear receptors, coregulators, ligands, and selective receptor modulators: making sense of the patchwork quilt. *Ann NY Acad Sci*. 2001;949:3–5.
11. **Smith CL, O'Malley BW.** Coregulator function: a key to understanding tissue specificity of selective receptor modulators. *Endocr Rev*. 2004;25:45–71.
12. **Métivier R, Penot G, Hübner MR, et al.** Estrogen receptor-α directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell*. 2003;115:751–763.
13. **Hewitt SC, Deroo BJ, Hansen K, et al.** Estrogen receptor-dependent genomic responses in the uterus mirror the biphasic physiological response to estrogen. *Mol Endocrinol*. 2003;17:2070–2083.
14. **Katzenellenbogen BS, Bhakoo HS, Ferguson ER, et al.** Estrogen and antiestrogen action in reproductive tissues and tumors. *Recent Prog Horm Res*. 1979;35:259–300.
15. **Griffith JS, Jensen SM, Lunceford JK, et al.** Evidence for the genetic control of estradiol-regulated responses. Implications for variation in normal and pathological hormone-dependent phenotypes. *Am J Pathol*. 1997;150:2223–2230.
16. **Hewitt SC, Kissling GE, Fieselman KE, Jayes FL, Gerrish KE, Korach KS.** Biological and biochemical consequences of global deletion of exon 3 from the ER α gene. *FASEB J*. 2010;24:4660–4667.
17. **Sinkevicius KW, Burdette JE, Woloszyn K, et al.** An estrogen receptor-α knock-in mutation provides evidence of ligand-independent signaling and allows modulation of ligand-induced pathways in vivo. *Endocrinology*. 2008;149:2970–2979.
18. **Arao Y, Hamilton KJ, Ray MK, Scott G, Mishina Y, Korach KS.** Estrogen receptor α AF-2 mutation results in antagonist reversal and reveals tissue selective function of estrogen receptor modulators. *Proc Natl Acad Sci USA*. 2011;108:14986–14991.
19. **Billon-Gales A, Fontaine C, Filipe C, et al.** The transactivating function 1 of estrogen receptor α is dispensable for the vasculoprotective actions of 17β-estradiol. *Proc Natl Acad Sci USA*. 2009;106:2053–2058.
20. **Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P, Mark M.** Effect of single and compound knockouts of estrogen receptors α (ERα) and β (ERβ) on mouse reproductive phenotypes. *Development*. 2000;127:4277–4291.
21. **Spurgeon SL, Jones RC, Ramakrishnan R.** High throughput gene expression measurement with real time PCR in a microfluidic dynamic array. *PloS one*. 2008;3:e1662.
22. **Laurell H, Iacovoni JS, Abot A, et al.** Correction of RT-qPCR data for genomic DNA-derived signals with ValidPrime. *Nucleic Acids Res*. 2012;40:e51.
23. **Watanabe H, Suzuki A, Kobayashi M, Takahashi E, Itamoto M, Lubahn DB, Handa H, Iguchi T.** Analysis of temporal changes in the expression of estrogen-regulated genes in the uterus. *J Mol Endocrinol*. 2003;30:347–358.
24. **Moggs JG, Tinwell H, Spurway T, et al.** Phenotypic anchoring of gene expression changes during estrogen-induced uterine growth. *Environ Health Perspect*. 2004;112:1589–1606.
25. **Suzuki A, Urushitani H, Watanabe H, Sato T, Iguchi T, Kobayashi T, Ohta Y.** Comparison of estrogen responsive genes in the mouse uterus, vagina and mammary gland. *J Vet Med Sci*. 2007;69:725–731.
26. **Waters KM, Safe S, Gaido KW.** Differential gene expression in response to methoxychlor and estradiol through ERα, ERβ, and AR in reproductive tissues of female mice. *Toxicol Sci*. 2001;63:47–56.
27. **Ivanga M, Labrie Y, Calvo E, et al.** Temporal analysis of E2 transcriptional induction of PTP and MKP and downregulation of IGF-I pathway key components in the mouse uterus. *Physiol Genomics*. 2007;29:13–23.
28. **McDonnell DP.** The molecular pharmacology of SERMs. *Trends Endocrinol Metab*. 1999;10:301–311.
29. **Heldring N, Nilsson M, Buehrer B, Treuter E, Gustafsson JA.** Identification of tamoxifen-induced coregulator interaction surfaces within the ligand-binding domain of estrogen receptors. *Mol Cell Biol*. 2004;24:3445–3459.
30. **Zhang H, McElrath T, Tong W, Pollard JW.** The molecular basis of tamoxifen induction of mouse uterine epithelial cell proliferation. *J Endocrinol*. 2005;184:129–140.

31. **Fong CJ, Burgoon LD, Williams KJ, Forgacs AL, Zacharewski TR.** Comparative temporal and dose-dependent morphological and transcriptional uterine effects elicited by tamoxifen and ethynylestradiol in immature, ovariectomized mice. *BMC Genomics*. 2007;8:151.
32. **Kazi AA, Koos RD.** Estrogen-induced activation of hypoxia-inducible factor-1α, vascular endothelial growth factor expression, and edema in the uterus are mediated by the phosphatidylinositol 3-kinase/Akt pathway. *Endocrinology*. 2007;148:2363–2374.
33. **Billon-Galés A, Krust A, Fontaine C, et al.** Activation function 2 (AF2) of estrogen receptor-α is required for the atheroprotective action of estradiol but not to accelerate endothelial healing. *Proc Natl Acad Sci USA*. 2011;108:13311–13316.
34. **Curtis Hewitt S, Goulding EH, Eddy EM, Korach KS.** Studies using the estrogen receptor α knockout uterus demonstrate that implantation but not decidualization-associated signaling is estrogen dependent. *Biol Reprod*. 2002;67:1268–1277.
35. **Kurita T, Lee KJ, Cooke PS, Lydon JP, Cunha GR.** Paracrine regulation of epithelial progesterone receptor and lactoferrin by progesterone in the mouse uterus. *Biol Reprod*. 2000;62:831–838.
36. **Tibbetts TA, Mendoza-Meneses M, O'Malley BW, Conneely OM.** Mutual and intercompartmental regulation of estrogen receptor and progesterone receptor expression in the mouse uterus. *Biol Reprod*. 1998;59:1143–1152.
37. **Winuthayanon W, Hewitt SC, Orvis GD, Behringer RR, Korach KS.** Uterine epithelial estrogen receptor α is dispensable for proliferation but essential for complete biological and biochemical responses. *Proc Natl Acad Sci USA*. 2010;107:19272–19277.
38. **Martin L, Das RM, Finn CA.** The inhibition by progesterone of uterine epithelial proliferation in the mouse. *J Endocrinol*. 1973;57:549–554.
39. **Mérot Y, Métivier R, Penot G, et al.** The relative contribution exerted by AF-1 and AF-2 transactivation functions in estrogen receptor α transcriptional activity depends upon the differentiation stage of the cell. *J Biol Chem*. 2004;279:26184–26191.
40. **Onate SA, Boonyaratanakornkit V, Spencer TE, et al.** The steroid receptor coactivator-1 contains multiple receptor interacting and activation domains that cooperatively enhance the activation function 1 (AF1) and AF2 domains of steroid receptors. *J Biol Chem*. 1998;273:12101–12108.
41. **Benecke A, Chambon P, Gronemeyer H.** Synergy between estrogen receptor α activation functions AF1 and AF2 mediated by transcription intermediary factor TIF2. *EMBO Rep*. 2000;1:151–157.
42. **Kobayashi Y, Kitamoto T, Masuhiro Y, et al.** p300 mediates functional synergism between AF-1 and AF-2 of estrogen receptor α and β by interacting directly with the N-terminal A/B domains. *J Biol Chem*. 2000;275:15645–15651.
43. **Métivier R, Penot G, Flouriot G, Pakdel F.** Synergism between ERα transactivation function 1 (AF-1) and AF-2 mediated by steroid receptor coactivator protein-1: requirement for the AF-1 α-helical core and for a direct interaction between the N- and C-terminal domains. *Mol Endocrinol*. 2001;15:1953–1970.
44. **Tzukerman MT, Esty A, Santiso-Mere D, et al.** Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. *Mol Endocrinol*. 1994;8:21–30.
45. **Métivier R, Stark A, Flouriot G, et al.** A dynamic structural model for estrogen receptor-α activation by ligands, emphasizing the role of interactions between distant A and E domains. *Mol Cell*. 2002;10:1019–1032.
46. **Ahlbory-Dieker DL, Stride BD, Leder G, et al.** DNA binding by estrogen receptor-α is essential for the transcriptional response to estrogen in the liver and the uterus. *Mol Endocrinol*. 2009;23:1544–1555.
47. **Levin ER.** Integration of the extranuclear and nuclear actions of estrogen. *Mol Endocrinol*. 2005;19:1951–1959.
48. **Chambliss KL, Wu Q, Oltmann S, et al.** Non-nuclear estrogen receptor α signaling promotes cardiovascular protection but not uterine or breast cancer growth in mice. *J Clin Invest*. 2010;120:2319–2330.
49. **Cooke PS, Buchanan DL, Young P, et al.** Stromal estrogen receptors mediate mitogenic effects of estradiol on uterine epithelium. *Proc Natl Acad Sci USA*. 1997;94:6535–6540.
50. **Kurita T, Lee KJ, Cooke PS, Taylor JA, Lubahn DB, Cunha GR.** Paracrine regulation of epithelial progesterone receptor by estradiol in the mouse female reproductive tract. *Biol Reprod*. 2000;62:821–830.
51. **Börjesson AE, Windahl SH, Lagerquist MK, et al.** Roles of transactivating functions 1 and 2 of estrogen receptor-α in bone. *Proc Natl Acad Sci USA*. 2011;108:6288–6293.
52. **Fujita T, Kobayashi Y, Wada O, et al.** Full activation of estrogen receptor α activation function-1 induces proliferation of breast cancer cells. *J Biol Chem*. 2003;278:26704–26714.
53. **Flouriot G, Brand H, Denger S, et al.** Identification of a new isoform of the human estrogen receptor-α (hER-α) that is encoded by distinct transcripts and that is able to repress hER-α activation function 1. *EMBO J*. 2000;19:4688–4700.
54. **Penot G, Le Péron C, Mérot Y, et al.** The human estrogen receptor-α isoform hERα46 antagonizes the proliferative influence of hERα66 in MCF7 breast cancer cells. *Endocrinology*. 2005;146:5474–5484.
55. **Katzenellenbogen BS, Katzenellenbogen JA.** Biomedicine. Defining the "S" in SERMs. *Science*. 2002;295:2380–2381.
56. **Sadar MD.** Small molecule inhibitors targeting the "achilles' heel" of androgen receptor activity. *Cancer Res*. 2011;71:1208–1213.