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LESSONS IN ESTROGEN BIOLOGY FROM KNOCKOUT AND TRANSGENIC ANIMALS

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■ **Abstract** Tremendous progress has been made in elucidating numerous critical aspects of estrogen signaling. New tools and techniques have enabled detailed molecular analysis of components that direct estrogen responses. At the other end of the spectrum, generation of a multiplicity of transgenic animals has allowed analysis of the physiological roles of the estrogen-signaling components in biologically relevant models. Here, we review the ever-increasing body of knowledge in the field of estrogen biology, especially as applied to the female reproductive processes.

THE ESTROGEN RECEPTOR MOLECULE: STRUCTURES LEAD TO MECHANISMS

Estrogens are essential hormones for successful reproduction in mammals. Although produced locally by the ovary, estrogens circulate systemically and exert selective effects on target tissues. This is mediated by the presence of estrogen receptors (ER), estrogen-regulated transcription factors. Estrogen receptors are members of a family of nuclear transcription factors including receptors for sex steroids, thyroid hormone, vitamin D, retinoids, as well as many orphan receptors, for which no ligands have been identified (1). A second ER gene was cloned from prostate tissue in 1996 (2), and thus there are two ER molecules: the originally described ERα and now the ERβ. By comparing the ER sequences, it is apparent that both share a general domain structure common to ligand-modulated nuclear transcription factors (3). The current understanding of ER mechanisms of action can be summarized with reference to the overall structure of the receptors (Figure 1). The functions of some regions of the ER molecules have been defined using deletion and mutation as well as structural analysis, as described by Nettles & Greene in this volume (3a). The best-characterized functions

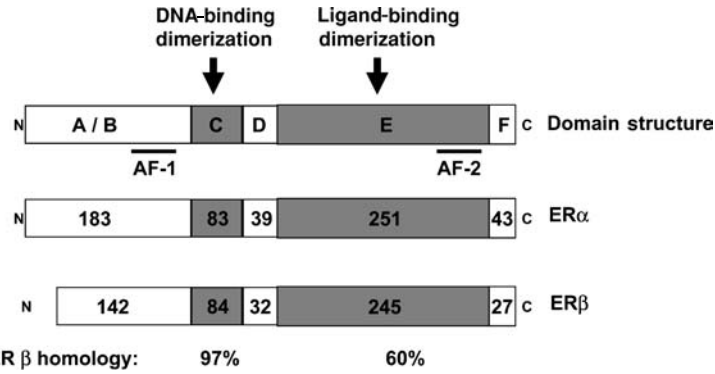


Figure 1 Comparison of domain structures of ERα and ERβ. The estrogen receptors are members of the nuclear receptor superfamily and share a domain structure, which is depicted schematically. The ERs have six domains, A–F, and the number of amino acids in these domains, as well as the functions associated with these domains, are indicated for each form of ER. AF-1 and AF-2 refer to regions that mediate the transcriptional activation functions of the ERs. The degree of homology between ERα and ERβ in the (C) and estrogen domains are indicated below these domains. The ERβ domains are derived from SeqWeb GAP analysis of mouse ERα amino acids 1–599 compared with mouse ERβ 1–530. Reproduced from (26).

include a zinc-finger-containing domain (C domain, Figure 1), which binds with high affinity and specificity to estrogen response elements (EREs) in target genes, and a ligand-binding domain (domain E), which binds estrogen as well as other estrogenic ligands. The consensus ERE is a 13-base pair inverted repeat sequence (GGTCAnnnTGACC); however, the majority of ERE sequences contain one or more variations from the consensus. In vitro DNA-binding studies have indicated that the ER binds as a dimer (4), with one ER molecule contacting each 5-base pair-inverted repeat (5). Although DNA binding is a dimerization stimulus, sequences in the ligand-binding domain are also involved in dimerization (4), and crystallized truncated ER containing only the ligand-binding domain is clearly shown to be a dimer in the presence of an agonist ligand (6).

The AF-1 region in the amino terminus and the AF-2 region within the ligand-binding domain are involved in ligand-independent and ligand-dependent transcriptional activation, respectively, as deletion or mutations of these regions result in a diminished ability to regulate estrogen-responsive genes (7, 8). The mechanism by which transcription is mediated by the ER is thought to occur via interaction of AF-1 and AF-2 with the transcriptional machinery, a general term referring to the complex of molecules that assembles and ultimately results in synthesis of mRNA (9). Much is now known about RNA polymerase II, and the enzymes and factors that orchestrate transcription as discussed in several recent reviews (9–11). Ligand-activated ERs bind to target genes and recruit coregulators and associated

chromatin remodeling complexes. Transcriptional coregulators mediate the interaction between ERs and the transcriptional machinery, and many coregulators have been isolated that interact with ER in a ligand-dependent manner. These include members of the steroid receptor complex (SRC)/p160 family or the thyroid receptor-associated protein (TRAP220) complex. Recent reports suggest that p160 and TRAP220 complexes are cyclically exchanged on and off estrogen-responsive genes in a process of repeated chromatin remodeling/transcriptional initiation and transcriptional reinitiation/maintenance, respectively. These mechanisms are more fully discussed elsewhere (11–13). The best-characterized coregulators interact with the AF-2 of the ER, although molecules that interact with the N terminus or AF-1 and AF-2 have also been described. Notably, the coactivator, p68, which interacts with the AF-1 region of ERα, has RNA helicase activity and associates with SRA, a RNA molecule with coactivator activity (14–16).

In the simplest models of estrogen action, estrogen binds to the receptor, which interacts with ERE DNA sequences in target genes. The estrogen-ER complex then recruits the transcriptional comodulators and consequently regulates transcription of target genes. However, numerous variations in this mechanism have been described. Many estrogen-responsive genes lack the canonical ERE sequence and interact with estrogen receptors via a tethering mechanism with a combination of estrogen receptor and SP1 or AP1 transcription factors (17–19). In addition, several mechanisms that account for very rapid nongenomic or nongenotropic estrogen responses indicate that estrogen receptors, either distinct or identical to the nuclear ERs, interact with and activate signal cascades at the cell membrane (20, 21). Finally, several activators of the growth factor receptor pathways, including IGF-1 and EGF, can result in activation of ER-mediated transcription in a ligand-independent manner (20). The mechanistic details of estrogen receptor–directed transcription are more fully described and discussed elsewhere.

USE OF MOUSE MODELS TO STUDY ESTROGEN BIOLOGY

The biological effects of estrogens occur predominantly in female reproductive tissues such as the reproductive tract and mammary glands. However, estrogen has also been shown to play roles in male tissues, as well as in nonreproductive tissues including the central nervous system, the skeletal system, and the cardiovascular system. Historically, surgical and/or pharmacological manipulation has been used to explore the roles of estrogen in reproduction and physiology. The development of knockout or transgenic mice with disruptions, mutation, or overexpression of molecules related to reproduction and hormone action has increased our understanding of their relative roles in developmental and biological processes in the mouse. For example ERα and ERβ knockout mice (αERKO and βERKO) exhibit overt phenotypes related to the essential roles of these receptors in certain tissues and biological responses. There are now hundreds of examples of gene disruptions

in mice that result in reproductive phenotypes, as discussed and summarized in recent reviews (22, 23). Here we provide an overview of phenotypes observed in some of these mouse models, particularly as they apply to estrogen biology and female reproduction. We also discuss applications of these models to study hormone responses or pathologic conditions.

Estrogen Biology 101: ERKO and PRKO Models

The description of the losses of function and pathologies in the ERKO model is an ongoing and basic lesson in estrogen biology studies. Disruption of ERα (24) resulted in infertility of both males and females and has been described in detail elsewhere (25, 26), but it is summarized in Table 1. In females, estrogen and progesterone are essential primarily in three biological processes: pubertal development, regulation of estrous cycles, and establishment and maintenance of pregnancy and lactation. At puberty, increasing estrogen directs the maturation of mammary tissue. At birth, the mammary tissue consists of an epithelial rudiment embedded in stromal tissue, and at puberty increased ovarian steroids induce outgrowth of the epithelial ducts until they reach the margins of the stroma (27). The importance of estrogen to this development is illustrated by the lack of mammary duct outgrowth in αERKO mice. In contrast, mice that lack ERβ (βERKO) or progesterone receptors (PR) (PRKO) develop full epithelial ductal structures during puberty (28).

The second biological process for which estrogen and progesterone are needed is regulation of the estrous cycle of the mouse. Normally, adult female mice undergo an estrous cycle every 4–5 days, under the control of gonadotropin-releasing hormone (GnRH) pulses from the hypothalamic region of the brain, which regulate the release of the gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), from the pituitary gonadotroph cells. The gonadotropins then direct the maturation of ovarian follicles, which produce estradiol and progesterone prior to and following ovulation, respectively (29). The interaction of these organs in the regulation of reproduction is termed the hypothalamic-pitutary-gonadal (HPG) axis. Estradiol produced by the ovary in response to the gonadotropin signals is also an essential regulator of the HPG axis, through both ERα and ERβ. Estradiol, for example, down-regulates LHβ gene transcription. αERKO females lack this estradiol-mediated negative feedback on LH and thus have chronically elevated LH, demonstrating the ERα dependence in this process (25). The αERKO females do not undergo estrous cycling and thus do not ovulate. When exogenous gonadotropins are administered to young αERKO females before the LH rises in an attempt to supply a corrected HPG environment (a course of treatment called superovulation), ovulation can be induced (30), indicating that the αERKO ovary responds to LH and FSH and that these processes do not depend on ERα per se, but successful ovulation requires appropriate regulation of HPG components, which does require the ERα.

The estrogen and progesterone produced by the ovary in each estrous cycle prepare the uterus for implantation of embryos (31). The increased weight of the

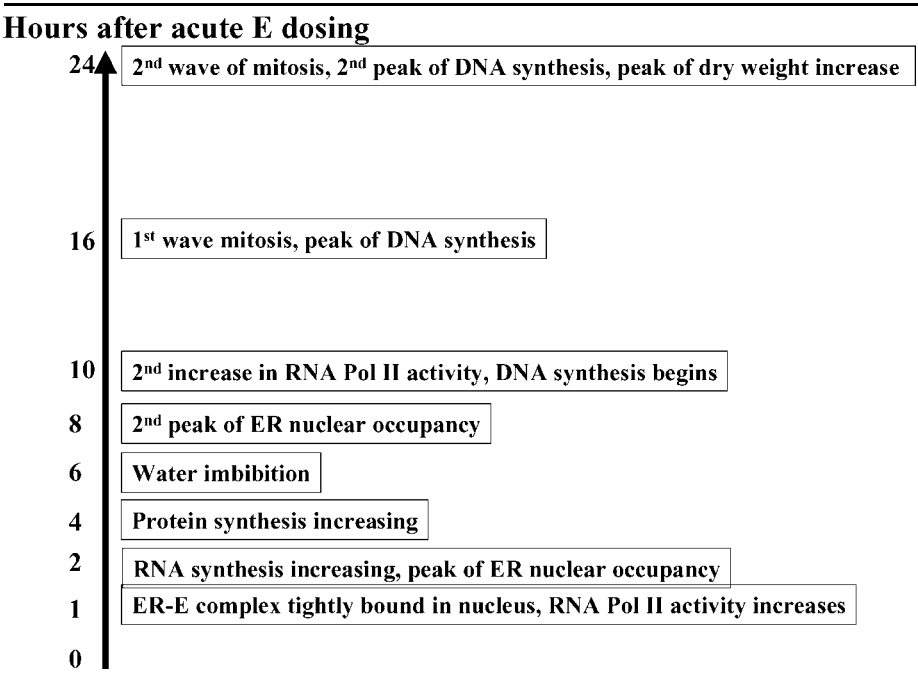
TABLE 1 Phenotypes leading to ERKO infertility[a]

Tissue	α	β	$\alpha\beta$
Mammary	Immature-ductal rudiment	Normal structure and lactation	Immature-ductal rudiment
Fertility	Both sexes are infertile	Fertile males Subfertile females: infrequent pregnancies, small litter sizes	Both sexes are infertile
Pituitary	LH production is elevated, low prolactin	Normal	Elevated LH production
Ovary	Estrogen and testosterone elevated Follicles don't mature; hemorrhagic cystic follicles begin developing at puberty as a result of chronic elevated LH. Reduced ovulations in superovulation trial, "trapped follicle" phenotype after superovulation	Reduced number of corpora lutea, inefficient ovulation in superovulation trial, "trapped follicle" phenotype at superovutation. Normal gonadotropins and steroids	Progressive degeneration of germ cells, dramatic loss of granulosa cells; appearance of Sertoli-like cells; elevated LH, elevated estrogen and testosterone
Uterus: estrogen responsiveness	Immature. Insensitive to estrogen, no epithelial proliferation or induction of estrogn-responsive genes	Normal responses to estrogen	Insensitive to estrogen-like αERKO
Uterus: progesterone responsiveness	PR present, progesterone-responsive genes induced, decidualization is estrogen independent	Nd	Nd
Uterus: implantation	No implantation	Not tested, but pregnancies occur and are carried to term, inferring implantation competence	Nd
Testes	Progressive fluid retention and dilation of seminiferous tubules, eventual loss of sperm	Normal	Progressive fluid retention and dilation of seminiferous tubules, eventual loss of sperm

[a]Adapted from (26).

ND: not determined.

TABLE 2



ovariectomized mouse uterus following a 3-day dosing of estrogenic compounds has long served as a measure of estrogen sensitivity, and the αERKO lacks this response (24). Additionally, following an acute dose of estradiol, the ovariectomized mouse uterus undergoes a series of biochemical and biological changes that are summarized in Table 2. The αERKO lacks these responses, indicating the requirement for ERα to mediate them. The uterine epithelial cells in the αERKO do not proliferate in response to estrogen, as measured by ^{3}H thymidine uptake or BrdU incorporation, nor is there an increase in uterine weight in this 24-h time period, illustrating the lack of water imbibition and hyperemia. Additionally, the infiltration of the uterine tissue by eosinophils following estrogen treatment does not occur in the αERKO (L.L. Hayes, A.-J. Lambert, C.R. Schmidt & H.H. Harris, personal communication). Uterine gene transcription in response to estrogen is robust, but as might be expected, the transcriptional response to estrogen in the αERKO is minimal (33, 34). Post-ovulatory progesterone is important in the regulation of the uterine proliferative response to estrogen. Rising progesterone shifts the proliferative response from the epithelial cells to the uterine stroma (35). Uteri of mice that lack the progesterone receptor (PRKO) develop hyperplasia in response to estrogen, indicating that PR is needed to regulate and temper the proliferative response to estrogen (36). Two PR isoforms are present in uterine tissue: PRA and PRB. Antiproliferative regulation of the estrogen response is recovered in a

PRB-selective knockout (PRBKO, has only PRA), indicating PRA is the isoform that mediates the antiproliferative effect (36). The diminished estrogen sensitivity of mice lacking the steroid receptor coactivator 1 (Src1) indicates uterine response to estrogen also depends on transcriptional coactivator molecules as well (37).

The third biological process that requires estrogen and progesterone is the establishment and maintenance of pregnancy. The αERKO is anovulatory (see Table 1); thus to determine whether the αERKO uterus is capable of implanting embryos, mice were treated with a hormonal regimen mimicking early pregnancy. Although similarly treated normal wild-type (WT) females could implant donor embryos, the αERKOs could not (38). Additionally, in response to implanting embryos, the uterine tissue normally undergoes a massive increase in size as implantation sites are formed in a process called decidualization. The αERKO uterus can be induced to decidualize with an artificial regimen mimicking early pregnancy (39). Together, these experiments indicate that although the uterus does not require ERα to decidualize, it must have ERα for implantation to occur. [Progesterone's role in implantation and decidualization is illustrated by studies in which the PRKO and PRAKO lack decidualization and implantation (36), indicating the essential role of PRA in both of these processes.]

During pregnancy, the progesterone level remains elevated, which not only maintains the pregnancy but also induces development of mammary gland structures necessary for lactation. In a nonpregnant mouse, during each estrous cycle, the mammary tissue is relatively quiescent and might be described as having the appearance of bare tree branches that occupy the extent of the underlying mammary stromal tissue (28). The quiescence of the mammary tissue is illustrated using microarray analysis to examine the global gene responses of the mammary tissue to acute estrogen. When the uterine and mammary gland gene regulation patterns are compared, it is apparent that fewer gene changes occur in the mammary gland, as eight times more uterine than mammary gland genes show significant changes 24 h after estrogen injection (S.C. Hewitt, unpublished data). In addition, 75% of the gene changes that occur in the mammary gland represent decreases in gene expression levels (S.C. Hewitt, unpublished data); in contrast, only 20% of the gene changes in the uterus reflect decreases (34). In response to pregnancy levels of progesterone and prolactin, extensive mammary epithelial proliferation occurs that greatly increases the complexity of the ducts by increasing side branches (which might be described as twigs on a tree branch) (28). Additionally, lobuloalveolar structures develop at the ends of the ducts, which have the appearance of buds on the tree branches and which will begin to fill with milk late in pregnancy. PRKO mice are infertile and thus never become pregnant, and although the ductal tree structure develops at puberty, treatment with pregnancy levels of progesterone does not induce the side-branching or lobuloalveolar development (28, 36). The PRAKO recovers this response, indicating the PRB is sufficient to mediate pregnancy-associated mammary development. The αERKO never develops a full underlying ductal tree structure. However, exogenous progesterone does increase the complexity of the rudimentary ductal structure (40). It has been reported that

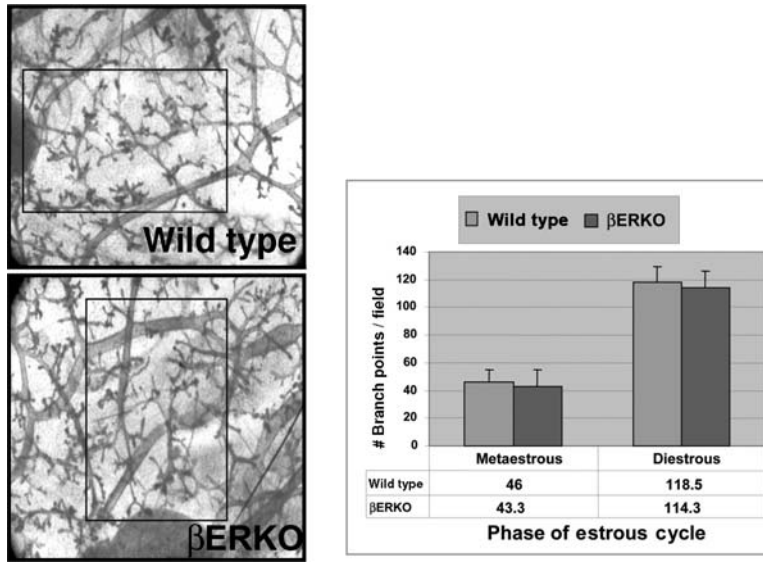


Figure 2 Mammary gland epithelial branching correlates with estrous stage. Comparable areas of wild-type (WT) or βERKO whole mounts (the rectangle shows the field) were analyzed by counting branch points. Each data point was associated with the estrous stage of the mouse, as determined by vaginal smear on the day of necropsy.

the βERKO ductal structures lack complexity (41); however, when correlated with the stage of the estrous cycle, we observed no difference between βERKO and WT structures (Figure 2). Therefore, the reported decreased complexity is a consequence of defects in βERKO estrous cycle leading to fewer individuals progressing to diestrous, the stage at which the greatest mammary complexity occurs. Overall, it is apparent that ERα is essential for pubertal ductal elongation, whereas PR is dispensable for this aspect of mammary gland development, but PRB is essential for pregnancy-associated alveolar formation and mammary gland development.

Estrogen Biology in the Ovary: A Complex Lesson

Study of the biological effects of estrogen on ovarian function is complicated by the level of estrogen synthesis inherent to the tissue. The ovary is made up of developing follicles that contain germ cells at different stages of maturity in a stromal/interstitial tissue (42). Within maturing follicles, the oocyte is surrounded by layers of granulosa cells that are rich in ERβ and are the primary source of estrogen biosynthesis. ERα, in contrast, is localized primarily to thecal and interstitial tissue components of the ovary (25). The ERKO models have proven valuable in that they provide an opportunity to study specific aspects of estrogen signaling within the ovary.

The chronically elevated LH in the αERKO leads to the development of hemorrhagic cysts in the αERKO ovary, an observation also reported in other knockout and transgenic mice that have elevated LH. These examples include mice overexpressing LH (43), FSH (44) or human chorionic gonadotropin (45) (HCG; an LH receptor agonist) transgenes, as well as inhibin α (46) and FSH receptor knockout mice (47), and those in long-term antiestrogen treatment (48, 49). GnRH antagonist treatment of prepubertal αERKO females prevents the rise in LH and the consequent formation of cysts (30). Thus the cysts are characteristic of elevated LH and occur in both the presence and absence of ERα. This is an important facet when drawing conclusions based on observed defects in knockout or transgenic mouse studies. Many homeostatic physiological systems, such as the HPG axis, are made up of interacting elements, and perturbation of components in one tissue (LHβ regulation in the pituitary) may result in physiological defects that are not a direct consequence of gene disruption in another tissue (ovarian cysts). Therefore, it is essential to be cautious in interpreting observations from knockout mice and to consider indirect effects on physiology. Newer models are now being developed to counteract the defect secondary to HPG problems of the ERKOs and allow study of estrogen biology and roles of ERs intrinsic to the ovary.

Lessons in Estrogen Biology: Estrogen Is Important to Male Fertility

The infertility exhibited by αERKO males was unexpected because estrogen was thought to have significant roles only in female reproduction. The αERKO males are infertile, in part, owing to the progressive degeneration of the testicular tissue and eventual loss of sperm because of fluid retention and dilation of the seminiferous tubules (25). αERKO females and males also fail to exhibit successful mating behaviors (25). The successful transmission of the ERα null trait by heterozygous breeders indicates the defect is not intrinsic to the ERα null sperm, but ERα is needed in somatic cells of the male reproductive tract for proper maturation and activation of sperm (50, 51).

Role of ERβ in Estrogen Biology as Revealed by the βERKO Mouse

Because the αERKO still retains ERβ function, the study of the βERKO highlights the contribution of ERβ to estrogen biology. The βERKO mice exhibit less profound phenotypes than the αERKO. The males retain full fertility but in some reported cases develop prostate hyperplasia with aging (52). The βERKO females are subfertile (25), and considering the profound ERβ expression in follicular granulosa cells, it is not surprising that the underlying cause of βERKO subfertility is inefficient ovulatory response. The ability of βERKO females to carry pregnancies to term and nurse their offspring indicates adequate uterine and mammary gland function, but it does not rule out more subtle effects on mammary or uterine responses. Microarray analysis of βERKO mice indicates a transcriptional

response to estrogen that is comparable to the WT in the uterine tissue (34). These findings are summarized in Table 1. Overall, the observations in the βERKO females indicate an important role for ERβ in achieving optimal fertility, yet successful pregnancies can occur, which indicates that ERβ is not required. Interestingly, in continuous mating studies, some βERKO females exhibited a normal frequency of pregnancy, with reduced litter sizes; some females had a reduced frequency of pregnancy, with reduced litter sizes; and a third group never became pregnant, suggesting individual differences in sensitivity to loss of ERβ (25, 53). One might hypothesize that all the processes required for successful ovulation are present in the βERKO; however, in some cases there is a defect in fully initiating the response. Indeed, superovulation studies resulted in a very low yield of oocytes, although pathology of the ovary showed many fully developed follicles seemingly ready to ovulate but apparently trapped on the verge of ovulation (54). Several other knockout models exhibit a similar defect, including cyclooxygenase 2 (55), PR (36), cyclin D2 (56), and RIP140 (57), and studies are underway to examine the regulation of these and other genes in superovulated βERKOs.

Male and female knockout mice that lack both ERα and ERβ (αβERKO) are infertile, and the underlying causes seem to reflect the previously observed defects in the αERKO (25), indicating the crucial contribution of ERα in mediating reproductive biological events. However, a unique phenotype was observed in the ovary, where a progressive loss of oocytes occurs, and an apparent transdifferentiation of granulosa cells into cells having the appearance of and expressing markers characteristic of Sertoli cells, which are normally found in seminiferous tubules (53, 58, 59). Such a unique ovarian phenotype suggests that maintenance of the proper differentiation state of granulosa cells requires the combined activity of both the ERα and ERβ in ovarian tissue.

Comparing ER-Null to Estrogen-Free Environment: ArKO

The enzyme responsible for synthesis of estradiol is a P450 enzyme, Cyp 19 also called aromatase, which converts testosterone to estradiol. The aromatase knockout (ArKO) mouse retains ERα and ERβ but does not synthesize any estrogen, and many of its observed phenotypes are similar to that of the αERKO, including immature mammary glands and uteri. Additionally, the ovaries progressively develop hemorrhagic cysts like those found in the αERKO mice, again consistent with a secondary effect as a consequence of elevated gonadotropins. Males are infertile owing to progressive loss of spermatids but do not develop the fluid distension seen in the αERKO, suggesting ligand-independent activity of the ERα in the ArKO might prevent the occurrence of the defect. ArKO males also exhibit deficiencies in sexual behaviors. In particular, the mounting response is impaired in both the ArKO and αβERKO males but is observed in the αERKO and βERKO males, although intromissions and ejaculations are deficient in the αERKO (60, 61). This indicates that mounting is dependent upon estrogen ligand synthesis but that

either ERα or ERβ is sufficient to mediate the mounting response (62). Estrogen replacement in ArKO females results in recovery of uterine weight (62, 63).

Biology Selective to the AP-1 Tethered Mode of ER Signaling: NERKI

A mouse has been engineered to express an ERα with mutations that selectively eliminate classical ERE-mediated signaling while retaining responses mediated via tethering through AP-1 sequences. Females heterozygous for this nonclassical ER knock-in (NERKI) are infertile; they are anovulatory and administering exogenous gonadotropins results in few ovulations (64). It is interesting that superovulation induced hemorrhagic cysts in the NERKI ovaries, yet prior to dosing, the LH levels are in the normal range, suggesting the presence of the nonclassical ER mutant somehow results in increased sensitivity to the gonadatropin, which causes a pathology characteristic of LH overstimulation; superovulation in WT and αERKO mice does not induce hemorrhagic cysts. The NERKI uteri progressively form enlarged hyperplastic endometrial glands, despite normal ovarian steroid levels, suggesting dis-regulated responsiveness of the tissue. The NERKI mammary glands have full ductal development but have decreased complexity, likely a result of anovulatory progesterone levels. The infertility of the heterozygotes has prevented generation of a mouse with two copies of the mutated ER. It is interesting that replacement of one copy of the WT ER results in such a pronounced phenotype, as mice heterozygous for the ERα null allele are fertile, indicating one copy of ERα is sufficient for reproduction. It seems that the combination of one copy of WT ERα and one copy of the nonclassical signaling ER mutation results in a unique perturbation of estrogen physiology. Future analysis of hemizygous NERKI mice produced by crossing the NERKI and the αERKO should indicate the biological consequences of exclusive expression of this nonclassical ER mutant in the animal. Similarly, we have generated a knock-in mouse that expresses ERα with a mutation in the ligand-binding domain of the receptor, which allows it to retain binding to estradiol, but prevents transcriptional activity (AF2ER). In our case, the mice expressing one copy of this mutant ER are fertile; however, embryos homozygous for this mutant ER die before implantation, indicating that unlike the ER-null mutant, the AF2ER mutant disrupts estrogen signaling at a critical point in embryogenesis (65).

APPLICATION OF MOUSE MODELS TO ESTROGEN MECHANISM STUDIES

As described briefly above, acute treatment of ovariectomized mice with estrogen has long served as a key experimental model in which to study the biochemical mechanisms underlying uterine responses. The events that occur following estrogen administration have been divided into those that occur early, within the

first hours following estrogen elevation, and subsequent responses that follow up to 24 h later. Thus this acute and rapid response of the uterus has been described as biphasic (66, 67). Early events include nuclear ER occupancy, transcription of early-phase genes such as c-*fos*, fluid uptake (termed water imbibition), hyperemia, and infiltration of immune system cells such as macrophages and eosinophils into the uterine tissue (68, 69). Later phase responses include the transcription of late-phase genes such as *lactoferrin*, increase in uterine wet weight, further accumulation of immune system cells, the development of the epithelial layer into columnar secretory epithelial cells, and subsequent mitosis, which occurs principally in the epithelial layer (70). Coordinated increased uterine DNA synthesis and mitosis are reported to begin 12–24 h following estrogen treatment of ovariectomized mice, indicative of synchronized entry into S phase (70). This is illustrated by the increased proliferating cell nuclear antigen (PCNA) detected in the epithelial cells (Figure 3). Microarray analysis of the global gene expression pattern in response to acute estrogen identified clusters of genes characteristic of these early and later responses, some of which overlap but some are distinct to the early or late time points of response (34) (Figure 4).

Significantly, estrogen decreased the expression of many genes (Figure 4), yet most mechanisms of ER-mediated gene regulation consider only increases in transcription. Our microarray data indicate ERα also mediates transcriptional repression because (*a*) antiestrogen treatment inhibited gene increases and decreases and (*b*) neither increased nor decreased gene levels were apparent in the αERKO microarray analysis (34). We have identified numerous endogenous uterine genes appropriate for investigation of the mechanisms involved in gene repression by estrogen.

Uterine epithelial cells not only must proliferate but must do so at the proper time as the estrous cycle progresses in preparation for implantation of embryos. Thus it is not surprising that the microarray analysis revealed regulation of several cell cycle modulators, including *p21*, *Cycin G1*, *cdc2*, *and Cyclin E1* (34), (Figure 5). Synchronous/coordinated regulation of the components modulating entry into S phase is one mechanism by which the acute exposure to estradiol might orchestrate the ordered biological response. Estrogen regulation of some cell cycle modulators in the uterus has been previously reported. For example, estrogen treatment induces nuclear relocalization of cyclin D1 protein and an increase in expression of cyclin A and E proteins in the uterine epithelium (35). In some cell types, cyclin D1 is an estrogen-responsive gene; however, in the uterus a minimal increase in transcript occurs (34). p21 inhibits progression into S phase, and its RNA and protein levels are maximally induced and localized to the epithelial cell nuclei 12 h following estradiol treatment (34) (Figures 3 and 5), just prior to the peak of entry into S phase, suggesting that the increased p21 may prevent S phase progression of the epithelial cells until the proper time, allowing coordinated proliferation of the epithelial cells. Thus the properly timed increase in *p21* may act as a gate, coordinating appropriate S phase progression.

A

Hours after E	0.5	2	6	12	24
p21	1.8	2.8	2.4	2.9	
Mad2		4.3	2.7		2.0
CycG				2.3	
Cdc2					1.7

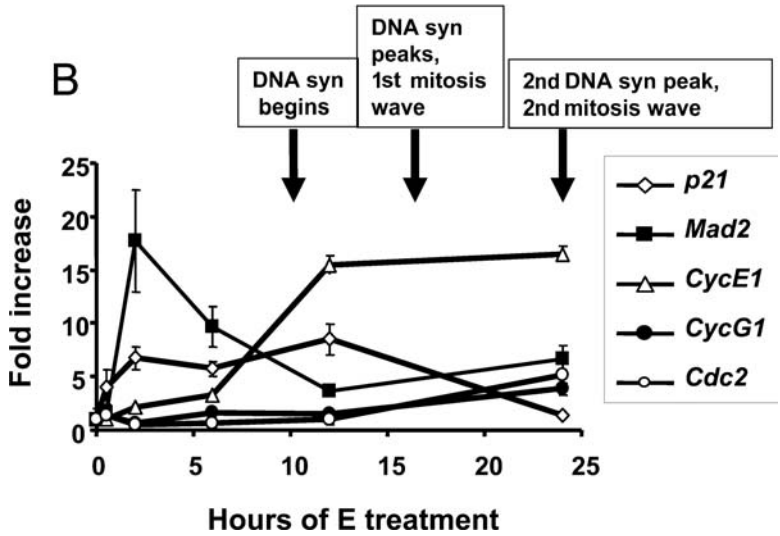


Figure 5 Coordinated regulation of cell cycle regulators by estrogen. (*A*) Table of values from microarray data. Fold increase versus vehicle control. (*B*) Transcripts for *p21*, *Mad2*, *Cyclins G1*, and *E1*, and *cdc2* were assayed following an acute dose of estrogen by RT-real time PCR. The observed times of biological events reflecting S and M phases are indicated. Adapted from (34).

Because the biphasic genomic pattern clearly mirrors the observed biological response, we considered whether the early gene changes were modulating the late genomic responses or whether the later responses depended on continuous ER-mediated activity. To test this, we injected the ER antagonist ICI 182–780 2 h after estrogen treatment to block any further ER activity subsequent to the initial early gene changes. Our preliminary results indicate that in some cases the late gene changes were blocked, suggesting that ER activity throughout the time course is necessary, whereas some late responses still occur, indicating they are most likely secondary responses mediated by early phase regulated genes (S.C. Hewitt, unpublished data).

Using the ERKO to Study ER-Growth Factor Cross Talk

Growth factors, including EGF and IGF-1, are present in uterine tissue, as are their respective receptors. The increases in these growth factors and activation of growth factor–signaling pathways by estrogen indicate roles in uterine biology. Additionally, activators of growth factor (GF) receptor pathways, including IGF-1 and EGF, can result in ER-mediated transcription (20). These cross talk mechanisms were demonstrated in vivo by showing an increase in uterine weight and proliferation of the uterine epithelial cells in ovariectomized mice following EGF or IGF-1 treatment, as illustrated by the Ki67-positive epithelial cells in Figure 6 (71–73). The lack of these responses in similarly tested αERKO mice indicated that the ERα is downstream of the growth factor receptor signaling in this response and that ERα is required (Figure 5) (72–74). The mechanism has been studied in vitro as well, using reporter gene assays, which similarly have shown the requirement for ERα for growth factor receptor activators to regulate estrogen-regulated reporter genes (75, 76). The approaches have been combined, and we showed that IGF-1 treatment increases expression of an estrogen-responsive luciferase reporter gene in transgenic mice (73), providing the first evidence in vivo of ligand-independent ER activation and support for the cross talk hypothesis.

Considering the model of growth factor receptor-ER cross talk, the uterine genomic response of WT and αERKO to EGF or IGF-1 would be expected to exhibit two patterns or clusters of genes with the following response profiles: The first would include genes regulated in response to estrogen or growth factor receptor signaling, representing the cross-talk response; because these responses require ERα, they would be lost in the αERKO. The second pattern would include genes that were directly regulated by growth factor receptor pathways. These genes would depend only on growth factor receptor pathways and therefore would be similarly regulated in the αERKO. Surprisingly, the global genomic response in the uterus did not fit these expected patterns (S.C. Hewitt, submitted manuscript). Clusters of genes that were regulated similarly by either estrogen or growth factors in the WT samples were observed, as described by the cross-talk mechanism. However, these genes retained growth factor responsiveness in the absence of ERα in the αERKO samples. ERα was required for estrogen regulation of these genes, as they were insensitive to estrogen in the αERKO. These responses to growth factors were not inhibited by antiestrogen (ICI 182,780). This seems to indicate that for these genes, growth factors can bypass the requirement for ERα, suggesting the responses are secondary to the increase of IGF by estrogen. However, these responses are occurring as early as 2 h subsequent to estrogen injection in the WT, prior to the peak of IGF induction. Clusters of genes that were regulated only by estrogen in the WT were also apparent. Additionally, genes regulated primarily by growth factors were seen in both WT and αERKOs. Although studies using model reporter genes and αERKO mice have previously demonstrated growth factor-mediated ERα responsiveness through a cross-talk model (72–75, 77), the global response of endogenous uterine genes to estrogen and growth factor appears to encompass greater complexity than this model describes.

Implantation-Associated Signals

In addition to responses initiated by estrogen or growth factors in the uterus, we have also examined responses to a stimulus intended to mimic early pregnancy. The uterine environment encounters a preovulatory estrogen surge, followed by increasing postovulatory progesterone that, as discussed above, prepares the uterus for embryo implantation. Experimental manipulation can mimic this process and elicit the responses of early pregnancy. For the uterus to decidualize, it is necessary to administer a stimulus, in our case infusion of inert oil into the uterus, which mimics the physical apposition of the embryo against the uterine wall. This stimulus must be administered during a specific time of responsiveness that reflects a window of receptivity to implantation (31). We became interested in the nature of the signals elicited by the oil infusion because historically the response to this stimulus had been shown to require prior priming with estrogen for decidualization to progress (78). However, in the αERKO uterus, we observed a relief of this estrogen requirement for decidualization. Use of ICI 182,780, an ER antagonist, also demonstrated that estrogen priming and ER signaling were required in the WT but not αERKO for decidualization responses (39). We examined some novel signals that were initiated and noted activation of phospho-STAT3, a transcriptional activator that is a target for cytokine signal pathways, in WT as well as in αERKO uteri following the oil infusion (38). Thus we evaluated the expression of leukemia-inhibitory factor (LIF), a cytokine increased by estrogen in the uterus and shown to be required for embryo implantation and decidualization (79, 80). We confirmed that estrogen increases uterine *Lif* transcripts, but not in the αERKO. Soon after infusion of oil into the uterus, we also observed an increase in Lif transcript and, unlike the estrogen-induced increase, this response also occurs in the αERKO (38), indicating an ERα-independent pathway. We have subsequently observed a similar regulation pattern in several additional genes, including *Connexin 26* (*Cx26*) (81), c-*Fos*, *cell division cycle 2 homolog A* (*Cdc2a*), and *cyclophilin* (*Cyc*) (S.C. Hewitt, unpublished observations). Therefore, it appears that some estrogen-responsive uterine genes are also regulated by the oil infusion and the latter mode of regulation is ERα independent, allowing uterine response without a need for estrogen priming. ER has been reported to interact with Stat3 (82), which suggests that activation of Stat3 might convey signals to ER-mediated responses.

Model: Converging Signals

Our studies examining uterine responses to acute estrogen, growth factors or the oil infusion stimulus mimicking early pregnancy indicate that divergent signal initiators converge at the level of gene regulation (see model, Figure 7). For example, estrogen initiates nuclear ERα recruitment of transcriptional coregulators (CoAc) and interaction with estrogen-responsive genes directly (ERE) or through tethering (AP-1/SP1), leading to ER-dependent responses. Some studies also indicate gene responses are initiated by estrogen activation of membrane-associated signals (nongenotropic signaling). Additionally, growth factors activate

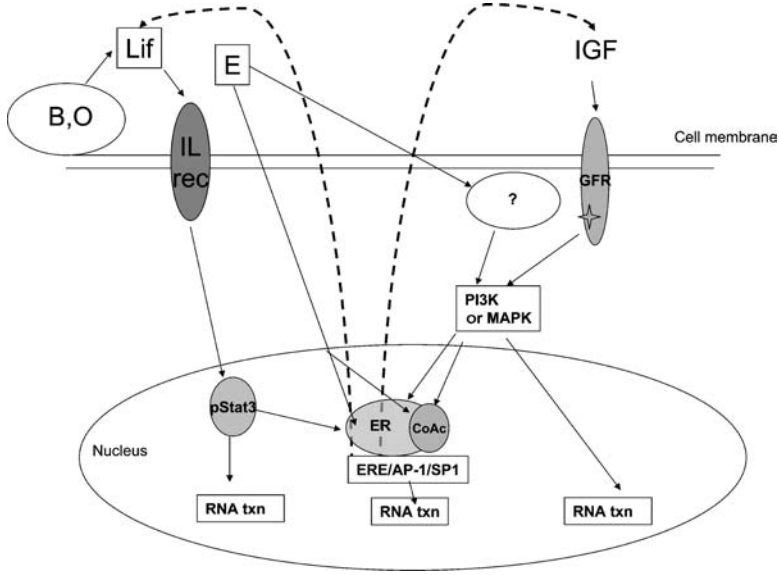


Figure 7 Model: Through divergent signals, genomic convergence. Estrogen (E) binds nuclear ERα, thus recruiting coregulators (CoAc) and modulating gene transcription by directly interacting with ERE DNA sequences or through tethered interaction with AP-1 or SP-1 transcription factors. Initiation of nongenotropic signals at the cell membrane is also depicted where estrogen activates signaling pathways such as the MAPK or PI3 kinase pathways. ERα, AR, or another estrogen-binding molecule may be involved in mediating this response (represented by ?). Growth factors also activate MAPK or PI3 kinase pathways by interacting with and activating their membrane receptors (GFR). Lif, blastocysts (B), or inert oil infusion (O) initiate cytokine-receptor signaling and activate pSTAT3-targeted transcription. These signals converge at the level of genomic modulation; this results in similar genomic responses to estrogen and growth factors or oil infusion either by direct activation of nuclear ER mediated transcription, or by other transcriptional mediators. *Igf-1* is increased by estrogen and further activates the growth factor receptor–mediated pathways, whereas *Lif* is increased by estrogen or oil infusion and activates cytokine signals.

their receptors' intracellular signals such as PI3 kinase and/or MAP kinase, resulting in direct regulation of growth factor–specific genes. Nongenotropic estrogen signaling and growth factor signaling converge as similar intracellular signaling is initiated by either factor and may further converge with nuclear ERα signaling by conveying this activity to the nuclear ERα and associated transcriptional modulators. The initiation of signal by estrogen is sensitive to ICI, as it utilizes ERα. When growth factors directly initiate the signal, ERα is bypassed, thus the initiation is not blocked by ICI and also occurs in the αERKO. In addition, IGF-1, but not EGF or TGFα, is induced by estrogen and can then initiate growth factor

receptor–mediated responses. Finally, initiation of a cytokine-like pathway by oil infusion, which results in activation of phospho-Stat3-mediated transcription, also converges with ER transcriptional responses.

STUDY OF ROLES OF ER IN BREAST CANCER MODELS

Knockout models can be applied to studies of pathological conditions such as cancer. As there is a strong correlation between exposure to estrogenic compounds and carcinogenesis and tumor growth in the mammary gland (83, 84), ERα has been a target of chemotherapeutic and chemopreventative therapies in breast cancer. Because ERα is involved in mammary development and its expression correlated with breast cancer growth, we have studied the effect of removing ERα from mice overexpressing mammary-tumor-inducing oncogenes in the mammary gland.

Expression of the MMTV-Wnt-1 transgene, a diffusible factor that signals through frizzled receptors, leads to mammary hyperplasia and tumors. Similarly, transgenic mouse lines that overexpress *erbB2* oncogene (also called neu), an epidermal growth factor receptor (EGFR)-like protein (85) reported to be overexpressed in 20–30% of human breast tumors (86), or a constitutively active mutant neu targeted to the mammary epithelium, exhibit an increased incidence of mammary tumors (87) compared with that of nontransgenic littermates. We further investigated the role of ERα in these transgenic models of mammary carcinogenesis by breeding the transgenes onto the αERKO and comparing tumor incidence and onset rates. In both cases, tumors occurred in both WT and αERKO; however, tumor onset was significantly delayed in the αERKO/MMTV-*Wnt 1* (88) and αERKO/MMTV-*neu* (Table 3) (89) mice in contrast to that in their WT transgenic counterparts. Therefore, functional ERα is not obligatory to MMTV-*Wnt-1* or MMTV-*neu* induced mammary tumors but contributes to the rate of tumor progression.

The effect of removing ERα signaling on mammary tumor induction has also been studied in transgenic mice that express large T antigen in developing mammary duct cells. However, when this transgene was introduced into the αERKO females, it was not expressed, presumably because there was no ductal development in the αERKO, and hence tumors did not occur (90).

Because αERKO mice are anovulatory and have persistent preovulatory progesterone levels, and because the mammary tissue contains only a rudimentary epithelial structure, several experiments were undertaken to determine whether increasing the progesterone level and/or the amount of mammary epithelium of αERKO/*neu* mice would accelerate tumor onset. This is of interest especially in light of the association between increased mammary duct tissue density and increased breast cancer risk in woman (91, 92). When the progesterone level in the αERKO/*neu* mice was increased, either by treatment with progesterone-release pellets or by pituitary xenografts, which secrete prolactin and result in stimulation

TABLE 3 Tumor onset age of neu mice[a]

Group	Onset age	p versus WT/neu
WT (ERα+/+)/neu	50% at 51 weeks	
αERKO (ERα−/−)/neu	50% at 105 weeks	<0.0001
WT/neu ovex	50% at 48 weeks	0.648
WT/neu multiparous	50% at 32 weeks	<0.0001
Pre-pubertal ovex WT/neu	50% at 62 weeks	.038
Pre-pubertal ovex+prog WT/neu	40% at 52 weeks	0.262
WT/neu+P	50% at 37 weeks	<0.0001
WT/neu ovex+P	50% at 40 weeks	0.88 versus WT/neu+P
αERKO/neu+P	50% at 70 weeks	0.0235 versus αERKO/neu without P
WT/neu +pit	50% at 37 weeks	<0.0001
αERKO+pit	50% at 43 weeks	<0.0001 versus αERKO non transplanted, 0.0941 versus WT/neu non transplanted

[a]Age at 50% onset was calculated from the Kaplan-Meier plots, and p values compared with WT/neu or indicated set were determined Adapted from (89).

of ovaian progesterone production, the mammary tumor onset rate equaled or exceeded that of unmanipulated WT/*neu* mice, despite a low content of epithelial tissue in αERKO/*neu* relative to WT/*neu* mice (89) (Table 3). Similarly, when WT/*neu* mice were exposed to elevated progesterone during pregnancy, by direct treatment with progesterone-releasing pellets or by stimulation of the ovaries with pituitary grafts, the onset rate was accelerated (89) (Table 3). In contrast, *Wnt-1* females did not display accelerated tumor onset following pregnancies, although prepubertal ovariectomy did cause some delay in onset (88). The ability of progesterone to accelerate tumor onset in WT/*neu* and αERKO/*neu* mice indicates that the underlying phenotypes of the αERKO (i.e., the lack of postovulatory progesterone, low volume of mammary epithelial cells in which to express transgene) contribute significantly to delayed tumor onset in the αERKO/*neu*. The more rapid onset following progesterone elevation may reflect progesterone's role in pregnancy-associated ductal proliferation and lobuloalveolar development (93) as a possible component to tumor progression.

PRKO mice have been utilized in a mammary tumorgenesis model in which a combination of dimethylbenzanthracene (DMBA) and a pituitary xenograft induce mammary tumors (28). Lack of PR reduced tumor incidence from 60% to 15%, indicating that tumors can occur in the absence of PR but suggesting a role for PR in susceptibility, which is attributed to the loss of epithelial proliferation in the PRKO mammary gland.

Studies in mouse mammary tissues indicate that the PR and ERα are present in a nonuniform pattern, selectively localized in nonproliferating cells, which suggests a paracrine mechanism of progesterone- or estrogen-stimulated mammary proliferation (28, 94, 95). Thus for ductal cells to be proliferative, they may contain neither PR nor ERα, but must respond to proliferative signals from PR- or Erα-positive cells, and it is the abundance of these proliferative cells that may be important indicators of cancer susceptibility, although their numbers may be increased by estrogen or progesterone exposures. If ERα does play such a role in human breast cancer, one might predict that ERα antagonists may delay tumor onset by limiting the population of proliferative cells; however, the identification of drug compounds targeted directly to the proliferative cells themselves, which abrogate the function of signaling molecules involved in breast tumorigenesis, might prevent rather than merely delay tumor formation effectively.

EPILOGUE: LESSONS LEARNED?

In this review we have summarized some of the important and interesting lessons learned utilizing engineered mice as experimental models to increase our understanding of estrogen biology at many levels. We have noted how perturbing homeostasis can reveal mechanisms of many components important in reproduction, both directly related to estrogen signaling and secondary to homeostatic disruptions. We discussed the application of the knockout models to pathologic states to study the role of estrogen signaling in cancer. The uterine model was utilized to examine mechanisms and converging pathways leading to estrogen-dependent and estrogen-independent gene regulation. Much has been learned, yet with every lesson, more questions continue to arise, and considering the techniques and technologies now available, the future promises to advance our understanding in this fascinating and important field of study.

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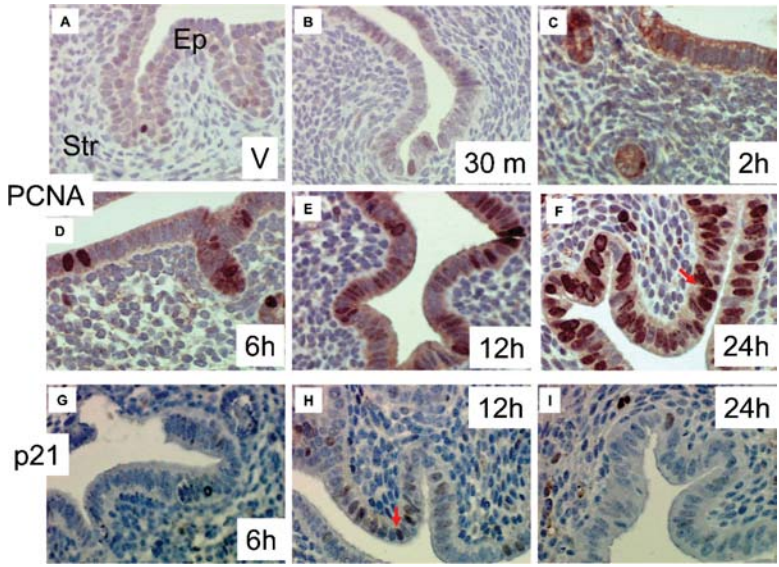

Figure 3 Uterine cross sections from mice treated with an acute dose of estrogen illustrate the synchronous proliferative response between 12 and 24 h. (*A–F*): Proliferating cell nuclear antigen (PCNA) expression in uterine epithelial cells. (*G–I*): p21 is increased in the nucleus of epithelial cells 12 h following estrogen treatment. (*Panel A*): vehicle treated. The stromal (Str) and epithelial (Ep) cells are indicated. (*Panel B*): 30 min estrogen. (*Panel C*): 2 h estrogen. (*Panel D*, *G*): 6 h estrogen. (*Panel E*, *H*): 12 h estrogen. Arrow highlights positive p21 epithelial cell in panel H. (*Panel F*, *I*): 24 h estrogen. Arrow highlights positive PCNA stain in epithelial cells in panel F.

Figure 4 Microarray analysis: genomic pattern mirrors biphasic biological response. Dendogram shows genomic response following estrogen treatment. Each horizontal row represents a comparison of a sample pair (vehicle treated compared with various times of estrogen treatment; 0.5–24 h). Each vertical line represents a single gene. Green indicates repression compared with vehicle; red indicates up-regulated genes. The groups of genes or clusters that are outlined in dark blue are characteristic of late responses; the clusters outlined in white are early genes, whereas others outlined in light blue occur throughout the 24 h. Adapted from (34).

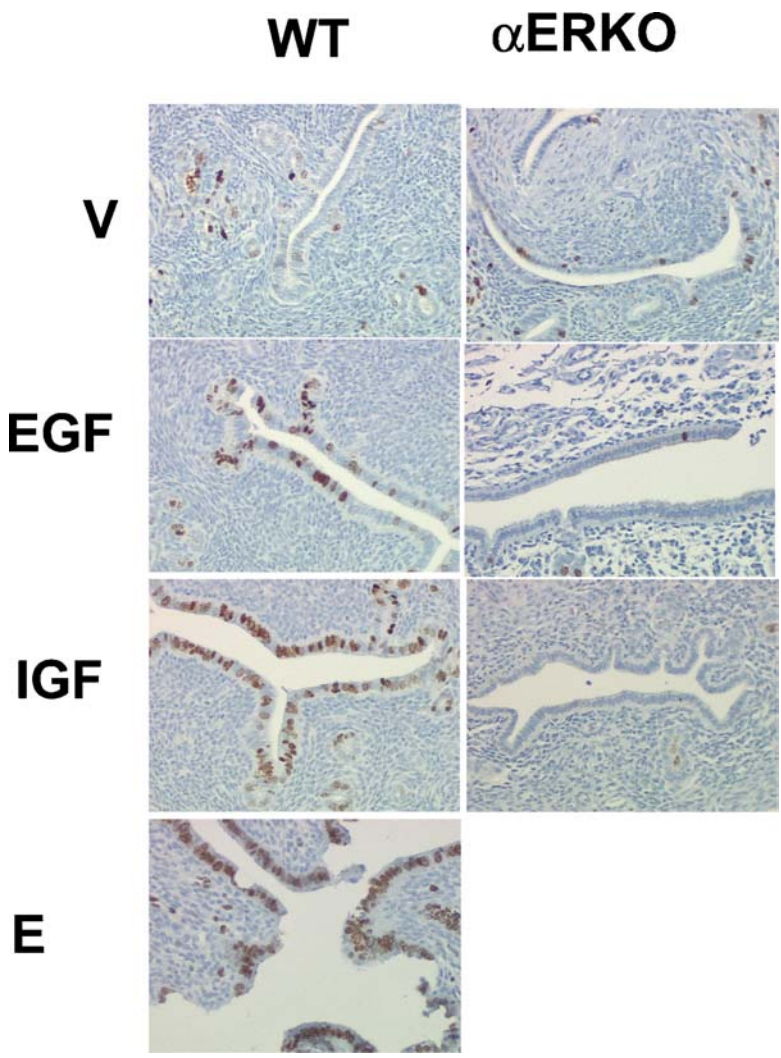

Figure 6 Ki67 expression in response to growth factors is ERα-dependent. Cross sections of uteri from WT or αERKO mice treated with vehicle (V) or for 24 h with EGF, IGF, or estrogen were analyzed for the expression of Ki67 antigen. The increase in Ki67 antigen, indicative of active, proliferative cells, following EGF or IGF treatment, occurs only in the ERα-containing tissue, thus indicating a cross talk mechanism.

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CONTENTS