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Estrogen Induces a Systemic Growth Factor Through an Estrogen Receptor-Alpha-Dependent Mechanism[1]

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ABSTRACT

Estrogen induces proliferation of uterine epithelium through a paracrine action of estrogen receptor (ERα) in the underlying stroma. In ovariectomized mice primed with progesterone, estrogen stimulates proliferation in both the epithelium and the stroma. We set out to test whether a paracrine mode of action is involved in estrogen-induced proliferation of the uterine stroma. Epithelial and mesenchymal tissues derived from uteri of neonatal ERα null mice (ERαKO) or wild-type mice were separated and recombined in all four possible configurations (ERα$^+$ or ERα$^-$ epithelium with ERα$^+$ or ERα$^-$ mesenchyme) and grafted into female athymic mice. After 5 wk, hosts were ovariectomized and challenged with hormone treatment, and cellular proliferation was monitored by thymidine autoradiography. Results showed that, although the full response of the epithelium was dependent on an ERα-positive mesenchyme, stromal cell proliferation was independent of tissue ERα. This latter observation suggests that the response of the stroma was due to a systemic factor induced in the ERα-positive hosts. To test this possibility, pieces of whole uterus from neonatal wild-type or ERαKO mice were grafted into syngeneic wild-type or ERαKO hosts. In these whole-uterus grafts, estradiol stimulated ERαKO uterine stroma when they were grown in wild-type hosts but not when grown in ERαKO hosts. The epithelium of whole-uterus ERαKO grafts did not respond to estrogen, regardless of the host phenotype. These observations suggest that treatment of progesterone-primed mice with estradiol stimulates production of a systemic factor that is capable of inducing uterine stromal cell proliferation and that this systemic factor is produced by an ERα-dependent mechanism.

estradiol receptor, growth factors, progesterone, uterus

INTRODUCTION

Sequential fluctuations in ovarian steroid hormone secretions prepare the uterus for pregnancy. In cycling women, the ovarian follicular phase is characterized by increasing secretion of estrogen with low constant levels of progesterone; this hormonal milieu produces endometrial hyperplasia and hypertrophy, which is particularly striking in the epithelium. After ovulation, the hormonal environment changes to one of high progesterone levels in a background of moderate estrogen blood concentration; this inhibits further proliferation of the epithelium, promoting a secretory phenotype. At approximately 10 days post ovulation, there is a round of proliferation in the subepithelial stroma that is a prelude to the decidual cell differentiation required for embryo implantation [1, 2]. Successful embryo implantation is strictly dependent on the sequential proliferation and differentiation events that occur in the endometrium, and it has been suggested that only a slight perturbation in their timing leads to a dysfunctional situation known as luteal phase deficiency [3].

The cell proliferation events in the human uterus can be mimicked in the ovariectomized rodent by administration of estradiol and progesterone. This model has long served as a means of studying the mechanisms involved in hormonal regulation of cellular physiology in the uterus. A single dose of estradiol stimulates a dramatic increase in proliferative activity within the uterine luminal epithelium [4]. If the animal has been primed with 2 days of progesterone treatment prior to administration of estradiol, then there is an increased proliferation of the endometrial stromal cells but a diminished proliferative response in the epithelium [5, 6]. The dual effects of progesterone are dose dependent; increasing the relative amount of estradiol can overcome the inhibitory effect of progesterone in the epithelium [6].

Using tissue recombination techniques, it was definitively proven that estrogen-induced proliferation in the uterine epithelium occurs through an indirect mechanism involving activation of estrogen receptor-alpha (ERα) in the underlying stromal cells [7]. Similarly, it was shown that progesterone inhibition of estrogen-induced epithelial cell proliferation occurs through an action of stromal cell progesterone receptors [8]. It has not been determined whether there is a paracrine component of the stromal response to estrogen following progesterone priming. An experimental system in which the endometrial epithelium is injured following progesterone and estrogen priming produces a dramatic overgrowth of the endometrial stroma known as the decidual cell reaction, and this has been shown to be dependent on signals derived from the epithelium [9]. In tissue recombination experiments, we showed that the stromal response to estrogen plus progesterone occurred only if the overlying epithelium was uterine in origin, suggesting that there may be an epithelially derived signal required [10].

In the present study, we set out to determine whether estrogen-induced stromal cell proliferation in the progesterone-primed uterus was mediated by paracrine signals induced by activation of ERα in the overlying epithelium.

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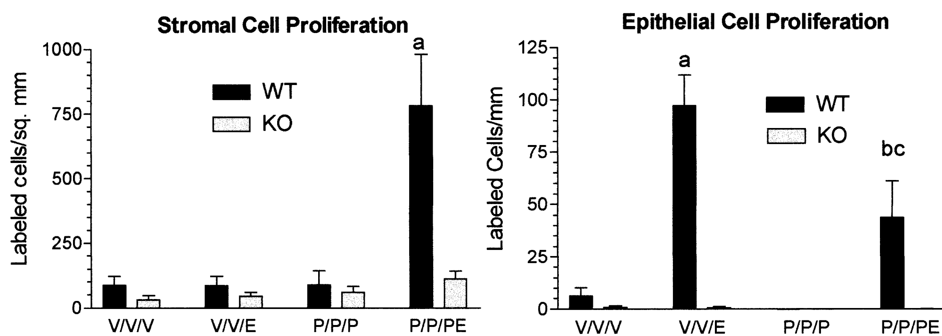

FIG. 1. Effects of estradiol and progesterone administered alone or together on cell proliferation of the wild-type and ERαKO mouse uterus. Adult wild-type (WT) or ERαKO (KO) mice were ovariectomized and treated with vehicle for 3 days (V/V/V), V for 2 days followed by a single dose of estradiol (V/V/E), progesterone for 3 days (P/P/P), or P for 2 days followed by P plus E (P/P/PE). Thymidine autoradiography was used to assess cell proliferation in the stromal and epithelial compartments. Values are means ± SEM, n = 4–6; a, $P < 0.001$, or b, $P < 0.05$ vs. vehicle control; c, $P < 0.01$ vs. V/V/E treatment.

We employed tissue recombinations and xenografting techniques used in earlier studies [7, 10]. The results suggest that there is an ERα-dependent, systemic factor capable of inducing uterine stromal cell proliferation.

METHODS AND MATERIALS

Animals

All animal experimentation and care was performed under protocols approved by the local Institutional Animal Care and Use Committee. An ERα knockout (ERαKO) colony was maintained by inbreeding adult male and female mice that were heterozygous for receptor ($ER\alpha^{+/-}$) [11]. At 1–2 days of age, the pups were genotyped by using polymerase chain reaction (PCR) on DNA derived from toe clippings, as described previously [11].

Tissue recombinants (see below) were grafted into 6- to 8-wk-old female athymic (Balb/c) mice (Harlan-Sprague, Indianapolis, IN) that were wild type for ERα. In addition, whole pieces of uterus from neonatal mice were grafted into 6- to 8-wk-old female wild-type, $ER\alpha^{+/+}$, or knockout $ER\alpha^{-/-}$ mice from the ERαKO colony. Host animals were anesthetized and their kidneys were exteriorized. Tissue pieces (2–3) were grafted under the capsule of each kidney, and the kidney was returned to the abdominal cavity. After 5 wk the host animals were ovariectomized. Three weeks after ovariectomy, hosts were treated as follows: three daily doses of vehicle (V); two daily doses of V followed by a single dose of estradiol (E, 4 μg/kg) on the third day; three daily doses of progesterone (P, 10 mg/kg); two daily doses of P followed by single doses of both P and E on the third day. At 20 h after the last treatment dose, each host was injected with 1.5 μCi/g body weight ^{3}H-thymidine (^{3}H-TdR) and then killed 1 h later by cervical dislocation.

Tissue Recombinations

The epithelial and mesenchymal components of uterus from neonatal mice were separated after enzymatic digestion as described previously [12], and these were recombined in all four possible combinations to make tissues that were ERα homotypic (epithelium/mesenchyme: $ER\alpha^+/ER\alpha^+$; $ER\alpha^-ER\alpha^-$) or ERα heterotypic (epithelium/mesenchyme: $ER\alpha^+/ER\alpha^-$; $ER\alpha^-/ER\alpha^+$). The recombined tissue pieces were placed on a medium-impregnated agarose gel, incubated at 37°C overnight and then grafted under the kidney capsule of athymic Balb/c hosts as described above.

Immunohistochemistry and Autoradiography

Host uterus and grafted tissues were fixed in formalin for 60–90 min at 4°C and then placed in an 8% sucrose/PBS solution overnight at 4°C. Tissue pieces were then flash frozen and embedded for cryosectioning. Alternate sections (8 μm) were prepared for either ERα immunohistochemistry using the monoclonal antibody H222 (kindly supplied by Dr. Geoffrey Greene, University of Chicago) or ^{3}H-TdR autoradiography as described previously [13].

Cell proliferation was assessed by counting the number of ^{3}H-TdR-labeled cells in 3–5 sections of each grafted tissue or of the host uterus. For the stroma, a circumscribed area was measured with an image analysis software package, IPLab Spectrum (Signal Analytics Corp., Vienna, VA), and the number of labeled cells within that area was determined; the labeling index (labeled cells/mm^2) was determined by counting cells in an area sufficient to include a total of at least 5000 stromal cells, as determined in a preliminary calibration study on four separate tissue sections. Care was taken to insure that the stromal area measured included neither glandular epithelium nor large blood vessels. The epithelial labeling index was determined as the number of labeled cells in a measured length of tissue, expressed as labeled cells per millimeter; sufficient length was analyzed to yield at least 500 total epithelial cells, as determined in a preliminary calibration analysis. All cell counting was performed twice in a blinded fashion by two technicians.

Statistical Analysis

Labeling indices were log transformed and analyzed for treatment effects by one-way ANOVA followed by Bonferroni multiple comparison tests. For the experiment in which wild-type and ERαKO uteri were grafted into either wild-type or ERαKO animals, host-treatment interactions were tested by two-way ANOVA followed by Bonferroni tests.

RESULTS

Thymidine autoradiography was used to assess the proliferative responses of the uterine stroma and epithelium. As described extensively in early literature, progesterone priming causes estrogen to stimulate uterine stromal cell proliferation [5, 6, 14–16]. When administered alone, estrogen induces a dramatic proliferative response in the luminal epithelium, which can be blocked by concomitant administration of progesterone but only if the progesterone: estradiol dose is high [6]. We found that when ovariectomized mice were primed with 10 mg/kg progesterone followed by a single dose of 4 μg/kg estradiol, cell proliferation was stimulated in both the stroma and the epithelium (Fig. 1). The effect of estradiol on the epithelium was significantly diminished by pretreatment with progesterone. Estrogen was ineffective in the ERαKO animals, with or without progesterone pretreatment (Fig. 1).

We set up tissue recombinations using epithelium and mesenchyme from ERαKO and wild-type mice. The recombinants were grown in athymic mice for testing the effects of hormone treatments. Immunohistochemistry was used to verify the ERα phenotype of the recombined tissues after growth in the hosts (Fig. 2). All recombined tissues did or did not express receptor as predicted from the genotype of the donor tissue as determined by PCR. There were no detectably ERα-positive blood cells present in the ERαKO stroma of the grafts. The tissue recombinants formed three tissue components, an epithelium, stroma, and smooth musculature, with the same architecture as a normal uterus.

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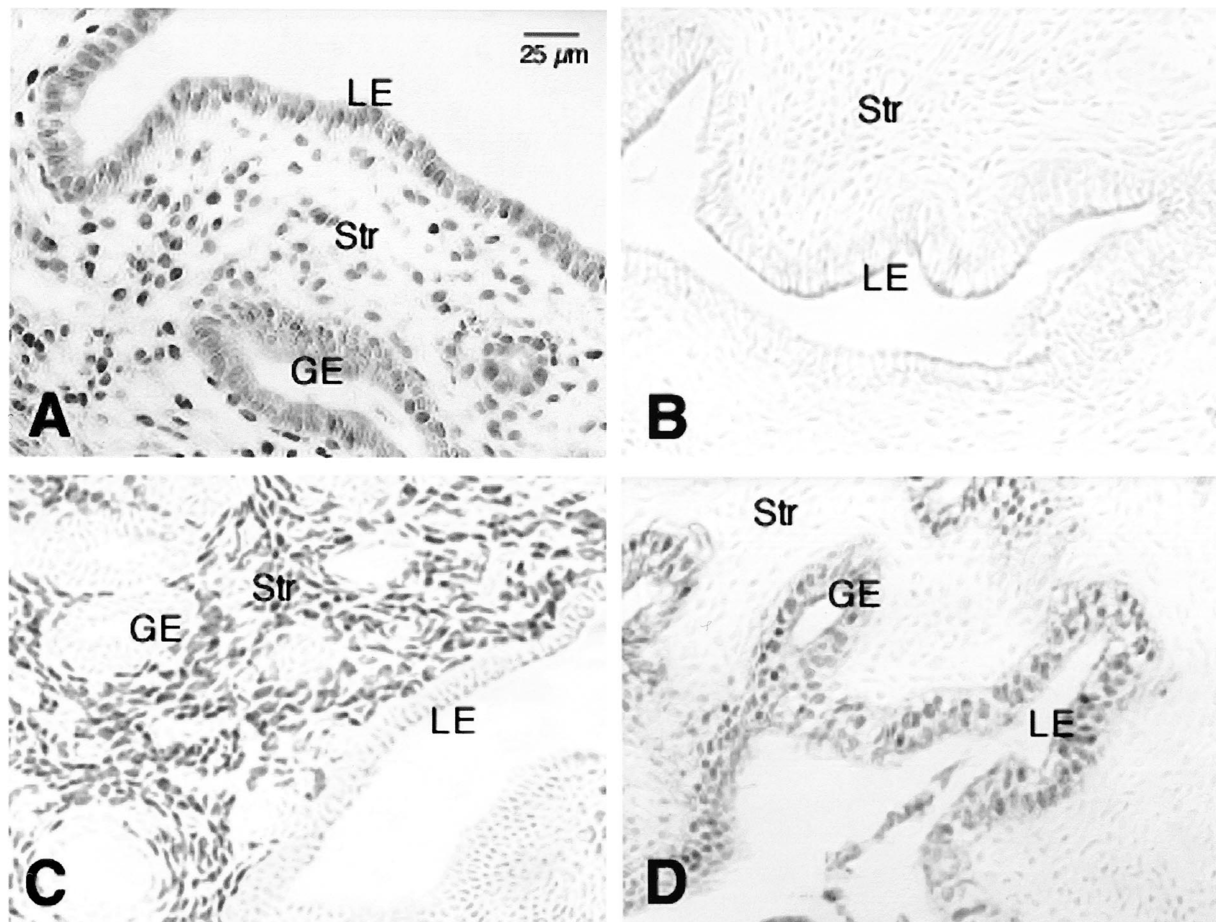

FIG. 2. Immunohistochemical verification of tissue phenotype in homotypic and heterotypic tissue recombinants. Epithelium and mesenchyme were derived from neonatal wild-type or ERαKO mice. The tissues were combined in all four possible configurations and grown in athymic female mice. Grafted tissues, harvested from hosts 3 wk after ovariectomy and following vehicle treatment, were processed for ERα immunostaining; the receptor-positive cells have dark nuclei. **A**) $ER\alpha^+$ epithelium + $ER\alpha^+$ mesenchyme. **B**) $ER\alpha^-$ epithelium + $ER\alpha^-$ mesenchyme. **C**) $ER\alpha^-$ epithelium + $ER\alpha^+$ mesenchyme. **D**) $ER\alpha^+$ epithelium + $ER\alpha^-$ mesenchyme. LE, Luminal epithelium; GE, glandular epithelium; Str, stroma. Bar = 25 μm (**A**) indicates magnification throughout.

The stroma of grafted tissue recombinants was stimulated slightly when either estradiol or progesterone was administered alone, but there was a dramatic, additional increase in stromal cell proliferation when the steroid hormones were combined (Fig. 3A). Surprisingly, the stromal response to progesterone plus estradiol was equivalent in all four tissue recombinations, including those that were composed entirely of ERα-negative tissues. In contrast to the stromal response, stimulation of epithelial proliferation required expression of ERα either in the mesenchyme or the epithelium (Fig. 3B). However, the full epithelial response occurred in the tissue recombinants only when the mesenchyme contained ERα; the response in the ERα-positive epithelium grown with ERα-negative mesenchyme was significantly higher than vehicle-treated controls but significantly lower than that of epithelium associated with ERα-positive mesenchyme in estradiol-treated hosts (Fig. 3B).

Because the stromal response to estrogen plus progesterone occurred in tissue recombinations composed entirely of $ER\alpha^{-/-}$ tissues and these grafts were grown in $ER\alpha^{+/+}$ hosts, we tested the possibility that estrogen plus progesterone induces a systemic factor that stimulates the uterine stroma. Whole pieces of uterus from neonatal wild-type or ERαKO mice were grafted into syngeneic hosts that were either wild type or ERαKO. After 5 wk, the hosts were ovariectomized, and the effects of progesterone and estrogen were tested as above. In these whole-uterus grafts, both the epithelium and the stroma were stimulated in the wild-type grafts, regardless of the ERα status of the host (Fig. 4), indicating a local effect of the hormone treatments. However, the observation that the stroma of the ERαKO whole-uterus grafts was also stimulated when grown in wild-type hosts but not in ERαKO hosts (Fig. 4) indicate that there was a systemic factor that had been induced by the estrogen activation of ERα in host tissues.

DISCUSSION

Results of the tissue recombination study suggest three possibilities for the action of estrogen on stromal cell proliferation: a form of ER other than ERα is responsible for stimulation of the endometrial stroma in the progesterone-primed uterus, the stromal response is independent of nuclear ER, or there is a systemic factor produced in the ERα-positive host animals and this stimulates the stroma. ERβ has been shown to mediate estrogen action in much the same way as ERα; however, it is doubtful that ERβ is responsible for the effect in the uterine stroma. Although there have been reports that ERβ is expressed in the rodent uterus [17–20], others have suggested that it is either present in insignificant quantities, playing only a minor role in uterine physiology [21–23], or is in fact not expressed at

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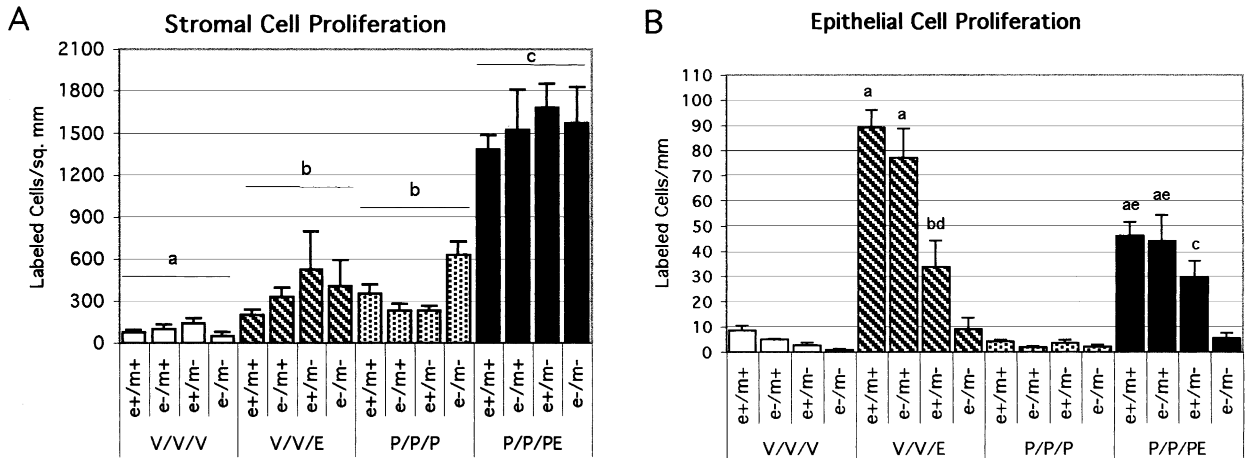

FIG. 3. Effect of hormone treatments on tissue recombinants made from wild-type and ERαKO uterus. The uterine epithelium (e) and mesenchyme (m) of wild-type (+) or ERαKO (−) mice were recombined and grown in athymic mice. After ovariectomy, the host mice were treated as described in Figure 1. Cell proliferation was assessed by thymidine autoradiography. Values are means ± SEM, n = 4–15. **A**) Stromal cell proliferation. Means below bars with different letters differ from their corresponding means in other treatment groups ($P < 0.05$). **B**) Epithelial cell proliferation a, $P < 0.001$, b, $P < 0.01$, or c, $P < 0.05$ vs. vehicle controls; d, $P < 0.01$ e+/m− vs. e−/m+ or e+/m+ recombinants within V/V/E treatment group; e, $P < 0.01$ corresponding recombinants in P/P/PE vs. V/V/E.

all [24, 25]. However, the fact that the uterus of an ERαKO animal does not respond to estrogen strongly argues against the presence of another receptor mediating the stromal effect in the uterus. On the other hand, Das et al. [26] found that estradiol is capable of stimulating expression of specific genes independently of ER, but again, the observation that the stroma of the ERαKO uterus did not proliferate in response to estrogen indicates that the ER-independent response is not sufficient to stimulate proliferation. Thus, induction of a systemic factor through stimulation of ERα in host tissues is the most likely explanation for the observations in the recombination study. Indeed, grafting whole pieces of ERαKO uterus into wild-type or ERαKO hosts proved this to be the case. The observation that the wild-type whole-uterus grafts showed no host dependence indicates that the systemic factor was sufficient but not required to stimulate the uterine stroma if ERα was present locally. In addition, the fact that only the stroma of the ERαKO graft responded suggests that this factor is specific to that tissue. The experimental system described has demonstrated the existence of an estrogen-induced, systemic factor that stimulates uterine stromal cells. Further experimentation will be needed to determine whether both the systemic and local factors are required for the full physiological effects of progesterone and estrogen on normal uterus.

The source and identity of the hormonally induced sys-

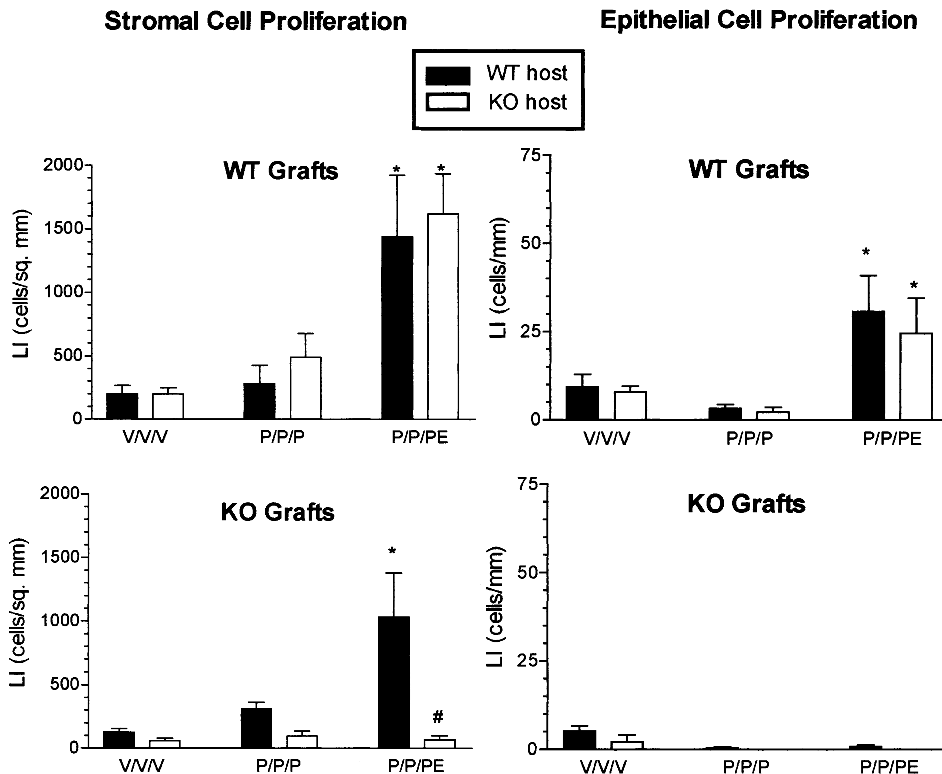

FIG. 4. Effect of combined progesterone and estrogen on cell proliferation in grafts grown in wild-type or ERαKO hosts. Pieces of whole uterus from neonatal wild-type mice (WT grafts) or ERαKO mice (KO grafts) were grown in wild-type (WT) or ERαKO (KO) hosts. Host treatment and thymidine labeling of tissue were as described in Figure 1. The stroma and the epithelium of grafted WT uterus responded to estradiol, regardless of the host in which it was grown. The KO stroma responded when the tissue was grafted in WT hosts but not when grafted into KO hosts. The epithelium of the KO uterine graft did not respond, regardless of the host phenotype. Values are means ± SEM, n = 4–7; * $P < 0.01$ vs. vehicle control; #, $P < 0.01$ vs. KO stroma grown in the wild-type host.

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temic factor(s) can only be speculated upon. Likely sources of such growth factors in our experimental system are the intact uterus or pituitary of the host animal. It is well documented that steroid hormones regulate expression of growth factors in the uterus [27], but it has been assumed that these growth factors behave in a paracrine fashion, affecting only proximal tissues but being diluted sufficiently in systemic circulation to be ineffective as endocrine factors. Estrogen, with or without concomitant progesterone administration, induces pituitary secretion of prolactin [28], which is known to stimulate a number of epithelial tissues, including uterine glands [29]; however, prolactin has never been implicated as a growth factor for stromal cells but is rather considered a marker of terminal differentiation that occurs during decidualization [30, 31]. Transforming growth factor-β2, a stromal cell growth factor, has been detected at appreciable levels in serum of pregnant rats [32]. Whether the 3-day treatment with progesterone used herein would induce sufficient levels to produce an endocrine growth effect needs to be determined. Several other growth factors have been implicated as paracrine mediators of growth in the endometrium [27], but systemic levels have not been examined. Alternatively, hormonal treatment may have increased sensitivity of the stromal tissue to a growth factor that is already circulating in the blood; for example, estrogen has been shown to increase expression of epithelial growth factor receptors in the uterus [33]. A systematic approach to identifying the factors involved is required.

The existence of a systemic growth factor induced by estrogen has clinical implications. Much of the research on hormonally responsive tissue growth, whether normal or pathological, has emphasized two modes of action: either direct stimulation of responding cells through their intracellular receptors or indirect action by induction of paracrine factors elaborated by neighboring tissues as a result of receptor activation within those cells. The observations reported in the present study suggest a third mode of action: induction of an endocrine growth factor. Furthermore, induction of this endocrine factor requires the combined action of progesterone and estrogen. In an earlier report, we showed that these two hormones behave synergistically in promoting carcinogen-induced mammary tumors in rats [34]. Similarly, epidemiological studies have suggested that combined progestin/estrogen hormone treatment pose a greater risk for breast cancer than estrogen alone [35, 36]. Although estrogen induction of pituitary prolactin secretion has long been considered to play a role in experimental mammary cancers in rodents, early clinical trials aimed at affecting the course of the disease in humans by altering pituitary prolactin secretion were uniformly unsuccessful [37]. Nonetheless, this type of systemic action of estrogen must still be considered in breast and other hormonally responsive cancers.

Whether estrogen acts through a direct uterine ER-mediated effect or through an indirect, systemic mediator, progesterone priming is required for estrogen induction of cell proliferation in the uterine stroma. Progesterone receptor is present and functional in the uterine cells of both wild-type and ERαKO mice [38]. Additional studies are underway to determine whether the uterine progesterone receptor mediates the priming action, allowing the systemic effect of estrogen to occur or whether its presence in another tissue that produces that systemic factor is required.

When ERα-negative epithelium was associated with ERα-negative stroma (tissue recombinant grafts or whole-uterus ERαKO grafts), it did not respond to estrogen in ERα-wild-type hosts (either athymic nudes or syngeneic wild types), with or without progesterone priming. On the other hand, the epithelium of the wild-type uterine grafts grown in ERαKO hosts was stimulated by estrogen. These results indicate a requirement for local mediators of estrogen-induced epithelial proliferation. The tissue recombinant study showed that estrogen stimulates epithelial cells via either a direct effect on epithelial ERα or a paracrine mediator elaborated by ERα-positive stromal cells. The observation that ERα-positive epithelium grown in association with ERα-negative stroma responded partially to estradiol contrasts the report by Cooke et al [7] in which the epithelial response was completely dependent on stromal ERα. The reason for this discrepancy may be the length of time the grafts were grown in the hosts before hormone challenge. In our study the grafts were in the host for a total of 8–9 wk (5 wk growth in the intact host plus 3 wk after ovariectomy of the host) prior to hormone treatment, whereas those in the earlier study were grown for 5 wk (4 wk growth in the intact host plus 1 wk after ovariectomy of the host). We lengthened the time the grafts were grown because preliminary studies (not shown) indicated that the tissues behaved as an immature uterus after only 5 wk of growth, i.e., estrogen-stimulated cell proliferation in both the epithelium and the stroma in the absence of progesterone priming, just as occurs in the immature rodent uterus [39]. Indeed, the micrographs presented in the study by Cooke et al. [7] suggest that estradiol had stimulated the stroma in their tissue recombinants. The stroma of the mature uterus does not respond to treatment with estrogen alone [39]. It may be that the additional length of time grown before hormonal challenge allowed the epithelium in our tissue recombinants to differentiate so that it was capable of responding to estrogen through a direct, or an autocrine, mechanism. Nonetheless, our data indicate that the ERα-positive stroma is capable of mediating the effect of estrogen on the epithelium and that this paracrine mode of action is required for maximal effect of the steroid.

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