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Evidence That Epithelial and Mesenchymal Estrogen Receptor-α Mediates Effects of Estrogen on Prostatic Epithelium

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In combination with androgens, estrogens can induce aberrant growth and malignancy of the prostate gland. Estrogen action is mediated through two receptor subtypes: estrogen receptors α (ERα) and β (ERβ). Wild-type (wt) and transgenic mice lacking a functional ERα (αERKO) or ERβ (βERKO) were treated with the synthetic estrogen diethylstilbestrol (DES). DES induced prostatic squamous metaplasia (SQM) in wt and βERKO but not in αERKO mice, indicating an essential role for ERα, but not ERβ, in the induction of SQM of prostatic epithelium. In order to determine the respective roles of epithelial and stromal ERα in this response, the following tissue recombinants were constructed with prostatic epithelia (E) and stroma (S) from wt and ERKO mice: wt-S+wt-E, αERKO-S+αERKO-E, wt-S+αERKO-E, and αERKO-S+wt-E. A metaplastic response to DES was observed in wt-S+wt-E tissue recombinants. This response to DES involved multilayering of basal epithelial cells, expression of cytokeratin 10, and up-regulation of the progesterone receptor. Tissue recombinants containing αERKO-E and/or -S (αERKO-S+αERKO-E, wt-S+αERKO-E, and αERKO-S+wt-E) failed to respond to DES. Therefore, full and uniform epithelial SQM requires ERα in the epithelium and stroma. These results provide a novel insight into the cell–cell interactions mediating estrogen action in the prostate via ERα.

Key Words: **prostate; ERα; tissue recombination; squamous metaplasia.**

INTRODUCTION

Androgen-dependent development of the male urogenital system occurs via stromal–epithelial interactions in which androgens elicit epithelial development through paracrine mechanisms mediated by androgen receptors in the stroma (Cunha *et al.*, 1992). While androgens play a central role in prostatic biology, estrogens also profoundly affect growth and differentiation of the prostate. Estrogenic effects on the prostate are complex; they may be indirect or direct. Since the pioneering work of Huggins and Hodges on hormonal therapy for prostate cancer (Huggins and Hodges, 1941), it has become evident that pharmacologic doses of estrogen elicit androgen deprivation because exogenous estrogens suppress pituitary gonadotrophin and thus reduce testosterone secretion by the testes (Cook *et al.*, 1998). This indirect effect of estrogen on the prostate results in "chemical castration."

Induction of squamous metaplasia (SQM) is a specific response to estrogen by the prostate gland and has been induced *in vivo* by a variety of estrogenic substances: estradiol-17β (Tunn *et al.*, 1979), diethylstilbestrol (DES) (Weijman *et al.*, 1992), and zeranol (Deschamps *et al.*, 1987). The effect is observed in a large number of animal species as well as humans, including mice, rats, dogs, beef bulls, monkeys, sheep, and goats (Andersson and Tisell, 1982; Aumuller *et al.*, 1982; Deschamps *et al.*, 1987; Driscoll and Taylor, 1980; Habenicht and Etreby, 1988; Kesteren *et al.*, 1996; Pylkkanen *et al.*, 1991; Randles, 1990; Sugimura *et al.*, 1988; Weijman *et al.*, 1992). Therefore SQM is consid-

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ered to be a reliable marker or endpoint for assessing estrogenic action on the prostate. In the rats and mice, SQM is readily observed in the anterior prostate following estrogen treatment (Arai, 1970; Pylkkanen *et al.,* 1991) and we have previously reported estrogen binding to mouse anterior prostate (Cooke *et al.,* 1991). Therefore, the mouse anterior prostate is an appropriate lobe to study the mechanism of estrogen action on the gland.

In humans, sensitivity of the prostate to estrogen begins during fetal development. Full-term human male fetuses and newborn male infants usually exhibit prostatic SQM (Andrews, 1951; Brody and Goldman, 1940), which is reversible and has been attributed to high levels of maternal estrogen in the last trimester of pregnancy (Andrews, 1951). Exposure of the human fetal prostate to DES also elicits SQM (Driscoll and Taylor, 1980; Sugimura *et al.,* 1988; Yonemura *et al.,* 1995). In the adult, SQM is commonly associated with estrogen treatment of men with benign and malignant prostate disease (Epstein and Murphy, 1997). Estrogen treatment also induced SQM in aging male transsexuals who had undergone orchiectomy (Kesteren *et al.,* 1996). Thus, despite considerable variations in the morphology of the prostate gland across species ranging from zonal anatomy in human to lobular anatomy in the rodents, a common response to estrogen is manifest in fetal and adult tissues: the induction of SQM.

Estrogenic effects on the prostate are elicited as a result of the binding of the estrogen to its receptor. There are two subtypes, ERα and the more recently described ERβ (Kuiper *et al.,* 1996). ERα and ERβ are predominantly expressed in stroma and epithelium, respectively. However, a causal link has not been established between estrogenic effects on prostatic epithelium and the requirement of ERα or ERβ in either prostatic epithelium or stroma. Indeed, the respective roles of ERα and ERβ in the induction of prostatic SQM are not known and are the subject of this report. An emerging body of evidence suggests that certain effects of estrogen on target epithelial cells are mediated totally, or in part, via ERα in stromal cells (Buchanan *et al.,* 1998, 1999; Cooke *et al.,* 1997, 1998). Creation of mice with nonfunctional genes encoding ERα (αERKO mice) or ERβ (βERKO mice) (Krege *et al.,* 1998) has provided a unique means to characterize the respective roles of ERα and ERβ in the prostate. In this study tissue recombination techniques were used to determine the respective roles of epithelial versus stromal ERα in DES induction of prostatic SQM.

METHODS

Animal Surgery and Hormone Treatment

αERKO and βERKO mice were obtained by mating the appropriate heterozygote mice as previously described (Krege *et al.,* 1998; Lubahn *et al.,* 1993). Genotypes of pups were determined by multiplex PCR (Krege *et al.,* 1998; Lubahn *et al.,* 1993), and homozygous ERα and ERβ null males were used. Athymic males were used as hosts for grafting tissue recombinants. Host males were treated with subcutaneous implants containing 2 mg DES and 18 mg cholesterol. These hormone pellets were made in the laboratory with a pellet press. All animals were maintained under controlled lighting and temperature conditions and housed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Animals were fed *ad libitum*.

Tissue Recombination

Prostates were removed from adult or neonatal (1 day old) wt or αERKO male mice that had been killed by CO_2 asphyxiation. Separation of epithelium and stroma was performed as described previously (Cunha and Donjacour, 1987). Tissue recombinants were prepared with newborn seminal vesicle mesenchyme (SVM) and surgically isolated ductal tips from the anterior prostate of adult mice. The rationale for using newborn SVM instead of urogenital sinus mesenchyme (UGM) as a prostatic inducer was: (a) *In situ* SVM is a mesenchyme that induces and supports the differentiation of both seminal vesicle (SV) and anterior prostate. Buds of anterior prostate and SV grow into and branch within a common mass of mesenchyme designated SVM (Cunha and Donjacour, 1987). (b) Experimental studies showed that both SVM and UGM are nearly equivalent in their ability to induce prostatic differentiation from a variety of endoderm-derived epithelia (Cunha, 1972; Cunha *et al.,* 1980; Donjacour and Cunha, 1995; Norman *et al.,* 1986). (c) The use of neonatal SVM versus embryonic UGM means that inductive mesenchyme can be obtained from neonatal instead of embryonic mice, thus preserving valuable breeder females.

Tissue recombinants were prepared as described previously (Cunha and Donjacour, 1987). Briefly, SVs were removed from newborn wt or αERKO mice and digested with 1% trypsin (Life Technologies, Gaithersburg, MD) in calcium- and magnesium-free Hanks' balanced salt solution for 60 min at 4°C. SVM was then separated from epithelium using a von Graefe knife and fine forceps. Anterior prostates were removed from adult male wt or αERKO mice, and ductal tips of ~300 μm in length were excised. Prostatic tissue recombinants were prepared by placing a prostatic ductal tip on top of a SVM in dishes containing nutrient agar medium as described previously (Cunha and Donjacour, 1987). Prostatic tissue recombinants were cultured overnight on 1% nutrient agar. The types of tissue recombinants constructed with wt or αERKO stroma (S) and wt or αERKO epithelia (E) were wt-S+wt-E, αERKO-S+αERKO-E, wt-S+αERKO-E, and αERKO-S+wt-E. All tissue recombinants were grafted under the renal capsules of intact nude male mice and allowed to grow for 4 weeks to allow prostatic development to proceed. Prostatic differentiation was assessed in the above tissue recombinants by histology and immunostaining with a mouse prostate-specific antibody (Donjacour *et al.,* 1990). Hosts were then treated for 3 weeks by subcutaneous implants of pellets containing 2 mg DES plus 18 mg cholesterol or were sham treated for the same period of time. Tissues were recovered from hosts and fixed in 2% paraformaldehyde for 4 h prior to processing. The number of tissue recombinants in each group ranged from 5 to 29.

Immunohistochemistry

A number of parameters were used to monitor the response to estrogen. Identification of basal cells was made by immunolocal-

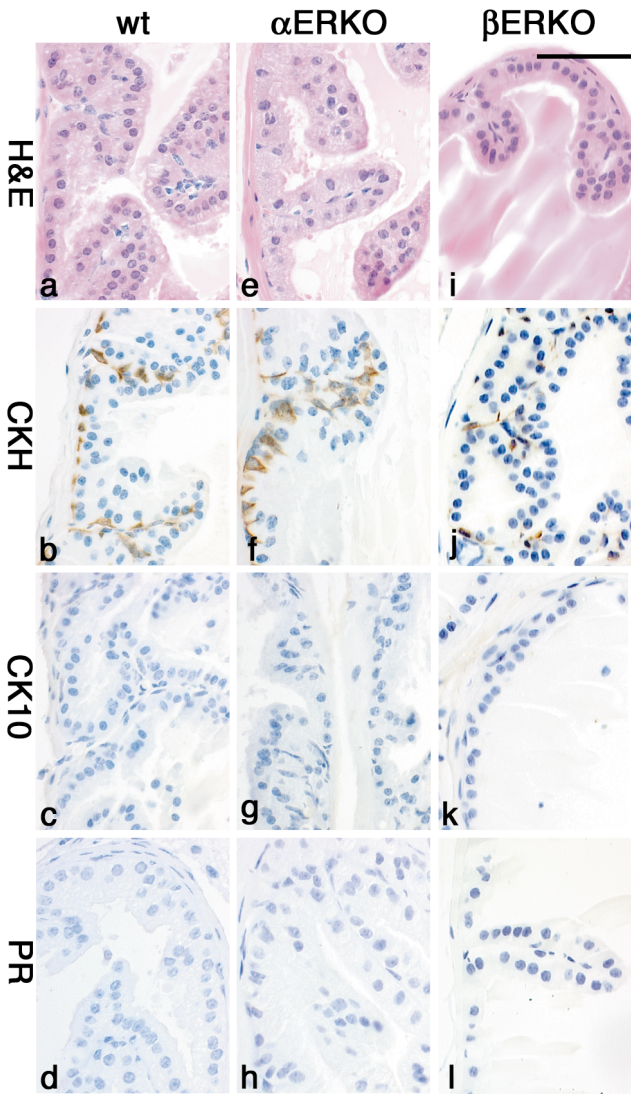

FIG. 1. The morphology of anterior prostate from control wt (a–d), αERKO (e–h), and βERKO (i–l) mice. (a, e, i) H&E staining; (b, f, j) CKH-positive basal cells; (c, g, k) CK10-negative staining; (d, h, l) PR. Only basal cells were identified in the epithelium of wt, αERKO, and βERKO mouse anterior prostate and not CK10 or PR. Bar, 50 μm.

ization of high-molecular-weight cytokeratin (CKH; DAKO-cytokeratin 34βE12; Carpinteria, CA) and cytokeratin 5 (CK5; Novocastra, UK) using these specific antibodies. Keratinization of the prostatic epithelium was detected by immunostaining for cytokeratin 10 (CK10; DAKO). Proliferating cells were identified by immunostaining for proliferating cell nuclear antigen (PCNA; DAKO). Progesterone receptor (PR) was immunostained with a specific polyclonal antibody (DAKO), and ERα was detected with ERα mouse monoclonal IgG 1D5 purchased from DAKO (Kurita *et al.*, 2000a). Both of these antibodies have been previously used in studies by this group (Buchanan *et al.*, 1999; Kurita *et al.*, 2000c). ERβ was detected with anti-human ERβ chicken polyclonal antibody (ERβ 503 IgY) as described previously (Saji *et al.*, 2000; van Pelt *et al.*, 1999). Mouse prostatic secretory proteins were detected using an antibody previously described (Donjacour *et al.*, 1990). Various protocols of pretreatment, with or without antigen retrieval, were employed. Briefly, CK10 immunoreactivity was detected in dewaxed paraffin sections after inactivation of endogenous peroxidase. CK5 and CKH staining was detected after pretreatment with 0.4% trypsin at 37°C for 10 min followed by inactivation of endogenous peroxidase. PR and PCNA were detected after antigen retrieval in 0.01 M citrate buffer, pH 6.0, for 15 min at 100°C followed by a period of cooling and inactivation of endogenous peroxidase. A common procedure for immunostaining was then used for all tissues. Nonspecific binding was blocked

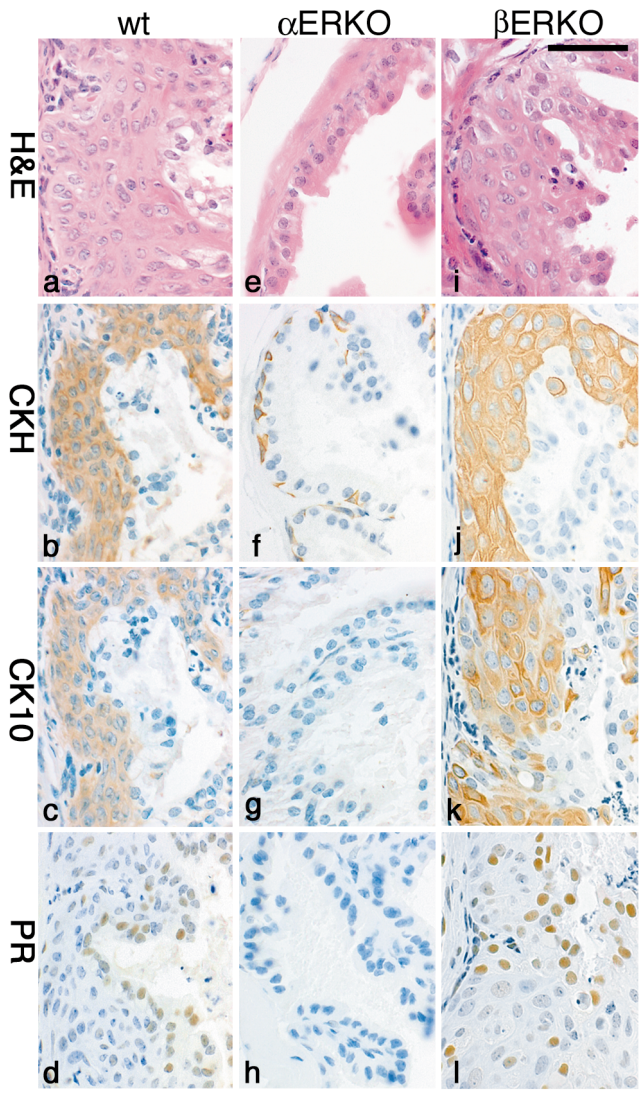

FIG. 2. Anterior prostate from wt (a–d), αERKO (e–h), and βERKO (i–l) mice. DES treatment induced SQM (a and i) with multilayer basal cells (b and j), CK10-positive staining (c and k), and PR expression (d and l) in wt and βERKO, respectively. No corresponding changes were observed in DES-treated αERKO tissue (e–h). Bar, 50 μm.

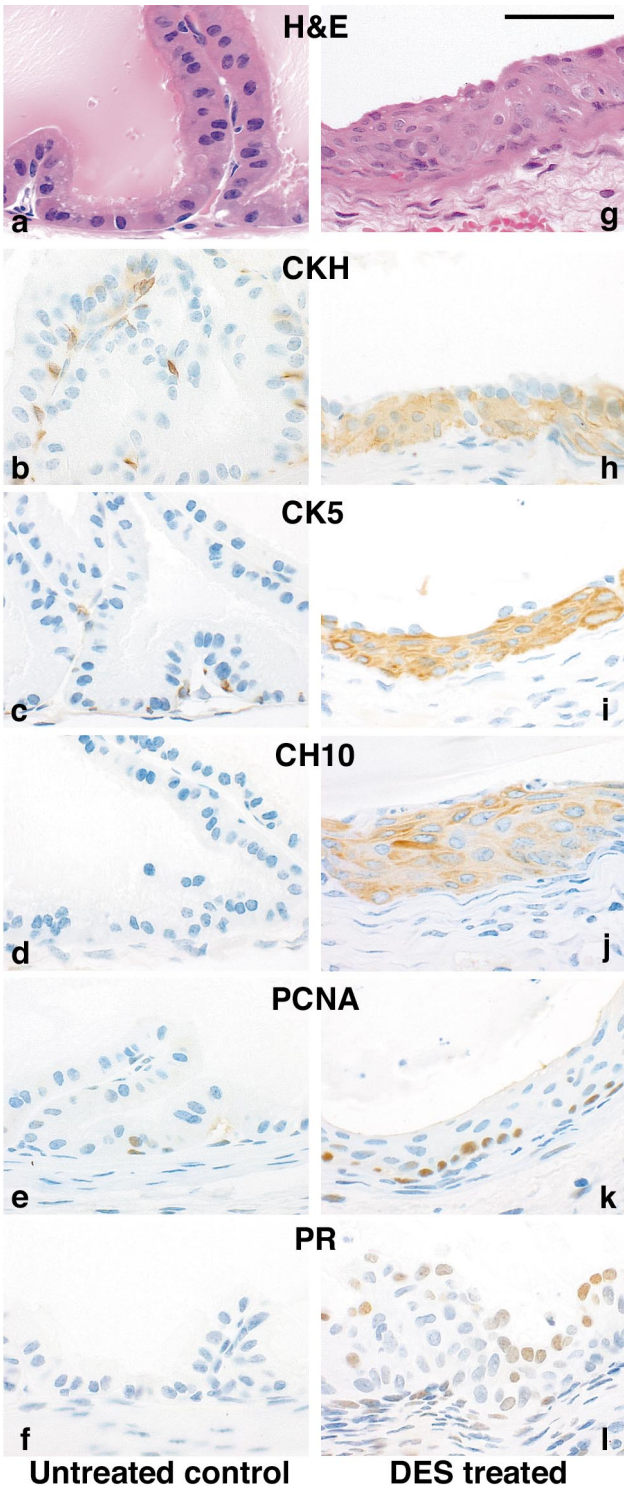

FIG. 3. The morphology of the wt-S+wt-E tissue recombinants was examined by H&E staining after 7 weeks growth in control host mice (a) or in mice treated with DES during the last 3 weeks of the grafting period (g). Immunostaining for basal cell CKH and CK5 (b and h, c and i, respectively), SQM CK10 (d and j), PCNA (e and k), and PR (f and l) is shown in control (b–f) and DES-treated tissues (h–l). In contrast to control tissues SQM, proliferating cells and PR were observed in DES-treated tissues. Bar, 50 μm.

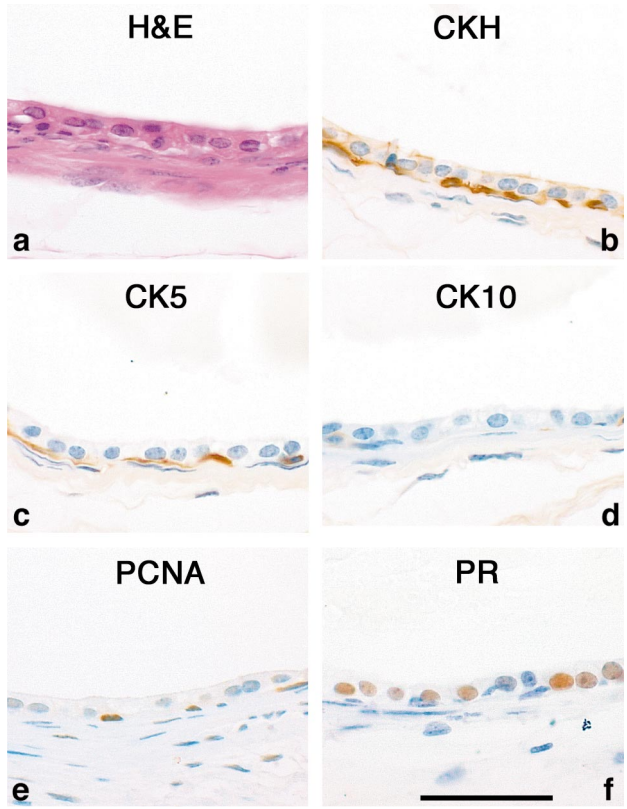

FIG. 4. αERKO-S+αERKO-E tissue recombinants failed to respond to DES (a–f), which is evident by H&E (a) and staining for CKH (b), CK5 (c), CK10 (d), and PCNA (e). However, PR expression was up-regulated (f). Bar, 50 μm.

using Super Block (Pierce, Rockford, IL), and sections were then incubated with primary antibodies for 1 h at room temperature. After being washed, sections were incubated for 30 min with biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) or biotinylated goat anti-rabbit IgG (Zymed, San Francisco, CA) followed by incubation with a Vectastain Elite ABC kit (Vector Laboratories) for 30 min. Immunostaining was visualized using DAB (3,3′-diaminobenzidine tetrahydrochloride).

RESULTS

Examination of ducts of the anterior prostate of control wt, αERKO, and βERKO mice revealed a simple columnar epithelium, lined with tall columnar secretory epithelial cells (Figs. 1a, 1e, and 1i). A discontinuous layer of basal cells, which exhibited immunostaining for CKH, was

present beneath the prostatic luminal cells of control mice (Figs. 1b, 1f, and 1j). Neither CK10 nor PR was detected by immunocytochemistry in either luminal or basal cells of control mice (Figs. 1c, 1g, 1k, and 1d, 1h, 1l, respectively). After 3 weeks of treatment with DES, epithelium of the anterior prostate of wt and βERKO mice had undergone uniform SQM, i.e., there were layers of stratified squamous cells throughout the epithelium (Figs. 2a and 2i). The anterior prostatic epithelium of αERKO mice remained unchanged and was a simple columnar epithelium (Fig. 2e). In anterior prostates of DES-treated wt and βERKO mice immunostaining for CKH revealed an increase in basal cells, which formed multiple cell layers (Figs. 2b and 2j), while in αERKO mice the discontinuous layer of basal cells remained unchanged (Fig. 2f). The thickened metaplastic epithelium of wt and βERKO anterior prostates was also positive for CK10 and PR (Figs. 2c, 2d, and 2k, 2l). In contrast, CK10 and PR were not detected by immunocytochemistry in αERKO anterior prostates (Figs. 2g and 2h). Anterior prostatic epithelium of wt and βERKO mice gave a uniform response to DES and SQM was observed throughout the gland. In contrast, anterior prostates of DES-treated αERKO mice maintained a simple columnar prostatic epithelium and were entirely devoid of SQM, epithelial stratification, and CK10 or PR expression (Figs. 2e–2h).

Since ERα is required for DES-induced SQM of the prostate, tissue recombinants were prepared to determine the respective roles of epithelial versus stromal ERα in the induction of SQM. The four types of tissue recombinants prepared as described under Methods were wt-S+wt-E, αERKO-S+αERKO-E, wt-S+αERKO-E, and αERKO-S+wt-E. Full growth and differentiation of the prostatic ducts occurred after 4 weeks of grafting of tissue recombinants into athymic male hosts and was confirmed by the expression of mouse prostate-specific protein as previously described (Donjacour and Cunha, 1995). Similarly, expression of mouse prostate-specific protein was detected in the host anterior prostate (data not shown).

H&E staining of wt-E+wt-S tissue recombinants grown for a further 3 weeks in control male hosts showed the continued presence of ducts lined with tall columnar secretory epithelial cells (Fig. 3a), which were underlain by a discontinuous layer of CKH-positive and CK5-positive basal epithelial cells (Figs. 3b and 3c). PCNA-positive epithelial cells were rare (Fig. 3e). Neither CK10 nor PR was detected in the control prostatic epithelium (Figs. 3d and 3f). In contrast, following 3 weeks of DES treatment epithelium of the wt-S+wt-E tissue recombinants (Fig. 3g) and the host's anterior prostate exhibited uniform SQM (data not shown). In wt-S+wt-E tissue recombinants the metaplastic epithelium was stratified squamous, five or more cell layers thick, and expressed CKH, CK5, and CK10 (Figs. 3h, 3i, and 3j, respectively). Many of the epithelial cells of DES-treated wt-S+wt-E recombinants expressed PCNA (Fig. 3k). PR expression was up-regulated in the epithelial cells of DES-treated wt-S+wt-E recombinants (Fig. 3l).

In αERKO-S+αERKO-E tissue recombinants treated for 3 weeks with DES the prostatic epithelium remained simple columnar, resembled that of control αERKO-S+αERKO-E tissue recombinants (data not shown), and was completely devoid of SQM (Fig. 4). Prostatic ducts were lined with columnar epithelial cells (Fig. 4a) underlain by a discontinuous layer of CKH-positive and CK5-positive basal cells in DES-treated αERKO-S+αERKO-E tissue recombinants (Figs. 4b and 4c, respectively). PCNA-positive epithelial cells were rare (Fig. 4e). CK10 was not detected in the prostatic epithelium (Fig. 4d). Surprisingly, PR was detected in many of the prostatic epithelial cells of DES-treated αERKO-S+αERKO-E tissue recombinants (Fig. 4f). This observation was confirmed in both laboratories by independent investigators.

Uniform SQM was not observed in ducts of wt-S+αERKO-E tissue recombinants, which instead were lined by columnar secretory prostatic epithelium in both control and DES-treated hosts (Figs. 5a and 5g). A discontinuous layer of CKH-positive and CK5-positive basal cells was present with (Figs. 5h and 5i) or without (Figs. 5b and 5c) DES treatment. CK10 was not detected in control or DES-treated specimens (Figs. 5d and 5j, respectively). The incidence of PCNA-positive basal prostatic epithelial cells was not affected by the presence or absence of DES (Figs. 5e and 5k). PR was detected in the prostatic epithelial cells of DES-treated wt-S+αERKO-E tissue recombinants (Fig. 5l) but not in control tissue (Fig. 5f).

αERKO-S+wt-E tissue recombinants also failed to develop mature SQM when treated for 3 weeks with DES. Prostatic tissue within αERKO-S+wt-E tissue recombinants consisted of ducts lined by a single layer of epithelial cells (Fig. 6a), which contained a discontinuous layer of basal cells positive for CKH and CK5 (Figs. 6b and 6c). CK10 was not detected (Fig. 6d) and PCNA-stained prostatic epithelial cells were rare (Fig. 6e), but PR nuclear immunostaining was detected in the prostatic epithelium (Fig. 6f). However, in this group two of five αERKO-S+wt-E recombinants exhibited small focal patches of epithelial stratification (Fig. 6g). These small foci of epithelial stratification were estimated to involve <10% of the epithelium and exhibited weak staining for PCNA, CKH, CK5 (data not shown), and CK10 and PR (Figs. 6h and 6i). Since prostatic SQM both *in situ* in the hosts's prostate and in wt-S+wt-E tissue recombinants is characterized as a profound uniform change in epithelial differentiation replete with strong expression of characteristic markers, none (5/5) of the αERKO-S+wt-E recombinants expressed SQM.

In summary, these results showed a requirement for both stromal and epithelial ERα to achieve full response to DES in terms of SQM (Table 1), although the up-regulation of PR expression did not have the same requirement and was observed in all recombinant tissues regardless of their composition. We examined the expression of ERα protein in anterior prostatic tissue of wt mice. In epithelium of anterior prostate, the detection of ERα was at the threshold of sensitivity and was not convincing prior to DES treatment of control mice (Fig. 7a), but after DES treatment, ERα

expression was observed, and ERα immunoreactivity was readily detected in distal tips of the prostatic epithelial cells of wt mice (Fig. 7b). As reported previously stromal cell staining for ERα was detected in proximal ducts near the urethra where ERα is not normally expressed in epithelium (Fig. 7c). ERα immunostaining was not detected in αERKO mouse prostate epithelium (Fig. 7d). Epithelial ERα was detected in αERKO-S+wt-E tissue recombinants, after DES treatment to host mice (Fig. 7d). As expected, ERβ was detected in the prostatic epithelium of control αERKO mice (Fig. 7e). Likewise, ERα was detected in the samples treated for 21 days with DES (Fig. 7f). It was noted that ERβ immunostaining was recorded in prostatic epithelium of βERKO mice and presumed to be due to the presence of a nonfunctional ERβ protein (Fig. 7h).

DISCUSSION

The mechanism of action of estrogens in male reproductive tract tissues has been advanced by the development of mouse models in which the estrogen receptor genes, ERα or ERβ, have been rendered nonfunctional by gene targeting (Couse and Korach, 1999; Krege *et al.,* 1998; Lubahn *et al.,* 1993). This study demonstrated that the prostate of the αERKO mouse (having only ERβ) failed to undergo SQM when treated with the potent synthetic estrogen, DES. In contrast, prostates of wt mice (having both ERα and ERβ) and βERKO mice (having only ERα) expressed SQM when treated with DES. Taken together these data demonstrate an essential role for ERα in estrogen-induced SQM of the prostate. These data, however, do not specify whether epithelial or stromal estrogen receptors are involved in the induction of prostatic SQM. ERα is expressed in both epithelial and stromal cells of the prostate although predominantly in the stroma (Lau *et al.,* 1998; Prins and Birch, 1997; Schulze and Barrack, 1987; and this study). Using tissue recombination we have demonstrated a requirement for both epithelial and stromal ERα to induce a complete estrogenic response in the prostatic epithelium. Collectively the results from this study reveal the importance of stromal–epithelial interactions and epithelial and stromal ERα in estrogen-induced SQM of the prostate gland.

A universal response to estrogen by the prostate gland is the induction of epithelial SQM in several species including mice and men (Andersson and Tisell, 1982; Aumuller *et al.,* 1982; Deschamps *et al.,* 1987; Driscoll and Taylor, 1980; Habenicht and Etreby, 1988; Kesteren *et al.,* 1996; Pylkkanen *et al.,* 1991; Randles, 1990; Sugimura *et al.,* 1988; Weijman *et al.,* 1992). Although this study uses high doses of DES, prostatic SQM is a physiological phenomenon in full-term human male fetuses and newborn male infants, which usually exhibit prostatic SQM (Andrews, 1951; Brody and Goldman, 1940), due to high levels of maternal estrogen in the last trimester of pregnancy (Andrews, 1951). In adult men, SQM is a common histologic finding that is usually associated with hormone treatment or infarction (Epstein and Murphy, 1997). In this study, the treatment of the transgenic αERKO and βERKO mice with estrogen showed that the induction of SQM was mediated through ERα. The current study identified a functional response to ERα, namely SQM, and this is consistent with localization of ERα in prostate epithelium and stroma. This raises the question of whether there is another specific response to estrogen that is mediated by ERβ. It is known that ERβ is localized to the epithelium, but a functional response to estrogen via ERβ has yet to be defined in the prostate. It was noted that immunoreactivity for ERβ protein was detected in wt and βERKO mouse prostate; this indicates that the antibody cannot discriminate between functional and nonfunctional protein in the tissues and emphasizes the need to identify a specific biological action of estrogen through ERβ.

The requirement for both stromal and epithelial ERα as mediators of estrogen action in the prostate implies both direct and paracrine mechanisms. ERα protein and mRNA and estrogen binding have been reported in prostatic stroma of several mammalian species, including mouse (Cooke *et al.,* 1991; Hatier *et al.,* 1990; Prins and Birch, 1997; Ruberg *et al.,* 1982; Schleicher *et al.,* 1984; Weaker and Sheridan, 1983). In prostatic epithelium ERα protein has been detected in rats following neonatal estrogen treatment (Prins and Birch, 1997); other studies failed to detect ERα mRNA in epithelium of the rat ventral prostate or primary cultures of human epithelial cells (Lau *et al.,* 1998, 2000). However, in the present study ERα protein was detected in wt stroma and epithelium of the mouse anterior prostate but only after administration of DES. In wt mouse the immunocytochemical staining for ERα is very weak in prostatic epithelium of control adult animals and shows lobular variation within the prostate, lowest in the ventral prostate and highest in the anterior prostate (data not shown). After treatment with DES the immunocytochemical signal for prostatic epithelial ERα is stronger in most areas, even though a subset of prostatic epithelial cells remain unstained after estrogen treatment, especially in the region of ductal tips (unpublished observations). It is possible that the up-regulation of ERα in the epithelium is mediated via ERα-positive stromal cells that were detected in the periurethral region of the prostate as reported earlier (Schulze and Barrack, 1987) and confirmed by steroid autoradiography (Cooke *et al.,* 1991; Schleicher *et al.,* 1985).

Since SQM was induced by DES only in wt-S+wt-E tissue recombinants, the interplay between stromal and epithelial ERα is required for induction of mature SQM by DES. After DES treatment, wt-S+wt-E tissue recombinants showed multilayering of epithelium with cells that were positive for CKH, CK5, and CK10. A substantial number of PCNA-positive cells were observed in the hyperplastic basal epithelial cells, and PR was induced by DES in the luminal cell layer of wt-S+wt-E tissue recombinants. DES induced a full and uniform response in the epithelium of wt-S+wt-E tissue recombinants only and not in any of the other tissue recombinants, thus demonstrating a requirement for both

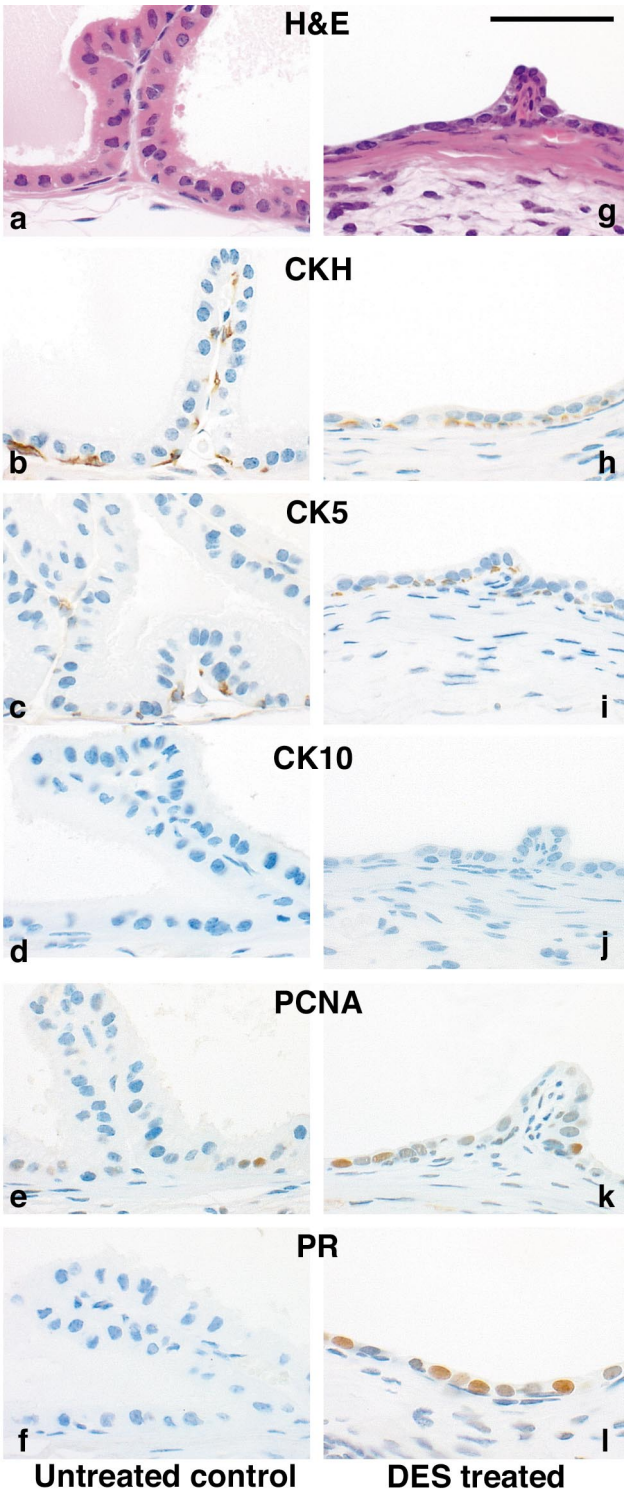

FIG. 5. The morphology of the wt-S+αERKO-E tissue recombinants was examined by H&E staining after 7 weeks growth in control host mice (a) or in mice treated with DES during the last 3 weeks of the grafting period (g). Immunostaining for basal cell CKH and CK5 (b and h, c and i, respectively), SQM CK10 (d and j), PCNA (e and k), and PR (f and l) is shown in control (b–f) and DES-treated tissues (h–l). No squamous epithelium was observed in both control and DES-treated tissues. DES-treatment up-regulated PR in the epithelium of wt-S+αERKO-E recombinant grafts (l). Bar, 50 μm.

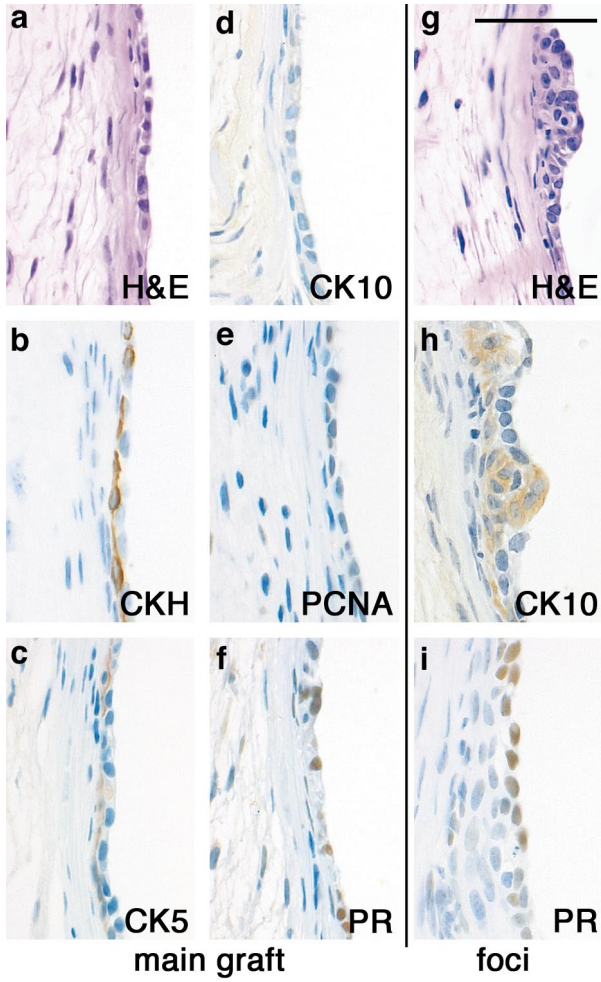

FIG. 6. DES-treated αERKO-S+wt-E tissue recombinants were examined by H&E (a and g) and immunostaining for basal cell CKH and CK5 (b and c), SQM CK10 (d and h), PCNA (e), and PR (f and i). The majority of tissue recombinant grafts failed to show any response to DES (a–e), although PR was up-regulated in the single-layer epithelium (f). In two of five recombinants, focal areas of stratification were detected as indicated by H&E (g), metaplastic CK10-positive cells (h), and PR up-regulation (i) compared to controls (data not shown). Bar, 50 μm.

epithelial and stromal ERα. DES-induced SQM was not observed if ERα was absent in either prostatic epithelium or stroma (or both) as is the case in wt-S+αERKO-E, αERKO-S+wt-E, and αERKO-S+αERKO-E tissue recombinants. Tissue recombinants consisting of wt-S+αERKO-E were

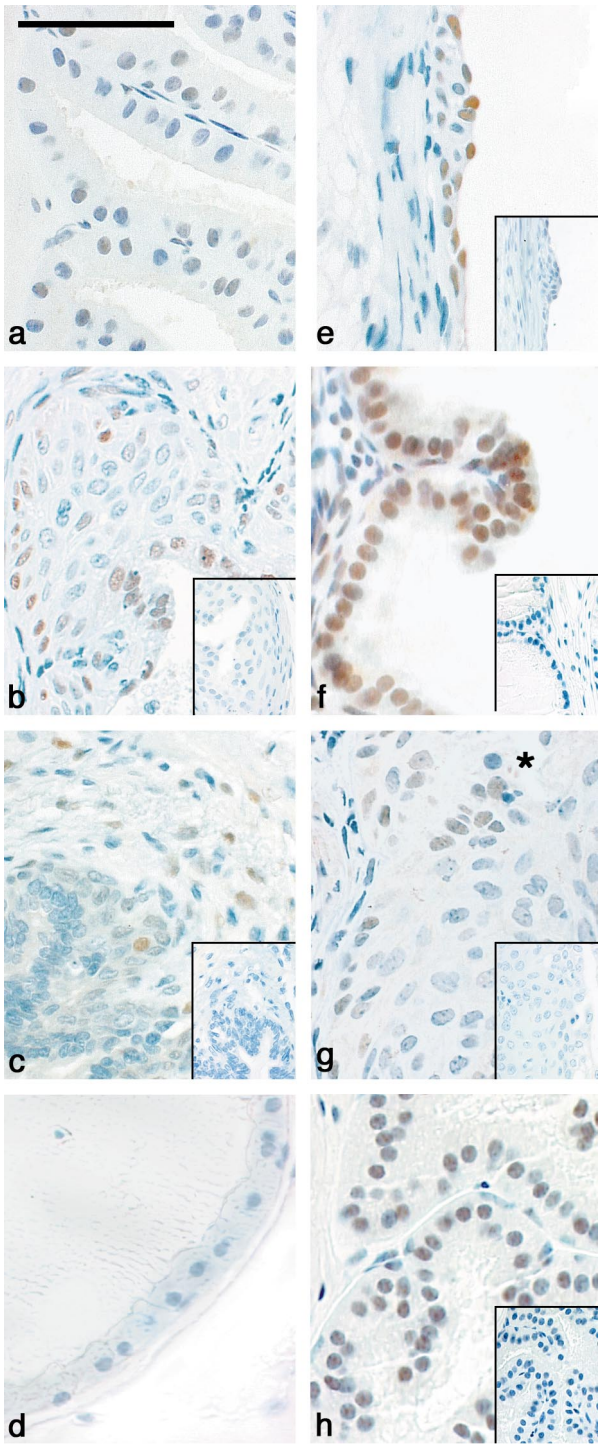

FIG. 7. Immunolocalization of ERα in anterior prostate epithelium of wt control mice (a), DES-treated wt mice (b, inset shows negative control), proximal stroma of wt DES-treated mice (c, inset shows negative control), αERKO mice (d), and DES-treated tissue recombinants of αERKO-S+wt-E (e, inset shows negative control). ERα is up-regulated following DES treatment in both normal (b) and recombinant (e) epithelium and not detected in αERKO mouse prostate (d). ERβ immunostaining of prostatic epithelium was detected in αERKO mice (f, inset shows negative control) and ERα immunostaining of prostate epithelium of DES-treated βERKO mice (g, inset shows negative control). It was also recorded that immunostaining for ERβ was detected in βERKO mouse prostate (h, inset shows negative control). Asterisk indicates the lumen. Bar, 50 μm.

TABLE 1

The Number of Tissue Recombinants Showing Uniform SQM and Up-regulation of CK10 and PR Expression

	SQM	CK10	PR
Wt-S+wt-E	20/21	20/21	12/12
Wt-S+αERKO-E	0/20	0/20	9/9
αERKO-S+wt-E	0/5[a]	0/5[a]	3/4
αERKO-S+αERKO-S	0/11	0/11	11/11

[a] As discussed in the text, two of five animals showed foci of cells that were positive for CK10 but the epithelium was not multilayered and the response was not observed throughout the recombinant grafted tissue. This response was not considered to be a full, uniform SQM.

composed of a single layer of epithelial cells that were not different from sham-treated controls. Thus, while αERKO-S+wt-E tissue recombinants express a limited spectrum of estrogen receptors in epithelium and stroma (see discussion above on expression of ERα and ERβ in the prostate), the specific absence of ERα in prostatic epithelium completely eliminated the development of SQM. Even though the epithelium of wt-S+αERKO-E tissue recombinants expressed ERβ (Kuiper *et al.*, 1996, 1997), it did not respond to DES. Likewise, in αERKO-S+wt-E tissue recombinants the specific absence of ERα in the prostatic stroma meant that a uniform mature prostatic SQM did not develop in response to DES. Thus, our data show that both epithelial and stromal ERα are required for full and uniform induction of SQM by DES.

Although the majority of αERKO-S+wt-E tissue recombinants failed to respond to DES, we observed that two of five αERKO-S+wt-E tissue recombinants showed small isolated foci of epithelial cells that were weakly positive for CKH, CK5, CK10, PCNA, and PR. These small foci of reactive epithelial cells comprised considerably less that 10% of the prostatic epithelial tissue of these αERKO-S+wt-E tissue recombinants. The vast majority of prostatic epithelium in αERKO-S+wt-E tissue recombinants was nonresponsive to DES. We suggest that these foci in two of five of the αERKO-S+wt-E tissue recombinants were contaminated with small amounts of wt-S carried over with the microdissected wt ductal tips used to prepare the tissue recombinants. In these regions a limited response of wt-E to DES treatment was initiated, but impaired due to an inad-

equate stroma. Immunostaining of these αERKO-S+wt-E tissue recombinants did not reveal ERα-positive stromal cells in these small foci. However, given the extremely weak ERα signal in wt prostatic stromal cells, this is not surprising.

Overall, our studies demonstrated that both stromal and epithelial ERα are required for a full uniform metaplastic response to DES in the mouse anterior prostate. The presence of ERα in both the epithelium and the stroma is also required for estrogen-induced vaginal cornification in which the full cornification response occurred only in tissue recombinants having ERα in both epithelium and stroma (Buchanan *et al.*, 1998). Thus, our findings in the prostate on DES-induced SQM corroborate earlier studies indicating that full estrogenic response requires both indirect paracrine mechanisms mediated by stromal ERα as well as direct estrogen action on the epithelium mediated by epithelial ERα. Tissue recombinant studies with steroid receptor null mutants have recently demonstrated that *in vivo* regulation of epithelial proliferation by androgen, estrogen, and progesterone is mediated by paracrine mechanisms in male and female hormone target organs (Cooke *et al.*, 1997; Kurita *et al.*, 1998; Sugimura *et al.*, 1986). Using similar techniques, the regulation of functional differentiation of epithelia of male and female genital tracts requires appropriate steroid receptors in both epithelium and stroma (Buchanan *et al.*, 1998, 1999; Cunha and Young, 1991).

The up-regulation of PR is a common marker of estrogen response in female reproductive tissues, and up-regulation of PR expression following estrogenic stimulation occurs in the human and rodent prostate (Kesteren *et al.*, 1996; West *et al.*, 1988). The results of this study showed that PR expression was detected in the luminal cell layer of all tissue recombinants, whether or not epithelial ERα was detected. This raises the possibility that PR is regulated in prostatic epithelium by ERβ. Up-regulation of PR in endometrial stroma is mediated through at least three mechanisms: classical estrogen signaling through ERα, estrogen signaling through ERβ, and mechanical stimulation plus progesterone (estrogen is not required) (Kurita *et al.*, 2000b). Other as yet undiscovered mechanisms of PR regulation may also exist. Further studies using recombinants of wt and βERKO mouse tissue could reveal the requirement for ERβ in mediating this response, but it is essential that a functional role for estrogens, mediated by ERβ, is identified. It is also essential that a role for progesterone in the prostate gland is determined. Together, this information could provide justification for further studies on the mechanism of action of estrogen via ERβ and of progesterone in the prostate.

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