Prostatic hormonal carcinogenesis is mediated by *in situ* estrogen production and estrogen receptor alpha signaling

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ABSTRACT **It was recently demonstrated that antiestrogens prevented prostate cancer (PRCA) in men. The source of estradiol (E2) that contributes to carcinogenesis, as well as the selected estrogen receptor (ER) signaling pathway, is unknown. To evaluate estrogen's effects in carcinogenesis, we developed a new model of PRCA utilizing testosterone and E2 to stimulate PRCA. To determine whether local *in situ* production of E2 affected incidence of PRCA, aromatase-knockout (ArKO) mice were evaluated. In contrast to the wild-type mice, ArKO mice had reduced incidences of PRCA, which implicates *in situ* production of E2 as an important determinant of PRCA. To determine whether E2-mediated responses were due to ERα or ERβ signaling, ERα-knockout (αERKO) or ERβ-knockout (βERKO) mice were used. Prostates from βERKO mice underwent biochemical and histological carcinogenesis similar to wild-type mice, whereas prostates from αERKO mice remained free of pathology. These data suggest that effective prevention of carcinogenesis will require antagonism of ERα but not ERβ. This mouse model provides a means to examine genetic gain and loss of function and determine the efficacy of therapeutics on prostatic carcinogenesis.—Ricke, W. A., McPherson, S. J., Bianco, J. J. Cunha, G. R., Wang, Y., Risbridger, G. P. Prostatic hormonal carcinogenesis is mediated by *in situ* estrogen production and estrogen receptor alpha signaling. *FASEB J.* 22, 1512–1520 (2008)**

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Androgens play a central role in the biology of the prostate gland, eliciting both normal development and adult function (1). Estrogens also affect prostatic growth and development (2). Low doses of estrogens given prenatally have been shown to enhance prostatic growth in mice (3). In contrast, high levels of estrogens administered perinatally generally elicit inhibitory effects on prostatic development, restricting growth and decreasing responsiveness to androgens in adulthood (3, 4).

When administered to adult animals, high doses of exogenous estrogens simultaneously have both indirect and direct effects on the prostate. An important indirect effect on the prostate is "chemical castration," which results from suppression of pituitary gonadotropin and thus reduction of testosterone (T) secretion by the testes (5, 6). The direct actions of E_2 on the adult prostate are mediated through the prostatic estrogen receptors (ERs) -alpha (ERα) and -beta (ERβ) (7, 8). Estrogens elicit various pathological changes in the prostate, the best characterized is the induction of squamous metaplasia, which has been reported in a number of different mammalian species (9–11). Estrogens have also been shown to act synergistically with androgens to induce benign prostatic hyperplasia in dogs (12) and prostatic cancer in adult Noble rats at an incidence of ~20% at 1 yr of treatment (13–15). In terms of human endocrinology, hormone profiles in T + E_2-treated mice maintain a dynamic hormonal milieu similar to that found in men as they age (16–18). Additionally, this hormonal environment stimulates the malignant transformation of human prostatic cells (17, 19).

ERα, the classic ER, is consistently reported in prostatic stroma in the rat (20, 21). A second ER, ERβ, was originally isolated from prostatic tissue and is expressed at high levels in prostatic epithelium. In adulthood ERβ is undetectable in prostatic stromal cells (8). Thus, ERs are present in both the stromal and epithelial tissues of the prostate. Prostatic development appears to be normal in mice deficient in either ERα or ERβ, suggesting that organogenesis of the prostate is independent of

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signaling through either of these ERs (7, 22). The role of estrogens in prostatic carcinogenesis remains to be clarified. However, several lines of evidence suggest that estrogen plays a major role in carcinogenesis of the prostate. Recent studies in humans show a significant decrease in early PRCA progression when men were administered the antiestrogen Toremifene (23). Although this is the first evidence that human PRCA can be prevented at very early stages, it is unclear as to where the estrogen is derived (systemic *vs.* local) as well as its mode of action (*via* ERα *vs.* ERβ). Indeed, the study prompted editorial comment that the results were intriguing but did not establish a clear rationale for "using SERMs for preventing prostate cancer" and it was the editor's opinion that "the action of estrogen must be further investigated through basic research before commencing further clinical trials." Thus, as industry, government, and academic institutions continue to develop new drugs, such as SERMs (selective estrogen receptor modulators) that specifically stimulate or antagonize ERα or ERβ, it is imperative to know how estrogens target the prostate as well as which ER-signaling mechanism is critical for carcinogenesis.

The studies presented here demonstrate that, as in the Noble rat, prostatic carcinogenesis can be elicited in mice by T + E_2. The present study additionally utilizes aromatase-knockout (ArKO), ERα-knockout (αERKO) and ERβ-knockout (βERKO) mice to investigate the influence of *in situ*-produced E_2 on carcinogenesis as well as to determine the mechanism of estrogenic signaling in eliciting prostatic carcinoma.

MATERIALS AND METHODS

Induction of carcinogenesis

Sexually mature wild-type (WT) CD-1, C57BL/6, and C57BL/6 × J129 mice were obtained from Charles River (Wilmington, MA, USA). αERKO and βERKO heterozygotes on a C57BL/6 background were obtained from Dr. Dennis Lubahn (University of Missouri, Columbia, MO, USA) and Dr. Jan-Ake Gustafsson (Karolinska Institute, Huddinge, Sweden), respectively. ArKO mice were generated by breeding mice heterozygous for the *cyp19* gene on a C57BL/6 × J129 background as we have previously described (24, 25). Genotypes of all mice were monitored by polymerase chain reaction (PCR) analysis as described previously (22, 25, 26). Mice were treated with T + E_2 for 4 months as we and others have described previously (14, 17, 19, 27). These methods have reliably reproduced circulating plasma levels of T + E2 similar to those found in aging men; specifically, E2 levels ranging from 20 to 120 pg/ml and T levels ranging from 1 to 12 ng/ml. Mice were treated hormonally by surgical implantation of a 1 cm Silastic capsule filled with testosterone and a 0.4 cm Silastic capsule filled with estradiol-17 (No. 602–305 Silastic tubing; 1.54 mm inside diameter, 3.18 mm outside diameter; Dow-Corning Corp., Midland, MI, USA). T and E2 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Mice were hormonally treated for 4 months. Control animals received empty Silastic tubing. All animals were housed with food and drinking water *ad libitum* under controlled conditions (12 h light, 12 h dark; 20±2°C).

Histopathological grading

Mice were euthanized by injection of an excess dose of Nembutal followed by cervical dislocation. Prostate glands were carefully collected and processed for histological analysis. Grading was performed as described by Shappell and colleagues (28).

Immunohistochemical staining

Immunohistochemical staining was performed as we have previously described (17, 19, 24). In these experiments, rabbit polyclonal antiandrogen receptor antibody (PA1–111A, 1:100) was purchased from Affinity BioReagents (Golden, CO, USA). Mouse monoclonal anticytokeratin 8 (CK8, LE41) was generously provided by Dr. Birgitte Lane, University of Dundee, Dundee, UK. Mouse antismooth muscle α-actin monoclonal antibody (A-2547, 1:500) was purchased from Sigma. Mouse anti-E-cadherin monoclonal antibody (C20820, 1:200) was purchased from Transduction Laboratories (San Diego, CA, USA). Antip63 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-human ERα (clone 1D5) was purchased from Dako (Carpenteria, CA, USA). Anti-ERβ was a gift from Dr. Jan-Ake Gustafsson. This antibody has been previously described (29) and was used at a dilution of 1:300 in PBS. The specificity of these ER antibodies was confirmed by staining prostatic tissue from αERKO and βERKO mice.

Quantification and imaging of prostates

Slides were examined histologically from serially sectioned mouse prostate to determine the incidence of prostatic lesions. The identity of each prostate was blinded from the reviewer. Every tenth section was examined (a separation of 50 μm). Five to eight sections per prostate were examined, dependent on the size of the harvested prostate. Slides were scored to determine the presence of normal, hyperplasia, atypical hyperplasia, or prostatic intraepithelial neoplasia (PIN).

Serum hormone levels

Plasma androgens were determined *via* RIA as we have described previously (24).

Statistics

Data analysis was performed by ANOVA; when statistical differences were observed ($P<0.05$), mean separation was performed using Tukey's test. Prostate pathologies were analyzed using χ^2.

RESULTS

Hormonal induction of PIN in WT mice

A total of 23 WT CD-1 mice, 25 WT C57BL/6 mice, 20 WT C57Bl/J129 mice, 10 αERKO (C57BL/6) mice, 15 βERKO mice, and 20 ArKO (C57Bl/J129) mice were analyzed after 4 months of T + E_2 treatment. No prostatic pathology was seen at either a gross or microscopic level in any WT untreated mouse in this study. Gross comparison of the WT (C57BL/6, CD-1, or

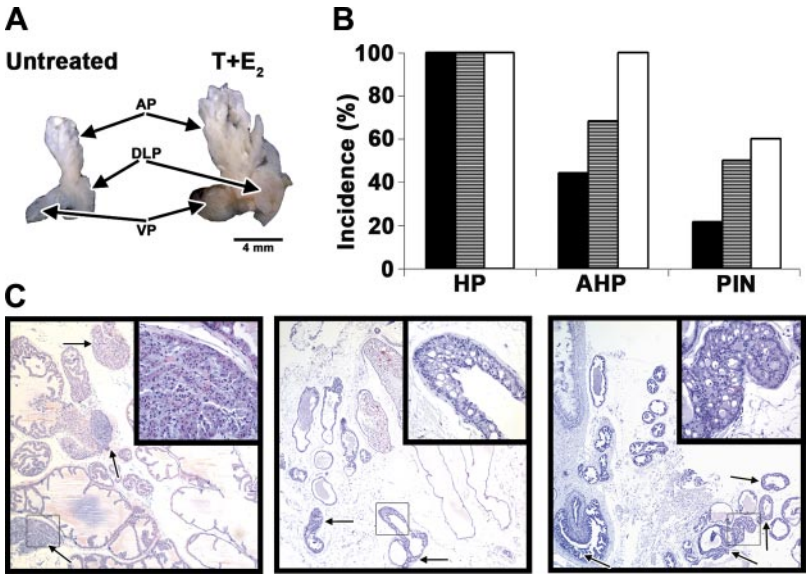

Figure 1. The effects of T and E_2 on mouse prostates. *A*) Whole mounts of untreated (left) and T + E_2-treated (4 months; right) C57BL/6 mouse prostatic complex. *B*) Incidence of prostatic pathologies in mice with different genetic backgrounds. Prostatic pathologies included hyperplasia (HP), atypical hyperplasia (AHP), and prostatic intraepithelial neoplasia (PIN). Solid = CD-1; slash = C57BL/6; open = C57BL/6 × J129. *C*) Histological analysis of H&E-stained prostates from T + E_2-C57BL/6 mice. Presence of PIN (arrows) was observed in AP, VP, and DLP. Micrographs were taken at ×100 and ×400 (insets).

C57BL/6×J129) prostates of treated and untreated animals revealed that T + E_2 treatment resulted in an overall increase in prostatic size (**Fig. 1*A***). Although hormonally treated WT prostates were larger, invasion into surrounding tissues or metastases were not observed by visual examination.

Comparison of the incidence of prostatic pathology in each mouse strain indicated differences in sensitivity, however all strains showed evidence of aberrant histology in response to T + E_2 treatment. Epithelial hyperplasia was found in ventral prostate (VP), dorsolateral prostate (DLP) and anterior prostate (AP) lobes of all WT CD-1, C57BL/6, and C57BL/6 × J129 strains treated with T + E_2. Atypical hyperplasia, was characterized by two or more epithelial cell layers with variable degrees of cytological abnormality (28). Foci of atypical hyperplasia were identifiable in all prostatic lobes following T + E_2 treatment; however, incidence of these lesions in the different mouse strains varied (44%, 68%, 100% in CD-1, C57BL/6, and C57BL/6×J129 strains, respectively; Fig. 1*B*). The cells within these areas had enlarged nuclei with prominent nucleoli and increased nuclear-cytoplasmic ratios compared to areas of normal histology. Multiple foci of atypia were often seen within a single section, and several foci were sometimes seen within a single large duct. Interestingly, many morphologically normal ducts were seen alongside atypically hyperplastic ducts, demonstrating that these effects were not uniform. It was also common to observe extensive infiltration of mononuclear cells around these atypical foci. As with atypical hyperplasia, all T + E_2-treated prostate lobes also contained areas of PIN as defined by Shappell and colleagues (Fig. 1*C*) (28).

Biochemical markers of prostate cancer progression in WT mice

The morphological characterization of prostatic response to T + E_2 treatment in mice was further evaluated in WT mice using immunohistochemical markers (**Fig. 2**). In untreated and T + E_2-treated mice, normal prostatic epithelial cells and cells within focal epithelial lesions were cytokeratin-8-positive, thus suggesting the formation of PIN. Androgen receptors were expressed in both epithelium and stroma of normal and pathological prostatic tissue (data not shown). In areas of PIN where luminal cells were piled into multiple layers, the majority of cells were both cytokeratin-8 and androgen receptor positive. The basal cell marker, p63, was found in normal areas in both treated and untreated animals; however, in areas of PIN, p63-positive basal cells were reduced in numbers or absent. Smooth muscle α-actin positive cells were evident within the stromal compartment of histologically normal-appearing tissue. However, in areas of pathology, localization of α-actin was dramatically reduced. Localization of E-cadherin was dramatically reduced and diffuse in areas of pathology, whereas in normal areas E-cadherin was intense and primarily found around the membrane. Increased numbers of proliferating PCNA-positive cells were observed in areas of pathology, yet the vast majority of normal cells were PCNA negative.

Absence of endogenous aromatase reduces incidence of prostatic pathologies

Compared to WT littermates, prostates of untreated ArKO mice are hyperplastic (**Fig. 3*A***). After treatment with T + E_2, weight of WT but not ArKO prostates was significantly increased (Fig. 3*B*, *C*). However, hyperplasia and focal PIN lesions were morphologically identifiable within both WT and ArKO prostates (Fig. 3*A*). PIN lesions derived from T + E_2-treated WT and ArKO mice showed increased proliferative activity, elevated ERα localization, and loss of E-cadherin compared to surrounding normal or hyperplastic tissues (data not shown). Although hyperplasia was induced in the WT prostates, due to the preexisting hyperplastic phenotype of the ArKO prostates (25) it was not possible to

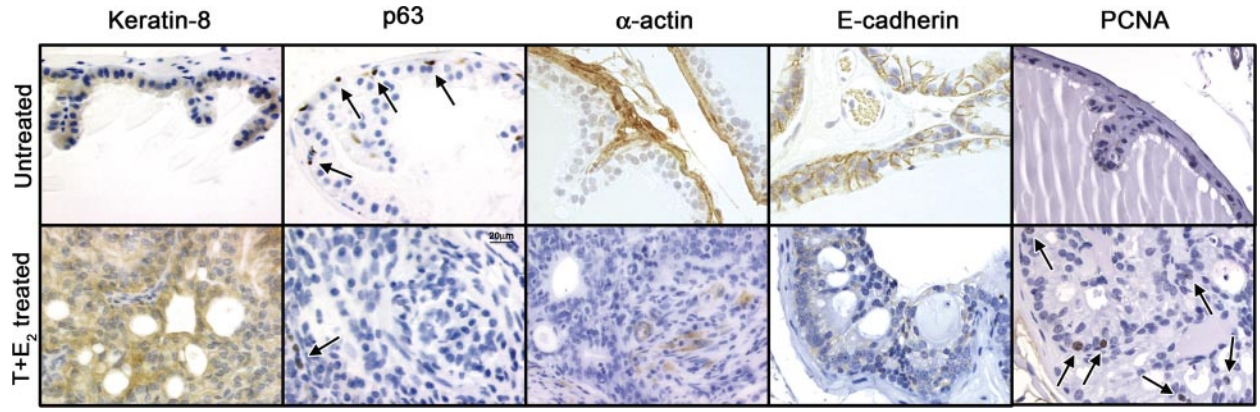

Figure 2. Immunohistochemical analysis of prostates from control untreated and T + E2-treated C57BL/6 mice. Localization of keratins-8 (luminal cell), p63 (basal cell, arrows), α-actin (smooth muscle stromal cells), E-cadherin (normal epithelial cells), and PCNA (proliferative cells, arrows) are all appropriately expressed for normal and pathological (PIN) states. Note specifically the loss of p63, α-actin, and E-cadherin as well as the increased expression of PCNA with progression to PIN.

accurately determine whether an increased hyperplastic response in the ArKO tissues had occurred.

A lobe-by-lobe comparison of WT prostates showed greatest susceptibility to the induction of dysplasia following T + E_2 treatment in the lateral prostate (LP) followed by VP, dorsal prostate (DP), and AP (**Table 1**). This pattern was also observed in ArKO prostates; however, incidence of atypical hyperplasia and PIN lesions was significantly reduced ($P<0.05$) in ArKO VP and LP lobes compared to corresponding WT tissues. Although the incidences of pathology in the DP from T + E2-treated ArKO mice were reduced, they were not significantly different than DP from WT animals. AP was least sensitive to the effects of T + E_2, and cancer incidence was not different between ArKO and WT.

Although ArKO mice normally show elevated levels of serum androgens (Fig. 3) compared to WT animals, administration of T + E_2 implants produces androgen profiles that are not different between these strains (Fig. 3*D*).

Distribution of prostatic ERα and ERβ in untreated and T + E_2-treated WT mice

Immunolocalization of ERα in untreated WT littermate and βERKO mouse prostates revealed ERα expression almost exclusively within the stroma, with little found within the epithelia. No ERα was observed in αERKO mice (data not shown). In contrast, when βERKO and WT littermate mice were treated with T + E_2 implants, ERα was detected within the prostatic epithelia as well as the stromal layer (**Fig. 4**).

Immunohistochemical detection of ERβ in untreated WT littermate and αERKO mouse prostates revealed that ERβ was expressed almost exclusively within the epithelia (Fig. 4). No ERβ was observed in βERKO mice (data not shown). In contrast, when WT littermates were treated with T + E_2 implants, ERβ was markedly decreased within areas of PIN but not in areas of normal histology.

Absence of ERα but not ERβ prevents hormonal induction of atypical hyperplasia and PIN

Comparison of the incidence of prostatic epithelial pathologies between T + E_2-implanted WT, αERKO, and βERKO mice demonstrated that WT, αERKO, and βERKO mice developed prostatic hyperplasia (**Fig. 5**). However, hormone-implanted αERKO mice exhibited no morphological evidence of atypical hyperplasia or PIN and showed no change in E-cadherin localization or cellular proliferation following T + E_2 treatment (Fig. 5). Additionally, ERβ expression appeared unaltered and was robustly localized in the prostatic epithelia of T + E2-implanted αERKO mice (data not shown). In contrast, βERKO mice treated with T + E_2 exhibited hyperplasia, atypical hyperplasia, and PIN. Additionally, βERKO prostates had decreased levels of E-cadherin and increased numbers of proliferating cells, comparable with WT animals treated with T + E_2. βERKO mice have been reported to have focal areas of hyperplasia within the adult prostate (30); therefore, it was expected that hormonal treatment might accelerate or amplify prostatic carcinogenesis within these areas. However, no such increase in advanced prostate cancer was observed for any prostate lobe collected from T + E2-treated βERKO mice.

DISCUSSION

For the past four decades, hormone-induced prostatic carcinogenesis has been studied primarily in a single key model, the Noble rat. For many scientists the Noble rat was believed to be a unique strain that was susceptible to hormones known to be important in the pathobiology of human disease. Data from the current study demonstrate for the first time the induction of malignant disease in the prostates of WT mice of different strain backgrounds (C57BL/6, CD-1, C57BL/6×J129) with a combination of T and E_2. Characterization of this response demonstrated that the incidence

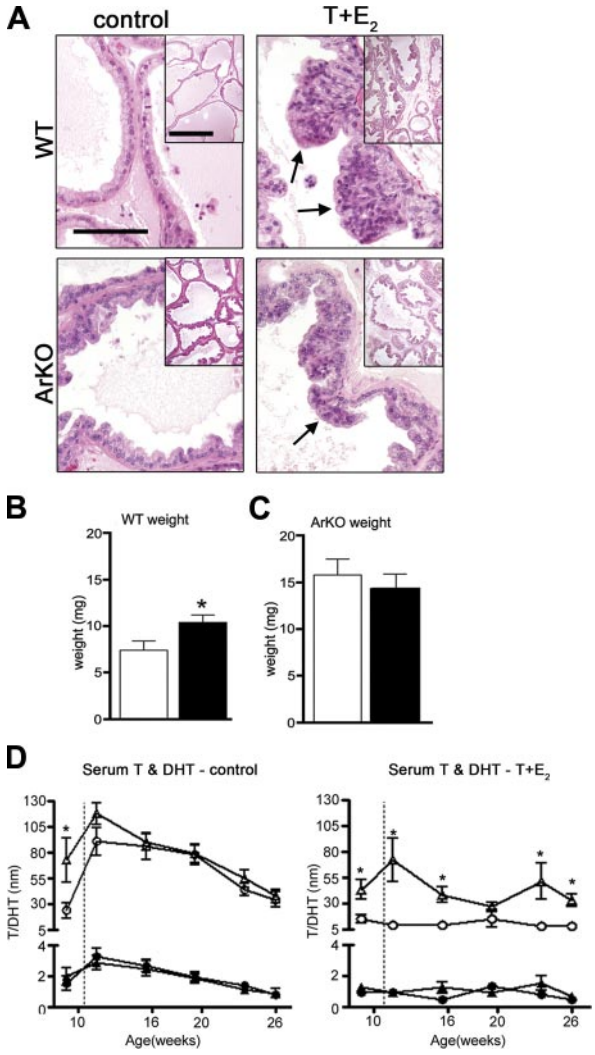

Figure 3. Effects of combined T + E_2 administration on ArKO mouse prostate. *A*) Representative prostate sections from T + E_2-treated ArKO and WT mice demonstrating the presence of hyperplastic and dysplastic lesions following T + E_2 treatment (arrows). *B, C*) Compared to untreated controls (open bars), T + E_2 treatment (solid bars) caused significant increase in weight of WT prostates (*B*) but had no significant effect on ArKO prostate weight (*C*). *D)* Serum androgen profiles demonstrate serum testosterone (△) but not DHT (▲) in untreated control ArKO mice was significantly elevated compared to untreated WT control T (○) and DHT (●); however, following administration of T + E_2, serum androgen profiles in both ArKO and WT mice were not significantly different. Dotted line indicates start of treatment. Scale bars = 100 μm (*A*); 500 μm (insets). $*P < 0.05$; $n \geq 10$.

of disease in the C57BL/6 strain (pure or mixed) is higher than in outbred CD-1 animals, consistent with the reported increased sensitivity of the C57BL/6 strain to estrogens and to carcinogenic influences (31, 32).

Although incidence of PIN due to T + E_2 treatment varies between the C57BL/6, CD-1, and C57BL/6 × J129 strains of mice, all animals showed clear morphological and immunohistochemical evidence comparable to progression of human prostatic carcinogenesis. These pathologies include: epithelial hyperplasia; atypical hyperplasia, and PIN. Additionally decreased numbers of smooth muscle cells and basal cells in our mouse models parallel the loss or dedifferentiation of smooth muscle in the development of human prostate cancer (17, 33, 34).

The implication drawn from these studies is that the combined effects of androgens and estrogens are important in inducing prostatic carcinogenesis, which is in agreement with studies performed on other species (14, 17, 27); extension of this hormonal carcinogenesis protocol to mice allows the use of transgenic and knockout mice to further dissect the contributions made by specific hormones, receptors, and other pathways involved in carcinogenesis. Given the speculation on the role of E_2 in prostate disease and the fact that the mechanism of action for the T + E_2 procedure is relatively poorly defined, we have used mice with aberrant estrogenic pathways to provide for the first time further insight into how estrogen may contribute to prostate carcinogenesis.

The response of the prostate to physiological doses of T is dependent on the developmental stage and pathological state of the organ. In fetal and immature animals, low titers of androgens act *via* receptors in the stromal compartment of the gland to induce proliferation of the prostatic epithelial cells (1). In the normal adult, high titers of androgens acting through receptors in the prostatic smooth muscle apparently maintain functional differentiation of a relatively growth-quiescent epithelium (35, 36). In the normal prostate, the direct action of androgens on the epithelial AR predominantly regulates the expression of secretory proteins (37). Androgens do not elicit the proliferation of normal adult prostatic epithelium in intact animals, but rather act to maintain a growth-quiescent functional prostatic epithelium *via* regulation of processes

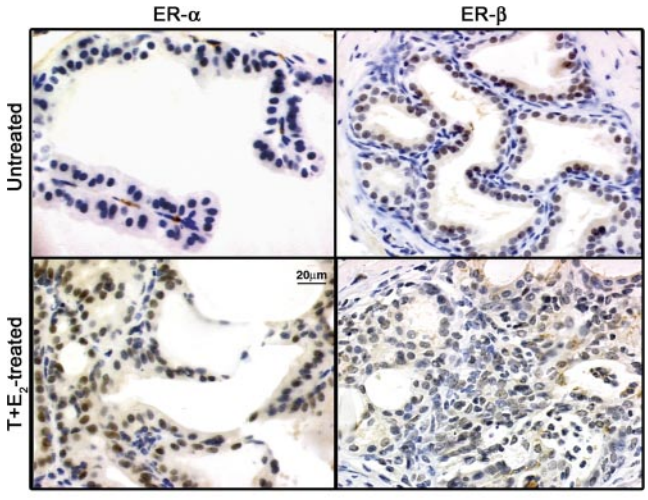

Figure 4. Presence of ERs in normal and neoplastic mouse prostates. In normal (control untreated) mouse prostates, ERα is restricted primarily to the prostatic stroma, whereas ERβ is localized to the epithelium. During prostate cancer (PRCA) progression in T + E_2-treated mice, ERα is dramatically increased within prostatic epithelia, whereas ERβ is considerably decreased within the epithelia.

TABLE 1. *Incidence of hormone-induced prostate pathologies is reduced in ArKO mice*

	VP		LP		DP		AP	
Pathology	WT	ArKO	WT	ArKO	WT	ArKO	WT	ArKO
Hyperplasia	100	100	100	100	100	100	67	44
Atypical hyperplasia	83	38*	100	60*	55	30	17	17
PIN	50	23*	90	50*	45	20	17	17

Values represent percentages; $*P < 0.05$.

such as apoptosis (38). Therefore, the role of androgens in T + E_2-treated mice appears to be to prevent prostatic atrophy and regression and preserve a glandular phenotype that otherwise would undergo squamous metaplasia if E_2 was unopposed by T. Thus, androgens may have a relatively passive role in the hormonal induction of carcinogenesis, while estrogens may play a critical role.

Evidence indicates that extremely high doses of estrogens, such as DES and E_2, may be genotoxic due to the generation of free radicals and epoxide derivatives that may be capable of chemical modification of DNA (39–41). Such events would presumably act in a random manner to genetically damage cells in androgen and E_2 target tissues such as prostate and mammary gland. This may explain the observation of focal tumors surrounded by phenotypically normal tissues within target organs following T + E_2 treatment rather than a homogeneous tissue-wide response. However, exposure to elevated androgen levels is not directly associated with DNA damage but is commonly associated with proliferative responses within the prostatic epithelium that result in pathologies such as benign prostatic hyperplasia. Indeed, prostates of ArKO mice, which maintain elevated levels of androgens in the absence of E_2 synthesis, or mice overexpressing AR demonstrate prostatic hyperplasia throughout the prostate (25, 42). Similarly, when androgen levels were increased by the T + E_2 treatment, all mice showed hyperplastic responses, irrespective of their ER status. Thus, it is possible that the observed hyperplasia may be a pathological consequence due solely to androgens rather than caused by the combined effects of T and E_2.

The linking of alterations in the ratio of estrogens to androgens in humans with prostate cancer and animal models, such as the Noble rat, implicates the role of E_2 in prostate malignancy (12, 18). Using three different transgenic mouse models, ArKO, αERKO, and βERKO,

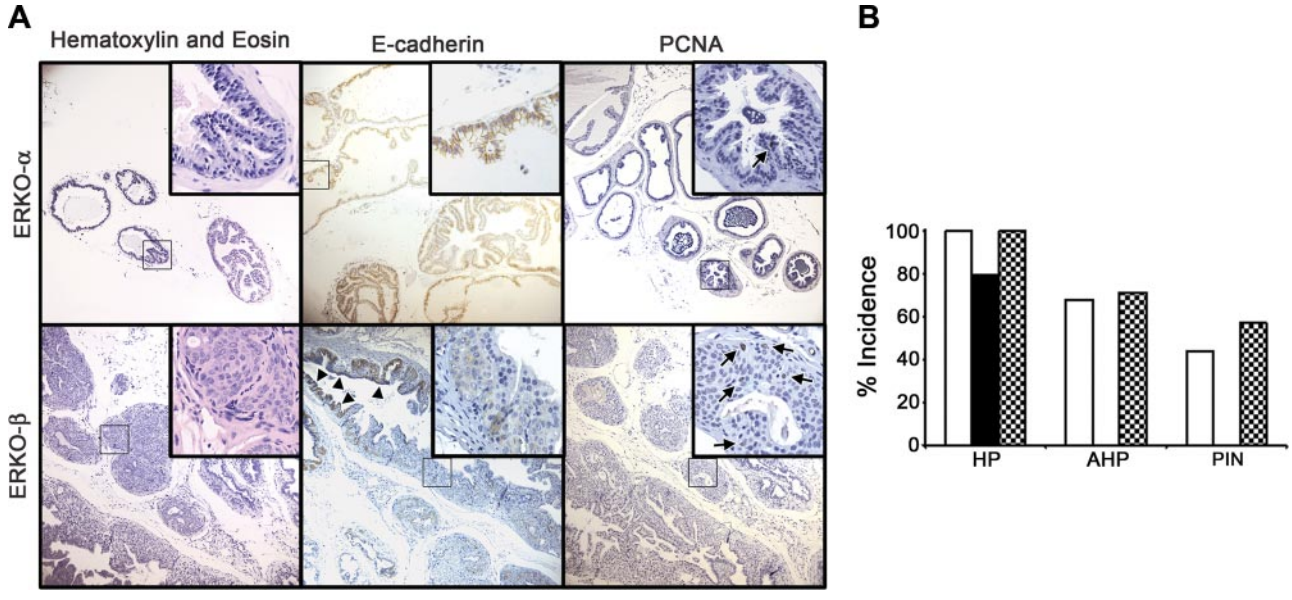

Figure 5. Estrogen mediates PRCA progression *via* ERα. To determine the mode of estrogen action in PRCA progression, ER-knockout mice (αERKO and βERKO) were hormonally implanted (T+E_2, 4 months) and evaluated. *A*) H&E analysis demonstrated that T + E_2-treated αERKO mice did not develop atypical hyperplasia or PIN, whereas βERKO mice exposed to exogenous hormones developed PIN. Hyperplasia, characterized by multilayered prostatic epithelium, was observed in all mice regardless of genetic background in response to T + E_2. In normal and hyperplastic areas epithelial cells were well organized and expressed E-cadherin (arrow heads) along the cell membranes. However, in areas of PIN, E-cadherin localization was diffuse, cytoplasmic, or completely lost. PCNA-positive cells (arrows) were rare in prostates collected from hormone-implanted αERKO mice but were abundant in epithelial cells of hormone-treated βERKO mice. *B*) Incidence of prostatic hyperplasia (HP), atypical hyperplasia (AHP), and PIN in mice treated with T + E_2 for 4 months. Open = WT; solid = αERKO; checkered = βERKO.

the role of E_2 in carcinogenesis was investigated. In ArKO mice, the absence of aromatase activity, and thus systemic and local estrogen production, results in a reduced sensitivity to hormonal induction of carcinogenesis compared to WT mice. Prior to these studies, the source of E_2 involved in prostatic hormonal carcinogenesis was thought to be solely provided *via* the E_2 implant. It is now clear that *in situ* E_2 production (most likely local) can have a profound effect on carcinogenesis. This may be clinically important because it suggests that local (prostatic synthesized) E_2 plays a central role in the etiology of PRCA. A number of clinically relevant inhibitors of estrogen synthesis or aromatase inhibitors exist; targeting these therapies to the prostate may enhance the efficacy of antiestrogenic SERMs. The decreased incidence of atypical hyperplasia and PIN was observed in all prostate lobes, with the greatest significant difference seen in the VP, which is considered the most androgen-responsive tissue. In contrast, the AP, which is considered the most E_2-sensitive prostatic lobe in rats, showed no difference in incidence of atypical hyperplasia or PIN compared to controls (20). Interestingly, ArKO LPs and DPs showed intermediate responses to T + E_2 and cancer incidence. This differential response of ArKO prostate lobes suggests that altering local production of E_2 may have significant consequences in an unbalanced hormonal environment.

The necessity of investigating both systemic and local *in situ* production of E_2 is important for determining the source of E_2. However, since receptor signaling is mediated *via* prostatic ERα and ERβ it was important to determine the mechanism of how estrogen elicited its carcinogenic effect. Therefore, αERKO and βERKO mice were used to dissect the mechanism of estrogenic action involved in hormonal carcinogenesis. Interestingly, mice lacking ERα but not ERβ showed a complete absence of the induction of carcinogenesis following T + E_2 treatment, although they did develop prostatic hyperplasia. Whether hyperplasias in αERKO mice were due to the effects of T/androgen receptor signaling or E2/ERβ signaling are not clear. As ERβ is still present in the αERKO prostate, this strongly implicates E_2 and its effects *via* ERα for the hormonal induction of carcinogenesis. Identification of ERα as the key mediator of E_2 action is further supported in hormone-treated βERKO mice, which lack ERβ but retain ERα, but show clear evidence of carcinogenesis both histopathologically and biochemically (*e.g.*, diffuse E-cad expression). This outcome suggests that ERα and ERβ signaling are responsible for mediating different cellular responses to E_2. For example, a recent report has demonstrated that ERβ signaling results in suppression of proliferative response within the prostatic epithelium (24). Furthermore, squamous metaplasia (11) and prostatic dysplasia caused by neonatal exposure to estrogens (43) have been shown to act *via* ERα and thus may share similar downstream pathways involved in carcinogenesis.

When administered to adult male animals, estrogens act locally on the prostate to induce rapid epithelial cell proliferation, resulting in squamous metaplasia, and act systemically to chemically castrate the animal (5, 6). High titers of sex steroids have a genotoxic potential, which may result in the initiation of prostatic carcinogenesis (39–41). However, carcinogenesis requires a collaboration between both androgens and estrogens (17, 19); furthermore, the nonaromatizable androgen DHT in combination with E_2 is not sufficient to induce carcinogenesis (14, 44, 45). The observation that ArKO mice are significantly less likely to develop malignancy is important, because this suggests that local estrogen production on top of an elevated circulating E_2 level is crucial for initiation and progression. Therefore, inhibition of local *in situ* production of E_2 by aromatase inhibitors might prove chemopreventative for prostatic carcinogenesis.

In the past decade, more than 2 million men were diagnosed with prostate cancer in the United States alone. Therefore, the continued development and testing of new models of early stages of prostate carcinogenesis will enhance the potential identification of the mechanisms involved in initiation and progression of prostate cancer and allow development and testing of new therapies for this disease. The importance of estrogen in hormonal carcinogenesis is underscored by the studies performed herein, and their relevance to human disease is supported by previous studies in which T + E_2 induced nontumorigenic human prostatic epithelial cells to undergo malignant transformation and, more importantly, by recent phase IIb clinical trials that successfully used Toremifene, a selective ERα modulator, as a chemopreventative agent (23). Understanding the mechanisms associated with antiestrogen treatment may lead to the prevention and or cure for this dreaded disease.

This investigation presents evidence of hormonal induction of carcinogenesis in the mouse prostate using combinatorial T + E_2 treatment and identifies altered susceptibilities of different strains of mice. The utility of this model for cancer researchers lies in the ability to incorporate mouse genetics to dissect the mechanisms involved in hormonal carcinogenesis. We demonstrated this point through the use of genetically modified mice lacking aromatase, ERα, and ERβ. We examined the specific role of *in situ* production of E_2 in hormonal carcinogenesis and came to an overall conclusion that locally produced E_2 plays an important role in T and E_2 induced prostatic carcinogenesis. Normally, in a balanced hormonal environment, androgens act on the adult prostate to maintain a differentiated, relatively growth-quiescent state (46). However, in the presence of elevated aromatizable androgens, prostatic hyperplasia can occur, presumably in synergy with locally produced E_2. We further conclude that local estrogens mediate their adverse effects *via* signaling through ERα to promote carcinogenesis and provide a basis for testing new-generation antiestrogens and SERMs as treatments for prostate cancer (47). Although complete loss of ERα prevents hormonal carci-

nogenesis, it is unclear if stromal or epithelial, or both stromal and epithelial ERα are responsible for the estrogenic mediation of carcinogenesis. Future research will evaluate the roles of stromal *vs.* epithelial ERα in prostatic carcinogenesis. FJ

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