Estrogen Imprinting of the Developing Prostate Gland Is Mediated through Stromal Estrogen Receptor α: Studies with αERKO and βERKO Mice

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ABSTRACT

Neonatal exposure of rodents to high doses of estrogen permanently implants the growth and function of the prostate and predisposes this gland to hyperplasia and severe dysplasia analogous to prostatic intraepithelial neoplasia with aging. Because the rodent prostate gland expresses estrogen receptor (ER)–α within a subpopulation of stromal cells and ERβ within epithelial cells, the present study was undertaken to determine the specific ER(s) involved in mediating prostatic developmental estrogenization. Wild-type (WT) mice, homozygous mutant ER (ERKO) α–/– mice, and βERKO β–/– mice were injected with 2 μg of diethylstilbestrol (DES) or oil (controls) on days 1, 3, and 5 of life. Reproductive tracts were excised on days 5 or 10 (prepubertal), day 30 (pubertal), day 90 (young adult), or with aging at 6, 12, and 18 months of age. Prostate complexes were microdissected and examined histologically for prostatic lesions and markers of estrogenization. Immunocytochemistry was used to examine expression of androgen receptor, ERα, ERβ, cytokeratin 14 (basal cells), cytokeratin 18 (luminal cells), and drosolarial protein over time in the treated mice. In WT-DES mice, developmental estrogenization of the prostate was observed at all of the time points as compared with WT-oil mice. These prostatic implants included transient up-regulation of ERα, down-regulation of androgen receptor, decreased ERβ levels in adult prostate epithelium, lack of DLP secretory protein, and a continuous layer of basal cells lining the ducts. With aging, epithelial dysplasia and inflammatory cell infiltrate were observed in the ventral and dorsolateral prostate lobes. In contrast, the prostates of αERKO mice exhibited no response to neonatal DES either immediately after exposure or throughout life up to 18 months of age. Furthermore, neonatal DES treatment of βERKO mice resulted in a prostatic response similar to that observed in WT animals. The present findings indicate that ERα is the dominant ER form mediating the developmental estrogenization of the prostate gland. If epithelial ERβ is involved in some component of estrogen imprinting, its role would be considered minor and would require the presence of ERα expression in the prostatic stromal cells.

INTRODUCTION

The rodent prostate gland is rudimentary at birth and undergoes extensive branching morphogenesis followed by functional differentiation during the first 15 days of life (1–3). Brief exposure of male rats or mice to high levels of estrogens during the neonatal period leads to permanent alterations in growth and function of the prostate gland and a reduced responsiveness to androgens during adulthood (4–7). This process, referred to as neonatal imprinting or developmental estrogenization, is associated with an increased incidence of prostatic lesions with aging, which include extensive immune cell infiltrate and epithelial cell hyperplasia and severe dysplasia similar to high grade prostatic intraepithelial neoplasia (8, 9). Thus, neonatal estrogenization of the rodent has evolved as a useful model for evaluating the role of endogenous and exogenous estrogens as a predisposing factor for prostatic tumor formation later in life (10–12). This time frame is distinct from the human, in which prostate morphogenesis occurs entirely during the fetal period (13–15). Whereas prostate morphogenesis is driven by testosterone produced by the fetal testes (16), there is clear evidence that maternal estrogens have a direct effect on the human prostate epithelium during fetal life as well (17, 18). In addition, maternal exposure to pharmacological levels of DES has been shown to induce prostatic abnormalities in human offspring (19). Consequently, it has been proposed that excessive estrogenization during prostatic development may contribute to the high incidence of benign prostatic hyperplasia and prostatic carcinoma currently observed in the aging male population (12).

A fundamental understanding of developmental estrogenization of the prostate requires knowledge of the immediate cellular and molecular changes induced by estrogens that, in turn, alter the course of prostatic development long after the withdrawal of estrogens. Toward that end, we have characterized previously several immediate and long-term prostatic alterations induced by neonatal estrogen exposure in the rat prostate model. Exposure to high levels of estrogens during the developmental critical period (days 1–5) initially blocks epithelial cells from entering a normal differentiation pathway (3, 20) resulting in permanent differentiation defects (7, 21, 22). Histologically, early estrogen exposure suppresses the formation of the distal prostate and extends the proximal phenotype into a larger portion of the tissue (23). The proximalized phenotype of the estrogenized prostate is characterized by a thick layer of periductal fibroblasts beneath the basement membrane and a continuous layer of basal epithelial cells between the basement membrane and the luminal cells (3, 22), which together may impede ductal branching and cell-cell interactions that are essential for normal morphogenesis. Because we have shown recently that neonatal estrogens alter box-13 gene expression in the prostate (23), we postulate that the effects of estrogen in creating a proximalized phenotype in the prostate gland may reflect a change in the positional identity of this structure, perhaps mediated by changes in the expression of distinct developmental genes.

The molecular mechanism whereby neonatal estrogen transmits its effects on the prostate gland is not completely understood. Although some of the effects of estrogen may be indirectly mediated through the hypothalamic-pituitary-testicular axis (24, 25), a direct response at the level of the prostate has also been documented (6, 26). Estrogen action is mediated through two distinct members of the nuclear steroid receptor gene superfamily, which possess high affinity for estrogenic ligands, namely, ERα (27) and β (28). Both ERα and β are expressed in the rodent prostate gland; thus, either one or both ERs could potentially mediate the effects of estrogens on this tissue. In the normal developing rodent prostate, ERα expression is confined to mesenchymal cells in the proximal region of the gland and is not present in the epithelial cells (29, 30). Whereas ERβ appears to be highly expressed in the epithelial cells of the adult prostate gland (28).
31), its expression in the undifferentiated epithelium of the neonatal prostate is relatively low (32). The different cellular localization of ERα and β in the prostate gland is particularly significant because action through ERα would imply a stromal-mediated estrogenization of the prostate, whereas action through ERβ would indicate an epithelial cell pathway for the initiation of the estrogenized phenotype. Although neonatal exposure to estrogens does not initially alter ERβ mRNA expression in the rat prostate (32), there is an immediate up-regulation of ERα mRNA and protein within periductal stromal cells along the length of the developing ducts, which allows for amplification of estrogenic signals in these stromal cells (29). Evidence that this transiently up-regulated ERα is functional comes from a concomitant, transient up-regulation of progesterone receptor, an estrogen regulated gene, in the same periductal cells (33). Whereas these findings implicate a specific role for ERα in the developmental estrogenization of the prostate gland, it is nonetheless possible that ERβ is also involved in this phenomenon.

The recent generation of transgenic mice with targeted deletion of the ERα (αERKO; Refs. 34, 35) or the ERβ (βERKO) gene (36) allows for the direct determination of the specific ER(s) involved in mediating the neonatal estrogen imprint on the prostate gland. In the present study, WT, αERKO and βERKO mice were administered DES on postnatal days 1–5, and prostatic cellular and molecular markers for estrogenization were monitored from day 5 through 18 months of age. The WT mice given neonatal DES exhibited an estrogenized phenotype similar to that characterized previously for rats. We envisioned three potential outcomes for the knockout mice. The lack of a DES effect in ERKO mice lacking either ERα or β would implicate the involvement of that specific ER in mediating the estrogen effect. Conversely, developmental estrogenization after neonatal DES in an αERKO or βERKO model would indicate the lack of involvement of that ER in estrogen imprinting. Alternatively, a partial DES effect in the ERKO models would suggest that both molecules may be involved in mediating estrogen action in the developing prostate. Our findings provide strong evidence that stromal ERα is the dominant ER form mediating the effects of early estrogen exposure on the prostate gland.

### MATERIALS AND METHODS

#### Animals

All of the procedures involving animals were performed under an approved animal protocol in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The generation of C57BL/6J/L29 black mice homozygous for the ERα gene (αERKO) and the ERβ gene (βERKO) have been described previously (34, 36). All of the mice for the present study were generated by breeding male and female mice heterozygous for the ERα gene or the ERβ gene to produce homozygous ERα−/− (αERKO) or ERβ−/− (βERKO) null male offspring. Genotyping of tail DNA was accomplished using PCR with primers, which spanned the site of the disrupted gene as described previously (34, 36).

Pregnant mice were monitored for the day of delivery, which was considered day 0. Pups from each litter were randomly assigned to one of two treatment groups and administered either 2 μg of DES in corn oil or vehicle alone daily on postnatal days 1–5 via injection at the nape of the neck. Male mice were killed by decapitation on postnatal day 5 or 10 (prepubertal), day 30 (pubertal), day 90 (young adult), or at 6, 12, and 18 months of age (aged). Reproductive tracts were immediately removed by gross dissection, and the seminal vesicles were separated and weighed. The remainder of the reproductive tract was placed in Bouin’s fixative for 48 h and transferred to 70% ethanol. Under a dissecting microscope, the entire prostatic complex was microdissected from adjacent organs. After dehydration in increasing concentrations of ethanol and clearance in xylene, the prostate complex was embedded in paraffin along a singular axis to allow simultaneous sectioning through the ventral and dorsolateral lobes on a single tissue section. This approach allowed for orientation of the prostatic ducts relative to the urethral openings and, thus, verification of distal, central, and proximal duct location.

#### Immunocytochemistry

Immunocytochemistry was performed according to methods published previously (37) with modifications for paraffin-embedded tissues. Briefly, 4-μm paraffin sections were mounted on Superfrost Plus glass slides (Fisher Scientific, Itasca, IL) and heated at 37°C overnight. The sections were deparaffinized in xylene, gradually hydrated with decreasing concentrations of ethanol, and subjected to antigen retrieval. Slides were immersed in 0.1 M citrate buffer (pH 6.0; for AR, ERα, CK14, CK18, and DLP) or in 0.05 M glycine and 0.01% EDTA (pH 3.5; for ERβ) and heated for 30 min in a Decloaking Chamber (Biocare Medical, Walnut Creek, CA). After a 10-min cooling period, the slides were slowly rinsed in running deionized water and endogenous peroxidases were removed with 3% H2O2 for 10 min. The slides were incubated with appropriate 2% blocking serum or SuperBlock blocking buffer (Pierce, Rockford, IL) for 30 min at room temperature and were subsequently incubated overnight at 4°C with primary antibody. The specific antibodies, sources, and concentrations used are presented in Table 1. As a negative control, normal rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) was substituted for primary antibody on separate sections of all of the tissues analyzed to determine nonspecific binding. The primary antibody was reacted with a species-appropriate biotinylated secondary antibody, and the biotin was detected with an avidin-biotin peroxidase kit (ABC-Elite; Vector Laboratories) using diaminobenzidine tetrachloride as a chromogen. As a final step, the sections were dehydrated gradually with alcohol, cleared with xylene, and coverslipped with Permount (Fisher Scientific). Some sections were stained with Gill’s #3 hematoxylin (1:4) as a blue nuclear counterstain. In addition to immunocytochemistry, sections from all of the time points and treatment groups were stained with H&E using standard technique to allow for examination of histological details.

The different regions of the resultant immunostained ducts were classified as either proximal, central, or distal depending on their distance from the urethra (38). Intensity of staining for each antibody-antigen reaction was subjectively characterized as negative, weak, moderate, or strong. For comparative studies with a single antigen, tissues from all of the time points for control and estrogenized WT, αERKO, and βERKO mice were always run in the same immunocytochemical assay to reduce discrepancies related to inter-assay variability in staining intensity. In many instances, the oil control prostate and the DES-exposed prostate from a given genotype and time point were processed on the same glass slide to allow for direct comparison of immunostain intensity between treatment groups. Images were digitally obtained with a Spot RT camera (Diagnostic Instruments, Inc.) attached to a Nikon E-400 microscope using a ×40 objective and ImagePro software. Photographic plates were assembled with Adobe PhotoShop software.

#### Statistical Analysis

All of the data sets were first tested for homoscedasticity of variance using the Levine’s test. In cases where data were found to lack homoscedasticity of variance, all of the data were log-transformed before additional statistical analysis. In all of the cases, data sets were analyzed by one-way ANOVA followed by individual post-hoc comparisons. Statistical analysis was carried out using the interactive statistical internet websites of the physics department of the College of Saint Benedict/St. John’s University4 and GraphPad2.

### Table 1 Various antibodies used for immunocytochemistry

<table>
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<tr>
<th>Antibody</th>
<th>Catalog no.</th>
<th>Animal source</th>
<th>Source</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
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<td>PG21</td>
<td>rabbit IgG</td>
<td>G. S. Prins (37)</td>
<td>2 μg/ml</td>
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<tr>
<td>anti-ERα</td>
<td>ER21</td>
<td>rabbit IgG</td>
<td>G. Greene (29)</td>
<td>5 μg/ml</td>
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<tr>
<td>anti-ERβ</td>
<td>CFK-E12</td>
<td>mouse IgG</td>
<td>B. Katzenellenbogen (42)</td>
<td>10 μg/ml</td>
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<tr>
<td>anti-C1-14</td>
<td>PRB-155P</td>
<td>rabbit</td>
<td>Covance Research Products, Inc, Richmond, CA</td>
<td>1:40,000</td>
</tr>
<tr>
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<td>PH504</td>
<td>sheep</td>
<td>The Binding Site Inc. San Diego, CA</td>
<td>1:800</td>
</tr>
<tr>
<td>anti-DLP</td>
<td>protein</td>
<td>rabbit</td>
<td>G. Cunha (48)</td>
<td>1:40,000</td>
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RESULTS

**Seminal Vesicle Weights.** To allow for optimal tissue and antigen preservation for the present studies, whole prostatic complexes were immediately fixed after removal from the animals, which precluded determination of prostate lobe weights. Thus, paired seminal vesicles were weighed in the WT and oERKO males to serve as a surrogate marker for neonatal DES effects on accessory sex gland growth. Previous studies have shown that similar to the prostate, the seminal vesicles are sensitive to high-dose neonatal estrogen exposures (39, 40). Fig. 1 shows the effects of neonatal DES treatment on seminal vesicle size in WT and oERKO mice at 4, 8, 12, and 18 months of age. At all ages, neonatal exposure of WT mice to 2 μg of DES on days 1–5 resulted in a significant reduction in seminal vesicle size as compared with oil-treated counterparts. In contrast, there was no difference in seminal vesicle weights between DES and oil-treated oERKO mice at each time point. It was observed that the seminal vesicles of oERKO mice exhibited a marked age-related increase in size as compared with WT males at each time point. This sustained seminal vesicle growth over time is thought to be a result of elevated circulating testosterone levels present in the oERKO male mice as compared with the WT animals (35).

**Histology.** The estrogenized phenotype of prostate glands treated neonatally with estradiol is best discerned in the aged animal where progressive epithelial hyperplasia and dysplasia and immune cell infiltrate is characteristically observed (9). Fig. 2 presents representative histology sections of ventral and dorsolateral prostates of aged WT, oERKO, and βERKO mice that had been neonatally treated with oil or 2 μg of DES on days 1–5 of life. The ventral prostate ducts of WT mice at 6 months and 1 year of age are characterized by a single cell layer of cuboidal to low cubular epithelial cells in the central ductal regions (Fig. 2, A and E) with infolding of this epithelial layer in the distal tips. The dorsolateral prostates of WT mice contain acini with marked infolding of the single-cell epithelial layer, which produces a complex-appearing structure throughout a typical cross-section of the tissue (Fig. 2, C and G). Neonatal exposure to DES resulted in significant histological alterations in the aged ventral and dorsolateral prostates of WT mice, which include a relative increase in stromal mass, large pockets of interstitial lymphocytic infiltrate, marked epithelial cell hyperplasia, and dysplasia characterized by nuclear enlargement, variable nuclear size, and hyperchromasia (Fig. 2, B, D, F, and H). The severity of these prostatic lesions increased with age in the WT animals and were most severe at 18 months of age. Unlike the rat prostate where lobe-specific differences were observed, the ventral and dorsolateral lobes appeared equally affected by neonatal estrogen exposure in the WT mice.

Ventral and dorsolateral prostates from oERKO mice appeared histologically identical to WT oil-control prostates at all of the time points including 1 year of age (Fig. 2, I and K). In contrast to the WT mice, neonatal exposure to DES had no effect on the histological appearance of the aged ventral or dorsolateral lobes in oERKO mice (Fig. 2, J and L). Prostates from oil-treated βERKO mice also appeared similar to oil-control WT and oERKO mice at 6 months of age with no evidence of epithelial hyperplasia when similar regions of the ducts and lobes were directly compared (Fig. 2, M and O). However, in contrast to the oERKO mice, prostates from βERKO mice neonatally exposed to DES exhibited all of the histological identifiers of an estrogenized prostate including increased stromal cells, lymphocytic infiltrate, epithelial hyperplasia, and dysplastic foci at 6 months of age (Fig. 2, N and P). One noted difference was that the dorsolateral prostates of βERKO mice appeared consistently more affected than the ventral lobe.

**Epithelial Cell Differentiation.** To better characterize differentiation defects induced by neonatal estrogen exposure and to determine the comparative extent of the DES effect in the different ERKO models, differentiation markers were used histochemically to identify basal cells (CK14), luminal cells (CK18) and DLP, the major secretory product of the mouse dorsolateral prostate and a marker of functional differentiation. The typical prostate of the adult WT mouse contains a limited number of basal epithelial cells, which are intermittently located along the basement membrane (Fig. 3A, arrowheads), differentiated luminal epithelial cells, which line the ducts in a single cell layer (Fig. 3B), and strong epithelial DLP stain in the dorsolateral prostate (Fig. 3C). Neonatal exposure to DES resulted in prostates with epithelial ducts and acini lined by a continuous layer of basal cells (Fig. 3D), extensive piling and hyperplasia of luminal cells (Fig. 3E, arrows), and loss of DLP expression in the dorsolateral lobe (Fig. 3F). In addition, a thick periductal layer of stromal cells was apparent along the lengths of the ducts (Fig. 3E, arrowheads). These hallmark indicators of estrogen imprinting were not evident in the oERKO mice where both oil and DES-treated prostates contained few basal cells (Fig. 2, G and J, arrows), a single cell layer of luminal cells (Fig. 3, H and K), and strong immunostain for DLP in the dorsolateral lobe (Fig. 3, I and L). Ventral prostates from day 90 oil-control βERKO mice were similar to wild type and oERKO controls with occasional basal cells (Fig. 3M, arrow) and a single cell layer of differentiated luminal cells (Fig. 3N). Similarly, the day 90 dorsolateral prostate was strongly stained for DLP protein (Fig. 3O). Neonatal exposure of βERKO mice to DES resulted in estrogen imprinting of prostatic epithelial differentiation as characterized by a continuous layer of basal cells along the basement membrane (Fig. 3P), extensive hyperplasia of the luminal cells (Fig. 3P–R, arrowheads), stromal lymphocytic infiltrate (Fig. 3, P and Q, arrows), and loss of DLP expression in the dorsolateral prostate (Fig. 3R). Also notable in the estrogen-exposed day 10 βERKO prostate was a thick periductal layer of fibroblasts and smooth muscle cells along the ductal length, which is an early marker of the estrogenized proximal phenotype (Fig. 4J, arrowheads).

**AR.** Previous work with the rat model has shown that an early and permanent effect of neonatal estrogen exposure is down-regulation of
AR in prostatic epithelial and stromal cells in a lobe-specific manner (6, 41). Thus, we have hypothesized that developmental estrogenization is attributable, in part, to the lack of appropriate androgenic signaling during the developmental critical period, which normally drives prostatic morphogenesis and differentiation. To determine whether similar mechanisms are operative in the murine model and to determine which ER was required for this AR down-regulation, we examined prostatic AR protein by immunocytochemistry at day 10 and day 90 in the WT, /H9251ERKO, and /H9252ERKO mice. At day 10, AR is strongly localized to the nucleus of periductal smooth muscle cells, whereas the epithelial cells stain at a moderate staining intensity in the WT mouse prostate (Fig. 4A). Treatment with DES from days 1 to 5 eliminated epithelial AR and severely reduced stromal cell AR labeling to a low stain intensity at day 10 (Fig. 4B). In the day 90 (adult) WT mouse prostate, epithelial cell nuclei and periductal smooth muscle cells were strongly AR-positive in oil-treated prostate (Fig. 4C), and AR down-regulation attributable to neonatal DES treatment persisted (Fig. 4D). AR immunostain profiles in the oil-control /H9251ERKO mice were indistinguishable from WT prostates at both day 10 and day 90 (Fig. 4E and G), and neonatal exposure of /H9251ERKO mice to DES had no effect on prostatic AR expression in the developing or the adult prostate gland (Fig. 4F and H). The day 10 and adult prostates of oil-treated /H9252ERKO mice expressed AR within stromal and epithelial cells similar to the WT animals (Fig. 4I and K). Neonatal exposure of /H9252ERKO mice expressed epithelial hyperplasia in the ventral lobe (N, short arrows) and dysplasia in the dorsolateral prostate (P, short arrows) at 6 months. Immune cells were present in lumens and stromal regions of DES-exposed /H9252ERKO prostates (N, P, long arrows), and stromal cells was increased as compared with oil-treated controls (M, O).
10 WT, αERKO, and βERKO mice. At day 5, ERα localized to a small number of mesenchymal cells in the proximal aspect of the ventral and dorsolateral prostate in the WT mice (Fig. 5A). Similar to rats, mesenchymal ERα was markedly up-regulated by neonatal DES exposure (Fig. 5B). This autoregulation of ERα was transient, because by day 30, prostates from WT DES-treated mice contained few ERα-positive cells. Similar to the WT prostate, ERα localized to the periductal stromal cells in the proximal prostatic region of day 10 βERKO mice (Fig. 5C). After DES treatment from days 1 to 5, the number of periducal stromal cells positive for ERα was greater in the βERKO mouse prostate as compared with oil controls (Fig. 5D). As expected, there was no specific ERα immunostain in the αERKO mouse prostate (Fig. 5E). It is important to note that ERα immunostain was not observed in the epithelial cells of the ventral or dorsolateral lobes in any of the mice in this study.

Immunolocalization of ERβ in Bouin’s fixed, paraffin-embedded tissue proved to be difficult with no less than 10 available antibodies from a variety of sources providing negative or nonspecific results.

Fig. 3. Differentiation markers of day 90 prostates of WT, αERKO, and βERKO mice that had been neonatally exposed to 2 μg DES or OIL. Left column, representative ventral lobe sections immunostained for CK14 as a marker of basal cells (A, D, G, J, M, P), whereas middle column shows ventral lobes immunostained for CK18 as a luminal cell marker (B, E, H, K, N, Q). Right column, representative dorsolateral lobe sections immunostained for DLP2, a major secretory product of that prostatic region and a marker of functional differentiation (C, F, I, L, O, R). See text for discussion. Note presence of leukocytes in ventral prostate lumens of WT DES mice (D arrow, E) and infiltrating lymphocytes in ventral lobes of βERKO-DES mice (P, Q, arrows).
With the use of a newly developed mouse monoclonal antibody against the human ER\(_{\beta}\) ligand-binding domain (CKF-E12, Ref. 42), a positive, specific, and competitive immunostain for ER\(_{\beta}\) was obtained in epithelial cell nuclei of adult mouse prostate sections using antigen retrieval methods under acidic conditions. As expected, the prostate of DES-exposed ER\(_{\alpha}\) mice was negative for ER\(_{\beta}\) at day 10 (Fig. 5F). Similarly, using the same conditions that provided positive results in the adult mouse prostate and ovary, we found no detectable ER\(_{\beta}\) protein in the neonatal prostate of WT mice treated with oil or DES (Fig. 5G and H). In contrast, nuclear immunostain was present at moderate-to-strong stain intensity within the luminal epithelial cells of the adult WT mouse ventral and dorsolateral prostate lobes (Fig. 5I). Treatment of WT mice with neonatal DES resulted in a significant reduction of nuclear ER\(_{\beta}\) throughout the epithelium of all prostate regions in the adult animals (Fig. 5J). In adult ER\(_{\alpha}\) mice, ER\(_{\beta}\) staining and localization was similar to the WT mouse (Fig. 5K) indicating that ER\(_{\alpha}\) is not required for normal ER\(_{\beta}\) expression within the prostate. However, in contrast to the WT mice, the ventral and dorsolateral prostate glands from DES-treated ER\(_{\beta}\) mice also exhibited strong nuclear immunostain for ER\(_{\beta}\) (Fig. 5L). As expected, there was no ER\(_{\beta}\) immunostain in the adult ER\(_{\beta}\) prostate glands with the use of the CFK-E12 antibody.

**DISCUSSION**

The present findings provide clear evidence that ER\(_{\alpha}\) is the dominant ER mediating developmental estrogenization of the rodent prostate gland after neonatal exposure to estrogens. The prostate glands of the WT C57BL/6J/129 black mice used in the present study exhibited...
all of the hallmarks of neonatal estrogen imprinting that have been characterized previously by this laboratory in the rat model as well as by others in different mouse strains (9, 11, 43). These features included a disruption in epithelial cell differentiation as evidenced by loss of its major secretory product as well as a proximalized phenotype characterized by an increased thickness of periductal stromal cells and a continuous layer of basal cells along the length of the ducts. Additionally, adult seminal vesicle weights were markedly smaller in estrogenized mice indicating that accessory sex gland growth was affected. Pathological sequella of neonatal estrogen exposure were evident in the adult WT mouse prostate and included extensive hyperplasia of the epithelium in the young adult, progression to epithelial dysplasia with aging, and severe inflammatory cell infiltrate. Similar to previous findings in the rat model (3, 6, 32), neonatal estrogens induced a down-regulation in epithelial cell AR and ERβ, which, themselves, are considered markers of a differentiated prostatic epithelium. Thus, it is reasonable to assume that similar molecular pathways are mediating the developmental estrogenization involved in the establishment of a prostatic phenotype.

Fig. 5. ERs in neonatal and adult mouse prostates. Neonatal ventral prostates were immunostained for ERα (A–E) and ERβ (F–H), whereas adult dorsolateral lobes were immunostained for ERβ (I–L). Day 5 prostates from WT mice contained scattered stromal cells in proximal region with positive ERα nuclei (A, arrow), whereas epithelial cell nuclei were ER negative. Exposure to DES resulted in a marked increase in number of ERα-positive stromal cells (B, arrows) in WT mice. Day 10 βERKO mice contained moderate ERα immunostain in periductal stromal cells in proximal prostate (C, arrows), and neonatal exposure to DES increased number of ERα-positive stromal cells in that region (D, arrows). Day 5 αERKO prostates were negative for ERα (E). Inset is normal rabbit IgG on an adjacent section to show background stain. Day 10 βERKO prostate was negative for ERβ (F, mouse IgG in inset shows background stain). In contrast to ERα, ERβ was not visualized in neonatal prostates from WT mice given either oil (G) or DES neonatally (H, mouse IgG in inset shows background stain). ERβ was present in epithelial cell nuclei (I, arrow) of adult prostates of WT mice (dorsolateral lobe shown in I). Neonatal exposure to DES resulted in a marked reduction of epithelial ERβ in WT prostates (J, arrow; mouse IgG in inset shows background stain). Adult prostates from nERKO mice were positive for ERβ in epithelial cell nuclei (K, arrow; mouse IgG in inset shows background stain) and this was not affected by neonatal exposure to DES (L, arrow; mouse IgG in inset).
of the prostate in both the rat and the mouse and that these responses are fully present in the C57BL/6J/129 black strain used to generate the knockout mice. Using this extensive panel of markers for developmental estrogenization of the prostate, we observed no evidence of estrogen imprinting in the αERKO mice either immediately after the estrogenic exposure (days 5–10) or throughout life up to 18 months of age. In contrast, the βERKO mice exhibited estrogenization of the prostate gland in response to estrogenic exposure with both early effects on stromal cells, AR and ERα levels at day 10, and progressive epithelial and immune cell pathology with aging. Thus, these combined results indicate that, whereas ERα is essential, the presence of ERβ is not required for the mediation of estrogen imprinting in the developing prostate gland. A similar conclusion was reached concerning the adult prostatic response to 3 weeks of DES treatment where squamous metaplasia is induced in WT and βERKO mice but not in the prostates of αERKO animals (44). Thus, it appears that in both the developing and the adult prostate gland, estrogenic responses that lead to prostatic lesions are mediated through the ERα.

ERβ protein was undetectable by immunocytochemistry in the developing prostate glands of WT and αERKO mice, and treatment with DES between days 1 and 5 did not induce its expression at that time. Thus, unlike ERα (29), ERβ does not appear to be directly autoregulated by estrogen in the developing mouse prostate. Previous work with the neonatal rat prostate had shown the presence of ERβ mRNA in mesenchymal and undifferentiated epithelial cells at day 5 of life; however, the levels were extremely low in comparison with expression observed in the adult prostate epithelium (32). It is possible that either this low mRNA level is not translated at that time or that the translation product is below the limit of detection by histochemistry. Strong nuclear stain for ERβ was observed in adult prostatic epithelial cells in both WT as well as αERKO mice, which confirms previous reports that ERβ expression does not require ERα in reproductive tissues (45). In the adult WT mice treated neonatally with DES, a marked reduction in nuclear ERβ immunostain was observed in both the ventral and dorsolateral prostate lobes. Importantly, this effect was absent in the αERKO adult prostate of DES-exposed mice, which indicates that ERβ down-regulation is likely to be the result of events initiated through ERα in the neonatal prostate. These findings are similar to what we observed previously in the neonatally estrogenized ventral prostate of the Sprague Dawley rat (32). There, ERβ mRNA levels were not initially autoregulated by neonatal estradiol; however, they failed to exhibit the differentiation-associated increase in epithelial expression between days 10 and 90. At day 90, estrogenized rat ventral lobes contained little ERβ mRNA, whereas in the dorsal and lateral lobes, strong signal was observed in the epithelium. It is important to note that unlike the present findings in the WT mouse prostate where the lobes were equally affected by estrogen, in the rat model, neonatal estrogen exposure results in a lobespecific effect where the ventral lobe is highly estrogenized, the dorsal lobe shows a reduced effect, and the lateral lobe exhibits normal epithelial cell differentiation (6). Thus, ERβ expression directly correlates with differentiation status in the rat prostate epithelium. Together with the present findings, we hypothesize that ERβ in the rodent prostate is a marker of a differentiated epithelial cell, and loss of ERβ expression in the estrogenized prostate is a result of differentiation defects in the epithelium. Alternatively, it could be argued that it is the lack of normal ERβ expression in the epithelial cells after neonatal estrogenization that leads to the differentiation defects and, eventually, the adult onset of hyperplasia and dysplasia, which progress with aging. However, the presence of normally differentiated epithelial cells in the adult βERKO mice as evidenced both morphologically and by the presence of DLP secretory protein with no signs of prostatic hyperplasia and dysplasia with aging suggests that ERβ is not required for epithelial differentiation and argues against that explanation. Nonetheless, it remains possible that lack of functional ERβ in the adult prostate of estrogenized rodents contributes to the progression of prostatic dysplasia initiated through ERα during the neonatal period.

Similar to the WT mice, neonatal DES exposure of βERKO mice resulted in marked suppression of AR immunostain in prostatic epithelial and stromal cells at day 10. This effect is apparently mediated through ERα, because AR down-regulation does not occur in the αERKO mouse prostate after estrogen exposure on days 1–5. One noticeable difference between the estrogenized WT and βERKO mouse prostates was the transient nature of AR down-regulation in the βERKO prostates, because strong AR expression was observed within adult ventral lobe epithelial cells of neonatally estrogenized βERKO mice. We have hypothesized previously that estrogenization is attributable, in part, to loss of prostatic AR during the critical developmental window, and the present findings would continue to support that theory. However, the presence of AR in the adult ventral prostate epithelium of estrogenized βERKO mice could indicate that continued down-regulation of AR expression is not required for the progressive pathology, which occurs with aging in the estrogenized prostate gland. The mechanistic basis for continued AR expression in neonatally estrogenized adult prostates that lack ERβ is presently unclear. Although not quantitated in the present study, it was consistently noted that AR immunostain was stronger in the oil-control βERKO ventral prostate epithelial cells as compared with those in WT mice. The present findings support the proposal that ERβ may be involved in some component of AR regulation in prostate epithelial cells. Nonetheless, it is important to note that AR is down-regulated in the adult WT mouse prostate in response to neonatal DES despite the loss of epithelial ERβ in the adult prostate gland. Thus, results from this treatment suggest that the presence of ERβ appears not to be a requirement for DES-induced AR suppression in the prostate.

In both the WT and βERKO mice, where developmental estrogenization of the prostate occurred, there was a transient up-regulation of ERα within periductal stromal cells, which allows for the amplification of estrogenic signals within that target population. ERα was not observed in the prostate epithelial cells of any of the mice in the present study, which confirms our previous observation in the rat prostate lobes (29) as well as RT-PCR analysis of the separate rat lobes where ERα mRNA was undetectable in the prostatic epithelial cells (31). Taken together with the lack of estrogenization in the αERKO model, these findings implicate the involvement of stromal-derived paracrine factors regulated by stromal ERα in mediating the estrogen imprint of the prostate epithelium. This contrasts with a recent study involving stromal-epithelial recombination techniques with tissues from WT and αERKO mice where it was concluded that ERα in both the epithelial and stromal fractions were required for estrogen induction of prostatic squamous metaplasia (44). However, in that study, epithelium was obtained from the adult anterior prostate (coagulating gland), which expresses epithelial ERα in the WT uninduced state in contrast with prostate lobes of which the epithelium are ERα negative. Additionally, the stromal component of the tissue recombinants was derived from seminal vesicle mesenchyme. Mesenchyme is a potent inducer of epithelial cell gene expression in a tissue-specific manner (46) and, unlike the prostate, seminal vesicle epithelium expresses ERα (47). Embryologically, the prostate gland is of urogenital sinus or endodermal origin, the seminal vesicle is of Wolffian duct or mesodermal origin, and the coagulating gland is a hybrid gland derived from urogenital sinus epithelium penetrating into seminal vesicle (Wolffian duct) mesenchyme. Thus, it is possible that the seminal vesicle mesenchyme is involved in the induction of ERα expression within epithelial cells. These differences in the embryological origin of the tissues used in that recombinant study and the
natural associated prostate tissue examined in present study, as well as the ability of the coagulating gland epithelium to express ERα may explain our divergent conclusions.

In conclusion, the present study demonstrates that developmental estrogenization of the prostate gland is mediated through ERα present within periductal stromal cells of the developing gland, which indicates that epithelial defects are initiated through a paracrine mechanism. If epithelial ERβ is involved in some component of prostatic estrogen imprinting, its role would be considered minor, and it appears to require the presence of ERα within the developing gland.

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Estrogen Imprinting of the Developing Prostate Gland Is Mediated through Stromal Estrogen Receptor α: Studies with α ERKO and βERKO Mice

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