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Developmental Estrogenization: Prostate Gland Reprogramming Leads to Increased Disease Risk with Aging

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Abstract

While estrogens are involved in normal prostate morphogenesis and function, inappropriate early-life estrogenic exposures, either in type, dose or timing, can reprogram the prostate gland and lead to increased disease risk with aging. This process is referred to as estrogen imprinting or developmental estrogenization of the prostate gland. The present review discusses published and new evidence for prostatic developmental estrogenization that includes extensive research in rodent models combined with epidemiology findings that together have helped to uncover the architectural and molecular underpinnings that promote this phenotype. Complex interactions between steroid receptors, developmental morphoregulatory factors, epigenetic machinery and stem-progenitor cell targets coalesce to hard wire structural, cellular and epigenomic reorganization of the tissue which retains a life-long memory of early-life estrogens, ultimately predisposing the gland to prostatitis, hyperplasia and carcinogenesis with aging.

Keywords

prostate; development; estrogen; estrogenization; reprogramming; HOXB13

Introduction:

The prostate gland is derived embryologically from the endodermal urogenital sinus and its development, growth and function throughout life are under tight androgenic regulation (1,2). Dihydrotestosterone (DHT), derived from intraprostatic metabolism of testosterone, is the primary androgen controlling prostate activity, mediated through the androgen receptor (AR) (3,4). It is also long recognized that estrogens can impact prostate growth, homeostasis and disease throughout life with actions mediated through multiple estrogens receptors (ERs), including ER α , ER β , GPER and estrogen-related receptors (ERR) that are expressed in a cell-specific manner in the prostate. During development, estradiol-17 β plays a physiologic role in modulating prostate branching morphogenesis and epithelial

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differentiation through ER α and ER β , respectively (5–8). However, inappropriate estrogenic exposures during development, in terms of dose, type and timing have been shown to reprogram the prostate gland resulting in developmental defects that set the stage for increased disease susceptibility, a phenotype referred to as estrogenic imprinting or developmental estrogenization. This process and implications for adult onset diseases are the focus of the present review.

Estrogen Receptors in the Developing Prostate

Rodent studies have shown robust stromal ER α expression during early-life prostate morphogenesis, which significantly declines with puberty as androgen levels rise, suggesting a specific developmental role (9,10). One study also identified transient epithelial ER α levels in the murine prostate at week 2 of life which thereafter declined (8); however, this was not observed in the neonatal or prepubertal rat ventral, dorsal or lateral lobes (personal observations). Elegant experiments in the Yeh laboratory using stromal cell-specific deletion of ESR1, the gene that encodes ER α , in murine prostates determined that fibroblast ER α modulates branching morphogenesis whereas smooth muscle ER α regulates stromal cell proliferation and ECM deposition (5,6). In humans, ER α is likewise expressed in stromal cells during fetal development (11,12) whereas at least one study identifies its localization in periurethral prostatic epithelium during mid-to-late gestation where it is associated with squamous metaplasia (12). Furthermore, recent studies have also identified ER α in human prostatic epithelial stem and progenitor cells, where they mediate estrogen-induced stimulation of stem cell self-renewal and progenitor cell proliferation (13–15). This implicates a potential estrogenic role in maintaining prostate epithelial homeostasis through this repopulating stem population. Of particular note, estrogen actions in the stem-progenitor cell pool are mediated through both genomic and membrane-initiated signaling pathways (16).

In contrast to ER α , ER β expression is localized almost exclusively to prostate epithelial cells with a putative role in differentiation of the luminal epithelium (17). In the rodent prostate, ESR2 expression, the gene that encodes ER β , steeply increases concomitant with luminal cell differentiation during the postnatal period implicating a role in that process (7). In humans, ER β is widely expressed in epithelial and stromal cells by gestational week 7 and is maintained throughout gestation and postnatally for several months, suggesting a specific developmental function (11,12). Further, ER β is found in human prostate stem and progenitor cells, localizing to both the nuclear and membrane compartments where it restrains stem cell symmetric self-renewal and promotes progenitor cell differentiation (14,16)

Estrogen Imprinting of the Prostate Gland

Epidemiologic evidence suggests that inappropriate estrogen exposures during development can lead to abnormal growth of the human prostate with predisposition to diseases with aging such as benign prostatic hyperplasia (BPH) and adenocarcinoma (18,19). Swedish cohort analysis found strong correlations between indicators of high levels of pregnancy estradiol, such as high birth weight and jaundice in the offspring, and increased risk for

prostate cancer whereas indicators of low maternal estrogens, like pre-eclampsia/eclampsia, were associated with decreased risk in offspring as they aged (20,21). African-American men have a two-fold higher risk of developing prostate cancer with aging than Caucasian men and evidence indicates this may be linked, in part, to elevated maternal estrogens during the first trimester (22). Further, sons of mothers who used diethylstilbestrol (DES) during pregnancy are predisposed to reproductive tract neoplasia (23) and exhibit excessive epithelial metaplasia (24) and structural abnormalities of the prostatic utricle at 1 month of age with persistent ectasia (25).

In contrast to humans where prostate development is largely completed *in utero*, the rodent prostate is rudimentary at birth and undergoes branching morphogenesis and cell differentiation postnatally (26). As such, the neonatal rodent prostate serves as a model for evaluating the effects of natural, pharmaceutical and environmental estrogens on prostate development. Studies from multiple laboratories have found that neonatal exposures to high levels of estrogens, modeling pharmaceutical exposures, leads to developmental and differentiation defects of the stroma and epithelium and onset of prostatic diseases with aging that include chronic prostatitis, stromal hyperplasia, epithelial dysplasia, adenomas, prostatic intraepithelial neoplasia (PIN) and over time, onset of adenocarcinoma (27–32). In the rat, lobe-specific effects were observed with the greatest perturbations in the ventral lobe (30) which was similar to that found in the estrogenized mouse prostate (33). These findings have led to the supposition that high-dose estrogenic exposures during development can directly predispose to prostate neoplasia and tumor formation with aging.

Increased concern in recent years regarding early-life exposures to endocrine disrupting chemicals (EDCs) has led to considerable research on prostate gland reprogramming by estrogenic EDCs. Bisphenol A (BPA) has been the most thoroughly investigated and serves as a model estrogenic EDC. The first report that developmental exposures to BPA altered the adult prostate came from the vom Saal laboratory which found that gestational low-dose BPA exposure led to larger dorsolateral prostates in adult mice (34). Underlying mechanisms include altered ER α and AR expression in mesenchymal cells through global epigenetic modifications (35) and elevated CYP19A1 activity and UGS estradiol levels (36). In the Sprague-Dawley rat model used in the Prins laboratory, brief neonatal low-dose BPA exposure was insufficient for inducing prostate pathology or weight changes in adulthood; however, it directly led to epigenetic reprogramming and stem cell modifications in the dorsolateral prostate that resulted in heightened susceptibility to estrogen-driven carcinogenesis with aging (37–41), an observation replicated by an independent group (42). Further, modeling with humanized prostate-like renal grafts in nude mice found similar carcinogenic responses to transient BPA exposures in the human-derived epithelium (15) indicating that the effects seen in rodent models are translatable to the human gland. Evidence with other estrogenic EDCs is limited but also points to prostate aberrations with aging that include hyperplasia and heightened carcinogenic risk (43,44).

Together, the phenotypic data in rodents and humanized models combined with epidemiology studies reveal that developmental exposures to multiple types of estrogens – elevated maternal estradiol, pharmaceutical estrogens, estrogenic EDCs – can increase disease propensity in the adult prostate gland, a concept that reinforces the developmental

basis of adult disease paradigm. The following sections will review the mechanistic underpinnings of these processes that include direct changes in steroid receptor expression profiles and activity, interference in morpho-regulatory gene expression resulting in distorted glandular architecture, epigenetic modifications leading to cellular reprogramming and altered cell memory, and aberrant stem cell reprogramming with altered self-renewal and lineage commitment leading to persistent life-long effects and tissue perturbations. Importantly, these mechanisms are not mutually exclusive and work together to drive heightened susceptibility to prostatic diseases with aging.

Altered Steroid Receptor and Developmental Gene Expression

Steroid Receptors

The developing rodent prostate responds to multiple steroidal signals which modulate development through specific steroid receptors (SRs). AR is the dominant receptor and is essential for ontogeny and development of the gland. At the early developmental stages, AR expression is confined to mesenchymal/stromal cells and when liganded, directs glandular morphogenesis and cellular differentiation through stimulation of stromal-derived secretory growth factors (1,45). As the epithelium differentiates, AR expression is induced in luminal cells, between postnatal days (PND) 5–15 in the rat prostate lobes (46), where it controls functional differentiation. As discussed above, ERs also contribute to normal prostate development as do other SRs including retinoid receptors RARs/RXRs whose levels decline post-pubertally implicating a specific developmental role (47–49). Studies in our laboratory have found that exposures to high levels of estrogens during the neonatal critical window (PND 1–5) markedly alter the expression profile of multiple SRs in the rat and mouse prostate glands. Some changes occur immediately (ER α , AR) and directly drive the early estrogenized phenotype whereas other SR changes appear later in development or adulthood (e.g. ER β). Furthermore, some of the initial SR alterations are transient (ER α , PR) whereas others are permanent (AR, ER β , RAR/RXR), lasting throughout life. We propose that these estrogen-induced changes in SR expression play a fundamental role in initiating growth and differentiation defects during early development and maintaining these phenotypes throughout life.

Studies with ER α KO and ER β KO mice determined that neonatal estrogenization of the rodent prostate is mediated entirely through ER α in the developing gland (50). Recent studies using membrane-only ER α and nuclear-only ER α mice (H2NES and NOER mice, respectively) revealed that both membrane-localized (mER) and nuclear ER pools are necessary for a full estrogenized phenotype, acting in a cooperative manner (Cooke & Prins, unpublished data). Soon after high-dose estrogen exposure, AR protein is sharply down-regulated in both stromal and epithelial cells (46) through heightened proteasomal degradation (51) and remains low throughout life leading to a reduced activational response to androgens (30,52). The estrogenic exposure leads to a transient up-regulation of ER α in periductal stromal cells which in turn, permits a transient induction of PR in these cells (10,53). In addition, RARs and RXRs and intraprostatic retinoid are immediately and permanently elevated allowing for the amplification of retinoid signaling during development and with aging (48,54). Other investigators have also examined AR and ER

levels following early-life estrogen exposure and found similar results (35,55,56). The prostatic steroid receptor changes during development due to early estrogens are summarized in Figure 1, bottom.

In the normal prostate, AR is the dominant SR dictating morphogenesis whereas lower levels of ER α are found in stromal cells surrounding the proximal ducts. Neonatal exposure to high-dose estradiol sharply reduces AR while amplifying ER α in periductal stromal cells along the length of the developing ducts. Additionally there is induction of PR and increased RAR α in stromal cells and upregulation of RAR β in epithelial basal cells. Thus the developing prostate is no longer under predominant androgen-AR regulation, but is rather driven by alternate steroids through estrogen-ER, progesterone-PR and retinoid-RAR signaling pathways. We propose the net effect of these changes is that programming and organizational signals that normally dictate and determine prostate development during discreet temporal windows are irretrievably altered.

Developmental Genes

Continuous branching morphogenesis of the prostate during development is dictated by time-specific and region-specific expression of master regulatory genes which form a prostatic code that includes hedgehogs, *Wnts*, *Bmps*, *Fgfs*, *Nkx3.1* and posterior *Hox* genes (see reviews in (2) and (58)). Importantly, there is clear evidence of a role for steroids in regulating developmental genes in hormone-sensitive tissues (59,60), including the prostate gland. Towards this end, our laboratory focused on determining whether neonatal exposure to estrogens altered rat prostate development through SR-induced changes in key developmental genes. Indeed, the expression of a number of prostate morphoregulatory genes, including secreted morphogens, their cognate receptors and developmentally critical transcription factors were either transiently or permanently altered by inappropriate early-life estrogen exposure, contributing to permanent structural reorganization and differentiation abnormalities. These included alterations in expression and/or activities of TGF β -TGF β R1 (61), SHH-PTC-GLI (62), FGF-10/FGFR2iib (63), BMP-4 and -7 (54,64), WNT5a (65), NKX3.1 (66), and HOXB13 genes (67,68) as schematized in Figure 1, top. In the normal prostate, mesenchymal secretion of FGF-10 acts on epithelial FGFR2iib to stimulate ductal outgrowth and branching while BMP4 and WNT5a act as localized inhibitors to restrict these events at specific sites. Distal tip epithelial cells secrete SHH which ligands to mesenchymal cell PTC receptors and activates GLI transcription factors that regionally restrict growth. Together these and other factors act in concert to tightly regulate branching morphogenesis. Transcription factors NKX3.1 and HOXB13 are expressed by developing epithelial cells to control differentiation. The alterations in steroidal signaling in response to neonatal estrogens redirect the expression of these morphoregulatory genes (Figure 1, top right). Specifically, BMP4 and WNT5a levels are markedly increased and remain elevated while FGF10/FGFR2iib levels and signaling are reduced. SHH signaling is heightened and together these factors act to suppress growth and branching of the ducts. Transient postnatal reduction in *Nkx3.1* expression, at the time of normal epithelial cell differentiation, combined with permanent *Hoxb13* gene suppression initiate differentiation defects that persist throughout life.

It is noteworthy that dysregulation of several of these morphoregulatory genes occurs during prostate carcinogenesis in humans (69–73), thus providing a mechanistic link between developmental reprogramming and tumor formation with aging. One in particular, HOXB13, is of specific interest since a novel HOXB13 G84E variant in humans is associated with increased risk of hereditary prostate cancer (74) and is the topic of intense research for its role in sporadic prostate cancer and metastasis. The Prins laboratory observed over 20 years ago that neonatal estradiol suppressed the *Hoxb13* levels in the developing rat ventral prostate which persisted throughout life (67). Further characterization of the rat prostatic Hox code found maximal *Hoxb13* expression in the adult rat ventral prostate epithelium, with decreasing expression in anterior direction; i.e. ventral > lateral > dorsal prostate lobes and minimal expression in the coagulating glands (anterior prostate) (68). Using *Hoxb13*-lentiviral vectors targeted to undifferentiated prostate epithelial cells, an essential role for HOXB13 in initiating and maintaining luminal cell differentiation was directly demonstrated, supporting earlier findings in the murine prostate (75). Prostatic *Hoxb13* expression in rats neonatally exposed to 25µg estradiol on PND 1, 3 and 5 was immediately and persistently suppressed in all three lobes with the greatest response noted in the ventral prostate (Figure 2A) (76). Further, immunohistochemistry of HOXB13, luminal cytokeratin 8, basal cytokeratin 5 and the secretory prostate binding protein (PBP) in serial sections of the day 90 ventral lobe revealed that suppressed *Hoxb13* levels directly overlapped with aberrant epithelial differentiation and loss of functional activity (Figure 2B). As such, we propose that life-long suppression of HOXB13 contributes to permanent differentiation defects of estrogenized prostate and its predisposition to cancer.

Structural Reorganization of the Estrogenized Prostate

Tissue development is an *einbeinstrasse* (one-way street) and as such, the early estrogen-induced reprogramming events lead to permanent alterations in prostate structure and activity throughout life. Prostates of rodents exposed to high-dose estrogen exhibit stunted growth and reconfigured stromal and epithelial organization (30). Normal prostatic ducts have a heterogeneous pattern of cell types and functions along the ductal length (77,78) and those exposed to high-dose estrogen retain an exaggerated proximal duct phenotype along the length of the prostatic ducts (79). This proximalized phenotype is characterized by a thick periductal fibroblast layer that extends to the distal tips and prevents interactions (e.g. secreted growth factors) between the central-distal epithelial cells and smooth muscle cells that normally lie adjacent to the basement membrane. Similarly, the epithelial cell organization retains a proximal pattern with a continuous layer of basal cells along the ductal length in contrast to the normal rodent prostate where the basal cell layer is discontinuous in the central-distal regions (46). This is accompanied by permanent shifts in epithelial cell adhesion and gap junction proteins (80) which further alter cell signaling. As such, in young adulthood, estrogenized prostate epithelial cells are disorganized, aberrantly differentiated and their secretory function is markedly compromised (Figure 2B) (52). We hypothesize that the estrogen-driven proximalized prostate phenotype reflects a change in proximal-distal positional identity within prostatic ducts, mediated by altered expression of genes that regulate proximal-distal axis specification.

It is important to note that these structural changes are quite different with low-dose estrogenic exposures, including EDCs where larger glands and hyperplasia in adulthood are reported (81). This underlines an established endocrinologic phenomenon where non-linear and bell-shaped dose response curves occur and stresses the importance of evaluating responses to estrogens based on dose (82).

Epigenetic Reprogramming by Early-life Estrogens

In addition to structural reorganization, prostate stromal and epithelial cells exposed inappropriately to estrogens during development retain a lifelong memory of the event such that they are sensitized to later life exposures which further promote disease. One identified mechanism that enables retained cellular memory is molecular reprogramming of the epigenome, first proposed by McLachlan for early-life estrogen and EDC exposures (83,84). Epigenetic modifications are *heritable* changes in gene expression not caused by nucleotide alterations in the genome. These include DNA methylation at CpG sites, histone methylation at lysine and arginine residues on histone tails, and noncoding RNAs with downstream epigenetic consequences (reviewed in (85)). Evidence has now emerged that all three of these epigenetic processes are involved in reprogramming of the developmentally estrogenized prostate gland.

The first evidence for epigenetic reprogramming of the prostate came from the Ho laboratory in collaboration with the Prins laboratory which, using genome-wide screening, identified differential methylation of > 50 genes in PND 10, 90 and 200 prostates following neonatal estradiol- or BPA-exposure as compared to controls (37). Persistent DNA hypomethylation resulting in increased gene transcription was detailed for nucleosome binding protein (*Nsfp1*) which plays a role in chromatin remodeling, phosphodiesterase 4 variant 4 (*Pde4d4*) which degrades cAMP, and Hippocampin-like 1 (*Hpcal1*), involved in cAMP formation (86). Of note, expression of both PDE4D4 and NSBP1 is associated with human prostate cancer cell growth (87,88) The reprogrammed DNA methylome is likely mediated by estrogen/BPA-induced permanent increases in expression of *de novo* DNA methyltransferases DNMT3a/b and methyl-CpG binding domain proteins (Mbd2/4) that have demethylating activities. In follow-up studies of young adult prostates neonatally exposed to estradiol or BPA, differentially methylated regions were identified for 111 estradiol-associated and 86 BPA-associated genes as compared to controls, with 20 in common for both treatment groups, thus uncovering unique DNA methylation fingerprints for specific estrogenic chemicals (40,89). Pathway analysis of the 20 common genes identified cancer as the top common disease pathway. The methylation status of 7 genes (*Pitx3*, *Wnt10b*, *Paqr4*, *Sox2*, *Chst14*, *Tpd52*, *Creb3l4*) showed inverse correlation with gene expression in tissue samples thus documenting functional relevance. Significantly, functional connectivity of these 7 genes was linked to embryonic stem cell pluripotency whereas clustering analyses using The Cancer Genome Atlas dataset discovered that expression of this 7 gene set was associated with recurrence-free survival of prostate cancer patients. Together, these results reveal that gene-specific promoter methylation changes resulting from early-life estrogen/EDC exposure in the rat may serve as predictive epigenetic biomarkers of prostate cancer recurrence and raise the possibility that such exposures may impact human disease. Several other research laboratories have similarly identified altered

DNA methylation patterns and altered gene expression in the prostate as a function of early-life estrogenic exposures (35,90–92) thus solidifying an epigenetic underpinning of prostatic developmental estrogenization.

The Walker laboratory extended the epigenetic reprogramming pathways involved in estrogenization by examining histone tail methylation marks in rat prostate tissue. They unequivocally demonstrated that brief estrogenic low-dose BPA exposure rapidly activated the mER α -PI3K-pAKT signaling cascade in the PND 6 rat prostates which in turn activated MLL1, a histone methyl transferase (HMT) component of the COMPASS complex responsible for laying down H3K4_{me3} marks on specific genes (93). Remarkably, these activational marks on multiple prostatic genes, including several KEGG prostate cancer genes, persisted into adulthood (not in controls) and exaggerated transcriptional responses to an adult estrogen/testosterone challenge. Similarly, DES and BPA activation of mESR1-PI3K-AKT signaling phosphorylated and down repressive H3K27_{me3} marks. The net result was a reduction in H3K27_{me3} levels with increased expression of downstream genes (94). Together, these important studies indicate that epigenetic reprogramming of histone methyl marks in the neonatal estrogen-exposed prostate, mediated through rapid mER α signaling, is a key mediator of life-long cellular imprinting and altered cell memory.

Modulation of ncRNAs was also found in human prostate progenitor cells enriched in 3-D prostasphere cultures and exposed to either estradiol or increasing doses of BPA for one week (95). Across all treatment groups, there was suppression of small nucleolar RNAs (SNORDS), a class of ncRNAs that guide methylation of rRNA/tRNA during protein biogenesis and regulate alternate RNA splicing. Notably, SNORD reprogramming was not mediated through DNA methylation but rather through modifications of H3K4_{me3}, H3K9_{me3} and H3K27_{me3} histone methyl marks.

As a continuing story of *Hoxb13* downregulation in estrogenized rat prostates, our laboratory explored possible epigenetic pathways that mediate its life-long suppression. First, we identified two CpG Islands (CGI) upstream of the rat *Hoxb13* promoter and, using Mass Sequenom, evaluated their methylation status in day 90 ventral prostates of rats exposed neonatally to 25 μ g estradiol or vehicle (Figure 3A). Methylation at both CGIs was relatively low, averaging 6–10% of available CpG sites in the control prostates. While CGI-2 methylation was not influenced by neonatal estradiol exposure, a small but significant increase in methylation (from 6 to 8%) of the proximal CGI-1 was noted. This mapped to a single CpG site that overlapped with an HMX2 binding site, a homeobox gene with yet unknown roles in prostate development. Next, we identified a polycomb group response element (PRE) and trithorax response element (TRE) positioned between CGI-1 and CGI-2 in the *Hoxb13* promoter region (Figure 3 B). Inactivating PRC2, which contains EZH2 that trimethylates H3K27, binds to PRE sites through SUZ12 while activating trithorax genes, UTX and JMJD3 which demethylate these sites, bind at TREs. Together, these complexes regulate the methylation status of H3K27. To determine whether neonatal estrogens influenced H3K27 methylation of the *Hoxb13* promoter region to silence prostatic expression, we used CHIP-PCR for selected histone methylation marks and for SUZ12 at the PRE and transcription start site (TSS) of the *Hoxb13* gene. H3K27_{me3} and SUZ12 were enriched at the PRE site in estrogen-exposed prostates versus oil controls (Figure 3B). At the

Hoxb13 TSS, H3K4_{me1}, H3K4_{me3} and H3K9_{me3} were unaffected by estrogen exposure; however, SUZ12 and H3K27di- and tri-methylation were significantly enhanced compared to oil controls (Figure 3B). Together, these data demonstrate that early-life estrogens epigenetically suppress prostate *Hoxb13* expression by laying down permanent silencing histone methylation marks as schematized in Figure 3C.

During organogenesis, establishment of DNA and histone methylation marks is a dynamic process that is vulnerable to endogenous and exogenous influences (85). The results summarized herein clearly show that early-life estrogens rewire or reprogram the developing prostate epigenome at multiple levels and in so doing, permanently alter tissue development which retains a memory of this exposure that renders the gland more responsive to subsequent exposures throughout life. It is particularly noteworthy that new research has revealed that prostate cancer reactivates developmental epigenomic programs during metastatic progression (96), affirming that altered epigenomic programs laid down during prostate development by brief estrogenic exposures can be later-life drivers of prostate cancer development and progression.

Prostate Stem-Progenitor Cells as Estrogen Targets

Stem cells are fundamental components of biological organization, responsible for the development and maintenance of tissues and organ systems (97). Embryonic stem cells (eSCs) are pluripotent cells with a robust proliferative capacity and ability to differentiate into the three germ cell layers. These subsequently give rise to unique organs, each with their own tissue-specific stem cell population, including for the prostate gland. Once tissues and organs are formed after morphogenesis, adult tissue-specific stem cells maintain homeostasis within that structure, providing cells for natural tissue turnover and regeneration in response to injury. While prostate stem cells have been identified in both rodents and humans (98,99), there are several unresolved issues including potential differences between stem cells at the developmental stage and adulthood, the lineage hierarchy of prostate stem cells to differentiated progeny, and differences in hierarchical lineage commitment in rodents versus humans. Nonetheless, Figure 4 presents a simplified schematic for the purposes of the present review. The highly plastic state of stem and daughter progenitor cells during development and tissue maintenance permits the needed flexibility for proper tissue formation and repair. Regrettably, this plasticity also provides an opportunity for aberrant cellular reprogramming due to inappropriate signals, both endogenous and exogenous, that can lead to persistent life-long effects and tissue perturbations.

While stem cells are tightly regulated by their niche microenvironment, hormonally sensitive tissues, including the prostate, have an additional layer of hormonal influences on the stem-progenitor populations. Recent research from our laboratory has identified human prostate stem and progenitor cells as direct hormone targets through the expression of multiple steroid receptors including ERs, RAR/RXRs, PPAR γ and GRs (100). Notably, AR is absent in both the prostate stem and early-stage progenitors thus any androgen actions on their activity must be directed through the niche cells. Regarding ERs and estrogenic control, the human prostate stem and progenitors express ER α and ER β at differing levels and with opposing actions (13,14,101). Specifically, ER β levels are relatively higher in the stem

compared to the daughter progenitor cells whereas ER α levels are higher in the progenitor cells. As previously mentioned, ER β restrains stem cell self-renewal and promotes progenitor cell differentiation whereas ER α stimulates stem cell self-renewal and progenitor cell amplification. Further, estrogen actions are mediated through both mER α and mER β that, through phosphorylation cascades, activate HMTs as well as through classical nuclear-localized signaling that drives gene transcription (16).

To directly interrogate whether early-life estrogenic exposures influence the prostate stem and progenitor cell populations, several approaches were utilized. In the BPA-Consortium Linking Academic and Regulatory Insights on Toxicity (CLARITY), Sprague Dawley rats were exposed from gestation day 6 through six months of age to ethinyl estradiol (0.5 $\mu\text{g}/\text{kg}$ BW/day) or BPA (2.5, 25 or 250 $\mu\text{g}/\text{kg}$ BW/day) at the FDA laboratories as described (41,102). Prostates were shipped overnight and the stem-progenitor cells were isolated and evaluated for numbers and lineage commitment through gene expression profiling. Both ethinyl estradiol and the 2.5 μg BPA dose doubled the total stem cell numbers in the 6 month dorsolateral prostate. Further, ethinyl estradiol and the two higher BPA doses stimulated progenitor cell proliferation and shifted their lineage commitment to basal progenitors at the expense of decreased luminal progenitor cells. These exposures, particularly 2.5 μg BPA/kg BW developmental exposure, also led to increased carcinogenic susceptibility in response to adult estradiol plus testosterone treatment in a separate animal group. As such, we propose that stem cell reprogramming by estrogens contribute to heightened carcinogenic susceptibility of the gland.

To directly assess the actions of estrogens on prostate stem and progenitor cells and to provide relevance of these findings to the human prostate gland, we utilized *in vitro* and *in vivo* systems using primary cells cultured from prostates of young, disease-free organ donors. In addition to estradiol, several EDCs that utilize ERs likewise stimulated stem cell self-renewal and progenitor proliferation including BPA (15), dioxin (101), and inorganic arsenic (103). Using these normal human stem-progenitor cells cultured in 3-D as prostaspheres, we grew humanized prostate-like structures by combining them with inductive rat embryonic UGS mesenchyme and grafting them under the renal capsule of adult male nude mice. To mimic developmental exposures, mice were fed low-doses of BPA during the first 2 weeks as the prostate-like tissues formed. At one month, the hosts were given testosterone plus estradiol pellets to drive carcinogenesis as described (13). After 3 months, the prostate-like structures were evaluated and results showed that the malignancy incidence in the human prostate epithelium significantly increased from 13% in oil-exposed controls to 33–36% in tissues exposed to BPA during the developmental window. This further increased to 45% cancerous lesions when the spheroids were precultured in BPA for 1 week prior to grafting followed by the *in vivo* BPA exposure. Together, these findings demonstrate that developmental BPA exposure increases the susceptibility of human prostate epithelium to estrogen-driven carcinogenesis and that the prostate stem-progenitor cells are the direct targets that propel this increased cancer risk.

One caveat of the above study is that young adult prostate stem cells were used which may differ from the developmental stage prostate stem cells. To address this, our laboratory established prostate organoids *in vitro* from directed differentiation of human eSC using

sequential exposure to stage-specific prostate growth factors and steroids (104). Following exposure of the differentiating eSC and prostate organoids to low-dose BPA, their phenotype and stem cell population was examined by immunohistochemistry with confocal microscopy (105). Although differentiation to mature organoids at day 30 was not affected by BPA, there was an increase in focal clusters of resident stem cells in the BPA-exposed organoids that was not observed in controls. Further, organoid expression of prostate stemness genes *OCT4*, *NANOG* and *CD49f* was increased by BPA exposure. This concurs with adult stem cell findings and shows that low-dose BPA exposures stimulate prostate stem cell symmetric self-renewal resulting in stem cell nests that do not properly enter lineage commitment. These results support our hypothesis that the human fetal prostate gland may be reprogrammed by estrogenic chemicals which may heighten carcinogenic risk. Similar conclusions were reached by an independent laboratory using human engrafted human fetal prostate tissue (91).

While discussing eSCs and prostate stem cells, it is critical to note that the epigenetic code is dynamically established in these cells and their progeny early in life, making them particularly vulnerable to epigenetic reprogramming from inappropriate estrogenic exposures. For mouse embryonic stem cells, H3K4_{me3} and H3K27_{me3} discriminate genes that are expressed, poised for expression, or repressed and reflect the stem-progenitor cell stage (106). Noteworthy is the discovery that several stem-progenitor cell genes possess bipotent chromatin marks with both H3K4_{me3} and H3K27_{me3} at their promoters, serving to poise key developmental genes for lineage-specific activation or repression. In this context, prostate reprogramming can be viewed as a combination of altered DNA methylation and histone modifications that are heritable as stem and progenitor cells self-renew, transmitting altered epigenomic information throughout the lifespan of the individual. Direct evidence that this occurs in prostate progenitor cells has been presented in the previous section where brief exposure of human prostaspheres to estradiol or BPA altered ncRNA (SNORD) expression through changes in H3K4_{me3}, H3K9_{me3} and H3K27_{me3}. Additionally, BPA-induced reprogramming of the neonatal rat prostate resulted in multiple genes retaining bipotent H3K4_{me3} and H3K27_{me3} marks in the adult prostate, perhaps a function of a rewired progenitor cell chromatin state (93). As such, we propose that the prostate epithelial stem-progenitor cells are functional targets of estrogens and environmental chemicals, as highlighted in Figure 4, mediated through coordinate alterations in DNA and histone methylation marks known to control stem and progenitor differentiation patterns during early development. Heritable perturbations in these cells would directly lead to aberrant differentiation. More importantly, continued proliferation of permanently reprogrammed prostate progenitor cells may contribute to heightened sensitivity to hormones throughout life and conceivably increased susceptibility to hormonal carcinogenesis in aging men.

Conclusions

In conclusion, the results summarized herein from decades of research in multiple laboratories including our own has documented the process of developmental reprogramming of the prostate gland by early-life estrogenic exposures that leads to increased disease risk with aging. As development is a stage of high plasticity influenced easily by endogenous and exogenous factors, perturbations set down early in life position the

gland on a life-long trajectory of structural and differentiation defects that contribute to heightened disease predisposition. Cellular and molecular underpinnings of developmental estrogenization, aka estrogen imprinting, include structural and epigenetic rearrangements mediated through dysregulated expression of steroid receptors, morphoregulatory genes and the epigenetic machinery that reprogram prostate cells, including the long-lived stem cell population, for life. The prostatic perturbations have dose-dependent trajectories that are typically non-linear, with responses varying between the types of estrogenic exposure. Understanding the developmental factors that contribute to BPH and prostate carcinoma with aging will be fundamental towards uncovering appropriate intervention strategies in future studies.

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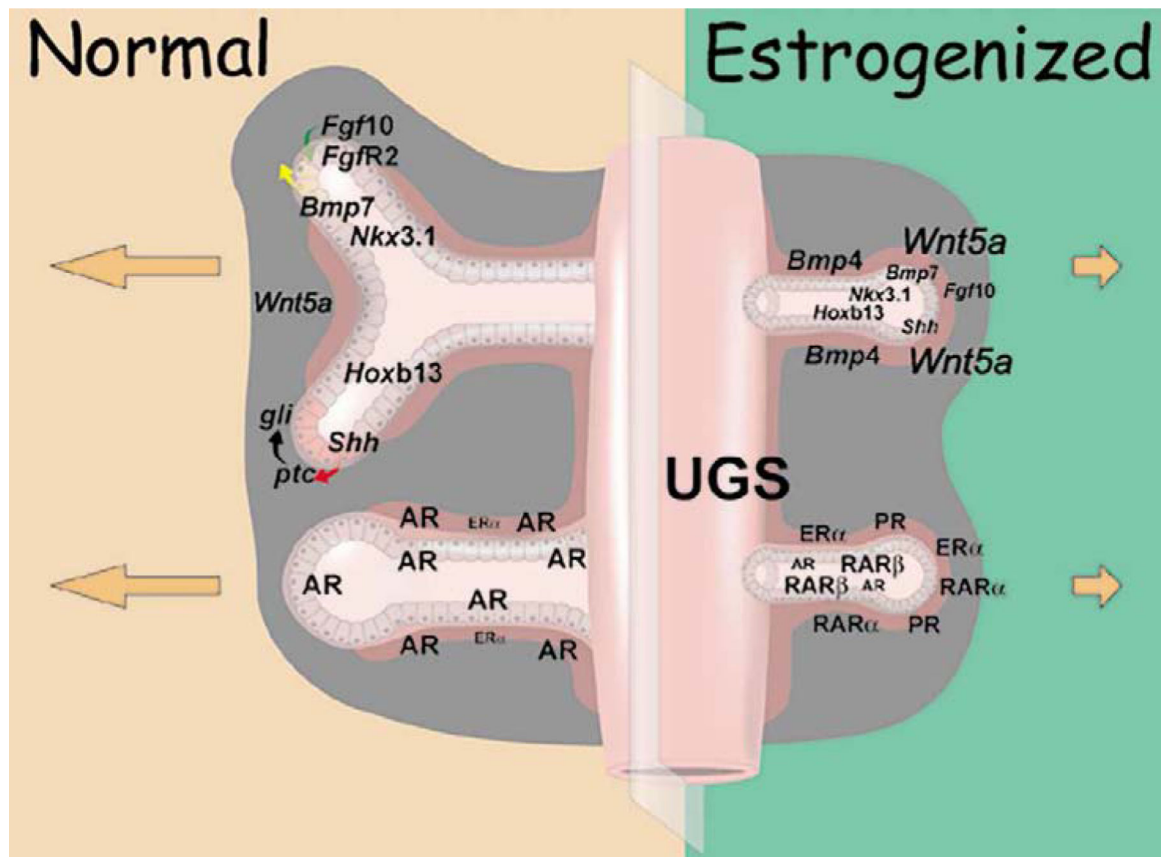


Figure 1: Schematic representation of the normal (left) and estrogenized (right) developing prostate gland that highlight essential morphogens and transcription factors (top) and steroid receptors (bottom) that coordinately regulate morphogenesis and differentiation. Relative gene expression levels are represented by type size. Dysregulation of steroid receptors and morphoregulatory gene expression following early estrogenic exposures results in stunted growth, structural rearrangements and aberrant cell differentiation. See text for details. Reproduced with permission from (57).

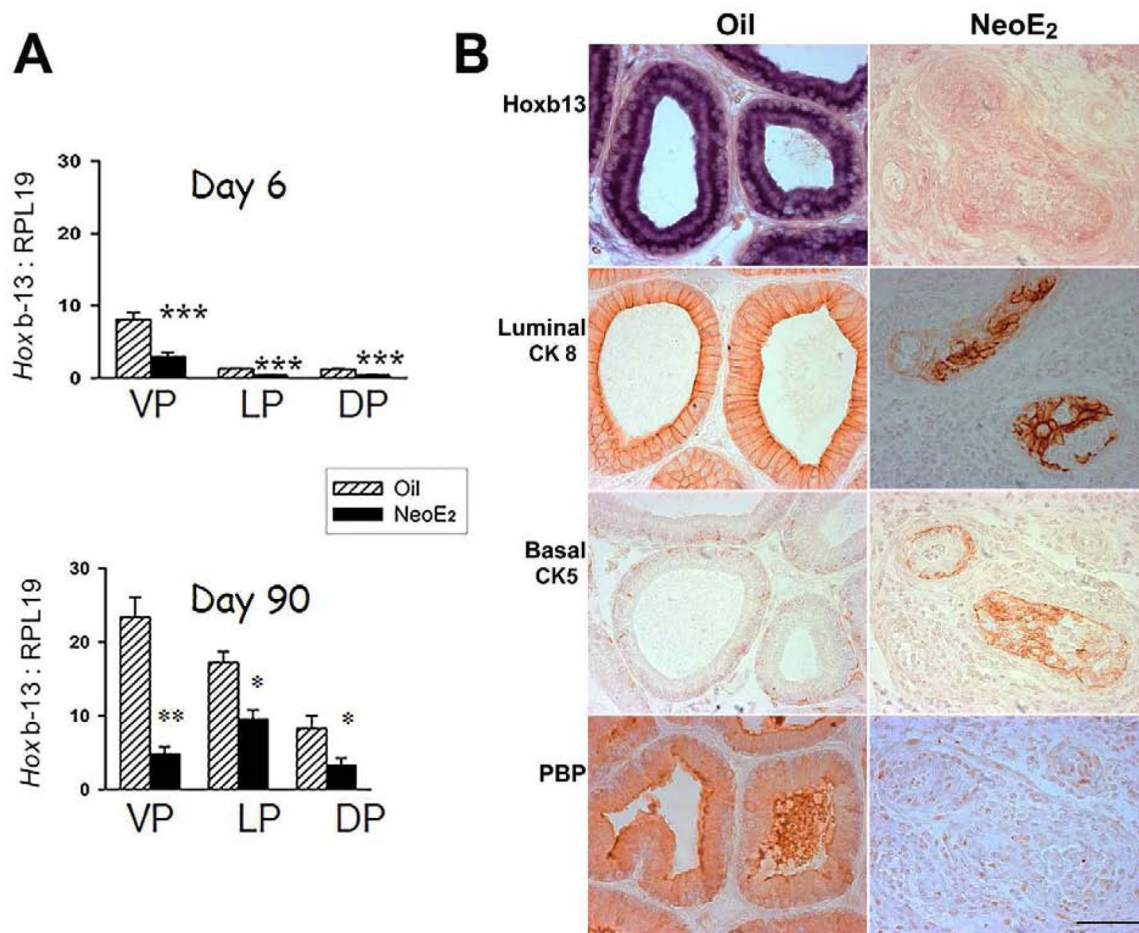
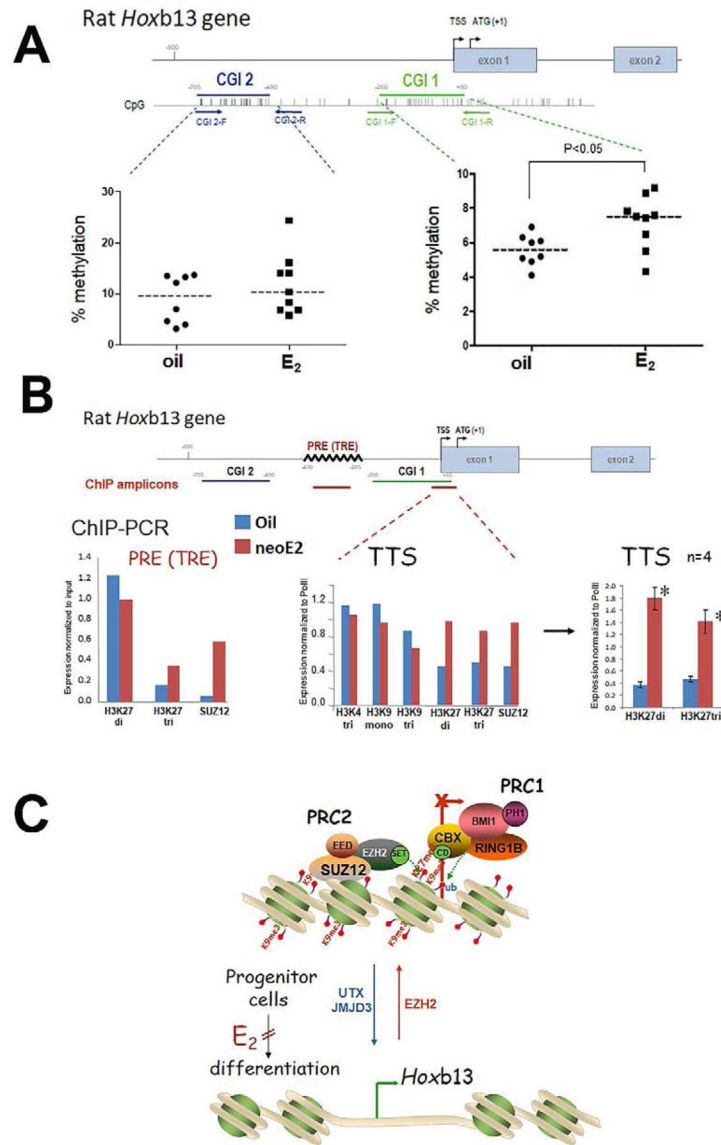


Figure 2:

Hoxb13 expression in the developing and adult prostate lobes of rats treated neonatally with oil or 25 μ g estradiol benzoate (NeoE₂) on PND 1, 3 and 5. **A:** Expression levels of *Hoxb13*, measured by RT-PCR, in the ventral (VP), lateral (LP) and dorsal (DP) lobes at day 6 and 90 in oil control and neoE₂ exposed rats. Expression is the highest in the VP with declining levels in the LP and DP of control prostates. Exposure to NeoE₂ immediately suppresses *Hoxb13* expression in the developing prostates which is maintained through adulthood. **B:** Immunohistochemistry in the adult VP for HOXB13, luminal cell CK8/18, basal cell CK5/15 and secretory prostate binding protein (PBP) on serial sections of both oil and NeoE₂ exposed prostates. Estrogenized prostates exhibit a disorganized epithelium with loss of luminal HOXB13, defective luminal and basal cell differentiation and orientation, and loss of luminal PBP secretions. See (63,68) for methodologic details.

**Figure 3:**

Hoxb13 epigenomic reprogramming in the adult rat ventral prostate by neonatal estradiol (neoE₂) exposure. **A:** *Hoxb13* promoter DNA methylation. A proximal CGI-1 and distal CGI-2 were identified in the first 800 bp upstream of the *Hoxb13* TSS. Mass Sequenom was used to identify DNA methylation at CpG sites in these CGIs from prostates of neonatal oil and neoE₂ treated rats (n=8–9 per treatment group). Average % methylation in CGI-2 was not affected by neoE₂ treatment whereas an increase was noted in CGI-1 from 6% to 8% (p<0.05). **B:** Histone methylation in the *Hoxb13* promoter. A PRE/TRE was identified between CGI-1 and CGI-2 of the rat *Hoxb13* promoter for PRC2 binding. ChIP-PCR was used to assess occupancy of the SUZ12, a PRC2 component, and methylated forms of H3K4, H3K9 and H3K27 at the PRE and TSS (amplicons marked in red bar on gene schematic). The PRE was enriched for SUZ12 and H3K27_{me3} in neoE₂ prostates vs oil controls. At the TTS, neoE₂ had no effect on H3K4_{me3}, H3K9_{me1} or H3K9_{me3} whereas

there was an increase in H3K27_{me2}, H3K27_{me3} and SUZ12 in the estrogenized prostates. The H3K27_{me2} and H3K27_{me3} occupancy at the TSS was repeated in 4 tissues per treatment group and the enrichment was highly significant ($p < 0.01$). **C:** Schematic representation of histone reprogramming of *Hoxb13* gene expression. In undifferentiated progenitor cells, PRC2 occupancy at the PRE and TSS permits EZH2 to lay down silencing H3K27_{me2} and H3K27_{me3} histone marks and recruit PRC1 to prevent *Hoxb13* gene transcription. With development, UTX and JMJD3 demethylases remove the H3K27 silencing methyl marks to permit *Hoxb13* transcription and epithelial differentiation. Exposure to neoE2 retains PRC2 occupancy and promoter H3K27 di- and tri-methylation during this critical developmental window, thus permanently blocking epithelial differentiation.

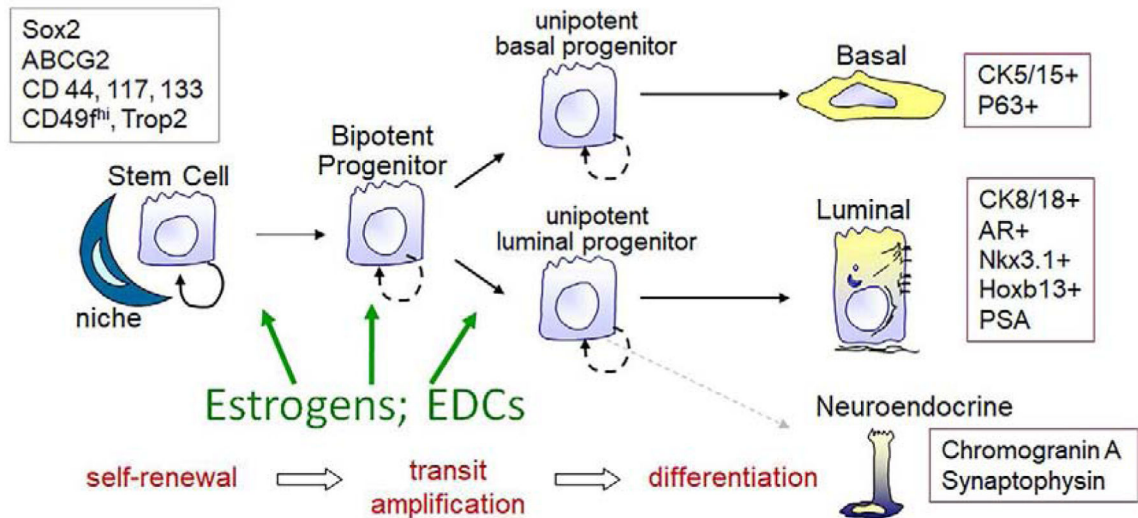


Figure 4:

Simplified schematic of prostate lineage hierarchy in the developing prostate. A rare stem cell population resides within the stem cell niche and in response to cues, undergoes asymmetric self-renewal, giving rise to a daughter stem and a daughter bipotent progenitor cell. The progenitor cell undergoes transit amplification and lineage commitment to unipotent basal and luminal progenitor cells that give rise, upon further divisions to differentiated basal, luminal and rare neuroendocrine epithelial cells. Markers for the stem and differentiated cells are shown in the boxes. We propose that estrogens and EDCs target the stem and progenitor cell populations to affect growth and lineage commitment pathways.