

**Perinatal Exposure to Estradiol and Bisphenol A Alters the Prostate Epigenome  
and Increases Susceptibility to Carcinogenesis**

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## **Abstract**

An important and controversial health concern is whether low dose exposures to hormonally active environmental estrogens such as bisphenol A (BPA) can promote human diseases including prostate cancer. Our studies in rats have shown that pharmacological doses of estradiol administered during the critical window of prostate development result in marked prostate pathology in adulthood which progress to neoplastic lesions with aging. Our recent studies have also demonstrated that transient developmental exposure of rats to low, environmentally relevant doses of BPA or estradiol increases prostate gland susceptibility to adult-onset precancerous lesions and hormonal carcinogenesis. These findings indicate that a wide range of estrogenic exposures during development can predispose to prostatic neoplasia which suggests a potential developmental basis for this adult disease. To identify a molecular basis for estrogen imprinting, we screened for DNA methylation changes over time in the exposed prostate glands. We found permanent alterations in DNA methylation patterns of multiple cell signaling genes suggesting an epigenetic mechanism of action. For phosphodiesterase type 4 variant 4 (PDE4D4), an enzyme responsible for intracellular cAMP breakdown, a specific methylation cluster was identified in the 5' flanking CpG island that was gradually hypermethylated with aging in normal prostates resulting in loss of gene expression. However, in prostates exposed to neonatal estradiol or BPA, this region became hypomethylated with aging resulting in persistent and elevated PDE4D4 expression. In total, these findings indicate that low-dose exposures to ubiquitous environmental estrogens impact the prostate epigenome during development and in so doing, promote prostate disease with aging.

## **Introduction**

Prostate cancer is the most common solid cancer in males and is the second leading cause of cancer deaths in American men. The most recent cancer statistics for 2007 indicate that prostate cancer incidence continues to rise in the United States [1]. The reason for this high propensity to develop cancer within the prostate is not well understood and is an area of intense investigation. It has been suggested that the unique embryological origin of the prostate gland - from the endodermal urogenital sinus, as opposed to the mesodermal Wolffian duct structures that form the other male accessory sex glands - may play a fundamental role in the high rates of abnormal growth as men age. During embryonic development, the prostate gland is highly dependent upon steroid hormones and it is notable that imbalances in steroid levels during early life can result in aberrant prostate growth [2, 3]. The present review will highlight data from several studies that support a hypothesis that early life exposures to estrogenic compounds, including the environmental estrogen bisphenol A (BPA), may predispose the prostate gland towards abnormal growth and carcinogenesis later in life.

### ***Influence of estrogen exposures during prostate development***

In humans, prostate development initiates towards the end of the first trimester in response to rising fetal androgen levels and glandular morphogenesis is largely completed during the second trimester as circulating androgen levels peak. During the third trimester of *in utero* development, fetal androgen production declines while maternal estrogen levels rise resulting in an increased estrogen/testosterone (E/T) ratio. This increased E/T ratio has been shown to directly stimulate extensive epithelial

squamous metaplasia which regresses after birth as estrogen levels rapidly decline [4]. Although the natural role for estrogens during prostatic development is unclear, it has been proposed that excessive estrogen exposures during development may contribute to the high incidence of prostate disease currently observed in the aging male population [5, 6]. The sons of women who took diethylstilbestrol (DES) during pregnancy were shown to have persistent abnormalities in prostate structure shortly after birth [7]. Further, indicators of pregnancy estrogen levels such as length of gestation, pre-eclampsia and jaundice have shown a high correlation between elevated estrogen levels and prostate cancer risk [8, 9]. Interestingly, African-American mothers have elevated levels of maternal estrogens and androgens during early gestation as compared to their Caucasian counterparts and it has been postulated that these elevated steroids may contribute to the two-fold increased risk of prostatic carcinoma in African-American men [10, 11].

Unlike humans, the rodent prostate gland is rudimentary at birth and undergoes morphogenesis and differentiation during the first two weeks of life [12]. Thus the neonatal rodent prostate gland is a useful model for fetal prostate development in humans. Our laboratory and others have shown that brief perinatal exposure of rats to high doses of natural or synthetic estrogens alters the prostate gland in a permanent manner resulting in reduced growth, differentiation defects, aberrant gene expression and perturbations in cell signaling mechanisms [13, 14]. This process, referred to as developmental estrogenization or estrogen imprinting, leads to prostatic lesions as the animals age including chronic immune cell infiltration, epithelial hyperplasia and prostatic intraepithelial neoplasia (PIN), the precursor lesion of prostate cancer [15]. We

thus propose that excessive estrogen exposure during the developmental critical period may be a predisposing factor for prostatic disease later in life. It is noteworthy that a comparable rodent model for female perinatal DES exposures accurately predicted the uterine and vaginal lesions found in daughters of DES-exposed pregnant women [16] which provides credibility for the rodent model system in assessing similar pathologies in males. Whether similar effects may be induced by low-dose estrogenic exposures has until recently remained unclear. This is currently a critical issue since hormonally active xenoestrogens are ubiquitous in the environment and have potential for adverse health outcomes in both humans and wildlife [17].

### ***Bisphenol A***

BPA, a synthetic polymer, is a prevalent environmental estrogen that was initially tested for efficacy as a synthetic estrogen in 1936 [18]. Shortly thereafter, Dodds synthesized DES which possessed much greater estrogenic potency and the use of BPA as a synthetic estrogen was set aside. Today, BPA is used as a cross-linking chemical in the manufacture of polycarbonate plastics, epoxy resins and several other common household products and is one of the highest volume chemicals produced worldwide ( $>6 \times 10^9$  lbs/yr). Unfortunately, BPA monomers leach from plastics and epoxy resins when heated or after repeated washings and BPA is now found at significant levels in environments throughout the world [19, 20]. As a result, unconjugated BPA is found in the serum of 95% of humans at levels ranging from 0.2 – 20 ng/ml [21, 22]. Importantly, BPA is found in 3-4 fold higher concentrations in amniotic fluid as compared to maternal serum [23] and placental and fetal tissue concentrations can exceed 100 ng/g with highest levels found in fetal males [24, 25].

While BPA binds to classical estrogen receptors (ER) with reduced affinity relative to 17 $\beta$ -estradiol [26], it possesses equivalent activational capacity of the nonclassical membrane ER [27]. Thus there is potential for this compound as a toxicant for developing human tissues, particularly the estrogen sensitive reproductive end organs. In this regard, fetal exposures to environmentally relevant doses of BPA in animal studies have been shown to advance puberty [28], increase prostatic growth [29], alter pubertal mammary gland development [30] and permanently change the morphology and functionality of female reproductive tract organs in mice [31].

***Developmental BPA exposure increases prostate gland susceptibility to hormonal carcinogenesis***

It has been shown that that developmental exposure to low-doses of estrogen augments the responsiveness of female reproductive end-organs to elevated estrogens at puberty and beyond. In this context, we asked whether low-dose estrogens during development might shift the sensitivity of the prostate gland to adult estrogenic exposures. This is highly relevant since prolonged adult exposure to estradiol is capable of driving prostatic carcinogenesis in the Noble rat model [32] and estrogens are associated with an increased prostate cancer risk in men [33]. Furthermore, the serum E:T ratio increases in aging men due, in part to increased body fat and aromatase activity [34], and this coincides with the increased propensity of aging men to develop prostate cancer.

We chose to work with Sprague-Dawley rats as an animal model since this strain is less sensitive than the Noble rat to adult estrogen-induced carcinogenesis [35]. A “two-hit” model for carcinogenesis was established. The “first hit” consisted of a brief

exposure to a low dose of estradiol (0.1  $\mu\text{g}/\text{kg}$  BW/day) or an environmentally relevant dose of BPA (10  $\mu\text{g}/\text{kg}$  BW/day) on neonatal days 1, 3 and 5 when the prostate undergoes branching morphogenesis and differentiation. This BPA dosage was chosen since it provides serum BPA concentrations that are similar to those measured in the blood of human fetuses at term (i.e. 0.2 to 9.2 ng unconjugated BPA/ml) [25, 36]. To avoid intake variability between pups associated with lactation, precise doses of estradiol and BPA were delivered via subcutaneous injections using oil as the vehicle which provides slow release of the compound over several hours. While this non-oral route avoids first-pass liver metabolism, it is noteworthy that neonatal rat pups have limited metabolic capacity for BPA [37]. When the neonatal-exposed rats in our study reached adulthood, they were given either oil (control group) or a “second hit” 4-month exposure to  $\sim 75$  pg/ml estradiol which is by itself able to drive carcinogenesis in 100% of Noble rats but only 33% of Sprague-Dawley rats [32, 35]. Our goal was to determine if neonatal low-dose estradiol or BPA exposure could increase the susceptibility of the prostate to adult-induced carcinogenesis.

Individual prostate lobes were histologically assessed at 7 months of age for proliferation, apoptosis and pathologic lesions including prostatic intraepithelial neoplasia or PIN, the precursor lesion for human prostate cancer [38, 39]. While neonatal high-dose estradiol exposure alone and to a lesser degree, early low-dose estradiol exposure increased the incidence of PIN lesions, early exposure to BPA alone had no effect on prostate pathology, proliferation or cell death as the animals aged. However, when rats were exposed to an environmentally relevant dose of BPA (10  $\mu\text{g}/\text{kg}$  BW/day) early in life followed by adult estradiol exposure for 4 months, the

incidence of PIN lesions significantly increased to 100% as compared to 40% in control Sprague-Dawley rats that received oil neonatally and estradiol in adulthood.

Importantly, the lesions were mostly classified as high-grade PIN and the severity and incidence was similar to that found in rats exposed neonatally to pharmacological levels of estradiol. As compared to controls, the PIN lesions in rats exposed to neonatal BPA and adult estradiol also exhibited significantly higher rates of epithelial cell proliferation and apoptosis which is considered key evidence that these are relevant precancerous lesions with similarity to human high-grade PIN, the precursor lesion to prostate cancer [40]. Taken together, these published findings suggest that early estradiol exposures predispose the prostate to PIN lesions with aging and that an environmentally relevant dose of BPA is capable of increasing susceptibility of the prostate gland to carcinogenesis brought on by elevated estradiol in the aging male animal.

### ***Developmental estradiol and BPA exposures alter the prostatic epigenome***

DNA methylation is one of three epigenetic systems that regulates mitotically heritable changes in gene expression that are not coded in the DNA sequence. DNA methylation occurs at the C<sup>5</sup> position of cytosine in CG dinucleotides (CpGs). In mammalian cells, CpGs are often found as aggregates, or *CpG islands* (CGI), in the promoter or 5' coding region of genes and methylation status at these sites can regulate gene transcription [41]. Simplistically, hypermethylation of CGIs will cause stable heritable transcriptional silencing while hypomethylation permits transcription. Once established in somatic cells, CpG methylation patterns within the genome remain relatively stable and are heritable through cell divisions except during early embryonic development and tumorigenesis when drastic alterations in DNA methylation occur.

Alterations in DNA methylation have been shown to contribute to both cancer initiation and promotion [42, 43] including prostate cancers [44, 45]. Furthermore, there is some evidence that early hormonal exposures can alter DNA methylation in reproductive tract tissues [46-48].

In this context we asked whether the molecular underpinning whereby brief exposure to estradiol or BPA during development could permanently affect prostate carcinogenic susceptibility might be a result of epigenomic alterations in DNA methylation of specific genes. To identify potential methylation-regulated genes in prostates exposed neonatally to estradiol and BPA, we used methylation-sensitive restriction fingerprinting (MSFR) which screens for novel CpG-rich sequences whose methylation status undergoes alterations following treatments [47, 49]. This approach allowed us to monitor epigenetic alterations over time as well as with different hormonal treatments. Importantly, this global screening method does not require the identities of the genes whose methylation status changes thus in addition to the suspected genes involved, one can identify novel genes that may play a role in developmental estrogenization of the prostate gland. Our preliminary screens identified over 50 DNA candidate sequences with repeatable methylation alterations across multiple samples and prostate lobes [38].

The candidate sequences were subsequently cloned and 28 unique candidate clones were identified (complete table in [38]) Sixteen candidates showed no homology with known rat genes while the remaining 8 genes were identified as PLC $\beta$ 3, NVP3, CARK, GPCR14, PDE4D4, PDGFR $\alpha$ , CAR-X1 and SLC12A2. Several of these genes are involved in signal transduction pathways including Na-K-Cl cotransport (SLC12A2),

MAPK/ERK pathway (PDGFR $\alpha$ ), phosphokinase C pathway (PLC $\beta$ 3), cAMP pathways (PDE4D4 and HPCAL1) and neural/cardiac development (CARX1, CARK). Since these signaling pathways play a role in cell cycle and/or apoptosis pathways within cells and tissues, it is intriguing to speculate that developmental estrogenic exposures may perturb proliferation/apoptosis equilibrium in the prostate gland through an epigenetic gene (de)regulation mechanism. These findings may also provide clues to previously unrecognized participants in prostate carcinogenesis.

We observed overlapping as well as unique methylation alterations for high-dose estrogen, low-dose estrogen and BPA. This suggests two important points. First, common prostatic genes may be epigenetically modified by different estrogenic compounds and doses suggesting common pathways which predispose to prostate carcinogenesis with aging. These identified candidates could be applicable not only to developmental estrogenic exposures but may provide clues to new participants in prostate cancer. Second, unique candidate genes specific to an estrogenic compound or dose may allow us to formulate specific epigenomic signatures which could serve as useful molecular markers for specific developmental exposures.

***Prostatic phosphodiesterase 4D4 (PDE4D4) expression is methylation-regulated by estradiol and BPA exposure***

We have initiated studies to determine whether altered DNA methylation due to neonatal estrogenic exposures results in altered gene expression. Phosphodiesterase type4, variant 4 (PDE4D4) was chosen for further characterization since the differentially methylated DNA fragment identified by MSRF corresponded to the 5' flanking region, the PDE4D4 fragment was consistently hypomethylated by all neonatal

estrogenic exposures and the changes were identified as early as day 10 of life. PDE4 is an intracellular enzyme that specifically degrades cAMP [50]. Downstream cAMP signaling pathways include PKA activation and phosphorylation of cAMP-responsive element binding protein (CREB) which regulates transcription of genes involved in cell growth and differentiation [51]. Thus persistent activation of cAMP pathways may contribute to neoplastic transformation. In this regard, recent studies have shown a tight association between PDE4 expression and cancer cell proliferation including gliomas [52], osteosarcomas [53] and chronic lymphocytic leukemia [54].

As previously detailed [38], PDE4D4 contains a 700 bp-CpG island with 60 CpG sites in the 5' regulatory region which encompasses the gene transcription and translation start sites. Methylation site mapping was performed by bisulfite genomic sequencing and a cluster was noted between 49-56 CG sites that became increasingly methylated in the normal control rat prostates with aging, reaching 100% methylation by 7 months of age. In contrast, these 49-56 CG sites remained hypomethylated in aging prostates exposed neonatally to high or low-dose estrogen or BPA. Importantly, these differential methylation patterns were inversely correlated to PDE4D4 gene expression as determined by real-time RT-PCR. Thus while normal aged rats contained low prostatic expression of PDE4D4, this gene was expressed at high levels in rats exposed to estradiol or BPA during development. Importantly, this differential gene expression pattern was observed prior to adult exposure to estradiol which indicates that molecular changes had occurred in the prostates of neonatal BPA-exposed rats which may have contributed to its increased predisposition to hormonal carcinogenesis as an adult. This later observation suggests that PDE4D4 methylation and/or gene expression may be a

useful early marker of adult-onset disease initiated by developmental estrogen exposures in the prostate gland.

### ***Conclusions***

In summary, we have shown that a range of estrogenic exposures during the early period of prostate development, from low-dose estradiol and environmentally relevant doses of BPA to pharmacological doses of estrogens, results in an increased susceptibility to preneoplastic lesions of the prostate gland with aging. Based upon these findings, we propose that estrogenic exposures during critical developmental periods may provide a fetal basis for adult prostatic diseases. Furthermore, we have obtained evidence that early exposures to estradiol or BPA can alter DNA methylation in a gene-specific manner which implicates epigenetic alterations as an underlying mechanism of action in developmental estrogenization. Since several of these genes are interconnected through similar signaling pathways, we predict that estrogen-induced alterations may produce complex changes within the prostatic cell that ultimately predispose the gland to carcinogenesis as the animal ages.

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