Early-life Estrogen Exposures Alter the Prostate Epigenome and Increase Prostate Cancer Risk

Gail S. Prins
University of Illinois at Chicago
The most prevalent non-cutaneous cancer in American males.

In 2006, 189,600 new cases; 26,900 deaths in US (2008 ACS statistics)

Second leading cause of cancer deaths in males – of similar magnitude to breast cancer in females and AIDS.

Ethnicity a major risk factor:
- 2:1 incidence ratio in African-American males vs Caucasian in US.
- Asian males have lowest incidence.

Age-related incidence: up to 30% of males over 50 years harbor silent prostate cancer.

% of men in US developing invasive PCa with age:
- Birth - 39 years: < 1 in 10,000
- 40 - 59 years: 1.83 % (1 in 45)
- 60 - 79 years: 14.79 % (1 in 7)
- Birth to Death: 17.00 % (1 in 6)
**Prostate Development**

**Male Accessory Sex Glands:**
- **Prostate:**
  - Urogenital Sinus *endodermal*
- **Seminal Vesicle, Vas Deferens, Epididymis:** Wolffian Duct *mesodermal*

**Human Fetal Prostate Development**

(from Cunha, *Endo Reviews*, 1987)

[Diagram showing the development of the prostate and related structures, including the endodermal and mesodermal origins of different organs, and the timeline of Androgen and Estrogen activity from birth to 40 weeks.)
Developmental Origins of Adult Disease Paradigm

Developmental Programming or Reprogramming: Critical periods (windows) in fetal and/or neonatal development when insults or stimuli have long-lasting (i.e. life-spanning) effects.
DEVELOPMENTAL ESTROGENIC EXPOSURES

Elevated Maternal Estrogens
- AA 40% higher E$_2$ during early PG vs Caucasians.
- ~ 2:1 AA-to-Caucasian ratio in PCa incidence. (Henderson, Ross, ‘88)
- Indicators of PG E$_2$ levels (pre-eclampsia, jaundice, gestation length): significant correlation between elevated E$_2$ levels and PCa risk (Ekbom, 1996, 2000)

DES Exposure
- Taken by PG women b/t 1950 - 1975
- Increase in vaginal clear-cell carcinoma in exposed daughters (Herbst, 1972)
- Neonatal prostatic abnormalities in humans (Driscoll & Taylor, ‘80)
- Rodent models: predisposed to male repro tract dysplasia, prostatic tumors (Arai, ’78; McLachlan, ‘75)

Endocrine Disrupting Chemicals (estrogen mimics)
- methoxychlor (Cooke,90); bisphenol A (Nagel, ’97) development exposure affects prostate growth.
- bioaccumulation in placenta and breast milk
Environmental Endocrine Disruptors

Sources for BPA

1. Polycarbonate plastic bottles
2. Dental sealings
3. Epoxy resins: Tin can linings

BPA monomers released upon heating

Welshons, W. V. et al. Endocrinology 2006
Human Exposures and Levels of BPA

- Most humans are exposed to BPA.
  - 93% of humans tested have BPA metabolites in urine (CDC, 2004)

- *Unconjugated* BPA in human serum is in the 0.3 to 5 ng/ml range.

- Present in breast milk, amniotic fluid, and placental tissue.

- BPA in fetal serum, umbilical cord blood, amniotic fluid and placenta indicate that the developing *human fetus* is chronically exposed to BPA:
  - 0.7-9.2 ng/ml range (*unconjugated* BPA)
Certainty Hierarchy for BPA and Carcinogenesis


1. What is certain
2. What is suggested
3. What is uncertain

What is certain:

1. Reproductive toxicity due to perinatal diethylstilbesterol (DES).
2. Prenatal DES exposure increases risk of breast cancer and uterine fibroids in women and animal models.
3. Estrogen is a carcinogen.
4. BPA acts as an endocrine disruptor with some estrogenic properties (among others).
What is suggested:

1. High-dose BPA increased hematopoietic cancers and Leydig cell tumors (testes) in rodents, suggestive of carcinogenesis. (NTP-1982)

2. Early life exposure to low-dose BPA may induce or predispose to pre-neoplastic lesions of mammary gland and prostate in adult rodents.

3. BPA alters microtubule function and can induce aneuploidy in some cells/tissues at environmentally relevant doses.

4. BPA may induce cellular transformation.

5. In advanced prostate cancers with specific AR mutations, BPA may promote tumor progression and reduce time to recurrence.
Certainty Hierarchy for BPA and Carcinogenesis

What is uncertain:

1. Whether developmental BPA exposures increase mammary or prostate carcinogenic risks in humans.
2. Whether BPA exposure directly induces or promotes cancers in mammary and prostate glands.
3. Whether BPA increases cancer susceptibility in all estrogen-target organs (prostate, mammary gland, uterus, vagina, testis, ovary, etc).
4. Mode of action for BPA effects. Estrogenic pathways? Epigenetics?
Conclusions:

1. **Some concern** for neural and behavioral effects of BPA in fetuses, infants and children at current human exposures.

2. **Some concern** for BPA exposure in fetuses, infants and children at current human exposures based on effects in the prostate gland.

3. **Minimal concern** for BPA exposure in same cohort based on effects in the mammary gland and early puberty in females. (Board of Scientific Counselors)

4. **Negligible concern** that BPA exposure of pregnant women will result in fetal/neonatal mortality, birth defects, ↓ birth weight and growth in offspring.

5. **Negligible concern** that BPA exposure causes reproductive effects in adults and **minimal concern** for workers exposed to higher levels in occupational settings.
Rat Prostate Development

Initiation & budding

- Determination
- Branching morphogenesis
- Differentiation
- Maturation

Birth Development

- Day 1: mesenchymal pad, UGS, proximal
- Day 2: mesenchymal pad, UGS, proximal
- Day 3: distal tips, proximal
- Day 4: distal tips, central, proximal
- Day 5: distal tips, central, proximal
- Day 6: distal tips, central, proximal

Birth

T A M SV P
Developmental Estrogenization of the Prostate Gland

McLachlan, et al. (1975)
Arai & Bern (1975)
Rajfer & Coffey (1978, 1979)
Prins (1992)

Prostate:
Lobe-specific
Developmental and Differentiation Defects

↑ Dysplasia Tumors

Birth PND 1-5 Puberty
Estrogen induced changes in prostate gene and protein expression

- **Steroid Receptors**
  - AR
  - ER$\alpha$, ER$\beta$
  - PR
  - RAR $\alpha$, $\beta$, $\gamma$, RXR $\gamma$
    - RALDH2, CYP26
    - CRABPs 1 and 2
    - retinoids

- **Homeobox Genes**
  - Hox-13b, Hox-13a, Hox-13d
  - Nkx3.1

- **Secreted Morphogens:**
  - TGF$\beta$
  - Bmp-4, Bmp-7
  - Fgf-10 + FgfR2iiib
  - Sonic Hedgehog + ptc & glis
  - Wnt5a

- **Extracellular matrix molecules:**
  - laminin, fibronectin, collagens,
  - MMPs, tPA

- **Cell Adhesion Molecules**
  - e-cadherins
  - gap junctions (Cx32, Cx 43)

- **Oxidative Stress Enzymes**
  - SOD
  - CYP1B1
  - COMT

Huang et al, *J Andrology*, 2004
Dose-response profiles

Vom Saal et al. (1997)

Low-dose non-monotonic dose-response of prostate to estradiol and DES, BPA
  - mice
  - fetal exposure

- Does low-dose effect really exist?
- Reproducibility?
- Species or even strain differences?
- Does it drive prostate pathology?
Dose-response profiles:

0.015 to 1500 estradiol μg/kg BW: SD rats

Prostate weights at day 90

Cellular differentiation in VP

<table>
<thead>
<tr>
<th>Control</th>
<th>Low-dose</th>
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<td>[Image]</td>
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</table>
Developmental Basis of Prostate Cancer: Two Hit Model
Animal model (Sprague-Dawley rats)

**First Hit:**
Day 1, 3, 5
1. Oil (acts as control)
2. Estradiol: high dose, 2500 μg/kg BW
3. Estradiol: low dose, 0.1 μg/kg BW
4. Bisphenol A: low-dose, 10 μg/kg BW

**Second Hit:** T + E, 16 weeks
Empty capsules

**predicted serum unconjugated BPA:** ~ 0.5 - 2ng/ml

Day 200: Histologic analysis (blinded)
- PIN scores (grade 0-3)
- PIN incidence
- proliferation & apoptosis
Dorsal Prostate PIN Scores

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<th>Oil</th>
<th>High EB</th>
<th>Low EB</th>
<th>BPA</th>
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* Indicates significant difference.
Dorsal Prostate PIN Scores

- Oil
- High EB
- Low EB
- BPA

**High EB**
**Low EB**
**BPA**

Adult T+E

Relative PIN Scores

![Images of tissue samples](High EB, Low EB, BPA)
Neonatal estrogens and BPA increase prostatic epithelial cell proliferation and apoptosis

- Developmental estrogenic exposures initiate or activate precancerous pathways resulting in an imbalance in cell proliferation and apoptosis which may contribute to prostate pathology with aging.
Developmental Estradiol/BPA Pathology Summary:

- A range of estradiol exposures - from “low dose” or biologically elevated levels to “high-dose” pharmacologic exposures predispose the prostate gland to PIN lesions with aging.

- Neonatal exposure to environmentally relevant doses of Bisphenol A may increase susceptibility of the prostate gland to carcinogenesis following additional adult insults.

How does a brief low-dose exposure permanently alter the “memory” of prostate cells long after hormone withdrawl?
Estrogen Action

ACTIVATION
– Adult effects: **Reversible**
– Response lasts as long as hormone exposure lasts.
– ex: cyclic changes in uterine receptivity

ORGANIZATION
– Developmental effects: **Irreversible**
– Structural organization; i.e. of tissues, circuitry, cell types
– Epigenomic organization: DNA & chromatin modifications which organize genetic information
**Epigenetic Modification:**
Mitotically *heritable* alterations in gene expression that are *not* caused by changes in the DNA sequence

I. DNA methylation at CpG sites, CpG islands
II. Histone methylation: H3K4 (activate), H3K27 (silence)
III. Non-coding RNAs: siRNA, micro RNA

**Hypothesis:**
Estrogen reprogramming of the prostate may be mediated, in part, by *epigenetic alterations* in developmental *methylation patterns* of prostatic genes.

- McLachlan demonstrated abnormal demethylation of CpG/-464 lactoferrin promoter of mouse uteri following neonatal DES
  (Li et al, Can Research 57:4356, 1997)
DNA methylation occurs at 5’ position of cytosine in CpG dinucleotides.
CpG often found as aggregates, CpG islands, which are methylation targets.
40-50% of all human genes are be associated with promoter CpG islands.
Catalyzed by DNA methyltransferases (DMNTs); SAM serves as methyl donor; methylated DNA binding proteins (MeCPs and MBDs) are involved.
DNA methylation in Cancer

- Alterations in DNA methylation can contribute to cancer initiation and promotion, including prostate cancers.

- Either hypomethylation or hypermethylation of DNA:
  - Global hypomethylation $\rightarrow$ chromosomal instability
  - Gene-specific hypo- or hypermethylation $\rightarrow$ inappropriate gene transcriptional activities:
    - hypermethylation $\rightarrow$ silencing of tumor suppressor genes
    - hypomethylation $\rightarrow$ expression of oncogenes
Experimental Work-Flow

Genomic DNA extracted from tissues

MSRF
(Methylation Sensitive Restriction Fingerprinting)

DNA extracted from gel and re-amplified by PCR

TA cloning (pCR2.1 vector)

Sequencing / BLAST / BLAT search

Data confirmation

Real-Time PCR

Bisulfite Sequencing Methylation-specific PCR
**MSRF (Methylation Sensitive Restriction Fingerprinting)**

- MseI digest:
  - TTAA
  - TTAA
  - TTAA
  - TTAA
  - TTAA
  - TTAA

- BstUI digest:
  - Red squares

- PCR product:
  - Black lines

- **MSRF screens for global methylation patterns** in CpG-rich DNA sequences.
- Gene identity not required to examine changes in methylation status.
## Candidate clones identified from MSRF

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<tr>
<th>Clone Name</th>
<th>Primer 1</th>
<th>Primer 2</th>
<th>Hypermethylation</th>
<th>Chromosome band</th>
<th>Gene homology</th>
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<th>Related pathways</th>
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**CAR-X1**: Carbonic anhydrase-related X1 protein  
**PLCbeta3**: Phospholipase C beta-3  
**SLC12A2**: Solute carrier family 12 member 2  
**HPCAL**: Neural visinin-like Ca2+ binding protein  
**CARK**: Cardiac ankyrin repeat kinase  
**GPCR14**: G-protein coupled receptor 14  
**PDE4D4**: Phosphodiesterase type 4 variant 4  
**PDGFRα**: platelet-derived growth factor receptor
**Phosphodiesterase type IV variant 4 (PDE4D4)**

- Functions in cAMP degradation
- Maintains cAMP in a narrow concentration range that is critical for growth and differentiation
- Involved in tumor growth by inhibiting cAMP activity in glioma, osteosarcoma and lymphocytic leukemia cells (Chen et al., 2002; Narita et al., 2003; Lerner et al., 2000)
Large CpG island (GC% >60%) was identified in rat PDE4D4 5’ flanking region.

By nested PCR, amplified a 690 bp region in promoter/exon1 of PDE4D4 with 60 CG dinucleotides.

Methylation status of CG sites in the PDE4D4 5’ regulatory region

Bisulfite genomic sequencing

DP: Day 200 with T+E₂

60 CG sites in 5’ end of PDE4D4
PDE4D4 gene: *Hyper*-methylated and silenced with aging

*Hypo*-methylated and up-regulated with neonatal $E_2$/BPA

Methylation-specific PCR

Real-time RT-PCR
Normal (NbE1) and tumorigenic (AIT) rat prostate cell lines: Methylation of PDE4D4 promoter mirrors estrogenized prostates

Demethylation with 5-aza-dC increases PDE4 gene expression
Direct effects of BPA or Estradiol on PDE4D4 in normal (NbE1) and tumorigenic (AIT) rat prostate cell lines:

- BPA and E2 increase PDE4D4 expression through gene hypomethylation in normal but not tumorigenic cells.
- This is blocked by ICI suggesting ER dependent mechanism.

MS-PCR of PDE4D4
Effects of BPA and E₂ on DNMT (DMNT1, DMNT 3a, DMNT3b) and Methylated Cytosine Binding Proteins: MBDs and MeCPs (MBD1, MBD2, MBD3, MBD4 and MeCP1 MeCP2)

MBD2: Methyl CpG Binding Domain Protein 2:
- Putative DNA *demethylase*.
- BPA and E₂ increase MBD2 expression in dose-dependent manner in normal rat epithelial cells (NbE-1) but not tumorigenic epithelial cells (AIT).
- ICI blocks this response; *ER-dependent process*. 

![Diagram showing DNA Methylation and Repression by MeCP2]
Does elevated MBD2 regulate PDE4D4 methylation and expression?

- In normal cells (NbE-1) where BPA and E₂ hypomethylate PDE4D4 and increase gene expression, *blockade of MBD2 with antisense RNA blocks this estrogen-induced effect.*

- **Conclude:** BPA and E₂-induced increase in MBD2 expression mediates PDE4D4 hypomethylation and ↑ gene transcription in prostate cells.
Human PDE4D4 in Normal PrEp cells: 5-aza-dC demethylated PDE4D4 promoter and reactivated PDE4D4 expression

PDE4D4 expression is regulated by methylation in human prostate cells
PDE4D4 is methylated in normal prostate and hypomethylated in tumor tissue
Hippocalcin-like protein 1 (HPCAL1): stimulates cAMP production

- HPCAL1 expression is regulated by methylation
- HPCAL1 is primarily unmethylated and expressed in normal prostate tissues and cells
- Early BPA exposure results in HPCAL1 methylation and reduced expression
- In prostate cancer cells and tissues with ↑ dysplasia, HPCAL1 becomes methylated with reduced gene expression
The prostate epigenome is altered by early life exposure to estradiol or bisphenol A.

By MSRF, more than 50 candidate clones were identified and found to be differentially methylated between oil-treated controls and tissues exposed neonatally to estradiol or bisphenol A.

Gene-specific changes in methylation of cAMP regulators resulted in altered gene expression with variations across doses and exposures:

- **PDE4D4**: hypomethylated by high and low estradiol, BPA
  → expression levels throughout life
- **HPCAL1**: hypermethylated with aging by neonatal BPA
  → expression in aged prostate and with carcinogenesis

These and other candidate genes may alter signaling transduction pathways that contribute to prostatic carcinogenesis with aging.
Working Model: Epigenetic basis for developmental reprogramming of the prostate gland

**Fetal**
- Determination
- Initiation & budding
- Branching morphogenesis
- Birth

**Neonatal**
- Progenitor cells
- Cytodifferentiation

**Pubertal**
- Functional differentiation
- Secretion

**Adult**
- Outbred Sprague-Dawley
- Adult insults
- PIN PCa

Exposure (E₂, BPA)
- Epigenetic changes
- Altered or imprinted progenitor cells
- Differentiation defects
ACKNOWLEDGMENTS

PRINS LAB
• Jessica Belmonte, BS
• Lynn Birch, MS
• Oliver Putz, PhD
• Yong-Bing Pu, MD, PhD
• Liwei Huang, MD, PhD
• Doug Luccia-Camelo, PhD
• Wen Hu, MD, PhD

Ho LAB University of Cincinnati
• Winnie Tang, Ph.D.
• Robert Cheng, D.V.M.