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TITLE: INVESTIGATION OF A PUTATIVE ESTROGEN-IMPRINTING GENE,
PHOSPHODIESTERASE TYPE IV VARIANT (PDE4D4), IN DETERMINING
PROSTATE CANCER RISK

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14. ABSTRACT: Estrogens are known to play a role in the initiation and progression of prostate cancer. Recently, environmental factors such as xenoestrogens have been reported on their prevalence of prostate diseases or cancers. Estrogen imprinting of the prostate gland is believed to associate with an increased incidence of prostatic lesions including inflammation, epithelial hyperplasia, squamous metaplasia, dysplasia and adenocarcinoma. And DNA methylation may be one of the possible mechanisms of the prostate reprogramming. By using one of the global methylation profiling techniques, MSRF, a gene called phosphodiesterase type IV variant 4 (PDE4D4) was shown to be hypomethylated following neonatal exposure to estradiol (EB) or bisphenol A (BPA). We further confirmed the persistence of PDE4D4 promoter hypomethylation and gene up-regulation in the adult life by using bisulfite genomic sequencing and real-time PCR. PDE4D4 has function of cAMP-degradation to maintain the second messenger, cAMP, in a narrow range of concentrations that is critical for growth and differentiation of the hormone target cells by activating several downstream signaling molecules. Taken together, these findings supported that PDE4D4 dysregulation, via CpG island hypomethylation, at its promoter regions in early life, by EB or BPA, can alter its expression and activity of the gene. Present data also suggested that PDE4D4 can be used as a biomarker for prostate cancer assessment.					
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Introduction

Background:

Steroid hormones such as estrogens are known to play a role in the initiation and progression of prostate cancer. Recent studies have also proposed environmental factors such as xenoestrogens on the prevalence of prostate diseases or cancers. Estrogen imprinting of the prostate gland is believed to associate with an increased incidence of prostatic lesions including inflammation, epithelial hyperplasia, squamous metaplasia, dysplasia and adenocarcinoma. However, there is little information about how estrogen imprints prostate gland.

DNA methylation induced by estrogens may be one of the possible mechanisms of the prostate reprogramming. Using the technique called Methylation-sensitive restriction fingerprinting (MSRF), I have successfully cloned and identified one candidate gene, phosphodiesterase type IV variant (*PDE4D4*), in lateral prostate lobes in Sprague Dawley rats. *PDE4D*, a cAMP-phosphodiesterase gene, has function of cAMP degradation to maintain the second messenger, cAMP, in a narrow range of concentrations that is critical for growth and differentiation of the hormone target cells by activating several downstream signaling molecules. My preliminary result data showed that *PDE4D4* was hypomethylated following neonatal exposure to estradiol (EB) or bisphenol A (BPA). Importantly, the expression remained at a high level during adult life. Taken together, these findings supported the possibility that *PDE4D4* dysregulation, via CpG island hypomethylation, at its promoter/exon regions in early life, by EB or BPA, can alter its expression and activity of the gene. These events could then activate several cAMP-dependent signaling pathways leading to abnormal cell proliferation or differentiation in prostate gland.

Hypothesis:

Early estrogen exposure was demonstrated to alter epithelial cell differentiation, predispose the gland to the development of hyperplasia, severe dysplasia and carcinoma. I hypothesize the *PDE4D4* gene dysregulation, via CpG island hypomethylation, at its promoter/exon regions in early life by estrogens or xenoestrogens can alter the expression and activity of the gene. It will then trigger

several cAMP-dependent signaling pathways that lead to cell proliferation and differentiation. Since the proliferative rat DP/LP lesion closely resembles human PIN that are the purported precursors of prostate cancer, I further hypothesize *PDE4D4* plays an important role in determining cell proliferation of human prostate gland to the development of prostatic dysplasia and carcinoma.

Specific Aim1: To investigate the gene regulation of *PDE4D4* under the influence of estrogen or BPA by studying its promoter activity

To achieve this aim, genomic DNA isolated from samples will be undergone bisulfite genomic sequencing to study the estrogenic effect on *PDE4D4* promoter methylation status. Moreover, using serial deletion analysis and luciferase reporter assay, I can determine how *PDE4D4* promoter is regulated by EB and BPA. Results from the transcription factors binding assays such as ChIP, I can identify and characterize which transcription factors regulate the promoter activity of *PDE4D4*.

Specific Aim2: To investigate whether *PDE4D4* is responsible for determining cell proliferation and can be used as a biomarker to diagnosis prostate diseases and prostate cancer.

Rat prostate epithelial cells (NBE-1) will be transiently transfected with full-length clone of *PDE4D4*. The intracellular cAMP level, PKA, Ras-PI3K, PKC activity and cell proliferation markers expression will be measured by cAMP assay, Western blot analysis and MTS assay. Also, by using technique of immunohistochemistry, localization of *PDE4D4* in normal or tumor epithelial cells of rat prostate can be examined so as to understand whether *PDE4D4* involves in tumor cell development.

Specific Aim3: To study whether *PDE4D4* expression is linked to human prostate disease.

Full-length clone transfection or suppression of *PDE4D4* variant by methylated oligonucleotides or siRNA will be performed in human normal prostate epithelial cells (NPrEC) and prostate cancer cells (LNCap, DU145 and PC3) to examine the role of *PDE4D4* in growth of human prostate cancer cells.

Relevance:

Recently, the National Toxicology Program released a report from the Endocrine Disruptors Low-Dose Peer Review Panel which sighted the need for the identification of molecular and biochemical markers as sensitive indicators of prostatic abnormalities following low-dose estrogen and xenoestrogen exposures during development. MSRF is a highly innovative and reliable technique to find out specific and novel genes undergone methylation or demethylation following estrogen exposure. My study in characterization on PDE4D4 in prostate cancer development will be the first to investigate how environmental factors may affect early gene imprinting and the incidence rate of prostate disease. By investigating whether PDE4D4 regulates the cAMP-dependent signaling pathways such as PKA and Ras in prostate cancer development, we can hopefully find a new paradigm for treating prostate cancer.

Body

I. Tasks outlined in the approved Statement of Work

Task 1

Target: a) To confirm the methylation status of *PDE4D4* and b) to study the relationships between transcription factors (TF) binding and promoter activity following exposure to low- or high-dose of estrogen (EB) or bisphenol A (BPA)

Time Period: Month 1-6

Method: a) Bisulfite Genome Sequencing will be applied. Genomic DNA will be first modified with sodium bisulfite using the CpGenome™ DNA Modification Kit (Intergen, USA). Bisulphite treatment of DNA specially converts unmethylated cytosine residues, but not 5-methylcytosine (5mC), to uracil by deamination. When modified DNA is subjected to nested PCR, uracil residues are amplified as thymine and unmodified 5mCs as cytosines. Using Primer3 and MethPrimer analysis, two pairs of primers were specially designed complementary only to the completely converted DNA to allow nested-PCR to amplify most of the putative CpG islands. The amplified products will be

cloned using pCR 2.1 TOPO vector (Invitrogen, USA) and 8-10 clones will be sequenced to reveal the methylation status of CpG dinucleotides in the CpG island of PDE4D4 in each genomic sample.

b) Rat prostate epithelial NBE1 cells are used to perform transient transfection with a plasmid construct of different deletions in the TF binding sites located on the promoter region. Plasmid construct is made after cloning nested PCR products with different deletions into pGL3 basic luciferase reporter vector and the estrogen-induced promoter activity will be examined by luciferase assay.

Task 2

Target: To investigate the expression of other *PDE4D* variants following neonatal exposure to EB or BPA

Time Period: Month 7-8

Method: I will identify which *PDE4D* variants expressed most in prostate after estrogen exposure by Western blot analysis or real-time PCR with specific antibody or pairs of primers complementary to each *PDE4D* variants respectively. Using the Genome Browser from the Center for Biomolecular Science and Engineering, University of California, I identified eight more *PDE4D* variants. Theoretically, only *PDE4D4* contains CpG island at its 5' flanking regions. However, I will design 8 exon-exon spanning variants-specific primer pairs (which ensure detection of RNA transcripts and not products from genomic DNA, if any) to detect all the variants expression. By using the SYBR Green Real Time PCR, I will detect expression of all 9 RNA transcripts in all tissue samples. Gene expression level among different variants will be analyzed by $\Delta\Delta C_t$ method and determined whether there is significantly changes among groups by use of ANOVA statistical analysis.

Task 3

Target: a) To investigate whether PDE4D4 variant is responsible for determining cell proliferation and b) to determine whether PDE4D4 can be used as a biomarker to diagnosis prostate diseases and prostate cancer.

Time Period: Month 9-18

Method: a) Rat prostate epithelial cells (NBE1) will be transiently transfected with full-length clone of *PDE4D4*. Intracellular cAMP in cells transfected with *PDE4D4* will be assayed. Furthermore, protein extracted from cells can be applied to the PKA assay kit. Beside cAMP-dependent PKA signaling pathway, Ras-PI3K, Ras-Raf or PKC signaling molecules will also be investigated by Western Blot analysis of the whole protein lysate. To see whether *PDE4D4* affects cell proliferation, MTS assay on the cells that transfected with the full-length *PDE4D4* clone will be examined. Also, gene expression level of proliferation markers such as p42/p44, cyclin A, cyclin D1, p21^{WAF}, and p27^{CIP1} will be studied by RT-PCR.

b) Immunohistochemistry (IHC) will be performed by using a specific antibody for *PDE4D4*. Different prostatectomy tissue sections from rat with BPH, PIN, HG-PIN and adenocarcinoma will be undergone IHC. Positive staining of *PDE4D4* is defined as continuous and dark cytoplasmic or apical granular staining pattern in the cells of the glands. Each positive case is evaluated for the percentage of the glands/cells that reacted with *PDE4D4* and scored as <5%, 6-50%, 51-75% and >75%. RNA will be extracted, reverse transcribed and undergone real-time PCR with specific primers of *PDE4D4* from laser capture microdissection (LCM) samples.

Task 4

Target: To study whether the *PDE4D* transcripts expression linked to human prostate disease

Time Period: Month 19-24

Method: *PDE4D* variants expression level in human normal prostate cells (NPrEC) or prostate cancer cells (DU145, PC3 and LNCap) will be illustrated by real time PCR. Thereafter, normal cells will be transfected with full-length clone of *PDE4D4*. I will examine the intracellular cAMP level, protein expressions of cAMP signaling molecules and cell proliferation markers in *PDE4D4*-transfected NPrEC and compare the results from that of prostate cancer cells. Suppression of *PDE4D* variants by methylated oligonucleotides or siRNA in cancer cells will be performed to see whether inhibition of *PDE4D* activity or expression can promote tumor cell death. Cell death can be illustrated by Annexin V binding assay or caspases 3, 8 and 9 assays.

II. Work accomplished under Task 1

Part a) Investigation of the methylation status of *PDE4D4* among all tissues samples

I successfully used the bisulfite genomic sequencing to identify the methylation status of *PDE4D4* in rat prostate that neonatally exposed to EB and BPA. Figure 1A indicated the gene organization of rat *PDE4D4* 5' flanking region and the schematic of CpG content in its 5' regulatory region. As shown is Figure 1B, although most CG sites were unmethylated, a methylated cluster was noted between CG sites 49 to 56. The methylation frequency at this cluster progressively increase in the oil-control prostate as

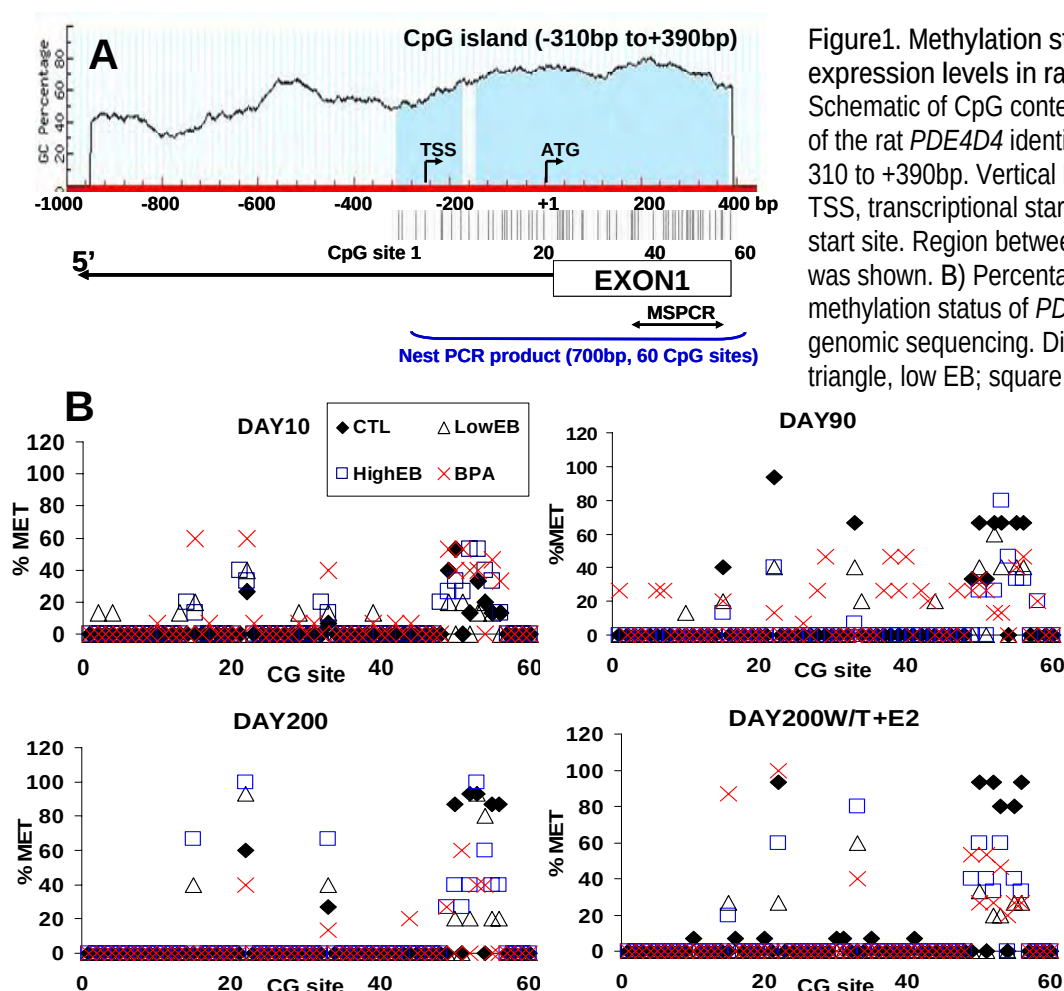


Figure1. Methylation status of *PDE4D4* and expression levels in rat prostate samples. A) Schematic of CpG content (%) in the 5' flanking region of the rat *PDE4D4* identifies a CpG island between -310 to +390bp. Vertical lines, individual CpG sites; TSS, transcriptional start site and ATG, translation start site. Region between -310to +390bp for BS-PCR was shown. B) Percentage methylation represents methylation status of *PDE4D4* assayed by bisulfite genomic sequencing. Diamond, oil-treated control; triangle, low EB; square, high EB and cross, BPA.

the animals aged, reaching by 100% methylation by day 200. In contrast, this cluster remained unmethylated throughout life in EB/BPA-treated prostates. The data suggested that EB or BPA hypomethylated *PDE4D4* promoter in rat prostate and this phenomenon persist in aging. It also associated with the incidence rate of dysplasia in rat prostate (Ho et al., 2006). In order to study the promoter activity and transcription factors binding assays *in vitro*, a cell line model including normal prostate epithelial NBE-1 cells and prostate tumor AIT cells should be included. We found that *PDE4D4* was hypomethylated (active transcribed) in tumor AIT cells while silenced in normal NBE-1 cells (Figure 2). It mirrored the data we obtained from *in vivo* studies. We further used this cell line model for experiment proposed in Task 1b and Task 3.

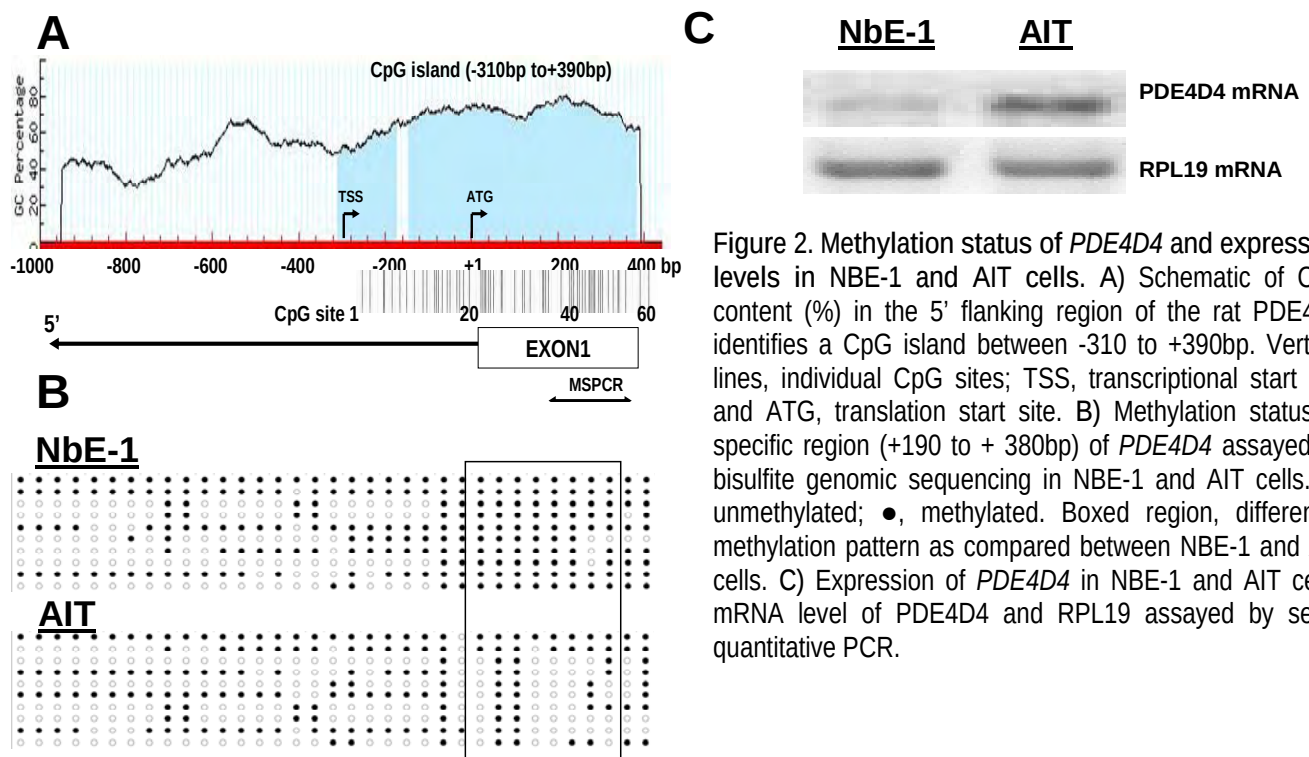


Figure 2. Methylation status of *PDE4D4* and expression levels in NBE-1 and AIT cells. A) Schematic of CpG content (%) in the 5' flanking region of the rat *PDE4D4* identifies a CpG island between -310 to +390bp. Vertical lines, individual CpG sites; TSS, transcriptional start site and ATG, translation start site. B) Methylation status of specific region (+190 to +380bp) of *PDE4D4* assayed by bisulfite genomic sequencing in NBE-1 and AIT cells. ○, unmethylated; ●, methylated. Boxed region, differential methylation pattern as compared between NBE-1 and AIT cells. C) Expression of *PDE4D4* in NBE-1 and AIT cells. mRNA level of *PDE4D4* and *RPL19* assayed by semi-quantitative PCR.

Part b) Characterization of *PDE4D4* promoter activity and transcription factors binding at its promoter region

I completed to characterize the promoter activity of NBE-1 cells by both *in silico* analysis and promoter luciferase reporter assay. I first used 5' Rapid amplification of cDNA ends (5'RACE) to characterize/localize the promoter region of *PDE4D4*. Transcription Start Site (TSS) was found at -251 away from the translation start site

(ATG). Next, I used the Promoter Scan Program provided by UCSC to identify the potential regulatory elements along the promoter. Estrogen response element (ERE) half site, Sp1 and cAMP response element binding protein (CREB) were computationally identified in the CpG island of *PDE4D4* (Figure 3).

Luciferase reporter plasmids containing different deletions in this 5' flanking region were constructed and transfected into NBE-1 cells to determine which region is specific for *PDE4D4* transcription. As shown in Figure 3, the promoter activity had a 30-fold increase in luciferase production as compared with a promoter-less construct. The promoter construct contained the entire CpG island (-310 to +350) therefore, suggested that this CpG island directed transcription more efficiently. Notably, construct encompassing CREB binding site (R4) showed a 20-fold luciferase production as compared to CREB-deleted construct (R2). It consistent to the data reported that *PDE4D* promoter could be induced by cAMP thru CREB binding (D'Sa C et al., 2002; Vicini E and Conti M 1997). Our findings confirmed this fact and suggested *PDE4D4* may involve in cAMP-dependent signaling pathways that may contribute to cell proliferation of prostate,

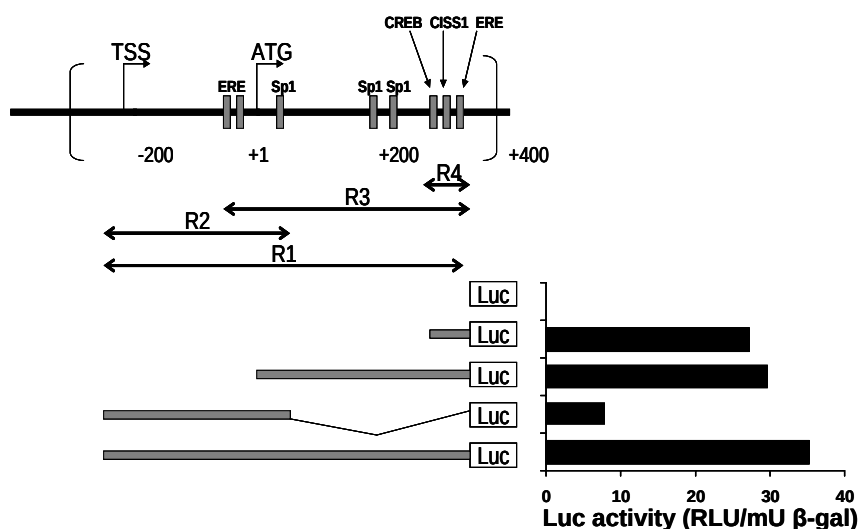


Figure 3. Characterization of 5' *PDE4D4* promoter. Upper panel, Schematic representation of the 5'-flanking region of *PDE4D4*. Bases are numbered from the translation start site. Potential regulatory elements are boxed. In lower left panel, a representation of the PDE-luc plasmids containing different 5'- and 3'-deletions of the -400 to +400bp genomic region is reported. Transient luciferase expression in NBE-1 cells transfected with the PDE-luc plasmids described in lower right panel. Luciferase activity is expressed as RLU normalized by β-gal activity.

III. Work accomplished under Task 2

Investigation of the expression of other *PDE4D* variants following neonatal exposure to EB or BPA

Based on the NCBI and UCSC nucleotides database, there are nine *PDE4D* variants in rat genome. 9 exon-exon spanning variants-specific primer pairs were used to detect all variants expression by real-time PCR. Gene expression level among different variants were analyzed by $\Delta\Delta C_t$ method and determined whether there were significantly changes among groups by use of ANOVA statistical analysis. Results from

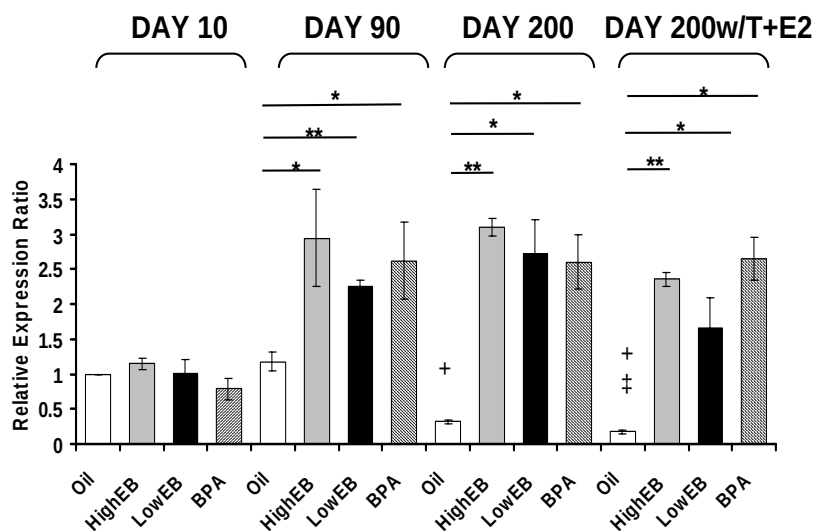


Figure 4. *PDE4D4* mRNA transcript levels as determined by real-time PCR. $\Delta\Delta C_t$ method was used to quantitate the mRNA transcript level. *PDE4D4* expression level was normalized by a house-keeping gene, *RPL19*. Relative expression ratio of *PDE4D4*/*RPL19* of day 10 oil samples was set as 1.0. Results represented the mean $\pm$ SD of three independent sets of experiment. All EB/BPA groups at day 90, 200, and 200/testosterone+estradiol were significantly different from the respective groups at day 10. *, $P < 0.05$; **, $P < 0.01$ versus oil controls at the same time intervals; +, $P < 0.05$ versus day 200 oil controls; ++, $P < 0.05$ versus day 10 oil controls.

real-time PCR showed that there was no difference in gene expressions of eight variants (*PDE4D1-3*, *5-9*) among the oil-control and estrogens-treated groups. Only up-regulation of *PDE4D4* occurred in EB/BPA-treated groups (Figure 4). It matched our preliminary results from *in silico* database analysis among nine variants, suggesting that only *PDE4D4* contains CpG island at its 5' flanking regions and its gene regulation can be regulated by DNA methylation. As shown in Figure 4, *PDE4D4* down-regulated in oil control samples with aging. In contrast, *PDE4D4* expression levels of prostate neonatally exposed to EB and BPA were markedly higher at Day 90 than oil control and remained elevated with aging. It concluded that *PDE4D4* is normally silenced with aging but remained active transcribed in the neonatally estrogenized prostates. Furthermore, I found that the elevated expression of *PDE4D4* in EB/BPA-treated prostates occurred before a second

hit of exposure to hormones (testosterone+estradiol). It suggested that *PDE4D4* may have potential as a marker for early prostate cancer risk assessment.

IV. Work accomplished under Task 3 (part a only)

Part a) Investigation on the role of PDE4D4 in cell proliferation.

Full-length transcript of *PDE4D4* was constructed in a pcDNA3.1 plasmid and transfected into normal and tumor cells followed by cell proliferation assay and cAMP assay. In Figure 5A, normal NBE-1 cells, having lower expression level of *PDE4D4*, increased not only *PDE4D4* mRNA level but also cell viability (>60%) after transfection. Transfected AIT cells were also found to increase cell proliferation (~30%) but the increase was less significant as compared to normal cells. *PDE4D4* is known to hydrolyze intracellular cAMP. Therefore, intracellular cAMP level after transfection with *PDE4D4* construct could reflect *PDE4D4* activity in cells. Cells transfected with *PDE4D4* construct showed decrease in cAMP level suggesting up-regulation of *PDE4D4* in cells resulted in greater amount of cAMP being hydrolysed. Although more functional assays such as cell death, PKA activity should be performed to study how *PDE4D4* regulates cell growth and involves in prostate carcinogenesis, present data indicated that *PDE4D4* could alter the cell proliferation in normal NBE-1 and tumor AIT cells.

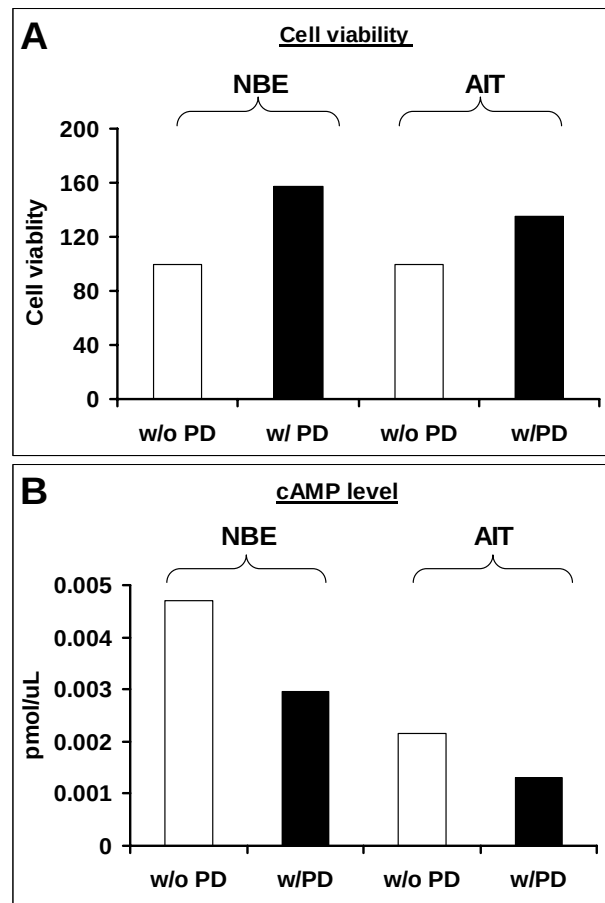


Figure 5. Cell viability and cellular cAMP level in NBE and AIT cells after transfection with full-length transcript of *PDE4D4*. Panel A. Cell viability assayed by MTS assay, Open bar, pcDNA3.1 plasmid only; solid bar, plasmid construct containing full-length transcript (2.4kb) of *PDE4D4*. Panel B, cAMP level assayed by cAMP HTS Immunoassay (Upstate) according the manufacturer's protocol. The chemiluminescence intensity was measured by a luminometer (Wallac 1420, Perkin Elmer) and represented as the Relative Light Units (RLU).

V. Remaining work to be performed under Task 3

Part a) Investigation on the role of PDE4D4 in cell proliferation.

I will compare the PKA, PKC and MAPK activity of cells transfected with full-length *PDE4D4* construct so as to determine which cAMP downstream signaling pathways is/are regulated by PDE4D4 and how these pathways involve in prostate carcinogenesis. The assay kits for these kinases are commercial available and so this task is believed to complete next year.

Part b) Determination of the possibility of using *PDE4D4* a diagnostic marker for prostate diseases and prostate cancer

Immunohistochemistry (IHC) will be performed using a specific antibody against PDE4D4. This antibody was custom-made by FabGennix International Inc and could be specific for both rat and human homologues. Different prostatectomy tissue sections from rat with BPH, PIN, HG-PIN and adenocarcinoma will be undergone IHC with PDE4D4. On the other hand, laser capture microdissection (LCM) will be performed on the sections showing positive staining of PDE4D4 in the cytoplasmic or apical granular cells of prostate. RNA will be extracted, reverse transcribed and undergone real-time PCR with specific primers of *PDE4D4*. Specific antibody against PDE4D4 was established in Jan this year and was ready for IHC use. Results from this task will help to examine the use of PDE4D4 as a diagnostic marker for prostate diseases/cancer. However, alternative approach will be employed by using DNA from LCM-samples for methylation specific PCR (Ho and Tang., 2007). Methylation status changes among normal and tumor will help to determine whether *PDE4D4* can be used as a marker for early prostate disease/cancer assessment.

VI. Remaining work to be performed under Task 4

Investigation of the role of human *PDE4D4* homolog on the human prostate cancer

Normal NPREC and DU145, PC3, LNCaP prostatic carcinoma will be used for cell proliferation assay, apoptosis assay, PKA Activity, PKC Activity assays. Cells will be transfected with 50nM siRNA oligonucleotides against *PDE4D4*. siRNA against

PDE4D4 will be designed by Dharmacon siDESIGN center (Dharmacon, Lafayette, CO). Negative control siRNA (siCONTROL pool), and transfection siRNA control (siTOX) will be included to ensure the specificity and transfection efficiency of siRNA. Same kinases assay kits proposed to be used in task 3 will be employed in this task also. Task 3 and 4 will be done in parallel manner. It helps to analyze the data and troubleshoot if problem exists.

Key research accomplishments

- By completing task 1 and 2, I successfully showed that *PDE4D4* is regulated by DNA methylation and its expression is associated with the incidence of prostate PIN in rat prostate.
- We published the results from task 1 and 2 in *Cancer Research* in June 2006 and *Reproductive Toxicology* in October 2006 and January 2007. We were the first group who reported neonatal estrogen exposure could reprogram the rat prostate via DNA methylation.
- I have already prepared the constructs specific for full-length *PDE4D4* transcript transfection or siRNA knockdown (task 3 and 4). They will be used in the functional assays I proposed to do. Studying function of *PDE4D4* in prostate carcinogenesis will help to understand the development of prostate cancer and tailor the treatment for prostate disease/cancer.
- Specific antibody against PDE4D4 was custom-made and it will be used for IHC studies for rat normal and tumor sections to find out whether PDE4D4 can be used a biomarker for prostate disease/cancer.

Reportable outcomes

- **Published papers:**
 1. Ho, S., **Tang, W.Y.** (2007) Techniques used in studies of epigenome dysregulation due to aberrant DNA methylation: an emphasis on fetal-based adult diseases, *Reproductive Toxicology*. 2007 Jan 8; [Epub ahead of print]
 2. Prins, G.S., Birch L., **Tang, W.Y.**, Ho, S. (2006) Developmental Estrogen

Exposures Predispose to Prostate Carcinogenesis with Aging, Reproductive Toxicology. 2006 Oct 10; [Epub ahead of print]

3. Ho, S.*, **Tang, W.Y.***, Belmonte, J. and Prins, G.S. (2006) Developmental exposure to estradiol and bisphenol A increases susceptibility to prostate carcinogenesis and epigenetically regulates phosphodiesterase type 4 variant 4, Cancer Research. 2006 June 66(11):5624-5632 * **co-authorship**

- **Abstract for national meetings:**

1. **Tang, W.Y.**, Prins, G.S., Belmonte, J. and Ho, S. (2006) Neonatal exposure to estradiol or bisphenol A epigenetically alters phosphodiesterase type IV variant 4 (PDE4D4) and increases susceptibility to adult-induced prostate carcinogenesis, 97th Annual Meeting of American Association for Cancer Research.

2. **Tang, W.Y.**, Prins, G.S., Belmonte, J. and Ho, S. (2005) Neonatal estradiol or bisphenol A (BPA) exposure increases susceptibility to adult-induced prostate carcinogenesis: association with epigenetic changes in gene methylation patterns and phosphodiesterase type 4 expression, Environmental Epigenomics Conference 2005.

3. **Tang, W.Y.**, Prins, G.S., Belmonte, J. and Ho, S. (2005) Discovery of phosphodiesterase type 4 variant (PDE4D4) as a gene susceptible to neonatal imprinting by estradiol or bisphenol A in the rat prostate, Endocrine descriptor Workshop, Endocrine Society Annual Meeting 2005.

- **Invited presentation at national meetings:**

1. **Tang, W.Y.** (2006) Early developmental exposures to estrogen/bisphenol A impact a specific prostate epigenome, platform session - environmental and physiological impact of the epigenome, Environmental Mutagen Society 37th Annual Meeting, Vancouver, British Columbia, Canada.

Conclusions

I have made significant progress during the first year of the funded period of this training award. In this year, I successfully found that *PDE4D4* is reprogrammed by early exposure to estrogen and bisphenol A and its expression associated with incidence of prostate cancer. This data not only earned me three papers in this year but also made me get into several national meetings. Moreover, our findings have already raised public concern about the effect of estrogen or environmental estrogens such as BPA in the prostate disease and cancer. This postdoctoral training experience helps me to equip myself with basic and clinical aspects of cancer research and to become an independent and highly competitive researcher on the prostate cancer. In the coming year, I will complete the work proposed in Task 3 and 4. First, I will investigate how *PDE4D4* regulate cAMP signaling pathways that involved in prostate development. Second, I will examine the possibility of using *PDE4D4* expression/methylation pattern as a biomarker for prostate cancer. I believe these findings will yield new approach that will be relevant to prostate cancer prevention and therapy.

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Developmental Exposure to Estradiol and Bisphenol A Increases Susceptibility to Prostate Carcinogenesis and Epigenetically Regulates Phosphodiesterase Type 4 Variant 4

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Abstract

Early developmental perturbations have been linked to adult-onset prostate pathology, including excessive exposure to estrogenic compounds; however, the molecular basis for this imprinting event is not known. An important and controversial health concern is whether low-dose exposures to hormonally active environmental estrogens, such as bisphenol A, can promote human diseases, including prostate cancer. Here, we show that transient developmental exposure of rats to low, environmentally relevant doses of bisphenol A or estradiol increases prostate gland susceptibility to adult-onset precancerous lesions and hormonal carcinogenesis. We found permanent alterations in the DNA methylation patterns of multiple cell signaling genes, suggesting an epigenetic basis for estrogen imprinting. For phosphodiesterase type 4 variant 4 (PDE4D4), an enzyme responsible for cyclic AMP 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. Early and prolonged hypomethylation at this site following neonatal estradiol or bisphenol A exposure resulted in continued, elevated PDE4D4 expression. Cell line studies confirmed that site-specific methylation is involved in transcriptional silencing of the *PDE4D4* gene and showed hypomethylation of this gene in prostate cancer cells. Importantly, the *PDE4D4* alterations in the estrogen-exposed prostates were distinguishable before histopathologic changes of the gland, making *PDE4D4* a candidate molecular marker for prostate cancer risk assessment as a result of endocrine disruptors. In total, these findings indicate that low-dose exposures to ubiquitous environmental estrogens affect the prostate epigenome during development and, in so doing, promote prostate disease with aging. (Cancer Res 2006; 66(11): 5624-32)

Introduction

There are increasing human health and wildlife concerns about low-dose estrogenic exposures because hormonally active xenoestrogens are ubiquitous in the environment (1). Bisphenol A, initially synthesized as a synthetic estrogen (2), is widely used as a cross-linking chemical in the manufacture of polycarbonate plastics and

epoxy resins. Although bisphenol A binds to classic estrogen receptors with reduced affinity relative to 17 β -estradiol (3), it possesses equivalent activational capacity of the nonclassic membrane estrogen receptors (4). Importantly, bisphenol A leaches from food and beverage containers as well as dental sealants and is found in the serum of humans with higher concentrations in placental and fetal tissues (5). Thus, there is potential for this compound as a toxicant for developing human tissues, particularly the sensitive reproductive end organs (6, 7).

Prostate gland development, which occurs during fetal life in humans and the perinatal period in rodents, is exquisitely sensitive to estrogen imprinting. The *in utero* estrogen environment of African-American mothers has been suggested to affect the elevated prostate cancer risk of their offspring because they have higher estradiol levels during pregnancy when compared with their Caucasian counterparts (8, 9). In rodent models, brief perinatal exposure to pharmacologic doses of natural or synthetic estrogens permanently alters prostate growth and differentiation (10–12) and results in precancerous lesions and tumors with aging (13). However, although perinatal exposures to environmentally relevant doses of bisphenol A or estradiol have been shown to augment prostatic size (14), they have not, as yet, been shown to induce pathologic prostatic lesions. Thus, it remains unclear whether low-dose exposures to estradiol or environmental estrogens can influence prostate cancer risk.

Because early exposure to low-dose estrogen augments estrogen responsiveness of adult female reproductive organs (15, 16), we asked whether analogous circumstances exist in the prostate. This is particularly relevant because relative estradiol levels increase in the aging male, partly due to increased body fat content and aromatase activity, at a time when prostate cancer incidence increases (17). Furthermore, estrogens have been associated with increased prostate cancer risk in men (18), whereas, in the Noble rat model, prolonged adult exposure to conjoint estradiol and testosterone drives prostatic carcinogenesis (19). In this context, we established a carcinogenesis model that involved neonatal exposure to high- or low-dose estradiol or low-dose bisphenol A followed by adult exposure to elevated but nonpharmacologic testosterone plus estradiol in the Sprague-Dawley rat, a strain less sensitive to hormone-induced prostate carcinogenesis. Our goal was to determine if neonatal low-dose exposures to estradiol or bisphenol A might increase cancer susceptibility as a result of adult exposure to elevated estradiol. We herein present the first evidence that indeed low-dose as well as high-dose estrogenic exposures predispose to neoplastic prostatic lesions in the aging male.

We next sought to determine the molecular underpinnings by which developmental estrogenic exposures can imprint or transform the prostate long after the initial hormone exposure. One distinct possibility is through permanent epigenetic modifications

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of the genome by DNA methylation at CpG-rich regions (CpG islands), which can silence (hypermethylation) or activate (hypomethylation) gene transcription (20). Once established in somatic cells, CpG methylation patterns within the genome are stable and heritable through subsequent cell divisions, except during early embryonic development and tumorigenesis. Importantly, alterations in DNA methylation have been shown to contribute to both cancer initiation and promotion (20, 21). Furthermore, previous studies have revealed an association between aberrant CpG methylation of specific genes in the reproductive tract and neonatal exposures to phytoestrogens, diethylstilbestrol, and the environmental toxicants vinclozolin and methoxychlor (22–25). To determine whether neonatal estrogenic exposures imprint the prostate gland via this epigenetic modification, we did methylation-sensitive restriction fingerprinting (MSRF) followed by specific methylation analysis to identify and characterize candidate genes as methylation targets in prostate glands exposed to our two-hit model. We herein present evidence for altered methylation patterns of several candidate genes and characterize phosphodiesterase type 4 variant 4 (PDE4D4), the enzyme involved in cyclic AMP (cAMP) degradation, as an estrogen-imprinted gene directly associated with preneoplastic prostatic lesions.

Materials and Methods

Animal housing and treatments. All animal treatments were approved by the Animal Use Committee at the University of Illinois (Chicago, IL). Pregnant Sprague-Dawley rats (Zivic-Miller Laboratories, Pittsburgh, PA) were shipped on gestation day 12 and immediately transferred to strict housing conditions. Rooms were maintained at 21°C with 50% relative humidity and a 14-hour-light/10-hour-dark schedule. To avoid bisphenol A leaching from polycarbonate plastic, all rats were housed in new polysulfone solid-bottom cages with steel covers, and water was supplied from glass bottles. Animals were fed *ad libitum* a special soy-free, phytoestrogen-reduced diet (Zeigler Reduced Rodent Diet 2, Zeigler Brothers, Inc., Gardners, PA) with 12 ppm phytoestrogens as determined by high-pressure liquid chromatography. To avoid variability, a single feed lot was purchased for the entire study, packaged in 25-pound bags, autoclaved, and stored at –30°C to minimize growth of microbes that might contribute estrogenic by-products. Pregnant dams were monitored, and the day of birth was designated postnatal day 0. The pups were sexed by anogenital distance, and each litter was culled to 10 pups by removing or adding female pups as necessary.

The hormonal treatment regime, consisting of newborn rats briefly exposed to estrogens followed by prolonged adult exposure to elevated estradiol with appropriate controls, resulted in a total of eight animal groups. Newborn pups were assigned to one of four neonatal treatment groups with 20 to 30 pups per group: (a) controls given tocopherol-stripped corn oil vehicle alone (ICN Biomedicals, Inc., Aurora, OH), (b) high-dose 17 β -estradiol 3-benzoate (EB) at 25 μ g/pup (or 2,500 μ g EB/kg body weight), (c) low-dose EB at 0.001 μ g EB/pup (or 0.1 μ g EB/kg body weight), or (c) bisphenol A at 0.1 μ g/pup (or 10 μ g/kg body weight). All steroids were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). The high-dose EB was chosen based on our published data of an estrogenized phenotype with adult-onset prostatic intraepithelial neoplasia (PIN; ref. 13). The low-dose EB was chosen because this dose delayed puberty and permanently affected other reproductive structures in our dose-response study (26). An environmentally relevant dose of bisphenol A was chosen based on a predicted exposure range from leached bisphenol A in the environment (27). To avoid litter effects, male pups within each litter were randomly assigned to a treatment and toe clipped for permanent identification. Treatments were given on postnatal days 1, 3, and 5 by s.c. injections in the nape of the neck. The pups were weaned at postnatal day 21, and siblings were housed three per cage until postnatal day 90 and individually thereafter. At postnatal day 90, half of the rats from each treatment group

were given implants of Silastic capsules (inside diameter 1.5 mm, outer diameter 2.0 mm; Dow Corning, Midland, MI) packed with estradiol (one 1-cm tube) and testosterone (two 2-cm tubes) for 16 weeks (replaced after 8 weeks), whereas the remaining half were given empty tubes. The testosterone capsules were necessary to maintain physiologic levels of testosterone because estradiol treatment alone results in hypothalamic-testicular feedback inhibition of endogenous testosterone secretion with resultant prostatic involution. These testosterone plus estradiol capsule lengths result in ~75 pg/mL serum estradiol and 3 ng/mL serum testosterone (28) and produce PIN in the dorsolateral prostates at 100% incidence in Noble rats (29) but only 33% incidence in Sprague-Dawley rats (30). At 28 weeks of age, the animals were sacrificed by decapitation, and prostate glands were quickly removed and microdissected into ventral, lateral, and dorsal lobes. Half of each lobe was snap frozen and stored in liquid nitrogen for subsequent methylation analysis, whereas the contralateral lobe was fixed in 10% buffered formalin overnight and stored in 70% ethanol for histopathologic diagnosis. In addition to the above animals sacrificed on day 200, rats from the four neonatal treatment groups were sacrificed, and prostates were removed on postnatal days 10 and 90 ($n = 5$ –7 per group) for DNA methylation analysis.

Histopathology. Fixed prostatic tissues were processed, paraffin embedded, and sectioned along the longitudinal axis at three levels of the tissue block (10 sections per lobe). The sections were coded to prevent reader bias and stained with H&E. Each lobe was scored in a blinded fashion for epithelial and stromal hyperplasia, inflammation, and the presence of PIN and other notable pathology (adenoma, metaplasia, basement membrane breakdown, microinvasion, etc). PIN lesions were characterized by the presence of nuclear atypia (enlarged and elongated nuclei, hyperchromasia, and prominent nucleoli) with or without aberrant cellular piling and ductal formation (31). PIN lesions were graded on a scale of 0 to 3 (0, no atypia; 1, low-grade PIN; 2, focal high-grade PIN; and 3, extensive high-grade PIN). For PIN lesions, the incidence and the mean PIN score per treatment group were determined. Incidence was analyzed by χ^2 , and PIN scores were analyzed by ANOVA after square root transformation of the data followed by Fisher's exact test with significance accepted at $P < 0.05$.

Immunohistochemistry and *in situ* apoptosis labeling. Proliferation was measured by immunohistochemistry using a polyclonal Ki-67 primary antibody (1:2,500; Novocastra, Newcastle upon Tyne, United Kingdom). For apoptosis assessment, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was used with ApopTag peroxidase *in situ* apoptosis detection kit (Chemicon International, Temecula, CA). To calculate the proliferation and apoptotic indices, multiple representative areas of each lobe were captured with a color digital AxioCam camera on an Axioskop microscope (Carl Zeiss, Inc., Thornwood, NY). Positive and negative Ki-67-stained or TUNEL-labeled epithelial cells were counted using Zeiss Image version 3.0 (Carl Zeiss), with an average of 1,000 cells counted per slide. Data were analyzed by ANOVA and *post hoc* Bonferroni tests, and $P < 0.05$ was considered significant.

Methylation-sensitive restriction fingerprinting. MSRF was done as described (32), with minor modifications. In brief, 1 μ g genomic DNA extracted from tissues with the DNeasy Tissue kit (Qiagen, Valencia, CA) was digested with *MseI* alone or double digested with *BstUI* and *MseI* (New England Biolabs, Beverly, MA). Digested DNA was amplified by PCR using 2 μ Ci [α -³²P] dCTP (3,000 Ci/mmol; NEN, Boston, MA) with various combinations of paired arbitrary primers chosen from the following: *Bs7*, 5'-GAGGTGCGCG; *Bs11*, 5'-GAGAGGCGCG; *Bs17*, 5'-GGGGACGCGA; *PCG1*, 5'-AAGGAAGACG; and *PCG4*, 5'-TCCTTCCTCG. PCR products were separated on 6% nondenaturing polyacrylamide gels, which were dried and exposed to Kodak MS film (Kodak, New Haven, CT) to visualize the labeled bands. Candidate bands displaying the appropriate differential methylation status among "control" and "comparative" samples were cut, reamplified, and cloned directly into pCR2.1 vector (Invitrogen, Carlsbad, CA) for sequencing. The sequence obtained was aligned with database from Genbank and RefSeq using BLAST, expressed sequence tag homology (National Center for Biotechnology Information), and BLAT search (University of California Genome Research, Santa Cruz, CA).

5'-Rapid amplification of cDNA ends. The first-strand cDNA of *PDE4D4* was amplified using a reverse specific primer (5'-AAAGACGAGGCCAGGACAT-3') and the GeneRacer 5' Primer (Invitrogen). Nested PCR was done, and products were subcloned into pCR4-TOPO vector (Invitrogen). At least 10 clones were chosen and sequenced.

Bisulfite genomic sequencing. Genomic DNA (200 ng) from rat prostate tissue samples or rat cell lines was modified with sodium bisulfite using CpGenome DNA Modification kit (Chemicon International) and used in nested PCR for bisulfite sequencing. Primers for amplifying the *PDE4D4* gene promoter/exon 1 region in completely converted DNA were designed with Primer3 and MethPrimer (33). First PCR was done using forward (5'-AGTGGTTTGGAGAAGTTAGAGTTTA-3') and reverse (5'-CCAAAACATCCTAAATTTCTTCAAA-3') primers. Nested PCR was done with forward (5'-TTATGTGTTGAAGAGTAGATTTGTG-3') and reverse (5'-ATCCTAAATTTCTTCAACCTAAC-3') primers. Both PCRs were done at 94°C for 9 minutes, 40 cycles of denaturing (94°C for 30 seconds), annealing (56°C for 1 minute), and extension (72°C for 1 minute) followed by a 12-minute final extension. The PCR product was gel purified and cloned into pCR2.1 vector. Six clones were picked from each sample for sequencing (Macrogen, Rockville, MD), and at least three sets of samples from each group were used. The DNA methylation data from sequencing were analyzed by BiQ Analyzer (34).

Methylation-specific PCR. PCR was done on bisulfite-treated DNA samples (40 ng) using primers specific for methylated (5'-GGTACGAGTAGTATTATTAGTATTCGTTTC-3' and 5'-CACGACAATACAAATAACGCTCCGT-3') or unmethylated (5'-GGTATGAGTAGTATTATTAGTATTTGTTT-3' and 5'-CACAACAATAACAATAACTCCAT-3') DNA. Forty PCR cycles were done with the following conditions: denature at 94°C for 30 seconds, anneal at 58°C for 1 minute, and extension at 72°C for 1 minute followed by 12-minute final extension. PCR products were separated on 2% agarose gel and visualized with ethidium bromide.

Real-time reverse transcription-PCR. Total RNA was isolated and reverse transcribed, and *PDE4D4* expression was quantitated by a fluorogenic method with 2× SYBR Green Master Mix using an iCycler iQ Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) as described previously (35). Primers specific for *PDE4D4* (AF031373) were designed in the exon/exon spanning region and were as follows: *PDE4D4*, 5'-ACGAGCAGCACCAGTA-3' (forward) and 5'-CTTGAGCGTAGC-GACCAC-3' (reverse). *PDE4D4* mRNA levels were normalized to RPL19 and the postnatal day 10 oil-treated control value was arbitrarily assigned an abundance value of 1. All data groups were analyzed by ANOVA followed by *post hoc* Bonferroni tests.

Prostate cell cultures and demethylation assay. Normal prostate epithelial NbE-1 cells and tumorigenic AIT cells were established from the Noble rat and immortalized as described previously (36). Cell cultures were treated with 0.5 or 1 μmol/L 5-aza-2'-deoxycytidine (5-Aza-dC; Sigma-Aldrich) for 8 days. Drugs were replenished every 4 days, and equivalent concentrations of DMSO were added in replicate control samples. At the end of the treatment, DNA and RNA were extracted from the cells and subjected to bisulfite genomic sequencing to determine the methylation status of the 5'-flanking region of the *PDE4D4* gene and real-time reverse transcription-PCR (RT-PCR) to quantitate *PDE4D4* gene expression.

Results

Low-dose estradiol and bisphenol A increase susceptibility to prostate neoplastic lesions. Responses to neonatal and adult hormone treatments were similar among the separate prostate lobes with only minor lobe-specific differences noted, and representative data are presented from the dorsal prostate (see Supplementary Fig. S1 for ventral and lateral data). Similar to our previous studies with rats (26), neonatal exposure to high-dose estrogen decreased adult prostate weights, whereas developmental exposure to low-dose estradiol or bisphenol A did not affect prostate size (Fig. 1A). Adult testosterone plus estradiol increased prostate weights but did not change the response pattern to

neonatal exposures (Fig. 1A). Prostate histopathology was assessed in a blinded manner for hyperplasia, chronic inflammation, and PIN, the precursor lesion of prostate cancer. PIN scores, based on grade and frequency, and PIN incidence showed marked differences across treatment groups (Fig. 1B-D). The oil-control group had a low PIN incidence (11%) and score (0.11) and normal prostate histology. Neonatal exposure to high-dose EB alone resulted in a 66% incidence of high-grade PIN and a markedly increased PIN score (1.12; $P < 0.05$). Areas of severe nuclear atypia, adenoma, and cellular piling were typically observed (Fig. 1D). Importantly, exposure to low-dose estradiol alone also increased the PIN incidence to 56% with mixed low-grade and high-grade PIN and elevated the PIN score (0.8). Focal areas of mild nuclear atypia were frequently observed in low-dose estradiol prostates. In contrast, neonatal low-dose bisphenol A alone did not induce PIN lesions in the aged prostates. Stromal and epithelial hyperplasia as well as inflammatory cell infiltration were observed in the high-dose EB prostate but not in the low-dose EB or the bisphenol A-exposed animals (data not shown).

As expected (19), prolonged adult testosterone plus estradiol exposure increased PIN incidence (40%) and score (0.52) in oil-control prostates (Fig. 1B and C), and this was further increased (incidence, 100% and score, 1.3; $P < 0.05$) by initial early exposure to high-dose EB. Neonatal low-dose EB before adult hormones did not augment PIN lesions further than that seen with adult testosterone plus estradiol or neonatal low-EB exposures alone. In contrast, neonatal exposure to low-dose bisphenol A significantly increased the PIN incidence (100%, mostly high-grade PIN) and score (1.3; $P < 0.01$) following adult exposure to elevated testosterone plus estradiol. Further, the neoplastic severity produced by bisphenol A was equivalent to high-dose EB exposures. Histologically, severe atypia was common with nuclear elongation and irregular size, cellular piling, and adenoma formation (Fig. 1D).

The prostatic tissues were assessed for alterations in epithelial cell proliferation and apoptosis, which are normally low in the adult prostate gland. Low rates of proliferation and apoptosis were consistently observed in all areas of the treated prostates, except for those exposed neonatally to high-dose EB or bisphenol A (Fig. 2A-B). Neonatal high EB treatment alone or with adult hormones increased basal proliferation rates throughout the tissue, with a higher rate observed in high-grade PIN regions (Fig. 2B, *inset*). Bisphenol A exposure followed by adult hormones also significantly increased proliferation in regions with high-grade PIN (Fig. 2A and B, *inset*). Similarly, low basal rates of apoptosis were detected throughout the prostate tissues, except for regions of high-grade PIN in the animals exposed neonatally to high-dose EB or bisphenol A with adult hormones (Fig. 2C-D). This provides support for the hypothesis that developmental estrogenic exposures initiate or activate precancerous pathways, resulting in an imbalance in cell proliferation and apoptosis that may contribute to prostatic pathology with aging. Taken together, the present experimental paradigm suggests that early low-dose estrogen exposures predispose the prostate to PIN with aging and that environmentally relevant doses of bisphenol A during development increase prostatic susceptibility to carcinogenesis following additional adult insults.

Neonatal estrogens epigenetically modify the prostate through alterations in DNA methylation. We sought to determine whether permanent alterations in prostate growth and carcinogenic susceptibility long after early estrogenic exposures could be mediated through epigenetic alterations. To examine genome-wide methylation changes, MSRF was done using DNA

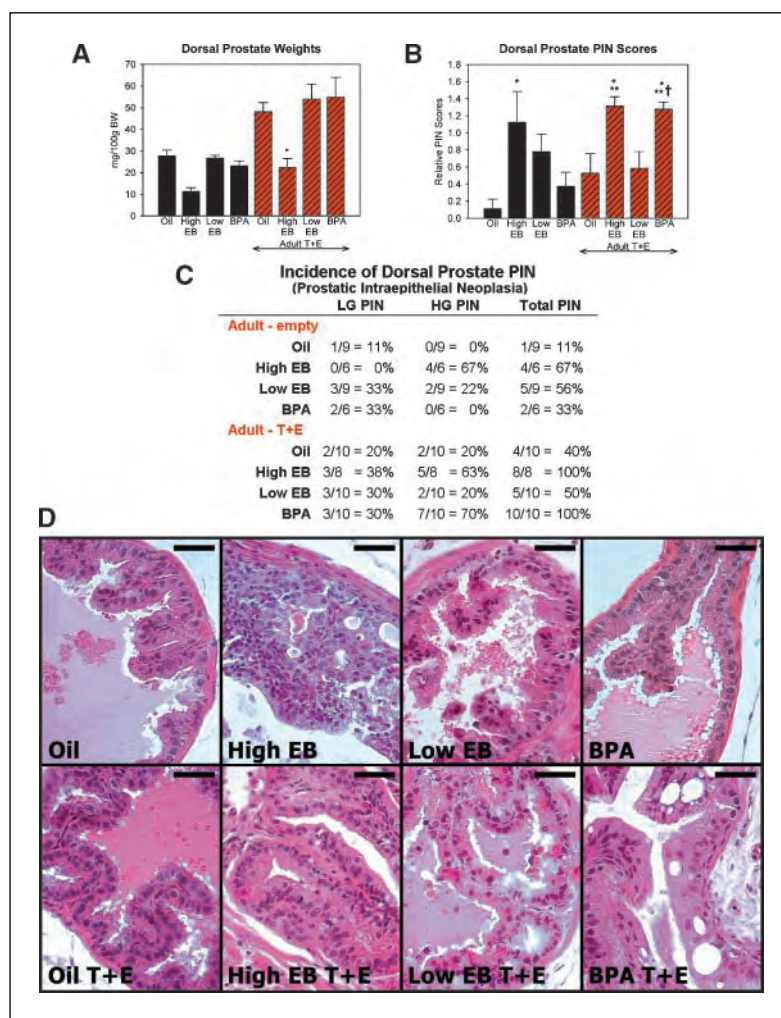


Figure 1. Effects of neonatal estrogens on adult prostate. Representative data for the dorsal prostate at 6 months. **A**, dorsal prostate weights at day 200. *, $P < 0.05$ versus oil/testosterone + estradiol (T + E). **B**, columns, mean PIN scores; bars, SE. *, $P < 0.05$ versus oil alone; **, $P < 0.05$ versus oil/testosterone + estradiol; †, $P < 0.01$ versus bisphenol A (BPA) alone. **C**, incidence of PIN lesions across treatment groups at day 200. LGPIN, low-grade PIN; HGPIN, high-grade PIN. **D**, representative H&E sections from dorsal prostates of the eight treatment groups. Bar, 50 μ m.

from the neonatally exposed tissues removed on days 10, 90 (before adult hormone treatment), and 200 (schematized in Supplementary Fig. S2). Differential methylation changes were sought between control versus neonatally treated tissues across time. We were additionally interested in identifying candidates with early onset methylation changes that can potentially be used as markers for risk assessment. More than 50 candidate bands were chosen for cloning and sequencing, and 28 unique DNA candidate clones were identified (Table 1). Of the identified candidates, 16 showed no homology with known rat genes, 6 were identified once, and 2 (*CAR-XI* and *SLC12A2*) were identified multiple times with similar methylation patterns observed each time. Importantly, these eight candidate genes were homologous (>95%) to known genes involved in signal transduction pathways: Na-K-Cl cotransporter (*SLC12A2*), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway (*GPCR14* and *PDGFR α*), phosphoinositide 3-kinase (PI3K) pathway (*PLC β 3*), cAMP pathways (*PDE4D4* and *HPCAL1*), and neural or cardiac development (*CARX1* and *CARK*).

PDE4D4, which breaks down intracellular cAMP, was chosen for further characterization because the differentially methylated candidate clone corresponded to the 5'-region of the gene, and the methylation differences between control and estrogen-exposed tissues were observed as early as postnatal day 10.

The 5'-flanking/promoter region of *PDE4D4* was first identified by 5'-rapid amplification of cDNA ends and a 700-bp CpG island with 60 CpG sites was found to encompass its transcription and translation start sites (Fig. 3A). Importantly, multiple transcription factor response elements, including cAMP response element, estrogen response element half-site, and Sp1, were computationally identified in this CpG island (Supplementary Fig. S3).

Methylation site mapping of this CpG island was done by bisulfite genomic sequencing in prostates from all treatment groups at postnatal days 10, 90, and 200. Figure 3B shows an example of methylation mapping at the 60 CpG sites in day 200 oil (Fig. 3B, top) and bisphenol A-treated prostates (Fig. 3B, bottom), whereas Fig. 3C shows the percentage methylation at the 60 CpG sites in all dorsal prostate tissues calculated over time for the different treatment groups. Although most CpG sites were unmethylated, a methylated cluster was noted between CpG sites 49 to 56 (Fig. 3B, boxed region), and methylation frequency at these sites progressively increased in the oil-control prostates as the animals aged, reaching 100% methylation by day 200 (Fig. 3C, solid diamonds). In contrast, the CpGs 49 to 56 remained relatively hypomethylated in aging prostates exposed neonatally to high- or low-dose estradiol or low-dose bisphenol A (Fig. 3B-C).

Direct association of DNA methylation at CpGs 49 to 56 and its resultant effect on *PDE4D4* gene expression were shown by using methylation-specific PCR and real-time RT-PCR. There were no differences in DNA methylation or gene expression between treatment groups at day 10 (Fig. 4A-B). However, as the animals aged, CpGs 49 to 56 became entirely methylated in oil-control prostates, whereas neonatal high EB-treated, low EB-treated, and bisphenol A-treated prostates possessed completely unmethylated sequences (Fig. 4A). Importantly, these differential methylation patterns were inversely correlated to *PDE4D4* gene expression (Fig. 4B). *PDE4D4* message levels in prostates exposed neonatally to estradiol or bisphenol A were markedly higher at day 90 than control tissues and remained elevated with aging. We thus conclude that the prostatic *PDE4D4* gene is normally silenced with aging through promoter hypermethylation but remains expressed in neonatally estrogenized prostates by virtue of hypomethylation at CpGs 49 to 56. Notably, this phenomenon was observed in all neonatal high- and low-dose EB and low-dose bisphenol A groups before the "second hit" of hormones and before adult-onset PIN lesions. Thus *PDE4D4* may have potential as a marker for prostate cancer risk assessment.

We further showed that *PDE4D4* transcription is dependent on the methylation status of its promoter region, specifically at CpGs 49 to 56, by using rat prostate NbE-1 cells, an immortalized normal epithelial cell line, and AIT, a dorsal prostate tumor-derived cell line. We found low levels of *PDE4D4* expression and a methylated 49 to 56 CpG cluster in NbE-1 cells but high levels of gene expression in AIT cells and unmethylated cluster at CpGs 49 to

56 (Fig. 5A-C). These *in vitro* findings mirror the *in vivo* data of control versus estrogenized prostate tissues. Treatment of NbE-1 cells with 5-Aza-dC induced loss of methylation at CpGs 49 to 56 and increased *PDE4D4* gene expression ($P < 0.01$; Fig. 5A and C). Similar treatment of AIT cells completely demethylated these previously hypomethylated CpG sites; however, it did not further increase the already high levels of *PDE4D4* expression (Fig. 5B-C). Taken together, these data provide direct evidence that hypermethylation of the *PDE4D4* promoter at CpGs 49 to 56 is involved in *PDE4D4* transcriptional silencing and that deregulation of methylation at this locus occurs in prostate cancer cells.

Discussion

The present findings provide the first evidence of a direct link between developmental low-dose bisphenol A or estradiol exposures and carcinogenesis of the prostate gland. Specifically, the data show that exposure to low doses of estradiol or environmentally relevant doses of bisphenol A during the neonatal developmental period in rats increases susceptibility to precancerous prostatic lesions as the animals aged and sensitizes the prostate gland to adult-induced hormonal carcinogenesis. This data thus contribute to the increasing body of evidence for a link between fetal exposures to endocrine disruptors and cancer (37–39). The human male fetus is exposed to elevated levels of maternal and exogenous estrogenic compounds, including bisphenol A (5, 40), and these estrogens could sensitize the prostate, perhaps through epigenetic mechanisms. Furthermore, relative increases in estradiol levels in the aging male

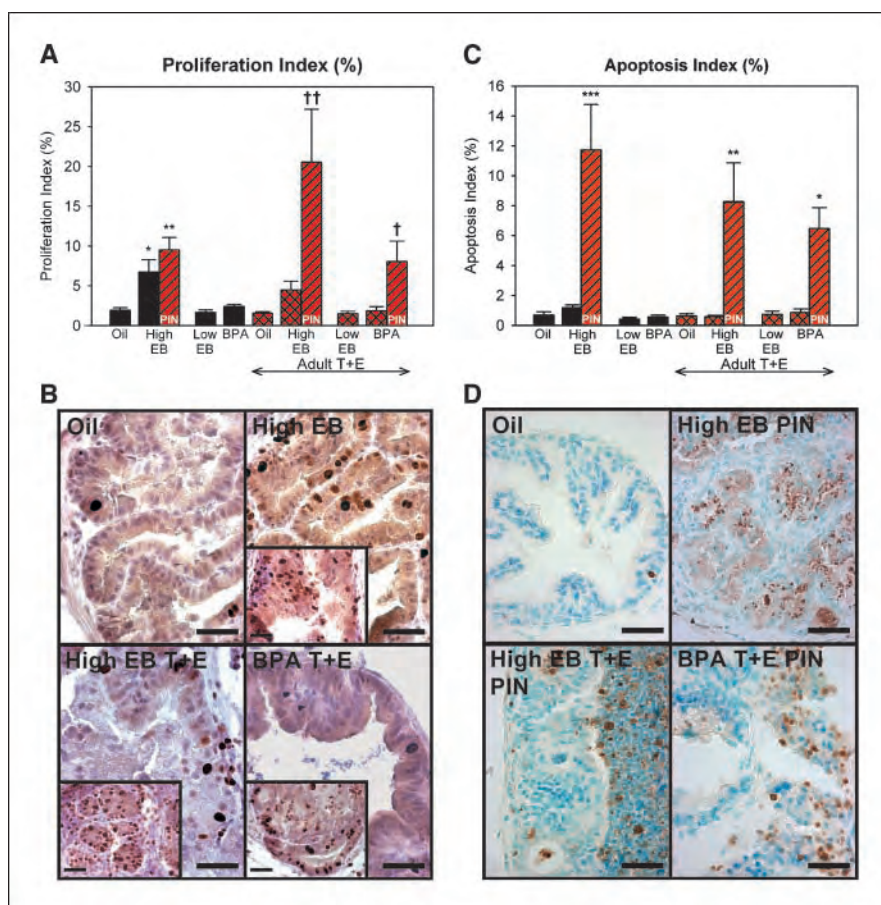


Figure 2. Proliferation and apoptosis rates following estrogenic exposures. **A**, proliferation index for dorsal prostate epithelial cells as determined by Ki-67 immunostaining. Black and red-hatched columns, counts in histologically normal regions; red-striped columns, areas of high-grade PIN. Basal proliferation was elevated in high-dose EB prostates. Proliferation rates were further elevated in high-grade PIN lesions of high-dose EB and bisphenol A/testosterone + estradiol prostates. *, $P < 0.05$ versus oil and low-dose EB; **, $P < 0.01$ versus oil and high-dose EB/testosterone + estradiol/PIN region; †, $P < 0.05$ versus bisphenol A/testosterone + estradiol/normal region; **, $P < 0.01$ versus normal regions of all testosterone + estradiol treatment groups. **B**, representative regions of histologically normal dorsal prostates immunostained for Ki-67. Inset, regions of high-grade PIN within each group. **C**, apoptotic index for dorsal prostate epithelial cells as determined by TUNEL. Areas of high-grade PIN in high-dose EB and bisphenol A/testosterone + estradiol tissues showed increased apoptosis. *, $P < 0.05$ versus bisphenol A/testosterone + estradiol; **, $P < 0.01$ versus normal regions of all testosterone + estradiol treatment groups; ***, $P < 0.001$ versus normal regions in all treatments. **D**, representative TUNEL-labeled dorsal prostates. Normal region for oil and high-grade PIN region for all others. Bar, 50 μ m.

Table 1. Differentially methylated candidate genes identified with MSRF

Clone name	Primer 1	Primer 2	Hypermethylation	Chromosomal band	Gene homology	Location	Related pathways
2p717	7	17	Low estradiol, high estradiol, bisphenol A (10, 90, 200)	1q22	CAR-XI	5'-End	Neural cell development
3p717	7	17	Low estradiol (200)	1q22	CAR-XI	5'-End	Neural cell development
1P11G1	11	G01	Low estradiol, bisphenol A (200)	1q43	PLC β 3	Exon19	PKC and phospholipase signaling
3p11G1	11	G01	Low estradiol, bisphenol A (200)	7q12	NA		
4p11G1	11	G01	Low estradiol, bisphenol A(90,200)	18q12.1	SLC12A2	Exon17	Na-K-2Cl cotransport
5p11G1	11	G01	Low estradiol, bisphenol A (200)	18q12.1	SLC12A2	Exon17	Na-K-2Cl cotransport
6p11G1	11	G01	Low estradiol, bisphenol A (200)	18q12.1	SLC12A2	Exon17	Na-K-2Cl cotransport
8p11G1	11	G01	Control (10, 90)	6q32	NA		
9p11G1	11	G01	Low estradiol, bisphenol A (200)	6q16	HPCAL1	Intron1	cAMP signaling
10p11G1	11	G01	Low estradiol, bisphenol A (200)	18p12	NA		
11p11G1	11	G01	Control (10, 90)	2q14	NA		
12p11G1	11	G01	Low estradiol (10, 90, 200)	6q32	NA		
3p11G4	11	G04	Low estradiol, high estradiol, bisphenol A (90,200)	7q34	NA		
5p11G4	11	G04	Low estradiol, high estradiol, bisphenol A (90, 200)	6q24	NA		
6p11G4	11	G04	Low estradiol, high estradiol, bisphenol A (90,200)	18q12.1	SLC12A2	Exon17	Na-K-2Cl cotransport
7p11G4	11	G04	Low estradiol, high estradiol, bisphenol A (90, 200)	8q22	NA		
8p11G4	11	G04	Low estradiol, high estradiol, bisphenol A (90, 200)	2q45	CARK	5'-End	Ca ²⁺ dependent signaling
9p11G4	11	G04	Low estradiol (90, 200)	4q31	NA		
10p11G4	11	G04	Low estradiol, bisphenol A (90, 200)	4q31	NA		
11p11G4	11	G04	Low estradiol, bisphenol A (90, 200)	4q31	NA		
14p11G4	11	G04	Low estradiol (90, 200)	4q31	NA		
15p11G4	11	G04	Low estradiol (90, 200)	19q12	NA		
17p11G4	11	G04	Low estradiol (90, 200)	8q32	GPCR14	5'-End	G-protein coupled receptor signaling
18p11G4	11	G04	Control (10, 90, 200)	2q14	PDE4D4	5'-End	cAMP signaling
2p1117	11	17	Low estradiol, high estradiol, bisphenol A (200 testosterone + estradiol)	20q13	NA		
3p1117	11	17	bisphenol A (10)	7q11	NA		
4p1117	11	17	Low estradiol, high estradiol, bisphenol A (10)	17p12	NA		
5p1117	11	17	Control (10)	114p11	PDGFR α	Intron4	MAPK, ERK signaling

NOTE: Fragments were identified based on SWISS-PROT, TrEMBL, mRNA, and RefSeq search. Shown for each candidates are MSRF primers used, the hypermethylation pattern observed in the control, high and low estradiol, and bisphenol A samples (days of adult testosterone + estradiol treatment), the chromosomal band to which the fragment localized, the gene homology, and the location of the methylated fragment on the gene and the known related pathways.

Abbreviation: NA, not available.

(17) are adequate to promote carcinogenesis in a sensitized organ. Alternatively, a neonatally sensitized organ may be more vulnerable to adult exposures to bisphenol A or other xenoestrogens that bioaccumulate in fat cells. In this regard, recent evidence has shown that low-dose bisphenol A inappropriately activates the androgen receptor and mitogenesis in prostate adenocarcinoma cells *in vitro* and sensitizes cells with previous mutations (41). It is particularly relevant that the developmentally estrogenized rodent has accurately modeled multiple male and female reproductive tract lesions in humans (42). Thus, the present findings may have implications for human prostatic adenocarcinoma, which occurs with a relatively high frequency in the aging population and whose etiology remains unclear.

Although the mechanism(s) by which developmental exposures to endogenous and environmental estrogens alter the carcinogenic potential of the prostate have not been fully clarified, the present findings support the hypothesis, initially proposed by McLachlan (43), that altered epigenetic memory by endocrine disruptors may play a critical role. In this study, we have provided direct evidence in support of this premise. Our data show that several genes exhibit methylation changes in response to the neonatal estrogen treatments, many of which are permanent. It is noteworthy that several of these genes encode signaling pathway proteins that are involved in cell cycle and/or apoptosis, suggesting that neonatal estrogen exposures may perturb proliferation/apoptosis equilibrium through epigenetic gene

(de)regulation. It is also interesting that overlapping as well as unique methylation alterations were observed for high- and low-dose estrogen and bisphenol A. This suggests two important points. First, common prostatic genes may be epigenetically imprinted by different estrogenic compounds and doses, suggesting common pathways that predispose to prostate carcinogenesis with aging. Second, unique candidate genes specific to the neonatal estrogenic exposure and/or dose may mediate the subtle differences in phenotypes that were observed following the separate neonatal exposures.

The epigenetic regulation of gene expression by neonatal estrogen exposure was confirmed by detailed analysis of the *PDE4D4* gene. The estradiol and bisphenol A-initiated alterations in *PDE4D4* gene methylation occurred at a CpG island that spans the promoter/exon 1 region, a site typically involved in epigenetic regulation. Importantly, the degree of methylation at

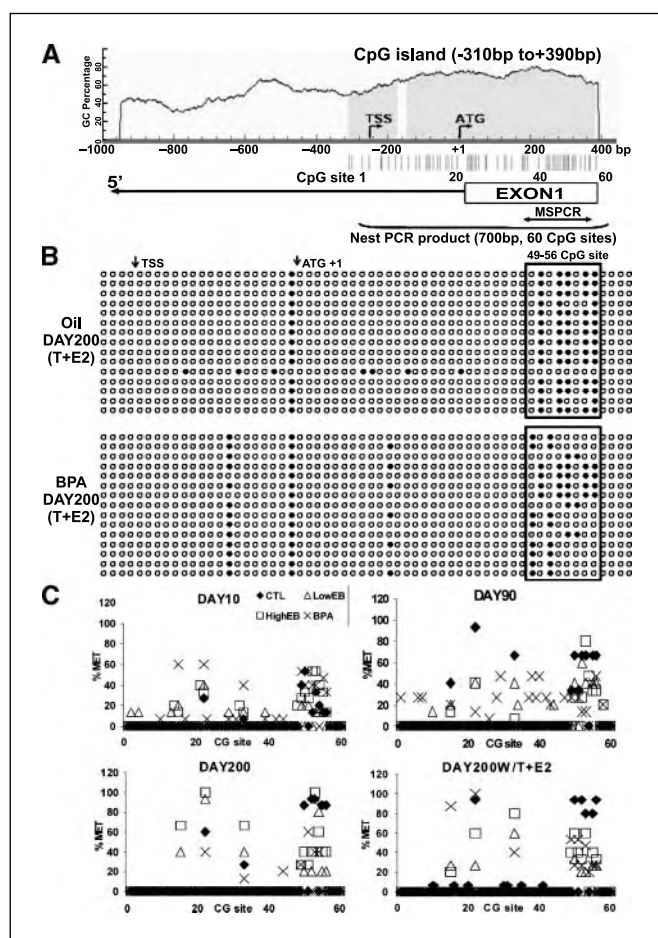


Figure 3. Bisulfite genomic sequencing of prostatic *PDE4D4* gene methylation. **A**, schematic of CpG content (%) in the 5'-flanking region of the rat *PDE4D4* gene identifies a CpG island (blue) between -310 to +390 bp. Vertical lines, individual CpG sites and the translation start site (ATG+1) and transcription start site (TSS). **Blanket**, 700-bp nested PCR-amplified region used for bisulfite sequencing. **B**, bisulfite genomic sequencing data from 4 to 6 clones each of three individual DNA samples taken from day 200 oil/testosterone + estradiol and bisphenol A/testosterone + estradiol dorsal prostates. Methylation status of specific CpG sites. ○, unmethylated; ●, methylated. **Boxed region**, potential CpG sites epigenetically altered by bisphenol A. **C**, percentage methylation at each CpG site within the 5'-CpG island of *PDE4D4* in the separate treatment groups at days 10, 90, and 200 with or without adult testosterone + estradiol. The percentage methylation at each site was averaged from three individual sample sets. ♦, oil control; □, high-dose estradiol; △, low-dose estradiol; ×, bisphenol A.

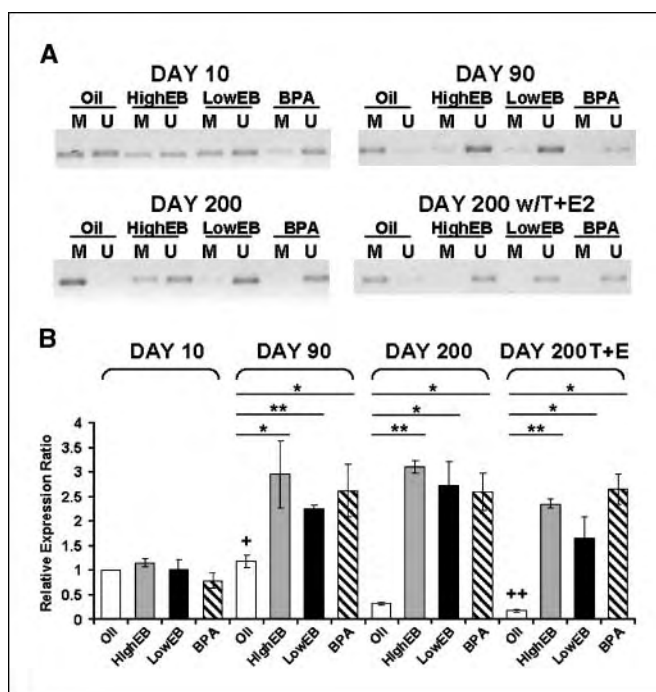


Figure 4. Comparison of *PDE4D4* CpG methylation and mRNA transcript levels. **A**, methylation-specific PCR analysis: dorsal prostate genomic DNA was bisulfite treated followed by methylation-specific PCR using methylated specific (M) or unmethylated-specific (U) primer sets. The amplified region is indicated in Fig. 3. The PCR products are representative data from three individual sets of samples. **B**, *PDE4D4* mRNA transcript levels as determined by real-time RT-PCR. Relative expression of day10 oil samples was set to 1. Columns, mean; bars, SD. All EB/bisphenol A groups at days 90, 200, and 200/testosterone + estradiol were significantly different ($P < 0.05$) from respective groups at day 10. *, $P < 0.05$; **, $P < 0.01$ versus oil controls at the same time interval; †, $P < 0.05$ versus day 200 oil controls; ††, $P < 0.05$ versus day 10 oil controls.

this site was inversely related to *PDE4D4* gene expression in the prostate tissues. Thus, the *PDE4D4* promoter undergoes gradual hypermethylation with aging in normal prostates, resulting in *PDE4D4* gene repression in the adult gland. In contrast, it remains hypomethylated in animals briefly exposed to neonatal estradiol or bisphenol A, thus engendering persistent *PDE4D4* overexpression throughout life. This pattern of *PDE4D4* methylation and transcriptional regulation was also observed in normal and malignant prostate epithelial cells where normal NbE-1 cells with hypermethylated *PDE4D4* gene had low gene expression, whereas tumorigenic AIT cells had hypomethylation at the CpG island and elevated *PDE4D4* expression. Taken together, these findings suggest the potential involvement of epigenetically mediated *PDE4D4* dysregulation in prostate epithelial cell transformation.

At present, it is premature to suggest that *PDE4D4* dysregulation is a direct mediator of the prostatic dysgenesis as a result of early exposures to low- and high-dose estradiol or low-dose bisphenol A, particularly because the phenotypic response to the hormonal agents has specific differences widely, whereas the *PDE4D4* methylation and expression alterations are quite similar. Nonetheless, PDE4 is a promising lead candidate that deserves further discussion. PDE4 is a member of a large family of intracellular PDE enzymes involved in cyclic nucleotide monophosphate breakdown, and it specifically degrades cAMP (44). There are multiple downstream signals for cAMP in the cell, including

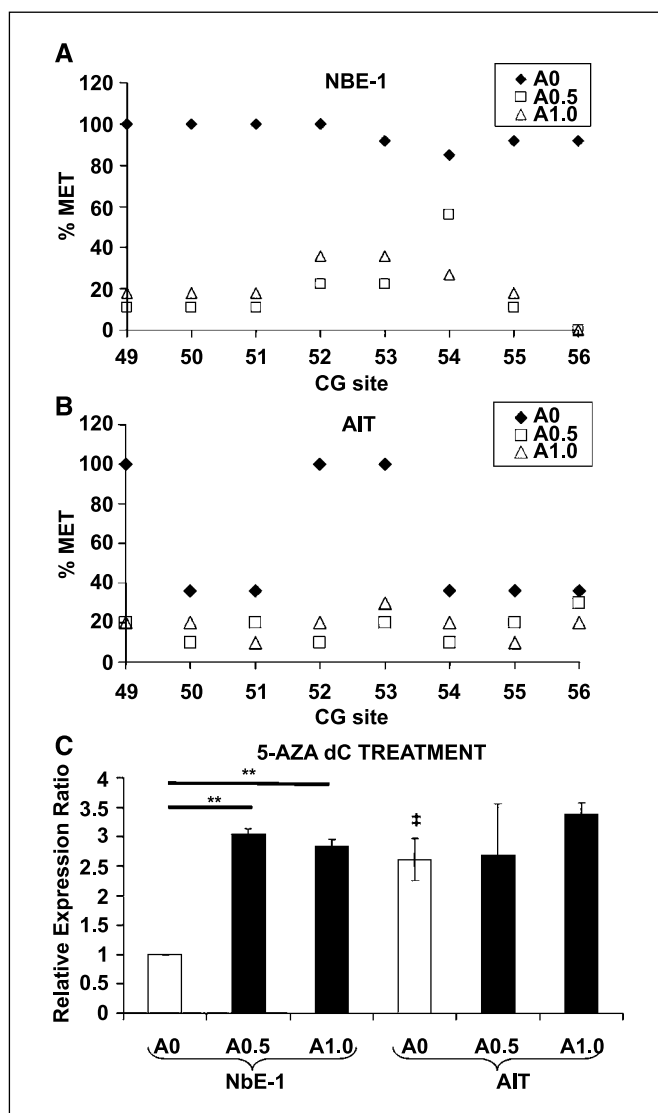


Figure 5. Alterations in *PDE4D4* CpG methylation and gene expression in NbE-1 and AIT cell lines by a demethylating agent. **A**, percentage CpG methylation for *PDE4D4* CpGs 49 to 56 in normal NbE-1 cells without (◆) or with 5-Aza-dC treatment (□, 0.5 μ mol/L 5-Aza-dC; △, 1 μ mol/L 5-Aza-dC). **B**, percentage CpG methylation for *PDE4D4* CpGs 49 to 56 in tumorigenic AIT cells without (◆) or with 5-Aza-dC treatment. **C**, relative *PDE4D4* mRNA levels in NbE-1 and AIT cells. White columns, control cells (A0); bars, SD. Black columns, cells treated with 5-Aza-dC. Expression of control NbE-1 cells was set to 1. **, $P < 0.01$ versus controls; †, $P < 0.05$ versus control NbE-1 cells.

activation of protein kinase A with resultant phosphorylation of cAMP-responsive element binding protein, which regulates transcription of genes involved in cell growth and differentiation (45). PDE4D has been shown to regulate cAMP levels in hormone-

targeted cells, and the PDE4D4 variant, which localizes to the cytoskeletal structures, is itself activated by hormones (46). Sustained expression of *PDE4D4* by hypomethylation could thus result in decreased intracellular cAMP in specific subcellular locations, creating a potential for aberrant cell signaling and potentially neoplastic transformation. In this regard, recent studies have shown a tight association between PDE4 expression and cancer cell proliferation, including glioma cells (47), osteosarcomas (48), and chronic lymphocytic leukemia (49). Importantly, PDE4 is currently being pursued as a possible chemotherapeutic target (50).

In addition to providing insight into the molecular underpinnings of estrogen imprinting, the methylated candidate genes identified herein have potential to serve as molecular markers for risk assessment of prostate disease due to early environmental exposures. *PDE4D4* shows particular promise in this regard because alterations in both gene methylation and expression were apparent before adult hormonal exposures and, importantly, before the onset of histopathologic changes in the prostate gland. This suggests that subtle alterations in gene expression may be more sensitive indicators of underlying pathology than the histologic alterations that occur when the disease is further progressed. Future studies are planned to develop a panel of methylated genes that may be used as markers for prostatic disease following early bisphenol A exposures.

In summary, we have shown that a range of estrogenic exposures during the developmental critical period, from environmentally relevant bisphenol A exposure to low-dose and pharmacologic estradiol exposures, results in an increased incidence and susceptibility to neoplastic prostatic lesions in the aging male, which may provide a fetal basis for this adult disease. Furthermore, the present findings provide evidence that developmental exposure to environmental endocrine disruptors (bisphenol A) and natural estrogens impacts the prostate epigenome during early life, which suggests an epigenetic basis for estrogen imprinting of the prostate gland. Methylation patterns and/or expression of candidate genes, such as *PDE4D4*, may serve as early biomarkers of prostate malignancy due to developmental exposure to endocrine disruptors or the *in utero* estrogenic environment.

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Developmental estrogen exposures predispose to prostate carcinogenesis with aging[☆]

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Abstract

Prostate morphogenesis occurs *in utero* in humans and during the perinatal period in rodents. While largely driven by androgens, there is compelling evidence for a permanent influence of estrogens on prostatic development. If estrogenic exposures are abnormally high during the critical developmental period, permanent alterations in prostate morphology and function are observed, a process referred to as developmental estrogenization. Using the neonatal rodent as an animal model, it has been shown that early exposure to high doses of estradiol results in an increased incidence of prostatic lesions with aging which include hyperplasia, inflammatory cell infiltration and prostatic intraepithelial neoplasia or PIN, believed to be the precursor lesion for prostatic adenocarcinoma. The present review summarizes research performed in our laboratory to characterize developmental estrogenization and identify the molecular pathways involved in mediating this response. Furthermore, recent studies performed with low-dose estradiol exposures during development as well as exposures to environmentally relevant doses of the endocrine disruptor bisphenol A show increased susceptibility to PIN lesions with aging following additional adult exposure to estradiol. Gene methylation analysis revealed a potential epigenetic basis for the estrogen imprinting of the prostate gland. Taken together, our results suggest that a full range of estrogenic exposures during the postnatal critical period – from environmentally relevant bisphenol A exposure to low-dose and pharmacologic estradiol exposures – results in an increased incidence and susceptibility to neoplastic transformation of the prostate gland in the aging male which may provide a fetal basis for this adult disease.

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Keywords: Prostate; Carcinogenesis; Estradiol; Estrogens bisphenol A; Development; Steroid receptor; Epigenetics; Methylation

1. Introduction

The prostate is a male accessory sex gland that receives a great deal of interest not because of its physiologic role, but rather due to the high incidence of abnormal growth and tumor formation with aging in humans. Currently, prostate cancer is the most common non-skin cancer in males and is the second leading cause of cancer deaths in American men [1]. According to the American Cancer Society, prostate cancer rates have been on the rise since 1975. With the 1987 introduction of PSA testing, the newly enhanced ability to diagnose the disease caused incidence to spike to 240 age-adjusted cases

per 100,000 men by 1992. After this “catch-up” period, rates dropped for 3 years, but have been rising again since 1998. Additionally, benign prostatic hyperplasia (BPH) is the most common benign neoplasm, occurring in ~50% of all men by the age of 60. Despite extensive research in the field, the basis for these high rates of abnormal prostatic growth is not well understood. It is recognized, however, that steroid hormones play a role in the initiation and progression of prostate cancer which is the basis for hormonal treatment strategies. Eunuchs with low levels of circulating testosterone do not develop prostatic carcinoma [2] and cancer regression can be initially achieved by castration and androgen blockade [3]. Although primarily under androgenic control, the prostate gland is also an estrogen target organ. Furthermore, estrogen involvement in the etiology of BPH and prostatic cancer has been postulated [4–6] and the use of antiestrogens has been recently recognized to have a therapeutic role in prostate cancer management [7,8].

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It has long been speculated that early developmental events which are regulated by steroids in the prostate gland may be linked to its predisposition to high rates of disease in adult men [9]. Thus, it is noteworthy that relative to adult estrogenic responses, the prostate gland is particularly sensitive to estrogen exposures during the critical developmental period. In this context, the present review will focus on the potential role of fetal or perinatal estrogens in permanently imprinting the prostate gland during development which in turn sets the stage for increased susceptibility to prostate carcinogenesis with aging.

2. Prostate gland development

Unlike other male accessory sex glands which develop embryologically from the mesodermal Wolffian ducts, the prostate gland originates from the urogenital sinus (UGS) and is endodermal in origin. It has been suggested for decades that the high rates of prostate cancer in men compared to the paucity of carcinoma in the seminal vesicles, vas deferens or epididymis may have a basis in this unique embryologic origin for an accessory sex gland. Prostate development commences *in utero* as UGS epithelial cells form outgrowths or buds that penetrate into the surrounding UGS mesenchyme in the ventral, dorsal and lateral directions posterior to the bladder. In humans, prostate morphogenesis occurs during the second and third trimester and is complete at the time of birth [10,11]. This contrasts with the rodent prostate gland where bud initiation commences in late fetal life and at the time of birth, a rudimentary structure is present consisting of a few main epithelial ducts. Extensive branching morphogenesis and cellular differentiation subsequently take place during the first 15 days of life [12]. Thus, the neonatal rodent prostate gland has emerged as a useful model for fetal prostate development in humans.

The initiation of prostatic development is dependent upon androgens produced by the fetal testes [9] and studies with 5α reductase inhibitors have shown that dihydrotestosterone (DHT) is the active androgen required for prostate formation [13]. Normal development, differentiation and onset of secretory activity requires the presence of androgens throughout the developmental process [14]. Androgen receptors (AR) are highly expressed in the UGS mesenchyme prior to and during prostate morphogenesis [15,16] and evidence by Cunha et al. [17] using mice has demonstrated that androgen-stimulated mesenchymal factors drive the morphogenetic process. Since AR are induced in rat prostate epithelium by postnatal days 1–3, it is possible that androgen-driven epithelial signals also contribute to morphogenesis and differentiation of the prostate [15].

The developing prostate gland also expresses other members of the steroid receptor superfamily including estrogen receptors $ER\alpha$ and $ER\beta$ and retinoic acid receptors $RAR\alpha$, β and γ which are liganded by all-trans or 9-*cis* retinoic acid as well as $RXR\alpha$, β and γ which are activated by 9-*cis* retinoic acid alone. Studies in rodent prostate glands have shown relatively high stromal cell $ER\alpha$ expression during perinatal morphogenesis of the gland which significantly declines thereafter suggesting a specific role for $ER\alpha$ in prostate development [16,18,19]. In the rat and murine prostate, $ER\beta$ is primarily localized to differen-

tiated luminal epithelial cells [20–22]. $ER\beta$ expression is low at birth, increases as epithelial cells cytodifferentiate and reaches maximal expression with onset of secretory capacity at puberty which suggests a role for $ER\beta$ in the differentiated function of the prostate [20]. In humans, $ER\alpha$ is also consistently observed in stromal cells during fetal development [23]. It is noteworthy, however, that the developmental pattern for $ER\beta$ in the human prostate differs markedly from the rodent. As early as fetal week 7, $ER\beta$ is expressed throughout the urogenital sinus epithelium and stroma [23]. This strong expression is maintained in most epithelial and stromal cells throughout gestation, particularly in the active phase of branching morphogenesis during the second trimester suggesting the involvement of $ER\beta$ and estrogens in this process [16,23]. While this pattern is maintained postnatally for several months, $ER\beta$ expression declines thereafter with a noticeable decrease in adluminal cells at puberty [16] again suggesting a specific developmental role for estrogens.

3. Estrogen imprinting of the developing prostate: fetal basis for adult disease

Similar to androgens, circulating levels of estradiol are high during the fetal and early neonatal life in both humans and rodent models [24] and there is compelling evidence that the developing prostate gland is particularly sensitive to these estrogens. During the third trimester of *in utero* development in humans, rising maternal estradiol levels and declining fetal androgen production result in an increased estrogen/testosterone (E/T) ratio. This relative increase in estradiol has been shown to directly stimulate extensive squamous metaplasia within the developing prostatic epithelium which regresses rapidly after birth when estrogen levels drop precipitously [25–27]. Although the natural role for estrogens during prostatic development is unclear, it has been proposed that excessive estrogenization during prostatic development may contribute to the high incidence of BPH and prostatic carcinoma currently observed in the aging male population [28,29]. African-American men have a two-fold increased risk of prostatic carcinoma as compared to their Caucasian counterparts and it has been postulated that this is related, in part, to elevated levels of maternal estrogens during early gestation in this population [30,31]. Indicators of pregnancy estrogen levels such as length of gestation, pre-eclampsia and jaundice indicate a significant correlation between elevated estrogen levels and prostate cancer risk [32,33]. Further, maternal exposure to diethylstilbestrol (DES) during pregnancy was found to result in more extensive prostatic squamous metaplasia in male offspring than observed with maternal estradiol alone [34]. While prostatic metaplasia eventually resolved following DES withdrawal, ectasia and persistent distortion of ductal architecture remained [35]. This has led to the postulation that men exposed prenatally to DES may be at increased risk for prostatic disease later in life although this has not been borne out in the limited population studies conducted to date [36]. However, extensive studies with rodent models predict marked abnormalities in the adult prostate including increased susceptibility to adult-onset carcinogenesis following early estrogenic exposures [28,37–39]. Although use of DES during pregnancy was discontinued in the early 1970s,

the recent realization that certain environmental chemicals have potent estrogenic activities [40] has led to a renewed interest in the effects of exogenous estrogens during prostatic development [41].

4. Rat model of developmental estrogenization

To carefully examine and elucidate a potential role for early-life estrogen exposures in adult prostate disease, we have made extensive use of the rat model for developmental estrogenization. The initial model used in our laboratory is the Sprague–Dawley rat given injections of 25 µg estradiol benzoate on neonatal days 1, 3 and 5 of life (Fig. 1). It is important to mention that while this is considered “high-dose”, the majority of neonatally administered estradiol is bound to α -fetoprotein which circulates at high levels in neonatal rat serum [42]. Consequently, neonatal estradiol is 75-fold less potent than an equivalent dose of DES [43] or, put another way, 25 µg estradiol/pup is equivalent to 0.33 µg DES/pup. As observed in earlier studies with mice and rats following early DES exposure [44,45], neonatal estradiol exposure consistently led to prominent pathology of the rat prostate gland. Histologic analysis of the young adult (day 90) ventral prostates of neonatally estrogenized prostates revealed disorganization of the epithelium with loss of basal/apical orientation, epithelial hyperplasia, inflammatory cell infiltrates and a relative increase in stromal elements [46–48]. Of significant interest, the pathologic lesions of the epithelium progress with aging such that by 18–22 months of age, ventral and dorsal lobes exhibited extensive hyperplasia (epithelial piling and cribriform patterning within the lumens), adenoma formation and moderate-to-high grade prostatic intraepithelial neoplasia (PIN) lesions characterized by nuclear enlargement, anisokaryosis and hyperchromasia [49]. Since neonatal estrogen exposure also results in decreased circulating testosterone (T) levels, a group of aged estrogenized rats were given 2 cm T implants for the last 6 months of life which restored T levels to normal. This treatment resulted in a 100% incidence of high-grade PIN throughout the ventral lobes by 18 months of age. Aged male rats exposed neonatally to DES have also been shown to develop profound squamous metaplasia in the dorsolateral prostate and development of solid tumors with highly invasive squamous cell carcinoma in some animals [44]. Similar results have been observed in neonatal DES-exposed mice [37,45]. Together these findings support the hypothesis

that early high-dose estrogen imprinting may be a predisposing factor to malignant transformation of the prostate gland in the aging male.

To better understand the processes by which high-dose neonatal estrogens drive hyperplasia and PIN lesions within the adult prostate gland, we further characterized the estrogenized phenotype at the anatomic and cellular level. Brief neonatal estrogen exposure permanently retards growth and development of the prostate gland such that all lobes are hypomorphic, reaching only 20–50% of normal adult prostate size [48]. In the dorsal and lateral lobes, not only is growth reduced but severe branching deficiencies exist such that elongating ducts fail to develop secondary and tertiary branch points and complex morphology [50]. While reduced growth is in part a function of reduced circulating T levels following neonatal estrogen exposure [48], organ culture studies also demonstrated a direct effect of estrogens in growth retardation as well as altering prostate differentiation [51,52].

Following neonatal exposure to high-dose estradiol, both epithelial and stromal cell proliferation and differentiation are markedly disturbed leading to defects that persist throughout the lifespan of the animal [15,53–56]. For epithelial cells, cytodifferentiation during development is perturbed or, for some end-points, permanently blocked by neonatal estrogens as determined by alterations in basal and luminal cell markers (p63, cytokeratins 5/15 and 8/18) and decreased production of secretory proteins (PBP, DLP proteins, urokinase, 26 kDa protease) [15,22,53]. Furthermore, alterations in the expression of e-cadherin and the gap junction proteins connexins 32 and 43 in the adult prostate epithelial cells result in impaired cell–cell adhesion and defective cell–cell communication [56]. In this regard, it is noteworthy that the epithelial cell differentiation defects are most prominent in the ventral prostate which also has the highest incidence of aging-associated PIN lesions [49].

Stromal-epithelial communication is also perturbed in the estrogen-exposed prostate through increased proliferation of periductal fibroblasts immediately adjacent to the outgrowing ducts during early development [53]. Direct cross-talk between epithelial cells and adjacent smooth muscle cells via secreted growth factors and extracellular matrix components is essential for normal prostate development [57]. The immediate proliferation and differentiation of mesenchymal cells into a multicellular fibroblast layer between the epithelial and smooth muscle cells

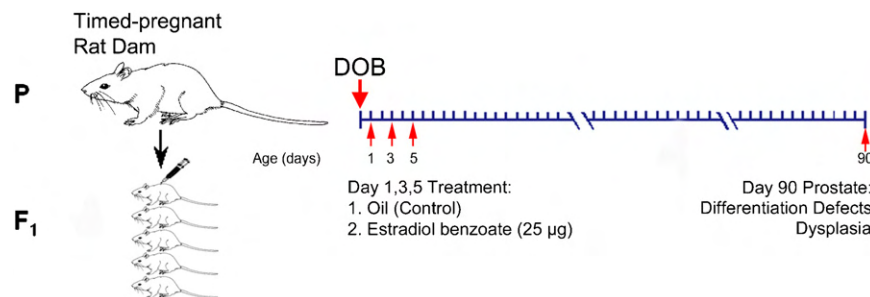


Fig. 1. Schematic representation of rat animal model for developmental estrogenization of the prostate gland. Day of birth (DOB) is considered day 0. Newborn male pups were given s.c. injections of high dose estradiol benzoate (25 µg) or oil as controls on days 1, 3 and 5. Rats are weaned on day 25. In young adulthood, significant differentiation defects and dysplasia are observed in the prostate gland as described in the text.

effectively blocks this critical stromal–epithelial cell interaction and interrupts growth factor communication as has been shown specifically for transforming growth factor $\beta 1$ (*Tgfb1*) [53]. Altogether, the above findings indicate that neonatal estrogen exposure interrupts intercellular communication and blocks certain epithelial cells within the rat prostate from entering a normal differentiation pathway. These persistent alterations in differentiation and gene expression may be a key mechanism through which changes towards a dysplastic state are mediated.

5. Molecular pathways for developmental estrogenization of the prostate

We next sought to determine the molecular pathways which mediate permanent alterations in prostate growth and function long after the hormone is withdrawn. It was observed in early studies that the activational response to androgens during adulthood is permanently blunted in estrogenized rats [46] and we determined that this effect is mediated, in part, through an immediate and permanent reduction in prostatic AR expression [15,48,58,59]. Furthermore, the temporal expression patterns and quantitative levels of several other members of the steroid receptor superfamily are dysregulated by early exposure to high doses of estradiol. Thus, ER α and progesterone receptor (PR) are transiently up-regulated in stromal cells [18,38], ER β is permanently down-regulated in luminal epithelial cells [20], RAR β is up-regulated in basal cells while RAR α is up-regulated in both epithelial and stromal cells [60]. This has led us to propose that early estrogen exposure effectively switches the developing prostate gland from an androgen-AR dominated tissue to one that is primarily regulated by estrogens and retinoids. We further hypothesize that this irretrievably alters the prostate by changing organizational signals that determine prostate behavior throughout life.

Developmental genes that dictate normal prostate gland morphogenesis were next examined as potential direct targets of the altered steroid signaling milieu. Specific perturbations in expression of key homeobox transcription factors as well as secreted morphoregulatory genes were observed which serves to explain some of the common and lobe-specific estrogenized phenotypes. As this has been the subject of two recent reviews [39,61], the findings will be briefly summarized here. In the prostate gland, the posterior *Hox13* genes are involved in positional identity and differentiation. Of these, *Hoxb13* is expressed in the epithelium where it plays a specific role in differentiation [62,63]. In the rat prostate, *Hoxb13* epithelial expression increased postnatally and was expressed at the highest levels in the ventral lobe [38]. Following neonatal exposure to high-dose estrogen, *Hoxb13* expression was immediately and permanently suppressed in all prostate lobes with the most significant reduction (80%) observed in the ventral prostate gland. Another critical homeobox gene, the androgen-regulated *Nkx3.1*, is normally expressed in UGS-derived prostate epithelium where it plays a role in differentiation and growth [64,65]. In control rats, a marked peak in *Nkx3.1* expression was observed postnatally between days 6 and 15 of life which subsequently declined to steady-state levels thereafter. While adult levels were not

disturbed, the transient postnatal *Nkx3.1* peak was completely abolished following high-dose neonatal estrogen exposure [61]. We propose that estrogen-initiated loss of prostatic epithelial *Hoxb13* and *Nkx3.1* genes may play a critical role in mediating the differentiation defects observed in the developmentally estrogenized prostate gland.

In addition to developmental regulation by homeobox genes, branching morphogenesis occurs as a complex interplay between epithelial and mesenchymal cells through secreted morphoregulatory genes [66]. We have recently examined the ontogeny and localization of bone morphogenic protein 4 (*Bmp4*), sonic hedgehog (*Shh*) and fibroblast growth factor-10 (*Fgf10*) in the normal developing rat prostate lobes and those exposed neonatally to estradiol to determine if alterations in their signaling pathways are involved in mediating specific aspects of the estrogenized phenotype. *Bmp4* has been implicated as a negative regulator of prostate growth [67] and levels in the rat prostate lobes rapidly decline postnatally. Following estrogen exposure however, *Bmp4* expression remained high through postnatal day 30 and we propose that this contributes to hypomorphic growth throughout the prostatic complex [61]. Our recent studies on epithelial *Shh* [50] and mesenchymal *Fgf10* [68] demonstrated a critical role for these two genes in regulating branching morphogenesis of the prostate gland. Interestingly, early estrogen exposure led to a lobe-specific reduction in *Shh* and *Fgf10* signaling in the dorsolateral prostate which is the site of severe branching deficiencies in response to estrogenization. Furthermore, the data suggest that reduced *Fgf10* expression in the stromal cells by estrogens is the proximate cause of *Shh* reductions and branching deficiencies [68]. Since a precise temporal expression pattern of these and other morphoregulatory genes is normally required for appropriate growth and differentiation of the prostatic epithelium and stroma, the estrogen-initiated disruption in this pattern would lead to permanent growth, branching and differentiation defects of the prostate gland. In summary, we propose that these and other yet unidentified molecular defects as a result of developmental estrogenization initiate permanent disturbances in prostate homeostasis which contributes to the development of prostatic neoplasia, PIN lesions and carcinoma as the animals age.

6. Neonatal exposure to low-dose estradiol and bisphenol A

The above effects of developmental estrogen exposures were in response to pharmacologic levels of estrogens as a model for early maternal exposures to agents such as DES or continued contraceptive use of ethinyl estradiol during pregnancy. A separate yet equally important issue is whether lower estrogenic exposures during development, such as elevated maternal estrogens or environmental estrogenic exposures, produce permanent prostatic abnormalities. Initial studies by vom Saal and colleagues to address the low-dose estrogen effects on the prostate gland found that in contrast to high-dose exposures, low-doses of estradiol or bisphenol A (BPA) during fetal life increased prostatic bud number, cell proliferation and adult prostate size in mice [69,70]. However, no histopathologic abnormalities were

observed in young adulthood. Furthermore, the low-dose estrogenic response in the prostate gland has not been consistently reported [71], due in part to species/strain differences, background estrogen levels and other experimental variables, and is a matter of considerable debate [72,73]. To examine this issue in the neonatal rat model, we administered estradiol over a 7-log range of doses on neonatal days 1, 3 and 5 in both Sprague-Dawley rats and the more estrogen-sensitive Fischer 344 rats [74]. We observed that only high-dose neonatal estradiol produced consistent prostatic pathology at 3 months whereas exposure to lower estradiol levels produced no permanent prostatic weight change or pathologic alterations [74] despite the advancement of puberty [75].

Although low-dose estrogens by themselves did not appear to drive prostate pathology in early adulthood, we asked whether low-dose exposures during development might shift the sensitivity of the prostate gland to adult estrogenic exposures as has been recently observed for some female reproductive endpoints [76,77]. This question is biologically relevant since circulating estradiol levels and the serum estrogen:testosterone ratio increase in aging men partly due to increased body fat content and aromatase activity, at a time when prostate cancer incidence rises [78]. Furthermore, prolonged adult exposure to estradiol at levels within a physiologic range is capable of driving prostatic carcinogenesis in the Noble rat model [4]. To address this possibility, we established a “second-hit” model as schematized in Fig. 2. Briefly, newborn male rats were exposed to either high-dose estradiol (2.5 mg/kg BW), low-dose estradiol (0.1 µg/kg BW), an environmentally relevant dose of BPA (10 µg/kg BW) or oil as controls on neonatal days 1, 3 and 5 as a “first hit”. At day 90 of life, a “second hit” of estradiol (E) was given by implanting T + E (or empty) capsules for 16 weeks. The T capsules result in 3 ng/ml serum T levels [79] and were necessary to maintain prostate homeostasis since E treatment alone results in feedback inhibition of endogenous T secretion with resultant prostatic involution. The E capsules produce serum levels of ~75 pg/ml in rats which, although elevated for males, is not considered pharmacologic [79]. These T + E capsule for 16 weeks produce PIN in the dorsolateral prostates at 100% incidence in Noble rats [4] but only 33% incidence in Sprague–Dawley rats [80]. At 28 weeks of age, the prostates were examined for hyperplasia, inflammation and PIN, the presumed precursor lesion of prostate cancer.

PIN scores, based on grade and frequency, and PIN incidence showed marked differences across treatment groups [81]. Neonatal exposure to either high-dose estradiol or to low-dose estradiol alone resulted in elevated PIN scores and incidence with aging (66% and 55%, respectively) while BPA alone had no effect on prostate pathology. As expected, prolonged adult T + E exposure increased PIN incidence to 40% in control rats given oil neonatally. This was further increased to a 100% incidence with significantly elevated PIN scores by initial early exposure to high-dose estradiol. Neonatal low-dose estradiol prior to adult hormones, however, did not augment PIN lesions further than that seen with neonatal low-dose estradiol alone. In contrast, neonatal exposure to an environmentally relevant dose of BPA produced a significant augmentation of PIN lesions to 100% incidence when followed by adult T + E exposure. The overall PIN score was significantly higher than both oil-treated rats ($P < 0.05$) and those given BPA alone ($P < 0.01$) and was equivalent to the PIN score observed following high-dose estradiol exposure. Histologically, severe atypia was common with nuclear elongation and irregular size, cellular piling, and adenoma formation. These findings are highly significant since they are the first observation of a link between developmental low-dose BPA exposure and adult prostatic pathologic lesions. Together, this new experimental paradigm suggests that low-dose exposures to estradiol alone increase susceptibility to adult onset prostate dysplasia while environmentally relevant doses of BPA increase the sensitivity of the prostate gland to carcinogenesis following additional adult insults such as elevated circulating estrogens.

7. Epigenetic changes in DNA methylation as a molecular mediator for estrogen imprinting of the prostate gland

While we have identified both transient and permanent alterations in the expression of multiple cell signaling pathways following high-dose estradiol exposure (see above), the molecular basis of these changes has remained elusive. One distinct possibility is through epigenetic modifications of DNA via cytosine methylation or demethylation which would result in aberrant and heritable silencing or activation of genes. Importantly, there is evidence that early hormonal exposures during developmental sensitive periods can permanently alter DNA

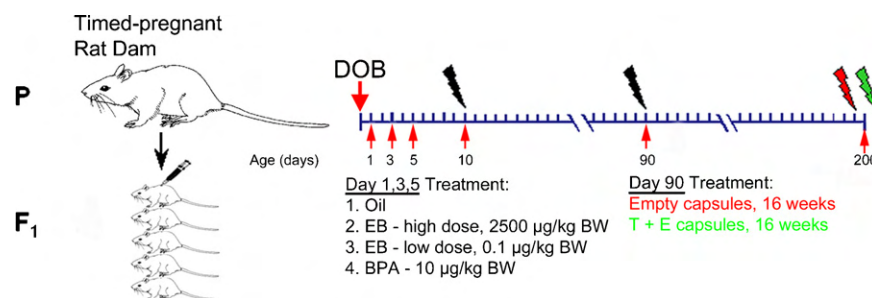


Fig. 2. Schematic representation of “two-hit” rat animal model for low-dose exposure to estradiol or bisphenol A (BPA) followed by second exposure to T + E implants on day 90. Newborn rats were injected with oil, high-dose estradiol benzoate (EB, 2500 µg/kg BW), low-dose EB (0.1 µg/kg BW) or BPA (10 µg/kg BW) on days 1, 3 and 5. At day 90, they were implanted with empty capsules or T + E capsules for 16 weeks. Arrowheads indicate times for tissue collection.

methylation of specific genes. McLachlan showed demethylation of CpG/–464 in the lactoferrin promoter of mouse uteri following neonatal high-dose DES exposure which persisted as tumors developed [82]. Similarly, neonatal phytoestrogen exposure was associated with hypermethylation of *c-H-ras* in the rat pancreas [83] while fetal DES and methoxychlor exposure resulted in altered methylation of ribosomal DNA in mouse uteri [84].

To examine whether epigenomic alterations in DNA methylation play a role in prostate imprinting by estrogens, we screened for global methylation changes using methylation sensitive restriction fingerprinting, or MSRF, using prostates exposed neonatally to high-dose estradiol, low-dose estradiol or environmentally relevant doses of BPA without or with adult T + E exposure (see above). As recently described [81], over 50 DNA candidates were identified as potential leads with repeatable methylation alterations across multiple samples and lobes. Of the identified candidates, 16 showed no homology with known rat genes, six were identified one time (PLC β 3, HPCAL1, CARK, GPCR14, PDE4D4 and PDGFR α) and two were identified multiple times (CAR-X1 and SLC12A2) with similar methylation patterns observed each time. It is noteworthy that several candidate genes involve signal transduction pathways: Na–K–Cl cotransport (SLC12A2), serotonin receptor/G-protein coupled receptor (GPCR14), MAPK/ERK pathway (PDGFR α), phosphokinase C pathway (PLC β 3), cAMP pathways (PDE4D4 and HPCAL1) and neural or cardiac development (CARX1, CARK). Furthermore, these signaling pathways are involved in cell cycle and/or apoptosis, suggesting that neonatal estrogen exposures may perturb proliferation/apoptosis equilibrium through epigenetic gene (de)regulation.

We initiated further studies to determine whether altered DNA methylation due to neonatal estrogenic exposures results in altered gene expression. Our initial studies focused on phosphodiesterase type 4, variant 4 (PDE4D4), an intracellular enzyme

that specifically degrades cAMP [85]. This gene was chosen since the differentially methylated DNA fragment identified by MSRF corresponded to the 5'-flanking region of the gene, it was consistently hypomethylated by all neonatal estrogenic exposures and the changes were identified by day 10 of life. Importantly, PDE4D4 controls the intracellular levels of cAMP which activates multiple downstream cell signaling pathways, regulating transcription of genes involved in cell growth and differentiation [86]. 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 glioma cells [87], osteosarcomas [88] and chronic lymphocytic leukemia [89]. Furthermore, PDE4 is currently being pursued as a possible chemotherapeutic target [90].

The 5'-flanking/promoter region of PDE4D4 was identified with a 700-bp CpG island that encompassed the transcription/translation start site [85]. Using bisulfite genomic sequencing, a specific methylation cluster was identified in the 5'-flanking region of this PDE4D4 CpG island that was gradually hypermethylated with aging in the normal prostates. It is significant that this age-related PDE4D4 hypermethylation was directly associated with loss of gene expression as determined by real-time RT-PCR. In contrast to normal prostates, the PDE4D4 CpG island became hypomethylated with aging in all prostates exposed neonatally to high or low-dose estradiol or to BPA. Furthermore, this was directly associated with continued elevated PDE4D4 expression throughout life. Cell line studies confirmed that site-specific methylation is involved in transcriptional silencing of the PDE4D4 gene and showed hypomethylation of this gene in the prostate cancer cells. Importantly, PDE4D4 hypomethylation with increased gene expression was distinguishable in all neonatally estrogen/BPA-exposed prostates as early as day 90 of age before any secondary exposure to estrogens had commenced. This raises the pos-

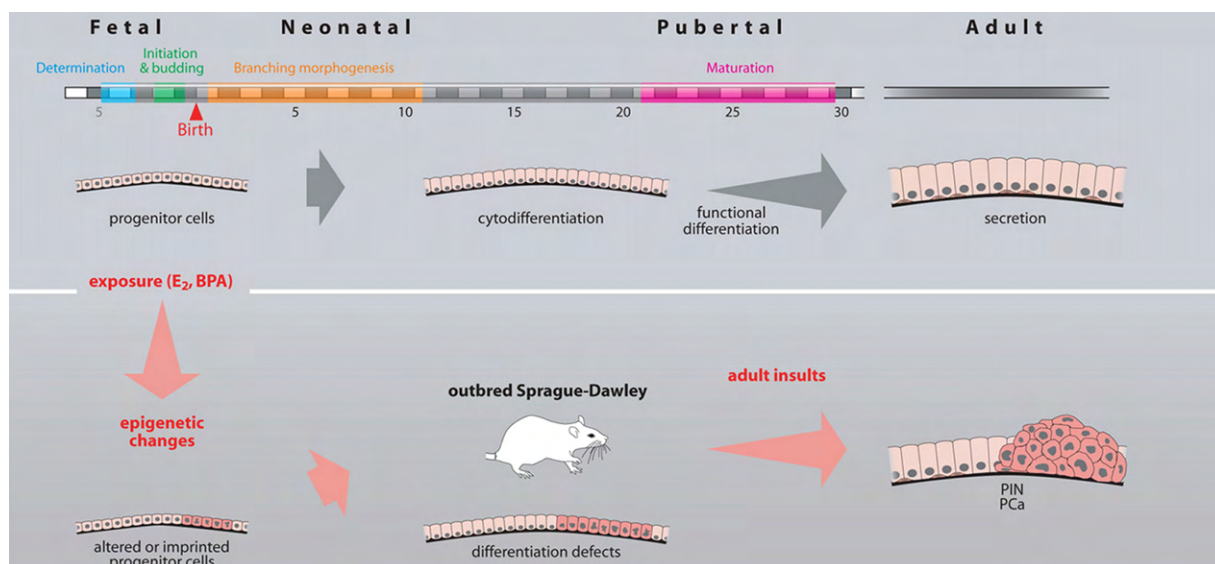


Fig. 3. Proposed model for epigenetic mechanism of developmental estrogenization of the prostate gland by exposures to estradiol, E₂ or environmental disruptors such as BPA. See text for description.

sibility that PDE4D4 could be used as a molecular marker for prostate cancer risk assessment as a result of endocrine disruptors.

8. Summary and conclusions

In summary, we have shown that a full range of estrogenic exposures during the developmental critical period – from environmentally relevant BPA exposure to low-dose and pharmacologic estradiol exposures – results in an increased incidence and susceptibility to neoplastic transformation of the prostate gland in the aging male which may provide a fetal basis for this adult disease. Our working hypothesis is that abnormal estrogenic exposures during developmental critical periods initiate permanent molecular and cellular changes early in life which predispose the prostate to neoplasia in adulthood. Further, our recent findings provides evidence that developmental exposures to environmental endocrine disruptors (such as BPA), pharmacologic and natural estrogens (E_2) impact the prostate epigenome during early life which suggests an epigenomic basis for estrogen imprinting of the prostate gland. Based upon these findings, we propose a model for prostate cancer susceptibility due to developmental estrogen exposures in Fig. 3. As depicted on the time-line, prostate development normally proceeds from fetal initiation and prostatic budding of progenitor cells, to neonatal morphogenesis and cytodifferentiation to pubertal growth and maturation with functional secretions which continue into adulthood. Developmental exposure to estrogens {high-dose pharmacologic exposure, low-dose exposures such as elevated maternal E_2 , or environmentally relevant xenoestrogens (e.g. BPA exposure)} lead to epigenetic changes (altered DNA methylation) which are heritable through subsequent cell divisions. This initially results in the expansion of progenitor cells with differentiation defects as the prostate develops. Subsequent adult insults such as rising E_2 with aging (or injury, inflammation or other mutation-generating events) are then required to promote these initial alterations leading to prostatic dysplasia and tumor-formation with aging.

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Review

Techniques used in studies of epigenome dysregulation due to aberrant DNA methylation: An emphasis on fetal-based adult diseases

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Abstract

Epigenetic changes are heritable modifications that do not involve alterations in the primary DNA sequence. They regulate crucial cellular functions such as genome stability, X-chromosome inactivation, and gene imprinting. Epidemiological and experimental observations now suggest that such changes may also explain the fetal basis of adult diseases such as cancer, obesity, diabetes, cardiovascular disorders, neurological diseases, and behavioral modifications. The main molecular events known to initiate and sustain epigenetic modifications are histone modification and DNA methylation. This review specifically focuses on existing and emerging technologies used in studying DNA methylation, which occurs primarily at CpG dinucleotides in the genome. These include standard exploratory tools used for global profiling of DNA methylation and targeted gene investigation: methylation sensitive restriction fingerprinting (MSRF), restriction landmark genomic scanning (RLGS), methylation CpG island amplification-representational difference analysis (MCA-RDA), differential methylation hybridization (DMH), and cDNA microarrays combined with treatment with demethylating agents and inhibitors of histone deacetylase. The basic operating principals, resource requirements, applications, and benefits and limitations of each methodology are discussed. Validation methodologies and functional assays needed to establish the role of a CpG-rich sequence in regulating the expression of a target or candidate gene are outlined. These include *in silico* database searches, methylation status studies (bisulfite genomic sequencing, COBRA, MS-PCR, MS-SSCP), gene expression studies, and promoter activity analyses. Our intention is to give readers a starting point for choosing methodologies and to suggest a workflow to follow during their investigations. We believe studies of epigenetic changes such as DNA methylation hold great promise in understanding the early origins of adult diseases and in advancing their diagnosis, prevention, and treatment.

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Keywords: Cytosine methylation; Chromatin remodeling; Fetal-based adult disease; Epigenetics; Genome-wide methylation profiling; Methylation sensitive restriction fingerprinting; Restriction landmark genomic scanning (RLGS)

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1. DNA methylation as an epigenetic mechanism of fetal-based adult diseases

1.1. Epigenetic modulation of fetal programming

Past research has identified mutations, deletions, gene fusion, tandem duplications, and gene amplifications as key mechanisms that dysregulate expression of disease-predisposing or disease-determining genes at the linear DNA level [1–7]. However, it has become clear that disruption of epigenetic regulation of gene expression plays an equally important role in the development of diseases [8–10].

The term *epigenetic* means *outside conventional genetics* [11]. Epigenetic changes are reversible, heritable modifications that do not involve alterations in the primary DNA sequence. Three distinct and intertwined mechanisms are now known to initiate and sustain epigenetic modifications: small-interfering RNAs, DNA methylation, and histone modification [12–14]. These processes affect transcript stability, DNA folding, nucleosome positioning, chromatin compaction, and ultimately nuclear organization. Singularly or conjointly, they determine whether a gene is silenced or activated. Dysregulation of these processes certainly is the possible mechanism underpinning the epigenetic basis of disease development [8,9]. Disease susceptibility, therefore, is a result of a complex interplay between one's genetic endowment and epigenetic modulations induced by endogenous or exogenous environmental cues.

Epigenetic modifications of disease risk could begin as early as during fetal development and be transmitted transgenerationally [15–20]. The paradigm of fetal basis of adult disease first emerged from large-cohort epidemiological studies linking poor growth *in utero* with adult diseases [16,21,22]. During pregnancy, maternal conditions such as nutritional deficits, infection, hypertension, diabetes, or hypoxia expose the fetus to hormonal and metabolic cues that induce “fetal programming.” It alters the courses of cellular and organ differentiation *in utero* and permanently affects the functional capacity of adult organs in later stages of life [15,22]. From an evolutionary perspective, fetal programming is an “adaptive” trait since it allows the fetus to make anticipatory responses to the external environment to

gain advantages for later life challenges. However, contemporary human life is greatly influenced by lifestyle choices that are in conflict with the programmed adaptive changes made during fetal development. In addition, synthetic agents that mimic internal cues can alter the course of fetal programming adversely. Both could cause insufferable consequences in later life, leading to heightened disease susceptibility. Classical examples include the association of lower birth weight with a greater risk for adult onset of cardiovascular disease [23], Type 2 diabetes mellitus [24], osteoporosis [25], and depression [26]. The link between exposure to the synthetic estrogen diethylstilbestrol (DES) *in utero* and increased incidence of reproductive tract cancers in “DES daughters” has been a difficult lesson learned by the health care community [27]. Genetic factors, such as telomere attrition [28] and polymorphisms in mitochondrial DNA [29], may in part mediate fetal programming. However, epigenetic dysregulation of gene expression is currently a widely accepted mechanism of fetal-based adult disease [15–20].

The two main epigenetic mechanisms currently recognized as playing a role in the fetal basis of adult disease are histone modification and DNA methylation [15–20]. A comprehensive review of how these processes affect gene transcription is beyond the scope of this review. In simple terms, the histone modification refers to post-translational modifications of histone tails, while DNA modification involves methylation of cytosine at the carbon-5 position in CpG dinucleotides. The two processes work together to affect chromatin packaging of DNA, which, in turn, determines which gene or gene set is transcribable. Changes mediated by either process are heritable, not only transmittable to the daughter cells, but to subsequent generations [19,30]. Thus, interest in the field of epigenetic control of fetal-based disease has increased dramatically within the last few years. Our intention with this review is to give readers a starting point for choosing methodologies and a workflow to follow during their investigations. The past decade has witnessed an exponential increase in novel approaches to the conduct of epigenetic analysis [31]. Methodologies applicable for the investigation of histone modifications have recently been reviewed [13]. This review will focus primarily on contemporary methods designed

to elucidate DNA methylation—regulated gene expression as a mechanism of early origins of adult diseases.

1.2. DNA methylation: chemistry, developmental dynamics, and proposed functions

DNA methylation refers to the covalent addition of a methyl group derived from *S*-adenosyl-L-methionine to the fifth carbon of the cytosine ring to form the fifth base, 5-methyl cytosine (5mC; Fig. 1) [32,33]. The reaction is catalyzed by DNA methyltransferases and accessory proteins (Dnmt1, Dnmt3a, Dnmt3b, Dnmt2, and Dnmt3L). Across eukaryotic species, methylation occurs predominantly in cytosines located 5' of guanines, known as CpG dinucleotides (CpGs), although methylation also takes place in non-CpGs such as CpNpG and nonsymmetrical CpA and CpT at a lower frequency.

In the mammalian genome, the distribution of CpG sequences is nonrandom [34]. Because of the high susceptibility of 5mC to undergo spontaneous deamination to yield thymine (Fig. 1), the mammalian genome has become progressively depleted of CpGs through the course of evolution. CpGs are normally under-represented, appearing at a low frequency of 1 per 80 dinucleotides in 98% of the mammalian genome. In contrast, CpGs are found as clusters known as CpG islands (CGIs) in 1–2% of the genome. Typically, a CGI ranges in length from 200 bp to 5 kb, has a high percentage of CG (>60%), and a ratio of CpG to GpC of at least 0.6. CGIs are normally unmethylated and presumably protected from spontaneous deamination. Important exceptions to the unmethylated status of CGIs include those that are associated with imprinted genes, genes subject to X-chromosome inactivation, and transposable elements [33,35]. About 70% of CGIs are associated with DNA sequences 1- to 2-kb long located in the promoter, the first and second exons, and the first intron regions of all genes (5' CGIs) [36]. Most 5' CGIs are found overlapping with the transcription start site, suggesting that they are important for gene regulation.

It has been proposed that over half of the 5' CGIs participate in the regulation of gene transcription [33,35], although, it is important to note, not all CGIs are involved in gene regulation. An inverse relationship usually exists between the extent of methylation of a regulatory CGI and gene transcription [35]. Two mechanisms have been proposed to explain how cytosine methylation leads to repression of gene transcription [33]. First, the methyl group of the 5mC extends into the major groove of DNA and inhibits binding of transcription factors (TFs) to their CpG-containing recognition sites. Second, a class of proteins known as methyl-binding proteins (MBDs) specifically bind methylated CGIs and create steric hindrance to access by TFs to their regulatory elements. Both scenarios will suppress gene transcription. Furthermore, upon binding to methylated CGIs, MBDs recruit histone deacetylases (HDACs) and histone methyltransferases (HMTs). These enzymes mediate complex histone modifications and result in the establishment of repressive chromatin structures that permanently silent gene transcription [35,37].

DNA methylation is viewed, at the global level, as a generalized repression system in more complex genomes [38]. It is thought to repress inappropriate expression of endogenous transposons that may disrupt the organization and integrity of the genome and to be the key mechanism responsible for X-chromosome inactivation and genomic imprinting [33,35]. Recent evidence suggests that DNA methylation plays a crucial role in the establishment and/or maintenance of cell- or tissue-specific gene expression in adult somatic tissues.

DNA methylation pattern is established through defined phases during the development of an organism. Gamete methylation patterns are erased by a genome-wide demethylation at around the eight-cell stage of blastocyst formation. During the implantation stage, methylation patterns are established via *de novo* methylation. In adulthood, the amount and pattern of methylation are tissue- and cell-type specific. Disruption of these preset patterns of DNA methylation in adult life has been linked to aging and disease development [39–41]. Furthermore, dereg-

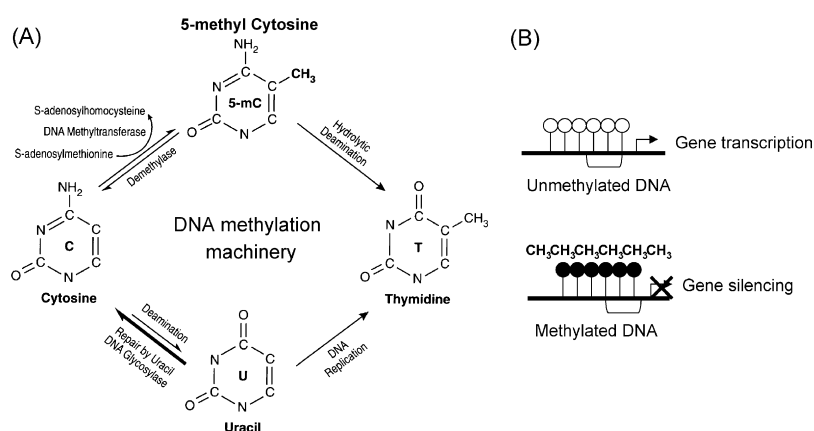


Fig. 1. DNA methylation machinery and transcriptional repression. (A) Diagrams showing the biochemical pathways for cytosine methylation, demethylation, and mutagenesis of cytosine and 5-metC [33]. DNA methylation by addition of a methyl group to carbon 5 position of the cytosine ring is catalyzed by DNA methyltransferases (DNMTs), and demethylation is catalyzed by demethylase. 5-Methyl cytosine (5mC) undergoes hydrolytic deamination to thymidine. Mutation at CpG occurs because 5mC is more susceptible than cytosine to deamination and because some of the T-G mismatches produced by deamination are poorly repaired. (B) Methylation at cytosine of DNA blocks transcription. Singal and Ginder have proposed three mechanisms of how methylation inhibits gene transcription [33]. Details are in the text.

ulation of fetal programming by suboptimal maternal factors or environmental mimics is now believed to involve abnormal DNA methylation of specific genes that permit them to undergo either inappropriate or untimely expression in adult life, leading to disease development. Recent and limited evidence supports this as a key mechanism of the fetal basis of adult disease (see below).

1.3. DNA methylation in fetal basis of adult disease

Several lines of direct and indirect evidence support the hypothesis that DNA methylation is a key mechanism mediating fetal-based adult disease development. Fetal exposure of agouti mice to methyl donors (folic acid, choline, and betaine), which is expected to induce global DNA methylation changes, causes shifts in the coat color from yellow to brown and reduces the risk of obesity, diabetes, and cancer [42]. Global gene profiling identified altered expression of 27 genes with regulatory 5' CGIs in mice born to mothers on a choline-deficient diet [43]. These mice also exhibited abnormal hippocampal development and loss of memory function in adult life.

McLachlan and associates [44] provided the first evidence in support of the effects of early-life exposure on DNA methylation of specific genes that may show dysregulated expression in disease target organs later in life. These investigators found a high incidence of uterine cancer and elevated expression of *c-fos* and *lactoferrin* genes in mice exposed neonatally to diethylstilbestrol (DES) [45]. In concordance with epigenetic regulation of gene expression as a underpinning the early origin of adult disease, these investigators reported demethylation of a single CpG site in the lactoferrin promoter and hypomethylation of the promoter and intron-1 regions of *c-fos* in the uterus of adult mice exposed neonatally to DES [46]. In a multi-generations study, Skinner and associates [19,47] demonstrated induction of 15 imprinted-like genes/DNA sequences with altered methylation patterns in sperm obtained from mice exposed developmentally to vinclozolin. Importantly, the altered methylation patterns in these sequences persist in sperm of subsequent non-exposed generations. Although these findings did not provide a direct link between changes in methylation status of the susceptible genes and disruption of their expression in later life, they are highly suggestive that such a relationship exists. More direct evidence linking epigenetic re-programming via DNA methylation and aberrant gene expression in adult tissues and altered disease susceptibility was provided by a recent study showing increased prostate cancer risk in rats exposed neonatally to bisphenol A or estradiol [20]. In this study, the investigators demonstrated concomitant, aberrant overexpression of *phosphodiesterase 4 variant 4 (PDE4D4)* and hypomethylation in a 5' CGI of *PDE4D4* in the prostate of rats exposed early in life to the estrogen and its mimic. Additional cell-culture experiments further substantiated the claim for this interrelationship.

Since the fetal basis of adult disease is a rapidly growing field, it would be timely to provide a comprehensive review of methods and techniques available to interested investigators. It is our intention to provide readers with a starting point in their choice of methodologies and a workflow to follow during

their investigations. The basic operating principals, resource requirements, applications, and benefits and limitations of each methodology are discussed below. Validation methodologies and functional assays necessary to establishing the role of a CpG-rich sequence in regulating the expression of a target or candidate gene are briefly outlined. We recognize that this is a rapidly growing field, with many innovative methodologies and approaches emerging daily, and thus that it will be impossible for us to cover this topic exhaustively.

2. Techniques and methods

In this review, we will outline methodologies that would allow investigators to globally discover CpGs or CGIs that are differentially methylated in different tissues/cells or under different experimental conditions. Many of the methods are also applicable to investigations in which researchers already have target genes in mind. In the course of describing methodologies, we will first introduce the operating principals, then give examples of applications, and finally discuss the benefits and limitations for each technique. Table 1 summarizes some of the key features of each methodology and their advantages and limitations for easy reading and for facilitating decision-making regarding the choice of a methodology for a particular study.

To identify candidates under methylation regulation and to determine whether a newly identified CGI is involved in gene regulation, one needs undertake a comprehensive and vigorous investigative approach (Fig. 2). The typical experimental workflow for a discovery platform involves: (1) identification of candidates through genome-wide screening methods, (2) in

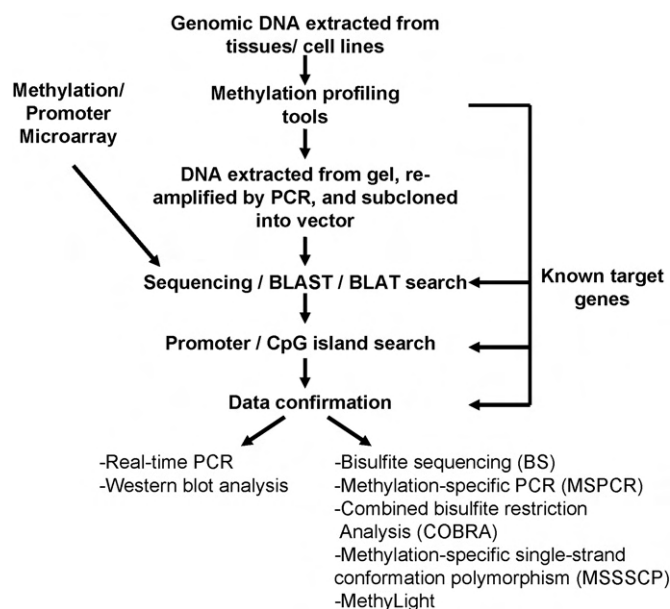


Fig. 2. The work-flow chart outlines how to discover the methylated genes in a step-by-step manner. First, one of the methylation profiling techniques is used to pool out several candidate clones showing differential methylation patterns. After subcloning and sequencing, candidate clones are identified by aligning sequences into BLAST (from NCBI) or BLAT (from UCSC) database. Next, *in silico* database analysis, such as promoter and CpG island search on the candidate clones, is done to characterize the gene structure and design primers for subsequent data validation assays. Several methods, such as RT-PCR, Western blotting analysis, bisulfite sequencing (BS), methylation-specific PCR (MS-PCR), combined bisulfite restriction analysis (COBRA), methylation-specific single strand conformation polymorphism (MS-SSCP), and MethylLight, are used to validate the methylation level and gene expression of the target genes. If target genes are ready the methylation studies, it is not necessary to start from the first step. Methylation studies start from any step, depending on the researcher's needs.

Table 1
Advantages and limitations of different approaches to methylation profiling

Methods	Global/gene specific	Sample amount	Advantages	Limitations	Examples
MSRF	Global	100 ng–1 µg	Simple set-up Screen for novel genes	Small scale	[20,49]
RLGS	Global	1–5 µg	High-throughput	Landmark site only Special gel electrophoresis set-up Software is not available for every species	[59–61]
MCA-RDA	Gene specific Global (RDA)	5 µg	High-throughput CpG islands specific	Need of prior knowledge of sequences (except RDA)	[63–65]
DMH/MSO	Gene specific	2 µg	High-throughput CpG islands specific	Need of prior knowledge of sequences	[71,81]
CGI/ChIP-chip Promoter array	Global	1–10 µg	High-throughput	High cost Platform specific Not popular	[79] [106,107]
LUMA	Global/gene specific	200–500 ng	High-throughput	Relative high cost Platform specific Limited length of sequence	[108,112,113]
MALDI-TOF/HPLC	Gene specific	10 ng–1 µg	High-throughput	Relative high cost Platform specific Still in stage of optimization of the protocol Mostly on specific target gene	[123,130] [131,132]

silico database analyses to shortlist candidates that contain 5' CGIs or CpGs, (3) confirmation of changes in methylation status in the candidate CpGs under the expected experimental conditions, (4) establishment of a causal or correlative relationship between increased cytosine methylation and gene silencing, and (5) other supportive functional assays to uncover the functionality of the cognate genes. However, if a target gene is to be studied, the entry point could begin at step (2). All the subsequent steps are applicable for target-gene studies.

2.1. Methods for identification of candidates

Currently, there is a wide range of approaches to obtaining quantitative and qualitative information on changes in genomic DNA methylation. Several standard exploratory tools, including methylation sensitive restriction fingerprinting (MSRF), restriction landmark genomic scanning (RLGS), methylation CpG island amplification-representational difference analysis (MCA-RDA), differential methylation hybridization (DMH), methylation-specific oligonucleotide microarrays (MSO), methylation target array (MTA), and cDNA microarrays combined with treatment of demethylating agents, are routinely used to identify putative regulatory CGIs at the genome-wide level, as well as to analyze methylation status of specific CGIs of known genes in large sample sets. Other

advanced techniques, such as chromatin immunoprecipitation (ChIP)-on chips, polymerase extension assay by pyrosequencing, mass spectrometry, and HPLC, have emerged as high-throughput methods for obtaining information of CpG methylation outside/inside CGIs. These techniques also have the potential for being used in a whole-genome screen.

2.1.1. Methylation sensitive restriction fingerprinting (MSRF)

MSRF (Fig. 3) is a sensitive PCR-based method that allows one to screen for novel CpG-rich sequences that exhibit differential changes in cytosine methylation under different physiological or pathological conditions [48]. Multiple samples can be compared simultaneously. Genomic DNA isolated from tissues is subjected to *MseI* restriction digestion. Since the restriction site of *MseI* is TTAA, which is rarely found in CG-rich regions, cellular DNA is digested into small fragments, leaving most of the CG-rich region intact. The *MseI*-digested DNA is then divided into two aliquots with one aliquot left unmodified and the other subjected to digestion with *BstUI*, a methylation-sensitive restriction enzyme. *BstUI* cuts at CGCG, a sequence that occurs in more than 80% of CGIs, and therefore will cut most CG-rich regions unless they are protected by methylation. Short DNA sequences with methylated *BstUI* sites are left uncut and can subsequently be amplified by PCRs, whereas sequences with

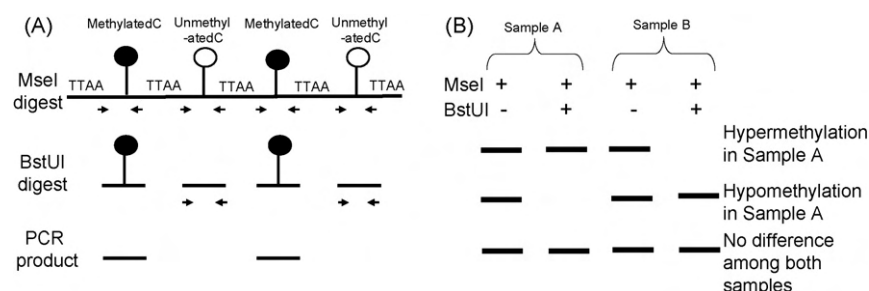


Fig. 3. Principle of methylation sensitive restriction fingerprinting (MSRF). (A) Methylated cellular DNA that will be digested by enzymes *MseI* and *BstUI* and DNA with an intact methyl group can be amplified in PCR. (B) A schematic diagram depicting expected results. In this comparison, the methylation status was defined by comparing samples A and B. If there is no difference in methylation status, no difference in methylation status will be observed for both cases. If that candidate is being methylated in sample A, none of the *BstUI* sites in the CpG-rich sequence will be digested and they can be amplified in PCR. If vice versa, DNA fragments will be absent in the lane of sample A (*MseI/BstUI*) if this candidate is unmethylated in sample A.

unmethylated *Bst*UI sites are cut and will not yield any PCR products. Aliquots of the single (*Mse*I)- or double (*Mse*I and *Bst*UI)-digested DNA are then subjected to PCR amplification using different pairs of short arbitrary primers (10mer with at least one CG site in the primer) [48] in the presence of radiolabeled [³²P]-dNTPs. Labeled PCR products are size-fractionated on 6% polyacrylamide gels. “Candidates” with differentially methylated patterns among different samples are visualized with autoradiography, which is used to guide excision of “candidates” from the dried sequencing gels. Amplification and sequencing of the candidates followed by sequence database analyses reveal the identities of the candidates and possibly their innate genes.

We have successfully used MSRF to globally profile the prostate epigenome for genes whose promoter/5′ CGIs undergo aberrant cytosine methylation following neonatal exposure to estrogens or bisphenol A [20]. Among the initial 50 candidate sequences identified, three were confirmed to be part of the promoter regions of genes regulated by methylation. One of the genes, phosphodiesterase 4D4, was further characterized for its functional role in modulating prostate cancer risk in adulthood [20]. In an earlier study [49], using a similar approach, we identified hypermethylation of peroxisome membrane protein 24 as a marker/modifier for the transition of prostate cancer cells from an androgen-dependent to an androgen-independent state. Others have used this method to identify genes whose promoters are aberrantly methylated in breast cancers [48], nasopharyngeal carcinoma [50], and hepatocellular carcinoma [51]. This method has also been used to identify novel epigenetically modified candidates during stem cell differentiation [52].

With radiolabeling of PCR products, only a small amount of genomic DNA from each sample is required (100 ng–1 μg) (Table 1). Therefore, this method is highly applicable to studies using laser-capture microdissected samples or for developmental studies in which tissue availability is a key concern. Usually products from 4–8 sets of arbitrary primers are run on each gel and only a limited number of candidates (30–40) can be identified in one autoradiograph. The ease of setting up MSRF for methylation profiling of multiple samples is definitely an advantage of this methodology, since it only requires the use of a simple sequencing gel set-up, standard PCR protocols, and routine amplification and sequencing techniques. By increasing the number of arbitrary primers used in MSRF, one can increase the coverage of the genome and the number of candidate sequences identified. For example, in two of the experiments we have conducted [20,49], both using 4 primers and 6 permutations of primer pairs, we identified approximately 50 candidate sequences. However, if 9 primers were to be used, a total of 40 [$n \times (n - 1)/2$; n is the number of primers] permutations for arbitrary PCRs could be achieved, likely yielding over 1400 candidates.

2.1.2. Restriction landmark genomic scanning (RLGS)

The RLGS approach was first developed to identify imprinting genes [53] and later adapted for genome-wide screening of methylation changes in CGIs (Fig. 4). It can evaluate the methylation status of thousands of CG-rich sequences and simultaneously obtain information on copy number of their cognate genes [53–56]. Methylation detection in RLGS profiles depends on the methylation sensitivity of the restriction enzymes used to cut the genomic DNA. *Not*I, which recognizes GC-rich sequences, is most commonly used to generate thousands of landmark sites on the gel. Differences in digestion are assessed by radiolabeling the DNA at cleaved *Not*I sites. Following further endonuclease digestion, two-dimensional electrophoretic separation, and autoradiography, the intensity of a DNA fragment on the resulting RLGS profile quantitatively reflects the copy number and methylation status of the *Not*I fragment (Fig. 4). Early applications of RLGS for identification of novel differentially methylated genes were hampered by the tedious task of having to obtain sequence information on the identified sequences. With the completion of several commonly used genome databases (human and mouse), development of interactive informatics tools, and tailored software programs, it is now possible to conduct automated RLGS fragment prediction and to download corresponding sequences for mouse [57] and human studies [58]. Users can directly upload or query RLGS databases at <http://genome.gsc.riken.go.jp/RLGS/RLGSHome.html> or <http://dot.ped.med.umich.edu:2000/VGS/index.html>.

RLGS has been used to analyze epigenetic changes due to aberrant DNA methylation for breast cancer [59], ovarian cancer [60], and hepatocellular carcinoma [61]. Recently, Sato and co-workers applied this method to illustrate the aberrant methylation of genomic DNA in the epididymis of mice neonatally exposed to DES [62]. Seven loci of the genomic DNA were found to be

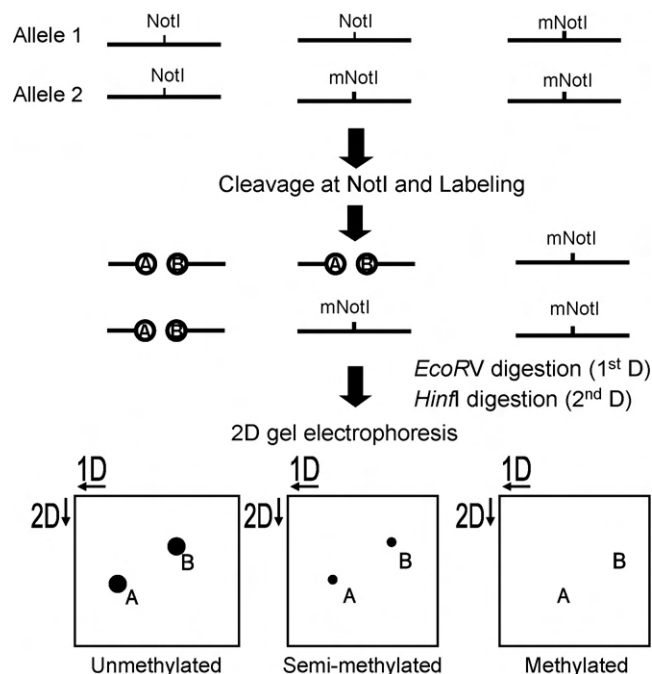


Fig. 4. Diagram showing the procedures of restriction landmark genomic scanning (RLGS). Methylation detection in the RLGS profile depends on the methylation sensitivity of *Not*I. *Not*I, which recognizes CG-rich regions and cannot cleave DNA sequences when 5-cytosine is methylated, acts as the landmark in the profiles. Three types of methylation status are expected, depending on which allele is methylated. m*Not*I represents the methylated site of the enzymes. Following further endonuclease digestion of *Eco*RV (1D gel electrophoresis) and *Hinf*I (2D gel electrophoresis), the RLGS profile will result in a change in spot intensity that quantitatively reflects the copy number and methylation status of the *Not*I fragment.

aberrantly demethylated, and one locus in the epididymis of DES-treated mice was found to be methylated. Among these putative candidates, four were confirmed to have promoter or 5′ CGIs. However, additional experiments necessary to establish the regulatory role of these CGIs on their cognate gene expression were not included in this study. This omission of validation experiments has prevented the authors from drawing definitive conclusions on the significance of DNA methylation changes in these CG-rich sequences or their cognate genes in mediating DES-induced abnormalities in this organ.

An obvious advantage of RLGS is its ability to identify thousands of loci/landmark fragments in a single run (Table 1). With continued improvements on the various RLGS sequence databases, sequence identification of the loci should no longer be a limitation of this methodology. At least for human and mouse studies, the current automated RLGS fragment prediction programs and sequence databases are quite adequate, yet challenges still exist for studies using samples from other species. Furthermore, since the methodology requires at least a few micrograms of DNA, sample availability may be a limitation for some investigations. Other demands for successful utilization of RLGS are the requirements for a fairly elaborate gel electrophoresis set-up and a powerful image analysis system. Finally, limited library coverage is another constraint for this approach unless new advanced genome sequence-based tools are developed in the future.

2.1.3. Methylation CpG island amplification-representational difference analysis (MCA-RDA)

Methylated CGI amplification (MCA) coupled with representational difference analyses (RDA) [63,64] provides a powerful approach [65] for simultaneous identification and cloning of novel differentially methylated sequences between two samples (Fig. 5). The fundamental principle of MCA involves amplification of DNA sequences with closely spaced (<1 kb)

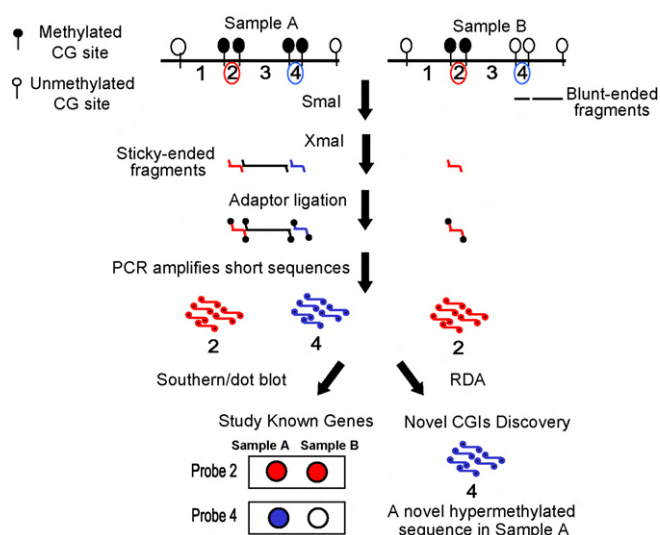


Fig. 5. Schematic diagram for methylation CpG island amplification (MCA) coupled with representational difference analysis (RDA). CpG sites are labeled as 1 to 4. In the comparison of the methylation status of sample A and sample B, CpG site 2 is methylated in both cases whereas CpG site 4 is methylated only in sample A. For MCA, unmethylated CpGs are digested by methylation-sensitive restriction enzyme (*SmaI*), resulting in the formation of the blunt end fragments. Those methylated CpGs are then digested with *XmaI* and generate sticky end fragments. Following ligation into linker and PCR amplification, amplicons of short sequences can then be directly hybridized to enable study of the methylation status of the gene of interest for which a probe is available. As shown in the figure, differential methylation of CpG sites 2 and 4 between samples A and B can be found in dot-blot analysis. Besides, MCA amplicons (i.e., fragments containing CpG site 4) showing differential methylation status in sample A can be cloned by RDA to identify any novel candidates.

methylated *SmaI* (CCCGGG) sites, which are commonly found in CGIs. Since only short fragments (400–600 bp) flanked by two *SmaI* sites are amplified, MCA ensures enrichment of CGIs [65]. A pair of enzymes, *SmaI* (methylation-sensitive) and *XmaI* (methylation insensitive), that cut the same recognition sequence CCCGGG are used in the protocol (Fig. 5). The two DNA samples under investigation are first subjected to *SmaI* digestion to remove unmethylated *SmaI* sites, generating only blunt end fragments in DNA regions that with no methylated *SmaI* sites; i.e., hypomethylated CGI regions. These blunt end sequences will not be amplified by subsequent steps of the protocol and thus are eliminated from the DNA pools. The samples are then digested with the *SmaI* isoschizomer *XmaI*, which cuts at methylated *SmaI* sites, generating fragments with a four-base overhang. Adaptors are ligated to this overhang before the fragments are amplified by PCR using primers complementary to the adaptors. Southern or dot blotting analysis is employed to determine if a candidate CGI is differentially methylated among samples.

To identify novel CGIs that are differentially methylated in two different samples, RDA, a subtraction technique developed to clone small differences between genomes [63], is used following MCA, a technique referred to as MCA-RDA. The RDA technique is based on subtraction of “tester” sequences from the “driver” pool of sequences, followed by PCR amplification of the tester sequences left un-hybridized. Differentially present clones, after the subtraction, are identified by subcloning and sequencing.

MCA-RDA was first used to identify novel hypermethylated CGIs in colorectal cancer [65]. More recently, several groups have used this method to identify genomic clones that are hypermethylated in microsatellite instability-positive sporadic colorectal cancers [66], hypermethylated DNA fragments as putative biomarkers of prostate cancer [67], and hypermethylated sequences uniquely expressed in pancreatic carcinoma but not in normal pancreatic cells [68].

The MCA assay can be adapted for medium-throughput determination of the methylation status of a large number of target genes through the utilization of “printed” membranes or highly reproducible dot-blot [69]. The costs and

efforts for these set-ups will depend largely on the numbers of samples and genes under investigation. Similarly, the combined MCA-RDA approach has proven to be highly effective in identifying hundreds of CGIs containing genomic fragments between two samples. However, its limitation resides in the inability to compare more than two samples in a single subtraction experiment. Furthermore, the method is not conducive to the identification of aberrantly hypomethylated sequences.

2.1.4. Differential methylation hybridization (DMH) using CGI arrays

An affinity column that contained the methyl-CpG binding domain of the rat MeCP2 protein was constructed to isolate CGIs from human genomic DNA [70]. Using this CGI library (close to 8000 CGIs), Huang et al. developed a novel array-based method called DMH that allows for a genome-wide screening for differentially methylated CGIs between two different samples [71]. The method has been widely used in the identification of aberrantly methylated gene promoters that are differentially expressed in various cancers [71–75]. In addition to its use in cancer research, DMH has been used to uncover specific CGIs that are altered in the prostates of mice fed genistein-rich diets [76]. Details of the methodology have been reviewed [77] and here we have illustrated only key steps and general principles underlying the procedures (Fig. 6). Genomic DNA isolated from two different samples is first digested with *MseI* to generate small DNA fragments (100–200 bp), leaving most CGIs intact. The cut ends of the DNA fragments are ligated to linkers. Repetitive sequences are removed from the digests using Cot-1 subtractive hybridization [78]. The remaining DNA fragments are then subjected to methylation-sensitive endonuclease *BstUI* digestion. Following *BstUI* digestion, DNA fragments in the two samples are amplified and labeled with cyanine (Cy) 3 or Cy5. The fluorescently-labeled amplicons, representing differentially methylated DNA fragments between the two samples, are co-hybridized to a high-density CGI microarray with approximately 8000 thousand probes. Cy3 and Cy5 fluorescence on each probe is detected in a two-channel scanner. Differences in methylation of a particular CGI between two samples are reflected in fluorescence intensities detected from the Cy3- and Cy5-labeled targets hybridized on the corresponding CGI probe in the microarray.

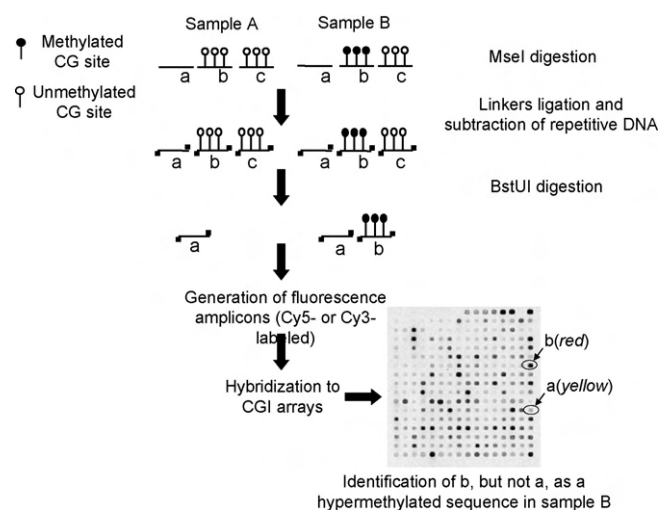


Fig. 6. Schematic diagram for differential methylation hybridization (DMH). In brief, genomic DNA is first digested with *MseI* and ligated to linkers and then digested with methylation-sensitive *BstUI*. Both *MseI* or *MseI/BstUI* digestion products are amplified to generate probes for hybridization to a microarray that is prescreened by their CGI library array. The hybridization output is the measured intensities of the two fluorescence reporters with red (sample B) and green (sample A). Yellow spots indicate equal amounts of bound DNA from each amplicon (i.e., amplicon a), signifying no methylation differences between sample A and B genomes. Spots hybridized predominantly with sample B amplicon but not with sample A amplicon would appear red (i.e., amplicon b), which is indicative of the presence of hypermethylated CpG island loci in the tumor genome. Methylation level of each CG clone can be analyzed.

Although the “first-generation” CGI arrays contain approximately 8000 probes, most published reports [71–75] found that only 0.5–1.5% (40–120 candidates) of the probes on the arrays were differentially methylated in the two samples under comparison. Following additional *post hoc* conformational analyses, most studies identified only a handful (8–15) of genes with promoter CGIs that play a role in gene regulation. The original DMH utilized CGIs dotted on nylon membranes, which have high background signal, thus creating problems with a high noise-to-signal ratio. The fabrication of current arrays on glass slides has minimized this problem. Recently, the availability of new high-density human and mouse CGI arrays have greatly expanded the power of using DMH for a whole-genome screen of differential methylation [79].

In summary, DMH is a useful tool for the discovery of differentially methylated CGIs in two samples. When combined with bioinformatic tools for analyzing expression microarray data, DMH can readily be used to classify samples into biologically or clinically relevant groups on the basis of their methylation profiles (see above references). However, the method does present some limitations for individual investigators if their institutional microarray core facility is not set up to run the arrays. On the technical side, since most genes are single-copy genes, the differences in Cy3 and Cy5 signal intensities for most loci (spots) may not be as strong and readily discernable as those in transcriptional profiling arrays in which fold changes for many probes could be more significant. Furthermore, problems arising from dye bias (Cy5 versus Cy3) would affect DMH more than transcriptional profiling. Furthermore, since the method requires approximately 2–5 μ g of high-quality genomic DNA as starting materials, it may put constraints on experiments in which sample availability is an issue (e.g., microdissected samples). Finally, only two samples can be compared at a time. If multiple samples or treatment groups (several experimental groups over multiple time points) were to be compared, high-order bioinformatic analysis tools would be needed to facilitate these comparisons.

2.1.5. Bisulfite sequencing

Bisulfite sequencing is a “gold-standard” method used to determine the methylation status of each cytosine over an amplified region of a given gene, a method now used routinely for studies of CGIs [80]. The underlying principle is based on the ability of sodium bisulfite to deaminate cytosine (C) residues into uracil (U) in genomic DNA, whereas the methylation cytosine residues are resistant to this modification. After PCR amplification, the Us are amplified as thymines (Ts). Cloning and subsequent sequencing of the DNA fragments containing the CGIs then provide information on the methylation status of each C within the island (Figs. 2 and 11).

The method is used routinely in analyzing the methylation status of any target or candidate DNA sequence containing CpGs. It has the advantage of revealing the methylation status of each CpG dinucleotide within the sequence and also the interrelationship between the methylation status of multiple CpG sites. However, it is more labor intensive than other shot-gun approaches, such as methylation-sensitive PCR (see below). Its successful application also depends on whether nested PCR primers could be designed to amplify the fragment of interest. Furthermore, DNA integrity that is less than optimal, as is the case of DNA isolated from microdissected samples or paraffin-embedded tissues, presents significant challenges for this application.

2.1.6. Methylation-specific oligonucleotide (MSO)/methylation target microarrays

As an alternative to bisulfite sequencing, methylation-sensitive oligonucleotide (MOS) array was initially designed to provide a high-throughput method for fine mapping of CpG sites in a known CGI using a hybridization-based microarray protocol [81–83] (Fig. 7). Subsequently, the methodology was adapted to interrogate simultaneously the methylation status of multiple CGIs [84]. Test DNA is restricted with *KpnI* and *NdeI*, bisulfite modified and amplified, and labeled with Cy5, resulting in a pool of labeled targets with altered nucleotide sequences due to their differential methylation status. Sets of short oligonucleotides (~21–25-mers), corresponding to the methylated and unmethylated versions of the CGI, are designed to provide coverage of the entire region. These pair of oligonucleotides are synthesized and immobilized in triplicates as probes on glass slides. After hybridization of the targets to the probes, hybridization signals are captured, quantified, and analyzed. The percentage of methylation for each short CG-rich fragment (2–4 CpG sites) is

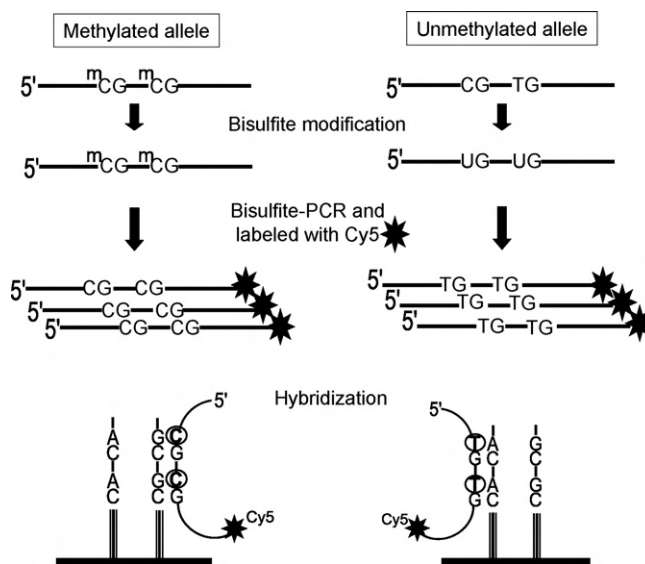


Fig. 7. Diagram illustrating how to analyze DNA methylation by methylation-specific oligonucleotide (MSO). Genomic DNA is first modified with sodium bisulfite before the assay. The probes on the MSO array are a set of short oligonucleotides (~20-mers) designed for specific methylated CG or unmethylated C/TG sites to test all the CpG sites within the CGI of a known gene. Both methylated and unmethylated DNA can be amplified, labeled with Cy5 dye, and then hybridized to oligonucleotide probes attached to the glass plate/membrane. Signals from MSO arrays can be recorded with a fluorescence scanner, and signal intensities between the pair of probes are compared to arrive at the percentage of methylation of the CpG within the short region represented by the oligonucleotides, usually 2–3 CpG sites [81].

determined by comparison of signal intensities between the paired “methylated” and “unmethylated” oligonucleotide probes.

Gitan et al. were successful in using this method to compare the methylation status of the estrogen receptor α CGI in breast cancer cell lines, normal fibroblasts, breast cancers, and control tissues [81]. Yang et al. used this method to examine the androgen receptor gene promoter in 76 cases of non-Hodgkin lymphoma [83]. Hou et al. also used MSO arrays to study the methylation status of *p16* in gastric carcinomas [84]. Yu and associates identified a subset of aberrantly methylated genes/ESTs (25/105) in prostate cancer using MSO arrays printed with probes for 105 CGIs [85]. Similarly, MSO arrays were used to examine 156 loci involving 38 genes to identify those with DNA methylation patterns that differentiate between three types of small B-cell lymphomas [86].

To increase the throughput for screening of methylation changes in the promoter of a given gene among multiple samples simultaneously, Zhou and co-workers have described two versions of methylation target arrays (MTAs) [87]. In their investigation of the promoter region of *IGFBP7* gene in ten cases of breast cancer tissues and six cases of responding normal breast tissues, the investigators have fabricated two arrays, a bisulfite PCR product array and a bisulfite genomic DNA array, and compared the sensitivity and reliability of these two arrays to detect methylation changes in this promoter. They found that the bisulfite genomic DNA array is less suitable than the bisulfite PCR product array for analyzing methylation changes using tissue samples. Hence, the latter methods warrant future development for high-throughput analysis of target genes.

Undoubtedly, MSO array hybridization is a versatile and high-throughput method for comparing the methylation status of known CGIs among multiple samples. Approximately 2–3 μ g of genomic DNA is needed as starting material [85]. However, reproducibility of results could be drastically affected by the performance of the bisulfite treatment, which would impact the quality of the targets. Furthermore, since only a few samples could be processed with a single hybridization, the method is insufficient for effective analyses of hundreds of clinical samples. The efficiency of the various hybridizations may vary significantly. The success of the methodology also depends on the specificity of the methylated/unmethylated oligonucleotides.

2.1.7. Transcriptional profiling of genes reactivated by demethylating agents or inhibitors of histone deacetylation

DNA methylation and histone deacetylation are two major mechanisms resulting in epigenetic silencing of genes through direct and indirect methylation of CGIs. Hence, several investigators have taken the approach of using DNA demethylating agents or inhibitors of histone deacetylation to reactivate epigenetically silenced genes and then using transcription profiling [88] to identify the reactivated genes. This approach allows a pool of candidates to be identified, i.e., those genes that are reactivated after the treatment. *Post hoc* validation methods (see below) are then used to identify among these candidates which promoters play a regulatory role in transcription through cytosine methylation [89–91].

This approach was proven successfully in identifying dysregulated genes caused by aberrant cytosine methylation in colorectal cancer and gastric cancer [89]. A recent study [91] compared the gene sets reactivated by 5-aza-2'-deoxycytosine (5-AZA), a DNA demethylating agent, and trichostatin (TSA), an inhibitor of histone deacetylation, in HepG2 cells. They found distinct and common gene sets that are reactivated by the individual agents and their combination, respectively, suggesting that the two epigenetic mechanisms may regulate different sets of genes. In contrast, Li and associates used an integrated "triple" microarray system to elucidate the "epigenetic hierarchies" on gene regulation. Analyses of data with established statistical models demonstrate that genes reactivated by DNA demethylation and histone hyperacetylation are highly correlative [92]. A comparable approach was recently applied to study the effects of maternal care, an early-life experience, in stable epigenetic programming of gene expression in the hippocampus that mediates stress responsivity. Adult offspring exhibiting anxiety behavior due to maternal care were centrally infused with TSA or methionine. Global transcriptional profiling identified over 900 genes that were reactivated by these two reagents, and these genes are likely as candidates mediating adult anxiety disorder caused by early life experience.

5-AZA is a commonly used DNA demethylating agent [93]; it causes cytosine demethylation by covalently binding to the maintenance DNA methyltransferase (DNMT1) and inhibiting its "proofreading" ability during DNA replication. In this manner, the hemimethylated DNA can escape methylation and thereby become fully unmethylated after one further round of DNA replication. Treatment of cells with 5-AZA has repeatedly been demonstrated to reactivate genes silenced by DNA methylation. However, a few precautionary notes are noteworthy regarding its use in the aforementioned approaches: (1) 5-AZA could cause a low frequency of mutations that affect patterns of gene expression; (2) the agent has a short half-life and must be freshly prepared and added to the cell cultures repeatedly; (3) 5-AZA works best when cells are replicating rapidly; and (4) a period of recovery after treatment is necessary for the new DNA methylation patterns to be established.

Trichostatin A (TSA) belongs to the hydroxamic acid type of histone deacetylase (HDAC) inhibitors with very high potency [94]. TSA is widely used in many experimental settings due to its potency and wide coverage, i.e., it inhibits both class I and II HDAC isoforms. Treatment of cells with TSA has been shown to reactivate about 10% of genes but also to cause silencing of close to an equal number of expressed genes in melanoma cell lines [95]. Most HDAC inhibitors are able to induce expression of genes related to the cell cycle and apoptosis, and therefore genes reactivated by HDAC inhibitors are not necessarily related to epigenetic regulation. Hence, similar to the use of DNA demethylating agents in expression array studies, *post hoc* experiments are needed to verify that the candidates identified are directly regulated by cytosine methylation of their promoter CGIs.

Finally, since the promoters of many genes and ESTs remain unknown, the use of transcriptional profiling to identify DNA methylation-regulated genes still faces significant challenges since confirmation of a regulatory CGI for a given gene is possible only if its promoter region is published or in public databases. The use of genome walking techniques to find the 5' upstream sequence of a gene of interest could be tedious.

2.1.8. Genome-wide methylation array/promoter arrays

The initial CGI library was first produced by Cross and co-workers by isolating CG-rich DNA fragments with methyl-CpG binding protein affinity columns (see above). Subsets of these CG-rich clones (9-k set or 12-k set) were used in the construction of several arrays for the detection of differentially methylated CGIs [70,71]. However, not all the CG-rich clones correspond to CGIs and not all

CGIs are in the promoter regions. Promoter arrays have recently become readily available through the University Health Network Microarray Center (UHNMC) in Toronto (www.microarray.ca). More than 20K CG-rich sequence clones can be assessed and used to distinguish promoter methylation status of human and mouse genome. The UHNMC's human CpG island array contains 12,192 spotted clones, while the mouse array contains 7296 probes. These probe sets cover a large percentage of CGIs found in the human and mouse genomes. Moreover, the UHNMC provides a web interface for users to download the corresponding sequences of the clones. These arrays clearly provide a high-throughput, genome-wide screen of promoters with CGIs undergoing methylation changes due to experimental or pathological conditions. Since these arrays have become commercially available, an increasing number of studies using this approach have been published and yield insightful data on epigenetic gene regulation [75,79,96–98]. A new approach that combines methylation-sensitive enzyme digestion with the comparative genomic hybridization (CGH) technique was developed to screen the entire genome for changes in methylation pattern [99].

The above technology remains most suitable for running by a core facility, where inter-array variations could be minimized and high-throughput could be achieved. The expenditure for setting-up routine hybridization is relatively high since it requires hybridization stations, equipment for quality controls of the targets (e.g., Nanodrop), and a scanner for capturing signals. In addition, because of the large volume of data generated in one experiment, pre- and post-experimental consultations on experimental design and data mining are critical elements for this kind of study. Specific software packages provided by the vendors and others developed in-house are required to generate meaningful results.

Custom designed or small-scale promoter methylation arrays have recently become commercial available that simultaneously profile the methylation status of the gene of interests from one sample (NimbleGen System; Panomics). This is a high-throughput analysis of promoter methylation that costs less than the genome-wide CGI array.

2.1.9. Chromatin immunoprecipitation (ChIP) on DNA microarray (ChIP-chip)

Chromatin immunoprecipitation (ChIP) was developed to identify and characterize the interactions of specific genomic DNA sequences associated with a target protein such as a transcriptional factor in the context of intact cells [100]. An antibody specific to the target protein is used to immunoprecipitate the protein–DNA complexes. After the crosslink between the two is reversed, the identities of the DNA sequences are then uncovered by amplification and sequencing. Recently, with the advent of bioinformatics on promoter/transcription start site [101] and DNA microarrays, it has become possible to use the DNA fragments isolated from a ChIP assay as targets to probe a microarray in a protocol known as the ChIP-chip [102–106] (Fig. 8). The logical approach to applying the ChIP-chip methodology to study the effects of DNA methylation/chromatin structure on gene expression is to use groups of antibodies specific for histone deacetylase/methylases to pull down DNA, followed by DNA array analysis. A human promoter array has recently been developed for ChIP experiments (Affymetrix). It is a single array comprising more than 4.6 million probes tiled through more than 25,500 human promoter regions proximal to transcriptional start sites and probes for approximately 59% CpG islands annotated by UCSC in the NCBI human genome assembly (Build 34).

An example of the use of the ChIP-chip approach for methylation studies is the identification of the "methylome," which refers to the complete set of DNA sequences susceptible to methylation in a cell [107]. Genomic DNA is sonicated into smaller fragments. Anti-5'-methylcytosine antibody is used to enrich methyl cytosine-rich genomic DNA fragments, a procedure known as methylated DNA immunoprecipitation (MeDIP). The MeDIP-enriched fragments are then labeled with Cy3, while the non-MeDIP DNA (or the input DNA) is labeled with Cy5. The two samples are co-hybridized to a sub-megabase resolution tiling (SMRT) array or a CGI array. Relative signal intensity at each locus (spot) represented on the array indicates methylation status. DNA methylation profiles could be obtained simultaneously at both genome-wide and locus-specific levels.

This approach provides an unbiased, whole-genome view of changes in methylation status that are mapped to chromosomal regions. It is one of the most exciting of the recently developed applications of the ChIP-chip technology for DNA methylation studies. However, the limitations in this technique remain the high cost of setting up the array technology platform and the dependence on the efficiency of the antibody to pull down DNA fragments. The

number of probes on the CGI arrays will continue to grow, hence increasing the coverage.

2.1.10. Luminometric methylation assay (LUMA) using pyrosequencing

The underlying principal of LUMA is based on DNA cleavage by a pair of isoschizomer endonucleases followed by bioluminometric polymerase extension to quantify the extent of restriction cleavage using pyrosequencing [108]. The method is a modern version of one of the earliest method used to detect methylated cytosine in genomic DNA [109] in which *HpaII* (methylation-sensitive) and *MspI* (methylation-insensitive) were used to cut at the recognition site CCGG. *HpaII* is not able to cut if the internal internal cytosine is methylated (C^m CCGG). After the DNA cleavage, the methylation status can be determined by Southern blotting or by PCR analysis [109]. With the advent of pyrosequencing [110], a new sequencing methodology that dispenses labeled primers, labeled nucleotides, and gel-electrophoresis, the extent of methylation at each CpG sites could be determined accurately and rapidly. The LUMA workflow (Fig. 9) started with DNA digestion in parallel reactions using either *MspI* or *HpaII* in combination with *EcoRI*. *MspI* or *HpaII* both leave 5'CG overhangs after DNA cleavage, but in the *HpaII* reaction, C^m CCGG sites are protected. As an internal reference, *EcoRI* generates 5'-AATT overhangs. These overhangs are then filled in a polymerase extension assay with stepwise addition of dNTPs (four steps: dATP α S, dGTP + dCTP, dTTP, and dGTP + dCTP). As each dNTP is extended, inorganic pyrophosphate (PPi) is released and converted to ATP by ATP-sulfurylase and adenosine-5-phosphosulfate. This reaction is coupled to the conversion of luciferin to oxyluciferin by luciferase and ATP to generate a proportional amount of visible light, which is quantified by a charge-coupled-device camera in the Pyrosequencer. The signals corresponding to dATP α S and dTTP additions both represent *EcoRI* cleavage and are equal in the two DNA samples (*MspI* + *EcoRI*-treated versus *HpaII* + *EcoRI*-treated). The signal intensities generated by the additions of dCTP and dGTP are added together, and the sum represents an *HpaII* or *MspI* cleavage. The degree of methylation at a CpG site can be derived from the (*HpaII/EcoRI*)/(*MspI/EcoRI*) ratio.

LUMA can be used to access cytosine methylation level of the whole genome [108]. However, the technology has more commonly applied to stud-

ies of targeted CGIs [111]. LUMA has been used to determine changes in methylation status associated with disease development [112–114] or as diagnostic/prognostic markers of cancers [115–118]. It is limited by the length of the sequence read and thereby the number of CpGs that can be analyzed in one sequencing reaction. In addition, the relatively high cost of the pyrosequencing machine has limited the use of the technology without the support of a core facility.

2.1.11. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)/high-performance liquid chromatography (HPLC)

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) [119,120] is a promising and powerful technique for DNA methylation analysis. Various MS approaches have been developed for the measurement of levels of DNA methylation, including rapid screening of single nucleotide polymorphisms (SNPs), and quantitative allele studies. It has been used to monitor nucleotide digestion and DNA sequencing [121,122]. The commonly used approach involves bisulfite conversion of genomic DNA [80] followed by DNA sequencing, resulting in accurate determination of methylation status in genomic DNA.

As an alternative, Schatz et al. introduced RNA-mediated analysis of methylation status of individual CpGs using MALDI-TOF-MS based on *in vitro* transcription of bisulfite PCR product and base-specific cleavage [123]. After treatment with sodium bisulfite, DNA samples are subjected to PCR in which the amplicons are tagged with T7 RNA polymerase promoter as well as with a complementary sequence with low guanosine content. After T7-mediated transcription, these guanosine residues in the newly transcribed RNA are subjected to cleavage by RNase T1 and the methylation fingerprint (RNA fragmentation pattern) is visualized by MALDI-MS (Fig. 10). This approach was applied to the analysis of artificially methylated and unmethylated DNA, mixtures thereof, and colon DNA samples.

Recently, Tos et al. combined the bisulfite conversion genomic DNA method with the GOODs [124,125] assay for accurate quantification of methylation status of CpG dinucleotides using MALDI-MS. They analyzed

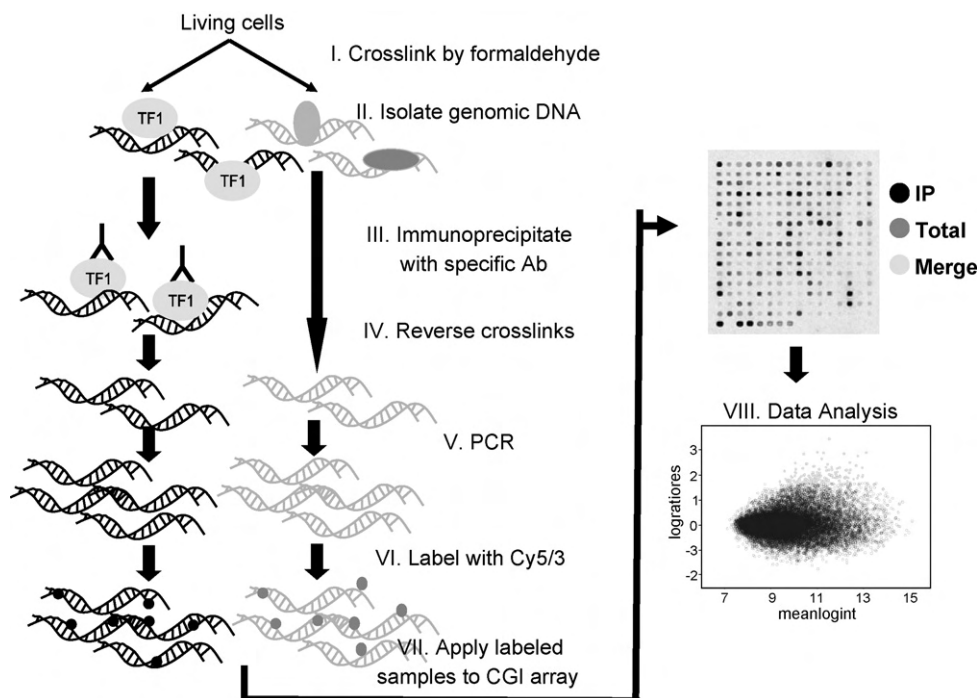


Fig. 8. Workflow of chromatin immunoprecipitation (ChIP) on DNA microarray (ChIP-chip) analysis. At first, living cells are fixed by formaldehyde crosslinking. Intact genomic DNA with transcription factors (TF) is then isolated, and the chromatin-protein complex of interest is immunoprecipitated (IP) with a specific antibody to that intact TF. After reverse crosslinking, DNA is extracted and purified before PCR to generate chromatin amplicons. Amplicons from experimental immunoprecipitation are labeled with Cy5, and Cy3 is used to label input reference amplicons. Labeled probes are applied on the CGI array for hybridization. Data can be utilized to study the interaction between particular TFs and specific CpG sites of genes.

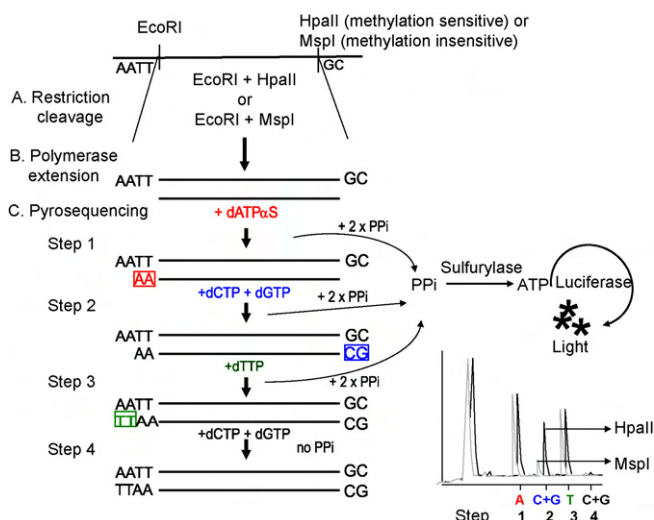


Fig. 9. Procedures for analysis global DNA methylation with luminometric methylation assay (LUMA). Genomic DNA is digested with combinations of restriction enzymes, *EcoRI*/*MspI* or *EcoRI*/*HpaII*, to leave the TTAA (*EcoRI*) and CG (*HpaII* [methylation-sensitive] or *MspI* [methylation-insensitive]) overhangs. Next, the extent of the cleavage is determined by a polymerase extension assay based on a four-step pyrosequencing reaction. Inorganic pyrophosphate (PPi) is generated at each nucleotide addition in the polymerase extension assay. Signals are generated based on the utilization of PPi in a luciferase-based reaction. The amount of light generated is directly proportional to the number of overhangs produced by respective restriction enzymes. The A and T peaks represent signals from Steps 1 and 3. The C + G peaks resulting from Step 2 illustrate *HpaII* or *MspI* cleavage. The second C + G peak originating from Step 4 is an internal control for the completion of Step 2. Unmethylated CG is cleaved by *HpaII*, and a CG overhang is left after the cleavage and amplified in polymerase extension. Methylation status can be determined by analyzing the *HpaII*/*MspI* ratio at CG peaks from Step 2. The *HpaII*/*MspI* ratio is close to 1 in the unmethylated sample but close to zero in the methylated sample.

several paraffin-embedded tissue biopsies and found the MS results comparable to those of chip hybridization. Furthermore, Ehrich et al. applied base-specific cleavage of DNA following MALDI-TOF-MS analysis to measure the degree of methylation in normal and neoplastic lung cancer tissue samples, allowing accurate classification of samples according to their histopathology [126]. This method is applicable to methylation studies of target genes.

Another related technology for global profiling involves the use of high-performance liquid chromatograph (HPLC). It is the routine and most widely used separation technique for the measurement of 5-methylcytosine and/or DNA methylation [127–129] involving the digestion of DNA to nucleotides, nucleosides, or bases, followed by high-resolution separation and quantification with UV detection. HPLC has been implicated in the study of p16 methylation status of gastric dysplasia/carcinomas [130,131] and a study of tamoxifen-induced hepatocarcinogenesis in rat [132]. Recently, Song et al. reported a novel method utilizing liquid chromatography-electrospray ionization tandem mass spectrometry to measure 5-methyl-2'-deoxycytidine levels [133]. Tissue-specific differences in DNA methylation in various mammals has been reported by using HPLC analysis [134]. Several other chromatographic and electrophoretic techniques, including gas chromatography, thin layer chromatography, and capillary electrophoresis, have also been used to determine DNA methylation [135–137].

Both MALDI-TOF-MS and HPLC are tools suitable for high-throughput, multi-channel detection with the benefits of high speed, accuracy, and automation. They either can measure the content of methyl-cytosine in the whole genome or can detect methylation patterns of specific target genes. Relatively high cost or the need for standardization of the protocols would be the major pitfalls of applying these techniques to methylation studies.

2.2. Characterization of a target or candidate sequence and validation of its involvement in gene regulation

The various global discovery platforms mentioned above would undoubtedly identify many novel genes that are epigenetically regulated and that might be important in the development of diseases. However, the major challenge remains in designing an efficient strategy for *post hoc* validation of the involvement of the CGI in gene regulation. It has to be re-emphasized that multiple validation approaches need to be conducted before one can lay claim to its role in gene regulation. These include, but are not limited to, database searches and *in silico* analyses, studies of methylation status using bisulfite sequencing (Section 2.1.5), combined bisulfite restriction analysis (COBRA), methylation-sensitive PCR (MS-PCR), methylation-sensitive single strand conformational polymorphism (MS-SSCP), MethylLight analysis, and gene expression studies (Figs. 2 and 11). Some of these techniques are summarized briefly below, and most of them can be used both for target gene studies and for studies of novel candidates identified by discovery platforms.

2.2.1. In silico database analysis

The first step in *post hoc* validation starts at *in silico* analysis of the candidate genes of interest. Detailed searches of public databases such as the National Centre for Biotechnology Information (NCBI) and the University of California Santa Cruz (UCSC) Genome Bioinformatics group are the logical first steps. If the CGI is located in the promoter or 5' region of a gene, it usually has a higher chance of being involved in transcriptional regulation. After promoter/5' sequences of a candidate gene are identified, the next step is to locate the CGI and discern its location relative to the transcriptional start site and to the 5' exons

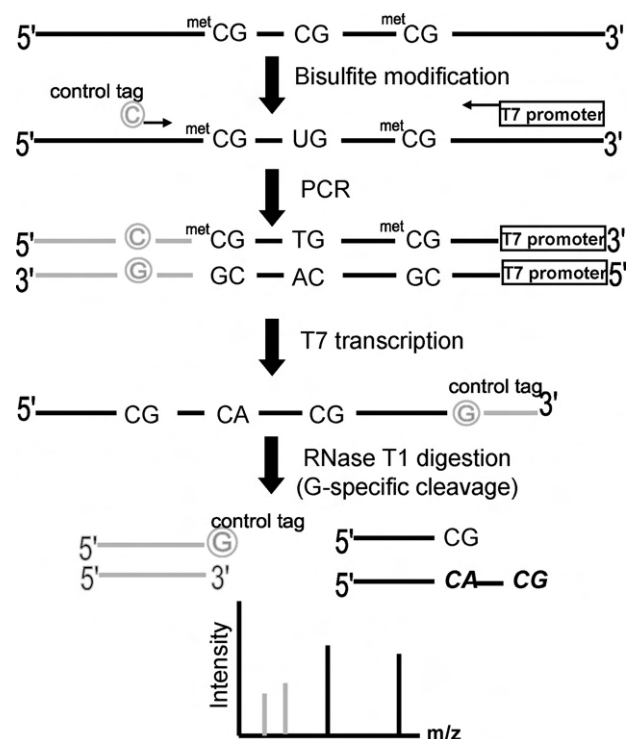


Fig. 10. Analysis of CpG methylation patterns using RNase T1 cleavage and Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Genomic DNA is modified by sodium bisulfite to convert unmethylated cytosines to uracils followed by PCR amplification with forward primer containing a control tag and reverse primer carrying T7 promoter. RNA transcription generates G-sites at originally methylated C sites and G-specific cleavage with RNase T1 is done. Control tag is used to monitor the successful full-length transcription and followed RNase T1 cleavage. RNA fragments are subjected to MALDI-TOF analysis. By comparison of the profile of *m/z* values of all fragments in the samples, methylation status of genes can be found.

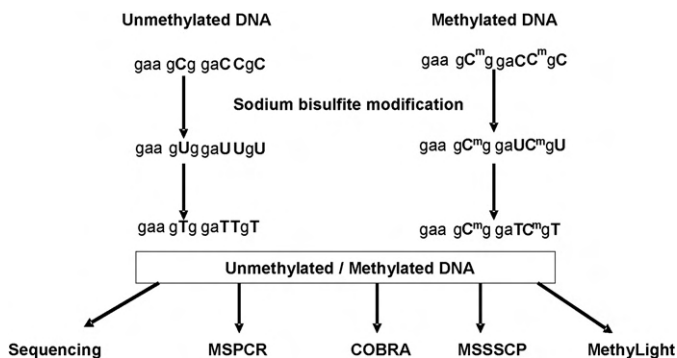


Fig. 11. Different approaches can be chosen to yield the information on the overall characterization of genes showing differential methylation status. Unmethylated DNA is distinguished from methylated DNA with a sodium bisulfite modification of DNA used as a standard procedure prior to validation assays. The underlying principle is based on the ability of sodium bisulfite to deaminate cytosine (C) residues into uracil (U) in genomic DNA, whereas the methylation cytosine residues are resistant to this modification. After PCR amplification, the U residues are amplified as thymines (Ts). Cloning and subsequent sequencing of the DNA fragments containing the CpGs then provide information on the methylation status of each C within the CpGs. Details of methylation-specific PCR (MS-PCR), combined bisulfite restriction analysis (COBRA), methylation-specific single-strand conformation polymorphism (MS-SSCP), and MethyLight are discussed in the text.

and introns. Information generated from these analyses provides clues as to (1) whether a CGI exists in the sequence and (2) if it has a high probability of playing a regulatory role in gene expression. These *in silico* analyses could be laborious, but several open-source tools are now available through the Internet: Met-Primer (<http://www.urogene.org/methprimer/index.html>), Promoter Inspector (<http://www.genomatix.de>), BIMAS (<http://thr.cit.nih.gov/molbio/index.shtml>), and Database of Transcription Start Sites (<http://dbtss.hgc.jp>).

2.2.2. Validation assays of gene of interest

Once a “potential” CGI has been identified in the 5′ region of a gene, physical and functional characterization of the CGI is in order. The first criterion of a regulatory CGI is that an inverse relationship can be established between its degree of methylation and the expression of its cognate gene. Determination of the methylation status of the CGI and its adjacent sequences could be achieved by one or more of the following methods: MS-PCR, COBRA, MS-SSCP, MethyLight, or bisulfite DNA sequencing. For cell-culture experiments, DNA could be extracted from cultures under different conditions or exposed to DNA-demethylating agents or inhibitors of histone deacetylase. By extracting both DNA for methylation studies and RNA for transcript quantification, one can determine if an inverse relationship exists between the extent of methylation in the CGI and gene transcription [20]. Bisulfite genomic sequencing [80] is the preferred method (see Section 2.1.5) of initial characterization because it can reveal the methylation status of individual CpG in the CGI. Detailed mapping of a CGI could uncover important “methylation hotspots” that could be used to design methylation oligonucleotides for use in gene silencing experiments by targeting these hotspot sequences [138].

After initial characterization of the putative CGI, other methods are available for more-rapid or higher-throughput analyses. MS-PCR [139,140] can be used to assess the methylation status of a cluster of CpGs within a CGI by conducting PCRs with primers designed for the methylated or unmethylated version of the sequence of interest. MS-PCR has the advantage of requiring small amounts of DNA, with a sensitivity of detecting 0.1% methylated alleles in a given sample. It can be applied to DNA extracted from paraffin-embedded samples which most of the DNA is fragmented.

One of the limitations of MS-PCR is that it is not highly quantitative. Hence, other more quantitative methods have recently been developed. COBRA is similar to MS-PCR but provides more quantitative information on the degree of methylation of the targeted sequence. Restriction-enzyme digestion is used to reveal methylation-dependent sequence differences in PCR products of sodium

bisulfite-treated DNA as described previously [141]. The method has the advantage of accessing methylation levels of a target sequence in a small amount of DNA samples and provides linear quantification across a wide spectrum of DNA methylation levels. The technique can be reliably applied to DNA obtained from microdissected paraffin-embedded tissue samples. COBRA thus combines the powerful features of ease of use, quantitative accuracy, and compatibility with archival samples. MS-SSCP also provides a fairly quantitative method to access the methylated and unmethylated allele populations. It uses high-resolution gel electrophoresis to generate a specific methylation pattern for determining the percentages of methylation in a targeted sequence. In SSCP, methylated alleles, due to the difference in conformation from the unmethylated alleles, can be separated by MDE-gel electrophoresis and visualized by SYBR-Gold staining or radiolabeling [142,143]. A high-throughput technology, known as MethyLight, has recently been developed for cytosine analysis. It utilizes fluorescence-based real-time PCR Taq-Man technology [144,145] and thus is highly quantitative. MethyLight assays have good precision and linearity. It can be used effectively in a high-throughput manner for analysis of DNA methylation of small amounts of DNA.

After the methylation status of the CGI has been established by the aforementioned methods, it is imperative to determine the expression of its cognate gene. Modern laboratory procedures such as quantitative reverse transcriptase-PCR or real-time PCR are most appropriate for such studies because they provide quantitative data and are adaptable to high-throughput application. If levels of transcripts can not be accessed directly (e.g., if only archival paraffin-embedded samples are available), then immunostaining of sections [138] or *in-situ* hybridization could be attempted to ascertain gene expression. In addition, cell cultures treated with DNA demethylating agents and inhibitors of histone deacetylase can further elucidate the regulatory role of the CGI. More recently, we have used a class of methylation oligonucleotides to induce sequence-specific methylation *in cellulo* that subsequently leads to gene silencing. In this manner, we have provided additional evidence that DNA methylation is involved in the regulation of estrogen receptor β [138].

3. Discussion

In this section, we would like to point out some important issues that should be noted when methylation techniques are employed in epigenetic studies. The above validation assays could be used for whole-tissue analyses [20]. However, a tissue contains multiple cell types and any change in the methylation status and gene expression of that CGI might occur in only one cell type. In this scenario, it would be beneficial to have laser-capture microscope-assisted microdissection to enrich a specific cell population [138]. Alternatively, enzymatic digestion of the tissue followed by isolation of the specific cell population would also enhance the experimental outcome.

Methods including BSPCR, MS-PCR, MS-SSCP, COBRA, or MethyLight depend on whether nested PCR primers could be designed to amplify the fragment of interest. Furthermore, if the integrity of the DNA is less than optimal, as is in the case of DNA isolated from microdissected samples or paraffin-embedded tissues, the amplified fragment would be limited in size. Also, efficiency of bisulfite modification on DNA samples would be another major pitfall affecting the sensitivity and accuracy of the PCR results.

On the other hand, if 5AZA and/or TSA treatments are performed to determine the relationship between the transcription level and methylation status of target genes, dosage of the modifiers and incubation period for the treatment are critical. Optimization of the protocol is necessary. Additional studies such as site-directed mutagenesis and luciferase reporter assay *in vitro* and *in vivo* can be involved to establish the gene regulatory

function of the CGIs. Briefly, deletion constructs at specific CG sites are generated by using a commercial site-directed mutagenesis kit according to the manufacturer's recommendations. Modified promoters are re-subcloned into reporter vector followed by a promoter reporter assay. ChIP or transcription-factors binding assay may also be included to determine which regulatory elements on promoters are associated with the methylation changes.

In summary, multiple methods and technologies are available to determine changes in global and regional methylation of cytosine in genomic DNA; each has advantages, disadvantages, and areas of applicability. Because of variations in sample size, the nature of the samples, the number of samples in the studies, the experience of the investigators, and the resources of the laboratory or the institution, there is no "gold" standard or "standard operation procedure" for conducting a DNA methylation analysis. This review simply attempts to provide an overview of the currently available techniques and to discuss some of the advantages and limitations of each technology. With the rapid growth in interest in understanding the epigenetic regulation of disease development, a variety of new and improved methodologies are certain to emerge in the coming years. These technologies will undoubtedly change the landscape of epigenetic studies and provide valuable new insights into areas such as the developmental basis of disease and reproductive toxicology.

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