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Androgen-induced Epigenetic Profiles of Polycomb and Trithorax Genes in Prostate Cancer Cells

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Abstract. *Background/Aim: Androgens are essential for the growth of most prostate cancers (PCa). As a result, androgen ablation is the mainstay of the treatment of PCa. Proteins of the polycomb and trithorax family are master epigenetic regulators of cell type specific gene expression including androgen receptor. Materials and Methods: We interrogated epigenetic changes of a 24-gene panel corresponding to polycomb and trithorax genes by PCR array and differential gene expression by quantitative real time-PCR on prostate cancer cell line (LNCaP) treated with the synthetic ligand R1881. Results: We observed the highest methylation for CBX2, PCGF6, PHC2, EZH2 and TRIM27 genes and the lowest methylation for CBX8 and PCGF2 ($p<0.05$), and a modest decrease in the expression of EZH2. Conclusion: Differential methylation profiles of polycomb and trithorax genes may contribute to the dynamics of prostate carcinogenesis.*

The biological roles of sex steroids in prostate cancer (PCa) are well established [for recent reviews see (1, 2). Specifically, a lot of attention has been given on the central role of androgen receptor (AR) in modulating the expression of genes in PCa. The AR is normally located in the cytosol and held in an inactive form by binding to the cytoplasmic chaperone protein HSP90. Upon binding and activation by androgen, AR dissociates from the HSP90 protein, undergoes self-dimerization and is translocated to the nucleus. In the nucleus, the androgen-AR complex functions as a transcription factor; it binds to androgen response elements to activate the transcription of target genes necessary for prostate development and maintenance (3). Undoubtedly, AR plays an essential role during the growth and development of the prostate from fetal life through adulthood and in PCa. Consequently, the molecular mechanisms involved in AR signaling have received a lot of attention in AR treatment. For instance, disrupting AR protein stability or AR co-factor interactions represents a therapeutic strategy to prostate cancer treatment.

Genes that are involved in androgen receptor (AR)-mediated signaling and AR-mediated metabolic pathways have been extensively characterized with regards to PCa susceptibility. For instance, allelic frequencies of genes in the AR signaling pathway have been associated with PCa development and resistance to therapy (4). Other findings support targeting AR signaling as important for PCa treatment and androgen deprivation therapy (5, 6). The mutational landscape in critical oncogenic or tumor suppressive loci including AR has for several years supported the hypothesis that PCa is a genetic disease (7). However, the current prevailing view is that there is a crosstalk between the genome and epigenome in PCa initiation and progression (8). Thus, the initiation and progression of PCa depends on not only genetic alterations but also aberrations in epigenetic factors (9). The Polycomb Group (PcG) of proteins is a family of proteins that were initially discovered to function in Drosophila melanogaster body patterning by acting as epigenetic transcriptional repressors of Homeobox gene (Hox) expression (10). Additional studies have shown that the Hox genes are regulated by the antagonistic function of PcG and another family of proteins; the Trithorax Group (TrxG) of proteins; these 2 families of proteins are key regulators of chromatin structure (11). It is apparent that epigenetic gene regulation includes the activity of different protein complexes in the nucleosome, which is the basic unit of the chromatin structure. A nucleosome is composed of approximately 150 bp DNA, which are wrapped around a cylindrical protein complex (histone core) (12). Seminal reports have demonstrated that histone undergoes a wide range of post-translational modifications that includes methylation, acetylation, sumoylation, phosphorylation and ADP-ribosylation to regulate gene expression (13). Despite the complexity of histone post-translational modifications, PcG and TrxG proteins has been shown to play roles in histone post-

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translational modifications. The emerging view is that an initial step in PCa development is epigenetic disruption of progenitor cells to create an abnormal polyclonal precursor population susceptible to additional aberrant genetic and epigenetic events (14). There are different classes of histone methylation marks in PCa: histone marks such as H3 lysine 4 dimethylation (H3K4me2) and H3 lysine 9 acetylation (H3K9acetyl) are commonly associated with active transcription whereas H3 lysine 9 dimethylation (H3K9me2), and H3 lysine 27 trimethylation (H3K27me3) are associated with transcriptional silencing. Several PcG protein complexes such as PRC1-PRC4 have been shown to control the transcriptional silencing: for instance, PRC1 contains BMI1 and RING1 domains that interact with H3K27me3, whereas PRC2-PRC4 contain EZH2 domain and expression of all these proteins (BMI1, RING1, and EZH2) is associated with aggressive PCa (14). The TrxG proteins contain domains (bromo, PWWP, chromo-PHD finger domains. Zinc fingers, AT hooks) that directly binds to histones such as BRPF1/3 (15). Overall these polycomb/trithorax complexes are fascinating chromosomal factors (11). Given the importance of PcG and TrxG gene products in the regulation of AR transcription, we were interested in studying the epigenetic profiles of a 24- gene panel of *PcG* and *TrxG* families and the corresponding gene expression in response to the synthetic ligand R1881 in PCa cells.

Materials and Methods

Cell culture, hormone treatment, and cell growth. The androgen receptor (AR) - positive prostate cancer LNCaP cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The LNCaP cells were grown in RPMI medium containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% Penicillin Streptomycin (GIBCO) and this is referred to as complete medium unless otherwise stated. For hormonal treatment, 1×10^6 LNCaP cells were seeded in each well of a 6-well plate in complete growth medium. After 24 h, cell cultures were refed with medium containing 2% charcoal-treated FBS to remove steroids, followed by incubation for an additional 72 h in RPMI containing 2% charcoal-treated FBS. The synthetic ligands: R1881 (10^{-10} M), 17β-estradiol (10^{-10} M), progesterone (10^{-10} M), testosterone (10^{-10} M) and dexamethasone (10^{-10} M) were obtained from Sigma Aldrich (St. Louis, MO, USA). Control, vehicle (DMSO)-treated cells were grown using the same medium without hormones. After 1-3 days in culture, cells were used for examining their proliferation (Coulter counter), or nucleic acids were extracted for methylation or gene expression analysis. The experiment was performed in triplicates.

Polycomb and trithorax gene expression. After R1881 or mock treatment for 3 days, the LNCaP cells were washed twice with 1× PBS and detached from the culture plate by trypsinization (1× trypsin–EDTA; GIBCO, Invitrogen Corporation). High molecular weight genomic DNA extracted from cells was used in Methyl-Profiler DNA methylation PCR array analysis according to the manufacturer's instruction (Methyl-Profiler DNA PCR array #MEAH-3520E-24 gene panel; Table I; Qiagen, Valencia, CA, USA). Briefly, equal amounts of each genomic DNA sample were added to components of the Methyl Profiler DNA Restriction Kit to set up 4 different restriction digests: mock (Mo), methyl-sensitive (Ms), methyl-dependent (Md), and double (Msd). After digestion and heat inactivation of the enzymes, the digested DNA samples were used for analysis of the methylation profile of the 24 genes mix of each digest with the appropriate RT^2 SYBR Green qPCR Mastermix, aliquoted into the appropriate wells of the Methyl Profiler PCR Array and run the recommended cycling program in the real-time PCR instrument. The C_T values were used to calculate differences in DNA methylation levels for each gene between R1881 treated and mock control (untreated) cells using the EpiTect Methyl Gene expression analyses software (SABiosceinces, Frederick, MD, USA). The experiment was performed in triplicate.

Gene expression. Total RNA extracted from R1881-treated and untreated LNCaP cells using TRIzoL Reagent (Invitrogen) was converted into cDNA by reverse transcription using Invitrogen SuperScript™ first strand synthesis system for RT-PCR and according to the manufacturer's protocol. The TaqMan™ assay used to quantitatively measure mRNA expression (GAPDH Forward primer 5'-GGACCTGACCTGCCGTCTAG-3', Reverse primer 5'-TCAGTGTAGCCCAGGATGCC-3' and Taqman probe 5'-TCCGA CGCCTGCTTCACCACCTTCT-3'; EZH2 Forward primer 5'-TCAAAACCGCTTTCCTGG-3' and Reverse primer 5'-TGTCC CAATGGTCAGCA-3' TaqMan probe 5'-AGTGTCCATGCT ACCTGGCTGT-3'. Real-time PCR was carried out in a Bio-Rad iCycler real-time thermal cycler (Hercules, CA, USA) as described previously (16) and by incorporating optimized PCR reaction conditions for each gene. The threshold PCR cycle (C_t) at which fluorescence exceeded the background was then converted to copy number based on a cDNA standard curve. Each experiment was carried out in duplicate.

Data analyses and statistics. Data analysis was performed using GraphPad Prism 6 software (GraphPad Software, Inc, La Jolla, CA, USA). One-way ANOVA test was used for statistical analysis of all experiments to determine the differences between DNA methylation levels of the tested genes. p-Values≤0.05 were considered statistically significant.

Results

To evaluate PCa cell proliferation in response to sex steroid hormonal signals, the androgen-dependent PCa LNCaP cells were treated with different sex steroid hormones over a 72 hour time period using previously established optimum concentrations (17). The LNCaP cells grown in complete medium that contained 10% FBS showed the highest growth rate over a 3-day period compared to the control, mock-treated, cells containing only 2% charcoal-treated FBS (Figure 1). Treatment of LNCaP cells with each individual sex steroid hormone stimulated LNCaP cell growth compared to the control (untreated, mock control) albeit not to the same extent as LNCaP cells cultured in complete medium. The data indicated that LNCaP cells treated with synthetic ligand (R1881; 0.1 nM) showed the highest cell

Table I. *Polycomb and Trithorax genes and their functions*.

Gene	Gene name	Gene function
ASXL1	Additional Sex Combs Like 1	Chromatin-binding protein. Required for normal determination of segment identity in the developing embryo.
ASXL2	Additional Sex Combs Like 2	An epigenetic regulator that bind various histone-modifying enzymes.
BRPF1	Bromodomain and PHD finger containing 1	Component of the MOZ/MORF histone acetyltransferase complexes which function as a transcriptional regulator.
BRPF3	Bromodomain and PHD finger containing 3	Histone H3 acetyltransferase activity.
CBX2	Chromobox 2	Maintain the transcriptionally repression *via* chromatin remodeling and modification of histones.
CBX4	Chromobox 4	E3 SUMO-protein ligase.
CBX8	Chromobox 8	Chromatin remodeling and modification of histones.
CTBP2	C-terminal binding protein 2	Act as a corepressor targeting diverse transcription regulators. Functions in brown adipose tissue (BAT) differentiation.
EED	Embryonic ectoderm development	Mediates repression of gene activity through histone deacetylation.
EPC1	Enhancer of polycomb homolog 1	Component of the NuA4 histone acetyltransferase complex and can act as both a transcriptional activator and repressor.
EPC2	Enhancer of polycomb homolog 2	Play a role in transcription or DNA repair.
EZH2	Enhancer of zeste 2 polycomb repressive complex 2 subunit	Associates with the embryonic ectoderm development protein, the VAV1 oncoprotein, and the X-linked nuclear protein.
PHF1	PHD finger protein 1	Transcriptional repression of homeotic genes.
PHF19	PHD finger protein 19	Recruits the PRC2 complex to CpG islands and contributes to embryonic stem cell self-renewal.
PCGF1	Polycomb group ring finger 1	Act as transcriptional repressors to regulate anterior-posterior patterning in early embryonic development
PCGF2	Polycomb group ring finger 2	A negative regulator of transcription and has tumor suppressor activity.
PCGF3	Polycomb group ring finger 3	Contains a C3HC4 type RING finger, which is a motif known to be involved in protein-protein interactions.
PCGF5	Polycomb group ring finger 5	Maintains the transcriptionally repressive state of many genes, including Hox genes, throughout development.
PCGF6	Polycomb group ring finger 6	Form protein complexes and function as transcription repressors. Interact with some PcG proteins and act as a transcription repressor.
PHC2	Polyhomeotic homolog 2	Heterodimerize with EDR1 and colocalize with BMI1 in interphase nuclei of human cells.
RING1	Ring finger protein 1	Transcriptional repressor
RNF2	Ring finger protein 2	Transcriptional repressor
SUZ12	Polycomb repressive complex 12 subunit	Zinc finger gene and identified at the breakpoints of a recurrent chromosomal translocation.
TRIM27	Tripartite motif containing 27	Repressor of gene transcription.

growth rate compared to the LNCaP cells treated with each of the other steroids; Estradiol (10 nM), testosterone (10 nM), Dexamethasone (10 nM). The LNCaP cells contain a mutation in the steroid binding domain of the AR and this allows broad binding specificity for progestogens, estradiol and several antiandrogens. In addition, the LNCaP cells also contain estrogen receptors that may mediate some of the effects of estrogen. Testosterone effects may be mediated through androgen and estrogen receptors in LNCaP cells. However, the LNCaP cells do not have glucocorticoid receptors (18), therefore, the synthetic glucocorticoid ligand dexamethasone may signal through the mutated AR in LNCaP cells. The results showed that R1881 can stimulate LNCaP cell proliferation and the maximum effect was observed following 72-h.

The PcG and TrxG proteins are critical regulators of target gene expression. We, therefore, assessed the methylation of the *PcG* and *TrxG* genes in LNCaP cells. To achieve this, we used real-time PCR to analyze the expression of a 24-gene panel of the *PcG* and *TrxG* complex array in LNCaP cells treated with R1881 for 72 h. The results of the methylation profiling in R1881-treated cells revealed that 18 genes showed more than 2-fold increase and 3 genes showed more than 2-fold decrease in methylation levels compared to the untreated control (Figure 2). Overall, the highest methylation was observed for *CBX*2, *PCGF*6, *PHC*2, *EZH*2 and *TRIM*27 and the lowest methylation for *CBX*8 and *PCGF*2 ($p<0.05$) in the R1881-treated cells compared to the mock untreated cells. The data suggests robust epigenetic alterations of several

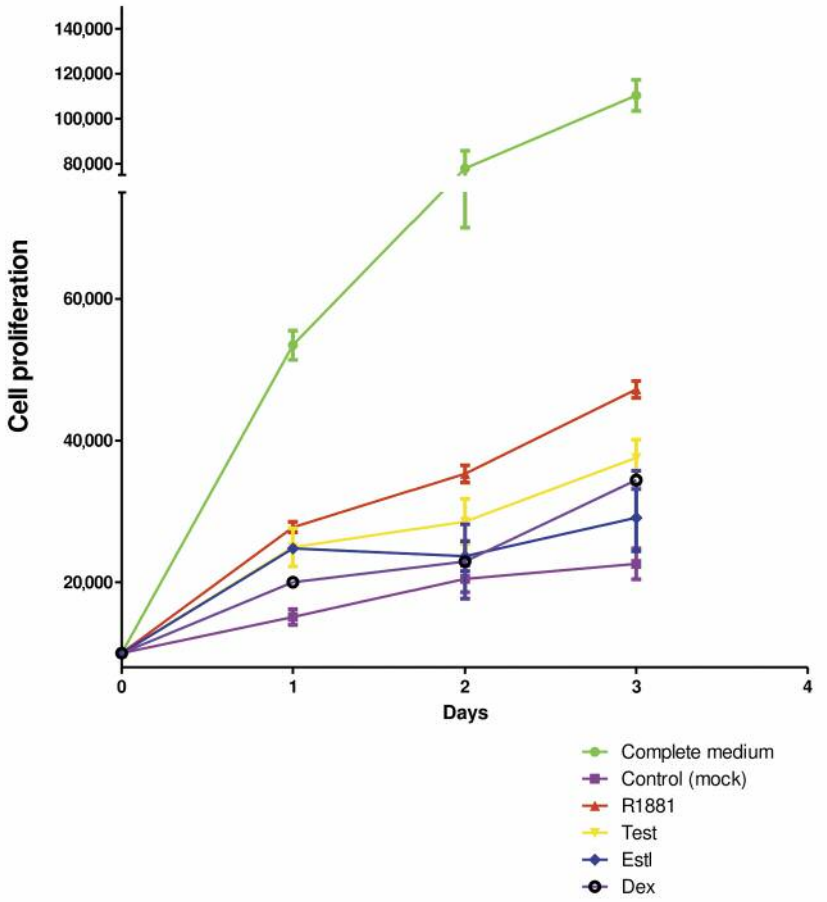

Figure 1. *Cell proliferation in response to incubation with sex steroid hormones. One million LNCaP cells were plated in each well of a 6-well plate containing complete growth medium. After 24 h, cell cultures were refed with medium containing 2% charcoal-treated FBS to remove steroids, followed by an additional 24 h in RPMI containing 2% charcoal-treated FBS. Twenty-four h later, cultures were treated with the synthetic ligands; R1881 (10^{-10}M), testosterone (10^{-8}M), 17β-estradiol (10^{-9}M), and dexamethasone (10^{-8}M) and mock (EtOH; Ctrl). Cell proliferation was assessed by Coulter counter. The data represents the mean of three independent experiments; bars represent standard error of mean.*

PcG and *TrxG* promoter gene methylation in prostate cancer cells in response to androgen stimulation.

We next examined the effect of R1881 on *EZH2* expression using semi-quantitative RT-PCR analysis. Treated of LNCaP cells with R1881 resulted in a very modest reduction in gene expression over a 3-day period compared to untreated control cells (Figure 3) suggesting that epigenetic mechanisms including DNA methylation are likely involved in the reduced expression of *EZH2* in LNCaP cells in response to R1881 stimulation.

Discussion

It is widely established that the PCa microenvironment is characterized by elevated levels of androgens capable of activating the AR (1) supporting androgen deprivation as the mainstay treatment in PCa (19). However, metastatic castration-resistant prostate cancer (CRPC) is an important concern since no agents are currently available to stop its progression, therefore, it is necessary to elucidate the molecular mechanisms associated with androgen expression and the role of regulatory genes, including *PcG* and *TrxG*. Recent studies have shown the global control of PcG and TrxG proteins in a plethora of cellular processes, ranging from modifying local chromatin structure to orchestrating the three-dimensional organization of the genome. Their importance is further underscored by dysregulation of multiple PcG and TrxG members in multiple diseases (20) including prostate cancer (21-24). The current findings suggest that dysregulation of PcG and TrxG may induce aberrant epigenetic alterations and this represents a key driver for PCa progression to metastatic disease making these proteins attractive targets for therapeutic intervention.

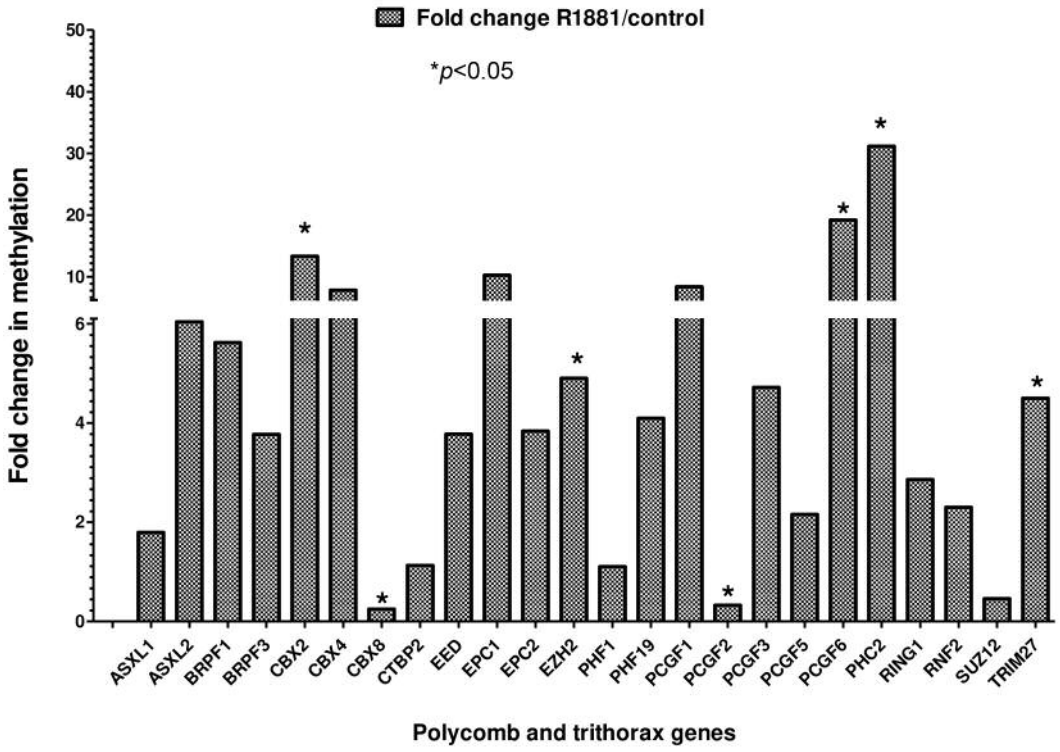

Figure 2. *DNA methylation levels of PcG and TrxG genes in response to R1881 treatment. The LNCaP cells were stimulated with R1881 (0.1 nM) or unstimulated (mock control) for 72 h and used for the Methyl-Profiler DNA methylation PCR array analysis. One-way ANOVA test was used for statistical analysis. Statistical significance (p-values<0.05) is shown as (*).*

In the present study, we observed higher methylation of several *PcG* and *TrxG* genes in LNCaP cells stimulated with the synthetic R1881 compared to untreated control. Alterations in several of these epigenetic master regulators leads to increased transcriptional activity of AR signaling. This is accomplished either by remodeling the chromatin to facilitate the assembly of the AR transcriptional complex and/or post-translational modifications in the AR itself or by allowing access to essential co-factors that enhance transcriptional features. Independent of AR signaling, the alterations in epigenetic master regulators may also activate other oncogenic signaling pathways and contribute to the aggressiveness and androgen independence in advanced PCa.

One well studied PcG gene is *EZH2*. For instance, overexpression of EZH2 has been detected in many clinical tumor specimens as a potential negative prognostic factor in these tumors (25, 26). In PCa, the expression levels of EZH2 correlate with the disease progression, where high levels of EZH2 significantly correlate with a greater risk for disease recurrence (27). A previous study by Kim *et al*. (28) has shown that EZH2 enhanced AR mRNA and protein expression levels by direct binding to the AR promoter and inducing gene transcription. The

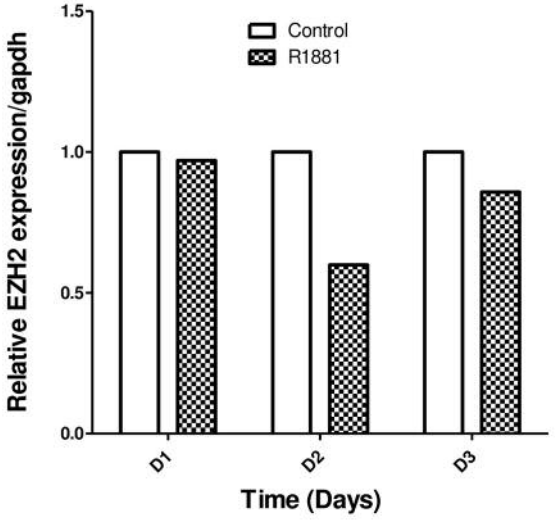

Figure 3. *R1881 stimulation and EZH2 gene expression. The prostate cancer cell line LNCaP was treated with R1881 or untreated for up to 3 days. The fold change in gene expression was assessed using quantitative RT-PCR and expressed relative to GAPDH to normalize differences in the amounts of reverse-transcribed RNA. The data derives from three independent experiments. The mean expression level (±SEM: standard error of mean) is indicated.*

activation of AR by EZH2 was found to be independent of PRC2 or histone methyltransferase activity. This suggests that EZH2 plays a dual role as histone methyltransferase that fits with its role as an epigenetic silencer and as an activator of gene promoters independently of methylation in PCa cells. In our current study, we observed a significant increase in *EZH2* DNA methylation in response to R1881 stimulation and this was accompanied by a decrease in gene expression albeit modest. In support of our observation, one previous report has also indicated that EZH2 expression was down-regulated in LNCaP cells treated with R1881 (29). Taken together, these observations suggest that DNA methylation may be a potential mechanism for down-regulating EZH2 expression in R1881-treated LNCaP cells. Some members of the PcG and TrxG may act in a feedback mechanism to regulate AR transcriptional activity and this is concordant with other reports regarding other coactivators (30). The accumulating evidence suggests that PcG and TrxG proteins are capable of stimulating or repressing gene expression *via* several mechanisms beyond PcG repressing complexes (PRCs) (31). Members of the TrxG family such as BRPF1/3 are able to bind to multiple histone acetylation marks (32) and are also implicated in multiple diseases including cancer. Because of the pivotal role of PcG and TrxG as master regulators of gene expression they are attractive candidates for therapeutic intervention in PCa. In agreement with this finding, Crea *et al*. (33) have found that targeted inhibition of PRC2 using the small molecule inhibitor 3-dezaneplanocin-A (DZneP) reduced tumor growth and invasion of two PCa models that resulted in a significant increase in tumor-free survival in these animal models. In conclusion our study suggests that androgen stimulation could potentially use epigenetic mechanisms to regulate the expression of PcG and TrxG and this may contribute to the dynamics of prostate carcinogenesis.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

Songping Wang: RT-PCR analysis. Krishma Tailor: Data analysis. Bernard Kwabi-Addo: Provided conceptual idea and wrote the article.

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