The E2F1/DNMT1 axis represses AR in both normal and malignant prostate epithelium

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Cellular and Molecular Biology)
in The University of Michigan
2011

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ACKNOWLEDGEMENTS

I would like to thank Dr. Mark Day for equipping me with the tools and insight necessary to pursue my personal scientific interests. He helped me realize my potential by constantly providing positive reinforcement. I would also like to thank my thesis committee; Dr. Daniel Bochar, Dr. Andrzej Dlugosz, Dr. Jill A Macoska, and Dr. Deneen Wellik for their willingness to meet and provide helpful suggestions that was pertinent to the conduction of this research. I am grateful to all the Day lab members past and present who have provided both technical assistance and constant encouragement. I am especially thankful to Janet Evangelista for going beyond her own administrative job responsibilities to take care of all exterior lab business.

A many thanks to the collaborators who have provided the reagents used in my studies. Thanks to William Kaelin, Diane Robins, Gerald Denis, Fazlul Sarkar, Donald Tindall, Evan Keller, William Cress, Michael Imperiale, Thomas Berton, and David Johnson. I appreciate the services provided by the Microarray, DNA sequencing, and Histology Core. I am grateful to Dr. Kirk Wojno and Dr. Priya Kunju for taking the time to diagnose and score the tissue arrays presented in my studies and appreciate the statistical guidance from Stephanie Daignault-Newton.

A special thanks to the Cellular and Molecular Biology Program for their efforts to provide an environment catering to my success as a grad student. I am thankful to Dr.

Jessica Schwartz for all her advice and encouragement. I am additionally grateful for Cathy Mitchell who was persistent in thoroughly taking care of all administrative responsibilities in the program. I would like to thank the CMB training grant and the DOD pre doctoral prostate training grant for the funds to execute my research.

I would have not been able to complete this thesis without the support of my family. My parents with unconditional love have continued to encourage my pursuits and provide endless support. My sister, Josefina, has additionally been there to listen to the pains and frustrations of research. My church family definitely provided the stability I needed to accomplish my goals in the lab. Finally, I would like to thank my soon to be wife, Jessica, who has fought through the hard times of our long distance relationship to support my endeavors here at the University of Michigan. As a recent Ph.D., she has been helpful with technical issues in the lab, and been able to commiserate with me during periods of failed experiments. She was the force that continued to inspire and encourage my completion of this thesis.

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ABSTRACT

The molecular events associated with the recurrence of castration resistant prostate cancer (CRPC) are of critical importance in prostate cancer research, as CRPC is associated with high morbidity and lethality. CRPC is associated with deregulated prostatic epithelium exhibiting decreased AR expression in over 30% of the cases and seems to mimic the proliferative AR-negative undifferentiated transit amplifying (T/A) cells of the developing prostate. In an effort to further characterize this proliferative and undifferentiated cell population, we evaluated possible mechanisms involved in AR gene repression.

We have shown that E2F1, a known transcriptional activator, represses AR expression. To explore this mechanism further, we overexpressed E2F1 in prostate epithelial cells and found that AR levels decreased while a dominant negative E2F1 construct reversed the inhibitory effects on AR transcription. E2F1 activates the transcription of DNMT1, a protein that typically silences genes through DNA methylation, however, we found that DNMT1 repressed the AR gene in a DNA methylation independent fashion.

We further explored the E2F1/DNMT1/AR regulatory axis in a CRPC mouse model. Heightened E2F1 expression was previously shown to be inversely correlated with AR expression during human prostate cancer progression to CRPC. We

demonstrated that DNMT1 nuclear staining significantly increased from benign tissue to treatment resistant, metastatic prostate cancer in humans. Considering that abnormal levels of DNMT1 may methylate and repress AR, we evaluated tissue from CRPC mice injected with a DNA methylation inhibitor, 5-aza. A rise in AR positive tissue corresponded with a decrease in the amount of DNMT1 nuclear staining following treatment. The immunohistochemical data suggests that hypermethylation mediated repression of the AR gene by DNMT1 during the development of CRPC may represent an important etiological aspect of this disease.

In summary, we have identified a mechanism of AR repression mediated by the E2F1/DNMT1 axis that results in methylation independent AR repression in proliferative, undifferentiated prostate epithelium. However, AR repression also identified in neoplastic cells appears to be dependent on DNA methylation during the emergence of CRPC. Our studies reveal novel epigenetic regulatory mechanisms involved in AR repression that may further elucidate the understanding of transcriptional regulation, particularly in CRPC.

CHAPTER I

INTRODUCTION

It is estimated that prostate cancer will be the second leading cause of cancer deaths in 2011 (1). As of 2006, the 5 year relative survival rate for patients with local and regional prostate cancer was 100%, while the survival rate for patients with metastatic disease was 30% (1). Elderly men continue to be at risk for the disease, considering that 97% of all prostate cancer cases occur at the age of 50 years or older (1). The heterogeneous pathology of prostate cancer has complicated efforts to classify the disease into treatable subtypes. Prostate tissue lesions already contain multiple foci with inconsistent allelic abnormalities during the pre-cancerous stage known as prostate intraepithelial neoplasia (PIN) (2). Prostate cancer additionally has an array of unusual morphological variants histologically defined as endometrioid adenocarcinoma, pseudohyperplastic carcinoma, foamy gland carcinoma, and adenocarcinoma with paneth-like cells (3). Continued analysis of the molecular events associated with prostate cancer initiation and progression may reveal targetable pathways that define specific prostate cancer subsets.

Prostate Development and Differentiation in an AR Dependent Context

A more thorough comprehension of the regulatory mechanisms during prostate development and differentiation might reveal processes that are potentially deregulated

during prostate tumorigenesis. Currently the prostate is known to emanate from the urogenital sinus below the bladder rudiment as small epithelial buds. The epithelium continues to branch out into the surrounding mesenchyme, developing solid cords of undifferentiated epithelium. The cells of each strand eventually stratify and form tubes composed of an inner luminal and outer basal layer. The mesenchyme surrounding the prostate epithelium differentiates into stromal smooth muscle (as reviewed in (4)). Androgenic hormones are required for the development of a fully functional prostate.

Prostate formation depends on adequate levels of the circulating androgen, testosterone (T). The prostate fails to grow in developing females that normally have low levels of T, however prostate epithelial buds emanate from extracted female mouse urogenital sinus (UGS) after 36h of T stimulation (5). The ability of T to establish a prostate is dependent on the enzymatic activity of 5-alpha-reductase. The reduction of T by this enzyme results in the formation of dihydrotestosterone (DHT), which is essential during prostate development (as reviewed in (6)). Both T and DHT function to regulate prostate growth and continued morphogenesis through the activation of the androgen receptor (AR).

AR is a member of the steroid receptor family and functions as a transcription factor upon hormone ligand binding. The receptor is composed of a transactivation domain at the N-terminal followed by 4 highly conserved regions (DNA binding, translocation, and ligand binding domain (as reviewed in (7)). In the absence of either T or DHT, AR localizes to the cytoplasm and associates with heat-shock proteins (HSP) that obstruct access to the DNA binding domain. Hormone interactions with the ligand binding domain induce conformational changes that facilitate hsp dissociation and

exposure of the translocation domain. The hormone bound receptor undergoes both dimerization and phosphorylation before entering the nucleus (as reviewed in (8)). Once in the nucleus, the transactivation domain interacts with co-regulators to direct the transcription of target genes involved in proliferation, differentiation, morphogenesis and cell signaling (9-11).

The embryonic development of the prostate requires AR expression in the stromal tissue surrounding the epithelial buds of the rudimentary prostate (as reviewed in (12)). Activated AR transcribes stromal autocrine and paracrine signaling factors, referred to as andromedins, that coordinate the stratification and canalization of UGS epithelium into a pre-pubertal prostate (as reviewed in (13)). Fibroblast growth factors (FGF) are suggested to function as andromedins that regulate cell growth and proliferation (Figure1.1). Treatment of rat ventral prostate, with FGF7 in culture, was shown to induce the growth of branching prostate epithelial ducts in the absence of androgens (14). The expression of unique receptors at the cell membrane further specifies the cellular response to FGF signaling. The expression of various FGF receptor-2 isoforms was specifically associated with different levels of cellular proliferation (15). The cell signaling events that are relayed between the mesenchyme and prostate epithelium are still ambiguous. However, the study of andromedins has helped define an intermediary mechanism utilized by the AR to stimulate cellular proliferation and differentiation.

It is generally agreed upon that the various populations of prostate epithelium share similar origins. English et al demonstrated that upon castration 94% of rat ventral prostate epithelium was lost due to cell death. However the remaining cells were still able to reproduce and remodel a functioning prostate after the re-installment of androgens

(16). The identification of an androgen independent, self-renewing cell within the basal epithelium implied that a prostate stem cell may exist. Many studies have tried to uniquely define a unique prostate epithelial stem cell through the use of molecular markers. The current molecular profile, CD133+CD44alpha2beta1hiCK5+/18+, distinguishes putative epithelial stem cells from others (as reviewed in (17)), but similarities with newly defined progenitors require identifiers with higher specificity. The requirement for androgens to regenerate castration depleted prostates suggests that the differentiation of prostate epithelial stem cells into the various cell types is dependent on androgen signaling. The actual signaling events involved in cell specific differentiation are unknown, but a stem cell population is assumed to re-establish the prostate.

Prostate stem cells differentiate into a set of cells that have become assigned to either the basal or luminal layer (Figure 1.1). The basal layer is defined by the expression of cytokeratins (CK) 14 and 5, while the luminal layer is predominantly CK8+/18+ (18). Neuroendocrine cells residing in the basal layer have long dendritic extensions that invade the luminal layer. Rare neuroendocrine cells secrete peptide hormones and biogenic amines that may stimulate proliferation (as reviewed in (19)). Transit amplifying (T/A) cells which also originate in the basal layer uniquely express p63 and have a limited number of cell divisions before transitioning to the next cell type (20). Intermediate cells, which express both basal (CK14+/5+) and luminal (express AR mRNA) markers, are characterized by the expression of prostate stem cell antigen (PSCA) (21). Functional AR expression is a defining characteristic of prostate luminal cells. Activation of AR within the cells is observed by the secretion of prostate specific

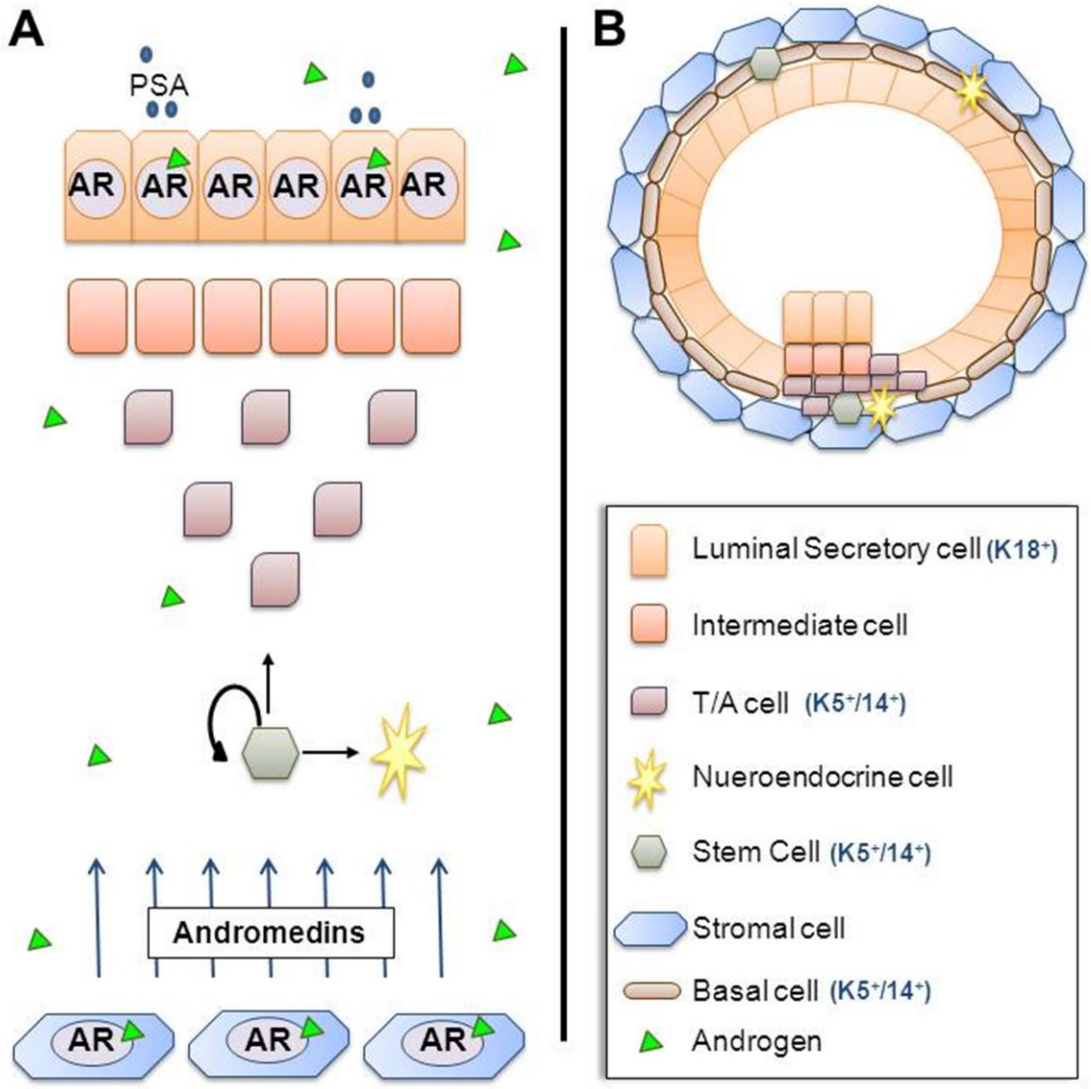

Figure 1.1 Model of epithelial cell differentiation in the prostate gland. (A) The activation of stromal expressed AR by DHT results in the release of paracrine factors (andromedins/growth factors) that facilitate epithelial differentiation. Prostate stem cells that are cytokeratin (K) $5^+/14^+$ either self-renew or differentiate into either T/A amplifying cells or neuroendocrine cells in the basal layer. T/A cells transition to intermediate cells that eventually terminally differentiate into the luminal epithelium ($K18^+$). (B) Schematic diagram of a prostate gland with a section undergoing cellular differentiation. The brown cells are representative of the basal layer. The graphical key defines the components depicted in (A) and (B).

antigen (PSA) which results from the targeted transcription of the Kallikrein 3 (KLK3) gene. A differentiation model presented by John Isaacs proposes two cell fate pathways for the prostate epithelial stem cell. One involves the differentiation of the cell into rare neuroendocrine cells, while the second transition into T/A cells further progresses through an intermediary state before terminally differentiating into luminal secretory cells (22). Further differentiation functions to limit the rates of cellular division observed in early development. AR negative T/A cells have a high proliferative index, while the terminally differentiated luminal cells express elevated AR levels.

High levels of activated AR in the luminal epithelium induce an anti-proliferative program. DHT was shown to slow the growth of AR overexpressing rat prostate cell lines, while AR negative cells were unaffected by the treatment (23). Directed expression of functional AR in both malignant and non-transformed AR negative human cell lines (PC-3 and BPH-1) reduced proliferation following androgen stimulation (24, 25). Activated AR in prostate epithelial cells may slow cellular division by stimulating pathways that upregulate either p27kip1 or p21/waf1 cyclin dependent kinases. A study demonstrated that the anti-proliferative effect of melatonin on minimally transformed prostate epithelial cells requires the transcriptional upregulation of $p27^{kip1}$ by AR (26). Additionally, reduced proliferation of LNCaP cells by vitamin D was mediated by p21/waf1 in an AR dependent manner (27). Considering that p21 is upregulated through the binding of activated AR to androgen response elements in the promoter (28), AR may target p21 to inhibit proliferation. The antagonistic effects of normal functioning AR on proliferation suggest that AR expression, during the differentiation of AR negative T/A cells, inhibits further cell division. AR may be silenced in T/A cells in order to maintain

a high proliferative potential. The mechanisms that allow for such a proliferative characteristic in T/A cells would likely benefit the survival of tumor cells as well.

Mechanisms of Cellular Proliferation

Cell division is regulated in a cyclical manner coordinated by the cooperation of a diverse set of genes. Periods of DNA replication and a cell division respectively known as the S and M-phase are separated by two preparatory G-phases (1 and 2), while non-dividing cells exist in a quiescent state known as G0 (as reviewed in (29)). Various checkpoints modulate cell cycle progression in order to reduce cellular abnormalities. One of the major cell cycle checkpoint regulators was discovered from studies of retinoblastomas. Genetic mutations in the RB1 gene were identified as the primary defect predisposing patients to retinal tumors (30) and have become associated with many human cancers (31-33). RB1 encodes a protein which may be involved in prostate cancer progression. Conditional RB1 allele deletions were shown to induce a pre-carcinogenic state in mouse prostate epithelium (34). Additionally, increased Rb protein (pRb) deficiencies were observed during metastatic prostate cancer (35). Studies showing that RB1 gene irregularities lead to abnormal cell division support a role for pRb in cell cycle checkpoint regulation.

Rb controls the G1/S-phase checkpoint by regulating the targeted transcription of S-phase activating genes. Rb, as a pocket protein (p107 and p130) family member, possesses a highly conserved binding domain capable of associating with multiple proteins (as reviewed in (36)). The interaction between hypophosphorylated pRb and E2F transcription factors (E2F1-3) (37) particularly inhibits cell cycle progression. The

transcription factors, while sequestered by hypophosphorylated pRb, are unable to target the transcription of genes required for the G1 to S-phase transition (38). Phosphorylation of pRb by cyclin dependent kinases disrupt pRb/E2F interactions and promote cell cycle progression (as reviewed in (39)). Rb protein plays a vital role in maintaining normal proliferation through control of the G1/S-phase checkpoint. Regulation of the E2F1 transcription factor by pRb is especially important, considering that the transcription factor is capable of inducing hyperproliferation.

E2F1 is a member of a functionally diverse set of transcription factors. E2Fs 1-3 generally activate transcription while E2Fs 4-8 function to repress transcription (as reviewed in (40)). E2F1 shares a set of conserved domains with E2Fs 2-3 that include a nuclear localization sequence, DNA binding, dimerization protein (DP), and Rb binding included transactivation domain (as reviewed in (41)). The heterodimerization of either DP-1 or 2 with E2F1 through the DP-dimerization domain facilitates transcriptional activity that is inhibited by additional interactions with pRb (42). Subsequent DP-1 phosphorylation is facilitated by the interaction between E2F1 and the cyclinA/cdk2 complex at the cyclinA/cdk2 binding domain and is required for normal S-phase progression (43). Although a majority of E2F1 initiated transcription occurs through contacts made between the DNA binding domain and the E2F responsive sequences (44), association with alternate factors may assist DNA binding independent transcription. E2F1 N-terminal interactions with Sp-1 (45) were shown to allow for the transcriptional activation of an Sp-1 consensus site containing c-myc promoter (46). Other undefined E2F1 interacting proteins may drive transcription by recruiting E2F1 to target gene promoters.

E2F1 targets a spectrum of genes with multiple cell functions. Transcriptional targets have been identified under varying cell context dependent conditions. A 2,500 gene microarray created from the livers of newborn mice demonstrated that gene expression, in most cases, was minimally altered between E2F1 mutant versus wild type mice. However, significant transcriptional changes resulting from the loss of E2F1 were observed in a small cohort of functionally undefined genes (47). Alternatively, induced expression of E2F1 in a non-transformed primary osteosarcoma cell line (Saos-2) increased the transcription of genes involved in cell cycle and growth regulation, angiogenesis, apoptosis, and cancer progression (48). Gene targets were additionally confirmed using chromatin immunoprecipitation (ChIP) analyses to identify sites in the upregulated gene promoters that were associated with E2F1. Using subtractive hybridization, Iwanaga et. al., further demonstrated that genes associated with DNA repair, transcription, signal transduction, and cellular metabolism were specifically upregulated in response to ectopic E2F1 expression in serum stimulated mouse fibroblasts (49). Altogether, there appears to be a large number of E2F1 genomic targets involved in a gamut of cell processes that both stimulate and inhibit cell growth.

E2F1 has a dual functional nature that appears to maintain balanced cell growth. Quiescent fibroblasts simultaneously entered S-phase and underwent p53 dependent apoptosis, after the induction of an overexpressing E2F1 plasmid (50). Although abnormal expression of E2F1 appears to initiate a governing mechanism to regulate cellular entry into an irregular proliferative state, the induction of cell cycle genes appears to overcompensate for the effects of activated apoptotic pathways. The expression of a constitutively active E2F1 protein in rat embryo fibroblasts resulted in the presence of

morphologicaly transformed foci (51). E2F1 possibly induces hyperproliferative states by driving cells into S-phase. E2F1 may upregulate cyclin E to phosphorylate and inactivate the pRb checkpoint (52). Mann et al. demonstrated that the expression of E2F1 in U2-OS cells (a human osteosarcoma cell line) blocks p16 mediated proliferative arrest (53), therefore propelling cells past the G1/S-phase checkpoint. Several factors are required during S-phase to insure correct DNA replication. DNA methyl transferase 1 (DNMT1), is a transcriptional E2F1 target (54) that plays a vital role in maintaining catalyticaly silenced genes during DNA replication (55).

DNMT1 is a member of the DNA methyltransferase family which includes DNMT3 (a,b) and DNMTL (as reviewed in (56)). DNMT3 (a,b) initiates *de novo* methylation (57) that is enhanced by DNMT3L (58) and maintained by DNMT1 (59). Full length DNMT1 (1616 amino acids) is the largest of the DNMT family members and contains a region within the catalytic domain is highly conserved across DNMT members (as reviewed in (60)). Less defined DNMT1 isoforms such as oocyte DNMT1 (DNMT1o) (-114 AA from N-terminal) (61) and somatic cell expressed DNMT1b (+48 nucleotides between exon 4 and 5) are cell specific (62). The DNMT1 structure enables interactions at the DNA replication fork that permit the methylation of newly synthesized DNA strands. Once the nuclear localization sequence has directed the enzyme to the nucleus, DNMT1 is recruited via the proliferating cell nuclear antigen (PCNA) binding domain to PCNA clamps located at the replication foci (63, 64). Additional DNA replication foci localization is mediated by the targeting sequence (65). At the DNA replication fork, the CXXC domain facilitates genomic contacts by penetrating the major groove and binding specifically to regions of unmethylated CpG dinucleotides (66)

(Figure 1.2). Two bromo-adjacent homology domains (BAH1 and 2) are located between the CXXC and catalytic motifs. BAH (1 and 2) tether the CXXC domain to the DNA, blocking access of the catalytic domain to the unmethylated sites. Certain structural confirmations of the BAH motifs additionally retain the target recognition domain in a restrictive structural confirmation. The BAH domains establish a large docking structure capable of facilitating protein interactions that may affect DNMT1 activity (66). Additionally the N-terminal interacts with histone deacetylase 2 (HDAC2) and contains a region capable of recruiting a co-repressor known as DNA methyltransferase associated protein 1 (DMAP1) (67). Methylation ensues once the catalytic domain gains access to DNA (66).

Methylation is a catalytic means by which DNMT1 modulates gene expression. DNMT1 catalyzes the transfer of methyl groups from S-adenosylmethionine to the 5'carbon of the cytosine ring located in Cytosine-phosphate-Guanine (CpG) dinucleotides (as reviewed in (68)). CpG dinucleotides are occasionally clustered in CpG islands (CGI) defined by a set of algorithmic parameters (%GC content $\geq$ 50%; CpG dinucleotides obsesrved/expected $\geq$ 0.6; and the CG rich sequence $\geq$ 200bps) (69). Under normal circumstances CGIs are predominantly unmethylated. However, increased gene promoter hypermethylation within CGIs tends to occur in certain cancer types (as reviewed in (70)). CGIs as a result, are prone to hypermethylation in environments with high DNA methyltransferase activity. In addition to CGIs, CpG dinucleotides are present in currently undefined arrangements that facilitate methylation in cell context dependent manners that possibly influence organismal development. A diverse CpG landscape provides a platform for complex DNA methylation profiles that differentially affect the

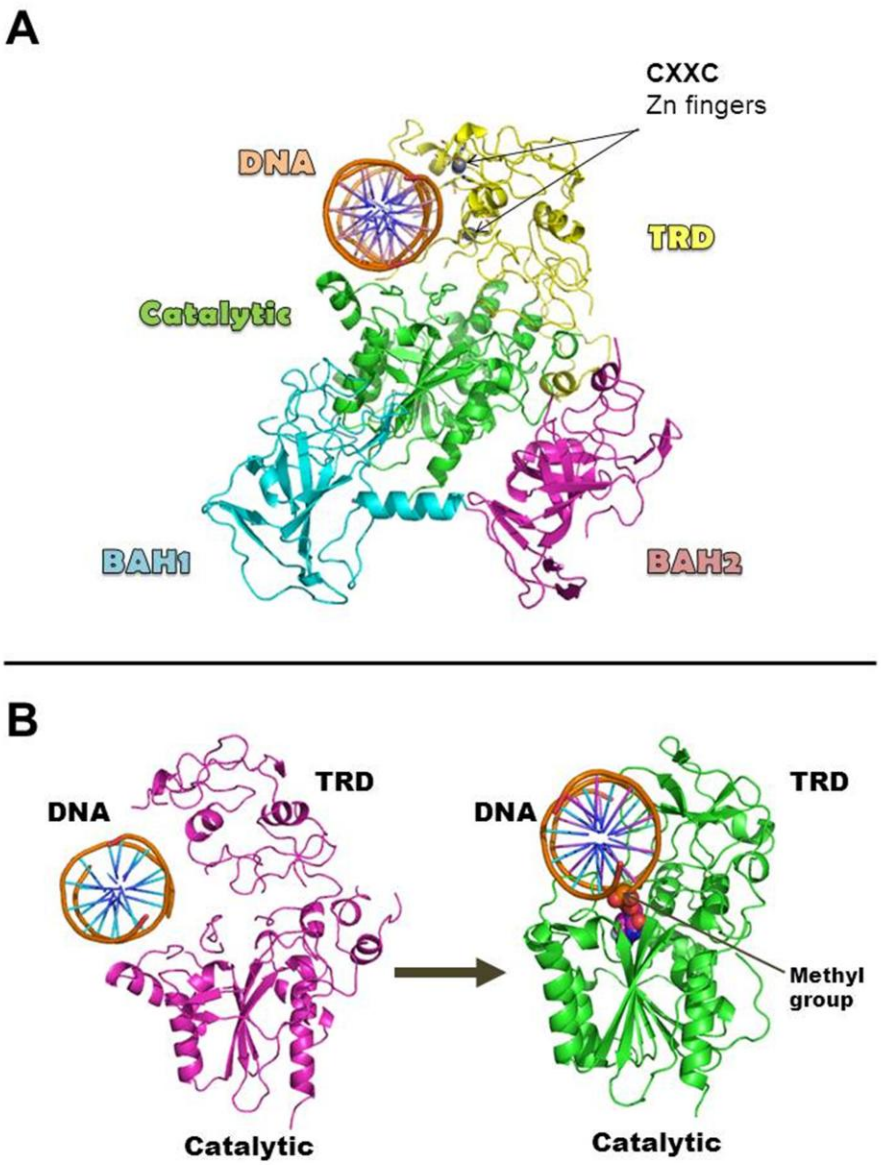

Figure 1.2 Cartoon representation of DNMT1 interacting with DNA. Cartoons of DNMT1 were created using protein database number 3TPA. (A) The Zn fingers of the CXXC domain are shown interacting with the unmethylated DNA CpG dinucleotides and block interaction with the catalytic site. The target recognition domain and BAH domains are additionally depicted in the cartoon. (B) A theoretical interpretation of cytosine methylation by the catalytic domain.

regulation of gene transcription. Generally thought to repress transcription, CpG methylation within intra- and inter-genic regions were observed in association with transcriptionally expressed genes (as reviewed in (71). Theories continue to evolve regarding the functional outcomes of positional CpG dinucleotide methylation.

Methylated DNA is usually bound by proteins that add another level of transcriptional regulation. The recruitment of functionally different methyl-CpG binding protein (MBD) family members (MeCP2 and MBD1-2 and 4) to the DNA, occurs in a cell context (species and developmental phase) dependent manner (as reviewed in (72)). The MBDs establish various complexes that may repress or induce transcription. Methylated DNA binding protein 2 (MeCP2) recognizes symmetrical methylation and facilitates repression through the compaction of the nucleosome (73). Conversely, a study, using a neuronal cell line, showed that 63% of the genomic promoters bound by MeCP2 were transcriptionally active (74). MBDs deacetylate histones by recruiting multi-subunit complexes. MBD2 and MBD3 were shown to respectively interact with NuRD and MeCP1 complexes that contain active histone deacetylases (75, 76) (Figure 1.3). The multilayered regulation of methylated DNA seems to support a fluid regulatory system that allows for flexible transitions between expressional programs.

Abnormal levels of methylation potentially promote oncogenesis. Although the hypermethylation of promoter CGIs occur naturally, tumor suppressor gene hypermethylation is observed more frequently in certain cancers ($P16^{INK4A}$ in solid lymphomas, BRCA1 in non-inherited breast cancer, and hMLH1 in MIN+ cancers) (as reviewed in (77). Hypomethylation assessed on a global genomic level was associated with hepatocellular and cervical cancers and breast ductal carcinomas ((as reviewed in

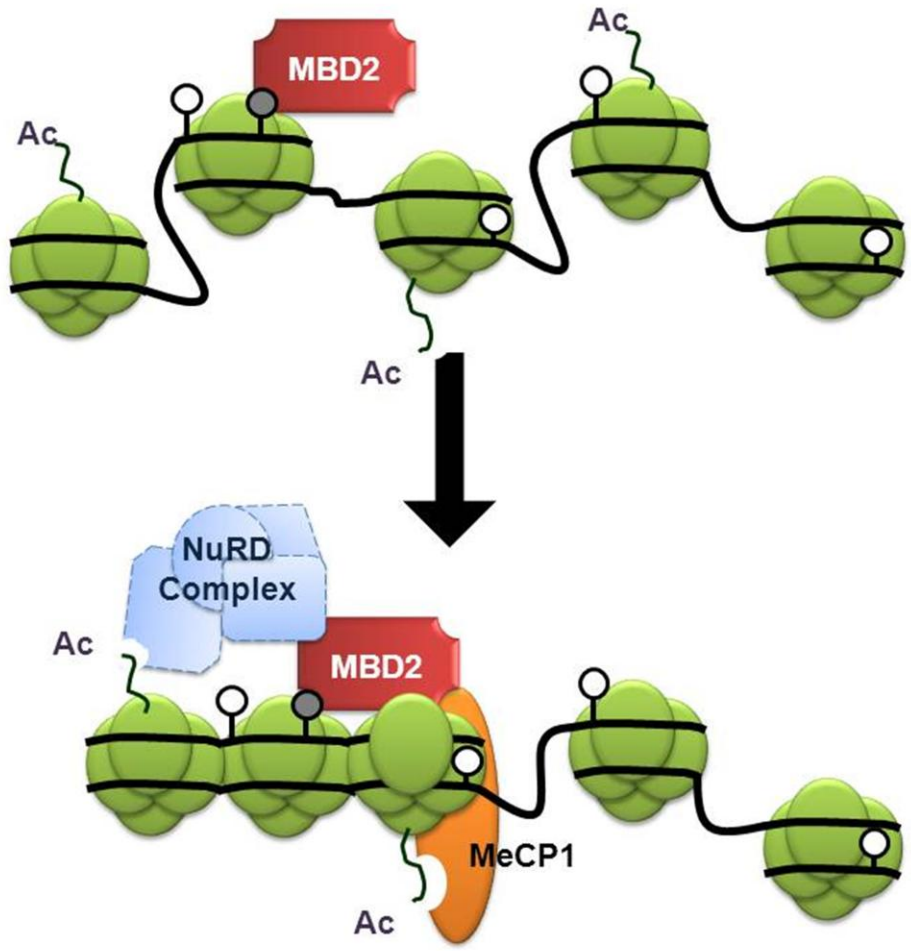

Figure 1.3 Methylation driven chromatin compaction. Bead-like units represent nucleosomes that are composed of eight histones. The nucleosomes are wrapped with DNA that has either methylated or unmethylated cytosines, respectively represented by a filled or open circle. Methyl binding protein 2 (MBD2) facilitates heterochromatin formation through interactions with MeCP1 and the NuRD complex to deacetylate histone tails. Acetyl groups are represented by "Ac".

(78)). Genomic hypermethylation may result from abnormal increases in DNMT1 expression. Induced overexpression of DNMT1 in a transgenic mouse resulted in the hypermethylation of a region located on the imprinted Igf2 allele (79). Binding of all the accessible CpG sites potentially occurs in the presence of elevated DNMT1 levels. The over abundant enzyme may seek available CpG sites and promote *de novo* methylation in an additional subset of genes. DNMT1 was shown to methylate nucleasomal DNA in a cell free based system (80). De novo methylation appears to be reliant on DNMT1 over expression and is CGI specific (81, 82). Although much focus has been placed on the catalytic function of DNMT1, the enzyme is observed to mediate methylation independent gene regulation.

The DNMT1 structure enables both known and potential protein interactions that may facilitate the regulation of targeted genes in a catalytically independent manner. Rountree et. al. demonstrated that a catalytically absent N-terminal region of DNMT1 was capable of repression through interactions with either HDAC2 or the DMAP1 co-repressor mentioned earlier (67). Another study also identified a homologus trithorax related protein HRX repressive domain upstream of the catalytic region capable of silencing transcription through active HDAC1 interactions (83). In addition to known domain interactions which facilitate methylation independent repression, the ability of DNMT1 to complex with known histone modifiers (HP1, G9a, and EZH2) may provide an alternate route for genetic regulation (as further discussed in chapter 2). DNMT1 as a result, is capable of dynamically modulating gene expression.

DNMT1 is responsible for maintaining appropriate cellular proliferation and development. Loss of DNMT1 function in homozygous mutant mice led to disruptions

of genomic imprints and resulted in embryonic death (84, 85). The requirement for DNMT1 in embryonic development suggests that the protein plays an essential role in cellular differentiation. DNMT1 downregulation induced epidermal progenitors to differentiate by abrogating self-renewing mechanisms (86). The protein also functions to limit replication errors during S-phase. In the presence of reduced DNMT1 levels, mismatch repair mechanisms are compromised and allow for the accumulation of mutations (87). While abnormally low expression of DNMT1 disrupts certain cellular processes, elevated levels tend to confer oncogenic properties. DNMT1 overexpression is associated with many cancers (88-91) and transforms NIH 3T3 mouse fibroblasts into cells capable of initiating xenograft tumors (92). DNMT1 as described maintains a delicate balance between proliferation and differentiation.

E2F1 and DNMT1 function to regulate the cell cycle and thus control a vital aspect of cellular proliferation. Abnormalities in either E2F1 or DNMT1 have severe consequences for both cellular differentiation and tumorigenesis. E2F1 may illicit transformative responses through the activation of and cooperation with DNMT1.

Castration Resistant Prostate Cancer

Although the mechanisms of prostate cancer progression are unclear, treatment options are much more optimistic for properly diagnosed lower stage cancers compared to advanced disease. In the 1940s, advanced prostate cancer was characterized as a hormone dependent carcinoma (93). Subsequent studies led to hormonal ablation treatments that seemed to cure patients by increasing wellness and decreasing tumor size. The short lived effects were met with the recurrence of an aggressive hormone

independent cancer, currently known as castration resistant prostate cancer (CRPC) (as reviewed in (94)). Many have tried to uncover the molecular events involved in the onset of the hormone insensitive disease using currently available models of CRPC.

CRPC models have been developed from prostate cancer cell lines and the manipulation of current genetic murine models such as Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP). Cell lines derived from pre-existing CRPC metastes (R 3327 MAT LyLu, PC-3,TsuPr1) (95-97) maintain androgen insensitive characteristics, while other prostate lines have been modified to acquire castration resistant properties (LNCaP C88 and LNCaP C4-2) (98, 99) (Table 1.1). Androgen independence is clearly evident in many cell lines. However, cells in culture have a limited ability to recapitulate human disease. Cells lines have altered molecular profiles in the absence of niche specific paracrine signals. While functional studies may become constrained by the features of the cell culturing environment, human tissue studies may further corroborate mechanistic findings. Animal models (castrated TRAMP, cre floxed Pten, and Pten/Nkx3.1 KO) (100-104) represent the standard of murine models available for the interrogation of niche specific cellular mechanisms *in vivo*. The emergence of CRPC was first observed in castrated TRAMP mice, although, TRAMP tumors rarely metastasize to the bone and show a predominantly neuroendocrine phenotype (105, 106). Both prostate cancer progression and CRPC were observed in Pten/NKx3.1 double knockouts and prostate specific cre floxed Pten mice. While Pten/NKx3.1 double knockout mice better recapitulated the bone metastasis observed in human cancer, the floxed Pten model show inconsistent bone met rates. The present models are variable and may simulate subtypes of CRPC.

Table 1.1 Currently Used Models of Castration Resistant Prostate Cancer

Models	Origin/ Generation	citation
Cell lines		
R 3327 MAT LyLu	Castration resistant Dunning Rat tumor	95
PC-3	Castration resistant bone metastases	96
TsuPr1	Castration resistant cervical lymph node metasteses	97
LNCaP C88	induced androgen insensitivity after several passages	98
LNCaP C4-2	second generation epithilail line derived from a castrated mouse xenoplant consisting of LNCaP cells and bone fibroblast	99
Mice		
Castrated TRAMP	Rb and p53 inactivation by SV-40 large T-antigen, expressed in the prostate epithelium of castrated mice.	100, 101
Cre floxed Pten	conditional knockout of Pten driven by the probasin promoter in the mouse prostate epithelium	102
Pten/Nkx3.1 KO	Double knock out of both Pten and Nkx3.1	103, 104

A number of pathways are associated with CRPC progression. Both increased AR expression and high PSA secretion during the emergence of CRPC (107, 108) suggested an oncogenic role for an active androgen independent AR. Studies have mainly focused on AR abnormalities (mutation, co-activation, growth factor stimulation, and amplification) that permit the unregulated induction of growth promoting genes in the absence of androgens (as reviewed in (109)). Studies continue to explore the AR independent pathways that support CRPC tumor progression. Marques et. al., using a castration induced cell line, identified significant expressional abnormalities in a subset of genes (TWIST1, VAV3, and DKK3) associated with alternate proliferative pathways (110). Neuroendocrine cells present during CRPC may also secrete growth promoting peptides, while the loss of Pten potentially increases bcl2 and inhibits apoptosis (as reviewed in (111)). An additional feature of CRPC to consider, is the significant decrease in AR expression during cancer progression studies (112, 113). We further explore the regulatory factors that facilitate AR repression utilizing a castrated TRAMP model of CRPC in chapter 3. Normal terminally differentiated prostate luminal epithelial cells depend on AR to transcriptionally activate programs that inhibit proliferation. These studies suggest that tumorigenic cells may repress normal functioning AR to maintain a high proliferative potential during CRPC.

Our work sought to define a specific molecular axis involved in AR repression and to evaluate a potential role for the AR regulatory mechanism during CRPC. The hyperproliferative nature of AR negative T/A cells led us to consider new roles that cell cycle regulators have in regulating AR expression. The S-phase inducing E2F1 transcription factor exhibits the ability to drive cells into the cell cycle and target the

transcription of DNMT1, a potent epigenetic silencer that is associated with cellular proliferation. We hypothesized that AR repression is mediated by an E2F1/DNMT1 axis within proliferative cells that are AR negative.

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CHAPTER II

REPRESSION OF ANDROGEN RECEPTOR TRANSCRIPTION THROUGH THE E2F1/DNMT1 AXIS

ABSTRACT

Although androgen receptor (AR) function has been extensively studied, regulation of the AR gene itself has been much less characterized. In this study, we observed a dramatic reduction in the expression of androgen receptor mRNA and protein in hyperproliferative prostate epithelium of keratin 5 promoter driven E2F1 transgenic mice. To confirm an inhibitory function for E2F1 on AR transcription, we showed that E2F1 inhibited the transcription of endogenous AR mRNA, subsequent AR protein, and AR promoter activity in both human and mouse epithelial cells. E2F1 also inhibited androgen-stimulated activation of two AR target gene promoters. To elucidate the molecular mechanism of E2F-mediated inhibition of AR, we evaluated the effects of two functional E2F1 mutants on AR promoter activity, and found that the transactivation domain appears to mediate E2F1 repression of the AR promoter. Because DNMT1 is a downstream target of E2F1, we examined DNMT1 function in AR repression. Repression of endogenous AR in normal human prostate epithelial cells was relieved by DNMT1 knock down. DNMT1 was shown to be physically associated within the AR minimal promoter located 22 bps from the transcription start site, however,

methylation remained unchanged at the promoter regardless of DNMT1 expression. Taken together, our results suggest that DNMT1 operates either as a functional intermediary or in cooperation with E2F1 to inhibit AR gene expression in a methylation independent manner.

INTRODUCTION

Androgens are required for prostate gland development and for prostatic function and glandular maintenance in the adult male (1). Androgen action is mediated through the androgen receptor (AR), a ligand-activated nuclear transcription factor. AR expression is found in a variety of tissues, including prostate and breast, and changes throughout development, aging and malignant transformation (reviewed in (2)). AR exists in the cytoplasm and is associated with at least three heat shock proteins, hsp56, hsp70 and hsp90 (3). Upon androgen binding and activation of the AR, heat shock proteins dissociate and expose the nuclear localization domain which directs the receptor to the nucleus (4). Prior to nuclear translocation, the androgen/AR complex undergoes dimerization and phosphorylation. Upon entering the nucleus, the AR binds to androgen response elements (ARE) in the promoter or enhancer region of numerous androgen-responsive genes. Several AR co-activators have been identified (ARA 70, ARA 55 and ARA 54) which also interact with and regulate AR gene transactivation (5, 6). Thus, ligand-activated AR may stimulate androgen-regulated genes through a variety of mechanisms. AR function and the signaling pathways regulated through androgen and AR interaction have been extensively studied for decades however; regulation of the AR gene itself is not clearly understood.

Studies have shown that transcriptional regulation of AR is cell specific and age-dependent (7, 8). The promoter region of the AR gene lacks transcriptional regulatory sequences (TATA and CAAT), but is rich in GC sequences (9, 10). There are at least two transcription initiation start sites whose use vary depending on cell type (11). Studies of the AR promoter have identified potential binding sites for several transcription factors, however, there have only been a few well characterized studies demonstrating transcriptional regulation of AR. For example, Sp1 (9, 11) has been shown to be a positive regulator of AR gene expression, whereas, NF-κB p50/p50 and NF-1 have been shown to be strong negative regulators of AR (12, 13). The mechanisms underlying the repression of the AR gene remain to be elucidated.

The E2F family of transcription factors control cell proliferation by regulating cell cycle progression (14-16). The E2F family has eight characterized family members (E2F1-E2F8) which can form heterodimers with DP family members (DP1, 2, and 3), giving rise to functional E2F activity (16). E2Fs control entry into the cell cycle and regulate G1/S phase transition by regulating the transcription of genes that encode cell cycle regulatory proteins including Cyclin E, Cyclin A, Cdc 2, Cdc 25A, and proliferating nuclear cell antigen (PCNA), as well as enzymes involved in nucleotide biosynthesis such as dihydrofolate reductase, thymidylate synthase and thymidine kinase (17). E2F1, E2F2 and E2F3 are traditionally thought of as transcriptional activators of E2F responsive genes, whereas E2F4, E2F5, E2F6, E2F7, and E2F8 act as transcriptional repressors. Overexpression of E2Fs 1-3 in serum starved cells induces S-phase entry and DNA synthesis by binding to DNA response elements and activating the transcription of E2F target genes (17-19). E2Fs 1-3 can also override growth-arrest signals induced by

Cdk inhibitors p16 (19) and can act as both oncogenes and tumor suppressors (20-22). E2F1 binding sites have been reported in the promoters of the breast cancer susceptibility gene BRCA1 (23), p73 (24, 25), the tumor suppressor gene $p14^{ARF}$ (26), and the gene for apoptosis protease-activating factor 1 (Apaf-1) (27). We have identified E2F binding sites in the promoter of DNA methyltransferase 1 (DNMT1) that allow for regulation by E2F1 (28). E2F1 has also been shown to act as a direct transcriptional repressor for several genes including urokinase-type PA (uPA) (29), the anti-apoptotic protein Mcl-1 (30), and human telomerase reverse transcriptase (31). These results suggest that E2F1 can have both positive and negative regulatory roles on gene transcription, however the molecular basis of these disparate functions is not known.

The DNA methyltransferases (DNMT 1, 3a, 3b and 3L) play an integral role in the epigenetic regulation of many genes. All DNMTs except for 3L have a catalytic domain that facilitates the transfer of methyl groups from S-adenosyl-methionine to cytosines located in CG dinucleotides. DNMT 3a and 3b are generally responsible for genome-wide *de novo* methylation during early embryogenesis (32). DNMT3a is shown to methylate both maternally and paternally imprinted genes in germ-line cells (33), while DNMT3b maintains the chromosomal stability of 6, 9, and 16 via centromeric methylation (34). Genomic methylation by DNMT3 (a and b) is enhanced in the presence of DNMT3L (35). Initial methylation by the DNMT3 family members is maintained by DNMT1, which has an affinity for hemimethylated DNA during replication and cell division. DNMT1 is necessary for mouse fetal development and the progenitor and self-renewing characteristics of somatic cells located in the epidermis (36). As mentioned, DNMT1 was characterized in our lab to be a direct transcriptional

target of E2F1 (28) and may mediate targeted repression by E2F1. Previous work in our lab has additionally shown that the DNMT1 is trans-activated by

In this study, we explored a regulatory mechanism that controls the endogenous expression of AR in prostate epithelium. We investigated the effects of the transcription factor E2F1 on AR mRNA and protein expression in both human and mouse prostate epithelial cells. We demonstrate how E2F-1, a classical transcriptional activator, might cooperate with DNMT1 to repress AR transcription in the prostate gland.

MATERIALS AND METHODS

Cell Culture. LNCaP and DU145 cell lines obtained from American Type Culture Collection, Rockville, MD and BPH-1 cells received from Dr. Simon Hayward, Vanderbilt University Medical Center (37) were cultured in RPMI 1640 medium supplemented with 8% fetal bovine serum (FBS), 0.1% penicillin/streptomycin and 0.1% L-glutamine. Human prostate epithelial cells (hPrEC) purchased from Lonza/Clonetics, Walkersville, MD were maintained in Prostate Epithelial Cell Growth Medium (Lonza/Clonetics). Mouse prostate epithelial (PrE) cells, previously described in reference (38) were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS), 0.1% penicillin/streptomycin and 0.1% L-glutamine.

For the development of the PrE-E2F1 stable cell lines, PrE cells were transfected with pcDNA3-E2F1 (kindly provided by W. Kaelin, (39) or empty vector pcDNA3 (Invitrogen, Carlsbad, CA) using Tfx50 (Promega, Madison, WI) according to the manufacturer's protocol. Stable clones were selected in RPMI 1640 media containing

5% FBS, 0.1 % penicillin/streptomycin, 0.1% L-glutamine and 200 μg/ml G418 (Sigma, St. Louis, MO). For the development of the DNMT1 knock-down stable BPH-1 and transient hPrEC cell lines, cells were lentivirally infected with a pLKO.1-puro vector either expressing DNMT1 specific short hairpin RNA (shRNA) (clone ID: NM_001379.1-1687s1c1), non-targeting shRNA (Cat#: SHC002) or no shRNA insert (Sigma/Mission, St. Louis, MO). Stable shRNA BPH-1 clones were selected in RPMI 1640 media containing 8% FBS, 0.1 % penicillin/streptomycin, 0.1% L-glutamine and 1 μg/ml puromycin (Sigma, St. Louis, MO).

Cell treatments. All synthentic androgen (R1881) treatments were done at 10^{-9}M for 24h after 15h of serum starvation. All 5-aza-2'-deoxycytidine (5Aza) (Sigma) treatments were done in either complete culturing media used for DU145 or hPrEc lines. Fresh media containing 1μM 5Aza was added every 24h for a total of 72h and DMSO treatments were matched as vehicle controls.

Northern Blot Analysis. Total RNA was prepared using QIAGEN RNA Easy kit per manufacturer's protocol (QIAGEN, Valencia, CA). Twenty micrograms of RNA was resolved by gel electrophoresis under denaturing conditions and RNA was transferred to a Duralon-UV membrane (STRATAGENE, La Jolla, CA) overnight by capillary action in 20 X SSC buffer (3 M NaCl and 0.3 M Na Citrate). RNA was crosslinked to the membrane by UV cross linking. A 1.6 kb human AR cDNA fragment was isolated from CMV3-hAR3.1 (kindly provided by D. Robins, University of Michigan, Ann Arbor, MI) using a HindIII and NheI restriction enzyme sites. A 1.5 mouse AR cDNA fragment was

isolated from CMV5-mAR (kindly provided by D. Robins, University of Michigan, Ann Arbor, MI) using HindIII restriction enzyme sites. The human and mouse AR cDNA fragments were gel purified using QIAGEN Gel Purification Kit, per manufacturer's protocol and subsequently labeled with [α-^{32}P] dATP using the random oligonucleotide-primer labeling kit (STRATAGENE) and purified on STRATAGNE Nucleotide Push Columns following manufacturer's protocol. The [α-^{32}P] dATP labeled probes were hybridized to a Duralon-UV membrane (STRATAGENE) at 65^{o}C overnight in hybridization buffer (0.25 M Na_2HPO_4, pH 7.2 and 7% SDS) while rotating. The membrane was subsequently washed twice for 45 min each in 20 mM Na_2HPO_4, pH 7.2 and 5% SDS followed by two additional washes for 45 min each in 20 mM Na_2HPO_4, pH 7.2 and 1 % SDS. The membranes were exposed to X-ray film (Kodak, Rochester, NY) overnight and visualized by autoradiography.

Western Blot Analysis. Cells were either trypsinized, centrifuged and washed one time with PBS then lysed with RIPA buffer (50 mM Tris pH 8.0, 120 mM NaCl, 0.5% Nonidet P40, 1.0 mM EGTA, 200 μg/ml PMSF, 50 μg/ml aprotinin, 5 μg/ml leupeptin, 200 μM sodium orthovanidate) or lysed directly in the plate. The hPrEC lines were harevested 4 days post infection, while stably infected BPH-1 cells were collected 4 days post selection in puromycin (1μg/ml). Protein concentrations were determined using Bradford Protein Assay Reagent (BIO-RAD, Hercules, CA), following manufacturer's protocol. For Western blot analysis, protein extract was subjected to gel electrophoresis either on a tris-glycine polyacrylamide gel (Invitrogen) (LNCaP and mPrE) or on a Nu Page tris-acetate polyacrylamide gel (Invitrogen) (BPH-1 and hPrEC). The gel was

transferred to Optitran nitrocellulose membrane (Schleicher & Schuell Biosciences Inc. Keene, NH) by electrophoresis for 1 hour at 45 V. The membrane was blocked in 10% nonfat dry milk in TBST (10 mM Tris, 250 mM NaCl, 1% Tween 20) for 1 hour at room temperature and immunoblotted with primary antibodies for AR (N-20, Santa Cruz, Santa Cruz, CA), E2F1 (KH-95, Pharmingen, Franklin Lakes, NJ), Rb (Pharmingen), PCNA (C-20, Santa Cruz), E-Cadherin (H-108 Santa Cruz), Cyclin E (M-20, Santa Cruz), DNMT1 (Raw M0231S prep gift from Dr. Sriharsa Pradhan, New England BioLabs Inc., Ipswich, MA), β-actin (C-11, Santa Cruz), or actin (AC-40 Sigma, St. Louis, MO). The membrane was incubated with secondary antibody conjugated to horseradish peroxidase (BIO-RAD, Hercules, CA) and the bands were detected using ECL (PIERCE, Rockford, IL) detection system, following manufacturer's protocol.

Luciferase Assay. LNCaP and PrE cells were plated at 2 x 10^5 cells per 6 well dish and incubated at 37^{o}C overnight. Stably infected BPH-1s with DNMT1 shRNAs were plated at a 1 to 60 passage into a 12 well dish and incubated at 37^{o}C overnight. Cells were co-transfected with 1 μg/ml of either of the following promoter-luciferase reporter constructs; DHFR-Luc, E2F-Luc and CRE-Luc were kindly provided by G. Denis, Boston University, Boston, MA (40), 2.0 kb human AR promoter-Luc (hAR-Luc) (kindly provided by F. H. Sarkar, Wayne State University, Detroit, MI), 1.5 kb mouse AR promoter-Luc (mAR-Luc) was kindly provided by D. J. Tindall, Mayo Clinic, Rochester, MN (41), MMTV-Luc (gift from E. Keller, University of Michigan, Ann Arbor, MI) and 3XHRE-Luc (gift from D. Robins, University of Michigan, Ann Arbor, MI). The promoter-reporter constructs were co-transfected in LNCaP and PrE cells with either

empty pcDNA3 vector, wild type E2F1 or the following E2F1 mutants ($E2F_{1-284}$, or Eco132) (gifts from W.D. Cress, Moffitt Cancer Center, Tampa, FL, (30, 42)), a dominant negative E2F1 was kindly provided by W. Kaelin, Harvard University, Boston, MA, (43) or Tag (gift from M. Imperiale, University of Michigan, Ann Arbor MI). The pSV-beta-galactosidase (β-gal, Promega) expression plasmid was co-transfected into LNCAP and PrE cell lines at 0.1 μg/ml and into hPrEC and BPH-1 cells at 1 μg/ml as an internal control. DNA was transfected using Tfx50 transfection reagent (Promega) at a ratio of ~3:1 (Tfx50: DNA) following manufacturer's protocol. After 72 hours of transfection, whole cell lysates were collected in lysis buffer. Luciferase expression was determined by adding 50 μl luciferase substrate (Promega) to 50 μl of lysate and luciferase was monitored using a Monolight 2010 luminometer. β-gal expression was monitored using β-gal Detection System (Tropix, Bedford, MA) following manufacturer's protocol using Monolight 2010 luminometer. Samples were assayed in triplicate and luciferase activity was normalized to β-gal activity.

qRTPCR and PCR Analysis. Total RNA was extracted by scraping and collecting cells in TRizol (Invitrogen, Carlsbad, CA) (1ml per 60mm dish). The lysate was added at 1ml to a pre-spun 2ml heavy phase lock gel tube (5 PRIME Inc., Gaithersburg, MD), incubated for 5min at room temperature, and combined with chloroform. After the mixture was centrifuged at 12,000xg for 10min at 4°C, the resulting aqueous mixture above the wax plug was removed and mixed together with 500 μl of isopropanol, and incubated for 10 min at room temperature. The mixture was centrifuged into a pellet at 12,000xg for 10min at 4°C and washed 1 time in 70% ethanol. RNA was reconstituted in 35 μl of

UltraPure Distilled Water (Invitrgen/GiBCO, Carlsbad, CA) and quantitated with the NanoDrop Spectrophotometer ND-1000 (Thermoscientific, Wilmington, DE), treated with DNase I (Invitrogen), then converted to cDNA using the Thermoscript RT PCR Reaction System (Invitrogen) according to the manufacture's protocol. The qRTPCR was conducted with the following primers:

human AR forward: 5'-GACCAGATGGCTGTCATTCA-3'
human AR reverse: 5'-GGAGCCATCCAAACTCTTGA-3'

human GAPDH forward: 5'-TGCACCACCAACTGCTTAGC-3'
human GAPDH reverse: 5'-GGCATGGACTGTGGTCATGAG-3'

A Mastercycler ep realpex^2 (eppendorf, Hamburg, Germany) using SYBR green PCR Master Mix (Applied Biosystems, Carlsbad, CA) was used to amplify the cDNA with the following PCR conditions; denatured at 95^{o}C for 3min and subjected to 40 cycles (95^{o}C 30 sec, 60^{o}C 30sec, and 72^{o}C 30sec). The primers were used in a separate PCR and electrophoresed on a gel to verify the presence of a single amplicon from the cDNA. Each sample reaction in the qRTPCR was done in triplicate in a 96 well plate format. Cycle threshold units were obtained using Mastercycler ep realpex^2 software. Data was analyzed using the $2^{-\Delta\Delta CT}$ method (44) relative to GAPDH values. PCR was conducted on cDNA using the human AR and GAPDH primers referred to above in combination with platinum PCR super mix (Invitrogen). Reactions were run in an epindorf thermocycler denatured at 95^{o}C for 3min, subjected to 35 cycles (95^{o}C 30 sec, 60^{o}C 30sec, and 72^{o}C 30sec) and processed on a 2% agarose gel.

Chromatin Immunoprecipitation qPCR Analysis. For each ChIP $1X10^7$ BPH-1 cells were cross-linked with 1% formaldehyde for 10 min at room temperature on a rocking platform. The reaction was quenched with 0.125 M glycine. Cells were scraped and collected in cold PBS containing protease inhibitors (200 ug/ml PMSF, 50 ug/ml aprotinin, 5 ug/ml leupeptin, and 200 uM sodium orthovanidate), following 2 washes in cold PBS. The harvested cells were pelleted at 5,000 rpm for 6 min at 4^{o}C and washed once with cold PBS containing protease inhibitors. Lysates were prepared using the reagents in the Magna ChIP A kit (Millipore, Temecula, CA) according to manufacture instructions, however, the lysis buffer available was substituted with 400ul of SDS lysis buffer (Millipore) containing kit supplied protease inhibitors. The chromatin in the lysate was sheared to ≤ 600bps in a 2ml tube placed in a Covaris S2 (Covaris Inc., Woburn, MA) water bath set to the following cavitation parameters: duty cycle, 20%; intensity, 5; cycles per burst, 200; cycle time, 30 sec; and cycles, 30. The sheared chromatin was processed and immunoprecipitated with 5μg of either DNMT1 (ab19505, abcam, Cambridge, MA) or rabbit IgG (sc-2027, Santa Cruz) using the reagents and instructions provided in the Magna ChIP A kit. The purified ChIP DNA was retrieved with 40μl of elution buffer C. The DNA sample was amplified with a two step PCR program (Denaturation at 95^{o}C for 10min and 40 cycles of 95^{o}C for 15 sec and 60^{o}C for 1 min) using SYBR green PCR Master Mix in a StepOnePlus Real-Time PCR thermocycler (Applied Biosystems) employing the following primers:

site A forward: 5'-GACTCGCAAACTGTTGCATT-3'
site A reverse: 5'-TACAGCACTGGAGCGGCTA-3'

site B forward: 5’-CCTAGCAGGGCAGATCTTGT-3’
site B reverse: 5’-TCCCCTTCTCTTGCTCAGAA-3’

site C forward: 5’-GGTAGGAAGTGGCTGAATTCTGGATGA-3’
site C reverse: 5’-CCCTGCCCATGCACCTGCTC-3’

human PS2 forward: 5’-TTCCGGCCATCTCTCACTAT-3’
human PS2 reverse: 5’-CGGGGATCCTCTGAGACA-3’

human ABCB1 forward: 5’-TCTAGAGAGGTGCAACGGAAGCCA-3’
human ABCB1 reverse: 5’-CCTGCCCAGCCAATCAGCCT-3’

An extended program (95^{o}C for 15min, 60^{o}C for 1min, and 95^{o}C for 15 sec) was used to create a melting curve that was analyzed with the StepOne software v2.1 package to verify that the primers only amplify a single amplicon from genomic DNA. Each sample reaction in the qPCR was done in triplicate in a 96 well plate format. Cycle threshold units were obtained using StepOne software v2.1. Data is represented as a percent of input using a derivation of the $2^{-\Delta CT}$ method (44).

Bisulfite sequencing. Genomic DNA (gDNA) was extracted from cells using the Wizard Genomic DNA Purification kit (Promega) and quantified with the NanoDrop Spectrophotometer ND-1000 (Thermoscientific, Wilmington, DE) . A 250 ng sample of DNA was bisulfite converted using the EZ DNA Methylation-Direct kit (Zymo Research, Orange, CA) according to the instructions provided by the manufacturer. The following bisulfite converted DNA specific primers, targeting a region in the AR (NM_000044) minimal promoter were created with Methyl Primer Express v1.0:

bisulfite sequence forward: 5'-GGGAGTTAGTTTGTTGGGAG-3'

bisulfite sequence reverse: 5'-TCCTACCAAACACTTTCCTTACT-3'

Amplification of the bisulfite converted gDNA was accomplished using special ZymoTaq PreMix (Zymo Research) polymerase to facilitate the production of aplicons with A overhangs using the following PCR program: denature at 95^{o}C for 10 minutes, run 35 cycles (95^{o}C 30 sec, 59^{o}C 30 sec, and 72^{o}C 60 sec), run a final extension at72^{o}C for 7 min, and hold at 4^{o}C. PCR product was combined with pCR8/GW/TOPO TA cloning vector (Invitrogen) in a mixture prescribed by the manufacture to facilitate the insertion of the amplified products into the plasmids which contain sequencing primer sites that flank the insert. Plasmids were transformed and plated in One Shot Top 10 chemically competent cells (Invitrogen) per manufacturer's instructions and at least 12 bacterial colonies were individually grown in 5ml of LB containing spectinomycin (100 μg/ml). Plasmids were harvested from the bacteria using the Wizard Plus SV Miniprep kit (Promega) and sequenced with the M13 forward and reverse primers at the University of Michigan DNA sequencing core.

Statistics- Data showing significance was analyzed using 2-tailed Student's t test. $P < 0.05$ was accepted as the level of significance.

RESULTS

Transgenic K5-E2F1 prostate glands exhibit hyper-proliferative epithelium and an atypical morphology. Accumulating evidence suggests that increased E2F1 activity reactivates several aspects of benign and malignant disease including increases in cellular proliferation (45, 46). We were curious as to the role of E2F1 in prostate gland disease processes. We have shown previously that normal human prostate gland expresses low levels of E2F1 (47). We were thus intrigued by the observation that keratin 5 promoter driven expression of the human E2F1 gene in the mouse prostate gland (48), resulted in hyperproliferative changes that were not detected in wild type mice (Figure 2.1A). This K5 promoter fragment is known to direct transgene expression to the basal cell compartment of stratified epithelia of several glandular tissues such a mammary gland, salivary gland and prostate (48, 49). In K5-E2F1 transgenic mice, the majority of glands appeared grossly normal and were lined with a single layer of epithelial cells, however there were focal areas of increased epithelial hyperplasia with abnormal gland architecture in the dorsolateral lobe of the prostate (Figure 2.1A). Some glands had increased stratification of epithelial cells that formed compact glands with a cribriform growth pattern, representative of prostatic intraepithelial neoplasia (PIN) and some nuclear atypia. Similar lesions were not detected in wild type animals. Prostate tissue from age and strain-matched wild type mice consisted of normal prostatic ducts lined with a single layer of epithelial cells surrounded by a thin layer of stroma (Figure 2.1A). To further define a role for E2F1 in prostate epithelial cell growth, we generated prostate epithelial cells lines from glands harvested from two wild type mice and three K5-E2F1

transgenic mice. Semi-quantitative RT-PCR analysis using specific primers for mouse and human E2F1 show the presence of endogenous mouse E2F1 in wild type and K5-E2F1 cells, however, human E2F1 was only detected in K5-E2F1 cell lines (data not shown). In agreement with the hyper-proliferative epithelial histology, the K5-E2F1 lines exhibited a 2-fold increase in proliferation compared to wild type cells (Figure 2.1C). We also observed a significant reduction of K5-E2F1 cells in G1 phase and a concurrent increase in the distribution of cells in G2/M and S phase (data not shown). Western blot analysis reflected E2F1 expression and revealed a significant increase in human E2F1 protein expression in all three K5-E2F1 transgenic lines compared to wild type controls (Figure 2.1B). To investigate the molecular events associated with increased E2F1 expression, we analyzed several regulators of prostate epithelium in addition to E2F1 target genes. Whole cell lysates prepared from log phase wild type and K5-E2F1 cells were analyzed for Cyclin E and PCNA protein levels. K5-E2F1 cells exhibited an aproximate 3 fold increase in Cyclin E and a 2 fold increase in PCNA (Figure 2.1B). Cyclin E and PCNA are E2F1 target genes that regulate DNA synthesis and promote G1/S transition, suggesting that E2F can control both DNA replication and mitotic activities in our transgenic prostate model and cell lines (50, 51). The prostate epithelial lineage of these cell lines was verified by the expression of the epithelial cell marker E-cadherin (Figure 2.1B) and the steroid hormone receptors estrogen receptor-beta (ER-β) (data not shown). Surprisingly, all 3 K5-E2F1 lines exhibited significant repression of AR protein compared to the wild type cells (Figure 2.1B). An unknown protein is additionally observed in the developed western blot for the K5-E2F1-2 line to run about 20 kDa lower than full length AR. The transgenic line may express the AR-A isoform,

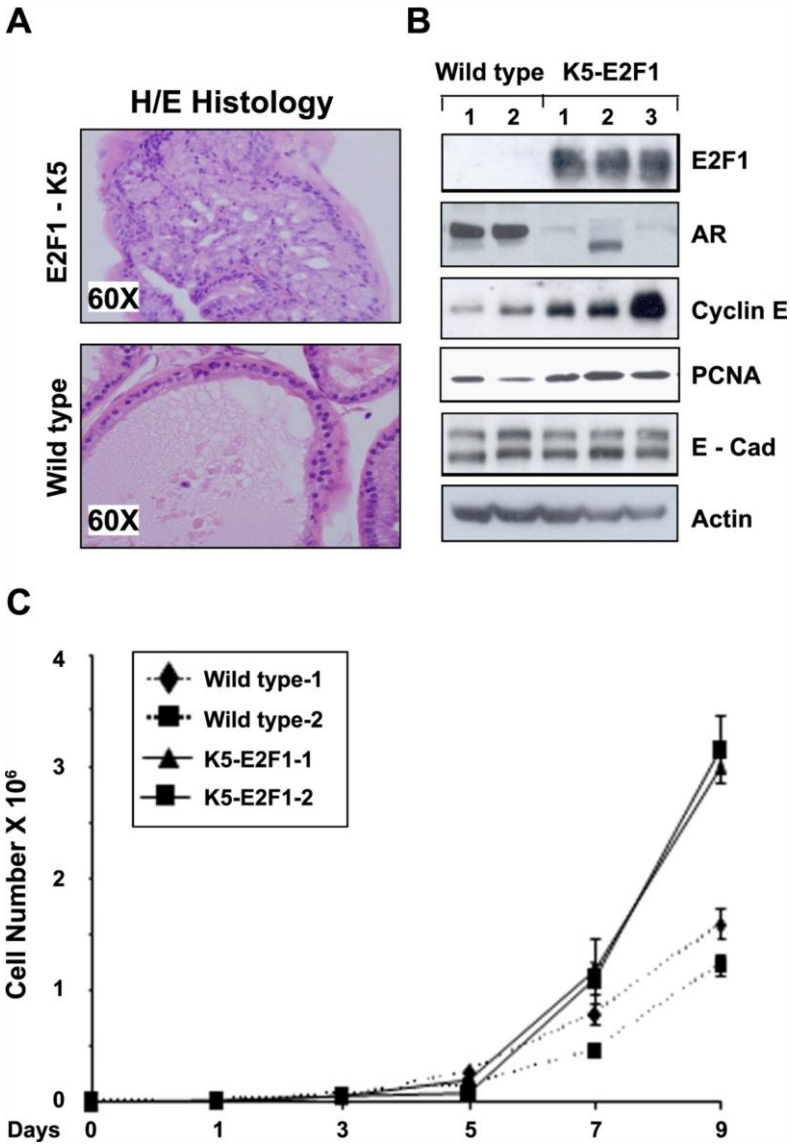


Figure 2.1 E2F1 leads to atypical prostatic morphology and increases prostate epithelial proliferation in a K5-E2F1 transgenic mouse. (A) Histology of prostate tissue taken from both K5-E2F1 transgenic and wild type mice. (B) Prostate epithelial cell lines established from both transgenic and wild type mice were analyzed by western blot for the expression of E2F1, cell cycle genes (Cyclin E and PCNA), the epithelial cell specific marker E-cadherin (E-cad), and Androgen Receptor (AR). Actin is shown as a loading control. (C) A trypan blue exclusion assay was implemented to measure the viability of cell lines obtained from the mouse models. Each point represents the mean of three independent experiments with the standard deviation.

characterized by an N-terminal domain that is 187 amino acids (21 kDa) shorter than the full length form (52).

E2F1 down regulates AR expression and the promoter activity of AR target genes. We were surprised by the reduction in AR expression in the K5-E2F1 transgenic lines compared to wildtype lines. To determine if E2F1 directly represses AR transcription, we examined whether exogenous expression of E2F1 reduces AR mRNA levels in prostate epithelial cells. Stable E2F1 over-expressing clones were established in mouse prostate epithelial cells (PrE) and two clones, PrE2F1-1 and PrE2F1-2, were expanded and characterized. Total RNA was harvested and subjected to Northern blot analysis for the detection of AR and E2F1 mRNA. Both clones exhibited increased E2F1 mRNA and significantly reduced AR mRNA (Figure 2.2A) and protein (Figure 2.2B). These cells exhibited increased E2F activity by exhibiting increased expression of Cyclin E and PCNA, two well described E2F-target genes (Figure 2.2B). These result demonstrated that exogenous E2F1 is involved in the repression of AR expression.

The findings from the transgenic animals indicated that E2F1 might be driving a proliferative and undifferentiated phenotype. We had previously observed the AR-regulated prostate specific antigen (PSA) gene was down regulated following E2F1 over expression suggesting a repressive activity of E2F1 on AR target genes through the repression of AR (47). To explore this possibility, we examined the effect of E2F1 on a hormone-responsive promoter/reporter construct (3XHRE-Luc) in the androgen-responsive prostate cell line, LNCaP. The 3XHRE-Luc construct has 3 hormone response elements cloned in front of a luciferase reporter gene and allows for the

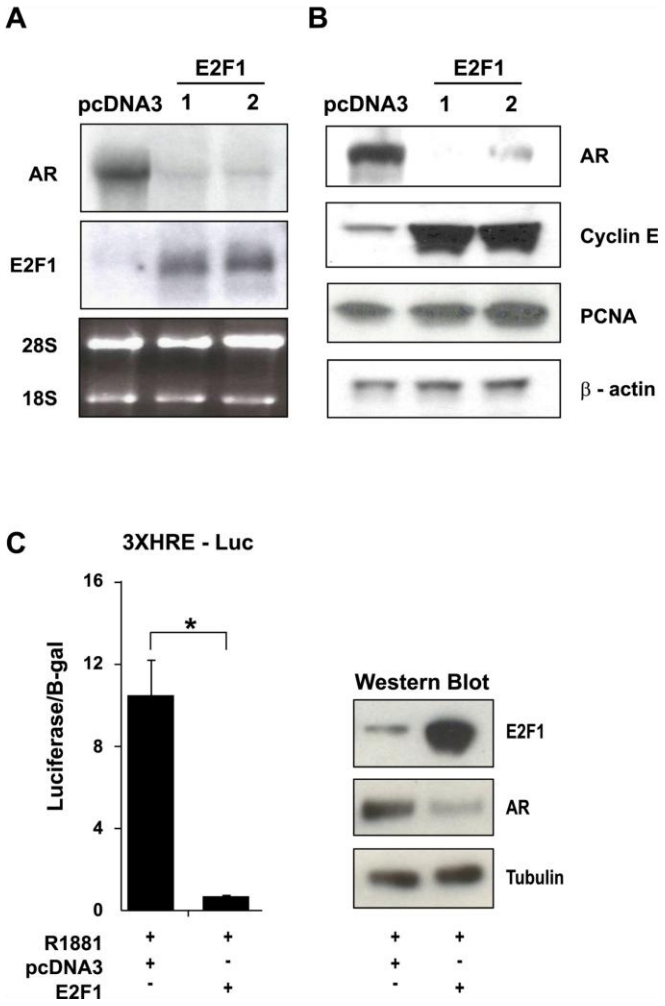


Figure 2.2 Exogenous E2F1 inhibits both AR expression and responsive promoters. (A) Northern blot analysis of stably transfected mouse prostate epithelial cells (PrE) with either pCDNA3 (control vector) or E2F1 shown as E2F1-1 and E2F1-2 to detect AR and E2F1 gene transcription. The 28S and 18S ribosomal bands are shown for loading comparison. (B) Western blot analysis of whole cell lysates harvested from PrE cells stably transfected with pcDNA3 (control) or E2F1 for the detection of AR, Cyclin E, and PCNA. β-actin is shown as a loading control. (A and B) The northern blot and western is representative of 3 separate experiments. LNCaP cells were co-transfected with 1 µg of ARE-Luc (C) and 500 ng of either empty pcDNA3 vector or E2F1 in the presence of 10^{-9} M R1881. Results were normalized to β-galactosidase (B-gal) from a co-transfected CMV promoter driven B-gal reporter construct. The histogram represents the mean value of three independent experiments with the indicated standard deviation. The western depicts the expression levels for E2F1 and AR relative to tubulin for the transfection and treatment conditions. * indicates $P < 0.05$ for the indicated comparison in brackets.

monitoring of directed hormone receptor activation. LNCaP cells were co-transfected with the 3XHRE-Luc construct with either pcDNA3 (empty vector) or E2F1. We treated cells with the synthetic androgen R1881 to specifically activate AR and observed activity from the 3XHRE-Luc reporter (Figure 2.2C). Co-transfection of E2F1 both abrogated 3XHRE-Luc activity in the presence of R1881 and downregulated AR protein expression (Figure 2.2C). These results demonstrate that E2F1 inhibits transcriptional regulation of AR target gene promoters by inhibiting AR expression.

Transcriptional repression of AR requires the transcriptional regulatory domain of E2F1. To determine if E2F1 exerts repressive activity directly on the AR promoter, we utilized a 1.5 kb (-1571 to +131 bp) mouse AR promoter construct (Figure 2.3A) cloned upstream of a luciferase reporter cassette (mAR-Luc). This plasmid was co-transfected into normal mouse prostate epithelial (mPrE) cells with either empty pcDNA3 vector (control) or wild type E2F1 along with a CMV promoter-driven β-galactosidase (B-gal) reporter plasmid as an internal control. Wild type E2F1 reduced mouse AR promoter activity 3.5 fold compared to cells transfected with empty pcDNA3 plasmid (Figure 2.3A). To assess AR promoter activity resulting from the direct disruption of E2F1 activity, we used a dominant negative E2F1 (DN E2F1) construct encoding a fusion cassette of the E2F1 DNA binding and the Rb pocket domain. This fusion binds to E2F consensus regions and blocks endogenous E2F1 activity at E2F1-responsive promoters (43), when employed in our system relieved repression of the AR promoter (Figure 2.3A). As a control, we demonstrated that E2F1 activates an E2F-inducible promoter containing 4 adjacent E2F consensus binding sites (E2F-Luc), while the DN E2F1

construct repressed promoter activity. We also demonstrated that E2F1 does not have an effect on an unrelated CRE-Luc promoter, which contains 4 adjacent cyclic AMP regulatory elements in front of a luciferase reporter construct (Figure 2.3A). We demonstrated that exogenous E2F1 repressed AR promoter activity. To assure that endogenous E2F1 carried out this repressive activity, we disrupted the inhibitory effect of endogenous Rb on E2F1 by co-transfection with SV-40 large T antigen (Tag) and assessed AR promoter activity. Ectopic expression of Tag led to 9 fold activation of the E2F-Luc promoter, but repressed mAR promoter activity nearly 4 fold (Figure 2.3A). These results confirm that E2F1, normally a transcriptional activator, participates in the repression of the AR promoter.

To elucidate the mechanism of E2F-mediated inhibition of AR, we examined the effects of two functionally debilitating E2F1 mutants on AR promoter activity. A mutation in the DNA binding domain (Eco 132) failed to significantly relieve E2F-mediated inhibition (Figure 2.3B). However, deletion of the transactivation domain of E2F1 ($E2F1_{1-284}$) abrogated the inhibitory effect of E2F1 on the AR promoter (Figure 2.3B). As expected, these E2F1 mutants did not activate the dihydrofolate reductase-luciferase reporter construct (DHFR-Luc), which is known to require both E2F1 transactivation and DNA binding domains (Figure 2.3B). These results indicate that the transactivation domain of E2F1 appears to be more essential than the DNA binding domain for E2F1 repression of the AR promoter. This observation prompted us to examine co-repressive factors that are involved in the E2F1 mediated repression of AR.

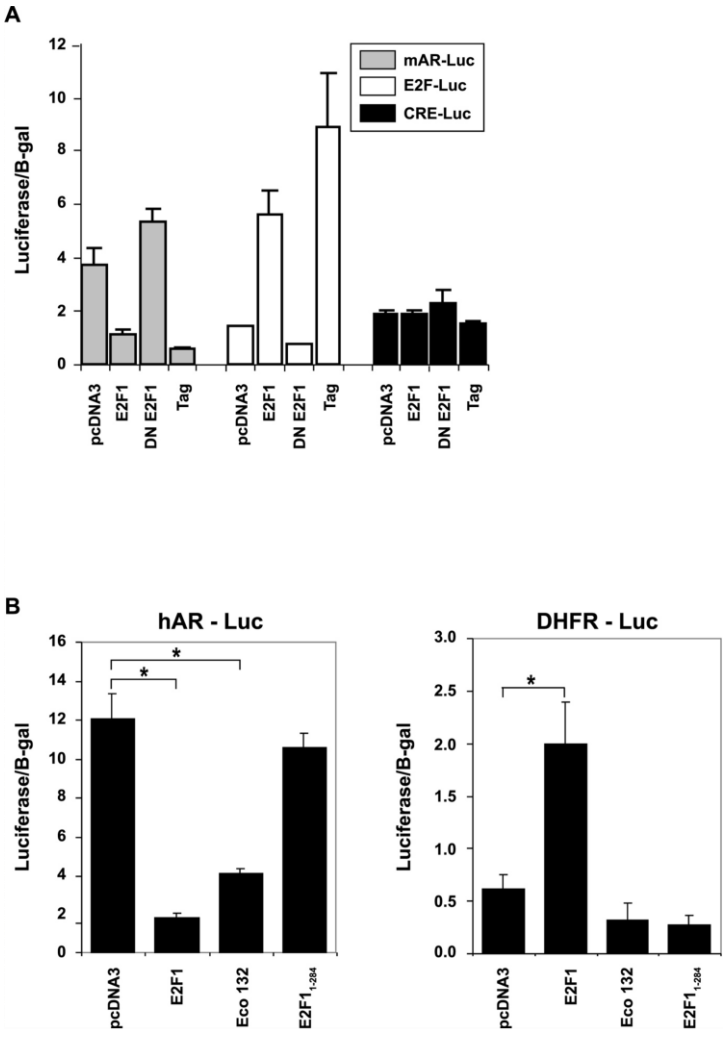


Figure 2.3 The E2F1 transactivation domain is required for AR promoter activity repression. (A) mPrE cells were co-transfected with 1 µg of mARp-Luc, E2F-Luc or CRE-Luc luciferase reporter constructs with 0.5 µg of empty vector (pcDNA3), wild type E2F1, dominant negative E2F1 (DN E2F1) or SV-40 Large T antigen (Tag). After 72 hours, cells were harvested and assayed for luciferase expression. The results are shown as averages of three independent experiments. Assays were done in triplicate and mean values are shown with standard deviation. The results were normalized to β-galactosidase (B-gal) expression from a co-transfected CMV promoter driven B-gal reporter construct. (B) LNCaP cells were co-transfected with either 1 µg of hAR-Luc or DHFR-Luc and 0.5 µg of either wild type E2F1or E2F11-284 mutant constructs. The histograms represent the mean value of three independent experiments with the indicated standard deviation. Results were normalized to β-galactosidase (B-gal) expression from a co-transfected CMV promoter driven by a B-gal reporter construct. * indicates $P < 0.05$ for the indicated comparison in brackets.

DNMT1 down regulation relieves AR repression in AR negative cells lines. We have previously shown that the DNA methyltransferase 1 (DNMT1) gene, which typically functions to maintain the methylation and repression of specific genes, was trans-activated by E2F1 (28). Interestingly, DNMT1 was shown to be part of an E2F1 containing complex that facilitated repression at E2F consensus sites (53). To determine if DNMT1 is involved in E2F1 dependent repression of AR transcription, we assessed whether AR expression is relieved in the AR negative defined human primary prostate epithelial cells (hPrEC) following DNMT1 shRNA knockdown. The hPrEC line is a model of transit/amplifying cells of the prostate gland and as such lacks markers of terminal differentiation such as AR expression (54, 55). These cells allow for the study of normal mechanisms that regulate AR expression. The hPrEC line was subjected to a transient transduction with either empty short hairpin RNA (shRNA), vector, non-targeting shRNA or DNMT1 targeting shRNA (4-1 and 4-2) (Figure 2.4A) and processed for both qRTPCR and western blot analysis. Compared to controls, the expression of the DNMT1 shRNA sequence resulted in a significant decrease in DNMT1 expression at both the transcriptional (data not shown) and protein level (Figure 2.4A). AR protein and transcription increased in response to decreases in DNMT1 expression, indicating that gene repression may also involve DNMT1. To assess the role of DNMT1 on AR promoter activity, we cloned a region of the AR gene containing a 2kb human AR promoter upstream of a luciferase reporter (hAR-Luc). Because primary hPrEC cells cannot withstand multiple passages required for stable shRNA transduction, we employed the immortalized human prostate epithelial line, BPH1, which still maintain a non-transformed phenotype (37). The hAR-Luc construct along with a CMV promoter-

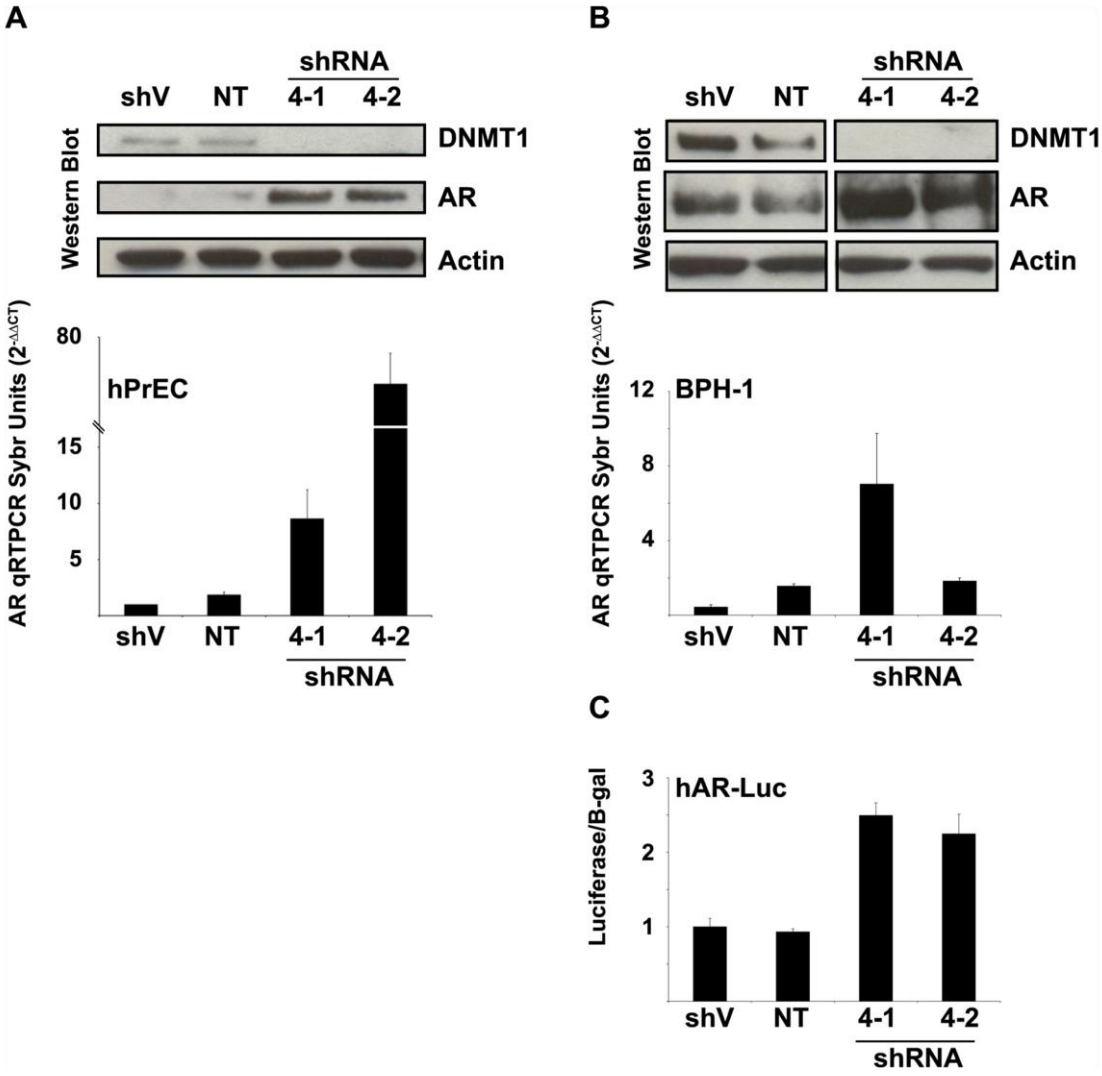


Figure 2.4 DNMT1 downregulation relieves AR Expression. (A) Primary cultures of human prostate epithelial cells (hPrEC) and (B) immortalized benign prostate epithelial cells (BPH-1) transduced with either control (shVector (shV) and shNon-targeting (NT)) or DNMT1 shRNA constructs. AR and DNMT1 protein expression relative to actin loading control were analyzed by Western blot. The exposures in (B) were taken from different sections of the same blot at the same intensity. AR transcription was analyzed using qRTPCR with readings done in triplicate (graphs A and B). Mean values are represented with standard error bars. (C) shRNA transduced BPH-1 cells (described in A and B) were transfected with the human AR promoter luciferase reporter (hAR-Luc) construct. The histograms represent the mean value of three independent experiments with the indicated standard deviation. Results were normalized to β-galactosidase (B-gal) expression from a co-transfected CMV promoter driven B-gal reporter construct. All western blots are representative of 3 separate experimental replicates.

driven β-galactosidase (B-gal) internal control reporter were co-transfected into BPH-1 cells that were previously transduced with DNMT1 shRNA targeting constructs (Figure 2.4B). DNMT1 shRNA relieved AR promoter activity (measured by increased luciferase activity) in BPH-1 cells when compared to controls (Figure 2.4C). These results suggest that DNMT1 contributes to the repression of AR promoter activity in normal prostate epithelial cells.

DNMT1 associates with the intronic and minimal promoter regions of the AR gene independent of methylation activity. To understand how DNMT1 functions to represses AR expression, we explored the possibility for DNMT1 to physically associate with the AR gene. Chromatin immunoprecipitation sequencing (ChIP-seq) analysis indentified both DNMT1 and E2F1 associated regions across the whole hPrEC genome (data not shown). H-peak analysis (56, 57) of the data revealed regions in the AR genomic structure exhibiting significant DNMT1 and E2F1 co-occupancy (Figure 2.5). Considering that DNMT1 has been reported to form complexes that bind to E2F responsive promoters, we designed primers flanking specific E2F consensus sequences (site A, B, and C) in the AR promoter. Site A and B were located within 1,000 bps of the transcription start site, while site C corresponded to a location in the ChIP-seq identified region of DNMT1 and E2F1 co-occupancy in the first intron (Figure 2.5A). Although the ChIP-seq demonstrated some E2F1 associations with the AR gene, we focused our ChIP analysis on DNMT1 interactions, considering that the region under analysis presented with weak E2F consensus sites and that the E2F1 DNA binding domain was not necessary for AR promoter repression (Figure 2.3B). Primers were used to analyze a

known binding target of DNMT1 located in the PS2 promoter (58) and a non-related DNA sequence located in exon 2 of the ABCB1 gene (59). Rabbit IgG was used to control for any non-specific DNA binding incurred by the antibodies. Quantitative PCR indicates that DNMT1 strongly associates with intronic region showing a $\geq$ 4-fold over enrichment at the ABCB1 genomic region. DNMT1 demonstrated some association with sites A and B in the AR gene, showing slightly increased levels of enrichment over ABCB1, that were similar to amplification levels at the PS2 promoter (Figure 2.5B). DNMT1, therefore, associates with the 5' UTR and a region in the first intron of the AR gene that has a possible affinity for E2F1.

DNMT1 is traditionally thought to facilitate the repression of target genes through a catalytic process that involves the transfer of methyl groups to cytosines located in CG dinucleotides present in the DNA sequence. Aberrant hyper-methylation of the AR promoter has been detected in the AR negative metastatic prostate cancer cell lines DU-145 and TSU-PR1 (60). ChIP analysis demonstrated that DNMT1 associated with a section of DNA spanning a region (+44 to +54) of heavy methylation conserved between DU145s and other transformed AR lacking cell lines (61). To determine whether methylation of the AR minimal promoter associated region (Figure 2.5A) is dependent on DNMT1, we sequenced a section (+22 - +293) of bisulfite converted DNA extracted from DU145s and hPrECs infected with DNMT1 shRNA. The methylation pattern remained unchanged in the absence of DNMT1 when compared to the cells infected with the non-targeting shRNA construct (NT) in DU145s, while subtle increases were observed in a single hPrEC DNMT1 knockdown cell line (Figure 2.5C). According to this data, methylation at the AR minimal promoter does not appear to rely on DNMT1.

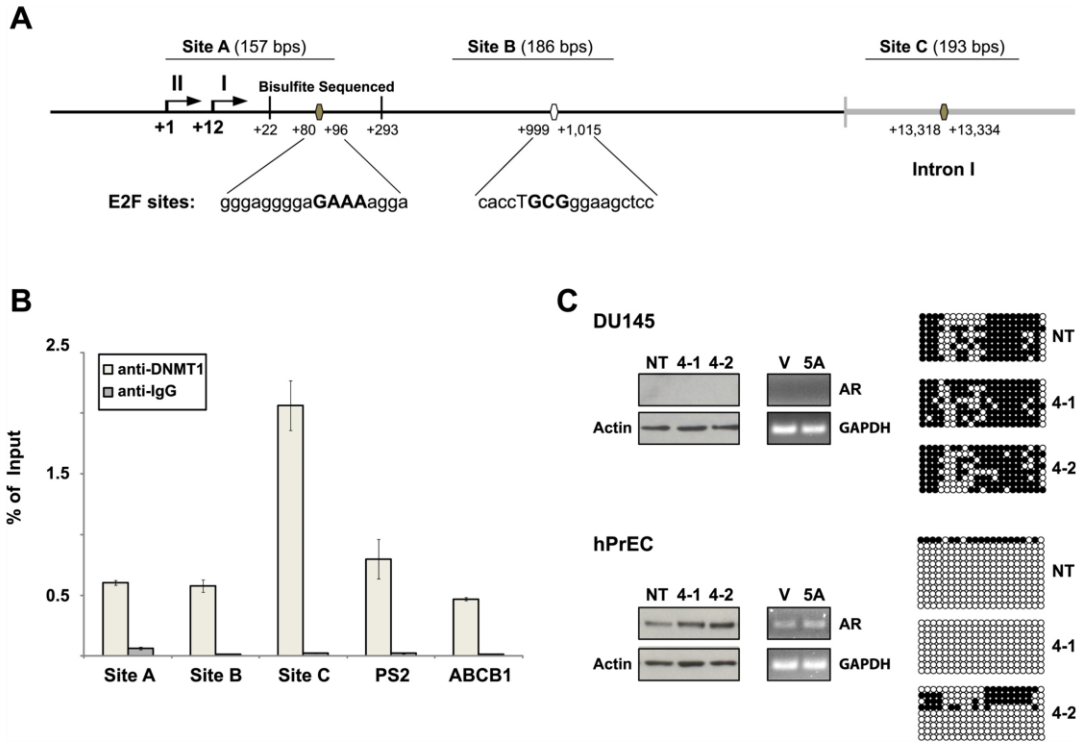


Figure 2.5 Methylation independent association of DNMT1 with the AR gene. (A) Map of a region of the AR promoter depicting two types of E2F consensus sequences +80 → +96 and +13,318 → +13,334 (shown as solid ovals) and +999 → +1,015 (shown as open ovals). Primer flanked regions are designated as sites A, B, and C. The 243 bp region (+22 → +293) analyzed by bisulfite sequencing is indicated. (B) qPCR analysis of target (DNMT1) and non-specific (IgG) ChIPed DNA from BPH-1 cells using primers that flank sites A, B, and C. Primers flanking a region in the PS2 (targeted DNMT1) promoter and ABCB1 (non-targeted DNMT1) region were used as ChIP controls. Data is representative of the mean from 3 qPCR reactions and shown as a percent of input with the standard error indicated. (C) Bisulfite sequencing analysis of the 243 bp region in the AR minimal promoter in hPrEC compared to DU145 cells infected with both non-targeting (NT) (control) and DNMT1 shRNA constructs (4-1 and 4-2). Solid circles (methylated) and open circles (un-methylated) were used to represent the methylation status of cytosines within CpG dinucleotides. Each horizontal strand of circles depicts a separate DNA clone. Lysates were probed on a western blot for AR and actin. Cell lines were also treated with either 1μM 5Aza (5A) or DMSO matched vehicle (V) and extracted cDNA was PCR amplified with both human AR and GAPDH. All data shown except for the 5Aza treatments are representative of 3 separate experimental replicates.

We additionally demonstrated that AR expression resulting from the downregulation of DNMT1 occurred regardless of methylation at the bisulfite sequenced region (+22 - +293) in hPrECs. To further asses the possibility for a methylation independent process we treated both DU145 and hPrEC lines with a global DNA methylation inhibitor, 5-aza-2'-deoxycytidine (5-Aza) and observed no change in AR transcription (Figure 2.5C). These data point to a possible mechanism of AR repression in normal prostate epithelial cell lines that utilizes methylation - independent DNMT1 activity.

DISCUSSION

In this study we have shown that E2F1 drives the expression of DNMT1 and may cooperate with DNMT1 to repress AR transcription in normal undifferentiated prostate epithelium. Specifically, exogenous E2F1 down-regulated AR promoter activity (decreases in luciferase activity) and mRNA and protein expression, while a dominant negative E2F1 construct (DN-E2F1) relieved AR promoter repression by inhibiting access of endogenous E2F1 to E2F targeted promoters. All of these observations correlated with variations in activation of the E2F-target gene promoter DHFR-Luc, and changes in expression levels of endogenous cell cycle regulatory proteins consistent with E2F1 activity. The use of functionally debilitating E2F1 mutants suggests that the transcriptional regulatory domain of E2F1 comprises this repressive activity possibly through the interaction of an intermediary co-repressor. Based on studies showing that DNMT1 is both a target of E2F1 and that it may co-repress some targets with E2F1 (28, 53), we decided to evaluate the role of DNMT1 in AR repression. Targeted knockdown of DNMT1 relieved AR promoter activity, mRNA and protein expression. Additionally,

DNMT1 directed ChIP showed association of DNMT1 with the AR promoter. The lack of *de novo* methylation at the minimal AR promoter following loss of DNMT1 suggests DNMT1 represses AR expression in a methylation independent manner.

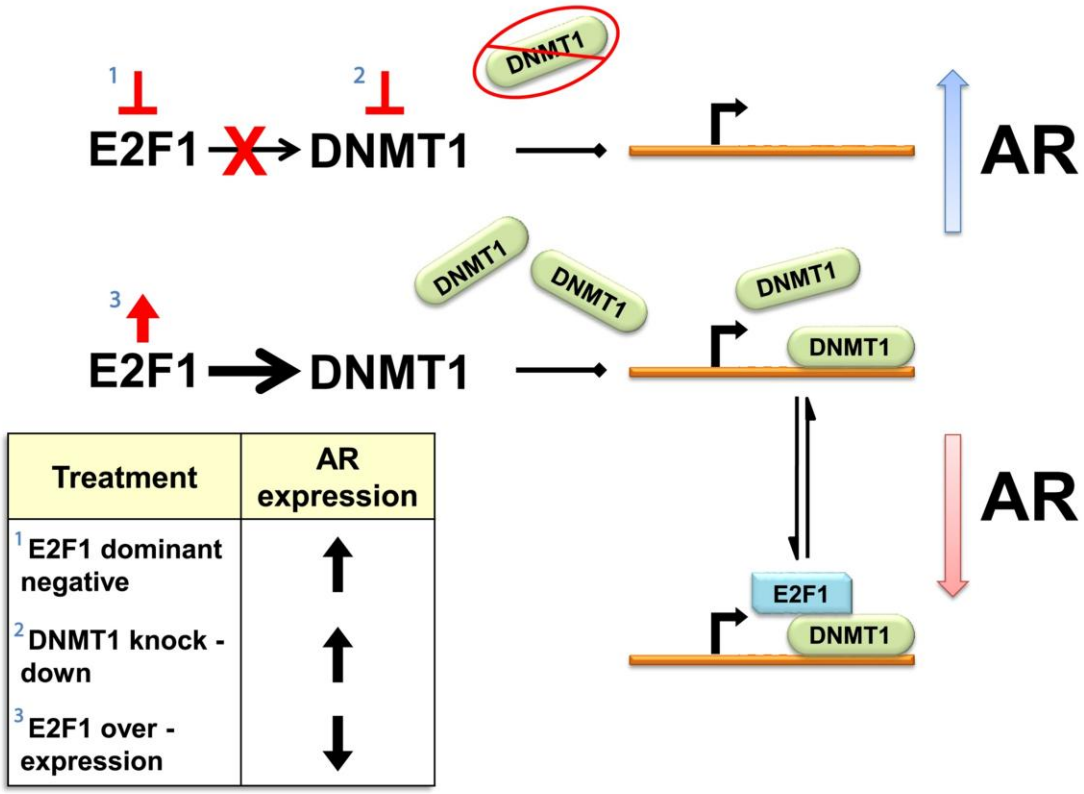
Sharma et al. have recently demonstrated that E2F1 transactivates the AR gene on a depleted RB1 background in an engineered model of castrate resistant prostate cancer (62). This observation is interesting in light of our findings in non-transformed prostate epithelium in which E2F1 represses AR transcription in the presence of functional pocket proteins. Our findings that the Large T antigen facilitates E2F1-mediated repression of AR, suggests that E2F1 has roles in both the activation and repression of AR transcription. While Sharma et al. provide evidence for a mechanism of AR activation that involves the association of E2F1 with specific regions of the AR promoter, our results did not find association of E2F1 with these reported sites, but revealed regions containing weak E2F1 consensus binding sites downstream of the AR transcription start site that demonstrated DNMT1 association instead. Sharma et al demonstrated a lack of E2F1 binding at the only site analyzed by both our labs. While E2F1 failed to promote at AR activation at this location, we demonstrated moderate DNMT1 binding associated with AR inhibition. These seemingly contradictory findings might begin to explain how E2F1 functions in a more traditional role to activate AR in the absence of RB in prostate cancer cells, yet represses AR transcription in normal (non-transformed and non-immortalized) prostate epithelium in the context of functional RB.

Although E2F1 is thought to primarily function as a positive regulator of transcription, a negative regulatory role of E2F1 has also been described for a number of genes (29). Unlike its positive regulatory function, which is mediated by the direct

interaction of E2F1 with DNA, the mechanism(s) for E2F1-mediated negative regulation are still largely unknown. Crowe et. al. identified two putative E2F binding sites in the hTERT promoter that were important for E2F1- mediated repression (31). The E2F1 mutant (E132) which lacks the DNA binding domain of E2F1 was inefficient at repressing hTERT promoter activity, suggesting that the DNA binding domain was essential for repression. In another study, direct repression of the Mcl-1 promoter by E2F1 also required the DNA binding domain, but not the transactivation domain (30). Koziczak et. al. demonstrated that both the DNA and transactivation binding domains of E2F1 were necessary for the negative regulation of uPA and the PA inhibitor (PAI-1) genes (29) however, E2F1 repressed promoter activity independently of the pocket protein Rb. We have shown here that E2F1-mediated repression independent of Rb pocket protein family members, suggesting that multiple mechanism(s) exist for E2F1-mediated repression. We noted that the AR promoter does not contain a strong E2F1 consensus binding site (TTTGCGG/CG/CAAA), furthermore the E2F1 DNA binding domain was not required for repression of the AR promoter suggesting that E2F1 does not bind directly to the AR promoter, but cooperates with other regulatory proteins to repress AR. Our data demonstrate that the carboxy-terminal transactivation domain was essential for E2F1 suppression of the AR promoter (Figure 2.3) and therefore supports two possible models of AR repression. Several proteins are known to bind to this region and regulate transcription including CREB-binding protein (63), MDM2 (64) and TRRAP/Tip60 complex (65). E2F1 may regulate AR promoter activity by forming a known repressive complex that includes Rb, HDAC1, and DNMT1 through an as of yet undefined domain. The transactivation domain also interacts with the basal transcription

factor IIH (TFIIH) (66) which may facilitate the E2F1 contacts necessary to induce transcription at the DNMT1 promoter, increasing DNMT1/AR gene interactions that lead to repression (Figure 2.6). Our findings, along with previous work in the lab, support a linear model of AR repression that is reliant on the positive transcription of DNMT1 by E2F1. However, the possibility remains for E2F1 to regulate AR expression through a complex involving DNMT1 (Figure 2.6).

We have shown through targeted knockdown of DNMT1 and ChIP analysis that the association of DNMT1 with weak E2F consensus sites in the AR gene inhibits transcription. A previous study has shown that estrogen receptor (ER) re-expression at both the transcriptional and protein level, results from the targeted inhibition of DNMT1 in ER negative breast cells (67). DNMT1 is traditionally thought to cause genetic repression through methylation, however, the current understanding of methylation facilitated repression continues to evolve in the field of epigenetics. Glypican 3 (GCP3), a developmentaly associated gene, is regulated by a promoter methylation independent mechanism in human fetal systems (68). Methylation sensitive restriction digests show that methylation at the GPC3 promoter is sex specific and occurs regardless of GPC3 expression status in females, but remains absent in males. Yakabe et. al. demonstrated through ChIP analysis that methyl CpG binding protein 2 (MeCP2), which is usually dependent on DNA methylation for genetic interaction, was able to both associate with unmethylated promoter sequences and regulate the expression of a subset of selected genes (69). Furthermore DNMT1 was reported to repress p21 and BIK in a methylation independent manner (70). Epigenetic regulation possibly involves the intercommunication of multiple epigenetic marks to orchestrate the regulation of genetic



Treatment	AR expression
[1] E2F1 dominant negative	↑
[2] DNMT1 knock - down	↑
[3] E2F1 over - expression	↓

Figure 2.6. Schematic representation of AR repression through the E2F1/DNMT1 axis. The use of either a dominant negative E2F1 construct or a shRNA to knock down DNMT1 both result in the downregulation of DNMT1 and subsequent rescue of AR expression. E2F1 overexpression experiments suggest that elevated E2F1 levels increase DNMT1 protein expression that associates directly with the AR promoter or possibly in complex with E2F1 to repress AR.

expression. The simultaneous employment of ChIP and methyl specific primer (MSP) analysis, verified the presence of transcription promoting histone modifications (acetyl-H3K9 and dimethyl-H3K4), associated with unmethylated regions responsible for facilitating hTERT expression from a heavily methylated promoter in cancer cells (71). The role of methylation independent DNMT1 regulation at the AR promoter may involve other epigenetic modifications.

DNMT1 appears to function in concert with other factors to regulate gene expression. Heterochromatin protein 1 (HP1) GST fusions pull down DNMT1, 3a, and 3b (72). Smallwood et. al. and colleagues further demonstrated that methylation by DNMT1 in complex with the HP1 proteins was dependent on G9a H3K9me2 using *in vitro* chromatin array methylation assays. DNMT1 is also known to directly interact with the enhancer of zeste 2 (EZH2) protein, which mediates H3K27me2/3, in the context of the Polycomb repressive complexes (PRC2/3) (73). Furthermore, DNMT1 was shown to associate with histone deacetylase activity during direct interaction with HDAC1 (74) and in complex with HDAC2 and corepressor DMAP1 (75). DNMT1 is likely to facilitate repression at the AR promoter through the interaction with many multi-subunit complexes.

Clearly, mechanisms for AR amplification and mutation play a role in prostate cancer progression, however, loss of AR has been reported in a subset of hormone-independent cancers, including a complete loss in some cases (76, 77). Highly proliferative cells that present with an AR negative phenotype are actually necessary for normal prostate development. Prostate stem cells differentiate into an AR negative transit-amplifying (TA) population that is known to transiently undergo multiple rounds

of cellular division before terminally differentiating into AR positive luminal epithelium (55). Certain prostate cancers may present with mutations that allow for unhindered TA cell proliferation as mentioned in a review by Paul C. Marker (78). Our findings demonstrate that exogenous E2F1 inhibited activation of the AR responsive promoter construct (3XHRE-Luc) in a minimally invasive cell line, and that the repression is possibly mediated by methylation independent DNMT1 activity at the AR promoter in TA cells. The long term effects of E2F1 overexpression in prostate epithelium is still not fully understood, however, we propose that the inhibition of AR expression by the E2F1/DNMT1 axis may be required for normal growth and development of the prostate gland.

CONTRIBUTIONS

I was responsible for producing a majority of the data presented in this chapter. Dr. Joanne Davis independently ran the northern blot assay and derived cell lines from tissue taken from wild type and K5E2F1 transgenic mice. Joanne additionally contributed greatly to the data derived from the mouse PrEC and LNCaP cell lines. Dr. Kirk Wojno provided the morphological descriptions for the wild type and transgenic mice tissues. Hana Odeh provided the ChIP-seq data that directed analysis of DNMT1 association within the first AR intron. Both Tristan Layfield and Craig Cousineau helped with the DNMT1 knockdown studies and bisulfite sequencing assay.

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CHAPTER III

THE E2F1/DNMT1 AXIS DRIVES AR NEGATIVE CASTRATION RESISTANT PROSTATE CANCER

ABSTRACT

The molecular events underlying the clinical recurrence of castration resistant prostate cancer (CRPC) remain vague. CRPC develops through mechanisms that bypass the cellular requirement for androgen, by either circumventing normal androgen receptor (AR) function or by reducing AR gene expression. Although AR function has been extensively studied in CRPC, less is known concerning the deregulation of AR gene transcription in this disease. We have previously demonstrated that the repression of AR gene transcription is mediated by E2F1-dependent induction of the DNA methyltransferase 1 gene (DNMT1). In the current study, we present the first comprehensive evaluation of DNMT1 protein expression during the progression of human prostate cancer. Immunohistochemical analysis revealed increased nuclear DNMT1 staining in localized prostate cancer ($p<0.0001$) and metastatic prostate cancer ($p<0.0001$) compared to normal tissue. Examination of specific diagnoses revealed that Gleason 7 tumors exhibited greater nuclear DNMT1 staining than Gleason 6 tumors ($p<0.05$) and that metastatic tissue exhibited higher DNMT1 levels than Gleason 7 tumors ($p<0.01$). Because CRPC is not uniquely represented in this tissue microarray

(TMA), we employed a murine model of prostate cancer to specifically evaluate levels of E2F1, DNMT1 and AR in experimentally induced CRPC. Only 20% and 30% of benign tissues stained for E2F1 and DNMT1 respectively, while 70% of these samples were positive for AR. Conversely, most cancers stained positive for E2F1 (81%) and DNMT1 (100%) compared to 18% of AR positive cores. Treating CRPC mice with the DNA methytransferase inhibitor 5'-Aza-2'-deoxycytidine (5Aza) reduced DNMT1 staining by 30%, while AR positive cores increased by 27%. These results demonstrated that an inverse correlation exists between DNMT1 and AR during progression of human prostate cancer that was also observed in experimentally-induced murine CRPC. Taken together these results indicate a functional role for DNMT1-depedent repression of AR expression during the emergence of CRPC.

INTRODUCTION

Castration resistant prostate cancer (CRPC) continues to be associated with significant morbidity and is the major cause of prostate cancer related deaths. Research on this disease has focused primarily on the function of androgen receptor (AR) during progression to CRPC, but few have considered the loss of AR gene expression itself (1, 2) and the possible contribution of AR negative cells to CRPC. A significant decrease in AR expression coincident with the onset of CRPC has been demonstrated in human prostate tumors recurring after combined androgen blockade (3). Immunohistochemical (IHC) analysis revealed a significant decrease in AR expression in malignant prostate tissue procured from patients who failed combination hormone therapy compared to untreated individuals (4). More recently, we reported an evaluation of metastatic lesions

from CRPC patients that revealed a significant reduction in AR staining when compared to hormone naïve cases (5). However, others have reported a general increase in AR expression in castration resistant tissues compared to hormone naïve samples, but continue to find CRPC tissue samples with a complete loss of AR expression (3, 6). Thus, the heterogeneous or complete reduction of AR expression appears to be a relevant incident during the development of CPRC. We have recently discovered a novel mechanism by which AR transcription is repressed through E2F1-mediated induction of the DNA methyltransferase 1 (DNMT1) gene. Based on these functional studies, we postulated that this mechanism may play a role in AR repression in a subset of CRPC patients.

E2F1 is a member of the E2F transcription factor family that plays a traditional role in driving S-phase transit of the cell cycle (7) and that functions mainly in proliferative cell states. A genome wide microarray study evaluating gene regulatory signatures associated with cancer verified that more than half of the programs analyzed were E2F target genes (8), supporting the possibility for E2F1 mediated oncogenic transitions. Both invasive ductal breast (9) and non-small cell lung (10) carcinomas were shown to have high levels of E2F1 expression associated with poor prognosis. Several studies have provided evidence supporting a relationship between E2F1 and prostate cancer progression. Our group reported findings from tissue microarray (TMA) evaluations of metastatic CRPC that revealed significant increases in E2F1 staining when compared to hormone naïve cases and that was inversely correlated with reductions in AR staining in the same patients (5). Zheng et. al. demonstrated that both DU145 and PC-3 prostate cancer cell lines rely on E2F1 expression to confer anti-apoptotic

characteristics in the presence of various chemotherapies (11). An expression microarray analysis measured high levels of E2F1 cDNA in a set of advanced prostate cancer tumors and lymph node metastases (12). The same study also noted that a majority of the cellular nuclei within tumors associated with biochemical recurrence exhibited strong E2F1 staining. The mechanism by which heightened E2F1 promotes the malignant progression of human prostate cancer is still unknown. It has been suggested that the hyper-proliferative state of malignant prostate cells is a consequence of unregulated S-phase induced by elevated E2F1. We postulate that abnormal E2F1 levels also induce the transcription of DNMT1 that leads to AR gene repression and facilitation of tumor progression.

DNA methyltransferase 1 (DNMT1) is a member of the DNA methyltransferase family that is responsible for maintaining methylation patterns located in CG dinucleotide-rich regions within the genomic DNA. Methylation of DNA contributes to genetic silencing by cooperating with other epigenetic mechanisms to either block transcriptional complexes or induce conformational changes in the DNA structure that inhibit transcription (as reviewed in (13)). Aberrant DNMT1 expression and function are associated with the progression of various human tumors. High DNMT1 expression levels were observed during the progression of colon (14), bladder (15), gastric (16), and pancreatic cancers (17). Little is known about the relationship between DNMT1 and prostate cancer progression; however, recent studies demonstrated increased expression in advanced disease. Chen et. al. observed increased DNMT1 levels as prostate cancer cell lines transitioned to CRPC (18). A genome wide cDNA microarray study conducted by Tomlins et. al. revealed substantial DNMT1 increases in CRPC compared to primary

prostate cancer (19). Another study reported that prostate tumors exhibited elevated DNMT1 immunoreactivity and protein content compared to benign prostate tissue (20). The TRAMP mouse model of prostate cancer demonstrated increased DNMT1 expression during progression to poorly differentiated and metastatic (21, 22). In addition, we have shown that 5-Aza treatment completely prevents the histopathological progression and its associated lethality in TRAMP mice (22). Other efforts to uncover common abnormalities amongst heterogeneous prostate cancer cases have identified a variety of consistently hyper-methylated genes that may represent the catalytic signature of DNMT1 in prostate cancer progression (23, 24). Collectively, these observations support a functional role for DNMT1 during prostate cancer progression that may include the repression of the AR gene in the development of CRPC.

This study will demonstrate a correlative relationship between E2F1, DNMT1, and AR during human prostate cancer progression to treatment-resistant and recurrent disease. We will also demonstrate the presence of this axis in experimentally induced CRPC using the TRAMP prostate cancer model that when considering our findings human samples supports a functional role for this axis during the emergence of AR-negative CRPC.

MATERIALS AND METHODS

Analysis of human prostate cancer progression tissue microarray. Tissue microarrays (TMA) containing cores procured from both human and mouse tissues during prostate cancer progression were immune-stained to detect the expression of E2F1 DNMT1 and

AR. The human TMA consists of 90 patients represented by 270 cores containing normal, bening prostatic hyperplasia (BPH), BPH stromal nodules, prostate intraepithelial neoplasia (PIN), prostatic atrophy, proliferative inflammatory atrophy (PIA), and both localized and metastatic prostate cancer tissues. Benign tissues were acquired by cystectomy, prostatectomy, and transurethral re-sectioning from 30 patients. Pre-cancerous (PIN and Hyperplasia) and localized disease samples were obtained from radical prostatectomy specimens from patients who had their prostate removed for prostate cancer. Metastatic sections from distant tissue sites were acquired through rapid autopsy from 10 patients who were non-responsive to cancer therapies.

Analysis of TRAMP tissue microarray. The 333 cores present in the mouse TMA were procured from a total of 111 wildtype and TRAMP mice that were either castrated, sham operated, or treated with 5' –Aza-2'-deoxycytidine (5Aza) during a cancer survival study (25). Treatments and operations were done either independently or in combination, while mice receiving mock operations or PBS treatments served as controls. Mice lived the longest after castration followed with 5Aza treatments and showed significantly higher survival rates compared to mice receiving castration alone. All mice tissues evaluated in the present study, however, were taken from mice sacrificed at 24 weeks of age.

RESULTS

Immunohistochemistry and quantitative analysis of DNMT1 in human tissues. We have previously reported the inverse correlation between E2F1 and AR during prostate cancer progression to castration resistant disease (5). Further studies by our group demonstrated that existence of an AR regulatory mechanism controlled by E2F1-dependent activation of the DNMT1 gene that acted as a functional intermediary during AR repression. To evaluate any patho-mechanical roles of DNMT1 in human prostate cancer and the emergence of CRPC, we evaluated a human TMA comprised of various stages or localized prostate cancer as well samples of metastatic disease. The mean nuclear intensity (MNI) scores for DNMT1 staining were determined in of 27 cores from normal adjacent prostate tissue, 59 Gleason scored cores, and 19 metastatic cores from distal tissue sites. DNMT1 nuclear staining significantly increased from normal to metastatic prostate cancer (Figure 3.1A). Metastatic cores with a Mean Nuclear Intensity (MNI) score of 1.95 had a significantly ($p<0.0001$) higher nuclear staining intensity than localized tissue cores with an MNI of 1.05. Normal tissue cores showed the least nuclear staining (MNI=0.19) and was significantly ($p<0.0001$) lower than levels observed in localized prostate cancer tissues (Figure 3.1B).

A detailed look at the progression from normal to metastatic prostate cancer revealed significant nuclear intensity changes amongst specific diagnoses. Tissues with a Gleason score of 7 (G7) showed significantly ($p<0.05$) greater nuclear DNMT1 staining (NMI=1.26) than G6 graded cores (MNI=0.5) (Figure 3.2A and 3.2B). Metastatic cancer samples (MI=1.95) with the highest nuclear DNMT1 levels were significantly ($p<0.01$)

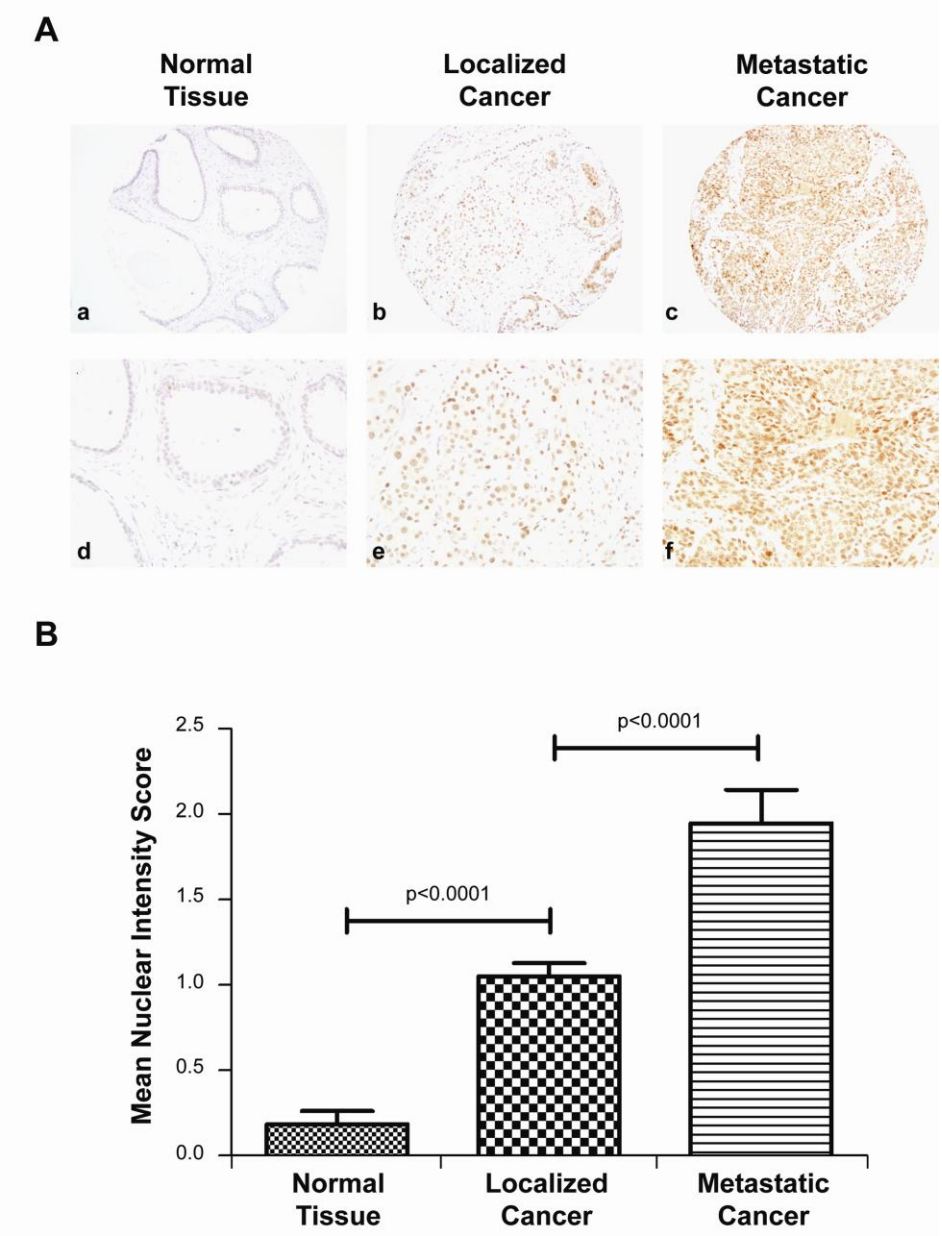

Figure 3.1 Nuclear DNMT1 expression during prostate cancer progression. A) Histological representation of human tissue cores recovered from the rapid autopsy program. Normal, localized, and metastatic prostate cancer representations magnified respectively both at 20X (a-c) and 40X (d-f). B) Mean nuclear intensity scores are presented in a bar graph for each diagnosis normal tissue (n=27 cores), localized cancer (n=59 cores), and metastatic cancer (n=19 cores). Tukey's multiple comparison test demonstrates statistical significance (p-value < 0.001).

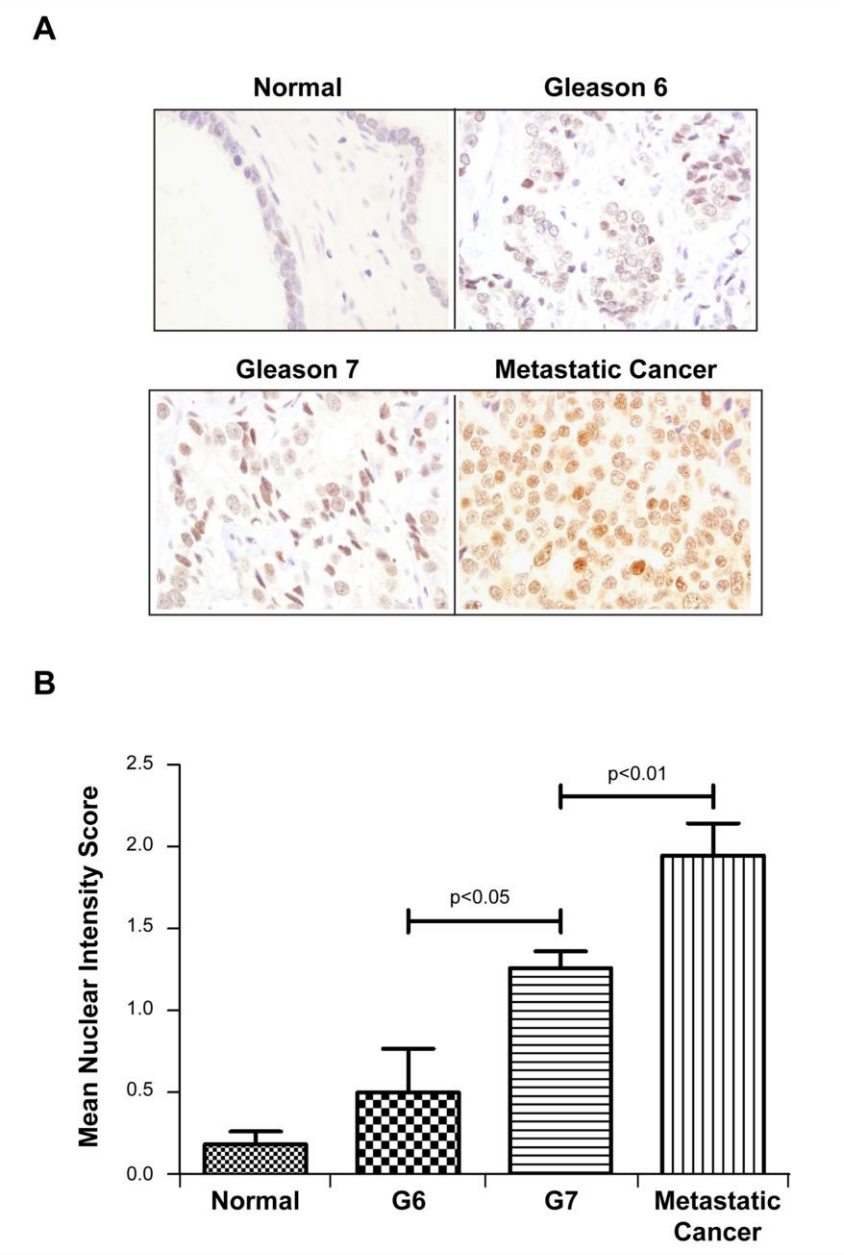

Figure 3.2 Nuclear DNMT1 expression according to Gleason score. A) Nucler DNMT1 staining across normal (n=27 cores), Gleason 6 (G6) (n=8 cores) and G7 (n=27 cores) localized prostate cancer, and metastatic categories (n=19 cores). B) The mean nuclear intensity score are shown via bar graph. Tukey's multiple comparison test demonstrates statistical significance (p-value<0.05, p-value < 0.01, and p-value < 0.0001).

higher than G7 staged localized prostate cancer (Figure 3.2B). These data demonstrated that localized prostate cancer samples show variations in DNMT1 immunostaining that are dependent on tissue grade. Nuclear DNMT1 staining was strongest in metastatic tissues procured from patients that were non-responsive to cancer therapies. The evidence supported a possible role for DNMT1 during CRPC as an intermediary involved in AR repression and prompted further analysis.

Analysis of AR Regulation through the E2F1/DNMT1 Axis in the TRAMP Mouse. We used the Large T antigen (Tag) mouse model of prostate cancer (TRAMP) for analysis of E2F1, DNMT1 and AR in tumorigenesis. SV-40 Tag is driven by the rodent probasin promoter that preferentially binds to hypophosphorylated Rb, thereby releasing E2F family members and disrupting G1 cell cycle checkpoints. We hypothesized that increased E2F1 activity in the TRAMP mouse, results in the modulation of downstream E2F1 target genes such as PCNA and the E2F-mediated repression of AR. To test this hypothesis, we examined prostate tissue from C57Bl/6 and TRAMP mice at 24 weeks of age with poorly differentiated prostate cancer. Detailed assessment of proliferation, E2F activation, and expression of either: E2F1, DNMT1, or AR was performed on paraffin-embedded tissue sections. Tag was present in prostate tissue of all transgenic animals with poorly differentiated prostate cancer, but absent in tissues from C57Bl/6 mice (Figure 3.3A (a,f)). Levels of E2F1 were also comparably higher during poorly differentiated cancer (Figure 3.3A (c,h)). E2F1 activity and cell proliferation were assessed by staining for proliferating cell nuclear antigen (PCNA). Epithelial cells in normal prostate tissues were predominantly negative for PCNA staining (Figure 3.3A (b)),

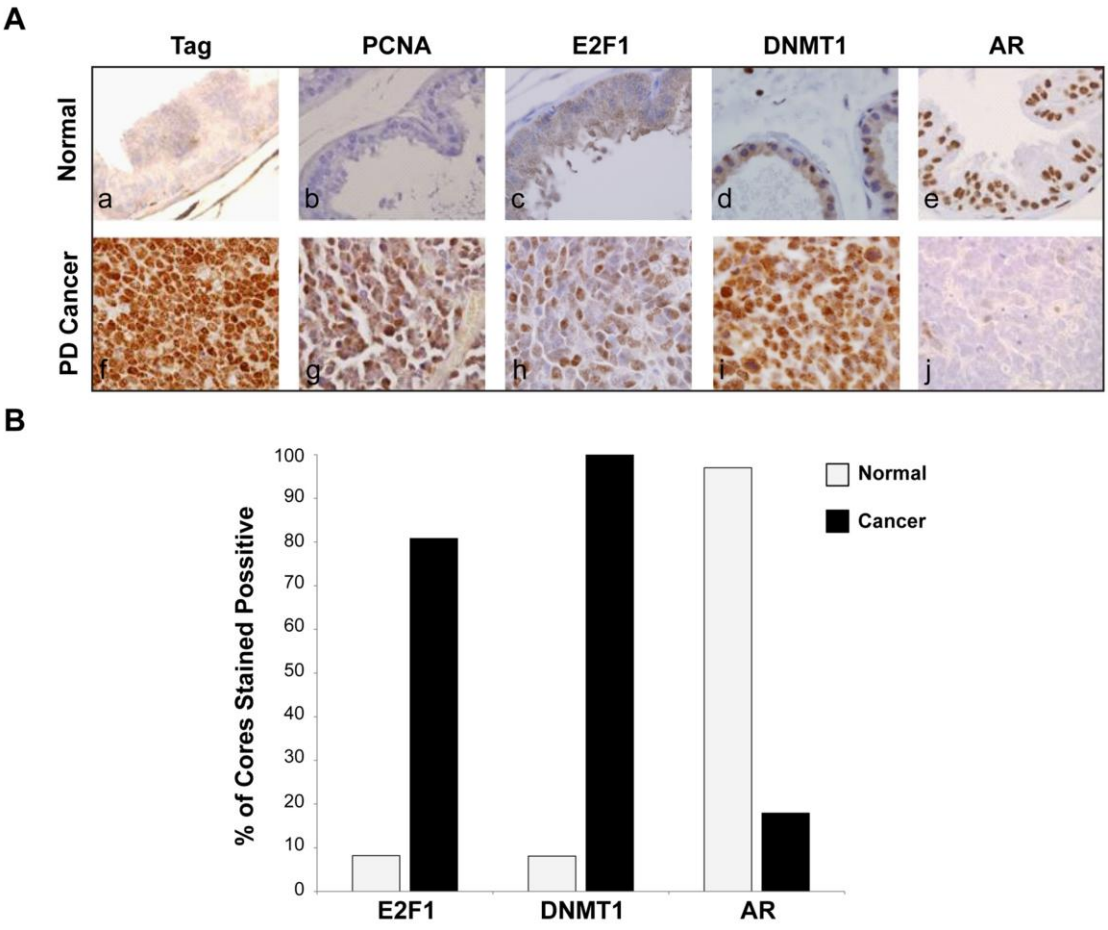

Figure 3.3 E2F1, DNMT1, and AR expression during tumorigenesis in the TRAMP model. A) Immunohistochemical staining of normal prostate tissue from C57Blk/6 control mice (panels a-e) and prostate cancer from a 24 week old TRAMP mouse (panels f-j) at 100X magnification. Sections were stained for Large Tag (panels a, f), PCNA (panels b, g), E2F1(c,h), DNMT1(d,i), and AR (panels e,j) B)The number of tissue cores staining positive for either E2F1 (n=49 normal and n=73 cancer), DNMT1 (n=62 normal and n=80 cancer), or AR (n=67 normal and n=67 cancer) are shown as a percent of the total available within each diagnosis (normal or cancer) group.

indicating both low E2F1 activity and a low proliferative index. However increases in PCNA staining (Figure 3.3A (g)) in addition to Cyclins A and E protein (data not shown) were observed in TRAMP mice with poorly differentiated tumors compared to C57Blk/6 mice at 24 weeks. This increase in both cell proliferation and E2F1 activity was associated with a concomitant increase in nuclear DNMT1 (Figure 3.3A (d,i)) expression with subsequent decreases in AR protein (Figure 3.3A (e,j)). Conversely, normal tissues showed strong AR nuclear staining. Together, these results demonstrate that increased E2F1 expression correlates with increased cell proliferation and expression of E2F-target genes (PCNA and DNMT1) with a concomitant down-regulation of AR in prostate epithelial cells during prostate cancer progression in the TRAMP model.

We constructed a TMA from tissues procured from a previous treatment study of TRAMP mice (25) (Figure 3.4). This TMA was used to evaluate normal and tumor diagnosed tissue by immunostaining of E2F1, DNMT1 and AR. Only 20 and 30% of all benign tissue cores stained positive for E2F1 and DNMT1 respectively, while 70% of the cores stained positive for AR. Conversely, a large proportion of the tissues diagnosed with cancer stained positive for E2F1 (80.8%) and DNMT1 (100%) compared to 17.9% of the cores that stained positive for AR (Figure 3.3B). The high levels of E2F1 and DNMT1 present during low AR expression continue to support a mechanism for the regulation of AR by the E2F1/DNMT1 axis *in vivo*.

Analysis of E2F1, DNMT1, and AR in CRPC of TRAMP mice. To assess AR repression in association with elevated expression of E2F1 and DNMT1 during CRPC in vivo, we measured expressional changes in tissue cores procured from a murine model of

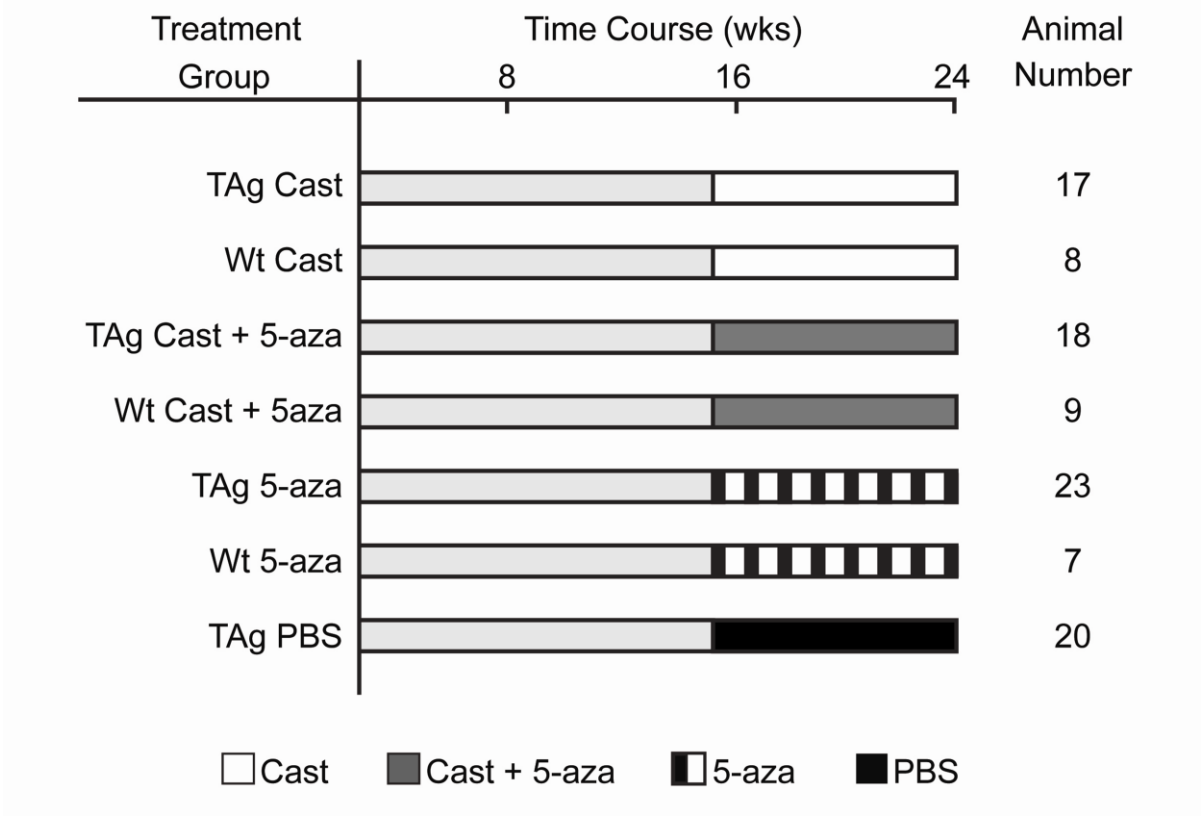

Figure 3.4 TMA treatment schedule. Tissue procured from treated wild type and TRAMP mice were used to construct the mouse TMA. Treatment with either vehicle (PBS), 5-aza, or castration were initiated at 15 weeks of age. Drug injections were administered 3 times a week for 10 weeks. An adaptation of a figure created by Zorn et. al. *Clin Cancer Res*, 2007.

CRPC (25) (Figure 3.4). CRPC was recapitulated in the TRAMP model by castrating mice with fully developed tumors at 15 weeks of age (25, 26). TRAMP mice undergoing castration were additionally treated with 5Aza, a DNA methylation inhibitor shown to enhance overall survival and decrease cancer pathology (25). We observed a 30% decrease in tissues stained with DNMT1 from castrated mice treated with 5Aza compared to those receiving PBS (Figure 3.5B). The percent of AR positive cores, however, increased by 27% after 5Aza treatments (Figure 3.5B). The number of tissues immunostained with E2F1 showed no significant change in cores taken from castrated mice treated with 5Aza (Figure 3.5B). Representative tissue from castrated mice that maintained normal glandular morphology following 5Aza treatment show intense nuclear AR staining compared to decreases in DNMT1 (Figure 3.5A). These data demonstrate that AR positivity increases in both the absence of methylation and in the presence of diminished DNMT1 levels in vivo.

DISCUSSION

AR repression through the E2F1/DNMT1 axis may be a mechanism by which cells maintain an undifferentiated yet aberrantly proliferative state during progression to CRPC. We demonstrated that nuclear DNMT1 like E2F1 increases during human prostate cancer progression to metastases. These expressional changes along with decreases in AR expression are recapitulated in TRAMP mice with poorly differentiated prostate cancer. We obtained a better understanding of the molecular changes occurring during CRPC after treating castrated TRAMP mice with 5Aza. The increase in epithelial AR levels following the inhibition of DNA methylation, suggests that AR repression may involve

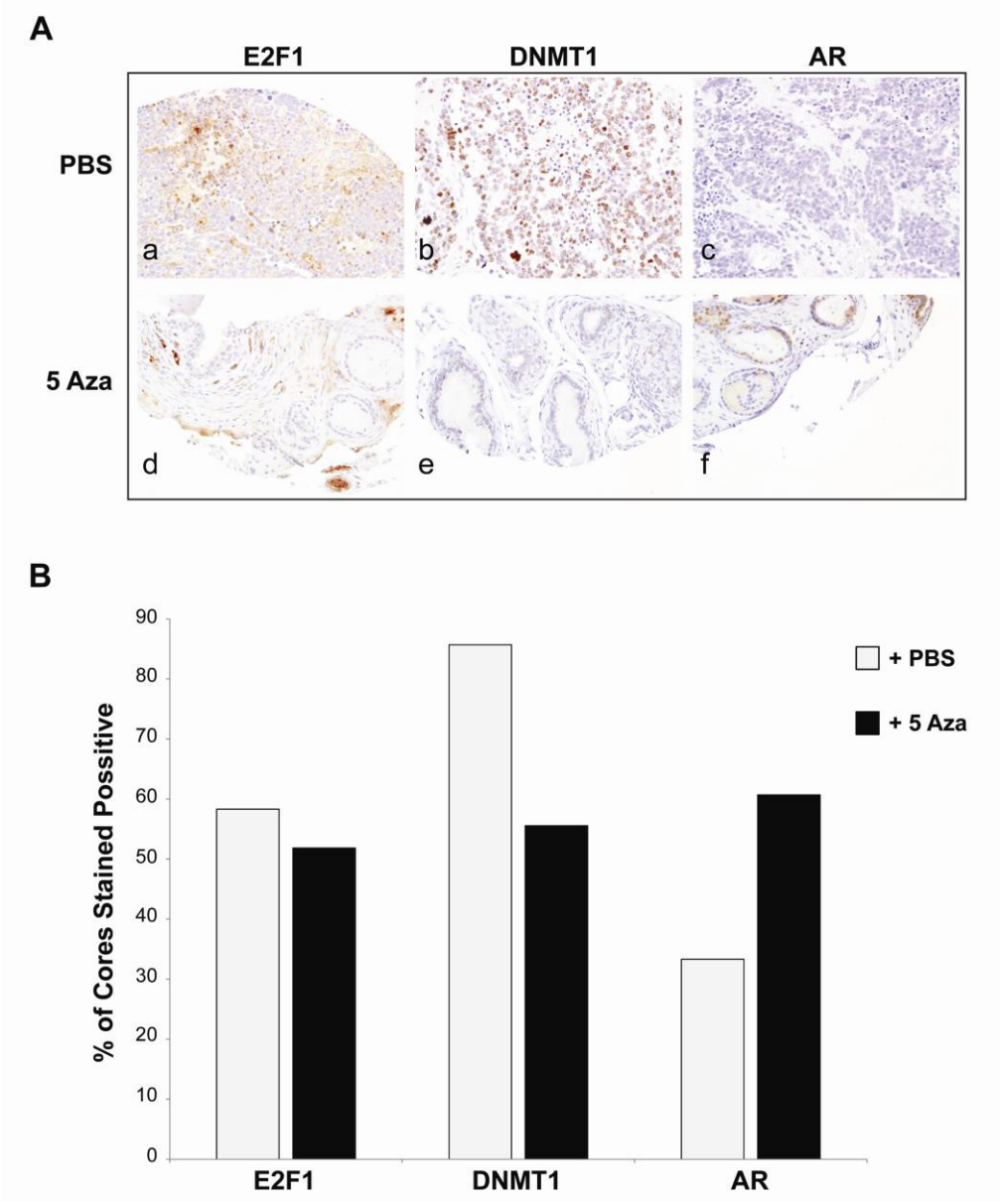

Figure 3.5 E2F1, DNMT1, and AR expression consequent to castration in the TRAMP model. A) The shown immunostained cores are representative of tissues taken from castrated TRAMP mice treated with either PBS (a-c) or 5-Aza (d-f). The histological expression of E2F1 (a,d), DNMT1 (b,e), and AR (c,f) were viewed at a 20X magnification. B) The castrated TRAMP cores staining positive for either E2F1 (n=24 PBS treated and n=28 5-aza treated), DNMT1(n=28 PBS treated and n=28 5-aza treated), and AR (n=21 PBS treated and n=29 5-aza treated) are shown as a percent of the total amount of cores available in either PBS or 5-Aza treated cohorts.

DNMT1 catalytic activity during CRPC. The correlation between late stage/metastatic prostate cancer and AR repression through the E2F1/DNMT1 axis presents possible prognostic and therapeutic targets.

This study is the first comprehensive histopathological evaluation of DNMT1 in human prostate cancer. The significant correlation between cancer progression and increased DNMT1 expression supports a possible use for DNMT1 as a diagnostic marker for certain prostate cancers. A previous study reports that gastric cancer patients with high epithelial DNMT1 expression have a significantly higher risk of death (27). Elevated DNMT1 expression was additionally shown to indicate the presence of metastatic prostate cancer in the TRAMP model. Kinney et al. observed a loss in prostate cancer metastases after DNMT1 levels were knocked down in TRAMP mice (21). Our study reveals DNMT1 expressional changes correlate with stage (localized vs. metastatic) and histological grade, with increased intensity for both more advanced disease and higher grade tumor. The significant difference in mean nuclear intensity (MNI) between Gleason grade 6 and 7 is of interest for potential clinical application in the setting of active surveillance. There is an ever expanding repertoire of markers being applied to small low grade (Gleason 6 and 7) tumors in an effort to triage patients into active surveillance and definitive treatment categories based on scientific data. Therefore DNMT1 staining of prostate biopsy samples alone or in combination with other markers may be of clinical value in this setting and deserves further study.

AR repression is one molecular event observed during CRPC. We show an increase in the amount of AR positive tissues acquired from castrated TRAMP mice (CRPC model) after treatments with 5Aza and suggest that AR repression is facilitated by

DNA methylation. AR has been shown to be methylated in a series of metastatic human prostate cancer cell lines. In addition to being methylated within specific regions, AR gene expression is rescued in M12 cells (AR negative metastatic lines) (28), androgen independent TSU-PR1 cells (29), and rat cells (androgen independent tumor cell line) (30) after 5Aza treatments. Kinoshita et. al. also identified hypermethylated regions within and downstream of the AR promoter that were common amongst AR negative castration resistant cell lines (31). A study of localized prostate cancer samples revealed that AR gene methylation was specifically occurring during late stage prostate cancer (32). A comparison of prostate cancer tissues showing AR methylaion demonstrated that 100% of the CpG sites analyzed were methylated in samples from CRPC patients compared to 50% of the sites being methylated in samples acquired from localized prostate cancer tumors (33). These data further support a mechanism for AR regulation via methylation in our model of CRPC.

DNMT1 facilitates genomic silencing by predominately maintaining the state of DNA methylaion but is also known to utilize de novo pathways. We have previously observed the repression of AR by E2F1 through DNMT1 as an intermediary (Valdez et al unpublished observations). Our current study demonstrated that DNMT1 increases during the progression of human prostate cancer to metastatic and CPRC disease. Higher DNMT1 levels may facilitate methylation dependent silencing and suppression of AR activity. Evidently, DNMT1 levels are important in maintaining abnormal levels of methylation. Robert et al. showed that the downregulation of DNMT1 with anti-sense oligonucleotides in the HCT116 colorectal cells significantly increased the amount of unmethylated DNA (34). High DNMT1 levels and increased genomic methylation have

also been reported during the metastatic progression of prostate cancer in the murine TRAMP model (21). Contrary to these findings, Hoffmann et al. reported that DNMT1 is not responsible for methylation during prostate cancer (35), however, this study did not globally assess genome-wide methylation. DNMT1 may therefore repress AR expression through methylation during late stage prostate cancer and CRPC.

The substantial increase in AR and decrease in DNMT1 following 5Aza treatment in our TRAMP CRPC model suggests that AR is regulated by DNMT1 dependent methylation, which is a conceivable therapeutic target. Methylation inhibitors such as 5-Aza, were shown to be effective in the treatment of myelodisplastic disease (36). The short half-life and high toxicity of 5Aza, however, create many complications for patient treatment. More suitable treatments may utilize less toxic methylation inhibitors such as Zebularine, but the drug is only effective at unreasonably high doses (37). Alternatives might directly target DNMT1 through the use of siRNA oligonucleotides. Knockdown of DNMT1 levels was shown to reduce proliferation and increase apoptosis in both gastric and esophageal squamous cell carcinoma cell lines (38, 39). The delivery of siRNA oligonucleotides to tumorigenic cells remains problematic. Future studies will need to be conducted to further evaluate the therapeutic potential of DNMT1 as well as the predictive value of DNMT1 in CRPC disease.

CONTRIBUTIONS

I was responsible for providing the scientific ideas that culminated in the creation of this chapter. I additionally coordinated the immunostaining of tissue microarrays by the

histology core. Tissue microarrays were analyzed by both Dr. Kirk Wojno and Dr. Lakshmi Kunju. Stephanie Diagnault provided biostatistical consultations.

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CHAPTER IV

DISCUSSION

Our studies sought to understand the proliferative nature of castration resistant prostate cancer (CRPC) by evaluating normal cellular division during prostate development. Considering that AR defines a differentiated and proliferative endpoint in prostate epithelium, we were curious how AR expression was repressed in the proliferative undifferentiated transit amplifying (T/A) population. An AR repressive mechanism may contribute to the proliferative progenitor-like phenotype of T/A cells. Results from our studies suggest that E2F1 represses AR through a DNMT1 mediated process. We assessed the potential for the E2F1/DNMT1 axis to repress AR during the development of castration resistant prostate cancer (CRPC), a cancer transition known to show decreases in AR expression. A substantially greater amount of castrated TRAMP CRPC tissue stained positive for E2F1 and DNMT1 compared to AR, consistent with a mechanism of AR repression by the E2F1/DNMT1 axis. We also demonstrate that contrary to the repression of AR in a model of CRPC, AR regulation by the E2F1/DNMT1 axis in non-malignant cells appears to be methylation independent. These findings imply that altercations in the E2F1/DNMT1/AR regulatory axis may lead to a methylation dependent mechanism during CRPC.

E2F1 mediated AR repression

Historically, E2F1 is thought to function as a transcriptional activator (1), however, recent research suggests E2F1 may function as a repressor of gene transcription. The E2F1 transactivation domain was required for the repression of both the PA inhibitor gene and the Mcl-1 gene (2, 3). All reported transcriptional inhibition by E2F1 was shown to be DNA binding domain dependent. Our studies demonstrated that the transactivation domain greatly contributed to the repression of AR by E2F1, while the DNA binding domain demonstrated little to no effect on transcriptional silencing. We considered that E2F1 either utilizes the transactivation domain to complex with a group of transcriptionally repressive proteins or promotes the expression of downstream genes that facilitate repression. As a result, we identified DNMT1 as a functional intermediary for AR repression by E2F1. We suggest that E2F1 may complex with DNMT1 and/or directly upregulate repressive DNMT1 levels.

E2F1 is shown to form a repressive complex with Rb. Rowland et. al. demonstrated that Rb binding domain mutants of E2F1 disrupt the endogenous repression of the p19ARF gene (4). The implications that E2F1 forms a repressive complex with Rb are further supported by evidence showing that reporters are silenced by the recruitment of Rb to E2F consensus site in the promoter (5). Similar repression of E2F binding site containing reporter constructs was facilitated by an E2F1/Rb complex that included DNMT1 and HDAC1 (6). These studies suggest that highly conserved E2F binding sites are required to recruit Rb repressive complexes to the promoter. Our studies, however, demonstrated that the AR promoter presents weak E2F consensus sites and that an E2F1

DNA binding mutant was still capable of partial AR gene repression. While our findings fail to support a mechanism that explains an Rb recruitment complex, the transactivation domain is clearly required for AR repression. Our explanation for this observation is that the E2F1 DNA binding mutant is still able to contribute to the formation of an as of yet undefined inhibitory complex that incorporates DNMT1, through transactivation domain contacts. In the absence of E2F1 facilitated gene targeting, DNMT1 may mediate multi-subunit recruitment to the DNA through CXXC domain interactions with CpG dinucleotides. Repression by the E2F1 DNA binding mutant reveals one possible mode of AR regulation by E2F1. In addition to facilitating repressive complexes, E2F1 may target the transcription of a genetic silencer.

E2F1 may directly interact with the gene that is transcribed or target the expression of an intermediary that regulates the expression of downstream genes. A microarray analysis of newborn mice livers demonstrated that endogenous E2F1, in addition to activating transcription, contributed to the repression of a small cohort of genes (7). E2F1 may either indirectly or directly mediate transcriptional repression. McCabe et. al. showed that E2F1 directly coordinates the transcription of DNMT1, a major epigenetic modifier involved in gene silencing. We showed that AR expression is re-expressed in AR negative cells following DNMT1 downregulation. We suggest that E2F1 upregulates DNMT1 to repress AR expression.

We provide further evidence to support transcriptionally repressive roles for E2F1. We propose two potential mechanisms by which E2F1 mediates the regulation of AR. 1) E2F1 either forms a complex containing DNMT1 and/or 2) transcriptionally upregulates DNMT1 to repress AR (Figure 4.1).

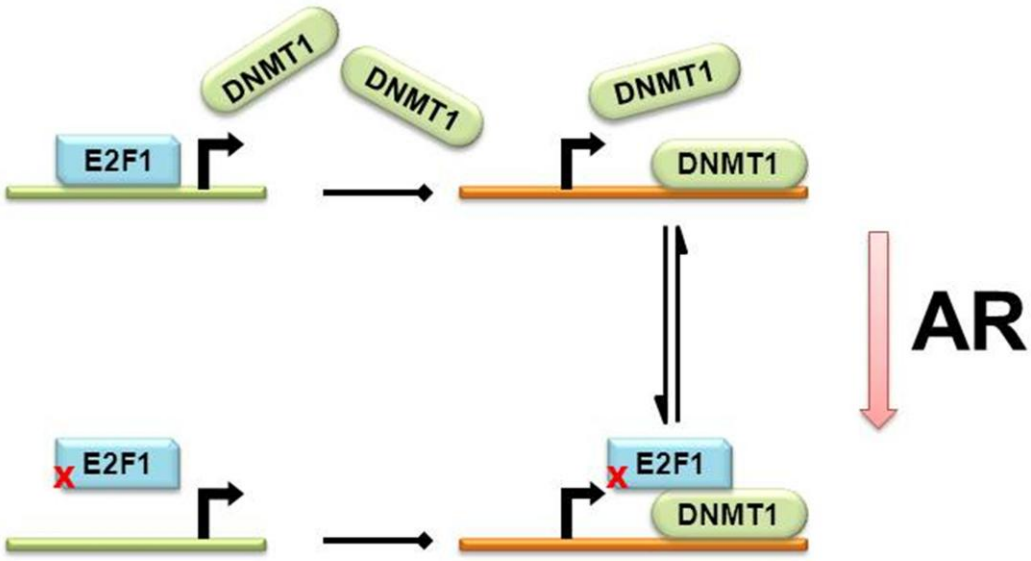

Figure 4.1 Schematic of AR repression by E2F1. E2F1 targets the transcription of DNMT1 which binds to and represses the AR gene. The E2F1 transactivation domain assists in the formation of a DNMT1 containing repressive complex that is still functional in the absence of the DNA binding domain.

Methylation Independent Regulation by DNMT1

DNMT1 is generally believed to function as an enzyme that regulates gene expression through the methylation of DNA. However, few have explored the possibilities for methylation-independent regulation. We showed that AR expression was unchanged following administration of the DNA methylation inhibitor, 5-aza. AR expression in AR negative cells was achieved through the downregulation of DNMT1, which additionally failed to change the methylation status of the minimal promoter. We postulate that DNMT1 regulates AR in a DNA methylation independent manner. Szyf et. al. demonstrated that DNMT1 utilizes a non-DNA-methylating alternative to regulate both BIK and p21 expression (8). The findings implied that suppressive DNMT1 interactions with promoter bound Sp1 and 3 factors inhibited the transcriptional activity required for gene expression. Similar to E2F1, DNMT1 regulatory activity in the absence of DNA methylation is potentially dependent on interactions with specific multi-unit protein subsets.

DNMT1 may facilitate DNA methylation independent AR repression through the establishment of chromatin modifying complexes (Figure 4.2). DNMT1 may function through known repressive mechanisms dependent on the recruitment of HDAC1 and 2 deacetylase activity by defined interaction domains (9, 10). DNMT1 is additionally found to directly interact with the polycomb repressive complex 2 (PRC2) protein EZH2, which silences gene expression through histone methylation (H3k27me2/3) (11). We observed the DNA methylation independent regulation of AR in cells that recapitulate a progenitor-like phenotype. EZH2 knockouts, resulting in embryonic lethality (12),

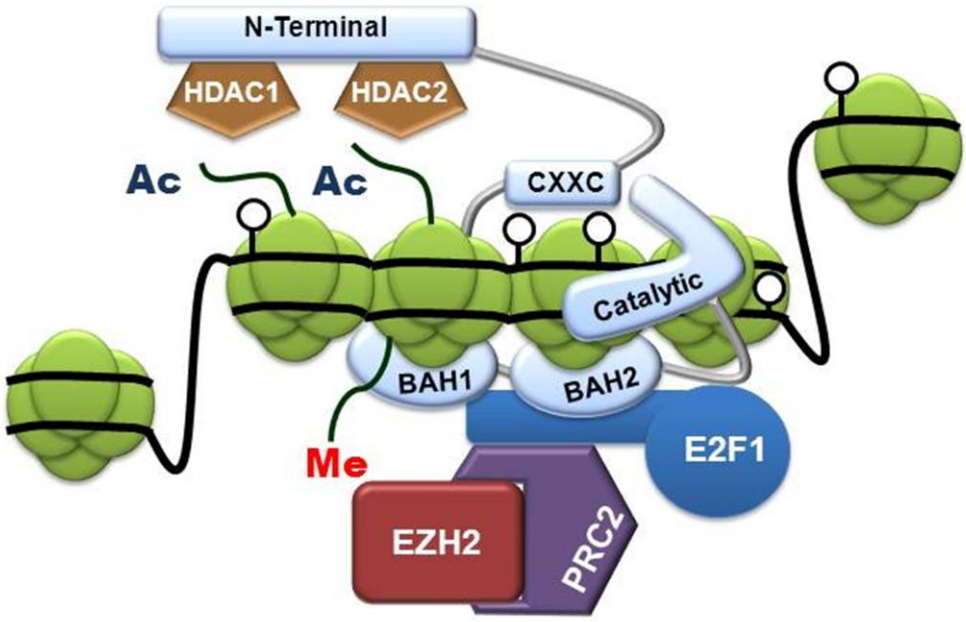

Figure 4.2 DNMT1 repressive complex stimulates the formation of heterochromation. The following DNMT1 domains (N-terminal, CXXC, BAH 1 and 2, and catalytic) are represented in the schematic. Unmethylated cytosines depicted by open circles and are shown in contact with the CXXC domain. E2F1 interactions with the BAH domains facilitates the recruitment of the PRC2 complex which guides histone tail methylation (Me) by EZH2. HDAC 1 and 2 remove of histone tail acetyl groups (Ac) by binding to the N-terminal domain.

suggest that the protein plays a critical role in development and functions to maintain progenitor-like properties. AR repression in our cell models may therefore occur through EZH2 facilitated histone methylation. The methylation of H3K27 by EZH2 may additionally cooperate with HDAC (1-2) deacetylation to facilitate AR silencing. The sequential treatment of cells with an inhibitor (DZNep) of EZH2 dependent methylation, followed by a histone deacetylase inhibitor (TSA), resulted in the re-expression of CDKN1C (13). Chromatin modification is a mechanism of repression that may operate independent of methylation.

DNMT1 has the potential to regulate gene expression through non-DNA methylating mechanisms. The structural domains facilitate interactions that promote chromatin condensation and gene silencing. We provide an example of gene regulation by non-methylating DNMT1 and suggest a mechanism that involves both histone methylation and deacetylation. DNMT1 targeted histone modifications, in the absence of DNA methylation, may enable a repressive state that is more suitable for temporally coordinated expression. Further studies might examine the epigenetic states of the AR gene during prostate epithelial differentiation.

Transcriptional regulation of the AR gene

A comprehensive understanding of AR gene repression is still lacking. The presence of cis-elements and multi-protein regulatory domains were observed to negatively regulate AR promoter activity (14, 15). We present evidence suggesting that DNMT1 regulates AR gene repression. The abundance of GC rich sequences in the AR promoter (16) provides sites for repressive DNMT1 interactions. Our studies further verify that the

regulation of AR transcription is cell-type specific (17). We postulate that DNMT1 facilitates DNA methylation independent repression in cells, recapitulating a non-malignant phenotype while silencing gene transcription by methylating the AR promoter in CRPC.

Our nonmalignant cell model demonstrated AR repression that was independent of DNA methylation. According to our DNMT1 directed ChIP findings DNMT1 demonstrated strong association with a section of the first AR intron. DNMT1 interactions at the intron may affect the overall regulation of the gene. Understanding that the AR expression is both tissue and differentiated state specific implies that intronic sequence elements may exist to coordinate appropriate spatial and temporal expression. The second intron of the AGAMOUS (AG) gene was shown to be required for normal patterns of expression for the AG:beta-glucoronidase gene fusion reporter in plants (18). Kamachi et. al. additionally defined a location in the third intronic region of the lens specific delta1-crystallin gene that facilitates regulatory factor interactions to restrict broad abnormal expression (19). The intron is also shown to enhance gene expression as demonstrated by the first intron in the rat growth hormone gene (20). DNMT1 may facilitate AR repression through interactions with a possible regulatory element in the first intron (Figure 4.3A).

DNMT1 might inhibit the binding of transcriptional activators to enhancer elements within the first intron (Figure 4.3). NF-kappa-B was shown to negatively regulate rat AR transcription by blocking the kappa-immunoglobulin light chain enhancer from interacting with a location 570 bps upstream of the transcription start site. However, no conservation was observed between the human kappa-light chain enhancer

consensus site (ATTTGCATA) (21) and a region spanning ~2,000 bp of the intronic site associated with DNMT1 in our ChIP analysis. A regulatory sequence may still exist considering the prevalence of numerous non-coding genomic sequences with unknown functions.

AR regulation during CRPC is alternatively shown to be associated with methylation that maybe mediated by DNMT1. Repressive AR gene methylation at the DNA level is directly shown through bisulfite sequencing or inferred from 5-aza (DNA methylation inhibitor) induced gene re-expression in many castration resistant cell lines (22-24). We demonstrated that 60.71% of the CRPC TRAMP mice treated with 5Aza showed AR positivity compared to 33.33% of the untreated cohort. The large increase in the amount of AR positive tissues from treated mice implied that AR repression was facilitated by DNA methylation during CRPC. DNMT1 is possibly responsible for methylating the promoter, considering the high amount of castration resistant tissue that stained positive for DNMT1. Feltus et. al. demonstrated that a group of CpG islands (CGI) were hypermethylated following the overexpression of DNMT1 (25). A CGI is present in the AR promoter region and is consequently susceptible to the hypermethylation observed at CGIs in genes during cancer. Abnormal levels of DNMT1 may therefore repress AR expression during CRPC by hypermethylating the promoter (Figure 4.3B).

The seemingly conflicting functional roles for DNMT1 during normal development and the onset of CRPC are reconciled within the context of protein complexes. DNMT1 was observed to methylate and silence genes while in complex with HP1 (26), but was also shown to facilitate methylation independent repression while

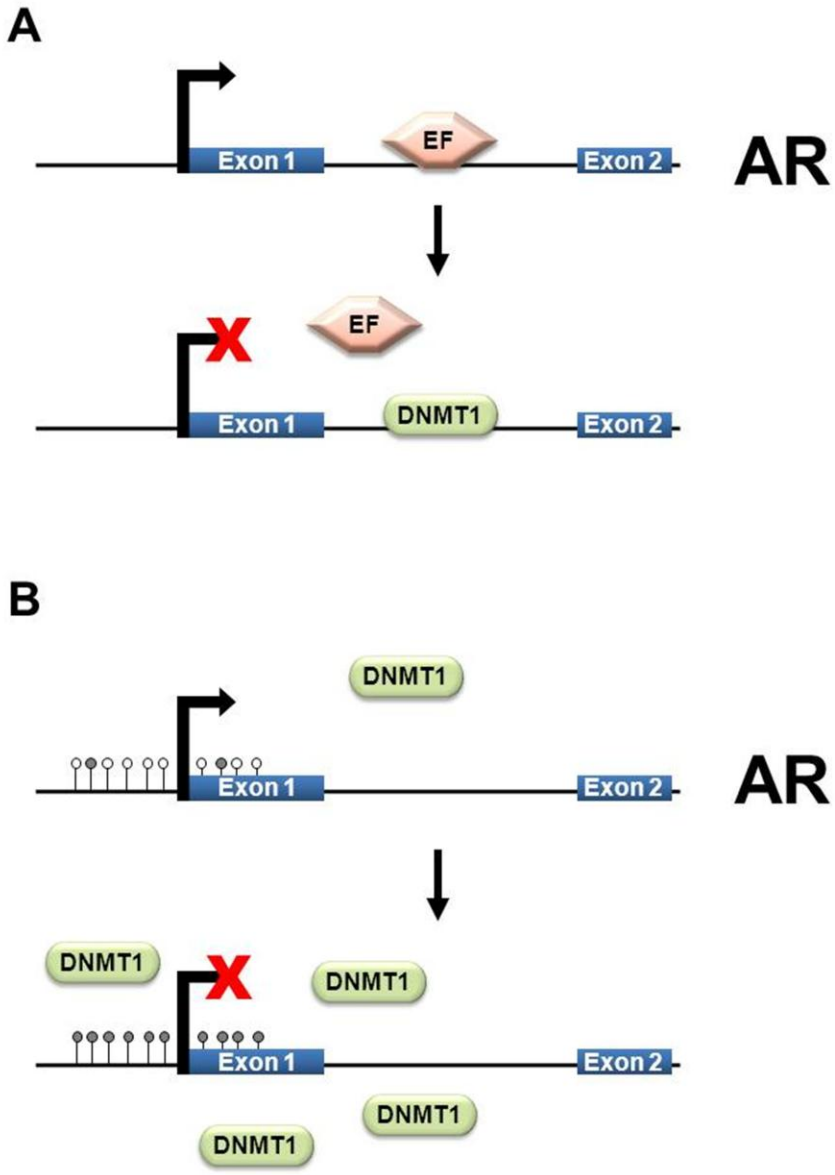

Figure 4.3 Two modes of AR repression facilitated by DNMT1. (A) DNMT1 competes with an enhancer factor (EF) at a site in the first intron of the AR gene and inhibits the expression of AR. (B) DNMT1 accumulation results in the hypermethylation of a CpG island spanning across transcription start site II.

interacting with an HDAC2/DMAP1 co-repressor complex (9). These findings suggest that specific protein interactions may regulate DNMT1 dependent methylation. Kar et. al. reported a number of mutated proteins expressed in various cancers that are able to form aberrant interactions (27). Compared to normal prostate development, abnormal complex formation during CRPC potentially recruits DNMT1 to hypermethylate and silence genes. As a result, DNMT1 facilitate repression appears to be both dependent and independent of methylation. We demonstrated that these divergent modulatory roles may correspond to a cell type specific environment.

Differentiation within the Context of AR Expression

We have defined an AR repressive mechanism that is associated with undifferentiated proliferative epithelium in the prostate and therefore suggest that AR expression may facilitate differentiation. AR expression has been shown in the terminally differentiated luminal epithelium of the developing prostate (28). Activated AR may promote the transcription of genes that drive epithelial differentiation. Alternatively, an established differentiated state may support the expression of AR. The latter alternative would suggest that AR is not required for epithelial differentiation. Although active AR may not be sufficient to induce differentiation, evidence exists to support the notion that epithelial differentiation requires AR activity.

The removal of AR from normal prostate epithelium results in a corresponding loss of differentiation. Wu et. al. knocked out (KO) AR in the luminal epithelium of mouse prostates and observed de-differentiating morphological changes in the ventral prostate (29). AR KO mice at 9 weeks of age had decreased glandular infolding and

showed a pronounced transition of luminal epithelium from tall columnar to small cuboidal cells typical of a less differentiated phenotype. The authors show a decrease in the expression of differentiation markers (PSP94 and NKX3.1), however, these genes are androgen activated and expressional levels coincide with AR activity. The re-expression of AR post de-differentiation might reverse the effects and demonstrate a substantial role for AR in differentiation.

AR expression is evidently required for the differentiation of the luminal epithelium in the ventral prostate. Further studies might determine how functional AR is involved in differentiation. Overexpression of AR in AR negative cell lines such as, BPH-1 and PC-3, was shown to reduce proliferation (30, 31), however differentiation was not evaluated in these studies. The directed expression of AR via a k5 promoter in the basal epithelium may induce differentiation *in vivo*. Although, transgenic overexpression of the AR gene may promote genetic abnormalities which further contribute to tumorigenesis. AR activation potentially facilitates differentiation during normal prostate development.

DNMT1 as a prognostic marker

Treatment plans for patients with prostate cancer are diagnosis dependent. A number of parameters are considered (Prostate specific antigen (PSA) levels, gleason score, staging, ect.) to prescribe the most efficient therapy. Active surveillance, an intensive monitoring program involving continual checkups and prostate screens, was recently shown to extend the quality of life for patients with localized low risk prostate cancer (32) by reducing overtreatment. The surveillance program is offered to patients that match the

established diagnostic criteria (PSA < 10ng/ml, stage ≤ T2a, and Gleason score ≤ 6) (32). Immediate treatment occurs at a Gleason score of 7 (G7). However, scores may mislead options to withhold treatment.

Although Gleason scores provide definitive information on the status of prostate progression from localized to metastatic cancer (as reviewed in (33)) errors may occur in the assignment of numerical values. Pinthus et. al. revealed that 61.8% of the G6 diagnosed biopsies were underscored compared to the G7 evaluated tissues following radical prostatectomy (34). The decision to either treat or enroll patients with G6 biopsies into the active surveillance program is confounded by these findings. Molecular panel studies have attempted to better define prostate cancer progression, however, the heterogeneous nature of prostate cancer continues to cause prognostic marker inconsistencies (35). We observed a significant increase in the DNMT1 nuclear staining of tissues procured from G7 graded tumors compared to G6. Our samples were obtained by radical prostatectomy and therefore provided more reliable Gleason scores. DNMT1, as a result, may be a candidate for a prognostic marker that better differentiates between G6 and 7 graded tumors evaluated by biopsies. An improved diagnosis will help to better manage treatment options for low grade prostate cancer.

Quantitative based analyses of DNMT1 may function as a predictive measurement for certain prostate cancer treatments. Specific levels of DNMT1 may help to predict a point at which methylation inhibiting drugs such as 5-aza are most effective in the treatment of certain prostate cancer subtypes. Treatments with 5aza were shown to significantly increase the survival of TRAMP mice (36, 37) but had subtle effects on human patients suffering from late stage CRPC (38). A study evaluating the methylation

of several tumor related genes noted that the highest frequency in promoter CGI hypermethylation occurred in cancer tissue with a Gleason score ≥ 7. DNMT1 levels assessed in biopsies may help to define a temporally finite point to initiate anti-methylating treatments that prevent the onset of hypermethylation confining the cancer to a more treatable state. Drug administration at the correct time may prevent the transition from G6 to G7 prostate cancers or divert androgen independent pathways that lead to CRPC.

Expressional measurements of DNMT1 may be used to determine appropriate treatment options. We have described a mechanism of AR repression facilitated by DNMT1 that may be characteristic of androgen independent prostate cancer cells. Further evidence suggests that silencing by DNMT1 in CRPC may occur in a methylation dependent fashion. More comprehensive studies on DNMT1 levels during prostate cancer may help to illuminate novel therapeutic pathways.

Future Directions

Our studies have established two possible mechanisms of AR regulation in prostate epithelium. We suggest that DNA methylation independent repression by DNMT1 contributes to the maintenance of the progenitor cell phenotype, while AR repression during CRPC via DNMT1 dependent hypermethylation facilitates an undifferentiated proliferative phenotype. Future studies might examine the stromal signaling factors that may utilize our mechanism of repression to regulate epithelial AR expression *in vivo*.

Additionally, regulation of AR at the chromatin level may reveal new regulatory complexes and epigenetic modifying interactions.

Much is unknown about the *in vivo* cross talk between the mesenchyme and epithelium of the prostate gland. Stromal signals may either induce or inactivate the E2F1/DNMT1/AR repression axis described herein. The use of tissue recombination techniques as described by Chuna et. al. (39) in the analysis of AR deficient epithelium, may provide a workable experimental model. The fibroblast growth factor receptor 2 (FGFR2) shown to mediate paracrine signaling (andromedin) from the stoma via FGF7 (40), would be targeted as a critical point for stromal and epithelial communication. FGFR2 knockout mouse epithelial cells that are AR negative would be mixed with normal mouse stromal cells and grafted on the renal capsule of the mouse kidney. Tissue sections of an either developed or undeveloped prostate would be stained with AR to determine the effect of a lack of andromedin signaling through the FGFR2 receptor on AR expression. If AR expression is deregulated, further analysis of DNMT1 and E2F1 expression may reveal mechanisms linking andromedin signaling to AR regulation. DNMT1 knockout epithelial cells that are AR negative would also be assessed within the context of the tissue recombination model. The analysis may provide further insight to the role that the mesenchyme has during prostate epithelial differentiation.

The androgen receptor regulation may involve epigenetic modifying mechanisms that occur at the chromatin level. We currently used DZNep (EZH2 inhibitor) and TSA (broad HDAC class I and II inhibitor) to determine the requirement for chromatin modifiers during AR repression in PrEC and BPH-1 cells. We demonstrated that DZNep in combination with TSA induce the expression of AR in AR negative cells (Figure 4.4).

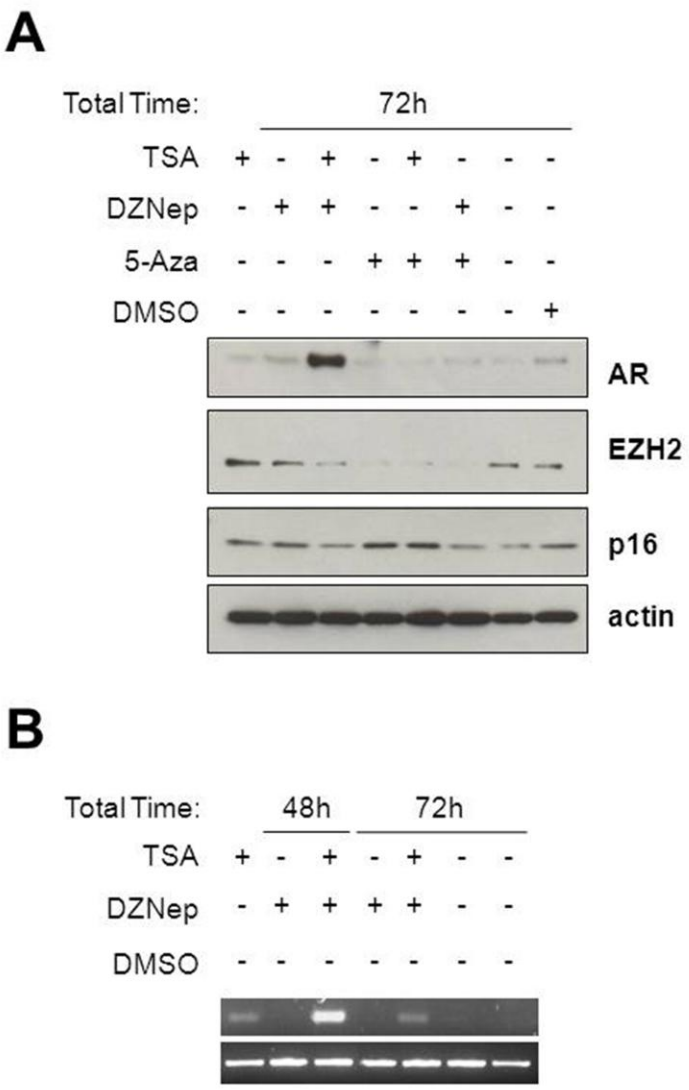

Figure 4.4 Treatment with both a histone methylation and deacetylation inhibitor relieves AR expression in PrECs. All TSA treatments were done at 100nM for the last 24h of treatment. Treatment durations are shown (48 or 72h). (A) Western blot presenting the expressional changes following treatment. (B) Changes in cDNA levels resulting from the transcriptional variation elicited by the treatments.

We additionally verified a lack of regulation by DNA methylation showing that treatment with the DNA methylation inhibitor, 5-aza, failed to rescue AR expression. A comparison of AR gene histone methylation at H3K27 and acetylation between AR expressing (LNCaP) and non-expressing (PrEC) may define regions of the AR gene undergoing chromosomal compaction during repression. Also of interest is the possibility for a functional chromatin regulating complex associated with DNMT1 and E2F1. Co-immunoprecipitation in addition to coulumn purification experiments would examine the potential endogenous interactions between E2F1, DNMT1, EZH2, and HDAC (1 and 2). These studies would further characterize the epigenetic regulation of the AR gene.

Our findings have brought new insights that challenge the current understandings of gene regulation and CRPC. Contrary to the traditional role of E2F1 in transcriptional activation, the regulatory factor was shown to repress AR transcription. We additionally demonstrated that the repression of AR by E2F1 in non-transformed cells was mediated by DNMT1 through an unconventional mechanism that is independent of DNA methylation. We further considered how the E2F1/DNMT1 axis might repress AR during CRPC. While many suggest that irregular AR activity contributes to CRPC, we provide data supporting a mechanism of AR repression through DNA methylation. We suggest that abnormally high levels of DNMT1 may hypermethylate promoter CGIs and repress transcription. Our studies reveal novel epigenetic regulatory mechanisms involved in AR repression that may further contribute to the understanding of transcriptional regulation and castration resistant disease.

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