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Androgen receptor-driven chromatin looping in prostate cancer

Dayong Wu[1,*], **Chunpeng Zhang**[1,*], **Yanping Shen**[1,2], **Kenneth P. Nephew**[3], and **Qianben Wang**[1,2]

[1]Department of Molecular and Cellular Biochemistry and the Comprehensive Cancer Center, The Ohio State University College of Medicine, Columbus, Ohio, 43210, USA

[2]Ohio State Biochemistry Graduate Program, The Ohio State University, Columbus, Ohio, 43210, USA

[3]Medical Sciences Program, Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Bloomington, IN 47405, USA

Abstract

Androgen receptor (AR) is important for prostate cancer development and progression. Genome-wide mapping of AR binding sites in prostate cancer have found that the majority of AR binding sites are located within non-promoter regions. These distal AR binding regions regulate AR target genes (e.g. *UBE2C*) involved in prostate cancer growth through chromatin looping. In addition to long-distance gene regulation, looping has been shown to induce spatial proximity of two genes otherwise located far away along the genomic sequence and the formation of double strand DNA breaks, resulting in aberrant gene fusions (e.g. *TMPRSS2-ERG*) that also contribute to prostate tumorigenesis. Elucidating the mechanisms of AR-driven chromatin looping will increase our understanding of prostate carcinogenesis and may lead to the identification of new therapeutic targets.

Androgen receptor (AR) signaling in prostate cancer

Although significant strides have been made in prostate cancer research and treatment, prostate cancer remains one of the most commonly diagnosed cancers and the second leading cause of cancer deaths in American men[1]. The “male” hormones or androgens, exerting their biological effect through androgen receptor (AR), play important role in prostate cancer development and progression[2, 3]. Therefore androgen ablation therapy, including surgical and chemical castration, is the first-line therapeutic approach for advanced androgen-dependent prostate cancer (ADPC). Although this therapy is initially effective, ADPC ultimately progresses into an incurable, castration-resistant stage of the disease (CRPC), involving the reactivation of AR signaling. The mechanisms for AR reactivation after castration include AR amplification, increased androgen sensitivity, increased intracellular synthesis of androgen, a constitutively active AR lacking ligand binding domain (LBD), activation of growth factor pathways, and retinoblastoma (RB) loss-induced, E2F1-mediated AR overexpression[3–6]. Thus, aberrantly active AR signaling exists in both ADPC and CRPC.

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Corresponding author: Wang, Q. (qianben.wang@osumc.edu).
*These authors contributed equally to this work

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AR is a ligand-dependent transcription factor belonging to the nuclear hormone receptor (NR) superfamily[7]. To understand how AR signaling contributes to ADPC and CRPC, recent studies have utilized genome-wide chromatin immunoprecipitation (ChIP) techniques to define AR binding sites across the entire human genome in prostate cancer cells[8–13]. In these studies, AR ChIP-enriched DNA was amplified and hybridized to tiling DNA microarrays (ChIP-on-chip), or subjected to massively parallel high throughput sequencing (ChIP-seq)[14, 15]. These studies have greatly advanced understanding of AR binding to chromatin, the functional interplay between AR and chromatin, and AR-mediated regulation of target genes involved in prostate tumorigenesis. In this review, we discuss how the majority of AR binding sites in the genome is located within non-promoter distal regions and the importance of this for transcriptional regulation. At these sites, several transcription factors act as positive or negative regulators of AR function and affect AR-mediated gene regulation. We then discuss two types of AR binding-driven chromatin looping in prostate cancer, the AR-driven chromatin looping leading to target gene expression (without genomic rearrangement) and the AR-driven chromatin looping that results in gene fusion (with genomic rearrangement). The elucidation of the mechanisms of AR signaling in prostate cancer has translational implications in the development of new therapies in prostate cancer.

Features of genome-wide AR binding atlas in prostate cancer

Distal AR binding sites: their location and importance

In the pre-genome-wide ChIP era, studies on the canonical AR target gene *PSA* (prostate specific antigen) found that AR primarily binds to the *PSA* enhancer rather than to the promoter region[16]. Consistent with the findings from the *PSA* gene, genome-wide mapping of AR binding sites in prostate cancer cells revealed that most AR binding sites are not within the promoter region of AR-regulated genes. Approximately 86%~–95% of AR binding sites identified in ADPC cell models (LNCaP[12, 13] and VCaP[13]) and CRPC cell models (LNCaP-abl[12] and C4-2B[11]) are located within non-promoter regions. Interestingly, similar distal AR binding patterns are also observed in an immortalized normal human prostate epithelium cell line (HPr-1)[17], and non-prostate androgen-responsive cells (human primary skeletal muscle myoblasts[18]) or tissues (mouse epididymis[19]). These studies strongly indicate that distal binding may be a general rule for AR action.

Several lines of evidence suggest that at least part of these distal AR binding regions are important for transcriptional regulation. First, some AR binding regions, when cloned upstream of minimal promoters, function as enhancers in reporter gene assays[8, 10, 11]. Second, transcriptional coactivators (e.g. the histone acetyltransferases [HAT], BRM-containing chromatin-remodeling complex and the Mediator complex) and RNA polymerase II (Pol II) bind to some distal AR binding sites[8, 10, 12, 13, 20, 21], indicating that these sites are transcriptionally active. Third, some distal AR binding sites are associated with active histone modifications including H3K4 mono- and di-methylation (H3K4me1 and H3K4me2) and H3 acetylation (H3Ac)[10–13], which create a permissive chromatin environment that facilitates active transcription. Fourth, the nucleosomes present at some distal AR binding regions contain the H2A.Z variant[22], which has been reported to correlate with gene activation[23, 24]. Collectively, these observations suggest that some distal AR binding sites act as transcriptional enhancers.

Transcription factors that positively or negatively affect AR mediated gene expression

De novo motif searching (i.e. searching for common sequence patterns) or known motif scanning within the AR binding regions across the genome has identified that, in addition to AR binding motifs, many other transcription factor motifs (e.g. Forkhead, GATA, OCT,

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ETS, and NKX3-1) are significantly enriched within the AR binding regions, compared with genomic background. These observations suggest that transcription factors recognizing these motifs may be recruited to AR binding regions and play important roles in AR-mediated gene expression in prostate cancer, either by enhancing or repressing AR action. Consistent with this hypothesis, the pioneer factors FoxA1 and GATA2 were reported to be recruited to a significant portion of AR binding sites in ADPC and CRPC cells, facilitating AR binding and enhancing AR-mediated transcription[8, 11, 13, 22]. In addition, OCT1, a member of the POU domain family of transcription factors, may facilitate Pol II binding to AR-bound regions after short-term and prolonged androgen stimulation[8, 22]. By contrast, E26 transformation-specific (ETS) family member ERG binding sites that significantly overlap with AR binding sites in VCaP cells, repress AR action and AR target gene expression[13]. These studies suggest that AR-mediated gene expression in prostate cancer involves combinatorial transcriptional regulation.

AR-driven chromatin looping leads to target gene expression

Distal AR binding regions communicate with target gene promoters through chromatin looping

As the majority of AR binding sites are located at distal regions, how these distal binding sites interact with their target gene promoters and regulate target gene expression remains an open question. The looping model proposes that distal transcription factor binding sites interact with the proximal promoter regions with the intervening DNA looped out[25, 26]. Chromosome conformation capture (3C) technology, developed almost a decade ago[27], allows for testing this proposed model. In this assay, the distal and proximal genomic regions, which have been cross-linked, are digested by the same restriction enzyme and re-ligated under extensively diluted DNA concentrations. The re-ligation of distal/proximal regions should be detected by PCR, if the distal region is physically associated with the proximal region[27, 28]. Using this assay and its derived ChIP-3C (ChIP combined with 3C) assay, recent studies have demonstrated that distal AR binding sites regulate well-known AR target genes such as *PSA*[16] and *TMPRSS2*[8, 21] through chromatin looping. Chromatin looping also drives the expression of critical AR target genes directly involved in prostate cancer growth. For example, the CRPC-specific AR target gene *UBE2C* (ubiquitin-conjugating enzyme E2C) has an essential role in promoting CRPC cell growth[12, 21] by inactivation of the M-phase checkpoint[29] or increasing the pool of active anaphase promoting complex/cyclosome (APC/C)[30]. It was recently demonstrated that two CRPC cell-specific AR-bound enhancers, located −32.8 kb and +41.6 kb from the transcription start site (TSS) of *UBE2C* gene, interact with the UBE2C promoters through chromatin looping in CRPC but not in ADPC cells, which is required for *UBE2C* gene expression and CRPC growth[12, 21].

Although the 3C assay is a powerful approach for studying enhancer/promoter interaction, it is not feasible to perform standard 3C for each AR binding site to identify or validate its target gene owing to more than 10,000 AR binding sites in the genome. The development of high-throughput adaptation of 3C assays, 4C (circular 3C or 3C-on-chip)[31], 5C (3C-carbon copy)[32], Hi-C[33] and CHIA-PET (chromatin interaction analysis by paired-end sequencing)[34], should allow for genome-wide assessment of AR-bound enhancer/promoter interactions in the near future.

Mechanisms for AR-mediated chromatin looping in CRPC

Chromatin looping is established and/or maintained by protein-protein interaction between enhancer-bound transcription factors and promoter-bound proteins[25, 26, 35]. While the distally bound transcription factors may directly interact with promoter-bound proteins, in

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many cases transcriptional coregulators or Pol II facilitate and/or mediate such interactions[26, 36]. Indeed, recent studies demonstrated that both transcription factors (e.g. AR, estrogen receptor [ER], FoxA1 and GATA-1)[8, 16, 21, 34, 37] and coactivators (e.g. Med12, SRC-1, p300/CBP and BRG1)[38–41] play essential roles in looping formation and/or maintenance. Among these coactivators, Mediator, an evolutionally conserved multiprotein complex including ~30 subunits[42], is of particular interest. While previous studies imply that Mediator is important for chromatin looping in several loci[41, 43], a recent study found that Mediator may mediate genome-wide enhancer/promoter interactions. The global Mediator-mediated looping is stabilized by cohensin, a protein which embraces the loop[39]. These studies suggest that Mediator is an essential chromatin architectural factor. Consistent with this notion, a recent study demonstrated that silencing of the Mediator subunit MED1, an AR coactivator[16, 44], significantly decreased the interaction between the AR-bound UBE2C enhancers and the UBE2C promoter in the CRPC cell model LNCaP-abl, leading to decreased UBE2C gene expression[21]. Importantly, phosphorylation of MED1 at threonine (T) 1032 by phosphatidylinositol 3-kinase (PI3K)/AKT was proposed as a mechanism for MED1-mediated looping in CRPC cells by enhancing interactions between enhancer-bound FoxA1/AR and promoter-bound Pol II and TATA binding protein[21]. Based on the findings from these studies, we propose a looping model for AR-mediated gene regulation in CRPC (Figure 1). Furthermore, it seems reasonable to hypothesize that other looping-related coactivators such as SRC-1, p300/CBP[38], and cohesin[39] may also be involved in AR-mediated looping in CRPC (Figure 1). Future studies are needed to test these hypotheses.

AR-driven chromatin looping results in gene fusion

Overview of AR-regulated fusion genes in prostate cancer

Although genomic rearrangements are important for physiological processes such as VDJ recombination and class switch recombination (CSR) in lymphocytes, aberrant genomic rearrangements may lead to gene fusions in prostate cells contributing to prostate cancer initiation and progression[45, 46]. For example, the *TMPRSS2-ERG* fusion gene, which is expressed and functional in ~50% of primary prostate cancer and ~30% of CRPC patients[45, 47, 48], is generated by the juxtaposition of the 5' untranslated region of the AR target gene *TMPRSS2* (21q22. 3)[8] and the 5' end of the oncogene *ERG* (21q22.2)[45, 49]. The juxtaposition of *TMPRSS2* and *ERG* occurs either through balanced translocations with a "closed chain" pattern (i.e. without loss of genetic material) or through interstitial deletions (Edel)[50–52].

The *TMPRSS2-ERG* fusion gene was first identified by Chinnaiyan's group using an integrative computational and experimental approach[49]. An algorithm, termed COPA (cancer outlier profile analysis), was used to identify *ERG* as an outlier gene (i.e., a gene overexpressed in a subset of patients) from gene expression datasets in the Oncomine database[53]. Exon-walk PCR and 5'-RNA-ligase-mediated rapid amplification of complementary DNA ends (5'-RLM-RACE) assays were then performed to identify the fusion between *TMPRSS2* and *ERG*[45, 49]. Using the same approach, many other androgen-regulated genes fused with members of the ETS gene family (e.g. *TMPRSS2*-ETV1[49], *SLC45A3-ETV1*[54], and *CANT1-ETV4*[55, 56]), have been identified. Recently, more androgen-regulated gene fusions including non-ETS fusions (e.g. *NDRG1-ERG*[57], *SLC45A3-BRAF*[58] and *TMPRSS2-FKBP5-ERG*[59]) have been identified in prostate cancer by using the newly developed paired-end RNA sequencing (PE RNA-seq)[60]. In this approach, the total RNA is fragmented and converted into double-stranded cDNA. The cDNA fragments go through adapter ligation and PCR amplification processes, and the final cDNA library is used for paired-end high throughput sequencing. By integrating publically available AR ChIP-seq data[13] and standard AR ChIP data[8, 61, 62] with gene expression and FISH (fluorescence *in*

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situ hybridization) analysis data, we have summarized the published AR-regulated fusion genes (Table S1).

In addition to AR-regulated fusion genes, many non-AR regulated fusion genes have been reported. For example, *ETV1* was found to be fused with the housekeeping gene *HNRPA2B1*[54]. In addition, none of the most recently identified 5' gene fusion partners (e.g. *ALG5*, *PIGU*, and *TNPO1*) by PE RNA-seq are androgen-regulated[59]. These findings suggest that multiple mechanisms exist for regulation of fusion gene expression in prostate cancer.

Function of AR-regulated fusion genes

The binding of liganded AR to the regulatory elements of the 5' partner of a fusion gene drives the overexpression of the 3' partner, which is often an oncogene (e.g. *ERG*), thus contributing to prostate carcinogenesis. For example, it has been reported that AR-driven expression of the ETS family members *ETV1*, *ETV5* and *ERG* promotes invasion of benign prostate cells (e.g. RWPE, PrEC and BPH-1) and ADPC cells (e.g. LNCaP and VCaP) by activating an invasion-associated transcriptional program and thus inducing the plasminogen activator pathway[13, 54, 63–65]. While overexpression of *ETV1* and *ETV5* showed no effect on cell proliferation[54, 63], recent studies found that *ERG* overexpression also suppresses the AR-mediated differentiation program to promote LNCaP and VCaP cell growth[13, 65]. Although *ERG* expression is necessary for prostate cancer cell invasion and growth, transgenic overexpression of *ERG* in mouse prostate is insufficient for inducing prostatic intraepithelial neoplasia (PIN) and prostate cancer[64, 65], suggesting that *ERG* overexpression cooperates with other genetic alterations in prostate tumorigenesis. Consistent with this hypothesis, recent studies using mouse models indicated that loss of the tumor suppressor gene *PTEN* collaborates with *ERG* overexpression to develop PIN and prostate cancer[66, 67]. In addition, assessment of human prostate samples identified an association of *TMPRSS2-ERG* with deletion of chromosome 3p14, which includes the potential tumor suppressor genes *FoxP1*, *RYBP* and *SHQ1*, suggesting a potential new cooperation in prostate tumorigenesis[68].

In addition to AR-regulated ETS fusion genes, AR-driven non-ETS fusion genes also have a critical role in prostate cancer growth and invasion. For example, the AR target gene *SLC45A3* was reported to be fused with *BRAF*, a gene encoding a serine/threonine-specific protein kinase involved in mitogen-activated protein kinase (MAPK) signaling pathway, in less than 2% of prostate cancer patients. Overexpression of *SLC45A3-BRAF* in RWPE cells increases cell proliferation and invasion, and formation of small tumors in immunodeficient mice. While ETS fusion genes are considered as poor therapeutic targets, the function of the *SLC45A3-BRAF* fusion gene can be readily inhibited by RAF and mitogen-activated protein kinase kinase (MAP2K1) inhibitors, indicating that this fusion gene is a druggable target[58].

Mechanisms for AR-mediated gene fusion

In general, gene fusions require the spatial proximity of two genomic regions otherwise located far away on the genome, the formation of double strand DNA breaks (DSB), and error-prone DNA repair, which together might lead to illegitimate DNA recombination[46, 69]. In addition to the ability of AR to directly regulate expression of fusion genes, as described above, recent studies focusing on the *TMPRSS2-ERG* fusion have found that androgen and/or AR might contribute to the processes that drive gene fusion. For example, androgen stimulation increases spatial association of *TMPRSS2* and *ERG*. Inspired by the finding that estrogen is able to induce chromatin looping[38], two independent studies[61,70] found that dihydrotestosterone (DHT) treatment of LNCaP cells induces colocalization of *TMPRSS2* and *ERG*. Further studies found that this androgen-induced chromatin looping is mediated

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by AR, which is recruited to the *TMPRSS2* and *ERG* breakpoints upon DHT treatment[61, 62] (Table S1). In agreement with the notion that breakpoints are associated with active histone modifications[46], these AR binding regions at the *TMPRSS2* and *ERG* breakpoints are enriched in histone H3K79 methylation and H4K16 acetylation[61]. A recent study that combined prostate cancer whole genome sequencing data with VCaP ChIP-seq data[13] further revealed a genome-wide association of AR binding and active histone modifications (H3K4me3, H3K36me3 and H3Ac) near breakpoints in *TMPRSS2-ERG* positive patients[50]. These active histone modifications may facilitate AR binding and gene fusions.

In addition, DHT-bound AR recruits enzymes capable of inducing DSB. DHT stimulation has been found to induce AR-mediated recruitment of topoimerase II beta (TOP2B) to breakpoints[62], an enzyme producing transient DSB that are required for ER-[71, 72] and AR-regulated transcription[62]. Of note is also the observation that in the presence of exogenous genotoxic stress (e.g. irradiation), DHT treatment markedly increases the mRNA and protein expression of activation-induced cytidine deaminase (AID)[61], a lymphoid-specific enzyme that changes cytosine to uracil and may ultimately lead to DSB[73]. AR then recruits AID to chromatin via its coactivator Gadd45[61]. Along these lines, genotoxic stress also increases the expression of long interspersed nucleotide element-1 (LINE-1) retroelement encoded open reading frame 2(ORF2) endonuclease. While ORF2 is recruited to translocation regions in a DHT-dependent manner, no physical interaction between AR and ORF2 has been detected[61].

Finally, androgen stimulation increases recruitment of proteins involved in non-homologous end joining (NHEJ). There are two main pathways for DSB repair, namely the homologous recombination (HR) pathway and the NHEJ pathway. The error-prone NHEJ is the major pathway for the repair of DSB in eukaryotes[74]. It has been reported that upon DHT stimulation and increased DSB, several proteins involved in NHEJ including Ku70–Ku80 and ataxia telangiectasia-mutated protein (ATM), are recruited to the breakpoints, resulting in *TMPRSS2-ERG* fusion[61, 62]. Significantly, pharmacologic inhibitors and small interfering RNAs (siRNAs) targeting NHEJ pathway components have been shown to reduce the formation of *de novo TMPRSS2-ERG* gene fusion[62]. Based on these findings, a model for AR-mediated *TMPRSS2-ERG* fusion in prostate cancer is proposed (Figure 2). Evidence suggests that this model is generalizable to other AR-regulated fusion genes, such as *SLC45A3-ETV1*[61].

Concluding remarks and future directions

AR expression and functionality have been well documented in both ADPC and CRPC[2, 3]. Recent studies have found that the distal binding AR transcription complex, including AR and associated transcription factors and coactivators, regulates expression of several AR target genes involved in prostate cancer growth through chromatin looping. By using a global 3C assay, future studies should address whether such a long-range combinatorial regulation can be generalized to include other AR-dependent genes in the genome. This would allow for the identification of all AR direct target genes involved in prostate tumorigenesis and might lead to the development of new therapies for the disease. In addition to the looping mechanism, future studies should also examine whether other mechanisms for enhancer function (e.g. spreading and non-coding RNA[26]) participate in AR-mediated long-range gene regulation.

With regard to AR-mediated gene fusion, significant progress has been made in our understanding of the mechanisms of AR-driven chromatin looping that leads to gene fusions. The finding that inhibition of the NHEJ pathway reduces *TMPRSS2-ERG* gene fusion[62] suggest that future clinical trials may consider combining agents targeting both the

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AR and NHEJ pathway proteins. Despite progress on gene fusion mechanisms, it is still unclear on why gene fusions only occur in a limited number of genomic regions. It has been proposed that histone modifications, coactivators or noncoding RNAs may assist in the selection of these regions[75]. Future work should test these possibilities. Finally, as gene fusion at the RNA level without genomic rearrangement has recently been reported[59, 76, 77], future investigations should include whether AR has a role in driving the formation of such chimeric RNAs that also promote prostate carcinogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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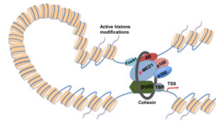

Figure 1.

A proposed looping model for AR-regulated gene expression in CRPC. Active histone modifications (e.g. H3K4me1 and H3K4me2) facilitate the binding of AR and its collaborating transcription factors (e.g. FoxA1) to distal nucleosome-depleted regions[12, 22]. p-MED1 mediates chromatin looping by facilitating interactions between distal transcription factors and the basal transcriptional apparatus[21, 39]. Other coactivators (e.g. p160 coactivators and p300[38]) may also enhance chromatin looping. Cohension may stabilize chromatin looping by embracing the loop[39]. AR, androgen receptor; H3K4me1 and H3K4me2, H3K4 mono- and di-methylation; p-MED1, PI3K/AKT phosphorylated MED1.

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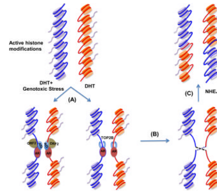

Figure 2.

AR-mediated *TMPRSS2-ERG* gene fusion in prostate cancer cells. **(A)** In the presence of genotoxic stress, AR binds to *TMPRSS2* and *ERG* breakpoints and recruits AID upon DHT treatment. Exposure of prostate cancer cells under genotoxic stress to DHT also increases ORF2 recruitment to breakpoints[61]. DHT stimulation only leads to AR-TOP2B complex loading on breakpoints[62]. **(B)** The recruitment of AID, ORF2 and TOP2B to breakpoints causes DSB[61, 62]. **(C)** NHEJ machinery is recruited to the breakpoints, leading to error-prone DSB repair and gene fusions such as *TMPRSS2-ERG* gene fusion[61, 62]. AID, activation-induced cytidine deaminase; DHT, dihydrotestosterone; ORF2, open reading frame 2; TOP2B, topoimerase II beta; NHEJ, non-homologous end joining.

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