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Inhibition of AR-mediated Transcription by Binding of Oct1 to a Motif enriched in AR-Occupied Regions

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Abstract

Background: The androgen receptor (AR) plays roles in prostate development and cancer (PCa). In response to androgens, the AR binds to androgen-response elements (AREs) to modulate gene transcription. The responses of such genes are dependent on the cellular milieu and on sequences around the AREs, which attract other transcription factors. Previously, bioinformatic analysis of 62 AR-occupied regions (ARORs) in PCa cells revealed enrichment for both AREs and a TTGGCAAATA-like motif. We undertook the present study to investigate the significance of the TTGGCAAATA-like motif.

Methods: Prostate cancer cell lines, LNCaP and C4-2B, were analyzed by transient transfections of wild type and mutant reporter constructs, electro-mobility shift assays (EMSAs), and RT-qPCR analysis of endogenous genes.

Results: In two of six tested ARORs, point mutations in the TTGGCAAATA-like motif resulted in inhibition of DHT-mediated enhancer activity. EMSA revealed that Oct1 bound the motif, and that the mutations that abolished DHT responsiveness in the transfection assays augmented Oct1 binding. These results suggest a role for Oct1 as a context-dependent negative coregulator of AR. Consistent with this, siRNA knockdown of Oct1 increased the DHT-mediated enhancer activity of transfected reporters as well as an endogenous AR target gene, transglutaminase 2.

Conclusions: Oct1 negatively regulates DHT-mediated enhancer activity in a subset of ARORs. The enrichment of ARORs for the Oct-binding, TTGGCAAATA-like motif may reflect a mechanism that utilizes Oct1 to keep AR activity in check at some ARORs, while augmenting AR activity in other ARORs. Therefore, Oct1 may have regulatory functions in prostate development and cancer progression.

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Keywords

prostate cancer; androgen receptor; transcription; repression; target genes; Oct1

Introduction

The androgen receptor (AR), a steroid-activated transcription factor belonging to the nuclear hormone receptor superfamily, is involved in normal prostate development and is the main driving force in prostate cancer (PCa) (1-7). AR activation by androgens causes receptor dimerization, translocation into the nucleus and genomic occupancy at androgen responsive elements (AREs) (8). Cofactors and coregulators collaborate with the AR to mediate subsequent transcriptional regulation of target genes. Cofactors bind to the AR, but not to DNA, and include coactivators [e.g. p160 family of proteins, mediator, ARA-54, -55 and -70, among many others, see review (9)] and co-repressors [e.g. SMRT and NCoR, (9)]. Coregulators, on the other hand, are DNA-binding proteins that recognize specific DNA sequences near the AR and modulate its activity either positively or negatively (10-15). Recent chromosome- and genome-wide location analyses of nuclear hormone receptors, particularly of AR and estrogen receptor (ER) have highlighted the existence of several such coregulators. For example, Carroll *et al* (11,12), showed that response elements for C/EBP, octamer transcription factor (Oct) and forkhead transcription factors cluster in ER-occupied regions. They also reported that FOXA1 facilitates ER-mediated transcription in MCF-7 breast cancer cells. Massie *et al* (14), found that AR-occupied promoters are enriched for ETS1-binding elements and that ETS-1 modulates AR activity at a subset of these promoters. Furthermore, Wang *et al* (15) identified GATA2 and Oct1 as AR coregulators, while the same group (13) later demonstrated that the combination of FoxA1 with AR and/or ER plays an important role in translating epigenetic signatures to enhancer-driven lineage-specific transcription. These findings indicate that an understanding of the roles of AR co-regulating transcription factors is critical to appreciate AR action in prostate biology.

The AR coregulator Oct1, also known as POU2F1, is a member of the POU homeodomain family of transcription factors. It is ubiquitously expressed and is involved in the regulation of a wide variety of processes, including embryogenesis (16). Several studies have demonstrated that Oct1 can also physically interact with various nuclear hormone receptors, including glucocorticoid receptor (GR), progesterone receptor (PR), retinoic receptor (RXR), thyroid hormone receptor (TR), estrogen receptor (ER) and AR (17). Interestingly, the associations between Oct1 and nuclear hormone receptors can impact their transcriptional activity either positively or negatively. For example, Oct1 interaction with the glucocorticoid receptor (GR) was required for transactivation of the mouse mammary tumor virus (MMTV) promoter (17). In contrast, GR sequestration of Oct1 prior to DNA binding led to histone H2b promoter repression (18). Oct1 also directly inhibits RXR-mediated transcription (19). AR/Oct1 interactions have been reported at the mouse Sex-limited protein (Slp) enhancer (20,21); this interaction was found to be DNA-dependent and led to the recruitment of the coactivator SRC-1 and to increased transcriptional activity (22). On the other hand, testosterone inhibited expression of the muscle atrophy factor MAFbx via AR interaction with Oct1 in cultured muscle cells (23). The molecular mechanisms causing the diverse transcriptional modulation by Oct1 are probably related to its DNA-binding domain, which is capable of forming varying DNA sequence-dependent dimer arrangements, allowing for different cofactor and coregulator assemblies (24-26).

We recently mapped 62 ARORs in C4-2B advanced prostate cancer cells (27). These ARORs were not only enriched for AREs. *De novo* motif finding revealed that they were also enriched for a motif conforming to the consensus TTGGCAAATA, with 26 of the 62

ARORs having at least one such motif (27). Furthermore, the presence of TTGGCAAATA-like motifs was highly correlated with DHT-mediated enhancer activity of the ARORs in reporter assays; a 3.2-fold enrichment of the TTGGCAAATA-like motifs was evident in 18 ARORs most responsive to DHT compared to 19 least responsive ones (27). These results implicate the TTGGCAAATA-like motif, and its corresponding binding proteins, in AR-mediated gene regulation. In the present study we found that Oct1 bound the TTGGCAAATA-like motifs and that augmentation of this binding repressed AR activity in two ARORs, one of which mediated DHT-responsiveness of the nearby transglutaminase 2 gene.

Materials and Methods

Cells and Reagents

C4-2B cells were obtained from ViroMed Laboratories (Minneapolis, MN) and LNCaP from ATCC (<http://www.atcc.org/Home/tabid/57/Default.aspx>). The cells were maintained in RPMI 1640 supplemented with 5% (v/v) fetal bovine serum (FBS). Monoclonal anti-Oct1 antibodies were obtained from Abcam Inc. (Cambridge, MA). Pre-designed SMARTpool siRNA reagents against Oct1 and nonspecific siRNA were purchased from Dharmacon (Lafayette, CO). PNS2 CMV-Oct1 was a kind gift from Dr. D.M. Robins (Michigan Medical School, MI).

Construction of Plasmids and Mutagenesis

Cloning of ARORs upstream of the thymidine kinase (TK) minimal promoter-luciferase vector has previously been described (27). Briefly, the AROR sequences (an average of 500bp) were PCR amplified from C4-2B genomic DNA and subcloned upstream of the TK-luciferase cassette. The primers for subcloning are listed in Supplemental Table 1. To generate mutations into the AROR-associated TTGGCAAATA-like motif in selected AROR-luc constructs we utilized PCR mutagenesis or the Quick Change II Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Mutations were verified by sequencing. The primers used to engineer the mutations are listed in Supplemental Table 1.

Luciferase Assay

C4-2B cells (1×10^5 per 1-cm well) or LNCaP cells (2.5×10^5 per 1-cm well) were cultured for 2 days in 12-well plates in RPMI 1640 containing 5% charcoal-stripped serum (CSS). Cells were transfected with 1 μ g/well of the indicated luciferase reporter plasmids using Lipofectamine LTX Reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol. Three hours post transfection, cells were treated with DHT (10 nM) or ethanol vehicle (0.01%) in 5% CSS for 16 hrs. Cells were then lysed in 250 μ l 1x passive lysis buffer (Promega Madison, WI) and luciferase activity was measured using the luciferase assay system (Promega).

Protein Extractions

C4-2B cells were grown in 100mm dishes in RPMI-1640 containing 5% CSS for two days followed by 10nM DHT treatment for 4hrs. Nuclear extracts were obtained by processing the cells with the Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to manufacturer's instructions. Protein concentration was measured using Micro BSA Protein Assay Kit (Pierce, Rockford, IL). Aliquots of nuclear extracts were kept at -80°C until further use in EMSAs.

EMSA

C4-2B cells were grown in 100 mm dishes in RPMI-1640 containing 5% CSS for two days followed by 10 nM DHT treatment for 4hrs. Nuclear extracts were obtained using the Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Protein concentration was measured using Micro BSA Protein Assay Kit (Pierce, Rockford, IL). EMSA was performed as previously described (28) with the oligonucleotides listed in Supplemental Table 1. Briefly, reactions containing 15 µg nuclear extracts from 4 hr DHT-stimulated C4-2B cells were incubated on ice in the presence of salmon sperm DNA, 28% glycerol and/or antibodies and/or unlabeled excess competition oligonucleotides as indicated. The binding reaction was initiated by adding the ³²P-labeled oligonucleotides as probes along with binding buffer followed by incubation for 15 minutes at room temperature and PAGE for 3 hrs at 200V. The gels were dried and exposed to a phospho-screen for 2 days followed by image acquisition by a phospho-imager.

Transfections

C4-2B cells (1×10^5 per 1-cm well) were grown in 12-well plates using phenol red-free RPMI 1640 containing 5% CSS for 2 days. To silence Oct1, cells were transfected with 100 nM siRNA duplexes and 1 µg/well of the indicated luciferase reporters using lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions. After transfection, cells were grown in phenol red-free RPMI 1640 containing 5% CSS for 48 hrs and then treated with DHT (10 nM) or ethanol vehicle for additional 18 hrs. For Oct1 over-expression analyses, C4-2B (1×10^5 cells/well) were grown in 12-well plates using phenol red-free RPMI-1640 containing 5% CSS for 2 days. Cells were subsequently transfected in triplicates with 0, 0.1, 0.3, 1.0 and 3.0 µg of a human pCMV-Oct1 (29), using Lipofectamine LTX reagent according to manufacturer's protocol. pCAT Basic and pCDNA3.1 were used to balance the absolute and molar amounts of DNA in the transfection mixtures. Three hours post transfection, cells were treated with 10 nM DHT or ethanol vehicle for 16 hrs, followed by analysis of gene expression.

RT-qPCR

RNA was extracted using Aurum Total RNA Kit (Bio-Rad, Hercules, CA). cDNA was prepared from 1 µg of RNA using iScript cDNA Synthesis Kit (Bio-Rad), and qPCR was conducted using SYBR Green PCR Master Mix (Applied Biosystems, Branchburg, NJ) and the primers listed in Supplemental Table 1.

Results

Contribution of AROR-associated TTGGCAAATA-like motifs to androgen responsiveness

We have recently found a motif, 5'-TTGGCAAATA-3' (Figure 1A), which was enriched in ARORs in the C4-2B prostate cancer cell line, and especially in ARORs that conferred responsiveness to DHT in transient transfection luciferase assays (27). In many cases, the TTGGCAAATA-like motif was adjacent to an ARE (27). To determine whether this motif contributes to AR-mediated transactivation, we mutated the motif in a number of AROR-luciferase reporters (Figure 1B) chosen based on the similarity of the wild type motif to the TTGGCAAATA consensus sequence (Figure 1A). In each case, the ARE adjacent to the TTGGCAAATA-like motif was left intact. The DHT responsiveness of the TTGGCAAATA-mutants was compared to the respective wild type AROR reporters (Figure 1C).

Initially, we mutated all ten bases of the TTGGCAAATA-like motif in the context of two ARORs, A020 and A078 (27). As shown in Figure 1C, these block mutations, designed to destroy interaction with any transcription factor with the wild type motif, inhibited DHT-

mediated transactivation at AROR A020, suggesting that the TTGGCAAATA-like motif recruits a coregulatory factor that positively interacts with the AR. However, the block mutation had no effect on DHT action at AROR A078. This could result from the presence of both positive and negative response elements within the TTGGCAAATA motif, mutation of both would eliminate the two opposing functions. We therefore proceeded with more subtle mutations. Furthermore, considering the possibility that the TTGGCAAATA motif may function differently in different contexts, we introduced the subtle mutations in different ARORs, A107, A046 and A080. In each of these cases, we mutated the nucleotides at positions 2 and 7, which constitute the second base of a TTnG sequence within the TTGGCAAATA consensus. In the case of A107 and A046, these mutations had no or little effect on DHT responsiveness (Figure 1C). However, the two-nucleotide mutation in A080 resulted in extensive loss of the DHT-mediated transcriptional enhancement, from 25-fold to 3.7-fold (Figure 1C). Similar results (not shown) were obtained in the LNCaP prostate cancer cell line, from which the C4-2B line was derived (30).

We were particularly interested in AROR A156 (27), located 55-kb upstream of the transcription start site of transglutaminase 2 (TGM2), a gene whose androgen responsiveness may contribute to prostate cancer progression (31-33). Because this AROR is exceptionally long (1360bp) (27), and because it contains two TTGGCAAATA-like motifs, each adjacent to an ARE (27), we first constructed two luciferase reporters, A156a and A156b, each with one TTGGCAAATA-like motif and one ARE (see Materials and Methods). Transient transfection assays showed that A156b was not responsive to DHT, whereas A156a was strongly responsive (Figure 1C). Because position 7 in the TTGGCAAATA-like motif of A156a is already dissimilar to the consensus, we introduced in this case mutations at positions 2 and 8. These mutations abrogated the DHT-responsiveness of A156a from 22-fold to only 2.1-fold in C4-2B cells (Figure 1C) with similar results obtained in LNCaP cells (not shown). Thus, although mutation of the TTGGCAAATA-like motif did not strongly influence responsiveness of the AROR to DHT in every case, we have identified two ARORs in which intact TTGGCAAATA-like motifs appear to be critical for DHT responsiveness and therefore may function as a critical response element for one or more transcription factors.

Mutations in the TTGGCAAATA-like motif that abolish DHT responsiveness lead to increased Oct1 binding

The two point mutations in the TTGGCAAATA-like motifs within each of ARORs A080 and A156a could have disrupted interaction with a modulating transcription factor that positively influences AR activity, or they could enhance interaction with a modulator that negatively influences the AR. To address these possibilities, we compared protein/DNA complexes formed in EMSA using oligonucleotide probes centered on the TTGGCAAATA-like motif or the respective mutant sequence. As shown in Figure 2A-C, the A080- and A156a-derived probes formed similar, slow-migrating complexes with a C4-2B nuclear protein(s), and in both cases formation of this topmost complex was much more robust with the mutant probe. Similar results were obtained with LNCaP nuclear extracts (data not shown). We also performed EMSA with C4-2B nuclear extracts and probes derived from ARORs A046 and A107, where DHT responsiveness in luciferase assays was not affected by the point mutations in the TTGGCAAATA-like motifs as shown in Figure 1C. Each of these probes formed a slow migrating protein/DNA complex similar to that observed with A080 and A156a (Figure 2D). However, in these cases, the two point mutations did not enhance, but instead diminished the protein/DNA interaction. These results suggest that the TTGGCAAATA-like motif constitutes a binding site(s) for a protein(s) that modulate AR activity in a context-dependent manner.

Because the logo representing the TTGGCAAATA-like sequences in our previous study (27) contained elements reminiscent of Oct1, FOXA1 and NF-1 binding sites (Figure 1A), we pursued identification of the band that intensified upon mutagenesis (Figure 2), initially using competition assays with oligonucleotide containing consensus binding sites for these transcription factors. The A080-derived sequence was first used as probe in EMSA. As seen in Figure 3A, the topmost complex was most strongly competed with an Oct1 consensus oligonucleotide whereas a similar oligonucleotide harboring mutations in the consensus Oct1 site did not compete for complex formation. Oligonucleotides with consensus binding sites for FoxA1 or NF-1 did not compete for formation of this topmost complex (Figure 3A). Because Oct1 is highly expressed in prostate cancer (15), including in the C4-2B cell line (27), we tested by supershift analysis whether the topmost complex formed with the A080-derived probe indeed contained Oct1. As shown in Figure 3B, addition of anti-Oct1 antibodies in the EMSA resulted in complete elimination of the topmost complex and the appearance of a faint band with slower migration (indicated by asterisks). This did not occur when the Oct1 antibody was denatured by boiling prior to the EMSA (Figure 3B). Furthermore, we confirmed using the same antibodies that the intensified band observed with the A080 mutant probe also contained Oct1 (Figure 3C). Together, the reporter analyses and the EMSA of the wild type and mutant A080 suggest that Oct1 plays a negative role in AR-mediated transcriptional activity at this AROR.

We next performed a similar EMSA to test whether the two point mutations, which abrogated DHT-responsiveness of A156a (Figure 1C) and resulted in enhanced complex formation (Figure 2B), also intensified the interaction with Oct1. Indeed, the topmost complex formed with the A156a mutant probe was supershifted by Oct1 antibodies (Figure 3D). Thus, increased Oct1 binding again appears to correlate with dampened DHT responsiveness.

The main complex formed on the A156a-derived probes, either wild type (Figure 2B) or mutant (Figure 3D), was relatively fast migrating and was neither Oct1, nor FoxA1 nor NF1; instead this complex appears to be GATA-related (Supplemental Figure S1). The functional significance of GATA to AR-mediated transcriptional enhancement has been recently studied (15) and is beyond the scope of the present work.

siRNA Knockdown of Oct1 Augments AR-mediated Transcriptional Enhancement

We next examined the role of Oct1 in modulating DHT responsiveness by manipulating Oct1 cellular levels. We refrained from an over-expression approach because we found that DHT treatment inhibited endogenous Oct1 expression, and even more strongly suppressed Oct1 mRNA transcribed from a transiently transfected vector (Figure 4A). Instead, we employed an Oct1 siRNA knockdown approach. We hypothesized that if Oct1 negatively modulated AR-mediated transcriptional enhancement, reduced Oct1 expression would augment DHT responsiveness. Indeed, siRNA-mediated depletion of Oct1 (Figure 4B) resulted in augmented DHT responsiveness of the co-transfected A080-luc (Figure 4C). The effect of siOct1 was specific for DHT-mediated transcriptional enhancement, because the basal luciferase activity in the absence of DHT was not affected by the siOct1 treatment (Figure 4C). Essentially the same results were obtained with A156a-luc (Figure 4D), confirming that in C4-2B cells, Oct1 negatively modulates AR-mediated transcriptional enhancement at least in a subset of ARORs. Furthermore, RT-qPCR analysis of the endogenous TGM-2 gene, whose transcription start site (TSS) resides 55-kb downstream of the A156a AROR (Figure 4E), revealed increased TGM-2 mRNA levels in cells treated with DHT and Oct1 siRNA versus DHT and control siRNA (Figure 4F). Figure 4G shows that expression of the DHT non-responsive C20RF77 gene, whose TSS resides 76-kb from AROR A156 (Figure 4E) was not influenced by the Oct1 siRNA knockdown.

Finally, we attempted to mimic the stimulatory effect of the Oct1 knock-down on the enhancer activity of AROR A080 by mutating the Oct1 response element in the AROR. Specifically, we altered the AAA in the TTGGCAAATA-like motif to TTT. However, this mutation, although destroying the Oct1 binding in EMSA (Figure 4H), had no impact on DHT-mediated transcriptional enhancement in luciferase assays (Figure 4I, and see discussion).

Discussion

Many auxiliary proteins modulate the activity of steroid hormone receptors, once bound to DNA. Some of them are cofactors (coactivators and corepressors) that bind to the receptors or receptor complexes but not to DNA [for review see (9)]. Others are transcriptional coregulators (positive- and negative-acting transcription factors) that bind to DNA elements in the near vicinity of the steroid hormone receptor and may or may not physically interact with the steroid receptor (10-15). In the work presented here we report that Oct1 acts as an AR coregulator by repressing AR-dependent transcriptional activity at two enhancers, so-called A080 and A156a. Leading to this conclusion, point mutations in the TTGGCAAATA-like motifs in the two enhancers caused a dramatic inhibition of DHT-mediated enhancer activity in reporter assays, concomitant with equally dramatic increase in binding of Oct1 to the respective mutant sequences in EMSA. Furthermore, siRNA knockdown of Oct1 augmented the DHT-mediated enhancer activity of the respective ARORs, as well as the DHT-mediated increase of TGM2 expression, which is likely regulated by a nearby A156 AROR (see Figure 4E). We conclude that Oct1 has the ability to repress AR-mediated transcriptional enhancement of some genes.

The present and other studies (20,21) demonstrate that AR and Oct1 collaborate in the regulation of gene expression. It is also known that they physically interact with each other (22,34). Several mechanisms could account for the Oct1-related repression of DHT-mediated transcriptional activation described in the present paper. First, by binding to its cognate site, Oct1 could be positioned in close proximity to the nearby bound AR, so that AR coactivators such as p160 proteins are no longer able to support AR-mediated transactivation. Alternatively, Oct1 binding may recruit corepressors to the site. Such cofactor-related mechanisms have been proposed for the AR-mediated, testosterone-dependent inhibition of the muscle atrophy factor MAFbx in muscle cells (23). On the other hand, Oct1-related positive regulation of AR-mediated *Slp* expression in mice has been demonstrated to involve the recruitment of p160 coactivator SRC-1 to the site (34). Despite the evidence for such cofactor-related mechanisms in some cases, one should not necessarily extrapolate that Oct1 binding will always have the same effect on a nearby bound AR. Indeed, block mutation of the TTGGCAAATA-like motif, including the Oct1-like site, in ARORs A078, A107 and A046 did not significantly affect DHT-mediated transcriptional activity. The more specific, AAA → TTT mutation of the Oct1-like site in AROR A080 also did not affect DHT-mediated responsiveness. Furthermore, deletion of the Oct1-binding site at the PSA enhancer did not augment, but instead compromised DHT-mediated transcriptional activity, indicating an Oct1 requirement for AR-mediated activation in this case (15); a similar phenomenology occurs at the A020 AROR (Figure 1C). However, the novel findings reported here indicate yet another possible mechanism where increased affinity of Oct1 to cognate sites near AREs virtually shuts down DHT-dependent transcriptional activity. Although this was exemplified here by mutagenesis of the binding site, strong binding of Oct1 near otherwise functional AREs could occur under pathological conditions at the same sites and under physiological conditions at other sites. Be that as it may, high affinity binding of Oct1 could sterically preclude AR or other obligate factors from assembling and interacting with the transcriptional preinitiation complex.

It is possible that the two point mutations introduced in each of the A080 and A156a ARORs exaggerated a situation already partially existing at the two relevant ARORs. In support of this notion, DHT-mediated enhancer activity of both ARORs was augmented upon siRNA-knockdown of Oct1. However, mutation of the AAA within the Oct1 element of A080 (to TTT) did not impact DHT responsiveness of this AROR. One interpretation of this data is that the AAA → TTT mutation eliminated binding of both Oct1 and a transcription factor that influences the nearby ARE in a manner opposite to Oct1. However, EMSA did not support the existence of such a transcription factor, and specific competition with FoxA1 or NF1-binding oligonucleotides did not suggest binding of these specific proteins to the TTGCAAATA-like motif of A080. Therefore, we prefer another interpretation to explain the lack of change in A080 DHT-responsiveness after elimination of Oct1 binding by the AAA → TTT mutation. We propose that under the experimental condition employed, the affinity of Oct1 to the wild type A080 sequence is too low to influence AR action at this AROR. Instead, under these conditions, Oct1 influences AR activity in a DNA-binding-independent manner, via direct protein-protein interaction. By the same token, the increased DHT-mediated expression of the nearby gene TGM2 after siRNA knockdown of Oct1 (Figure 4F) was achieved in the face of barely detectable binding (indicating low affinity) of Oct1 to the wild-type A156a sequence (EMSA analysis, Figure 2 B & C) and lack of demonstrable Oct1 occupancy by ChIP at A156 [Figure 5 reference (27)]. Although the latter could result from low antibody sensitivity, or even significant epitope masking at the site, it could simply reflect low occupancy that is much dependent on interaction with the AR, not with DNA. In the *in vivo* situation, even low Oct1 occupancy through the AR may have significant consequences as it may facilitate histone modifications (e.g. acetylation) necessary for efficient AR-mediated gene expression (35). Overall, our results indicate that perhaps at many sites Oct1 subtly keeps AR-mediated signaling in check to achieve nuanced modulatory responses to androgens (see below). Taken together it is also clear that context-dependent effects of Oct1 may segregate AR target genes into subgroups with respect to their responses related to the combined Oct1 and AR transcriptional activities. This is consistent with findings that the specific architecture of the octamer motif regulates the function of Oct transcription factors, enabling them to either activate or repress transcription in a context dependent manner (25).

We further propose that specific DNA sequences present at different ARORs result in site-specific interplay between the AR, Oct1, and other transcription factors (both DNA-binding and non-DNA-binding), such that the contribution of each factor to overall enhancer activity is different in each case. Such combinatorial modulation of DHT action would facilitate the integration of androgen signaling with other developmental and environmental cues, to produce individualized physiological response to androgens in a manner dependent on specific contexts, such as age, tissue and nutrition state. Since our previous work (27) has indicated that AR occupancy at many genomic sites does not necessarily lead to gene modulation under all physiological conditions, coregulators such as Oct1 may become rate-limiting for AR-mediated effects. Therefore the traditional premise that AR occupancy is necessary and sufficient for gene activation is probably incorrect.

Similar to the combinatorial regulation of AR target genes in health, such genes are most likely regulated in a combinatorial and dynamic manner during prostate cancer progression. This notion has important implications to targeting Oct1 (and other AR modulators) for PCa therapy. Because of changes in the status of AR coregulators and cofactors during different stages of the disease, we believe that drugs targeting AR/Oct1 interaction may be beneficial only during specific stages of disease progression. Furthermore, we envision that cells may alter the expression of AR coregulators and cofactors in response to such therapy. Consequently, further work is required to unravel the very complicated network of AR-mediated responses, which probably evolved to yield integrated responses to androgens, but

at the same time add to the complexity of rational intervention strategies in diseases such as PCa.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Balk S. Androgen receptor as a target in androgen-independent prostate cancer. *Urology*. 2002; 60(3 Suppl 1):132–138. [PubMed: 12231070]
2. Buchanan G, Irvine RA, Coetzee GA, Tilley WD. Contribution of the androgen receptor to prostate cancer predisposition and progression. *Cancer Metastasis Rev*. 2001; 20(3-4):207–223. [PubMed: 12085963]
3. Chen CD, Welsbie DS, Tran C, Baek SH, Chen R, Vessella R, Rosenfeld MG, Sawyers CL. Molecular determinants of resistance to antiandrogen therapy. *Nat Med*. 2004; 10(1):33–39. [PubMed: 14702632]
4. Debes JD, Tindall DJ. Mechanisms of androgen-refractory prostate cancer. *N Engl J Med*. 2004; 351(15):1488–1490. [PubMed: 15470210]
5. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. *Nat Rev Cancer*. 2001; 1(1):34–45. [PubMed: 11900250]
6. Scher HI, Buchanan G, Gerald W, Butler LM, Tilley WD. Targeting the androgen receptor: improving outcomes for castration-resistant prostate cancer. *Endocr Relat Cancer*. 2004; 11(3):459–476. [PubMed: 15369448]
7. Scher HI, Sawyers CL. Biology of progressive, castration-resistant prostate cancer: directed therapies targeting the androgen-receptor signaling axis. *J Clin Oncol*. 2005; 23(32):8253–8261. [PubMed: 16278481]
8. Gelmann EP. Molecular biology of the androgen receptor. *J Clin Oncol*. 2002; 20(13):3001–3015. [PubMed: 12089231]
9. Heemers HV, Tindall DJ. Androgen Receptor (AR) Coregulators: A Diversity of Functions Converging on and Regulating the AR Transcriptional Complex. *Endocr Rev*. 2007
10. Bolton EC, So AY, Chaivorapol C, Haqq CM, Li H, Yamamoto KR. Cell- and gene-specific regulation of primary target genes by the androgen receptor. *Genes Dev*. 2007; 21(16):2005–2017. [PubMed: 17699749]
11. Carroll JS, Liu XS, Brodsky AS, Li W, Meyer CA, Szary AJ, Eeckhoutte J, Shao W, Hestermann EV, Geistlinger TR, Fox EA, Silver PA, Brown M. Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. *Cell*. 2005; 122(1):33–43. [PubMed: 16009131]
12. Carroll JS, Meyer CA, Song J, Li W, Geistlinger TR, Eeckhoutte J, Brodsky AS, Keeton EK, Fertuck KC, Hall GF, Wang Q, Bekiranov S, Sementchenko V, Fox EA, Silver PA, Gingeras TR, Liu XS, Brown M. Genome-wide analysis of estrogen receptor binding sites. *Nat Genet*. 2006; 38(11):1289–1297. [PubMed: 17013392]

13. Lupien M, Eeckhoutte J, Meyer CA, Wang Q, Zhang Y, Li W, Carroll JS, Liu XS, Brown M. FoxA1 translates epigenetic signatures into enhancer-driven lineage-specific transcription. *Cell*. 2008; 132(6):958–970. [PubMed: 18358809]
14. Massie CE, Adryan B, Barbosa-Morais NL, Lynch AG, Tran MG, Neal DE, Mills IG. New androgen receptor genomic targets show an interaction with the ETS1 transcription factor. *EMBO Rep*. 2007
15. Wang Q, Li W, Liu XS, Carroll JS, Janne OA, Keeton EK, Chinnaiyan AM, Pienta KJ, Brown M. A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth. *Mol Cell*. 2007; 27(3):380–392. [PubMed: 17679089]
16. Phillips K, Luisi B. The virtuoso of versatility: POU proteins that flex to fit. *J Mol Biol*. 2000; 302(5):1023–1039. [PubMed: 11183772]
17. Prefontaine GG, Walther R, Giffin W, Lemieux ME, Pope L, Hache RJ. Selective binding of steroid hormone receptors to octamer transcription factors determines transcriptional synergism at the mouse mammary tumor virus promoter. *J Biol Chem*. 1999; 274(38):26713–26719. [PubMed: 10480874]
18. Kutoh E, Stromstedt PE, Poellinger L. Functional interference between the ubiquitous and constitutive octamer transcription factor 1 (OTF-1) and the glucocorticoid receptor by direct protein-protein interaction involving the homeo subdomain of OTF-1. *Mol Cell Biol*. 1992; 12(11):4960–4969. [PubMed: 1406672]
19. Kakizawa T, Miyamoto T, Ichikawa K, Kaneko A, Suzuki S, Hara M, Nagasawa T, Takeda T, Mori J, Kumagai M, Hashizume K. Functional interaction between Oct-1 and retinoid X receptor. *J Biol Chem*. 1999; 274(27):19103–19108. [PubMed: 10383413]
20. Scarlett CO, Robins DM. In vivo footprinting of an androgen-dependent enhancer reveals an accessory element integral to hormonal response. *Mol Endocrinol*. 1995; 9(4):413–423. [PubMed: 7659085]
21. Scarlett CO, Scheller A, Thompson E, Robins DM. Involvement of an octamer-like sequence within a crucial region of the androgen-dependent Slp enhancer. *DNA Cell Biol*. 1997; 16(1):45–57. [PubMed: 9022044]
22. Gonzalez MI, Robins DM. Oct-1 preferentially interacts with androgen receptor in a DNA-dependent manner that facilitates recruitment of SRC-1. *J Biol Chem*. 2001; 276(9):6420–6428. [PubMed: 11096094]
23. Zhao W, Pan J, Wang X, Wu Y, Bauman WA, Cardozo CP. Expression of the muscle atrophy factor MAFbx is suppressed by testosterone. *Endocrinology*. 2008
24. Remenyi A, Tomilin A, Pohl E, Lins K, Philippsen A, Reinbold R, Scholer HR, Wilmanns M. Differential dimer activities of the transcription factor Oct-1 by DNA-induced interface swapping. *Mol Cell*. 2001; 8(3):569–580. [PubMed: 11583619]
25. Remenyi A, Tomilin A, Scholer HR, Wilmanns M. Differential activity by DNA-induced quaternary structures of POU transcription factors. *Biochem Pharmacol*. 2002; 64(5-6):979–984. [PubMed: 12213595]
26. Tomilin A, Remenyi A, Lins K, Bak H, Leidel S, Vriend G, Wilmanns M, Scholer HR. Synergism with the coactivator OBF-1 (OCA-B, BOB-1) is mediated by a specific POU dimer configuration. *Cell*. 2000; 103(6):853–864. [PubMed: 11136971]
27. Jia L, Berman B, Jariwala U, Yan X, Cogan JP, Walters Allison, Chen T, Buchanan G, Frenkel B, Coetzee GA. Genomic Androgen Receptor-occupied Regions with Different Functions, Defined by Histone Acetylation. *Coregulators and Transcriptional Capacity Submitted*. 2008
28. Luppen CA, Leclerc N, Noh T, Barski A, Khokhar A, Boskey AL, Smith E, Frenkel B. Brief bone morphogenetic protein 2 treatment of glucocorticoid-inhibited MC3T3-E1 osteoblasts rescues commitment-associated cell cycle and mineralization without alteration of Runx2. *J Biol Chem*. 2003; 278(45):44995–45003. [PubMed: 12933820]
29. Segil N, Roberts SB, Heintz N. Mitotic phosphorylation of the Oct-1 homeodomain and regulation of Oct-1 DNA binding activity. *Science*. 1991; 254(5039):1814–1816. [PubMed: 1684878]
30. Thalmann GN, Anezinis PE, Chang SM, Zhau HE, Kim EE, Hopwood VL, Pathak S, von Eschenbach AC, Chung LW. Androgen-independent cancer progression and bone metastasis in the

- LNCaP model of human prostate cancer. *Cancer Res.* 1994; 54(10):2577–2581. [PubMed: 8168083]
31. Boehm JE, Singh U, Combs C, Antonyak MA, Cerione RA. Tissue transglutaminase protects against apoptosis by modifying the tumor suppressor protein p110 Rb. *J Biol Chem.* 2002; 277(23):20127–20130. [PubMed: 11956182]
 32. Mangala LS, Fok JY, Zorrilla-Calancha IR, Verma A, Mehta K. Tissue transglutaminase expression promotes cell attachment, invasion and survival in breast cancer cells. *Oncogene.* 2007; 26(17):2459–2470. [PubMed: 17043648]
 33. Mehta K, Fok J, Miller FR, Koul D, Sahin AA. Prognostic significance of tissue transglutaminase in drug resistant and metastatic breast cancer. *Clin Cancer Res.* 2004; 10(23):8068–8076. [PubMed: 15585642]
 34. Gonzalez MI, Tovaglieri A, Robins DM. Androgen receptor interactions with Oct-1 and Brn-1 are physically and functionally distinct. *Mol Cell Endocrinol.* 2002; 190(1-2):39–49. [PubMed: 11997177]
 35. Jia L, Shen HC, Wantroba M, Khalid O, Liang G, Wang Q, Gentzsch E, Pinski JK, Stanczyk FZ, Jones PA, Coetzee GA. Locus-wide chromatin remodeling and enhanced androgen receptor-mediated transcription in recurrent prostate tumor cells. *Mol Cell Biol.* 2006; 26(19):7331–7341. [PubMed: 16980632]

Figure 1. TTGGCAAATA-like motif logo, endogenous sequences within ARORs and consequences of mutations on DHT-responsiveness

A) Logo representing the TTGGCAAATA-like AROR-associated motifs (27). Within this logo are sequences resembling binding sites for the indicated transcription factors. B) Schematic representation of the AROR containing the TTGGCAAATA-like sequence and ARE upstream from the basal TK-driven luciferase reporter. C) Luciferase assays were conducted with ARORs (column 1) cloned upstream of a minimal thymidine kinase (TK) promoter (see Materials and Methods) with the wild type TTGGCAAATA-like sequences or mutants thereof (column 2). Bold lower case letters indicates the mutations. C4-2B cells were grown in 5% charcoal stripped serum (CSS) for 2 days followed by transient transfections of AROR-TK-luc constructs and 10nM DHT or ethanol (0.01%) treatment for 16 hrs. Bars in column 3 represent fold change in luciferase activity (DHT/Ethanol) (Mean $\pm$ SD; n $\geq$ 3 independent transfections). Dotted vertical line depicts 1-fold. Results are from one representative of 3 independent experiments.

Figure 2. Mutations that diminish DHT-responsiveness augment interaction with a TTGGCAAATA-binding protein

EMSAs were conducted with nuclear extracts from C4-2B cells that had been treated with 10 nM DHT for 4 hrs. Probes were 30-bp long, centered on the wild type or mutant TTGGCAAATA-like motifs (see Figure 1C) from AROR A080 (A & C) and A156a (B & C), A046 and A107 (D). Arrowhead indicates the slow-migrating, top-most complex that was enhanced upon mutation of the TTGGCAAATA-like motif in A080 and A156, but not in A046 or A107. C) Side-by-side EMSAs preformed as in A and B demonstrating co-migration of the topmost complexes formed with the A080 and A156a probes. This gel was overexposed to clearly demonstrate the top-most complex formed with the A156a wild-type probe. WT = wild type, MUT = mutant, -NE = no nuclear extracts, +NE = with nuclear extracts. Results are representative of at least 3 experiments.

Figure 3. Oct1 is the protein whose binding to the TTGGCAATA-like motif increases upon mutagenesis that decreases DHT-responsiveness

A) EMSA was performed with the WT A080 probe and C4-2B nuclear extracts. Competition assays employed oligonucleotides containing consensus-binding sites for Oct1, FOXA-1 and NF-1 (WT, wild type), or similar oligonucleotides where these sites were mutated (MUT). B & C) Supershift assays were performed by conducting EMSAs in the same manner as above using either the A080 TTGGCAAATA-like motif (B), or the mutant (C) in the presence of monoclonal antibodies against Oct1. D) Supershift analysis performed as in B and C, showing that the topmost complex formed with the AROR 156a mutant sequence is Oct1. -NE=no nuclear extracts, NE = nuclear extracts, Ab. = antibody, B=boiled antibody.

Figure 4. siRNA-mediated inhibition of Oct1 augments DHT-mediated enhancer activity and AROR-associated gene expression

A, B) C4-2B cells grown for two days in 5% CSS containing RPMI-1640 were transfected with either the indicated amounts of the pNS2-CMV-Oct1 expression vector (**A**) or with 100 nM siRNA duplexes, either non-specific (n.s.) or directed against Oct-1 (**B**). Cells were then treated for 18 hrs with either 10 nM DHT (black bars) or ethanol vehicle (0.01%; white bars) and Oct1 mRNA levels were quantified using RT-qPCR and normalized to 18s rRNA levels. Values are mean $\pm$ SD ($n=3$), where basal Oct1 mRNA level is defined as 1. **C, D)** Cells were transfected with the non-specific and Oct1-specific siRNA as in **B**, along with either A080-TK-Luc (**C**) or A156a-TK-Luc (**D**). They were treated with ethanol (white bars) or 10nM DHT (black bars) for 16 hrs, followed by measurement of luciferase activity. Values are depicted as fold change (DHT/Ethanol $\pm$ SD, $n = 3$). **E)** Genomic map showing two endogenous genes (TGM2 and C20ORF77) close to A156. **F, G)** RNA was collected from cultures transfected and treated as in **B**, and the mRNA levels of TGM2 (**F**) and C20ORF77 (**G**) were examined by RT-qPCR. Values were normalized to 18S rRNA and are mean $\pm$ SD of triplicate transfections from one representative of at least 3 independent experiments. **H)** EMSA was performed with the WT A080 (TTTGCAAAGA), mutant A080 (TaTGCAAtAGA, with mutations in lowercase, see Figure 1) and mutant1 A080 (TTTGCAAttGA) using 30 bps-long radiolabeled probes and C4-2B nuclear extracts treated with 10nM DHT for 4hrs. The arrowhead points to the Oct1 band, which is gained in A080mutant (MUT) and lost in A080 mutant1 (MUT1). –NE=no nuclear extracts, NE = nuclear extracts, **I)** Luciferase assays were conducted with A080, A080 mut and A080 mut1 containing ARORs cloned upstream of a minimal-TK promoter (see Materials and Methods for A080 mutant1 construction). C4-2B cells were grown in 5% charcoal stripped serum (CSS) for 2 days followed by transient transfections of AROR-TK-luc constructs and 10nM DHT or ethanol (0.01%) treatment for 16 hrs. Bars in column 3 represent fold change in luciferase activity (DHT/Ethanol).