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Receptor-Mediated Transcriptional Regulation

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13. ABSTRACT (Maximum 200 Words) Recent studies have implicated N-CoR and SMRT in repression by antagonist-bound AR, and corepressors modulate the action of various antiandrogens in a different way. For example, it has been reported that SMRT binds to AR only after treatment with the progestagenic antiandrogen cyproterone acetate but not in the presence of nonsteroidal antagonists hydroxyflutamide or bicalutamide. What is even more intriguing is the finding that both N-CoR and SMRT are negative regulators of agonist bound AR transcriptional activity. Consistent with this, our recent study confirmed that both N-CoR and SMRT were recruited to the endogeneous promoter region of the AR regulated gene, prostate-specific antigen (PSA), in the presence of either agonist or antagonists. Thus, corepressors SMRT and N-CoR may have important role in modulating both agonist- and antagonist-regulated function of AR. Understanding the mechanism responsible for transcriptional regulation by corepressor complexes will help to develop more effective antagonists for treating androgen-independent prostate cancer. We first selected a set of androgen-regulated genes and determine their promoter regions by RT-PCR and TRANSFAC database mining. Next, we confirmed the recruitment of corepressor N-CoR to the AREs of endgeneus AR-regulated genes, TSC22, NKX3-1 and TMPRSS2 in the presence of agonist and antagonist. It suggests that N-CoR are generally involved in agonist-regulated transcription by AR.				
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Principal Investigator: HoGeun Yoon, Ph.D.

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Introduction

Androgens are critical in the development and maintenance of the male reproductive system, and for growth of normal prostate and prostate cancer (1-2). The effects of androgens are mediated through the androgen receptor (AR), a member of a large family of ligand-dependent transcriptional factors that belong to the steroid receptor superfamily (3-5). AR is also involved in the development and progression of prostate cancer, which is one of the most frequent diagnosed cancers in males (6). Indeed, somatic mutations in the *AR* gene have been found in prostate tumors, which may contribute to androgen-independent growth of the cancer cell (7). X-ray crystallographic studies indicate that the AR LBD adopts a similar structural fold as other NRs, suggesting that the regulatory mechanisms for AR activity may be conserved among NRs (8-9). In the past few years, it has become clear that the transcriptional activity of AR, as well as other members of the NR superfamily, is modulated by coregulatory proteins known as coactivators (10-15) and corepressors (16-20).

The nuclear receptor corepressor (N-CoR) (21) and the related silencing mediator for retinoid and thyroid hormone receptors (SMRT) (22) were originally isolated as RAR- and TR-interacting proteins that form complexes with receptors in the absence of ligand. More recently, N-CoR and SMRT were found to interact with antagonist-bound progesterone receptor (PR) (23), glucocorticoid receptor (GR) (24), and estrogen receptor (ER) (25) to repress transcription, and they also serve as corepressors for several additional members of the NR superfamily, including RevErb (26), peroxisome proliferator-activated receptor (PPAR) α (27), chicken ovalbumin upstream promoter (COUP)-transcription factor I (28), and δ and the orphan receptor DAX1. In addition, SMRT and N-CoR have also been implicated as corepressors for diverse transcription factors including Mad/Mxi, BCL6/LAZ3, ETO, CBF, and REST/NRSF in a wide array of biological processes (29-30). Elegant studies with N-CoR knockout mice have revealed the functional importance of N-CoR in early embryonic development with defects in neural cell differentiation and development progression of specific erythrocytes and thymocytes (31-32).

To understand the molecular mechanisms by which corepressors SMRT and N-CoR mediate repression function, several groups including us have carried out biochemical purification of SMRT and N-CoR proteins. These efforts collectively have led to the conclusion that both SMRT and N-CoR exist as large protein complexes within cells and associate with HDAC3, GPS2 (a cell signaling protein) and TBL1 and TBLR1 (two highly related WD-40 repeat proteins). In addition, we have reported recently that N-CoR is specifically associated with a methyl-CpG binding protein, Kaiso, and is functionally required for DNA methylation-dependent repression by Kaiso. On the basis of recent biochemical and functional studies, it has become clear that SMRT and N-CoR are class I HDAC-containing corepressor complexes that are distinct from the other HDAC-containing corepressor complexes such as Sin3A and NuRD.

SMRT and N-CoR have been recently implicated in both agonist and antagonist regulated transcription by AR. AR was shown to interact with N-CoR in HEK293 cells in the presence of agonist DHT (33). Interestingly, this interaction is enhanced substantially by an acetylation site mutation at the hinge/D region of AR that affected transactivation function of AR without affecting its transrepression activity. However, in a different study, SMRT was shown to interact with AR only after treatment with progestagenic antiandrogen cyproterone acetate but not in the presence of nonsteroidal antagonists hydroxyflutamide or bicalutamide (34). Thus, despite of the established function of SMRT and N-CoR in regulating transcriptional repression of many NRs, the role SMRT and N-CoR in regulating AR transcriptional activity is currently unclear.

Main Body

Task 1. Identification and characterization of AREs (Month 1-6).

a. Identification of AREs by database mining using TRANSFAC program (Month 1-3).

To gain an understanding of the molecular mechanism regulating ARG transcription, we have searched for putative AREs (androgen response elements) in the 5'UTR of ARGs by TRANSFAC database, a program allowing identification of transcription factor binding sites by sequencing comparison. The putative AREs have been identified for TSC22, NKX3A and TMRSS2 genes, in positions -16,782/12,871/1,902, -3,013, and -8,381/-4,937 bp (relative to transcription start sites), respectively, which were also confirmed by our database mining (Fig. 1). The PSA gene has been shown to contain multiple functional AREs. A recent study clearly demonstrated that transcription factors and their coactivators or Pol II recruited to a distant enhancer could act on the promoter by either a looping mechanism or tracking along the chromatin. Consistent with that study, we confirmed the robust recruitment of AR, N-CoR and SMRT to the distant enhancer region between -4006 and -4115 bp in the presence of antagonist.

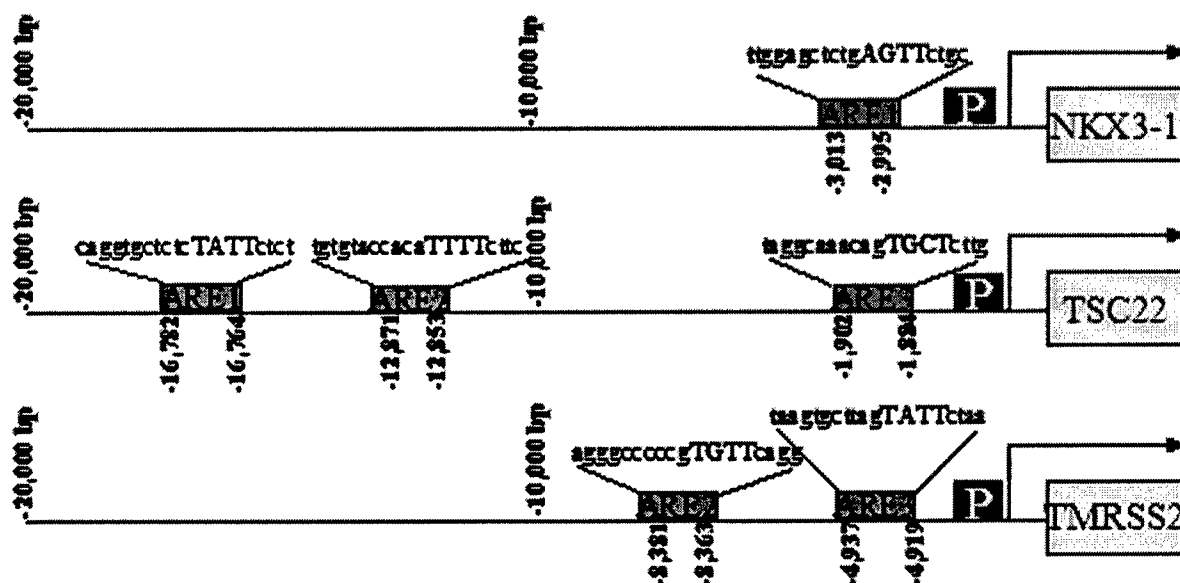


Fig. 1. Identification of putative ARE in NKX3-1 TSC22 and TMRSS2 by TRANSFAC database

b. Test and verify various AREs by ChIP assays (Month 1-6).

We found that both SMRT and N-CoR were targeted to the PSA distant enhancer in the presence of agonist R1881 as well as antagonists flutamide and casodex. To determine whether SMRT and N-CoR are targeted to different ARGs in the presence of agonists and antagonists by ChIP assays, we first examined whether AR binds to the previously identified putative AREs in

TSC22, NKX3A and TMRSS2 genes by ChIP assay. In short, specific PCR primers were designed to amplify sequences (100-150 bp) surrounding the putative AREs. LNCaP cells were treated with or without agonist R1881 or antagonists flutamide and casodex for 1 hr and then processed for ChIP assays using antibodies against AR, N-CoR and SMRT.

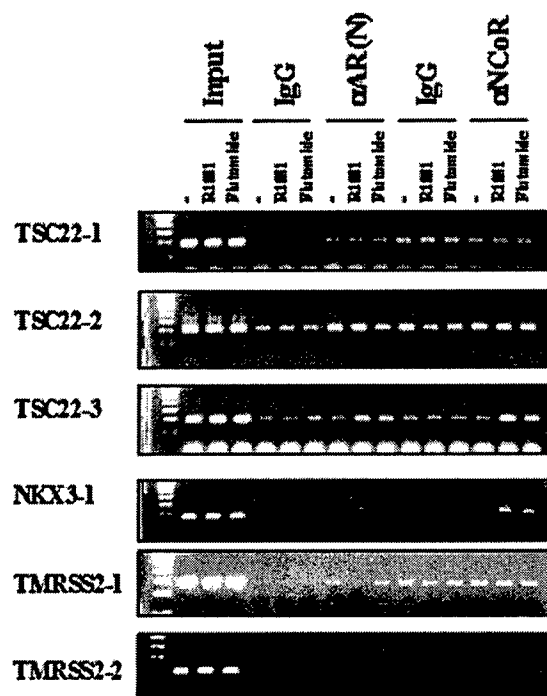


Fig. 2. N-CoR are targeted to the ARGs in the presence of agonist and antagonist. ChIP assay were performed after treatment of 1 nM R1881 or 1 μ M flutamide.

By using ChIP assays, the first key question we like to address is whether in the presence of agonist R1881 N-CoR and SMRT are targeted to the AREs where AR is found to associate with. ChIP assays clearly revealed that N-CoR are recruited to the AREs of all four ARGs. Thus we concluded that N-CoR are generally involved in agonist-regulated transcription by AR.

The second issue we like to address is whether N-CoR are recruited to various AREs together with AR after treatment with antagonists such as flutamide and casodex. Our preliminary study on PSA gene indicates that both N-CoR were targeted to the distant enhancer when LNCaP cells were treated with these antagonists. Similar analysis on TSC22, NKX3A and TMRSS2 revealed that SMRT and N-CoR are also recruited to these genes after antagonist treatment.

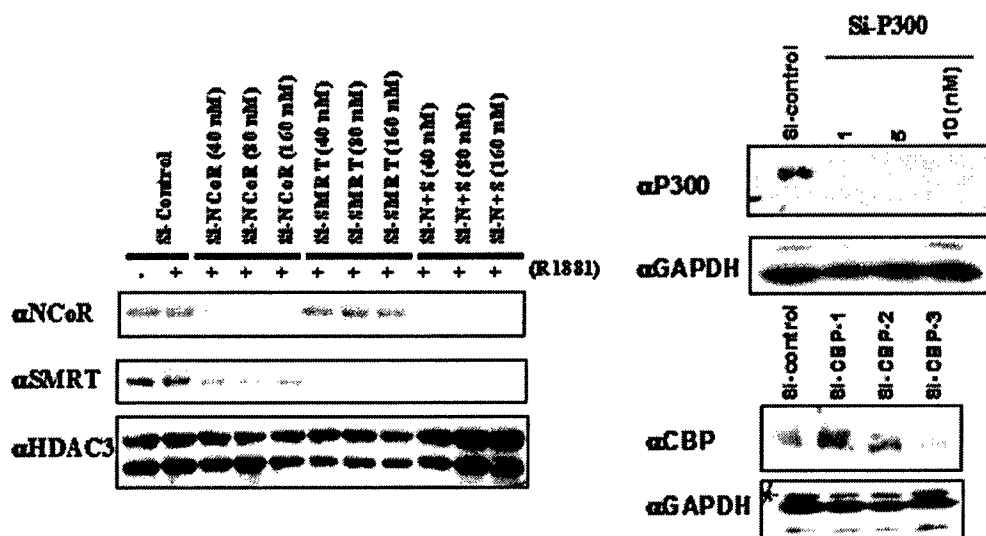
Task 2. Use small interference RNA (siRNA) and chromatin ChIP analyses to study the mechanisms by corepressors SMRT and N-CoR regulate transcription by AR (Month 1-12).

a. Optimize the use of siRNAs against corepressor complex subunits including N-CoR, SMRT, HDAC3, TBL1, TBLR1 and coactivators CBP/P300 and PCAF in LnCaP cells (Month 1-6).

- Establish a siRNA system against corepressor and coactivator complexes.

Recent studies indicate that 21–23 nucleotide double-stranded RNA (siRNA) can act as a guide sequence within a multicomponent nuclease complex to target complementary mRNA for degradation. We already published two studies (18, 20) using siRNA against SMRT and N-CoR to study the structural and function of SMRT and N-CoR complexes in HeLa cells. All siRNAs will be designed according to instructions and synthesized by Dharmacon Research Inc, a leading company in providing service for chemical synthesis of siRNA. According to our experience, the synthetic siRNA is more efficient and less time-consuming in comparison to other vector-based RNA interference techniques. In addition, we find that approximately 50% of siRNAs we have designed so far work efficiently.

We have test and confirmed the effect of each siRNA by examining the level of its corresponding proteins by Western blot analysis.



The optimal concentration of siRNA to knocking-down of N-CoR protein is 80 nM, however in the case of SMRT, the optimal concentration of SMRT is less than 40 nM. The selected siRNA sequences are as follows: N-CoR, 5'-AAGACGAGTCAAGTTCATTAA-3' + 5'-AAAATGAT-ACTTCTCGAGGAA-3'; SMRT, 5'-AAGGGTATCATCACCGCTGTG-3'; CBP, 5'-AAAG-AGAGCATTAAGGAAGTAG-3'; P300, SMRT Pool from Dharmacon Inc.

Key Research Accomplishments

1) Verification of various AREs by ChIP assays

- The AREs have been identified for TSC22, NKX3A and TMRSS2 genes, in positions – 1,902, -3,013, and -4,937 bp (relative to transcription start sites). N-CoR and AR are targeted t these AREs in the presence of agonist and antagonists.

2) Establishments of siRNA system against corepressor N-CoR and SMRT and coactivators CBP and P300

Reportable Outcomes

N/A

Conclusions

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b. Test and verify various AREs by ChIP assays (Month 1-6).

To determine whether SMRT and N-CoR are targeted to different ARGs in the presence of agonists and antagonists by ChIP assays, we first examined whether AR binds to the previously identified putative AREs in TSC22, NKX3A and TMRSS2 genes by ChIP assay. ChIP assays clearly showed that N-CoR and AR are recruited to the AREs of all four ARGs. Thus we concluded that N-CoR are generally involved in agonist-regulated transcription by AR. SMRT and N-CoR are also recruited to these genes after antagonist treatment.

Task 2. Use small interference RNA (siRNA) and chromatin ChIP analyses to study the mechanisms by corepressors SMRT and N-CoR regulate transcription by AR (Month 1-12).

a. Optimize the use of siRNAs against corepressor complex subunits including N-CoR, SMRT, HDAC3, TBL1, TBLR1 and coactivators CBP/P300 and PCAF in LnCaP cells (Month 1-6).

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