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14. ABSTRACT My study involved two aspects of androgen receptor (AR) regulation-1) molecular mechanism underlying negative regulation of AR expression and activity, 2) microRNA-mediated regulation of prostate cancer cell proliferation. My data establish that the human AR level is negatively regulated by nuclear factor κ B (NF- κ B) following its activation by TNF- α -induced signaling. I have defined the regulatory region in the human AR promoter that responds to the TNF- α -controlled negative transcriptional regulation. I show that TNF- α -controlled inhibition of AR expression occurs in androgen-dependent LNCaP human prostate cancer cells, but not in the androgen-independent C4-2 human prostate cancer cells. I further show that TNF- α treatment caused recruitment of corepressor complexes on negative response region in LNCaP cells, but not in C4-2 cells. To search for the microRNA effect on prostate cancer, scanning of the cancer microRNA array shows that miR-454 is up regulated in androgen-independent C4-2 cells and overexpression of miR-454 enhanced prostate cancer cell proliferation. In addition, Slain1 is identified as a miR-454 target protein using bioinformatics approaches.					
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Introduction

The androgen receptor (AR) plays a central role in the initiation, development and progression of prostate cancer. AR is expressed at all stages of this malignancy, and gene amplification along with increased prostatic AR levels is frequently associated with androgen-independent, therapy resistant prostate cancer. In experimental models, knockdown of AR using various approaches is known to cause growth inhibition and apoptosis of androgen-dependent (AD) and androgen-independent (AI) prostate cancer cells. Therefore, inhibition of AR expression is a potentially viable therapeutic approach to control growth and proliferation of prostate tumors regardless of its androgen dependence status. Significant reduction of the receptor is likely to sensitize tumor cells to chemo-and/or radio-induced apoptosis, even in the absence of a complete loss of AR.

MicroRNAs (miRNA) are a class of 21-25 nucleotide small, noncoding RNAs which base pair with mRNAs at 3'UTR and reduced protein levels by inhibiting translation of target messenger RNAs. It has been reported that temporal and spatial expression of miRNAs are related with various cancers and genes both tumour suppressors and oncogenes. For example, PC3 cells, which are AR negative, metastatic and advanced prostate carcinoma cells were overexpressed for miR-221 and miR-222 compared to LNCaP and CWR22, both of which are AR positive and less aggressive prostate cancer cells (1). Inhibition of the expression of the miR-221/222 target p27^{Kip1} in PC3 cells enhanced cell proliferation rate and cell cycle (1).

Based on these conditions, this project is designed to explore mechanisms that cause tumor necrosis factor α (TNF α)-mediated suppression of AR levels and apoptosis in AD human prostate cancer cells and resistance of AI cells to TNF α -induced inhibition of AR expression and to apoptosis. In addition, I demonstrate the identification of miRNA which is differently expressed in AD vs AI and the function of target miRNA. These studies are expected to provide insights that can potentially open up new approaches to prostate cancer therapy, especially for hormone refractive tumors.

Result

I. Altered coregulator dynamics at the negative site in AI C4-2 cells

In previous report, I have shown that the TNF α , which is a pro-inflammatory cytokine, can induce apoptosis of LNCaP AD human prostate cancer cells, whereas the AR-expressing C4-2 and C4-2B AI prostate cancer cells (descendants of LNCaP cells) were protected against the TNF α -induced apoptosis. AR mRNA and protein expression in LNCaP cells declined upon TNF α treatment, causing reduced androgen sensitivity of these cells. Reduced AR expression in TNF α -treated LNCaP cells was due to transcription repression that requires a combined action of activated NF- κ B and the B-myb transcription factor in the 5'-UTR of the AR gene promoter.

Given that NF- κ B interaction with B-myb is an essential aspect for the TNF α -controlled down regulation of AR, and that NF- κ B activity is induced equally well by TNF α in C4-2 and LNCaP cells, I determined how B-myb expression levels and coregulator dynamics at the negative site in the genomic AR would compare between the two cell lines (Fig. 1). Probing of LNCaP and C4-2 cells using Western blot assay shows similar B-myb expression levels (Fig. 1A). B-myb levels also remained same in the two cell lines after treatment with TNF α . On the other hand, real time qPCR assay of DNAs from chromatin IP shows that upon TNF α treatment, the negative regulatory site in the C4-2 cells was not

enriched for p65, p50, B-myb and the components of the corepressor complex that were tested (HDAC1, SMRT, mSin3A). Signal for c-myb also remained at the background level (Fig. 1B). The results in C4-2 cells contrast to those in LNCaP cells where TNF α induced robust recruitment of p65, B-myb and HDAC1 (1B, left panel). Similar results are also seen with the semi-quantitative analysis of the PCR-amplified DNAs from immunoprecipitated chromatin fragments (Fig. 1C). Therefore, the absent negative regulation of AR in C4-2 cells is due to the inability of the required regulatory factors to occupy the negative regulatory site. Studies are under way to identify the cellular changes that prevent TNF α -induced engagement of relevant regulators at the negative site.

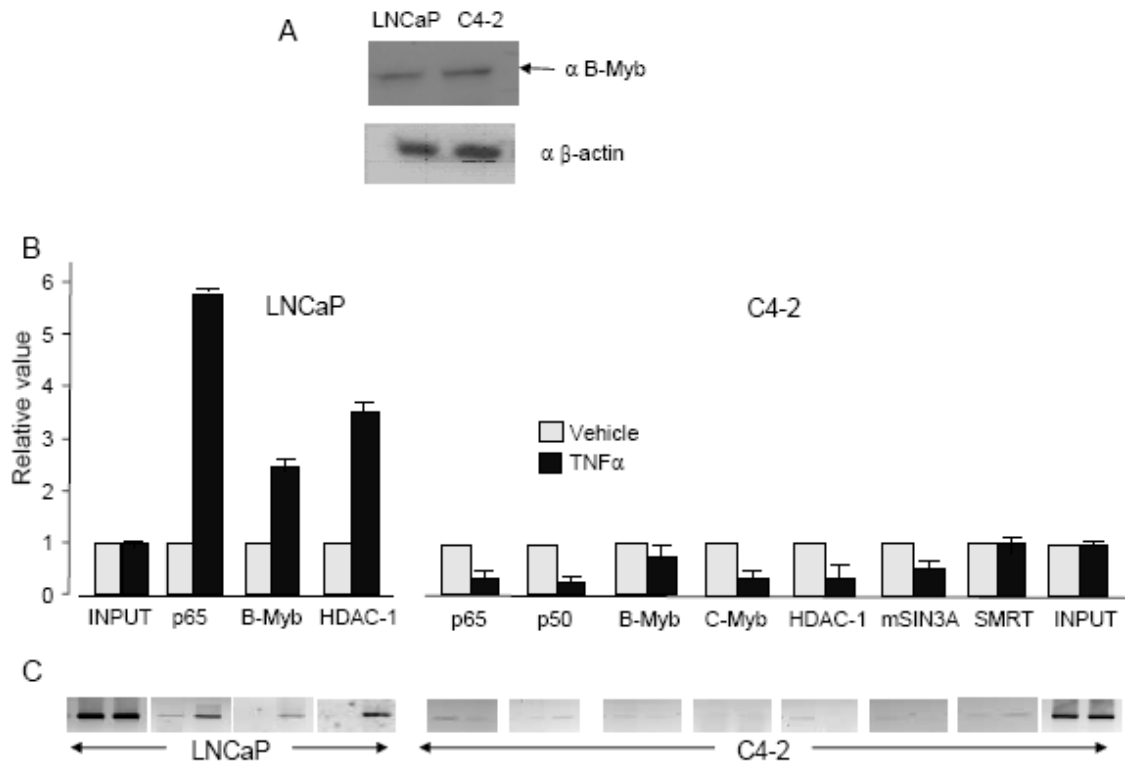


Figure 1. B-myb expression and TNF α -induced coregulator dynamics at the negative regulatory site in the AR gene in LNCaP and C4-2 cells. (A) Western blot of B-myb, using β -actin as the normalization control. (B) ChIP in LNCaP and C4-2 cells treated with TNF α (20 ng/ml for 30 min) or vehicle. Real-time qPCR quantified PCR signals. In each case, the value from vehicle-treated cells was set to unity. (C) PCR products (285 bp) from the same ChIP analysis as in panel B, visualized as ethidium bromide-stained bands, on agarose gel.

II: Examine roles of selected miRNAs, displaying distinct expression profile in LNCaP cells versus C4-2 cells before and after TNF α treatment, in cell proliferation and apoptosis.

In previous report, Baltimore and colleagues (2) showed that miR-146 is highly expressed following by TNF α treatment in THP-1 cells. However, 95 miRNAs (System Biosciences), expressed differentially in LNCaP vs C4-2 cells under TNF α treatment, didn't show any significant difference in expression including mir-146. Based on this result, I focused to the different miRNA expression profile in LNCaP vs C4-2 cells.

1. MiR-454 and miR-222 are overexpressed in C4-2 cells.

To search for miRNAs differentially expressed in androgen-dependent LNCaP cells and androgen-independent C4-2 cells, total RNAs from LNCaP and C4-2 cells were polyadenylated and PCR amplified in real-time to quantify relative levels of each of the 95 cancer-related miRNAs (System Biosciences) in LNCaP versus C4-2 cells. Results from three independent screening revealed that miR-454 and miR-222 were markedly elevated in C4-2 cells compared to LNCaP cells (Fig.2). Although differential expression was observed for several additional miRNAs, most robust changes were seen for miR-454 and miR-222. MiR-222 has been reported to enhance prostate tumor growth in a mouse xenograft model. However, miR-454 is an unexplored miRNA so far. Therefore, this study focused on miR-454.

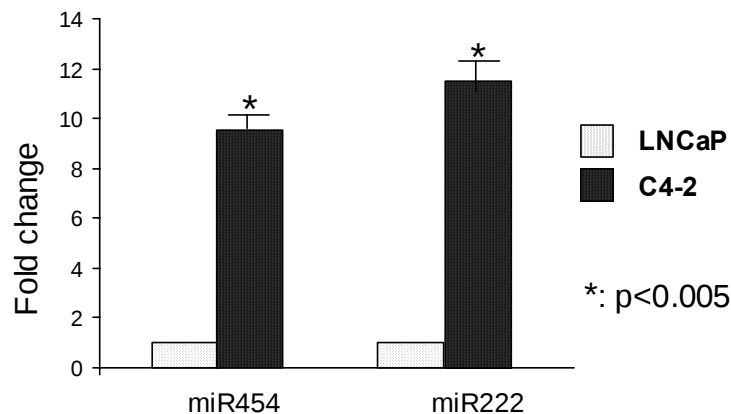


Figure 2. Differential levels of miR-222 and miR-454 in LNCaP and C4-2 cells. The miRNAs were quantified by real-time q-PCR using miRNA sequence-specific forward primers along with an oligo dT-linked universal reverse primer. Results are normalized to the human U6 transcript as the invariant control. Data represent average $\pm$ SD from three independent assays, each with n=3 replicates. P values are determined using student's t-test.

2. Ectopic expression of miR-454 stimulates prostate cancer cell proliferation.

The influence of the miR-454 on proliferation rates of LNCaP and E006AA prostate cancer cells in culture was examined. Fig. 3A shows that overexpression of miR-454 accelerated cell proliferation compared to the vector transfected (pcDNA3.1) cells. Enumeration of viable LNCaP cells at day-3 and day-6 following miR-454 overexpression versus pcDNA3.1 overexpression also demonstrated the proliferation enhancing effect of miR-454 (Fig. 3B).

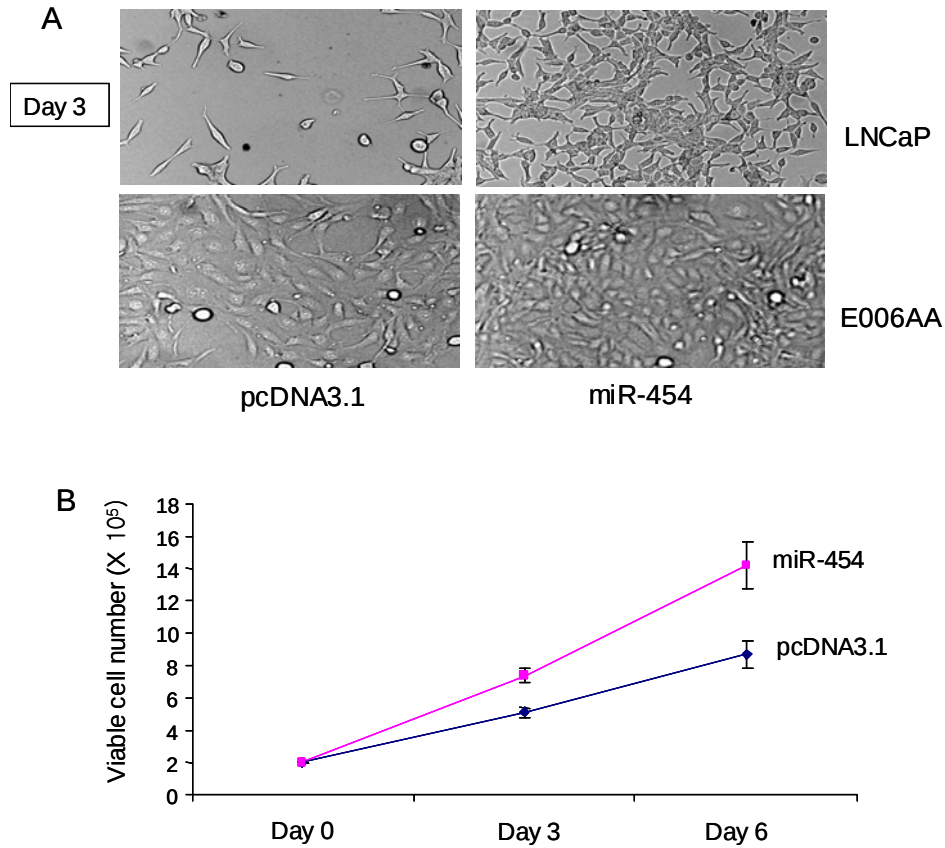


Figure 3. Overexpression of miR-454 enhanced cell proliferation in prostate cancer cells. (A) LNCaP and E006aa cells were transfected with the pcDNA3.1 or miR-454 expression plasmid. Photomicrographs of the cells after 3 days of culture are shown. (B) LNCaP cells were transfected with miR-454 expression plasmid or vector alone (pcDNA3.1). Transfected cells were counted for trypan blue excluded viable cells at day-3 and day-6. Increased proliferation rate of LNCaP cells in response to miR-454 overexpression is clearly evident. Data represent average $\pm$ SD from three independent assays.

3. Identification of Slain1 as a miR-454 target

To identify the target protein for miR-454, bioinformatics search was performed (TargetScan, miRanda and miRBase). 3'UTR of Slain1 carries three binding sites for miR-454, suggesting that Slain1 might be a target of miR-454. To verify the putative miR-454 binding site in the 3'UTR of Slain1, I cloned 3' UTR of Slain1 mRNA into the pMIR luciferase vector. Overexpression of miR-454 inhibited luciferase activity expressed from the Slain1 3'UTR-pMIR construct in LNCaP cells (Fig.4). However, when the putative miR-454 target sites in Slain1 were mutated, inhibition of luciferase activity was abolished, indicating specificity of the miR-454 targeting of the 3'UTR of the Slain1 mRNA (Fig.4). This result indicates that Slain1 is targeted by miR-454 for translational suppression.

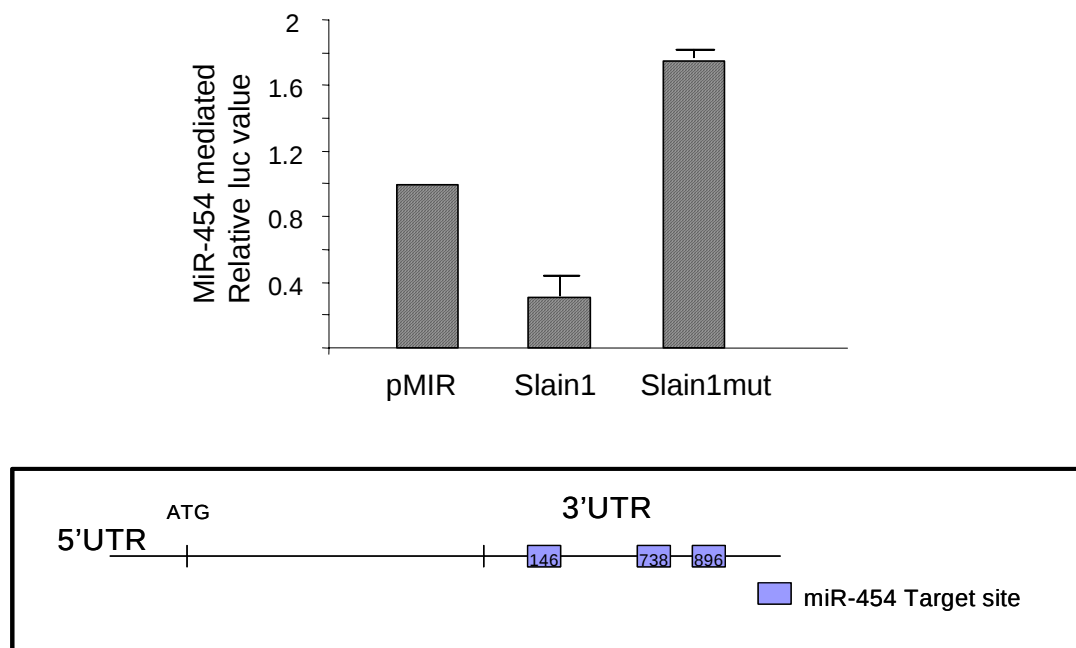


Figure 4. Slain1 is a target of miR-454. Wild type Slain1-3'UTR in pMIR plasmid or mutant Slain1-3'UTR plasmid were transfected into LNCaP cells with pcDNA3.1 or miR-454 plasmid. Luciferase activity was determined after 48 h. Data represent average $\pm$ SD from three independent assays, each with n=3 replicates.

Key research accomplishments

- Demonstration of the potential mechanisms of differential TNF α response of AD LNCaP cells from that of the AI C4-2 cells.
- Identification of miR-454 which is highly expressed in C4-2 cells compare to LNCaP cells and Slain1 as a miR-454 target.

Reportable outcomes

Publication

1. **Ko, S.**, Shi, L., Kim, S., Song, C.S., and Chatterjee B. Interplay of NF- κ B and B-myb in the negative regulation of androgen receptor expression by TNF α . *Mol. Endo.* 2008 Feb;22(2):273-86.

Abstracts for national meeting

1. Kim S, Ahn, J, Echchgadda, I, **Ko S**, Kim, SJ, Song C and Chatterjee B. The WINAC Chromatin Remodeling Complex and the bHLH Protein E47 Regulate Androgen-stimulated and Vitamin D₃-inhibited Expression of the Cell Cycle Regulator E2F1 in Prostate Cancer Cells. 90th Endocrine Society Meeting, June 15-18, San Francisco, 2008.

2. **Ko S**, Song CS, Chatterjee B. Inhibition of Androgen Receptor Function through Lysine Methylation. 90th Endocrine Society Meeting, June 15-18, San Francisco, 2008. (*Selected for Oral Presentation*).

Conclusion

My results linking inflammation and cancer through the NF- κ B and B-myb pathway is an advance toward illuminating role of inflammation and cancer. Studies on the mechanism for the TNF- α -controlled differential transcription regulation of AR in AD versus AI cells are partially identified. These results may suggest that NF- κ B-directed repression of the AR gene activity and AR function may signify an anti-neoplastic, growth-inhibitory response of cells against prostate cancer progression to androgen independence that is normally associated with NF- κ B activation. I have also identified that miR-454 are underexpressed in AD LNCaP cells compare to AI C4-2 cells and ectopic expression of miR-454 accelerated proliferation of prostate cancer cells. It may explain the important role of miR-454 in prostate cancer cell progression and growth.

References

1. Galardi, S., Mercatelli, N., Giorda, E., Massalini, S., Frajese, G.V., Ciafrè, S.A., and Farace, M.G. (2007) miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27kip1. *J. Biol. Chem.* 282(32):23716-24
2. Taganov, K.D., Boldin, M.P., Chang, K.J., and Baltimore, D. (2006) NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc. Natl. Acad. Sci. USA.* Aug 15;103(33):12481-6

Appendices

Original abstract for Endocrine Society Meeting

[OR19-6] Inhibition of Androgen Receptor Function through Lysine Methylation.

S Ko, CS Song, B Chatterjee. Molecular Med, Univ of Texas Hlth Sci Ctr at San Antonio, San Antonio, TX; South Texas Veterans Hlth Care Syst, San Antonio, TX

Protein methylation generally serves as a cue for transcriptional switch in mammalian gene regulation. Protein methyltransferases, initially identified for activity on specific lysine or arginine residues of histone H3 and H4, also act on non-histone substrates, notably on transcription factors and coregulators. For example, Set9, a lysine-4 H3 histone methyltransferase can potentiate DNA damage-induced expression of the cell cycle inhibitor p21, a target gene for p53, which is a transcription factor and tumor suppressor. Enhanced transcription was due to Set9-mediated methylation of p53 (1). Here we report that Set9

methylates androgen receptor (AR) at two specific lysine residues within an internal domain, causing attenuation of AR-mediated transactivation. AR is methylated *in vitro* by recombinant Set9 in the presence of S-adenosyl-L-methionine. AR is also methylated *in vivo*, since 1) AR, after immunoprecipitation from prostate cell lysates, was recognized by antibody specific to methylated lysine; 2) cotransfection of the AR-negative 293 kidney cells with Set9 and AR expression plasmids showed methylation of wild-type AR but not mutant AR carrying lysine to alanine substitutions at the two Set9-targeted residues. Set9 can be co-immunoprecipitated with AR from LNCaP prostate cancer cell lysate, indicating that these two proteins can interact directly or indirectly within cells. Androgen-induced transactivation of the probasin promoter decreased several folds in Set9-cotransfected 293 cells. No such decrease was caused by a mutant Set9 lacking methyltransferase activity. Lysine to alanine substitution of AR at the two Set9-targeted residues protected AR from Set9-mediated reduction of AR transactivation function. The same AR mutant showed augmented functional interaction with coactivators compared to the wild-type counterpart, evident from increased coactivation of mutant AR activity by p160 family of coactivators and by p300 and p/CAF acetylases. The H3 lysine-9 methyltransferase G9a, which is generally a corepressor for transcription factors, serves as an AR coactivator in transfected cells, although direct methylation of AR by G9a was not demonstrated (2). Our finding that Set9 inhibits AR function contrasts with the potentiating effect of Set9 on the transcriptional activity of p53, indicating that protein methyltransferases assume a context-dependent positive or negative role in the functional regulation of AR and other transcription factors.

1) Chulkov et al, Nature 432: 353, 2004

2) Lee et al, J. Biol. Chem. 281: 8476, 2006

[P2-50] The WINAC Chromatin Remodeling Complex and the bHLH Protein E47 Regulate Androgen-Stimulated and Vitamin D₃-Inhibited Expression of the Cell Cycle Regulator E2F1 in Prostate Cancer Cells.

S Kim, J Ahn, I Echchgadda, S Ko, S-J Kim, C Song, B Chatterjee. Molecular Med/IBT, Univ of Texas Hlth Sci Ctr at San Antonio, San Antonio, TX; South Texas Veterans Hlth Care Syst, San Antonio, TX

Androgen-stimulated and androgen-independent prostate cancer cell proliferation is inhibited *in vitro* and *in vivo* by 1,25(OH)₂ vitamin D₃ (D₃). Therapeutic benefits of D₃/D₃ analogs, however, are limited by hypercalcemia-associated toxicity from high doses of these hormones. To identify new molecular targets for prostate cancer therapeutics, we set out to examine the regulatory factors that coordinate androgen-stimulated proliferation and D₃-mediated inhibition of prostate cancer cells. Here we report that androgen-stimulated expression of the key cell cycle regulator E2F1 declines markedly in LNCaP prostate cancer cells in the presence of 1,25(OH)₂ vitaminD₃ due to transcription repression. LAPC4 prostate cancer cells, expressing wild type androgen receptor (AR), similarly showed androgen-mediated induction and D₃-regulated de-induction of E2F1. AR was recruited to the hormone-responsive E2F1 promoter in androgen-stimulated LNCaP cells without direct DNA binding. WINAC, an ATP-dependent chromatin remodeling complex, appears to be part of the regulatory scheme that defines the hormonal control of E2F1 expression. Thus, two core components of WINAC, WSTF (a transcription factor deleted in the Williams Syndrome) and SWI/SNF-related ATPase BRG1, along with AR, p/CAF histone acetylase and RNA polymerase II, occupied the regulated chromatin in androgen-stimulated cells. WSTF co-immunoprecipitated with AR

from LNCaP cells, implying that WINAC, or specific WINAC components, are part of an AR-associated coactivator complex. E2F1 de-induction correlated with depletion of WSTF, p/CAF and Pol II from the regulated chromatin, whereas the presence of AR and BRG1 persisted and an NCoR and HDAC-2 deacetylase associated co-repressor complex occupied the repressed promoter. Vitamin D receptor (VDR) was chromatin-bound both in the absence and presence of D₃ through a VDR-binding element. E47, a basic helix-loop-helix protein bound to an E-box DNA sequence in the regulated chromatin in androgen-stimulated cells; furthermore, E47 transiently associated with the D₃-repressed E2F1 promoter. Since E47 can interact with ligand-bound VDR, E47 may be involved in the coactivator to corepressor switch resulting in the D₃-regulated inhibitory response. Results on the functional consequences of depleting one or more of these regulatory factors may reveal a novel therapeutic approach to inhibit E2F1 expression and reduce proliferation of prostate cancer cells *in vitro* and *in vivo*.

Interplay of Nuclear Factor- κ B and B-myb in the Negative Regulation of Androgen Receptor Expression by Tumor Necrosis Factor α

Soyoung Ko, Liheng Shi, Soyoung Kim, Chung S. Song, and Bandana Chatterjee

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Increased androgen receptor (AR) levels are associated with prostate cancer progression to androgen independence and therapy resistance. Evidence has suggested that chronic inflammation is closely linked to various cancers including prostate cancer. Herein we show that the proinflammatory cytokine TNF α negatively regulates AR mRNA and protein expression and reduces androgen sensitivity in androgen-dependent LNCaP human prostate cancer cells. Decreased AR expression results from transcription repression involving essential *in cis* interaction of nuclear factor- κ B (NF- κ B) with the B-myb transcription factor at a composite genomic element in the 5'-untranslated region of AR. The negative regulation was abrogated when NF- κ B activity was inhibited by a superrepressor of the inhibitory κ B protein. In contrast, androgen-independent C4-2 (LNCaP-derived) cells fail to show AR down-regulation by TNF α , despite expression of B-myb and TNF α -induced NF- κ B

activity similar to that in LNCaP cells. The negatively regulated AR gene chromatin region showed TNF α -dependent enrichment of B-myb and the NF- κ B proteins p65 and p50. In parallel, the histone deacetylase 1, corepressor silencing mediator of retinoid and thyroid hormone receptor and the corepressor-associated scaffold protein mSin3A were recruited to the inhibitory site. In C4-2 cells, neither NF- κ B and B-myb, nor any of the corepressor components, were detected at the negative site in response to TNF α . Apoptosis was induced in TNF α -treated LNCaP cells, likely in part due to the down-regulation of AR. The androgen-independent, AR-expressing C4-2 and C4-2B (derived from C4-2) cells were resistant to TNF α -induced apoptosis. The results linking androgen dependence to the NF- κ B and AR pathways may be insightful in identifying novel treatment targets for prostate cancer. (*Molecular Endocrinology* 22: 273–286, 2008)

DIVERSE PHYSIOLOGY, MOST notably that related to male reproduction, muscle growth, and cognitive vigor, is dependent on the intracellular androgen signaling coordinated by the androgen receptor (AR), which is an inducible transcription factor of the nuclear hormone receptor family. The androgen-induced AR pathway plays a key role in the structural and functional integrity of the prostate, whereas misregulation of AR signaling leads to aberrant growth and malignant progression of prostate cells (1, 2). In a

wide variety of examples, the transcription regulatory function of AR is known to be modulated by multiple kinase-regulated signaling cascades such as those involving protein kinases A and C, MAPKs, and phosphatidylinositol 3-kinase/Akt-activated kinase pathways (3). Attenuation of AR activity due to transrepression by the nuclear factor- κ B (NF- κ B) has also been reported (4). Apart from its activity, the expression level of AR is another important determinant of androgen-regulated tissue functions. For example, moderate increases in the AR mRNA and protein levels were observed to associate with the transition of human prostate xenograft tumors from hormone dependence to hormone resistance (5). Auto-induced AR gene expression, observed in transfected cells (6), would conceivably amplify target gene responses to AR signaling and change AR-dependent cell/tissue functions. Furthermore, knockdown of AR using various approaches [antisense oligo; AR antibody; AR-specific hammerhead ribozyme; AR mRNA-targeting small interfering RNAs (siRNAs)] caused growth inhibition and apoptosis of both androgen-dependent and androgen-independent prostate cancer cells (7–10). In clinical samples, AR is expressed at nearly all stages of prostate cancer (2), and gene amplification along with elevated

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Abbreviations: AR, Androgen receptor; ChIP, chromatin immunoprecipitation; DNase I, deoxyribonuclease I; GFP, green fluorescent protein; GST, glutathione-S-transferase; HDAC, histone deacetylase; I κ B, inhibitory κ B; moi, multiplicity of infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; N-CoR, nuclear receptor corepressor; NTD, N-terminal domain; PI, propidium iodide; PSA, prostate-specific antigen; qPCR, quantitative PCR; siRNA, small interfering RNA; SMRT, silencing mediator of retinoid and thyroid hormone receptor; SR, superrepressor; TUNEL, terminal deoxynucleotide transferase-mediated dUTP nick end labeling; UTR, untranslated region.

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tissue AR levels are frequently observed in hormone-refractory cancers (11, 12). Above experimental and clinical findings highlight the need to understand the molecular events that control prostate cell AR expression in the background of normal and pathogenic stimuli.

Accumulated evidence strongly implicates chronic inflammation as a key player in tumorigenesis (13). Malignant tissues, including prostate tumors, are normally under inflammatory stress rendered by the cytokines released from infiltrated inflammatory cells such as monocytes and macrophages (14). In an experimental model, prostate cancer cell interaction with monocytic cells converted the antiandrogen bicalutamide (casodex) to an androgen agonist (15). The functional impact of the antagonist to agonist conversion for bicalutamide was amplified when cancer cells were pretreated with the proinflammatory cytokine TNF α , indicating that the TNF α -elicited signaling profoundly impacts androgen-dependent prostate cell function. NF- κ B is rapidly activated by TNF α in various cell types after a receptor-mediated transmembrane activation response that culminates in phosphorylation, ubiquitination, and proteasomal degradation of the NF- κ B-associated inhibitory κ B (I κ B) protein and release of the cytosol-retained NF- κ B for nuclear translocation and transcription regulation (16). The mammalian NF- κ B/Rel protein family has five members, of which p65/RelA, c-Rel and Rel B possess transactivation activity at the carboxyl terminus. The proteins p50 and p52 lack activation function, bearing only the sequence-specific DNA-binding activity. Using the common Rel homology domain, individual NF- κ B members form homo- or heterodimers, generating functionally distinct NF- κ B complexes. Upon inflammatory stimulation, the p65/p50 complex is the predominantly induced NF- κ B in most cells. Diverse processes such as immune activation, cell growth, proliferation, differentiation, and apoptosis are impacted by target gene responses to the activated NF- κ B.

In the majority of examples, NF- κ B functions as a positive regulator of transcription. Nevertheless, we had previously shown that the rat AR gene activity in transfected HepG2 human hepatoma cells was inhibited by p65/p50-NF κ B. The inhibition resulted from direct binding of NF- κ B to a 21-bp DNA element at an upstream promoter location (17). Progressive loss of AR mRNA expression in the rat liver during aging in parallel to increased age-associated NF- κ B activity led us to speculate that similar negative regulation of rat AR can be expected *in vivo* in the liver (17). Additional examples of NF- κ B-repressible targets include the growth arrest- and DNA damage-inducible GADD 45 genes - α and - γ ; glutamate transporter gene EAAT-2; and long-terminal repeat of the latent HIV virus (18–20). Inhibition of EAAT-2 expression in astrogloma cells by TNF α required juxtaposition of NF- κ B and the oncoprotein N-Myc at the regulatory site.

Herein we present evidence that in androgen-dependent malignant (LNCaP) and nonmalignant (RWPE-1) human prostate cells, TNF α -activated NF- κ B inhibits AR expression. In contrast, the inhibitory effect is absent in the LNCaP derived C4-2 and C4-2B prostate cancer cells that have progressed to androgen independence. TNF α caused apoptosis in LNCaP cells but not in the androgen-independent C4-2 and C4-2B cells. The reduced AR level in TNF α -treated LNCaP cells is due to transcription repression involving an essential interaction *in cis* between NF- κ B and the transcription factor B-myb (a protooncoprotein) at a composite binding sequence in the genomic 5'-UTR (untranslated region) of AR. A silencing mediator of retinoid and thyroid hormone receptor (SMRT)/histone deacetylase (HDAC)1/mSin3A-containing corepressor complex is involved in the negative regulation. In androgen-independent prostate cancer cells, TNF α did not induce recruitment of NF- κ B, B-myb, and corepressors to the repressor-responsive site. Delineation of the factors regulating AR gene repression, or lack of it, in response to inflammation-induced NF- κ B, may provide important insights on the search to identify new prostate cancer therapeutics.

RESULTS

TNF α -Controlled Inhibition of AR Expression in Human Prostate Cancer Cells and a Role of NF- κ B in the Negative Regulation

Because prostate tumor tissues are frequently infiltrated with inflammatory cells, and TNF α treatment of malignant or nonmalignant prostate epithelial cells can convert an antiandrogen to an AR agonist, we sought detailed insights into the effect of TNF α on AR expression in prostatic cells. Consistent with an earlier report (21), TNF α caused a dose-dependent reduction of the AR mRNA and protein levels in LNCaP prostate cancer cells (Fig. 1, A and B). The TNF α effect is rapid, because AR mRNAs declined markedly within 30 min of the cytokine treatment (data not shown). As expected, the reduced AR level was associated with attenuation of AR-mediated transactivation, because androgen-induced prostate-specific antigen (PSA) mRNA expression declined markedly in TNF α -treated cells (Fig. 1C).

NF- κ B activity has an essential role in the TNF α -regulated inhibition, because adenovirus-mediated expression of the I κ B- α superrepressor (I κ B-SR), which specifically blocks NF- κ B activity, prevented reduction of the AR level in TNF α -treated LNCaP cells (Fig. 1D). The SR with Ser $\rightarrow$ Ala mutational changes (amino acids 32 and 36) is resistant to TNF α -induced phosphorylation and proteolysis (22), thus preventing the nuclear translocation and activity of NF- κ B. The short-wavelength UV radiation UV-C (<290 nm), another activator of NF- κ B, also reduced the immunoreactive AR level in LNCaP cells, whereas estrogen

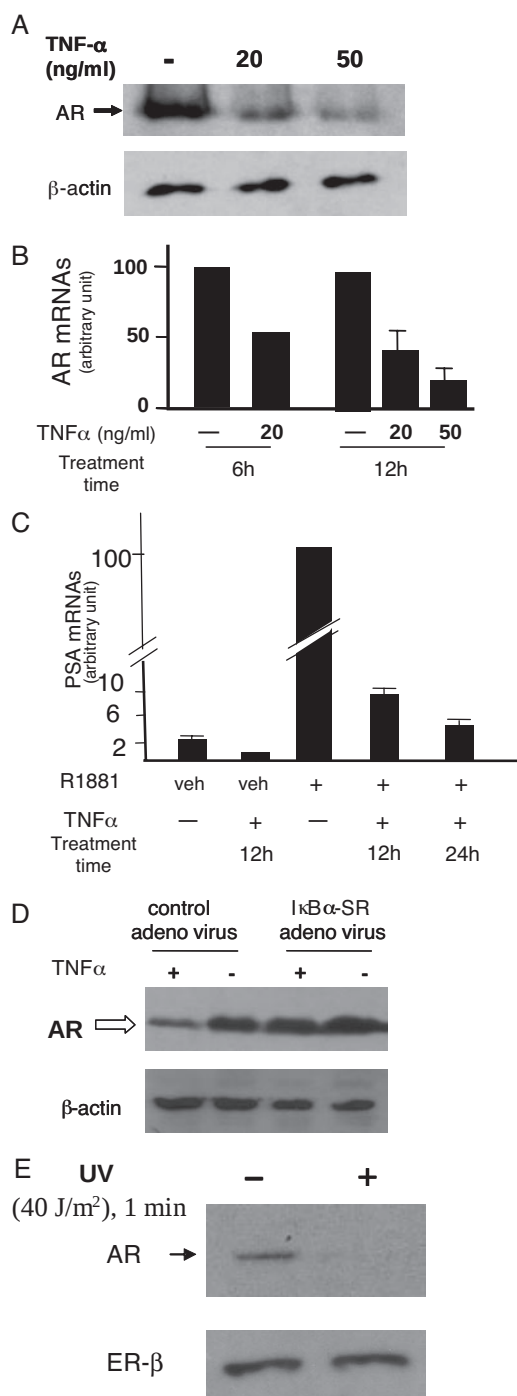


Fig. 1. TNF α Suppressed AR Expression and Activity in LNCaP Human Prostate Cancer Cells

A, Reduced AR protein level. Cells were treated with TNF α at indicated doses for 12 h, and AR and β -actin proteins were assayed by Western blot. **B**, Reduced AR mRNAs in cells treated with TNF α for 6 h (at 20 ng/ml) or 12 h (at 20 ng/ml and 50 ng/ml). AR mRNAs were assayed by real-time RT-qPCR, normalized to β -actin mRNAs. Minus TNF α control cells were treated with vehicle (PBS). **C**, Reduced androgen sensitivity indicated by decreased androgen-induced PSA mRNA expression. Cells were incubated with 10 nM R1881 (or 0.001% ethanol as vehicle) for 24 h. Thereafter, TNF α (20 ng/ml) was

receptor- β (ER- β) expression was unaffected (Fig. 1E). These results support the conclusion that NF- κ B is a negative regulator of AR gene expression in LNCaP cells.

Lack of TNF α Response in Androgen-Independent Prostate Cancer Cells

Given that the AR is expressed at all stages of prostate malignancy and the majority of clinical prostate tumor specimens are AR positive, we examined the impact of TNF α on AR expression and cell viability in androgen-independent, AR-positive human prostate cancer cells (Fig. 2). TNF α did not reduce AR levels in C4-2 and C4-2B androgen-independent prostate cancer cells (Fig. 2A). The lack of AR down-regulation correlated with almost a complete protection of C4-2 and C4-2B cells against loss of cell viability (Fig. 2, B and C). C4-2 cells originated from tumor xenografts in castrated mice produced by a mixture of LNCaP cells and bone stromal cells (23). The androgen-independent C4-2B cells were isolated from the bone metastasis of C4-2-derived xenograft tumors (24). In contrast to C4-2 and C4-2B cells, the viability of parental LNCaP cells decreased steadily with increasing doses of TNF α (Fig. 2D, *open bars*), consistent with a previous report (25). The essential role of NF- κ B in the TNF α -induced cell death is evident from the result that stable expression of I κ B- α -SR protected LNCaP cells from the loss of viability at all tested doses of TNF α (Fig. 2D, *solid bars*). Resistance of C4-2 cells to TNF α -induced apoptosis is also evident from *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (Fig. 3). TNF α -treated parental LNCaP cells (Fig. 3A), but not the C4-2 cells (Fig. 3B) showed TUNEL staining. Taken together, these results suggest that the prostate cancer cells that proliferate indepen-

added to the culture medium. Control cells (minus TNF α) received PBS. Incubation continued for 12 additional hours, after which cells were harvested for total RNA preparation. In one indicated case, TNF α incubation continued for additional 12 h (total TNF α treatment time: 24 h), thus extending R1881 incubation period to 48 h in this case. PSA mRNAs relative to the invariant β -actin mRNAs were assayed by real-time RT-qPCR. Error bars are averages of three experiments $\pm$ SD. PCR primers for AR, PSA, and β -actin mRNAs are described in *Materials and Methods*. Error bars are not shown in cases where assays were repeated twice, each in duplicate. Data in these cases are presented as average values. **D**, I κ B-SR blocked TNF α -induced AR down-regulation. LNCaP cells were infected with I κ B-SR-expressing adenovirus (at 20 moi) or the control GFP-expressing adenovirus (at 20 moi), and 48 h later cells were treated with TNF α (20 ng/ml) or PBS (vehicle) for an additional 24 h. Cell lysates were immunoblotted with anti-AR or anti- β -actin. **E**, Reduced AR expression in UV-C-irradiated LNCaP cells. UV-C (<290 nm) exposure was at 40 J/m² (1 min). AR and ER- β protein levels were analyzed by Western blot. ER- β , Estrogen receptor- β ; veh, vehicle.

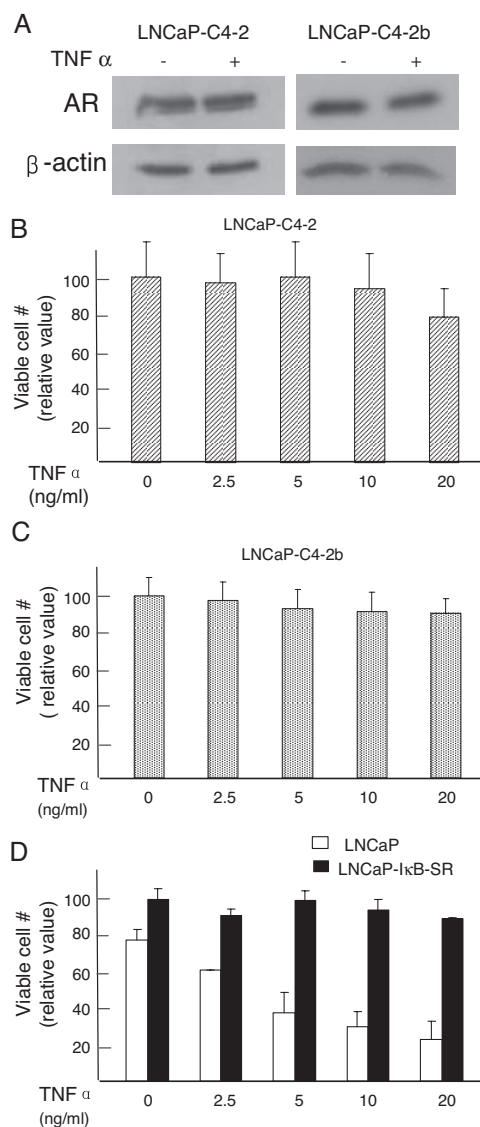


Fig. 2. Resistance of Androgen-Independent Prostate Cancer Cells to TNF α -Induced AR Down-Regulation and to Apoptosis

A, Unaltered AR protein levels (analyzed by Western blot) in LNCaP-derived androgen-independent C4-2 and C4-2b cells treated with TNF α (20 ng/ml). B and C, Sustained viability of C4-2 and C4-2b cells against TNF α . The cells were treated with increasing doses of TNF α for 5 d, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay determined the number of viable cells. D, Reduction in the number of viable cells for the parental LNCaP (open bars). Absent loss of viability for LNCaP-IκB-SR cells that stably express the IκB-SR (dark-filled bar). Viable cell numbers are averages of three experiments $\pm$ SD.

dently of androgens are unresponsive to TNF α -induced apoptosis, and this failure may be due, in part, to the absence of the TNF α -regulated reduction of AR expression in androgen-independent cells.

Transcriptional repression accounted for the TNF α /NF- κ B-mediated reduction of AR expression in

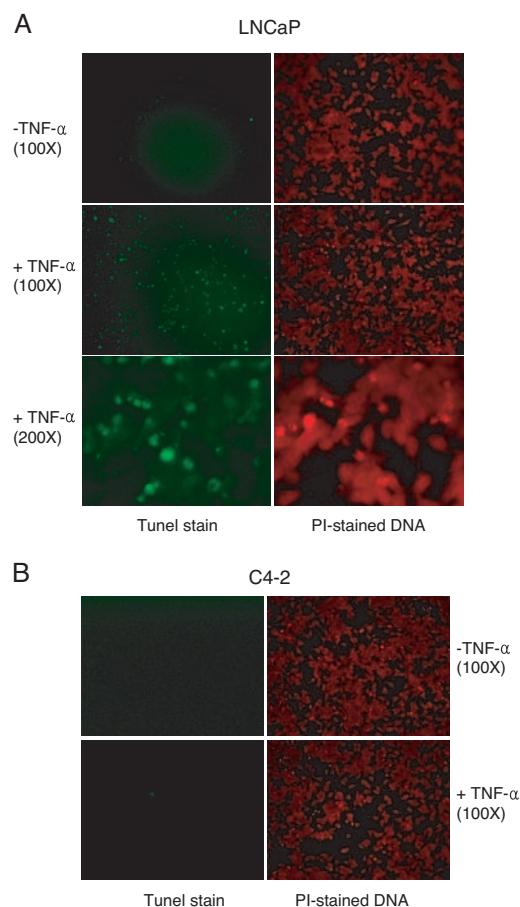


Fig. 3. Apoptosis of Androgen-Dependent (LNCaP) But Not Androgen-Independent (C4-2) Prostate Cancer Cells after TNF α Treatment

Cells were incubated with TNF α (or PBS for minus TNF α) for 5 d, and TUNEL staining was used to examine apoptosis. Representative cells within a microscopic field (at $\times 100$ or $\times 200$ magnification, as indicated) displaying TUNEL staining (left) and PI staining (right) are shown. TUNEL-stained LNCaP cells emitting green fluorescence are visible at $\times 100$ and $\times 200$. No TUNEL staining for C4-2 cells was detectable. Data are representative of green fluorescence in five different fields.

LNCaP cells, revealed from reporter assay in transfected cells (Fig. 4, A and B). Genomic fragments of the human AR cloned into the promoter-less pGL3b luciferase vector were examined for activity to direct reporter expression in p65- and p50-cotransfected LNCaP cells (Fig. 4A). The pGL3b vector itself was strongly stimulated upon overexpression of NF- κ B/p65 and NF- κ B/p50, due to the presence of an NF- κ B-inducible sequence in the firefly luciferase cDNA. However, for the reporter constructs containing genomic AR fragments up to -726 , -311 , or -110 nucleotide of the upstream sequences and intragenic sequences to various lengths (shown in Fig. 4A), co-transfection of p65 and p50 reduced luciferase expression by 50% or more relative to the pcDNA3.1-transfected control cells (Fig. 4A). Therefore, an NF-

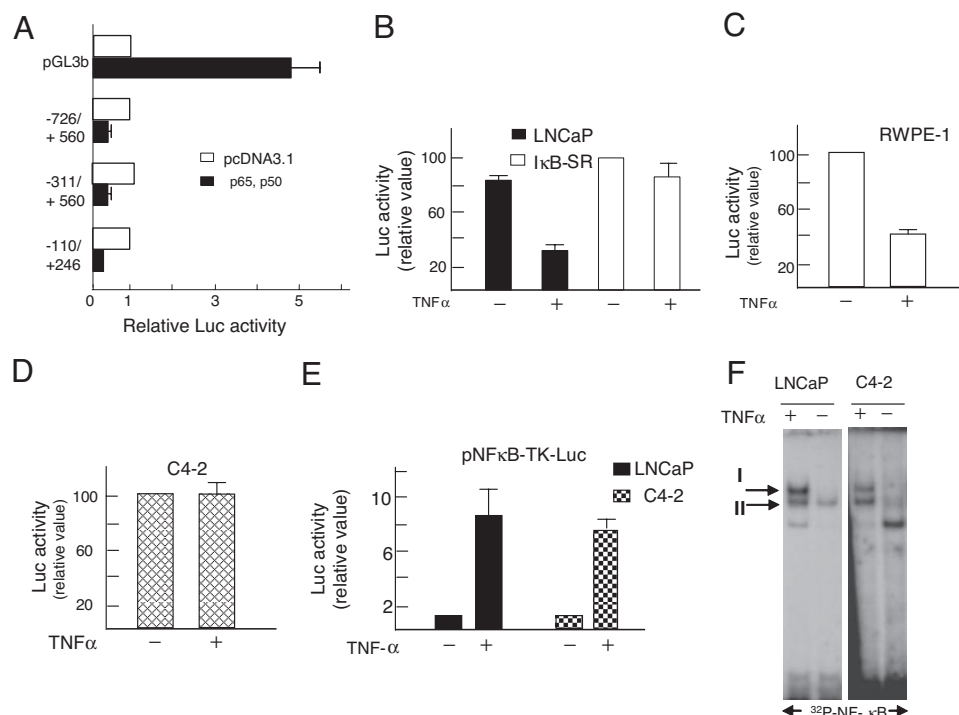


Fig. 4. AR Promoter Repression by NF- κ B-p65/p50 in Androgen-Dependent LNCaP and RWPE-1 Prostate Cells and Lack of the Repression in Androgen-Independent C4-2 Cells

A, Luciferase reporter activity is shown for each construct in p65 and p50 cotransfected LNCaP cells relative to pcDNA3.1-transfected cells. Reporter expression was directed by the human AR promoters (–110/+246, –311/+560, –726/+560) or from the pGL3b vector itself. Data are averages with SD from three independent transfections. B, Repression of the –110 to +246 AR promoter by TNF α in LNCaP cells, and lack of the negative regulation in the presence of I κ B-SR. The AR promoter-reporter was transfected into LNCaP (solid bar) or LNCaP-SR-I κ B cells (open bars), and 24 h later the cells were treated with vehicle or TNF α (20 ng/ml) for an additional 24 h before the cells were analyzed for luciferase. Luciferase values are presented relative to the 100% value set for LNCaP-I κ B-SR cells in the absence of TNF α . C, Repression of the AR promoter (–110 to +246) in RWPE-1 (AR-positive, nontumorigenic) cells treated with TNF α . Transfection conditions were same as in panel B. D, Absence of the TNF α effect on the AR promoter (–110 to +246) in C4-2 cells. It should be noted that the luciferase values were comparable in LNCaP and C4-2 cells in the absence of TNF α treatment. In contrast to LNCaP cells, C4-2 cells showed no reduction of luciferase activity after TNF α treatment. E, Comparable NF- κ B activation by TNF α in LNCaP and C4-2 cells. Transactivation of a canonical NF- κ B element was assayed in transfected LNCaP and C4-2 cells with or without TNF α treatment. F, NF- κ B DNA-binding activity in TNF α -treated LNCaP and C4-2 cells. The NF- κ B element from the IL-6 gene was used as the EMSA probe. TNF α treatments in panels B–F were for 24 h at 20 ng/ml.

κ B-responsive negative regulatory region maps to a location delimited by –110 and +246 positions. Indeed, TNF α treatment itself also inhibited the activity of the –110 to +246 AR genome fragment in LNCaP cells, and the inhibition was completely blocked in the presence of I κ B-SR (Fig. 4B). Of note is the finding that TNF α did not induce the pGL3b vector in LNCaP or RWPE-1 or C4-2 cells (data not shown). Therefore, in Fig. 4A, pGL3b stimulation by NF- κ B is a result of p65/p50 overexpression.

Similar to LNCaP cells, RWPE-1 nontumorigenic prostate epithelial cells were inhibited for AR promoter function by about 60% upon treatment with TNF α (Fig. 4C), indicating that the molecular machinery coordinating NF- κ B-mediated repression of AR is not limited to LNCaP cells but is present also in nonmalignant, normal type prostate cells. In contrast, androgen-independent C4-2 cells were refractory to the TNF α -mediated inhibition of the AR promoter activity (Fig.

4D). The luciferase activity driven by the AR promoter in the absence of TNF α was comparable in C4-2 vs. LNCaP cells, indicating that the sensitivity of the repression assay is not limiting in C4-2 cells. Thus, we conclude that the lack of AR down-regulation in C4-2 cells reflects a block at the level of transcription.

Interestingly, NF- κ B activation in LNCaP and C4-2 cells is similar, evident from: 1) comparable TNF α -induced luciferase expression from the NF- κ B-responsive reporter construct in the two cell lines (Fig. 4E); 2) comparable NF- κ B DNA-binding activity in the two cell lines (note EMSA complex I and II) in response to TNF α treatment (Fig. 4F). Thus, the lack of TNF α -induced apoptosis in androgen-independent prostate cancer cells correlated with the lack of repression of the AR gene by NF- κ B. To elucidate the molecular basis for the differential NF- κ B regulation of the AR gene in the androgen-dependent vs. androgen-independent prostate cells, we characterized the TNF α -

responsive negative element in the human AR gene and explored the coregulator dynamics at the regulatory site.

TNF α -Responsive, NF- κ B-Regulated Negative Element in the Human AR Gene

To pinpoint the TNF α /NF- κ B-responsive negatively acting element, deoxyribonuclease I (DNase I) footprinting was conducted. The footprint within a segment of the 5'-UTR (from +110 to just beyond +235) produced by the rat liver nuclear extract revealed four protein-binding sites (Fig. 5A). Site I is especially noteworthy because it includes an NF- κ B-like element (GGGGCTGGCGT) at +154 to +144, in the opposite strand. The adjacent site II includes the consensus binding sequence (AACGCC) for the Myb family of transcription factors. The mouse and rat AR genes show 100% (excluding the 6-base deleted gap area) sequence homology with the human gene at site I, and the entire site II is 100% conserved with correspond-

ing sequences in the rodent genes (Fig. 5B). Given the high across-the-species sequence conservation at sites I and II in the 5'-UTR, it is highly likely that this region is involved in regulatory events with important physiological implications.

The chromatin area harboring site I and site II was explored for TNF α -regulated specific enrichment of NF- κ B and possibly the proteins of the myb family, using chromatin immunoprecipitation (ChIP) (Fig. 6A). Within 30 min of exposing LNCaP cells to TNF α , NF- κ B/p65 and NF- κ B/p50 had associated with chromatin fragments containing site I and site II, because the immunoprecipitated DNA could be PCR amplified with a primer set specifying -38 to +246 positions. B-myb was also recruited to this region within 30 min. The closely related c-myb did not associate with this region, although c-myb and B-myb can bind to the same DNA elements *in vitro*. Recruitment of several known components of corepressor complexes, such as the histone deacetylase HDAC1, the transcription silencer SMRT, and the corepressor complex scaffold protein mSin3A, was also observed within 30 min of TNF α treatment. ChIP with unrelated anti-Cox-2 antibody (Fig. 6A), and with a primer set at -760 (forward), -460 (reverse) (Fig. 6B) did not generate any PCR signal. Association of acetylated histones and phospho-RNA polymerase II (two markers of transcription-active chromatin) with the negatively acting chromatin region was also markedly diminished in the presence of TNF α (Fig. 6C). Therefore, we conclude that B-myb and NF- κ B p65, p50 are involved in the TNF α -regulated AR gene repression.

A Stabilizing Role of B-myb for NF- κ B Binding to the Negative Element

Whether recruitment of NF- κ B and B-myb to the AR chromatin reflected direct binding of these transcription factors to 5'-UTR-located site I and site II, respectively, was investigated in EMSA using recombinant p65, p50, and B-myb and the DNA probe spanning both site I and site II (Fig. 7A). Recombinant p65 or p50 by themselves (lanes 1 and 2) or together (data not shown) did not produce any EMSA complex. In DNase I footprinting assay also, the combined presence of p65 and p50 did not reveal any protected region within the site I- and site II-included 5'-UTR (data not shown). B-myb (amino-terminal fragment containing the entire DNA-binding domain) produced a strong gel-shifted band (complex I, lane 3). B-myb together with p50 produced complex I and a second gel-shifted band (complex II, lane 4), indicating that complex II likely contains both p50 and B-myb. In the presence of p65 and B-myb, only complex I was detected (lane 5), indicating that B-myb did not produce a new complex in association with p65. When all three transcription factors were included in EMSA, it appears that a new poorly resolved complex III (in addition to I and II) might have appeared just above complex I (lane 6, arrowhead). Antibody supershift (lanes 11–14) shows

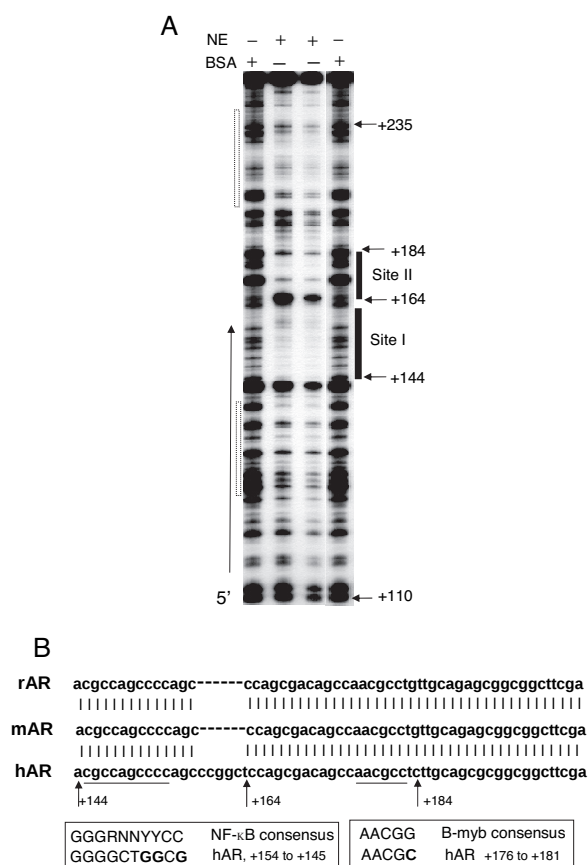


Fig. 5. Protein-Binding Sites at the 5'-UTR of the Human AR Promoter

A, DNase I footprinting of human AR gene from -110 to +246 with rat liver nuclear extract. B, Sequence conservation of rat and mouse AR promoters with the human AR promoter at +144 to +204. An NF- κ B-like element in site I and myb consensus sequence in site II are shown. hAR, human AR; mAR, mouse AR; rAR, rat AR; NE, nuclear extract.

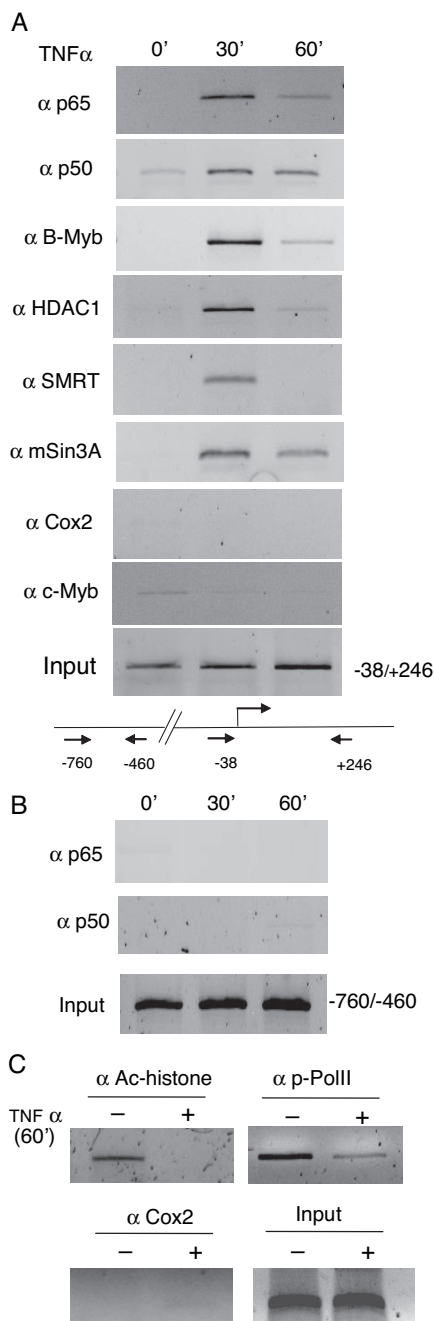


Fig. 6. TNF α -Responsive Enrichment of NF- κ B, B-myb, HDAC1, SMRT, and mSin3A at the Chromatin Region Containing Site I and Site II of the AR Promoter in LNCaP Cells. Soluble chromatin fragments (average, $\sim$ 500 bp) from LNCaP cells treated with TNF α (for 30 min or 60 min), or with vehicle (PBS), were analyzed by ChIP. A, The immunoprecipitated DNAs were amplified with AR-specific PCR primers (forward at -38; reverse at +246). These primers are described in *Materials and Methods*. B, PCR of the immunoprecipitated DNAs using upstream primers corresponding to the AR promoter from -760 to -460 (negative control). C, ChIP using antibodies to acetylated histones and to phospho-RNA polymerase II. AR-specific primers as in panel A were used in PCR amplification. Anti-Cox2 served as a negative control antibody. Amplified PCR products of 1% input DNAs from cells with or without TNF α treatment are shown. Ac-histone, Acetylated histone; Pol II, polymerase II.

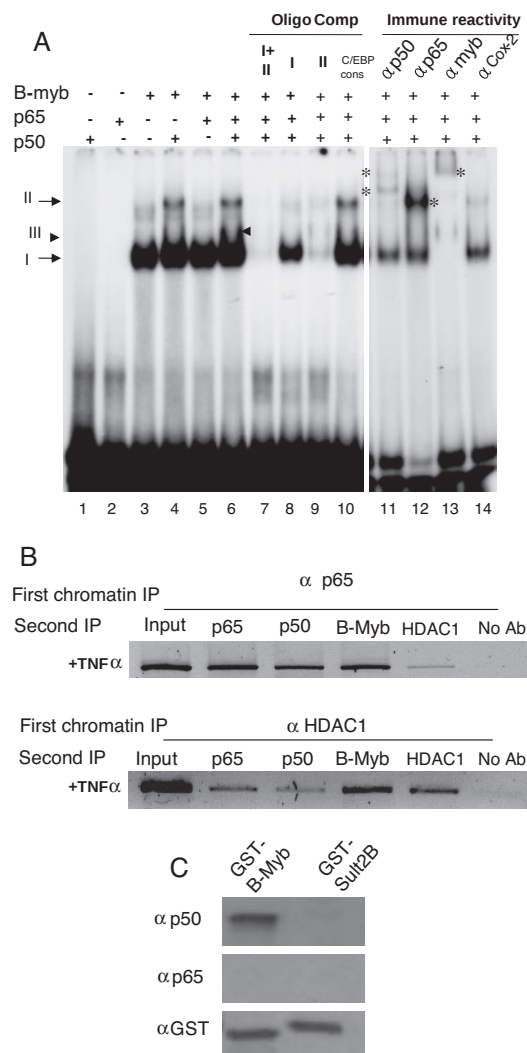


Fig. 7. Stabilization of NF- κ B Binding to the Negatively Acting Composite Site by the Adjacent Bound B-myb, and Concurrent Occupancy of the Regulatory Factors at the Negative Site in LNCaP Cells

A, Recombinant p65, p50, and B-myb singly, or in combination, were used in EMSA. Binding specificity was verified by competition with unlabeled double-stranded oligos. Identity of individual EMSA complexes was shown by antibody supershifts. Supershifted bands (lanes 11–13) are designated using asterisks. B, Two-step ChIP showing concurrent occupancy of p65, p50, B-Myb, and HDAC1 at the same genomic AR chromatin fragments harboring the negative site. LNCaP cells were treated with TNF α (20 ng/ml) for 30 min and then processed for two-step ChIP as described in *Materials and Methods*. C, GST-B-myb (NTD) bound to glutathione sepharose beads was mixed with recombinant p50 or p65, and retained proteins were identified (Western blot). GST-Sult2B (a GST fusion of the mouse dehydroepiandrosterone-sulfotransferase isoform Sult2B) is a negative control. Ab, Antibody; IP, immunoprecipitation; Comp, competitor; cons, consensus.

that B-myb is part of both complex I and II (lane 13); complex II additionally contained p50 (lane 11; two supershifted bands, noted with asterisks). In the presence of the anti-p65 antibody, a strong band appeared

at the complex II position, indicating the recognition of complex II by the p65 antibody (lane 12). Oligonucleotide competition showed specificity of individual EMSA complexes. Unlabeled oligo sequence covering sites I + II (lane 7) competed out all EMSA complexes, whereas only B-myb-containing complex I survived (lane 8) in the presence of unlabeled site I (+144 to 158). The unlabeled site II (+164 to 184) abolished all complexes (lane 9), indicating that p50 and p65 binding to site I is stabilized by the site II-bound B-myb.

Evidence for the concurrent occupancy of p65, p50, B-myb, and HDAC1 in the same chromatin fragments of the genomic AR was provided by two-step ChIP on TNF α -treated LNCaP cells (Fig. 7B). Only TNF α -treated cells were analyzed, because in the absence of TNF α none of the regulatory factors were present at the negative site. The chromatin fragments immunoprecipitated by anti-p65 were reimmunoprecipitated by anti-p65 (positive control), as well as by anti-p50 and anti-B-myb (*upper panel*). Re-ChIP with anti-HDAC1 also pulled down the same fragments, indicating association of HDAC1 with the same region of AR that was enriched for p65, p50, and B-myb. Co-occupancy of HDAC1 with p65, p50, and B-myb is further shown from another series of two-step ChIPs, in which anti-HDAC1 was used as the first antibody for the immunoprecipitation (*lower panel*). Absent PCR signals in no-antibody lanes confirmed specificity of the results.

B-myb is likely to directly associate with p50 at the TNF α -responsive negative regulatory site, whereas p65 appears to be part of this complex through its interaction with p50, because p50 and B-myb together formed a protein-DNA complex (*i.e.* complex II; Fig. 7A, lane 4), but no EMSA complex was detected with p65 and B-myb (Fig. 7A, lane 5). Interaction *in vitro* between B-myb and p50 and lack of an interaction between B-myb and p65 were observed in glutathione-S-transferase (GST) pull-down assay (Fig. 7C). Recombinant p50 bound to GST-B-myb, because immunoblotting detected p50 after its release from the glutathione beads. Absence of B-myb interaction with p65 is shown by the lack of Western blot signal for p65, indicating that p65 was not retained by the glutathione bead-immobilized GST-B-myb.

Interdependence of NF- κ B and B-myb in Mediating TNF α -Directed Negative Regulation

ChIP and EMSA (Figs. 6 and 7) indicated that functional interaction *in-cis* between NF- κ B and B-myb at the negative response region may be a prerequisite for the inhibition of AR expression. Functional assay with the wild-type and mutant AR promoters showed that the requirement is indeed an important controlling feature (Fig. 8). In the natural –110 to +246 AR gene background, point mutations at a single site (site I or site II) led to higher luciferase

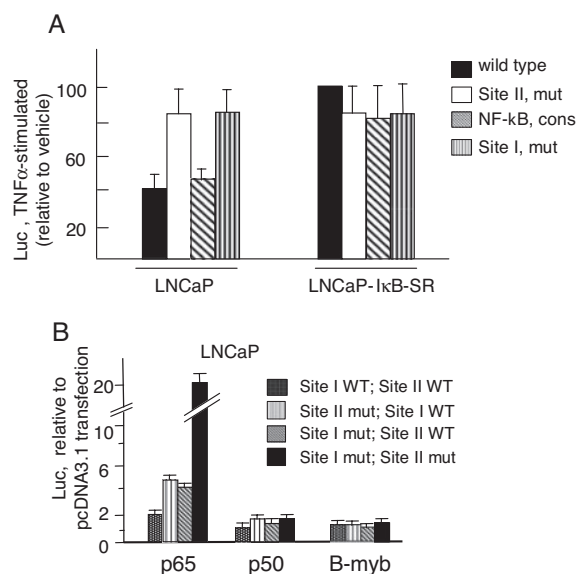


Fig. 8. Essential Involvement of NF- κ B (Site I) and B-myb (Site II) in the Repression of the AR Promoter

A, AR promoter (–110 to +246)-driven luciferase expression in TNF α -treated parental LNCaP or LNCaP-IκB-SR stable cells, relative to the corresponding cells treated with vehicle (PBS). All values are presented as percent of the luciferase expression from the wild-type promoter in LNCaP-IκB-SR cells, for which the value was set at 100%. The NF- κ B consensus indicates replacement of site I with a consensus NF- κ B element. Bar graphs indicate averages from three experiments ($\pm$ SD). B, Negative regulation of the TNF α /NF- κ B-responsive region in a heterologous promoter context. Averages of triplicate independent experiments, each performed in duplicate ($\pm$ SD). Luciferase activity in vector (pcDNA3.1)-transfected cells was set to a value of unity. Mut, Mutated; cons, consensus; WT, wild type.

expression compared with the wild-type promoter in TNF α -treated LNCaP cells. Thus, the repressive effect of activated NF- κ B on the wild-type promoter is released upon mutational inactivation of either site I or site II (Fig. 8A). Notably, changing site I to an NF- κ B consensus sequence (*third bar graph from the left*) retained TNF α -induced repression similar to that with the wild-type promoter. In the presence of IκB-SR (LNCaP-IκB-SR cells), TNF α did not repress the AR promoter function and, furthermore, the wild-type and mutant promoters were similarly active (Fig. 8A). Taken together, Fig. 8A shows that site I and site II act cooperatively to negatively regulate the AR gene activity.

The combined site I and site II activity also conferred NF- κ B-mediated negative regulation to a heterologous promoter (Fig. 8B). The reporter constructs contained two copies each of site I and site II sequences (combined as either both sites wild type or mutated, or mutated at only site I or site II). The heterologous sequences were cloned at the 5'-end of the minimal promoter vector (MCS-TATA-Luc). Upon p65 cotransfection, each single mutant construct caused derepression of luciferase activity by approximately 2-fold

relative to the wild-type construct. Mutations at both sites (double mutant construct) led to about 10-fold higher luciferase expression compared with the wild-type construct (Fig. 8B). The results are presented as relative values and compared the luciferase activity of individual constructs in p65-cotransfected vs. pcDNA3.1-cotransfected cells. Because p65 cotransfection induced the vector itself (due to the NF- κ B-inducible site in the luciferase cDNA), the construct with the wild-type site I plus site II was also induced ($\sim$ 2-fold) by p65 compared with pcDNA3.1. Nevertheless, sequence mutation at site I and/or site II was associated with much higher luciferase induction, indicating robust derepression of the negative activity manifested in the wild-type sequence (site I + site II). The mutant constructs did not derepress AR gene activity after cotransfection of p50 and B-myb, either individually (Fig. 8B) or in combination (data not shown). This is expected, because the stimulatory activity of NF- κ B requires the p65 transactivation domain.

Knock down of p65/NF- κ B in LNCaP cells caused approximately 1.5-fold increase in the endogenous AR level, further indicating that AR expression is under negative regulation by the NF- κ B/p65 (Fig. 9). The endogenous p65 level in p65-siRNA-treated cells was reduced by 90% or more (Fig. 9A). Knock down of c-Rel did not alter the endogenous AR level, suggesting that c-Rel/NF- κ B is not involved in the negative regulation. The endogenous AR level was also not altered by p50 knock down (Fig. 9, A and B), most likely due to the sustained NF- κ B activity of the p65/p52 heterodimer complex. Even in unstimulated

LNCaP cells, AR expression appears to be under NF- κ B-directed repression because 1) activated NF- κ B is detectable at a low level in untreated LNCaP cells (complex II, Fig. 4F); and 2) autocrine production of TNF α has been reported in unstimulated LNCaP cells (21).

Due to the presence of multiple positively acting myb-binding sites in the human AR gene, knock down of B-myb led to decreased expression of endogenous AR in LNCaP cells (data not shown). Nevertheless, functional assays in Fig. 8, A and B, clearly show that B-myb is an essential partner of NF- κ B in the TNF α -regulated transcription repression of AR. Furthermore, ChIP results (Figs. 6A and 7B) show that B-myb is part of the corepressor complex assembled at the negative site in response to TNF α .

Altered Coregulator Dynamics at the Negative Site in Androgen-Independent C4-2 Cells

Given that NF- κ B interaction with B-myb is an essential aspect for the TNF α -controlled down-regulation of AR, and that NF- κ B activity is induced equally well by TNF α in C4-2 and LNCaP cells, we determined how B-myb expression levels and coregulator dynamics at the negative site in the genomic AR would compare between the two cell lines (Fig. 10). Probing of LNCaP and C4-2 cells using Western blot assay shows similar B-myb expression levels (Fig. 10A). B-myb levels also remained the same in the two cell lines after treatment with TNF α (data not shown). On the other hand, real-time quantitative PCR (qPCR) assay of DNAs from ChIP shows that upon TNF α treatment, the negative regulatory site in the C4-2 cells was not enriched for p65, p50, B-myb, and the components of the corepressor complex that were tested (HDAC1, SMRT, mSin3A). The signal for c-myb also remained at the background level (Fig. 10B). The results in C4-2 cells are in contrast to those in LNCaP cells in which TNF α induced robust recruitment of p65, B-myb, and HDAC1 (Fig. 10B, *left panel*). Similar results are also seen with the semi-quantitative analysis of the PCR-amplified DNAs from immunoprecipitated chromatin fragments (Fig. 10C). Therefore, the absent negative regulation of AR in C4-2 cells is due to the inability of the required regulatory factors to occupy the negative regulatory site. Studies are under way to identify the cellular changes that prevent TNF α -induced engagement of relevant regulators at the negative site.

Figure 11 presents a model consistent with the notion that TNF α -induced interaction between NF- κ B and B-myb would facilitate corepressor assembly at the negatively responsive composite NF- κ B/B-myb element and initiate molecular events leading to AR gene repression.

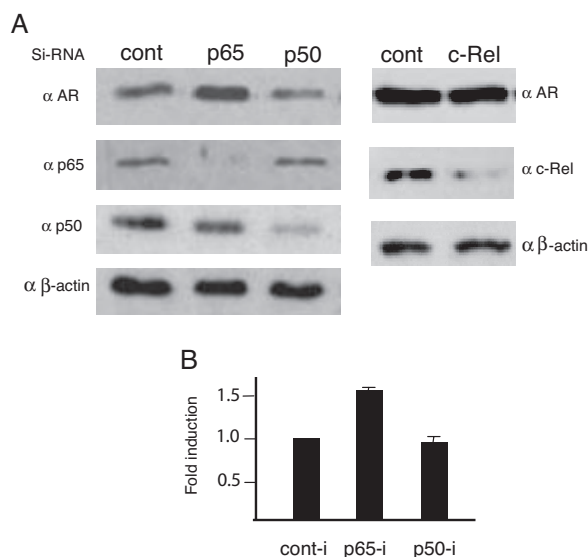


Fig. 9. Released Repression of Endogenous AR in p65 Knocked Down LNCaP Cells

A, Western blot of AR, p65 and p50 (*left*), and AR, c-Rel (*right*). B, Quantification of AR levels in the immunoblots using quanti-software. Bar graphs are averages $\pm$ SD from triplicate experiments. cont-i, Control RNA interference.

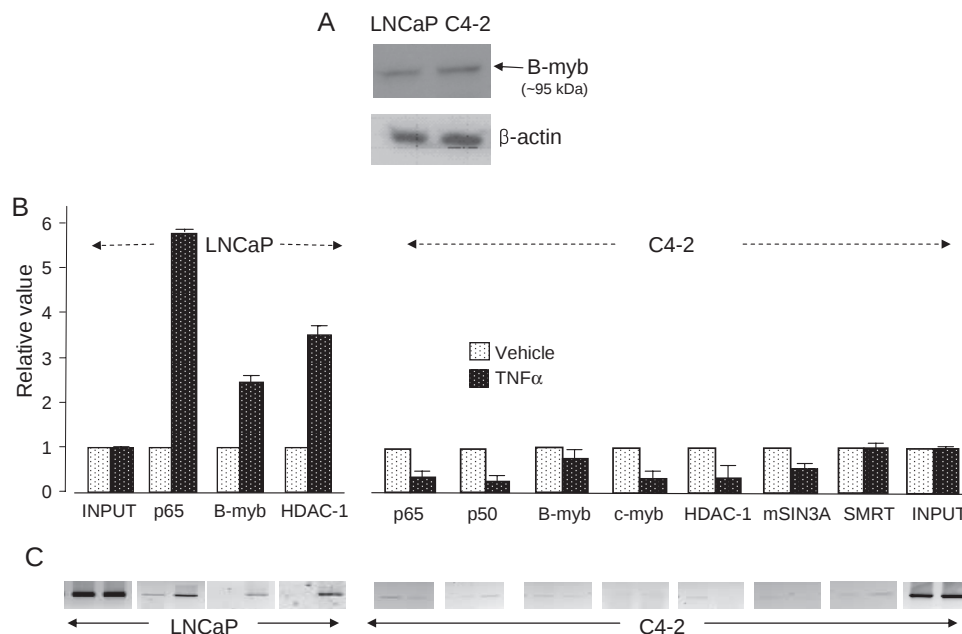


Fig. 10. B-myb Expression and TNF α -Induced Coregulator Dynamics at the Negative Regulatory Site in the AR Gene in LNCaP and C4-2 Cells

A, Western blot of B-myb, using β -actin as the normalization control. B, ChIP in LNCaP and C4-2 cells treated with TNF α (20 ng/ml for 30 min) or vehicle. Real-time qPCR quantified PCR signals. In each case, the value from vehicle-treated cells was set to unity. C, PCR products (285 bp) from the same ChIP analysis as in panel B, visualized as ethidium bromide-stained bands, on agarose gel.

DISCUSSION

TNF α is an autocrine and paracrine stimulator of prostate cancer cells *in vivo*, because prostate tumors, not the surrounding normal tissues, express TNF α (21), and local enrichment of this proinflammatory cytokine at tumor sites is brought about by infiltrating monocytes and macrophages (14, 15). The present study shows that the endogenous AR in androgen-dependent LNCaP prostate cancer cells is reduced by TNF α . Additionally, TNF α -regulated repression of AR was observed in nonmalignant RWPE-1 androgen-dependent prostate cells. In contrast, the negative regulation is absent in the LNCaP descendant C4-2 and C4-2B cells, which are androgen independent. Importantly, TNF α inhibited proliferation and induced apoptosis of LNCaP cells but not C4-2 and C4-2B cells. The absent AR down-regulation appears to explain, at least in part, the resistance of androgen-independent prostate cancer cells to the cytokine-induced apoptosis, because AR knock down can trigger apoptosis of both androgen-dependent and androgen-independent prostate cancer cells (7–10).

Reduced AR expression in TNF α -treated LNCaP cells is due to transcription repression that requires a combined action of activated NF- κ B and the B-myb transcription factor at a composite regulatory site in the 5'-UTR of the AR gene. Reduction of AR leads to decreased androgen sensitivity, evident from the inhibition of the androgen-induced PSA expression in

LNCaP cells after TNF α treatment. Previously, a post-transcriptional mechanism for the TNF α action to suppress AR was suggested, based on the result that AR mRNA levels in TNF α -treated LNCaP cells declined even after actinomycin D inhibition of transcription (21). Our results unambiguously establish a transcription mechanism for the negative regulation of AR, because 1) TNF α inhibited activity of the transfected AR promoter in reporter assay; 2) TNF α induced recruitment of NF- κ B and B-myb to the chromatin region in the genomic AR harboring the negative regulatory site; 3) NF- κ B and B-myb specifically bound to cognate DNA sequences within the composite site; and 4) TNF α induced corepressor occupancy at the negative site, evident from the enriched presence of HDAC1, SMRT, and mSin3A in parallel to the recruitment of NF- κ B and B-myb.

Analysis of the activity of the mutagenized AR promoter showed that a functional interplay between NF- κ B and B-myb at the composite regulatory site is essential for AR gene repression. Negative regulation by the composite site was also observed in a heterologous promoter background. EMSA results suggest that B-myb bound to the target regulatory site can stabilize NF- κ B binding to the adjacent imperfect NF- κ B-binding sequence. Two-step ChIP showed that the same population of genomic AR fragments are enriched for NF- κ B, B-myb, and the corepressor components HDAC1, SMRT, and mSin3A in response to TNF α signaling. It appears from the GST pull-down

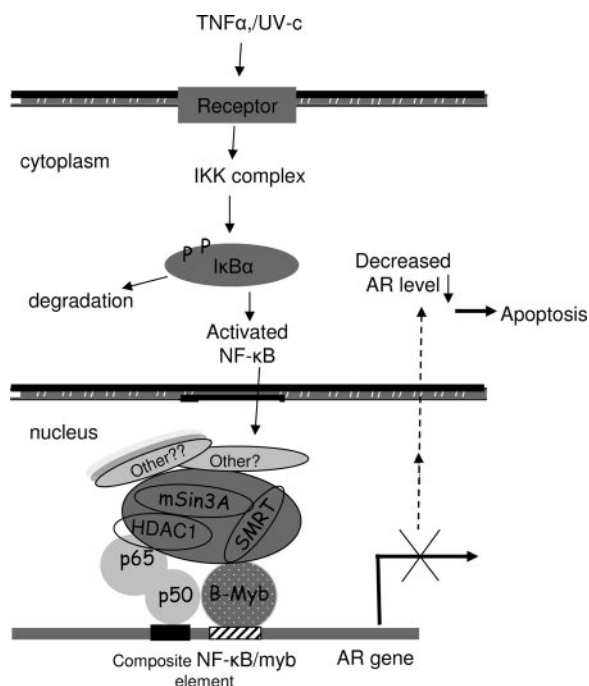


Fig. 11. Model for the Role of NF- κ B and B-myb in the TNF α -Controlled Transcription Repression of AR

Based on our results we propose: 1) NF- κ B and B-myb are recruited to the composite element at the AR promoter in response to TNF α ; 2) NF- κ B binding to the composite site is dependent upon B-myb binding to this site; and 3) B-myb physically associates with p50, not p65; the trimeric B-myb/p50/p65 complex further recruits components of corepressor complex (SMRT, HDAC1, mSin3A, and others) to orchestrate AR gene repression.

result that B-myb engages in protein-protein interaction with p50 but not p65. Nevertheless, p65 should be an essential component of the negative regulation because two-step ChIP showed concurrent occupancy of p65, p50, and B-myb at the regulated site. Additionally, the endogenous AR level in LNCaP cells was repressed by NF- κ B/p65, because the AR level increased after p65 knock down.

Despite the absent AR down-regulation, NF- κ B was activated in androgen-independent C4-2 cells by TNF α similarly to LNCaP cells. B-myb expression levels were also similar in these two cell lines. Resistance of androgen-independent prostate cancer cells to the negative regulation of AR manifests a block in transcriptional regulation, because TNF α failed to inhibit the AR promoter activity in C4-2 cells (Fig. 4D). ChIP showed that neither the NF- κ B subunits (p65, p50) and B-myb nor the corepressor components HDAC1, SMRT, and mSin3A were detected at the target-responsive region in C4-2 cells after TNF α stimulation. The failure to assemble coregulatory factors along with NF- κ B and B-myb in C4-2 cells at the negative site may be due to one or more of the following possibilities: 1) the composite site is altered in C4-2 cells through mutation or altered DNA methylation; 2) post-

translational modification of a critical regulatory factor is altered in C4-2 cells due to changes in a specific intracellular signaling pathway (such as a specific kinase pathway), preventing assembly of required regulatory factors at the negative site; and 3) the expression of one or more yet-to-be identified factor(s) essential for transcription repression is lacking in C4-2 cells. These possibilities are currently under investigation.

The protooncogene product B-myb is an important regulator of G1 $\rightarrow$ S progression of cell cycle (26). A role for B-myb in prostate cancer progression is likely, because it can transactivate the gene for the antiapoptotic protein clusterin, which confers resistance of prostate cancer cells to apoptosis (27, 28). The essential role of B-myb in the NF- κ B-directed AR gene repression is reminiscent of the interdependence of N-myc and NF- κ B in the TNF α -controlled negative regulation of the glutamate transporter gene (19).

NF- κ B targeting of HDAC1, as seen here for the negative regulation of AR, has also been described in several other settings. For example, HDAC1 plays a role in the NF- κ B-mediated transcriptional repression of the long-terminal repeat of the HIV genome, causing viral latency (20). HDAC1 appears to interact with NF- κ B/p50 on the latent promoter. In another example, negative gene regulation by NF- κ B involves direct association of HDAC1 with p65 via the Rel homology domain (29). The corepressor SMRT and the corepressor complex-associated scaffold protein mSin3A were recruited to the negative regulatory region in the AR gene under TNF α stimulation. SMRT (silencing mediator of retinoid and thyroid receptor) was originally identified as a coregulator involved in the transcription repression mediated by unliganded retinoic acid and thyroid hormone receptors (30). The role of SMRT and the related corepressor N-CoR (nuclear receptor corepressor) in transcription repression extends beyond nuclear receptors to other transcription factors (31). Sin3A is a large multidomain protein, which acts as a scaffold upon which other components of the corepressor assembly congregate (32). Transcription repression mediated by the unliganded thyroid hormone receptor as well as by the MAD family of transcription factors was shown to require interaction of these transcription factors with corepressor assembly containing mSin3, N-CoR, and HDAC1 (33, 34). We did not detect enrichment of N-CoR at the genomic AR, indicating its absence in the corepressor assembly that plays a role in suppressing the AR gene in prostate cancer cells in response to TNF α -induced inflammation.

It has been reported that reduced AR expression through antisense oligos caused overexpression of the cell cycle-regulatory cyclin-dependent kinase 2 inhibitor p21 (Waf1/Cip1) in prostate cancer cells, and increased p21 expression partially restored androgen dependence of cell growth in androgen-independent prostate cancer cells (35). The androgen-independent cells used in this study were derived from androgen-dependent LNCaP cells that were passaged under

chronically androgen-deprived culture conditions. Whether p21 expression would increase in LNCaP cells in association with TNF α -regulated AR repression needs to be assessed.

We may speculate that suppression of AR in androgen-dependent prostate tumors by inflammatory agents would lead to enhanced sensitivity of the cancer cells to apoptotic stimuli. An intriguing recent finding in this regard is that down-regulation of AR expression by the antitumor antibiotic Mithramycin, which inhibits binding of the Sp1 transcription factor to the 5'-UTR in the human AR promoter, reversed the resistance of AR-positive androgen-independent cells to the antiandrogen bicalutamide and increased sensitivity of these cells to the chemotherapeutic drug paclitaxel (36). Detailed knowledge of the role of specific factors and/or converged signal transduction pathways in AR gene repression by TNF α may reveal novel approaches to knock down AR in prostate tumors and increase tumor cell sensitivity to apoptotic stimuli.

MATERIALS AND METHODS

Cells, Culture Conditions, and UV Irradiation

The sources for different cell lines are as follows: LNCaP, RWPE-1 (American Type Culture Collection, Manassas, VA); C4-2, C4-2B (ViroMed Laboratories, Inc., Minnetonka, MN); LNCaP-SR- $\text{I}\kappa\text{B}$ (37), a gift from S. Maheswaran at Harvard University, Cambridge, MA). LNCaP and LNCaP-SR- $\text{I}\kappa\text{B}$ were cultured in RPMI; C4-2 and C4-2B were cultured in DMEM, and RWPE-1 were cultured in keratinocyte serum free medium. Media contained penicillin, streptomycin, and 10% fetal bovine serum. For androgen induction of PSA, LNCaP cells were cultured in RPMI containing 5% charcoal-stripped serum for 3 d, and changed to a fresh medium before hormone treatment. LNCaP cells (80% confluence) were irradiated with UV at 40 J/m² for 1 min. After irradiation, medium was replaced, cells were further incubated (12 h, 37 C, 5% CO₂), and cell lysate was analyzed.

Plasmids, Cell Transfections with Plasmids and siRNAs, and Adenovirus Infection

A human AR promoter (−1040 to +560), generated by PCR amplification of the HepG2 cell genomic DNA was sequence confirmed and used to prepare AR reporter constructs by inserting restriction enzyme-digested AR promoter fragments into *KpnI/HindIII* sites in pGL3b (Promega Corp., Madison, WI). Heterologous constructs were generated by cloning two copies of an oligonucleotide (separated by TTT) covering site I and site II (from +144 to +184) into the MCS-TATA-Luc vector (at the *HindIII/EcoRI* site). Wild-type or mutant oligos are as follows: 1) wild-type: 5'-ACGCCAGCCCCAGCCCG-GCTCCAGCGACAGCCAACGCCTCT; 2) site I (NF- κB) mutant: 5'-ACGCCATCTCCAGTCTGGCTCCAGCGACAGCCA-ACGCCTCT; 3) site II (Myb) mutant: 5'-ACGCCAGC-CAGCCCGGCTCCAGCGACAGCCAATCGTCTCT; and 4) double mutant (site I + site II): 5'-ACGCCATCTCCAGTCT-GGC-TCCAGCGACAGCCAATCGTCTCT. The base mutations can inactivate binding of NF- κB or Myb to the cognate site.

Cells were seeded in 24-well plates, cultured overnight, and then transfected with plasmids for 48 h using Fugene6 liposome (Roche Molecular Biochemicals, Indianapolis, IN). Lysates from washed cells were assayed for luciferase ac-

tivity and protein. Transfection of siRNAs was performed as per vendor recommendation (Invitrogen, Carlsbad, CA). Cells at 10⁵ per well (six-well plate, medium, no antibiotics) were transfected with 20 μM siRNA oligo, using oligofectamine. Culture medium was changed to a fresh medium without serum, oligofectamine-siRNA complexes were added onto cells, and serum was added after 4 h. At 48 h after transfection, harvested cells were processed for Western.

For $\text{I}\kappa\text{B}$ -SR expression, cells were infected with the adenovirus at 20 multiplicity of infection (moi). After 48 h, cells were treated with TNF α for 12 h. Total cell lysates were used for Western blot. Viral amplification and titer determination were done as per vendor recommendation (Qbiogene, Irvine, CA). Other reagents are as follows: B-myb cDNA (full-length; ATCC); p65 and p50 cDNA plasmids (from Dr. Steve Harris, UTHSCSA, San Antonio, TX; originally from Dr. Albert Baldwin, Chapel Hill, NC). The p65 and p50 cDNAs were cloned in our laboratory into pcDNA 3.1. Adenovirus $\text{I}\kappa\text{B}$ -SR (and control virus) were gifts from Dr. Santanu Bose.

Chromatin Immunoprecipitation (ChIP), Re-ChIP

ChIP was performed as described elsewhere (38). Cells were seeded onto 10-cm dishes at 1×10^7 cells and incubated in medium with or without TNF α (20 ng/ml) for 30 min or 60 min. Control cells were treated with vehicle for 30 min. After formaldehyde-mediated cross-linking of the cells, chromatin fragments were sheared by sonication to approximately 500-bp size, and solubilized chromatin was incubated at 4 C with approximately 2–5 μl of antibody, followed by incubation with 40 μl protein-A sepharose (Upstate Biotechnology, Inc., Lake Placid, NY). Protein complexes were washed (twice) with low-salt, high-salt, and LiCl solution, and Tris-EDTA buffer (twice). Precipitated complexes were eluted (0.1 M sodium bicarbonate, 1% sodium dodecyl sulfate). A portion of the eluate (90 of 100 μl) was saved for re-ChIP. Eluate (10 μl) was brought to 0.2 M sodium chloride and incubated at 65 C (≤ 4 h). DNA was extracted by a DNA clean kit (Zymo Research, Inc., Orange, CA). For re-ChIP, 90 μl eluate was diluted 40-fold with ChIP dilution buffer and then incubated first with 2–5 μl of the second-step antibody and then with 40 μl protein-A sepharose. The washed precipitated complex was digested with proteinase K, and DNA was purified as above. DNA (3 μl of 100 μl total) was used for PCR amplification using primers corresponding to 5'-GACCCGACTCG-CAAACCTGTT at −38 (forward) and 5'-CCTCCGAGTCTT-TAGC-AGCT at +246 (reverse).

GST-Pull Down

The N-terminal domain (NTD) of B-myb was fused in frame to the 3'-end of GST cDNA (pGEX-4T1), and GST-B-myb NTD expressed in *Escherichia coli* (BL21) were purified by glutathione-sepharose affinity chromatography. Recombinant p65 and p50 were commercially obtained [Active Motif (Carlsbad, CA) and Promega Corp., respectively]. GST-B-Myb NTD was incubated first with either p65 or p50 at 4 C for 4 h, and protein complexes were pelleted out after incubation with glutathione-Sepharose beads. Washed beads were boiled with Laemmli's buffer and pelleted, and the supernatant was analyzed by Western blot using antibodies to p50 or p65 or GST.

DNase I Footprinting, EMSA, and Quantification of mRNAs

A radiolabeled coding strand probe for DNase I footprinting was generated by PCR of the human AR promoter using a ³²P-end-labeled forward primer (at +50) and an unlabeled reverse primer from +240 site. Conditions for DNase I footprinting, EMSA, and nuclear extract preparation from rat liver were described else-

where (39). LNCaP cell nuclear extract was prepared as described (40). Total RNAs (TRIzol extraction) were used to quantify AR and PSA mRNAs by RT-qPCR using SYBR Green PCR reagents kit (Bio-Rad Laboratories, Hercules, CA). Primer pairs are as follows. AR: 5'-TTCACCAATGTCAACTCCAGGA (+2262, forward); 5'-CTTGCACAGAGATGATCTCTG (+2689, reverse). PSA: 5'-TATTTCCAATG-ACGTGTG (+533, forward), 5'-TGCACCA-CCTTGGGTGTACAGG (+722, reverse). β -actin-sense: 5'-CGTACCCTGGCATCGTGAT-3'; antisense: 5'-GTGTTGGCGTACAGGTCTTT-3'. A single melting curve peak in qPCR assay ensured specificity of the PCR.

MTT Assay and TUNEL Staining

Cells (5000/well) were seeded in 96-well plates and treated with different amounts of TNF α for 5 d. Cells were incubated with Cell Titer⁹⁶ Aqueous One solution reagent (Promega) for 2 h at 37°C, 5% CO₂. The blue color was quantified from absorbance at 490 nm using a 96-well plate reader (Victor³, PerkinElmer, Norwalk, CT).

For TUNEL staining, LNCaP and C4-2 cells were seeded onto 60-mm plate (coated with poly-L-lysine, glass-bottom culture dish) at 50% confluence, incubated with vehicle or TNF α for 5 d and stained using vendor-recommended protocol (Dead End Fluorometric TUNEL System protocol; Promega). Briefly, after TNF α (or vehicle) treatment, the cells were washed (PBS) and fixed with 4% methanol-free formaldehyde solution. Cells were then permeabilized by 0.2% Triton X-100 solution. Cells were incubated with recombinant TdT and nucleotide-mixed reagents (Promega) for 1 h at 37°C, after which the cells were washed with PBS and 2 $\times$ SSC. Cells were then incubated with propidium iodide (PI) solution. Signals from green fluorescent protein (GFP) fluorescence and red fluorescence (PI-emitted) were analyzed under a fluorescence microscope.

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