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LEF1 in androgen-independent prostate cancer: regulation of androgen receptor expression, prostate cancer growth and invasion

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Abstract

A major obstacle in treating prostate cancer is the development of androgen-independent disease. In this study, we examined LEF1 expression in androgen-independent cancer as well as its regulation of androgen receptor (AR) expression, prostate cancer growth and invasion in androgen-independent prostate cancer cells. Affymetrix microarray analysis of LNCaP and LNCaP-AI (androgen-independent variant LNCaP) cells revealed 100-fold increases in LEF1 expression in LNCaP-AI cells. We showed that LEF1 overexpression in LNCaP cells resulted in increased AR expression and consequently enhanced growth and invasion ability, while LEF1 knockdown in LNCaP-AI cells decreased AR expression and subsequently the growth and invasion capacity. Chromatin immunoprecipitation, gel shift, and luciferase assays confirmed LEF1 occupancy and regulation of the AR promoter. Thus, we identified LEF1 as a potential marker for androgen independent disease and a key regulator of AR expression and prostate cancer growth and invasion. LEF1 is highly expressed in androgen-independent prostate cancer, potentially serving as a marker for androgen-independent disease.

Keywords

androgen receptor; LNCaP; LNCaP-AI; prostate cancer growth and invasion

Introduction

A variety of theories have been proposed regarding the mechanism by which prostate cancer cells progress to aggressive phenotypes, including increased invasive and metastatic potentials, and developing resistance to androgen ablation therapy (1-3). Of great importance, increasing evidences fortify that increased androgen receptor (AR) expression and altered AR function, due to amplification, overexpression and mutation, play a dominant role in the progression and treatment failure of advanced prostate cancer (4-8). The role of

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AR in the invasive and metastatic potential of prostate cancer is suggested by several (9-11), including a recent (12), studies. However, the direct role AR plays in the invasion and metastasis of prostate cancer remains under-studied.

Regulation of AR expression is complex, involving positive (13,14) and negative (15) control by transcription factors. AR expression is also linked to cross-talk with growth factor (GF) receptor or other signaling pathways (16-24). LEF1, lymphoid enhancer-binding factor 1 in the Wnt signaling pathway, has been indicated to regulate AR expression (25). The LEF1/TCFs are the nuclear transducers on activated Wnt pathway. Members of the family represents a group of proteins with DNA-binding activities that specifically recognize and bind to a contiguous 6 base consensus sequence, 5'-a/ta/ t-C-A-A-G-3' within the 5' promoter regions of Wnt target genes (26). There are 8 putative LEF1 binding sites described at the AR promoter region (25) regulating its expression.

In addition to its function in a variety of developmental processes including stem-cell renewal, embryonic development, and tissue differentiation, more recent evidences indicate that aberrant activity of the Wnt signaling pathway is involved in tumorigenesis of several organ systems including androgen-independent prostate cancer (27-31). Androgen-independent prostate cancer cells are defined by their ability to survive testosterone deprivation by castration and to sustain AR activation through ligand-dependent and independent mechanisms (32-34). Several AR positive androgen-independent prostate cancer cell lines have been established (12,35,36). In comparison with traditional AR negative PC3 (or low AR) and DU145 cells, AR-positive androgen-independent cell lines are very useful in studying growth and invasion characteristics of androgen-independent prostate cancer. Like other androgen-independent prostate cancer cell lines, LNCaP-AI cells can grow both in the presence and absence of androgen, whereas its parental LNCaP cells can only grow in the presence of androgen (15,35,37-40). This study used androgen-independent prostate cancer cells to determine the function and mechanism of LEF1 in regulation of AR expression, prostate cancer growth and invasion.

Materials and Methods

Cell culture, dual luciferase assays, cell proliferation and *in vitro* Matrigel invasion assays

LNCaP cells were maintained in RPMI 1640 (GIBCO, Gaithersburg, MD) with 10% heat-inactivated bovine serum (FBS). The androgen independent LNCaP-AI cells were maintained in RPMI 1640 medium containing 10% charcoal-stripped, heat-inactivated FBS (CSFBS) (Hyclone Laboratories, Inc., Logan, UT) and 5 μg/ml of insulin, as described previously (38). Dual luciferase assay was performed as previously described (38) with luciferase reporter pAR-Luc (with 1.7 kb AR promoter sequence) and pCMV:LEF1 (Origene, MD). Cell proliferation by cell counting, flow cytometry and Matrigel invasion assays were performed as described previously (41). For invasion assays, percent invasion was expressed as the ratio of invading cells over cell number normalized on day 2 growth curve.

Immunofluorescence microscopy

The paraformaldehyde (4%) fixed LNCaP and LNCaP-AI cells were permeablized in methynol:acetone (1:1) for 10 minutes at -20°C. The cells were incubated overnight at 4°C with anti-AR and anti-LEF1 specific antibodies diluted 1:500 in 5% BSA buffer followed with anti-rabbit and anti-mouse IgG-Cy2-conjugated antibodies (Molecular Probes, Portland, OR) (1:300 in 5% BSA) incubation for 45 min at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; 10μg/mL; Molecular Probes, Carlsbad, CA) according to manufacturer's instructions.

Chromatin immunoprecipitation (ChIP) assays

CHIP was performed as described (42) with the following modifications: cross-linking was initiated with 11% formaldehyde solution at room temperature for 10 min and then stopped by addition of 0.125M glycine. The cross-linked chromatin was sonicated with Sonifer 450 microtip (Branson Ultrasonic Corp., Danbury, CT) at power setting 2 for 10 × 25sec on ice, produced DNA fragments with an average size of 1-3kb. For immunoprecipitation, 2μg of anti-AR antibody, anti-LEF1 antibody and IgG were mixed with 25μg of the purified cross-linked chromatin and incubated overnight at 4°C. Immunocomplexes were washed with RIPA buffer and TE buffer. After reversal and recovery of the immunoprecipitated chromatin DNA, the final DNA pellets were dissolved with 100μl water. Immunopurified DNA (4 μl) was used for a PCR with primers described in Table 1.

Gel shift assay

The assay was performed as previously described (43). Nuclear proteins extracted from LNCaP or LNCaP-AI cells were reacted with ^{32}P-labeled LEF1 binding sequence oligos corresponding to the promoter of AR gene (25), in binding buffer containing 1 μg of poly (dIdC) for 30 min at room temperature. The reaction mixtures were then subjected to electrophoresis and the binding complexes visualized by exposure on x-ray films. Specificity of the LEF binding complexes were established using specific and non-specific competitions (43), and LEF1 antibody.

Western blot and Immunohistochemistry analysis

Whole cell or cell fraction extracts (42) were subjected to SDS-PAGE and transferred to a nitrocellulose membrane for western blot analysis. Blots were incubated with primary antibodies (AR, LEF1, and β-actin Cell Signaling Technology, Inc., Danvers, MA) for 2h at room temperature, washed with TBST, and incubated for 1.5h with the horseradish peroxidase-conjugated secondary antibody (1:5,000, Amersham Biosciences). The protein bands were detected by an enhanced chemiluminesence kit (Amersham Biosciences). Immunohistochemistry for LEF1 and AR was performed using single label IHC on a NexES automated immunostainer as described previously (44).

Results

High levels of LEF1 expression in androgen-independent disease

In Affymetrix microarray with U133A GeneChip (15), the ratio of LEF1 expression between LNCaP-AI (an androgen-independent variant of LNCaP)(35) and LNCaP was 100-fold. To confirm this dramatic change of LEF1 expression and its celluar localization between LNCaP and LNCaP-AI cells, we performed RT-PCR, Western blot analysis and immunofluorescent microscopy. LEF1 showed sharply increased expression in LNCaP-AI at the mRNA level by RT-PCR (Fig. 1 A, *Left*, panel 1-2) and at the protein level by Western blot analysis (Fig.1 A, *Left*, panel 3-4). Cell fractionation revealed that LEF1 is localized to the nucleus in LNCaP-AI cells (Fig.1A, *Right*, panel 1) with nuclear co-localization of AR (Fig. 1A, *Right*, panel 2) and Histone 4 (Fig. 1A, *Right*, panel 4), serving as controls for the nuclear localization and β-actin (Fig. 1A, *Right*, panel 3) as control for the cytoplasmic localization. Immunofluorescent microscopy confirmed nuclear localization of LEF1 in the majority (98%) of LNCaP-AI cells (Fig. 1 B). In contrast, there is minimal LEF1 expression in LNCaP cells. As a control, AR was localized to the nucleus in both LNCaP and LNCaP-AI cells (Fig. 1B, mid panels). We also examined the levels of LEF1 expression in several prostate cell lines, including benign immortalized RC165 and RC170 (45), PC3 (low or negative AR) and DU145 (negative for AR) cells and did not detect LEF1 expression by Western blot analysis (data not shown).

Since LEF1 is specifically increased in LNCaP-AI prostate cancer cells, we examined the expression of LEF1 in androgen-independent cancer using a tissue microarray consisting of 99 cases, including 24 benign, 56 androgen-dependent, and 19 androgen-independent cases of prostate cancer. Androgen-dependent specimens were derived from patients who were diagnosed with prostate cancer by transurethral resection of the prostate (TURP), having high grade (Gleason 8 or higher) and volume of disease. Androgen-independent samples were derived from patients who underwent TURP at least 6 months after surgical orchiectomy. The immunostaining signal is scored as a combination of intensity (0 as negative, 1+ as weak, 2+ as moderate and 3+ as strong expression) and percentage of positive cells (<10% as 1+ and ≥10% as 2+) as in any given case with highest score as 5. Of 24 benign cases, LEF1 is not expressed in luminal cells in all cases and is only expressed in basal cells in certain cases (Fig. 1C, *Left*). Lef1 is expressed in 9 of 56 (16%) androgen-dependent cases ranging from 20% to 60% positive cells for a given case. We observed a statistically significant ($p<0.01$) difference of LEF1 expression between androgen-dependent and -independent cases. LEF1 is expressed (Fig. 1C, *Right* and Fig. 1D, *Left*) in 13 of 19 (66%) androgen-independent cases. The expression of combined score is statistically significantly higher in androgen-independent than -dependent cases ($p=0.016$) (Fig. 1D, *Right*). The IHC was performed without primary antibody and find no staining above background. The antigen peptide was also able to compete off the IHC signal.

Increased growth and invasion of LNCaP-AI is AR-dependent

AR is expressed in both LNCaP and LNCaP-AI cells, with greater levels in LNCaP-AI cells (Fig. 2A, *Left*). The increase in cell proliferation among LNCaP-AI cells is prominent in androgen free, though androgen can stimulate its growth (Fig. 2B, *Left* and *right*, respectively), validating the androgen-independent nature of LNCaP-AI cells. Flow cytometric analysis of LNCaP-AI cells with reduced levels of AR by siRNA knock down showed a 5.1% of decrease of cells in the S-phase cell cycle. Further, we used Matrigel invasion assays to determine the difference of invasion ability between LNCaP and LNCaP-AI cells, both in androgen medium and androgen-free medium. LNCaP and LNCaP-AI cells showed comparable minimal invasion in androgen free medium (Fig. 2C and 2D, *left*). While androgen increased the invasion ability of both LNCaP and LNCaP-AI cells, LNCaP-AI cells showed greater increase in invasive ability (up to 4-fold) compared to LNCaP cells (1.5 fold) in presence of 10 nM R1881 (Fig. 2D, *Left*), suggesting that androgen and AR plays a key role for invasion in LNCaP-AI cells. We employed small RNA interference technology to knock down AR in LNCaP (Fig. 2A, *Middle*) and LNCaP-AI-cells (Fig. 2A, *right*) followed by invasion assays to confirm AR-dependent nature of invasion. In the presence of 10 nM R1881, there is a 50% reduction in invasion by LNCaP cells and even greater reduction (up to 3 fold) of invasive LNCaP-AI cells (Fig. 2D, *Right*). There was no statistical difference in invasiveness of both LNCaP and LNCaP-AI cells with (Fig. 2D, *Right*) or without (Fig. 2D, *Left*) AR knockdown in androgen-free medium. These results strongly indicate that increased invasiveness of LNCaP-AI cells is dependent on AR.

LEF1 promotes growth and invasion of LNCaP-AI cells

LEF1 is highly expressed in LNCaP-AI but not LNCaP cells (Fig. 1A, *left*). To determine whether LEF1 is involved in the increased growth and invasion of LNCaP-AI cells, we performed cell proliferation and invasion assays of LNCaP with LEF1 over-expression and of LNCaP-AI cells with decreased LEF1 expression by siRNA knock down. The levels of LEF1 expression in LNCaP cells transfected with pCMV:LEF1 is comparable with LEF1 in LNCaP-AI cells (data not shown). With LEF1 overexpression (Fig. 3A, *Left*, upper panel), increases were observed in both LNCaP-LEF1 cell proliferation, up to 4.1% in S-phase cell population by flow cytometry (Fig. 3B, *Left*) and invasion, up to 2.5-fold by Matrigel invasion assays (Fig. 3B, *Right*). On the other hand, with reduced levels of LEF1 by siRNA

(Fig. 3A, *Right*), LNCaP-AI cell growth was reduced as indicated by a 6% reduction in S-phase cell population in androgen media (Fig. 3C, *Left*). Similarly, the invasion ability of LNCaP-AI cells was also reduced 3-fold in androgen media (Fig. 3C, *Right*). To examine the specificity of the LEF1 knockdown and to guard against off target effects, we have repeated this experiment using two additional LEF1 siRNAs and obtained similar effects in cell growth and invasion assay (data not shown). Importantly, these distinct LEF1 siRNAs also substantially reduce AR expression in LNCaP-AI cells. Taken together, the results show that LEF1 is involved in growth and invasion of LNCaP-AI cells.

LEF1 regulates expression of AR in LNCaP-AI cells

AR is expressed at greater level in LNCaP-AI than LNCaP cells. In parallel, LEF1 is expressed at high levels in LNCaP-AI and at minimal levels in LNCaP cells. We determined the relationship between the expression of LEF1 and AR in LNCaP and LNCaP-AI cells by siRNA knockdown of either AR or LEF1, coupled with Western blot analysis to detect LEF1 and AR expression, respectively. The LNCaP-AI cells with LEF1 knocked down by 10 nM siRNA exhibited a complete block in AR expression, shown by RT-PCR at mRNA levels (Fig. 4A, *Left*) and Western blot analysis at the protein level (Fig. 4A, *Middle*). In contrast, knockdown of AR did not affect the levels of LEF1 expression in both LNCaP and LNCaP-AI cells (Fig. 4A, *Right*), indicating that LEF1 regulates expression of AR in LNCaP-AI cells.

We performed ChIP and gel shift assays to examine the direct occupancy of LEF1 on the eight putative LEF1 binding sites of AR promoter of both LNCaP and LNCaP-AI cells. In LNCaP cells, we first confirmed the promoter occupancy of the three reported binding sites (25), sites 2, 3, 4/5 (Fig. 4B, *upper panels*), validating our ChIP assay data. In LNCaP-AI cells in the absence of androgen, LEF1 was readily recruited to the sites 2, 3, 4/5 of the AR promoter (Fig. 4B, *Lower part*). After treatment of LNCaP-AI cells with androgen, LEF1 recruitment increased in both sites and intensity on the promoter of AR (Fig. 4B, *Lower panels*).

The specific binding of LEF1 on the AR promoter region was confirmed by gel shift assays using both wild type and mutant probes. Data indicate that LEF1 binds to site 2 binding region in LNCaP (Fig. 4C, lane 2) and LNCaP-AI cells (Fig. 4C, lane 3). The specificity of binding was confirmed as the complex does not form in the absence of probe (Fig. 4C, lane 1) and in the presence of mutant probes (5’-CCAGACTCTCGACGGCAAAATC-3’) (Fig. 4C, lane 4-6). Binding at sites 3 and 4/5 were also confirmed (data not shown), and there is no complex binding for site 6 (Fig. 4C, lane 7-9).

To correlate the physical occupancy of LEF1 on the AR promoter with transcriptional activation of AR, we performed dual luciferase assays with a luciferase reporter containing a 6 kb AR promoter region in LNCaP cells. Transcriptional activity was increased up to 2-fold with LEF1 overexpression (Fig. 4D). The results of the above studies strongly support the direct regulation of AR expression by LEF1.

Discussion

The AR expressing androgen-independent prostate cancer cells can grow in the absence of androgen but also they grow faster in the presence of androgen (32). Our data show enhanced growth of LNCaP-AI cell in both androgen-free and androgen media, indicating possible androgen-independent function of AR in this process. We also investigated the invasion of LNCaP and LNCaP-AI cells with a Matrigel invasion chamber. Both LNCaP and LNCaP-AI cells have higher invasive abilities in the presence of androgen, suggesting that androgen and AR are involved in invasion. In addition, LNCaP-AI cells showed over 4-

fold increases of invasion compared to LNCaP cells in androgen media. AR knockdown by siRNA revealed reduced invasion of LNCaP-AI cells, indicating that the increased invasiveness of LNCaP-AI cells is androgen receptor-dependent, consistent with recent reports (12).

Interestingly, the enhanced invasion ability is also LEF1-dependent and our data showed this is due to regulation of AR expression by LEF1. Similar effects were observed with another set of androgen-dependent and -independent, MDA and MDA-HR cells (Li Y and Lee P, unpublished data). Wnt signaling has been shown to be involved in prostate cancer development and progression (27,28,31,46) with cross-talk between the Wnt and androgen signaling pathways in prostate cancer (47,48). β-Catenin, a key component of the Wnt signaling pathway, serves as a coactivator for LEF/TCF transcriptional activator proteins and a ligand-dependent coactivator of AR (49,50). Wnt signals are transmitted to the nucleus through β-catenin and lead to active transcriptional regulation by LEF1 (51). There is very low expression of LEF1 in LNCaP cells but over 100-fold increases of LEF1 expression in LNCaP-AI cells. On the protein level, LEF1 is localized to the nucleus in LNCaP-AI cells and binds to promoter regions of AR shown by ChIP and gel shift assays. Further, when overexpressed, LEF1 increases levels of AR expression in LNCaP cells by Western blot analysis, and when knocked down by siRNA, LEF1 reduces the levels of AR expression in LNCaP-AI cells. Functionally, dual luciferase assays showed that LEF1 increased luciferase activity using a reporter expressing AR promoter. These results of this study provide a novel mechanism for the crosstalk between AR and Wnt pathways in androgen-independent prostate cancer cells. Note worth mention, however, it does not seem that AR is regulated by LEF1 at all times. For example, AR is expressed in prostate stromal cells (52), whereas LEF1 is not (Fig. 1C), suggesting the AR expression can be LEF1 independent. Also, during prostate development, AR is expressed in both epithelial and stromal cells; however, LEF1 is only expressed in a subset of epithelial cells (Daniels and Lee, unpublished data). These data indicates spatial, temporal, and tissue-specific regulation of AR by LEF1.

The Affymetrix study with LNCaP anCaP-AI cells also revealed increased expression of other LEF1 target genes such as MMP7 (X. Liu and A. Ferrari, unpublished data). It is of great interest to determine the contribution of these LEF1 target genes in the growth and invasion of androgen-independent cancer cells. In addition, our data confirming LEF1 expression in both human androgen-independent prostate cancer and AR-positive, androgen-independent prostate cancer cell lines strongly indicate that LEF1 is associated with androgen-independent prostate cancer and may potentially serve as a marker for androgen-independent disease. Given the importance of LEF1 in androgen-independent LNCaP-AI cell growth and invasion it will be important to test and compare the growth of LNCaP-AI, LNCaP-LEF1 and LNCaP-AI-LEF1siRNA cells in an animal model in future. This could lead to a new mouse model for metastatic and androgen-independent prostate cancer.

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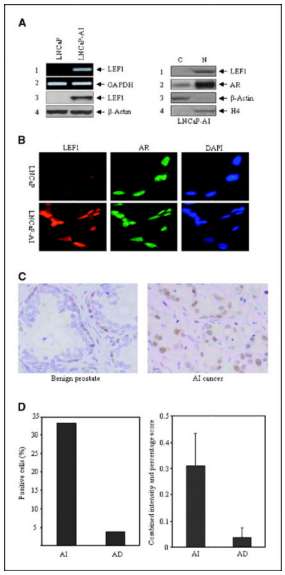

Figure 1. LEF1 expression in androgen-independent disease

(A) *Left*, RT-PCR (panel 1 and 2) and immunoblots (panel 3 and 4) for LEF1 of whole cell extracts from LNCaP and LNCaP-AI. LEF1 was undetectable in LNCaP cell lysates but gave strong signal in LNCaP-AI cell lysates. *Right*, immunoblots for LEF1 of nuclear (N) and cytoplasmic (C) fractions from LNCaP-AI, with β-actin as internal control for cytoplasmic fraction (Middle) and histone H4 as internal control for nuclear fraction (Bottom). AR is mainly expressed in the nucleus. (B) Immunofluorescent microscopy for LEF1 and AR in LNCaP and LNCaP-AI. *Left*, low immunofluorescent density of LEF1 was seen in the cytoplasm of LNCaP cells while strong immunofluorescent density of LEF1 is in nucleus of LNCaP-AI cells. *Middle*, androgen caused AR relocalization from cytoplasm to nucleus in LNCaP cells and increased the intensity of fluorescence in the nuclei of LNCaP-AI cells. (C) *Left*, LEF1 expression in Basal cells. *Right*, LEF1 expression in AI cells. (D) *Left*, statistically significant ($p<0.01$) difference of LEF1 expression between androgen-dependent and -independent cases. *Right*, the expression of combined score is statistically significantly higher in androgen-independent than -dependent cases ($p=0.016$). Cells were grown in media with 10 nMR1881 for (A) and (B).

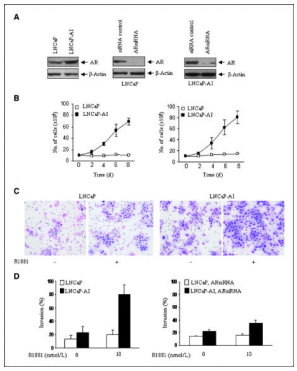

Figure 2. Increased growth and invasion of LNCaP-AI is AR-dependent

(A) *Left*, immunoblots for AR (Upper band) from LNCaP and LNCaP-AI whole cell extracts, with β-actin as control (Bottom band). *Middle*, Immunoblots for AR of whole cell extracts from LNCaP treated with 10 nM siRNA for AR knockdown, with β-actin as control (Bottom). *Right*, immunoblots for AR of whole cell extracts from LNCaP-AI, treated with 10 nM siRNA for AR knockdown with β-actin as control (Bottom). (B) *Left* and *right*, growth kinetics of LNCaP (open square) and LNCaP-AI (solid square) in the absent or presence of 10 nM R1881. (C) Photomicrograph of LNCaP and LNCaP-AI cells in Matrigel invasion assays. (D) *Left*, invasiveness of LNCaP and LNCaP-AI in androgen medium and androgen free medium. Treatment with 10 nM R1881 induced increases in invasion for LNCaP-AI cells of over 4 fold, although the parental LNCaP cells also showed a slight increase in invasion. (D) *Right*, Reduction of invasiveness of LNCaP-AI cells due to decreased AR levels. AR was knocked down with siRNA at a final concentration of 10 nM. In the presence of 10 nM R1881, LNCaP-AI cells exhibited a 2.5-fold reduction in invasiveness.

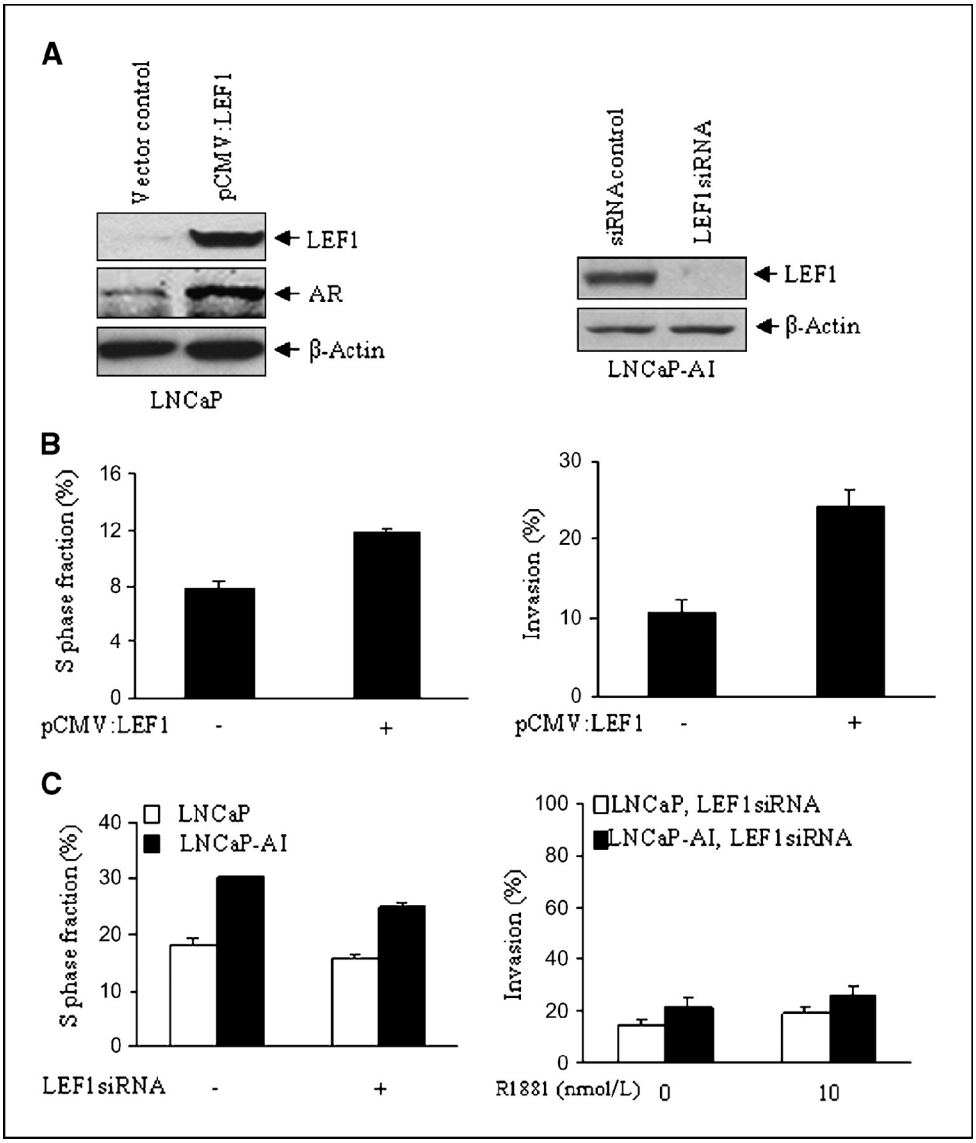

Figure 3. LEF1 promotes growth and invasion of LNCaP-AI cells

(A) *Left*, immunoblots of whole cell extracts from LNCaP overexpressing LEF1. Upper panel, exogenous LEF1. Mid panel; increased AR expression due to LEF1 expression. Low panel: β-actin as loading control. *Right*, immunoblots of whole cell extracts from LNCaP-AI, treated with 10 nM siRNA. (B) *Left*, 4.1% increase in S phase cell fraction was obtained with overexpression of LEF1 in LNCaP. *Right*, 2.3-fold increase in invasiveness with overexpression of LEF1 in LNCaP cells. (C) *Left*, reduction of S phase cell fraction occurred when LEF1 was knocked down in LNCaP-AI (6%), but not LNCaP cells. Right, Decrease (3.0-fold) in invasiveness of LNCaP-AI with LEF1 knockdown in androgen medium (compared to Fig. 2D).

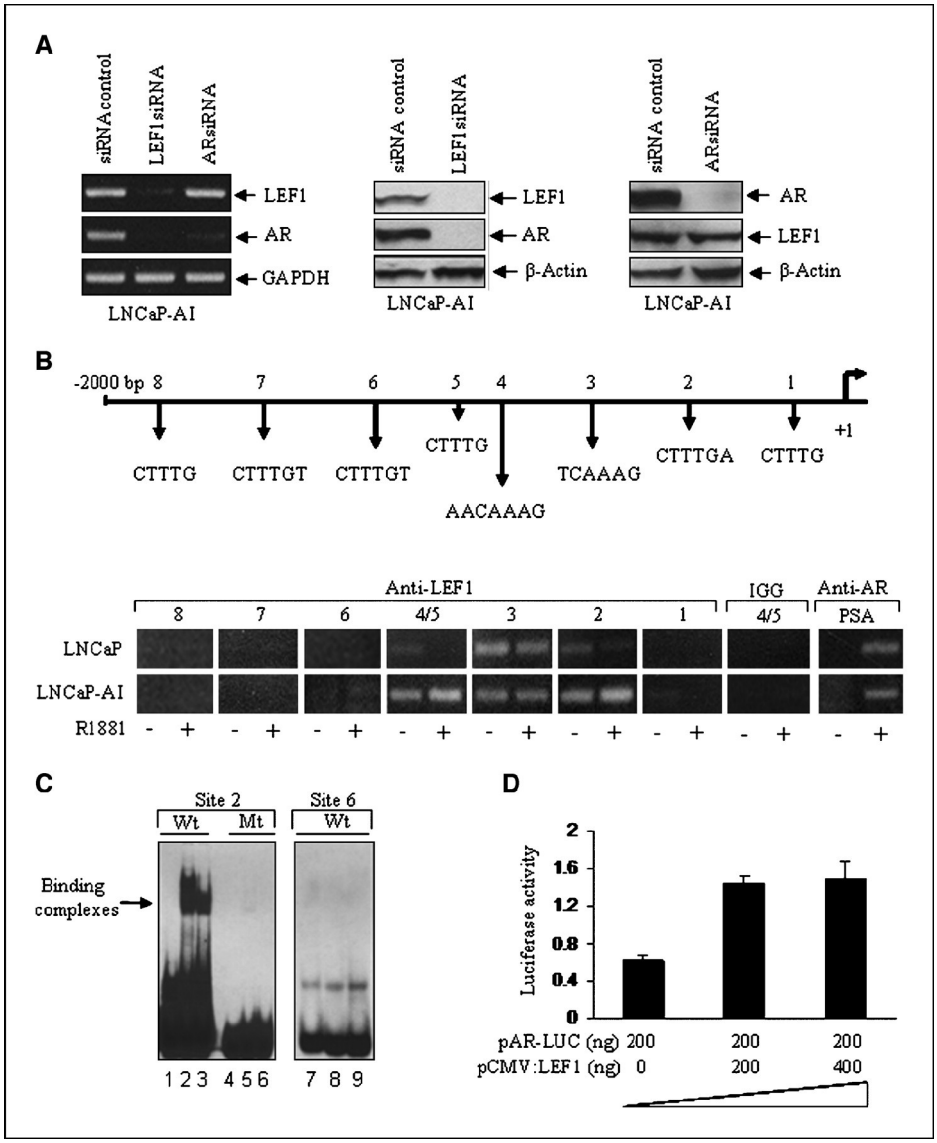

Figure 4. LEF1 regulates the expression of AR in LNCaP-AI

(A) *Left*, regulation of AR expression by LEF 1 in LNCaP-AI cells by RT-PCR. When LEF1 was knocked down, transcription of AR was blocked. In contrast, AR knockdown did not affect the expression of LEF1. *Middle* and *Right*, regulation of AR expression by LEF1 on the protein level by Western blot. LEF1 knockdown resulted in undetectable AR in the lysates while AR knockdown did not affect the expression of LEF1, consistent with the RT-PCR results. (B) *Upper*, scheme identifies relative sites of potential LEF-1/TCF binding sites within the first 2000 bp upstream of the 5' start of transcription for the hAR gene and sites of primer amplification products used to analyze DNA extracted from immunoprecipitated chromatin. *Lower*, ethidium bromide-stained agarose gel profiles of PCR reaction products from LEF1 antibody immunoprecipitated LNCaP and LNCaP-AI cell chromatin DNA. Three regions of the hAR promoter were immunoprecipitated by LEF1 antibody and gave positive signals for both LNCaP and LNCaP-AI. (C) Gel shift confirmed the positive occupation at site 2 (lane 1-6) and negative occupation (lane 7-9) at site 6 of LEF1 on the promoter of AR. Lane 1: no probe added. Lane2-3: wild type (wt) probe added in the presence and absence of androgen. Lane 4-5: no probe (lane 4) or mutated (Mt) probe

added. Lane 7: no probe added. Lane 8-9: Wt probe added. (D) LEF1 enhanced activity of AR promoter by luciferase assay. Over 2-fold increases were obtained in transcriptional activity.

Table 1

Primers used in this study

RT-PCR	AR	5’-GCCAGGGACCATGTTTTGCC-3’, 5’-CCTCCTGTAGTTTCAGATTAC-3’
	LEF1	5’-TTCAAGGACGAGGGCGAT-3’, 5’-TGTACCCGGAATAACTCG-3’
	GAPDH	5’-GACAGTCAGCCGCATCTTCTT-3’, 5’-CAATACGACCAAATCCGTTGAC-3’
CHIP	Site1	5’-TTAGATTGGGCTTTGGAACC-3’, 5’-GCTTCCTGAATAGCTCCTGCT-3’
	Site2	5’-CAAAATTGAGCGCCTATGTG-3’, 5’-TTGCTCTAGGAACCCTCAGC-3’
	Site3	5’-GGCAAAAATCTCGGAATGAC-3’, 5’-AAAGGTGGAGATGCAAGTGG-3’
	Site4/5	5’-ATCCAGTCTTCCTTGCCTTT-3’, 5’-TTCTGGGAGGCTCTCTGTTC-3’
	Site6	5’-CAGGTGAAAGGGTCTTCAGG-3’, 5’-AGGACATAATTTGTTCTATGTTCCAC-3’
	Site7	5’-TTTTTCAGGCCTCTTTGTGTC-3’, 5’-TGTGTCTACACACTAACAGTGAAGGA-3’
	Site8	5’-TGGTGATGTGGAAGCAACATA-3’, 5’-AAGGTGAGAAATAATGCTCTGAAGTT-3’
	PSA	5’-TCTGCCTTTGTCCGCTAGAT -3’, 5’-AACCTTCATTCCCCAGGACT-3’
	CyclinD1	5’-GGGAGGAATTCACCCTGAAA-3’, 5’-CCTGCCCCAAATTAAGAAAA-3’