



Published in final edited form as:

Prostate. 2014 February ; 74(2): 201–209. doi:10.1002/pros.22741.

Inhibition of constitutively active Stat3 reverses enzalutamide resistance in LNCaP derivative prostate cancer cells

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Abstract

Purpose—Use of enzalutamide has produced a revolutionary change in the treatment of advanced prostate cancer. However, clinical resistance to enzalutamide can develop swiftly in initial responders. This study aimed to test whether overexpression of IL-6 and constitutive activation of Stat3 in prostate cancer cells increase resistance to enzalutamide.

Experimental design—Sensitivity of prostate cancer cells to enzalutamide was tested using cell growth assays and clonogenic assays. Quantitative reverse transcription-PCR, ELISA and Western blotting were performed to detect expression levels of IL-6, c-Myc, survivin and AR. Expression of Stat3 was downregulated using siRNA specific to Stat3. ChIP assay was performed to examine recruitment of AR to the PSA promoter.

Results—Prostate cancer cells expressing autocrine IL-6 are resistant to enzalutamide and autocrine IL-6 leads to constitutive activation of Stat3 and its target genes. Down regulation of Stat3 led to an increase in sensitivity of prostate cancer cells to enzalutamide. Overexpression of constitutively active Stat3 in prostate cancer cells induced resistance to enzalutamide treatment. Constitutively active Stat3 also enhanced the recruitment of AR to PSA promoter which could not be disrupted by enzalutamide. The Stat3 inhibitor AG490 reversed enzalutamide resistance in prostate cancer cells, while combination treatment with enzalutamide and AG490 significantly inhibited cell growth and induced cell apoptosis.

Conclusions—This study demonstrates that the autocrine IL-6 pathway induces enzalutamide resistance in prostate cancer cells via the constitutive activation of Stat3. Co-targeting IL6-Stat3 pathway with enzalutamide may be utilized for treatment of advanced prostate cancer.

Keywords

Prostate cancer; Interleukin-6; Enzalutamide; Stat3

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Introduction

Prior to the approval of the second generation androgen antagonist Enzalutamide (MDV3100), few treatment choices existed after the progression of many patients to castration resistant prostate cancer (CRPC). Enzalutamide created a revolutionary change in the treatment of advanced prostate cancer, whereby it not only prolonged the survival of men with metastatic CRPC after chemotherapy, but also significantly improved their quality of life (1). Unfortunately, on the other hand, resistance to enzalutamide can develop in the initial responders (2). There is an urgent need to understand the mechanisms of resistance to enzalutamide which would provide more information to clinical investigators applying better intervention strategies to those patients.

Interleukin-6 (IL-6) is a pleiotropic cytokine that plays a central role in host defense mechanisms. The biological activities of IL-6 are mediated by the IL6 receptor which is composed of an IL6-specific receptor subunit and a signal transducer, gp130. The binding of IL-6 to its receptor results in activation of several intracellular signaling cascades including JAK-Stat and MAPK pathways (3,4). IL-6 production correlates with tumor progression in many human cancers, such as ovarian and prostate cancers (5–9). The serum levels of IL-6 are significantly elevated in many men with advanced, hormone-refractory prostate cancer and are associated with progression and poor prognosis (10–12). IL-6 also regulates the expression of genes encoding many steroidogenic enzymes involved in androgen synthesis (13). Autocrine IL-6 promotes androgen independent growth in prostate cancer cells (14). Constitutively active Stat3 is a part of the positive autocrine IL-6 loop and Stat3 activation in human tumors is often observed at the invasive front of tumors adjacent to inflammatory cells, which suggests that Stat3-dependent tumorigenesis is mediated by IL-6 (15). In the current study, we demonstrate that autocrine IL-6 in prostate cancer cells induces resistance to enzalutamide and that constitutive activation of Stat3 maybe one of the underlying mechanisms. Suppression of constitutively active Stat3 by Stat3 siRNA or a Stat3 inhibitor-AG490, could overcome enzalutamide resistance in prostate cancer cells. The IL-6-Stat3 feed forward axis in prostate cancer patient maybe one of the critical mechanisms of enzalutamide resistance.

Materials and Methods

Reagents and Cell Culture

LNCaP cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 0.1 mg/ml streptomycin. LNCaP-neo (LNCaP cells stably expressing pcDNA3.1 control vector), LNCaP-s17 (LNCaP cells stably expressing IL-6) (16), and LNCaP-Stat3C cells (LNCaP cells stably expressing constitutively active Stat3) (17) were described previously. The cells were maintained at 37°C in a humidified incubator with 5% carbon dioxide. Human recombinant IL-6 was obtained from R&D Systems (Minneapolis, MN. Cat. NO.: 206-IL-050). AG490 was purchased from Sigma-Aldrich, St. Louis, MO (Cat. NO.: T3454) and dissolved in DMSO.

Plasmids and cell transfection

For small interfering RNA (siRNA) transfection, cells were seeded at a density of 1×10^5 cells per well in 12-well plates and transfected with siRNA (Cell Signaling #6582) targeting Stat3 and a control siRNA targeting the luciferase (Luc) gene, using Lipofectamine-RNAiMAX (Invitrogen). Cells were transiently transfected with the indicated expression plasmids using Attractene (QIAGEN).

Chromatin immunoprecipitation assay

LNCAp-neo, LNCAp-s17 or LNCAp-Stat3C cells were treated with DMSO or 20 μ M enzalutamide overnight. DNA-AR protein complexes were cross-linked inside the cells by the addition of 1% formaldehyde. Whole-cell extracts were prepared by sonication, and an aliquot of the cross-linked DNA-protein complexes was immunoprecipitated by incubation with the AR-specific antibody (AR-C19; Santa Cruz Biotechnology) overnight at 4°C with rotation. Chromatin-antibody complexes were isolated from solution by incubation with protein A/G agarose beads for 1 hour at 4°C with rotation. The bound DNA-protein complexes were washed and eluted from beads with elution buffer (1% SDS and 0.1 mol/L NaHCO₃), cross links were reversed, and DNA was extracted. The resulting chromatin preparations were analyzed by PCR using primers spanning either the proximal or the distal enhancer AREs of the PSA promoter. Primers used for ChIP assay were: AREI/II: 5'-CCTAGATGAAGTCTCCATGAGCTACA, AREI/II -3'-GGGAGGGAGAGCTAGCACTTG (Proximal); ARE-III: 5'-CATGTTACATTAGTACACCTTGCC, ARE-III: 3'-TCTCAGAT CCAGGCTTGCTTACTGTC (Distal). Isotype-matched IgG was used as control.

Western blot analysis

Whole cell protein extracts were resolved on SDS-PAGE and proteins were transferred to nitrocellulose membranes. After blocking for 1 hour at room temperature in 5% milk in PBS/0.1% Tween-20, membranes were incubated overnight at 4°C with the indicated primary antibodies. [p-Stat3(Tyr705) (sc-7993-R, rabbit polyclonal antibody, 1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA); stat3 (C-20, sc-482, rabbit polyclonal antibody, 1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA); AR (441, sc-7305, Mouse monoclonal antibody, 1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA); c-myc (sc-764, rabbit polyclonal antibody, 1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA); Survivin (FL-142, rabbit polyclonal antibody, 1:1000 dilution, Sigma-Aldrich, St. Louis, MO) Bcl-2 (human specific) (#2872, rabbit polyclonal antibody, 1:1000 dilution, Cell Signaling Technology); Tubulin (T5168, Monoclonal Anti- α -Tubulin antibody, 1:5000 dilution, Sigma-Aldrich, St. Louis, MO)]. Tubulin was used to monitor the amounts of samples applied. Following secondary antibody incubation, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Millipore, Billerica, MA), the western blot was quantified by Image J software.

Cell growth assay

LNCAp neo, LNCAp-s17, LNCAp-IL6+ or LNCAp-Stat3C cells were seeded on 12-well plates at a density of 1×10^5 cells/well in RPMI 1640 media containing 10% FBS. LNCAp-neo and

LNCaP-s17 cells were treated with different concentration of enzalutamide (0 μ M, 5 μ M, 10 μ M, 20 μ M or 40 μ M) for 48 hours; LNCaP and LNCaP-IL6+ cells or LNCaP-neo and LNCaP-Stat3C cells were treated with 0 μ M, 10 μ M or 20 μ M enzalutamide for 48 hours; LNCaP-s17 cells were treated with 10 μ M AG490 with or without 20 μ M enzalutamide for 48 hours, total cell numbers were counted and the cell survival rate (%) was calculated. Cell survival rate (%) = (Treatment group cell number / Control group cell number) \times 100%.

Cell death ELISA

LNCaP-neo or LNCaP-s17 cells were seeded on 12-well plates (1×10^5 cells/well) in RPMI 1640 media containing 10% FBS and treated with 10 μ M AG490 with or without 20 μ M enzalutamide for 48 hours. Mono- and oligonucleosomes in the cytoplasmic fraction were measured by the Cell Death Detection ELISA kit (Roche, Cat. NO. 11544675001) according to the manufacturer's instructions. Briefly, floating and attached cells were collected and homogenized in 400 μ L of incubation buffer. The wells were coated with antihistone antibodies and incubated with the lysates, horseradish peroxidase-conjugated anti-DNA antibodies, and the substrate, and absorbance was read at 405 nm.

Clonogenic Assay

LNCaP-neo cells or LNCaP-s17 stable clone cells were treated with DMSO or 20 μ M enzalutamide in media containing 10% complete FBS. Cells were plated at equal density (1000 cells/dish) in 100 mm dishes for 14 days; the medium was changed every 7 days. The colonies were rinsed with PBS before staining with 0.5% crystal violet/4% formaldehyde for 30 min and the number of colonies was counted.

Real-Time quantitative RT-PCR

Total RNAs were extracted using TriZOL reagent (Invitrogen). cDNAs were prepared after digestion with RNase-free RQ1 DNase (Promega). The cDNAs were subjected to real-time reverse transcription-PCR (RT-PCR) using Sso Fast Eva Green Supermix (Bio- Rad) according to the manufacturer's instructions and as described previously (18). Primers used for Real-time PCR were: IL6: 5'-AAGCCAGAGCTGTGCAGATGA, 3'-TGTCCTGCAGCCACTGGTTC; c-Myc: 5'-TGAGGAGACACCGCCAC, 3'-CAACATCGATTCTTCTTCAT; Survivin: 5'-AGAAGCTGGCCCTTCTTGGAGG, 3'-GTTTTATGTTCTCTATGGG; Actin: 5'-CCCAGCCATGTACGTTGCTA, 3'-AGGGCATAACCCTCGTAGATG. Each reaction was normalized by co-amplification of actin. Triplicates of samples were run on default settings of Bio-Rad CFX-96 real-time cyclers.

Measurement of IL6

Secretion of IL-6 by LNCaP-neo and LNCaP-s17 cells was determined by Human IL6 ELISA Ready-SET-Go (eBioscience, San Diego, CA. Cat.NO. 88-7066) according to the manufacturer's protocol. Briefly, LNCaP neo and LNCaP-s17 cells were cultured in RPMI 1640 media containing 10% FBS for 2 days, 50 μ l of cell culture supernatants were collected to determine levels of IL-6 secretion, The wells were coated with capture antibody and

incubated with the standard or supernatants samples, Avidin-HRP and the substrate, and absorbance was read at 450 nm.

Statistical Analysis

All data are presented as means \pm standard deviation of the mean (SD). Statistical analyses were performed with Microsoft Excel analysis tools. Differences between individual groups were analyzed by one-way analysis of variance (ANOVA) followed by the Scheffé procedure for comparison of means. $P < 0.05$ was considered statistically significant.

Results

Overexpression of IL-6 increases LNCaP cell resistance to enzalutamide

Our previous data demonstrated that autocrine expression of IL-6 in LNCaP (LNCaP-s17) cells promotes cell growth and increases resistance to bicalutamide treatment (14,19). To test whether expression of IL-6 affects the response of prostate cancer cells to enzalutamide, LNCaP-s17 cells were treated with increasing doses of enzalutamide and cell numbers were counted. As shown in Fig.1A, LNCaP-neo cells were highly sensitive to enzalutamide treatment compared to LNCaP-s17 cells. Enzalutamide at a concentration of 5 μ M reduced the growth of LNCaP-neo cells by more than 30%, while it had almost no effect on the growth of LNCaP-s17 cells. Even at a higher concentration of enzalutamide (40 μ M), the growth of LNCaP-s17 cells was only reduced by about 30% compared to almost 60% reduction in LNCaP-neo cells. To further confirm these results, clonogenic assay was performed. LNCaP-neo cells and LNCaP-s17 cells were treated with 20 μ M enzalutamide and clonogenic ability was determined. As shown in Fig.1B and 1C, the colony formation ability was significantly inhibited in LNCaP-neo cells treated with 20 μ M enzalutamide, while LNCaP-s17 cells continued to grow and form colonies. To further confirm that overexpression of IL-6 is involved in enzalutamide resistance, LNCaP-IL6+ cells, LNCaP cells expressing IL-6 by long-term culture of LNCaP cells in media containing IL-6 (20), were treated with 10 μ M and 20 μ M enzalutamide in media containing complete FBS for 48 hours. As shown in Fig.1D, enzalutamide significantly inhibited growth of LNCaP cells. In contrast, enzalutamide had little effect on the growth of LNCaP-IL6+ cells. Collectively, these data suggest that overexpression of IL-6 in prostate cancer cells is associated with enzalutamide resistance.

Autocrine IL-6 constitutively activates Stat3 pathway and enhances androgen receptor transactivation in prostate cancer cells

Numerous reports have demonstrated that constitutive Stat3 activation is oncogenic and contributes to tumor progression and metastasis (21–23). Previous studies showed that Stat3 is constitutively activated in LNCaP-s17 cells (14). To test whether LNCaP-s17 cells exhibit elevated Stat3 signaling, we examined the levels of expression of several Stat3 target genes including c-Myc, survivin, and Bcl-2. As shown in Fig.2A, LNCaP-s17 cells express constitutively activated Stat3 (Stat3 phosphorylated at Tyr705) and express higher levels of AR, c-Myc, survivin, and Bcl-2 proteins than LNCaP-neo cells. Consistent with the protein levels, LNCaP-s17 cells express higher levels of c-Myc and survivin mRNA than LNCaP-neo cells (Fig. 2B and 2C). We also confirmed that LNCaP-s17 cells expressed higher levels

of IL-6 mRNA and protein than LNCaP-neo cells (Fig.2D and 2E). In our previous study, we showed that over expression of IL-6 enhances AR-ARE DNA binding activity in LNCaP cells (14). To determine whether constitutively active Stat3 increases the recruitment of AR to the ARE sites, ChIP assay was performed in LNCaP, LNCaP-s17 and LNCaP-Stat3C cells. As shown in Fig.2F, both LNCaP-s17 cells and LNCaP-Stat3C cells showed enhanced recruitment of AR to both the proximal binding site (AREI/II) and the distal enhancer binding site (AREIII) of the PSA promoter compared to LNCaP-neo cells. Collectively, these data demonstrate that overexpression of IL-6 activates Stat3 and AR signaling pathways in prostate cancer cells.

Knockdown of Stat3 expression restores the sensitivity of LNCaP-s17 cells to enzalutamide treatment

To determine whether Stat3 is involved in enzalutamide resistance in IL-6 expressing prostate cancer cells, LNCaP-neo and LNCaP-s17 cells were transfected with siRNA specific to Stat3. The cells were then treated with 20 μ M enzalutamide for an additional 3 days and the cell numbers were determined. As shown in Fig 3A, knockdown of Stat3 expression in LNCaP-s17 cells resensitized the cells to enzalutamide treatment. The knockdown effects were confirmed by Western blotting using p-Stat3 (Tyr705) and Stat3 antibodies (Fig. 3B). In addition, LNCaP cells expressing constitutively active Stat3 (Stat3C) exhibited increased resistance to enzalutamide (Fig. 3C). These results suggest that stat3 activation is involved in enzalutamide resistance in prostate cancer cells.

One of the mechanisms of action of enzalutamide is to inhibit the recruitment of the AR to AREs in promoters of target genes. To test whether Stat3-mediated enzalutamide resistance affects the recruitment of AR to AREs, ChIP assay was performed. As shown in Fig. 3D, enzalutamide significantly inhibited recruitment of the AR to AREs in the PSA promoter (both proximal binding site and distal binding site) in LNCaP-neo cells. In contrast, enzalutamide had little effect on the recruitment of the AR to the AREs in LNCaP-s17 cells and LNCaP-Stat3C cells. These results suggest that Stat3-mediated enzalutamide resistance may be involved in affecting the recruitment of the AR to the AREs in the PSA promoter.

AG490, a Stat3 inhibitor, reverses enzalutamide resistance in prostate cancer cells

We next tested whether AG490, which has been known as a Jak2-Stat3 inhibitor, is able to reverse enzalutamide resistance in prostate cancer cells. As shown in Fig 4A, enzalutamide treatment at 20 μ M had minimal effect on LNCaP-s17 cell growth. However, enzalutamide significantly inhibited cell growth in the presence of 10 μ M AG490, possibly through induction of apoptotic cell death (Fig 4B). Collectively, these data demonstrate that using Jak2-stat3 inhibitor can overcome enzalutamide resistance through inhibition of Stat3 activation.

Discussion

Enzalutamide is a second generation, non-steroidal AR antagonist rationally designed utilizing the AR crystal structure and cell based screening (24,25). It was approved by U.S. Food and Drug Administration (FDA) in September 2012 based on significant improvement

in overall survival (18.4 vs. 13.6 months, $p = 0.001$) in a phase III randomized study in men with progressive castration-resistant prostate cancer (CRPC) who were previously treated with docetaxel (1). In vitro, enzalutamide binds to the ligand binding domain of the AR with 5 fold higher affinity than bicalutamide and impairs AR nuclear translocation and DNA binding (25). However, despite its significant clinical effects and being the first choice after docetaxel failure in CRPC patients, resistance to enzalutamide would eventually occur in many patients (2). A better understanding of the therapeutic effects of enzalutamide and the mechanisms of resistance would be very important for patient treatment in the future. A recent study showed expression of AR variants driven by AR gene rearrangements can mediate resistance to therapies targeting full-length AR, including enzalutamide (26). But many questions remain to be answered, including the absence of expression of AR variants in many patients who may still develop resistance to enzalutamide. In the present study, we report that the axis between IL-6 and persistent Stat3 activation may be one of the critical mechanisms involved in enzalutamide resistance.

Our previous studies demonstrated that IL-6 overexpression enhances AR-ARE DNA binding activity and enhances AR nuclear translocation in LNCaP cells (14) and that IL-6 up regulates intraprostatic androgen synthesis in the absence of exogenous steroid precursors (13). Here we found that autocrine IL-6 in LNCaP cells activates Stat3 pathway and enhances resistance to enzalutamide, while downregulation of Stat3 in LNCaP-s17 cells dramatically resensitized LNCaP-s17 cells to enzalutamide. In addition, transfection of constitutively active Stat3 into LNCaP cells induced resistance to enzalutamide, which indicates that enzalutamide resistance may be driven by Stat3 activation in prostate cancer cells. Enzalutamide as a second generation androgen antagonist is superior to bicalutamide, in that enzalutamide does not promote translocation of AR to the nucleus, and in addition, it prevents binding of AR to DNA and AR to co-activator proteins, which abrogates agonist activity (25). However, our study showed that in prostate cancer cell lines expressing constitutively active Stat3, enzalutamide treatment only slightly reduced recruitment of AR to the PSA promoter compared with LNCaP parental cells, which suggests that persistent Stat3 activation may counteract enzalutamide action in blocking the recruitment of AR to target gene promoters.

Constitutive activation of Stat3 has been shown in many advanced cancers and is associated with poor prognosis (27). Constitutive activation of Stat3 is often accompanied by high levels of IL-6 expression, which implicates IL-6 as the main driver of persistent activation of Stat3(28). Recently, a group reported enzalutamide in prostate cancer patients might lead to unwanted side effects of enhanced macrophage infiltration/prostate cancer metastasis through modulation of CCL2-STAT3 signaling (29), which suggested inhibition of the IL-6-Stat3 axis appears to be a potent strategy to overcome enzalutamide resistance in prostate cancer cells. AG490 is a specific inhibitor of the Janus kinase 2 protein (JAK2) which is the upstream regulator of stat3 signaling. In the current study, we found that AG490 significantly reversed enzalutamide resistance in prostate cancer cells exhibiting constitutive activation of Stat3. Combination treatment with enzalutamide and AG490 not only inhibited cell proliferation but also induced apoptosis, co-targeting IL-6/Stat3 pathway using stat3

inhibitor and androgen signaling by enzalutamide may be logical for treatment of advanced prostate cancer.

In summary, our study demonstrates that the IL6-Stat3 axis is involved in the development of resistance to enzalutamide in prostate cancer. In addition, our results imply that targeting the IL-6-Stat3 Axis may be a potential therapeutic strategy in patients resistant to enzalutamide.

Acknowledgments

Grant Support: This work is supported in part by grants NIH/NCI CA140468, CA168601, DOD PCRP PC080538 (A.C. Gao), US Department of Veterans Affairs, Office of Research and Development VA Merits I01 BX000526 (A.C. Gao), and by resources from the VA Northern California Health Care System, Sacramento, California.

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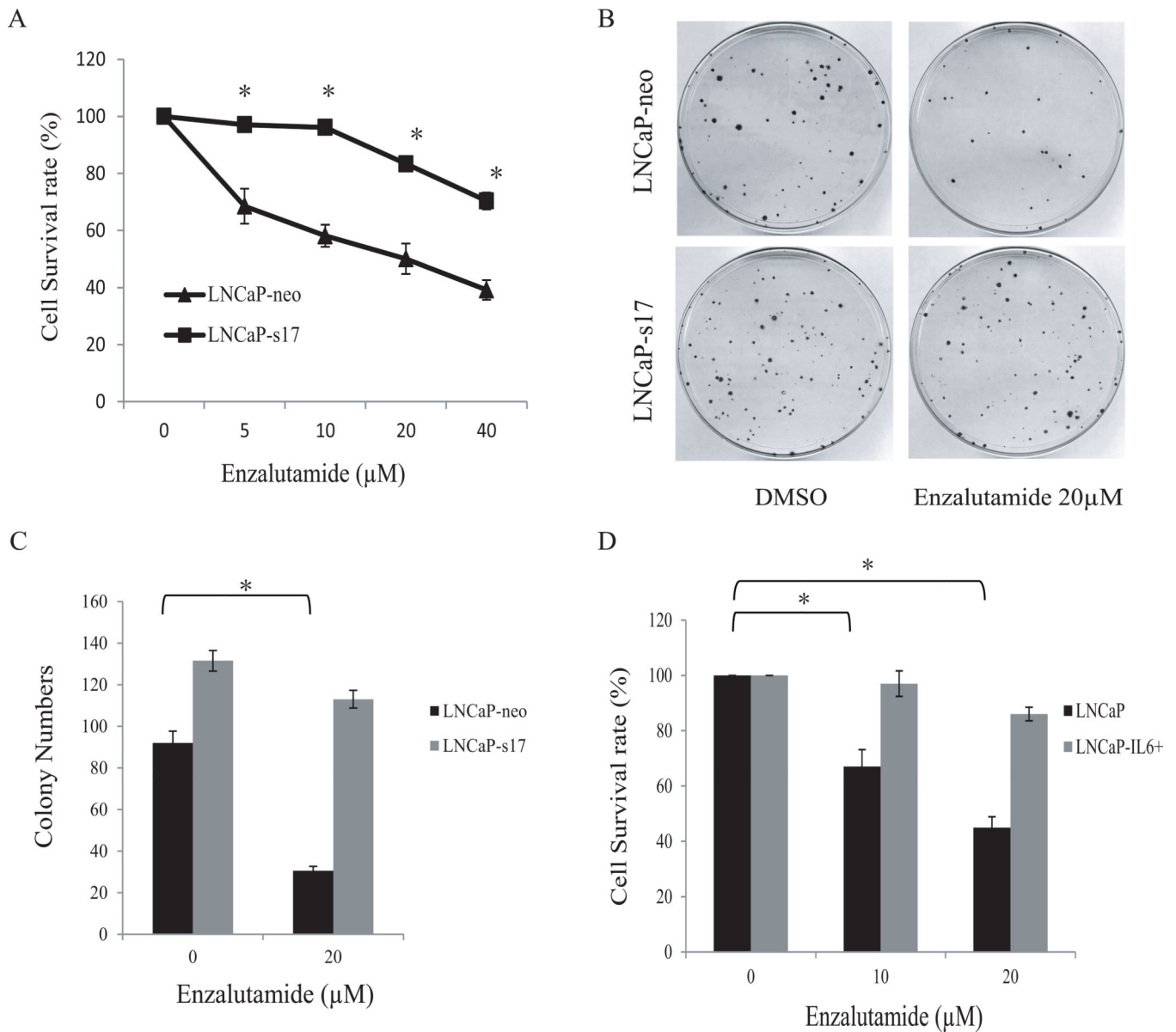


Figure 1. Overexpression of IL-6 increases LNCaP cell resistance to enzalutamide

A: LNCaP cells stably expressing IL6 (LNCaP-s17) and control LNCaP cells (LNCaP-neo) were treated with different doses of enzalutamide in media containing complete FBS. Total cell numbers were counted after 48 h and cell survival rates were calculated as described in the methods. Results are presented as means \pm SD of 3 experiments performed in duplicate. **B:** LNCaP-neo and LNCaP-s17 cells were treated with 0 or 20 μM enzalutamide and clonogenic assays were performed. **C:** Colonies were counted and results are presented as means \pm SD of 2 experiments performed in duplicate. LNCaP-s17 cells formed higher numbers of colonies compared to LNCaP-neo cells when treated with enzalutamide. **D:** LNCaP-IL6+ cells (continuous culture in medium containing 10% FBS and 5 ng/mL IL-6) and control LNCaP cells were treated with 0, 10 or 20 μM enzalutamide in media containing

complete FBS and cell numbers were counted after 48 h. Results are presented as means \pm SD of 3 experiments performed in duplicate. * $P < 0.05$.

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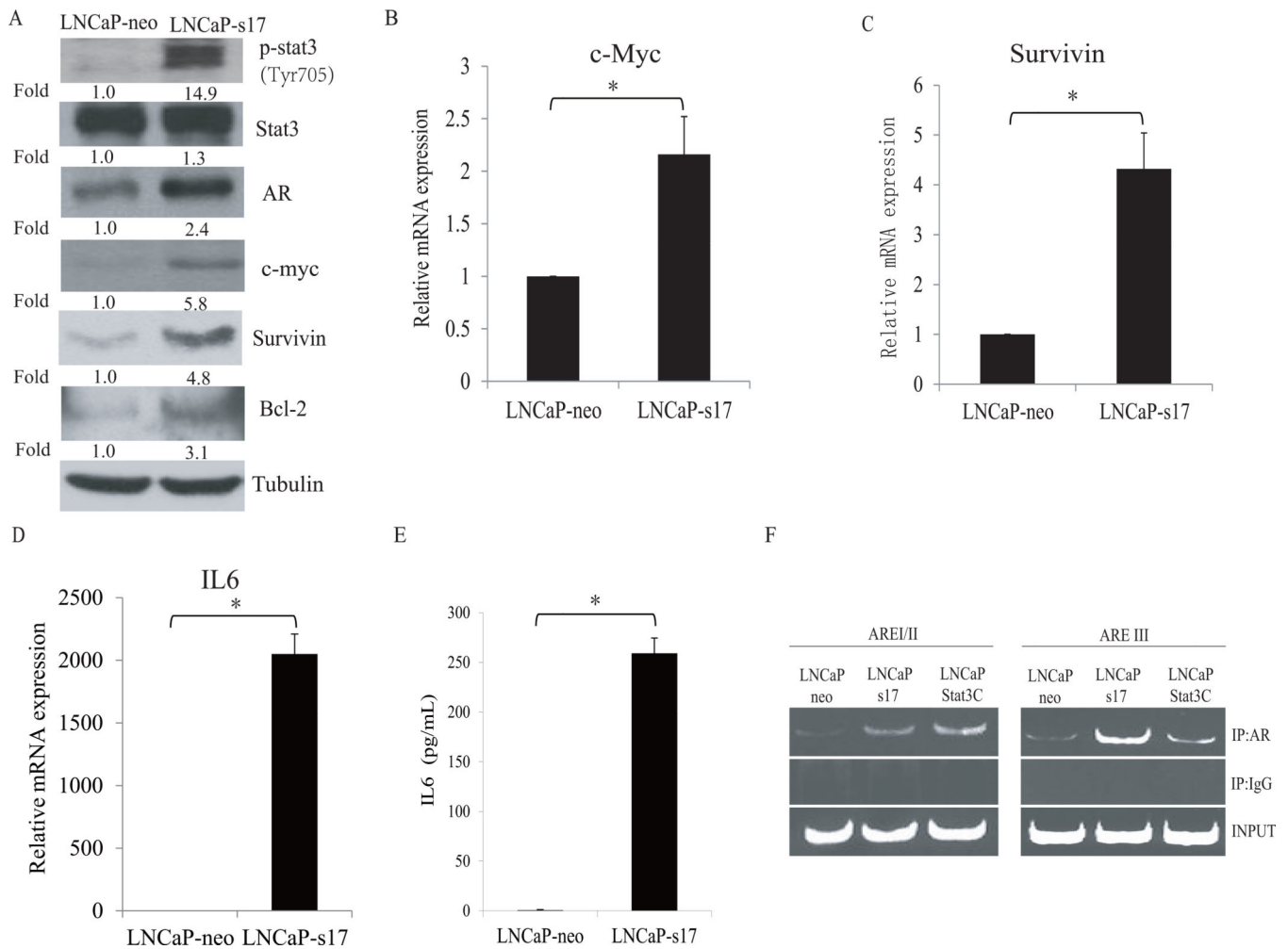


Figure 2. Autocrine IL-6 constitutively activates Stat3 pathway and enhances androgen receptor transactivation in prostate cancer cells

A: Prostate cancer cells with autocrine IL-6 exhibit constitutive activation of Stat3. LNCaP cells and LNCaP-s17 cells were cultured in RPMI 1640 media containing 10% FBS condition for 24 hours and the lysates were immunoblotted with the indicated antibodies. **B:** LNCaP cells and LNCaP-s17 cells were cultured in RPMI 1640 media containing 10% FBS condition for 24 hours, total RNAs were extracted and c-Myc mRNA level was analyzed by qRT-PCR. **C:** Survivin mRNA level of LNCaP-neo and LNCaP-s17 cells were analyzed by qRT-PCR. **D:** IL6 mRNA level of LNCaP-neo and LNCaP-s17 cells were analyzed by qRT-PCR. Each reaction was normalized by co-amplification of actin. **E:** LNCaP-neo and LNCaP-s17 cells were cultured in RPMI 1640 media containing 10% FBS condition for 24 hours, the supernatant were collected and subjected to IL6 ELISA detection. Results are presented as means \pm SD of 3 experiments performed in duplicate. **F:** Recruitment of AR to AREs in PSA promoter was analyzed by ChIP assay, LNCaP-s17 and LNCaP-Stat3C cells enhanced recruitment of AR to PSA promoter compared to LNCaP-neo cells. * $P < 0.05$

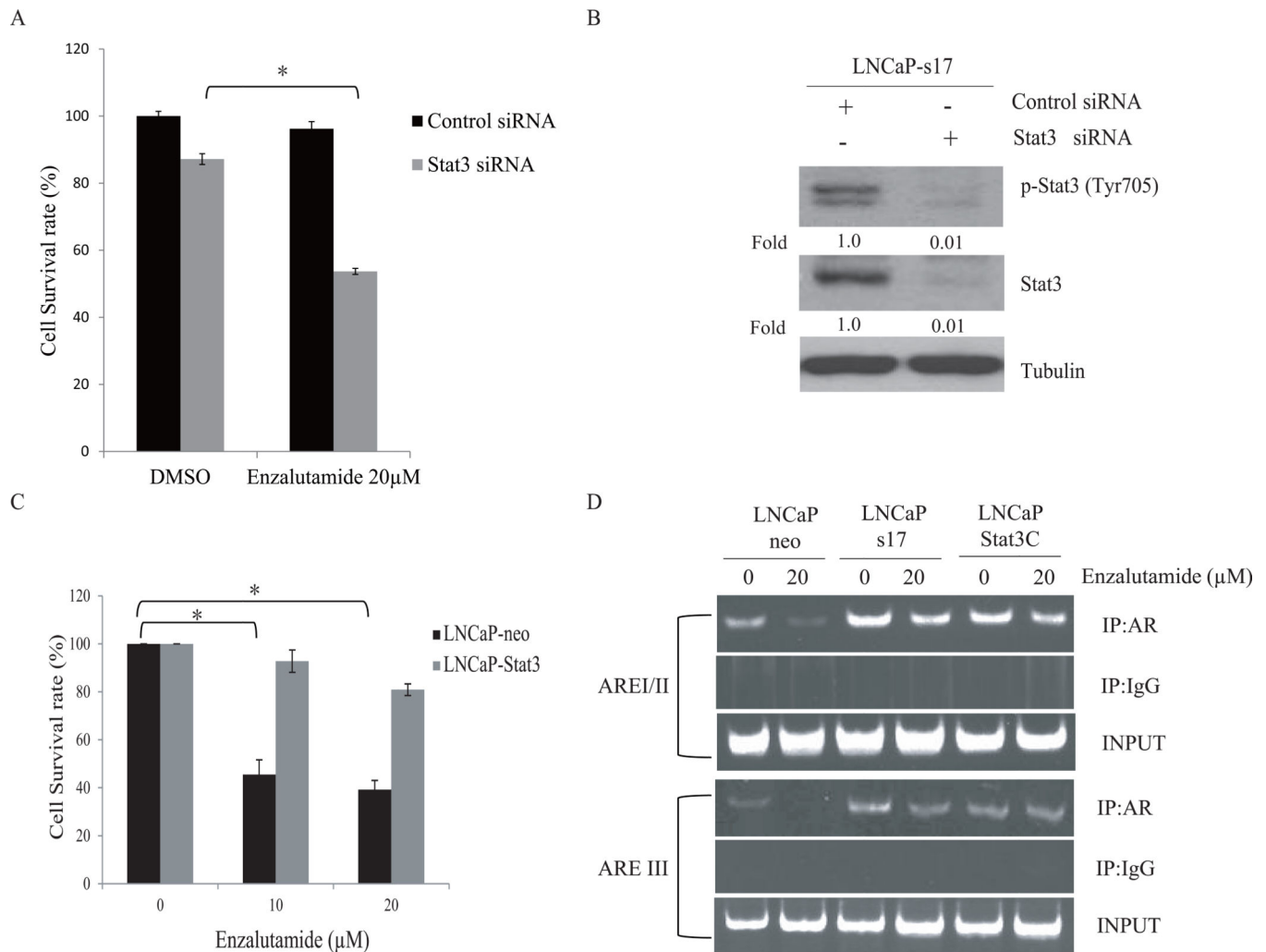


Figure 3. Downregulation of Stat3 expression restores the sensitivity of LNCaP-s17 cells to enzalutamide treatment

A: Down regulation of Stat3 expression increased sensitivity of LNCaP-s17 cells to enzalutamide. LNCaP-s17 cells were transfected with siRNAs specific to Stat3 or control siRNA and were treated with 0 or 20 µM enzalutamide. Cell numbers were counted and cell survival rates were calculated after 3 days. Results are presented as means ± SD of 3 experiments performed in duplicate. **B:** The knock down effects were examined by western blot; whole cell lysates were collected and subjected to Western blot. **C:** LNCaP-neo and LNCaP-Stat3C cells were treated with DMSO, 10 or 20 µM enzalutamide in media containing FBS for 48 hours, cell numbers were counted and cell survival rates were calculated. Results are presented as means ± SD of 3 experiments performed in duplicate. **D:** Recruitment of AR to AREs in PSA promoter was analyzed by ChIP assay, LNCaP-neo, LNCaP-s17 and LNCaP-Stat3C cells were cultured in FBS condition with or without 20 µM enzalutamide overnight, cross-linked with 1% formaldehyde and the resultant DNA-protein complexes were subjected to ChIP assay. * $P < 0.05$

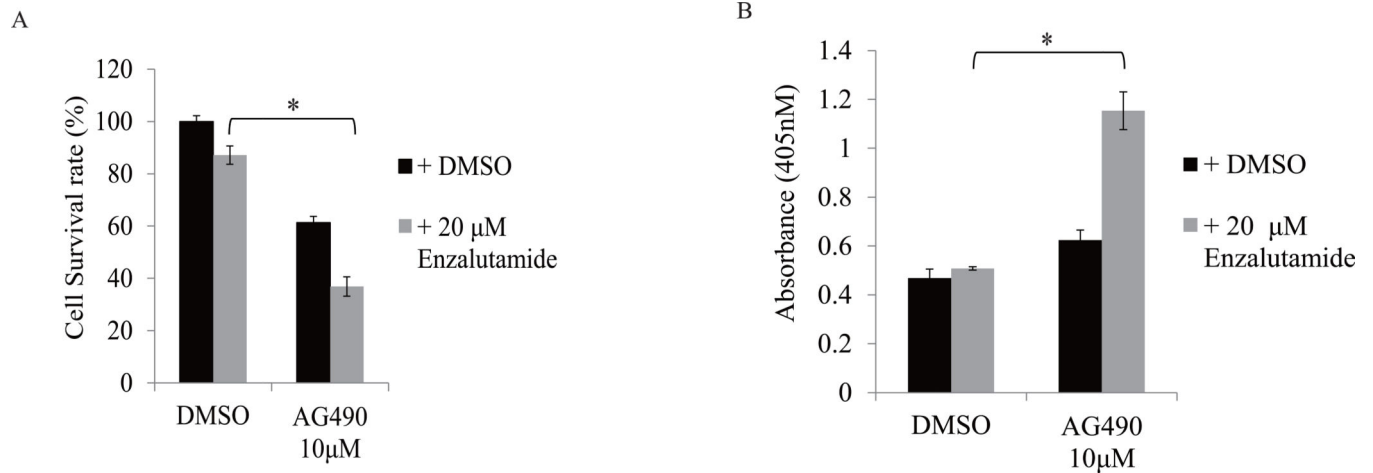


Figure 4. Stat3 inhibitor-AG490, reverses enzalutamide resistance in prostate cancer cells
A: LNCaP-s17 cells were treated with 10 μ M AG490 combined with or without 20 μ M enzalutamide in media containing FBS, cell numbers were counted and cell survival rate was calculated after 48 h, the apoptosis was analyzed by Cell death ELISA (**B**). Results are presented as means \pm SD of 3 experiments performed in duplicate. * $P < 0.05$