

July 2007

Autocrine prolactin promotes prostate cancer cell growth via Janus kinase-2-signal transducer and activator of transcription-5a/b signaling pathway.

Ayush Dagvadorj

Sean Collins

Jean-Baptiste Jomain

Junaid Abdulghani

James Karras

See next page for additional authors

[Let us know how access to this document benefits you](#)

Follow this and additional works at: <http://jdc.jefferson.edu/cbfp>

 Part of the [Amino Acids, Peptides, and Proteins Commons](#)

Recommended Citation

Dagvadorj, Ayush; Collins, Sean; Jomain, Jean-Baptiste; Abdulghani, Junaid; Karras, James; Zellweger, Tobias; Li, Hongzhen; Nurmi, Martti; Alanen, Kalle; Mirtti, Tuomas; Visakorpi, Tapio; Bubendorf, Lukas; Goffin, Vincent; and Nevalainen, Marja T, "Autocrine prolactin promotes prostate cancer cell growth via Janus kinase-2-signal transducer and activator of transcription-5a/b signaling pathway." (2007). *Department of Cancer Biology Faculty Papers*. Paper 2.
<http://jdc.jefferson.edu/cbfp/2>

Authors

Ayush Dagvadorj, Sean Collins, Jean-Baptiste Jomain, Junaid Abdulghani, James Karras, Tobias Zellweger, Hongzhen Li, Martti Nurmi, Kalle Alanen, Tuomas Mirtti, Tapio Visakorpi, Lukas Bubendorf, Vincent Goffin, and Marja T Nevalainen

Autocrine Prolactin Promotes Prostate Cancer Cell Growth via Janus Kinase-2-Signal Transducer and Activator of Transcription-5a/b Signaling Pathway

Ayush Dagvadorj, Sean Collins, Jean-Baptiste Jomain, Junaid Abdulghani, James Karras, Tobias Zellweger, Hongzhen Li, Martti Nurmi, Kalle Alanen, Tuomas Mirtti, Tapio Visakorpi, Lukas Bubendorf, Vincent Goffin, and Marja T. Nevalainen

Department of Cancer Biology (A.D., J.A., M.T.N.), Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania 19107; Department of Oncology (S.C., H.L.), Lombardi Comprehensive Cancer Center, Georgetown University, Washington, D.C. 20057; Institut National de la Santé et de la Recherche Médicale (J.-B.J., V.G.), Unité 808, Laboratory “PRL, GH, and Tumors” and University Paris Descartes, Faculty of Medicine Rene Descartes, Paris 5-Necker site, F-75015 Paris, France; ISIS Pharmaceuticals (J.K.), Carlsbad, California 92008; Department of Urology (T.Z.), University of Basel, CH-4056 Basel, Switzerland; Department of Surgery (M.N.), University Hospital of Turku, 20014 Turku, Finland; Department of Pathology (K.A., T.M.), Institute of Biomedicine, University of Turku, 20520 Turku, Finland; Institute of Medical Technology (T.V.), University of Tampere and Tampere University Hospital, Tampere 33520, Finland; and Institute for Pathology (L.B.), Basel CH-4003, Switzerland

The molecular mechanisms that promote progression of localized prostate cancer to hormone-refractory and disseminated disease are poorly understood. Prolactin (Prl) is a local growth factor produced in high-grade prostate cancer, and exogenously added Prl in tissue or explant cultures of normal and malignant prostate is a strong mitogen and survival factor for prostate epithelium. The key signaling proteins that mediate the biological effects of Prl in prostate cancer are Signal Transducer and Activator of Transcription (Stat)-5a/5b via activation of Janus kinase-2. Importantly, inhibition of Stat5a/in prostate cancer cells induces apoptotic death. Using a specific Prl receptor antagonist ($\Delta 1-9G129R$ -hPRL), we demonstrate here for the first time that autocrine Prl in androgen-independent human prostate cancer cells promotes cell viability via Stat5 signaling pathway. Furthermore, we examined a unique clinical material of human hormone refractory prostate cancers and metastases and show that autocrine Prl is expressed in 54% of hormone-refractory clinical human prostate cancers and 62% prostate cancer metastases. Finally, we demonstrate that autocrine Prl is expressed from both the proximal and distal promoters of the Prl gene in clinical human prostate cancers and *in vivo* and *in vitro* human prostate cancer models, independently of pituitary transcription factor-1 (Pit-1). Collectively, the data provide novel evidence for the concept that autocrine Prl signaling pathway is involved in growth of hormone-refractory and metastatic prostate cancer. The study also provides support for the use of Prl receptor antagonists or other therapeutic strategies to block the Prl-Janus kinase-2-Stat5 signaling pathway in advanced prostate cancer.

The central problem in clinical management of prostate cancer is the development of hormone-refractory and metastatic disease. The molecular mechanisms underlying the progression of prostate cancer to disseminated disease and the growth of prostate cancer in an androgen-deprived milieu are largely unknown. Adaptation of prostate cancer cells to androgen deprivation may involve both amplification and mutations of androgen receptor (AR) (1). In addition to alterations of AR function, protein kinase signaling pathways activated by peptide hormones and local growth factors are known to promote proliferation and survival of prostate cancer cells either directly or through stimulation of AR action (2).

One such local growth factor-initiated protein kinase signaling pathway in prostate cancer is the prolactin (Prl)-Janus kinase (Jak)-2-Signal Transducer and Activator of Transcription (Stat)-5a/b signaling cascade. We

have previously demonstrated that receptors for Prl are expressed in human prostate epithelial cells (3, 4) and that Prl protein is produced in both normal and malignant prostate epithelium (4, 5). We also showed that expression of Prl and activation of Stat5a/b in clinical human prostate cancer specimens are associated with high histological grade (5), suggesting that the autocrine Prl-Jak2-Stat5a/b signaling pathway might be involved in clinical progression of human prostate cancer to advanced disease. The human Prl gene is regulated at the transcriptional level by two distinct promoters (6, 7). The proximal promoter, also referred to as the pituitary promoter, is located in exon 1b. The distal promoter is located 5.8 kb upstream of the pituitary transcription start site, resulting in the transcription of an extra noncoding exon 1a (6), which is known to direct Prl gene transcription in extrapituitary sites such as lymphoid and decidual cells (8, 9). The transcription from the alternative promoter does not lead to generation of different protein isoforms but provides an additional level of transcriptional regulation of the Prl gene (10). The promoter that drives Prl gene expression in human prostate cancer has not been identified.

Prl added exogenously to organ explant cultures of normal and malignant prostate tissue promotes proliferation and inhibits apoptosis of prostate epithelial cells (11–18). The key signaling proteins that are activated by Prl in prostate cancer cell lines and clinical human prostate cancer specimens are Stat5a and Stat5b via the Jak2 tyrosine kinase (5, 19). Importantly, we have shown that inhibition of Stat5a/b induces rapid death of human prostate cancer cells (20), a finding that was later confirmed in transgenic adenocarcinoma of mouse prostate (TRAMP) mice by another laboratory (21). Transgenic mice overexpressing the Prl gene develop hyperplasia of the prostate (14–16), and, correspondingly, Prl-null mice have smaller prostates than their wild-type (WT) counterparts (18). Even if the effects of exogenously added Prl on prostate cells have been extensively investigated using various experimental models, the biological significance of locally produced Prl by prostate cells has so far not been established. Pseudophosphorylated Prl has been demonstrated to inhibit growth of human prostate cancer cells (22), but the mechanisms of action of pseudophosphorylated Prl are largely unclear. Specifically, the antagonistic properties of pseudophosphorylated Prl are not based on competitive inhibition of Prl receptor (23) but may involve induction of alternative splicing of Prl receptor transcripts (24). This effect on Prl receptor isoforms varied among different cell lines, which further complicates the interpretation of the results and development of this agent for clinical use. Here we use a competitive Prl receptor antagonist (Δ 1–9G129R-hPRL) to block autocrine Prl in human prostate cancer cells to determine the biological effects of prostate epithelial Prl. The Prl receptor antagonist Δ 1–9G129R-hPRL, which was developed based on rational drug design, inhibits ligand-induced Prl receptor activation and therefore Prl-activated intracellular signaling pathways (23, 25, 26).

In this work, we demonstrate that Prl gene transcription is driven by both the proximal and distal promoters in human prostate cancer cell lines, prostate xenograft tumors, and clinical human prostate cancer specimens, and this occurs independently of pituitary transcription factor (Pit)-1. We show that the Prl protein is expressed in a large proportion of hormone-refractory clinical human prostate cancers and in prostate cancer metastases. We compare different approaches of pharmacological inhibition of the Prl-Jak2Stat5a/b axis in prostate cancer cells and establish that the Prl receptor antagonist Δ 1–9G129R-hPRL (23) inhibits constitutive activation of Stat5a/b by autocrine Prl in androgen-independent human prostate cancer cells and induces death of the cells. The work presented here provides the first evidence of potential involvement of autocrine loop of Prl in androgen-independent growth of clinical human prostate cancer. Moreover, identification of the promoters driving Prl gene expression in prostate cancer provides the basis for the identification of coactivators and signaling pathways that may represent additional molecular targets for drug development for prostate cancer.

Materials and Methods

Cell culture

CWR22Rv, LNCaP, DU145, and PC-3 cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 (Biofluids, Gaithersburg, MD) containing 10% fetal calf serum, 2.5 mM L-glutamine, and penicillin-streptomycin (100 IU/ml and 100 µg/ml, respectively) at 37 C with 5% CO₂. LNCAP cells were cultured in the presence of 0.5 nM dihydrotestosterone (5 α -androstan-17 β -ol-3-one; Sigma, St. Louis, MO).

In the experiments testing the effects of AG490 or the Prl receptor (PrlR) antagonist on Stat5a/b phosphorylation, CWR22Rv cells were transfected with 1.0 µg of plasmids encoding WT PrlR and WTStat5a or WTStat5b using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Twenty-four hours after the transfection, the cells were pretreated for 1 h with AG490 or the PrlR antagonist (Δ 1–9G129R hPRL) at the indicated concentrations with appropriate controls and subsequently stimulated with 10 nM human Prl (hPrl) for 15 min. In the experiments testing the effects of the dominant-negative (DN) Jak2 or DNPrIR on Stat5a/b phosphorylation, CWR22Rv cells transfected with 1.0 µg of pWTPrIR and pStat5a or pStat5b were cotransfected with pWTJak2 or pDNJak2 or pWTPrIR or pDNPrIR or empty control plasmid as indicated using Lipofectamine 2000 (Invitrogen). Then 24 h after transfection, cells were serum starved for 24 h and stimulated with hPrl (10 nm) for 15 min.

Production of the hPRL receptor antagonist Δ 1–9G129R hPRL

Recombinant WTHPRL and mutated hPRL (Δ 1–9G129R) were produced in *Escherichia coli* as inclusion bodies and purified as we have reported previously (26).

Clinical human prostate cancer specimens and prostate cancer metastases

Recurrent human prostate cancer specimens (n = 183) were obtained from Tampere University Hospital in Finland (n = 73) (27) and the Institute for Pathology (University of Basel, Basel, Switzerland; n = 110) (28). All samples were transurethral resections from local recurrences. Of the patients, 116 had received androgen ablation therapy (orchiectomy, n = 71; LHRH, n = 19; estrogen, n = 2; antiandrogen, n = 1; orchiectomy + estrogen, n = 2; maximal androgen blockade, n = 20; maximal androgen blockade + estramustine, n = 1), whereas the rest (n = 67) had received no hormonal treatment. The fresh benign prostate hyperplasia and prostate cancer specimens were obtained from 10 patients undergoing radical prostatectomy at the Turku University Hospital by the urologist (M.N.). The prostate tissues were obtained after informed consent of the patient and approval of the Ethical Committee of the Turku University Hospital. Within 1–3 h of the surgery, a board-certified pathologist (K.A.) made a selection of the tissue slices of prostate cancer nodules that were available for the analysis. The selection of the area was assisted by frozen sections and the clinical information of the localization of the cancer based on the location of the needle biopsy taken at the time of the diagnosis.

Paraffin-embedded prostate cancer metastases were obtained from the Turku University Hospital (n = 93) (lymph node metastases, n = 38; metastases to other organs, n = 55) and the Institute for Pathology, University of Basel (lymph node metastases, n = 19; metastases to other organs, n = 69). The use of the de-identified archival tissue specimens in research was approved by the Thomas Jefferson University Institutional Review Board. All tissue sections (prostate cancer recurrences and metastases) were on tissue microarrays.

Immunohistochemical detection of Prl in human prostate cancer

Formalin-fixed prostate cancer sections were immunostained for Prl as described previously (5). Tissue sections were deparaffinized and treated with pepsin (2.5 mg/ml; BioGenex, San Ramon, CA) for 10 min at 37 C to unmask the epitopes. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide and the primary antibody recognizing Prl (mAb) (BioGenex)

was used at concentration of 1:40. Antigen-antibody complexes were detected using a biotinylated goat secondary antibody followed by streptavidin-horseradish-peroxidase complex (BioGenex). 3,3'-Diaminobenzidine was used as chromogen and hematoxylin as counterstain. For controls, subtype-specific IgG was used as appropriate. Human pituitary prolactinoma was used as a positive control tissue.

Scoring of levels of active Stat5a/b in primary and recurrent clinical human prostate cancer

Individual prostate tumor samples on tissue microarrays were scored (M.T.N., J.A., and A.D.) for Prl level on a scale from 0 to 1, where 0 was undetectable and 1 represented positive immunostaining.

Human prostate cancer xenograft tumors

Castrated male athymic mice were purchased from Taconic (Germantown, NY) and cared for according to the institutional guidelines. Briefly, 1×10^6 human prostate cancer cells (CWR22Rv, LNCAP, DU145, and PC-3) were mixed with half of the total injection volume of 0.2 ml with Matrigel (BD Bioscience, Palo Alto, CA). Simultaneously with the tumor cell inoculation (two sites/mouse), sustained-release testosterone pellets (12.5 mg/pellet, one pellet/mouse; Innovative Research of America, Sarasota, FL) were implanted sc. When the tumors reached 12–15 mm in diameter, mice were killed and the tumor tissues were harvested.

Stat5a/b antisense transfections

CWR22Rv cells were transfected with Stat5a/b antisense oligodeoxynucleotides (900 pmol) with mismatch oligodeoxynucleotides (ODNs) (ISIS Pharmaceuticals, Carlsbad, CA) as control using jetPEI (QBiogene Inc., Carlsbad, CA) (900 pmol per 1×10^6 cells) according to manufacturer's instructions. This yielded a transfection efficiency of 50–60% (our unpublished data). Specifically, Stat5a/b antisense ODN (5'-GGG CCT GGT CCA TGT ACG TG-3') (a shared sequence within both human Stat5a and Stat5b transcripts) (bp 2153–2173 in open reading frame) were synthesized using a phosphorothioate backbone with 2'-O-methoxyethyl modification of five terminal nucleotides (underlined) to increase their stability (ISIS 130826) as described before (29). Mismatch ODN for the same chemistry was synthesized as a mixture of all four bases. After 24 h, the cells were harvested for Western blotting and the cell viability assays were carried out 72 h after the transfection.

RT-PCR

Total RNA was isolated using TRIZOL reagent (Invitrogen) and reverse transcribed with Super-Script II reverse transcriptase (Invitrogen) using oligodeoxythymidylic acid primers. The conditions for PCR for all reactions were 94 C for 2 min, followed by 30 sec of denaturation at 94 C, 30 sec of annealing at 60 C, 30 sec of extension at 72 C, and final extension period of 10 min. The PCR products were size separated on a 2% Tris-borate EDTA-agarose gel. For detection of Prl mRNA, we used the following primer pair (30): forward primer (exon 2), 5'-CTCTCCTCAGAAATGTTTCAGC-3' and reverse primer (exon 4) 5'-GGTTTGCTCCTCAATCTCTAC-3'. The size of the PCR product yielded by this primer pair was 276 bp.

To identify the promoter used for regulation of Prl gene expression in human prostate cancer, we used two different primer pairs. Specifically, for distal promoter (exon 1a), we used 5'-CATTCCAGAAGTACCCTCAAAGAC-3' as the forward primer and 5'-GGTTTGCTCCTCAATCTCTAC-3' (exon 4) as the reverse primer, which yields 618-bp PCR product. For proximal promoter we designed

forward primer between -315 and -295 upstream of transcription start codon, which was a unique sequence for the proximal promoter driven Prl mRNA and was confirmed by sequencing. 5'-GGTTTGCTCCTCAATCTCTAC-3' (exon 4) was used as the reverse primer, and the size of the PCR product yielded was 768 bp for the mRNA transcribed from the proximal promoter (see Fig. 2).

For detection of Pit-1 mRNA, we used the following primer pair: forward primer Pit1-F 5'-GATAATGCATCACAGTGCTG-3' (in exon 1; position between 200 and 219 in open reading frame) and reverse primer Pit1-R 5'-GGCAGATTGTTGTTTGGACTG-3' (in exon 4; position between 639 and 620 in open reading frame) (GenBank accession no. NM_000306 for human Pit1). The size of the PCR product yielded by this primer pair was 440 bp. Human brain pituitary total RNA was purchased from BioChain Institute, Inc. (Hayward, CA) and was used as positive control.

Luciferase reporter gene assay

CWR22Rv cells were transfected with 1.0 µg of pCDNA-WTPrlR and pCDNA-WTStat5a or pCDNA-WTStat5b plasmid, 2.0 µg of pZZ1, and 0.1 µg of pRL-TK as an internal control using Lipofectamine 2000 (Invitrogen). In addition, cells were cotransfected with 1.0 µg of pCDNA3.1-WTJak2 or pCDNA3.1-DNJak2 or pCDNA3.1-DNPrIR in some of the experiments. Dominant-negative mutant of Jak2 was created by deletion of the JH1 domain and dominant-negative PrIR lacked the B1-box, which mediates the binding of Jak2 to PrIR. Twenty-four hours after the transfection, cells were stimulated with hPrl (10 nM) for 12 h in starvation medium before the assay for the firefly and Renilla luciferase activity.

In the experiments testing the efficacies of pharmacological Jak2 inhibitor or Prl receptor antagonist in inhibition of the transcriptional activity of Stat5a/b, CWR22Rv cells cotransfected with pCDNA-WTPrlR and pCDNA-Stat5a or pCDNA-Stat5b were pretreated 24 h after transfection with AG490 (Tyrphostin 42; Calbiochem, San Diego, CA) for 30 min at indicated concentrations with AG9 (Tyrphostin 1, inactive control compound; Calbiochem) as a control before 16 h stimulation of the cells with hPrl (10 nM) in the presence of the compound in the starvation medium. Alternatively, CWR22Rv cells were pretreated 24 h after the transfection with indicated concentrations of hPrIR antagonist, Δ1–9G129R hPRL, for 30 min before the 16 h hPrl stimulation (10 nM) of the cells in the presence of the hPrIR antagonist in the starvation medium. The cells were then assayed for firefly and Renilla luciferase activities. Three independent experiments were carried out, and the firefly luciferase activity was normalized to the Renilla luciferase activity of the same sample, and the mean was calculated from the parallel. From the mean values of each independent run, the overall mean and SE were determined.

Cell viability assay, DNA fragmentation assay, and flow cytometry

Nontransfected CWR22Rv cells were treated with the PrIR antagonist for indicated times and the media were changed every day. Cell viability was determined by counting attached cells by hemacytometer and trypan blue exclusion. For flow cytometry, CWR22Rv cells (1×10^6 cells/sample) were stained with 100 µg/ml propidium iodide (Roche Applied Science, Mannheim, Germany) and treated with RNase A (Invitrogen) for 30 min at 37 C. The cells were analyzed by flow cytometry using a Coulter EPICS XL cell analyzer (Beckman-Coulter, Fullerton, CA). Fragmentation of DNA was determined by photometric enzyme immunoassay according to the manufacturers' instructions (cell death detection ELISA^{PLUS}; Roche Molecular Biochemicals, Indianapolis, IN). Briefly, cells were centrifuged at 200 x g, and cytoplasmic fractions containing fragmented DNA were transferred to streptavidin-coated microtiter plates that had been incubated with biotinylated monoclonal antihistone antibody. The amount of fragmented DNA bound to anti-histone antibody was evaluated by peroxidase-conjugated monoclonal anti-DNA antibody.

Protein solubilization and immunoblotting

Pellets of prostate cancer cells were solubilized in lysis buffer [10 mM Tris-HCl (pH 7.6), 5 mM EDTA, 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, 1 µg/ml pepstatin A, and 2 µg/ml leupeptin], rotated end over end at 4 C for 60 min, and insoluble material pelleted at 12,000 x g for 30 min at 4 C. In some of the experiments, protein concentrations of the clarified lysates were determined by a simplified Bradford method (Bio-Rad Laboratories Inc., Hercules, CA) before Western blotting of the cell lysates. One milliliter of the cell lysates was used for immunoprecipitation for 3h at 4C with polyclonal rabbit antisera against either Stat5a or Stat5b (2 µl/ml; Advantex Bioreagents, Conroe, TX). Antibodies were captured by incubation for 60 min with protein A-Sepharose beads (Pharmacia Biotech, Piscataway, NJ). Samples were run on a 4–12% SDS-PAGE under reducing conditions. The primary antibodies were used at the following concentrations: antiphosphotyrosine-Stat5a/b (Y694/Y699) mAb (Advantex BioReagents; 1 µg/ml), anti-Stat5a pAb (Advantex BioReagents; 1:3000), anti-Stat5b pAb (Advantex BioReagents; 1:3000), and anti-Stat5ab mAb (1:1000; Transduction Laboratories, Inc., Lexington, KY) and detected by horseradish peroxidase-conjugated secondary antibodies in conjunction with enhanced chemiluminescence.

Results

Prl protein is expressed in hormone refractory human prostate cancers and prostate cancer metastases

Based on our previous results showing that activation of the Prl-signaling molecule Stat5a/b in primary human prostate cancer predicts early prostate cancer recurrence (31) and that Prl protein expression is elevated in high-grade prostate cancer (5), we hypothesized that autocrine Prl contributes to progression of prostate cancer to androgen-independent and disseminated disease. As a first step to test this hypothesis, we determined whether Prl protein is expressed in clinical human prostate cancer during long-term androgen deprivation. Specifically we first assessed the frequency of Prl expression in 183 local recurrences of prostate cancer obtained by transurethral resection of prostate (Table 1) and next focused on recurrent prostate cancers from patients that had been treated with hormone therapy before the recurrence occurred. Prl protein expression was analyzed by immunohistochemical detection in paraffin embedded tissue sections. The specificity of anti-Prl mouse monoclonal antibody has been previously validated by peptide competition (5). Representative prostate cancers of positive (Fig. 1, left panel) or negative (Fig. 1, right panel) Prl expression status are presented in Fig. 1, A and B. Human prolactinomas were used as positive control tissues and sections of human prostate cancer or human prolactinoma were stained with subtype-specific mouse IgG for negative controls (data not shown).

Significant expression of Prl was detected in 91 (50%) of the entire collection of 183 recurrent human prostate cancer specimens (Table 1). Of these 183 patients, 116 had been treated with androgen deprivation before the recurrence occurred (see *Materials and Methods*). Prl protein was expressed in 63 (54%) of the 116 recurrent prostate cancers that had been treated with hormone therapy (Table 1). In summary, the Prl protein is expressed in a large fraction of hormone-refractory recurrent human prostate cancers. To address the question whether the Prl protein is also expressed in clinical prostate cancer metastases, we analyzed Prl protein expression in paraffin-embedded sections of prostate cancer metastases (total n = 181) to regional lymph nodes (n = 57) and other organs (n = 124). Overall, a positive immunoreaction for Prl protein was detected in 62% (112 of 181) of the prostate cancer metastases. In prostate cancer metastases to lymph nodes, a clear positive immunoreaction in the cytoplasm of prostate cancer cells (Fig. 1B) was detected in 38 of 57 (67%) of the specimens, whereas 74 of 124 (60%) of the distant prostate cancer metastases showed positive immunostaining

for Prl. In conclusion, the Prl protein is expressed at high frequency in hormone-refractory human prostate cancers and clinical human prostate cancer metastases to both lymph nodes and other organs.

Prl gene expression in human prostate cancer is driven by both the proximal and distal promoters

Prl gene expression is driven by two different promoters. In the pituitary, the hPrl mRNA starts with exon 1b at the pituitary start site and transcription is controlled by the pituitary (proximal) promoter (32). The proximal promoter contains binding sites for the pituitary-specific transcription factor Pit-1 and several binding sites for ubiquitous binding factors (33). The distal promoter is located 5.8 kb upstream of the pituitary start site in the exon 1a and produces an mRNA, which is 150 nucleotides longer than the transcript from the proximal promoter (6). To identify the promoter that drives Prl gene expression in human prostate cancer, we designed primer pairs specific for transcripts expressed from the distal promoter or for the proximal promoter (Fig. 2, A and B). Specifically, the primer pair detecting Prl mRNA transcribed from the distal promoter was located next to exon 1a (forward primer) and exon 4 (reverse primer) with a RT-PCR product of 618 bp (Fig. 2A). The forward primer detecting the Prl mRNA transcribed from the proximal promoter was located to a proximal promoter-specific sequence 518 bp upstream of the exon 1b, and the reverse primer was located to exon 4 producing a RT-PCR product of 768 bp (Fig. 2B).

In the first RT-PCR experiments with a primer pair (forward primer: exon 2; reverse primer: exon 4), which detects Prl mRNA regardless of the promoter usage, we showed for the first time Prl gene transcription in clinical human prostate cancer specimens (Fig. 2C, *top panel*). We also show Prl gene transcription in all human prostate cancer cell lines grown in tissue culture (LNCaP, DU145, PC-3, and CWR22Rv) and LNCaP, DU145, and PC3 xenograft tumors. The xenograft tumors are thought to reflect better the *in vivo* growth environment of prostate cancer, and our results indicated that Prl is expressed in both the primary (CWR22p) and recurrent CWR22 (CWR22r) tumors. CWR22 xenograft prostate cancer model closely mimics clinical human prostate cancer. Specifically CWR22 transplantable tumors were originally established from a stage D primary human prostate cancer with bone metastasis (34). Primary CWR22 tumors (CWR22p) are androgen dependent and regress in male mice after orchiectomy (35). However, within 7–9 months of androgen deprivation, the tumors start to regrow and the recurrent CWR22 tumors (CWR22r) are not dependent on androgens for growth (36). Because of varying AR status in different prostate cancer cell lines (LNCaP and CWR22Rv are AR positive, whereas Du145 and PC-3 are AR negative) and because circulating testosterone (Te) levels are highly variable in nude mice, we normalized the Te levels inserting sustained-release Te pellets to nude mice that had been castrated.

In the second set of experiments, RT-PCR was run using the two primer pairs specific for the proximal or distal promoters. The RT-PCR results show that Prl gene transcription in clinical human prostate cancer samples, human prostate cancer cells lines, and xenograft tumors is driven by both the proximal and distal promoters (Fig. 2C, *middle and bottom panels*). Pit-1 is a POU-domain transcription factor of the anterior pituitary that specifies cell lineage differentiation and is required for Prl gene expression in lactotropes (37). Because the proximal promoter, which drives Prl gene transcription in pituitary gland under control of Pit1, is also active in human prostate cancer, we next determined whether Pit-1 is expressed in human prostate cancer. Pit-1-specific RT-PCR was negative for all prostate-derived samples, whereas pituitary mRNA showed the expected 440 bp Pit-1 RT-PCR product (Fig. 2D). In summary, the results presented here indicate that Prl mRNA is transcribed in not only prostate cancer cell lines but also clinical human prostate cancer specimens. Furthermore, Prl gene transcription in prostate cancer is driven by the proximal and distal promoters, independently of Pit-1.

Inhibition of Stat5a/b by Stat5 antisense oligodeoxynucleotides induces death of CWR22Rv cells

We have previously shown by adenoviral gene delivery of a dominant-negative mutant of Stat5a/b that

inhibition of Stat5a/b-induced apoptotic death of prostate cancer cells within a few days (20). To validate this finding by an alternative method, we set up Stat5a/b inhibition by Stat5a/b antisense ODNs (900 pmol per 1×10^6 cells). Transfection of CWR22Rv cells with antisense ODNs targeted against a homologous region between both Stat5a and Stat5b (29) resulted in a decrease in Stat5a and Stat5b protein expression at 24 h, as demonstrated by Western blotting of the cell lysates (Fig. 3A, *upper panel*). Reblotting of the filters with antiactin antibody demonstrated equal loading of the gel (Fig. 3A, *lower panel*). Stat5a/b inhibition induced significant death of CWR22Rv cells by 72 h after transfection as demonstrated by cell morphology (Fig. 3B, *upper panel*). Specifically, inhibition of Stat5a/b protein expression induced extensive cell rounding, detachment, shrinkage, and blebbing, mismatch control oligonucleotides. In addition, nucleosomal which are morphological changes consistent with apoptotic DNA fragmentation was increased by 6-fold on average in cell death. In contrast, there was no evidence of reduced cell Stat5a/b antisense-treated cells 72 h after transfection (Fig. viability in response to the transfection reagent itself or to 3B, *lower panel*). These results indicated that antisense inhibition of Stat5a/b protein expression induces rapid apoptotic death of human prostate cancer cells.

The $\Delta 1-9G129R$ -hPRL Prl antagonist disrupts constitutive activation of Stat5a/b in human prostate cancer cells effectively and induces death of the cells

Given that the key Prl-signaling protein Stat5 is critical for viability of prostate cancer cells and that the components of autocrine Prl-Stat5a/b signaling pathway are expressed and active at high frequency in high-grade prostate cancers (5,31), hormone-refractory prostate cancers, and prostate cancer metastases (Table i), we wanted to determine the involvement of autocrine Prl in prostate cancer growth. We chose to use a specific competitive Prl receptor antagonist ($\Delta 1-9G129R$ -hPR) developed based on rational drug design (23) to inhibit autocrine Prl in human prostate cancer cells. To set up a reference for the pharmacological Prl receptor antagonist, we first inhibited phosphorylation of stat5a/b by a CNPrIR. This is a PrIR mutant lacking both the cytoplasmic box 1 and internalization motif and therefore is unable to bind Jak2 and accumulates on the cell surface. CWR22Rv cells transfected with STPrIR and Stata or Stat5b were cotransfected with DNPrIR or empty control vector. Then 24 h after the transfection, the cells were serum starved for 24 h and stimulated with 10 nM hPrl for 15 min. Expression of DN-PrIR in CWR22Rv cells inhibited Prl-induced phosphorylation of Stat5a and Stat5b, as shown by Western blotting by approximately 80-90% (Fig. 4A). In line with these results, DN-PrIR inhibited ligand-induced transcriptional activity of Stat5a and Stat5b by approximately 80-90% when tested in β -casein-luciferase of CWR22Rv cells cotransfected with WTPrIR, Stat5a, Stat5b, DNPrIR, or empty control vector (Fig. 4B).

$\Delta 1-9-G129R$ -hPRL acts as a competitive PrIR antagonist because the mutation of glycine 129 to arginine impairs the interaction of hPrl with a second PrIR molecule and prevents functional receptor dimerization. This mutation is further enhanced by deletion of the first nine N-terminal residues, which is thought to prevent additional hormone-ligand interactions presumably involved in receptor activation (23). Next, we wanted to establish the efficacy of a specific PrIR antagonist in disruption of autocrine Prl-Jak2-Stat5 signaling pathway in prostate cancer cells. CWR22Rv cells were cotransfected with WPPrIR and Stat5a or Stat5b, and 24 h after the transfection the cells were pretreated for 30 min with the PrIR antagonist and stimulated with 10 nM hPrl for 16 h in the presence of the PrIR antagonist. $\Delta 1-9-G129R$ -hPRL inhibited transcriptional activity of Prl-induced Stat5a and Stat5b in β -casein reporter gene assay in a dose-dependent manner (Fig. 4C). We have shown previously that hPrl activated both Stat5a and Stat5b in CWR22Rv cells (5). To test whether $\Delta 1-9-G129R$ -hPRL will inhibit Prl-induced phosphorylation of Stat5a and Stat5b, CWR22Rv cells were transfected with WTPrIR and Stat5a or Stat5b and pretreated with the PrIR antagonist at the indicated concentrations for 1 h before stimulation of the cells with 10 nM hPrl for 15 min. $\Delta 1-9-G129R$ -hPRL inhibited phosphorylation of Prl-induced Stat5a/b in a dose-dependent manner shown by Western blotting (Fig. 4D). A 100-nM concentration of the PrIR antagonist-induced inhibition of Stat5a/b activity after stimulation of the CWR22Rv

cells with 10 nM exogenous Prl (Fig. 4, C–E). These experiments concluded that $\Delta 1-9$ -G129RhPRL is a potent inhibitor of Prl-activated Stat5a/b signaling in human prostate cancer cells.

Next, we aimed to test whether autocrine Prl activates Stat5a/b and promotes prostate cancer cell viability using the specific Prl receptor antagonist $\Delta 1-9$ -G129R-hPRL. Because autocrine peptide hormones require higher concentrations of respective antagonist for effective inhibition compared with concentration needed for antagonizing the exogenously added hormones (38) and because 100 nM $\Delta 1-9$ -G129R-hPRL showed residual Stat5a/b phosphorylation after Prl-induction in Western blotting (Fig. 4D), we chose to use 500 nM concentration of $\Delta 1-9$ -G129R-hPRL to disrupt autocrine Prl in prostate cancer cells. In the first set of experiments, $\Delta 1-9$ -G129R-hPRL was tested for inhibition of autocrine Prl activation of Stat5a/b in nontransfected androgen-independent human CWR22Rv prostate cancer cells. CWR22Rv cells were chosen as an experimental model because exogenously added Prl activated Stat5a/b in CWR22Rv cells (5). Moreover, CWR22Rv cells produce autocrine Prl (Fig. 2C), and Stat5a/b is constitutively active in this cell line (Fig. 5A) potentially due to autocrine Prl. Treatment of nontransfected CWR22Rv cells with $\Delta 1-9$ -G129R-hPRL for 10 d resulted in 46% inhibition of basal phosphorylation of Stat5a/b, which was reversed by addition of exogenous hPrl (Fig. 5A). Filters were stripped and reblotted for total Stat5a/b to verify equal loading of the gel. At the same time point, the number of attached cells after washing of the wells with medium was determined in parallel sets of wells ($n = 8$) by trypan blue exclusion accompanied by manual counting. This indicated a 64% decrease in attached and viable CWR22Rv cells in the Prl antagonist treatment group (Fig. 5B). The cell death induced by the Prl-antagonist was partly prevented by addition of hPrl to the cells. The result was further confirmed by cell cycle analysis (Fig. 5C) in which $\Delta 1-9$ -G129R-hPRL treatment of nontransfected CWR22Rv cells induced a 2-fold increase in dead cells (preG1 peak) at d 10 (Fig. 5C). However, addition of exogenous hPrl did not affect the volume of the preG1-peak, which might be due to saturation of the PrlRs with autocrine Prl. These data indicated that $\Delta 1-9$ -G129R-hPRL disrupts autocrine Prl-Jak2-Stat5a/b signaling in CWR22Rv human prostate cancer cells and decreases the viability of the cells.

DNJak2 and small-molecule inhibitor of Jak2 in suppression of Stat5a/b phosphorylation and transcription

To set up an alternative strategy to pharmacologically mpanied inhibit Prl-Jak2-Stat5 signaling in human prostate cancer cells, we decided to inhibit tyrosine kinase Jak2 by a small-molecule Jak2-inhibitor, AG490 (Tyrphostin 42). As a reference, we first established inhibition of Stat5a/b phosphorylation and transcriptional activity by a dominant-negative mutant of Jak2 (DNJak2), which lacks the kinase domain JH1 (5, 39) (Fig. 6, A and B). CWR22Rv cells were cotransfected with WTPrlR, Stat5a, and Stat5b and cotransfected with WT-Jak2 or DNJak2 or empty control vector, starved for 24 h and stimulated with 10 nM hPrl for 15 min. Western blotting with antiactive Stat5a/b antibody shows 90–100% inhibition of Prl-induced Stat5a/b phosphorylation in CWR22Rv human prostate cancer cells cotransfected with PrlR, Stat5a/b, and WTJak2 increased phosphorylation of Stat5a/b, compared DNJak2 (Fig. 6A, *upper panel*), whereas the Stat5a/b protein with empty control vector-transfected cells. In the second set levels remained constant (Fig. 6A, *lower panel*). In addition, of experiments, CWR22Rv cells transfected with WTPrlR, Stat5a, or Stat5b were cotransfected with either WTJak2 or DNJak2 or empty control vector and stimulated 24 h after the transfection with 10 nM hPrl for 12 h in starvation medium. Prl-induced transcriptional activation of Stat5a/b on genomic β -casein promoter-Luc was 100% inhibited by cotransfection of CWR22Rv cells with DNJak2, whereas WTJak2 increased transcriptional activity of Stat5a/b. (Fig. 6B). The β -casein promoter reporter gene was chosen for these experiments because it contains a Stat5-binding site flanked by a nonconsensus binding site and therefore constitutes a strong tetrameric Stat binding promoter (40). The results of these experiments serve as standard for testing the efficacy of pharmacological inhibitor of Jak2 in disruption of PrlJak2-Stat5 signaling pathway with Stat5a/b activation as the readout.

First, we established a dose-response curve for inhibition of transcriptional activity of Prl-induced Stat5a/b by a small-molecule inhibitor of Jak2, AG490. CWR22Rv cells were transfected with WTPrlR and Stat5a or Stat5b and pretreated with AG490 for 30 min with dimethylsulfoxide as a control before hPrl (10 nM) stimulation of the cells for 16 h in the presence of AG490 or control. We determined that 100 μ M concentration of AG490 resulted in 50–60% inhibition of Stat5a (Fig. 6C) and 30–40% inhibition of transcriptional activity of Stat5b (Fig. 6D). We then compared the efficacy of 100 μ M AG490 in inhibition of Prl-induced phosphorylation of Stat5a/b with AG9 (inactive compound provided by the supplier) as a control compound in Western blotting. CWR22Rv cells transfected with WTPrlR and Stat5a or Stat5b were serum starved for 24 h and pretreated with AG490 for 1 h before 15 min stimulation of the cells with 10 nM hPrl. AG490 inhibited phosphorylation of Stat5a and Stat5b by approximately 50%, whereas the control compound AG9 also resulted in Stat5a/b inhibition of approximately 20–30% in CWR22Rv cells (Fig. 6E). Reblotting of the stripped filters with anti-Stat5ab antibody demonstrated equal loading of the gel. Comparison of AG490 in inhibiting transcriptional activity of Stat5a/b to the control compound at 100 μ M concentration in β -casein luciferase reporter gene assay showed consistent inhibition of transcriptional activity of Stat5a and Stat5b by approximately 45 and 35%, respectively (Fig. 6F). Treatment of nontransfected CWR22Rv cells with AG490 did not cause changes in the viability of the cells (data not shown).

Collectively, these results show that AG490 (Tyrphostin 42) is a relatively weak inhibitor of Prl-activated Jak2Stat5a/b signaling pathway in prostate cancer cells.

Discussion

Identification of the molecular mechanisms that promote proliferation and survival of prostate cancer cells is required for the development of new therapies for prostate cancer. Protein kinase signaling pathways, often activated by local growth factors, may provide the critical growth signals for premalignant lesions to progress to clinical prostate cancer and organ-confined primary prostate cancer to progress to hormone-refractory disseminated disease. In this work, we show that Prl protein is produced by 54% of hormone-refractory human prostate cancers and 62% of clinical prostate cancer metastases and that Prl gene transcription in clinical human prostate cancer is regulated by both the proximal and distal promoters. Using a competitive Prl receptor antagonist, we demonstrate that autocrine Prl activated Stat5a/b in prostate cancer cells and that disruption of autocrine-PrlStat5a/b signaling by Δ 1–9-G129R-hPRL induces death of androgen-independent CWR22Rv human prostate cancer cells.

This is the first time that disruption of autocrine Prl has been shown to result in decrease of Stat5a/b activation in human prostate cancer cells. This is important because active Stat5a/b is highly critical for survival and proliferation of prostate cancer cells (20, 21), and therefore, the Prl-Jak2Stat5a/b signaling pathway provides several different therapeutic target molecules for human prostate cancer. These include Prl-receptor inhibitors and antibodies, Stat5a/b phosphatases, and Stat5a/b proteases as well as small-molecule inhibitors for Jak2 and Stat5a/b. Here our results demonstrate that the competitive PrlR antagonist Δ 1–9-G129RhPRL decreased transcriptional activity of Stat5a/b and the viability of the androgen-independent CWR22Rv human prostate cancer cells. Pseudophosphorylated Prl (S179D Prl) has previously been shown to decrease growth of DU145 cells *in vitro* and *in vivo* (22), but no data were shown on the effects of pseudophosphorylated Prl on PrlR-Jak2-Stat5a/b activation in prostate cancer cells. The mechanism of antagonist action of the pseudophosphorylated Prl has been reported to be based on induction of altered expression pattern of the different PrlR forms (24), not rational drug design. Future studies need to determine the efficacy of the competitive PrlR antagonist, Δ 1–9-G129R-hPRL, on inhibition of tumor growth of human prostate cancer xenografts *in vivo* and the effects of Δ 1–9-G129R-hPRL on prostate cancer growth in mouse prostate cancer models. Furthermore, our data on the efficacy of AG490 (Tyrphostin 42) in inhibiting Stat5a/b activation indicated that there is a clear need for development of more efficient small molecule inhibitors for

Jak2.

The second key finding of this work was the demonstration of Prl gene transcription in prostate cancer being driven by both the proximal and the distal promoters. Prl gene expression from the proximal and distal promoters was shown in not only human prostate cancer cell lines but also prostate xenograft tumors and, more importantly, in clinical human prostate cancer samples. Transcription factor Pit-1, which regulates Prl gene expression in the pituitary gland, was not expressed in prostate cancer cells or in prostate cancer specimens, suggesting that factors other than Pit-1 control the proximal promoter activation in prostate cancer. Future work will focus on identifying the transcription factors that are crucial for proximal and distal promoter-driven Prl gene transcription in human prostate cancer. Some of these factors may be prostate specific, and therefore, they will potentially provide therapeutic target proteins for prostate cancer. Of special interest will be evaluation of androgens as regulators of proximal and distal promoter-driven Prl gene transcription in normal and malignant prostate cells. This is based on the hypothesis that liganded ARs repress Prl gene transcription in prostate cancer leading to increased Prl expression during androgen deprivation.

Autocrine Prl protein was expressed in 54% of hormone-refractory human prostate cancers and 62% of lymph node and distant prostate cancer metastases. Our previous data showed that both autocrine Prl and active Stat5 expression correlated with high histological grade of prostate cancer (5, 31). These findings together support the concept that autocrine Prl-Stat5a/b signaling pathway is associated with advanced prostate cancer (high grade, hormone refractory, and metastatic disease). It is possible that autocrine Prl-PrlR-Jak2 STAT5a/b signaling pathway provides prostate cancer cells with the ability to survive in a growth environment lacking androgens. This hypothesis needs to be tested using $\Delta 1-9$ -G129R-hPRL in appropriate human xenograft prostate cancer models such as primary and recurrent CWR22 or LAPC prostate tumors (34, 41, 42). We have previously shown that active Stat5a/b in primary human prostate cancer predicts early disease recurrence (31). Because autocrine Prl is one of the main activators of Stat5a/b in prostate cancer, it is likely that autocrine Prl in primary prostate cancer could provide a prognostic marker to assist clinical decision making in addition to active Stat5a/b. Finally, involvement of autocrine Prl in metastatic behavior and homo/heterotypic cell adhesion should be investigated to identify the biological functions of autocrine Prl in distant prostate cancer metastasis.

In summary, this work establishes that autocrine Prl in CWR22Rv prostate cancer cells promotes cell viability via Stat5a/b signaling pathway. Moreover, this work shows autocrine Prl expression in 50–60% of hormone-refractory clinical prostate cancers and prostate cancer metastases. This work demonstrates that Prl gene in prostate cancer is transcribed from both the proximal and distal promoters, which establishes the platform for future studies on the identification of prostate-specific transcriptional regulators of Prl gene. In conclusion, this study provides support for therapeutic strategies for prostate cancer based on inhibition of Prl-Jak2-Stat5 signaling pathway.

Acknowledgments

We thank Dr. Hallgeir Rui for providing us with DNJak2 and DNPrIR cDNA constructs. The National Institute of Diabetes and Digestive and Kidney Diseases National Pituitary Hormone Program and Dr. A. F. Parlow (Pituitary Hormone and Antisera Center, Torrance, CA) are gratefully acknowledged for providing hPRL. We also thank Dr. Julian Davis for discussions and advice and Dr. Shyh-Han Tan and Ms. Jacqueline Lutz for critical reading of the manuscript.

Address all correspondence and requests for reprints to: Marja T. Nevalainen, M.D., Ph.D., Department of Cancer Biology, Kimmel Cancer Center, Thomas Jefferson University, BLSB 309B, 233 South 10th Street, Philadelphia, Pennsylvania 19107. E-mail: marja.nevalainen@jefferson.edu.

This work was supported by American Cancer Society (RSG-04-19601-MGO), Department of Defense Prostate Cancer Grant W81XWH-0501-0062, and National Institutes of Health, National Cancer Institute Grant 1RO1CA113580-01A1, and Association for International Cancer Research Grant 05-603. J.-B.J. was supported by the Ministry of Research and Technology of France and the Association pour la Recherche Contre le Cancer. Shared resources of the Kimmel Cancer Center are partially supported by National Institutes of Health Grant CA56036-08 (Cancer Center Support Grant to Kimmel Cancer Center).

Disclosure Summary: The authors have nothing to disclose, except J.K., who has equity interests in ISIS Pharmaceuticals.

References

1. Linja MJ, Visakorpi T 2004 Alterations of androgen receptor in prostate cancer. *J Steroid Biochem Mol Biol* 92:255–264
2. Feldman BJ, Feldman D 2001 The development of androgen-independent prostate cancer. *Nat Rev Cancer* 1:34–45
3. Nevalainen MT, Valve EM, Ingleton PM, Harkonen PL 1996 Expression and hormone regulation of prolactin receptors in rat dorsal and lateral prostate. *Endocrinology* 137:3078–3088
4. Nevalainen MT, Valve EM, Ingleton PM, Nurmi M, Martikainen PM, Harkonen PL 1997 Prolactin and prolactin receptors are expressed and functioning in human prostate. *J Clin Invest* 99:618–627
5. Li H, Ahonen TJ, Alanen K, Xie J, LeBaron MJ, Pretlow TG, Ealley EL, Zhang Y, Nurmi M, Singh B, Martikainen PM, Nevalainen MT 2004 Activation of signal transducer and activator of transcription 5 in human prostate cancer is associated with high histological grade. *Cancer Res* 64:4774–4782
6. DiMattia G, Gellersen B, Duckworth ML, Friesen HG 1990 Human prolactin gene expression. *J Biol Chem* 265:16412–16421
7. Ben-Jonathan N, Mershon J, Allen DL, Steinmetz RW 1996 Extrapituitary prolactin: distribution, regulation, functions, and clinical aspects. *Endocr Rev* 17:639–669
8. Baudhuin A, Manfroid I, Van De Weerd C, Martial JA, Muller M 2002 Transcription of the human prolactin gene in mammary cells. *Ann NY Acad Sci* 973:454–458
9. Ben-Jonathan N, Liby K, McFarland M, Zinger M 2002 Prolactin as an autocrine/paracrine growth factor in human cancer. *Trends Endocrinol Metab* 13:245–263
10. Gerlo S, Davis JR, Mager DL, Kooijman R 2006 Prolactin in man: a tale of two promoters. *Bioessays* 28:1051–1055
11. Nevalainen MT, Valve EM, Ingleton PM, Nurmi M, Martikainen PM, Harkonen PL 1997 Androgen-dependent expression of prolactin in rat prostate epithelium in vivo and in organ culture. *FASEB J* 11:1297–1308
12. Nevalainen MT, Valve EM, Makela SI, Blauer M, Tuohimaa PJ, Harkonen PL 1991 Estrogen and

prolactin regulation of rat dorsal and lateral prostate in organ culture. *Endocrinology* 129:612–622

13. Ahonen TJ, Harkonen PL, Laine J, Rui H, Martikainen PM, Nevalainen MT 1999 Prolactin is a survival factor for androgen-deprived rat dorsal and lateral prostate epithelium in organ culture. *Endocrinology* 140:5412–5421
14. Wennbo H, Kindblom J, Isaksson OG, Tornell J 1997 Transgenic mice over-expressing the prolactin gene develop dramatic enlargement of the prostate gland. *Endocrinology* 138:4410–4415
15. Kindblom J, Dillner K, Ling C, Tornell J, Wennbo H 2002 Progressive prostate hyperplasia in adult prolactin transgenic mice is not dependent on elevated serum androgen levels. *Prostate* 53:24–33
16. Kindblom J, Dillner K, Sahlin L, Robertson F, Ormandy C, Tornell J, Wennbo H 2003 Prostate hyperplasia in a transgenic mouse with prostate-specific expression of prolactin. *Endocrinology* 144:2269–2278
17. Janssen T, Darro F, Petein M, Raviv G, Pasteels JL, Kiss R, Schulman CC 1996 In vitro characterization of prolactin-induced effects on proliferation in the neoplastic LNCaP, DU145, and PC3 models of the human prostate. *Cancer* 77:144–149
18. Steger RW, Chandrashekar V, Zhao W, Bartke A, Horseman ND 1998 Neuroendocrine and reproductive functions in male mice with targeted disruption of the prolactin gene. *Endocrinology* 139:3691–3695
19. Ahonen TJ, Harkonen PL, Rui H, Nevalainen MT 2002 PRL signal transduction in the epithelial compartment of rat prostate maintained as long-term organ cultures in vitro. *Endocrinology* 143:228–238
20. Ahonen TJ, Xie J, LeBaron MJ, Zhu J, Nurmi M, Alanen K, Rui H, Nevalainen MT 2003 Inhibition of transcription factor Stat5 induces cell death of human prostate cancer cells. *J Biol Chem* 278:27287–27292
21. Kazansky AV, Spencer DM, Greenberg NM 2003 Activation of signal transducer and activator of transcription 5 is required for progression of autochthonous prostate cancer: evidence from the transgenic adenocarcinoma of the mouse prostate system. *Cancer Res* 63:8757–8762
22. Xu X, Kreye E, Kuo CB, Walker AM 2001 A molecular mimic of phosphorylated prolactin markedly reduced tumor incidence and size when du145 human prostate cancer cells were grown in nude mice. *Cancer Res* 61:6098–6104
23. Goffin V, Bernichtein S, Touraine P, Kelly PA 2005 Development and potential clinical uses of human prolactin receptor antagonists. *Endocr Rev* 26: 400–422
24. Wu W, Ginsburg E, Vonderhaar BK, Walker AM 2005 S179D prolactin increases vitamin D receptor and p21 through up-regulation of short 1b prolactin receptor in human prostate cancer cells. *Cancer Res* 65:7509–7515
25. Goffin V, Touraine P, Culler MD, Kelly PA 2006 Drug insight: prolactin-receptor antagonists, a novel

approach to treatment of unresolved systemic and local hyperprolactinemia? *Nat Clin Pract Endocrinol Metab* 2:571–581

26. Bernichtein S, Kayser C, Dillner K, Moulin S, Kopchick JJ, Martial JA, Norstedt G, Isaksson O, Kelly PA, Goffin V 2003 Development of pure prolactin receptor antagonists. *J Biol Chem* 278:35988–35999
27. Saramaki O, Willi N, Bratt O, Gasser TC, Koivisto P, Nupponen NN, Bubendorf L, Visakorpi T 2001 Amplification of EIF3S3 gene is associated with advanced stage in prostate cancer. *Am J Pathol* 159:2089–2094
28. Zellweger T, Ninck C, Bloch M, Mirlacher M, Koivisto PA, Helin HJ, Mihatsch MJ, Gasser TC, Bubendorf L 2005 Expression patterns of potential therapeutic targets in prostate cancer. *Int J Cancer* 113:619–628
29. Behbod F, Nagy ZS, Stepkowski SM, Karras J, Johnson CR, Jarvis WD, Kirken RA 2003 Specific inhibition of Stat5a/b promotes apoptosis of IL-2-responsive primary and tumor-derived lymphoid cells. *J Immunol* 171:3919–3927
30. Stevens A, Ray D, Alansari A, Hajeer A, Thomson W, Donn R, Ollier WE, Worthington J, Davis JR 2001 Characterization of a prolactin gene polymorphism and its associations with systemic lupus erythematosus. *Arthritis Rheum* 44:2358–2366
31. Li H, Zhang Y, Glass A, Zellweger T, Gehan E, Bubendorf L, Gelmann EP, Nevalainen MT 2005 Activation of signal transducer and activator of transcription-5 in prostate cancer predicts early recurrence. *Clin Cancer Res* 11:5863–5868
32. Truong AT, Duez C, Belayew A, Renard A, Pictet R, Bell GI, Martial JA 1984 Isolation and characterization of the human prolactin gene. *EMBO J* 3:429–437
33. Van De Weerd C, Peers B, Belayew A, Martial JA, Muller M 2000 Far upstream sequences regulate the human prolactin promoter transcription. *Neuroendocrinology* 71:124–137
34. Pretlow TG, Wolman SR, Micale MA, Pelley RJ, Kursh ED, Resnick MI, Bodner DR, Jacobberger JW, Delmoro CM, Giaconia JM, et al 1993 Xenografts of primary human prostatic carcinoma. *J Natl Cancer Inst* 85:394–398
35. Wainstein MA, He F, Robinson D, Kung HJ, Schwartz S, Giaconia JM, Edgehouse NL, Pretlow TP, Bodner DR, Kursh ED, et al 1994 CWR22: androgen-dependent xenograft model derived from a primary human prostatic carcinoma. *Cancer Res* 54:6049–6052
36. Nagabhushan M, Miller CM, Pretlow TP, Giaconia JM, Edgehouse NL, Schwartz S, Kung HJ, de Vere White RW, Gumerlock PH, Resnick MI, Amini SB, Pretlow TG 1996 CWR22: the first human prostate cancer xenograft with strongly androgen-dependent and relapsed strains both in vivo and in soft agar. *Cancer Res* 56:3042–3046
37. Ryan AK, Rosenfeld MG 1997 POU domain family values: flexibility, partnerships, and developmental codes. *Genes Dev* 11:1207–1225

38. Kaulsay KK, Zhu T, Bennett W, Lee KO, Lobie PE 2001 The effects of autocrine human growth hormone (hGH) on human mammary carcinoma cell behavior are mediated via the hGH receptor. *Endocrinology* 142:767–777
39. Xie J, LeBaron MJ, Nevalainen MT, Rui H 2002 Role of tyrosine kinase Jak2 in prolactin-induced differentiation and growth of mammary epithelial cells. *J Biol Chem* 277:14020–14030
40. Gouilleux F, Wakao H, Mundt M, Groner B 1994 Prolactin induces phosphorylation of Tyr694 of Stat5 (MGF), a prerequisite for DNA binding and induction of transcription. *EMBO J* 13:4361–4369
41. Craft N, Chhor C, Tran C, Beldegrun A, DeKernion J, Witte ON, Said J, Reiter RE, Sawyers CL 1999 Evidence for clonal outgrowth of androgen-independent prostate cancer cells from androgen-dependent tumors through a two-step process. *Cancer Res* 59:5030–5036
42. Nickerson T, Chang F, Lorimer D, Smeekens SP, Sawyers CL, Pollak M 2001 In vivo progression of LAPC-9 and LNCaP prostate cancer models to androgen independence is associated with increased expression of insulin-like growth factor-I (IGF-I) and IGF-I receptor (IGF-IR). *Cancer Res* 61:6276 – 6280

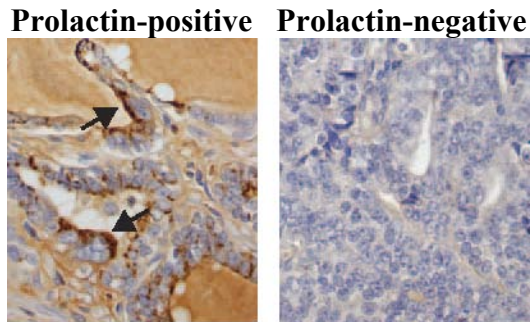
Tables and Figures

TABLE 1. Prl expression in recurrent hormone-refractory prostate cancers and prostate cancer metastasis

	No. of patients	%
Recurrent prostate cancers		
Prl protein expression status	183	100
Negative	92	50
Positive	91	50
Recurrent prostate cancers treated with hormone therapy		
Prl protein expression status	116	100
Negative	53	46
Positive	63	54
Prostate cancer metastases (lymph node metastases and metastases to other organs)		
Prl protein expression status	181	100
Negative	69	38
Positive	112	62
Prostate cancer metastases to regional lymph nodes		
Prl protein expression status	57	100
Negative	19	33
Positive	38	67
Prostate cancer metastases to other organs		
Prl protein expression status	124	100
Negative	50	40
Positive	74	60

A

Recurrent Human Prostate Cancer



B

Prostate Cancer Metastases

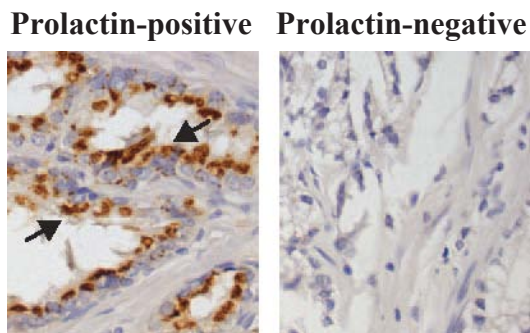
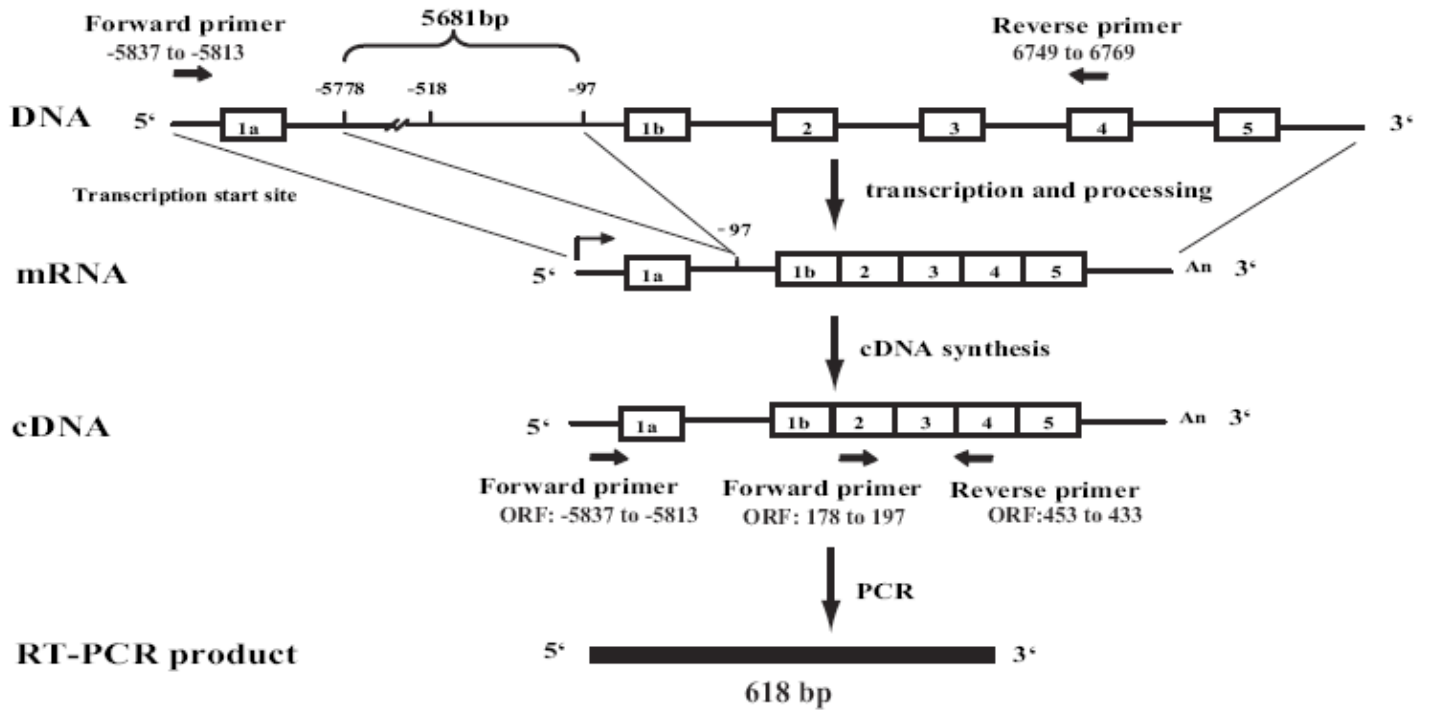
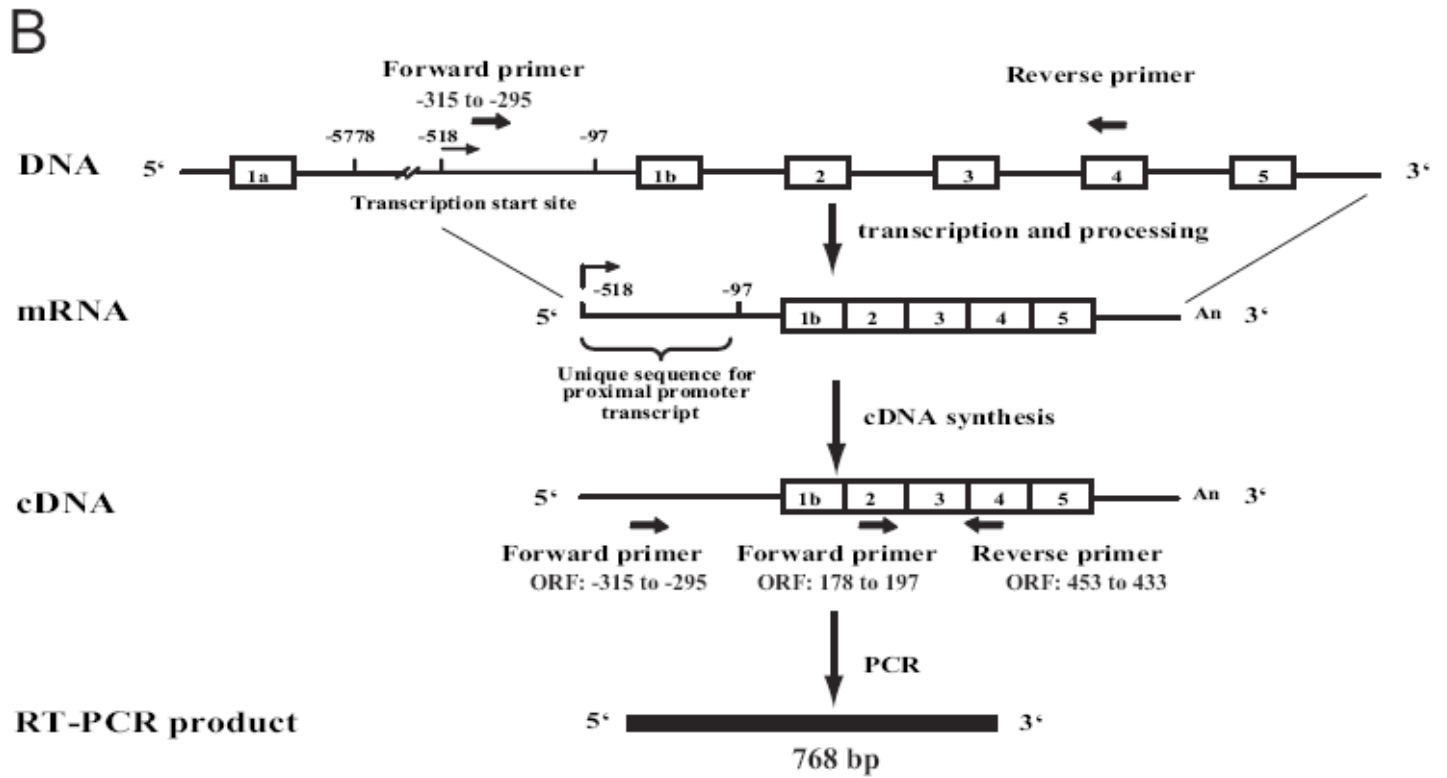


FIG. 1. Prl protein in hormone-refractory recurrent human prostate cancers and distant prostate cancer metastases. Prl protein is expressed in the majority of hormone-refractory recurrent (A) prostate cancers and prostate cancer metastases (B). Prl protein expression in recurrent human prostate cancers and prostate cancer metastases was analyzed by immunohistochemical staining using a monoclonal anti-Prl antibody in paraffin-embedded tissue sections. 3,3'-DAB was used as a chromogen and Mayer hematoxylin as counterstain. Biotinstreptavidin amplified peroxidase-antiperoxidase immunodetection shows intense positive reactions for Prl in the cytoplasm of epithelial cells of a representative hormone-refractory prostate cancer (A) and prostate cancer metastases (B) that had a high level of Prl protein expression (*left panels*), compared with specimens that were considered negative for Prl (*right panels*).

A

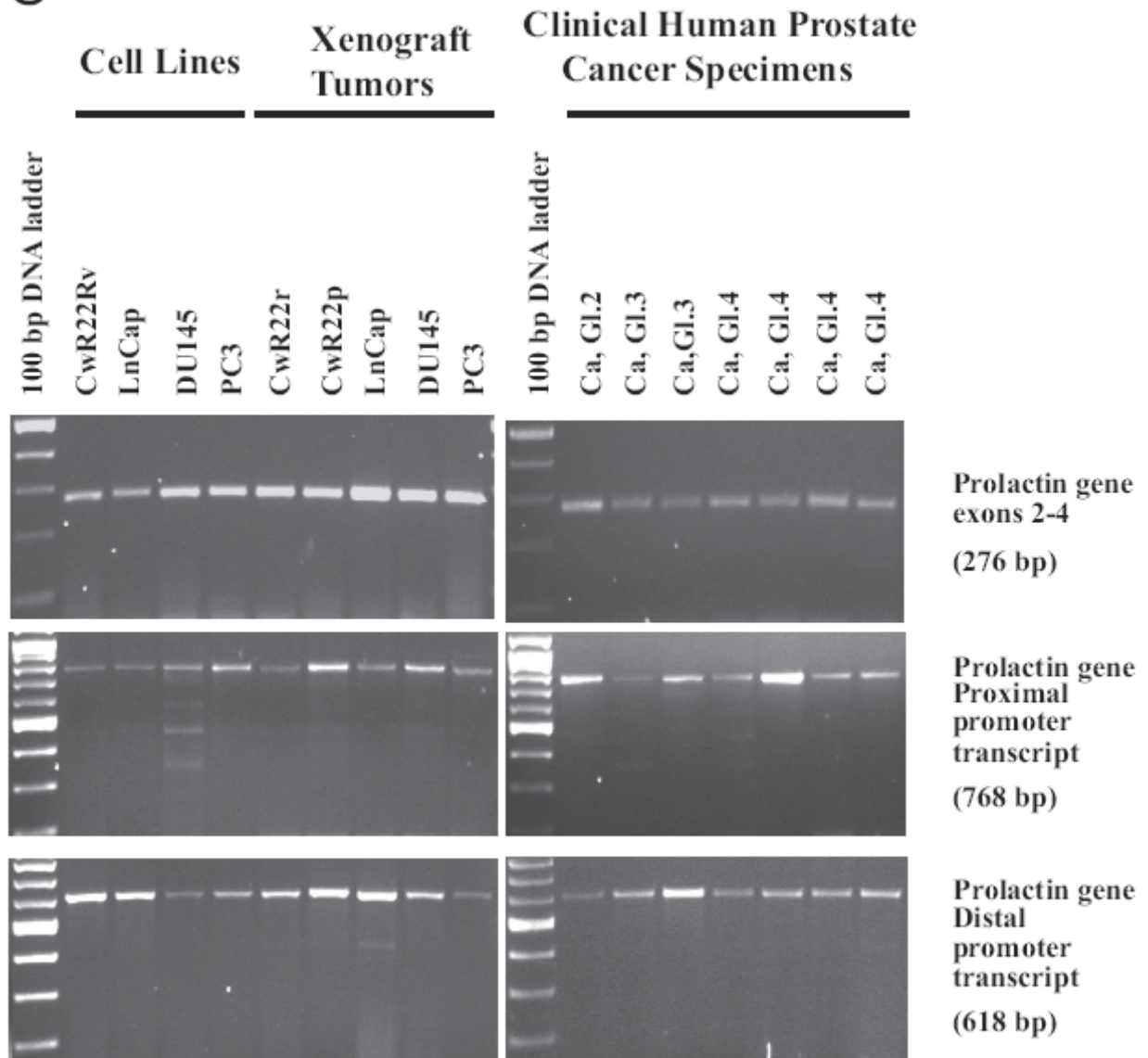


RT-PCR for Prl proximal promoter transcript



RT-PCR for Prl proximal promoter transcript

C



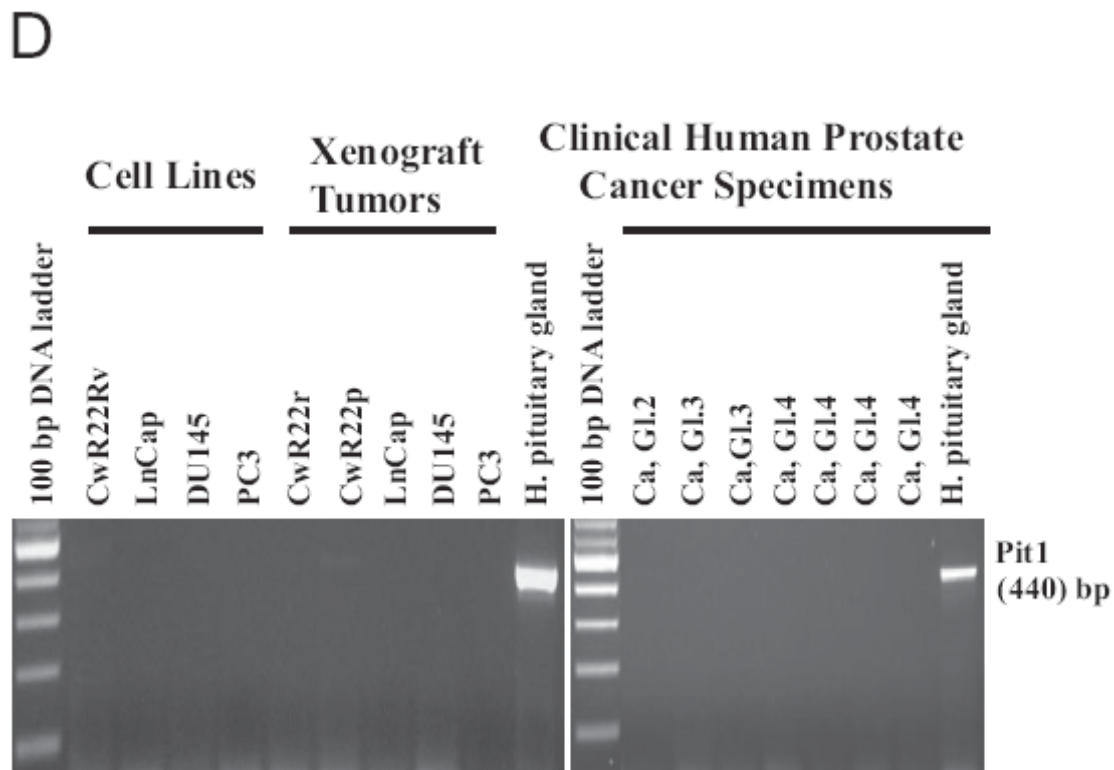


FIG. 2. Prl gene transcription in human prostate cancer is driven by both the distal and proximal promoters. Schematic illustration of the design of the RT-PCR primers for exclusive detection of Prl mRNA transcribed from the distal (A) or proximal (B) promoter. A, The primer pair detecting Prl mRNA transcribed from the distal promoter was upstream of exon 1a (forward primer) and the reverse primer was in exon 4 with a RT-PCR product of 618 bp. B, The distal promoter is located 5.8 kb upstream of the pituitary start site in exon 1a. The sequence from 97–518 upstream of exon 1b is a unique sequence for the mRNA transcribed from the proximal promoter and is spliced out from the distal promoter derived Prl transcript. The primer pair detecting the Prl mRNA transcribed from the proximal promoter was located to the proximal promoter-specific transcript 518 bp upstream of the exon 1b (forward primer) and the reverse primer was in exon 4 with a RT-PCR product of 768 bp. ORF, Open reading frame. C, RT-PCR with a primer pair detecting Prl mRNA regardless of the promoter usage (forward primer: exon 2; reverse primer: exon 4) (*top panel*) or with proximal/distal promoter-specific primer pairs (*middle and bottom panels*, respectively) show Prl gene transcription in human prostate cancer cell lines (LNCAP, DU145, PC-3, and CWR22Rv); xenograft prostate tumors [primary (CWR22p) and recurrent CWR22 (CWR22r)] tumors; LNCAP, DU145, and PC3 tumors; and clinical human prostate cancer specimens (Ca) of different Gleason (G1) grades. D, For RT-PCR detection of transcription factor Pit-1 mRNA, we used the following primer pair: forward primer Pit1-F, 5'-GATAATGCATCACAGTGCTG-3' (in exon 1; position between 200 and 219 in open reading frame) and reverse primer Pit1-R, 5'-GGCAGATTGTTGTTGACTG-3' (in exon 4; position between 639 and 620 in open reading frame). The size of the PCR product yielded by this primer pair is 440 bp. Human brain pituitary total RNA was used a positive control. Pit-1-specific RT-PCR was negative for all prostate-derived samples, whereas pituitary mRNA showed the expected 440 bp Pit-1 RT-PCR product.

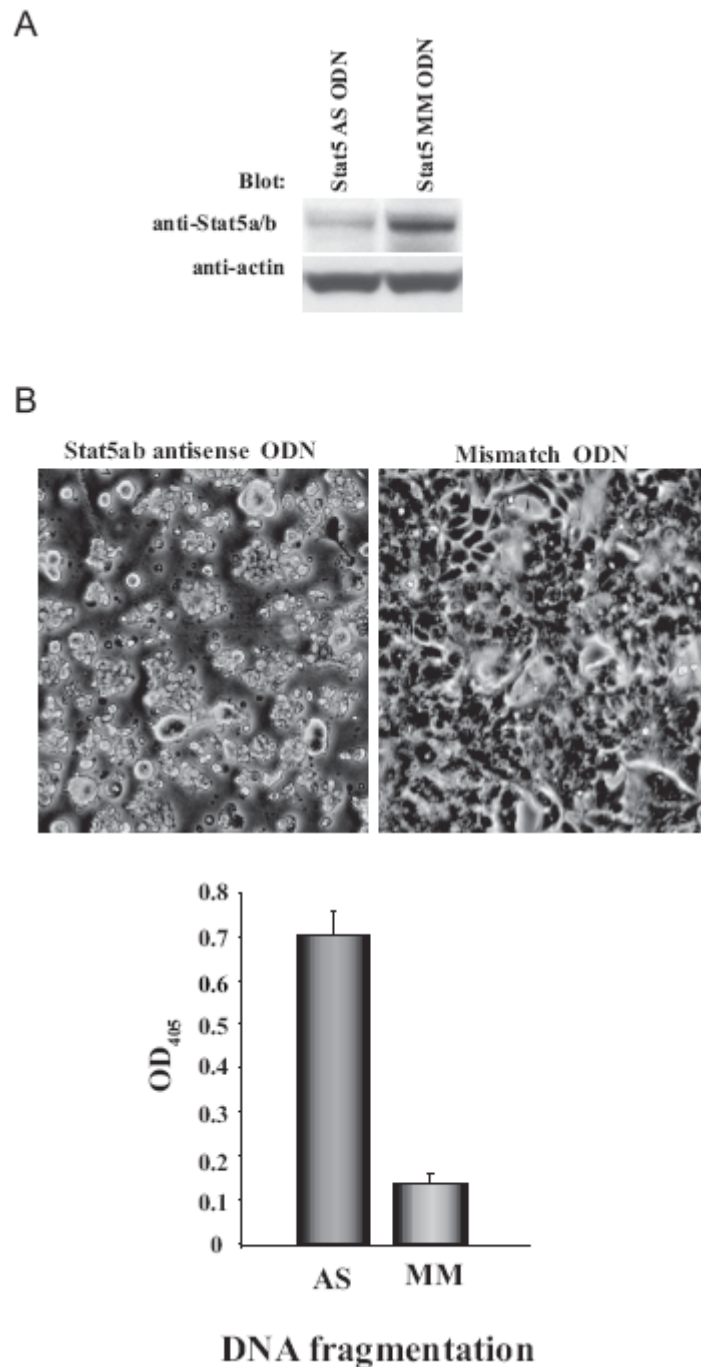
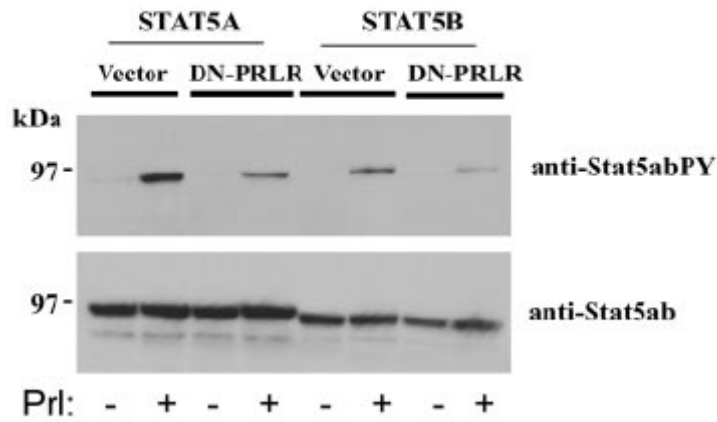
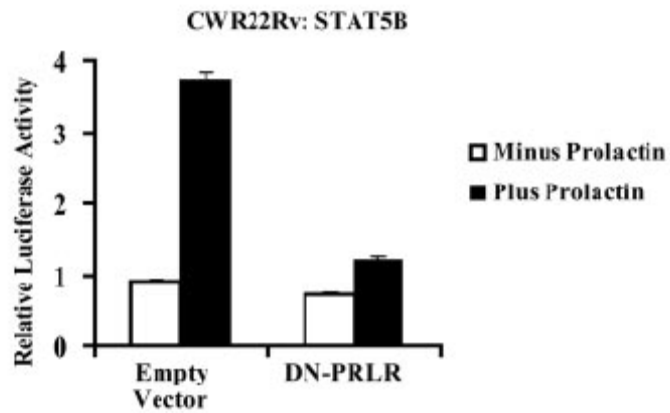
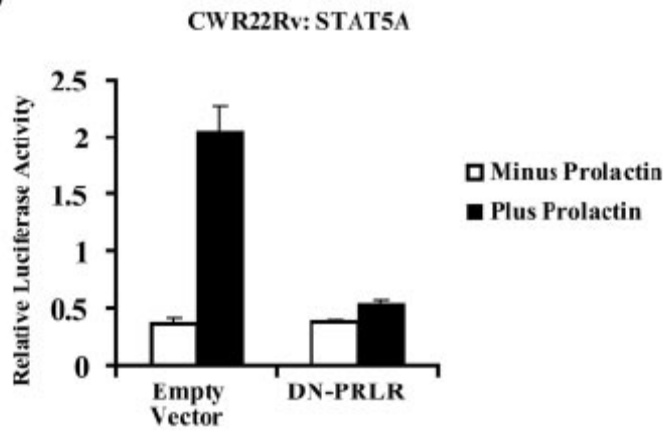


FIG. 3. Inhibition of Stat5a/b in CWR22Rv cells by antisense oligonucleotides (ODNs). CWR22Rv cells transiently transfected with Stat5a/b antisense ODNs or mismatch ODNs (900 pmol per 1×10^6 cells) as a control. *A*, At 24 h, whole-cell extracts were immunoblotted with anti-Stat5a/b antibody (*upper panel*) and stripped filters were reblotted with antiactin antibody to demonstrate equal loading. *B*, *Upper panel*, Microscope photography of Stat5a/b antisense and mismatch ODN-treated cells 3 d after transfection. Morphology of cell death induced by Stat5a/b antisense ODN in CWR22Rv cells is consistent with apoptosis. *Lower panel*, Antisense inhibition of Stat5a/b expression induced DNA fragmentation analyzed by nucleosomal ELISA 72 h after transfection of the cells with Stat5a/b antisense (AS) or mismatch (MM) oligonucleotides (900 pmol per 1×10^6 cells).

A

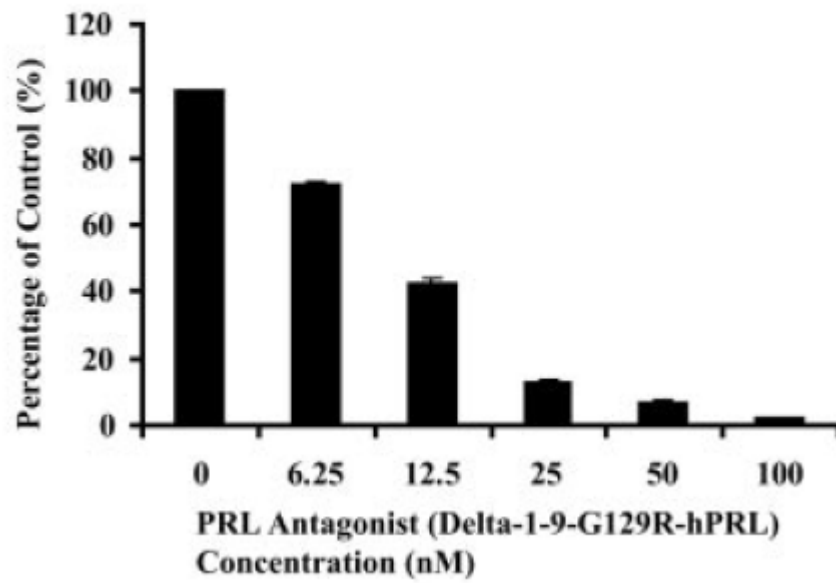


B

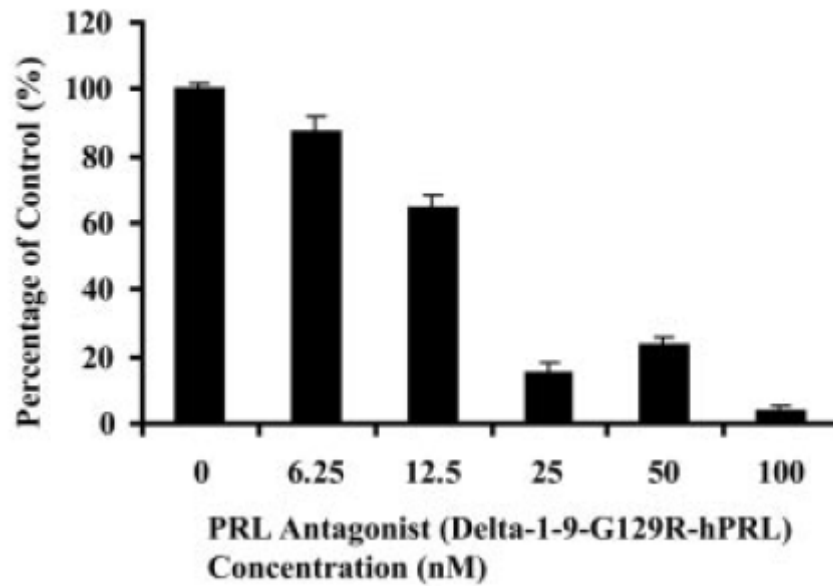


C

CWR22Rv: STAT5A



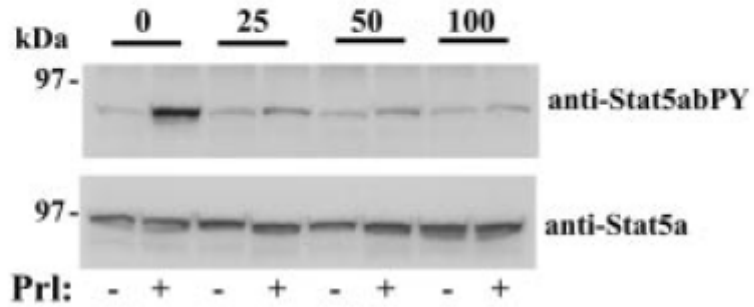
CWR22Rv: STAT5B



D

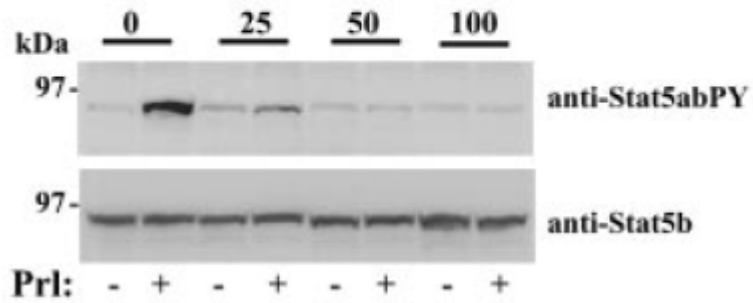
CWR22Rv: STAT5A

**PRL Antagonist (Delta-1-9-G129R-hPRL)
Concentration (nM)**



CWR22Rv: STAT5B

**PRL Antagonist (Delta-1-9-G129R-hPRL)
Concentration (nM)**



E

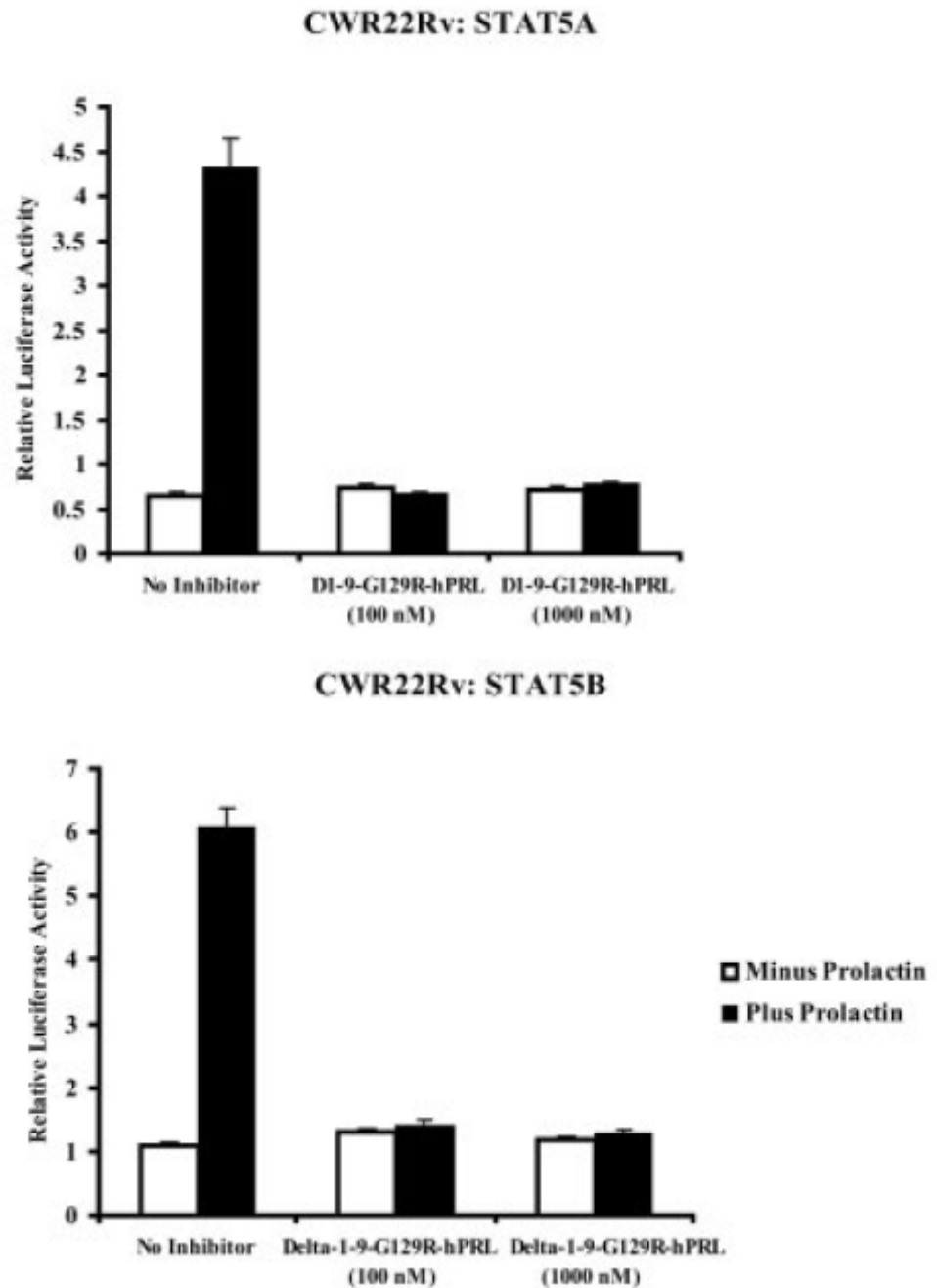
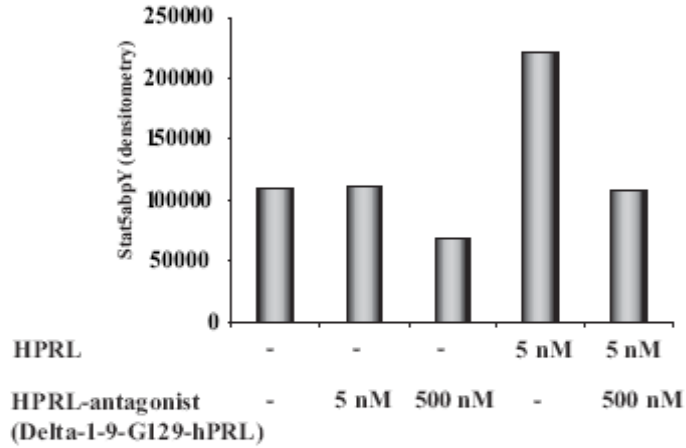
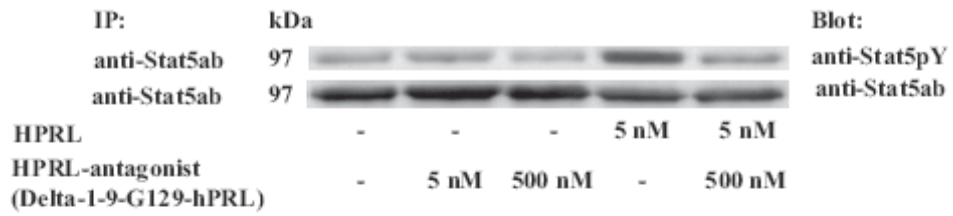


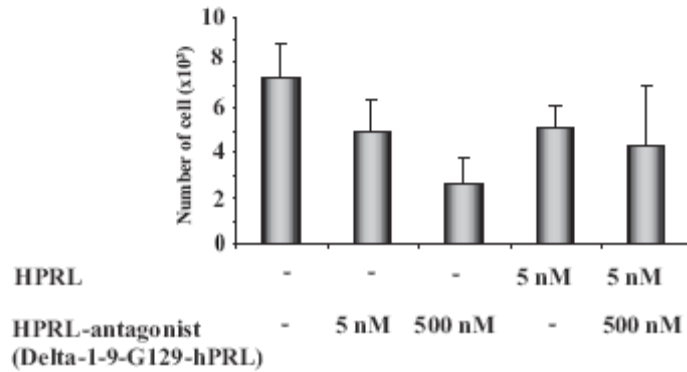
FIG. 4. Dominant-negative PrIR and a competitive PrIR antagonist $\Delta 1-9G129R-hPR$ inhibit Stat5a/b phosphorylation and transcriptional activity. A, CWR22Rv transiently transfected with pWTPrIR and pWTStat5a or pWTStat5b was cotransfected with pDNPrIR or empty plasmid as a control, starved for 24 h in serum-free media, and stimulated with hPrI (10 nM) for 15 min before harvesting. Western blotting of whole-cell extracts with antiactive Stat5a/b antibody (*upper panel*) with reblotting of the stripped filters with anti-Stat5a/b antibody (*lower panel*). B, Transiently transfected CWR22Rv cells with genomic β -casein-promoter-luciferase plasmid, pWTPrIR and pWTStat5a or pWTStat5b, were cotransfected with pDNPrIR or empty plasmid and stimulated with 10 nM hPrI or vehicle for 12 h in the starvation medium. The mean values of three independent experiments are presented and SD values are indicated by *bars*. C, CWR22Rv cells transiently transfected with genomic β -casein-promoter-luciferase plasmid and pWTPrIR, pWTStat5a or pWTStat5b,

pretreated for 30 min with indicated concentrations of $\Delta 1-9$ -G129R-hPRL, and stimulated with hPrl (10 nM) for 16 h in the presence of the PrlR antagonist in the starvation medium. The mean values of three independent experiments are presented and SD values are indicated by *bars*. D, CWR22Rv cells were transiently transfected with pWTPrIR and pWTStat5a or pWTStat5b, starved for 24 h, pretreated with indicated concentrations of $\Delta 1-9$ -G129R-hPRL for 60 min, and stimulated with hPrl (10 nM) for 15 min before Western blotting of whole-cell extracts with antiactive Stat5a/b antibody (*upper panel*) and anti-Stat5a or anti-Stat5b antibodies (*lower panel*). E, β -Casein-promoter-luciferase assay of CWR22Rv cells transfected and treated with $\Delta 1-9$ -G129R-hPRL at indicated concentrations as described in C. The mean values of three independent experiments are presented and SD values are indicated by *bars*.

A



B



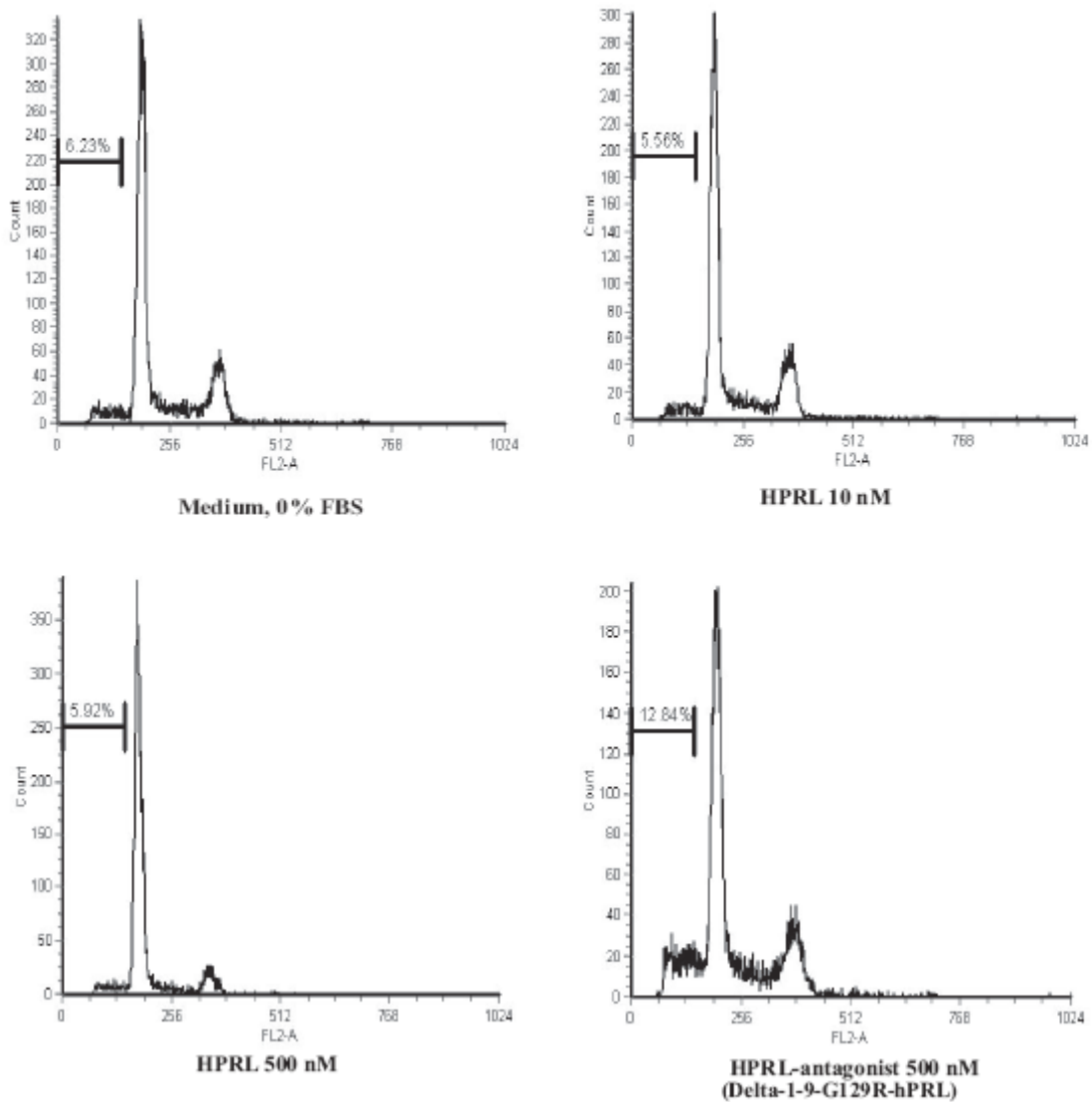
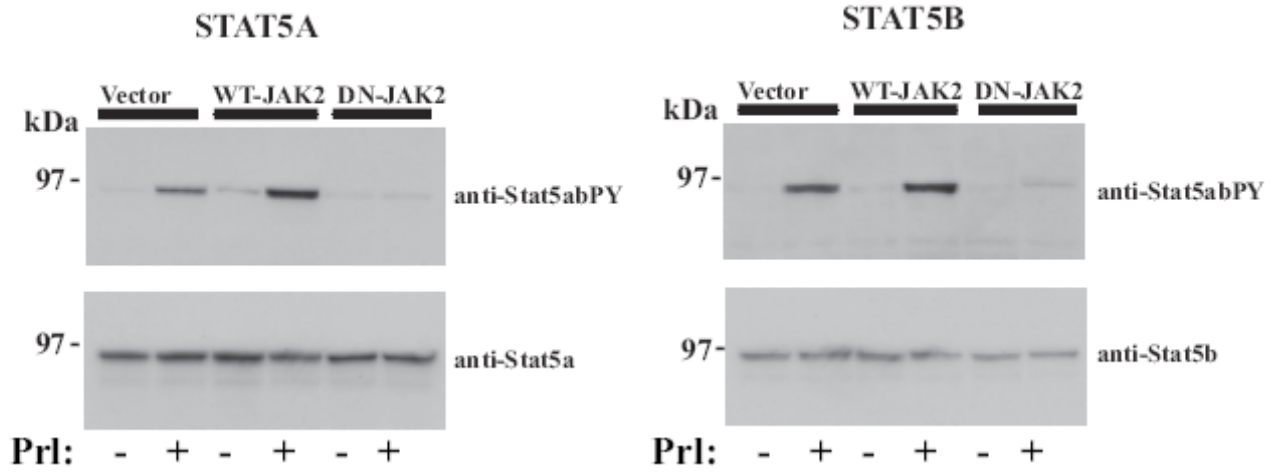
C

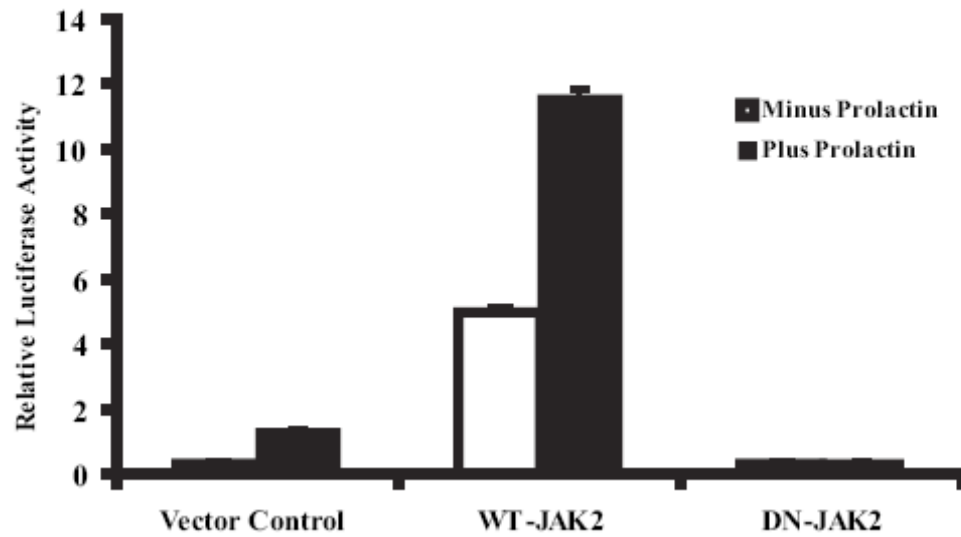
FIG. 5. Disruption of Prl-Stat5a/b autocrine loop in prostate cancer cells by the PrIR antagonist, $\Delta 1-9$ -G129R-hPRL, decreases viability of androgen-independent CWR22Rv human prostate cancer cells. A, $\Delta 1-9$ -G129R-hPRL inhibits constitutive activation of Stat5a/b in androgen-independent CWR22Rv human prostate cancer cells. CWR22Rv cells were treated with $\Delta 1-9$ -G129R-hPRL and/or hPrl in serum-free medium at indicated concentrations for 10 d. Stat5a and Stat5b were immunoprecipitated (IP) and blotted with antiactiveStat5a/b antibody (*upper panel*) and anti-Stat5a/b antibody to demonstrate equal loading of the gel (*lower panel*). Densitometric normalization and comparison of inhibition of Stat5ab activation is shown at the *bottom*. B, Parallel wells (eight wells per treatment) were harvested for counting of attached viable cells. Bars, \pm SD. C, $\Delta 1-9$ -G129R-hPRL treatment-induced cell death was determined by flow cytometry on d 10. Cellular apoptosis was detected as increased hypodiploid fraction (pre-G1 peak) after treatment of the cells with $\Delta 1-9$ -G129R-hPRL with bacterially produced recombinant hPrl as a control at indicated concentrations. FBS, Fetal bovine serum.

A

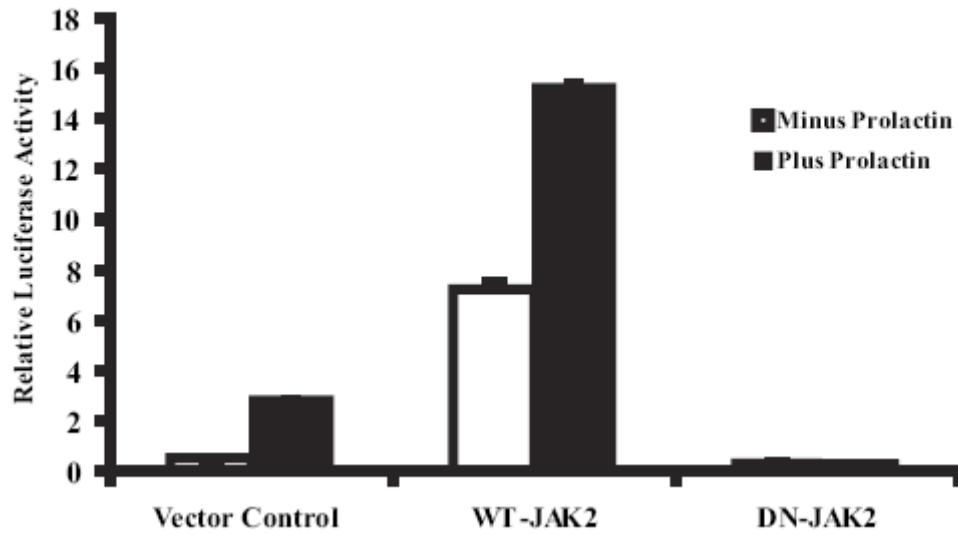


B

CXWR22Rv: STAT5A

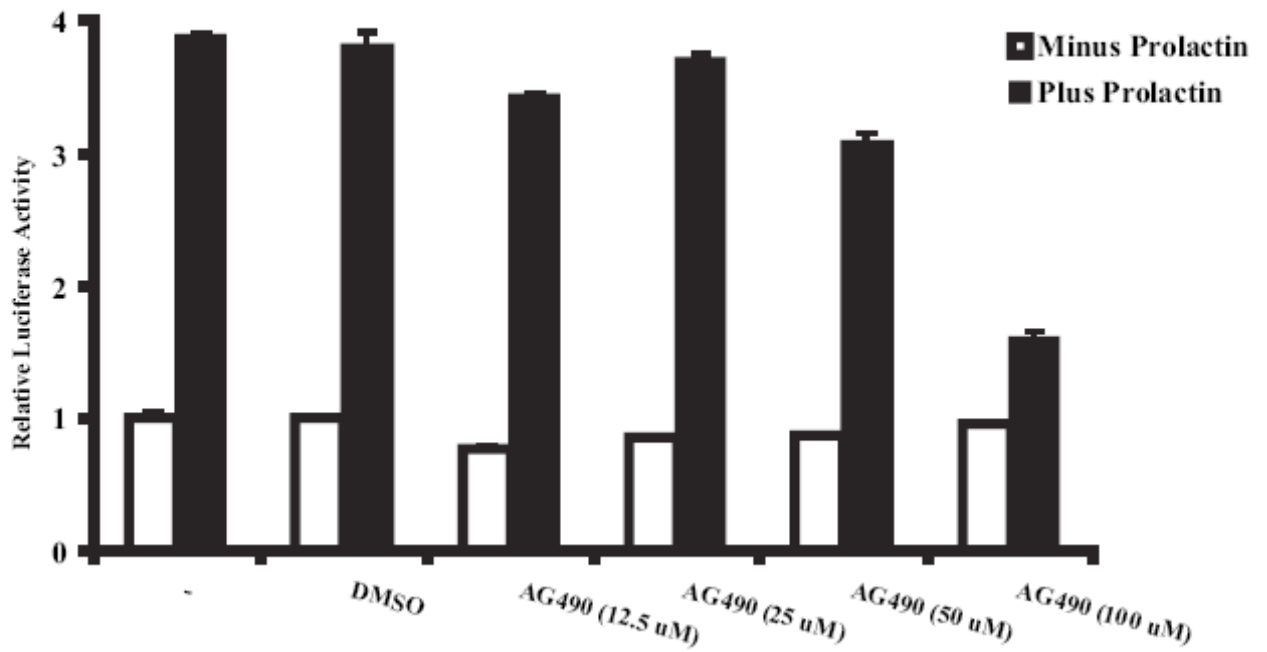


CXWR22Rv: STAT5B



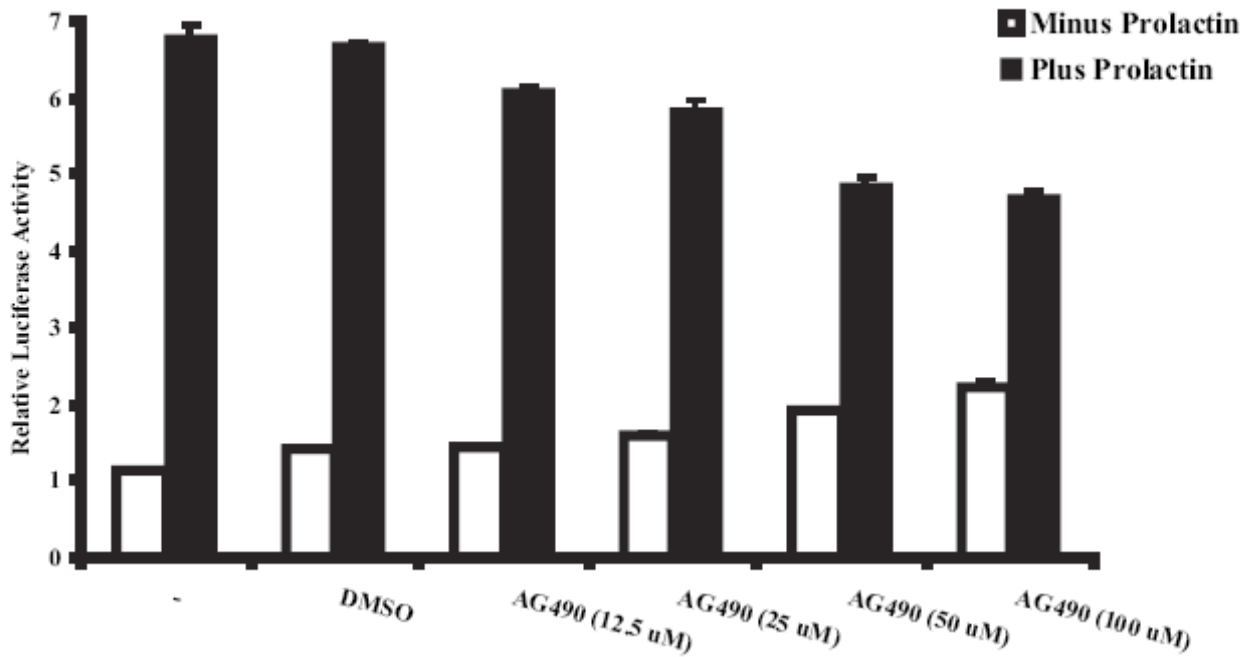
C

CWR22Rv: STAT5A



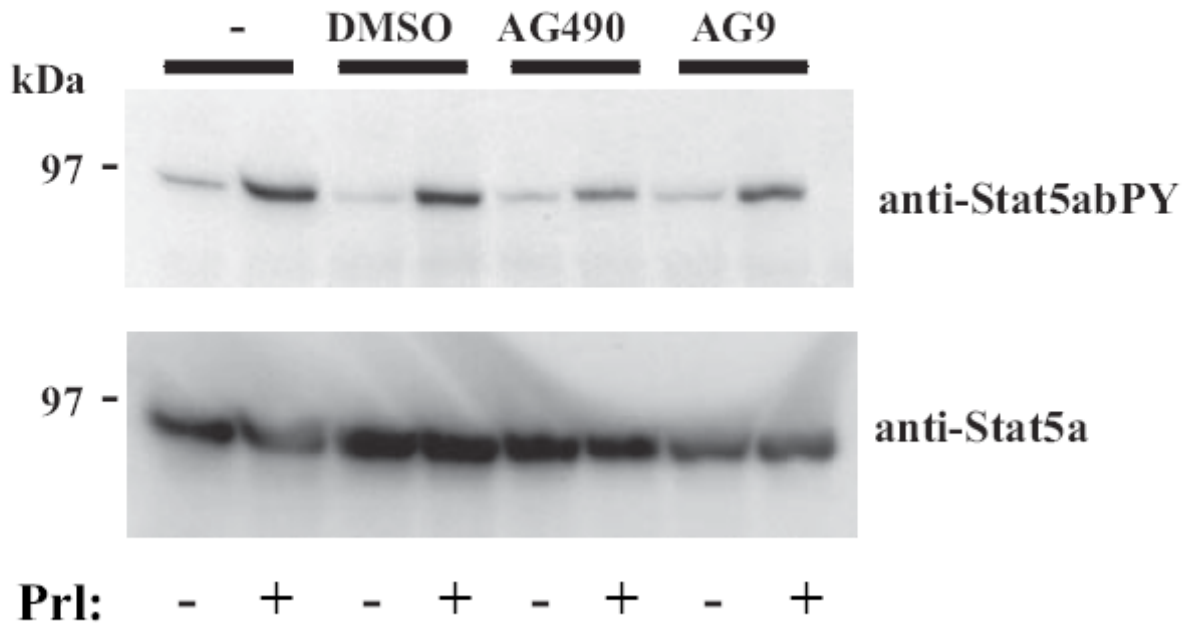
D

CWR22Rv: STAT5B

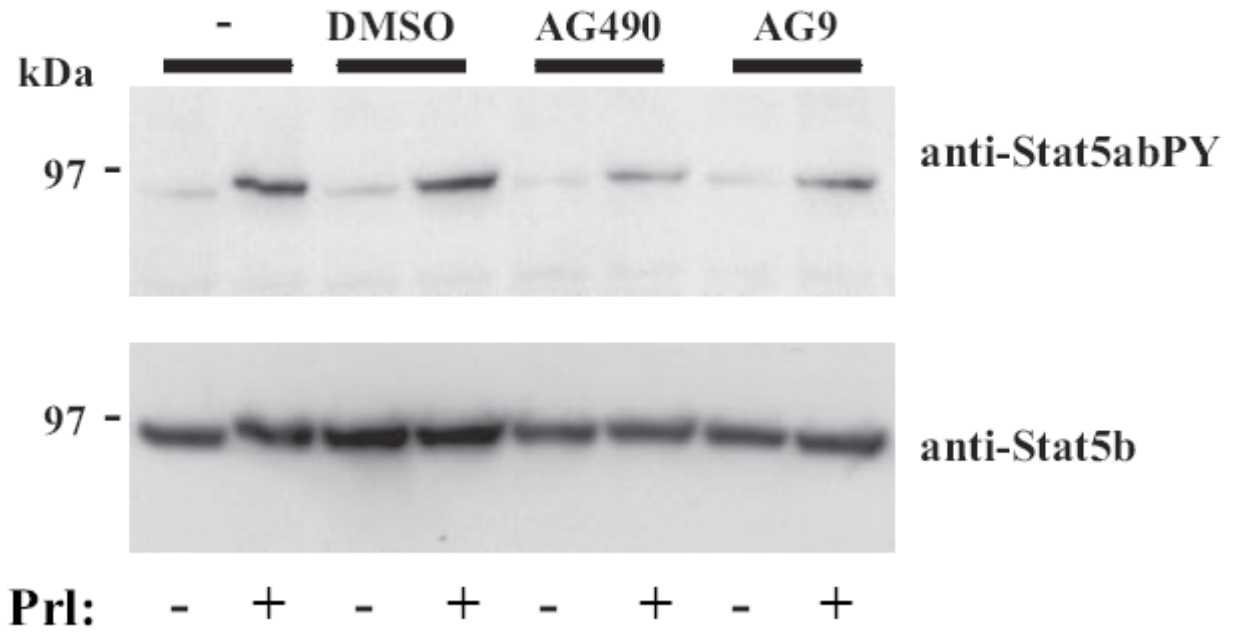


E

STAT5A

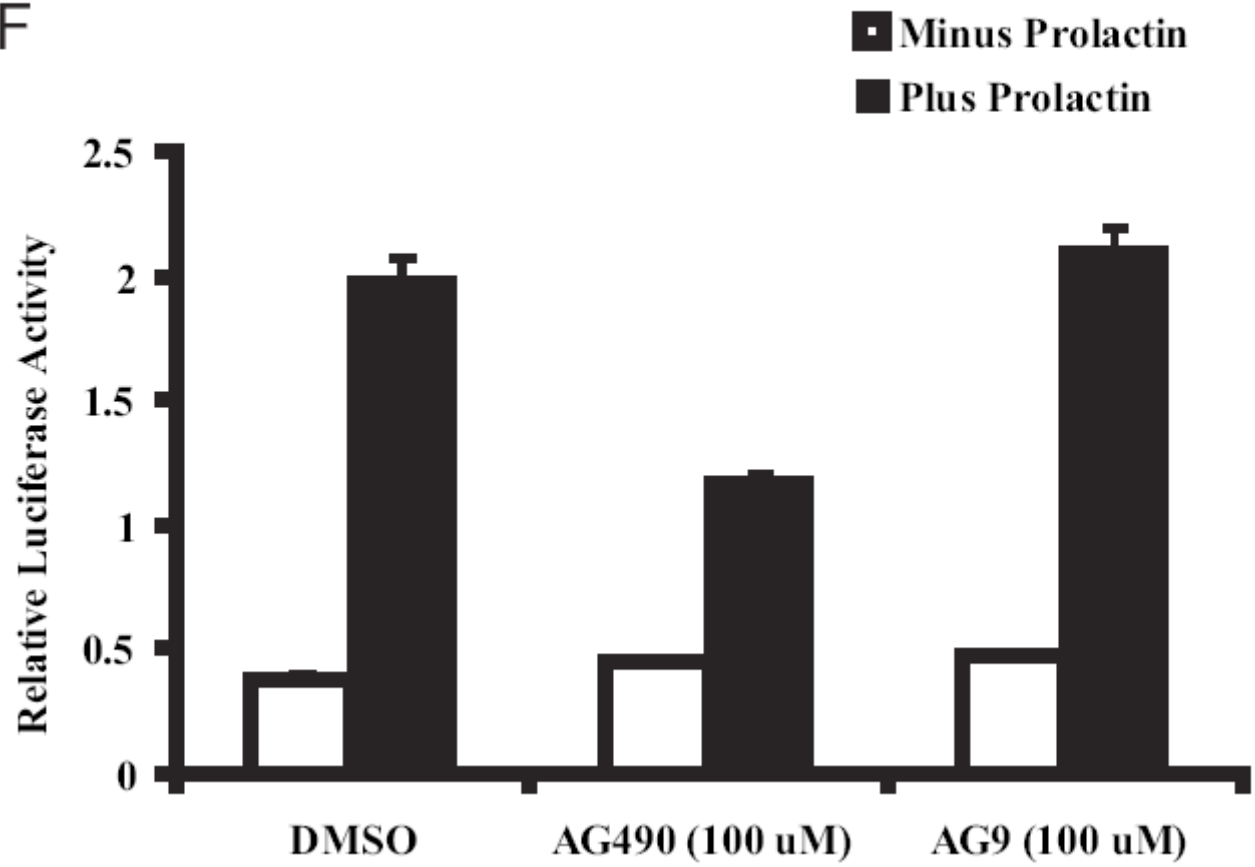


STAT5B



CWR22Rv: STAT5A

F



CWR22Rv: STAT5B

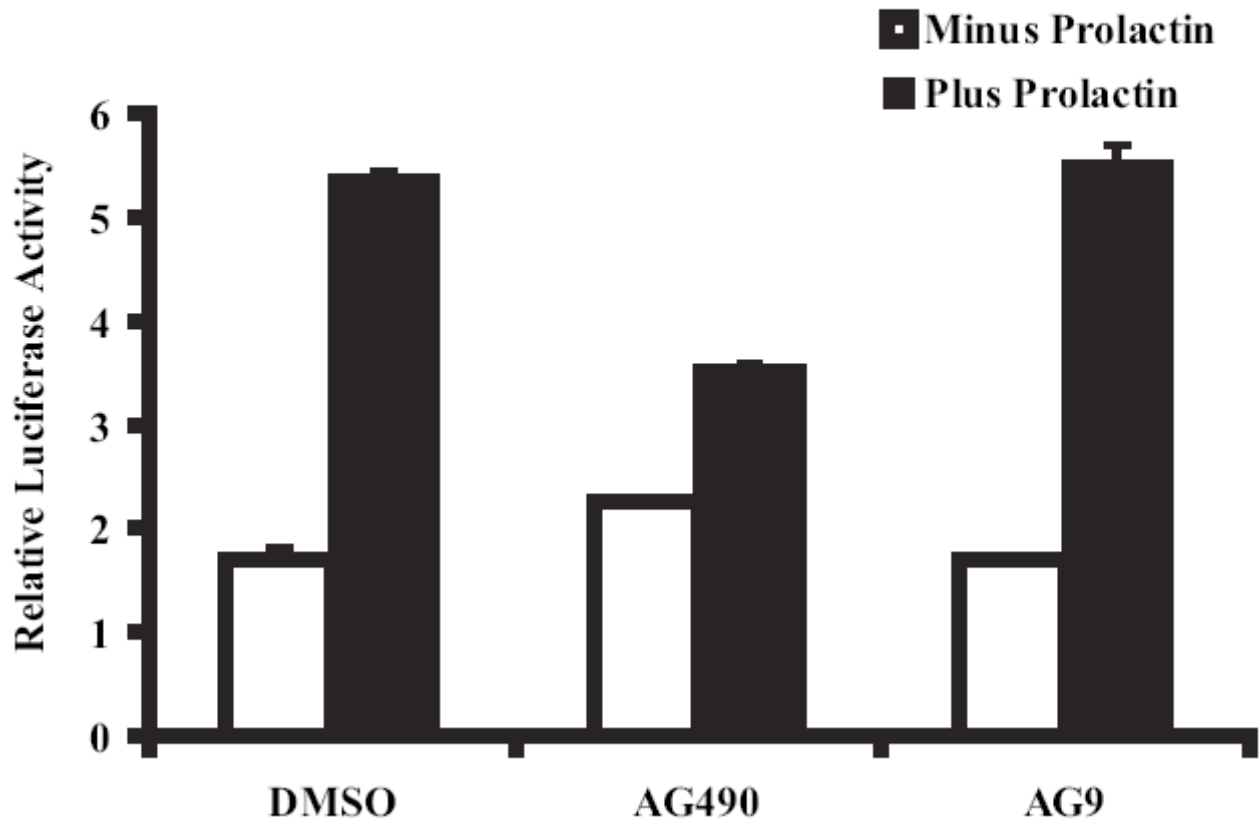


FIG. 6. Inhibition of Stat5a/b phosphorylation and transcriptional activity by DNJak2 and a small molecule inhibitor of Jak2. A, CWR22Rv cells transiently transfected with pWTPrlR and pWTStat5a and pWTStat5b were cotransfected with pWTJak2, pDNJak2, or empty control plasmid, starved for 24 h, and stimulated with hPrl (10 nM) for 15 min before harvesting. Western blotting of whole-cell extracts with antiactive Stat5a/b antibody shows inhibition of Prl-induced Stat5a/b phosphorylation in CWR22Rv human prostate cancer cells (*upper panel*), whereas the Stat5a/b protein levels remained constant (*lower panel*). B, CWR22Rv cells transiently transfected with genomic β -casein-promoterluciferase plasmid, pWTPrlR, and pWTStat5a or pWTStat5b were cotransfected with pWTJak2, pDNJak2, or empty plasmid as a control as indicated, with or without 10 nM hPrl for 12 h in starvation medium. The mean values of three independent experiments are presented and SD values are indicated by *bars*. Prl-induced transcriptional activation of Stat5a/b on genomic β -casein promoter-luciferase was 100% inhibited by cotransfection of CWR22Rv cells with DNJak2. Dose-response curve for inhibition of transcriptional activity of Prl induced Stat5a (C) and Stat5b (D) by AG490 in CWR22Rv cells. CWR22Rv cells transfected with pWTPrlR and pWTStat5a or pWTStat5b were pretreated with AG490 for 30 min and then stimulated with hPrl (10 nM) for 16 h in the presence of the compound in the starvation medium. E, The efficacy of AG490 at 100 μ M concentration to inhibit of Prl-induced phosphorylation of Stat5a/b and Stat5a/b in CWR22Rv cells shown by Western blotting of whole-cell extracts. CWR22Rv cells transfected with pWTPrlR and pWTStat5a or pWTStat5b were pretreated for 1 h with AG490 or AG9 at 100 μ M concentration with dimethylsulfoxide (DMSO)-treated cells as an additional control before stimulation of the cells with 10 nM hPrl for 15 min. F, The efficacy of AG490 at 100 μ M concentration to

inhibit transcriptional activity of Prl-induced Stat5a/b in β -casein-luciferase assay with AG9 as a control compound. The transfections for the luciferase assay and Prl stimulation were performed as described in D.