[CANCER RESEARCH 64, 4774–4782, July 15, 2004]

Activation of Signal Transducer and Activator of Transcription 5 in Human Prostate Cancer Is Associated with High Histological Grade

Hongzhen Li,[1] Tommi J. Ahonen,[4] Kalle Alanen,[5] Jianwu Xie,[1] Matthew J. LeBaron,[1] Thomas G. Pretlow,[7] Erica L. Ealley,[1] Ying Zhang,[2] Martti Nurmi,[6] Baljit Singh,[3,1] Paula M. Martikainen,[8] and Marja T. Nevalainen[1]

Departments of [1]Oncology, [2]Biostatistics Unit, and [3]Pathology, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington DC; Departments of [4]Anatomy and Cell Biology and [5]Pathology, Institute of Biomedicine, University of Turku, Turku, Finland; [6]Department of Surgery, University Hospital of Turku, Turku, Finland; [7]Department of Pathology, Case Western Reserve Medical Center, Cleveland, Ohio; and [8]Deptartment of Pathology, Tampere University Hospital, Tampere, Finland

ABSTRACT

We have recently identified signal transducer and activator of transcription 5 (Stat5) as a critical survival factor for prostate cancer cells. We now report that activation of Stat5 is associated with high histological grade of human prostate cancer. Specifically, immunohistochemical analysis demonstrated a strong positive correlation with activation of Stat5 and high Gleason score in 114 human prostate cancers. To investigate the mechanisms underlying constitutive activation of Stat5 in prostate cancer, a dominant-negative mutant of Janus kinase 2 (Jak2) was delivered by adenovirus to CWR22Rv cells. Dominant-negative-Jak2 effectively blocked the activation of Stat5 whereas wild-type Jak2 enhanced activation, indicating that Jak2 is the main kinase that phosphorylates Stat5 in human prostate cancer cells. A ligand-induced mechanism for activation of Stat5 in prostate cancer was suggested by the ability of prolactin (Prl) to stimulate activation of both Jak2 and Stat5 in CWR22Rv human prostate cancer cells and in CWR22Rv xenograft tumors. In addition, Prl restored constitutive activation of Stat5 in five of six human prostate cancer specimens in *ex vivo* long-term organ cultures. Finally, Prl protein was locally expressed in the epithelium of 54% of 80 human prostate cancer specimens with positive correlation with high Gleason scores and activation of Stat5. In conclusion, our data indicate that increased activation of Stat5 was associated with more biologically aggressive behavior of prostate cancer. The results further suggest that Jak2 is the principal Stat5 tyrosine kinase in human prostate cancer, possibly activated by autocrine/paracrine Prl.

INTRODUCTION

Ablation of androgens, the primary pharmacological treatment of prostate cancer (1), inhibits androgen-dependent proliferation of prostate cancer cells and induces tumor cell apoptosis. However, androgen ablation provides only a transient remission of the disease, because androgen-independent regrowth of prostate cancer is, in most cases, inevitable (1). The molecular mechanisms of androgen-independent growth of prostate cancer are still largely unclear. Androgen-independent proliferation and survival of prostate cancer cells are thought to be mediated by several different mechanisms, such as the following: (*a*) by increased expression of androgen receptor (AR; Ref. 2); (*b*) by altered ligand specificity of AR attributable to mutations in AR (3); and (*c*) by activation of kinase signaling cascades, which stimulate prostate cancer cell growth either independently of AR or by influencing the activation of AR (4–9).

We have recently identified transcription factor Signal Transducer and Activator of Transcription 5 (Stat5) as a critical survival protein for human prostate cancer cells (4). Stat5 is a latent cytoplasmic protein, which is comprised of two highly homologous isoforms, 94-kDa Stat5a and 92-kDa Stat5b (10). Stat5 becomes activated by phosphorylation of a specific tyrosine residue (11, 12), after which phosphorylated Stat proteins dimerize and translocate to nucleus where they bind to specific response elements of target gene promoters to regulate transcription (11, 12). Stat5 does not possess intrinsic tyrosine kinase activity and, therefore, requires an upstream kinase for activation (13). Members of Janus kinase (Jak)-kinase protein family are known to phosphorylate Stat5 in mammary and hematopoietic cells (11, 13). In addition to being activated by Jak kinases, Stat5 can be activated in some cell types by receptor and nonreceptor tyrosine kinases (14–17) and by tyrosine kinase fusion oncogenes such as BCR-ABL and Tel-Jak2 (18, 19).

The factors involved in activation of Stat5 in human prostate cancer cells are unclear. In normal rat prostate epithelium, we have shown previously that transcription factor Stat5 is the central signaling protein activated by prolactin (Prl; Ref. 20). Prl, at the same time, is known to inhibit androgen deprivation-induced apoptotic cell death of prostate epithelium (21) and promote proliferation of both normal (21–23) and malignant prostate epithelial cells (24, 25). Receptors for Prl are expressed in human prostate cancer (26), and a Prl antagonist has been reported to inhibit prostate xenograft tumor initiation and growth in nude mice (27). Moreover, Prl-transgenic mice develop significant enlargement of prostate (28–30). However, clinical studies have not revealed differences in serum Prl levels of men with prostate disease compared with age-matched controls (31, 32).

On the basis of our previous findings of a survival function of Stat5 in human prostate cancer cells (4), we hypothesized that activation of Stat5 may be associated with more biologically aggressive behavior of prostate cancer. Furthermore, we wanted to determine the mechanisms underlying continuous activation of Stat5 in prostate cancer cells, because additional molecular targets might prove useful for therapy development for prostate cancer based on inhibition of Stat5. Here, we show that activation of Stat5 strongly correlates with high histological grade of human prostate cancer. We also show that Prl activates Stat5 in human prostate cancer specimens and in CWR22Rv cells both *in vitro* and *in vivo* when grown as tumors in nude mice. Both basal and Prl-induced activation of Stat5 in CWR22Rv human prostate cancer cells were blocked by adenoviral expression of dominant-negative (Dn) mutant of Jak2, suggesting that Jak2 is the upstream protein kinase that activates Stat5 in human prostate cancer. Finally, we showed that Prl protein was expressed in 54% of 80 human prostate cancers with increased expression in high-grade prostate cancer and correlation with activated Stat5. Collectively, our results indicate that Stat5 is primarily active in high-grade prostate cancers and that Jak2 is the key upstream kinase responsible for activating Stat5 in prostate cancer cells, possibly by autocrine/paracrine Prl.

Received 11/7/03; revised 4/6/04; accepted 5/10/04.

Grant support: This work was supported by ACS grants IRG-97-152-11 and RSG-04-196-01 MGO, NIH Grant RO1-CA-83813, Department of Defense Prostate Cancer Grant DAMD-17-01-1-0059, Turku University Graduate School of Biomedical Sciences (University of Turku, Turku, Finland), and the Foundation for the Finnish Cancer Institute. Shared Resources of Lombardi Comprehensive Cancer Center are partially supported by NIH Grant 1P30-CA-51008 (Cancer Center Support Grant, to Lombardi Comprehensive Cancer Center).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Marja T. Nevalainen, Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, NRB E508, 3970 Reservoir Road NW, Washington DC 20057. Phone: (202) 687-2043; Fax: (202) 687-7830; E-mail: mtn3@georgetown.edu.

MATERIALS AND METHODS

Prostate Cancer Samples. For immunohistochemical analysis, we obtained 103 human prostate cancer specimens (Georgetown University Hospital) in formalin (Table 1). The Gleason (Gl.) grades and the Gl. score (33) of each sample were verified by a board-certified pathologist (B. S.).

For organ cultures, the prostate cancer specimens were obtained from 11 patients (age range 54–69 years; mean age ± SD, 62.5 ± 4.5 years) with localized or locally advanced prostate cancer (Table 2) undergoing radical prostatectomy. The prostate tissues were obtained after informed consent of the patient and the approval of the Ethical Committee of University Hospital of Turku. 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 to be available for *ex vivo* organ cultures or to be frozen for other analysis. The selection of the area was assisted by the clinical information of the localization of the cancer based on the location of the needle biopsy taken at the time of diagnosis and by frozen sections. The rest of each prostate was fixed in formalin, and the whole prostate cross-sections were used for histological grading, for confirming that the areas cultured in organ culture were cancer, and for immunohistochemical stainings.

***Ex vivo* Long-Term Organ Cultures of Human Prostate Cancer.** The prostate organ cultures were performed as described previously (20–23, 34–36). The explants were cultured for 7 days in the presence of 100 nM dihydrotestosterone (5α-androstan-17β-ol-3-one, Sigma; Refs.23 and 34), and on the 7th day of culture, half of the explants were stimulated with 100 nM hPrl (a gift from Dr. A. F. Parlow; The National Pituitary Hormone Program, National Institutes of Diabetes, Digestive and Kidney Diseases; Ref. 20) for 1 h, after which the explants were fixed in formalin. Four parallel dishes each containing 20-prostate cancer explants were cultured in both treatment groups.

Xenograft Tumors and Cell Culture. Castrated male athymic mice were purchased from Taconic (Germantown, NY) and cared for according to the institutional guidelines. Briefly, 5×10^6 CWR22Rv cells were mixed with one quarter of the total injection volume of 0.2 ml with Matrigel. Simultaneously with the tumor cell inoculation (2 sites/mouse), sustained-release testosterone pellets (12.5 mg/pellet, 1 pellet/mouse; Innovative Research of America, Sarasota, FL) were implanted s.c. When the tumors ($n = 12$) reached 12–15 mm in size, half of the mice were injected i.p. with human Prl (hPrl; 3.0 μg/g body weight; $n = 3$) or PBS ($n = 3$). The mice were sacrificed, and the tumor tissues were harvested 30 min after the injection.

For immunoprecipitation of Stat5a and Stat5b, CWR22Rv cells were harvested at 50% or 100% confluency. For adenoviral gene delivery, CWR22Rv cells were infected with AdDNJak2 or AdWTJak2 at multiplicity of infection 10 for 90 min after which RPMI 1640 containing 10% FCS was added. Before the stimulation of CWR22Rv and T47D breast cancer cells with hPrl, the cells were starved in serum-free medium for 16 h (37). For Stat5 immunoprecipitations, cells were incubated for 15 min, and for Jak2 immunoprecipitations, CWR22Rv and T47D cells were incubated for 10 min in the presence or absence of 10 nM hPrl, and the cell pellets were frozen at −70°C or fixed with 4% paraformaldehyde for immunocytochemistry.

Immunohistochemistry. Regular paraffin sections of 103 prostate cancer samples (Table 1), cross-sections of the entire prostate (Fig. 1*I*; Table 2) of 11 patients with localized or locally advanced prostate cancer, and paraffin sections of cultured explants of human prostate cancer tissues were immunostained for activated Stat5, total Stat5, or Prl. Immunohistochemistry of activated Stat5 was performed as described previously (38). Briefly, tissue sections or paraformaldehyde-fixed cells were microwave-treated with antigen retrieval solution AXAR1 (Advantex BioReagents, Conroe, TX). For the detection of total Stat5, parallel tissue sections were microwave-treated in citrate solution (BioGenex Laboratories, San Ramon, CA). Sections immunostained for Prl were treated with pepsin (2.5 mg/ml; BioGenex) for 10 min at 37°C to unmask the epitopes. Endogenous peroxidase activity was blocked by incubating the slides in 0.3% hydrogen peroxide. The primary antibodies recognizing phosphorylated (Y694/Y699) Stat5 monoclonal antibody (mAb; Advantex BioReagents), total Stat5 (mAb; Santa Cruz Biotechnology, Santa Monica, CA), and Prl (mAb; BioGenex) were diluted in 1% BSA in PBS at concentrations of 0.6 μg/ml, 2 μg/ml, and 1:40, respectively. For immunohistochemical detection of prostate-specific antigen in cultured human prostate cancer explants, a polyclonal antiprostate-specific antigen antibody was used (BioGenex, ready-to-use preparation). Antigen-antibody complexes were detected using appropriate biotinylated goat secondary antibodies followed by streptavidin-horseradish-peroxidase complex (BioGenex). 3,3′-Diaminobenzidine was used as chromogen and hematoxylin was used as counterstain. For controls, subtype-specific mouse IgG or normal rabbit serum was used as appropriate. Lactating human breast tissue (38) was used as a positive control tissue for total and activated Stat5 immunohistochemistry and human pituitary prolactinoma for Prl immunohistochemistry, respectively. For immunohistochemical detection of Prl protein, additional control sections of human prostate cancer were immunostained with anti-hPrl mAb in the presence or absence of 100-fold excess of hPrl.

Scoring of Levels of Active and Nuclear Stat5 in Prostate Cancer Specimens. Individual prostate cancer samples were scored (M. T. N. and H. L.) for active Stat5 and Prl levels at three semi-quantitative steps of increasing staining intensity, where 0 was undetectable, low immunostaining gave 1+, intermediate immunostaining gave 2+, and high immunostaining gave 3+ as a score.

Statistical Analysis. The Gl. scores of the prostate cancer specimens immunostained for activated Stat5 (114 records) and for Prl protein (80 records) were classified into three groups (4–6, 7, 8–9). Because both the Gl. score and immunostaining intensity (Stat5pY, Prl) are ordinal categorical variables, the Spearman's rank-order correlation coefficients (39) were calculated, and the test of the correlation coefficient equals zero was performed. All of the analyses were performed using SAS software (40).

Solubilization of Proteins, Immunoprecipitation, and Immunoblotting. Prostate cancer cells and tissues were lysed in lysis buffer [10 mM Tris-HCl (pH 7.6), 5 mM EDTA, 50 mM NaCl, 30 mM sodium PP_I, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1% Triton X-100, 1 mM phenylmethylsulphonylfluoride, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, and 2 μg/ml leupeptin]. The protein concentrations of clarified tissue lysates were determined by a simplified Bradford method (Bio-Rad Laboratories Inc., Hercules, CA). Depending on the experiment, 4 mg of tissue protein and 1 ml of the cell lysates were used for immunoprecipitation for 3 h at 4°C with polyclonal rabbit antisera against either Stat5a, Stat5b, or Jak2 (2 μl/ml; Advantex Bioreagents). Both Stat5a and Stat5b were simultaneously immunoprecipitated from the human prostate cancer samples. Antibodies were captured by incubation for 60 min with protein A-Sepharose beads (Pharmacia Biotech, Piscataway, NJ).

Table 1 *Relationship between active Stat5 and Gleason score* [a]

Activation of Stat5 positively correlated with Gleason score of prostate cancer.

	Active Stat5 immunostaining [b]				
Gleason score	−	+	++	+++	*n*
4–6	32	15	2	0	49
7	17	11	14	1	43
8–9	1	2	7	12	22
n	50	28	23	13	114

[a] Spearman's rank-order correlation coefficient for active Stat5 *versus* Gleason score was 0.6088 ($P < 0.0001$).

[b] Absence of immunostaining had a score 0, weak positive immunostaining had a score 1+, intermediate had a score 2+, and strong staining intensity had a score 3+.

Table 2 *Prostate cancer samples cultured in organ culture*

Age at the day of operation [a]	Gleason grade	Gleason score	WHO grade	Stage	Volume % of the tumor [b]
54 [c,d]	(2 + 2)	4	I	T_{3a}	10–15
60	(2 + 2)	4	I	T_{2a}	15–20
65	(2 + 2)	4	I	T_{2a}	5–10
69 [c,d]	(2 + 3)	5	I	T_{2a}	5–10
64 [c,d]	(2 + 3)	5	I	T_{2a}	10–15
65	(2 + 3)	5	I	T_{2a}	15–20
59 [c]	(3 + 2)	5	II	T_{2a}	5–10
63	(2 + 4)	6	II	T_{2a}	10–15
60 [c,d]	(3 + 3)	6	II	T_{3a}	5–10
60 [c,d]	(5 + 2)	7	III	T_{3c}	75–80
69 [d]	(5 + 2)	7	III	T_{2a}	5–10

[a] Ages of the patients ranged from 54 to 69 yrs. at the time of the operation (mean age 62.5 ± 4.5 SD yrs).

[b] The volume of the tumor is an estimated value that describes the percentage of the total prostate volume taken over by the malignant tissue.

[c] Prostate cancers in which Stat5 was activated by Prl in organ culture.

[d] Prostate cancers that had constitutive activation of Stat5 prior to culture.

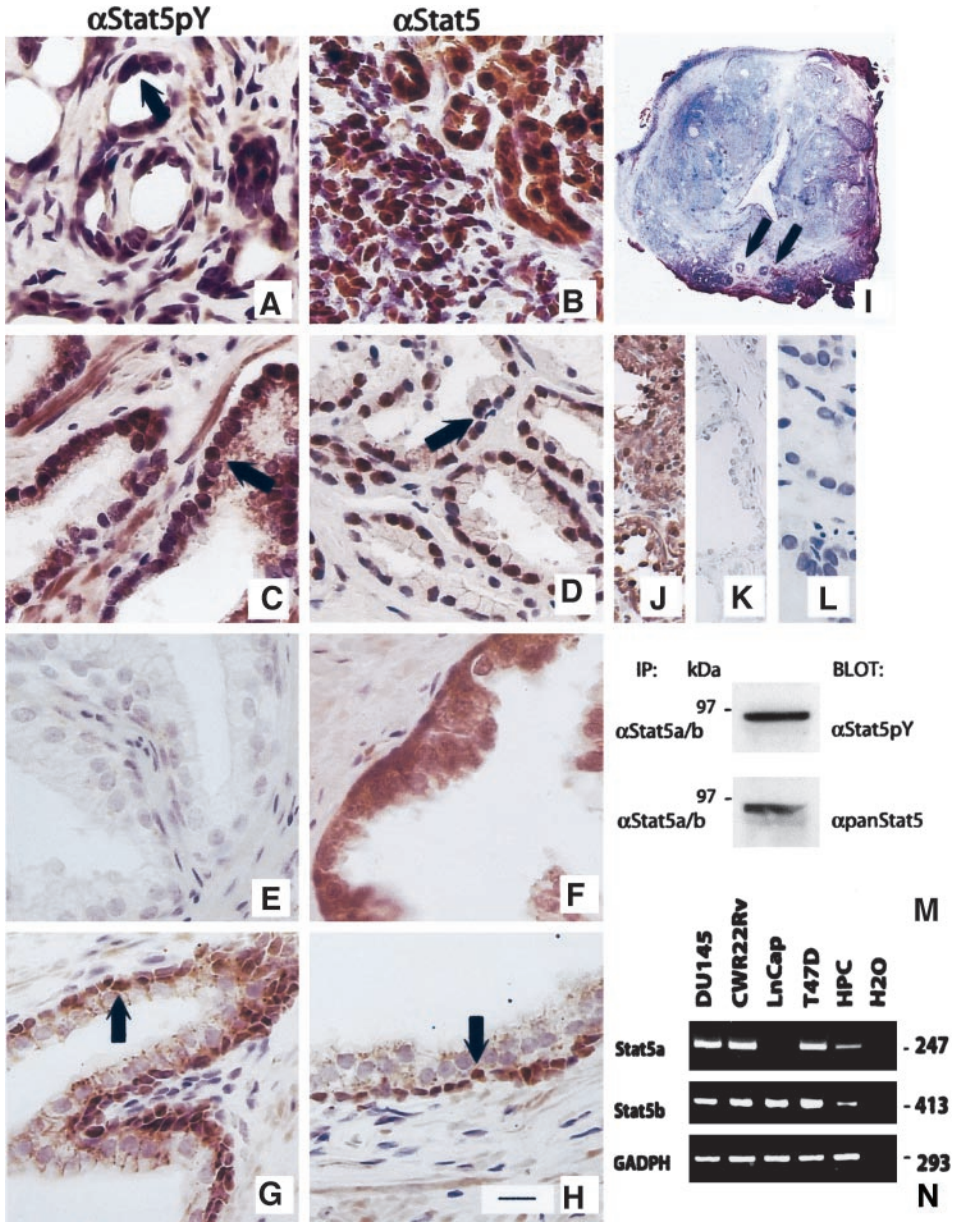

Fig. 1. Signal transducer and activator of transcription 5 (Stat5) is active in human prostate cancer. Paraffin-embedded tissue sections of human prostate specimens were immunostained with a monoclonal activation-state-specific anti-pTyrStat5 antibody (*A, C, E, G*) and with a monoclonal anti-Stat5 antibody (*B, D, F, H*). 3,3′-diaminobenzidine was used as a chromogen and Mayer hematoxylin as counterstain. Biotin-streptavidin amplified peroxidase-antiperoxidase immunodetection shows intense positive reactions for active Stat5 in the nuclei of epithelial cells of poorly differentiated prostate cancer acini (Gleason 4 + 5 = 9, patterns 4 and 5 shown; *A, B*) and moderately differentiated prostate adenocarcinoma (Gleason 3 + 4 = 7, pattern 3 shown; *C, D*), whereas adjacent normal secretory prostate epithelium was negative for phosphorylated Stat5 (*E*), and predominant nuclear localization of immunostaining for Stat5 protein was absent (*F*). Cross-sections of entire prostate (*I*; cross-sections were taken proximal to verumontanum; *arrows* point at the ejaculatory ducts) from 11 patients with localized or locally advanced prostate cancer were analyzed by immunohistochemistry for activation of Stat5. Basal epithelial cells of periurethral prostate glands with normal acinar morphology in transition zone stained positively for activated nuclear Stat5 (*G, H*). Lactating human breast is presented as a positive control tissue (*J*), and parallel sections of lactating human breast (*K*) and moderately differentiated human prostate cancer (*L*) stained with subtype-specific mouse IgG were negative. Stat5a and Stat5b were collectively immunoprecipitated from tissue homogenate of poorly differentiated human prostate cancer sample, resolved on SDS-PAGE, and immunoblotted with a monoclonal anti-phosphoTyr-Stat5 (αStat5pY) antibody (*M, top panel*). The blot was stripped and reblotted with a monoclonal antibody recognizing both Stat5a and Stat5b (αpanStat5; *M, bottom panel*). *N*, reverse transcription-PCR analysis of mRNA from human prostate cancer cells and prostate cancer specimens with Stat5a- and Stat5b-specific primers demonstrated the expression of Stat5a and Stat5b mRNA in human prostate cancer cells (HPC). The reverse transcription-PCR products were size-separated on 2% Tris-borate EDTA-agarose gel. The glyceraldehyde-3-phosphate dehydrogenase gene was amplified in a separate reaction to confirm the integrity of the first-strand cDNA of each sample, and H_2O was used as a negative control. *A-H* and *J-L bar*, 15 μm. *Arrows*, positive nuclear immunostaining for activated Stat5 (*A* and *C*), nuclear localization of Stat5 protein (*D*), and positive immunostaining of basal epithelial cells (*G* and *H*).

Samples were subjected to 7.5% SDS-PAGE under reducing conditions. The primary antibodies were used at the following concentrations: anti-phosphotyrosine-Stat5a/b (Y694/Y699) mAb (1 μg/ml; Advantex BioReagents); anti-Stat5a pAb (1:3000; Advantex BioReagents); anti-Stat5b pAb (1:3000; Advantex BioReagents); antiphosphotyrosine mAb (1:1000; Upstate Biotechnology, Lake Placid, NY); anti-Jak2 pAb (1:3000; Upstate Biotechnology); and anti-panStat5 mAb (1:1000; Transduction Laboratories, Inc.). Antigen-antibody complexes were detected by horseradish peroxidase-conjugated secondary antibodies in conjunction with enhanced chemiluminisence substrate mixture (Amersham, Piscataway, NJ) and exposed to film.

Generation of Adenovirus for Gene Delivery of DnJak2 and Wild-Type Jak2 (WtJak2). Replication-defective human adenovirus (Ad5) carrying DnJak2 or WtJak2 was generated using the AdEasy Vector system (Qbiogene, Carlsbad, CA). Briefly, V5/His epitope-tagged WtJak2 and DnJak2 expression vectors were derived from rat Jak2 cDNA (41), as described previously (42), and were subsequently released by *Apa*I and *Not*I digestion and subcloned into the *Not*I and *Eco*RV sites of Adv-shuttle vector under cytomegalovirus promoter. Before *Not*I digestion, the *Apa*I digested ends were blunt-ended by T4 DNA polymerase. Homologous recombination of WtJak2 and DnJak2 transfer vectors with the AdEasy vector was performed in BJ5183 *Escherichia coli* by electroporation. Recombined clones were screened by kanamycin-resistant growth, and the recombinant viruses were packaged in QBI-293 cells. Resulting clones were selected from plaques and amplified. Expression of WtJak2 and DnJak2 from adenoviral stocks was verified by Western blotting using an anti-Jak2 antibody (Upstate Biotechnology). Selected recombinant viral stocks were expanded in large-scale cultures, purified by double-cesium chloride gradient centrifugation, and titered side-by-side by a standard plaque assay method in QBI-293 cells as per the manufacturer's instructions.

Reverse Transcription (RT)-PCR. For detection of Stat5a, we used the following primer pair: forward primer (exon 18), 5′-CACAGATCAAGCAAG TGGTC-3′ and reverse primer (exon 20), 5′-CTGTCCATTGGTCGGCG-TAA3′. The size of the PCR product yielded by this primer pair is 247 bp. For detection of Stat5b, we used 5′-GATTCTCAGGAAAGAATGTT-3′ (exons 15 and 16) as the forward primer and 5′-TGTGTCCTCCAGATCGAA G-3′ (exon 19) as the reverse primer. The size of the PCR product yielded by this primer pair is 413 bp. Total RNA was isolated from CWR22Rv, LnCap, and Du145 prostate cancer cells, from fresh human prostate cancer specimens and from T-47D breast cancer cells using Trizol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was reverse transcribed from 1 μg of total RNA with Super-Script II reverse transcriptase (Invitrogen) using oligodeoxythymidylic acid primers. The RT reaction was incubated at 42°C for 50 min followed by heat inactivation for 15 min at 70°C. The conditions for PCR for all reactions

were 94°C for 2 min, followed by 1 min of denaturation at 94°C, 2 min of annealing at 55°C, 2 min 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. The glyceraldehyde-3-phosphate dehydrogenase gene (forward primer, 5′- AACATCATCCCTGCCTCTCCTACTG-3′; reverse primer, 5′-TTGACAAAGTGGTCGTTGAGG-3′) was amplified in a separate reaction to confirm the integrity of the first-strand cDNA of each sample.

RESULTS

Activation of Stat5 Is Associated with High Histological Grade of Human Prostate Cancer. Activation of Stat5 was evaluated in 114 prostate cancer specimens obtained from patients with localized or locally advanced prostate cancer (Table 1). Prostate carcinomas were diagnosed with Gl. tumor scores (33), which, in our material, ranged from 4 to 9 (Table 1). Activation of Stat5 was analyzed in paraffin-embedded tissues by a highly sensitive *in situ* detection method, which is based on immunohistochemical detection of phosphorylated Stat5 (Ref. 38; Fig. 1, *A*, *C*, *E*, and *G*). The specificity of antiphosphoStat5 mouse monoclonal antibody AX-1 to tyrosine-phosphorylated Stat5 in immunohistochemistry and immunoblotting has been validated by peptide competition, inducible phosphorylation studies, site-directed mutagenesis, and Stat5 knock-out model analyses (38). This method was complemented with immunohistochemical detection of nuclear Stat5 protein (Fig. 1, *B*, *D*, *F*, and *H*). Activation of Stat5 was also shown in human prostate cancer by immunoprecipitation of Stat5 followed by Western blot analysis (Fig. 1*M*). Expression of Stat5a and Stat5b mRNA in human prostate cancer cell lines and prostate cancer specimens was demonstrated by RT-PCR analysis (Fig. 1*N*).

The immunostained tissue sections were evaluated using a semiquantitative scoring method, which was based on the intensity of nuclear phosphorylated Stat5 staining. The data in Table 1 show that the activation of Stat5 has a strong positive correlation ($r = 0.6088$; $P < 0.0001$) with the Gl. score of prostate cancer, which is further illustrated in Fig. 2. Specifically, as shown in Fig. 2, the percentage of prostate cancer specimens with high immunostaining intensity for active Stat5 increased as the Gl. score increased, whereas the percentage of prostate cancer specimens with low immunostaining intensity or absence of immunostaining for activated Stat5 decreased as Gl. score increased.

Representative samples of poorly differentiated adenocarcinoma (Gl. 4 + 5 = 9, patterns 4 and 5 shown; Fig. 1, *A* and *B*) and moderately differentiated adenocarcinoma (Gl. 4 + 3 = 7, pattern 3 shown; Fig. 1, *C* and *D*) illustrating Stat5 activation by phosphotyrosine detection (Fig. 1, *A* and *C*) or nuclear anti-Stat5 detection (Fig. 1, *B* and *D*) are presented. Adjacent normal secretory epithelium was negative for Stat5 phosphotyrosine staining (Fig. 1*E*), and predominant nuclear localization of Stat5 immunostaining was absent (Fig. 1*F*). Interestingly, immunohistochemical analysis of the cross-sections of entire prostate of 11 patients with localized or locally advanced prostate cancer (Fig. 1*I*; Table 2) showed that basal epithelial cells of periurethral prostate glands, in particular in the transition zone (Fig. 1*I*; *arrows* point at ejaculatory ducts indicating a cross-section proximal to verumontanum), were positive for activated nuclear Stat5 (Fig. 1, *G* and *H*). Lactating human breast epithelium is presented as positive control for immunostaining of tyrosine phosphorylated, activated Stat5 (Ref. 38; Fig. 1*J*), and parallel sections of lactating human breast and human prostate cancer specimens (Fig. 1, *K* and *L*, respectively) stained with subtype-specific mouse IgG were negative. We conclude that activation of Stat5 was positively associated with high Gl. score of human prostate cancer.

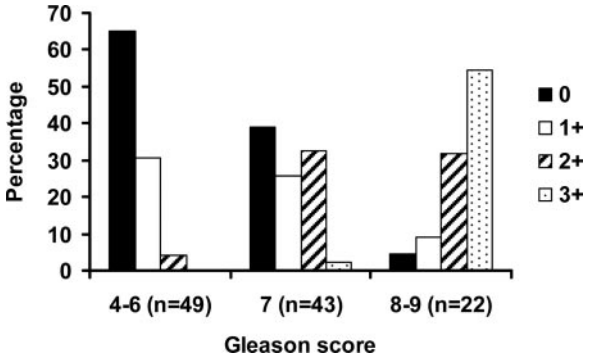

Fig. 2. Relationship between active nuclear Stat5 and Gleason score. Immunostaining for active Stat5 was associated with high Gleason (Gl.) scores of prostate cancer samples. The Gl. scores of the prostate cancer specimens immunostained for activated Stat5 (114 records) were classified into three groups (4–6, 7, 8–9). Individual prostate cancer samples were scored for active Stat5 immunostaining at three semi-quantitative steps of increasing staining intensity, where 0 was undetectable, low immunostaining gave 1+, intermediate immunostaining gave 2+, and high immunostaining gave 3+ as a score.

Prl Activates Stat5 in Androgen-Independent CWR22Rv Cells and in CWR22Rv Xenograft Tumors Grown in Nude Mice. The fact that transcription factor Stat5 is the key signaling protein activated by Prl in normal rat prostate epithelium prompted the hypothesis that Stat5 is activated by Prl also in human prostate cancer. First, we tested the hypothesis in CWR22Rv cells grown *in vitro* (Fig. 3, *A* and *B*). CWR22Rv is an androgen-independent human prostate cancer cell line that has been established from one of the hormone-refractory CWR22R tumors (43). The original tumor was derived from a Gleason score 9 primary prostate cancer with osseous metastases (44), and the primary CWR22 prostate tumor is highly responsive to androgen deprivation with marked tumor regression after castration, mimicking the course of human disease (45). After androgen deprivation-induced regression of the original tumor, the recurred tumors (CWR22R) are not dependent on androgens and are able to grow in female or castrated male mice (45).

In the preliminary experiments, we showed that Prl induced nuclear immunostaining of activated Stat5 in CWR22Rv cells by using activation state-specific anti-pTyrStat5 antibody (Fig. 3*A*). To study whether activation of Stat5 induced by Prl was attributable to activation of Stat5a and/or Stat5b, Stat5a and Stat5b were immunoprecipitated from Prl-stimulated and unstimulated exponentially growing (low density) or confluent (high density) CWR22Rv cells and immunoblotted with the anti-pTyrStat5 antibody (Fig. 3*B*). In exponentially growing CWR22Rv cells, Prl activated both Stat5a and Stat5b (Fig. 3*B*), whereas in confluent CWR22Rv cells, only phosphorylation of Stat5b, and not Stat5a, was induced by Prl (Fig. 3*B*). Reblotting of the membranes with corresponding Stat5a and Stat5b antibodies verified equal levels of Stat5 protein loaded per lane (Fig. 3*B*).

After showing that Prl activates Stat5 in CWR22Rv cell line, we next examined whether Prl activates Stat5 in CWR22Rv cells grown as s.c. tumors in nude mice. Male mice carrying CWR22Rv prostate tumors were injected i.p. with hPrl or vehicle for 30 min. Nuclear immunostaining for activated Stat5 sharply increased in the epithelial cells of CWR22Rv tumors of Prl-treated mice (Fig. 3*C*). On the basis of these experiments, we concluded that Prl activates Stat5 in CWR22Rv human prostate cancer cells *in vitro* and *in vivo* when grown as tumors in nude mice.

Stat5 Is Activated by Prl in *ex Vivo* Long-Term Organ Cultures of Human Prostate Cancer Specimens. Because Stat5 is the key signal transduction protein activated by Prl in rat prostate epithelium and because Prl activated Stat5 in androgen-independent CWR22Rv prostate cancer cells *in vitro* and in CWR22Rv cells grown as tumors

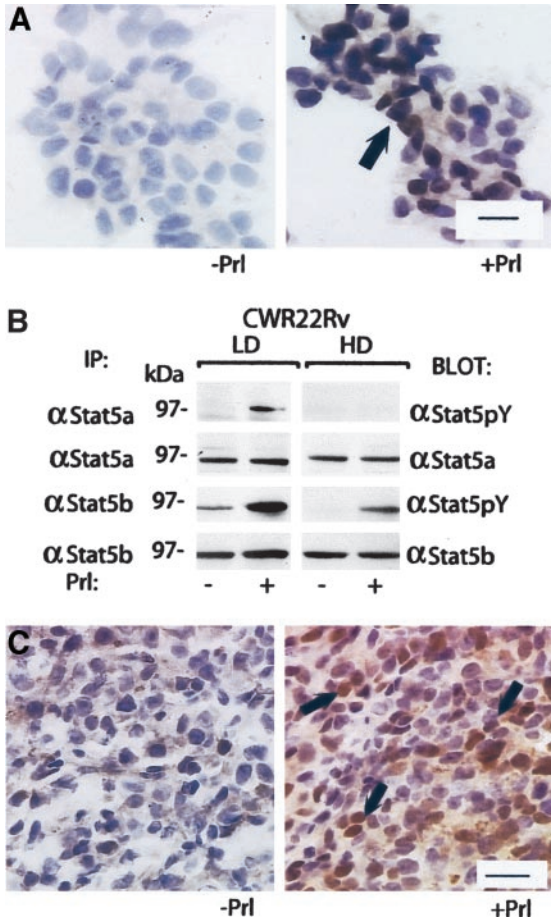

Fig. 3. Prl activates Stat5 in androgen-independent CWR22Rv prostate cancer cells and in CWR22Rv xenograft tumors grown in athymic mice. *A*, androgen-independent CWR22Rv cells were cultured exponentially, starved in serum-free medium for 16 h, and incubated with (+Prl) or without (−Prl) human (h)Prl (10 nM) for 15 min and fixed in 4% paraformaldehyde. Cells were immunostained with anti-pTyrStat5 as described in the legend to Fig. 1. Biotin-streptavidin amplified peroxidase-antiperoxidase immunodetection shows intensive positive nuclear immunostaining in CWR22Rv cells stimulated with Prl (*arrow*). *Prl*, prolactin. *B*, Prl activates both Stat5a and Stat5b in exponentially growing CWR22Rv prostate cancer cells. Stat5a and Stat5b were immunoprecipitated from CWR22Rv cells grown at low (LD; 50% confluency) or at high density (HD; 100% confluency), starved in serum-free medium for 16 h, incubated with (+) or without (−) human Prl (10 nM) for 15 min, resolved on SDS-PAGE, and blotted with a monoclonal anti-pTyrStat5 antibody (αStat5pY; *B, top panel* and *the third panel from the top*). Filters were stripped and reblotted with polyclonal antisera against Stat5a (*B, the second panel from the top*) or Stat5b (*B, bottom panel*). *C*, Prl activated Stat5 in CWR22Rv prostate cancer cells grown as xenograft tumors in nude mice. CWR22Rv cells were grown as s.c. tumors ($n = 12$) in castrated athymic male mice that had been supplied with testosterone implants. Mice received i.p. injection with human Prl (0.3 μg/g body weight) or with vehicle (PBS) for 30 min and sacrificed, and paraffin-embedded tissue sections of tumors were immunostained with a monoclonal anti-pTyrStat5 antibody as described in the legend to Fig. 1. *Arrows* indicate intense positive immunostaining for active Stat5 in CWR22Rv tumor cells of Prl-treated mice. *A* and *C*, *bar* 20 μm.

in nude mice, we next wanted to determine whether Prl activates Stat5 also in human prostate cancer samples. To address this question, *ex vivo* long-term organ cultures of human prostate cancer specimens were established (Fig. 4), as shown previously (23, 34). We have demonstrated the use of organ cultures of normal and malignant human prostate for studies of hormonal regulation of prostate epithelium (23, 34), and recently we demonstrated the use of organ culture of rat prostate as an experimental model for identification of specific intracellular signaling proteins in prostate epithelium activated by hormones and growth factors (20).

Here, we cultured 11 human prostate cancer samples with Gl. scores ranging from 4 to 7 (Table 2) for 7 days in organ culture. At the end of each culture, half of the explants were stimulated with 100 nM hPrl for 1 h (Fig. 4). Activation of Stat5 was analyzed by immunohistochemical staining of cultured human prostate cancer explants with monoclonal activation state-specific antibody for Stat5. Also, parallel sections were immunostained for Stat5 protein (data not shown).

Stat5 was constitutively activated in 6 of 11 prostate cancer specimens before organ culture (Table 2). In five of those six prostate cancer samples, Prl restored activation of Stat5 in prostate cancer acini of prostate tissue explants when cultured in low serum conditions in organ culture (Table 2). Representative prostate cancer samples illustrating activation of Stat5 by Prl within malignant epithelium of cultured prostate explants are presented (Fig. 4, *A* and *B*). In addition, a prostate cancer specimen that did not respond to Prl stimulation by activation of Stat5 is shown in Fig. 4*C*. Development of squamous metaplasia in prostate cancer acini of human prostate cancer explants cultured in organ culture was often noted. All prostate cancer samples cultured in organ culture were positive for prostate-specific antigen immunostaining (data not shown).

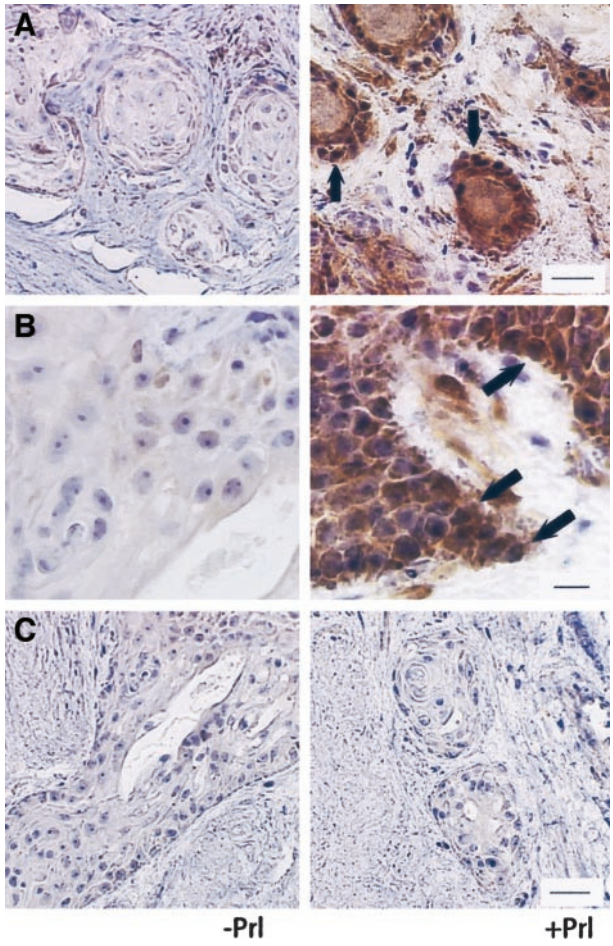

Fig. 4. Prl activates Stat5 in *ex vivo* long-term organ cultures of human prostate cancer. To test whether Prl activates Stat5 in prostate cancer, 11 localized or locally advanced human prostate cancers (Table 2) were cultured for 7 days in organ culture in the presence of androgens (100 nM dihydrotestosterone). At the end of each organ culture, half of the explants were stimulated with human Prl (100 nM) for 1 h. Paraffin-embedded tissue sections of cultured prostate cancer explants were immunostained with a monoclonal anti-pTyrStat5 antibody. The immunohistochemistry was performed as described in the legend to Fig. 1. Biotin-streptavidin amplified peroxidase-antiperoxidase immunodetection shows intensive positive nuclear immunostaining in the epithelial cells of Prl-stimulated explants of human prostate cancer (*A* and *B*, +Prl, *arrows*) compared with epithelial cells of unstimulated explants (*A* and *B*, −Prl). A representative prostate cancer that did not respond to Prl stimulation by activation of Stat5 is presented in *C*. *B*, *bar*, 10 μm. *A* and *C*, *bar*, 25 μm.

Activation of Stat5 in Prostate Cancer Cells Is Blocked by Inhibition of Jak2. To identify the tyrosine kinase that phosphorylates Stat5 in human prostate cancer, we hypothesized that Jak2 is the upstream kinase that activates Stat5 in prostate cancer cells. To directly test whether Jak2 is required for phosphorylation of Stat5 in human prostate cancer cells, a DnJak2 or WtJak2 was introduced into exponentially growing CWR22Rv cells at multiplicity of infection 10. Twenty-four h later, cells were harvested for immunocytochemistry for activated Stat5. DnJak2 effectively blocked, whereas WtJak2 enhanced, basal activation of Stat5 in exponentially growing CWR22Rv prostate cancer cells (Fig. 5*A*). To determine whether Jak2 also mediated Stat5 activation induced by Prl, we first showed that Prl stimulation of CWR22Rv cells activates Jak2 (Fig. 5*B*). Next, CWR22Rv cells were mock-infected or infected with adenovirus carrying DnJak2 or WtJak2 at multiplicity of infection 10, and 24 h later, cells were starved in serum-free medium for 16 h and then stimulated with Prl. Prl-induced phosphorylation of Stat5 was completely inhibited by DnJak2 (Fig. 5*C*). Furthermore, activation of Stat5 was significantly increased in both exponentially growing and quiescent CWR22Rv cells overexpressing WtJak2 (Fig. 5, *A* and *C*). On the basis of these three sets of experiments, we conclude that Jak2 phosphorylates transcription factor Stat5 in CWR22Rv human prostate cancer cells. RT-PCR analysis of prostate cancer cells (CWR22Rv, LnCap, and Du145) and xenograft tumors showed no evidence for TEL-Jak2 or BCR-ABL chromosomal translocations, which are known to cause constitutive activation of Stat5 in leukemia cells (data not shown).

Prl Is Locally Produced in the Epithelial Cells of Human Prostate Cancer. Because phosphorylation of Stat5 by Prl in prostate cancer cells and in human prostate cancer samples suggested a ligand-induced mechanism for Stat5 activation in human prostate cancer, we hypothesized that Prl is locally produced by the epithelial cells of human prostate cancer. We have shown previously, at both mRNA and protein level, that Prl is locally produced in the epithelium of normal rat (36) and human (23) prostate. In this study, we used immunohistochemical detection of Prl to examine Prl protein expression in human prostate cancer samples of different Gl. scores.

Specifically, we analyzed Prl protein expression in 80 human prostate cancer specimens of different Gl. scores and evaluated the immunostaining with the same semi-quantitative scoring method as was used for the analysis of immunohistochemistry of activated Stat5. A mAb specific to hPrl showed strong immunostaining for Prl in normal human prostate epithelium (Fig. 6*A*) and in the cytoplasm of the epithelial cells (Fig. 6, *B* and *C*) of 54% of 80 prostate cancers analyzed (Table 3). In addition to mAb against Prl, highly corresponding results were obtained by using a polyclonal anti-Prl antibody (data not shown). The highest expression of Prl protein was observed in Gl. score 7, 8, and 9 prostate cancers (Fig. 7; Table 3). Specifically, the intensity of immunostaining for Prl showed significant positive correlation with the Gl. scores (Spearman's rank-order correlation coefficient 0.3297, $P = 0.003$). Moreover, comparison of prostate cancer specimens positive for activated Stat5 with prostate cancer specimens expressing Prl protein also revealed a positive correlation between immunostaining for activated Stat5 and for Prl protein (Spearman's rank-order correlation coefficient 0.2680, $P = 0.0162$; Table 4).

In addition to human prostate cancer samples, approximately 20–30% of the epithelial cells of both primary (Fig. 6*D*) and recurrent (data not shown) CWR22 prostate cancer xenografts grown in nude mice showed positive immunostaining for Prl. Human pituitary prolactinomas were used as positive control tissues (Fig. 6*E*), and immunostaining of parallel sections with a subtype-specific mouse IgG were negative (Fig. 6*F*). Furthermore, preincubation of anti-Prl antibody with a 100-fold excess of hPrl blocked the immunostaining for Prl in human prostate cancer cells showing the specificity of the immunostaining reaction (Fig. 6, *G* and *H*). Thus, we conclude that Prl protein is expressed in the acinar cells of human prostate cancer with increased expression in high-grade prostate cancers.

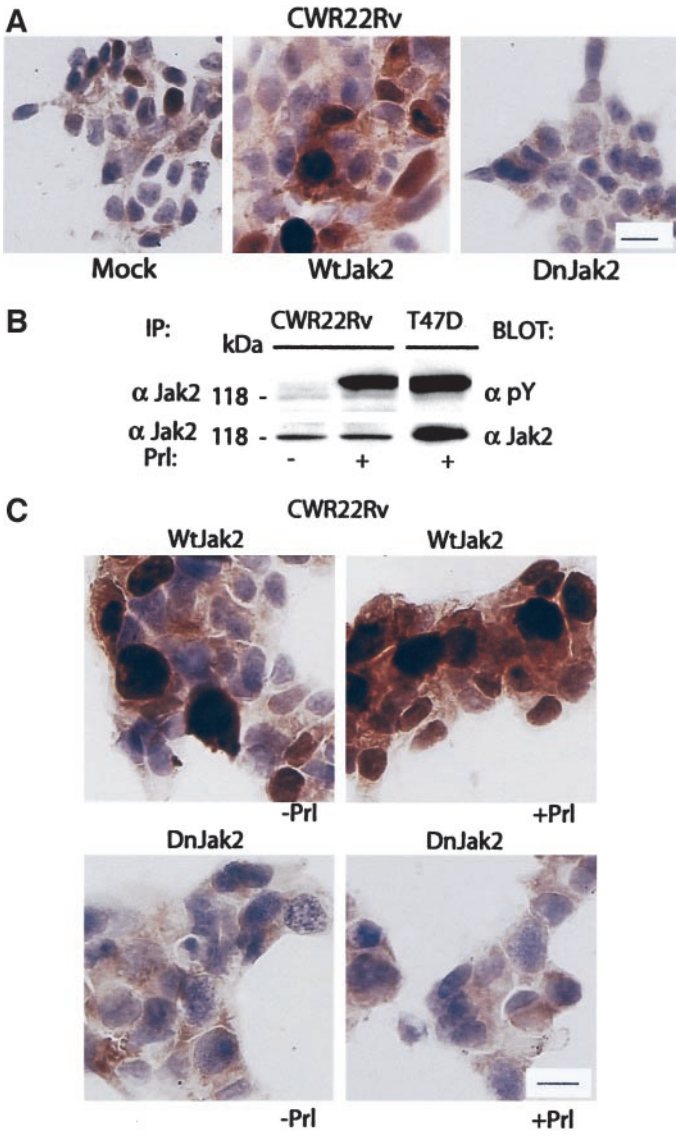

Fig. 5. Inhibition of Janus kinase 2 (Jak2) blocks activation of Stat5 in CWR22Rv prostate cancer cells. *A*, adenoviral expression of dominant-negative Jak2 (DnJak2) blocks activation of Stat5 in exponentially growing CWR22Rv prostate cancer cells. Exponentially growing CWR22Rv cells were mock infected or infected with adenovirus carrying DnJak2 or wild-type Jak2 (WtJak2) at multiplicity of infection 10, and 24 h later, cells were harvested for immunocytochemistry for activated Stat5. Cells were immunostained with anti-pTyrStat5 as described in the legend to Fig. 1. *B*, Prl induces activation of Jak2 in CWR22Rv prostate cancer cells. Confluent CWR22Rv prostate cancer cells were starved in serum-free medium for 16 h, incubated with (+) or without (−) human Prl (10 nM) for 10 min, resolved on SDS-PAGE, and blotted with a monoclonal anti-pTyr antibody (αpY) with T47D breast cancer cells as positive control (*top panel*). Filters were stripped and reblotted with a polyclonal anti-Jak2 antiserum (αJak2; *bottom panel*). *C*, adenoviral delivery of DnJak2 blocks Prl-induced activation of Stat5 in CWR22Rv cells. CWR22Rv cells were mock infected or infected with adenovirus carrying DnJak2 or WtJak2 at multiplicity of infection 10, and 24 h later, cells were starved in serum-free medium for 16 h, stimulated with human Prl (10 nM) for 15 min, and harvested for immunocytochemistry performed as described in the legend to Fig. 1. *A* and *C*, *bar* 15 μm.

DISCUSSION

In the present work, we show that activation of transcription factor Stat5 is associated with a high histological grade of human prostate

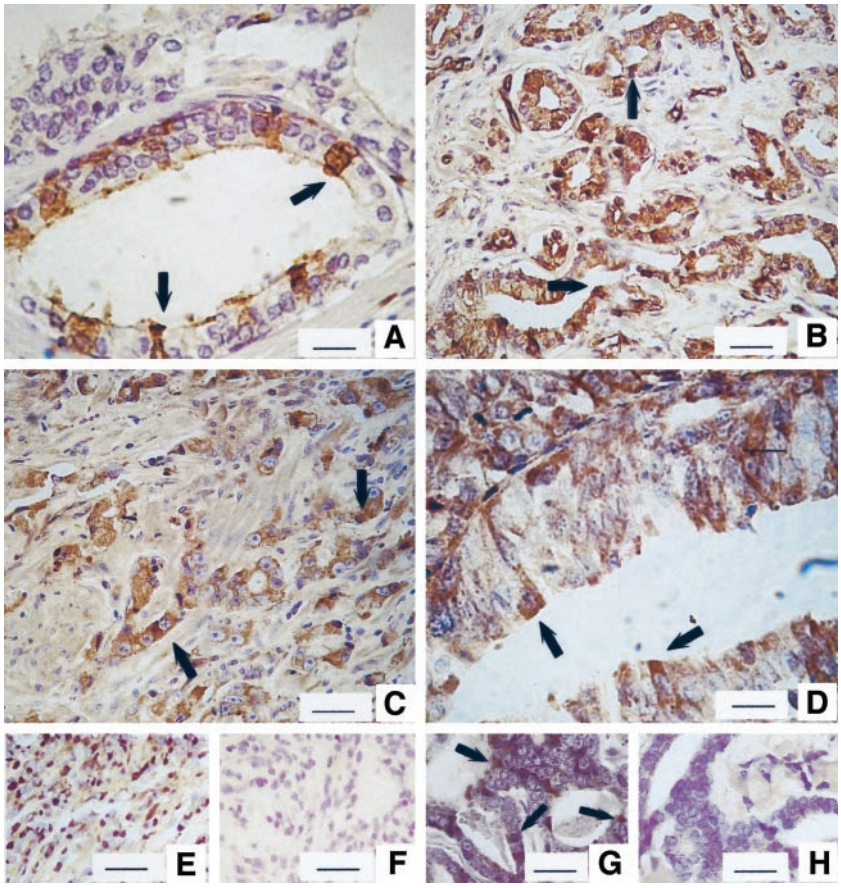

Fig. 6. Prolactin protein is locally expressed in the epithelial cells of human prostate cancer. Paraffin-embedded sections of prostate cancer from 80 patients and from CWR22 human prostate cancer xenografts grown in nude mice were immunostained with a monoclonal anti-hPrl antibody. Cytoplasm from a number of epithelial cells (*arrows*) in normal human prostate acini (*A*) and in prostate cancer acini (*B*, Gleason pattern 3; *C*, Gleason pattern 4) showed intense positive immunostaining for Prl protein. Also, epithelial cells of CWR prostate tumors grown in nude mice stained positive for Prl protein (*D*). Human pituitary prolactinomas were immunostained for Prl as positive control tissues (*E*), and parallel sections stained with subtype-specific mouse IgG were negative (*F*). Immunohistochemistry of sections of human prostate cancer with anti-hPrl monoclonal antibody in the absence (*G*) and in the presence of 100-fold excess of hPrl (*H*). Excess of hPrl blocked the positive immunostaining for Prl in human prostate cancer cells. *A* and *D*, *bar* 10 μm and *B*, *C*, *E*, *F*, *G*, and *H*, *bar*, 20 μm. *h*, human; *Prl*, prolactin.

cancer. Also, we demonstrate that both Jak2 and Prl are components of a signaling pathway that leads to activation of Stat5 in a large fraction of human prostate cancers. The rationale behind this work centers on our recent identification of Stat5 as a critical survival factor for prostate cancer cells (4).

The prevalent detection of activated Stat5 in high-grade human prostate cancer specimens indicates that Stat5 could be a candidate therapeutic target protein, specifically in advanced prostate cancer. This is of importance for the development of new therapies for prostate cancer, because there are only a few treatment options currently available, particularly for advanced prostate cancer. Positive association of activation of Stat5 and high Gl. grade of prostate cancer raises a question of possible connection of Stat5 with prognosis of prostate cancer. Ongoing work in our laboratory seeks to investigate whether detection of activation of Stat5 in a prostate cancer specimen improves the predictive value of Gl. score in identification of prostate cancer patients who would need more aggressive intervention.

Our finding of preferential activation of Stat5 in high-grade prostate cancers suggests that activation of Stat5 could be one of the molecular mechanisms involved in progression of human prostate cancer. Activation of Stat5 in prostate cancer cells could represent a gain of function event, which may contribute to development of prostate cancer cell clones that are able to grow independently of androgens. In addition to affecting gene transcription directly, Stat5 might interact with androgen receptor and influence its activation in prostate cancer cells, as has been shown for Stat5 interaction with glucocorticoid receptor in mammary epithelial cells (46). As well as being potentially involved in androgen-independent growth of prostate cancer, Stat5 might also contribute to invasion of prostate cancer. On the basis of the present observations, evaluation of Stat5 target genes in

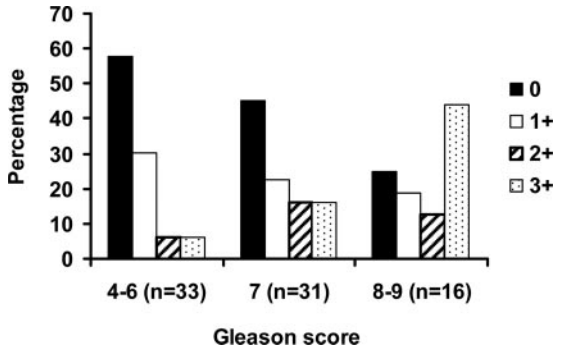

Fig. 7. Immunostaining for Prl was associated with high Gleason (Gl.) scores of prostate cancer samples. The Gl. scores of the prostate cancer specimens immunostained for Prl (80 records) were classified into three groups (Gleason scores 4–6, 7, and 8–9). Individual prostate cancer samples were scored for Prl protein immunostaining at three semi-quantitative steps of increasing staining intensity, where 0 was undetectable, low immunostaining gave 1+, intermediate immunostaining gave 2+, and high immunostaining gave 3+ as a score. *Prl*, prolactin.

Table 3 *Relationship between prolactin immunostaining and Gleason score* [a]

Prolactin protein expression in human prostate cancer positively correlated with the Gleason score.

	Prolactin immunostaining[b]				
Gleason score	−	+	++	+++	*n*
4–6	19	10	2	2	33
7	14	7	5	5	31
8–9	4	3	2	7	16
n	37	20	9	14	80

[a] Spearman's rank-order correlation coefficient for prolactin immunostaining *versus* Gleason score was 0.3297 ($P = 0.003$).

[b] Absence of immunostaining had a score 0, weak positive immunostaining had a score 1+, intermediate had a score 2+, and strong staining intensity had a score 3+.

Table 4 *Relationship between active Stat5 immunostaining and prolactin (Prl) protein expression* [a]

Activation of Stat5 in human prostate cancer samples positively correlated with prolactin protein expression.

	Active Stat5				
	−	+	++	+++	*n*
Prl					
−	18	7	9	3	37
+	6	8	5	1	20
++	3	2	3	1	9
+++	2	3	5	4	14
n	29	20	22	9	80

[a] Spearman's rank-order correlation coefficient for immunostaining for active Stat5 *versus* prolactin protein was 0.2680 ($P = 0.0162$).

prostate cancer will be important, because they are likely to have further therapeutic implications.

A recent study by Gao *et al.* (47) indicated that in human prostate tissue homogenates there was only a low level of Stat5 binding to an oligonucleotide probe corresponding to the Stat5-response element of the rat β-casein gene promoter in electrophoretic mobility shift assays. In this work we show, by both immunohistochemical staining of phosphorylated nuclear Stat5 protein and by immunoblotting of phosphorylated Stat5, that Stat5 is in the activated state in human prostate cancer. It is possible that when whole tissue homogenates are used in electrophoretic mobility shift assays, prostate-specific or stromal factors interfere with binding of Stat5 to β-casein promoter. In this regard, using our highly sensitive *in situ* technique, which detects activation of Stat5 at a single cell level, we have demonstrated recently that Stat5 is activated in normal human and mouse mammary epithelial cells outside pregnancy, an activity that is difficult to detect by electrophoretic mobility shift assays using tissue homogenates of whole mammary glands (38).

Because activation of Stat5 was increased in high-grade human prostate cancers, it would be interesting to determine whether increased activation of Stat5 is attributable to alteration of the level of Stat5 protein itself in malignant prostate epithelial cells. However, the protein levels of Stat5 in different Gl. grade prostate cancers would need to be analyzed by immunoprecipitation and immunoblotting of fresh prostate cancer specimens, because immunohistochemical determination of total Stat5 protein levels in prostate epithelium is difficult if Stat5 is not activated and located in the nucleus. As illustrated in Fig. 1*F*, immunohistochemical detection of nonactivated Stat5 protein appears as diffuse and weak immunostaining in both cytoplasm and nuclei of prostate epithelial cells, which is complicated to quantitate reliably. Interestingly, in contrast to secretory epithelium, basal epithelial cells of normal prostate epithelium showed intense activation and nuclear localization of Stat5, particularly in periurethral prostate glands. The basal epithelial compartment of prostate acini has been shown to contain a population of pluripotent progenitor cells that proliferate and differentiate into mature basal and secretory cells (48). We do not know what the role of activation of Stat5 in basal prostate epithelial cells is.

A ligand-induced mechanism for activation of Stat5 in prostate cancer was suggested by the fact that Prl stimulated activation of both Jak2 and Stat5 in CWR22Rv human prostate cancer cells and in CWR22Rv tumors grown in nude mice. Inhibition of Stat5 activation by adenoviral overexpression of Jak2 verified Jak2 as the principal tyrosine kinase that activates Stat5 in CWR22Rv human prostate cancer cells. We also analyzed Prl-induced activation of Stat5 in human prostate cancer samples using organ culture as an experimental model. Prostate organ culture is an *in vitro* culture system for fresh prostate tissue samples for studies on hormone regulation and signaling in normal and malignant prostate tissue (20–23, 34–36). The principal difference of this model system and cell cultures of primary prostate epithelial cells or cancer cell lines is that in prostate organ culture the whole tissue architecture with critical interactions of epithelium and stroma are maintained. Specifically, we cultured 11 prostate cancer specimens of Gl. scores 4–7 in organ culture (Table 2). In 6 of 11 prostate cancer samples, Stat5 was constitutively activated before culture. Prl restored activation of Stat5 in five of six of these prostate cancer specimens in organ culture, which has low serum conditions. Collectively, these data suggest that Prl-induced activation of Stat5 is one of the mechanisms responsible for Stat5 activation in human prostate cancer.

A study by Leav *et al.* (26) evaluating Prl-receptor expression in developing normal human prostate and in prostate neoplasia reported similar expression levels of Prl receptors in Gl. grade 3 and 4 prostate cancers with foci of cells with diminished Prl-receptor expression in Gl. grade 4 cancers. In our material, there were moderate regional variations in Stat5 activation in Gl. grade 4 prostate cancers. However, the general level of Stat5 activation was clearly increased in Gl. grade 4 cancers compared with grade 3 prostate cancers. In Gl. grade 5 prostate cancers, Stat5 was highly activated, and the pattern of Stat5 activation was uniform. It is important to note that the material of the study reported by Leav *et al.* (26) included only a limited number of prostate cancer specimens of Gl. grade 4, and moreover, their material did not contain prostate cancer specimens of Gl. grade 5.

The role of Prl as one of the key ligands that activate Stat5 in human prostate cancer was further supported by the finding of local expression of Prl protein in 54% of prostate cancer specimens and positive correlation with Gl. scores of prostate cancer samples. In addition, expression of Prl protein in prostate cancer specimens correlated positively with activation of Stat5. Previously, using *in situ* hybridization, Northern blotting, and RT-PCR, we have shown high expression of Prl mRNA in both normal rat and human prostate epithelium (23, 36). Prl has been shown in numerous *in vitro* studies and mouse model systems to promote prostate epithelial growth (20–24, 26–30, 35, 49, 50). Neutralizing anti-Prl-receptor antibodies may provide a therapeutic approach to inhibit Jak2-Stat5 signaling pathway in human prostate cancer. Moreover, identification of Jak2 as the major kinase that phosphorylates Stat5 in prostate cancer cells provides an alternative approach to block Stat5 in human prostate cancer by small molecule inhibitors of Jak2.

In conclusion, positive correlation of activation of Stat5 with the histological grade of prostate cancer suggests that activation of Stat5 is associated with biologically aggressive behavior of prostate cancer. Prl and tyrosine kinase Jak2 were identified as components of a signaling cascade that activates Stat5 in human prostate cancer cells, and thus, they are potential molecular targets for pharmacological intervention.

ACKNOWLEDGMENTS

We thank Dr. Hallgeir Rui [Lombardi Comprehensive Cancer Center (LCCC), Georgetown University] for helpful discussions and support; The National Institute of Diabetes and Digestive and Kidney Diseases National Hormone (Pituitary) Program and Dr. A. F. Parlow for providing polypeptide hormones; Dr. Edmund Gehan in Biostatistics Unit of LCCC for advice and assistance in statistical analysis; Jennifer Johnson and Michael Messuri for technical assistance in immunohistochemical analyses; Dr. Hannu Kalimo (University of Turku, Turku, Finland) for the control samples for Prl immunohistochemistry; Dr. Lukas Bubendorf (Institute of Pathology, University of Basel, Basel, Switzerland) for providing normal human breast tissue; Dr. Mao-Zheng Dai in the Microscopy and Imaging Shared Resources for kind help; Eric Williams and Mark Lloyd in Histopathology and Tissue Shared Resources of LCCC; and Tissue Culture and Animal Shared Resources of LCCC.

REFERENCES

1. DeMarzo AM, Nelson WG, Isaacs WB, Epstein JI. Pathological and molecular aspects of prostate cancer. Lancet 2003;361:955–64.
2. Visakorpi T, Hyytinen E, Koivisto P, et al. In vivo amplification of the androgen receptor gene and progression of human prostate cancer. Nat Genet 1995;9:401–6.
3. Taplin ME, Bubley GJ, Shuster TD, et al. Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. N Engl J Med 1995;332:1393–8.
4. Ahonen TJ, Xie J, LeBaron MJ, et al. Inhibition of transcription factor Stat5 induces cell death of human prostate cancer cells. J Biol Chem 2003;278:27287–92.
5. Mora LB, Buettner R, Seigne J, et al. Constitutive activation of Stat3 in human prostate tumors and cell lines: direct inhibition of Stat3 signaling induces apoptosis of prostate cancer cells. Cancer Res 2002;62:6659–66.
6. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2002;2:489–501.
7. Gioeli D, Mandell JW, Petroni GR, Frierson HF Jr., Weber MJ. Activation of mitogen-activated protein kinase associated with prostate cancer progression. Cancer Res 1999;59:279–84.
8. Bakin RE, Gioeli D, Sikes RA, Bissonette EA, Weber MJ. Constitutive activation of the Ras/mitogen-activated protein kinase signaling pathway promotes androgen hypersensitivity in LNCaP prostate cancer cells. Cancer Res 2003;63:1981–9.
9. Chen T, Wang LH, Farrar WL. Interleukin 6 activates androgen receptor-mediated gene expression through a signal transducer and activator of transcription 3-dependent pathway in LNCaP prostate cancer cells. Cancer Res 2000;60:2132–5.
10. Liu X, Robinson GW, Gouilleux F, Groner B, Hennighausen L. Cloning and expression of Stat5 and an additional homologue (Stat5b) involved in prolactin signal transduction in mouse mammary tissue. Proc Natl Acad Sci USA 1995;92:8831–5.
11. Gouilleux F, Wakao H, Mundt M, Groner B. Prolactin induces phosphorylation of Tyr694 of Stat5 (MGF), a prerequisite for DNA binding and induction of transcription. EMBO J 1994;13:4361–9.
12. Wakao H, Gouilleux F, Groner B. Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. EMBO J 1994;13:2182–91.
13. Ihle JN. The Stat family in cytokine signaling. Curr Opin Cell Biol 2001;13:211–7.
14. David M, Wong L, Flavell R, et al. STAT activation by epidermal growth factor (EGF) and amphiregulin. Requirement for the EGF receptor kinase but not for tyrosine phosphorylation sites or JAK1. J Biol Chem 1996;271:9185–8.
15. Chen J, Sadowski HB, Kohanski RA, Wang LH. Stat5 is a physiological substrate of the insulin receptor. Proc Natl Acad Sci USA 1997;94:2295–300.
16. Klejman A, Schreiner SJ, Nieborowska-Skorska M, et al. The Src family kinase Hck couples BCR/ABL to STAT5 activation in myeloid leukemia cells. EMBO J 2002; 21:5766–74.
17. Welte T, Leitenberg D, Dittel BN, et al. STAT5 interaction with the T cell receptor complex and stimulation of T cell proliferation. Science (Wash D C) 1999;283:222–5.
18. Ho JM, Beattie BK, Squire JA, Frank DA, Barber DL. Fusion of the ets transcription factor TEL to Jak2 results in constitutive Jak-Stat signaling. Blood 1999;93:4354–64.
19. Shuai K, Halpern J, ten Hoeve J, Rao X, Sawyers CL. Constitutive activation of STAT5 by the BCR-ABL oncogene in chronic myelogenous leukemia. Oncogene 1996;13:247–54.
20. Ahonen TJ, Harkonen PL, Rui H, Nevalainen MT. PRL signal transduction in the epithelial compartment of rat prostate maintained as long-term organ cultures in vitro. Endocrinology 2002;143:228–38.
21. Ahonen TJ, Harkonen PL, Laine J, Rui H, Martikainen PM, Nevalainen MT. Prolactin is a survival factor for androgen-deprived rat dorsal and lateral prostate epithelium in organ culture. Endocrinology 1999;140:5412–21.
22. Nevalainen MT, Valve EM, Makela SI, Blauer M, Tuohimaa PJ, Harkonen PL. Estrogen and prolactin regulation of rat dorsal and lateral prostate in organ culture. Endocrinology 1991;129:612–22.
23. Nevalainen MT, Valve EM, Ingleton PM, Nurmi M, Martikainen PM, Harkonen PL. Prolactin and prolactin receptors are expressed and functioning in human prostate. J Clin Investig 1997;99:618–27.
24. Janssen T, Darro F, Petein M, et al. In vitro characterization of prolactin-induced effects on proliferation in the neoplastic LNCaP, DU145, and PC3 models of the human prostate. Cancer (Phila) 1996;77:144–9.
25. Melck D, De Petrocellis L, Orlando P, et al. Suppression of nerve growth factor Trk receptors and prolactin receptors by endocannabinoids leads to inhibition of human breast and prostate cancer cell proliferation. Endocrinology 2000;141:118–26.
26. Leav I, Merk FB, Lee KF, et al. Prolactin receptor expression in the developing human prostate and in hyperplastic, dysplastic, and neoplastic lesions. Am J Pathol 1999;154:863–70.
27. Xu X, Kreye E, Kuo CB, Walker AM. 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 2001;61:6098–104.
28. Kindblom J, Dillner K, Ling C, Tornell J, Wennbo H. Progressive prostate hyperplasia in adult prolactin transgenic mice is not dependent on elevated serum androgen levels. Prostate 2002;53:24–33.
29. Kindblom J, Dillner K, Sahlin L, et al. Prostate hyperplasia in a transgenic mouse with prostate-specific expression of prolactin. Endocrinology 2003;144:2269–78.
30. Wennbo H, Kindblom J, Isaksson OG, Tornell J. Transgenic mice overexpressing the prolactin gene develop dramatic enlargement of the prostate gland. Endocrinology 1997;138:4410–5.
31. Eaton NE, Reeves GK, Appleby PN, Key TJ. Endogenous sex hormones and prostate cancer: a quantitative review of prospective studies. Br J Cancer 1999;80:930–4.
32. Harper ME, Wilson DW, Jensen HM, Pierrepoint CG, Griffiths K. Steroid hormone concentrations in relation to patient prognosis and prostate tumour grade. J Steroid Biochem 1987;27:521–4.
33. Gleason DF. Classification of prostatic carcinomas. Cancer Chemother Rep 1966;50: 125–8.
34. Nevalainen MT, Härkönen PL, Valve EM, Ping W, Nurmi M, Martikainen PM. Hormone regulation of human prostate in organ culture. Cancer Res 1993;53:5199–207.
35. Nevalainen MT, Valve EM, Ingleton PM, Harkonen PL. Expression and hormone regulation of prolactin receptors in rat dorsal and lateral prostate. Endocrinology 1996;137:3078–88.
36. Nevalainen MT, Valve EM, Ahonen T, Yagi A, Paranko J, Harkonen PL. Androgen-dependent expression of prolactin in rat prostate epithelium in vivo and in organ culture. FASEB J 1997;11:1297–307.
37. Yamashita H, Xu J, Erwin RA, Larner AC, Rui H. A lymphoma growth inhibitor blocks some but not all prolactin-stimulated signaling pathways. J Biol Chem 1999; 274:14699–705.
38. Nevalainen MT, Xie J, Bubendorf L, Wagner KU, Rui H. Basal activation of transcription factor signal transducer and activator of transcription (stat5) in nonpregnant mouse and human breast epithelium. Mol Endocrinol 2002;16:1108–24.
39. Siegel S, Castellan NJ. Nonparametric statistics for the behavioral sciences, 2nd edition. New York: McGraw-Hill; 1988.
40. The SAS System. Cary, NC: SAS Institute; 1999.
41. Duhe RJ, Rui H, Greenwood JD, Garvey K, Farrar WL. Cloning of the gene encoding rat JAK2, a protein tyrosine kinase. Gene 1995;158:281–5.
42. Xie J, LeBaron MJ, Nevalainen MT, Rui H. Role of tyrosine kinase Jak2 in prolactin-induced differentiation and growth of mammary epithelial cells. J Biol Chem 2002;277:14020–30.
43. Sramkoski RM, Pretlow TG 2nd, Giaconia JM, et al. A new human prostate carcinoma cell line, 22Rv1. In Vitro Cell Dev Biol Anim 1999;35:403–9.
44. Pretlow TG, Wolman SR, Micale MA, et al. Xenografts of primary human prostatic carcinoma. J Natl Cancer Inst (Bethesda) 1993;85:394–8.
45. Nagabhushan M, Miller CM, Pretlow TP, et al. CWR22: the first human prostate cancer xenograft with strongly androgen-dependent and relapsed strains both in vivo and in soft agar. Cancer Res 1996;56:3042–6.
46. Stöcklin E, Wissler M, Gouilleux F, Groner B. Functional interactions between Stat5 and the glucocorticoid receptor. Nature (Lond) 1997;383:726–8.
47. Ni Z, Lou W, Lee SO, et al. Selective activation of members of the signal transducers and activators of transcription family in prostate carcinoma. J Urol 2002;167: 1859–62.
48. Wang Y, Hayward S, Cao M, Thayer K, Cunha G. Cell differentiation lineage in the prostate. Differentiation 2001;68:270–9.
49. Hostetter MW, Piacsek BE. The effect of prolactin deficiency during sexual maturation in the male rat. Biol Reprod 1977;17:574–7.
50. Robertson FG, Harris J, Naylor MJ, et al. Prostate development and carcinogenesis in prolactin receptor knockout mice. Endocrinology 2003;144:3196–205.