Down-regulation of Suppressor of Cytokine Signaling-3 Causes Prostate Cancer Cell Death through Activation of the Extrinsic and Intrinsic Apoptosis Pathways

Martin Puhr,[1] Frédéric R. Santer,[1] Hannes Neuwirt,[1] Martin Susani,[2] Jeffrey A. Nemeth,[4] Alfred Hobisch,[5] Lukas Kenner,[2,3] and Zoran Culig[1]

[1]Department of Urology, Innsbruck Medical University, Innsbruck, Austria; [2]Institute of Clinical Pathology, Medical University of Vienna; [3]Ludwig Boltzmann Institute for Cancer Research, Vienna, Austria; [4]Centocor Research and Development, Malvern, Pennsylvania; and [5]Department of Urology, General Hospital Feldkirch, Feldkirch, Austria

Abstract

Suppressor of cytokine signaling-3 (SOCS-3) acts as a negative feedback regulator of the Janus-activated kinase/signal transducers and activators of transcription factors signaling pathway and plays an important role in the development and progression of various cancers. To better understand the role of SOCS-3 in prostate cancer, SOCS-3 expression was down-regulated in DU-145, LNCaP-IL-6+, and PC3 cells by consecutive SOCS-3 small interfering RNA transfections. SOCS-3 mRNA and protein expression as measured by quantitative reverse transcription-PCR and Western blot, respectively, were decreased by ~70% to 80% compared with controls. We observed a significant decrease in cell proliferation and viability in all SOCS-3–positive cell lines but not in the parental LNCaP cell line, which is SOCS-3 negative. In this study, we show that down-regulation of SOCS-3 leads to an increased cell death in prostate cancer cell lines. We found a considerable increase in the activation of the proapoptotic caspase-3/caspase-7, caspase-8, and caspase-9. A significant up-regulation of cleaved poly(ADP-ribose) polymerase and inhibition of Bcl-2 expression was observed in all SOCS-3–positive cell lines. Overexpression of Bcl-2 could rescue cells with decreased SOCS-3 levels from going into apoptosis. Tissue microarray data prove that SOCS-3 is highly expressed in castration-refractory tumor samples. In conclusion, we show that SOCS-3 is an important protein in the survival machinery in prostate cancer and is overexpressed in castration-resistant tumors. SOCS-3 knockdown results in an increase of cell death via activation of the extrinsic and intrinsic apoptosis pathways. [Cancer Res 2009;69(18):7375–84]

Introduction

Dysregulation of the pathway of signal transducer and activator of transcription (STAT) factors has been implicated in various diseases. In many types of carcinomas, the oncogenic role of STAT factors, especially of STAT3, is well documented (1–3). Its overexpression and constitutive activation are correlated with an increased proliferation and metastatic progression. However, in prostate cancer the role of STAT3 is a subject of investigation because its activation is associated with either inhibition or promotion of tumor growth (4, 5). To explain these controversial results, negative feedback regulators of the Janus-activated kinase (JAK)–STAT pathway, especially suppressors of cytokine signaling (SOCS), have become a focus of interest. The SOCS family consists of eight members, SOCS-1 to SOCS-7 and cytokine-inducible SH2 protein. All these proteins contain a NH_2 terminal region of variable length, a central SH2 domain, and a conserved COOH terminal region called SOCS box. This region interacts with Elongin-B, Elongin-C, Cullin-5, and RING-box-2. The complex can recruit E2 ubiquitin transferases (6). Thus, the proteins can act as E3 ubiquitin ligases and mediate the proteasomal degradation of associated proteins. SOCS-1 and SOCS-3 are especially induced by interleukin-6 (IL-6). IL-6 is a multifunctional cytokine that plays an important role in chronic inflammation of the prostate (7, 8) and in prostate carcinogenesis (9). SOCS-1 and SOCS-3 can inhibit IL-6–mediated JAK activation directly through their kinase inhibitory region as evidenced for the first time in mouse BF-ER cells (10). In addition, it was shown in renal cancer cells that SOCS-3 can also bind with high affinity to phosphothyrosine 757 within the IL-6 receptor subunit glycoprotein 130 (11). It is generally accepted that SOCS-3 acts as an important negative feedback regulator of the IL-6-JAK-STAT signaling pathway (12–14) and plays a crucial role in many biological processes. SOCS-3–deficient mice die as a result of placental defects, whereas enforced expression of SOCS-3 also results in embryonic death (15–17). SOCS-3 is involved in the development and progression of several malignancies. There are indications that SOCS-3 has different functions depending on the tumor origin. In human lung (18), hepatocellular (19), and head and neck cancer (20), SOCS-3 is silenced by hypermethylation, which causes a growth advantage for cancer cells. In contrast, SOCS-3 is detectable in breast cancer (21). In previous studies, we showed that SOCS-3 is expressed in prostate cancer and its expression is inversely correlated with STAT3 phosphorylation, suggesting an inactivation of the JAK-STAT pathway (22). Overexpression of SOCS-3 differentially affects proliferation of prostate cancer cells, depending on androgen sensitivity (22, 23). However, effects of SOCS-3 down-regulation on apoptosis have not been investigated in detail yet.

In this study, we show for the first time that sustained down-regulation of SOCS-3 results in reactivation of IL-6–induced STAT3 and in decreased cell proliferation and viability of SOCS-3–positive DU-145, LNCaP-IL-6+, and PC3 prostate cancer cell lines. Increased apoptosis is associated with activation of proapoptotic caspase-3, caspase-8, and caspase-9, increased levels of cleaved

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

L. Kenner and Z. Culig contributed equally to this work.

Requests for reprints: Zoran Culig, Department of Urology, Innsbruck Medical University, Anichstrasse 35, A-6020 Innsbruck, Austria. Phone: 43-512-504-24717; Fax: 43-512-504-24817; Email: zoran.culig@i-med.ac.at.

doi:10.1158/0008-5472.CAN-09-0806

poly(ADP-ribose) polymerase (cPARP), and a significant decrease of Bcl-2. Immunohistochemical staining proves that SOCS-3 expression increases in castration-refractory prostate cancer (CRPC). Thus, in the present manuscript, we show that SOCS-3 interference with apoptotic pathways is relevant to prostate cancer development and progression.

Materials and Methods

Cell culture and chemicals. LNCaP American Type Culture Collection (ATCC), DU-145, and PC3 prostate cancer cells were obtained from American Type Culture Collection. The LNCaP subline LNCaP-IL-6+ was derived in the presence of IL-6, as described elsewhere (24). VCaP cells were a kind gift from Dr. Kenneth Pienta (University of Michigan). All cell lines were cultured in RPMI 1640 (HyClone). Media were supplemented with 10% FCS and 1% penicillin/streptomycin and glutamin.

Short interfering RNA transfection. The short interfering RNA (siRNA) sequence used for targeting human SOCS-3 was previously published (ref. 25; sense 5-CCAAGAACCUGCGCAUCCAdTdT-3, antisense, 5-UGGAUGCG-CAGGUUCUUGGdTdT-3) and synthesized by Biomers. A nontargeting siRNA pool (Dharmacon) was used as a negative control. siRNA transfections were performed with Lipofectamine 2000 (Invitrogen) reagent according to the manufacturer's protocol. All cell lines were transfected with either 2 nmol/L siRNA against SOCS-3 or control siRNA during a period of 6 d. Two days after the first transfection, the cells were seeded for [^{3}H]thymidine incorporation assay, Western blots, flow cytometry, or caspase assay, respectively. The second transfection was done with the same amount of siRNAs.

RNA isolation and reverse transcription-PCR. Cells were grown on six-well plates. Subsequently, total RNA was isolated using the RNeasy mini kit (Qiagen) following the manufacturer's protocol. cDNA synthesis was performed using iScript select cDNA synthesis kit (Bio-Rad). Real-time PCR conditions were as follows: one cycle of denaturing at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All amplifications were performed in triplicates. TATA-Box binding protein (TBP) was chosen as an endogenous expression standard (26). Primer and probe sequences were as follows: SOCS-3 (forward 5-TGATCCGCGACAGCTCG-3, reverse 5-TCCCAGACTGGGTCTTGACG-3, TaqMan probe 5-FAM-CCAGCGC-CACTTCTTCACGCTCA-TAMRA-3) and TBP (forward 5-CACGAACCACGG-CACTGATT-3, reverse 5-TTTTCTGCTGCCAGTCTGGAC-3, TaqMan probe 5-FAM-TCTTCACTCTTGGCTCCTGTGCACA-TAMRA-3). Each reaction was performed in a 11-μL volume containing 50 ng of cDNA and 5 μL of 2× ABI Mastermix (Applied Biosystems). The primers were added to a final concentration of 900 nmol/L, and the final probe concentration was 150 nmol/L. PCR products were measured using the ABI Prism 7500 Fast RT-PCR System (Applied Biosystems). C_t values of SOCS-3 and TBP, as assessed by ABI Sequence Detection Software (version 1.3), were used to calculate the ΔC_t using Microsoft Excel. Values obtained in control cells were defined as 100%, and those from treated cells were expressed as a percentage of control.

Western blot. Cells were harvested, washed with PBS, and lysed. The total protein was quantified using the Bradford method (27). Protein (50 μg) per lane was then resolved using a 4% to 12% bis-Tris gel (Invitrogen) and transfered onto a nitrocellulose membrane (Invitrogen). The membrane was blocked for 1 h using Starting Block buffer (Pierce Biotechnology) and incubated at 4°C overnight with a primary antibody followed by incubation for 1 h at room temperature with fluorescence-labeled secondary antibodies (Molecular Probes). The membranes were scanned and quantified using the Odyssey IR Imaging System (LiCor Biosciences). The following antibodies were used for Western blots: SOCS-3 (1:1,000; Acris Antibodies), glyceraldehyde-3-phosphate dehydrogenase (1:100,000; Chemicon), phosphorylated STAT3 (1:200; Upstate Cell Signaling Technology), STAT3 (1:500; Santa Cruz Biotechnology), phosphorylated STAT1 (1:500; Santa Cruz Biotechnology), STAT1 (1:500; Santa Cruz Biotechnology), Mcl-1 (1:1,000; Becton Dickinson), Bcl-2 (1:200; Cell Signaling), and cPARP (1:1,000; Promega).

[^{3}H]Thymidine incorporation assay. DU-145, LNCaP-IL-6+, and PC3 cells were seeded 2 d after the first transfection at a density of 3.5×10^3 per well, and LNCaP cells were seeded at a density of 1.5×10^4 per well in triplicates onto 96-well plates. On the next day, the cells were treated with 2 nmol/L of siRNA against SOCS-3 or with negative control siRNA for 72 h; 25 μL/well of [^{3}H]thymidine (1 μCi/well) were added, and DNA was harvested on 96-well filter plates (UniFilter; Perkin-Elmer) after a 24-h incubation. Scintillation fluid (50 μL) was added, and radioactivity was quantified using Chameleon 5025 liquid scintillation counter (HVD Life Sciences).

Flow cytometry. DU-145, LNCaP-IL-6+, and PC3 cells were seeded 2 d after the first transfection at a density of 2.5×10^5 per well, and LNCaP cells were seeded at a density of 3.5×10^5 per well onto six-well plates. On the next day, the cells were treated with 2 nmol/L of siRNA against SOCS-3 or with negative control siRNA for 72 h. The pellet was resuspended in propidium iodide (PI) buffer (0.2% Triton-X-100, 2 ng/mL Na-Citrate, and 0.1 mg/mL PI) and kept light-protected at 4°C for 1 h. Apoptosis was analyzed using FACS Calibur (Becton Dickinson). For the experiments in which Bcl-2 cDNA was overexpressed, the cells were seeded onto six wells and cotransfected on the following day with 2 nmol/L control siRNA, siRNA against SOCS-3, or mock and 0.5 μg/mL pEGFP-Bcl-2 or empty vector as indicated using Lipofectamine 2000 (Invitrogen). Two days after transfection, the cells were split and the second transfection was performed on the next day as described above. Cells were grown for additional 3 d and then trypsinized and collected. Cell pellets were lyzed in PI buffer and analyzed for DNA content by flow cytometry.

Caspase assay. For the measurement of the caspase-3/caspase-7, caspase-8, and caspase-9 activity, DU-145 and LNCaP-IL-6+ cells were seeded 2 d after the first transfection at a density of 3.5×10^3 per well and LNCaP cells were seeded at a density of 5×10^3 per well in triplicates onto 96-well plates. On the next day, the cells were treated with 2 nmol/L of siRNA against SOCS-3 or with negative control siRNA for 72 h. On day 6, assays for caspase activity were performed with the respective Caspase-Glo assay kits (Promega) according to the manufacturer's protocols. PC3 cells were not used in those experiments because of excessive cell death.

Patient samples. We established tissue microarrays (TMA) using samples from prostate cancer patients comparing normal, prostate intraepithelial neoplasia (PIN), and cancer areas with Gleason scores ranging from 6 to 8. From each patient, two different samples of normal, two samples of high-grade PIN, and four samples of prostate cancer were available. To compare the expression levels from patients without prostate cancer, normal prostate tissue was included from prostates of patients who underwent cystoprostatectomy. We introduced four tissue arrays of eight prostate cancer patients. Additionally, six samples were from CRPC. All those patients have received therapy with luteinizing hormone-releasing hormone antagonists. In three cases, docetaxel was given after failure of hormonal therapy.

Immunohistochemistry and TMA. TMAs were assembled using a manual tissue puncher. The different samples were punched out from selected regions of donor tissue and assembled into a new paraffin block. Tissue cores were 2 mm in diameter, and the length ranged from 4 to 6 mm. TMAs were sectioned (4 μm) and stained with H&E to verify histology. The immunohistochemistry stainings were performed on 4-μm sections. For the antigen retrieval, the slides were microwaved for 7 min in 0.01 mol/L citrate buffer (pH 6) and then allowed to cool for 30 min. After washing with PBS (pH 7.4), the endogenous peroxidase activity was inactivated in a solution containing 3% hydrogen peroxidase in PBS. The pretreated slides were blocked with an Avidin/Biotin Blocking kit (SP-2001, Vector Laboratories) and a Super block (IDST1007, IDetect Superstain System HRP, ID Labs.) between washing steps. The primary anti–SOCS-3 antibody (Santa Cruz) was diluted 1:300 in PBS containing 1% bovine serum albumin and incubated overnight at 4°C. For Bcl-2 immunohistochemistry, an antibody from DAKO (M0887) was used at a dilution of 1:80 and staining was performed after microwave treatment in citrate buffer. On the next day, the slides were washed with PBS, and the IDetect Superstain System HRP was used for immunodetection. The substrate detection was performed using the AEC Chromogen/Substrate kit (BP1108, ID Labs.). Slides were counterstained in Mayer's hematoxylin, cleared, and coverslipped. The percentage

of SOCS-3–positive cells was determined. Pictures were acquired using Tissuequest software (TissueGnostics). The percentage of SOCS-3–positive cells was determined using Histoquest software (TissueGnostics).

Statistical analysis. For each treatment group, statistical distribution was determined using Kolmogorov-Smirnov test. Because of non-Gaussian distribution, nonparametric tests were applied as follows. To assess the overall significance for experiments with more than one treatment group, we used the Kruskal-Wallis test. To confirm statistically significant findings in the Kruskal-Wallis test, the Mann-Whitney *U* test was applied. *P* values of <0.05 in the Mann-Whitney *U* test were defined as statistically significant and encoded in figures (*, $P < 0.05$; **, $P < 0.01$). All statistical analyses were performed using SPSS 12.0 software (SPSS).

Results

Inhibition of SOCS-3 expression in prostate cancer cells. To investigate the role of SOCS-3 in regulation of prostate cancer cell death, we used a siRNA approach. To obtain a high-transfection efficiency and maintain low SOCS-3 mRNA and protein levels, we treated SOCS-3–positive DU-145, LNCaP-IL-6+, and PC3 cell lines twice with either 2 nmol/L of specific siRNA against SOCS-3 (25) or control siRNA during 6 days. On day 6, the cells were harvested and SOCS-3 mRNA and protein expression levels were measured by quantitative reverse transcription–PCR (qRT-PCR) and Western blot, respectively. We found a 70% to 80% decrease of SOCS-3 mRNA (Fig. 1*A*) and protein (Fig. 1*B*) compared with the controls in all three cell lines.

Decrease of SOCS-3 leads to reactivation of STAT3 and STAT1. In previous studies, we showed that STAT3 could not be activated by IL-6 in SOCS-3–positive prostate cancer cell lines (22). To elucidate if SOCS-3 down-regulation is sufficient for STAT3 reactivation, we treated DU-145 and LNCaP-IL-6+ cells with 20 ng/mL IL-6 for 15 min (Fig. 1*C*). The SOCS-3–negative parental cell line LNCaP ATCC was used as a positive control. In that cell line, phosphorylation of STAT3 can be induced by IL-6. SOCS-3 down-regulation was sufficient to reactivate STAT3 in DU-145 and LNCaP-IL-6+ cells. Because modulation of SOCS-3 expression may affect other members of the STAT family, we examined the implications of siRNA treatment on the levels of phosphorylated STAT1. Interestingly, we found that down-regulation of SOCS-3 enhances STAT1 phosphorylation in LNCaP-IL-6+ and DU-145 cells (Supplementary Fig. S1).

SOCS-3 down-regulation causes growth inhibition. To investigate the consequences of SOCS-3 down-regulation, cell proliferation was measured using [^{3}H]thymidine incorporation assay for DU-145, LNCaP-IL-6+, and PC3 cells (Fig. 2). The LNCaP ATCC cell line was used as a negative control (Fig. 2). All three cell lines responded to a down-regulation of SOCS-3 by a significant 50% to 80% decrease in cell proliferation. As expected, there was no change in the proliferation of the LNCaP ATCC cell line. In concordance with a significant decrease in proliferation, we also observed a change in cell morphology and decreased viability of cells treated with specific siRNA, compared with cells treated with control siRNA or with LNCaP ATCC cells, which did not change in morphology or viability at all (Fig. 3*A*).

Cells with decreased SOCS-3 levels undergo apoptosis. Because most of these cells seemed dead under the microscope, we hypothesized that changes in the apoptosis rate occur. Apoptosis was measured with PI staining 3 days after the second transfection. We found that a significant number of DU-145, LNCaP-IL-6+, and PC3 cells treated with specific siRNA against SOCS-3 underwent apoptosis, whereas only a few apoptotic cells were observed in the controls (Fig. 3*B*). LNCaP ATCC cells did not show an increase of apoptosis after treatment with SOCS-3–specific siRNA. Furthermore, the activities of the key executioners of both apoptotic pathways, caspase-3/caspase-7, caspase-8, and caspase-9, significantly increased in DU-145 and LNCaP-IL-6+ (Fig. 4*A*) but not in the LNCaP ATCC cell line. In the cell lines, the activity of caspase-3 was four times, the activity of caspase-8 was up to four times, and that of caspase 9 was about twice higher than in the controls. The p85 fragment of PARP, cPARP, which is cleaved by activated caspase-3 and represents the end point of the intrinsic apoptosis pathway, was also significantly increased in cells treated with specific siRNA in all three SOCS-3–positive cell lines, whereas no cPARP expression was detectable in LNCaP ATCC cells (Fig. 4*B*). To understand the mechanism, we looked at expression levels of the antiapoptotic proteins Mcl-1 and Bcl-2. In DU-145 and PC3 cells, there were no significant changes in Mcl-1 expression levels, whereas we could observe a significant decrease of the protein in LNCaP-IL-6+ cells (Fig. 5*A*). Furthermore, Bcl-2 protein levels decreased significantly in DU-145 and PC3 cells to 50% and in LNCaP-IL-6+ cells to ~30% compared with the control siRNA treatment (Fig. 5*B*).

Overexpression of Bcl-2 rescues cells with low SOCS-3 levels from apoptosis. To determine if Bcl-2 is crucial for the survival of cells with low SOCS-3 expression levels, we cotransfected DU-145 and LNCaP-IL-6+ cells with 2 nmol/L control siRNA, siRNA against SOCS-3, or mock and 0.5 μg/mL pEGFP-Bcl-2 or empty vector as indicated twice and measured apoptosis afterwards (Fig. 5*C*). In both cell lines, we observed a significant decrease in the percentage of apoptotic cells that were transfected with the Bcl-2 vector and siRNA against SOCS-3 together compared with cells that were treated with siRNA against SOCS-3 alone.

SOCS-3 is overexpressed in CRPC. To get a better insight into SOCS-3 expression levels during cancer development and progression, we implemented TMAs using patient samples (Fig. 6). Only a weak expression of SOCS-3 (29.66% positive cells) was detected in the basal prostate epithelial cell layer. In PIN and carcinoma samples, we could observe an increase of SOCS-3–positive epithelial cells (PIN 58.68%, carcinoma 89.32%; Fig. 6*A*) compared with the benign prostate tissue. These differences were found to be statistically significant ($P < 0.001$). In samples from CRPC patients, expression of SOCS-3 was even higher (benign tissue 46.63%, carcinoma 93.80%, $P < 0.001$; Fig. 6*B*). To better understand the relationship between SOCS-3 expression and programmed cell death *in vivo*, we have performed TMAs for Bcl-2. The highest expression of Bcl-2 was detected in PIN samples obtained from cancer patients, followed by benign tissue (Fig. 6*C*). A small percentage of Bcl-2–positive cells was detected in tumors, whereas no Bcl-2 positivity was detected in specimens from CRPC patients (Fig. 6*D*).

Discussion

Deregulation of expression of STAT3 or activation of the JAK-STAT pathway are implicated in many diseases including cancer. Although recent studies have linked STAT3 to advanced tumor growth and metastatic progression of several carcinomas (28–32), the function of STAT3 in prostate cancer seems to be more complex. Differences in STAT3 phosphorylation reported by various laboratories may be in part explained by results from a recent publication of Kreis and associates. The authors showed that STAT3 activity depends on cell density in melanoma models,

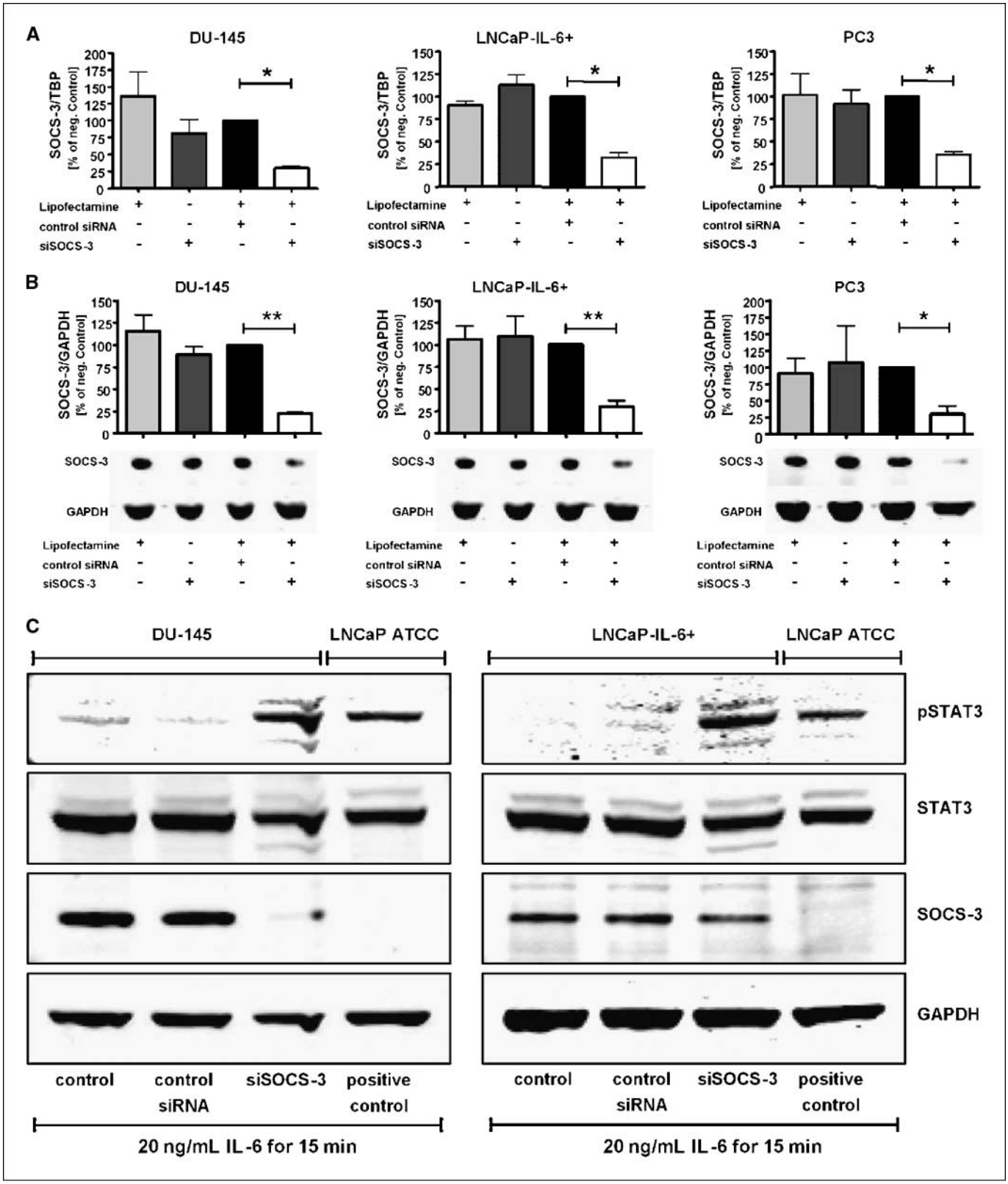

Figure 1. *A,* SOCS-3 mRNA down-regulation after treatment with siRNA. DU-145, LNCaP-IL-6+, and PC3 cells were treated twice with 2 nmol/L siRNA against SOCS-3 during a period of 6 d. The cells were then harvested, and qRT-PCR was performed. The results are expressed in relation to values measured in cells treated with negative control siRNA and represent mean values ± SEM from at least three independent experiments (*, $P < 0.05$; **, $P < 0.01$, Mann-Whitney *U* test). *B,* SOCS-3 protein down-regulation after treatment with siRNA. DU-145, LNCaP-IL-6+, and PC3 cells were treated with SOCS-3 siRNA as described above. The cells were then harvested, and Western blots were performed. The results are expressed in relation to values measured in cells treated with negative control siRNA and represent mean values ± SEM from at least three independent experiments (*, $P < 0.05$, Mann-Whitney *U* test). *C,* STAT3 phosphorylation after SOCS-3 protein down-regulation. After two consecutive transfections with SOCS-3 siRNA, DU-145 and LNCaP-IL-6+ cells were treated with 20 ng/mL IL-6 for 15 min.

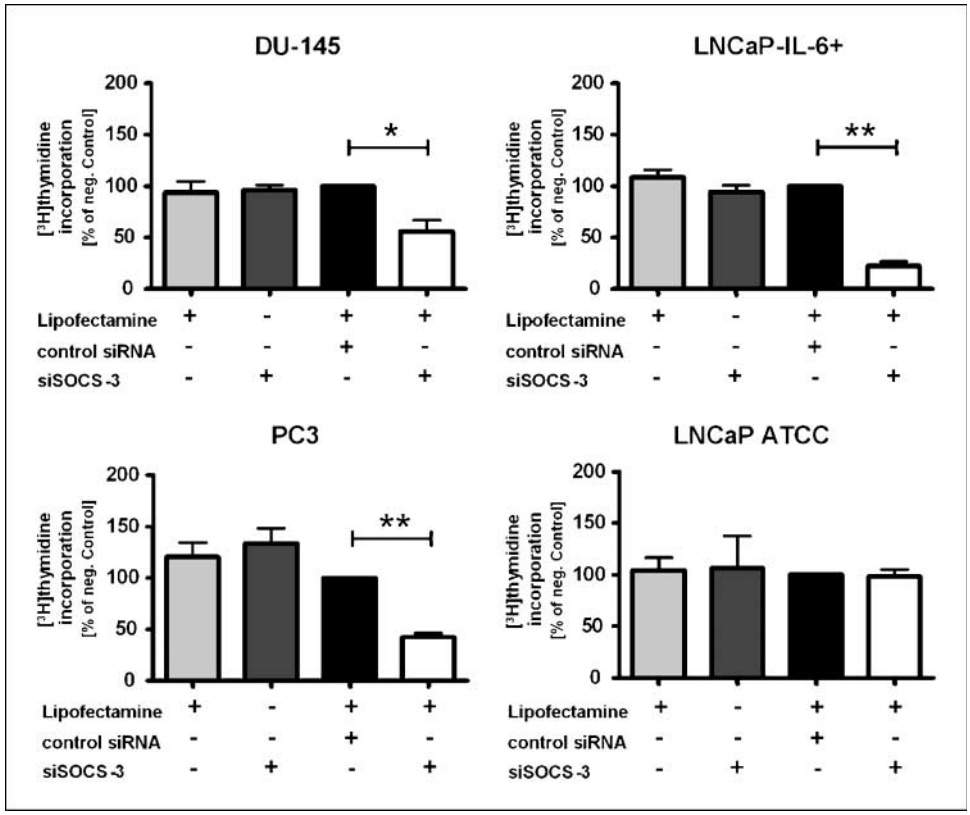

Figure 2. Regulation of cellular proliferation in DU-145, LNCaP-IL-6+, PC3, and LNCaP ATCC cells. Down-regulation of SOCS-3 causes a decrease in proliferation in all SOCS-3–positive cell lines. The treatment of cells was the same as described above. Cellular proliferation was assessed by measurement of [^{3}H]thymidine incorporation (mean values ± SEM at least three independent experiments; *, $P < 0.05$; **, $P < 0.01$, Mann-Whitney *U* test). The percentage of cells treated with control siRNA was set at 100%.

and these findings may also be relevant to carcinoma of the prostate (33). The transcription factor has been implicated in promotion of apoptosis or growth arrest under certain conditions (4, 34, 35). However, the molecular basis for such a dual function of STAT3 has not been clarified yet. It is known that various regulators of STAT3, such as protein inhibitors of activated STAT (PIAS) and the family of SOCS proteins, can determine the activation status of this transcription factor. In previous studies, we were able to show that STAT3 phosphorylation inversely correlates with SOCS-3 expression *in vitro*. In prostate cancer cell lines that express SOCS-3, STAT3 could not be phosphorylated (22). In the present manuscript, we investigated biological effects of SOCS-3 down-regulation in DU-145, LNCaP-IL6+, and PC3 prostate cancer cell lines, which are considered models for an advanced disease. Down-regulation of SOCS-3 with specific siRNA resulted in restoration of STAT3 phosphorylation induced by IL-6 in DU-145 and LNCaP-IL-6+ cells. Thus, down-regulation of SOCS-3 seems to be crucial for STAT3 phosphorylation in these cell lines. These data are consistent with the *in vitro* results of Lu and colleagues, who showed STAT3 phosphorylation after down-regulation of SOCS-3 in mouse embryonic fibroblasts. Furthermore, they proved that loss of SOCS-3 gene expression converts the antiapoptotic function of STAT3 into proapoptotic (35). It is known that SOCS-3 is an important feedback regulator of the IL-6-JAK-STAT pathway and SOCS-3 expression can be induced by IL-6, cyclic AMP, and androgens (22, 23). Cell lines in the present study in which apoptosis was induced are IL-6–positive, and therefore, our results support the contention that IL-6–induced apoptosis through STAT3 phosphorylation occurs after SOCS-3 inhibition.

In prostate cells with elevated levels of SOCS-3, we observed a suppression of androgen-regulated cell growth (23). In the present manuscript, we show for the first time that down-regulation of SOCS-3 leads to a decrease in proliferation of prostate cancer cells. Taken together with the results of our previous study (23), one could hypothesize that different functions of SOCS-3 in prostate cancer depend on the presence of the androgen receptor (AR). Decreased SOCS-3 levels result in an increase of apoptosis in all tested SOCS-3–positive prostate cancer cell lines in which AR expression is absent. SOCS-3 expression in AR-positive cell lines is heterogenous; we show in the present paper that VCaP cells are, similar as LNCaP, SOCS-3 negative (Supplementary Fig. S2), whereas LAPC-4 cells express SOCS-3 protein (22). In VCaP cells, induction of pSTAT3 after IL-6 treatment was observed.

Other studies have indicated that SOCS-3 effects are not limited solely to IL-6 signaling. Masuhiro and associates showed that SOCS-3 is an important key regulator in cell cycle progression (36). It is influenced by mutual interaction between SOCS-3 and the transcriptional factor DP-1. Therefore, it seems that SOCS-3 is implicated in the regulation of cell cycle and programmed cell death. We also showed that increased apoptosis after SOCS-3 down-regulation is associated with a significant increase of active proapoptotic caspase-8, caspase-9, and caspase-3/caspase-7, key proteases of both apoptosis pathways. Induced caspase activity was correlated with increased levels of cPARP. Cell survival is dependent on a complex interplay between proapoptotic and antiapoptotic proteins. To understand the mechanism responsible for increased apoptosis after SOCS-3 down-regulation, we looked for changes in the expression levels of the antiapoptotic proteins of the Bcl-2 family, Mcl-1, and Bcl-2. In previous studies, it was shown that Mcl-1 expression increases during tumor progression, whereas contradictory results on Bcl-2 are presented in the literature (37–40). In a recent publication, we showed that Mcl-1 is regulated by

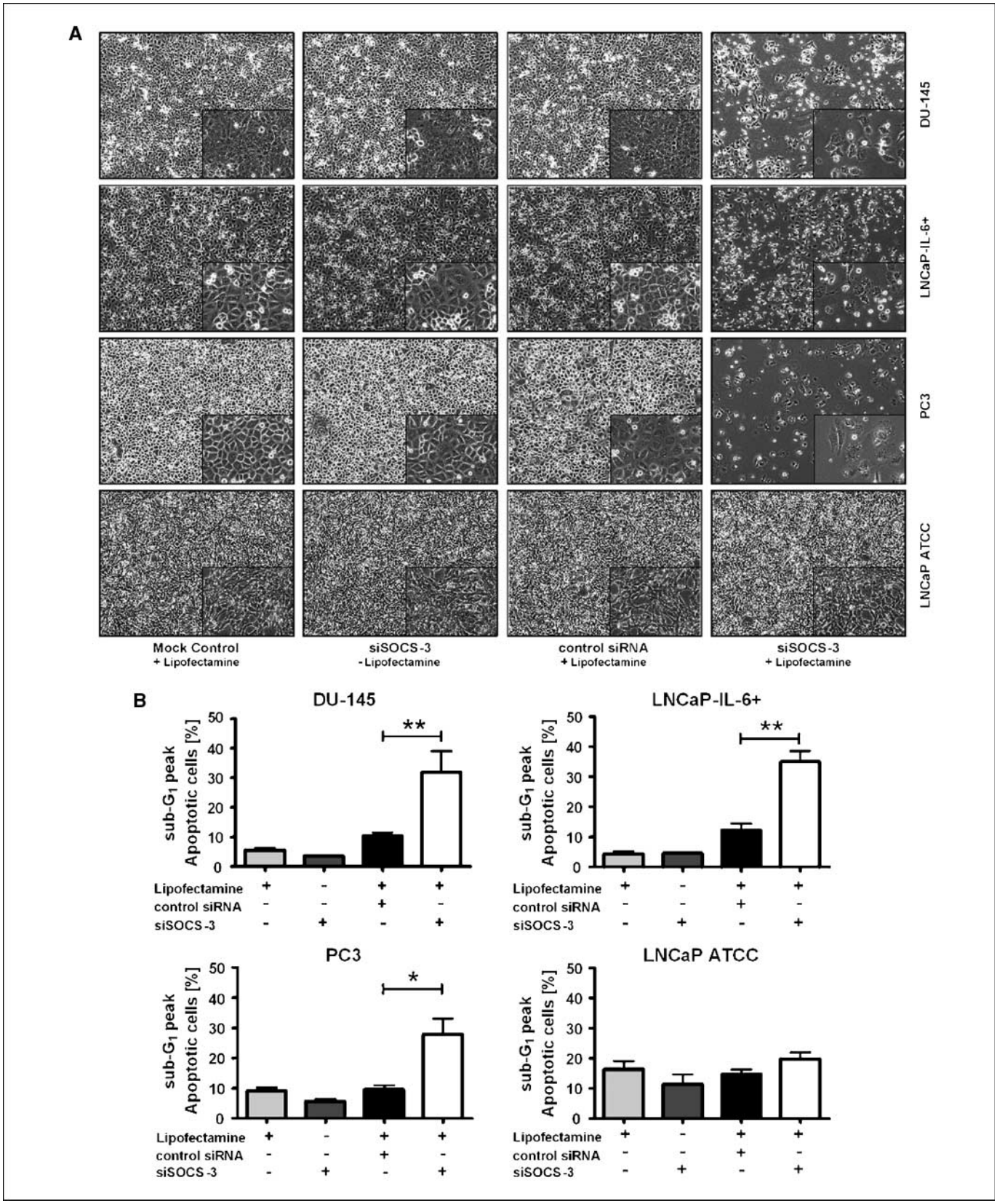

Figure 3. SOCS-3 protein down-regulation causes a decrease in cell viability and increase in the apoptotic rate after treatment with specific siRNA in all SOCS-3–positive cell lines but not LNCaP ATCC cells. *A,* DU-145, LNCaP-IL-6+, PC3, and LNCaP ATCC cells were treated twice with control siRNA or siRNA against SOCS-3. On day 6, the pictures of all cells were captured (magnifications, 40× and 200×). *B,* determination of the apoptosis rate in DU-145, LNCaP-IL-6+, PC3, and LNCaP ATCC cells. The apoptotic rate was assessed by PI staining and flow cytometry (mean values ± SEM from at least three independent experiments; *, $P < 0.05$; **, $P < 0.01$, Mann-Whitney *U* test).

IL-6 and mediates survival activity of the cytokine in models of late stage prostate carcinoma (41). In the present study, no alteration in expression levels of Mcl-1 could be observed in DU-145 and PC3 cells after SOCS-3 down-regulation. Only in the LNCaP-IL-6+ cell line, a down-regulation of SOCS-3 was associated with a decrease of Mcl-1. Thus, regulatory mechanisms in prostate cancer differ from those in cholangiocarcinoma cells in which SOCS-3 inhibits IL-6 induction of Mcl-1 (42). In contrast, Bcl-2 levels decreased significantly in all tested prostate cancer cell lines. These findings could be discussed in view of published results of Vanasse and colleagues (43). They showed that overexpression of Bcl-2 results in induction of SOCS-3 in mouse embryonic fibroblasts and hematopoietic cell lines suggesting a Bcl-2–associated mechanism underlying SOCS-3 induction. A colocalization of Bcl-2 and SOCS-3

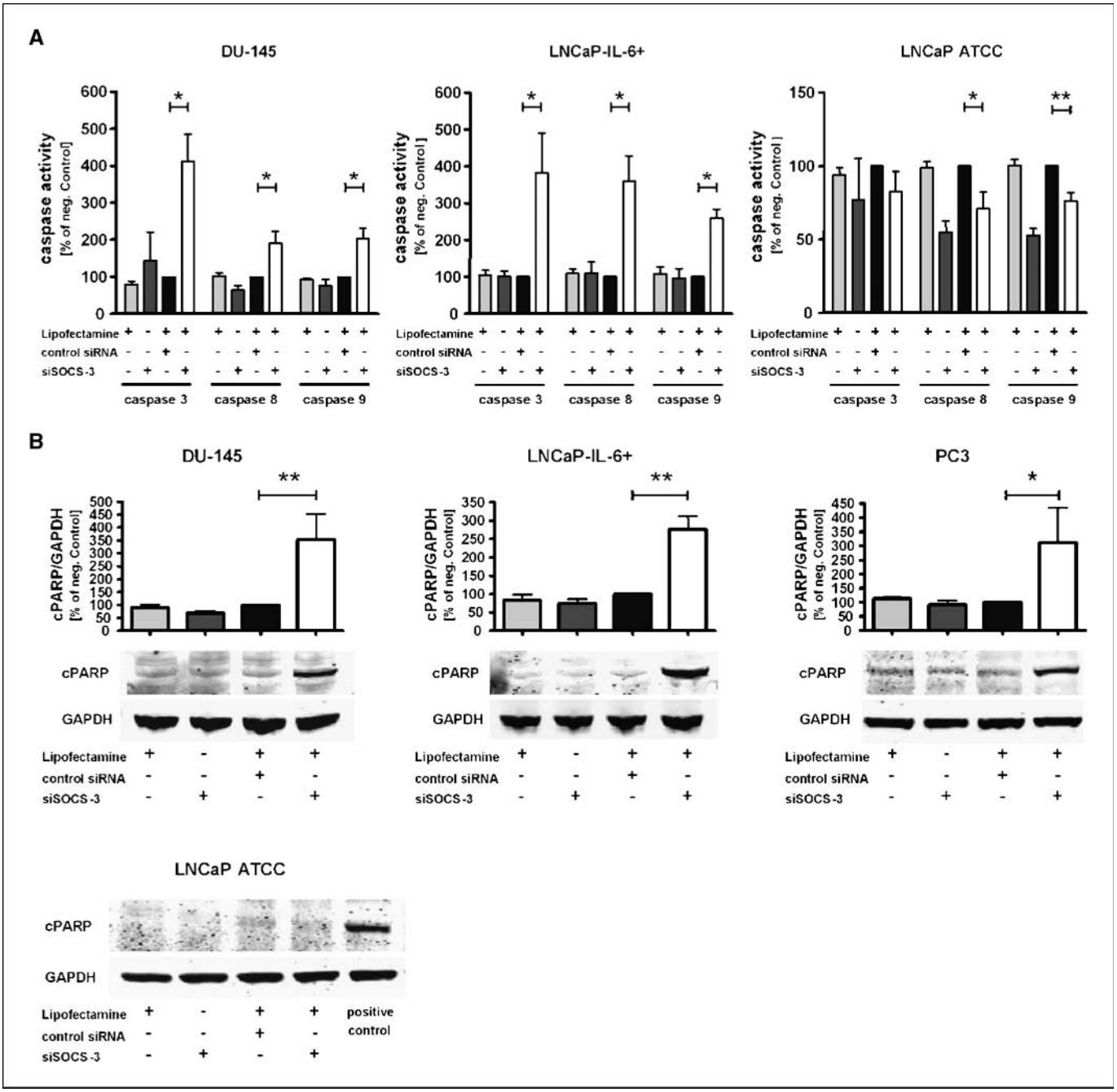

Figure 4. Increased activation of the proapoptotic caspase-3, caspase-8, and caspase-9 and PARP cleavage after SOCS-3 down-regulation. *A,* DU-145, LNCaP-IL-6+, and LNCaP ATCC cells were treated with SOCS-3 siRNA. A significant increase of the caspase-3/caspase-7, caspase-8, and caspase-9 activity could be observed in the SOCS-3–positive cell lines LNCaP-IL-6+ and DU-145, but not in the control cell line LNCaP ATCC. The results are expressed in relation to values measured in cells treated with negative control siRNA and represent mean values ± SEM from at least three independent experiments (*, $P < 0.05$; **, $P < 0.01$, Mann-Whitney *U* test). *B,* cPARP expression levels after SOCS-3 down-regulation. DU-145, LNCaP-IL-6+, PC3, and LNCaP-ATCC cells were treated with siRNA against SOCS-3. cPARP expression significantly increased in all three SOCS-3–positive cell lines. The results are expressed in relation to values measured in cells treated with negative control siRNA and represent mean values ± SEM from at least three independent experiments (*, $P < 0.05$; **, $P < 0.01$, Mann-Whitney *U* test).

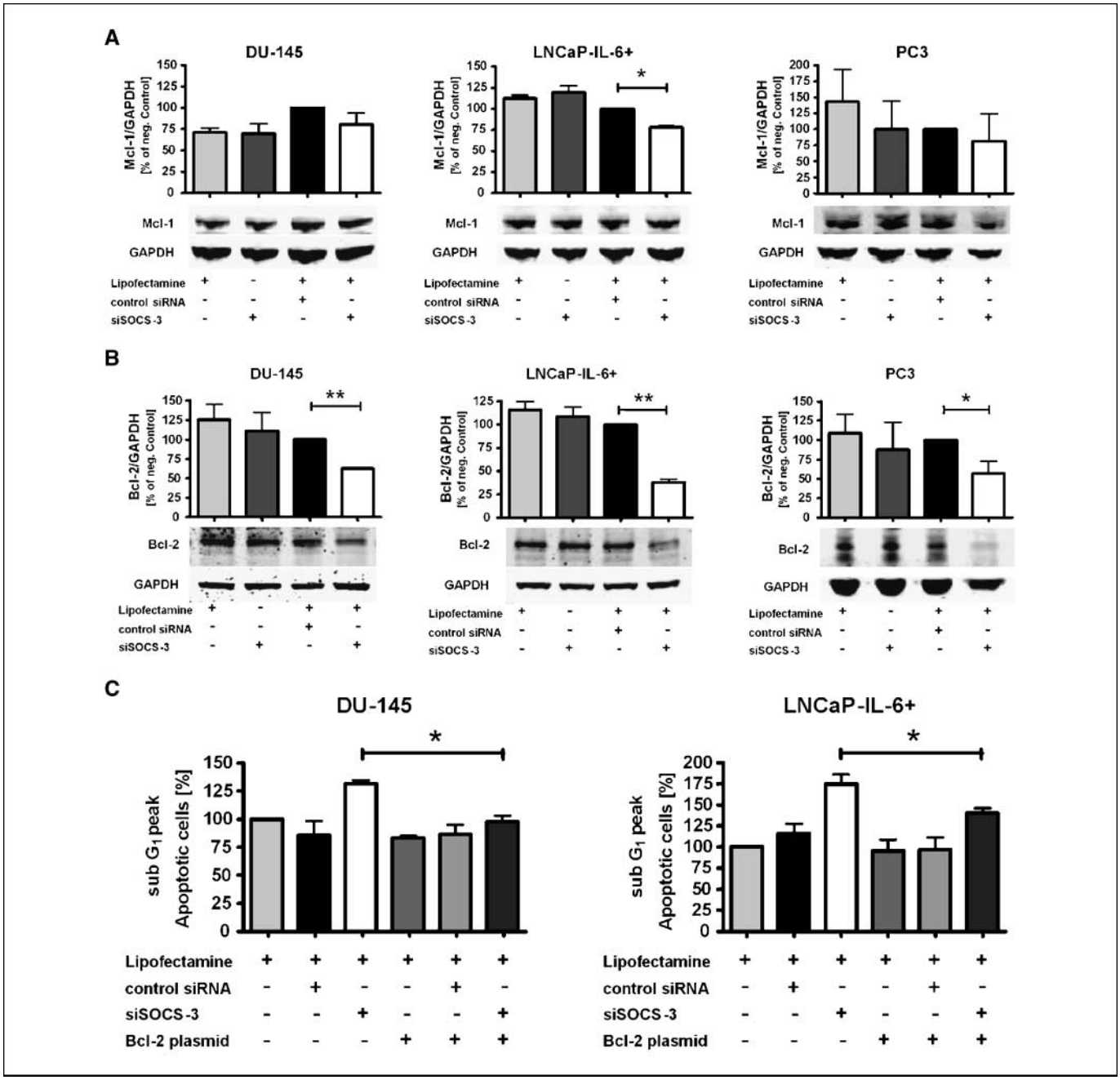

Figure 5. Expression of Mcl-1 (*A*) and Bcl-2 (*B*) after SOCS-3 down-regulation. DU-145, LNCaP-IL-6+, and PC3 cells were treated with siRNA against SOCS-3 as described above. The cells were then harvested, and Western blots for Mcl-1 and Bcl-2 were performed. The results are expressed in relation to values measured in cells treated with negative control siRNA and represent mean values ± SEM from at least three independent experiments (*, $P < 0.05$; **, $P < 0.01$, Mann-Whitney *U* test). *C,* determination of apoptosis in DU-145 and LNCaP-IL-6+ cells. Cells were seeded in six wells and cotransfected on the following day with control or SOCS-3 siRNA and 0.5 μg/mL pEGFP-Bcl2 or empty vector as indicated. Cell pellets were lyzed in PI buffer and analyzed for DNA content by flow cytometry (mean values ± SEM from at least three independent experiments; *, $P < 0.05$, Mann-Whitney *U* test).

could be observed in *de novo* follicular lymphoma but not in benign tissue (43). Thus, it was concluded that induction of SOCS-3 is an early genetic event, influencing pathways important for the pathogenesis of follicular lymphoma. Nevertheless, we did not observe an induction of SOCS-3 after overexpression of Bcl-2 in prostate cancer cell lines (data not shown). To investigate if down-regulation of Bcl-2 is a trigger for increased apoptosis in SOCS-3–deficient cells, we overexpressed Bcl-2 in DU-145 and LNCaP-IL-6+ cells. A decrease in the percentage of apoptotic cells in both cell lines indicates that restored Bcl-2 expression levels could prevent cells lacking SOCS-3 from undergoing apoptosis. In PC3 cells in which Bax expression was shown, the Bcl-2/Bax ratio is therefore altered (44). In contrast, DU-145 and LNCaP-IL-6+ cells express Bcl-2 but not Bax (45). In future studies, it may be determined how SOCS-3 interacts with various proteins in the apoptosis pathways and which apoptosis pathway is initially activated.

In a previous study, we reported on increased SOCS-3 staining intensity in prostate cancer versus benign tissue (22). We could confirm and extend those previous data. In PIN and malignant tissue, the expression of SOCS-3 was up-regulated. The highest expression was found in tissue specimens of CRPC patients. An increase of SOCS-3 in prostate cancer development and progression can be explained by elevated IL-6 levels during carcinogenesis. It is well known that IL-6 exhibits pleiotropic effects *in vitro* and *in vivo* and SOCS-3 is the main negative feedback regulator of IL-6–mediated JAK-STAT activation. On the basis of the *in vitro* and *in vivo* results of the present study, we hypothesize that SOCS-3 not only has a classic negative feedback regulator function, but it is crucial for cancer cell survival in CRPC. Interestingly, Tu and colleagues did not detect Bcl-2 in metastatic prostate cancer (40). On the basis of increased expression of Bcl-2 and SOCS-3 in PIN, we hypothesize that the inhibition of apoptosis by SOCS-3 as described in the present manuscript may be particularly important during early malignant transformation in the prostate.

Accumulated evidence suggests that proapoptotic or antiapoptotic effects of SOCS-3 are cell type specific. In mammary gland development, SOCS-3 is essential for cell survival. Sutherland and colleagues could prove that silencing of the SOCS-3 gene leads to accelerated apoptosis through activation of STAT3 and induction of proapoptotic Bak and Bax proteins (46, 47). In many tumors, SOCS-3 is silenced through gene promoter hypermethylation. This leads to an increased proliferation and tumor growth (18–20). In those carcinomas, overexpression of SOCS-3 results in suppression of cell growth. Thus, it has been proposed that SOCS-3 has a tumor suppressor function. In contrast to those data, SOCS-3 is constitutively expressed in breast cancer and does not suppress growth of MCF-7 tumor cells (48). In a recent publication, it was however shown that SOCS-3 inhibits proliferation of T47D cells (49).

Taken together, in the present manuscript, we show for the first time that SOCS-3 is important for the survival machinery of prostate cancer cells and its down-regulation leads to increased

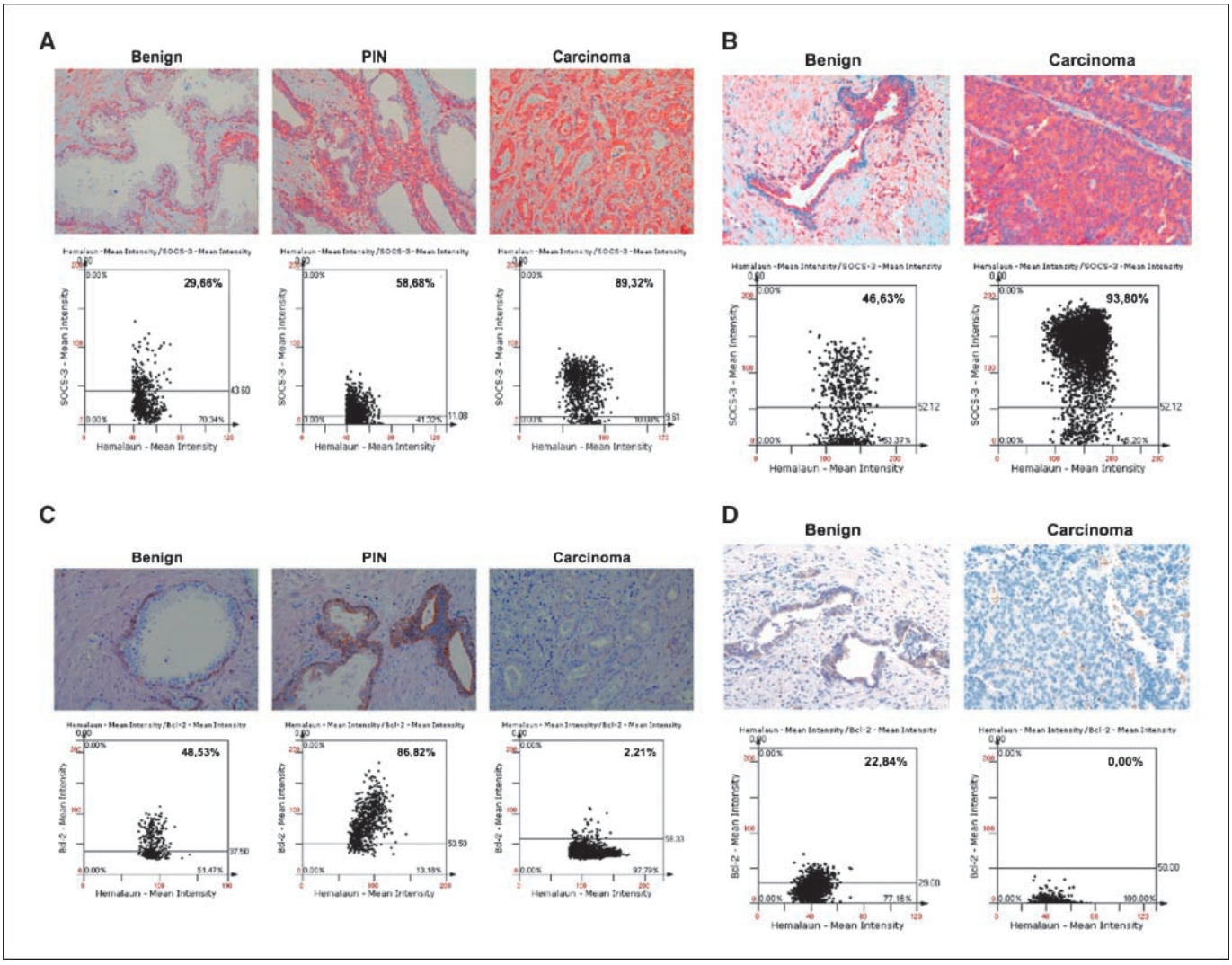

Figure 6. Expression of SOCS-3 (*A* and *B*) and Bcl-2 (*C* and *D*) in normal, PIN, and cancer tissue from a patient with prostate cancer who did not receive endocrine therapy (*A* and *C*) and normal and cancer tissue from a patient who failed therapy (*B* and *D*). Immunohistochemistry for SOCS-3 was performed using the primary antibody from Santa Cruz and IDetect Superstain System HRP for immunodetection. SOCS-3 expression levels were analyzed quantitatively using a single-cell resolution technique.

cell death through activation of the extrinsic and intrinsic apoptosis pathways. SOCS-3 expression is increased during development and progression of prostate cancer in clinical specimens. On the basis of the present results, we suggest that SOCS-3 may be a potential target for future therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 3/4/09; revised 6/16/09; accepted 7/13/09; published OnlineFirst 9/8/09.

Grant support: Austrian Science Fund FWF grants W1101, 19933, and 18193 (Z. Culig).

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.

We thank Prof. Pradip Roy-Burman, Dr. Ilaria T. Cavarretta, and Dr. Kamilla Malinowska for helpful discussions; Gertraud Sierek and Michaela Schlederer for excellent technical assistance; Robert Schober for the editorial assistance; Dr. Michele Barry for kindly providing the pEGFP-Bcl-2 construct used in the present study; Dr. Kenneth Pienta for providing VCaP cells; and Prof. Gero Kramer for providing patient information.

References

1. Hodge DR, Hurt EM, Farrar WL. The role of IL-6 and STAT3 in inflammation and cancer. Eur J Cancer 2005; 41:2502–12.

2. Klein B, Tarte K, Jourdan M, et al. Survival and proliferation factors of normal and malignant plasma cells. Int J Hematol 2003;78:106–13.

3. Burke WM, Jin X, Lin HJ, et al. Inhibition of constitutively active Stat3 suppresses growth of human ovarian and breast cancer cells. Oncogene 2001;20:7925–34.

4. Spiotto MT, Chung TD. STAT3 mediates IL-6-induced growth inhibition in the human prostate cancer cell line LNCaP. Prostate 2000;42:88–98.

5. Giri D, Ozen M, Ittmann M. Interleukin-6 is an autocrine growth factor in human prostate cancer. Am J Pathol 2001;159:2159–65.

6. Kamura T, Maenaka K, Kotoshiba S, et al. VHL-box and SOCS-box domains determine binding specificity for Cul2-Rbx1 and Cul5-Rbx2 modules of ubiquitin ligases. Genes Dev 2004;18:3055–65.

7. Wong CP, Bray TM, Ho E. Induction of proinflammatory response in prostate cancer epithelial cells by activated macrophages. Cancer Lett 2009;276:38–46.

8. DeMarzo AM, Nelson WG, Isaacs WB, Epstein JI. Pathological and molecular aspects of prostate cancer. Lancet 2003;361:955–64.

9. Hammacher A, Thompson EW, Williams ED. Interleukin-6 is a potent inducer of S100P, which is up-regulated in androgen-refractory and metastatic prostate cancer. Int J Biochem Cell Biol 2005;37:442–50.

10. Sasaki A, Yasukawa H, Suzuki A, et al. Cytokine-inducible SH2 protein-3 (CIS3/SOCS3) inhibits Janus tyrosine kinase by binding through the N-terminal kinase inhibitory region as well as SH2 domain. Genes Cells 1999;4:339–51.

11. Nicholson SE, De Souza D, Fabri LJ, et al. Suppressor of cytokine signaling-3 preferentially binds to the SHP-2-binding site on the shared cytokine receptor subunit gp130. Proc Natl Acad Sci U S A 2000;97:6493–8.

12. Kubo M, Hanada T, Yoshimura A. Suppressors of cytokine signaling and immunity. Nat Immunol 2003;4: 1169–76.

13. Alexander WS, Hilton DJ. The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response. Annu Rev Immunol 2004;22:503–29.

14. Larsen L, Ropke C. Suppressors of cytokine signalling: SOCS. APMIS 2002;110:833–44.

15. Roberts AW, Robb L, Rakar S, et al. Placental defects and embryonic lethality in mice lacking suppressor of cytokine signaling 3. Proc Natl Acad Sci U S A 2001;98: 9324–9.

16. Takahashi Y, Carpino N, Cross JC, Torres M, Parganas E, Ihle JN. SOCS3: an essential regulator of LIF receptor signaling in trophoblast giant cell differentiation. EMBO J 2003;22:372–84.

17. Marine JC, McKay C, Wang D, et al. SOCS3 is essential in the regulation of fetal liver erythropoiesis. Cell 1999;98:617–27.

18. He B, You L, Uematsu K, et al. SOCS-3 is frequently silenced by hypermethylation and suppresses cell growth in human lung cancer. Proc Natl Acad Sci U S A 2003;100:14133–8.

19. Niwa Y, Kanda H, Shikauchi Y, et al. Methylation silencing of SOCS-3 promotes cell growth and migration by enhancing JAK/STAT and FAK signalings in human hepatocellular carcinoma. Oncogene 2005;24:6406–17.

20. Weber A, Hengge UR, Bardenheuer W, et al. SOCS-3 is frequently methylated in head and neck squamous cell carcinoma and its precursor lesions and causes growth inhibition. Oncogene 2005;24:6699–708.

21. Evans MK, Yu CR, Lohani A, et al. Expression of SOCS1 and SOCS3 genes is differentially regulated in breast cancer cells in response to proinflammatory cytokine and growth factor signals. Oncogene 2007;26: 1941–8.

22. Bellezza I, Neuwirt H, Nemes C, et al. Suppressor of cytokine signaling-3 antagonizes cAMP effects on proliferation and apoptosis and is expressed in human prostate cancer. Am J Pathol 2006;169:2199–208.

23. Neuwirt H, Puhr M, Cavarretta IT, Mitterberger M, Hobisch A, Culig Z. Suppressor of cytokine signalling-3 is up-regulated by androgen in prostate cancer cell lines and inhibits androgen-mediated proliferation and secretion. Endocr Relat Cancer 2007;14:1007–19.

24. Hobisch A, Ramoner R, Fuchs D, et al. Prostate cancer cells (LNCaP) generated after long-term interleukin 6 (IL-6) treatment express IL-6 and acquire an IL-6 partially resistant phenotype. Clin Cancer Res 2001;7: 2941–8.

25. Komyod W, Bohm M, Metze D, Heinrich PC, Behrmann I. Constitutive suppressor of cytokine signaling 3 expression confers a growth advantage to a human melanoma cell line. Mol Cancer Res 2007;5:271–81.

26. Savinainen KJ, Saramaki OR, Linja MJ, et al. Expression and gene copy number analysis of ERBB2 oncogene in prostate cancer. Am J Pathol 2002;160:339–45.

27. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

28. Xie TX, Huang FJ, Aldape KD, et al. Activation of STAT3 in human melanoma promotes brain metastasis. Cancer Res 2006;66:3188–96.

29. Li WC, Ye SL, Sun RX, et al. Inhibition of growth and metastasis of human hepatocellular carcinoma by antisense oligonucleotide targeting signal transducer and activator of transcription 3. Clin Cancer Res 2006; 12:7140–8.

30. Silver DL, Naora H, Liu J, Cheng W, Montell DJ. Activated signal transducer and activator of transcription (STAT) 3: localization in focal adhesions and function in ovarian cancer cell motility. Cancer Res 2004;64:3550–8.

31. Kusaba T, Nakayama T, Yamazumi K, et al. Expression of p-STAT3 in human colorectal adenocarcinoma and adenoma; correlation with clinicopathological factors. J Clin Pathol 2005;58:833–8.

32. Abdulghani J, Gu L, Dagvadorj A, et al. Stat3 promotes metastatic progression of prostate cancer. Am J Pathol 2008;172:1717–28.

33. Kreis S, Munz GA, Haan S, Heinrich PC, Behrmann I. Cell density dependent increase of constitutive signal transducers and activators of transcription 3 activity in melanoma cells is mediated by Janus kinases. Mol Cancer Res 2007;5:1331–41.

34. Nakajima K, Yamanaka Y, Nakae K, et al. A central role for Stat3 in IL-6-induced regulation of growth and differentiation in M1 leukemia cells. EMBO J 1996;15: 3651–8.

35. Lu Y, Fukuyama S, Yoshida R, et al. Loss of SOCS3 gene expression converts STAT3 function from anti-apoptotic to pro-apoptotic. J Biol Chem 2006;281: 36683–90.

36. Masuhiro Y, Kayama K, Fukushima A, et al. SOCS-3 inhibits E2F/DP-1 transcriptional activity and cell cycle progression via interaction with DP-1. J Biol Chem 2008; 283:31575–83.

37. Krajewska M, Krajewski S, Epstein JI, et al. Immunohistochemical analysis of bcl-2, bax, bcl-X, and mcl-1 expression in prostate cancers. Am J Pathol 1996;148: 1567–76.

38. Krajewski S, Bodrug S, Krajewska M, et al. Immunohistochemical analysis of Mcl-1 protein in human tissues. Differential regulation of Mcl-1 and Bcl-2 protein production suggests a unique role for Mcl-1 in control of programmed cell death *in vivo*. Am J Pathol 1995;146:1309–19.

39. Colombel M, Symmans F, Gil S, et al. Detection of the apoptosis-suppressing oncoprotein bcl-2 in hormone-refractory human prostate cancers. Am J Pathol 1993; 143:390–400.

40. Tu H, Jacobs SC, Borkowski A, Kyprianou N. Incidence of apoptosis and cell proliferation in prostate cancer: relationship with TGF-β1 and bcl-2 expression. Int J Cancer 1996;69:357–63.

41. Cavarretta IT, Neuwirt H, Untergasser G, et al. The antiapoptotic effect of IL-6 autocrine loop in a cellular model of advanced prostate cancer is mediated by Mcl-1. Oncogene 2007;26:2822–32.

42. Isomoto H, Mott JL, Kobayashi S, et al. Sustained IL-6/STAT-3 signaling in cholangiocarcinoma cells due to SOCS-3 epigenetic silencing. Gastroenterology 2007;132: 384–96.

43. Vanasse GJ, Winn RK, Rodov S, et al. Bcl-2 overexpression leads to increases in suppressor of cytokine signaling-3 expression in B cells and *de novo* follicular lymphoma. Mol Cancer Res 2004;2:620–31.

44. Rokhlin OW, Bishop GA, Hostager BS, et al. Fas-mediated apoptosis in human prostatic carcinoma cell lines. Cancer Res 1997;57:1758–68.

45. Steiner H, Cavarretta IT, Moser PL, et al. regulation of growth of prostate cancer cells selected in the presence of interleukin-6 antibody CNTO 328. Prostate 2006;66: 1744–52.

46. Sutherland KD, Vaillant F, Alexander WS, et al. c-myc as a mediator of accelerated apoptosis and involution in mammary glands lacking Socs3. EMBO J 2006;25:5805–15.

47. Sutherland KD, Lindeman GJ, Visvader JE. Knocking off SOCS genes in the mammary gland. Cell Cycle 2007; 6:799–803.

48. Sutherland KD, Lindeman GJ, Choong DY, et al. Differential hypermethylation of SOCS genes in ovarian and breast carcinomas. Oncogene 2004;23:7726–33.

49. Barclay JL, Anderson ST, Waters MJ, Curlewis JD. SOCS3 as a tumor suppressor in breast cancer cells, and its regulation by PRL. Int J Cancer 2008;124: 1756–66.