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Tumorigenesis and Neoplastic Progression

Suppressor of Cytokine Signaling-3 Antagonizes cAMP Effects on Proliferation and Apoptosis and Is Expressed in Human Prostate Cancer

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Interleukin-6, levels of which are elevated in prostate cancer, activates different signal transduction pathways including that of Janus kinases/signal transducer and activator of transcription (STAT)3. However, phosphorylation of STAT3 has been reported to be associated with either stimulatory or inhibitory effects on cellular proliferation. To better understand the mechanisms of STAT3 regulation in benign and malignant prostate, we have investigated the role of suppressor of cytokine signaling (SOCS)-3. Cell lines that did not express phosphorylated STAT3 were found to be SOCS-3-positive. SOCS-3 was re-expressed in LNCaP cells after treatment with a demethylating agent. SOCS-3 immunohistochemistry revealed a negative or weak reaction in benign areas, whereas its expression was detected in tumor tissue. To investigate the involvement of SOCS-3 in regulation of cellular events, we incubated cancer cells with a cAMP derivative. This treatment yielded higher SOCS-3 levels, reduced [^{3}H]thymidine incorporation, and increased percentage of apoptotic cells. However, down-regulation of SOCS-3 by a short interfering RNA approach resulted in inhibition of proliferation and an increased apoptotic rate. Collectively, our results show that SOCS-3 antagonizes regulation of cellular events by cAMP and is expressed in human prostate cancer. ***(Am J Pathol 2006, 169:2199–2208; DOI: 10.2353/ajpath.2006.060171)***

Interleukin-6 (IL-6) expression and signaling in carcinogenesis have been thoroughly investigated because of the cytokine's association with chronic inflammation and its effects on various cellular functions. IL-6 is a 21- to 28-kd cytokine that is elevated in tissue extracts and sera from prostate cancer patients.[1,2] It has a pleiotropic role in carcinoma of the prostate. In a cell line derived from prostate intraepithelial neoplasia, primary prostate cancer cultures, and androgen-insensitive PC3 and DU-145 cells, IL-6 stimulates proliferation or acts as an anti-apoptotic cytokine.[1,3–5] In contrast, studies from various laboratories on IL-6 regulation of growth of androgen-sensitive LNCaP cells did not yield unequivocal results.[1,6] Treatment of prostate cells with IL-6 leads to stimulation of the transcription function of the androgen receptor, which is expressed in most prostate cancers and regulates cellular events.[7,8]

The IL-6 receptor, which is composed of the ligand-binding gp80 and the signal-transducing subunit gp130, is expressed in prostate cancer cell lines and tissue specimens.[9] It is evident that signaling pathways of Janus kinase (JAK)/signal transducer and activator of transcription (STAT)3, mitogen-activated protein kinase (MAPK), and phosphoinositol 3-kinase are implicated in IL-6 signal transduction in prostate cancer cells in different ways. Activation of MAPK occurs in various cell lines associated with a proliferative response, whereas phosphorylation of Akt indicates the role of the cytokine in inhibition of apoptosis.

In various types of cancer, the oncogenic role of STAT3 is well documented.[10,11] STAT3 may prevent death of tumor cells, stimulates expression of angiogenetic factors, and induces expression of extracellular matrix degradation enzymes, thus promoting metastasis. However, it seems that the role of STAT3 in prostate

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cancer is more complex because its phosphorylation in association with either promotion or inhibition of growth has been observed.[1,6] One possible reason for variable outcome of experiments in which IL-6 regulation of growth was investigated are differences in requirement of the tyrosine phosphatase SHP-2 or the adaptor protein Shc that give rise to activation of intracellular signaling pathways to the gp130 subunit.[12] In addition, STAT function is regulated by suppressors of cytokine signaling (SOCS), protein inhibitors of activated STAT, caveolin-1, and members of the Rho family of small GTP-binding proteins.[13]

The role of negative regulators of cytokine signaling, ie, SOCS, has been investigated in several malignant diseases. SOCS are induced by cytokines in a rapid manner and exert their action through different mechanisms such as binding to the phosphorylated cytokine receptor or JAK.[14] The SOCS family has eight members, SOCS 1 to 7 and CIS. They contain a variable amino-terminal region, a central SH2 domain, and a conserved carboxy-terminal domain termed the SOCS box. Alterations in expression of SOCS proteins were observed in human lung, liver, and squamous head and neck cancer.[15–17] Those studies revealed that SOCS proteins are implicated in regulation of cellular proliferation and apoptosis. Furthermore, SOCS-3 has been identified as a regulator of myoblast differentiation.[18]

To understand better the mechanisms of IL-6/JAK/STAT3 signaling in benign and malignant prostate tissue, we have assessed expression of SOCS-3 in prostate cells and in material obtained from patients. The mechanisms responsible for changes in SOCS-3 expression were investigated. We have addressed the question of whether modulation of SOCS-3 levels has an impact on prostate cancer cellular events.

Materials and Methods

Cell Culture and Chemicals

Prostate cancer cells were obtained from American Type Culture Collection (Rockville, MD). LNCaP sublines LNCaP-IL-6$^+$ and LNCaP-IL-6$^-$ were derived in the presence or absence of IL-6, respectively, as described elsewhere.[19] BPH-1 cells were a kind gift from Dr. Jack Schalken (University Medical Center St. Radboud, Nijmegen, The Netherlands), and LAPC-4 cells were kindly provided by Dr. Charles Sawyers (University of California, Los Angeles, CA). LAPC-4 cells were maintained in IMDM medium containing 10 nmol/L R1881 (Perkin Elmer, Montreal, QC, Canada). LNCaP cells were cultured in MCDB-131 supplemented with sodium pyruvate, glucose, and HEPES buffer, pH 7.2 (Invitrogen, Leek, The Netherlands). We found that attachment of LNCaP cells is improved when MCDB-131 is used.[20] All other cell lines were cultured in RPMI 1640 (HyClone, Logan, UT). Media were supplemented with 10% fetal calf serum and penicillin/streptomycin. Dibutyryl adenosine 3′5′-cyclic-phosphate was purchased from Sigma (Deisenhofen, Germany) and Fugene 6 from Roche (Vienna, Austria).

Real-Time Polymerase Chain Reaction (PCR)

Cells were grown in 100-mm Petri dishes. For studies on promoter methylation, LNCaP and LNCaP-IL-6$^-$ cells were maintained in medium supplemented with 5-aza-2′-cytidine (1 and 5 μmol/L) for 4 days followed by a 1-hour stimulation with IL-6 (10 ng/ml). Subsequently, total RNAs were isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. cDNA synthesis was performed using Superscript III RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR conditions were as follows: one cycle of denaturing at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. All amplifications were performed in triplicates. TATA-Box binding protein (TBP) was chosen as an endogenous expression standard.[21] Primer and probe sequences were as follows: SOCS-3 (forward 5′-TGATCCGCGACAGCTCG-3′; reverse 5′-TCCCAGACTGGGTCTTGACG-3′; TaqMan probe 5′-FAM-CCAGCGCCACTTCTTCACGCTCA-TAMRA-3′), TBP (forward 5′-CACGAACCACGGCACTGATT-3′; reverse 5′-TTTTCTGCTGCCAGTCTGGAC-3′; TaqMan probe 5′-FAM-TCTTCACTCTTGGCTCCTGTGCACA-TAMRA-3′). Each reaction was performed in a 20-μl volume containing 50 ng of cDNA and 10 μl of 2× ABI Mastermix (Applied Biosystems, Foster City, CA). 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, Rotkreuz, Switzerland). Ct values of SOCS-3 and TBP as assessed by ABI sequence detection software (version 1.3) were used to calculate the ΔCt using Microsoft Excel 2002. Values obtained in control cells were defined as 100%, and those from treated cells were expressed as percentage of control.

Western Blot

Cells were harvested, washed twice with phosphate-buffered saline (PBS), and lysed. The total protein was quantified using the Bradford method.[22] Fifty μg of protein per lane were then resolved using a 4 to 12% Bis-Tris gel (Invitrogen, Leek, The Netherlands) and transferred onto a nitrocellulose membrane (Invitrogen). The membrane was blocked for 1 hour using Starting Block (TBS) buffer (Pierce Biotechnology, Rockford, IL) and incubated at 4°C overnight with a primary antibody. This step was followed by incubation for 1 hour at room temperature with fluorescence-labeled secondary antibodies (Molecular Probes, Eugene, OR). The membranes were scanned and quantified using the Odyssey infrared imaging system (LiCor Biosciences, Lincoln, NE). The following antibodies were used for Western blots: SOCS-3 (1:1000; Acris Antibodies, Hiddenhausen, Germany), GAPDH (1:100,000; Chemicon Int., Temecula, CA), phospho-STAT3 (1:1000; Upstate Cell Signaling Technology, Beverly, MA), and STAT3 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA).

Patient Samples

Samples were obtained from 18 patients with diagnosed prostate cancer by radical prostatectomy. Two samples were obtained by transurethral resection of the prostate, and two were biopsy samples.

Immunohistochemistry

SOCS-3 and phosphorylated STAT3 (pSTAT3) immunostaining were performed using the Ventana Discovery autostainer (Ventana Medical Systems, Tucson, AZ). For deparaffinization of slides, Ventana EZ prep buffer was used at 76°C. For antigen retrieval, Cell Conditioner 1 mild reagent was applied at 95°C for 30 minutes, followed by 30 minutes at 100°C. Then the samples were treated with EZ prep cell conditioner volume adjusted for 4 minutes, followed by two additions of Cell Conditioner 1 mild reagent. The primary anti-SOCS-3 antibody from Santa Cruz was applied manually at 1:100 dilution and incubated for 30 minutes. This step was followed by incubation with the biotinylated IgG for 8 minutes. The RedMap kit was used for detection. The sections were then counterstained with hematoxylin and bluing reagent (0.1 mol/L lithium carbonate, 0.5 mol/L sodium bicarbonate). For immunodetection of $pSTAT3^{tyr705}$, a monoclonal antibody from Santa Cruz (clone B-7) was applied at 1:20 dilution.

[^{3}H]Thymidine Incorporation Assay

Cells (LNCaP-IL-6$^+$, PC3) were seeded at a density of 1×10^4/well and incubated with db cAMP (1 to 5 mmol/L; Sigma, Vienna, Austria) for 48 and 72 hours in triplicates in 96-well plates, and 50 μl of [^{3}H]thymidine (1 μCi/well) were added. After a 16-hour incubation, cells were frozen at −18°C. After thawing, DNA was harvested on 96-well filter plates (UniFilter; Perkin Elmer, Boston, MA). Fifty μl of scintillation fluid were added, and radioactivity was quantified using Chameleon 5025 liquid scintillation counter (HVD Life Sciences, Vienna, Austria).

Flow Cytometry

Cells (LNCaP-IL-6$^+$, PC3) were seeded onto 12-well plates at a density of 8×10^4/well and incubated with db cAMP (1 to 5 mmol/L) for 48 and 72 hours. Trypsinized cells were washed once in ice-cold PBS and centrifuged at $200 \times g$ for 5 minutes. The pellet was resuspended in labeling buffer (50 μg/ml propidium iodide, 1 mg/ml sodium citrate, and 1 μl/ml Triton X-100 in PBS) and kept light protected at 4°C for 2 hours. Cell cycle and apoptosis were analyzed using FACS-Calibur (Becton Dickinson, San Jose, CA) with gating set on FL3-A and FL3-W to exclude doublets and forward and side scatter to exclude cell debris.

Short Interfering RNA (siRNA) Transfection

Cells were grown as described before for Western blot, [^{3}H]thymidine incorporation assay, and flow cytometry, respectively. siRNA transfections were performed with Fugene 6 reagent according to the manufacturer's protocol. Both control siRNA (catalog no. 4611) and SOCS-3 siRNA (catalog no. 16708A) were purchased from Ambion (Austin, TX). The SOCS-3 siRNA sequence was as follows: sense 5′-GAACCUGCGCAUCCAGUGUtt-3′ and anti-sense 5′-ACACUGGAUGCGCAGGUUCtt-3′.

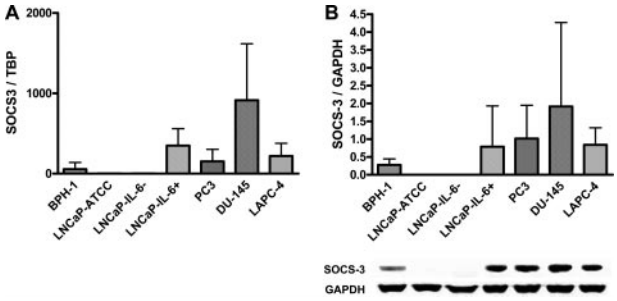

Figure 1. Basal expression of SOCS-3 mRNA (**A**) and protein (**B**) in benign and malignant prostate cells. SOCS-3 was measured by real-time PCR and Western blot, respectively. The values were normalized according to the housekeeping genes TBP and GAPDH, respectively ($n = 3$, mean values ± SD).

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 < 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, Chicago, IL).

Results

SOCS-3 Is Expressed in the Majority of Prostate Cancer Cell Lines

To evaluate the expression of SOCS-3 in prostate cell lines, we first measured its mRNA by real-time PCR and normalized its values according to those of the housekeeping gene TBP. As shown in Figure 1A, the highest levels were found in DU-145 cells, followed by LNCaP-IL-6$^+$, LAPC-4, PC3, and BPH-1 cells. SOCS-3 mRNA expression was undetectable in the LNCaP subline established by passaging cells in the absence of IL-6 (LNCaP-IL-6$^-$). The pattern of SOCS-3 protein expression in these cells was very similar to that of SOCS-3 mRNA. The highest expression of the SOCS-3 protein was detected in DU-145 cells (Figure 1B), whereas LNCaP-IL-6$^-$ cells do not express SOCS-3. Because of the origin of LNCaP-IL-6$^-$ cells, we were interested to know whether lack of expression of SOCS-3 is a consequence of serial passaging. For this reason, we determined SOCS-3 expression in parental LNCaP cells and found that they are also SOCS-3-negative. To investigate the correlation between expression of SOCS-3 and pSTAT3, we treated cell lines with 10 ng/ml IL-6 for 15 to 45 minutes.

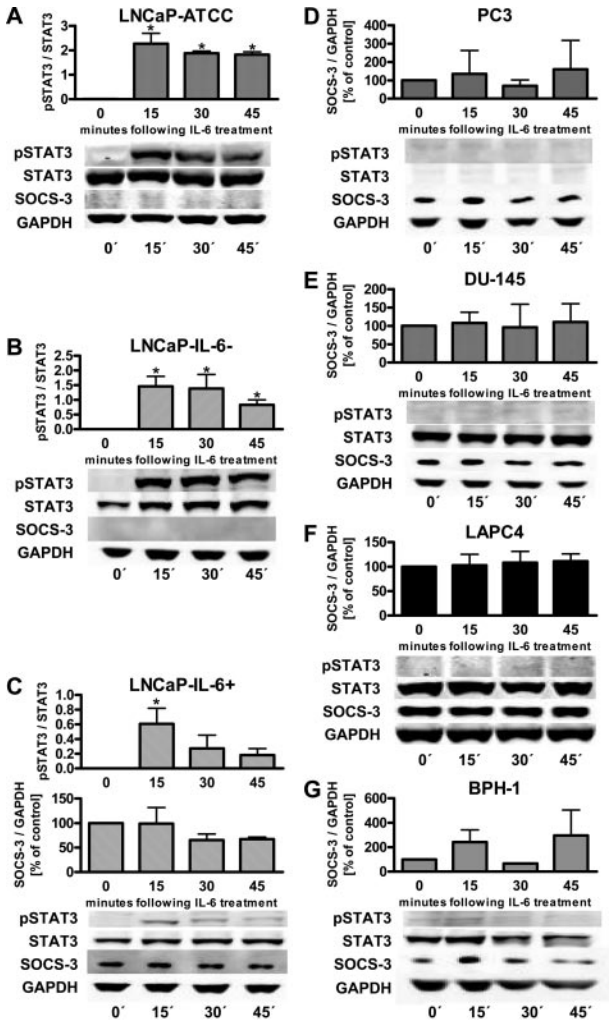

Figure 2. Correlation between expression of SOCS-3 and expression and phosphorylation of STAT3 in prostate cells. SOCS-3, pSTAT3, STAT3, and GAPDH were determined by Western blotting after treatment with IL-6. Densitometric analysis and results of representative experiments are shown ($n = 3$, mean values ± SD, $^*P < 0.05$, IL-6-treated versus untreated cells, Mann-Whitney *U*-test).

There was no induction of pSTAT3 in DU-145, LAPC-4, or BPH-1 cells, whereas a minimal stimulation was observed in LNCaP-IL-6$^+$ cells (Figure 2). In contrast, tyrosine phosphorylation of STAT3 was strongly induced at three time points (15, 30, and 45 minutes) in LNCaP-IL-6$^-$ and parental LNCaP cells. The maximal effect was observed after 15 minutes of supplementation of IL-6 to culture media. PC3 cells do not express STAT3 at all. Taken together, these data show that there is an inverse correlation between the expression of SOCS-3 and IL-6-induced phosphorylation of STAT3 in prostate cancer *in vitro*. SOCS-3 levels varied in cell lines after the treatment with IL-6; however, the differences between treated and untreated cells did not reach statistical significance.

SOCS-3 Is Re-Expressed in Prostate Cells after Treatment with the Demethylating Agent

We hypothesized that hypermethylation of the SOCS-3 gene promoter is a mechanism responsible for lack of its expression in parental LNCaP cells and their derivative passaged in the absence of IL-6. To address this issue, we treated the cells with the demethylating agent 5-aza-2′-cytidine for 4 days and measured SOCS-3 mRNA after subsequent incubation with IL-6. We show a statistically significant increase in expression of SOCS-3 mRNA. Maximal induction was similar in parental LNCaP cells and in the LNCaP-IL-6$^-$ derivative (Figure 3).

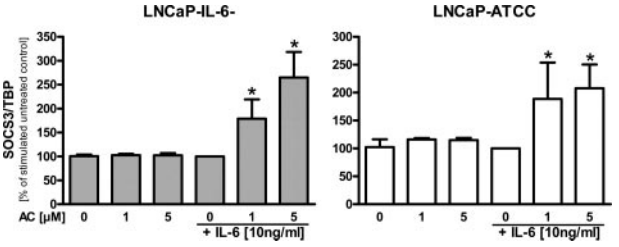

Figure 3. Re-expression of SOCS-3 after treatment with the demethylating agent 5-aza-2′-cytidine (AC). LNCaP-IL-6$^-$ and LNCaP cells were treated with increasing concentrations of 5-aza-2′-cytidine for 4 days followed by a 1-hour incubation with 10 ng/ml IL-6. The cells were then harvested, and RNA was isolated and transcribed in cDNA. qRT-PCR was then performed. The results are expressed in relation to values measured in untreated cells and represent mean values ± SD from at least three independent experiments ($^*P < 0.05$, Mann-Whitney *U*-test).

SOCS-3 and pSTAT3 Detection in Prostate Cancer Specimens

To assess the relevance of findings obtained with prostate cell lines, immunohistochemical analysis was performed in specimens obtained from patients. SOCS-3 expression in benign, premalignant, and malignant prostate tissue was evaluated on the basis of staining intensity. It was classified as absent (0), weakly positive (1+), moderately positive (2+), and strong (3+). Immunohistochemical reaction for SOCS-3 in the cytoplasm of endothelial cells (Figure 4A) was considered as a positive control.[23] The use of nonimmune serum resulted in no staining. Immunohistochemical procedures were performed two times and similar results were obtained. In most normal glands and in adjacent stromal cells, there was an absent or weak SOCS-3 immunoreactivity (Table 1). In contrast, SOCS-3 was detected in the cytoplasm in more than 50% of prostate intraepithelial neoplasia samples (Figure 4B). In the vast majority of tumor specimens, SOCS-3 was also expressed in the cytoplasm (Figure 4, A and C). Interestingly, six of seven Gleason pattern 4 and all four cases of Gleason grade 5 were SOCS-3-positive. These data are in concordance with those obtained *in vitro* and indicate an increase in expression of SOCS-3 during prostate carcinogenesis. In addition to SOCS-3, we also examined expression of pSTAT3 (Table 2). Interestingly, nuclear pSTAT3 was detected in normal tissue (Figure 4D) and in 50% of prostate intraepithelial neoplasia lesions investigated. Heterogeneous expression of nuclear pSTAT3 was observed in prostate cancer specimens.

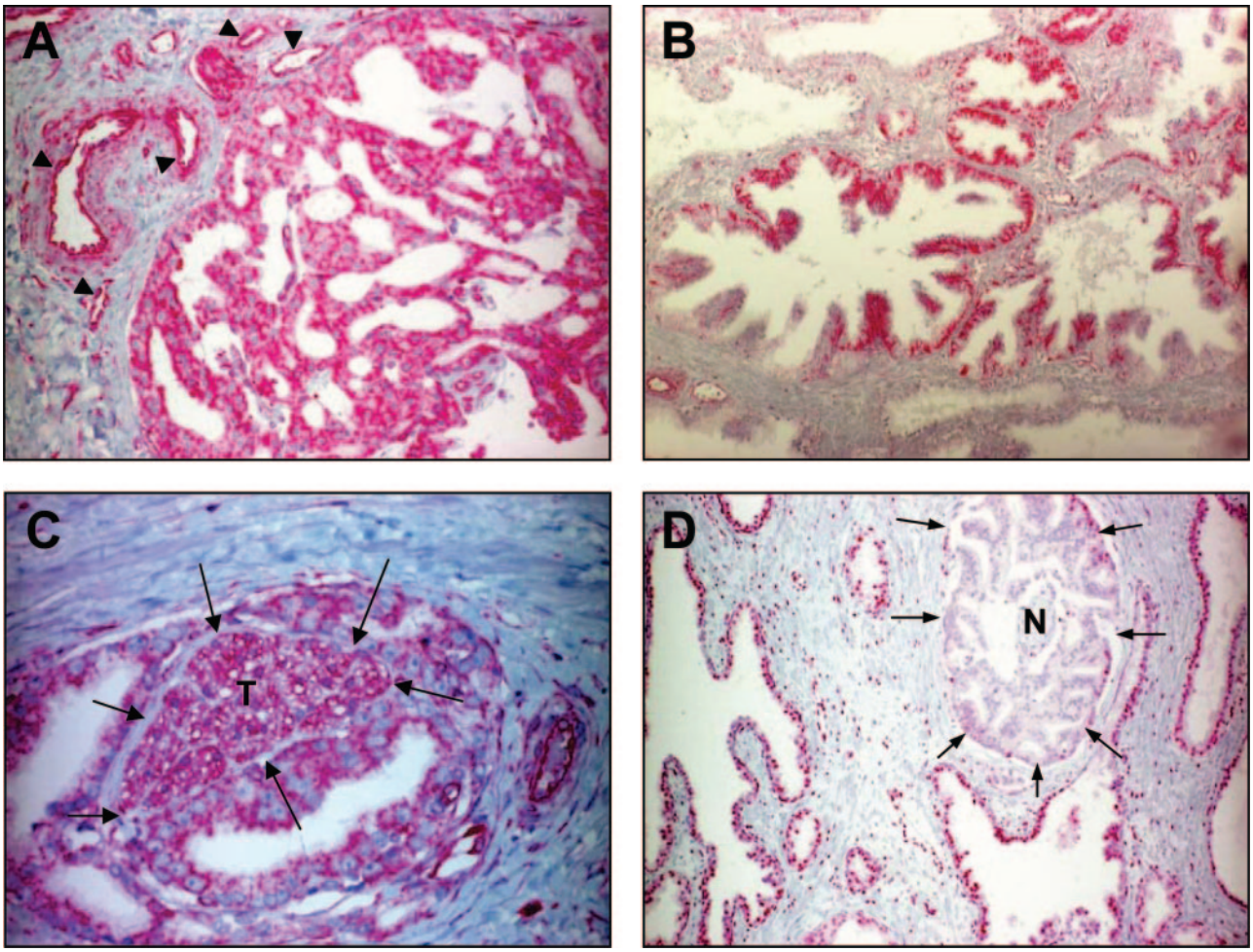

Figure 4. Immunohistochemical expression of SOCS-3 (**A–C**) and pSTAT3 (**D**) in benign and malignant prostate tissue. Specimens obtained from patients were stained with the anti-SOCS-3 antibody diluted 1:100 or anti-pSTAT3 antibody diluted 1:20. Expression of SOCS-3 in endothelium (**A**, **arrowheads**) was regarded as a positive control. SOCS-3 expression is detected in Gleason pattern 3 prostate cancer specimen (**A**), high-grade prostate intraepithelial neoplasia (**B**), and tumor (T, **arrows**) that invades perineural space (**C**). pSTAT3 immunostaining is shown in benign tissue but not in tumor cells (**D**) (**arrows**, tumor; N, nerve). Original magnifications: ×20 (**A**, **B**); ×40 (**C**); ×10 (**D**).

SOCS-3 Is Up-Regulated by a cAMP Derivative in Prostate Cancer Cell Lines

To investigate involvement of SOCS-3 in regulation of cellular events in prostate cancer, we first asked whether SOCS-3 protein levels are influenced by treatment with a compound that leads to activation of the protein kinase A pathway. Our assumption was based on a previous report showing increased levels of SOCS-3 mRNA in pituitary cells after treatment with the cAMP derivative db cAMP.[24] We show that either 48- or 72-hour treatment of PC3 and LNCaP-IL-6^{+} cells with db cAMP leads to a statistically significant concentration-dependent increase in expression of SOCS-3 in both cell lines (Figure 5). In

Table 1. Immunohistochemical Expression of SOCS-3 in Benign, Premalignant, and Malignant Prostate Tissue

Tissue	Immunohistochemical staining				
	n	0	1	2	3
Benign	20	14	4	2	0
PIN	8	3	3	1	1
Gleason pattern					
2	3	1	1	1	0
3	17	3	8	4	2
4	7	1	3	2	1
5	4	0	2	2	0

PIN, prostate intraepithelial neoplasia.
Immunohistochemical staining: 0, no reaction; 1, weakly positive reaction; 2, moderately positive reaction; 3, strongly positive reaction.

Table 2. Immunohistochemical Expression of pSTAT3 in Benign, Premalignant, and Malignant Prostate Tissue

Tissue	Immunohistochemical staining				
	n	0	1	2	3
Benign	19	3	0	3	13
PIN	16	8	0	3	5
Gleason pattern					
2	3	3	0	0	0
3	17	9	0	3	5
4	7	4	0	0	3
5	5	4	0	0	1

Immunohistochemical staining: 0, no reaction; 1, weakly positive reaction; 2, moderately positive reaction; 3, strongly positive reaction.

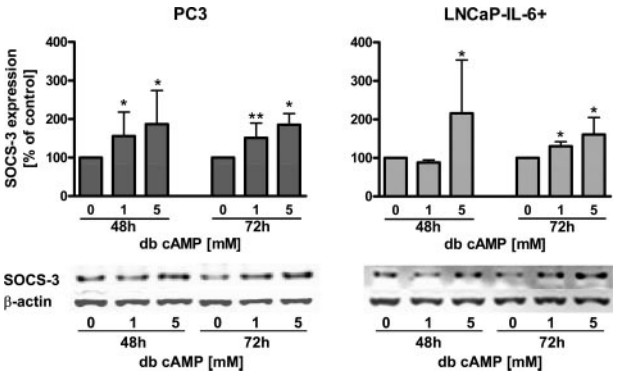

Figure 5. Expression of SOCS-3 after treatment with the cAMP analogue db cAMP. PC3 and LNCaP-IL-6$^+$ cell lines were treated with increasing concentrations of db cAMP for 48 and 72 hours before determination of SOCS-3 protein levels. Results of densitometric analysis and representative Western blots are shown ($n \geq 4$, mean values ± SD, $^*P < 0.05$, db cAMP-treated versus untreated cells, Mann-Whitney *U*-test).

contrast to these two cell lines, treatment of SOCS-3-protein-negative LNCaP-IL-6$^-$ cells with the protein kinase A activator did not lead to re-expression of SOCS-3.

We noted a reduced incorporation of [^{3}H]thymidine in both cell lines after treatment with db cAMP (Figure 6A). The effect of db cAMP was more pronounced in PC3 than in LNCaP-IL-6$^+$ cells. This treatment resulted in a decreased percentage of PC3 cells in the S phase of cell cycle after 48 and 72 hours. In LNCaP-IL-6$^+$ cells, a significant S phase reduction was observed only 48 hours after addition of db cAMP (data not shown). Results of fluorescence-activated cell sorting analysis showed an increase in the percentage of apoptotic cells by db cAMP in PC3 and LNCaP-IL-6$^+$ cells (Figure 6B).

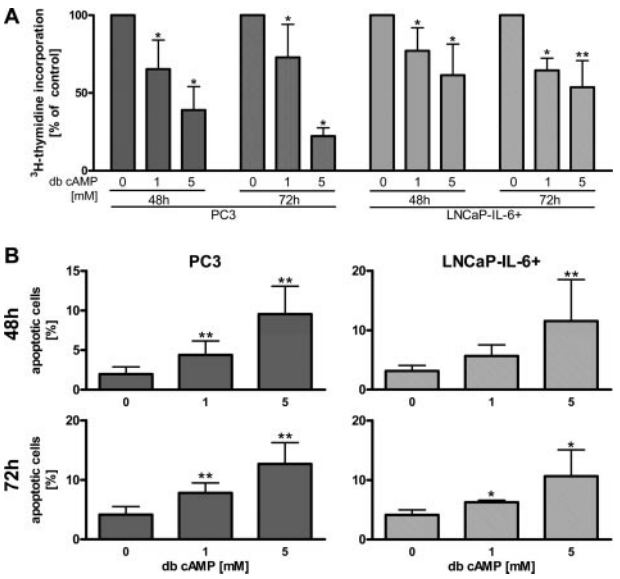

Figure 6. Regulation of cellular proliferation (**A**) and apoptosis (**B**) in PC3 and LNCaP-IL-6$^+$ cells. The two cell lines were treated with db cAMP (1 and 5 mmol/L) for 48 and 72 hours. Cellular proliferation was assessed by measurement of [^{3}H]thymidine incorporation, and the percentage of apoptotic cells was determined by flow cytometry ($n \geq 4$, mean values ± SD, $^*P < 0.05$, $^{**}P < 0.01$, db cAMP-treated versus untreated cells, Mann-Whitney *U*-test). In **A**, the percentage of untreated cells was set at 100.

Effects of SOCS-3 siRNA on Prostate Cancer Cell Proliferation and Apoptosis

To regulate directly the levels of SOCS-3, PC3 cells were transfected with siRNA. We found a strong down-regulation of SOCS-3 protein by ~75% after 48 hours with both concentrations used (10 and 25 nmol/L) (Figure 7A). [^{3}H]Thymidine incorporation did not differ between control siRNA and SOCS-3 siRNA treatments in the absence of db cAMP. Growth inhibition by 5 mmol/L db cAMP increased in SOCS-3 siRNA-transfected cells (Figure 7B). In addition, we found a significantly increased induction of apoptosis by db cAMP in SOCS-3 siRNA- compared with control siRNA-transfected cells, whereas basal apoptotic rate was not substantially changed (Figure 7C). No difference in proliferation or apoptosis between mock- and control siRNA-transfected cells was observed (data not shown).

Discussion

Although there is accumulating evidence showing that the transcription factor STAT3 may exhibit either prodifferentiation or prosurvival action in human prostate cancer, the mechanisms governing its expression are not well understood. Proteins of the SOCS family, in particular SOCS-3, the subject of investigation in our study, have been identified as important regulators of JAK/STAT signaling in various types of malignant tumors. On the other hand, IL-6, which selectively activates STAT3 in prostate cancer, is a valid therapy target.[25] Therefore, we reasoned that studying expression and function of SOCS-3 in carcinoma of the prostate may provide a basis for a more rational design of therapies aimed to interfere with IL-6/JAK/STAT signaling in prostate cancer. Our data show that there is an inverse correlation between the expression of SOCS-3 and IL-6-induced phosphorylation of STAT3 in prostate cancer cells. SOCS-3 mRNA and protein were not detected in parental LNCaP cells and their derivative LNCaP-IL-6$^-$, in which the addition of IL-6 causes growth arrest and terminal neuroendocrine differentiation.[6,19,26] In contrast to SOCS-3-positive cell lines, most of which express IL-6, parental LNCaP and LNCaP-IL-6$^-$ cells are IL-6-negative.[2,9,19] LAPC-4 is the only cell line used in the present study that is SOCS-3-positive but IL-6-negative (our unpublished observation). Thus, one possibility to explain the regulatory mechanism of JAK/STAT signaling in prostate cell lines is that SOCS-3 expression is a consequence of IL-6 production. Of note, data on expression and constitutive activation of STAT3 reported in the literature differ between various laboratories. Our results obtained with prostate cancer cell lines are very similar to those reported by Spiotto and Chung,[6] who were also unable to detect STAT3 in PC3 cells and did not observe its transcriptional activity in the DU-145 cell line. Absent expression of STAT3 and STAT5 in PC3 cells could be explained by complete loss at 17q21.31.[27] In contrast, other researchers were able to demonstrate constitutive activity of STAT3 in all prostate cancer cell lines. Mora and colleagues[5] reported that reduction of

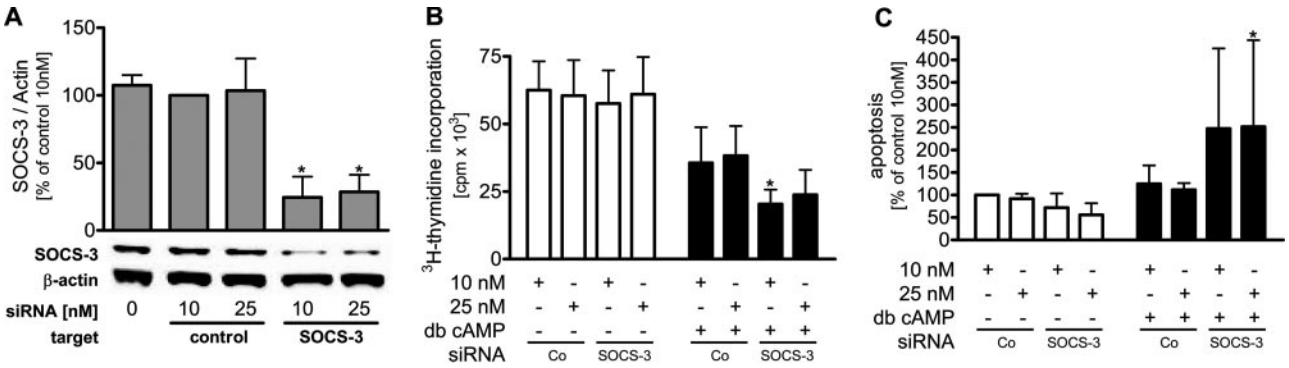

Figure 7. A: Expression of SOCS-3 after transfection of PC3 cells with control or SOCS-3 siRNA (10 or 25 nmol/L). Cells were transfected using Fugene 6, and protein expression was analyzed by Western blot. Mock transfection (first column) was performed as an additional control. Cellular proliferation was assessed by [^{3}H]thymidine incorporation (**B**) and flow cytometric analysis of sub-G_1 peak cells was performed to measure apoptosis (**C**). Mann-Whitney *U*-test was used ($n \geq 4$, mean values ± SD, control siRNA- versus SOCS-3 siRNA-treated cells in the presence of 5 mmol/L db cAMP, $^*P < 0.05$).

pSTAT3 in DU-145 cells is associated with growth inhibition and induction of apoptosis. However, it is not known whether SOCS-3 is expressed in those cells that might be cultured under different experimental conditions.[5,28] On the basis of these divergent results, one can conclude that cell culture conditions and passage numbers influence STAT3 activation. Likewise, both growth-stimulatory and -inhibitory responses were observed after rapid induction of pSTAT3 in the LNCaP cell line by IL-6 treatment.[1,6,29] Our results showing an inverse correlation between SOCS-3 expression and STAT3 activation contrast with those obtained in a cutaneous T-cell lymphoma cell line in which there was an apparent paradox of simultaneous SOCS-3 expression and STAT3 activation.[30] In those cells, SOCS-3 acted as a tumor cell protector against growth inhibition by interferon-α.

Data of the present study demonstrating restoration of SOCS-3 expression in LNCaP and LNCaP-IL-6$^-$ cells after treatment with the demethylating agent reveal that a mechanism similar to that previously described in other tumors is operative in some prostate cancers.[15–17] Hypermethylation of CpG islands of the promoter correlates with silencing of SOCS-3 in various cancer cell lines. It seems, however, that in lung, hepatocellular and breast tumor cells, head and neck squamous cell carcinoma, and mesothelioma, SOCS-3 silencing and promoter hypermethylation occur more frequently than in prostate malignancies. Our *in vitro* data are further supported by immunohistochemical analyses of SOCS-3 expression in benign and malignant prostate. It increased in prostate intraepithelial neoplasia as well as in tumor tissue compared with normal gland. This finding is in contrast to that obtained by immunohistochemistry in head and neck squamous cell carcinoma.[17] Only 18 of 94 tumor samples were found to be SOCS-3-positive, and it was concluded that SOCS-3 is a potential tumor suppressor in that type of tumor. Promoter methylation was also observed in those tumor samples and, to a lesser extent, in dysplastic lesions. In the present study, we have not established a correlation between SOCS-3 and prostate tumor grade or stage. A possible impact of SOCS-3 on prostate cancer prognosis could be assessed in future studies with a larger number of specimens.

Previous studies did not yield unequivocal results on expression of pSTAT3 in prostate cancer.[5,28,31] According to Dhir and colleagues,[31] there are similarly high levels of the activated transcription factor in tumor and adjacent benign tissue, in contrast to prostate samples from normal donors. Other publications suggested that nuclear pSTAT3 is elevated in tumors compared with nonmalignant glands.[5,28] Our data indicate a potential inverse correlation between SOCS-3 and pSTAT3 in benign prostate. In tumor tissue, the expression of pSTAT3 in patient specimens is heterogeneous. There might be several reasons for variable results on pSTAT3 expression between our and previous studies; for instance, different antibodies were used. We hypothesize that SOCS-3 and pSTAT3 regulation in prostate cancer are more complex than that in nonmalignant prostate tissue. On one hand, increased levels of SOCS-3 in prostate cancer could be partly explained by up-regulation of IL-6 and its receptor that was measured in tissue specimens obtained by radical prostatectomy.[1] One should also keep in mind that SOCS-3 binds to the insulin-like growth factor-I receptor.[32] It is therefore possible that in STAT3-negative cells SOCS-3 regulates cell growth through interaction with the insulin-like growth factor pathway. In the present study, we demonstrate up-regulation of the SOCS-3 protein by the cAMP derivative.[24] Little is known about expression of other STAT3 regulators with the exception of caveolin-1 in prostate cancer clinical specimens. Of note, SOCS-3 might be involved in the control of expression of STAT5, a transcription factor that inhibits cell death of human prostate cancer cells. Its activation in the prostate cancer correlates with high Gleason grade.[33,34]

We and others demonstrated STAT3 phosphorylation in LNCaP cells in which growth was inhibited by IL-6 and found that 22Rv1 cells stimulated by IL-6 do not exhibit any phosphorylation of STAT3.[6,29,35] As noted by Clevenger,[13] the role of STAT3 is in breast cancer, similar to that in prostate cancer, incompletely understood. There is evidence that breast cancer patients with increased pSTAT3 have a longer survival. This fact contradicts oncogenic properties of STAT3 observed in other tumors.[36] SOCS-3 transcript levels were significantly increased *in*

situ and in infiltrating ductal carcinomas in comparison to normal breast tissue.[37] Collectively, these findings imply that there might be very similar STAT3 regulation by SOCS in breast and prostate cancer.

SOCS-3 protein is up-regulated by the cAMP derivative in the two prostate cancer cell lines. Bousquet and associates[24] demonstrated that activation of the protein kinase A pathway through corticotropin-releasing hormone leads to an increased SOCS-3 promoter activity and gene expression. We have extended those previous data, thus showing that SOCS-3 protein is expressed after 48 and 72 hours of treatment with the protein kinase A activator. In the present manuscript, we used the same cAMP derivative as Bousquet and colleagues.[24] We cannot, however, completely exclude the possibility that released butyrate moiety is in part responsible for the effects of the compound on SOCS-3 expression, cell proliferation, and apoptosis.[38] On the other hand, our results are supported by a recent publication showing that SOCS-3 could be induced in endometrial stromal cells by 8-bromo-cAMP, in the absence of butyrate.[39] So, there might be at least two different kinetics of SOCS-3 induction in biological systems. After treatment with IL-6, its highest level is reached within minutes. In case of SOCS-3 elevation caused by a compound that increases intracellular cAMP levels, SOCS-3 protein could also be detected after several days. Our results showing cAMP-induced growth arrest in PC3 cells are in agreement with a previous study by Bang and associates[40] who proposed that growth retardation occurs because of an increased expression of the transforming growth factor (TGF)-β2 gene. Consistent with data by Farini and colleagues,[41] sustained accumulation of cAMP in prostate cancer cells leads to growth arrest and terminal differentiation, in contrast to a transient cAMP elevation that activates the signaling pathway of MAPK. It is well established that hormones that elevate intracellular cAMP levels activate androgen receptor in prostate cancer cells and increase the levels of the prostate-specific antigen gene.[42,43] Importantly, cAMP could enhance the effects of growth factors on MAPK activity in prostate cancer cells.[44] Invasiveness of prostate cancer cells is increased by calcitonin through the cAMP-dependent protein kinase A.[45] However, experiments in which SOCS-3 protein levels were down-regulated by siRNA showed further inhibition of tumor proliferation and an increase in the percentage of apoptotic cells in the presence of db cAMP. We therefore propose that SOCS-3 antagonizes the effects of cAMP on proliferation and programmed cell death in prostate cancer. Compounds that increase intracellular cAMP up-regulate expression of both growth-stimulatory and -inhibitory molecules. In case of prostate cancer cells, the net effects appear to be induction of growth arrest and cell death. It is important to emphasize that TGF-β, that inhibits proliferation of prostate cancer cells, is known as a growth enhancer *in vivo* because of stimulation of angiogenesis and suppression of immune response.[46] For this reason, it will be important to perform studies with the aim to investigate the role of SOCS-3 in appropriate *in vivo* models. On the basis of our results, one could also speculate that synergistic induction of neuroendocrine phenotype in prostate cancer cells by IL-6 and cAMP is modulated by SOCS-3.[47] This hypothesis deserves further investigation.

SOCS-3 levels are also elevated in the LNCaP-IL-6$^+$ cells that were derived during prolonged treatment with IL-6 *in vitro* and are representative of alterations that may occur during prostate carcinogenesis. They show an up-regulation of cyclin-dependent kinases, whereas the tumor suppressors p27 and pRb were undetectable.[29] Because that LNCaP cell subline has a clear growth advantage, it could be used for development of novel experimental therapy strategies. In the present study, a minimal elevation in STAT3 phosphorylation after treatment with IL-6 was observed, whereas in a previous work from our group no tyrosine phosphorylated STAT3 was detected. However, it should be noted that these minor variances may occur because of use of different passages. They do not contradict the results of the previous study because comparison of IL-6-induced STAT3 phosphorylation at three time points shows a substantially higher STAT3 activation in the LNCaP-IL-6$^-$ cells.[29] An example of a growth inhibition by a member of SOCS family was recently reported. The tyrosine kinase inhibitor peptide Tkip, which is a mimetic of SOCS-1, reduced progression of prostate cancer cells into S phase.[48] Recently, a cell-penetrating form of SOCS-3 was developed for intracellular anti-inflammatory therapy.[49]

In summary, the results of the present study show that SOCS-3 is expressed in the majority of cancer cell lines and clinical specimens, in contrast to findings obtained with various other solid tumors with the exemption of breast cancer. Having in mind the importance of the IL-6/JAK/STAT3- and protein kinase A-signaling pathways, further studies on SOCS-3 in carcinoma of the prostate is warranted.

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