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Tumorigenesis and Neoplastic Progression

Suppressor of Cytokine Signaling (SOCS)-1 Is Expressed in Human Prostate Cancer and Exerts Growth-Inhibitory Function through Down-Regulation of Cyclins and Cyclin-Dependent Kinases

Hannes Neuwirt,* Martin Puhr,* Frédéric R. Santer,* Martin Susani,† Wolfgang Doppler,‡ Gemma Marcias,* Veronika Rauch,* Maria Brugger,* Alfred Hobisch,§ Lukas Kenner,†¶ and Zoran Culig*

From the Department of Urology and Division of Biological Chemistry,‡ Biocenter, Innsbruck Medical University, Innsbruck; the Institute of Clinical Pathology,† Medical University of Vienna, Vienna; the Department of Urology,§ General Hospital Feldkirch, Feldkirch; and the Ludwig Boltzmann Institute for Cancer Research,¶ Vienna, Austria*

Suppressor of cytokine signaling (SOCS) proteins play a pivotal role in the development and progression of various cancers. We have previously shown that SOCS-3 is expressed in prostate cancer, and its expression is inversely correlated with activation of signal transducer and activator of transcription factor 3. We hypothesized that SOCS-1, if expressed in prostate cancer cells, has a growth-regulatory role in this malignancy. The presence of both SOCS-1 mRNA and protein was detected in all tested cell lines. To assess SOCS-1 expression levels *in vivo*, we analyzed tissue microarrays and found a high percentage of positive cells in both prostate intraepithelial neoplasias and cancers. SOCS-1 expression levels decreased in samples taken from patients undergoing hormonal therapy but increased in specimens from patients who failed therapy. In LNCaP-interleukin-6− prostate cancer cells, SOCS-1 was up-regulated by interleukin-6 and in PC3-AR cells by androgens; such up-regulation was also found to significantly impair cell proliferation. To corroborate these findings, we used a specific small interfering RNA against SOCS-1 and blocked expression of the protein. Down-regulation of SOCS-1 expression caused a potent growth stimulation of PC3, DU-145, and LNCaP-interleukin-6− cells that was associated with the increased expression levels of cyclins D1 and E as well as cyclin-dependent kinases 2 and 4. In summary, we show that SOCS-1 is expressed in prostate cancer both *in vitro* and *in vivo* and acts as a negative growth regulator. ***(Am J Pathol 2009, 174:1921–1930; DOI: 10.2353/ajpath.2009.080751)***

Prostate cancer is the second most common cause of tumor-related deaths in the Western world. Although localized tumors can be successfully treated with surgery or radiotherapy, clinically approved therapy for advanced prostate cancer is limited to androgen ablation, blockade of the androgen receptor (AR) or chemotherapy. Recent modest improvements in chemotherapy have been achieved with the anti-microtubule agent docetaxel.

Prostate cancer initiation and progression strongly depend on activation of the AR, but chronic inflammation of the prostate may also play an important role.[1] Therefore, it is not surprising that the role of the proinflammatory cytokine interleukin-6 (IL-6) in prostate carcinogenesis has received a considerable interest. IL-6 is a multifunctional cytokine that acts in a cell type-specific manner through activation of signaling pathways of Janus kinases/signal transducer and activator of transcription factors (STAT), mitogen-activated protein kinases, and/or phosphotidylinositol 3-kinase. In prostate cancer cells, either pro-differentiation or survival effects of IL-6 have been described.[2] The mechanisms responsible for differential activation of IL-6 signaling pathways in prostate

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Address reprint requests to Zoran Culig, M.D., Department of Urology, Innsbruck Medical University, Anichstrasse 35, A-6020 Innsbruck, Austria. E-mail: zoran.culig@i-med.ac.at.

tumor cells are being investigated. It is assumed that various regulators of phosphorylation of STAT3, in particular suppressors of cytokine signaling (SOCS) and protein inhibitors of activated STAT, determine activation status of this transcription factor. The SOCS family comprises eight members, SOCS 1 through 7 and CIS.[3] SOCS family members share the central Src homology 2 domain and SOCS box in the carboxy-terminal end, which plays a crucial role in proteasomal degradation of binding partners. SOCS-1 and -3 contain a kinase inhibitory region, which has a pivotal function in antagonizing activation of Janus kinases.

The role of SOCS-1 and -3 in carcinogenesis is of interest since it was shown by several groups that their expression may be altered in head and neck cancer,[4,5] gastric carcinoma,[6] chronic myeloid leukemia,[7] melanoma,[8] or prostate cancer.[9,10] There is an increasing evidence showing that SOCS have different functions depending on the origin of the tumor. Tannapfel and colleagues have shown that methylation-dependent silencing of the SOCS-1/3 genes in head and neck squamous cell[4] and Barrett's adenocarcinoma[11] is associated with tumor growth *in vitro* and *in vivo*. On the other hand, it was demonstrated that SOCS-1 is constitutively expressed in patients with chronic myeloid leukemia [7] or in human melanoma.[8] Our previous studies revealed that SOCS-3 is increasingly expressed in prostate cancer and can exert inhibitory effects on induction of apoptosis by cAMP.[9] Other researchers have reported that SOCS-1 can also act as an inhibitor of phosphorylation of STAT3.[5] In particular, IL-4 and IL-13 stimulate expression of SOCS-1 in keratinocytes, which in turn inhibits phosphorylation of STAT3.[12] The two cytokines' receptors were detected in prostate cells.[13,14] Furthermore, in breast cancer a N-Myc downstream-regulated gene can induce SOCS-1, which negatively regulates STAT3 activation.[15] Thus, we have asked whether SOCS-1 is expressed in prostate cancer cell lines and patient samples and what impact it has on tumor cell proliferation.

In this study, we report that SOCS-1 is expressed at similar levels in all tested prostate cancer cell lines at mRNA and protein level. SOCS-1 can be up-regulated by IL-6 in LNCaP cells. Consistent with the *in vitro* data, we have found that SOCS-1 is present in tissue specimens from prostate cancer patients. Furthermore, using SOCS-1-regulated expression approach and small interfering (si)RNA technique, we show a growth-inhibitory effect of SOCS-1 in three prostate cancer cell lines.

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 during chronic treatment in the absence or presence of IL-6, respectively, as described elsewhere.[16] LAPC-4 cells were kindly provided by Dr. Charles Sawyers (University of California, Los Angeles, CA) and PC3-AR cells were donated by Dr. Andrew Cato (Research Center Karlsruhe, Germany). Lipofectamine2000 (LF2000) was purchased from Invitrogen (Carlsbad, CA) and doxycycline from Sigma-Aldrich (St. Louis, MO). The synthetic androgen methyltrienolone (R1881) is a product of DuPont NEN (Boston, MA) and IL-6 was purchased from R&D (Minneapolis, MN).

Quantitative Real-Time PCR

Quantitative real-time PCR was performed as previously published.[9] The primer/probe sequences for SOCS-1 were as follows: forward primer: 5′-TTTTCGCCCTTAGCGTGAAG-3′; reverse primer: 5′-CATCCAGGTGAAAGCGGC-3′; Taqman-probe: FAM-5′-CCTCGGGACCCACGAGCATCC-3′-TAMRA.[17] TATA-Box binding protein was used as a housekeeping gene.[18]

Western Blot

Western blotting analysis, including harvesting and cell lysis, was performed as previously reported.[9] The expression of SOCS-1 was investigated after culturing the cells in charcoal-stripped fetal calf serum with increasing concentrations of androgen as described for SOCS-3.[10] The following antibodies were used: rabbit anti-human SOCS-1 antibody (Acris Antibodies, Hiddenhausen, Germany), mouse anti-human glyceraldehyde 3-phosphate dehydrogenase (Chemicon International, Temecula,

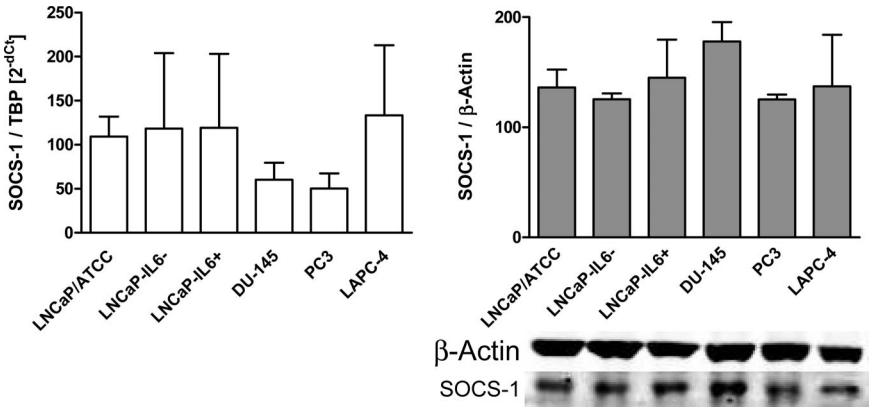

Figure 1. SOCS-1 mRNA and protein levels in prostate cancer cell lines. Cells were grown under standard conditions and SOCS-1 expression levels were measured using quantitative real-time PCR and Western blot, respectively. SOCS-1 mRNA levels were normalized to levels of the housekeeping gene encoding TATA-box binding protein and those of SOCS-1 protein to the value of β-actin. Data represent mean value ± SE; $n = 4$ independent experiments carried out in triplicate.

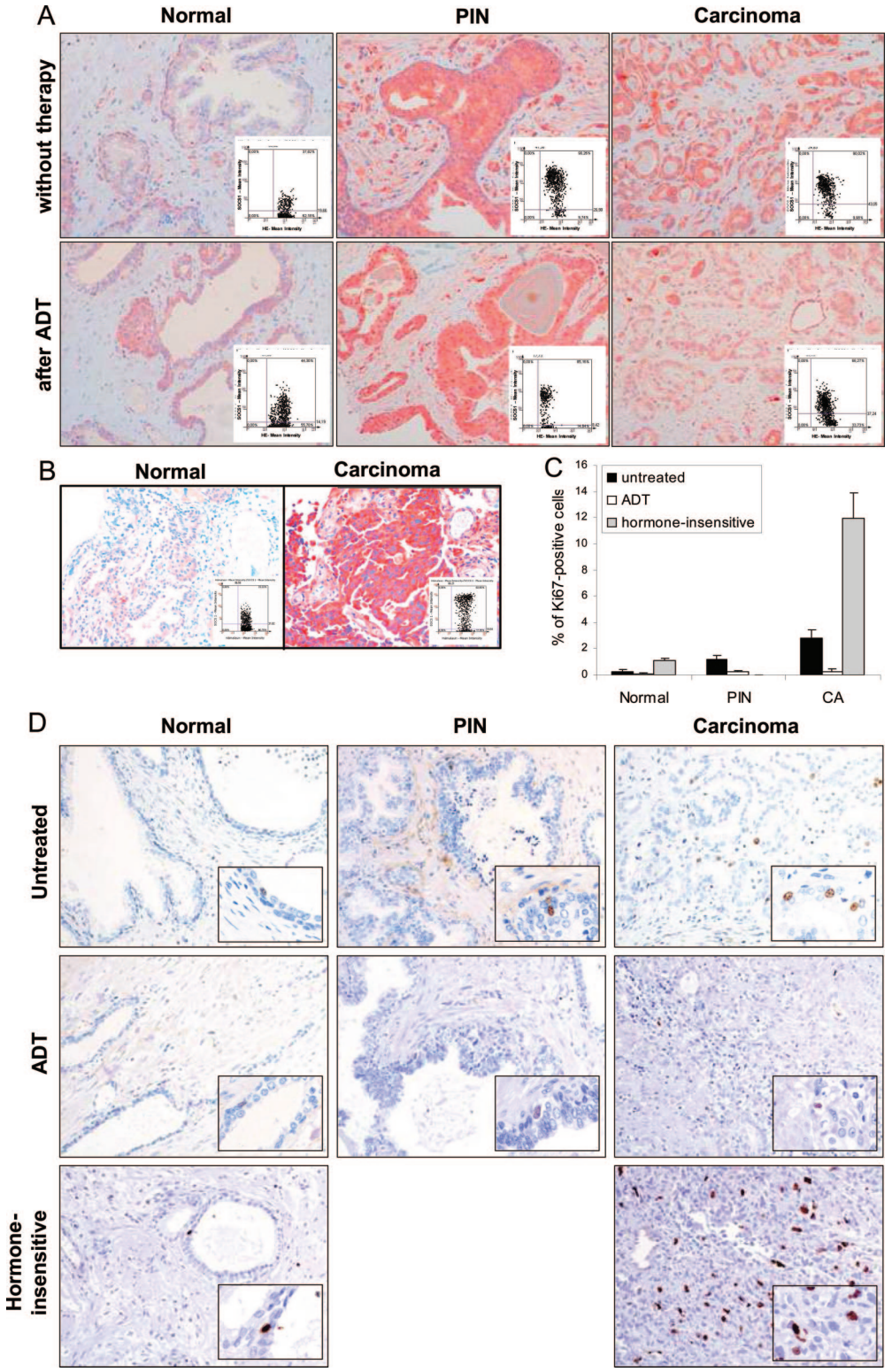

Figure 2. Expression of SOCS-1 and Ki-67 in normal prostate tissue, PIN (prostate intraepithelial neoplasia) and carcinoma obtained from individuals before or after androgen-deprivation therapy (ADT). **A:** SOCS-1 immunoreactivity in samples from patients with prostate cancer and (**B**) SOCS-1 staining in human prostate cancer from a patient who failed endocrine therapy. Immunohistochemistry for SOCS-1 was performed using the ABC staining kit according to the manufacturer's recommendations. SOCS-1 expression levels were analyzed quantitatively using a single cell resolution technique with the HistoQuest software. **C:** Percentage of Ki-67-positive cells in untreated, treated, and therapy-resistant prostate cancer and adjacent PIN and normal tissue. Data represent mean ± SE. **D:** Ki-67 staining in representative samples of human prostate cancer, adjacent PIN, and normal tissue.

Table 1. Percentage of SOCS-1-Positive Cells in Normal Prostate Tissue, PIN, and Cancer Tissue Obtained from Patients with Untreated, Treated, and Therapy-Resistant Cancer

	Untreated patients	Patients treated with ADT	Patients with therapy-resistant cancer
Normal tissue	37.82	44.3	33.22
PIN	90.26	85.16	n.d.
Cancer tissue	90.02	66.27	82.65

n.d., not determined.
PIN, prostate intraepithelial neoplasia.

CA), mouse anti-human β-actin (Chemicon), mouse anti-c-Myc and anti-cyclin E (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-cyclin-dependent kinase (cdk 2) and cdk 4 (Biosource International, Camarillo, CA), and rabbit anti-cyclin D1 (LabVision Neomarkers, Fremont, CA).

Patient Samples

We established tissue microarrays using samples from prostate cancer patients (mean age 62, age range 47 to 73) comparing normal, prostate intraepithelial neoplasia (PIN), and high- and low grade cancer areas. To compare the expression levels of proteins of interest from patients without prostate cancer, normal prostate tissue from patients who underwent cystectomy was included. We introduced four tissue arrays of eight patients with prostate cancer before, and eight patients after androgen ablation therapy. Tumor grade and stage were determined independently by two pathologists (M.S., L.K.). Four patients whose samples were obtained before therapy presented with Gleason score 7, two with Gleason 6, and one with Gleason 9 and 5, respectively. All those patients had tumor stage pT2c, whereas patients who received therapy presented with tumor stage 3a (three cases), 4a (two cases), and either 2a, 2b, or 3b (one case each). Samples from five patients with therapy-resistant tumors were also used. Target gene expression analysis was performed using immunofluorescence microscopy and our digital image techniques. The procedure concerning patients' data protection was according to regulations of Medical University of Vienna.

Immunohistochemistry

For histological analysis and immunohistochemistry, tumors were fixed with neutral buffered 4% paraformaldehyde. The tissues were then embedded in paraffin. The sections (5 μm) were stained either with hematoxylin and eosin or processed further. Immunohistochemistry staining for SOCS-1 (H93 antibody, Santa Cruz Biotechnology, Santa Cruz, CA) and Ki-67 (MIB-1 antibody, DAKO, Glostrup, Denmark) was performed using the ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's recommendations. As a negative control, staining was performed on selected sections without the primary antibodies.

Image Acquisition and Protein Quantification in Vivo

Samples were analyzed with a Zeiss AxioImager Z1 microscope system with CCD camera and an automated acquisition system (TissueGnostics, Vienna, Austria).[19,20] The percentage of SOCS-1- and Ki-67-positive cells was determined. Pictures were acquired using Aquest software. Statistics flow analysis was done with software WinMDI.

Plasmid and Short Interfering RNA Transfections

PC3, DU-145, and LNCaP-IL-6− cells were seeded onto six-well plates and transfected with either expression vector or siRNA using Lipofectamine2000 according to the manufacturer's protocol. For SOCS-1 overexpression, PC3 and DU-145 clones transfected with 1 μg of the pBig2i plasmid per well containing a tetracycline-regulated tricistronic transactivator/repressor and the tetracycline-responsive cMyc-tagged SOCS-1. Medium was changed 8 hours later and doxycycline (2 μg/ml) was added. cMyc-tagged-SOCS-1 expression levels were measured 48 and 72 hours after transfection. For ^{3}H-thymidine incorporation assay, the conditions were adapted to 96-well plates and proliferation was measured 48 hours after transfection.

For siRNA experiments, we have used the modified RNAi protocol supplied by Invitrogen with siRNA concentrations between 10 and 100 nmol/L. The following siRNA

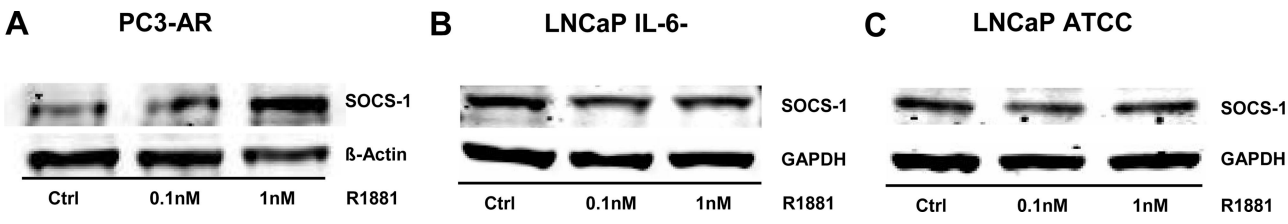

Figure 3. Regulation of expression of SOCS-1 by androgen in (**A**) PC3-AR, (**B**) LNCaP-IL-6− and (**C**) parental LNCaP ATCC cells. The cells were treated with increasing concentrations of the synthetic androgen methyltrienolone (R1881) for 72 hours and SOCS-1 levels were determined by Western blot. The results are representative of three independent experiments.

sequences were previously published[21]: SOCS-1 sense strand: 5′-GCAUCCGCGUGCACUUUCAUU-3′; SOCS-1 antisense strand: 5′-AAUGAAAGUGCACGCGGAUGC-3′; β-Galactosidase sense strand: 5′-UUAUGCCGA-UCGCGUCACAUU-3′; β-Galactosidase antisense strand: 5′-AAUGUGACGCGAUCGGCAUAA-3′. Mock-transfected cells were grown in the presence of Lipofectamine only. mRNA levels were read 48 hours after transfection, whereas protein levels were measured 72 hours later. For long-term experiments, the following protocol was applied: the cells were seeded on day one, the first transfection was carried out on the next day and medium was changed on day three followed by a 48 hours incubation period. Then the second transfection was performed with the subsequent change of medium 24 hours later and additional incubation for 48 hours. ^{3}H-thymidine incorporation was measured 72 and 144 hours after siRNA treatment.

Statistical Analysis

The following procedure was carried out to test significance of findings: all treatment and control groups were tested for Gaussian distribution using Kolmogorov-Smirnov-Test. As non-Gaussian distribution was found in all groups, non-parametric unpaired Kruskal-Wallis test was applied to compare more than two and Mann-Whitney-*U*-test was used to compare two groups. All *P* values were corrected according to Bonferroni. *P* values below 5% were defined as statistically significant and encoded as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Results

SOCS-1 Expression Is Detected in Prostate Cancer Cell Lines

To evaluate the expression of SOCS-1 in human prostate cell lines, we first measured its mRNA by quantitative real-time PCR and normalized the values according to those of the housekeeping gene encoding TATA-box binding protein. We found no significant differences in mRNA levels between the cell lines. SOCS-1 mRNA levels of DU-145 and PC3 cells were slightly lower (Figure 1, left panel). Using Western blot technique, however, we found slightly higher protein levels in DU-145 cells in comparison with the other cell lines (Figure 1, right panel). These changes were not statistically significant.

SOCS-1 Expression Is Decreased in Patients after Androgen Ablation Therapy and Is Elevated in Recurrent Cancer

To obtain a deeper insight into the regulation of SOCS-1 during carcinogenesis and therapy, we conducted tissue microarrays using patients′ samples (Figure 2, see supplementary Figure 1, *http://ajp.amjpathol.org* and Table 1). In normal tissue, SOCS-1 was mostly detected in the cytoplasm of basal cells. There was a high percentage of SOCS-1-positive cells in PIN and carcinoma (normal tissue: 37.82%, PIN: 90.26%, carcinoma: 90.02%) (Figure 2A). To investigate possible changes in SOCS-1 expression levels during androgen ablation, we also analyzed samples from patients who received this therapy. SOCS-1 expression was down-regulated in those samples. The percentage of SOCS-1-positive cells in carcinoma decreased to 66% in samples from patients who underwent therapy. However, the percentage of SOCS-1 in specimens obtained from patients who failed therapy raised to 82.65% (Figure 2B). To better understand the relationship between SOCS-1 expression and tumor cell proliferation *in vivo*, we performed tissue microarray and determined the percentage of Ki-67-positive cells (Figure 2, C and D). The Ki-67 index was the highest in therapy-resistant tumors, followed by levels measured in untreated cancer. Low percentages of Ki-67-positive cells were detected in tumor tissue during androgen withdrawal therapy similar to the level seen in normal tissue. On the basis of these *in vivo* results, we hypothesized that

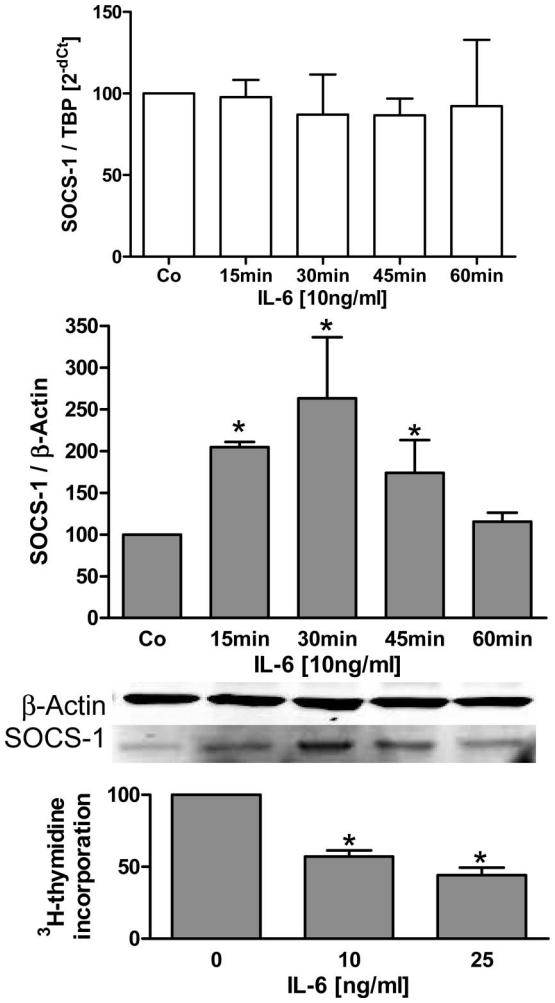

Figure 4. IL-6 induces SOCS-1 protein but not mRNA in LNCaP-IL-6− cells and inhibits cellular growth. Cells were seeded onto six-well plates and treated with 10 ng/ml of IL-6 for the indicated time periods. RNA was isolated and transcribed into cDNA or whole cell lysates were prepared. Graphs show levels of SOCS-1 mRNA (**top**) and protein (**middle**), respectively. Inhibition of proliferation by IL-6 is also shown (**bottom**); $n = 3$ independent experiments carried out in triplicate. *$P < 0.05$.

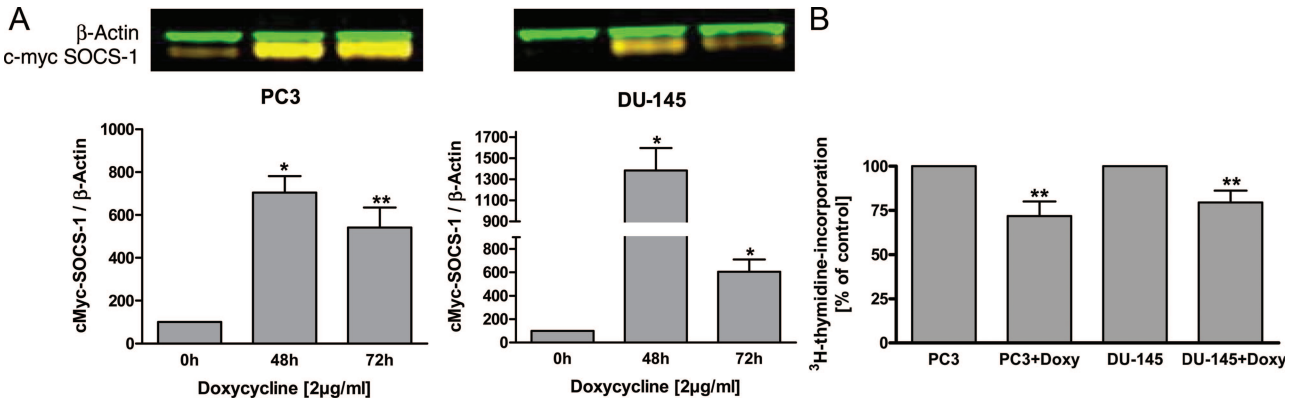

Figure 5. A: Up-regulation of SOCS-1 expression by transient transfection. PC3 and DU-145 cells were seeded onto six-well plates and transfected after 24 hours with a tetracycline-responsive SOCS-1 expression vector. Protein levels of SOCS-1 were measured 48 and 72 hours later. Controls included the cells grown in vehicle (PBS) only; $n = 5$ independent experiments carried out in duplicate. **B:** SOCS-1 overexpression leads to a reduced ^{3}H-thymidine uptake. Cells were seeded onto 96-well plates and transfected with the pBig2i plasmid. Vehicle or doxycycline (2 μg/ml) were supplemented and ^{3}H-thymidine was added after 48 hours. The assay was analyzed 24 hours later; $n = 4$ independent experiments carried out in triplicate. $^*P < 0.05$, $^{**}P < 0.01$.

SOCS-1 expression in prostate cancer cells is regulated by androgen. This hypothesis was tested in PC3 cells stably transfected with the AR cDNA (PC3-AR), parental LNCaP, and LNCaP IL-6− cells. As shown in Figure 3A, the treatment of PC3-AR cells with increasing concentrations of R1881 yielded a higher expression of the SOCS-1 protein. However, up-regulation of SOCS-1 in prostate cancer cells seems to be cell type-specific. In LNCaP cells and in their derivative LNCaP-IL-6− (Figure 3, B and C), the levels of SOCS-1 did not change after androgen treatment. Taken together, our results suggested that alterations in expression of SOCS-1 in tissue specimens obtained from prostate cancer patients are context dependent.

SOCS-1 Is Up-Regulated by IL-6 in LNCaP Cells and Inhibits Proliferation

To elucidate the influence of IL-6 on SOCS-1 expression, we treated LNCaP cells with IL-6 (10 ng/ml). Using quantitative real-time PCR we found no significant alteration of SOCS-1 mRNA levels (Figure 4, top). However, at the protein level, a significant increase of SOCS-1 already occurred after 15 to 30 minutes of treatment, although followed by a rapid decline to almost basal levels after 60 minutes (Figure 4, middle). Whether the transient increase of SOCS-1 protein could be a result of reduction in the rate of degradation remains to be determined. This effect was functionally associated with a significant inhibition of proliferation (Figure 4, bottom). PC3 and DU-145 cells were also tested under the same conditions. However, because of the presence of endogenous autocrine IL-6 loop, these cells apparently did not further respond to exogenous IL-6 either by the effect on SOCS-1 levels or on proliferation (data not shown).

Up-Regulation of SOCS-1 in Prostate Cancer Cells Causes Growth Inhibition

To directly examine the effect of SOCS-1 on growth of androgen-insensitive prostate cancer cells, we transfected these cells with a tetracycline-responsive SOCS-1 expression plasmid. Doxycycline enhanced SOCS-1 expression with the maximal effect seen after 48 hours. The induction was eight- and 16-fold in PC3 and DU-145 cells, respectively (Figure 5A). Under those conditions, proliferation, as assessed by the ^{3}H-thymidine incorporation assay, was significantly reduced by 25% in both cell lines (Figure 5B). In control experiments, doxycycline did not induce any effect on proliferation of prostate cancer cells DU-145 and PC3, which were similarly transfected with the empty vector (supplementary Figure 2, see *http://ajp.amjpathol.org*).

Down-Regulation of SOCS-1 Stimulates Cellular Growth through Effect on Expression of cdk and Cyclins

To corroborate our above findings, we used siRNA against SOCS-1 that was published by Zitzmann and associates.[21] SOCS-1 mRNA and protein levels were lowered to approximately 50% after 72 hours. We assessed cellular proliferation by ^{3}H-thymidine uptake and found a significant up-regulation in PC3 and DU-145 cells (Figure 6). A stronger down-regulation of SOCS-1 protein was achieved by transfection performed at two consecutive time points. The levels of SOCS-1 decreased to 20 and 40% in DU-145 and PC3 cells, respectively. In support of our results reported above, cellular growth was markedly stimulated in PC3 and DU-145 cells (Figure 7). To understand the mechanism underlying growth inhibition of prostate cancer cells by SOCS-1, we measured expression levels of cyclins and cdk that regulate G1 to S phase progression of the cell cycle. DU-145 and PC3 (Figure 8, A and B) cells were treated with increasing concentrations of either SOCS-1 or control siRNA (50 to 100 nmol/L). Following siRNA treatment, there was an increase in expression of cyclins D1 and E as well as cdk 2 and 4 in DU-145 cells. With the exception of cyclin D1, these cell cycle regulators were also up-regulated in PC3 cells. Taken together, these data are in concordance with the results obtained in our proliferation assays. To test

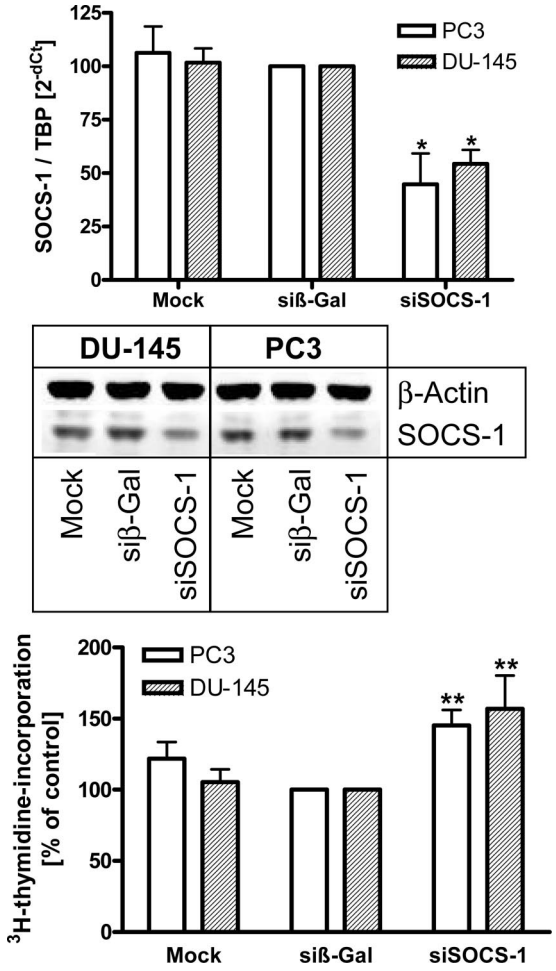

Figure 6. Inhibition of SOCS-1 expression causes a moderate growth advantage. Cells (DU-145 and PC3) were seeded onto six- and 96-well plates for quantitative real-time PCR, Western blot or proliferation assays, respectively. 72 hours after mock or siRNA transfection (final concentration 100 nmol/L), SOCS-1 levels (**top**, **middle**) and proliferation status (**bottom**) were measured; $n = 3$ to 5 independent experiments carried out in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$.

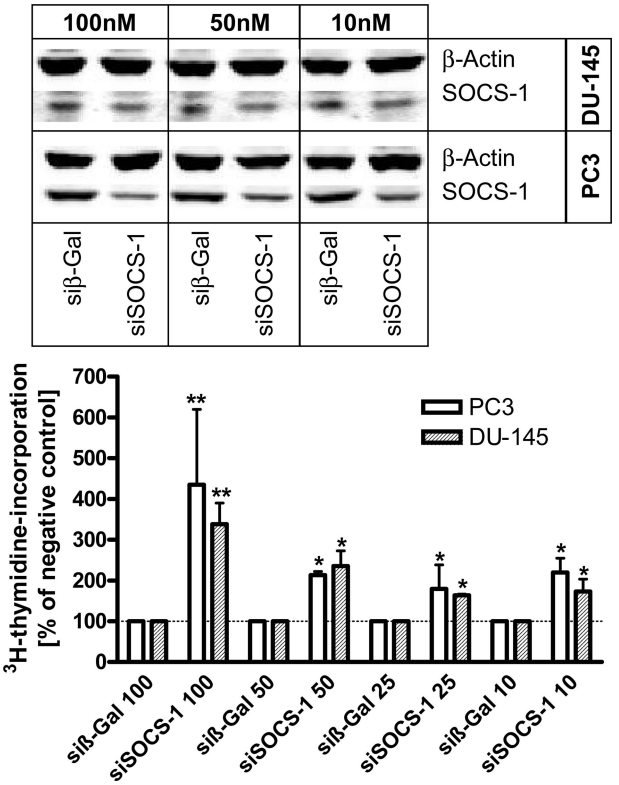

Figure 7. Long-term knock-down of SOCS-1 causes a strong stimulation of proliferation. Cells were seeded onto six- and 96-well plates for Western blot and ^{3}H-thymidine uptake measurement, respectively. Increasing concentrations of siRNA were transfected at two consecutive time points; $n = 6$ independent experiments carried out in triplicate; siRNA concentration is given 1 nmoL/L. $^{*}P < 0.05$, $^{**}P < 0.01$.

whether SOCS-1 knock-down affects growth of AR-positive cells in the same way as in androgen-insensitive ones, we repeated siRNA experiments in LNCaP-IL-6− cells. Growth stimulation in that cell line observed after SOCS-1 siRNA treatment was higher than fivefold (Figure 9). Interestingly, inhibition of proliferation of LNCaP-IL-6− cells by IL-6, which was reported previously was also observed after down-regulation of SOCS-1.[16]

Discussion

The important role of SOCS-3 was recently demonstrated in prostate cancer cells in which STAT3 is not phosphorylated and in tissue specimens obtained from patients.[9] SOCS-3 is induced not only by IL-6, but also by androgens and cAMP and acts as a negative feedback regulator of cellular events induced by hormones.[9,10] Expression levels and/or functional significance of SOCS-1 and -3 were investigated in some premalignant conditions and malignant tumors. In Barret's adenocarcinoma, they exhibit a similar pattern of expression.[11] However, reciprocal expression of SOCS-1 and -3 is associated with resistance to radiation therapy in human glioblastoma multiforme cell lines.[22] Those authors reported an in-

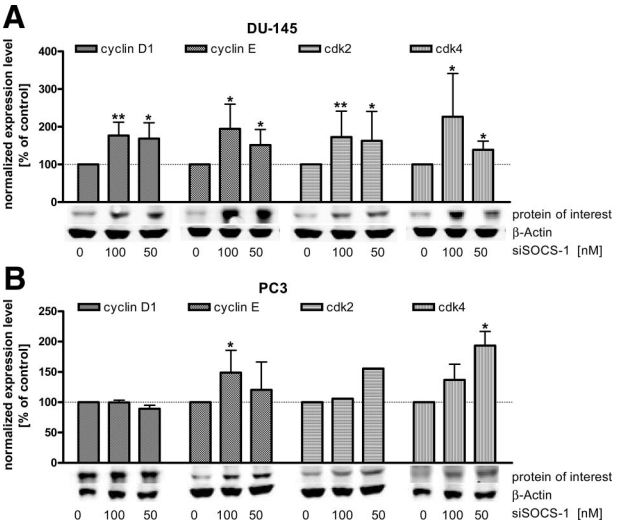

Figure 8. Inhibition of SOCS-1 expression by siRNA leads to elevation of intracellular levels of cyclins D1 and E and cdk 2 and 4 as determined by Western blot. Experimental conditions for DU-145 (**A**, 3 to 4 independent experiments) and PC3 cells (**B**, 2 to 4 independent experiments) were the same as those described in the legend for Figure 7. Data represent mean value ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$.

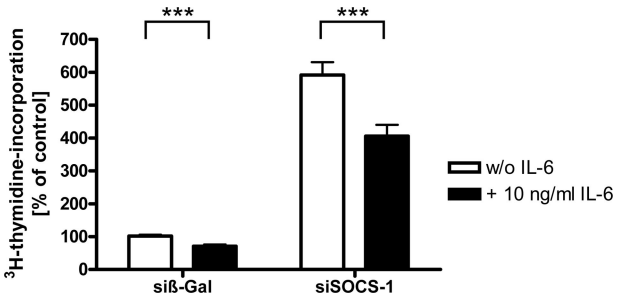

Figure 9. Inhibition of SOCS-1 expression in LNCaP-IL-6− cells leads to stimulation of proliferation but does not interfere with IL-6-induced growth inhibition. The cells were transfected with either SOCS-1 or control siRNA in the presence or absence of IL-6. Proliferation was measured by ^{3}H-thymidine incorporation. Data represent mean values ± SE from three independent experiments. $^{***}P < 0.001$.

creased expression of SOCS-3 and down-regulation of SOCS-1 levels in glioblastoma cells. Re-expression of SOCS-1 and inhibition of SOCS-3 yielded increased sensitivity to radiation. Therefore, we asked whether SOCS-1 levels are altered in prostate cancer cell lines and investigated the functional significance of SOCS-1 expression. We also hypothesized that SOCS-1 expression inversely correlates with phosphorylation of STAT3. We determined the levels of SOCS-1 mRNA and protein in six prostate cancer cell lines that showed differences in STAT3 phosphorylation.[9] However, all cell lines have similar levels of both SOCS-1 mRNA and protein. Our results do not establish a correlation between activation of STAT3 and SOCS-1 expression. It is possible that SOCS-1 inhibits phosphorylation of other STAT factors, such as STAT5 and 6, in selected human prostate cancer cell lines.[23,24]

IL-6, whose levels increase during prostate carcinogenesis, is known to exhibit pleiotropic effects *in vitro* and *in vivo*.[25,26] We demonstrated that IL-6 inhibits cellular proliferation of parental LNCaP cells.[16] After chronic treatment with IL-6, newly generated LNCaP-IL-6+ cells gain a growth advantage and increase activation of the MAPK pathway.[27] As expected, IL-6 induced SOCS-1 expression in cells that respond to the exogenous cytokine is associated with growth inhibition. The effect of IL-6 on up-regulation of SOCS-1 protein but not mRNA may not be surprising. Recently we demonstrated that androgen induces expression of SOCS-3 protein without an effect on mRNA.[10] This experiment, however, did not definitively answer the question whether SOCS-1 directly inhibits proliferation of prostate cancer cells. Thus, SOCS-1 expression was modulated by two different approaches, ie, introduction of a doxycycline-responsive SOCS-1 vector or siRNA, in prostate cancer cell lines. Consecutive transfections of SOCS-1 siRNA yielded an inhibition of SOCS-1 expression by up to 80%. The results of those experiments are consistent in terms of retardation of *in vitro* growth by SOCS-1. The growth-inhibitory effect of SOCS-1 in human prostate cancer cell lines is in concordance with a recent paper in which the authors described that the SOCS-1 mimetic peptide Tkip is a negative growth regulator of DU-145 cells.[28] It was demonstrated that the SOCS-1 inhibitory effect is mediated through negative regulation of STAT3 phosphorylation. DU-145 cells used in our laboratory and those subjected to treatment with Tkip differ in the phosphorylation of STAT3.[9] Our results are similar to those of Spiotto and Chung who did not detect phosphorylated form of STAT3 in DU-145 cells after treatment with IL-6.[29] Differences in data on phosphorylation of STAT3 reported by various laboratories may be explained by results from a recent publication of Kreis and associates. Those authors demonstrated that STAT3 activity depends on cell density in melanoma models.[30] The fact that inhibition of expression of SOCS-1 did not prevent growth inhibition by IL-6 in a LNCaP subline leaves the possibility that other members of the SOCS family such as SOCS-3 are more important for growth regulation by IL-6.

As new clues to the effect on proliferation, we demonstrate that down-regulation of SOCS-1 by siRNA yields increased expression of cyclins D1 and E and cdk 2 and 4, which drive cell cycle progression to the S phase. Inhibition of expression of cyclin D1 by SOCS-1 is in concordance with the report of Flowers and associates.[28] We have extended those previous findings and showed the effect of SOCS-1 on inhibition of critical cell cycle regulators in the two cell lines, which either lack phosphorylated STAT3 (DU-145) or are STAT3-null (PC3). Thus, our important new finding is that SOCS-1 can act as a negative growth regulator independently of its inhibitory role on the JAK-STAT pathway. Investigations on interference of SOCS with other signaling pathways in human prostate cancer seem to be justified. In this context, it is worthwhile to mention that another negative regulator of cytokine signaling, protein inhibitors of activated STAT 3 increases sensitivity of lung cancer cells to chemotherapeutic agents through inactivation of the phosphotidylinositol 3-kinase/Akt pathway.[31] Similarly, multiple nuclear factor kappa B-regulated pathways are inhibited by SOCS-3 in pancreatic cells.[32]

The results from other studies investigating the levels of SOCS-1 in malignant disease suggest that tumor type-specific differences exist. Liu and colleagues reported that the SOCS-1 gene is hypermethylated and consequently silenced *in vitro* and *in vivo*.[33] On the other hand, Hatirnaz and associates did not confirm those previous results.[34] A role of SOCS-1 as a tumor suppressor is evident from experiments with knockout fibroblasts, which are more sensitive to both spontaneous or oncogene-induced transformation.[35] In melanomas SOCS-1 represses the expression of matrix metalloproteinase, basic fibroblast growth factor, and vascular endothelial growth factor thus contributing to inhibition of invasion and angiogenesis.[36] In future studies it may be determined how SOCS-1 interacts with various partner proteins in human cancer.

Our tissue microarray data revealed the presence of SOCS-1 in pre-malignant and malignant lesions in the prostate. Although the presence of a protein that inhibits proliferation in cancer cells in patients' samples may be regarded as a contradiction, other possible functions of SOCS-1 have to be considered. SOCS-1 may regulate prostate cancer immune response. Shen and associates showed that SOCS-1 silencing results in enhanced antigen presentation by dendritic cells.[37] This finding implies

that the presence of SOCS-1 impairs immune response to a tumor. In this context, it should be kept in mind that transforming growth factor-β, a cytokine that is a potent growth inhibitor *in vitro*, is increasingly expressed in prostate cancer in which it has angiogenic and immunosuppressive properties.[38,39] Implications of enhanced expression of SOCS-1 *in vivo* may be better understood in the future when animal models in which its expression could be regulated are available.

We also show that SOCS-1 levels decreased in patients undergoing anti-androgen therapy. Explanations for these *in vivo* findings are partly offered by results of experiments performed in the prostate cancer cell line PC3-AR. In those cells, there was a concentration-dependent stimulatory effect of androgen on the SOCS-1 protein. These data are similar to our previous findings showing that SOCS-3 expression in prostate cancer cells is up-regulated by androgens.[10] The effect of androgen is, however, cell type-specific. Interestingly, androgens repressed the expression of human telomerase reverse transcriptase and telomerase activity in PC3-AR but not in LNCaP cells.[40] Differential regulation of SOCS-1 in prostate cancer cell lines may be explained by either presence of AR mutation or insufficient expression of a coactivator, which is critical for SOCS-1 expression. It was for example documented that PC3 but not DU-145 cells express the coactivators required for tumor suppressor ability of AR.[41] At present, it could be hypothesized that SOCS-1 down-regulation affects growth regulation by androgens in prostate cancer. However, limitations with available *in vitro* models prevented us to study this regulation further. In PC3-AR cells, androgenic hormones show a minor inhibitory effect on proliferation (data not shown) and in LNCaP cells SOCS-1 protein was not regulated by androgen. Increased expression of SOCS-3 in specimens obtained from patients who did not respond to endocrine therapy may reflect activation of the AR by non-steroidal compounds such as proinflammatory cytokines or HER-2/neu.[42–45] There may be a mechanism by which steroids regulate SOCS expression in selected cell lines. It was demonstrated that estrogenic steroids up-regulate SOCS-3 in hepatic and breast cancer cells.[46,47] SOCS-1 expression did not change in normal tissue or PIN after therapy compared with that in samples obtained before endocrine treatment. This finding could be explained by SOCS-1 expression in the basal cells, which are androgen-insensitive.

Experiments reported in the present manuscript were carried out with prostate cancer cell lines that are considered models for an advanced disease. It has been proposed that proinflammatory cytokines have a role in early prostate carcinogenesis.[1] For further analysis of the function of IL and SOCS in chronic inflammation that may lead to cancer, appropriate models should be developed.

In conclusion, in the present manuscript we show for the first time that SOCS-1 is expressed in all tested prostate cancer cell lines and in clinical specimens. It is up-regulated by IL-6 and inhibits proliferation of prostate cancer cells. Thus, we have demonstrated a growth-inhibitory effect of SOCS-1 in prostate cancer through negative regulations of cyclins and cdk. Other possible aspects of SOCS-1 action in carcinoma of the prostate, such as regulation of the immune response, should be addressed in future experimental studies.

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