Suppressor of cytokine signalling-3 is up-regulated by androgen in prostate cancer cell lines and inhibits androgen-mediated proliferation and secretion

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Abstract

Suppressors of cytokine signalling (SOCS) are induced by interleukins (ILs) and various peptide hormones and may prevent sustained activation of signalling pathways. We have previously shown that SOCS-3 antagonizes regulation of cellular events by cAMP and is expressed in human prostate cancer. To investigate possible effects of androgen on SOCS-3 protein expression, two prostate cancer cell lines (PC3-AR and LAPC4) were treated with different concentrations of R1881. Western blot analyses revealed induction of SOCS-3 protein expression in both cell lines by androgen, an effect which can be blocked by the anti-androgen bicalutamide. To further characterize the effects of R1881 on the SOCS-3 gene, promoter–reporter assay and real-time PCR were performed. We found no influence of androgen on promoter activity or SOCS-3 mRNA levels, thus suggesting a post-transcriptional effect of androgen. Concordant with our previous findings, we show a significant increase of SOCS-3 protein after androgen treatment in cells in which transcription was blocked, but not in those with impaired translation. In order to understand implications of SOCS-3 regulation by androgen, we used SOCS-3-negative LNCaP–IL-6 cells and stably transfected them with a tetracycline-responsive SOCS-3 Tet-On plasmid. We report that androgenic effects on cell proliferation and prostate-specific antigen secretion are significantly diminished following up-regulation of SOCS-3. In conclusion, androgen up-regulates SOCS-3 protein via post-transcriptional effects. SOCS-3 inhibits androgen-stimulated proliferation by influencing cell cycle regulation. Taken together with previous findings showing androgen receptor activation by IL-6, our results imply that androgen and cytokine signalling pathways interact at multiple levels in prostate cancer.

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Introduction

Prostate cancer is the most common malignant disease in men, except for non-melanoma skin cancer (Jemal et al. 2006). Its development and progression are intimately connected with androgenic stimulation because of expression of the functional androgen receptor (AR) protein in androgen-dependent and independent tumour stages (Hobisch et al. 1996, Linja et al. 2001). AR down-regulation in cell lines that represent androgen-independent prostate tumours slows down cancer cell growth (Zegarra-Moro et al. 2002). Moreover, AR is active as levels of its target protein, prostate-specific antigen (PSA), are measurable in sera of patients with prostate cancer (Grossmann et al. 2001). In general, AR is activated in a ligand-dependent manner. Increased activation because of receptor mutations, AR amplification and the presence of growth factors or cytokines that act in a ligand-independent or synergistic manner occurs in prostate cancer (Culig et al. 2003). AR coactivators that potentiate ligand-dependent activation may be increasingly expressed in prostate cancer. Ligand-independent activation of the AR by ErbB-2 contributes to the in vivo progression of the LAPC4 prostate tumour (Craft et al. 1999). Several groups showed that interleukin-6 (IL-6) activates AR-mediated transcription in a ligand-independent
manner (Hobisch et al. 1998, Chen et al. 2000, Lin et al. 2001). Additionally, IL-6 potentiates the effects of low concentrations of androgen. Implications of AR activation by IL-6 on regulation of cellular events are not fully understood. Although IL-6 inhibits LNCaP prostate cancer cell proliferation, a subline selected in its presence acquires a growth advantage (Hobisch et al. 2001). Higher tissue levels of IL-6 measured in prostate cancer patients may reflect decreased androgenic suppression of the promoter or loss of the tumour suppressor retinoblastoma that is a negative regulator of its expression (Bellido et al. 1995, Steiner et al. 2003). Pleiotropic effects of IL-6 in target tissues occur because of differential activation of signalling pathways of Janus kinase (JAK)/signal transducer and activators of transcription (STAT), MAP kinases and phosphatidylinositol 3-kinase. STAT3, which is phosphorylated and translocated into the nucleus upon IL-6 treatment, is known to act as an oncogene in most malignancies. It may prevent tumour cell death, stimulate expression of angiogenic factors and induce expression of extracellular matrix degradation enzymes thus resulting in enhanced metastatic potential (Turkson & Jove 2000, Buettner et al. 2002). Its role in prostate cancer is still controversially discussed.

Suppressor of cytokine signalling (SOCS)-3 belongs to the SOCS family, which consists of eight members, named SOCS 1–7 and CIS. These proteins contain a central SH2 domain and the SOCS box, a conserved 40-residue C-terminal motif (Yoshimura et al. 1995, Endo et al. 1997, Naka et al. 1997, Starr et al. 1997, Hilton et al. 1998). In addition, SOCS-1 and SOCS-3 have a kinase inhibitory region constituted of 12 amino acids (Sasaki et al. 1999). Cytokines like IL-6 stimulate a rapid induction of SOCS proteins, which inhibit various steps of the pathway, in particular in the case of IL-6 the JAK/STAT3 signalling. They exert their action through different mechanisms such as binding to the phosphorylated cytokine receptor or JAK (Tan & Rabkin 2005). SOCS can reduce phosphorylation of JAKs or STATs, STAT dimerization or import to the nucleus and target the complex of SOCS-3 with JAK/STAT for proteasomal degradation (Naka et al. 1997, Starr et al. 1997, Song & Shuai 1998, Larsen & Ropke 2002). Thus, SOCS proteins are believed to be negative feedback loop regulators of cytokine-mediated signalling pathways.

Expression and function of SOCS proteins have been investigated in several malignant diseases. Those studies revealed that SOCS are implicated in regulation of cellular proliferation and apoptosis. In squamous cell carcinoma (SCC; Weber et al. 2005) or lung cancer (He et al. 2003b), SOCS-3 is lost because of gene promoter methylation. Hence, silencing of the SOCS-3 gene causes a sustained activation of STAT3, resulting in enhanced proliferation and a more malignant phenotype. Introducing SOCS-3 in SCC yielded inhibition of cell proliferation and enhanced apoptotic rate accompanied by decreased protein expression levels of Bcl-2.

However, in breast and prostate cancers, the two most common hormone-dependent malignancies in female and male SOCS-3 respectively is not lost. In prostate cancer cells, SOCS-3 antagonizes the effects of cAMP resulting in inhibition of apoptosis and promotion of cellular proliferation (Bellezza et al. 2006). In breast cancer, it was shown that oestriadiol up-regulates SOCS-3 mRNA levels (Leong et al. 2004, Matthews et al. 2005). In this context, it is of interest that SOCS-3 causes enhanced phosphorylation of ERK kinases and therefore may exert proliferative effects on breast cancer cell lines (Raccurt et al. 2003). On the basis of those results, we hypothesized that androgens are implicated in regulation of SOCS-3 expression in prostate cancer. In this study, we show that SOCS-3 is up-regulated by androgen via a post-transcriptional effect on mRNA translation. We report that up-regulation of SOCS-3 using a tetracycline-responsive construct inhibits androgen-stimulated proliferation and secretion. Moreover, SOCS-3 significantly diminishes stimulation of cell cycle regulatory proteins cdk2, cdk4, cyclins E and D1 by androgen.

**Materials and methods**

**Cell culture and chemicals**

Prostate cancer PC3-AR cells were donated by Dr Andrew Cato (Research Center Karlsruhe, Germany). LAPC4 cells were kindly provided by Dr Charles Sawyers (University of California, Los Angeles). CV1 cells were purchased from ATCC (Rockville, MD, USA). LNCaP–IL-6 cells were maintained in IMDM containing 15% foetal calf serum (FCS) supplemented with 10 nM R1881 (Perkin–Elmer, Montreal, Canada). All other cell lines were cultured in RPMI 1640 (HyClone, Logan, UT, USA). Media were supplemented with 10% FCS and penicillin/streptomycin (PAA Laboratories, Pasching, Austria). Medium for LNCaP–IL-6/B and D cells was additionally supplemented with 70 μg/ml hygromycin B (Merck). These clones derived from LNCaP–IL-6 cells which were described previously (Hobisch et al. 2001). DMSO, cycloheximide and actinomycin D were purchased from Sigma. The synthetic androgen methyltrienolone (R1881) is a product of DuPont NEN Products.
(Boston, MA, USA), and IL-6 was purchased from R&D (Minneapolis, MN, USA). The SOCS-3 promoter construct was a kind gift of Dr David Jablons (University of California, San Francisco). All experiments were performed in media containing either 3% or in case of clones 0.2% steroid-free FCS (HyClone). Controls were treated with vehicle only, which was either DMSO or ethanol. None of these solvents exceeded a dilution of 0.1% either in controls or in treated samples.

Real-time PCR

Cells were grown in six-well plates. Upon treatment with R1881, RNA isolation, cDNA synthesis and quantitative real-time PCR were performed as previously published (Bellezza et al. 2006). Each experiment was carried out in triplicates. TATA box-binding protein (TBP) was chosen as an endogenous expression standard (Savinainen et al. 2002). 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 (Version 1.3) were used to calculate the dCt using Microsoft Excel 2002. Values obtained in control cells were defined as 100% and those from treated cells were expressed as percent of control.

Western blot

Western blots, including harvesting and cell lysis, were performed as previously reported (Bellezza et al. 2006). In the same experiments, protein levels of SOCS-3 and AR were analysed in cytosolic and nuclear fractions. The following antibodies were used: anti-SOCS-3 (1:1000; Acris Antibodies, Hiddenhausen, Germany), anti-AR (1:200; Biogenex, San Ramon, CA, USA), anti-AR were analysed in cytosolic and nuclear fractions. The following antibodies were used: anti-SOCS-3 (1:1000; Acris Antibodies, Hiddenhausen, Germany), anti-AR (1:200; Biogenex, San Ramon, CA, USA), anti-cyclin D1 (1:750; LabVision, Fremont, CA, USA) and anti-cyclin E (1:500; Santa Cruz). The membranes were scanned and quantified using Odyssey Infrared Imaging System (LiCor Biosciences, Lincoln, NE, USA).

Immunofluorescence

PC3-AR cells were grown on microscope slides for 48 h and stimulated with 1 nM R1881 for 48 h. Cells were fixed with buffered 5% paraformaldehyde for 20 min. After incubations with primary antibodies, slides were washed four times with PBS followed by a secondary antibody step (Alexa Fluor 555, Invitrogen) for 30 min. Primary antibodies used were: anti-SOCS-3 (1:200; Acris Antibodies), anti-AR (1:200; Biogenex) and mouse negative control (Dako, Vienna, Austria). After rinsing the slides twice with PBS, fluorescence was analysed using a Zeiss Axio Imager M1 microscope (Carl Zeiss, Oberkochen, Germany).

Luciferase activity assay

PC3-AR, CV1 and PC3 cells were seeded at a density of 1×10⁴ cells per well in black-walled 96-well plates (Corning, Corning, NY, USA). Cells were transfected using Lipofectamin2000 (Invitrogen) according to the manufacturer’s protocol with the following plasmids: SOCS-3 promoter construct as published by He et al. (2003a), pHRL-null plasmid as a control for transfection efficiency and in case of AR-negative cells pSG5-AR expression plasmid (Hobisch et al. 1998). Cells were then stimulated with either IL-6 (25 ng/ml) as a positive control or with 1 nM R1881. Luciferase activity was assessed using Dual-Glo luciferase activity assay (Promega) according to the manufacturer’s protocol with a luminometer (Chameleon Microplate Reader, Hidex, Turku, Finland).

Generation of stably transfected cell lines

For inducible expression of N-terminal myc-tagged SOCS-3 in LNCaP–IL-6 cells, a tetracycline-responsive expression vector pBIG2i (Strathdee et al. 1999) containing the coding region of SOCS-3 and the myc tag was used (Tonko-Geymayer et al. 2002). The plasmid was a kind gift of Prof. Wolfgang Doppler (Division of Medical Biochemistry, Innsbruck Medical University). In order to obtain stably transfected cells, 1×10⁶ LNCaP–IL-6 cells were transfected with 3 μg plasmid using Amaxa electroporation (Amaxa, Cologne, Germany) according to the manufacturer’s protocol. Transfected cells were selected in RPMI 1640 medium containing 70 μg/ml hygromycin B, 10% FCS, 1% penicillin/streptomycin and 1% glutamine (Invitrogen). Single cell clones were obtained using clone cylinders (Sigma–Aldrich).

Selected clones were cultivated in RPMI 1640 medium containing 10% tetracycline-free FCS (HyClone) followed by treatment with 2 μg/ml doxycycline (Sigma–Aldrich) for 2 days. SOCS-3 levels were measured by western blot. Two clones, LNCaP–IL-6/B and D (from now referred to as clone B and D) were chosen for further experiments because of similar expression levels of the AR and phenotype when compared with parental LNCaP–IL-6 cells.
[\textsuperscript{3}H]Thymidine incorporation assay

Thymidine incorporation assay was carried out as reported previously (Bellezza et al. 2006). The treatment times were as follows: after 24 h of stimulation with doxycycline (2 \( \mu \text{g/mL} \)), cells were treated with different concentrations of R1881 (1, 10, 100, 500 and 1000 pM) for 48 h.

PSA measurement

For measurement of PSA secretion, clones B and D were grown in 24-well plates (Corning) for 24 h with or without doxycycline and subsequently stimulated with different concentrations of R1881 (10, 100 and 1000 pM) for 48 h. PSA content was measured using Advia CentaurXP Immunoassay System (Siemens, Munich, Germany), and cell number was assessed by Casy Cell Counter and Analyser (Schaerfe Systems, Reutlingen, Germany). PSA levels were finally adjusted to cell number.

Statistical analysis

For each treatment group, statistical distribution was determined using Kolmogorov–Smirnov test. In case of non-Gaussian distribution, non-parametric tests were applied as follows: to assess the overall significance for experiments with more than one treatment group, we used Kruskal–Wallis test; additionally, Mann–Whitney U test was applied when appropriate. In case of Gaussian distribution two-sided Student’s t-test was used. For comparing datasets in PSA and proliferation experiments, two-way ANOVA for non-repeated measurements was applied. Bonferroni’s post hoc test was used to correct for multiple testing. Corrected P values <0.05 were defined as statistically significant and encoded in figures (*\( P<0.05 \), **\( P<0.01 \), ***\( P<0.001 \)). All statistical analyses were performed using SPSS 12.0 software (SPSS, Chicago, IL, USA). For detailed description of statistics, please refer to the figure legends.

Results

SOCS-3 is up-regulated by androgen in two prostate cancer cell lines

In order to investigate the influence of androgenic stimulation on protein expression of SOCS-3, two prostate cancer cell lines, in particular PC3-AR and LAPC4 were used. PC3-AR cells were treated with increasing concentrations of the synthetic androgen R1881, whereas in LAPC4, which are maintained in medium supplemented with 10 nM R1881, levels of androgen were decreased to 1 and 0.1 nM. In the PC3-AR cell line, we report that with increasing concentrations of androgen SOCS-3 expression levels increase significantly by threefold in the cytosol (Fig. 1A) without affecting SOCS-3 levels inside the nucleus (data not shown). In the case of LAPC4 cells, we evince a decrease of SOCS-3 (Fig. 1B) protein levels with decreasing concentrations of R1881 (10, 1 and 0.1 nM; \( P=0.011 \)).

To further confirm these results, we stimulated both cell lines with the same concentrations of R1881 in the presence of 1 and 5 \( \mu \text{M} \) anti-androgen bicalutamide. Corroborating our findings, under those conditions changing amounts of androgen did not yield an alteration of SOCS-3 protein in cytosol (Fig. 1A) and nucleus (data not shown). In LAPC4 cells, treatment with bicalutamide decreased SOCS-3 levels to about 60% of control. Additionally, we treated AR-negative PC3 cells with 0.1 and 1 nM R1881 and found no change in SOCS-3 levels (Fig. 1C). Taken together, these results strongly suggest an androgenic effect on SOCS-3 expression.

To analyse the time course of androgen-mediated SOCS-3 up-regulation, we performed experiments by stimulating PC3-AR cells for 24, 48 and 72 h with different concentrations of R1881 (0.1 and 1 nM). As shown in Fig. 2, we found the strongest induction at 48 h (\( P=0.0064 \)). AR levels were assessed in all experiments mentioned above. As expected, we found higher AR expression after androgenic stimulation in a dose-dependent manner with increasing concentrations of R1881 (data not shown). In LAPC4 cells, this regulation was also found, meaning a dose-dependent decrease to \( \sim 60\% \) of control with decreasing androgen concentrations. In nuclear extracts, this alteration was more prominent, for example, 13-fold AR increase in PC3-AR cells (data not shown). To corroborate our findings with western blot, we performed immunofluorescence experiments using PC3-AR cells. Both SOCS-3 and AR levels were enhanced in cells stimulated with 1 nM R1881 for 48 h (Fig. 1D).

Up-regulation of SOCS-3 by androgen is dependent on translation

To further investigate androgen-mediated SOCS-3 up-regulation, we questioned whether SOCS-3 promoter coupled with a firefly luciferase reporter can be directly stimulated by R1881 in PC3-AR and CV1 cells. For this purpose, the cells were transfected with a plasmid containing the 5\(^\text{th} \) region of the human SOCS-3 promoter (He et al. 2003a). CV1 cells, which lack AR, were co-transfected with the pSG5-AR plasmid (Hobisch et al. 1998). IL-6 stimulation was
considered a positive control for activation, whereas AR-negative PC3 cells were used as a negative control. Androgenic effect was proved by western blotting, showing a substantial increase of AR and translocation to the nucleus (data not shown). As demonstrated in Fig. 3, we found that neither in PC3-AR nor in CV1 cells androgenic stimulation yields a significant activation of the SOCS-3 promoter. Then, we asked whether SOCS-3 mRNA is regulated after androgen treatment in PC3-AR cells. For this purpose, the cells were treated with 0.1 and 1 nM R1881 at different time points (30 min, 1, 2, 8 and 24 h). We found no up-regulation of SOCS-3 mRNA at any time point (Fig. 4). In fact, mRNA levels were decreased after androgen treatment; however, due to the correction for multiple testing (Bonferroni’s post hoc test), these results are not statistically significant. Taken together, these experiments demonstrate that androgen does not have a direct effect on the SOCS-3 promoter, thus not enhancing mRNA transcription.

Hence, we asked whether androgen could up-regulate SOCS-3 protein levels when transcription is inhibited. Therefore, PC3-AR cells were pre-treated with actinomycin D, followed by a 48-h stimulation with 0.1 and 1 nM R1881. Actinomycin D, used at a concentration of 10 nM, did not increase apoptotic cell death, as measured by flow cytometry using Nicoletti’s method, over a time period of 72 h (data not shown). Compared with control conditions, under which SOCS-3 was up-regulated by twofold with

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**Figure 1** SOCS-3 expression levels assessed by western blot. (A) PC3-AR cells were treated for 48 h with the indicated concentrations of R1881 with or without pre-treatment with bicalutamide (1 and 5 μM). Results represent mean ± s.d. (n=8; number of independent experiments carried out in duplicates). (B) LAPC4 cells, maintained in medium supplemented with 10 nM R1881, were incubated for 48 h with decreasing concentrations of androgen with or without bicalutamide. Results shown are mean ± s.d. (n=8). (C) PC3 cells were treated with different concentrations of R1881 for 48 h. Results represent mean ± s.d. (n=4). (D) Immunofluorescence of PC3-AR cells grown on microscope slides and treated with 1 nM R1881 for 48 h. Statistics: (A) Mann–Whitney U: Co versus 0.1 nM R1881: P=0.000095***; Co versus 1 nM R1881: P=0.00266**; (B) Mann–Whitney U: Co versus 1, 0.1 nM R1881, 10 nM R1881+5 μM Bic: P=0.0117*, 0.0117*, 0.0049**.

**Figure 2** Time course of SOCS-3 expression after androgenic stimulation. PC3-AR cells were treated with R1881 for 24, 48 and 72 h. Results show mean ± s.d. (n=5). Statistics: Mann–Whitney U: 48 h Co versus 48 h 0.1, 1 nM R1881: P=0.0106*, 0.0064**; 72 h Co versus 72 h 0.1, 1 nM R1881: P=0.0208*, 0.0106**.
1 nM R1881 \((P = 0.0277)\), SOCS-3 protein levels were similarly increased in cells pre-treated with actinomycin D \((P = 0.0385; \text{Fig. 5})\). In conclusion, these data suggest that androgen-stimulated SOCS-3 protein up-regulation is not dependent on transcription.

To further elucidate these findings, we blocked translation by pre-treating the cells with cycloheximide \((1 \mu\text{g/ml})\) for 1 h followed by R1881 stimulation for 48 h. This concentration of cycloheximide did not alter the apoptotic rate (data not shown). We report that blocking translation significantly inhibits androgen-mediated SOCS-3 up-regulation, thus demonstrating the need for a functional translation apparatus to enhance SOCS-3 expression by androgen (Fig. 5).

**Up-regulation of SOCS-3 inhibits androgen-stimulated cell proliferation and PSA secretion**

To investigate the implications of SOCS-3 regulation on cellular function, we intended to use specific siRNA and knock-down SOCS-3 expression. Although SOCS-3 siRNA significantly lowered SOCS-3 levels in PC3 cells (Bellezza et al. 2006), it was not effective in PC3-AR or LAPC4 cells. To establish an appropriate model for studies of cellular events, we generated two sublines, derived from LNCaP–IL-6 cells (Steiner et al. 2003), which were transfected with a tetracycline-inducible construct encoding for SOCS-3. In two clones, in particular LNCaP–IL-6/B and D (clone B and D), doxycycline at a concentration of 2 \(\mu\text{g/ml}\) up-regulated SOCS-3 levels by about four- and sixfold \((B: P = 0.0138; D: P = 0.0243)\) respectively. After stimulation for 24 h, the cells were allowed to partially degrade SOCS-3 under non-stimulating conditions (without doxycycline) with or without 1 nM R1881. When compared with cells cultured without R1881, SOCS-3 levels in cells treated with androgen increased by \(\sim 1.5\)-fold \((B: P = 0.0084; D: P = 0.0063; \text{Fig. 6})\). Androgens exert a biphasic effect on cellular proliferation of prostate cancer cells (Sonnenschein et al. 1989, Lee et al. 1995). It was shown that low picomolar concentrations cause a maximal stimulation of cell growth. In order to test possible implications of SOCS-3 regulation for androgen-mediated growth effects, clones B and D were cultured in the absence or presence of doxycycline for 24 h, followed by a 48 h incubation with different concentrations of R1881 \((0.001, 0.01, 0.1, 0.5 \text{ and } 1 \text{nM})\). We confirm a four- to sixfold increase of cMyc-SOCS-3 after doxycycline stimulation as shown above (Figs 6 and 7A, right panel). Under those conditions, cellular proliferation was not altered (Fig. 7A, right panel). As shown in Fig. 7A (left panel), treatment with androgen yielded stimulation of cell proliferation at concentrations up to 0.1 nM thus confirming previous results (Sonnenschein et al. 1989, Lee et al. 1995). However, in cells in which SOCS-3 expression was induced by tetracycline,
changes in proliferation rate were substantially abolished. In particular, neither clone B nor D could be stimulated by R1881 (\(P < 0.0001\)). Inhibitory concentrations of androgen yielded a more pronounced repression of cell growth.

Additionally, we tested PSA production as a parameter for androgen-stimulated transcription under the same conditions as mentioned above. PSA levels increased by five- to sevenfold in clones B and D respectively. Confirming our previous results, PSA secretion was increased 2- to 2.5-fold in the presence of SOCS-3 (\(P < 0.001\); Fig. 7B).

SOCS-3 negatively influences androgen-mediated stimulation of cell cycle regulators

Molecules that have been reported to play a pivotal role in androgen-stimulated growth include cdk2, cdk4, cyclins D1 and E (Lu et al. 1997, Xu et al. 2006). To substantiate our findings from proliferation assays, we investigated expression of these cell cycle regulatory proteins after 48 h of androgen stimulation (1 and 100 pM R1881). We found that stimulation with R1881 resulted in a significant increase of cdk2, cdk4, cyclins D1 and E expression levels by about 1.8-, 1.5-, 1.6- and 2.2-fold respectively, in clone B (\(P = 0.044, 0.0107, 0.044\) and 0.011; Fig. 8). In clone D, similar significant regulations were found; however, the increase was lower than that in clone B (\(P = 0.046, 0.013, 0.013\) and 0.0233).

In order to elucidate implications of androgenic regulation of SOCS-3, the same experiments were performed in the presence of 2 µg/ml doxycycline. Interestingly, none of the cell cycle regulators was increased after the treatment with R1881 when SOCS-3 was up-regulated thus confirming our results from proliferation assays (Fig. 8). These data show that SOCS-3 significantly impairs androgen-stimulated cell proliferation by interfering with regulation of cell cycle.

Discussion

There is an increasing evidence that SOCS-3, beside its function as a negative feedback regulator of cytokine signalling, may exert pleiotropic effects on cell proliferation and apoptosis. We have previously shown that SOCS-3 is expressed in prostate cancer in vitro and in vivo and antagonizes cAMP-mediated apoptosis in cell lines (Bellezza et al. 2006). Furthermore, it is known that in breast cancer and melanoma (Raccurt et al. 2003, Komyod et al. 2007) levels of SOCS-3 are elevated and contribute to growth advantage and higher malignant phenotype. Based on previous data showing regulation of SOCS-3 by oestrogen (Matthews et al. 2005), we questioned whether there is an interplay between androgen and SOCS-3 in prostate cancer cell lines. Furthermore, it was previously shown that IL-6 acts as a ligand-independent activator of the AR (Hobisch et al. 1998, Chen et al. 2000, Lin et al. 2001).

In order to test our hypothesis, we used PC3-AR cells and the AR-positive cell line LAPC4. The reasons for choosing these two cell lines were on one hand that both express wild-type AR and on the other hand that LNCaP cells, which express a mutated AR, are SOCS-3 negative. In both cell lines, we found a significant androgenic stimulation of SOCS-3 protein expression after 48 h. These data strongly corroborate our hypothesis that SOCS-3 indeed is an androgen-responsive protein. The lowest concentration of R1881 for treatment of LAPC4 cells in our experiments that could be used without induction of apoptosis was 0.1 nM.

To further elucidate these findings, we investigated androgenic effects on SOCS-3 promoter activity and mRNA levels using luciferase assay and quantitative real-time PCR respectively. Neither promoter activity nor mRNA levels increased after androgen treatment.
Interestingly, our results obtained with prostate cells differ from those published in hepatoma or breast cancer cell lines in which oestrogen up-regulated SOCS-3 through transcriptional activation (Leong et al. 2004, Matthews et al. 2005). It is generally accepted that AR not only may act as a transcription factor but also has direct effects on protein translation or stability. Perry & Tindall (1996) showed that proliferating cell nuclear antigen, which is required for DNA replication, is up-regulated by androgen by increasing protein stability. Our data are comparable with those of Xu et al. (2006) who reported that D-type cyclins are up-regulated upon androgen treatment via post-transcriptional mechanisms. It was demonstrated that androgens are also capable of activating signalling pathways, such as that of phosphoinositol 3-kinase/AKT by stimulating AR interaction with the p85α kinase subunit and Src in the absence of endogenous receptor transcriptional activity (Castoria et al. 2003, Sun et al. 2003). Additionally, upon androgenic stimulation, Src and protein kinase C α mediate the phosphorylation of ezrin, a key signalling molecule that regulates cell survival, adhesion, migration and invasion of prostate cancer cells (Pang

Figure 7 (A) Left panel: [3H]thymidine incorporation assay after androgenic stimulation of clone B and D. Cells were seeded at a density of $1 \times 10^5$ cells per well in 96-well plates. After a 24-h incubation with or without doxycycline, the cells were treated with different concentrations of R1881. Means ± S.E.M. (n=4). Right panel: Clones B and D were treated with doxycycline for 48 h, followed by quantification of CMyc-SOCS-3 protein levels by western blots and measurement of cell proliferation by [3H]thymidine incorporation. Mean ± S.E.M. (n=4). (B) PSA levels after treatment with or without doxycycline under androgenic stimulation. Mean ± S.E.M. (n=3) Statistics: (A) Two-way ANOVA – overall difference between B versus B+: $P<0.0001^{***}$; D versus D+: $P<0.0001^{***}$; corrected $P_c$ values for B versus B+ and D versus D+ for each concentration: >0.05, <0.01**, <0.05*, <0.05*, >0.05 and >0.05, <0.05*, <0.05*, >0.05, >0.05, >0.05, >0.05, >0.05, >0.05, >0.05, >0.05. (B) Two-way ANOVA – overall difference between B versus B+: $P=0.0019^{**}$; D versus D+: $P=0.0011^{***}$; corrected $P_c$ values for B versus B+ and D versus D+ for each concentration: >0.05, >0.05, <0.05*, <0.05* and >0.05, >0.05, >0.05, >0.05, >0.01**.
et al. 2004, Chuan et al. 2006). To clarify the mechanism of regulation of SOCS-3, we blocked transcription and translation using actinomycin D and cycloheximide respectively. We found that blockade of transcription did not significantly inhibit androgen-mediated up-regulation of SOCS-3, whereas impeding translation substantially blocked this increase. Taken together, those data show that SOCS-3 is up-regulated by androgen in prostate cancer cell lines (PC3-AR and LAPC4) via post-transcriptional effects. Future studies may address an issue of androgenic interference with proteasomal degradation of SOCS-3. In this context, it is of interest that Zhang et al. (1999) have reported that such degradation plays a crucial role in regulating SOCS-3 expression after induction by IL-6.

Up-regulation of SOCS-3 by androgen might have clinical implications, due to the fact that AR is expressed at all stages of prostate cancer development and plays a pivotal role in carcinogenesis. Furthermore, we have reported that SOCS-3 is expressed at a higher level in malignant than in benign prostate tissue samples (Bellezza et al. 2006).

To establish a novel cellular model for studies on implications of SOCS-3 up-regulation, we stably transfected LNCaP–IL-6 cells with a tetracycline-responsive plasmid encoding for SOCS-3. We report that induction with doxycycline increased SOCS-3 levels in clones B and D. Moreover, SOCS-3 levels were elevated when compared with controls after subsequent treatment with R1881 for 24 h. Using previously published SOCS-3-specific siRNA (Bellezza et al. 2006), we aimed to address the question whether SOCS-3 has an impact on androgen-stimulated proliferation. However, although SOCS-3 levels were significantly lowered in PC3 cells, we were not able to reproduce those results in PC3-AR or LAPC4 cells. Hence, we tested various other SOCS-3 targeting siRNAs and oligonucleotides from different manufacturers (Dharmacon, Ambion) and others that have previously been published (Leung et al. 2003, Gomez-Guerrero et al. 2004, Calegari et al. 2005) using four different transfection reagents/methods. Although transfection efficiency as assessed using siRNA against GAPDH as a positive control was satisfactory, we were not able to significantly knock-down SOCS-3 levels. Thus, we used our stably transfected LNCaP–IL-6/B and D cells. Androgen treatment was applied with or without doxycycline-stimulated SOCS-3 expression.
Interestingly, we found that SOCS-3 substantially diminishes androgen-mediated cell proliferation at concentrations up to 100 pM R1881, whereas effects of inhibitory concentrations were even more pronounced when SOCS-3 expression was elevated. It was previously shown that D-type cyclins and cdks are induced by androgen thus resulting in enhanced G1–S phase transition and increased proliferation rate (Xu et al. 2006). To test whether SOCS-3 can impair the stimulation of protein expression levels of cyclins D1 and E, cdks2 and cdks4, we cultured clones B and D with or without doxycycline followed by androgenic stimulation. It is generally accepted that cell cycle regulatory genes are direct or post-transcriptional targets of the AR. Many publications report up-regulation of G1 cyclins, cdks or reduced expression of retinoblastoma, p16 or cdks inhibitors in vitro and in vivo (Chen et al. 1996, Lu et al. 1997, Ye et al. 1999, Taneja et al. 2001). Similar to results published by Xu et al. (2006), we found a significant up-regulation of all tested cell cycle regulatory molecules by androgen when SOCS-3 was not induced. However, after induction of SOCS-3 by doxycycline, these up-regulations were abolished, thus confirming a negative role of SOCS-3 on androgen-mediated proliferation.

Taken together with results of our previous publication (Bellezza et al. 2006), the present study suggests that SOCS-3 may have cellular context-dependent effects on tumour growth. Therefore, it is not surprising that both tumour-suppressive (He et al. 2003b, Niwa et al. 2005, Weber et al. 2005) and tumour-promoting (Bellezza et al. 2006, Fojtova et al. 2007, Zhou et al. 2007) roles were reported in the literature.

In conclusion, we have shown that androgen is capable of up-regulating SOCS-3 protein levels without affecting transcription. Furthermore, [3H]thymidine incorporation assays have proven that SOCS-3 can antagonize proliferative effects of androgen. Confirming these findings, western blot analyses showed that up-regulation of cyclins and cdks after androgenic stimulation can be effectively inhibited by SOCS-3. To our knowledge, this is the first study reporting a negative feedback loop in androgenic regulation of cell growth and secretion.

The results of our study, in conjunction with previous work on androgen and IL-6 signalling in prostate cancer, confirm a common negative regulator for androgen and IL-6 pathways in prostate cancer. IL-6 may cause a prodifferentiation effect as evidenced by PSA increase. Sustained activation of the JAK/STAT pathway is prevented by up-regulation of SOCS-3 by IL-6. SOCS-3 is also a negative feedback regulator for androgen signalling in prostate cancer cells.

Figure 9 SOCS-3 is a common negative regulator for androgen and IL-6 pathways in prostate cancer. IL-6 may cause a prodifferentiation effect as evidenced by PSA increase. Sustained activation of the JAK/STAT pathway is prevented by up-regulation of SOCS-3 by IL-6. SOCS-3 is also a negative feedback regulator for androgen signalling in prostate cancer cells.
prostate cancer, may lead to establishment of a novel concept in growth and differentiation regulation (Fig. 9). It is based on the fact that androgen and IL-6 induce the same negative regulator of signalling, SOCS-3. Thus, the hypothesis that SOCS-3 has an important role in prostate cancer in vivo warrants further investigation.

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References


(IL-6) treatment express IL-6 and acquire an IL-6 partially resistant phenotype. Clinical Cancer Research 7 2941–2948.


cancer cell line LNCaP is associated with reduced retinoblastoma protein expression. *Journal of Cellular Biochemistry* **84** 188–199.


