SOCS3 Modulates the Response to Enzalutamide and Is Regulated by Androgen Receptor Signaling and CpG Methylation in Prostate Cancer Cells

Florian Handle, Holger H.H. Erb, Birgit Luef, Julia Hoefer, Dimo Dietrich, Walther Parson, Glen Kristiansen, Frédéric R. Santer, and Zoran Culig

Abstract

The proinflammatory cytokine IL6 is associated with bad prognosis in prostate cancer and implicated in progression to castration resistance. Suppressor of cytokine signaling 3 (SOCS3) is an IL6-induced negative feedback regulator of the IL6/janus kinase (JAK)/STAT3 pathway. This study reveals that the SOCS3 promoter is hypomethylated in cancerous regions compared with adjacent benign tissue in prostate cancer using methylation-specific qPCR. A series of in vitro experiments was performed to assess the functional impact of low SOCS3 expression during anti-androgen treatment. Using lentivirus-mediated knockdown, it was demonstrated for the first time that SOCS3 regulates IL6/JAK/STAT3 signaling in androgen receptor–positive LNCaP cells. In addition, SOCS3 mRNA is upregulated by the anti-androgens bicalutamide and enzalutamide. This effect is caused by androgen receptor–mediated suppression of IL6ST and JAK1 expression, which leads to altered STAT3 signaling. Functionally, knockdown of SOCS3 led to enhanced androgen receptor activity after 3 weeks of enzalutamide treatment in an inflammatory setting. Furthermore, the stemness/self-renewal associated genes SOX2 and NANOG were strongly upregulated by the long-term treatment, and modulation of SOCS3 expression was sufficient to counteract this effect. These findings prove that SOCS3 plays an important role during anti-androgen treatment in an inflammatory environment.

Implications: SOCS3 is frequently inactivated by promoter hypermethylation in prostate cancer, which disrupts the feedback regulation of IL6 signaling and leads to reduced efficacy of enzalutamide in the presence of inflammatory cytokines.

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Introduction

Prostate cancer is the most common type of noncutaneous cancer in men in the developed countries (1). Localized prostate cancer is typically treated by surgery or radiation therapy, whereas treatment of advanced stages is predominantly based on inhibition of androgen receptor activity (2). Although, endocrine therapy is initially effective, most patients progress to castration-resistant prostate cancer (CRPC) within a few years (3).

The proinflammatory cytokine IL6 has been implicated in the development of castration resistance (4, 5) and ligand independent trans-activation of the androgen receptor (6, 7). In addition, IL6 has been demonstrated to enable androgen-sensitive cell lines to grow in castrated mice (8). Furthermore, IL6 is a predictive marker for poor prognosis in prostate cancer (9, 10).

IL6 binds to the IL6 receptor subunit IL6R (gp80), which leads to a complex formation with the signal transduction subunit IL6ST (gp130) and subsequent activation of the Janus kinase (JAK) tyrosine kinases by autophosphorylation (5). This in turn leads to the activation of STAT3 by phosphorylation of Tyr705 and subsequent translocation to the nucleus, where it can bind DNA and act as a transcription factor (11, 12). Activated STAT3 has been found in primary as well as in metastatic prostate cancer and correlates with Gleason score, tumor stage, and castration resistance (11, 13). Constitutively active STAT3 has been shown to confer enzalutamide resistance (14), and STAT3 has been implicated in promoting metastasis during treatment with the anti-androgens bicalutamide and enzalutamide (15). Furthermore, STAT3 has been shown to bind and trans-activate the N-terminal domain of the AR (7), which may lead to enhanced activation of the androgen receptor and drive progression to castration resistance. Thus, the IL6/JAK/STAT3 and AR signaling pathways, which are investigated in this study, are linked to prostate cancer progression and the development of castration resistance. In addition, several groups have demonstrated that activation of the
SOCS3 and Androgen Receptor Signaling in Prostate Cancer

Table 1. Primer sequences for real-time PCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Assay ID</th>
<th>Gene name</th>
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<td>Hs01075666_m1</td>
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IL6/JAK/STAT3 pathway is required for maintenance of stem cell properties in human malignancies (5, 16–18), whereas androgen receptor activity is known to induce differentiation and suppress self-renewal and proliferation of prostate cancer cells (19).

One of the downstream target genes of the IL6/JAK/STAT3 signaling cascade is the negative feedback regulator suppressor of cytokine signaling 3 (SOCS3). SOCS3 is able to inhibit STAT3 activation by various mechanisms (12). Previous publications could show that SOCS3 is an essential survival factor in androgen receptor–negative prostate cancer cell lines with an autocrine IL6 loop and that its RNAi-mediated knockdown led to cell death (20). In contrast to this, several publications could demonstrate that SOCS3 is frequently inactivated by promoter hypermethylation in different types of cancer, including prostate cancer (21–23). In addition, homozygous deletion of SOCS3 in a murine pancreatic intraepithelial neoplasia model led to accelerated cancer progression and reduced survival (24). So far, the role of SOCS3 has not been studied in androgen receptor–positive prostate cancer cells due to the low basal expression of SOCS3.

In the present study, we investigated the epigenetic regulation of SOCS3 in prostate cancer tissue and analyzed the functional impact of low SOCS3 expression in androgen receptor–positive prostate cancer cell lines. Taken together, we could show that SOCS3 is not only regulated by promoter hypermethylation but also by androgenic signaling and that knockdown of SOCS3 enhances androgen receptor activity. This influences the expression of stem cell–associated genes during treatment with the second-generation anti-androgen enzalutamide in an inflammatory setting. These findings demonstrate an intricate bidirectional crosstalk between androgen receptor and IL6/JAK/STAT3 signaling and may provide the biochemical rationale for the development of novel therapies employing DNA methyltransferase inhibitors in prostate cancer.

Materials and Methods

Patients and samples

Patient samples included cancerous and adjacent benign areas of 25 selected radical prostatectomy specimens (discovery cohort). The confirmation cohort comprised 300 patients undergoing radical prostatectomy at the University Hospital of Bonn between 1998 and 2008. The study has been approved by the Institutional Review Board (IRB) at the University Hospital of Bonn. The IRB waived the need for written informed consent from the donors.

DNA methylation analysis

Methylation analysis of the SOCS3 promoter region was performed as previously described (25, 26). Briefly, DNA was isolated from formalin-fixed, paraffin-embedded (FFPE) tissue blocks and subsequently converted with bisulfite using the InnuCONVERT Bisulfite All-In-One Kit (Analytik Jena) as previously described (27). Quantification of methylated DNA was performed by duplex qPCR using methylation-specific primers for SOCS3 and methylation-insensitive primers for ACTB as a reference (Table 1). All qPCRs were run in triplicate. Relative methylation levels were computed using the ΔΔCt method as previously described (25).

Analysis of public Bisulfi te-Seq and androgen receptor ChIP-Seq data

Publicly available Bisulfi te-Seq data from the "DNA Methylation by Reduced Representation Bisulfi te Seq from ENCODE/HudsonAlpha" track (28) was evaluated in the UCSC browser. Publicly available androgen receptor ChIP-Seq data from public datasets (GSE62442, GSE65478, and GSE70679; refs. 29, 30) were analyzed in the UCSC browser. Detailed analysis was performed by calculating the coverage of aligned reads on 50-bp windows using bedtools (31).

Reagents and chemicals

RPMI1640 (product number: BE12-167F), DMEM low glucose (BE12-707F), sodium pyruvate (BE13-115E), and penicillin/streptomycin (17-602E) were purchased from Lonza. Normal FCS (RAT1003033) was purchased from PAA and charcoal/dextran (CSS)-treated FCS (SH130068.03) from GE Healthcare. GlutaMAX (#35050-038) was ordered from Thermo Fisher Scientific and poly-o-lysine hydrobromide (P6407) from Sigma-Aldrich. The DNA methyl transferase inhibitor 5′-aza-2′-deoxycytidine (A3656) was purchased from Sigma-Aldrich. The anti-androgens bicalutamide (B9061) and enzalutamide (T7002) were...
purchased from Sigma-Aldrich and MedChemexpress, respectively. The synthetic androgen R1881 was obtained from Organon and the STAT3 inhibitor galichalcone (sc-202165) was ordered from Santa Cruz Biotechnology. Recombinant human IL6 was purchased from R&D Systems (#206-IL-050).

Cell culture

The cell lines LNCaP-FGC, MDA PCa 2b, VCaP, PC3, and Du145 were purchased from ATCC. DuCaP were a kind gift from Prof. J. Schalken (Department of Urology, Radboud UMC, Nijmegen, The Netherlands), and LAPC4 were obtained from Prof. A. Cato (Institute of Toxicology and Genetics, Karlsruhe Institut für Technologie, Karlsruhe, Germany). The LNCaP-derived cell lines LNCaP abl, LNCaP Bic, and LNCaP IL6+ were established in our laboratory as previously published (32-34). Identity of the used cell lines was confirmed by short tandem repeat analysis in the last 6 months. DuCaP cells were grown in RPMI-1640 supplemented with 10% FCS, 1% penicillin/streptomycin, and 1× Glutamax. All other cell lines were cultured as recommended by the ATCC or as published previously (32–34). For experiments with MDA PCa 2b, LAPC4, LNCaP, and its derived sublines multi-well plates were precoated with poly-0-lysine hydrobromide. For steroid starvation experiments, the cells were cultivated in RPMI-1640 supplemented with 3% charcoal/dextran-treated FCS (CSS-FCS), 1% penicillin/streptomycin, and 1× Glutamax. Knockdown of androgen receptor was performed by transfecting 30 nmol/L of siRNA (target sequence 5′-GCACUGCUACUCUUCAGCA-3′) or a control siRNA with Lipofectamine 2000 (Thermo Fisher Scientific, #11668019).

Lentivirus production and generation of stable cell lines

For production of lentiviral particles, HEK293FT cells were transfected with pSAX2, pSV-G, and the corresponding pLenti6.4 expression vector using the calcium phosphate transfection method (35). Viral supernatants were harvested 48 hours after transfection and passed through a 0.45-μm filter. The target cells were incubated for 8 hours with the virus in the presence of 2 μg/mL polybrene and subsequently selected with 4 μg/mL blasticidin. For overexpression of SOCS3 shRNA with Lipofectamine 2000 (Thermo Fisher Scientific, #11668019).

Plasmids

For knockdown of SOCS3, the BLOCK-it HiPerform Lentiviral Pol-II miR RNAi Expression System with EmGFP was used (Thermo Fisher Scientific, K4934-00). Briefly, three artificial shRNAs targeting SOCS3 (2 commercial mir RNAi Select oligos from Thermo Fisher Scientific Hm421395 and Hm421393 and one self-designed oligo with the antisense sequence: 5′-ACGTGATGGCCAGTTCTCTGG3′) were separately cloned into the pcDNA6-2-GW/EmGFP-miR vector according to the manufacturer’s instructions and then shuttled into the pDONR221 vector using the Gateway system. For overexpression of SOCS3, the EmGFP sequence in the non-targeting control vector was replaced with the SOCS3 CDS by conventional cloning procedures using the Ncol and SalI sites. For overexpression of the full-length androgen receptor and the V7-splice variant, the CDS was excised from the corresponding pEGFP-AR vector, which were a kind gift from Prof. J. Céraline (University of Strasbourg, Strasbourg, France) and recloned into the pDONR221 vector. All vectors were then shuttled into the expression vector using MultiSite Gateway recombination with pLenti6.4/R4R2/V5-DEST vector and pENTR5/EF1 rop vectors.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from the cells with the RNeasy Mini Kit (Qiagen, #74106) and digested with RNase-free DNase (Qiagen, #79254) on the column according to the manufacturer’s instructions. The RNA was reverse-transcribed with the iScript Select cDNA Synthesis Kit (Bio-Rad, #1708897) using oligo-dT primers. Real-time PCR was performed on an ABI 7500 Fast system (Thermo Fisher Scientific) using the TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, #4444965). The assay IDs for the TaqMan Gene Expression Assays (Thermo Fisher Scientific) and the sequences for our custom primers are shown in Table 1. Custom primers were used at a concentration of 800 nmol/L each and the FAM-TAMRA-labeled probes at 150 nmol/L. Thermo-reacting conditions were as recommended by the manufacturer. All expression data were normalized to the geometric mean of the three reference genes HPRT1, TBP, and HMBS, and the expression was calculated using the ΔΔCt method.

Protein isolation and Western blotting

For protein isolation, cells were sonicated in sample buffer containing 250 mmol/L Tris-HCl, pH 8.5, 2% LDS, 10% glycerol, and 0.5 mmol/L EDTA. Protein concentration was measured by BCA (Thermo Fisher Scientific). Total protein concentration was measured by BCA (Thermo Fisher Scientific, #23225), and 10 to 50 μg total protein was mixed with sample reducing agent (Thermo Fisher Scientific, #NP0335BOX). The gels were transferred to methanol-activated PVDF membranes (GE Healthcare, #78833) according to the manufacturer’s instructions and the protein expression was normalized to lamin-A.

Statistical analysis

GraphPad Prism 4 and Excel were used for the statistical analysis. Mann–Whitney U test (patient data) and Student t test (cell culture experiments) were used for evaluation of statistical significance between two groups. Correlation was analyzed by Spearman method. For curve fitting of dose–response curves, nonlinear regression was used in GraphPad Prism 4. P < 0.05 was considered significant and indicated as follows: *, P < 0.05; **, P<0.01; ***, P<0.001. All experiments have been performed in at least three biological replicates.

Results

The SOCS3 promoter region is frequently hypermethylated in prostate cancer patients

Epigenetic regulation of SOCS3 expression by promoter hypermethylation has been reported in a variety of cancer types,
including prostate cancer and it has been correlated with reduced SOCS3 mRNA expression (21–23). The SOCS3 gene contains a CpG island in the published promoter region of SOCS3 (Fig. 1A; ref. 36), and treatment of several androgen receptor–positive prostate cancer cell lines with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (decitabine) led to increased SOCS3 mRNA expression (Supplementary Fig. S1A). To investigate the epigenetic regulation of SOCS3 in prostate cancer patient samples, a region of the CpG island with evident hypermethylation in endocrine-related, prostate and breast cancer cell lines, was selected on the basis of publicly available Bisulfi-Seq data (Supplementary Fig. S1B).

First, matched cancerous and adjacent benign tissue samples from a discovery set of 25 patients who underwent radical prostatectomy were analyzed for SOCS3 promoter hypermethylation. Methylation measurements from two patients had to be excluded because of failed qPCR amplification. In cancerous areas, SOCS3 promoter methylation was found to be significantly increased compared with adjacent benign tissue (Fig. 1B). On the basis of the median methylation degree in benign samples, a SOCS3 promoter methylation level of 10% was selected as a cutoff to define samples as methylation positive. More than 95% of the cancerous regions were methylation positive, compared with only 13% in the adjacent benign tissue samples. To validate the results of our discovery dataset, we next employed a validation cohort comprising cancer tissue samples from 300 prostate cancer patients (Table 2, Fig. 1C). Methylation measurements from 31 patients had to be excluded because of failed qPCR amplification. Using the previously defined cutoff, 85.5% of the patients in the validation cohort showed SOCS3 promoter hypermethylation. In addition, we discovered a weak although significant positive correlation with Gleason score and tumor stage. The whiskers represent the 10% to 90% percentile.

Table 2. Patient statistics for the SOCS3 promoter methylation analysis

<table>
<thead>
<tr>
<th>N</th>
<th>Median methylation percentage</th>
<th>Methylation-positive percentage</th>
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<tr>
<td>Total</td>
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</tr>
<tr>
<td>Gleason score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(&lt;5)</td>
<td>73</td>
<td>27.6%</td>
</tr>
<tr>
<td>6</td>
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<tr>
<td>(\geq 9)</td>
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</tr>
<tr>
<td>Total</td>
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\(r = 0.205, \text{ }P = 0.0008\)

<table>
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<tr>
<th>Tumor stage</th>
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<tr>
<td>T2</td>
<td>185</td>
<td>32.2%</td>
</tr>
<tr>
<td>T3</td>
<td>79</td>
<td>48.1%</td>
</tr>
<tr>
<td>Total</td>
<td>264</td>
<td>40.2%</td>
</tr>
</tbody>
</table>

\(\text{Biochemical recurrence: } N = 184, \text{ }r = 0.0005\) | | |
| **Yes** | 49 | 41.34% | 87.8% |
| Total | 233 | 40.2% | 85.3% |

NOTE: 300 patients were analyzed with methylation-specific PCR and the median methylation of one region in the SOCS3 promoter was given. A cutoff of 10% methylation was used to stratify the patients into methylation-positive and -negative. Correlation analysis was performed with Spearman and comparison of two groups with Mann–Whitney U test.
correlation between Gleason score and SOCS3 methylation ($^{**}$, $P = 0.0008$; $r = 0.205$). The number of methylation-positive samples increased with Gleason score from 74% (GS ≤ 5) to 93.3% (GS ≥ 9). Moreover, samples with tumor stage T3 displayed significantly higher levels of SOCS3 promoter methylation than tumor stage T2 samples ($P < 0.001$). However, there was no significant association between methylation and the occurrence of biochemical relapse ($P = 0.62$).

Taken together, this demonstrates a strong epigenetic downregulation of SOCS3 in prostate cancer.

**SOCS3 is expressed in androgen receptor–positive prostate cancer cell lines**

Because of the hypermethylation of the SOCS3 promoter in the majority of patients with prostate cancer and in androgen receptor–positive prostate cancer cell lines, we decided to study the functional impact of reduced SOCS3 levels in prostate cancer cell lines. Androgen receptor–positive prostate cancer cell lines do not express IL6 and thus have very low basal expression levels of SOCS3 mRNA compared with the IL6-secreting androgen receptor–negative prostate cancer cell lines (Fig. 2A, Supplementary Fig. S2). Since a major origin of IL6 in the prostate is from paracrine sources (37), we evaluated the expression of SOCS3 in androgen receptor–positive prostate cancer cell lines in the presence of recombinant IL6. The majority of androgen receptor–positive prostate cancer cell lines responded to IL6 with massive upregulation of SOCS3 mRNA, approaching mRNA levels comparable with those found in androgen receptor–negative cell lines (Fig. 2B). Next, we aimed to assess the ability of SOCS3 to inhibit IL6 signaling in androgen receptor–positive prostate cancer cell lines. To this end, LNCaP sublines with stable overexpression or knockdown of SOCS3 were generated using lentiviral vectors. SOCS3 knockdown efficiency was rather weak in presence of IL6 due to the activated feedback loop (Supplementary Fig. S3).
Nevertheless, upon knockdown of SOCS3, the activation of STAT3 (Tyr705 phosphorylation) and the nuclear localization of STAT3 were both enhanced, whereas overexpression of SOCS3 had the opposite effects (Fig. 2C). In addition, the IL6-induced transcriptional activity of STAT3 signaling upon SOCS3 manipulation was measured by qPCR on two known IL6-regulated genes (38), TGFBR3 and vimentin (VIM). Again knockdown of SOCS3 significantly enhanced the effects of IL6 treatment with the anti-androgens bicalutamide or enzalutamide and upon downregulation of the androgen receptor by siRNA was measured by qPCR after 72 hours. LNCaP cells were transfected either with full-length (FL) androgen receptor or the constitutively active AR-V7 splice variant and treated for 72 hours in 3% CSS-FCS with and without 1 nmol/L R1881. In addition, the endogenous expression level in the V7-positive cell lines DuCaP, VCaP, and 22Rv1 is shown. Expression of SOCS3, PSA, and FKBP5 was determined by qPCR.

Therefore, we conclude that SOCS3 is functionally active in androgen receptor–positive prostate cancer cell lines after IL6 treatment, despite promoter methylation.

SOCS3 is regulated by androgenic signaling

IL6/JAK/STAT3 signaling has been implicated in progression to castration resistance and correlates with bad prognosis in prostate cancer (8–10). Therefore, we measured mRNA expression of SOCS3 in two LNCaP sublines which are either resistant to steroid deprivation (LNCaP abl) or bicalutamide treatment (LNCaP Bic). It has to be noted that both resistant sublines and parental LNCaP cell line do not express IL6 (Supplementary Fig. S2), which allows us to analyze SOCS3 expression in a CRPC-like context independently of the influence of this cytokine. In both resistant sublines, SOCS3 mRNA was significantly increased compared with the parental LNCaP cell line, whereas androgen receptor activity was strongly reduced, as shown by decreased expression of prostate-specific antigen (PSA; Fig. 3A).

To elucidate whether SOCS3 is regulated by androgenic signaling, we treated two androgen receptor–positive and androgen-sensitive prostate cancer cell lines (LNCaP and DuCaP) with increasing concentrations of the synthetic androgen R1881. Androgen treatment led to a dose-dependent downregulation of SOCS3 mRNA in both cell lines. The decrease in SOCS3 showed an almost perfect negative correlation with PSA mRNA, which underlines the tight regulation of SOCS3 expression.
SOCS3 by androgens (Fig. 3B). Direct inhibition of the androgen receptor with the two clinically used anti-androgens bicalutamide and enzalutamide as well as androgen receptor downregulation by siRNA led to an increase in SOCS3 expression (Fig. 3C). In addition, we tested whether the constitutively active AR-V7 splice variant, which is expressed in DuCaP cells, is able to regulate SOCS3 expression. To this end, we transfected AR-V7-negative LNCaP cells with either full-length androgen receptor (AR-FL) or the AR-V7 variant to achieve an expression level equivalent to cell lines with endogenous AR-V7 expression (Fig. 3D). To verify the functionality of the AR-V7 vector, two differentially regulated androgen receptor target genes, PSA and FKBP5, were selected on the basis of published whole-genome expression array data (39). In agreement with that previous study, the AR-V7 splice variant led to a significant increase in FKBP5 expression but was unable to activate PSA mRNA expression. Notably, SOCS3 mRNA expression was not downregulated by the AR-V7 splice variant, neither in absence nor in presence of R1881.

Altogether, we conclude that SOCS3 expression levels are regulated by the full-length androgen receptor signaling axis.

The androgen-induced regulation of SOCS3 is a secondary effect of altered IL6 signaling

Since IL6 treatment led to a remarkable induction of SOCS3, we were interested whether androgenic signaling is able to influence SOCS3 in the presence of IL6-activated STAT3. Indeed, LNCaP and DuCaP cells co-treated with IL6 and R1881 displayed significantly lower SOCS3 levels than the IL6-only treated controls (Fig. 4A). Furthermore, inhibition of androgen receptor by enzalutamide was able to increase SOCS3 expression approximately 3- to 4-fold in the presence of different IL6 concentrations. To further investigate the mechanism of SOCS3 regulation by the androgen receptor, we screened for androgen receptor–binding sites in the SOCS3 gene using 12 published ChIP-Seq datasets (29, 30). No androgen receptor–binding element could be found in the vicinity of the transcription start site of SOCS3 (Supplementary Fig. S4A). The closest consistent androgen receptor–binding site was 50 kb upstream in the body of another gene and could not be found in datasets from primary patient material. This suggests that SOCS3 is not directly influenced by androgen receptor. Furthermore, no effect of R1881 on the posttranscriptional regulation of SOCS3 mRNA could be observed (Supplementary Fig. S5). Therefore, we analyzed whether the regulation of SOCS3 following R1881 or anti-androgen treatment might be due to a change in the functional activity of STAT3 signaling. Androgen receptor inactivation by enzalutamide was able to potentiate the IL6-mediated STAT3 phosphorylation in LNCaP and DuCaP cells (Fig. 4B). This finding suggests that the effect of enzalutamide on SOCS3 is mediated via STAT3. We confirmed this hypothesis by blocking the transcriptional activity of STAT3 with the specific inhibitor gafilatolactone (40). Inhibition of STAT3 signaling was able to completely inhibit the effect of enzalutamide on SOCS3 expression (Fig. 4C), demonstrating that androgen receptor inactivation by enzalutamide enhances STAT3 signaling and thus potentiates the IL6-mediated SOCS3 upregulation. To identify the mechanism by which androgenic signaling influences STAT3 signaling, we screened for androgen receptor–binding sites in the proximity of genes of the IL6 signaling pathway. The two subunits of the IL6 receptor, IL6R and IL6ST, and one of the kinases responsible for STAT3 activation, JAK1, were found to be in close proximity of consistent androgen receptor–binding sites in several cell lines and patient samples (Supplementary Fig. S4B). In addition, androgen receptor binding was observed in close proximity to the IL6 gene in cell lines. Consequently, we found that enzalutamide is able to enhance expression of IL6ST and JAK1 in two cell lines (Fig. 4D). Expression of IL6R was regulated differently in LNCaP and DuCaP cells, whereas mRNA expression of IL6 itself remained undetectable in androgen receptor–positive cell lines after enzalutamide treatment (Supplementary Fig. S2 and data not shown).

Taken together, this demonstrates that two key components of the IL6/JAK/STAT3 signaling cascade, IL6ST and JAK1, are targets of the androgen receptor and upregulated in response to enzalutamide. In consequence, this leads to a potentiation of STAT3 signaling in the presence of IL6 and increases SOCS3 expression (Fig. 4E).

SOCS3 plays an important role in the response to enzalutamide

Because of the strong SOCS3 induction in response to anti-androgens (Fig. 3C), we were interested in the biologic role of SOCS3 in the presence of inflammatory cytokines during anti-androgen treatment. Therefore, we performed a prolonged combined treatment with IL6 and enzalutamide in LNCaP. To address the role of SOCS3 in this context, stable up- or downregulation of SOCS3 was performed by lentiviral gene transfer. To increase SOCS3 knockdown efficiency, a pool of three shRNAs targeting SOCS3 was used. Knockdown efficiency of SOCS3 was about 70% even after 3 weeks of combined IL6/enzalutamide treatment (Supplementary Fig. S6). On protein level, overexpression of SOCS3 led to an almost complete loss of STAT3 phosphorylation, but knockdown of SOCS3 could not potentiate phosphorylation of STAT3 any further (Fig. 5A). Noteworthy, STAT3 and AR protein levels were not altered by SOCS3 modulation. Since it is has been shown by several groups that IL6 could trans-activate the androgen receptor, we were interested in the effect of SOCS3 on androgen receptor activity during enzalutamide treatment. As expected, combined treatment with enzalutamide and IL6 led to reduced expression of the well-known androgen receptor target genes PSA and KLK2 in cells with normal or overexpressed SOCS3. Notably, knockdown of SOCS3 was sufficient to completely abrogate this effect (Fig. 5B).

IL6 is also known to be a key regulator of stem cell activity and to a small yet significant increase in POU5F1 (OCT-3/4). Interestingly, both overexpression and knockdown of SOCS3 blocked the IL6/enzalutamide-mediated induction of SOX2 and NANOG, thus restoring nearly baseline mRNA levels (Fig. 5C). The complex interactions of the androgen receptor and IL6/JAK1/STAT3/SOCS3 pathways in response to IL6 and enzalutamide are depicted in Fig. 5D.

These results indicate that SOCS3 has to be strictly fine-tuned especially during IL6/enzalutamide treatment to prevent deregulation of genes associated with self-renewal and stemness.

Discussion

In this study, we provide evidence that the SOCS3 promoter is frequently hypermethylated in prostate cancer tissue samples and furthermore demonstrate that SOCS3 affects androgen receptor activity and expression of stem cell–associated genes during...
SOCS3 treatment in an inflammatory environment in prostate cancer cell lines.

In previous publications, SOCS3 promoter hypermethylation was found in a wide range of cancer types. Methylation-positive tissue samples were shown to have 4- to 7-fold lower SOCS3 mRNA expression levels (21–23), and use of a DNA methyltransferase inhibitor can increase SOCS3 mRNA expression in prostate cancer cell lines. This demonstrates a robust epigenetic regulation.

Figure 4.
SOCS3 is regulated by androgenic signaling in the presence of IL6 due to altered JAK/STAT3 signaling. A, the influence of R1881 (left) and enzalutamide (right) on SOCS3 mRNA expression in presence of 5 ng/mL IL6 was measured after 72 hours by qPCR. B, LNCaP and DuCaP cells were treated for 72 hours with 1 or 10 μmol/L enzalutamide in the presence of increasing IL6 concentrations. The effect on JAK/STAT3 signaling was measured by determining STAT3 Tyr705 phosphorylation via Western blotting and calculation of dose-response curves. C, LNCaP cells were treated for 48 hours with 5 ng/mL IL6 and 1 μmol/L enzalutamide in the presence or absence of 10 μmol/L of the STAT3 inhibitor galiellalactone. D, mRNA expression of key components of the IL6 pathway was measured after treatment with the anti-androgen enzalutamide for 72 hours. E, proposed mechanism of the interaction between androgen receptor and IL6/JAK/STAT3 signaling and SOCS3 expression.

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of SOCS3 expression. Previously, Pierconti and colleagues detected SOCS3 promoter hypermethylation in 39.2% of analyzed prostate cancer tissues, and an increased methylation was associated with higher Gleason score, tumor stage, and reduced progression-free survival (21). Because of this, the group proposed SOCS3 promoter methylation as a potential biomarker to identify more aggressive prostate cancer. Our study could confirm that the SOCS3 promoter is frequently hypermethylated in prostate cancer samples, whereas adjacent benign areas are unmethylated (95.7% vs. 13%). This finding could be confirmed in a validation cohort. The higher frequency of SOCS3 promoter hypermethylation in the present study may be explained by the selected cutoff and the use of a more sensitive detection method compared with the previously published study. However, we cannot exclude that patient age, inflammation, androgen receptor status, and other confounding factors may play a role. In concordance with Pierconti and colleagues we found a weak positive correlation with Gleason score and tumor stage. Although

Figure 5.
SOCS3 influences androgen receptor activity and stem cell/self-renewal genes during combined long-term anti-androgen and IL6 treatment. LNCaP cells with stable overexpression (oe-S3) or knockdown (sh-S3) of SOCS3 were treated for 3 weeks with a combination of enzalutamide (1 μmol/L) and IL6 (1 ng/mL). A, the effect on STAT3 activity and androgen receptor expression were measured by Western blotting. The influence on androgen receptor target genes (B) and stem cell/self-renewal genes (C) was measured by qPCR. Statistical significances are calculated against the long-term IL6/Enz-treated cells transduced with the control vector. D, schematic summary of the interaction between androgen receptor and IL6/JAK/STAT3 signaling and SOCS3 expression and the effect on stemness markers and androgen receptor activity.
significant, the differences in hypermethylation between the groups were only minor, and no association of SOCS3 promoter methylation and biochemical recurrence-free survival could be found. Therefore, we suggest that SOCS3 promoter hypermethylation is not suitable as a prognostic biomarker for prostate cancer. Nevertheless, the fact that the vast majority of prostate cancer samples displayed epigenetic silencing of SOCS3 via promoter hypermethylation underlines the important role of SOCS3 in this malignancy.

To shed light on the clinical relevance of the frequent SOCS3 promoter hypermethylation in prostate cancer, we have decided to investigate the functional significance of low SOCS3 expression in the presence of androgen receptor. SOCS3 has been previously studied in androgen receptor–negative cell lines in which siRNA-mediated knockdown of SOCS3 led to massive cell death (20). Androgen receptor–negative prostate cancer cell lines harbor an autocrine IL6 loop and secrete large amounts of IL6 into the supernatant (42). Therefore, SOCS3 may be necessary in these cell lines to counteract the high expression of IL6 and keep the autocrine IL6 loop in balance. In contrast, androgen receptor–positive prostate cancer cell lines do not express IL6 and the basal SOCS3 expression is very low. In patients with prostate cancer, however, autocrine as well as paracrine sources of IL6 have been described (37). When mimicking this situation in cell culture by adding exogenous IL6, SOCS3 was massively induced in the majority of androgen receptor–positive prostate cancer cell lines. The lack of SOCS3 induction in androgen receptor–positive LAPC4 and MDA PCa 2b can be explained by an aberrant IL6/JAK/STAT3 signaling pathway which results in a loss of STAT3 activation (8). This finding clearly demonstrates that a functional IL6/JAK/STAT3 signaling pathway is necessary for the induction of SOCS3 in prostate cancer cell lines and that there is no major difference in SOCS3 expression between androgen receptor–positive and -negative cell lines in the presence of IL6.

To gain more insight into the effect of SOCS3 in androgen-sensitive cells, knockdown of SOCS3 was performed in LNCaP cells. We could demonstrate an increase in IL6/JAK/STAT3 signaling activity upon knockdown of SOCS3, which proves that SOCS3 regulates STAT3 activity in LNCaP. In patients with prostate cancer, activated STAT3 has been found in the majority of lymph node and bone metastases and has been associated with high histologic grade and disease stage (11). This finding may be explained by the strong epigenetic downregulation of SOCS3, which we detected in prostate cancer tissue. However, future studies are needed to clarify the association of IL6-activated STAT3 and SOCS3 promoter hypermethylation and expression in cancerous tissue.

Since the proinflammatory cytokine IL6 is known to play an important role in the progression to castration resistance (4, 8), and given the importance of SOCS3 in the fine tuning of IL6 signaling, we screened for changes in SOCS3 expression upon modulation of androgen signaling. Interestingly, we found SOCS3 to be upregulated by treatment with anti-androgens and this could be confirmed in two LNCaP sublines, which mimic the castration-resistant state and have reduced androgen receptor activity. These facts clearly demonstrate that SOCS3 is an androgen-regulated gene. To further investigate the effect of the androgen receptor on SOCS3 expression in CRPC, we performed experiments in presence of the constitutively active androgen receptor splice variant V7 which frequently contributes to the development of castration resistance (43, 44). It is still a matter of debate, whether the AR-V7 variant has a different target gene specificity (39, 45). Therefore, we investigated the influence of exogenous AR-V7 expression on SOCS3 expression in AR-V7–negative LNCaP cells. In concordance with the publication of Hu and colleagues (39) we could confirm that AR-V7 can regulate only a subset of the AR target genes and is unable to downregulate SOCS3 expression. This clearly demonstrates that the full-length androgen receptor is necessary for the androgenic regulation of SOCS3 and that the constitutively active AR-V7 splice variant is unable to influence the STAT3 pathway.

To elucidate the mechanism underlying the androgenic regulation of SOCS3, we first considered a direct binding of the androgen receptor to the promoter region of SOCS3. However, extensive analysis of published ChIP-Seq datasets did not reveal any androgen receptor–binding site in the vicinity of SOCS3. We thus conclude that SOCS3 is not directly regulated by the androgen receptor. Recent publications demonstrated that anti-androgen treatment can enhance STAT3 activity (15, 46). Indeed, we could not only confirm an increased STAT3 activation after treatment with enzalutamide but also achieve complete reversal of the effect on SOCS3 by blocking the transcriptional activity of STAT3. Furthermore, we could show that the signal transducer of the IL6 receptor and one of the kinases that phosphorylate STAT3 are target genes of the androgen receptor and were upregulated in response to enzalutamide. This increased expression of elements of the signaling cascade may lead to a potentiation of STAT3 activity and thus SOCS3 expression. These findings demonstrate that the effect of enzalutamide on SOCS3 is mediated via altered STAT3 signaling. On the basis of our results, we propose that SOCS3 is responsible for counteracting excessive STAT3 signaling upon treatment with anti-androgens in an inflammatory setting. Epigenetic silencing of SOCS3, as frequently found in prostate cancer tissue, may cause a breakdown of this negative feedback loop and consequently lead to hyperactivated STAT3 during anti-androgen treatment.

To investigate the functional consequences of an activated STAT3 pathway during anti-androgen therapy, a long-term treatment with enzalutamide and the proinflammatory cytokine IL6 was performed in combination with overexpression and knockdown of SOCS3. We first investigated the effect of SOCS3 on androgen receptor, because IL6 has previously been shown to trans-activate the androgen receptor (6, 7). Interestingly, knockdown of SOCS3 was able to completely abrogate the effect of enzalutamide treatment on the two well-studied androgen receptor target genes PSA and KLK2 in this high inflammatory environment. This remarkable finding indicates an important role for SOCS3 in the development of anti-androgen resistance in presence of inflammatory cytokines.

In addition to its effect on androgen receptor signaling, IL6 is also important for the maintenance of stem cell properties in several cancers (5, 16–18), and blocking STAT3 activity has been shown to inhibit prostate cancer stem-like cells (5, 16). Treatment of LNCaP with a combination of IL6 and enzalutamide led to a significant increase in stemness/self-renewal genes. The importance of these genes in prostate cancer could be demonstrated by several groups (47, 48). Overexpression of SOCS3 was able to completely block the IL6/enzalutamide-mediated induction of the stemness genes, most likely due to the inhibition of STAT3 activity. Surprisingly, knockdown of SOCS3 also reduced the induction of the stemness genes. This paradoxical effect could be explained by the increased androgen receptor activity upon
knockdown of SOCS3, which is known to induce differentiation and suppress self-renewal (19). On the basis of these findings, we speculate that patients with SOCS3 promoter hypermethylation undergoing anti-androgen therapy could benefit from combination treatment with DNA methyltransferase inhibitors, such as azacitidine and decitabine, clinically approved drugs for treatment of myelodysplastic syndromes and acute myeloid leukemia (49). This might lead to a reactivation of SOCS3 which consequently could both reduce androgen receptor activity and downregulate stromal/self-renewal genes.

In conclusion, our experiments demonstrate that SOCS3 is frequently inactivated by promoter hypermethylation in prostate cancer, which inhibits SOCS3 induction during anti-androgen treatment. In presence of inflammatory cytokines, low SOCS3 expression reactivates androgen receptor activity during enzalutamide treatment, whereas high SOCS3 expression represses the induction of stem cell–associated genes. On the basis of these data, we propose that in an inflammatory background, demethylating agents may provide a beneficial effect by restoring SOCS3 levels during anti-androgen treatment.

Disclosures of Potential Conflicts of Interest

Z. Culig is a consultant/advisory board member of Astellas. No potential conflicts of interest were disclosed by the other authors.

References

SOCS3 and Androgen Receptor Signaling in Prostate Cancer


