SOCS2 mediates the cross talk between androgen and growth hormone signaling in prostate cancer

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Anabolic signals such as androgens and the growth hormone/insulin-like growth factor 1 (GH/IGF-1) axis play an essential role in the normal development of the prostate but also in its malignant transformation. In this study, we investigated the role of suppressor of cytokine signaling 2 (SOCS2) as mediator of the cross talk between androgens and GH signals in the prostate and its potential role as tumor suppressor in prostate cancer (PCa). We observed that SOCS2 protein levels assayed by immunohistochemistry are elevated in hormone therapy-naive localized prostatic adenocarcinoma in comparison with benign tissue. In contrast, however, castration-resistant bone metastases exhibit reduced levels of SOCS2 in comparison with localized or hormone naive, untreated metastatic tumors. In PCa cells, SOCS2 expression is induced by androgens through a mechanism that requires signal transducer and activator of transcription 5 protein (STAT5) and androgen/erbB-dependent transcription. Consequently, SOCS2 inhibits GH activation of Janus kinase 2, Src and STAT5 as well as both cell invasion and cell proliferation in vitro. In vivo, SOCS2 limits proliferation and production of IGF-1 in the prostate in response to GH. Our results suggest that the use of GH-signaling inhibitors could be of value as a complementary treatment for castration-resistant PCa.

Summary: Androgen induced SOCS2 ubiquitin ligase expression and inhibited GH signaling as well as cell proliferation and invasion in PCa, whereas reduced SOCS2 was present in castration-resistant cases. GH-signaling inhibitors might be a complementary therapeutic option for advanced PCa.

Abbreviations: AR, androgen receptor; BrdU, bromodeoxyuridine; CRPC, castration-resistant prostate cancer; GH, growth hormone; GHR, growth hormone receptor; IGF-1, insulin-like growth factor; IR, immunoreactivity; JAK2, Janus kinase 2; mRNA, messenger RNA; PCa, prostate cancer; PRL, prolactin; PSA, prostate-specific antigen; siRNA, small interfering RNA; SOCS2, suppressor of cytokine signaling 2; Sox22−/−, SOCS2 knockout; STAT5, signal transducer and activator of transcription 5 protein; TMA, tissue microarray.

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Introduction

Androgens are essential for both the normal development of the prostate and also participating during prostate cancer (PCa) progression. This is demonstrated by the effectiveness of androgen deprivation as therapy in the late stage of the disease (1). However, some tumors relapse into a more aggressive castration-resistant prostate cancer (CRPC) state for which only palliative treatments are available. Although the causes behind PCa metastatic progression are not yet fully understood, androgen receptor (AR) signaling is thought to play a key role. AR gene amplification, expression of splice variants, mutations or phosphorylation can all lead to the activation of AR in metastatic tumors on conditions of low circulating levels of androgens (2).

The growth hormone/insulin-like growth factor 1 (GH/IGF-1) axis has also been implicated in PCa development. Human PCa xenografts grow slower in the GH deficient lig/lig mice (3). Disruption of GH signaling also reduces prostate carcinogenesis in mice and rats harboring the SV40 large T antigen, which develop prostatic intraepithelial neoplasia that progresses to invasive prostate carcinoma (4,5). In humans, however, the best evidence for the following involvement comes from the study of Laron dwarfism patients. These patients carry an inactivating mutation in the GH receptor (GHR) and do not develop PCa in their lifetime (6). Furthermore, elevated levels of IGF-1 is a risk factor for PCa (7) and acromegalic patients commonly exhibit both enlarged prostates and prostatic disorders (8).

GH intracellular signaling relies primarily on the activation of the protein tyrosine kinase Janus kinase 2 (JAK2) and the signal transducer and activator of transcription 5 protein (STAT5), which promotes the transcription of several GH-regulated genes. The JAK2/STAT5 pathway is also employed by other cytokines such as prolactin (PRL), which acts as survival factor for prostate cells (9). STAT5 activity increases in PCa (10,11), where it synergizes with the AR and mediates the cross talk between cytokine and androgen signaling (12). GHR actions are negatively regulated by intracellular feedback mechanisms that include the intervention of the suppressor of cytokine signaling 2 (SOCS2) (13). In response to GH, STAT5 induces the expression of SOCS2, which in turn acts on the receptor to limit STAT5 activation. Thus, SOCS2 knockout (Sox22−/−) mice are larger than the wild-type (WT) due to enhanced GH activity (14–16). Similarly, SOCS2 also negatively regulates the PRL-signaling pathway (17). SOCS2 belongs to a family of ubiquitin ligases consisting of eight members, SOCS1–7 and the cytokine-inducible SH2-containing protein. SOCS proteins are characterized by two distinctive domains: (i) the SOCS box, which mediates the formation of cullin/-ring E3 ligase complex and (ii) an Src homology 2 (SH2) domain able to interact with phosphorylated tyrosine and promote their ubiquitination (18,19). SOCS2 is known not only for targeting both the GH and PRL receptors but also for participating in the negative regulation of toll-like receptor 4-activated signaling pathways (20).

Interestingly, DNA-microarray-based studies suggest SOCS2 as an androgen-induced gene in prostate cells (21–23). Therefore, SOCS2 could potentially mediate the cross talk between steroids and cytokine signals in prostate cells. Reduced levels of SOCS2 messenger (mRNA) expression correlated with poor outcomes lead to the suggestion that SOCS2 is both a tumor suppressor and a potential prognostic marker in PCa (22,23). However, the functionality of SOCS2 in the prostate gland and the mechanisms behind its regulation are not known. In this study, we investigated functional aspects of SOCS2 in PCa growth and mapped the expression of SOCS2 protein in different stages of PCa. In addition, we also evaluated its potential value as a prognostic marker for PCa.
Androgens regulate GH actions through SOCS2

Materials and methods

Cell culture, hormone and drug treatments
LNCaP-FGC, PC-3 and 22Rv1 PCa cell lines were purchased from ATCC (Rockville, MD) in 2010 and cultured as described previously (24,25). C4-2B cells were obtained from MD Anderson Cancer Center (Houston, TX) and cultured in 10% fetal bovine serum RPMI medium. R1881 was obtained from Amershams (Braunschweig, Germany), human-GH from Novo Nordisk (Copenhagen, Denmark) and they were used at 10 nm and 1 μg/ml, respectively. Bicalutamide (Cadoxet), kindly donated by AstraZeneca (Stockholm, Sweden) and AG490, purchased from Sigma (St Louis, MO), were used at 10 μM. PP2 (Calbiochem, Neu Isenburg, Germany) was used at 50 μM. All the inhibitors were added to the media 2h before hormone stimulation. Charcoal/Dextran Treated Fetal Bovine Serum was purchased from Hyclone (Logan, UT).

Plasmids and cell transfection

Transfections were performed using the Amaxa Nucleofector (Amaxa, Gaithersburg, MD) according to the manufacturer’s instructions. SOCS2, elongin B and eloncin C expression vectors used for transfections were described earlier (14). Small interfering RNA (siRNA) directed against SOCS2 (siSOCS2_1, siSOCS2_2, STAT5 siSTAT5_2) and control siRNA were purchased from Qiagen (Hilden, Germany) and described previously (20). AR expression vector (pSVARo) was a kind gift from Dr Brinkmann (26).

Western blot analysis

Western blotting and immunoprecipitation procedures were carried out as described earlier (27). Rabbit anti-Jak2 and mouse anti-β-actin antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Rabbit anti-STAT5, phospho-STAT5 (Y694) and SOCS2 antibodies from Cell Signaling Technology (Danvers, MA). Mouse antiangiatic and total Src were obtained from Invitrogen (Carlsbad, CA) and mouse antiphosphotyrosine antibody (4G10) from Millipore (Billerica, MA).

In vitro cell proliferation and matrigel invasion

Matrigel invasion assays were carried out as described previously (27). Four independent experiments were performed. Each experimental group was measured in triplicate. The Student’s t-test was applied for statistical analyses. Cell proliferation assays were performed using the xcelligence system (Roche), following manufacturer’s recommendations. C4-2B cells were incubated in RPMI medium containing 1% fetal bovine serum and 1 μg/ml GH. Experiments were carried out in quadruplicates and repeated three times. Two-way analysis of variance test was used for statistical analyses.

Animal experiments

Twenty 11-month-old C57BL/6 WT and Soc2−/− male mice were housed at the Animal Core Facility of the University of Las Palmas de Gran Canaria (Spain) on a 12-h light, 12-h dark schedule with free access to food and water, according to Spanish guidelines for animal welfare. Mice were injected subcutaneously with 0.2 mg/kg/day recombinant human GH (Pfizer, Alcobendas, Spain) in 0.9% NaCl or vehicle, twice daily for 3 weeks. Thirty minutes prior to killing by CO2, half of the mice in each group were intraperitoneally injected with bromodeoxyuridine (BrdU; Sigma) (40 μg/kg body wt). Prostates were collected and either formalin-fixed for later evaluation of cell proliferation or RNA purified using SV Total RNA Isolation System (Promega, Madison, WI) following manufacturer’s respective guidelines.

Analysis of cell proliferation in vivo

The proliferation index was calculated as number of BrdU positive cells per total RNA Isolation System (Promega, Madison, WI) following manufacturer’s respective guidelines.

Reverse transcription–polymerase chain reaction and primers

Total RNA from cell cultures was purified using RNeasy kit (Qiagen) and reverse transcribed using iScript (Bio-Rad, Sundbyberg, Sweden). Reverse transcription–polymerase chain reaction was performed as described previously (28). The primers used for experimental purposes, expression vectors and specific antibodies as primers for reverse transcription–polymerase chain reaction were human-β-actin, 5′-CTGGTCTGCTGA-CGGAGG-3′ and 5′-GAAAGGCTCTCAAACATG ATCCGGT-3′; human-SOCS2, 5′-GAGTCGCCTGCAGACAGGATG-3′ and 5′-GATTGAGCCTCCGATGTTT-3′; human-prostate-specific antigen (PSA), 5′-CACTGCCTGGGTGATCTGATT-3′ and 5′-CCACCTCCGGTAAT GCAACA-3′; mouse-β-actin, 5′-GTCGAGTCTGACCTGCTGAA-3′ and 5′-GTCGATGCTGACCTGCTGAA-3′; mouse-SOCS2, 5′-AGGTCACAGTTGCA GACATCC-3′ and 5′-TCCAGATGGCAAGAGTAAC-3′; mouse-IGF-1, 5′-GGTTGGACAGAGGCCCCTTCTC-3′ and 5′-GCTTCA GTGGGACAGTA-CATCCT-3′. The annealing temperature was 58°C.

Immunohistochemistry

Tissue microarray (TMA) sections were deparaffinized in xylene and rehydrated through graded ethanol. For antigen retrieval, however, the slides were steamed for 20 min in 10 mM citrate buffer at pH 6.0. Immunohistochemistry was performed in one batch. The slides were incubated with 6 μg/ml mouse monoclonal antibody against SOCS2 (clone 4B6) (a kind gift from Dr Hilton, MD Anderson Cancer Center, Melbourne, Australia) overnight at 4°C. For detection purposes, Vectorstain ABC Elite Kit (Vector Laboratories, Burlingame, CA) and DAB (DAKO) were used following manufacturer’s recommendations. Slides were counterstained with hematoxylin. A multiorgan TMA was used as both positive (with primary antibody) and negative (with omitted or mouse immunoglobulin G, DAKO, instead of primary antibody) control. When staining SOCS2 in bone metastasis samples (and in radical prostatectomy specimens that were compared with non-cancer tissue), the rabbit polyclonal antibody ab74533 anti-β6 antibody (diluted 1:75, Abcam, Cambridge, UK) was used after antigen retrieval in 0.1 M Tris/ethylenediaminetetraacetic acid, pH 9.0, as it’s important to note that this but not the 4B6 antibody was found to work in decalcified tissue samples (data not shown).

Radical prostatectomies and PCa bone metastases

A TMA included paired tumor and benign samples from 21 radical prostatectomy specimens collected in 2003 at the Karolinska University Hospital, Solna, Sweden. None of the patients had received either hormonal therapy or radiotherapy prior to surgery. After overnight fixation in 4% buffered formalin, the specimens was inkered, sliced horizontally at 4 μm and totally embedded. The specimens were dehydrated, paraffin-embedded, cut at 4 μm and stained with hematoxylin/eosin. TMAs were constructed using a Beecher Manual Arrayer I (Beecher Instruments, Silver Spring, MD). One core with a diameter of 1 mm was collected from cancerous tissue from each specimen and another one from benign tissue. After exclusion of cases with no cancer in the cores, 19 complete pairs of cancer and benign tissues still remained for analysis. The mean age at surgery was 62 years (range 48–75), mean serum PSA was 9 ng/ml (4.3–16.7) and clinical stage was T1c in 10 cases and T2 in 9 cases. The Gleason scores were 5, 6, 7 and 9 in 1, 8, 2 and 8 cases, respectively. Bone metastasis specimens (n = 24) were obtained from biopsies collected from PCa patients operated either for metastatic spinal cord compression or pathologic fractures at Umeå University Hospital (2004–10) and are described in reference (29). Samples were formalin-fixed, dehydrated in 20% formic acid at 37°C for 1–3 days and embedded in paraffin. Seven bone metastases were collected from previously untreated, hormone-naive PCa patients (mean age at surgery 71, range 60–82; mean serum PSA 2340 ng/ml, range 21–10 000) and 17 metastases were obtained from patients defined to have CRPC due to disease progression after long-term androgen deprivation therapy (mean age at surgery 66, range 51–78; mean PSA 780 ng/ml, range 0.06–5140). SOCS2 staining in the metastases was compared with staining in non-malignant and malignant areas of radical prostatectomy specimens from 17 patients treated in Umeå (2004–10). Median age for the patients was 60 years (range 48–72 years) and PSA was 14 ng/ml (3.5–29 ng/ml). Gleason scores were 6, 7 and 8 in 1, 15 and 3 cases, respectively, and clinical stage was T1c, 2, 3 and unknown in 7, 7, 2 and 1 cases, respectively. Additional information about tissue handling has been described previously in reference (30).

Prognostic TMA

A TMA included tumor samples from 185 prostatectomy specimens collected from 1998 to 2002 at the Karolinska University Hospital. None of the patients had received either hormonal therapy or radiotherapy prior to surgery. Three cores with a diameter of 1 mm were collected from each specimen (two from the primary Gleason pattern and one from the secondary). Eight cases were excluded because of lost cores or insufficient amount of cancer for evaluation and 177 cases remained for analysis. Prostatectomy specimens were handled as described above.

The mean age of the patients at the time of the surgery was 62 years (range 48–74). Mean preoperative serum PSA was 11.1 ng/ml (median 9.2, range 1.1–58). Patients were followed in average 59.9 months (range 1–144) until a biochemical recurrence, death of unrelated causes or last recurrence-free follow-up. The clinical stage was T1c in 109 (60.9%), T2 in 67 (37.4%) and T3 in 3 (1.7%) men. Extrastrophic extension, positive surgical margins and seminal vesicle invasion were seen in 50 (30.3%), 39 (24.0%) and 29 men (16.2%), respectively. Gleason scores were 5–6, 7 and 8–10 in 80 (44.7%), 77 (43%) and 22 (12.3%), respectively. Of all men, 81 (45.3%) were uncensored.

Evaluation of immunoreactivity and statistical analysis

Both intensity and extent of immunoreactivity (IR) were either evaluated by two observers in an open discussion (D.D. and T.K.) or evaluated by a single observer (K.E.) at 4°C for comparisons of IR between bone metastases and primary tumor tissue by P.W. The intensity of the cytoplasmatic staining of epithelial cells was scored from 0 (no staining) to 3 (the most intense staining) based on the strongest staining of the core. The
staining extent (percentage of the core stained by the strongest intensity) was evaluated using a 3-tier scale: 1 was 1–33%, 2 was 34–66% and 3 was 67% of the core. An IR score from 0 to 9 was obtained by multiplying the intensity and extent scores. Paired or unpaired t-tests were used when appropriate for comparison of mean IR scores. A P value <0.05 was regarded as significant.

A univariate Cox proportional hazards model was used to compare prognostic parameters. Survival was analyzed in Kaplan–Meier plots with log-rank comparison between groups.

Ethics committee decision
The study for prostatectomy analysis was approved by the Regional Ethics Committee, Stockholm (2006/4:10) and the local ethic review board of Umeå University (03-482, 04-026M). Participants gave written or documented verbal consent.

Results
SOCS2 is overexpressed in hormone naive PCa
Previous studies have shown reduced SOCS2 mRNA levels in metastatic PCa compared with primary tumors and normal prostate tissue (22,31). We further confirmed these observations by analyzing a high quality data set of transcript profiles obtained from an independent sample cohort of 131 primary tumors, 29 of which were paired with benign tissue, and 19 metastatic tumors (32). Paired samples revealed no significant variation of SOCS2 expression (P = 0.2; t = 1.29), whereas a highly significant reduction (53% mean reduction) in the metastatic compared with the primary tumors (P < 0.0001) was seen (Figure 1A and B). This behavior resembles the evolution of the androgen-regulated gene KLK3 (PSA) expression during PCa progression (Figure 1C and D).

Afterward, we analyzed the expression of SOCS2 by immunohistochemistry in 19 human PCas with Gleason scores of 5 (n = 1), 6 (n = 8), 7 (n = 8) and 9 (n = 2). SOCS2 is expressed in the cytoplasm of epithelial cells with a higher IR score in cancer than in luminal cells of benign glands of the same origin (Figure 1E), with a mean IR score of 5.2 [standard error (SE) = 0.52] and 3.5 (SE = 0.56), respectively (P = 0.02, t = 2.54) (Figure 1F). The mean IR score in low-grade (Gleason score 5–6) and high-grade (Gleason score 7–9) cancers was 4.6 (SE = 0.80) and 5.8 (SE = 0.66), respectively (P = 0.24, t = 1.20). Finally, basal cells in benign glands often had a slightly stronger expression of SOCS2 than the luminal cells (Figure 1E). These results consequentially revealed that in contrast to the mRNA expression, SOCS2 protein levels are increased in primary prostate tumors independent of Gleason score.

To further study SOCS2 expression during PCa progression, we analyzed 17 castration-resistant and 7 hormonal therapy naive PCa bone metastases and compared SOCS2 expression with a cohort of 17 primary tumors (Figure 1G). Interestingly, therapy naive tumors showed similar levels of SOCS2 (primary tumors mean score 5.64; SE = 0.24 and hormone naive metastases mean score 5.14; SE = 0.55; P = 0.35), whereas castration-resistant tumors exhibited significantly reduced SOCS2 expression compared with localized tumors (mean score 4.5; SE = 0.41; P = 0.027). These results suggest that androgen signaling modulates SOCS2 expression in human tumors and that SOCS2 is part of a larger signature of androgen-induced genes whose expression is reduced in advanced tumors. Therefore, SOCS2 could be an indicator of the status of the AR activity during PCa progression.

Androgens inhibition of GH signaling in PCa cells is mediated by SOCS2
In order to determine whether SOCS2 expression was induced by androgens in PCa cells, we treated the AR positive LNCaP cells with the synthetic dihydrotestosterone analog, R1881. SOCS2 mRNA levels were induced up to 8-fold after 20 h of R1881 treatment (Figure 2A). These effects are not cell specific because similar results were obtained when the AR positive 22Rv1 cells were treated with R1881 (Supplementary Figure 1A, available at Carcinogenesis Online).

SOCS2 is known to inhibit GH signaling in different tissues (14). In order to investigate whether SOCS2 might modulate GH actions in prostate cells, we used the GH responsive LNCaP cells (33). GH treatment rapidly increases SOCS2 mRNA levels in LNCaP cells with a maximum induction of 2-fold observed after 2 h (Figure 2B). Inhibition of SOCS2 expression by siRNA both enhanced and extended the activation of JAK2 and STAT5 upon GH stimulation.
Androgens regulate GH actions through SOCS2

in LNCaP (Figure 2C, upper panel). Interestingly, reduced expression of SOCS2 also increased the basal activation of the Src kinase (Figure 2C, lower panel). Similar effects were observed in the 22Rv1 cell line (Supplementary Figure 1B and C, available at Carcinogenesis Online). Accordingly, transient overexpression of SOCS2 in LNCaP cells reduced JAK2 and STAT5 activation upon GH stimulation (Figure 2D), demonstrating that SOCS2 inhibits GH signaling in PCa cells.

We reasoned that SOCS2 induction by androgens could potentially affect GH actions in prostate cells. To test this hypothesis, LNCaP cells were treated with R1881 or vehicle for 20 h and then stimulated with GH for the indicated times. Figure 2E shows a repression in the GH-induced activation of STAT5 in cells treated with R1881 in parallel to a significant elevation of SOCS2 protein levels. This result mimics the one obtained by the overexpression of SOCS2 (Figure 2D) and suggests that androgens might control the GH/IGF-1 axis in the prostate by inducing SOCS2. To further demonstrate the involvement of SOCS2 in the inhibition of GH signaling by androgens, we transfected LNCaP cells with the same siRNAs targeting SOCS2 or a control siRNA as above, treated with R1881 (10 nM) for 20 h and then with GH (1 μg/ml) for the indicated times (F). Student’s t-test was applied for statistical analyses comparing each time point with the control (*P < 0.05; **P < 0.01; ***P < 0.001). All experiments were performed at least three times.

AR and STAT5 cooperate to induce SOCS2 expression

Although we demonstrated SOCS2 to be an androgen-regulated gene, little is known about the mechanism behind this regulation. To the best of our knowledge, no androgen response element has been found in the vicinity of the SOCS2 gene transcription start site (34,35). However, cotreatment with the AR inhibitor Bicalutamide inhibits SOCS2 (as well as KLK3) transcriptional induction by androgens in LNCaP cells. This indicates that the AR is critical for
the upregulation of SOCS2 by androgens (Figure 3A). On the other hand, AR negative PC-3 cells transfected with AR failed to induce the expression of SOCS2 after R1881 stimulation, whereas KLK3, a direct target of AR transcriptional activity (36,37), was indeed induced under these conditions (Figure 3B). These results suggest that the AR would require the presence of additional cofactors to induce SOCS2 expression after stimulation by androgens.

The GH- and PRL-activated transcription factor STAT5 regulates the transcription of SOCS2 (13). It is known that PC-3 cells over-expressing both AR and STAT5 transcription factors synergize to promote expression of luciferase reporter genes driven by STAT5-binding elements (12). Endogenous STAT5 expression, however, is barely detectable in PC-3 cells suggesting that the lack of SOCS2 induction by androgens in AR-transfected PC-3 cells is due to STAT5 deficiency. To investigate this hypothesis, we knocked down STAT5 expression in LNCaP cells by siRNA as above. Figure 3C shows that reduced levels of STAT5 lead to a lower induction of SOCS2 expression by androgens, whereas KLK3 (PSA) expression was not influenced. These results consequently suggest that the induction by androgens of SOCS2 transcription from its endogenous promoter requires both AR and STAT5.

SOCS2 inhibit GH actions in PCa cells

The ability of cells to migrate out from the prostate gland is an indicative feature of metastatic behavior during PCa progression. Therefore, we investigated if SOCS2 could influence the invasive capacity of PCa cells. LNCaP cells have a limited capacity to invade a matrigel layer and we have shown previously that this activity can be stimulated by androgens (Figure 4A and ref. 38). GH also induces invasiveness of LNCaP cells to a lesser extent, but not of 22Rv1 or PC-3 cells, likely due to a constitutively elevated invasive capacity of these cells (data not shown). In agreement with its role as negative regulator of GH action, depletion of SOCS2 in LNCaP cells further increased (~2-fold) the number of invading cells after GH stimulation (Figure 4A). On the other hand, however, SOCS2 does not influence the invasion induced either by androgen treatment (Figure 4A) or by the combination of GH and androgens (data not shown).

SOCS2 modulates GHR turnover (39) and through this mechanism regulates the activation by GH of downstream signaling proteins such as JAK2, STAT5 and Src. Therefore, we reasoned that inhibition of these proteins would result in an opposite phenotype to the one obtained from SOCS2 depletion. In order to test our hypothesis, we treated LNCaP cells with either JAK2 (AG490) or Src (PP2) inhibitors and stimulated with GH to evaluate their invasive capacity. JAK2 inhibition resulted in reduction of STAT5 phosphorylation after GH stimulation (Figure 4A). On the other hand, however, SOCS2 does not influence the invasion induced either by androgen treatment (Figure 4A) or by the combination of GH and androgens (data not shown).

SOCS2, JAK2, Src and STAT5 modulate GH proinvasive effects on PCa cells. (A) Matrigel invasion assays of LNCaP cells transfected with siRNAs against SOCS2 as in Figure 2 and stimulated with GH. Student’s t-test was applied for statistical analyses. #GH stimulated cells compared with untreated controls (**P < 0.05). *GH stimulated siSOCS2 depleted cells compared with untreated controls and GH-treated WT cells (**P < 0.05). (B) Effect of the anti-SOCS2 siRNAs on SOCS2 protein levels. (C) Left, Western blot analysis of the effect of AG490 and PP2 in the GH-activation of STAT5 and Src. Right, Matrigel invasion assays of GH-stimulated LNCaP cells treated with AG490 or PP2. (D) Matrigel invasion assays of LNCaP cells transfected with siRNA against STAT5 or control and treated with GH or vehicle. Effect of siRNA in STAT5 protein levels is shown on the left. Student’s t-test analysis was used to compare the effect of AG490, PP2 and STAT5 depletion in GH-induced cell invasiveness (**P < 0.05; ***P < 0.001). All the experiments were performed at least three times.
Androgens regulate GH actions through SOCS2

Treatment (Figure 4B, top panel) and Src inhibition caused reduction of both Src and STAT5 activation by GH (Figure 4B, lower panel). Moreover, both compounds totally abolished GH proinvasive effects suggesting that JAK2 and Src are essential factors in the protumorigenic action of GH in PCa cells (Figure 4B, right panel). Similar results were obtained when STAT5 expression was depleted from the cells by siRNA targeting (Figure 4C). Finally, the combination of SOCS2 depletion in the cells with the inhibition of JAK2, Src or STAT5 did not restore the LNCaP invasive capacity in any case (data not shown), thereby supporting the idea that SOCS2 does indeed act upstream of these proteins in the GH-activated pathway leading to cell invasion.

SOCS2 inhibits GH-induced proliferation of prostatic cells in vivo

In order to study the role of SOCS2 in prostate cells proliferation in vivo, we took advantage of the Soc2−/− mouse model. These mice tend to grow larger than the WT due to enhanced GH sensitivity (16,40). Eleven-month-old Soc2−/− and WT mice were treated with either human GH or vehicle for 3 weeks and cell proliferation was estimated by BrdU labeling followed by histological examination. Despite of their advanced age, no evidence of neoplastic transformation was detected in any of the animals, suggesting that lack of SOCS2 is insufficient to start a tumorigenic process in the prostate of these mice. Vehicle treated Soc2−/− mice showed higher prostate weight than the WT (P < 0.05; Figure 5A). This enlargement correlates with the general increase in body weight in these mice because no differences were observed when the data were corrected by the total body weight (Figure 5B). Interestingly, 3 weeks of GH treatment increased prostate weight in Soc2−/− animals but not in the WT (P < 0.05; Figure 5A). In addition, this was a trend that was maintained when the measurements were made relative to the body weight (P < 0.06; Figure 5B). These data suggest that GH preferentially increases prostatic growth in the absence of SOCS2 in addition to a generalized effect on body growth. Immunohistochemical analysis of the BrdU staining of prostate sections revealed that GH treatment increased proliferation rates in Soc2−/− mice prostates but had no effect on the WT animals (Figure 5C). Interestingly, of all the prostate lobes, both the anterior and dorsal prostate lobes appear to be more sensitive to growth regulation by SOCS2. It is important to notice the low basal levels of proliferation exhibited by the knockout mice in the absence of GH treatment. This effect is in line with the reduced circulating levels of GH of these animals (41). These results support the notion of GH as a contributing factor to prostatic growth and its action being modulated by SOCS2 in vivo.

The effects of SOCS2 in prostate cells proliferation may be of importance in the context of CRPC where SOCS2 expression is drastically reduced (Figure 1). Thus, we investigated whether SOCS2 affected cell proliferation of the CRPC cell line C4-2B. Figure 5D shows that transient overexpression of SOCS2 in C4-2B cells has significant inhibitory effects on the proliferation. This result suggests a possible role of SOCS2 as tumor suppressor in CRPC.

GH is known to induce IGF-1 gene transcription in several tissues and may act as a read out for GHR activity (42,43). We investigated whether SOCS2 might influence IGF-1 production in the prostate in vivo. Total RNA was obtained from the anterior prostate lobe of Soc2−/− and Soc2−/− mice treated as above and assayed for the expression of SOCS2 and IGF-1. GH treatment increased SOCS2 expression ~1.8-fold in WT animals (Figure 5E). On the
other hand, only in the absence of SOCS2, GH was able to induce IGF-1 expression in the prostate (Figure 5F). Together, these results indicate that SOCS2 plays a key role in inhibiting GH proliferative actions and IGF-1 expression in the prostate gland in vivo. Noticeably, we were not able to demonstrate IGF-1 induction by GH in any of the GH and SOCS2 responsive cell lines tested (LNCaP, 22rv1 and C4-2B; Supplementary Figure 2A, available at Carcinogenesis Online and refs 44,45) suggesting that some of the actions of SOCS2 may be independent of its ability to modulate IGF-1 production.

**Elevated SOCS2 protein levels correlate with early biochemical recurrence in PCa patients**

To evaluate the potential utility of SOCS2 as a prognostic marker in PCa, TMAs containing samples from 185 prostatectomy specimens were analyzed for SOCS2 expression as described in the Materials and methods. Cox univariate analysis of IR score against biochemical recurrence-free survival gave $P = 0.08$, hazards ratio $= 1.28$ (0.97–1.70). However, when the immunostaining was dichotomized at the median IR score of 4 (4 or less versus >4), Kaplan–Meier analysis using log-rank regression gave a $P=0.007$ (Figure 6). Although a validation from a larger study would be necessary, SOCS2 upregulation in hormonal therapy naive tumors may be relevant as an early predictor of disease recurrence after radical prostatectomy.

**Discussion**

In this study, we report that SOCS2 protein levels are reduced in CRPC and show that SOCS2 has novel tumor suppressor functions in the prostate. SOCS2 inhibits invasion and proliferation of PCa cells in culture and prostate cells proliferation and IGF-1 production in vivo (Figures 4 and 5). In PCa cells, SOCS2 expression is induced by GH and by androgens and plays a key role in mediating the cross talk between these anabolic pathways (Figure 2). The androgen regulation of SOCS2 gene expression in the prostate is mediated by a mechanism that involves the AR and STAT5 transcription factors (Figure 3). Interestingly, SOCS2 protein levels are elevated in hormone therapy naive prostate adenocarcinoma compared with benign tissue suggesting that it may serve as a sensor of androgen activity during the different stages of PCa progression.

The effects of androgens on prostate cells vary through the evolution of PCa (Figure 6B and ref. 35). In normal prostate epithelium, androgens have prodifferentiation properties. In this context, induction of SOCS2 by androgens could prevent tumorigenic actions of...
other growth factors (e.g. GH and PRL). Transformed prostate carcinoma cells are dependent on androgens for cell proliferation as indicated by the effectiveness of the androgen deprivation therapy as first-line treatment for PCs. In localized, treatment naive tumors, this increased androgen sensitivity would result in increased expression of AR targets such as SOCS2 (Figure 1). The fact that SOCS2 is overexpressed in localized tumors suggests that SOCS2 is unable to antagonize growth of androgen-sensitive tumors, as we have observed by SOCS2 inability to inhibit androgen-dependent proliferation in vitro.

The defective androgen-signaling consequence of androgen ablation therapy is likely to explain reduced expression of SOCS2 in CRPC. This in turn may contribute to activation of some of the alternative pathways that can sustain growth in conditions of low circulating androgen levels. Although the mechanisms driving proliferation of castration-resistant prostate tumors are not fully understood, it presumably involves the activation of action of several tyrosine-kinases-mediated signaling pathways (e.g. JAK2, Src, etc., refs 46–48) known to act upstream STAT5; some of which can be inhibited by SOCS2 (Figure 2). The ability of SOCS2 to modulate tyrosine-kinase signaling (Figure 2 and Supplementary Figures 1B and 2B, available at Carcinogenesis Online) and to inhibit proliferation of the androgen-independent cell line C4-2B (Figure 3D) provides strong support for this hypothesis.

SOCS2 is the target recognition subunit of an E3 ubiquitin ligase complex (19). However, the identification of proteins directly targeted for proteasomal degradation by SOCS2 has remained elusive. Recently, we have identified GHR as a direct target for SOCS2 actions leading to the silencing of the GH signal (39). Our demonstration of the inhibitory function of SOCS2 on GHR both in vivo and in vitro suggests that SOCS2 tumor suppressor functions in the prostate may also involve the antagonism of GH actions. However, our findings do not preclude the existence of additional targets. In humans, GH closely resembles PRL and binds to the PRL receptors (49). Because SOCS2 also inhibits PRL signaling in other tissues (e.g. breast) (50), it is highly likely that the PRL receptor is also targeted by SOCS2 in the prostate. Moreover, we also observed that alteration in SOCS2 protein levels in PCs cells influences STAT5 protein expression levels but it has no effect on JAK2 levels (Figure 2). Whether SOCS2 directly targets STAT5 for ubiquitination and degradation remains to be elucidated but if confirmed, it would reinforce the notion that SOCS2 has a role as tumor suppressor because STAT5 is thought to contribute to CRPC growth (11).

The role of GH and PRL as contributing factors in PCA development is controversial. On one hand, models for PCs (3–5) develop at a slower rate when GH availability is reduced, whereas PRL transgenic mice develop prostate hyperplasia at advanced ages (51). Likewise, a role for GH in prostate carcinogenesis is supported by the positive correlation between IGF-1 plasma levels (which are dependent on GH actions in liver) with PCA incidence (52). On the other hand, however, GH does not induce cell proliferation of PCA cells in vitro (ref. 33 and Supplementary Figure 2C, available at Carcinogenesis Online). Moreover, circulating GH levels (as well as testosterone levels) decrease with age, whereas PCs is mainly a disease of the elderly, and there is no statistical support for an increased incidence of PCs in acromegalic or prolactinoma patients (53). The lack of correlation between GH or PRL blood levels and PCA incidence may reflect the importance of paracrine actions of hormones produced locally in the prostate as recently demonstrated (54,55). Here, we present evidence for an alternative explanation where the tumor promoting actions of GH are regulated intracellularly by the inhibitory actions of SOCS2 and, therefore, are less dependent on fluctuations in GH levels. We observed that SOCS2 levels are reduced in CRPC in direct comparison with primary tumors suggesting that castration-resistant tumors are hypersensitive to GH and that SOCS2 might exert tumor suppressor functions specifically on this disease stage. Indeed, our data demonstrate that SOCS2 inhibits GH-induced invasion of PCs cells, whereas studies in vivo demonstrate that SOCS2 limits the prostate proliferation in response to GH. Increased expression of the polycomb repressor complex has been proposed to induce a reprogramming of AR transcriptional targets in advanced PCs (56). Overexpression of the polycomb complex protein EZH2 is often detected in CRPC (57,58). Previous studies have identified SOCS2 as a repressed target gene of the polycomb complex (59), suggesting that this mechanism may participate in the downregulation of SOCS2 in CRPC. Analysis of microarray data reveals a significant inverse correlation between SOCS2 and EZH2 expression in human prostate tumors (Spearman r = −0.22; Supplementary Figure 3, available at Carcinogenesis Online) suggesting that some of the EZH2 actions in CRPC may be mediated by the inhibition of SOCS2.

One striking observation of this work is the positive correlation between SOCS2 protein levels in localized tumors from prostatectomy samples and disease relapse (Figure 6A). As explained above, this may be a reflection that more aggressive tumors may exhibit enhanced activity of STAT5 and AR, which we have demonstrated to regulate the expression of SOCS2 in the prostate. This finding is the opposite of what has been previously reported for measurements of SOCS2 mRNA levels (22). Although this apparent lack of concordance between protein and mRNA levels would need to be confirmed by parallel measurements of proteins and mRNA levels in a sizable sample cohort, it is also intriguing that a similar disagreement has also been observed with another androgen-induced gene, KLK3 (PSA). Only 26% of the tumors positive for KLK3 proteins were as well positive for KLK3 mRNA (60). Genome-wide, the correlation between protein and mRNA levels can be modest (61) and specifically for SOCS2, differences between mRNA and protein levels have been observed before in liver tissue (62). SOCS2 posttranscriptional regulation is yet not fully understood but it is determined by interactions with the cullin 5 subunit of the SOCS2–E3 ubiquitin ligase complex and unknown mechanisms that influence the SH2 and N-terminal domain of SOCS2 (39). Future studies will help to clarify the potential role of SOCS2 protein or RNA levels as a prognostic marker for PCs. Specifically, it will be important to analyze additional cohorts that would allow one to study the correlation between SOCS2 expression and other end points of tumor aggressiveness such as appearance of distant metastasis or overall survival.

In summary, our study demonstrates that SOCS2 is a negative regulator of GHR actions in prostate where it mediates the cross talk between androgen and GH-signaling pathways. Loss of SOCS2 expression, as observed in castration-resistant tumors, may contribute to their increased proliferative rate through the activation of tyrosine-kinase-involving pathways. Our results would support the investigation of GHR and tyrosine kinase antagonists (e.g. JAK2) as a complementary treatment for CRPC, specifically in the subset of patients with low SOCS2 expression levels.

Supplementary material

Supplementary Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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References


32


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