Tumor and Stem Cell Biology

Loss of Androgen Receptor Expression Promotes a Stem-like Cell Phenotype in Prostate Cancer through STAT3 Signaling

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Abstract

Androgen receptor (AR) signaling is important for prostate cancer progression. However, androgen-deprivation and/or AR targeting-based therapies often lead to resistance. Here, we demonstrate that loss of AR expression results in STAT3 activation in prostate cancer cells. AR downregulation further leads to development of prostate cancer stem-like cells (CSC), which requires STAT3. In human prostate tumor tissues, elevated cancer stem-like cell markers coincide with those cells exhibiting high STAT3 activity and low AR expression. AR downregulation-induced STAT3 activation is mediated through increased interleukin (IL)-6 expression. Treating mice with soluble IL-6 receptor fusion protein or silencing STAT3 in tumor cells significantly reduced prostate tumor growth and CSCs. Together, these findings indicate an opposing role of AR and STAT3 in prostate CSC development. Cancer Res; 74(4); 1227–37. © 2013 AACR.

Introduction

Prostate cancer is the most common cause of male cancer-related deaths in Western countries. Current treatment relies on targeting androgen receptor (AR) signaling by hormone deprivation or antiandrogen therapy. However, tumors are heterogeneous and hormone depletion often results in the selection of drug resistant, highly metastatic tumor cells that survive targeted therapy. Hence, the initial response to treatment is often followed by tumor recurrence (1, 2). The development of recurring prostate cancer is not well understood and remains a challenge for more effective therapeutic intervention. Even with new therapies including abiraterone and enzalutamide targeting AR signaling, some cancers do not respond and ultimately recur. One suggested model to explain recurrence proposes the selection of cancer stem-like cells (CSC) that survive drug therapy. Because selected CSCs are thought to be resistant to conventional therapy, and have been suggested to resupply the highly proliferative tumor cell pop-

ulation, failure to eliminate this cell population might result in tumor relapse (3). Cancer cells with stem-cell like properties share phenotypic features with somatic stem cells and are characterized by self-renewal and multilineage differentiation (4). Importantly, the development of CSCs is not well understood and identification of signaling pathways that regulate phenotypic and tumorigenic potential of CSCs might provide new insights for drug development to prevent tumor drug resistance and relapse (5).

STAT3 protein has been implicated in maintaining pluripotency and self-renewing processes in embryonic stem cells and glioblastoma stem cells (6–8). STAT3 is a member of the STAT family of transcription factors that transduce signals from cytokine and growth factor receptors on the cell surface and regulate gene expression responses in the nucleus (9). In particular, STAT3 regulates the expression of genes that control cell proliferation, survival, and immune responses (10). Persistent activation of STAT3 signaling is oncogenic and has been demonstrated in a wide variety of human tumor specimens and tumor cell lines, including leukemias, lymphomas, and a variety of solid tumors, such as head and neck cancer, colon, breast, and prostate cancer (11–14). Activated STAT3 signaling contributes to oncogenesis by inducing cell proliferation, preventing apoptosis, and suppressing antitumor immune responses (15–20). Moreover, constitutive activation of STAT3 has been shown to be important in tumor metastasis and angiogenesis (21–23).

Besides growth factor receptors and nonreceptor tyrosine kinases, including EGF receptor (EGFR), platelet—derived growth factor receptor (PDGFR), and SRC, activation of STAT3 is mediated by cytokine receptors such as interleukin-6 (IL-6) receptor (9, 24). IL-6 signal transduction is initiated by ligand binding to the IL-6R/gp130 receptor complex followed by activation of an intracellular signaling cascade in which
receptor-associated Janus kinases (JAK) phosphorylate STAT3 on a single tyrosine residue. Upon STAT3 phosphorylation at Y705, STAT3 forms stable dimers and translocates to the nucleus, where it binds to its specific promoter sequences to induce target gene expression (9, 24).

The IL-6/STAT3 signaling pathway has been implicated in the progression of prostate tumors (25). Clinical studies demonstrated elevated levels of IL-6 in blood plasma and blood serum of patients with hormone-refractory prostate cancer (HRPC) or metastatic prostate cancer, compared with benign or nonmalignant forms (26, 27). Thus, high IL-6 expression (>7 pg/mL) has been suggested to participate in malignant progression from hormone-sensitive to HRPC (28). Moreover, IL-6 has been implicated in the maintenance of stem-like cancer cells. In gene expression profiles of CD44+/CD24−/CD133 breast CSCs, IL-6 has been demonstrated to be upregulated (29). Genetic signatures of prostate CSCs revealed activation of JAK/STAT3 signaling pathway as a potential target for prostate cancer and prostate CSCs (31). The IL-6 receptor fusion protein (IL-6RF) has been described as a cytokine ‘trap’ that efficiently sequesters soluble IL-6 and thereby prevents activation of downstream IL-6 signaling (32–34).

On the basis of these observations, we investigated the role of activated STAT3 signaling upon androgen blockade on CSC development in prostate cancer. Our findings provide new mechanistic insights for prostate cancer tumor relapse and CSC development involving STAT3 signaling. We further propose the IL-6RF as a potential approach to inhibit IL-6/STAT3 signaling and overcome recurrent prostate cancer.

Materials and Methods

Cells

Munire epithelial prostate cancer cells TRAMP-C1 (TC1) were obtained originally from American Type Culture Collection, TC1 control-, STAT3-, or AR-shRNA–engineered cell lines were generated by transducing lentiviral particles containing pLKO1-STAT3 mouse short hairpin RNAs (shRNA: TRCN0000071456, TRCN0000071453, and TRCN0000071455) or pLKO1-AR mouse shRNAs (TRCN0000026189, TRCN0000026195, and TRCN0000026211; Sigma). Pooled populations of transduced cells were used to avoid selection for clonal variants. Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 5% FBS (Gibco), 5% Nu-Serum IV, 0.005 mg/mL bovine insulin, 10 nmol/L dehydroisoandrosterone (Sigma), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Gibco). DU145 cells expressing pLKO1-human STAT3 shRNA (TRCN0000020840) or pLKO1-nonsilencing control shRNA were maintained in RPMI supplemented with 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2 μg/mL puromycin. Hek293 cells were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin. HEK-IL-6RF cells were stably reconstituted with a cDNA encoding murine IL-6RF in pcDNA5 FRT/TO using the Flp-In technique (Invitrogen) and selected with Hygromycin (1:100). All cells were obtained before 2008.

In vivo experiments

The Il2-rg(ko)/NOD-SCID mice were kindly provided by Dr. Jun Wu (City of Hope, Duarte, CA). Bag(ko)Mom/B6.129S7 mice were purchased from The Jackson Laboratory. Mouse care and experimental procedures were performed under pathogen-free conditions in accordance with established institutional guidance and approved protocols from the Institutional Animal Care and Use Committee of Beckman Research Institute at City of Hope National Medical Center. Approximately 2–2.5 × 106 TC1, TC1 cells expressing nonsilencing control-, STAT3-, or AR-shRNA were implanted in mice subcutaneously with Hank’s Balanced Salt Solution. Palpable tumors were treated peritumorally with either vehicle control (10% EtOH, 90% corn oil), 100 μL flutamide (25 mg/kg), or bicalutamide (50 mg/kg) every other day. Of note, 200 μL vehicle control (conditioned medium of parental HEK cells) or IL-6RF (1.8 μg/kg) were injected twice daily. Mice were sacrificed 3 to 4 weeks after tumor challenge; tumor specimens were harvested and prepared 2 hours after last drug treatment for necrotic analysis.

Immunofluorescence

For immunofluorescence staining of frozen Matrigel plugs, tissue sections were fixed with 2% paraformaldehyde, permeabilized in methanol, and blocked in PBS supplemented with 10% goat serum and 2.5% mouse serum (Sigma). Sections were incubated overnight with primary antibodies (pYSTAT3; Santa Cruz Biotechnology), AR, Nanog, CD44 (Epitomics), CD31 (BD Biosciences), cleaved caspase-3, His (Cell Signaling Technology), Ki-67, MSI-1 (Abcam), and secondary antibodies conjugated to fluorescein isothiocyanate (FITC), Alexa Fluor 488, Alexa Fluor 555, or Alexa Fluor 647 (Invitrogen). Immunofluorescence stainings were analyzed by confocal microscopy (LSM510Meta Zeiss). To acquire mean fluorescence intensities (MFI), single fluorescence intensities of fluorophore-conjugated secondary antibodies in regions of interest were recorded by total fluorescence intensity (FITC, Alexa Fluor 488, Alexa Fluor 555, and Alexa Fluor 647) and normalized by total fluorescence per field of view in a 12-bit mode and relative units were quantified and averaged.

Archival human prostate carcinoma tissues from an anonymous group of patients were provided by the Pathology Core of City of Hope Comprehensive Cancer Center. Tumor tissues were formalin-fixed and paraffin-embedded. Sections of 3 μm were deparaffinized and processed as described earlier. These sections were stained with antibodies raised against pYSTAT3 (1:50; Santa Cruz Biotechnology), AR (1:50; Epitomics), MSI-1 (1:50; Bioscience), Sox2, Nanog, CD44, integrin α2β1 (1:50; Abcam), and secondary antibodies conjugated to fluorophores diluted 1:100 (Invitrogen), additionally incubated with Hoechst33342 (Invitrogen), then mounted with Mowiol, and analyzed by confocal microscopy.

Intravital multiphoton microscopy

Mice were anesthetized with an isoflurane/oxygen gas and prepared for surgery. Then, mice were injected with 100 μg dextran-rhodamine and 25 μg of Hoechst33342 (Invitrogen), intravenously. Tumor-bearing mice continued to receive

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isofluorane/oxygen anesthesia while 2-photon live imaging was performed using Prairie Technologies Ultima microscope. To record rhodamine, signals were measured at λ(excit) = 660 nm with emission between 565–615 nm (rhodamine). For recording Hoechst 33342, emission signals were recorded at λ(excit) = 730 nm with emission between 435–485 nm. Extracellular matrix (ECM) emission was given by second harmonic generation at λ(excit) = 890 nm (Coherent Chameleon Ultra II Ti:Sa laser). Images were acquired using an Ultima Multiphoton Microscopy System (Prairie Technologies) equipped with Prairie View software and non-descanned Hamamatsu photomultiplier tubes. Images were collected in 512 × 512 pixels, 16-bit resolution. TIFF formatted images were analyzed by Image-Pro Plus professional imaging software (Media Cybernetics).

**Western blot analysis**

Cell lysates were prepared and protein concentrations were determined by Bio-Rad protein assay. Equivalent amounts of total proteins were separated by 10% SDS-PAGE (Invitrogen), subjected to immunoblotting, probed with the respective antibodies, and detected for signals using horseradish peroxidase–conjugated secondary antibodies by enhanced chemiluminesence (Amersham).

**Tumor sphere formation**

Cells were resuspended and dissociated into single cells by using a 40-μm cell strainer. To remove remaining serum, cells were washed 3-times with Hank’s Balanced Salt Solution (Invitrogen). Then, tumor cells were resuspended in 3 mL tumor sphere medium (TSM) consisting of DMEM F-12, L-glutamine (1%), 1 mol/L Hepes (2.5%), B-27 (50×), heparin sodium, and plated at a density of 4 × 10^4 cells per 6-well plate. Of note, 20 ng/mL fibroblast growth factor (FGF)-2 and EGF were added freshly. Tumor spheres with at least 20 cells per sphere were counted every other day using a transmitted light microscope for 6 days.

**Results**

**Loss of AR expression activates STAT3 signaling in prostate cancer cells**

Treatment with antiandrogens is one of the standard therapies for androgen-sensitive prostate cancer. Development of androgen insensitivity is the major obstacle for therapeutic intervention in recurrent prostate cancer. To determine the role of STAT3 in the transition from androgen-sensitive to HRPC upon hormone deprivation, mice bearing TCI murine prostate tumors derived from transgenic spontaneous adenocarcinoma of the mouse prostate (TRAMP) transgenic mice were treated with the AR antagonist flutamide. TCI cells implanted subcutaneously into Rag1−/− mice, established tumors within 4 weeks after implantation and expressed AR. Treatment with flutamide resulted in an immediate growth suppression (Fig. 1A, F1). However, repeated flutamide administration did not result in tumor rejection, but rather continued tumor growth (Fig. 1A, F2). Compared with vehicle-treated control, AR expression was significantly decreased at the onset of flutamide administration (Fig. 1B, F2; Fig. 1C, F2) as well as in advanced tumor growth (Fig. 1B, F1; Fig. 1C, F1). In contrast, STAT3 expression and activation significantly increased in TCI tumors challenged with flutamide, as shown by Western blot analysis and immunofluorescence staining (Fig. 1B, FI F2; Fig. 1C, FI F2).

Using RNA interference (RNAi) approaches to silence either AR or STAT3 expression in prostate cancer cells, tumor growth kinetics of engrafted engineered cells indicate that STAT3 knockdown delays tumor growth significantly (Fig. 1D). Conversely, silencing AR expression had no effect on tumor growth (Fig. 1D), tumor vasculature (Supplementary Fig. S1A), or proliferative activity (Supplementary Fig. S1B). Furthermore, AR knockdown resulted in a more robust STAT3 activation, suggesting that STAT3 signaling compensates for silenced AR and promotes tumor growth (Fig. 1E). In all RNAi experiments, pooled populations of transduced cells were used to avoid selection of clonal variants and RNAi specificity was validated. Hence, downregulation of AR signaling achieved either by antagonizing AR with flutamide or silencing AR expression through RNAi is accompanied by enhanced STAT3 activity, thereby favoring disease progression and tumor growth. Correspondingly, AR mRNA expression is significantly decreased in association with enhanced STAT3 activity in TC2 murine prostate cancer cells (Supplementary Fig. S1C). TC2 cells are considered to represent more advanced prostate cancer than TCI cells, which is reflected by significantly improved tumor growth (Supplementary Fig. S1D) and elevated expression of Ras and Myc similar to high-grade human prostate cancer (Supplementary Fig. S1E).

**AR downregulation-associated STAT3 activation promotes the development of prostate CSCs**

Because enhanced STAT3 activity in cancer cells has been suggested to impact the tumorigenic potential by unbalancing differentiation and promoting the expansion of CSCs, we analyzed the potential of STAT3 and AR to induce a CSC phenotype. Antagonizing AR in murine prostate tumors with flutamide resulted in significantly elevated protein expression of Musashi-1, Sox2, and CD44 (Fig. 2A). Furthermore, AR silencing promoted tumor sphere formation of prostate cancer cells (Fig. 2B, left), associated with increased STAT3 activity as well as increased expression of Musashi-1 and Sox2 (Fig. 2B, right). Thus, AR inhibition selected for a shift from AR+/pSTAT3+/MSI+/Sox2+ expression to a cancer cell stem-like phenotype with AR+/pSTAT3+/MSI+/Sox2+ expression as seen both in vitro and in vivo (Fig. 2C). In striking contrast, genetic silencing of STAT3 suppressed tumor sphere formation significantly, concomitant with reduction of CSC marker expression (Fig. 2B and C). This indicates that AR signaling downregulation, achieved by either androgen antagonists or genetic silencing, favors STAT3 activity and consequently expression of CSC markers, leading to the induction of a prostate CSC phenotype. As shown in Fig. 2D, AR is not associated with CD44 expression (top), whereas pSTAT3+/CD44−expressing cells represent the major population (bottom) in untreated prostate tumors. Hence, reduced AR activity promotes the CSC phenotype at least in part through activation of STAT3.
Human prostate CSCs are characterized by low AR and high p-STAT3 levels

To elucidate the role of STAT3 activity in mounting the CSC repertoire resulting in sustained malignancy of human prostate tumors, we investigated the coexpression of STAT3 and AR with CD44, which is thought to impact tumor initiation as well as homing/metastasis to the bone marrow, and Sox2 and integrin α2β1, both of which are involved in self-renewal. As shown by analysis of patient biopsies, activated STAT3 is associated predominantly with elevated Sox2 and integrin α2β1 expression (Fig. 3A), as well as with CD44, Musashi-1, and Nanog expression (Supplementary Fig. S2A and S2B). In contrast, lack of pSTAT3 is associated with substantially decreased expression of Sox2 and integrin α2β1. Notably, pSTAT3 does not coincide with AR expression (Fig. 3B). Accordingly, CD44 or Sox2 protein expression is not accompanied by AR expression, which is instead expressed locally, constrained and excluded from CD44+ or Sox2+ cell clusters (Fig. 3C). Thus, Sox2+ human CSCs of the prostate characteristically express elevated pSTAT3 but not AR (Fig. 3D and Supplementary Fig. S2B), which emphasizes a critical role for STAT3 in tumor progression.

Blocking IL-6/STAT3 signaling inhibits development of prostate CSCs

Because IL-6 mRNA expression was elevated in AR-targeted tumors by either antiandrogen or silencing of AR (Fig. 4A), we evaluated the role of IL-6 as a potential mediator of STAT3 activation in prostate CSCs. We used the IL-6 cytokine "trap" IL-6RFP, a soluble fusion protein of the ligand-binding domains of the IL-6 receptor subunits gp130 and IL-6Rα (32). In vitro characterization of IL-6RFP verified IL-6 binding capability and IL-6–neutralizing activity in prostate cancer cells (Fig. S3A-G). Although systemic in vivo delivery of IL-6RFP induces a significant delay in tumor growth kinetics, local administration of IL-6RFP shows an improved antitumoral efficacy early on...
Most importantly, comparison of mice treated with equimolar concentrations of IL-6RFP or anti-IL-6 antibodies revealed that antitumoral efficacy of IL-6RFP is improved by 3-fold over anti-IL-6 antibodies (Fig. 4B and Supplementary Fig. S3I). Moreover, compared with anti-IL-6 cytokine depletion antibodies, IL-6RFP–driven inhibition of STAT3 signaling is considerably enhanced (3.5-fold; Supplementary Fig. S3G).

In murine 3D cell culture in vitro, we evaluated the maturation of CD44+/Sox2+ tumor spheres upon IL-6RFP treatment, which resulted in decreased sphere formation and STAT3 activity (Supplementary Fig. S3J). Therefore, we assessed the therapeutic efficacy of blocking IL-6 signaling by administering IL-6RFP in vivo targeting the "stemness" phenotype in prostate cancer. Treatment with IL-6RFP resulted in significantly reduced STAT3 activity, concomitant with decreased protein expression levels of MSI-1, Sox2, CD44, and Nanog (Fig. 4C–E and Supplementary Fig. S3J and S3K).

As shown by immunofluorescence staining of tumor microsections, the phosphorylation of STAT3 (Fig. 4D) and expression of stem-cell markers, such as MSI-1 and Nanog (Fig. 4E, Supplementary Fig. S3K), is locally inhibited by tumor-permeating...
IL-6RFP. This finding indicates that blocking IL-6-mediated STAT3 signaling prevents maturation of multipotent CSC populations by inhibiting the expression of critical "stemness" regulators. Moreover, the regenerative potential of human prostate CSCs was considerably reduced after blocking IL-6/STAT3 signaling by IL-6RFP. Human tumor sphere formation was significantly inhibited, accompanied by diminished expression of pSTAT3, MSI-1, and Sox2 (Fig. 4F). As anticipated from rodent prostate CSCs populations, the presence of STAT3 is essential to mediate the expression of human CSC...
Importantly, whereas bicalutamide administration alone had tumor growth delay (Fig. 4H), tumor-permeating IL-6RFP efficiently inhibited STAT3 activation (Fig. 4I and J) and led to dramatically reduced expression of Sox2 and MSI-1 as shown by Western blot analysis from tumor homogenates (Fig. 4K). Thus, blocking IL-6/STAT3 signaling substantially diminishes the CSC phenotype in the prostate.

**Targeting both IL-6/STAT3 and AR signaling pathways gives stronger antitumor effects**

Both therapeutic interventions, androgen depletion and inhibition of IL-6 triggered STAT3 signaling, exert antitumor activity as monotherapy. Furthermore, silencing IL-6/STAT3 signaling prevents prostate CSC maturation. Therefore, we combined the clinically more relevant antiandrogen, bicalutamide, and IL-6RFP treatments to assess their combined potential for therapeutic benefit. Combinatorial therapy significantly reduced tumor growth but showed only slightly improved antitumor efficiency compared with treatment with IL-6RFP alone, whereas antiandrogen administration alone had a minor effect on tumor growth kinetics (Fig. 5A) Tumors treated with IL-6RFP alone or in combination with bicalutamide were positive for apoptotic cell death associated with downregulation of Bcl-2 and Bcl-xl (Fig. 5B), and exhibited a significant decrease in proliferative activity as shown by Ki-67 staining (Fig. 5C). Interestingly, androgen depletion alone led to accumulation of a CD44+ cell population in the tumor, as shown by 2-photon imaging in vivo (Fig. 5D). This indicates that bicalutamide treatment contributes to undesired CD44 expression suggestive of CSC development in prostate tumor tissue. In contrast, combination with IL-6RFP or IL-6RFP treatment alone resulted in significantly decreased amounts of CD44+ cells (Fig. 5D). Importantly, whereas bicalutamide administration alone had no beneficial effect on CSC marker expression, combined androgen depletion and IL-6RFP treatment enhanced suppression of MSI-1 and Sox2 expression (Fig. 5E).

**Discussion**

Standard treatment for prostate cancer targets the AR signaling pathway. However, therapeutic intervention often results in tumor relapse due to drug resistance and mechanistic explanations for this failure are diverse. A model based on the presence of prostate CSCs that are selected for drug resistance and allow sustained tumor progression could provide an explanation (35). Nevertheless, the underlying mechanisms for development of CSCs are not well understood. The cell populations initiating tumor growth and driving tumor relapse as a consequence of conventional therapies remain undefined. It has been suggested that CSCs may derive from somatic stem cells, progenitor, or differentiated cells, as well as highly proliferative, differentiated cancer cells by genetic or tumor microenvironment alterations (4). In these models, CSCs have been proposed to induce tumor growth and differentiate into the various cell types within a tumor. For the identification of CSCs, several markers have been suggested, including Sox2, Nanog, CD44, integrin-α2β1, and MSI-1 (36–38).

Here, we mainly use murine tumor cells derived from TRAMP model, which are of luminal origin, mirror primary prostate cancer (39), and express AR and STAT3. We demonstrate that chemotherapeutic intervention targeting the AR signaling pathway, which is important for differentiation and survival, promotes the development of a CSC phenotype and that this may, at least in part, be responsible for tumor relapse. Our data further indicate that inhibition of AR by either antiandrogens or gene silencing results in upregulation of the IL-6 cytokine (Fig. 4A). Although antiandrogen slows tumor growth more effectively than AR knockdown, this may reflect a delay in elevated IL-6 induction by antiandrogen treatment. Importantly, IL-6–induced activation of STAT3 signal transduction coincides with augmented CSC marker expression. We show that lack of AR is associated with this elevated STAT3 activity and augmented expression of CSC markers. In particular, AR-negative tumor cells exhibited increased MSI-1, Sox2, and CD44 expression, which was associated with activated STAT3 signaling. In human prostate cancer tissues, elevated CSC markers coincided with those cells exhibiting high STAT3 activity and low AR expression, indicating an inverse correlation between AR and pSTAT3 expression in the maturation of CSC in human prostate cancer progression. This counter-regulation of the AR and STAT3 signaling pathways in both mouse and human tumor cells provides a potential explanation for failure of monotherapies inhibiting the AR signaling pathway.

To effectively target the maturation of CSCs in prostate cancer, we applied a chimeric soluble IL-6RFP with improved inhibitory activity compared with IL-6 depletion antibodies. Inhibition of the STAT3 signaling pathway by gene silencing or using IL-6RFP in murine and human prostate cancer cells resulted in decreased CSC phenotype development in vitro and in vivo. Moreover, IL-6RFP-positive areas in tumor tissue revealed specific reduction of CSC markers, including MSI-1 and Nanog, suggesting that STAT3 activity is directly associated with CSC marker expression. Recent studies imply the IL-6 cytokine to be essential to enter senescence (40, 41) and subsequently maintain dedifferentiation/pluripotency (42). This is consistent with studies in glioblastomas showing that inhibition of the IL-6Rα expression diminished STAT3 activation, which was associated with reduced tumor growth and number of glioblastoma stem cells (7). Our data are consistent with and further expand on the studies of Korkaya and colleagues (43). Although previous studies have suggested that prostate CSCs are AR negative (44, 45), our findings indicate IL-6 secretion and downstream STAT3-mediated signaling as a critical pathway after blockade of AR, thereby maintaining potential toward development of the CSC phenotype as well as initiating tumorigenic progression.

The efficacy of targeting CSCs is often hampered by drug resistance, which is a challenge for therapeutic intervention using small-molecule drugs that exert their inhibitory activity intracellularly. Previous studies have demonstrated elevated levels of proteins exerting multidrug resistance such as ABCB1 and ABCG2 (46). Hence, inhibiting IL-6 with the IL-6RFP has several attractive aspects for targeting CSCs. Depletion of IL-6...
Figure 4. Decreased CSC phenotype formation upon IL-6RFP treatment. A, tumor homogenates of mice treated with either vehicle control or flutamide (left) or homogenates from engineered TC1 tumor cells as indicated (right) were prepared for real-time RT-PCR. Bar graphs show fold inductions of IL-6 mRNA levels assessed in triplicate. (Continued on the following page.)
is thought to impact the induction of cell senescence, which is considered one of the initial steps in the transition to a CSC phenotype (41). Blocking IL-6 would inhibit IL-6-mediated STAT3 signaling, which diminishes the CSC phenotype and downregulates STAT3 target gene induction such as Bcl-xL, survivin, and c-Myc. Finally, IL-6 signaling repression is thought to negatively impact IL-6R-mediated mitogen—activated protein kinase (MAPK) signaling and phosphoinositide 3-kinase (PI3K) signaling. The PI3K pathway is deregulated in many human tumors, including prostate cancer, and inhibition
revealed promising results in cancer therapy (47). Therefore, our data suggest that IL-6RFP or similar strategies are promising therapeutic agents for prostate cancer to reduce tumor growth and prevent recurring prostate cancer by targeting the CSC population.

It is unlikely that mono-therapies targeting the IL-6/STAT3 signaling pathway will be effective for complete tumor regression. It has been shown that first-line treatment inhibiting AR results in tumor regression, since the tumor bulk is eliminated. However, it seems possible that AR+/pSTAT3+ expressing CSCs can survive and differentiate into AR-dependent tumor cells even with low levels of androgens. Thus, targeting the tumor bulk with an AR inhibitor and at the same time preventing the dedifferentiated CSC cell population with high STAT3 activity is likely advantageous. Combining inhibition of IL-6/STAT3 with androgen blockade resulted in improved therapeutic benefit compared with the respective monotherapies. Importantly, IL-6 inhibition by IL-6RFP prevails over the antiandrogen-induced CSC phenotype development. Hence, our data support the concept that combination of AR and STAT3 inhibitors may be the most effective prostate cancer therapy. A similar reciprocal regulation between AR and PI3K signaling has been found in Pten-deficient mice (48).

In summary, our results demonstrate that blockade of AR mediates activation of STAT3 signaling through upregulation of IL-6, which is associated with the development of a CSC phenotype. Conversely, inhibition of the IL-6/STAT3 signaling pathway with IL-6RFP diminishes the CSC population, which results in reduced tumor growth. These findings provide new mechanistic insight into the emergence of drug-resistant prostate cancer. Moreover, combination of IL-6RFP and anti-androgens inhibited CSCs and differentiated AR-positive cancer cells, and therefore provides a promising treatment of prostate cancer to eventual tumor relapse.

Disclosure of Potential Conflicts of Interest
S. Pal has honoraria from Astellas. No potential conflicts of interest were disclosed by the other authors.

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