"Inhibition of the IL-6/STAT3 Signaling Pathway for Therapeutic Intervention in Prostate Carcinogenesis and Cancer Cell Differentiation"

Von der Fakultät für Mathematik, Informatik und Naturwissenschaften der RWTH Aachen University zur Erlangung des akademischen Grades einer Doktorin der Naturwissenschaften genehmigte Dissertation

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Other publications:


**The JAK2 Inhibitor AZD1480 Potently Blocks Stat3 Signaling and Oncogenesis in Solid Tumors**
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**Sorafenib Induces Growth Arrest and Apoptosis of Human Glioblastoma Cells through the Dephosphorylation of Signal Transducers and Activators of Transcription 3**
Mol Cancer Ther, 9:953-962


**Alkylation of cysteine 468 in Stat3 defines a novel site for therapeutic development**
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1 Introduction

1.1 The Family of the STAT Transcription Factors

Over 50 years ago, interferon has been described as a founder of the cytokine family [1]. In 1990, the first signal transducer and activator of transcription (STAT) protein was discovered in the course of research on interferon (IFN) signal transduction. The stimulation with IFN-alpha/beta resulted in the formation of a complex of transcription factors also known as ISGF-3 [2, 3], which consists of STAT1, STAT2 and IRF9, formerly termed p48 [4]. Subsequently, five more STAT proteins have been identified. So far, the STAT protein family consists of seven family members namely STAT1, STAT2, STAT3, STAT4, STAT5A and 5B, and STAT6 [5, 6]. Moreover, up to 11 additional splicing isoforms have been discovered, but a physiologic function is still unclear [7-9]. The STAT factors range in size from 750 – 900 amino acids and contain six conserved domains [10].

<table>
<thead>
<tr>
<th>STATs</th>
<th>Ligands</th>
<th>Phenotype of STAT deficient mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAT1</td>
<td>IFN-α/β, EGF</td>
<td>Loss of IFN-dependent immune response Increased susceptibility for bacterial and viral infections</td>
</tr>
<tr>
<td>STAT2</td>
<td>IFN-α/β</td>
<td>Defect in type-I-IFN dependent immune response</td>
</tr>
<tr>
<td>STAT3</td>
<td>IL-2, IL-6, IL-7, IL-9, IL-10, IL-11, IL-15, IL-19, IL-20 IL-21, IL-23, IL-27, IL-31, EGF, HGF, OSM, CNTF, CT-1, Leptin, G-CSF, TPO, LIF, FLT3L</td>
<td>Embryonic lethality</td>
</tr>
<tr>
<td>STAT4</td>
<td>IL-12, IL-23</td>
<td>Defect in T_{H}1 cell-differentiation</td>
</tr>
<tr>
<td>STAT5A and STAT5B</td>
<td>IL-2, IL-3, IL-5, IL-7, IL-9, IL-15, G-CSF, GM-CSF, EPO, TPO, GH, Prolactin, OSM</td>
<td>Infertility Defect in NK-cell development Defect in T-cell proliferation Impaired hematopoiesis Defect in development of mammary glands and lactation</td>
</tr>
<tr>
<td>STAT6</td>
<td>IL-4, IL-13</td>
<td>Impaired differentiation of T_{H}2 cells</td>
</tr>
</tbody>
</table>

Table 1: STATs are activated by the listed ligands. Genetic knock-out of STAT factors results in described phenotype (modified from [6, 9, 12, 13]).

Studies with genetically manipulated mice, lacking one or more Stat genes, have shown a relevance of STAT factors in fundamental cellular processes such as proliferation, differentiation, apoptosis, and inflammation [11-13] (Table 1).

Activation of the STAT signal transduction is a result of cytokine- or growth factor- binding at their specific receptors such as cytokine receptors (i.e. IL-
6Rα/gp130, OSMR), receptor tyrosine kinases (i.e. EGFR, PDGFR) and G-protein coupled receptors (i.e. S1P receptor) [14-17]. Ligand binding to the receptor leads to the activation of receptor associated kinases such as the Janus kinases (JAKs) [18], intrinsic receptor tyrosine kinases [14-16], or Src family kinases (SFKs) [19] which is followed by the phosphorylation of specific tyrosine motives at a cytoplasmic receptor domain. The phosphorylated receptor complexes direct SH-2-dependent recruitment of STAT proteins resulting in phosphorylation of a single tyrosine residue. Non-receptor tyrosine kinases such as the oncoproteins v-Src and BCR-Abl are able to phosphorylate STAT factors independently of receptors [20, 21]. The phosphorylated STAT factors dimerize through intermolecular SH2 domain/phosphotyrosine interactions resulting in nuclear translocation and DNA-binding to promote target gene induction [22-24]. Nuclear translocation has been described as a diffusion-driven process as well as a process in which STAT factors are associated with endocytotic vesicles [25]. Especially STAT3 has been shown to be activated by cytokine or growth factor receptors that were internalized upon ligand binding and continued signaling along the endocytotic pathway [26]. While STAT activation is usually transient and tightly regulated, persistent activation of cytokine receptors, receptor tyrosine kinases and non-receptor tyrosine kinases in cancer cells leads to a constitutive activation of STAT molecules, particularly of STAT3 and STAT5. Constitutive STAT activity results in an enduring change of gene expression [27]. Posttranslational modifications besides the tyrosine phosphorylation critical for activation have been characterized including serine phosphorylations which are important for an improved gene induction (Ser727 in STAT1 and STAT3; Ser721 in STAT4; Ser725 in STAT5A; Ser730 in STAT5B) [28]. Moreover, modifications such as acetylation of STAT3 at lysine 685 [29] and STAT6 [30], methylation at arginine residues [31], ubiquitination [32], and sumoylation [33] have been described.
1.1.1 Function of STAT3.

STAT3 was initially identified as an IL-6 dependent acute phase response factor (APRF) that binds to enhancer elements in the promoter of acute phase genes upon interleukin-6 (IL-6) stimulation in hepatocytes [34]. Unlike other STAT factors, STAT3 knock-out revealed early embryonic lethality and therefore shows the most serious phenotype. STAT3 deficient murine embryos died before gastrulation, of about 6.5 – 7.5 days post coitum [35]. STAT3 knock-out by conditional gene ablation revealed severe defects in targeted tissues. Affected functions upon loss of STAT3 involve impaired acute phase response (liver), migration (keratinocytes), survival (thymic epithelium, sensory-/motor-neurons, and T-lymphocytes), proliferation (T-lymphocytes) and apoptosis (mammary epithelium) [36-40] (Table 2).

<table>
<thead>
<tr>
<th>STAT3 deficient tissue</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keratinocytes</td>
<td>Defect wound healing by impaired migration of keratinocytes impaired hair cycle</td>
</tr>
<tr>
<td>Thymic epithelium</td>
<td>Hypersensitivity to stress Age-dependent thymic hypoplasia</td>
</tr>
<tr>
<td>Mammary epithelium</td>
<td>Impaired involution due to defect apoptosis of epithelial cells Impaired activation of mammary macrophages and number of mast cells during involution</td>
</tr>
<tr>
<td>Liver</td>
<td>Impaired acute-phase response</td>
</tr>
<tr>
<td>Lung</td>
<td>Impaired homeostasis Increased inflammation</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>IL-6 mediated IFN-γ-like response by extended STAT1 activity</td>
</tr>
<tr>
<td>T lymphocytes</td>
<td>Impaired IL-6 dependent survival Impaired IL-2Ra expression Defect Th17 cell-development</td>
</tr>
<tr>
<td>Monocytes/Neutrophils</td>
<td>Enhanced Th1 differentiation and activity Increased inflammatory response Development of chronic colitis</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>Enhanced proliferation due to impaired negative feedback Impaired acute neutrophil mobilization</td>
</tr>
<tr>
<td>Neurons</td>
<td>Enhanced neuronal apoptosis</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>Impaired astrocyte hypertrophy Defect inflammatory response</td>
</tr>
</tbody>
</table>

Table 2: Role of STAT3 in different tissues revealed by conditional gene silencing in mice. (modified from [36-40]).

So far, 4 isoforms of STAT3 have been identified (STAT3α, β, γ, δ). STAT3β (82 kDa) is a splicing variant of STAT3α (≈89 kDa). It is C-terminally truncated by 55 amino acids, which are replaced by 7 unique amino acids (FIDAVWK). Thus, STAT3β lacks the transactivation domain which implies no role in gene
expression, and therefore reveals dominant negative potential [41]. However, recent studies using specific gene ablation of either STAT3α or STAT3β indicated that STAT3β can rescue embryonic lethality induced by lack of STAT3α [42]. Moreover, STAT3β seems to be an important negative regulator of STAT3α by induction of specific target genes related to acute phase response [43, 44]. STAT3γ (72 kDa) and STAT3δ (64 kDa) are proteolytically truncated at the carboxy-terminus of STAT3α. So far, no function has been described for these isoforms [7, 45].

Mutations in one of the STAT3 alleles, which had dominant-negative effects, have been described in the context of the IgE-syndrome. The mutations were mainly located in the SH2-domain and the DNA binding domain [46].

1.1.2 The IL-6/gp130/STAT3 signaling pathway.

The IL-6-type cytokines interleukin-6 (IL-6), oncostatin M (OSM), leukemia inhibitory factor (LIF), IL-11, IL-27, cardiotrophin-1 (CT-1), Neuropoietin, ciliary neutrophic factor (CNTF) and cardiotrophin-like cytokine (CLC) are polypeptides of about 15 – 25 kDa that form a subfamily of the helix bundle cytokines consisting of four α-helices. IL-6-type cytokines feature pleiotropic functions and stimulate cells in an autocrine or paracrine manner [24, 47-49]. All these cytokines signal through the transmembrane protein gp130 (glycoprotein 130), whereby gp130 forms either homodimers or heterodimers with LIF-R, OSM-R or WSX-1. IL-6, IL-11, CT-1 and CNTF recruit additional specific α-receptors [23].

In the event of the IL-6/gp130/STAT3 signaling pathway, IL-6 binds to IL-6Rα resulting in the recruitment of gp130. The extracellular part of the IL-6Rα consists of three domains, whereby an Ig-like domain (D1) is followed by two FNIII (fibronectin type III)-like domains (D2, D3) which form the cytokine binding module (CBM). The extracellular part of gp130 consists of six domains (D1 – D6). Domain D1 represents an Ig-like domain which is followed by five FNIII domains (D2 – D6). The CBM is formed by domains D2 and D3 [50].

Binding of IL-6-type cytokines to plasma membrane receptor complexes results in the activation of two signaling pathways, the JAK/STAT pathway and the MAPK (mitogen-activated protein kinase) cascade [23].
Since cytokine receptors lack an intrinsic kinase activity, receptor engagement leads to the activation of intracellular, receptor associated Janus kinases (JAK).

**Figure 1: IL-6/gp130/STAT3 signaling pathway.** Stimulation with IL-6 results in the activation of an intrinsic signaling cascade, in which receptor associated JAKs phosphorylate the receptor resulting in the recruitment of STAT3 to the receptor. Receptor bound STAT3 gets phosphorylated by the JAKs which leads to the dissociation of STAT3 from the receptor, followed by dimerization and nuclear translocation. In the nucleus, STAT3 promotes gene expression upon which dephosphorylated STAT3 leaves the nucleus. Termination can be mediated by STAT3 induced SOCS proteins, PIAS or phosphatases. Abbreviations: IL-6-interleukin-6; gp130-glycoprotein 130; JAK-Janus Kinase; SOCS-suppressor of cytokine signaling; PIAS-protein inhibitor of activated STAT; PTPase-protein tyrosine phosphatase. (modified from [24, 50]).

The family of Janus kinases comprises JAK1, JAK2, JAK3 and TYK2. Janus kinases consist of two kinase domains of which only the carboxy terminus shows enzymatic activity [51, 52]. Upon activation JAKs phosphorylate collectively five tyrosine residues of the gp130 receptors. Tyrosine phosphorylation results in the
recruitment of STAT3 which binds with its SH-2-domain (src-homology-2-domain) at amino acid motives YXPQ or YXXQ [53-56]. Binding of STAT3 to the receptor leads to the phosphorylation at tyrosine 705 by JAKs [3, 57], which is followed by the dissociation of STAT3 from the receptor. Phosphorylated tyrosine 705 interacts with the SH-2 domain of another STAT3 molecule. Dimerized STAT3 translocates to the nucleus where it binds to enhancer elements of the DNA to promote induction of target genes [22, 29, 58]. One of many STAT3 target genes is SOCS3 (suppressor of cytokine signaling 3). This negative regulator can bind with its SH2 domain at Tyr759 of gp130 and inhibit JAK activity by direct interaction [59, 60]. Additional proteins for the negative regulation of IL-6/gp130/STAT3 signal transduction have been characterized. PIAS (protein inhibitors of activated STATs) binds to activated STAT factors and prevents DNA binding possibly by recruitment of co-repressor proteins or by modulation of STAT functionality through their SUMO ligase activity [61]. Moreover, STAT signaling can be regulated by tyrosine phosphatases such as SHP2 and TC-PTP which are able to dephosphorylate and inactivate STAT3. Similar to SOCS3, SHP2 binds to a phosphotyrosine motif (Tyr759) of the gp130 receptor and is able to dephosphorylate JAKs [62, 63]. Furthermore, nuclear translocation of STAT3 has been found to be inhibited by Grim-19 [64]. The IL-6/gp130/STAT3 signaling pathway is a transient process of about one hour (Figure 1).

1.1.3 Dysregulation of STAT3 signal transduction in tumorigenesis.

In the early 90s, the importance of STAT3 in oncogenesis was recognized. In contrast to benign cells, STAT3 has been found constitutively active in v-Src transformed cells [20]. Inhibition of STAT3 signal transduction reversed transformation of murine fibroblasts by the v-Src oncoprotein and resulted in impaired proliferation or apoptosis [65, 66]. Moreover, a constitutively active mutant of STAT3 (STAT3C) transformed murine fibroblasts in culture which allowed them to form tumors in mice [67]. Aberrant signaling by STAT3 has been detected in a wide variety of human tumor specimens and tumor cell lines including multiple myeloma, leukemias, lymphomas, and a variety of solid tumors such as, breast, melanoma, head and neck, lung, ovarian, pancreatic and
prostate cancer. Besides STAT3, other STAT factors such as STAT1 and STAT5 have been described to play a role in tumorigenesis as well [68].

In the scope of STAT3 several upstream activators have been identified to be responsible for aberrant activation of STAT3 in tumor cells such as cytokines [69] or growth factor [70, 71]. Constitutive active kinases such as v-Src, TEL-JAK, JAK2-V617F, BCR-Abl, v-Abl, NPM-Alk, v-Ros and v-Eyk can function independently of extracellular stimuli and persistently phosphorylate STAT3 at Tyr705 [27]. Insufficient induction or gene silencing of negative regulators such as PIAS3, SOCS proteins or phosphatases like SHP-2 might result in constitutive activation of STAT3 as well [72, 73]. In addition, a constitutive phosphorylation of STAT3 is mediated by some tumor viruses such as the human-T-lymphotrophic virus-1 (HTLV-1) [74], polyomaviruses [75], Epstein-Barr virus (EBV) [76] and Herpes virus samiri [77], which activate JAKs or tyrosine kinases of the Src family directly or indirectly.

<table>
<thead>
<tr>
<th>Genes induced by STAT3</th>
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<tbody>
<tr>
<td><strong>Acute-phase</strong></td>
</tr>
<tr>
<td>α2-Macroglobuline (α2M)</td>
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<tr>
<td>Antichymotrypsine (ACT)</td>
</tr>
<tr>
<td>Serum amyloid A (SAA)</td>
</tr>
<tr>
<td>SOCS3</td>
</tr>
<tr>
<td>C-reactive protein (CRP)</td>
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<tr>
<td>Fibrinogen</td>
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**Table 3:** Differences of STAT3 regulated genes in inflammation and oncogenesis. (modified from [27, 58]).

In contrast to the transient activation of STAT3 in inflammation, signal transduction of STAT3 is dysregulated in cancer and results in constitutive activation, which is followed by altered regulation of STAT3 target genes.

While inflammation results in the upregulation of acute phase proteins, continuous gene expression of proliferative, angiogenetic, and anti-apoptotic proteins is induced in oncogenesis [27]. STAT3 has been shown to contribute to the transformation of cells in vitro, the induction of Cyclin D1 and D2 (proliferation) [78, 79], c-myc (transformation) [80], Bcl-xL, Mcl-1, Survivin (anti-
apoptotic signals) [81], MMP2, MMP9 (metastasis) [82, 83], VEGF, FGF2 and HIF1α (angiogenesis) [84] (Table 3). Processes in cancer cells such as uncontrollable proliferation, resistance to apoptosis, angiogenesis and metastasis are necessary for the formation and progression of tumors. Thereby, tumor cells interact with the microenvironment or tumor stroma which consists of the basement membrane, extracellular matrix, vasculature, fibroblasts and immune cells. The communication is mediated by the expression, secretion and autocrine or paracrine action of soluble factors such as cytokines and growth factors. In this context STAT3 plays an important role [85]. On the one hand, activated STAT3 promotes proliferation and survival in tumor cells. On the other hand, STAT3 drives the production of cytokines and growth factors by tumor cells that activate STAT3 in non-transformed cells of the microenvironment.

STAT3 is involved in the tumor induced immunosuppression by induction of cytokines. In the presence of IL-6, IL-21 and IL-23, STAT3 promotes the differentiation of TH17 cells. In the tumor microenvironment, TH17 cells secrete IL-17 in a STAT3 dependent manner [86]. IL-17 production results in TH17 cell expansion and the activation of STAT3 in other stromal cells, which can further enhance tumor growth [87]. The soluble factors IL-6, IL-10 and VEGF stimulate STAT3 activation in dendritic cells (DC) which inhibits their maturation and results in the inactivation of effector T-cells [85, 88]. Negative regulation of effector T cells results in reduced cytotoxicity. Moreover, activated STAT3 favors the emergence of regulatory T cells (Treg), which suppress activity of cytotoxic CD8+ T cells in the tumor [89, 90]. It has been shown that STAT1 is involved in the immune evasion regulated by STAT3. STAT1 is thought to be a tumor suppressor which counter-regulates STAT3. While STAT1 is overexpressed in STAT3 knock-out cells, minor expression of STAT1 was observed in cells with constitutive active STAT3. Contrary to STAT3, STAT1 is essential in signal transduction upon IFNγ stimulation and therefore regulates cell cycle arrest, pro-apoptotic pathways and immune surveillance [91].
1.1.4 STAT3 as a target for cancer therapy.

A limited number of transcription factors are constitutively active in human cancers. Since transcription factors play a central role as mediators between oncogenic kinases and aberrant gene regulation, they are promising targets for therapeutic intervention [92]. In the context of STAT3, several indirect and direct approaches have been emphasized in diminishing constitutive activation or protein expression to prevent malignant transformation and are summarized in figure 2. On protein level, one promising strategy is the application of antisense oligonucleotides [93] and small interfering RNA [94]. Other direct approaches involve the development of specific STAT3 inhibitors that prevent receptor recruitment, dimerization or DNA binding. Peptides, peptidomimetics, peptide-aptamers, non-peptide analogs, and platin (IV)-complexes have been studied regarding STAT3 inhibition [95].

![Figure 2: Strategies for STAT3 inhibition. These include the inhibition of STAT3- gene expression, dimerization, DNA-binding and the inhibition of tyrosine kinases (modified from [91]).](image)

Moreover, engineered oligonucleotides such as guanine-rich G-quartets and alcyators have been identified to bind at phosphorylated STAT3 to prevent binding of STAT3 on target gene promoters [91]. An indirect strategy is the
application of small molecule inhibitors to diminish the activity of dysregulated kinases upstream of STAT3. AG490 [96], AZD1480 [97], and JSI-1 (cucurbitacin-1) [98] are inhibitors of the Janus kinases and prevent activation of down-stream effectors including STAT3, leading to growth arrest and apoptosis of tumor cells. Additional inhibitors of tyrosine kinases involve the BCR-Abl inhibitor Imatinib (gleevec, Novartis, USA) [91], the Src-inhibitor Dasatinib and the VEGFR/EGFR inhibitor Sorafenib [99]. All these inhibitors target intracellular proteins which is problematic, since they have to pass the hydrophobic cell membrane. Therefore, another strategy to prevent STAT3 activation is the inhibition of extracellular binding of cytokines or growth factors to their cognate receptor. This can be achieved by the application of recombinant proteins such as monoclonal antibodies, cytokine antagonists, soluble receptors, or engineered receptor fusion proteins.

**Figure 3: The IL-6**<sub>RFP</sub>. **A**: Domain structure of the human IL-6 receptor fusion protein (left), and bound to IL-6 (right) **B**: hexameric structure of IL-6<sup>RFP</sup> bound to IL-6. (modified from [100]).

The receptor fusion proteins were developed for the inhibition of cytokine receptor signaling like the IL-6/STAT3 signal transduction [100]. The human IL-6 receptor fusion protein (hIL-6<sup>RFP</sup>) consists of the three extracellular domains of human gp130 and two domains D2-D3 located in the N-terminus of human IL-6Rα fused by a linker (33 – 49 Aa). The linker provides flexibility and stability for optimal ligand-binding [101] (Figure 3A). It has been implicated that IL-6 and the IL-6<sup>RFP</sup> form a tetrameric complex, similar to the hexameric IL-6 receptor complex, consisting of two molecules IL-6 bound to two receptor fusion proteins (Figure 3B). The IL-6<sup>RFP</sup> has been shown to prevent IL-6 induced STAT3 phosphorylation *in vitro* and does not interact with other IL-6 type cytokines such as OSM, LIF, and IL-11 [101, 102].
1.2 Initiation of Prostate Cancer

The normal human prostate is a tubular–alveolar gland consisting of an epithelium surrounded by a stromal compartment. The adult prostate is located at the base of the bladder and its function is the secretion of proteins to the seminal fluid consisting of organic solutes, lipids, and cholesterol [103]. The prostate is described by three distinct morphological regions: the peripheral zone, the transition zone, and the central zone. Benign prostate hyperplasia (BPH), which is a non-cancerous enlargement, occurs in the transition zone. Prostatic intraepithelial neoplasia (PIN) is a precursor of invasive carcinoma and most commonly found in the peripheral zone (Figure 4A) [104, 105]. Histologically, the prostate gland consists of an epithelial and a stromal compartment, separated by the basement membrane. The prostatic epithelium is composed of three different cell types including luminal cells, basal cells and neuroendocrine cells. Luminal cells are terminally differentiated cells with limited proliferative activity. They express the androgen receptor (AR) and secrete prostate fluid containing fatty acids such as prostaglandins and proteins including prostate specific antigen (PSA), prostate specific acid phosphatase (PAP) androgen dependently [106].

![Figure 4](image.png)

**Figure 4: Schematic structure of the prostate.** A: Schematic illustration of the human prostate with its different zones. B: Graphic of the cell-types within a prostatic duct (modified from [104]).

Basal cells rarely express AR, do not require androgens for survival, and are highly proliferative. The basal layer is less differentiated and defined as the precursor compartment of luminal cells [107, 108]. The role of neuroendocrine cells in the prostatic epithelium is considered to produce soluble factors that paracrine supports the growth of luminal cells. Neuroendocrine cells are
androgen independent [109, 110] (Figure 4B). The stromal compartment contains nerves, fibroblasts, infiltrating lymphocytes and macrophages, endothelial cells and smooth muscle cells. Chronic inflammation might facilitate prostate cancer initiation. Infections by bacteria and viruses, toxins or uric acid can cause prostate inflammation and might lead to DNA-damage or cell death in epithelial cells. Defective DNA-repair results in enhanced risk of mutations or chromosomal alterations [111]. Amplification of Myc [112] and AR [113] has been emphasized to play a role in prostate cancer initiation. Moreover, inflammation leads to up-regulation of cytokines that promote epithelial cell proliferation [114].

In 1940 it has been demonstrated that the prostate is androgen dependent [115]. Presumably, another possibility for the initiation of prostate cancer is the up-regulation of androgens followed by the constitutive activation of the androgen receptor. This hypothesis has been supported by studies showing that eunuchs do not develop prostate cancer and that men who use anabolic steroids have a higher risk of prostate cancer initiation [116, 117].

1.2.1 Function of AR.

As a member of the nuclear hormone receptor superfamily, the androgen receptor (AR) is a hormone dependent transcription factor [118]. In the absence of androgens, AR is primarily localized in the cytoplasm of stromal and secretory luminal cells, bound to heat shock proteins (HSPs) which stabilize the tertiary structure of inactive AR and protect AR from proteolysis [119]. Activation of AR is initiated by androgens. Androgens are important for sexual differentiation, development and maintenance in males. Testosterone is an androgen, which like other steroid hormones is derived from cholesterol. Testosterone is primarily synthesized in the Leydig cells of the testis, and to a lesser extent by the adrenal glands. In serum, testosterone is bound to the sex-hormone-binding globulin (SHBG) and albumin. Only 2 % of total testosterone is unbound in serum [120]. As a hydrophobic steroid hormone free testosterone passes the cell membrane. In the cytoplasm, testosterone gets reduced to 5α-dihydrotestosterone (DHT) by the 5α-reductase [119, 121]. Binding of DHT to AR results in dissociation from HSPs, causing homo-dimerization, subsequent phosphorylation and nuclear
Introduction

Translocation of AR [122-124]. In the nucleus, AR binds to androgen responsive elements (AREs) of target genes resulting in recruitment of co-regulatory proteins such as SRC-1 (steroid receptor coactivator), p160, ARA (androgen receptor activator), CBP (CREB-binding protein), and p300 to form an active transcription complex [125] (Figure 5).

Figure 5: The androgen receptor signaling pathway. The androgen receptor (AR) is bound to heat-shock protein 90 (HSP90) in a confirmation that prevents DNA binding. Free testosterone passes the cell membrane and is converted to dihydrotestosterone (DHT) by the 5α-reductase. DHT binding to AR induces a conformational change in AR which leads to the dissociation of HSP90. Then, AR homo-dimerizes, gets phosphorylated and translocates to the nucleus. Phosphorylated, DNA-bound AR homodimer recruits co-regulators such as steroid receptor coactivator 1 (SRC-1), ARA (Androgen receptor activator), or CBP (CREB-binding protein) (modified from [119]).

Posttranslational modifications have been characterized and include eight serine phosphorylations (Ser16, 81, 213, 256, 308, 424, 515 and 650) and two tyrosine phosphorylations (Tyr267, 543) [124, 126-128]. Among other kinases, AKT (Ser213) and MAP kinases have been suggested to phosphorylate AR [128]. The Src kinase has been implicated to phosphorylate AR at Tyr543 [127]. Serine and tyrosine phosphorylations of the androgen receptor are important for improved gene induction [129, 130].

AR driven target gene expression is involved in diverse biological processes such as proliferation, differentiation and secretion. In prostate cancer the androgen receptor regulates genes involved in differentiation and prostate function (e.g. PSA, kallikrein 2, PAP) [131, 132], cell cycle (Cyclin A, B1, D1, CDK2, 4, p27,
The androgen receptor is predominantly expressed in the male sexual organs. Other tissues including bone, skin, thyroid, hepatic, pancreatic, gastrointestinal, neuronal, muscular, and female reproductive system show weak expression of AR [138]. Studies of conditional AR knock-out in mice demonstrated 80% smaller testes associated with decreased serum testosterone concentrations, arrested spermatogenesis, and decreased bone volume [139, 140].

1.2.2 Targeting the androgen receptor – Hormone deprivation therapy.

Early stage prostate cancer has been emphasized to be a hormone-dependent cancer requiring androgens as well as AR for cell growth and oncogenic progression. So far, hormone deprivation therapy is considered a typical treatment either by surgical or chemical means resulting in tumor regression in most patients [141]. However, most prostate cancers escape hormone ablation therapy after a limited duration of one to three years which is called androgen independent prostate cancer (AIPC) or hormone refractory prostate cancer (HRPC) [142, 143].

Androgen independence defines a clinical stage in which prostate cancer cells have developed different mechanisms which enables them to survive and proliferate in an androgen-poor microenvironment. This development occurs in late stage prostate cancer and in HRPC. Numerous mechanisms that lead to HRPC have been proposed including the hypersensitive, promiscuous, outlaw, bypass, and cancer stem cell pathways [142]. In most cases, AR is suggested to remain functional, either in a ligand-dependent or ligand-independent manner.

**Table 4:** AR regulated genes in prostate cancer. (modified from [131-137]).

<table>
<thead>
<tr>
<th>AR gene regulation</th>
<th>Secretion</th>
<th>Proliferation/Apoptosis</th>
<th>Metastasis</th>
<th>Angiogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>kallikrein 2 (hK2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>prostatic acid phosphatase (PAP)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin A, cyclin B1, cyclin D1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDK2, CDK4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p27, p21, p16↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF-C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p21, p16,) [133, 134], metastasis (MMP-2) [135] and angiogenesis (VEGF-C, FGF2) [136, 137] (Table 4).
In the hypersensitive pathway, prostate cancer cells adapt to low levels of androgens by clonal selection of cells with amplified AR-gene, or by local increases in androgen levels in the tumor tissue due to up-regulation of enzymes involved in the conversion of adrenal precursors to testosterone [113, 144, 145].

Figure 6: Possible mechanisms leading to HRPC. 1. One mechanism in the hypersensitive pathway is the amplification of the AR-gene. 2. In the promiscuous pathway, AR-specificity to its cognate ligand is widened to the stimulation by non-androgenic ligands due to mutations. 3. In the outlaw pathway, AR can be phosphorylated by RTKs resulting in a ligand-independent activation of AR. 4. AR signaling can be bypassed by other signaling pathways e.g. the Map kinase, AKT, and STAT3 signal transduction. 5. Due to clonal selection, epithelial cancer stem cells survive and promote proliferation and anti-apoptosis. Abbreviations: GF – growth factors; CR – cytokine receptor; RTK - receptor tyrosine kinase, (modified from [142]).

The promiscuous pathway involves mutations in AR that effect structure and function, i.e. the T877A mutation in the ligand binding domain of AR. This mutation broadens ligand specificity of AR to other steroid hormones such as progestin and estrogen, as well as anti-androgens like flutamide. Flutamide
action changes from antagonistic to an agonistic resulting in activation of AR [146].

In the outlaw pathway it has been suggested that the androgen receptor can be activated by cytokines (IL-4, IL-6) and growth factors (KGF, EGF, IGF-1, FGF2, HGF, and TGFβ) in the presence of low level androgens [147, 148]. Elevated IL-6 serum concentrations are associated with higher prostate-specific antigen (PSA) levels and tumor metastasis [149]. IL-6 stimulation leads to synergistic activation of AR in the presence of low androgen concentrations [150]. In this context, it has been implicated that STAT3 interacts with AR at the DNA to promote AR-target-gene expression [151]. Other signaling pathways that have been emphasized to play an important role in the ligand-independent activation of the androgen receptor are the MAP kinase and the AKT/PI3-kinase signal transduction [152, 153].

The bypass pathway encompasses signaling pathways that are up-regulated in HRPC and promote oncogenic target gene induction such as the STAT3, MAP kinase and the AKT/PI3-kinase pathways. These alternative signaling pathways are capable of bypassing AR. In this context, IL-6 has been suggested to participate in malignant progression of prostate cancer. Bypass of AR signaling pathway in the absence of androgens and AR may occur due to selection of cancer clones during hormone ablation therapy [154-156].

The prostate cancer stem cell model suggests that the failure of HRPC is a result of the survival of a subpopulation of androgen independent cancer cells. These epithelial stem cells or progenitor cells remain viable upon HRPC and continue to proliferate and differentiate into either androgen-dependent or –independent cells [157, 158] (Figure 6).

1.2.3 Therapeutic intervention in prostate cancer.

Primary or local prostate cancer is usually treated by radical prostatectomy, radiation or bilateral orchiectomy combined with hormone deprivation therapy using either anti-androgens or androgen lowering agents. Hormone deprivation therapy is the standard therapy in metastatic prostate cancer as well. Beside the possibility of existent androgen independent cancer clones many cancer cells seem to remain dependent on the AR signaling pathway in HRPC. Thus,
treatment of HRPC encompasses a second-line hormone deprivation therapy and the inhibition of receptor tyrosine kinases such as EGFR, VEGFR, or the non-receptor tyrosine kinase Src [159]. Chemotherapeutical agents of the androgen receptor signaling pathway include anti-androgens, HSP90 inhibitors, HDAC inhibitors, and androgen lowering agents [160].

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HSP90 inhibitors:</strong></td>
<td>Inhibition of HSP90 to prevent proper conformation of AR and others</td>
</tr>
<tr>
<td>Geldanamycin</td>
<td></td>
</tr>
<tr>
<td>Tanespimycin</td>
<td></td>
</tr>
<tr>
<td>Celastrol</td>
<td></td>
</tr>
<tr>
<td>Gedunin</td>
<td></td>
</tr>
<tr>
<td>AR siRNA</td>
<td>Downregulation using small antisense oligonucleotides</td>
</tr>
<tr>
<td><strong>Anti-androgens:</strong></td>
<td>Inhibition of AR transcriptional engagement</td>
</tr>
<tr>
<td>Flutamide</td>
<td></td>
</tr>
<tr>
<td>Bicalutamide (Casodex)</td>
<td></td>
</tr>
<tr>
<td>Nilutamide</td>
<td></td>
</tr>
<tr>
<td>MDV-3100</td>
<td></td>
</tr>
<tr>
<td><strong>Androgen lowering agents:</strong></td>
<td>Inhibition of androgen synthesis and processing by targeting enzymes such as p450, 5α-reductase, or using Gonadotropin-releasing hormone (GnRH) antagonists</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td></td>
</tr>
<tr>
<td>Abiraterone</td>
<td></td>
</tr>
<tr>
<td>Finasteride</td>
<td></td>
</tr>
<tr>
<td>Dutasteride</td>
<td></td>
</tr>
<tr>
<td><strong>HDAC inhibitors:</strong></td>
<td>Downregulation of approximately 50 % AR target-genes Preventing HSP90 binding to AR resulting in destabilized AR</td>
</tr>
<tr>
<td>Trichostatin A</td>
<td></td>
</tr>
<tr>
<td>Depsipeptide</td>
<td></td>
</tr>
<tr>
<td>Suber-oylanilide hydroxamic acid (SAHA)</td>
<td></td>
</tr>
<tr>
<td>LBH589</td>
<td></td>
</tr>
<tr>
<td><strong>EGFR/ErbB2 inhibitors:</strong></td>
<td>Among others, inhibition of EGFR results in down regulation of the MAP kinase and AKT/PI3 kinase pathways</td>
</tr>
<tr>
<td>Small molecule inhibitors (Gefitinib, Erlotinib, Lapatinib)</td>
<td></td>
</tr>
<tr>
<td>Monoclonal antibodies (Trastzumab (Herceptin), Pertuzamab)</td>
<td></td>
</tr>
<tr>
<td><strong>Src inhibitors:</strong></td>
<td>Down regulation of the MAP kinase and AKT/PI3 kinase and STAT3 pathways</td>
</tr>
<tr>
<td>Dasatinib</td>
<td></td>
</tr>
<tr>
<td><strong>VEGFR inhibitors</strong></td>
<td>Inhibition of neo-vascularization</td>
</tr>
<tr>
<td>Sunitinib</td>
<td></td>
</tr>
<tr>
<td>Bevacizumab</td>
<td></td>
</tr>
</tbody>
</table>

Table 5: Chemotherapeutical agents used in prostate cancer therapy. (modified from [159]).

Anti-androgens compete with testosterone at the ligand binding domain to prevent AR activity [161]. However, mutations in AR result in the conversion of the antagonistic to an agonistic action of anti-androgens [162].

HSP90 is required for accurate folding and stabilization of proteins. Inhibitors of HSP90 compete with ATP for N-terminal binding. In addition to AR, kinases such as ErbB2, and AKT as well as p53 are inhibited by HSP90 targeting [163].
Histone deacetylases (HDACs) remove acetyl groups from acetylated lysines. Deacetylated lysins of histone proteins interact with the negatively charged DNA and therefore down-regulate transcription. Moreover, HDAC inhibitors seem to destabilize AR by deacetylizing HSP90 which interferes with the binding of HSP90 to AR [164]. Androgen lowering agents target androgen synthesis or antagonize soluble factors that stimulate androgen synthesis such as GnRH (Gonadotropin-releasing hormone) [165]. Moreover, antisense oligonucleotides have been developed to downregulate AR protein. However, delivery of these siRNAs still needs to be addressed [166]. Agents that are in pre-clinical investigations or clinical studies are summarized in table 5.

1.3 Cancer Cell Differentiation and Cancer Stem Cells (CSCs) in Prostate Cancer

1.3.1 The cancer stem cell theory.

Tissue regeneration is regulated by a subset of cells called ‘somatic stem cells’. The stem cell population possesses properties of self-renewal and differentiation. Stem cells can either divide into one or two daughter cells with the same stem cell characteristics (self-renewal) or they can generate transit amplifying or progenitor cells that differentiate into specialized, less proliferative cells with defined life-spans (differentiation) [167].

The first connection between stem cells and cancer was proposed in the 19th century. Due to similarities between tumor and embryonic tissue, Rudolf Virchow hypothesized that cancer arises from activation of embryonic stem like cells that are present in mature tissue [168].

It has been suggested that cancer stem cells or cancer stem-like cells can arise from normal stem cells, progenitor cells, and differentiated cells by genetic alterations and influences of the microenvironment. In this context, progenitor and mature cells de-differentiate to regain stem-like properties [169]. Clonal selection by mutations or microenvironmental changes may result in some cancer stem cell clones with growth and survival advantages and therefore drive tumor malignancy (Figure 7).
Moreover, the cancer stem cell model proposes that tumorigenic CSCs may generate tumors through the stem cell processes of self-renewal and differentiation into multiple cell types. Cancer stem cells are described as the tumor initiating population that drives tumor growth [169]. This observation was supported by analysis of neuroblastomas. Highly differentiated neuroblastomas are usually locally confined and therefore generally curable by surgery [170]. In contrast, less differentiated tumors are often more aggressive and have fatal results despite intensive therapeutical intervention [171].

The first subpopulation of cancer cells with tumorigenic potential was isolated by flow cytometry. This technique made it possible to distinguish between phenotypically distinct cells. The CSC subpopulation, which expressed CD34, but lacked CD38, was capable of initiating an acute myeloid leukemia (AML) in immunocompromised NSG NOD mice [172]. Other studies were able to provide evidences about the existence of a cancer stem cell population that is able to initiate tumor growth, including colon, pancreatic, brain, ovarian and breast cancer. These subpopulations were characterized by distinct protein expression profiles called cancer stem cell markers. Some of the stem-like cell indicating markers that have been identified in prostate CSCs are summarized in table 6. The most prominent cell-surface proteins that have been used for the isolation of prostate CSCs are CD44, integrin α2β1 and CD133 (Prominin 1). Among others, Sox2, Musashi-1 (MSI-1), Nanog and the Aldehyde dehydrogenase 1 (ALDH1) are expressed in prostate CSCs [158, 173-176]. Since these stem-like cell indicating markers comprise multi-functional proteins, it is necessary to analyze

Figure 7: Cancer stem cell development. Shown are normal stem cells, progenitor and differentiated cells. Internal factors such as genetic alterations and external factors such as the secretion of soluble factors by the microenvironment (indicated by the bolt of lightning) can result in conversion, or de-differentiation, into cancer stem cells. Cancer stem cells then drive tumor progression, (modified from [169]).
and confirm the up-regulation of several markers to identify cancer cells with stem-like properties.

<table>
<thead>
<tr>
<th>CSC marker</th>
<th>Function</th>
<th>Tumor type</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44</td>
<td>Cell-surface glycoprotein involved in cell-adhesion, cell-cell interaction, migration and homing Receptor for hyaluronic acid</td>
<td>Breast, Head and Neck, Prostate</td>
</tr>
<tr>
<td>Integrin α2β1</td>
<td>Extracellular matrix receptor for collagen and laminin.</td>
<td>Prostate</td>
</tr>
<tr>
<td>CD133 (Prominin-1)</td>
<td>Five-transmembrane glycoprotein involved in plasma membrane topology</td>
<td>Prostate, Breast, Brain, Colon, Pancreas, Lung, Liver</td>
</tr>
<tr>
<td></td>
<td>Expressed in fetal neural stem cells, progenitor cells in fetal liver, endothelial precursor and developing epithelium</td>
<td></td>
</tr>
<tr>
<td>Sox2</td>
<td>Transcription factor essential for stem cell maintenance and self-renewal of embryonic stem cells</td>
<td>Prostate, Breast, Brain</td>
</tr>
<tr>
<td>Musashi-1 (MSI-1)</td>
<td>RNA binding protein that is important for post-transcriptional gene regulation Plays a role in normal stem cell maintenance and differentiation Regulates Notch signaling during asymmetric cell division</td>
<td>Breast, Colon, Prostate, Brain</td>
</tr>
<tr>
<td>Nanog</td>
<td>Transcription factor with roles in self-renewal and maintenance of pluripotency of embryonic stem cells by target gene regulation (upregulation of ALDH1 and CD133) Interactions with other stem cell regulators such as Sox2 and Oct3/4</td>
<td>Breast, Prostate, Brain</td>
</tr>
<tr>
<td>Aldehyde dehydrogenase1 (ALDH1)</td>
<td>Cytoplasmic enzyme involved in oxidation of aldehydes, and conversion of retinol to retinoic acid in normal stem cells</td>
<td>Bladder, Lung, Head and Neck, Breast, Prostate</td>
</tr>
</tbody>
</table>

Table 6: Prostate cancer stem-like cell markers. Listed are identified CSC indicating markers for prostate cancer stem cells, their function and tissues in which they have been noted to be upregulated in CSCs (modified from [158,173-176]).

1.3.2 Implications for prostate cancer therapy.

Tumors consist of phenotypically and functionally heterogeneous cancer cells. In prostate cancer, CSCs comprise only a small subset (0.1 – 1 %) of tumor cells. Since CSCs have been shown to be highly drug- and radiation- resistant, failure to eliminate this cell-population might result in tumor relapse. Drug-resistance might be a result of up-regulated ABC transporters which decrease intracellular drug concentration by pumping substrate drugs out of the cell [177]. The overexpression of anti-apoptotic proteins such as Bcl-2 and survivin as well as the up-regulation of DNA-repair apparatuses have been implicated to play a role in CSC drug resistance [178, 179]. Furthermore, the slow proliferation rate of CSCs in the context of self-renewal (symmetric division)
results in escape from chemotherapeutics that preferentially target high proliferative cells [180]. Conventional therapies transiently reduce tumors by targeting the tumor bulk, but survival of CSCs leads to recurring tumor growth. In order to prevent recurrence it is important to target all heterogenous cell populations. Therapies targeting the CSCs can be designed by inhibiting the maintenance of pluripotency or by inducing differentiation of these cells. Strategies to eliminate CSCs involve targeting ABC-transporters, transcription factors that induce anti-apoptotic proteins, and signaling pathways which regulate self-renewal [181].

One of the signaling pathways that has been suggested to play a role in CSC phenotype development is the IL-6/STAT3 signal transduction. Gene signatures of prostate CSCs revealed activation of JAK/STAT3 signal transduction in this cell population [182]. Moreover, STAT3 has been emphasized to play a role in maintaining pluripotency [183] and self-renewing processes [184] in embryonic stem cells and glioblastoma stem cells [185].

1.4 Scientific goals

Prostate cancer is initially androgen dependent. Androgen deprivation therapy, as the standard therapy for progressive tumor growth, targets the androgen receptor signaling pathway. Despite initial regression of androgen dependent tumors, hormone refractory prostate cancer recurs. HRPC might be a result of cancer stem cells that survive targeted therapy. Most often, HRPC is a lethal form of prostate cancer that progresses and metastasizes.

The transcription factor STAT3 plays an important role in tumorigenesis by enhancing gene-induction of proteins involved in proliferation, angiogenesis, metastasis, anti-apoptosis and immune evasion [27, 186]. In prostate cancer, the IL-6/STAT3 signaling pathway has been suggested to correlate with malignancy and metastatic behavior of tumor cells [155, 187]. Elevated levels of IL-6 have been noted in patients with metastatic disease or HRPC [188-190]. Activated STAT3 has been found in several metastatic cancer cells e.g. DU145 and PC-3 cells [191]. So far, no connection between STAT3 activity and the development of
a CSC phenotype as a potential reason for aggressive tumor growth and HRPC has been established.

In this study, a murine epithelial prostate cancer cell line was used to establish and to verify the suggested role of IL-6/STAT3 signaling pathway in the transition to hormone refractory prostate cancer. It was planned to evaluate the oncogenic potential of STAT3 and the androgen receptor by comparing tumor cells gene silenced for STAT3 and AR by determining proliferative, angiogenic and apoptotic activity. A gene-expression profile gave more insights into the genetic alterations induced by genetic knock-down of STAT3 and AR. The impact of STAT3 on angiogenic and metastatic processes in prostate cancer was evaluated by using cells stably expressing the fusion proteins STAT3α-YFP or the dominant-negative form STAT3β-YFP.

It was planned to characterize the IL-6 receptor fusion protein (IL-6RFP) in order to evaluate its functionality in vitro and in vivo. The impact of IL-6RFP on inhibiting IL-6/STAT3 signaling was investigated by determining tumor growth and connected to proliferation, apoptosis and vascularization.

The role of STAT3 dependent CSC phenotype development was evaluated in primary, metastatic, recurring, and targeted prostate cancer by IL-6RFP. It was planned to determine the correlation of STAT3 or AR and CSC phenotype development in murine and human prostate cancer samples. Moreover, the impact of a combined therapy using IL-6RFP and the anti-androgen bicalutamide on tumor growth and CSC phenotype development was compared to the respective mono-therapies.

The aim of this study was to evaluate the impact of the IL-6/STAT3 and the androgen receptor signaling on the development of a CSC phenotype in the progression and recurrence of prostate cancer upon hormone ablation therapy, and to introduce the IL-6 receptor fusion protein as a potential therapy to target the CSC population in prostate cancer.
2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Chemicals were used in pro analysis quality. Buffers and media were prepared with double destilled water.

2.1.2 Cytokines, inhibitors and enzymes

Recombinant murine and human IL-6 were supplied from R+D systems and its median effective dose was 0.02 – 0.06 ng/ml, Flutamide with an IC50 of 1.3 μM and bicalutamide with an IC50 of 0.16 μM were supplied from Sigma (Saint Louis, USA). The IC50 represents the molar concentration of an antagonist, which produces 50% of the maximal possible inhibitory response for that antagonist. The murine IL-6 receptor fusion protein (IL-6RFP) as an inhibitor of the IL-6/Stat3 signaling pathway was produced and secreted in Hek<sup>IL-6RFP</sup> cells. 80 – 100 % confluent Hek cells stably expressing IL-6RFP were plated with 4 ml medium without FBS and antibiotics for 48 h. Afterwards, conditioned medium was sterile filtered with a filter containing a PVDF membrane with 0.45 μm pores (Millipore, Billerica, USA) and stored at 4°C for 2 – 3 days. The neutralizing anti-IL-6 antibody (MP5-20F3) was obtained from BD bioscience.

If not indicated differently enzymes were used by Roche or New England Biolabs.

2.1.3 Antibodies

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Reactivity</th>
<th>Isotype</th>
<th>Source</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-STAT3</td>
<td>N-Terminus (Aa 50-240) of STAT3</td>
<td>mouse</td>
<td>St. Cruz Biotechnology, St. Cruz, USA (F-2)</td>
<td>WB</td>
</tr>
<tr>
<td>α-STAT3</td>
<td>C-terminus</td>
<td>rabbit</td>
<td>St. Cruz Biotechnology, St. Cruz, USA (C-20)</td>
<td>EMSA</td>
</tr>
<tr>
<td>α-pY-STAT3</td>
<td>p-tyrosine-motive of STAT3</td>
<td>mouse</td>
<td>St. Cruz Biotechnology, St. Cruz, USA (B-7)</td>
<td>WB, IF</td>
</tr>
<tr>
<td>α-pY-STAT3</td>
<td>p-tyrosine-motive of STAT3</td>
<td>rabbit</td>
<td>St. Cruz Biotechnology, St. Cruz, USA (Tyr705)-R</td>
<td>IF</td>
</tr>
<tr>
<td>α-AR</td>
<td>C-terminus of AR</td>
<td>rabbit</td>
<td>St. Cruz Biotechnology, St. Cruz, USA (C-19)</td>
<td>WB</td>
</tr>
<tr>
<td>α-AR</td>
<td>N-terminus of AR</td>
<td>rabbit</td>
<td>St. Cruz Biotechnology, St. Cruz, USA (N-20)</td>
<td>WB</td>
</tr>
</tbody>
</table>
### Materials and Methods

<table>
<thead>
<tr>
<th>Primary Ab</th>
<th>Target</th>
<th>Secondary Ab</th>
<th>Source</th>
<th>Fluo</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-AR</td>
<td>Internal residues of AR</td>
<td>Rabbit</td>
<td>Epitomics, Burlingame, USA (3165-1)</td>
<td>WB, IF</td>
</tr>
<tr>
<td>α-β-Actin</td>
<td>N-terminus of actin β isoform</td>
<td>Mouse</td>
<td>Sigma, St. Louis, USA</td>
<td>WB</td>
</tr>
<tr>
<td>α-GAPDH</td>
<td>C-terminus GAPDH</td>
<td>Rabbit</td>
<td>Cell signaling, Danvers, USA (#2118)</td>
<td>WB</td>
</tr>
<tr>
<td>α-IL-6</td>
<td>C-terminus of IL-6</td>
<td>Goat</td>
<td>St. Cruz Biotechnology, St. Cruz, USA (M-19)</td>
<td>IF</td>
</tr>
<tr>
<td>α-IL-6</td>
<td>C-terminus of IL-6</td>
<td>Mouse</td>
<td>ABCAM, Cambridge, USA (ab9324)</td>
<td>IF</td>
</tr>
<tr>
<td>α-His</td>
<td>6x His epitope</td>
<td>Rabbit</td>
<td>Cell signaling, Danvers, USA (#2365)</td>
<td>WB, IF</td>
</tr>
<tr>
<td>α-His</td>
<td>6x His epitope</td>
<td>Goat</td>
<td>ABCAM (ab9136)</td>
<td>IF</td>
</tr>
<tr>
<td>α-Ki-67</td>
<td>Internal residues of Ki-67 (1200 – 1300)</td>
<td>Rabbit</td>
<td>ABCAM, Cambridge, USA (ab15580)</td>
<td>IF</td>
</tr>
<tr>
<td>α-CD31</td>
<td>Internal residues of CD31</td>
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<td>APC-conjugated integrin β1</td>
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<td>Goat</td>
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<td>Heavy chain of rabbit Ig</td>
<td>Rabbit</td>
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Materials and Methods

### 2.1.4 Plasmids

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<td>Stat3α-YFP</td>
<td>Andreas Herrmann, CITI Duarte, USA</td>
</tr>
<tr>
<td>pcDNA5/FRT/TO</td>
<td>Stat3β-YFP</td>
<td>Andreas Herrmann, CITI Duarte, USA</td>
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<tr>
<td>pSVL</td>
<td>Stat3β</td>
<td>Andreas Herrmann, CITI Duarte, USA</td>
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<tr>
<td>pcDNA3.1</td>
<td>mIL-6&lt;sup&gt;H&lt;sup&gt;†&lt;/sup&gt;-V5-His</td>
<td>Gerhard Müller-Newen, Institut für Biochemie, Uniklinikum Aachen</td>
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<tr>
<td>pcDNA5/FRT/TO</td>
<td>mIL-6&lt;sup&gt;H&lt;sup&gt;†&lt;/sup&gt;-V5-His</td>
<td>Gerhard Müller-Newen, Institut für Biochemie, Uniklinikum Aachen</td>
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<td>pLKO.1-puro</td>
<td>Stat3-shRNA</td>
<td>Sigma, Saint Louis, USA</td>
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<tr>
<td>pLKO.1-puro</td>
<td>AR-shRNA</td>
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<td>pLKO.1-puro</td>
<td>Non-silencing control-shRNA</td>
<td>Sigma, Saint Louis, USA</td>
</tr>
</tbody>
</table>

### 2.1.5 Oligonucleotides

The used oligonucleotides were supplied from IDT (Integrated DNA Technologies, Coralville, USA). GAPDH primers were used of the universal probe library mouse GAPDH assay (Roche Applied Science, USA)

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>IL-6</td>
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<td>5’-caggttagctatggtactccagaa</td>
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<tr>
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<td>VEGF</td>
<td>5’-actggacccgtgcttacttg</td>
<td>5’-tctgtctccctgcttgcttg</td>
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<tr>
<td>EGF</td>
<td>5’-catgcccacaggattg</td>
<td>5’-gggcaggaacaggtcgt</td>
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<td>AR</td>
<td>5’-ccagttccaatgtgcataaa</td>
<td>5’-tccctggtactgtcacaacg</td>
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</table>
2.2 Methods of Molecular Biology

2.2.1 Growth and storage of bacteria

One-shot-TOP-10 E. coli bacteria (Invitrogen, Carlsbad, USA) were either cultured in LB-medium, supplemented with a selection marker such as ampicillin (50 µg/ml) and incubated rocking with 250 rpm or on LB-agar (1,5%) at 37°C. Storage of bacteria followed at –80°C in LB-Medium with 20%Glycerol.

| LB-Medium: | 10 g/l | Tryptone |
|           | 5 g/l  | Yeastextract |
|           | 10 g/l | NaCl |

| LB-Agar: | 15 g/l | Agar (in LB-Medium) |

2.2.2 Isolation of plasmid-DNA

Small scale isolation of plasmid-DNA

To control cloning procedures plasmid-DNA was isolated of 5 ml bacterial over night culture by means of Mini Plasmid Kit (Qiagen, Maryland, USA) according to manufacturer’s recommendations.

Large scale isolation of plasmid-DNA

For transfection of eucaaryotic cells as well as sequencing of plasmid-DNA in a preparative manner, DNA (50-500 µg) was purified of 250 ml bacterial overnight culture by means of the Plasmid Maxi Kit (Qiagen, Maryland, USA) according to manufacturer’s recommendations. Purified DNA was solved in Nuklease free water.

2.2.3 Quantitative analysis of nucleic acids

Quantities of DNA were determined by photometric measurement of the absorption at 260 nm ($A_{260}$). An absorption-value of $A_{260}$=1.0 is equal to an amount of 50 µg double stranded DNA. Purity of prepared nucleic acids was analyzed by measuring absorption at 280 nm ($A_{280}$) additionally. For pure DNA the quotient of $A_{260}/A_{280}$ should correspond to 1.8 – 2.0.
2.2.4 DNA digest with restriction endonucleases

Enzymatic restriction of DNA was performed according to manufacturer’s recommendations.

2.2.5 Fractionation of nucleic acids by agarose gel electrophoresis

To verify clonings and for preparations, digested DNA fragments were separated via gel electrophoresis and compared with a marker to determine their size. Digested DNA was compounded with 5x DNA probe mixture (Bio-Rad, Hercules, USA) and subjected to a 0.7-2% (w/v) Agarose gel. To display DNA at a UV transilluminator (λ=366 nm) ethidium bromide was added to the gel as well as the TAE running buffer.

\[
\text{TAE (1x):} \quad 242 \text{ g Tris base} \\
57.1 \text{ ml glacial acetic acid} \\
100 \text{ ml } 0.5 \text{ M EDTA (pH } 8.0) \\
in 1000 \text{ ml ddH}_2\text{O}
\]

2.2.6 Isolation of DNA-fragments from agarose gels

Gel electrophoretic separated DNA-fragments were cut of the agarose gel, then purified according to manufacturer’s recommendations by means of the Qiaquick gel extraction kit (Qiagen, Maryland, USA) and finally dissolved in 20 µl nuclease-free water.

2.2.7 DNA ligation

The DNA rapid ligase (Roche, Mannheim, Germany) was used to ligate double stranded DNA fragments. According to strength and the supposed size of the DNA band in the agarose gel, 10 - 100 ng of linearized vector was incubated with a 2 - 10 molar excess of the insert for 30 - 60 min at room temperature. Then ligation preparations were transformed into competent bacteria.

2.2.8 Transformation of competent bacteria

For transformation purposes One-shot-TOP-10 E.coli (Invitrogen, Carlsbad, USA) were mixed with either 0.5 - 2 µl plasmid or with 10 µl ligated product, and incubated for 25 min on ice. Afterwards bacteria were heat shocked for 30 s at 42°C. After heat shock 250 µl SOC-Medium was added to bacteria-DNA mixture.
and subsequently rocked with 250 rpm for 1 h at 37°C. Up to 100 µl of the bacterial suspension was plated on agar plates containing ampicillin (50 µg/ml). Agar plates were incubated over night at 37°C and subsequently grown bacterial colonies were analyzed.

2.2.9 Cloning of mIL-6 RFP and STAT3β-YFP

mIL-6 RFP:
The expression vector pcDNA3.1-mIL-6 RFP V5/his as well as pcDNA5/FRT/TO were digested with restriction endonucleases Nhe I and Pme I. Afterwards the DNA fragment mIL-6-RFP was integrated into the pcDNA5/FRT/TO vector.

Stat3β-YFP:
Expression vectors pSVL-STAT3β and pcDNA5/FRT/TO-STAT3α-YFP were digested with endonucleases Xho I and Age I. Then STAT3β was ligated with the pcDNA5/FRT/TO backbone vector.

2.2.10 QRT-PCR and QPCR-array

QRT-PCR:
Total RNA was isolated using the RNeasy 96 kit (Qiagen, Valencia, CA). The qScript One-Step Fast qRT-PCR kit (Quanta, Gaithersburg, MD) was used to reverse transcribe and amplify 25 ng of total RNA per reaction, according to the manufacturer's protocol. ProbeFinder software (Roche Applied Science, Indianapolis, Indiana, USA) was used to design primer sets for IL-6, OSM, VEGF, EGF, STAT3 and AR and to select the respective probes from the Universal Probe Library (Roche Applied Science). All samples were run in triplicates. Amplifications were performed on a Bio-Rad iCycler iQ5 Multiple-Color Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, California, USA). Data were normalized to the GAPDH expression. Expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method.

QPCR arrays:
Total RNA was isolated using the RNeasy 96 Kit (Qiagen, Valencia, CA). 500 ng of each RNA sample were converted to cDNA with the RT² First Strand Kit (Qiagen) and then combined with the RT² SYBR Green qPCR Mastermix (Qiagen) according to the manufacturer’s instructions. Equal volumes (25 µl) of
this mixture were loaded into each well of the mouse JAK-STAT or angiogenesis RT² Profiler PCR array plates (Qiagen). The qPCR was performed on an iQ5 Real-time PCR Detection System (Bio-Rad, Hercules, CA). Relative gene expression levels were calculated using the ΔΔCt method.

2.3 Culture and Transfection of eukaryotic cells

2.3.1 Culture of eucaryotic cells

Murine epithelial prostate cancer cells TRAMP-C1 (TC1), TRAMP-C2 (TC2) (American Type Culture Collection (ATCC), Manassas, USA), TC1- control-, STAT3-, or AR-shRNA cell lines, as well as TC1 Stat3α-YFP and TC1 Stat3β-YFP cell lines were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Gibco, Grand island, USA) medium supplemented with 5 % FBS, 5 % Nu-Serum IV, 0.005 mg/ml bovine insulin, and 10 nM Dehydroisoandrosterone (SIGMA, Saint Louis, USA). EC line, derived from prostate, was kindly provided by S. Huang and J. Fidler (M.D. Anderson Cancer Center, Houston, Texas). This cell line was maintained in RPMI medium (Gibco, Grand island, USA) supplemented with 10 % FBS. Parental Hek293 and Hek293-IL-6RFP cells were cultured in DMEM containing 10 % FBS. Additionally, all media were supplemented with 100U/ml penicillin, 0.1 mg/ml streptomycin (Gibco, Grand island, USA). Cells were incubated at 37°C and 5 % CO₂ in a water-saturated atmosphere. For subculture adherent growing cells were detached from cell culture plates with a trypsin/EDTA solution.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Description</th>
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<tbody>
<tr>
<td>TC1</td>
<td>adherent murine prostate cancer cells</td>
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<tr>
<td>TC2</td>
<td>adherent murine prostate cancer cells</td>
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<tr>
<td>TC1 Stat3α-YFP</td>
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<td>TC1 Stat3β-YFP</td>
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<td>TC1 nt-control&lt;sup&gt;shRNA&lt;/sup&gt;</td>
<td>adherent murine prostate cancer cells</td>
</tr>
<tr>
<td>EC</td>
<td>adherent murine endothelial cell derived from prostate</td>
</tr>
<tr>
<td>Hek293</td>
<td>adherent human embryonic kidney cells</td>
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<tr>
<td>Hek293&lt;sup&gt;IL-6RFP&lt;/sup&gt;</td>
<td>adherent human embryonic kidney cells</td>
</tr>
<tr>
<td>MFib</td>
<td>adherent murine fibrosarcoma cells</td>
</tr>
</tbody>
</table>
Materials and Methods

DMEM Medium supplemented with 4.5 g Glukose/l, L-Glutamin und 110 mg/l Natrium-Pyruvat (Gibco, Paisley, UK)

RPMI 1640 Medium with L-Gutamin (Gibco, Paisley, UK)

OPTIMEM Medium (Gibco, Paisley, UK)

FBS mycoplasma free fetal bovine serum (Sigma, Saint Louis, USA)

HBSS⁻⁻ Medium without Ca²⁺ and Mg²⁺ and Phenolrot (Gibco, Paisley, UK)

HBSS⁺⁺ Medium with Ca²⁺ and Mg²⁺

Trypsin (0.05%)/ EDTA (0.02%) (Gibco, Paisley, UK)

Penicillin/Streptomycin/Amphotericin (Gibco, Paisley, UK)

PBS 200 mM NaCl
2.5 mM KCl
8.0 mM Na₂HPO₄
1.5 mM KH₂PO₄

2.3.2 Storage of eucaryotic cells

Eucaryotic cells were stored in media containing 3.3 % FBS and 10 % DMSO (freezing medium). Cells were detached from cell culture plate using Trypsin/EDTA, affiliated in media and then spun down at 4°C. Sedimented cells were resuspended with cold freezing medium, frozen for a couple of days at – 80°C and subsequently stored in liquid nitrogen at – 196°C.

2.3.3 Transfection and preparation of stable expression cell lines

Transfection with Lipofectamine 2000:

One day before transfection, cells were plated on 6-well plates and cultured to a confluency of 50 - 70 %. For transfection, complexes were prepared as follows:
A. 4 µg DNA was diluted in 250 µl OPTIMEM (Gibco, Grand island, USA) without FBS. B. 250 µl OPTIMEM lacking FBS were mixed with 10 µl Lipofectamine. Upon an incubation of complexes A and B at RT for 5 min, they were mixed, and then incubated for another 20 - 30 min at RT. Afterwards complexes were added
to cells grown in medium without antibiotics. 24 h later, transfection-mixture was aspirated and replaced with new medium.

Generating Flp-In host cell lines and stable expression cells:
To generate Flp-In Host Cell Lines cells of interest were transfected with pFRT/lacZeo plasmid. Maintenance and Zeocin selection of transfected cells were performed according to manufacturer’s recommendations (Invitrogen, Carlsbad, USA). Once, the Flp-In Host cell line was generated, cells were co-transfected with pOG44 vector that expresses the Flp recombinase as well as the pcDNA5/FRT/TO vector containing the insert of interest in a ratio of 9:1. Maintenance and selection of stable expressing cells by Hygromycin was performed according to manufacturer’s recommendations (Invitrogen, Carlsbad, USA).

2.3.4 Development of shRNA cells
293T cells were plated at a density of $4 \times 10^6$ cells per 10-cm dish. Cells were co-transfected by calcium phosphate co-precipitation with 10 mg of pPACK packaging plasmid mix (SBI, Mountain View, USA) and 15 mg of the respective transfer plasmid (pLKO1-mouse Stat3 shRNA (TRCN0000071456, Sigma, St. Louis, USA), pLKO1-AR mouse shRNA (TRCN0000026189), pLKO1-human Stat3 shRNA (TRCN0000020840) or pLKO1-non-silencing shRNA (Sigma)). The culture medium was replaced with fresh medium after 6 h. Supernatants were collected 24 h and 48 h after transfection. To determine the viral titers, $10^5$ HT1080 cells were seeded in a six-well plate and transduced with various dilutions of the vectors in the presence of 4 mg of Polybrene/ml (Sigma). The culture medium was replaced 48 h later with fresh medium containing puromycin (Sigma) at a concentration of 1.5 mg/ml. Puromycin-resistant colonies were counted 10 days after transduction. TC1 cells or DU145 cells were transduced with the viral vectors at an MOI of 0.5. Transduced cells were selected by adding 1.5 mg/ml puromycin to the culture medium.
2.3.5 Preparation of cells for injection in mice

For injection of mice designated cells were amplified \textit{in vitro} and counted by means of trypan-blue staining in a hemacytometer. Between \(0.5 \times 10^6\) and \(2.5 \times 10^6\) cells were diluted in HBSS\(^{-/-}\), and 100 µl cell-suspension was implanted in mice ventral, subcutaneously. TC1, TC2, TC1 cells expressing control-, STAT3-, or AR-shRNA, TC1 cells stably expressing STAT3\(\alpha\)-YFP, or STAT3\(\beta\)-YFP were implanted in immunocompromised mice.

To analyze metastatic potential of tumor cells, \(5 \times 10^4\) cells were injected retro-orbitally into mice. Lungs of mice were excised every other day after injection for 7 days, stored in HBSS\(^{-/-}\) and analyzed by IVMPM \textit{ex vivo} shortly after excision.

2.4 Biochemical Methods

2.4.1 Preparation of cell-lysates

All following preparations were performed at \(4^\circ\text{C}\) with pre-chilled buffers. Cells were washed with PBS once, then scratched from cell-culture dish in 300 µl Lysis-buffer, and transferred to an Eppendorf Tube. Upon an incubation of 30 min cell lysates were vortexed for about 10 s and then centrifuged at 13,000 rpm for 15 min and \(4^\circ\text{C}\). The supernatant (=lysate) was transferred to a new Eppendorf tube. Protein concentration was determined by Bradford. Lysates were stored at \(-20^\circ\text{C}\).

2.4.2 Preparation of tissue-lysates

For preparation of lysates, tumors were excised, transferred to an eppendorf tube, and frozen in liquid nitrogen immediately. Frozen tissue was grinded with mortar and pestle while adding liquid nitrogen continously. When tissue was homogenized, 1 ml pre-chilled RIPA-lysisbuffer was added and mixed carefully. Then, lysate was transferred to an eppendorf tube and incubated for 30 min on ice. Upon vortexing, lysates were centrifuged for 20 min at 13,000 rpm and \(4^\circ\text{C}\). Supernatant was transferred to a new eppendorf tube and stored at \(-20^\circ\text{C}\).

RIPA-Lysis-buffer: 50 mM Tris-HCl, pH=7.4
150 mM NaCl
Materials and Methods

1 mM EDTA
0.5 % Nonidet P-40
1 mM NaF
15 % Glycerol
20 mM b-Glycerophosphat

Protease- and Phosphatase-Inhibitors (Pierce, Rockford, USA) were added fresh:

Protease-Phosphatase-Inhibitor-Cocktail:
- 1 mM AEBSF-HCl
- 0.8 µM Aprotinin
- 5 µM Bestatin
- 15 µM E-64
- 5 mM EDTA
- 20 µM Leupeptin
- 10 µM Pepstatin A
- 1 mM Na-Vanadat

2.4.3 Quantitative protein determination

The Bradford-reagent (Bio-Rad, Hercules, USA) was used to assess quantities of soluble proteins. 5 µl of a protein-solution was dissolved in 200 µl of the Bradford-reagent mixed with 800 µl water and incubated for 5 min at RT upon the sample was vortexed shortly. The absorption was evaluated with a photometer at 595 nm. By means of a calibration curve which was determined for BSA the factor for an exact calculation of protein concentrations could be assessed.

2.4.4 Discontinuous SDS polyacrylamide gel electrophoresis

The discontinuous SDS polyacrylamide gel electrophoresis involves the separation of proteins based on their size. Lysates were mixed with 4x Laemmli-sample buffer containing β-mercaptoethanol to reduce possibly existing disulfide bonds. Additionally, proteins were denatured by heating at 95 °C for 10 min. Therefore the size of negatively charged proteins defines their electrophoretic mobility which is about to be proportional to the molecular weight. The gel electrophoresis was performed in a vertical gel chamber (Invitrogen, Carlsbad, USA) at 190 V for approximately 45 min. The Rainbow-Marker (Amersham, Buckinghamshire, UK) was applied as a size standard.
Materials and Methods

SDS-running buffer:
1.5 % Tris-Base, pH=8.3
7.2 % Glycin
0.5 % SDS

4x Laemmli-sample buffer:
40 % Glycerin
8 % SDS
250 mM Tris-HCl, pH=6.8
0.4 % Bromphenolblau
120 % β-Mercaptoethanol

2.4.5 Western blot and immunodetection

Gel electrophoretic separated proteins were transferred to a nitrocellulose-membrane (Amersham, Buckinghamshire, UK) by use of a tank blot chamber. For that the gel as well as the nitrocellulose membrane were dipped in water first, and afterwards incubated with two whatman paper and two sponges in transfer-buffer for 10 min. Gel and membrane were layered in the blotting cassette, air bubble-free, in between the two sponges and whatman paper, and then placed in the tank blot chamber which contained transfer-buffer and ice. Transfer of proteins from the gel to the membrane was performed at 110 V for 75 min or 30 V over night, and at 4°C.

To suppress unspecific bindings the membrane was blocked with 5 % milkpowder-solution in TBS-T for 1 h upon transfer. Afterwards the membrane was incubated with a solution containing the antibody of interest (1 µg/ml in TBS-T) over night by rocking evenly at 4°C. The other day the membrane was washed once for 15 min and twice for 5 min with TBS-T. Then TBS-T was replaced with a corresponding solution of a HRP-conjugated secondary antibody for 1 h upon which the three-step washing procedure followed once again. The detection is based on Horseradish Peroxidase (HRP) labelled secondary antibodies. Solutions for detection contain a substrate, luminol, which in its oxidated form results in exposition of chemiluminescence. Thus, occuring antibody-protein complexes could be visualized on autoradiography films.

Transfer-buffer: 14.4 g/l Glycerin
3.03 g/l Tris-HCl
Materials and Methods

20 % Methanol

TBS-T: 50 mM Tris-HCl
150 mM NaCl
0.1 % Tween20

For counterstainings the nitrocellulose membrane was covered with stripping buffer (Pierce, Rockford, USA), rocked for 10 min at RT, and then washed with TBS-T twice for 10 min. After a block with 5 % milkpowder solution for 1 h follow up procedures were performed as described above.

2.4.6 Protein crosslinking

To detect ligand-protein interaction, proteins were crosslinked to the ligand by using DSS or DTSSP. Both crosslinkers form covalent amide bonds between primary amino groups of lysine residues or the N-terminus of polypeptides and NHS-esters. The reaction results in the release of N-hydroxysuccinimide. While DSS binds ligand to protein covalently, DTSSP contains a disulfide bond and is therefore cleavable by β-mercaptoethanol or Dithiothreitol (DTT). 90 µl CM of HekIL-6RFP cells was mixed with 10 µl IL-6 representing a final concentration of 20 ng/ml interleukin-6. DSS or DTSSP were added to a final concentration of 5 mM and whole mixture was incubated for 2 h at RT. Then, samples were mixed with 4x Laemmli-sample buffer containing β-mercaptoethanol and subjected to SDS-polyacrylamide gel electrophoresis and subsequent western blot.

2.4.7 Preparation of nuclear extracts

All following procedures were performed at 4°C with pre-chilled buffers. Cells were washed with PBS/Vanadate (0.1 mM) twice, detached from the cell culture dish and then transferred to an eppendorf tube. Upon centrifugation at 1500 rpm for 2 min sedimented cells were resuspended in 400 µl hypotonic Buffer A and incubated for 10 min. Then, cells were mechanically lysed by vortexing for 10 s followed by another centrifugation upon which the sediment was resuspended in 100 µl Buffer C and incubated for 20 min. The nuclear fraction encompassed the supernatant after centrifugation at 14000 rpm for 2 min.

Buffer A: 10 mM Hepes-KOH pH=7.9
Materials and Methods

1.5 mM MgCl₂
10 mM KCl

Buffer C:
20 mM Hepes-KOH pH=7.9
420 mM NaCl
1.5 mM MgCl₂
0.2 mM EDTA
25 % (v/v) Glycerin

Protease-Inhibitors were added freshly:
0.2 mM PMSF
0.5 mM DTT
1 mM Na-Vanadate

2.4.8 Electrophoretic-Mobility-Shift-Assay (EMSA)

EMSA was performed to determine binding of proteins to specific DNA-sequences. For that nuclear extracts were incubated with radioactive labelled DNA-oligonucleotides and electrophoretic separated.

Up to 10 µg of nuclear extract were mixed with 9.5 µl probe mixture and radioactive probe with 10000 cpm and incubated for 10 min at RT. The probe mixture consists of 10 mM HEPES, 10 % Glycerol, 1 mM DTT, 0.1 µg/µl poly (dI:dC), 0.5 µg/µl BSA, and 0.2 pmols radiolabeled probe. For supershift controls STAT-specific antibodies were added additionally. The antibodies bind the examined protein whereby a complex of protein, DNA and antibody emerged. This complex should migrate slower in a native gel than DNA-protein complexes, what enables the identification of STAT-proteins. The separation of complexes was performed in 5.5 % native polyacrylamide gels at 220 V. Upon electrophoresis gels were fixed in 10 % methanol/10 % acetic acid for 30 min, and dried by vacuum at 80°C. Protein-DNA-complexes were visualized by autoradiography.

Gel:
6.75 ml Acrylamide (40/2 %)
4.5 ml 99 % glycerol
3 ml 5X TBE
43.5 ml Water
400 µl 10 % APS
40 µl TEMED
To measure DNA binding of STAT3 a radioactive labeled double stranded m67SIE-DNA-oligonucleotide was used, which is a synthetic DNA sequence containing the sis inducible element (SIE) of the c-fos promoter. The DNA sequence contains a mutation (m67) which allows STAT1 and STAT3 to bind.

m67SIE: \[5'\text{GAT TGA CGG GAA CTG} \] 3'

(STAT binding site is underlined)

### 2.4.9 Gelatin Zymography

Zymography was performed in order to detect secreted matrix metalloproteinases (MMP) 2 and MMP-9. MMP-2 and MMP-9 are gelatinases. Conditioned medium of cells was mixed with an equal amount of Tris-Glycine sample buffer for 10 min at RT. Then, samples were applied to a Ready Gel Zymogram Gel with 10 % gelatin (Bio Rad Hercules, USA). The gel electrophoresis was performed in a vertical gel chamber with 1x Tris-Glycine Running Buffer at 125 V for approximately 80 min. After running, gel was incubated with Zymogramm Renaturing Buffer with gentle agitation for at RT. 30 min later, Renaturing Buffer was replaced with Zymogramm Developing Buffer. Gel was equilibrated with gentle agitation for 24 h at 37°C. After washing thrice with ddH\(_2\)O to remove SDS and buffer salts, which interfere with binding of the dye to the protein, gel was incubated with Simply Blue Safe Stain (Invitrogen, Grand island, USA) for 60 min at RT with gentle shaking. Then, gel was washed with ddH\(_2\)O for 60 min and subsequently photographed at the \(\alpha\)-imager.

### Sample-Buffer (2x):

- 2.5 ml 0.5 M Tris-HCl, pH = 6.8
- 2 ml Glycerol
- 4 ml 10 % SDS
- 0.5 ml 0.1 % Bromphenol Blue
- 1 ml ddH\(_2\)O

### Running-Buffer:

- 2.9 g Tris Base
- 14.4 g Glycerol
- 1 g SDS
Materials and Methods

To 1 L ddH$_2$O

Renaturing-Buffer: 25 % Triton X-100

Developing-Buffer: 50 mM Tris-HCl, pH = 7.5
0.2 M NaCl
5 mM CaCl$_2$
0.2 % Brij 35

2.4.10 Preparation of single cell from tissue

Excised tumors were washed with HBSS$^{+/−}$ (Ca$^{2+}$, Mg$^{2+}$) and treated with a homogenizing-buffer in which tissue was fragmented manually. Afterwards, homogenized tissue was incubated for 30 min at 37 °C and then filtered by a cell strainer (BD bioscience, Durham, USA). Interfering erythrocyte contaminations were removed by Lysis. For this purpose, cell suspension was sedimented at 4°C with 1500 rpm for 5 min, resuspended in ACS, and incubated for 3 min at RT. By adding an excess of HBSS$^{−/−}$ Lysis was finalized. Upon a centrifugation with 1500 rpm for 5 min cell sediment was resuspended in 10 ml HBSS$^{+/−}$.

For analysis of apoptosis by flow cytometry 1 ml of cell-suspension was transferred to FACS-tube and following a further centrifugation with 1500 rpm for 5 min at 4°C cells were resuspended in 500 µl Binding-buffer containing 5 µl AnnexinV-FITC (Biovision, Milpitas, USA. Samples were analyzed within 30 min after staining at the Accuri C6 of Accuri Cytometers (Ann Arbor, USA). Evaluation was performed with C-plus.

For analysis requiring living cells 5 ml pre warmed Histopaque (Sigma, Saint Louis, USA) were pipetted below the cell-suspension, and centrifuged at 600 x g, no acceleration nor deceleration for 20 min. After density-gradient centrifugation opaque phase containing living cells was transferred to a new tube and washed with HBSS$^{−/−}$ thrice. Then cells were subjected to either flow cytometry or RT-PCR.

Homogenizing-buffer:
DNase 100 U/ml
Collagenase 400 U/ml
in HBSS$^{+/−}$
Materials and Methods

<table>
<thead>
<tr>
<th>ACS:</th>
<th>NH₄Cl</th>
<th>9 g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KHCO₃</td>
<td>1 g/l</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>37 mg/l</td>
</tr>
</tbody>
</table>

2.5 Methods of Cell Biology

2.5.1 Migration assay

To analyze migration of cells a Boyden chamber assay was performed. A Boyden chamber consists of a transwell insert, with a porous filter (pore size: 8 µm), that is set inside a culture plate. Migratory cells then move from the apical area of the transwell through the filter towards the lower compartment below, where they can be quantified.

For this purpose, Collagen was diluted with medium to a final concentration of 1.2 mg/ml, and was added to the cell culture insert (Corning, Tewksbury, USA). To harden the collagen, Boyden Chamber was incubated for approximately 30 min at 37°C. Then, 150 µl of 2 x 10⁵ cells were plated on collagen. 48 h later, cells that migrated to the lower compartment were collected, centrifuged for 5 min at 1500 rpm, and counted by trypan-blue staining in a hemacytometer.

2.5.2 Tube formation assay

Angiogenesis is defined as the formation of new blood vessels from existing vasculature. Endothelial cells are the key cell type involved in this process. During angiogenesis, endothelial cell migrate toward angiogenic stimuli such as FGF or VEGF, proliferate, and differentiate into new capillaries. In order to determine the formation of three-dimensional vessels the tube formation assay is used as a model for studying endothelial differentiation into vasculature. For this purpose, ECs were stained with Cell Tracker Orange (CMTMR) (Invitrogen, Grand island, USA). 5 µM CMTMR was pre-warmed in serum-free medium to 37°C. Afterwards, culture medium of ECs was replaced by medium containing CMTMR, incubated for 30 min at 37°C, and washed twice with medium containing FBS and antibiotics. Meanwhile, 1 ml collagen that was diluted with conditioned medium to a final concentration of 1.2 mg/ml was plated on a 6-well plate to achieve a neutral cellular environment and to enhance tube formation.
After collagen hardened at 37°C for approximately 30 min, 1.5 x 10^5 cells diluted in 150 µl were plated on collagen. Then, additional 2 ml collagen was diluted to a final concentration of 1.2 mg/ml and added to the 6-well plate. 24 h later, cell formation was analyzed at the confocal microscope LSM510.

2.5.3 Tumor sphere formation assay

Normal proliferating tissues are maintained by a stem cell population. Recent studies suggest that tumors as well are derived from a similar self-renewing subpopulation of undifferentiated cancer stem cells. The tumor sphere formation assay measures the ability to form stem cell colonies. These non-adherent spheres termed tumor spheres are enriched with cells containing stem cell-like properties. To evaluate the capability of cells forming tumor spheres, cells were resuspended, singularized by using a 40 µm cell strainer, and then counted by trypan blue staining with a hepacytometer. To remove remaining serum, cells were washed thrice with Hank's balanced salt solution (HBSS, Invitrogen, Grand island, USA). Then, 5 x 10^4 cells were plated in 3 ml tumor sphere medium on a 6-well plate. 20 ng/ml FGF-2 and EGF were added freshly. Formed tumor spheres with at least 20 cells per sphere were counted every other day using a transmitted light microscope for six days.

Tumor sphere medium:

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 ml</td>
<td>DMEM F-12 50:50 (1X) w/o Glutamine</td>
</tr>
<tr>
<td>5 ml</td>
<td>200 mM L-Glutamine: (1%)</td>
</tr>
<tr>
<td>13.7 ml</td>
<td>1M Hepes: (2.5%)</td>
</tr>
<tr>
<td>10 ml</td>
<td>B-27 supplement (50X)</td>
</tr>
<tr>
<td>420 ul</td>
<td>Heparin Sodium(1,000 U/ml)</td>
</tr>
<tr>
<td>5 ml</td>
<td>Pen/Strep (10,000 U Pen/ml, 10,000 ug Strep/ml)</td>
</tr>
</tbody>
</table>

2.5.4 Flow cytometry

Flow cytometry is a technology that measures multiple physical characteristics such as light scattering, light excitation, and emission of single particles, usually cells, in the size range of 0.5 µm to 150 µm diameter. The measured characteristics describe relative size, granularity, and fluorescence intensity of particles. For this purpose, particles are transported under pressure in a fluid stream through a beam of light with constant velocity to record how the particle
scatters incident laser light and emits fluorescence. The forward scatter (FSC) is used as a measure for particle’s size, while the side scatter (SSC) describes the granularity of particles. Fluorescence emission is processed by appropriate optical filters. The electronic system converts the detected light signals into electronic signals that can be processed by the computer.

For analysis of cell surface proteins, cells were washed with HBSS once, detached from cell culture dish with 1 ml PBS/EDTA (10 mM) and transferred to a pre-chilled FACS tube. Cells were centrifuged at 1500 rpm (600 x g) for 5 min at 4°C, and sediment was resuspended with 500 μl FACS Buffer twice. Afterwards, cells were blocked with Fcγ antibodies diluted 1:100 in FACS Buffer for 10 min at 4°C. Then, cells were washed with 4 ml FACS Buffer twice and stained with fluorophore conjugated antibodies diluted 1:50 to 1:100 in FACS Buffer for 30 min on ice. Subsequently, cells were washed twice with 4 ml FACS Buffer. Sediment was resuspended in 300 μl FACS Buffer. The measurement was performed at the Accuri C6 (BD Accuri cytometers, Ann Arbor, USA) and analysis was performed with C plus.

### FACS-Puffer:

<table>
<thead>
<tr>
<th></th>
<th>5% FBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0,1% NaN3</td>
<td>in PBS</td>
</tr>
</tbody>
</table>

#### 2.5.5 Enzyme-linked immunosorbent assay (ELISA)

The sandwich ELISA is a method for detecting and quantifying a specific protein in a complex mixture. It involves the attachment of a capture antibody to a solid phase, like polystyrene microplate wells. Afterwards, samples containing known or unknown antigen, and then enzyme-labeled antibodies are added. The detection antibody is either directly labeled with a signal-generating enzyme or fluorophore or it is probed with an enzyme- or fluor-labeled secondary antibody. For enzymatic detection, the appropriate enzyme substrate is added. The observed signal is proportional to the amount of antigen in the sample (Figure 8).

In order to quantify a protein, the 100 μl capture antibody (goat anti mouse IL-6Rα) was coated on a 96-well microplate and incubated over night at RT. The
other day, plate was washed with 400 μl Wash Buffer twice and then blocked by adding 300 μl Blocking Buffer for 1 h at RT.

After another wash 100 μl sample was added per well and incubated for 2 h at RT. Afterwards plate was washed and incubated with 100 μl Detection antibody at RT. 20 min later, plate was washed again and incubated with 50 μl substrate solution for 20 min at RT. The substrate solution consists of a 1:1 mixture of H$_2$O$_2$ and tetramethylbenzidine. After adding Stop solution to each well, the optical density was measured with $\lambda = 450$ nm using a microplate reader. All reagents were supplied from R&D Systems (Minneapolis, USA) and stored according to manufacturer’s recommendations.

2.6 Handling and Treatment of Mice

2.6.1 Mice

The following mouse strains were used:

- B6.129S7-Rag1$^{tm1Mom}$/J mice have a C57BL/6J background and lack B- and T- cells.
- Il2-rg(ko)/NOD-SCID mice are IL2 receptor gamma negative and therefore lack mature B-, T- and natural killer cells (NK).

Treatment of mice with putative tumor growth inhibitors:

- Flutamide was diluted in 10 % EtOH and 90 % neobee oil and injected into mice in a concentration of 25 mg/kg.
- Secreted IL-6$^{RFP}$ was gathered in blank DMEM medium and injected in a concentration of 3.5 μg/kg.
Bicalutamide was diluted in 10 % EtOH and 90 % corn oil and injected into mice with a concentration of 50 mg/kg.

2.6.2 Observation of tumor growth

Tumorgrowth was observed every 2 to 3 days using a vernier caliper (size = length x width x height) starting after a tumor volume of about 50 mm$^3$ and until tumors reached sizes up to 1400 mm$^3$. Growth curves were depicted by averaged values of 6 to 8 mice per group. Tumors of interest were excised and supplied to necroptic analysis.

2.7 Microscopic Methods

2.7.1 Indirect Immunofluorescence

Indirect immunofluorescence in cells:

Cells were plated on cover slips and washed with PBS$^{++}$ after 24 h or 48 h. Fixation of cells was performed with 2 - 4 % paraformaldehyde at RT for 20 min. Afterwards, cells were washed with PBS$^{++}$ and permeabilized with PBS$^{T++}$ for 5 min at RT. To prevent unspecific bindings of antibodies, remaining aldehyde groups of permeabilized cells were saturated with 50 mM NH$_4$Cl (dissolved in PBS$^{T++}$). Then, specimens were blocked with blocking-buffer for 60 min at RT. About 30 µl primary antibody, diluted 1:100 in 0.2 % BSA/PBS$^{T++}$ were pipetted on parafilm in a prepared wet chamber, cover slips were placed with cells upside down on antibody solution, and then incubated over night at 4°C. After washing with 0.2 % BSA/PBS$^{T++}$, cells were incubated with a secondary, fluorophore-conjugated antibody (1:100 in 0.2 %BSA/PBS$^{T++}$) and Hoechst33342 to stain the nuclei for 1 h at RT. Then, specimens were washed thrice, whereby coverslips were dipped in 0.2 % BSA/PBS$^{T++}$ first, then in PBS$^{T++}$, followed by a dip in water. Finally, coverslips were mounted with Mowiol (Calbiochem, La Jolla, USA) on slides. Immunofluorescent stainings were analyzed by confocal microscopy (LSM510Meta, Carl Zeiss, Jena, Germany).

$$\text{PBS}^{++}: \begin{align*} &1 \text{ mM} \quad \text{MgCl}_2 \\ &0.1 \text{ mM} \quad \text{CaCl}_2 \end{align*}$$
Indirect immunofluorescence in OCT frozen sections:
For immunofluorescent staining of tumors, excised tissue was mounted with OCT and frozen at -80°C. Sections of frozen tissue were prepared at the Cryostat (Leica CM 3050S, Germany). After air drying at RT for 30 min, tissue sections of 10 µm were fixed with 2% paraformaldehyde for 15 min, permeabilized in ice cold methanol for 10 min, and washed thrice in PBS for 5 min at RT. Then, sections were incubated with Image Enhancer (Invitrogen, Carlsbad, USA) for 30 min followed by a block in PBS supplemented with 10% goat serum/ 2.5% mouse serum (Sigma, Saint Louis, USA) for 1 h at RT. Incubation with primary antibodies diluted 1:50 – 1:100 in PBS containing 10% goat and 2.5% mouse sera was performed over night at 4 °C. The other day, sections were washed with PBS thrice for 5 min, and incubated for 1 h with fluorophore-conjugated secondary antibodies in PBS and Hoechst33342, followed by another wash with PBS thrice. Afterwards, tissue sections were mounted with Mowiol, covered with a coverslip and analyzed by confocal microscopy.

Indirect Immunofluorescence in paraffin embedded slides:
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. To soften paraffin slides of 3 µm were heated for 30 min at 42°C on a heating plate. Sections were deparaffinized in xylene thrice for 10 min, in 100 % ethanol for 10 min, once in 100 % ethanol for 5 min, twice in 95 % ethanol for 5 min, then in 70 % ethanol for 5 min, and finally in 50 % ethanol for 5 min. Afterwards sections were rinsed in water for 5 min and then boiled in 1x Antigen Unmasking Solution (Vector, Burlingame, USA) at 121°C for 10 min to retrieve antigens. Upon a
PBS-wash for 10 min sections were processed as described above (Indirect immunofluorescence of OCT frozen sections).

Live cell imaging:
For the upregulation of CD44 in tumor spheres, cells were incubated with APC conjugated CD44 antibodies 30 min before imaging. Plates were transferred to the confocal microscope and images were acquired within 10 min to avoid influences on cells by temperature changes.

Instructions and settings:
Hoechst33342 diluted (Invitrogen, Carlsbad, USA) to a final concentration of 100 ng/ml was added to secondary antibody solution and used to stain DNA. It binds preferably at AT-rich regions in the small groove of DNA and it has an emission maximum at 460 nm. The excitation of the fluorophore was performed by means of a 2-photon-laser at 790 nm. With 2-photon-technique fluorescent molecules were excited by effectively simultaneous absorption of two almost infrared photons. Detection of fluorophores was performed employing different filters. In case of Hoechst33342 fluorescence a bandpassfilter with $\lambda = 390 - 465$ nm was applied.
In proof of YFP-fusionproteins an argon laser with $\lambda = 514$ nm was chosen. Detection of YFP emission was performed by a bandpassfilter, $\lambda = 535 - 590$ nm. AlexaFluor488, Cy2, or FiTC conjugated secondary antibodies were excited with $\lambda = 488$, and emissions were detected by a bandpassfilter with $\lambda = 500 - 530$. Secondary antibodies conjugated to AlexaFluor546, AlexaFluor555, as well as CellTracker Orange -CMTMR (Invitrogen, Carlsbad, USA) were excited with $\lambda = 543$ nm, and detected between $\lambda = 565 - 615$ nm.
Mean fluorescence intensities were used for quantifications of fluorescence intensities. For this purpose single fluorescence intensities of fluorophore-conjugated secondary antibodies in regions of interest were recorded in 12 bit mode and arbitrary units were quantified and averaged. Microscopic settings for optimal fluorescence intensities were adapted for each experiment. Among each experiment, microscopic settings were kept consistently to achieve comparable results. Moreover, nuclear fluorescence intensity profiles were generated at the
confocal microscope in which Hoechst staining was used as a marker for the nuclei.

Recording of fluorescence intensities was performed at the confocal laser scanning microscope LSM 510 (Carl Zeiss, Jena, Germany) with objectives such as 10x (Fluar 10x/0.5), 20x (Plan-Apochromat 20x/0.8), 40x (C-Apochromat 40x/1.20 W corr), or a 63x (C Apochromat 63x/1.20 W corr) and a constant slice thickness (1 µm).

2.7.2 Intravital Multi Photon Microscopy (IVMPM)

Background:
In conventional linear (i.e. single-photon) fluorescence imaging, short wavelength light is used to excite fluorophores. After relaxation (NR) excited electrons drop from an energetic higher state (S₁) back to default (S₀). The resulting energy or emission at a slightly longer wavelength due to the Stokes shift is used to form the image. For this purpose, the excitation light is focused into the specimen. The fluorescence from the focal plane passes through the pinhole and arrives at the detector.

Since single photons are used for the excitation of the fluorophore, light is emitted along the laser-beam (Figure 9A). This results in several disadvantages of which one general problem is that the short (high-energy) excitation wavelengths enhance phototoxicity resulting in fluorophore bleaching. To exclude light scattering confocal microscopy utilizes a pinhole. Thus, this technique allows 3D sectioning (x,y,z) into thicker tissue. However, high-energy wavelength causes tissue damage and therefore decreases penetration depth. The application of non-linear multiphoton microscopy provides 3D optical sectioning without phototoxicity below and above the focal plane, because two-photon excitation occurs only at the focal spot. Thus, depth of tissue penetration is improved compared to single-photon microscopy (500 µm vs 80 µm). The principle of intravital multiphoton microscopy (IVMPM) is that an atom or molecule can absorb near-simultaneous energy from two photons, each of which contributes one half of the energy required to induce fluorescence. The electrons of a fluorophore are transferred from default (S₀) to a higher energy state (S₂). The following relaxation results in energy loss (NR), and upon drop of electrons
from $S_1$ to default ($S_0$), light of longer wavelength is emitted (Stokes shift). The resulting fluorescence emission varies with the square of the excitation intensity. Due to this quadratic relationship between excitation and emission, fluorescence is constrained to a focal spot formed by the microscope objective. Typically, each pulse lasts of about 100 fs, with intervening gaps of 10 ns compared to a continuous laser as used in single photon microscopy (Figure 9B).

![Figure 9: Comparison of single- and multi-photon microscopy.](image)

For intravital Multi Photon Microscopy tumor bearing mice were anesthetized with a 2 % isoflurane/oxygen gas, and prepared for surgery. Then, mice were injected with dextran-rhodamine (100 µg/200 µl HBSS−) (Invitrogen, Carlsbad, USA) to visualize the vasculature, AnnexinV-FITC (BioVision), or CD44-FITC (50...
μg/200 μl) (Biolegend, USA) in complex with an equal amount of an iRGD peptide with the sequence: CRGDKGPDC (synthesized by Piotr Swiderski at the City of Hope), and/or Hoechst33342 (250 μg/200 μl HBSS\(^{\text{+}}\)) intravenously. Anesthesized mice were opened ventrally and the subcutan growing tumor was exposed. Afterwards, the region of interest was coated with a coverslip and analyzed by IVMPM. Tumor bearing mice continued to receive 2 % isofluorane/oxygene anesthesia while imaging was performed using the Prairie Technologies Ultima microscope (Middleton, USA). To record rhodamine- and YFP- emission, fluorophores were excited with λ = 860 nm. Emission was recorded by a filter with λ = 500 nm – 550 nm (YFP), and 565 nm - 615 nm (rhodamine). For recording Hoechst33342 emission signals were recorded at λ = 730 nm with emission between 435 nm – 485 nm. Extracellular matrix (ECM) emission was given by second harmonic generation at λ = 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, and objectives such as 40x (Olympus LUMPlanFL/IR 40x/0.80W) or 10x (Olympus U PLanFLN 10x/0.30). Images were collected in a 512x512 pixels, 16-bit resolution. TIFF formatted images were analyzed by Image Pro Plus professional imaging software (Media Cybernetics, Bethesda, MD).
3 Results

3.1 Critical Role of STAT3 in Prostate Tumorigenesis

3.1.1 IL-6-STAT3 signaling pathway is important for prostate cancer progression upon hormone deprivation.

In primary prostate cancer oncogenic cells expressing the androgen receptor (AR) depend on androgens to survive. Therefore, in this androgen-sensitive state treatment with anti-androgens is one of the standard therapies. Among others, flutamide is an anti-androgen that competes with testosterone for the ligand-binding domain of AR, inhibiting its transcriptional activity. As such, flutamide is used for hormone deprivation therapy. However, after one to three years cancer frequently recurs even more aggressively and is considered to be androgen-independent [142].

To investigate the role of STAT3 in the transition from androgen-sensitive to androgen-independent prostate cancer upon hormone deprivation therapy, TRAMP-C1 (TC1) cells were engrafted in immunocompromised Rag1−/− mice. Murine TC1 cells are epithelial prostate cancer cells of luminal origin and represent the primary tumor site. Rag1−/− mice lack B- and T-cells and were used to avoid immune responses that could impact tumor growth.

Once the TC1-tumors were palpable and reached a volume of 200 – 300 mm³, mice were injected intraperitoneally with either 25 mg/kg flutamide or a vehicle control. Relative to the control-vehicle treated group, flutamide administration resulted in significantly delayed tumor growth initially. However, subsequent treatment with flutamide was insufficient to reduce growth kinetics, but rather resulted in tumor growth progression. On day 36, eight days after start of treatment, the vehicle treated mice (V) reached a tumor volume of 885 mm³, while flutamide injected mice (Fl1) obtained mean tumor sizes of 372 mm³.

Subsequent continuing flutamide administration of group Fl2 resulted in tumor sizes of 856 mm³ after day 46 (Figure 10A). On the 36th day, tumor-bearing mice from group V and Fl1 were euthanized to perform necroptic analysis. The
remaining mice treated with flutamide were resected 46 days after tumor cell engraftment (Fl2).

Figure 10: Impact of anti-androgen therapy on tumor growth, AR expression and STAT3 activity. A: 3 groups of Rag1−/− mice (V, Fl1 and Fl2, with n = 8) were injected with 2.5 × 10⁶ TC1 cells subcutaneously (s.c.). Treatment with 25 mg/kg flutamide or vehicle started on day 28 (indicated by the black arrow) and was performed intraperitoneally (i.p.) every other day. Mean tumor volumes are shown in graph (n = 6–7). On days 36 and 46 tumors were harvested 2 hours after last treatment (indicated by red boxes). B: Lysates of excised tumors were analyzed by western blot with antibodies raised against indicated proteins. C: Frozen sections of isolated tumors were prepared and stained with primary antibodies raised against pYSTAT3 or AR. Secondary antibodies conjugated to AF488 were used for immunodetection. pYSTAT3 and AR are depicted in green. Hoechst dye was added for nuclear staining and is depicted in blue. (Scale: 100 µm) D: Shown are fluorescence intensities of tyrosine phosphorylated STAT3 or AR from 3 different tumors. Bar graphs show quantified mean fluorescence intensities (MFI) (n = 3). Error bars represent standard deviation.

The excised tumors were prepared for western blot analysis, indirect immunofluorescence and RT-PCR.

In order to determine patterns in AR and STAT3 protein expression upon flutamide treatment, lysates were subjected to western blot and analyzed with antibodies as indicated.
Since AR binds to its own promoter to induce AR expression [192], anti-androgen treatment resulted in decreased AR protein-level (Fl1 and Fl2) compared to vehicle control. Conversely, flutamide administration led to elevated STAT3 total protein. Moreover, tyrosine phosphorylation of STAT3 was increased in Fl2 relative to vehicle control. No significant change in STAT3 phosphorylation was detectable when comparing V and Fl1. Since phosphorylated STAT3 was elevated in Fl2 but not in Fl1, it seemed that STAT3 activity might be critical for anti-androgen escape. Staining with antibodies against β-actin was performed as a loading control (Figure 10B). To confirm this result, tumor sections were immunostained for pYSTAT3 or AR. Relative to vehicle control, images showed decreased fluorescence intensities in Fl1 and Fl2 sections stained for AR, while fluorescence of pYSTAT3 was elevated upon flutamide treatment, especially in Fl2. Moreover, immunofluorescent staining revealed that tyrosine phosphorylated STAT3 was predominantly localized in the nucleus. AR was co-localized with the nuclear staining as well, suggesting AR activity (Figure 10C). Mean fluorescence intensities (MFIs) of obtained images were calculated to quantify protein expressions of AR and pYSTAT3. The bar graph showed a steady increase of tyrosine-phosphorylated STAT3 during flutamide treatment. In contrast, MFIs revealed decreases of AR expression in samples Fl1 and Fl2 compared to vehicle control (Figure 10D).

**Figure 11: IL-6, Osm, Vegf and Egf mRNA levels in vehicle-control and flutamide treated tumors.** Harvested tumors of mice treated with either vehicle control or Flutamide were prepared for RT-PCR. Extracted mRNAs were reverse transcribed to cDNA, quantified by PCR and normalized to GAPDH. Bar graph shows IL-6, OSM, VEGF and EGF mRNA levels normalized to vehicle control. IL-6 mRNA expression level was performed in triplicates. Error bars represent standard deviation.
In order to detect potential ligands responsible for STAT3 activation upon flutamide administration, mRNA expressions from members of IL-6 type cytokines and the growth factor family were analyzed by RT-PCR. Flutamide-treatment did not affect VEGF or EGF mRNA expression levels considerably. In contrast, OSM expression was augmented 2.24 fold in Fl1, but dropped to 1.31 in Fl2. Relative to vehicle control, mRNA expression of IL-6 was elevated in both samples, Fl1 and Fl2 (Figure 11). Thus, IL-6 might be the activator of STAT3 under anti-androgen treatment.

3.1.2 STAT3 is relevant for the initiation of prostate cancer.

In order to evaluate the oncogenic potential of STAT3 and AR in primary prostate cancer, knock-downs were engineered in TC1 cells by using shRNAs for STAT3, AR as well as a non-silencing control. Gene silencing of STAT3 and AR was validated on protein level by western blot analysis. Relative to non-silencing control, the knock-down of STAT3 did not affect the AR expression level.

Conversely, AR-silenced cells revealed elevated levels of total and tyrosine-phosphorylated STAT3 protein. β-actin was used as a loading control (Figure 12A). Moreover, relative to non-silencing control TC1 cells, AR knock-down led to increased interleukin-6 mRNA expression (3.53 fold) (Figure 12B). This is
consistent with previous studies showing that AR represses the IL-6 promoter [193, 194]. Therefore, up-regulation of phosphorylated STAT3 and STAT3 protein might be a result of increased IL-6, correlating with previous results shown in figure 10 and 11. There, anti-androgen treatment by flutamide revealed diminished AR protein expression, followed by up-regulation of the IL-6/STAT3 signaling pathway. Thus, TC1-AR^shRNA cells might represent prostate cancer cells in hormone deprivation state. In STAT3-silenced TC1 cells, mRNA expression of IL-6 decreased to 0.52-fold induction compared to non-silencing control. This might be a result of an improper induction of the IL-6 promoter by NFκB. NFκB induced IL-6 expression has been suggested to be promoted by STAT3 [195] (Figure 12B).

To further investigate the impact of STAT3 on prostate tumorigenesis in vivo, immunocompromised NSG/NOD mice lacking B-, T- and natural killer (NK) cells were engrafted with TC1 cells expressing shRNAs for STAT3, AR or non-silencing control. Tumor growth was observed for 29 days. Growth kinetics revealed that knock-down of AR did not influence tumor progression compared to control tumors. On the 29th day, AR-silenced tumors reached a mean volume of 649 mm^3 in comparison to a mean volume of 627 mm^3 for control tumors. Conversely, knock-down of STAT3 resulted in growth delay with tumors obtaining a median volume of 161 mm^3 after 29 days (Figure 13A). In order to validate gene silencing of AR and STAT3 in vivo, sections of frozen tumor tissue were stained for tyrosine phosphorylated STAT3 and AR. Confocal images revealed decreased fluorescence intensities of phosphorylated STAT3 and AR protein in their respective shRNA tumors (Figure 13B). Additionally, mean fluorescence intensities of pYSTAT3 or AR from images acquired at the confocal microscope confirmed decreased expression of either pYSTAT3 (1.9 fold) or AR (10.4 fold) in TC1^STAT3^shRNA or TC1^AR^shRNA tumors compared to MFIs in TC1 tumors expressing non-silencing control shRNAs. Moreover, the fluorescence intensity of phosphorylated STAT3 was elevated 1.38-fold in tumors silenced for AR compared to control tumors, confirming in vitro results shown by western blot in figure 12A (Figure 13C). Interestingly, as shown by immunofluorescent staining, expression of CD31 was down-regulated in STAT3 shRNA tumors (Figure 13B). CD31 or PECAM-1 is a member of the cell adhesion family of
proteins and is predominantly expressed by endothelial cells. Therefore, it is used as a marker for vascularization.

![Graph showing tumor volume over time](image)

**Figure 13:** Analysis of STAT3 and AR knock-down in TC1 tumors *in vivo*. **A:** $2 \times 10^6$ TC1 cells virally transfected with shRNAs for STAT3, AR or non-silencing control were injected s.c. into 3 groups of NSG-NOD mice with $n = 7$. Graphs show quantified tumor volumes over time ($n = 6$). **B:** After 29 days excised tumors were prepared for immunofluorescent staining using antibodies raised against pYSTAT3 or AR and secondary antibodies conjugated to Cy2 (green). Additionally, CD31 was immunostained with a specific primary and AF555-conjugated secondary antibody (red). Hoechst33342 was added to stain the nuclei. Scale: 100 µm. **C:** Fluorescence intensities of phosphorylated STAT3 (left) or AR (right) were acquired at the confocal microscope. Bar graphs show quantified mean fluorescence intensities (MFI) ($n = 3$). Error bars represent standard deviation.

To further evaluate evidence of diminished vascularization upon STAT3 knock-down in tumor tissue, intra-vital multiphoton microscopy (IVMPM) was performed on living animals. Tumor-bearing mice were anesthesized and injected with a mixture of Hoechst33342 and dextran-rhodamine. Subsequently, tumors were exposed for imaging purposes. IVMPM was performed at the Prairie microscope using a 2-photon laser.
Figure 14: Analysis of STAT3- or AR- knock-down on angiogenesis, proliferation and apoptosis. 

**A:** Intra vital multiphoton microscopy (IVMPM) on living animals. Tumor bearing mice as indicated were retro-orbitally injected with a mixture of dextran-rhodamine [100 µg/200 µl] and Hoechst33342 [250 µg/200 µl]. Then, tumors were exposed. Imaging was performed using a two-photon laser with $\lambda = 730$ nm for detection of Hoechst, and $\lambda = 860$ nm for Rhodamine. Scale bar represents 200 µm.

**B:** Frozen sections of excised tumors were immunostained with fluorophore-conjugated secondary antibodies recognizing specific primary antibodies raised against Ki-67 and CD31. Hoechst was added to stain the nuclei. Scale: 100 µm.

**C:** Single-cell suspensions of excised tumors were prepared for FACS analysis. Cells of non-silencing control tumors were either stained with AnnexinV-FITC or left unstained as a blank control. Tumor cells silenced for STAT3 or AR were stained with AnnexinV-FITC. Five min later, cell suspensions were analyzed by flow cytometry.

**D:** Tumor sections were immunostained for cleaved caspase 3 and CD31 with specific primary and AF488- or AF555-conjugated secondary antibodies. Hoechst33342 was added 1:500 to stain the nuclei. Scale: 100 µm.
Results

Acquired images revealed a proper and functional vasculature in both non-silencing control and AR-silenced tumors. In contrast, no vascularization was detectable in tumors silenced for STAT3. Here, blood vessels were either leaky or collapsed, because the dextrane-rhodamine appeared to be distributed ubiquitously in the tissue and was not constrained to the vasculature. Nuclear staining with Hoechst was used as a tissue marker (Figure 14A).

Furthermore, tumor sections were stained for the proliferative marker Ki-67. Ki-67 is a nuclear protein and not expressed in non-proliferating, G(0) resting cells. It turned out that Ki-67 was present in both, non-silencing control and AR shRNA tumors, but was diminished in tumors silenced for STAT3. Additional staining for CD31 showed decreased fluorescence intensity in STAT3 knock-down tumors, which is consistent with previous results as shown in figures 14B and 15A (Figure 14B).

To determine apoptosis induced by knock-down of either AR or STAT3 in vivo, single cell suspensions of excised tumors were stained with AnnexinV conjugated to fluorescein isothiocyanate (FITC) and analyzed by flow cytometry (FACS). The FACS analysis revealed slightly increased apoptotic events in tumors silenced for AR as compared to non-silencing control tumor cells. However, knock-down of STAT3 resulted in the highest detectable apoptotic events. The increase of stained non-silencing control tumor cells versus non-stained cells (blank) is considered to be background fluorescence (Figure 14C).

To confirm flow cytometry results, sections of frozen tumor tissue were stained for cleaved caspase 3. Caspase 3 is a central protein of apoptosis. For its activation, caspase 3 is cleaved into activated p17 and p12 fragments. It turned out that cleaved caspase 3 was elevated in tumors silenced for STAT3. It was mainly located in the cytoplasm of tumor cells and appeared in patches in the tumor tissue.

To determine the impact of STAT3 and AR on gene induction, gene expression profiles were analyzed by qPCR arrays that represent the angiogenic and IL-6/STAT3 signaling pathways. TC1 cells silenced for STAT3 or AR were compared to non-silencing control cells. While STAT3-silenced TC1 cells showed decreased mRNA expression of inflammatory genes, knock-down of AR led to elevated levels of α2M (α2-Macroglobuline), CRP (C-reactive protein) and IL-6. Since α2M and CRP are target genes of STAT3, the increase of these
inflammatory genes might be a result of up-regulated STAT3 activity upon AR gene silencing. Moreover, since SOCS3 is induced by STAT3, SOCS3 mRNA was down-regulated in TC1 cells silenced for STAT3 (- 7.32). SOCS3 mRNA level was reduced in AR knock-down cells as well (- 5.35). The decrease in SOCS3 expression favors the IL-6/STAT3 signaling pathway. Moreover, knock-down of STAT3 resulted in decreased expression of metastatic (MMP2) and angiogenic (HIF1α, FGF1, FGF2) factors (Table 7).

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Table 7: QPCR arrays to measure mRNAs of the IL-6/STAT3 signaling pathway and angiogenic pathway. Total RNA of TC1 cells expressing shRNAs for STAT3, AR, or non-silencing control was isolated, converted to cDNA, and then analyzed by qPCR using SYBR Green. Relative gene expression was normalized to GAPDH. Expression levels above + 2 or below – 2 were considered to be regulated.

In summary, STAT3 seems to be important for the initiation and progression of primary prostate cancer. RNAi-induced knock-down of STAT3 resulted in decreased proliferation, angiogenesis, and anti-apoptosis. Conversely, knock-down of AR had no effect on progression of tumor growth, but rather promoted the IL-6/STAT3 signaling pathway.

3.1.3 STAT3 signal-transduction impacts angiogenesis and metastasis in prostate cancer.

To further determine the role of STAT3 in angiogenic and metastatic processes in prostate cancer, TC1 cells were stably reconstituted with STAT3\textalpha-YFP or STAT3\textbeta-YFP. The functionality of STAT3\textalpha fused to YFP has been demonstrated previously [196]. The fusion protein STAT3\textbeta-YFP was cloned by excising STAT3\textalpha from the pcDNA5/FRT/TO-STAT3\textalpha-YFP vector and inserting STAT3\textbeta. STAT3\textbeta is a splicing isoform of STAT3\textalpha, and is considered to act dominant-negative on STAT3 target-gene induction [42].
To evaluate the impact of STAT3α on angiogenesis, endothelial cells (EC) derived from prostate were cultured on collagen to mimic the natural environment. Cells were cultured with conditioned medium (CM) of TC1 cells expressing either STAT3α- or STAT3β-YFP.

Figure 15: Impact of STAT3α and STAT3β on angiogenic processes

A: Endothelial cells derived from prostate (ECs) were labeled with CMTMR, plated on collagen, and covered with conditioned medium (CM) of TC1-STAT3α-YFP or STAT3β-YFP cells. Tube formation was analyzed by confocal microscopy (left) and quantified per field of view (FOV) after 24 and 48 h (right). Error bars represent standard deviation.

B: ECs labeled with CMTMR were co-cultured with TC1 cells stably expressing STAT3α-YFP or STAT3β-YFP on collagen-containing CM of TC1-STAT3α-YFP or STAT3β-YFP cells. Tube formation was analyzed by confocal microscopy.

C: 3 groups of NSGNO mice (n = 8) were injected subcutaneously with 2 x 10⁶ TC1 cells, or TC1 cells stably expressing STAT3α-YFP or STAT3β-YFP. Tumor growth was observed for 33 days. Graph shows quantified tumor volume [mm³] (n = 6) over time. Error bars represent standard deviations.

D: To record vascularization, anesthetized mice were injected with Dextran-rhodamine [100 µg/200 µl] and exposed tumors were analyzed by IVMPM, ECM is shown by second-harmonic generation (2HG) (blue). Scale bar represents 200 µm.

Tube formation of EC cells was imaged and quantified after one and two days. It turned out that factors secreted by TC1-STAT3β-YFP cells decreased tube
formation of ECs considerably compared to cells expressing STAT3α-YFP as shown by confocal images (left) and quantifications (right) (Figure 15A). Moreover, in co-cultures of CellTracker Orange (CMTMR) -labeled ECs and TC1 cells expressing STAT3α or STAT3β revealed that TC1-STAT3α-YFP cells seemed to support the tube formation of ECs. Here, TC1-STAT3α-YFP cells showed branches and formed tubes. Conversely, STAT3β-YFP cells were distributed homogenously and did not seem to promote tube formation (Figure 15B). To determine the impact of STAT3 protein on vascularization in vivo, parental TC1 cells, TC1-STAT3α-YFP or TC1-STAT3β-YFP were injected into NSGNO mice s.c.. Tumor growth kinetic revealed that STAT3α enhances tumor progression compared to control TC1 tumors. In contrast, the presence of over-expressed STAT3β resulted in considerable tumor growth delay (Figure 15C). Furthermore, in vivo imaging of the vasculature stained by dextran-rhodamine showed no detectable vascularization in TC1 STAT3β-YFP tumors as compared to tumors over-expressing STAT3α-YFP. The extracellular matrix (ECM) consists of collagen and fibrinogen and represents stromal stability. The ECM was used as a tissue marker and was visualized by second harmonic generation (2HG). Second harmonic generation describes a non-linear optical phenomenon where photons interact with a non-linear material such as the collagen fibers in the ECM. By using a two-photon laser with $\lambda = 890$ nm the autofluorescence of the collagen fibers is detectable (Figure 15D).

To assess the role of STAT3 protein in metastasis of prostate cancer cells, conditioned medium of TC1 cells and TC1 cells expressing STAT3α-YFP or STAT3β-YFP was analyzed by zymography. Secretory factors MMP2 and MMP9, which are produced and secreted in metastatic tumor cells, were measured. MMP2 and MMP9 are gelatinases and are important for ECM degradation as one of the first steps in metastatic processes [197]. MMP2 and MMP9 are secreted as inactive pro-proteins and get activated through cleavage by extracellular proteinases. Zymography analysis revealed no detectable MMP9 or MMP2 in CM of TC1 control and TC1 cells stably expressing STAT3β-YFP. Conversely, active MMP2 and MMP9 were secreted by TC1 cells expressing STAT3α-YFP.
Figure 16: Role of STAT3 in prostate cancer metastasis

A: TC1, TC1-STAT3α- or TC1-STAT3β-YFP cells were cultured with 4 ml medium for 48 h. 30 µl conditioned medium (CM) was subjected to Zymography using a gelatin-gel. B: Boyden chamber assay was performed with TC1 cells, TC1-STAT3α- and TC1-STAT3β-YFP cells. 250,000 cells were cultured on collagen-covered cell-culture inserts with 8 µm pores (n = 3). After 48 h, migrated cells were quantified. Error bars represent standard deviation. C: TC1-STAT3α-YFP and TC1-STAT3β-YFP cells were stained with APC-conjugated specific antibodies as indicated. The expression of CD44, Galectin-3 and integrin β1 was analyzed using fluorescence-activated cell sorting (FACS). The YFP positive cell population was gated. D: TC1-STAT3α-YFP and TC1-STAT3β-YFP cells were injected into NSG NOD mice intravenously (i.v.). Two mice each were prepared for imaging on day 1, 3, 5 and 7. Lung colony formation was visualized by 2-photon imaging using a laser for YFP (λ = 860 nm). ECM is shown by 2HG with λ = 890 nm and depicted in blue. Scale bar represents 200 µm.
Since only cleaved MMP2 and MMP9 among the matrix metallopeptidases exert collagenase activity, any additional detected band is considered to be artificial (Figure 16A).

In order to assess migration as part of the metastatic processes, parental TC1 cells, TC1 cells expressing STAT3α- or STAT3β-YFP were analyzed in a boyden chamber assay for 48 h. It turned out that considerably more TC1-STAT3α-YFP cells (4704) migrated to the bottom of the well as compared to parental TC1 cells (1926). In contrast, stably expression of STAT3β-YFP repressed cell migration (Figure 16B). In the process of metastasis, tumor cells adhere to distant tissue. For that purpose metastatic cells express adhesion molecules such as Galectin-3, CD44, or integrin β1 to modulate cell-to-cell and cell-to-extracellular matrix interaction. To determine protein regulation of adhesion molecules, TC1 cells expressing STAT3α-YFP or STAT3β-YFP were stained with specific APC-conjugated antibodies for integrin β1, Galectin-3 and CD44. Cell-suspensions were analyzed by flow cytometry (FACS). The results demonstrated that protein expression of integrin β1, Galectin-3 and CD44 was elevated in TC1 cells expressing STAT3α-YFP compared to STAT3β-YFP TC1 cells, suggesting that inhibiting STAT3 activity led to decreased expression of adhesion molecules (Figure 16C). To further evaluate the effect of STAT3 on metastasis in vivo, TC1 cells expressing STAT3α- or STAT3β-YFP were injected intravenously into immunocompromised Rag1-/- mice. In this assay, tumor cells with metastatic activity will infiltrate the lungs. In a time dependent manner, lungs were extracted and subsequently imaged using 2-photon microscopy. Acquired images revealed that TC1 STAT3α-YFP cells infiltrated the lungs already after 24 h. Moreover, overexpression of STAT3α resulted in nodule formation after 3-5 days, and colonies expanded up to day 7. Conversely, TC1 cells expressing STAT3β-YFP did not infiltrate the lungs before day 5. No colony formation was detectable up to day 7 (Figure 16D).

In summary, inhibition of STAT3α action seems to repress angiogenic and metastatic processes in prostate cancer.
3.2 Targeting the IL-6/STAT3 Signaling Pathway using the IL-6 Receptor Fusion Protein (IL-6RFP)

3.2.1 Functional characterization of the IL-6 receptor fusion protein.

Previous experiments have shown that the IL-6/STAT3 signaling pathway might be important for the initiation and progression of prostate cancer. On the one hand, the IL-6/STAT3 signaling pathway was up-regulated upon anti-androgen treatment or knock-down of AR (Figure 10, 11, 12). This is consistent with previous studies demonstrating that IL-6 was elevated in patients with advanced prostate cancer or HRPC [188, 189]. On the other hand, gene silencing of STAT3 reduced tumor growth of prostate cancer cells considerably (Figure 13A).

Therefore, IL-6/STAT3 signaling seemed to be a promising target for prostate cancer therapy. In order to determine a possible therapeutic agent, the efficiency of the IL-6 receptor fusion protein (IL-6RFP) as an inhibitor of the IL-6 signaling pathway was assessed. The IL-6RFP competes with cognate transmembrane receptors for IL-6-binding. IL-6RFP consists of the three extracellular domains of murine IL-6Rα and three N-terminal domains of murine gp130 (IL-6Rβ) fused by an artificial, flexible peptide linker. C-terminally, a histidine tag (His-tag) is fused to the murine IL-6RFP [102, 198] (Figure 17A).

To produce soluble IL-6RFP, HEK cells were stably transfected with the IL-6RFP. Assessing the expression and secretion of IL-6RFP in HEKIL-6RFP cells, CM and lysates of parental HEK and HEKIL-6RFP cells were compared by western blot analysis as indicated in figure 17B. Two protein bands were detectable in lysates of HEKIL-6RFP using antibodies against His and gp130. The detection of two IL-6RFP protein isoforms might be a result of glycosylation processes. Furthermore, secreted IL-6RFP was detectable in CM of HEKIL-6RFP cells as a single band. Here, the protein band of IL-6RFP was slightly shifted compared to cellular receptor fusion protein which is assumingly a result of glycosylation as well. In lysates and CM of non-transfected HEK cells no protein was measurable using His antibody. Actin was used as a loading control (Figure 17B).

To evaluate concentrations of secreted IL-6RFP, HEKIL-6RFP cells were cultured with 4 ml Medium for two days. IL-6RFP concentration of HEKIL-6RFP CM was quantified by ELISA and amounted to 196.54 ng/ml (Figure 17C).
Figure 17: Functional characterization of IL-6<sup>RFP</sup> in vitro. A: Domain structure of murine IL-6<sup>RFP</sup>. B: Lysates and conditioned medium (CM) of HEK cells expressing IL-6<sup>RFP</sup> (HEK<sub>IL-6RFP</sub>) and parental HEK cells were subjected to western blot and analyzed with antibodies as indicated. C: HEK<sub>IL-6RFP</sub> and HEK cells were cultured with 4 ml DMEM medium for 48 h. Bar graph shows concentrations of mIL-6Rα in HEK<sub>IL-6RFP</sub> CM and HEK<sub>C</sub>M quantified by ELISA. Error bars represent standard deviation. D: For 4 h HEK<sub>IL-6RFP</sub> CM [0.071 pmol] was incubated with IL-6 [4.54 pmol] and two crosslinkers, DSS [5 mM] or DTSSP [5 mM], as indicated. Mixtures were subjected to western blot and analyzed with antibodies against His-tag and IL-6.
Moreover, the IL-6 binding capability of IL-6^{RFP} was determined in vitro by adding recombinant IL-6 to IL-6^{RFP}-conditioned medium. As shown in figure 17D, IL-6 was detectable in complex with IL-6^{RFP} by irreversible crosslinking with DSS (lane 3). Two protein bands were detectable using His-tag antibodies representing free and IL-6 bound to the receptor fusion protein. Since the lower protein band runs on the same height as plain IL-6^{RFP} (lane 2), the upper band represents the complex of IL-6 and IL-6^{RFP}. Western blotting with antibodies raised against IL-6 detected protein band at about 130 kDa (lane 3). This band represented IL-6^{RFP} crosslinked to IL-6. The slight shift of the IL-6 band in lane 4 compared to lane 5 is probably a result of reversible crosslinking between IL-6 and IL-6^{RFP} by DTSSP (Figure 17D).

In order to assess the inhibition of IL-6 downstream events by administration of IL-6^{RFP}, TC1 cells were stimulated with either conditioned medium (TCM) of murine fibrosarcoma cells (MFib) that secrete high amounts of IL-6 (600 pg/ml, data not shown) or left unstimulated. Stimulation of TC1 cells with MFib^{TCM} containing 27.27 pM IL-6 led to augmented levels of phosphorylated STAT3. Conversely, stimulated cells that were treated with a 65-fold molar surplus of IL-6^{RFP} CM (1.787 nM) showed reduced STAT3 tyrosine phosphorylation. Expression of STAT3 total protein was augmented in MFib^{TCM}-stimulated cells, but was not affected upon IL-6^{RFP} administration (Figure 17E).

To analyze nuclear accumulation of phosphorylated STAT3, TC1 cells were stimulated with 25 % MFib^{TCM} and treated with ascending concentrations of IL-6^{RFP} CM for 24 h, as indicated. Cells were then fixed and immunostained against tyrosine-phosphorylated STAT3. Recorded images showed dose-dependent decrease of phosphorylated STAT3 colocalized with Hoechst, which was used to stain the nuclei (Figure 17F). Nuclear fluorescence intensity profiles were
acquired at the confocal microscope. Relative fluorescence intensities were averaged and normalized with a fluorescence intensity profile of unstimulated cells. Quantified and normalized nuclear fluorescences are depicted in a graph to support recorded images showing diminished nuclear translocation of activated STAT3 upon IL-6RFP administration (Figure 17G). Finally, DNA-binding ability of STAT3 was analyzed after treatment with IL-6RFP. Therefore, nuclear extracts of TC1 cells stimulated with MFib	extsuperscript{TCM} (25 %) and treated with increasing concentrations of IL-6RFP CM (0 %, 25 %, 50 %) were prepared and subjected to EMSA analysis. Administration of IL-6RFP resulted in a dose dependent decrease of STAT3 DNA-binding. Supershift analysis was performed with a STAT3 specific antibody (Figure 17H).

![Image](image.png)

**Figure 18: Comparison of the IL-6 inhibitory activity of IL-6RFP to a neutralizing antibody.** TC1 cells were treated with HEK-IL-6RFP CM [1.78 nM], or an anti-IL-6 antibody with increasing concentrations (0.17, 1.78, 17.8, 178, and 1780 nM) as indicated, or left untreated for 2 h. 90 min after treatment started, cells were stimulated with IL-6 [10 ng/ml] or left unstimulated for 30 min. **A:** Lysates were analyzed by western blot with antibodies raised against pYSTAT3 and GAPDH. **B:** The intensity of pYSTAT3 bands were quantified with ImageQuant, corrected with background and normalized with the intensities of stimulated, untreated cells in lane 2.

In order to compare the inhibitory activities of IL-6RFP to a neutralizing antibody (MP5-20F3), IL-6 induced tyrosine phosphorylation of STAT3 was determined. TC1 cells were stimulated with IL-6 and treated with 1.78 nM IL-6RFP or varying concentrations of anti-IL-6 antibody. Staining for GAPDH was used as a loading
control. The western blot analysis revealed that 1.78 nM IL-6RFP diminished IL-6 induced activation of STAT3 considerably. Moreover, application of a neutralizing antibody resulted in a dose-dependent decrease of tyrosine phosphorylated STAT3. However, similar concentrations of IL-6RFP and anti-IL-6 antibody showed different inhibitory activities on STAT3 phosphorylation (Figure 18A). The quantification of pYSTAT3 band-intensities revealed a 3.5 fold increased IL-6 inhibitory activity of IL-6RFP relative to the neutralizing antibody using concentrations of 1.78 nM (Figure 18B).

Taken together, the IL-6RFP is properly processed and secreted, exerts the desired biological activity of capturing interleukin-6, and therefore reduces IL-6-induced STAT3 activation. The inhibitory activity of IL-6RFP is 3.5 fold increased compared to a neutralizing antibody.

3.2.2 Determination of the best delivery route of IL-6RFP in vivo.

In order to assess the best delivery route of IL-6RFP in vivo it seemed necessary to first evaluate the biostability of the receptor fusion protein in mouse serum. For this purpose IL-6RFP-conditioned medium was incubated with an equal volume of mouse serum for different times. The mixture was analyzed by western blot with antibodies raised against His-tag.

![Figure 19: Biostability of IL-6RFP in mouse serum. IL-6RFP CM was incubated with the same volume mouse serum in a time-dependent manner as indicated. Mixture was subjected to western blot and analyzed with antibodies raised against His-tag.](image)

Relative to IL-6RFP at 0 min, the receptor fusion protein amount decreased time dependently. After 24 h, IL-6RFP protein was barely detectable. Additional protein bands that were detectable 1 min after mixing the receptor fusion protein with mouse serum seemed to hint at proteolytic degradation of IL-6RFP in mouse serum (Figure 19).
Since the IL-6<sup>RFP</sup> protein seemed to be degraded within 24 h, IL-6<sup>RFP</sup>-conditioned medium (CM) was injected into tumor bearing mice with a concentration of 1.8 µg/kg twice daily (3.6 µg/kg per day).

To determine the best biological efficiency of the IL-6<sup>RFP</sup> in vivo, immunocompromised Rag1−/− mice were injected with TC1 cells s.c.. Upon palpable tumors reached a volume of about 350 mm<sup>3</sup>, 3 cohorts of mice were injected with either IL-6<sup>RFP</sup> CM, CM of parental HEK cells or blank medium. HEK<sup>CM</sup> and medium in the absence of antibiotics and bovine serum were used as vehicle control. The injections were placed either subcutaneously peritumoral, intraperitoneal or intravenously. IL-6<sup>RFP</sup> treatment started 27 days after tumor cell engraftment. Growth kinetics revealed that peritumoral (s.c.) administration of IL-6<sup>RFP</sup> resulted in the most significant tumor growth inhibition. Intravenous treatment of IL-6<sup>RFP</sup> by retro-orbital injections delayed tumor growth kinetics, and

**Figure 20: Delivery routes of IL-6<sup>RFP</sup> in vivo.** 2 x 10<sup>6</sup> TC1 cells were injected into 3 groups of Rag1−/− mice s.c. (n = 8). 27 days after cell engraftment, tumors were treated with IL-6<sup>RFP</sup> CM [1.8 µg/kg], HEK<sup>CM</sup>, or blank medium. Injections were placed either A: i.p., B: s.c., C: or i.v. (indicated by the black arrows). Tumor growth was observed for 37 days. On day 37, tumor tissue was excised and prepared for necroptic analysis. Error bars represent standard deviation.
intraperitoneal administration of the receptor fusion protein had the least effect on tumor growth. On day 37, in all three different delivery routes, mice treated with vehicle (HEK\textsuperscript{CM} or blank medium) reached tumor volumes between 700 – 900 mm\textsuperscript{3}. Intraperitoneal IL-6\textsuperscript{RFP} administration resulted in mean tumor sizes of 572 mm\textsuperscript{3}; i.v. injected mice obtained mean tumor volumes of 417 mm\textsuperscript{3}, and peritumoral injections led to mean tumor sizes of 316 mm\textsuperscript{3} (Figure 20).

After 37 days, tumor-bearing mice from s.c., i.p., and i.v. injected mice were euthanized two hours after last treatment to perform necroptic analysis. Tyrosine phosphorylation of STAT3 and STAT3 total protein was analyzed by western blot. Relative to vehicle-treated mice, administration of IL-6\textsuperscript{RFP} reduced phosphorylated STAT3 and total STAT3 protein considerably when tumors were injected subcutaneously.

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\caption{Inhibition of STAT3 phosphorylation by IL-6\textsuperscript{RFP} in vivo. Tumors of i.p., s.c., or i.v. injected mice either treated with blank medium (M), HEK\textsuperscript{CM} (CM), or IL-6\textsuperscript{RFP} CM (IL-6\textsuperscript{RFP}) were harvested. Tumor tissue of each group was pooled and frozen in liquid nitrogen. Lysates were prepared and subjected to western blot analysis. STAT3, pYSTAT3 and Actin were stained with specific primary antibodies and HRP-conjugated secondary antibodies.}
\end{figure}

Decrease in total STAT3 expression might be a result of diminished STAT3 activation upon IL-6\textsuperscript{RFP} treatment, because phosphorylated STAT3 induces its own promoter. Systemic, intravenous administration of IL-6\textsuperscript{RFP} appeared to result in reduced expression and phosphorylation of STAT3 as well, but not as effective as in s.c. injected mice. Conversely, intraperitoneal administration of IL-6\textsuperscript{RFP} had no considerable impact on STAT3 expression or phosphorylation which seemed consistent with the tumor growth kinetic. β-actin was used as a loading control (Figure 21). Taken together, subcutaneous, peritumoral administration of IL-6\textsuperscript{RFP} twice daily had significant impact on tumor growth of primary prostate cancer cells. Moreover, efficient IL-6\textsuperscript{RFP} treatment correlates with diminished tyrosine phosphorylated STAT3 and total STAT3 expression. Hence, reduced
tumor growth might be due to inhibition of IL-6 signal transduction and decreased STAT3 activity.

3.2.3 The IL-6 receptor fusion protein decreases prostate cancer progression by inhibiting tumorigenic processes.

The IL-6/STAT3 signaling pathway is known to induce a number of oncogenic factors which are involved in proliferation, survival, angiogenesis, and anti-apoptosis. Therefore, the impact of IL-6RFP on tumor promoting processes in primary prostate cancer was determined. Peritumoral injections of IL-6RFP appeared the most promising delivery route to affect tumor growth of TC1 engrafted cells. Therefore, tumor tissue was excised from subcutaneous administered HEKCM and IL-6RFP groups of mice for immunofluorescent stainings.

To evaluate the infiltration of IL-6RFP and occurrence of IL-6 in the tumor, frozen sections were dried, fixed and immunostained with antibodies against His-tag and IL-6. Hoechst was used for nuclear staining and as a tissue marker. As shown by immunofluorescent staining, IL-6 is expressed ubiquitously in the tumor tissue of vehicle- (HEKCM) treated mice. Here, staining for IL-6RFP by His-tag antibodies was negative. In IL-6RFP treated mice, tumor infiltration of administered IL-6RFP as well as IL-6 staining turned out to be confined. The strongest IL-6RFP fluorescence appeared at the edge of the IL-6-positive area and seemed partly co-localized with IL-6 as indicated by the circles. Moreover, areas lacking IL-6, but partly positive for IL-6RFP revealed porous Hoechst staining, possibly indicating instability of the tissue (indicated by the arrows). In contrast to vehicle treated tumors, occurrence of porous Hoechst staining and lack of IL-6 was observed at the edge of the tumor. The edge of the tumor is marked by a dot. Since IL-6RFP administration was performed peritumoral, porous Hoechst and lack of IL-6 at the tumor edge might be a result of the inhibitory activity of the IL-6 receptor fusion protein, infiltrating the tumor from the edge to the center, presumably causing tissue instability (Figure 22A).

To assess activation of STAT3 upon IL-6RFP tumor infiltration, tissue sections were stained for tyrosine phosphorylated STAT3 and His-tag. Similar to IL-6, activated STAT3 is distributed ubiquitously in vehicle-treated tumor sections.
while equal fluorescence intensities of pSTAT3 were locally restricted upon IL-6 RFP treatment as indicated by the arrows.

**Figure 22: Tumor infiltration of IL-6 RFP.** Frozen sections of isolated tumors from subcutaneous IL-6 RFP treated tumor-bearing mice were prepared for immunofluorescent stainings. **A:** Tumor sections were stained with fluorophore-conjugated secondary antibodies recognizing specific primary antibodies raised against IL-6 and His-tag. **B:** Sections of isolated tumors were stained for tyrosine phosphorylated STAT3 and His-tag by fluorophore conjugated secondary antibodies. Edge of tumor tissue is indicated by the dot. **C:** Mean fluorescence intensities (MFI) were obtained from images recorded at the confocal microscope; MFI bars represent average of 3 different tumor sections. **D:** AR was stained in sections by specific primary and AF488 conjugated secondary antibodies. Hoechst was used as nuclear staining (left). Fluorescence intensities of AR were quantified and depicted in the graph (right). Scale: 100µm. Error bars represent standard deviation.
Images of phosphorylated STAT3 and IL-6<sup>RFP</sup> revealed that activated STAT3 is reduced in all areas positive for IL-6<sup>RFP</sup>. This confirms the biological activity of IL-6<sup>RFP</sup> <em>in vivo</em> (Figure 22B). Quantified mean fluorescence intensities of activated STAT3 supported depicted images by revealing a 4.16-fold decrease of tyrosine phosphorylated STAT3 upon IL-6<sup>RFP</sup> injection, relative to vehicle control (Figure 22C).

Furthermore, locally constrained peritumoral treatment with IL-6<sup>RFP</sup> did not affect protein expression of AR as shown by immunofluorescent staining and quantified MFIs (Figure 22D). This indicates that <em>in vivo</em> IL-6<sup>RFP</sup> specifically inhibits signaling events downstream of the IL-6 receptor complex.

To evaluate the impact of IL-6<sup>RFP</sup> on proliferative activity of engrafted TC1 tumors, Ki-67 was stained in frozen tumor sections of isolated tumor tissue. Immunofluorescent staining revealed that Ki-67 was mainly localized in the nucleus. Moreover, the marker for proliferation was decreased upon IL-6<sup>RFP</sup> administration (Figure 23A, left). Additional quantifications of mean fluorescence intensities from three different tumor sections supported representative images showing an inhibitory effect of IL-6<sup>RFP</sup> on proliferation (Figure 23A, right). Furthermore, administration of IL-6<sup>RFP</sup> resulted in reduced vascularization as shown by immunofluorescent staining of CD31, an endothelial marker. Hoechst dye stained the nuclei and was used as a marker for the tissue (Figure 23B, left). Manual calculation of CD31 stained blood vessels revealed a diminished number of blood vessels upon IL-6<sup>RFP</sup> treatment (Figure 23B, right).

Finally, <em>in vivo</em> imaging by 2-photon microscopy was performed to analyze apoptosis in living, animals. For this purpose, two mice of each, the vehicle- and the IL-6<sup>RFP</sup> treated group were anesthesized, injected with AnnexinV-FITC, and then prepared for imaging. AnnexinV staining occurred in tumors injected with IL-6<sup>RFP</sup>. Imaging of the extracellular matrix (ECM) was performed as a tissue marker. The ECM indicates tissue stability whereby disarranged ECM hints to instable tissue. Relative to vehicle control, IL-6<sup>RFP</sup> administration resulted in decreased organization of ECM, indicating tissue instability (Figure 23C). Additionally, staining of tumor sections from vehicle- and IL-6<sup>RFP</sup> treated mice showed elevated levels of cleaved Caspase 3 upon inhibition of the IL-6/STAT3 signaling pathway (Figure 23D).
Hence, immunofluorescent stainings could demonstrate the infiltration of IL-6\textsuperscript{RFP} into the tumor tissue, in which it partially co-localized with IL-6. Locally confined IL-6\textsuperscript{RFP} resulted in diminished tyrosine phosphorylation of STAT3, but did not affect expression levels of AR, indicating biological specificity \textit{in vivo}. Moreover, inhibition of the IL-6/STAT3 signal transduction pathway by IL-6\textsuperscript{RFP} reduced tumorigenic processes such as proliferation, angiogenesis and anti-apoptosis.
3.3 STAT3 promotes Cancer Stem Cell Phenotype Development

3.3.1 Hormone deprivation therapy promotes cancer stem cell phenotype.

Cancer stem cells (CSCs) are a subpopulation of tumor cells. They possess the ability of self-renewal and differentiation and they are characterized by the expression of overlapping markers with normal stem cells from the same tissue. Among others, CSC markers that have been identified for prostate cancer are CD44, Sox2, MSI-1, Nanog and integrin α2β1 [158, 173]. Based on the CSC theory, conventional cancer therapy results in tumor regression initially, but since CSCs seem to be highly drug resistant, tumor relapse frequently occurs.

As most cancer cells, TC1 cells generally express the established CSC marker. Therefore, the focus of the investigations laid on the regulation of CSC marker

Figure 24: Impact of flutamide treatment on CSC marker expression. Tumors of either flutamide or vehicle injected mice were excised and prepared for immune-fluorescence. Sections of isolated tumors were stained with specific primary and AF488 conjugated secondary antibodies. Hoechst was added to stain nuclei. Images were obtained at the confocal microscope (left). Mean fluorescence intensities were quantified and depicted in a graph (n = 3) (right). A: Staining and quantifications for Sox2. B: CD44. C: MSI-1. Scale bars: 100 µm. Error bars represent standard deviation.
expression under different conditions such as anti-androgen treatment, gene silencing of STAT3 or AR, and treatment with IL-6<sup>RFP</sup>. Since hormone deprivation therapy most often results in tumor recurrence, the impact of flutamide on CSC phenotype development was assessed. Therefore, the expression of various biomarkers putatively related to the cancer stem cell phenotype was determined. Tumors of vehicle and flutamide treated groups of mice were excised and prepared for immunofluorescent staining. Images and mean fluorescence intensities of Sox2 staining revealed an increase of the stained CSC marker upon flutamide treatment. Sox2 turned out to be ubiquitously expressed in flutamide injected tumor tissue and its cellular localization was equally detectable in the cytoplasm and the nucleus (Figure 24A). Similar results were achieved by staining of tumor sections with antibodies raised against CD44 and MSI-1. Immunofluorescent stainings and quantifications of mean fluorescence intensities of both cancer stem cell markers, CD44 and MSI-1, were elevated in flutamide administered tumor bearing mice relative to vehicle treated mice (Figure 24B and 24C).

These results demonstrated that hormone deprivation therapy by flutamide treatment resulted in elevated expression of three cancer stem cell markers, Sox2, CD44, and MSI-1. This might indicate that hormone deprivation therapy triggers the development of cancer stem cell phenotype which might be the reason for cancer recurrence.

3.3.2 Knock-down of STAT3 resulted in decreased cancer stem cell phenotype

Since hormone deprivation therapy led to the development of a CSC phenotype, the impact of STAT3 and AR on the up-regulation of CSC markers was determined. For this purpose, TC1 cells expressing shRNAs for STAT3, AR or non-silencing control were plated in conditions enhancing tumor-sphere formation.

It has been shown that undifferentiated multipotent tumor cells could be grown and maintained in suspension using the tumor sphere assay. The tumor sphere assay measures the ability of tumor cells to form stem cell colonies and enriches tumor cells with stem-like properties. Thus, sphere formation enables to isolate, enrich and to enumerate the potential CSC subpopulations.
Figure 25: Effect of STAT3 and AR knock-down on CSC development. A: TC1 cells expressing STAT3 or AR shRNA, or non-silencing were cultured in tumor sphere medium for 6 days. Every other day sphere formation was counted at a brightfield microscope. B: On the 6th day, lysates of tumor spheres were prepared, subjected to western blot and analyzed with antibodies as indicated. C/D: Tissue of TC1 tumors silenced for STAT3, AR or non-silencing control tumors were excised and prepared for immunofluorescence. Sections were stained with specific primary antibodies for Sox2, MSI-1 (C), CD44 or Nanog (D) and fluorophore-conjugated secondary antibodies (AF488). Images were obtained at the confocal laserscanning microscope. Scale bars: 100 µm. (left). Mean fluorescence intensities of Sox2, MSI-1 (C), and CD44 or Nanog (D) were averaged from 3 different sections. Error bars represent standard deviation (right).
As shown, relative to non-silencing control (32), AR knock-down cells formed significantly more tumor-spheres (109) while cells silenced for STAT3 did not show considerable sphere formation capability (7) after six days (Figure 25A). Lysates of tumor-spheres subjected to western blot and analyzed with antibodies as indicated supported the results from the sphere formation assay. This more detailed analysis demonstrated that TC1 cells generally express the established CSC markers such as MSI-1 and Sox2. More importantly, expressions of MSI-1 and Sox2 were elevated in tumor spheres formed by AR-silenced cells relative to non-silencing control or TC1 cells silenced for STAT3. The up-regulation of MSI-1 and Sox2 correlated with elevated tyrosine phosphorylated and total STAT3 expression in TC1 cells upon knock-down of AR (Figure 25B).

To investigate the role of STAT3 and AR in the up-regulation or development of cancer stem cell markers in vivo, mice were injected with TC1 cells silenced for STAT3, AR, or non-silencing control. Palpable tumors were excised and prepared for necroptic analysis. Sox2, MSI-1, CD44, and Nanog were stained in tumor sections, and fluorescence intensities obtained from confocal microscopy were quantified. Representative images and MFIs revealed diminished expression levels of the mentioned cancer stem cell markers in STAT3 silenced tumors compared to TC1 tumors silenced for AR or non-silencing control. Consistent to the tumor sphere formation assay and the subsequent western blot analysis, fluorescence intensities of CSC markers were slightly elevated in AR silenced tumors relative to non-silencing control. However, increased CSC marker expression did not turn out to be as dramatic as in vitro analysis (Figure 25C, D).

Taken together, knock-down of AR by RNAi resulted in enhanced CSC phenotype development. Conversely, gene silencing of STAT3 led to diminished expression of CSC markers such as Sox2, MSI-1, CD44, and Nanog, indicating that STAT3 impacted CSC phenotype development. Thus, increased CSC phenotype upon AR inhibition might be a result of elevated interleukin-6 expression (Figure 11 and 12B) and subsequent enhanced STAT3 activity.
3.3.3 Inhibition of the IL-6/STAT3 signaling pathway by IL-6\textsuperscript{RFP} leads to reduced cancer stem cell phenotype.

Since the IL-6/STAT3 signaling pathway might be critical for the development of cancer stem cell phenotype, the IL-6 receptor fusion protein seemed a promising inhibitor to target CSCs. In order to determine inhibitory effects of IL-6\textsuperscript{RFP} on prostate cancer stem cells, TC1 cells were cultured in tumor sphere-promoting conditions.

Figure 26: Impact of IL-6\textsuperscript{RFP} on CSC development \textit{in vitro}. A: TC1 cells were cultured in tumor sphere medium for 6 days. Cells were treated with either IL-6\textsuperscript{RFP} CM or HEK\textsuperscript{CM} every other day. Formed tumor sphere were counted at a brightfield microscope. B: Lysates of tumor spheres were prepared, subjected to western blot and analyzed with antibodies as indicated. Exposure time was adjusted to avoid blackshield effect. C: On the 6\textsuperscript{th} day of the sphere formation assay cells treated with either HEK\textsuperscript{CM} or IL-6\textsuperscript{RFP} CM were incubated with an APC-conjugated CD44 antibody and prepared for flow cytometry. D: TC1 cells treated as indicated were incubated with a CD44 antibody conjugated to APC. 30 min later cells were analyzed at the confocal microscope. Formed tumor spheres are indicated by a black circle. Scale: 100 \textmu m.
Relative to vehicle control (HEK\textsuperscript{CM}) (754 spheres), application of IL-6\textsuperscript{RFP} resulted in decreased sphere formation (325 spheres) (Figure 26A). Reduced tumor sphere formation upon IL-6\textsuperscript{RFP} application was associated with reduced Sox-2 and diminished levels of pYSTAT3 and STAT3 expression as detected by western blot analysis (Figure 26B). Assessing CD44 expression, TC1 tumor spheres treated with either HEK\textsuperscript{CM} or IL-6\textsuperscript{RFP} were stained with specific antibodies conjugated to APC and analyzed by flow cytometry and live cell imaging. Quantifications by FACS analysis showed a decrease in CD44 expression from 34.2 % to 23.3 % upon IL-6\textsuperscript{RFP} administration (Figure 26C). Furthermore, inhibition of IL-6/STAT3 signaling reduced CD44 expression visualized in living cells by confocal microscopy. Acquired images of vehicle treated cells showed non-adherent tumor spheres as indicated by the circles. These spheres revealed up-regulation of CD44. In contrast, IL-6\textsuperscript{RFP} treatment resulted in no detectable tumor sphere formation accompanied by diminished CD44 expression (Figure 26D).

To evaluate the impact of IL-6/STAT3 signaling inhibition on CSC phenotype development \textit{in vivo}, Rag1\textsuperscript{-/-} mice were injected with TC1 cells and treated with either HEK\textsuperscript{CM} or IL-6\textsuperscript{RFP} twice daily. Once vehicle treated tumors reached a volume of about 800 mm\textsuperscript{3}, tumor tissues were excised and prepared for necroptic analysis. Lysates were subjected to western blot and analyzed with antibodies as indicated. Western blot analysis revealed that vehicle treated TC1 tumors expressed the CSC markers MSI-1 and Sox2. IL-6\textsuperscript{RFP} administration demonstrated inhibitory effects on Sox2, and MSI-1 expression. Moreover, decreases in CSC marker expression correlated with reduced levels of phosphorylated STAT3. In addition, a weak reduction in STAT3 expression was detectable. Actin was used as a loading control (Figure 27A).

To confirm CSC marker expression, frozen sections of isolated tumor tissue were prepared for immunofluorescent staining. Representative images as well as mean fluorescence intensities (MFIs)\textsuperscript{v} showed reduced expression of Sox2 upon IL-6\textsuperscript{RFP} administration. Here, Sox2 expression was distributed ubiquitously, similar to vehicle control (Figure 27B). In contrast, MSI-1 appeared to be expressed in distinct areas of IL-6\textsuperscript{RFP} treated tumors only. Fluorescence intensities of MSI-1-positive areas were not decreased when compared to vehicle control (indicated by the circle). The distribution of IL-6\textsuperscript{RFP} in the tumor...
tissue was not ubiquitously as well, similar to previous results shown in figures 22A and 22B. However, tumor areas infiltrated by IL-6^{RFP} revealed reduced MSI-1 fluorescence as indicated by the arrows (Figure 27C, left). Quantifications of MSI-1 fluorescence intensities showed decreased CSC marker expression upon IL-6^{RFP} treatment (Figure 27C, right).

The 1.45-fold decrease of MSI-1 fluorescence intensity upon IL-6^{RFP} treatment seemed moderate in comparison to the diminished expression of MSI-1 shown
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by western blot analysis in figure 27A. MSI-1 expression was detected as a locally confined event in immunofluorescent stainings, but up-regulation of the CSC marker in few areas of the tumor tissue might not have been sufficient enough to show a signal in the western blot analysis.

Moreover, fluorescence staining of Nanog was similar to that of MSI-1. While Nanog-fluorescence was distributed homogenously in vehicle treated tumors, IL-6\textsuperscript{RFP} administration resulted in decreased fluorescence intensity of Nanog in areas infiltrated by the receptor fusion protein (Figure 28A, left).

**Figure 28:** Protein regulation of CD44 and Nanog upon IL-6\textsuperscript{RFP} administration \textit{in vivo}. \textbf{A:} Tumor sections were incubated with primary antibodies against Nanog and His-tag, and stained with fluoroophore conjugated secondary antibodies. Hoechst was used for nuclear staining (left). Mean fluorescence intensities of Nanog were quantified and depicted in graph (right). Scale: 100 μm \textbf{B:} Sections were stained for CD44 and Hoechst was added to stain nuclei (left). Quantifications of CD44 fluorescence intensities were depicted in the graph (right). Scale bars: 100 μm. Error bars represent standard deviation.

MFIs showed overall decrease (2.71 fold) of Nanog fluorescence intensity in IL-6\textsuperscript{RFP} treated mice relative to vehicle injected tumors (Figure 28A, right). The fluorescence intensity of the CSC marker CD44 was 2.28 fold decreased upon IL-6\textsuperscript{RFP} administration when compared to vehicle control, as shown by immunofluorescence and quantifications of MFIs (Figure 28B). In summary, inhibition of STAT3 activity using IL-6\textsuperscript{RFP} \textit{in vitro} and \textit{in vivo} resulted in diminished CSC phenotype as shown by decreased expression of Sox2, MSI-1, CD44 and Nanog.
3.3.4 Up-regulated STAT3 activity coincides with CSC marker expression in advanced prostate cancer cells.

Since CSCs are believed to drive tumor progression and possess enhanced metastatic potential, AR and STAT3 expression levels were correlated with the expression levels of CSC markers in primary and advanced or metastatic prostate cancer.

While murine TC1 cells represent early stage prostate cancer, TRAMP-C2 (TC2) cells correspond to advanced prostate cancer cells. TC1 and TC2 cells were established from a 32-week-old prostatic adenocarcinoma of a C57BL/6 male TRAMP mouse. The cell lines were generated based on different growth rates and cell morphology [199].

To evaluate the expression of AR and STAT3 in both cell lines, AR mRNA expression was determined by RT-PCR initially. AR mRNA level was decreased in TC2 (0.97 fold) cells compared to TC1 cells (6.22 fold) (Figure 29A).

Comparing lysates of TC1 and TC2 cells by western blot analysis, no expression of AR protein was detected. Importantly, detection of tyrosine-phosphorylated STAT3 revealed elevated STAT3 activation in TC2 cells relative to TC1 cells. Conversely, STAT3 total protein was reduced in TC2 cells. Actin was used as a loading control (Figure 29B). In order to compare cells representing primary and advanced prostate cancer in vivo, TC1 and TC2 cells were injected into
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NSGNOD mice. Growth kinetics revealed significantly increased tumor growth of TC2 cells. While TC1 tumors reached mean tumor volumes of 1332 mm$^3$ after 45 days, TC2 tumors obtained a volume of 1314 mm$^3$ already after 14 days (Figure 30A). When palpable tumors reached volumes of about 1300 mm$^3$, tissue was excised and prepared for immunofluorescent stainings.

![Graph showing tumor growth kinetics](image)

**Figure 30: Comparison of TC1 and TC2 cells in vivo.** A: 1.5 x 10$^6$ TC1 and TC2 cells were injected into 2 groups of 7 NSGNOD mice s.c.. Tumor growth was observed for 45 days. Graph shows mean tumor volumes (n = 6) over time. Once tumors reached a tumor volume of about 1300 mm$^3$, tumor tissue was harvested and prepared for necrotic analysis. B: Sections of isolated tumor tissue were stained for Ki-67 and CD31. Hoechst was added to stain nuclei. C: Tumor sections were stained with specific primary antibodies raised against AR (left) or pYSTAT3 (right) and AF488-conjugated secondary antibodies. Nuclei were stained by Hoechst. Scale bars: 100 µm.

In order to determine proliferation, tumor sections were stained for Ki-67. It turned out that Ki-67 fluorescence was elevated in TC2 tumor sections relative to TC1 tumors, which correlated with the growth kinetic. CD31 staining did not show considerable differences between TC1 and TC2 tumor sections (Figure
Results

Moreover, similar to in vitro experiments (Figure 29B), down-regulation of AR and elevated STAT3 phosphorylation was detectable in tumor sections of TC2 tumors (Figure 30C).

![Figure 31: CSC marker expression in TC1 and TC2 tumors.](image)

To evaluate and compare the expression of cancer stem cell markers in TC1- and TC2-tumors, sections of excised tumors were stained with antibodies raised against Sox2 and MSI-1. Stainings revealed up-regulated CSC marker expression of Sox2 (left) and MSI-1 (right) in TC-2 tumors relative to TC1 tumor sections (Figure 31).

Taken together, advanced prostate cancer cells expressed elevated levels of activated STAT3, but not AR, and proliferated significantly faster than primary prostate cancer cells in vivo. This might correlate with the enhanced expression of CSC markers such as MSI-1 and Sox2 in metastatic TC2 tumors.

3.3.5 Inhibition of STAT3 causes CSC phenotype reduction in human prostate cancer cells.

Similar to TC-2 cells, many advanced human prostate cancer cells are AR-negative but express high levels of tyrosine-phosphorylated STAT3, such as DU145, and PC-3 cells. DU145 cells express an IL-6 autocrine loop and it has been shown that targeting the IL-6/STAT3 signaling pathway was promising for cancer therapy [93].

Assessing the impact of STAT3 inhibition on human prostate cancer cells by IL-6RFP, binding of murine IL-6RFP to human IL-6 was determined by western blot.
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analysis first. For this purpose, conditioned medium of HEK<sup>IL-6RFP</sup> cells was incubated with 20 ng/µl murine or human IL-6 and the non-cleavable crosslinker DSS or the cleavable crosslinker DTSSP.

Western blot analysis revealed that mIL-6<sup>RFP</sup> binds to murine as well as human IL-6, since in samples incubated with DSS a second band representing IL-6 covalently bound to IL-6<sup>RFP</sup> was detectable (Figure 32).

To determine the impact of IL-6<sup>RFP</sup> on CSC-indicating marker expression in human prostate cancer cells, DU145 cells were cultured in tumor sphere condition medium and treated with either HEK<sup>CM</sup> as vehicle control or murine IL-6<sup>RFP</sup>. Indeed, relative to vehicle control (326 spheres), application of IL-6<sup>RFP</sup> resulted in decreased number of tumor spheres (95 spheres) (Figure 33A).

**Figure 32: Binding of IL-6<sup>RFP</sup> to murine or human IL-6.** Conditioned medium of HEK<sup>IL-6RFP</sup> cells were incubated with 20 ng/µl murine or human IL-6 and 5 mM DSS or DTSSP for 4 h at 4°C. Mixture was subjected to western blot and analyzed with antibodies raised against His-tag.

**Figure 33:** Impact of IL-6<sup>RFP</sup> treatment on CSC phenotype development in human DU145 cells. A: Parental HEK cells and HEK<sup>IL-6RFP</sup> cells were covered with 4 ml tumor sphere medium for 48 h. For 6 days, DU145 cells were cultured in 50% tumor sphere medium and 50% tumor sphere CM of either HEK<sup>IL-6RFP</sup> or parental HEK cells (vehicle). Tumor spheres were quantified every other day. B: On day 6, tumor spheres of vehicle or IL-6<sup>RFP</sup> treated cells were imaged at a brightfield microscope. C: Lysates of tumor spheres were subjected to western blot and analyzed with antibodies raised against pYSTAT3, STAT3, MSI-1, Sox2 and GAPDH.

Live cell imaging of formed tumor spheres in vehicle control compared to IL-6<sup>RFP</sup> treated cells confirmed the results obtained by the tumor sphere formation assay. In vehicle treated cells, distinct spheres were detectable consisting of at least 20 cells that were detached from the cell culture dish. Conversely, inhibition
of the IL-6/STAT3 signaling pathway resulted in settled, single cells (Figure 33B). More detailed analysis of formed spheres by western blot analysis revealed that MSI-1, Sox2 as well as pYSTAT3 and total STAT3 expression is decreased upon IL-6<sup>RFP</sup> treatment. GAPDH was used as a loading control (Figure 33C). Thus, inhibiting IL-6/STAT3 signal transduction in metastatic DU145 cells by IL-6<sup>RFP</sup> resulted in diminished CSC phenotype development.

To confirm that suppression of CSC phenotype predominantly was a result of STAT3 inhibition, and not due to inhibition of other pathways stimulated by IL-6, such as the MAP kinase pathway, DU145 cells were stably transfected with a STAT3 shRNA or non-silencing control. DU145 cells silenced for STAT3 or non-silencing control cells were cultured in conditions enhancing tumor sphere formation and analyzed regarding their sphere formation ability for six days. STAT3-silenced cells formed significantly less tumor spheres (68 spheres) than non-silencing control DU145 cells (178 spheres) (Figure 34A). Furthermore, Sox2 and MSI-1 expression were abolished in lysates of these tumor spheres, as analyzed by western blot. Knock-down of STAT3 in DU145 cells was validated by staining for tyrosine phosphorylated STAT3 and STAT3 total protein. Actin was used as a loading control (Figure 34B).

**Figure 34:** Gene silencing of STAT3 reduces expression of CSC markers in tumor spheres. A: DU145 cells expressing a STAT3 shRNA or non-silencing control were culture in tumor sphere medium for 6 days. Sphere formation was quantified every other day. Error bars represent standard deviation. B: After 6 days, lysates of tumor spheres were analyzed by western blot using antibodies against pYSTAT3, STAT3, MSI-1, Sox2 and β-Actin.

In summary, inhibition of STAT3 in DU145 cells either by gene silencing or IL-6<sup>RFP</sup> delivery resulted in decreased development of a CSC phenotype, as
determined by reduced CSC marker expression such as MSI-1 and Sox2. Hence, targeting STAT3 by administration of IL-6RFP seemed to be a promising treatment for the CSC population in metastatic human prostate cancer as well.

3.3.6 CSC phenotype occurs in areas with activated STAT3 in human prostate cancer.

To further assess the correlation between the IL-6/STAT3 signaling pathway and increased CSC phenotype development, human prostate cancer tissues were analyzed. The tumor tissue represents primary prostate cancer of human patients.

![Image](image.png)

**Figure 35: Phosphorylated STAT3 is up-regulated in IL-6 positive areas of human prostate cancer tissue.** Shown are representative images of human prostate cancer tissue, that was immunostained for pYSTAT3 (green) and IL-6 (red). Hoechst was added to stain the nuclei, as a tissue marker. Tissue sections were analyzed by confocal microscopy. Circle indicates stromal cells, arrows indicate glandular cells. Scale bar: 100 µm

In order to evaluate the impact of IL-6 signal transduction in human prostate cancer, malignant tissue was stained for IL-6 and STAT3 phosphorylation. Distribution of phosphorylated STAT3 and IL-6 was constrained to few areas in the tissue. Areas positive for phosphorylated STAT3 revealed elevated levels of IL-6 suggesting interleukin-6 as the upstream ligand responsible for STAT3 activation. Nuclear staining with Hoechst was used as a tissue marker (Figure 35, left). IL-6 appeared to be mainly located in the cytoplasm and interstitially of stromal cells as indicated by the circle. Tyrosine phosphorylated STAT3 was exclusively detectable in the nucleus of stromal cells and glandular cells. Glandular cells are indicated by the arrows (Figure 35, right).

In order to assess the expression of CSC markers in a STAT3-dependent manner, human prostate cancer sections were stained for MSI-1, Sox2, CD44, integrin α2β1, or Nanog. Sections were co-stained for pYSTAT3, and Hoechst
dye was used as a tissue marker. Tyrosine-phosphorylated STAT3 was not detectable ubiquitously, but was confined in areas of the prostatic tissue as shown in the left panels of figure 36. Moreover, in areas with high levels of phosphorylated STAT3, expression of CSC-indicating markers like CD44, integrin α2β1, MSI-1, Sox-2, and Nanog were elevated as well. Areas positive for tyrosine phosphorylated STAT3 and the corresponding CSC marker are indicated by the arrows (Figure 36, left panels).
Interestingly, most cells with up-regulated CSC marker expression revealed increased tyrosine-phosphorylated STAT3 as shown by magnified field of views in the double-positive areas (Figure 36, right panels). MSI-1 and CD44 were mainly localized in the cytoplasm (Figure 36A, 36C). MSI-1 expression coincided with pYSTAT3 up-regulation in stromal (indicated by the circle) and glandular cells (indicated by the arrows) of the malignant tissue (Figure 36A, right image). CD44 was predominantly expressed in stromal cells and to a lesser extent in glandular cells, as indicated by the arrows in figure 36C (right image). While integrin α2β1 was detected at the cell membrane (Figure 36D, right image), the transcription factors Sox2 and Nanog were located in the cytoplasm and the nucleus as indicated by the arrows. Moreover, Sox2 and Nanog did not seem to be expressed in the stroma (Figure 36B, 36E, right images). A quantitative
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approach by calculating cells single-positive for either pYSTAT3 or CSC-indicating marker, or double-positives showed that STAT3 activity is primarily associated with the expression of CSC markers. Tyrosine phosphorylated STAT3 could also be found as an isolated event in human prostate cancer sections, but to a lower extend than double-positives, as shown in the bar graphs (Figure 36, right). Moreover, the number of tumor cells testing positive for the expression of CSC markers alone were significantly decreased. Taken together, human prostate cancer tissue revealed elevated CSC marker expression of Sox2, MSI-1, integrin α2β1, CD44 and Nanog coincided with those cells exhibiting high STAT3 activity.

3.3.7 Elevated CSC marker expression does not coincide with AR positive tumor cells.

In order to determine the role of the androgen receptor on the expression of CSC-indicating markers, CD44 expression was analyzed in frozen sections of TC1 tumors stained for AR or tyrosine phosphorylated STAT3. Tissue areas with a partial up-regulation of pYSTAT3 or AR were picked, and cells single positive for CD44, pYSTAT3, or AR and double positive cells were quantified.

![Figure 37: Comparison of CD44 expression in pYSTAT3 positive and AR positive murine tumor cells. A: Shown is a representative image of a murine TC1 prostate cancer section stained for AR (green) and CD44 (red); Hoechst was used for nuclear staining (left). Double- and single-positive cells were quantified from 3 different tumor sections (right). B: Representative image of TC1 tumor tissue section stained for CD44 (red) and pYSTAT3 (green). For nuclear staining Hoechst was added (left). Quantifications of double and single-positive cells are shown (right). Error bars represent standard deviation. Scale bar: 100 μm.](image-url)
Similar to human prostate cancer slides, CD44 was detected at the cell membrane while the two transcription factors, STAT3 and AR, were located in the nucleus (Figure 37). Magnified field of views of immunofluorescent stainings revealed that CD44 expression was mainly up-regulated in AR negative tumor cells (Figure 37A, left). Quantifications of single- and double-positive cells showed that the minority of tumor cells (9%) were double positive for AR and CD44. The CSC marker and AR expressions were primarily found as solitary events (Figure 37A, right). In contrast, tyrosine phosphorylated STAT3 correlated with CD44 expression in immunofluorescent stainings (Figure 37B, left), which was supported by quantifications of double and single positive cells. The majority of tumor cells were positive for both, CD44 and tyrosine phosphorylated STAT3 (68%) (Figure 37B, right).

To determine a correlation of AR and CSC marker expression in human samples, tumorigenic tissue from patients was stained for AR and two CSC markers, CD44 and Sox2. Similar to murine TC1 tumors, AR was detected in the nucleus suggesting that the hormone receptor is transcriptionally active. CD44 and Sox2 were mainly localized in the cytoplasm. In contrast to the co-staining of CD44 and phosphorylated STAT3, the highest detectable fluorescence intensity of CD44 did not overlap with areas positive for AR (indicated by arrows, Figure 38A, left). Instead, AR and CD44 positive areas seemed to exclude each other, except in few regions as indicated by the circle (Figure 36A, left). As shown in the bar graph of quantified double- and single-positive cells, 17.2 % of CD44 and AR double-positive cells were detected in representative regions of the tissue (Figure 38A, right). Moreover, tumor sections stained for AR and Sox2 revealed a similar distribution. Beside regions with upregulated AR and Sox2, single-positive areas were predominantly detectable for Sox2 as indicated by the arrows (Figure 38B). In a more detailed analysis, magnified field of views from double-positive areas were examined regarding the expression level of AR and Sox2, and compared to tissue sections stained for STAT3 and Sox2. The protein expression level is relative to the fluorescence intensity, which was depicted in the rainbow-colours. In the rainbow-colour mode blue represents low fluorescence intensity and red indicates high fluorescence intensity. Acquired images revealed that Sox2 was highly expressed in cells negative for AR.
suggesting a negative correlation between AR and Sox2 expression (Figure 38C, upper panel). Conversely, cells with elevated levels of tyrosine phosphorylated STAT3 showed high Sox2 fluorescence intensity (Figure 38C, lower panel).

In summary, while tumor cells that tested negative for the androgen receptor revealed elevated levels of CSC marker expression, tyrosine-phosphorylated STAT3 is accompanied with the expression of CSC markers, providing further evidence that STAT3 might be a promising target for CSC phenotype deprivation in prostate cancer.

Figure 38: CSC markers are up-regulated in cells with diminished AR expression. A: Shown is a representative image of a human prostate cancer section stained for AR (green) and CD44 (red). Hoechst was used for nuclear staining (left). Double- and single-positive cells were quantified from 3 different patients (right). Error bars represent standard deviation. B: Tissue sections from patients with primary prostate cancer were immunostained with specific primary antibodies for AR and Sox2 and fluorophore-conjugated secondary antibodies. For nuclear staining Hoechst was added. C: Detailed analysis of Sox2 and AR stained tumor sections. Fluorescence intensities of AR, Sox2 and pYSTAT3 are depicted in the rainbow colours as indicated (left). Original image with stainings for AR or pYSTAT3 (green) and Sox2 (red) are depicted (right). Hoechst was added to stain the nuclei. Scale bar: 100 μm.
3.4 Treatment of TC1 Tumors with a Combined Therapy using Bicalutamide and IL-6\textsuperscript{RFP}.

3.4.1 The impact of combining bicalutamide and IL-6\textsuperscript{RFP} on tumor growth.

So far, treatment of prostate cancer relies on targeting the androgen receptor signaling with considerable primary effect on tumor growth. However, anti-androgen therapy leads to recurring prostate cancer. In chapter 3.1, downregulation of AR upon application of anti-androgens or gene silencing of AR has been shown to result in up-regulation of the IL-6/STAT3 signaling pathway. Since STAT3 is an oncoprotein, up-regulation of the IL-6/STAT3 signaling pathway might be a potential reason for tumor relapse. In order to reduce tumor growth and prevent recurring prostate cancer, a combined therapy targeting AR and IL-6/STAT3 signaling pathway was applied in mice. Instead of flutamide as used in previous experiments (Chapter 3.1), the anti-androgen bicalutamide was administered to evaluate whether the mode of action of anti-androgens on AR and IL-6/STAT3 signaling pathway is universally applicable. Both anti-androgens have similar mechanistical inhibitory activity, but bicalutamide is used in the clinics due to reduced side effects.

Four groups of immunocompromised Rag1\textsuperscript{-/-} mice were engrafted with TC1 cells. Once TC1-tumors were palpable and reached a volume of 100 – 200 mm\textsuperscript{3}, mice were injected with either vehicle control, 50 mg/kg bicalutamide, 1.8 μg/kg IL-6\textsuperscript{RFP}, or a combination of bicalutamide and IL-6\textsuperscript{RFP}. While the anti-androgen bicalutamide was injected intraperitoneally every other day, conditioned medium of HEK\textsuperscript{IL-6RFP} cells was administered subcutaneously, in close proximity to the tumor twice daily. Drug delivery started after 18 days. While IL-6\textsuperscript{RFP} treatment delayed tumor growth compared to vehicle control, administration of bicalutamide had no considerable impact on tumor growth. However, the combination of IL-6\textsuperscript{RFP} and bicalutamide improved tumor growth delay (Figure 39A).

Tumors were resected 34 days after tumor cell engraftment. The excised tumors were prepared for western blot analysis and indirect immunofluorescent. Moreover, one mouse per group was used for IVMPM as shown in figure 40A. In order to determine tumor cell death, lysates of tumor tissue were subjected to
western blot and analyzed with antibodies raised against pro- and anti- apoptotic proteins. PARP (poly (ADP-ribose) polymerase) is involved in DNA repair. Cleavage of PARP is mainly performed by cleaved caspase 3 and results in its inactivation. Cleaved PARP facilitates cellular disassembly and is therefore involved in cell death.

Figure 39: Combining IL-6RFP and bicalutamide improves tumor growth delay. A: 4 groups of Rag1<sup>−/−</sup> mice (n = 6) were injected with 2 x 10<sup>6</sup> TC1 cells subcutaneously (s.c.). Treatment with vehicle control (10% EtOH in corn oil and CM of parental HEK cells), 50 mg/kg bicalutamide, 1.8 μg/kg IL-6RFP or IL-6RFP and bicalutamide (bic) combined started on day 18 (indicated by the black arrow). Injections of bicalutamide were performed intraperitoneally (i.p.) every other day. IL-6RFP was administered s.c. twice daily. Mean tumor volumes are shown in graph (n = 5). 34 days after cell engraftment, tumors were harvested 2 hours after last treatment. B: Tissue lysates were subjected to western blot and analyzed with antibodies against cleaved PARP, cleaved caspase 3, Bcl-2, Bcl-xL and GAPDH. C: Sections of frozen tumor tissue were stained with Ki-67 (green). Secondary antibodies conjugated to AF488 were used for immunodetection and quantifications of MFIs. Hoechst dye was added for nuclear staining and is depicted in blue. Scale: 100 μm (left). Bar graphs show quantified mean fluorescence intensities (MFI) of Ki-67 (n = 3). Error bars represent standard deviation (right).

The pro-apoptotic proteins cleaved caspase 3 and cleaved PARP were down-regulated upon IL-6RFP administration and mice treated with IL-6RFP and bicalutamide combined relative to vehicle control. No significant change in cleaved caspase 3 and cleaved PARP expression was detected in bicalutamide
injected in comparison to vehicle treated mice. In contrast, expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL was decreased upon IL-6<sup>RFP</sup> and the combined IL-6<sup>RFP</sup>/bicalutamide treatment relative to vehicle control. Bcl-2 and Bcl-xL as a member of the Bcl-2 family exert pro-survival activity. They inhibit caspase activity through inhibition of mitochondrial cytochrome c release and by binding of APAF-1 (apoptosis-activating factor 1). Both anti-apoptotic proteins were up-regulated in mice treated with bicalutamide compared to vehicle control. The inverse expression of pro- and anti-apoptotic proteins correlated with the levels of tyrosine phosphorylated STAT3. While phospho-tyrosine STAT3 was increased upon bicalutamide injection, diminished STAT3 activity was detected in tumor bearing mice treated with IL-6<sup>RFP</sup> or a combination of bicalutamide and the IL-6<sup>RFP</sup>. Total STAT3 protein was not changed considerably upon drug delivery. GAPDH was used as a loading control (Figure 39B).

In order to determine proliferative activity in the tumor tissue, frozen sections were stained for Ki-67. Immunofluorescent stainings and quantifications of mean fluorescence intensities revealed that Ki-67 was up-regulated in vehicle and bicalutamide administered tumor sections, but was decreased in sections from mice treated with IL-6<sup>RFP</sup> and IL-6<sup>RFP</sup>/bicalutamide (Figure 39C).

These results demonstrated that, similar to flutamide, application of the anti-androgen bicalutamide resulted in increased tyrosine phosphorylation of STAT3 which correlated with decreased apoptosis. Inhibition of the IL-6/STAT3 signaling pathway using IL-6<sup>RFP</sup> lead to decreased proliferation, but elevated apoptosis. The combination of IL-6<sup>RFP</sup> and bicalutamide revealed similar effects on apoptosis and proliferative activity of tumor cells as IL-6<sup>RFP</sup> administration. Moreover, combining the IL-6 and AR inhibitor resulted in a slight improvement of tumor growth delay compared to the mono-therapy using IL-6<sup>RFP</sup>.

### 3.4.2 CSC marker expression upon combined treatment of IL-6<sup>RFP</sup> and bicalutamide.

In order to evaluate the impact of a combined treatment including bicalutamide and IL-6<sup>RFP</sup> on the expression of CSC markers such as CD44, MSI-1 and Sox2, tumor bearing mice were prepared for IVMPM and indirect immunofluorescent 34 days after tumor cell engraftment (Figure 39A).
Results

Mice were injected *i.v.* with a mixture of CD44-FITC and iRGD peptide 30 min before surgical preparation for IVMPM. The iRGD peptide was added to improve tissue penetration of CD44-FITC. Two-photon microscopy on living animals and quantifications of CD44 positive cells per field of view revealed that few cells in vehicle treated mice were CD44 positive (17.75). Compared to vehicle control, the CSC marker expression of CD44 was reduced in TC1 tumor-bearing mice treated with IL-6<sup>RFP</sup> (8.5) and IL-6<sup>RFP</sup>/bicalutamide (4.25). In contrast, bicalutamide injections resulted in elevated CD44 positive cells (29.75). The ECM was detected as a tissue marker (Figure 40A).

**Figure 40: CSC phenotype is inhibited upon combining IL-6<sup>RFP</sup> and bicalutamide.** A: Tumor-bearing mice treated with vehicle, bicalutamide, IL-6<sup>RFP</sup> or bicalutamide and IL-6<sup>RFP</sup> combined were injected with 50 μg CD44-FITC per mouse. CD44-FITC was in complex with an iRGD peptide for better tissue infiltration. 30 min after injection, mice were prepared and analyzed by intra-vital multi photon microscopy (IVMPM). ECM is shown by 2HG. Scale bar: 200 μm.B: Lysates from tissue were subjected to western blot analysis and analyzed with antibodies against MSI-1, Sox2 and GAPDH.

Lysates of tumor tissue were subjected to western blot and analyzed with the indicated antibodies. Similar to the expression profile of tyrosine phosphorylated STAT3 as shown in figure 39B, the CSC markers MSI-1 and Sox2 were diminished in tumors treated with IL-6<sup>RFP</sup> and a combination of IL-6<sup>RFP</sup> and bicalutamide. In contrast, MSI-1 and Sox2 expression was up-regulated in bicalutamide injected mice compared to vehicle control (Figure 40B).

In summary, the combined treatment of IL-6<sup>RFP</sup> and bicalutamide resulted in decreased expression of the CSC markers CD44, Sox2 and MSI-1, similar to the
mono-therapy using IL-6RFP. Inversely, bicalutamide application leads to up-regulation of a CSC phenotype confirming results as shown in figure 24 using the anti-androgen flutamide.
4 Discussion

4.1 The IL-6/STAT3 Signaling Pathway as a Target for Prostate Cancer Therapy

The prostate is not dependent on androgens alone, but also on cytokines and growth factors, mainly secreted by stromal and basal cells [200]. The cytokine interleukin-6 is involved in cellular processes regulating inflammation, proliferation, anti-apoptosis, angiogenesis, differentiation and immune responses [201]. IL-6 is expressed and secreted by fibroblasts, endothelial and mesothelial cells, keratinocytes, B- and T- cells, macrophages, monocytes, and certain tumor cells such as prostate cancer cells [202]. In immunohistochemical studies interleukin-6 and its receptor was predominantly expressed in basal cells of the benign prostate. In prostate cancer tissue, IL-6 is up-regulated in all cell types, stromal cells and basal as well as luminal cells of the prostatic epithelium, which has been shown by immunostainings of sections from human patients [200]. 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 to earlier stages such as benign prostate hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN), or non-malignant forms [203]. Thus, high IL-6 expression (> 7 pg/ml) has been correlated with poor prognosis in human patients [204]. However, approaches of targeting IL-6 signal transduction in prostate cancer are rare. The monoclonal IL-6 antibody siltuximab (CNTO 328) has been used in vivo and clinical studies [205, 206]. Other approaches involve IL-6 antisense oligonucleotides or IL-6 antagonists like Sant-7, a modified IL-6 which binds to the IL-6Rα, but does not induce recruitment of gp130 [207].

The goal of this study was to evaluate the IL-6/STAT3 signaling pathway in prostate cancer as a potential target for therapeutic agents such as the IL-6 receptor fusion protein (IL-6\textsuperscript{RFP}). In this context, one focus laid on influences of the androgen receptor and the IL-6/STAT3 signal transduction on proliferative, apoptotic, angiogenic, and metastatic processes. Furthermore, since tumor relapse upon AR down-regulation by hormone deprivation therapy might be a
result of cancer stem cell (CSC) survival, the impact of IL-6 induced STAT3 activation on CSC development was determined in prostate carcinogenesis. Finally, in order to prevent tumor relapse the effect of a combined therapy using anti-androgens and the IL-6\textsuperscript{RFP} was evaluated in regard to tumor growth and CSC phenotype development.

4.1.1 IL-6/STAT3 Signaling in Prostate Cancer.

In order to evaluate the impact of IL-6/STAT3 signaling on tumor relapse, the connection between elevated levels of IL-6 and HRPC had to be confirmed by establishing a murine tumor model. For this purpose murine TRAMP-C1 cells (TC1) of luminal origin were used. Similar to the course of hormone deprivation therapy in patients, flutamide treatment of TC1 tumor bearing mice resulted in initial tumor growth delay, but tumors progressed and grew out. Analyzed tumors revealed down-regulation of AR and elevated levels of IL-6 as well as total and phosphorylated STAT3 upon flutamide administration. Thus, the suggested up-regulation of the IL-6/STAT3 signaling pathway in the transition from androgen-dependent to HRPC was confirmed and established with the TC1 tumor model. Hence, increased IL-6 induced STAT3 tyrosine phosphorylation seemed to be important for tumor progression upon anti-androgen treatment. Besides its role in tumor relapse, STAT3 was crucial for the initiation of prostate cancer. Tumors silenced for STAT3 showed considerable tumor growth delay. Reduced tumor growth correlated with decreased proliferation, angiogenesis and increased apoptosis. Interestingly, gene silencing of AR did not affect tumor growth compared to non-silencing control. This was accompanied by elevated proliferative activity, a proper vascularization, and reduced apoptotic events. Similar to recurring prostate cancer upon anti-androgen treatment, knock-down of AR favored the IL-6/STAT3 signaling pathway. Up-regulation of the STAT3 signal transduction as a result of AR silencing was supported by the finding that SOCS3 was down-regulated in AR negative TC1 cells. Since SOCS3 is a STAT3 target gene, this result was unanticipated. However, one could imagine that gene methylation of SOCS3 might be a result of AR knock-down. Epigenetic silencing of SOCS3 has been described in several tumors including prostate cancer [208, 209].
The reduction of the androgen receptor by either hormone deprivation therapy or gene silencing was accompanied by the up-regulation of CSC indicating markers such as Sox2, MSI-1, Nanog and CD44 in TC1 tumors. Since cancer stem cells have been implicated to be the major cause for tumor relapse, the over-expression of CSC markers upon anti-androgen treatment seemed to indicate one possible reason for recurring prostate cancer. Interestingly, CSC phenotype development was a result of the synergistic effect of decreased AR expression and elevated IL-6/STAT3 signaling. Indeed, knock-down of STAT3 by RNAi resulted in decreased CSC phenotype development as indicated by down-regulation of the previous mentioned CSC markers in immunofluorescent stainings of tumor sections and \textit{in vitro} tumor spheres. It has not been addressed yet how and whether AR and STAT3 affect gene-regulation of these CSC markers in prostate cancer.

However, STAT3 activity correlated with CSC phenotype development, as constitutively active STAT3 in cells with diminished AR expression resulted in the overexpression of CSC markers, and lack of STAT3 activity led to down-regulated CSC markers. The correlation between up-regulated IL-6/STAT3 signaling pathway and CSC marker expression was confirmed in human prostate cancer tissue derived from patients. Tissue-areas positive for tyrosine-phosphorylated STAT3 revealed elevated expression of IL-6 and CSC-indicating markers. On the other hand, CSC markers seemed to overlap with up-regulated AR as well. However, magnified field of views from tissue regions positive for AR and Sox2 showed that AR-negative cells revealed considerable increased Sox2 expression, which suggests an inverse correlation between AR expression and the regulation of CSC-indicating markers. Similar results were achieved by staining murine TC1 tumors for CD44 and AR or tyrosine phosphorylated STAT3. While expressions of CD44 coincided with phospho-tyrosine STAT3, AR positive cells did not reveal CD44 up-regulation.

Based on these observations, the following hypothesis was proposed: In the normal prostate, STAT3 signaling is tightly regulated and the transcription factor acts as an inflammatory response factor exclusively. The androgen receptor is responsible for differentiation and the secretion of seminal fluid components. Initiation of prostate cancer by genetic alterations or microenvironmental changes is promoted by overactive AR. However, prostate cancer initiation might
Discussion

not necessarily depend on AR activity, but is as well a result of aberrant STAT3 activity. Hormone ablation therapy inhibits AR activity which results in decreased expression of AR (Figure 41A, 41B). Diminished AR protein activity leads to the up-regulation of the IL-6/STAT3 signaling pathway suggesting a counter-regulatory process between AR- and IL-6/STAT3-signaling (Figure 41C).

Figure 41: Hypothesis about the regulation of the AR- and the IL-6/STAT3 signaling pathways in prostate cancer progression. A: Shown is a scheme of the development of hormone refractory prostate cancer starting with a normal prostate, primary prostate cancer (PC) followed by an invasive carcinoma as a result of hormone ablation therapy as indicated. Tumor-heterogeneity is indicated by different colors of cancer cells. CSCs are indicated in green. B: Expressions of STAT3 and AR in correlation to the occurrence of CSCs are summarized in the different stages. While ‘-‘ represents downregulation, ‘+’ and ‘++’ represent the grade of upregulation. C: Depicted is a suggested signaling network representing the crosstalk of AR and STAT3 in primary PC and HRPC or Metastasis. The changes in the transition from androgen dependent to androgen-independent prostate cancer are summarized by down-regulation of AR and up-regulation of the IL-6/STAT3 signaling pathway as a counter-regulating process (middle). Abbreviations: PC-prostate cancer; HRPC-hormone refractory prostate cancer.

Elevated STAT3 activity is associated with the development of a CSC phenotype (Figure 41B). CSC phenotype development is a result of CSC survival or altered gene-expression which might be induced by IL-6/STAT3 signal transduction. Constitutive activation of STAT3 and CSC phenotype development results in the recurrence of prostate cancer upon hormone ablation therapy (Figure 41A, 41B).

4.1.2 STAT3 in Prostate Cancer Metastasis

Since HRPC might exert a similar phenotype as metastatic prostate cancer, the impact of STAT3 on metastasis was determined by using metastatic TC2 cells
Discussion

and primary TC1 cells in which the STAT3 signaling pathway was enhanced by stable over-expression of STAT3α-YFP. STAT3 has been demonstrated to play a role in angiogenesis and metastasis in a variety of tumors promoting neo-vascularization, extravasation and invasion of metastatic cells into target tissue [27]. A gene-expression profile of TC1 cells silenced for either STAT3 or AR revealed, that knock-down of AR promoted IL-6 induced inflammatory response as shown by up-regulated STAT3 target genes α2-Macroglobuline and CRP. Interestingly, AR did not impact STAT3 target genes involved in angiogenesis (HIF-1α, FGF1, FGF2) and metastasis (MMP2, MMP9) (Table 7). Particularly MMP2, which has been described as an AR target-gene, was not regulated upon AR gene silencing. The lack of MMP2 down-regulation in AR knock-down cells might be a result of up-regulated STAT3 which compensates MMP2 gene regulation by AR. However, down-regulation of angiogenic and metastatic factors upon STAT3 gene silencing suggested a role of STAT3 in neo-vascularization and metastasis in prostate cancer.

In order to assess the impact of STAT3 on angiogenesis and metastatic processes, TC1 cells stably expressing STAT3α-YFP were compared with cells stably transfected with the dominant-negative form STAT3β fused to YFP. The induction of secretory factors that induce neo-vascularization is partially regulated by STAT3 signaling. The STAT3 target gene HIF1α encodes a transcription factor essential for oxygen homeostasis. HIF1α is activated by hypoxic conditions and promotes VEGF induction [210]. The soluble factors VEGF as well as FGF bind to endothelial cells and promote sprouting, migration and microvascular tube formation [211, 212].

In this study, STAT3β, as a negative regulator of STAT3α, inhibited microvascular tube formation of endothelial cells considerably. As another consequence, overexpression of STAT3β resulted in significantly decreased tumor growth. The tumors revealed no detectable vascularization compared to TC1 tumors stably expressing STAT3α-YFP. Since STAT3α enhances different cellular processes important for tumor growth, other target genes beside angiogenic factors might be responsible for STAT3β induced tumor growth delay as well.

In the course of cell metastasis neo-vascularization of the tumor is essential. Metastasis involves the migration of tumor cells to blood vessels, invasion,
circulation of tumor cells in blood vessels, adherence at secondary organ sites, and finally the evasion of host immune defense [197]. Migratory processes of prostate cancer cells were assessed by detecting STAT3 dependent secretion of gelatinases MMP2 and MMP9, and a boyden chamber assay. Since STAT3α induces gene expression of MMP2 and MMP9, it was consistent that TC1 cells stably expressing STAT3α-YFP migrate faster. Conversely, STAT3β reduced induction of these matrix metalloproteases that are important for ECM degradation which resulted in decreased migratory activity of TC1-STAT3β-YFP cells compared to parental TC1 cells. Moreover, the expression of adhesion proteins such as integrin β1, Galectin3, and CD44 were elevated in prostate cancer cells expressing STAT3α-YFP compared to STAT3β-YFP expressing cells. In vivo, the metastatic activity was assessed by retro-orbital injection of TC1 cells expressing STAT3α-YFP or STAT3β-YFP. Lung-infiltration of STAT3α-YFP cells was already observed one day after injection. Colony formation started on day 3, expanded on day 5, and seven days after tumor cell challenge a nodule outgrowth was observed. Conversely, overexpression of STAT3β-YFP inhibited this process considerably, indicating that STAT3α plays a substantial role in metastasis of prostate cancer cells.

This might explain the observation that several metastatic prostate cancer cells such as DU-145 and PC-3 express high levels of tyrosine phosphorylated STAT3. Since prostate tumor metastasis seems to develop similar as recurring prostate cancer, these results underline the importance of targeting STAT3 to prevent aggressive tumor progression.

Similar results to recurring prostate cancer upon hormone deprivation therapy were observed in metastatic TC2 cells. In comparison to TC1 cells, tumors derived from TC2 cells grew considerably faster due to increased proliferative activity. These TC2 cells showed no AR protein expression, but elevated levels of tyrosine phosphorylated STAT3 which was associated with up-regulation of the CSC markers MSI-1 and Sox2. These observations correlate with HRPC showing a potential counter-regulation between AR- and IL-6/STAT3-signaling pathways.
4.1.3 Targeting the IL-6/STAT3 Signaling Pathway with the IL-6 Receptor Fusion Protein

Since hormone ablation therapy has shown to enhance IL-6 induction in this study, the IL-6/STAT3 signaling pathway is a promising target for recurring prostate cancer. The IL-6 receptor fusion protein (IL-6RFP) has been engineered and tested in vitro. It revealed high specificity and potency towards IL-6 [101, 102]. In order to assess the IL-6RFP as a potential therapeutic agent for prostate cancer, the IL-6RFP plasmid was stably introduced into HEK293-FRT cells. The expression and secretion of the receptor fusion protein from HEK(IL-6RFP) cells was confirmed, and the concentration of IL-6RFP in conditioned medium was about 200 ng/ml. The functionality was assessed using TC1 cells revealing inhibitory effects of IL-6RFP on tyrosine-phosphorylation, nuclear translocation, and DNA-binding of STAT3 suggesting that the IL-6RFP exerts the desired activity by binding IL-6 and inhibiting STAT3 activity. Moreover, comparing the IL-6RFP with a commercially available neutralizing IL-6 antibody on the inhibition of STAT3 activity revealed a 3.5 fold higher inhibitory efficiency of the IL-6 receptor fusion protein.

The subcutaneous, peritumoral administration of IL-6RFP in TC1 tumor bearing mice resulted in the most efficient tumor growth delay. Reduced tumor growth was associated with decreased total and tyrosine-phosphorylated STAT3 protein, but did not affect AR protein level. A more detailed analysis by immunofluorescent staining of phosphorylated STAT3 and IL-6RFP showed a locally confined distribution of the receptor fusion protein in the tumor tissue. While tissue-areas negative for IL-6RFP revealed tyrosine-phosphorylated STAT3, tumor tissue infiltrated by IL-6RFP showed reduced levels of activated STAT3 indicating proper functionality in vivo. Diminished STAT3 activity was accompanied by reduced proliferation, vascularization and elevated apoptosis.

Since up-regulated IL-6/STAT3 signaling correlated with CSC phenotype development, the inhibitory impact of IL-6RFP on CSC-indicating marker expression was analyzed in tumor sections and tumor spheres. In fact, inhibition of the IL-6/STAT3 signaling pathway by IL-6RFP resulted in decreased levels of CSC markers such as Sox2, CD44, MSI-1 and Nanog in vitro and in vivo. Additionally, the impact of IL-6RFP on CSC marker expression in human prostate
cancer cells was confirmed in human DU145 cells. Tumor sphere formation of DU145 cells was considerably reduced upon IL-6RFP treatment. This was accompanied by decreased MSI-1 and Sox2 expression. The inhibition of CSC marker expression by IL-6RFP was STAT3 dependent, as DU145 cells silenced for STAT3 revealed a down-regulation of MSI-1 and Sox2 as well.

Despite HRPC might be a result of AR down-regulation coinciding with CSC development and up-regulated IL-6/STAT3 signaling, targeting AR results in reduced tumor growth initially. Prostate cancer has been shown to be AR dependent [116]. A combined therapy including the anti-androgen bicalutamide and the IL-6RFP resulted in improved tumor growth delay compared to a mono-therapy using IL-6RFP. Decreased proliferation and survival upon IL-6 and AR inhibition was similar to IL-6RFP treated tumor tissue. Moreover, IL-6RFP mono-therapy and the combined treatment resulted in decreased CSC phenotype development. The anti-androgen bicalutamide led to elevated CSC phenotype. Thus, additional application of IL-6RFP seemed to overwrite the bicalutamide induced CSC marker up-regulation in the combined therapy suggesting that the development of CSCs is an IL-6/STAT3 dependent process.

Taken together, the inhibition of the IL-6/STAT3 signaling pathway in prostate cancer did not affect expression of AR, but reduced tumor growth and CSC phenotype development. Hence, the IL-6RFP is a promising agent for therapeutic intervention in prostate cancer by targeting the proliferative tumor cells as well as the CSC population.

### 4.2 Crosstalk of STAT3 and AR

In the ligand-independent activation of the androgen receptor in HRPC, STAT3 has been implicated to promote AR regulated gene-induction in an IL-6 dependent manner [155]. AR activation by phosphorylation through the Ras/MAPK and PI3K/AKT pathways has been reported, though the occurrence of direct phosphorylation by MAP kinases is still unclear. In this context, it has been shown that STAT3 physically interacts with AR at the N-terminal region (234-558) in an IL-6 dependent but androgen-independent manner in human prostate adenocarcinoma cells (LNCaP) [151]. Additionally, the IL-6 induced activation of STAT3 was required for IL-6 to activate AR in LNCaP cells. STAT3
has been shown to enhance AR-mediated gene expression and AR transcriptional activity in prostate cancer cells [213]. It has been suggested that activated STAT3 can act synergistically with other coactivators such as CBP, p300 and SRC-1 to enhance AR transcriptional activity [214]. This crosstalk of STAT3 and AR by direct interaction and the subsequent enhancement of AR-target genes in the course of ligand-independent signaling in HRPC is the prevalent opinion, but remains controversial.

![Figure 42: Nuclear translocation of STAT3-DsRed and GFP-AR upon different activators.](image)

In an artificial cell-model using STAT3 deficient MEF cells, nuclear translocation of AR which is critical for target gene induction was determined upon either stimulation with IL-6, R1881 or overexpression of v-Src. The agonist R1881 is a synthetic derivate of testosterone, and v-Src has been suggested to phosphorylate STAT3 at Y705 and AR at Y534 to promote STAT3- and AR-signal transduction [20, 127]. MEF$_{\text{STAT3A}}$ cells were transiently transfected with GFP-AR and STAT3-DsRed or co-transfected with v-Src as indicated in figure 42. Transfected cells were stimulated with IL-6, R1881 or left unstimulated. Nuclear translocation of both transcription factors was analyzed by confocal microscopy. STAT3-DsRed was mainly localized in the nucleus after IL-6 stimulation and in v-Src co-transfected cells. The testosterone derivate R1881 did not result in nuclear localization of STAT3-DsRed, but led to translocation of GFP-AR to the nucleus. Interestingly, neither IL-6 stimulation nor v-Src co-transfection resulted in a nuclear localization of AR (Figure 42). Hence, these results did not support the hypothesis of IL-6 induced AR activation.
A possible explanation for AR activation might involve an IL-6-induced promotion of AR signal transduction by phosphorylation in an androgen poor environment in which androgens stimulate nuclear translocation and DNA-binding of AR homo-dimers.

On the other hand, Keller et al. pointed out that the androgen receptor represses NFκB dependent IL-6 induction [215, 216], which would rather underline a crosstalk in which AR and STAT3 inversely-regulate each other. This was supported by the finding in TC1 cells which were stimulated with IL-6 or R1881 in a time-dependent manner as indicated in figure 43.

The western blot analysis revealed an increase of tyrosine-phosphorylated and total STAT3 protein upon IL-6 stimulation, while protein-level of AR decreased time-dependently (Figure 43A). In contrast, testosterone stimulation resulted in an up-regulation of AR protein, but decreased levels of phosphorylated and total STAT3 protein (Figure 43B). The connection between decreased AR expression and elevated IL-6 levels in many advanced prostate cancers seems to contribute to the hypothesis of an inverse regulation between the AR- and STAT3-signaling pathways. However, since a tumor is heterogeneous one could imagine that both, the interaction of STAT3 and AR to enhance AREs and a counter-regulation between AR- and STAT3-signaling, might play a role in tumor progression and HRPC. Therefore, the TC1-tumor-model might not fully display the heterogeneity in patients, but rather shows the situation in a subpopulation of tumor cells.

Moreover, Heisler et al. indicated that AR might as well function as a tumor repressor. They demonstrated, that stable expression of AR in androgen-
independent and AR-negative prostate cancer cells called PC-3 revealed reduced proliferation and invasion [217, 218]. Moreover, it has been demonstrated that IL-6 acted as an autocrine growth and survival factor in androgen-independent DU-145 and PC-3 cells [219]. Thus, reduced IL-6 induction followed by decreased pYSTAT3 might be a potential reason for reduced proliferation and invasion upon AR overexpression.

On the contrary, androgen sensitive LNCaP cells did not secrete detectable amounts of IL-6. Interestingly, LNCaP cells were derived from lymph node metastasis, which is usually the first site metastasis in prostate cancer. Conversely, androgen-independent PC-3 cells were derived from bone and DU145 from brain metastasis which represent second or late stage metastasis (Figure 44). Since LNCaP, DU145, and PC-3 cells were originated from different tissue, it is to be expected that these cancer cells exert altered gene expression profiles. Thus, one could imagine that prostate cancer cells loose AR function, and lack of AR might lead to a more aggressive cell phenotype which results in metastasis to secondary sites due to growth advantage.

4.3 Limitations of IL-6RFP Treatment

The binding of IL-6 to its cytokine receptor results in the activation of intracellular signaling cascades including JAK/STAT and MAPK [24]. The activation of the
PI3K/AKT pathway has been suggested to be activated by IL-6 stimulation as well [220]. Both pathways, the MAPK and the PI3K/AKT signal transduction, have been demonstrated to be dysregulated in a variety of cancers. Similar to the STAT3 signaling pathway, they play critical roles in the promotion of proliferation, survival, and anti-apoptosis (Figure 45). Thus, inhibition of the IL-6 signaling pathway using IL-6RFP lacks STAT3 specificity, but prevents activation of three oncogenic pathways, which might be advantageous for cancer therapy.

High levels of autocrine and paracrine acting IL-6 were measured in conditioned medium of androgen-independent prostate cancer cells [221]. Increases in IL-6 expression have been associated with several regulatory processes. TGFβ, which is up-regulated in advanced prostatic tumors, loss of Rb, and the AP-1 complex consisting of c-Jun and c-fos have been suggested to play a role in the induction of IL-6 [222]. Moreover, the androgen receptor has been observed to repress NFκB mediated IL-6 induction [191, 215]. The dysregulation of IL-6 expression in prostate cancer suggests that the cytokine might be a target for therapy in this disease, but therapeutic agents are rare and revealed mixed effects. The anti-IL-6 antibody siltuximab (CNTO328) has been applied in patients with recurring prostate cancer. Tumor growth had progressed beyond several chemotherapeutical treatments. Siltuximab administration showed limited clinical efficiency, despite achieved IL-6 inhibition [223]. Since tumors are heterogeneous, especially in advanced disease, it seems unlikely that a single agent would be effective. Application of a combined therapy using IL-6RFP and

![Figure 45: Activation of the MAPK, STAT3, and PI3K signaling pathways via IL-6.](image)
the anti-androgen bicalutamide in this study resulted in improved tumor growth delay compared to IL-6RFP mono-therapy. However, inhibition of the IL-6/STAT3 and AR signaling pathway did not lead to tumor rejection and an entire cure of the disease. Hence, administration of different inhibitor combinations might be valuable to determine.

Despite the inhibition of STAT3, MAPK and PI3K signaling by IL-6RFP, these pathways can get activated by receptor tyrosine kinases as a response to EGF or VEGF and non-receptor tyrosine kinases such as v-Src as well.

Interestingly, comparing TC1 cells which represent primary prostate cancer with advanced TC2 cells, differences in IL-6 and Src expression were observed. The RT-PCR analysis revealed that IL-6 mRNA levels were 34-fold increased in TC1 cells compared to TC2 cells (Figure 46A). Instead, a different tyrosine kinase was responsible for elevated activation of STAT3 in TC2 cells. Src expression was considerably increased in TC2 tumor sections. Hoechst and CD31 were used as markers for the tissue (Figure 46B). These results indicate that in the transition from primary (TC1) to metastatic (TC2) tumors, cell phenotypes change due to altered gene-expression. When tumor cells change their intracellular signal transduction network in the course of metastasis, therapeutical intervention becomes challenging. Thus, targeting the IL-6 signaling pathway might not be sufficient for tumor rejection and therefore, tumor relapse of selected cancer clones might occur. Instead of a mono-therapy, poly-therapies combining anti-IL-6 therapy such as IL-6RFP with tyrosine kinase inhibitors (i.e. dasatininb) might be more promising.

This observation seems to be consistent with the IL-6RFP treatment in this study, which did not result in complete tumor rejection, but tumor growth delay.

**Figure 46: Counter-regulation of IL-6 and Src in TC1 and TC2 cells.**

**A:** Shown is a RT-PCR of TC1 and TC2 cells analyzed for IL-6 mRNA induction. IL-6 mRNA induction of TC1 cells was normalized to TC2 IL-6 mRNA levels. Error bars represent standard deviations. **B:** Tumor sections of TC1 and TC2 tumor bearing mice were immunostained for pSrc (green) and CD31 (red). Hoechst dye was used to stain the nuclei. Scale: 100 µm.
Discussion

However, lack of tumor rejection might be a result of too low IL-6<sup>RFP</sup> concentrations. IL-6<sup>RFP</sup> was injected into tumor bearing mice in a concentration of 1.8 nM. TC1 tumors showed IL-6 concentrations of approximately 3.2 pM per 1x10<sup>6</sup> tumor cells. To determine intratumoral IL-6 concentrations, TC1 tumors were excised, single cell suspensions were prepared and 1x10<sup>6</sup> tumor cells were cultured on a 12 well plate for 24 h. Then, conditioned medium was subjected to a murine IL-6 ELISA (data not shown).

Considering that each molecule IL-6<sup>RFP</sup> can bind one molecule IL-6, approximately 560-fold more IL-6<sup>RFP</sup> molecules were given daily to prevent IL-6 action in a tumor consisting of 1x10<sup>6</sup> cancer cells. *In vitro* analysis revealed that a 65-fold molar surplus was sufficient to reduce tyrosine phosphorylated STAT3 in IL-6 stimulated TC1 cells (Figure 17E). A 560-fold molar surplus of IL-6<sup>RFP</sup> *in vivo* led to tumor growth delay, but not tumor rejection. The distribution of peritumoral injected IL-6<sup>RFP</sup> was locally confined and IL-6<sup>RFP</sup> negative areas revealed up-regulated STAT3 activity (Figure 22B). Thus, IL-6<sup>RFP</sup> concentrations might have been too low to reach complete inhibition of the IL-6 signaling pathway. However, used concentrations resulted in tumor regression, and application of increased IL-6<sup>RFP</sup> concentrations after proper purification might lead to tumor rejection and prevention of tumor relapse.

4.4 STAT3 in Cancer Stem Cells

One critical aspect for the evaluation of CSC phenotype development is the identification of CSC markers such as CD44, Sox2, MSI-1, Nanog, and integrin α2β1. However, probably all of these markers are expressed in a variety of cancer stem cells. As summarized in table 6, CSC markers have more functions than maintaining pluripotency and self-renewal. CD44, for instance, is involved in cell-adhesion, and is therefore also used as a marker for metastasis. This involvement in cancer functionality might explain why these CSC-indicating markers were up-regulated in many cells in this study, instead of only few cells as proposed in the cancer stem cell theory. Hence, it is important to determine the up-regulation of several CSC markers (>3) to make conclusions regarding the development of a CSC phenotype.
In the course of prostate CSC development it has been proposed that somatic stem cells, progenitor cells and differentiated prostate cancer cells can evolve into CSCs upon genetic alterations or microenvironmental influences (Figure 7). In turn, CSCs have the ability to differentiate into a variety of tumorigenic cells that promote tumor growth.

**Figure 47:** Over-expression of STAT3α-YFP promotes an endothelial phenotype of TC1 cells. A: TC1 cells stably expressing STAT3α-YFP or STAT3β-YFP were cultured on collagen containing CM of TC1-STAT3αYFP or –STAT3β-YFP cells. After 48 h cells were analyzed at the confocal microscope at 37°C and 5% CO₂. Scale: 100 μm. B: TC1-STAT3α-YFP and TC1-STAT3β-YFP cells were incubated with an APC-conjugated CD31 antibody for 30 min. Then, cells were prepared for FACS analysis. YFP-positive cells were gated and analyzed for APC fluorescence.

The de-differentiation of cancer cells to CSCs and their subsequent differentiation to tumor cells with a changed phenotype might have been observed in the following experiment: TC1-STAT3α-YFP and TC1-STAT3β-YFP cells were cultured on collagen mixed with CM of STAT3α or STAT3β-YFP expressing cells for 48 h. Live cell imaging was performed via confocal microscopy and revealed that TC1-STAT3α-YFP cells were able to form tubes similar to endothelial cells, while cells over-expressing the dominant-negative form STAT3β-YFP were distributed homogenously (Figure 47A). In a further analysis by flow cytometry, YFP positive cells revealed that CD31 expression is elevated in STAT3α-YFP expressing cells relative to TC1-STAT3β-YFP cells. CD31 is a marker for endothelial cells that is indicating blood vessels (Figure 47B). One could imagine that STAT3 drives de-differentiation of luminal prostate cancer cells to CSCs, due to up-regulation of CSC markers such as Sox2, MSC-1, CD44, Nanog, and integrin α2β1. Hypothetically, CSCs differentiated then STAT3 dependently into tumor cells with endothelial features. These endothelial cells might either stabilize or promote neo-vascularization in tumors.
Furthermore, one could imagine that tumor cells need to adapt endothelial features temporarily in the course of angiogenesis during metastasis and tumor growth.

The STAT3 dependent up-regulation of CD31 and the ability of TC1-STAT3α-YFP cells to form tubes might be a result of the microenvironment induced by collagen or secreted autocrine acting soluble factors. Hence, it may be possible that over-activation of signaling pathways such as the STAT3 signaling pathway results in phenotypical changes of cells that might not only include transformation, but de-differentiation to CSCs, and a subsequent induction of genes that enhance an endothelial phenotype as well. Moreover, one could imagine that, in a different microenvironment, CSCs could differentiate into phenotypically changed tumor cells i.e. luminal or basal prostate cancer cells, tumor cells with endothelial features, or even osteoblasts or astrocytes in the course of metastasis (Figure 48A).

**Figure 48:** Potential role of STAT3 in de-differentiation and differentiation of tumor cells. 

**A:** Prostate cancer cells can de-differentiate to CSCs (indicated by 1). CSCs may differentiate into various tumor cells with different phenotypes: Basal, luminal, endothelial, and metastatic prostate cancer cells (indicated by 2). **B:** STAT3 dependent de-differentiation and CSC development due to the up-regulation of CSC markers such as Sox2, CD44, MSI-1, Nanog and Integrin α2β1, as well as Bcl-2 and FGF2. **C:** Role of STAT3 in differentiated tumor cells including the up-regulation of c-myc, Cyclin D1, D2, HIF1α, VEGF, MMP2,9, BclXl, Bcl-2, and FGF2.

In this context, STAT3 might be important due to the following reasons: on the one hand, STAT3 has been suggested to play a role in CSC phenotype development in glioblastomas by promoting self-renewal and maintaining pluripotency (Figure 48B) [224]. On the other hand, STAT3 has been...
demonstrated to play a critical role in tumor growth by inducing genes involved in proliferation, anti-apoptosis, angiogenesis and metastasis (Figure 48C). The differences in STAT3 function in CSCs and differentiated tumor cells, probably due to altered gene expression profiles, could be a result of changes in secreted soluble factors by the microenvironment, epigenetic alterations and mutations. Posttranslational modifications of STAT3 or the crosstalk with other signaling pathways might play a role as well. For instance, it has been shown that STAT3 interacts with Nanog in embryonic stem cells to maintain pluripotency [225].

Whether the STAT3 dependent balance between CSC development and tumorigenic processes in differentiated cancer cells is mechanistically and/or temporally excluded is unclear. Since the STAT3 target genes Bcl-2 and FGF2 have been implicated to be important in CSC maintenance [181], as well as anti-apoptosis and angiogenesis, one could imagine that the processes involved in de-differentiation and differentiation of tumor cells are inter-connected and rather fluent than chronologically excluded (Figure 48).

In the context of cancer treatment, the prevalent drug-resistance and the potential ability of CSCs to avoid treatment by adapting differentiation processes to microenvironmental changes make tumor targeted therapy challenging. Thus, treatment of the cancer stem cell population seems critical to prevent recurring tumors.

In prostate cancer, the expression of AR in CSCs is controversially discussed, but most evidence hints to an AR negative phenotype [181]. Thus, androgen ablation therapy which affects AR positive cells results in the survival of CSCs. Immunotherapy targets differentiated cells, but CSCs are undifferentiated. Radiotherapy as well as most chemotherapies target rapidly dividing cells, but CSCs usually show low proliferative activity during self-renewing processes [226]. Since STAT3 plays a critical role in differentiated, highly proliferative tumor cells, as well as in de-differentiated CSCs, the STAT3 signaling pathway seems to be a promising target. Moreover, IL-6 has been implicated in the maintenance of stem-like cancer cells. In gene-expression profiles of CD44 positive breast CSCs, IL-6 has been demonstrated to be up-regulated [227]. Thus, targeting the IL-6/STAT3 signaling pathway seems to be a promising therapy to abolish tumor cells and CSCs in prostate cancer.
5 Synopsis

Prostate cancer is the most common cause of male cancer-related deaths in Western countries. Current treatment relies on targeting the androgen receptor (AR) signaling by hormone deprivation therapy. However, hormone depletion results in the selection of drug resistant, highly metastatic tumor cells that survive targeted therapy. Hence, the initial response to treatment is almost always followed by tumor relapse, which is called hormone refractory prostate cancer (HRPC) [142]. The development of HRPC is not well understood and remains a challenge for prostate cancer therapy. One model to explain HRPC development proposes the survival of so called cancer stem cells (CSC). CSCs share phenotypical features with embryonic stem cells, are highly drug resistant, and have been suggested to resupply the highly proliferative tumor cell population.

STAT3 has been shown to maintain the pluripotent state of glioblastoma stem cells. Moreover, the IL-6/STAT3 signaling pathway has been emphasized to play a critical role in the progression of prostate tumors [27].

In this study, the role of the IL-6/STAT3 signaling pathway in the initiation, progression and recurrence of prostate cancer upon hormone ablation therapy was determined. It was demonstrated that down-regulation of AR by either hormone deprivation therapy or gene-silencing results in the up-regulation of IL-6/STAT3 signaling. Overactive IL-6/STAT3 signal transduction coincided with CSC phenotype development. CSC marker expressions were up-regulated in tumors treated with anti-androgens, metastatic tumor cells as well as human prostate cancer. In human prostate cancer tissues, elevated cancer stem cell markers coincided with those cells exhibiting high STAT3 activity and low AR expression, supporting an opposing role of AR and STAT3 in prostate cancer stem cell development.

Targeting the IL-6/STAT3 signaling pathway by gene silencing or using an IL-6 receptor fusion protein (IL-6RFP) resulted in significantly reduced tumor growth which was associated with decreased proliferation, angiogenesis and anti-apoptosis. It was found that overexpression of STAT3β, a dominant-negative
form of STAT3α, resulted in diminished prostate tumor growth, reduced angiogenesis and metastasis. Moreover, STAT3 inhibition by either using IL-6RFP or gene-silencing resulted in decreased CSC phenotype development in vitro and in vivo. Application of a combined therapy using the IL-6RFP and the anti-androgen bicalutamide improved tumor growth delay compared to the respective mono-therapies. In addition, IL-6 inhibition by IL-6RFP overwrote the anti-androgen induced CSC phenotype development.

More specifically the following results were obtained:

➢ The up-regulation of the IL-6/STAT3 signaling pathway upon hormone ablation therapy was determined by using the TC1-tumor model. Anti-androgen treatment with flutamide resulted in decreased AR protein expression, but increased IL-6 mRNA levels, total and tyrosine phosphorylated STAT3. Since growth factor expression levels were not affected by flutamide administration, IL-6 seemed to be the trigger for elevated STAT3 activation. Moreover, hormone ablation therapy using flutamide led to up-regulated CSC marker expression of Sox2, CD44 and MSI-1.

➢ Gene silencing of STAT3 in TC1 cells diminished the initiation of prostate cancer and resulted in considerable tumor growth delay and decreased CSC phenotype development in vitro and in vivo. Western blot analysis of tumor spheres and tumor sections stained for CSC markers revealed reduced expression of Sox2 and MSI-1. CD44 and Nanog were down-regulated in immunofluorescent stainings of frozen tumor sections as well. Conversely, AR knock-down did not affect tumor growth, but rather enhanced the IL-6/STAT3 signaling pathway by up-regulation of IL-6 mRNA, elevated levels of total and tyrosine-phosphorylated STAT3 protein and down-regulation of SOCS3, the feedback inhibitor of STAT3. Moreover, CSC phenotype was up-regulated in tumor sections and tumor spheres of TC1 cells silenced for AR.

➢ Tissue sections from human prostate cancer patients were stained for CSC markers such as Sox2, MSI-1, CD44, integrin α2β1 or Nanog and tyrosine phosphorylated STAT3. Those cells with elevated pYSTAT3 coincided with increased IL-6 and cancer stem cell marker expression. In
contrast, despite co-expression of AR and CSC markers in cancerous tissue, up-regulated AR was associated with decreased CD44 and Sox2 expression in murine and human tumorigenic tissue.

- In order to achieve tumor growth reduction and diminished CSC phenotype development, the IL-6 receptor fusion protein was evaluated as a potential therapeutic agent for prostate cancer. The functionality of IL-6RFP was characterized in vitro showing a proper binding of secreted IL-6RFP to murine and human IL-6. The IL-6RFP inhibits phosphorylation of STAT3 and prevents nuclear translocation as well as DNA-binding of STAT3 in a dose-dependent manner. The IL-6RFP was 3.5 fold more efficient in inhibiting STAT3 phosphorylation relative to a neutralizing antibody.

- In the course of a functional characterization in vivo, 1.8 µg/kg IL-6RFP was injected into TC1-tumor bearing mice twice daily. Peritumoral administration of IL-6RFP revealed the highest inhibitory impact on tumor growth. The IL-6RFP did not affect expression of AR, but reduced activation of STAT3, which was accompanied by decreased proliferation, vascularization and enhanced apoptosis.

- IL-6RFP treatment of TC1 tumor spheres and tumor bearing mice showed decreased expression of MSI-1, CD44, Nanog and Sox2 which was associated with reduced total and tyrosine phosphorylated STAT3. Application of IL-6RFP on human, metastatic DU145 cells inhibited tumor sphere formation as well, and was associated with Sox2 and MSI-1 down-regulation.

- Combining IL-6RFP and an anti-androgen bicalutamide resulted in improved tumor growth delay relative to monotherapies including IL-6RFP or bicalutamide. Increased apoptosis, and reduced proliferation and CSC marker expression was observed upon IL-6RFP and IL-6RFP/bicalutamide treatment. Similar to flutamide, bicalutamide application resulted in increased proliferation and anti-apoptosis accompanied by elevated levels of CSC marker expression.

- Gene-silencing of STAT3 in TC1 cells resulted in decreased proliferation and increased apoptosis of tumor bearing mice. The neo-vascularization was impaired upon knock-down of STAT3 as well. This was associated
with decreased mRNA levels of HIF1α, FGF1 and FGF2. MMP2 and MMP9, which are involved in metastatic processes, were down-regulated in STAT3 silenced cells as well. To support the impact of STAT3 on angiogenic and metastatic processes TC1 cells stably expressing STAT3α-YFP or STAT3β-YFP were developed. Overexpressed STAT3β-YFP diminished tube-formation of endothelial prostate cancer cells and reduced tumor growth considerably, which was associated with no detectable vascularization. Moreover, STAT3β-YFP decreased STAT3α-YFP induced expression of MMP2 and MMP9, repressed migration and the expression of adhesion molecules such as integrin β1, CD44 and Galectin3. Finally, STAT3β-YFP reduced infiltration and colony formation of murine prostate cancer cells in a time-dependent manner.

Similar to recurring prostate cancer upon hormone ablation therapy and tumor cells silenced for AR, metastatic TC2 cells revealed decreased AR expression, but elevated levels of phosphorylated STAT3. Mice injected with TC2 cells showed considerable enhanced tumor growth, which correlated with increased proliferation as compared to TC1 tumor development. Moreover, the expression of CSC indicating markers was elevated in advanced TC2 tumor sections stained for MSI-1 and Sox2.

In summary, it was found that down-regulation of AR mediates activation of STAT3 signaling through increasing IL-6 expression, which coincided with increased CSC phenotype development, and that the IL-6RFP is a potential therapeutical agent for prostate cancer to reduce tumor growth and to prevent recurring prostate cancer by targeting the CSC population.
6 Future Directions

In this study, the importance of the IL-6/STAT3 signaling pathway in prostate cancer development was determined and treatment using the IL-6 receptor fusion protein was successful to reduce tumor growth and CSC development. Since the TC1 tumor model does not represent the heterogeneity of tumor cells in human patients, it would be preferable to include more tumor models to confirm general validity of the shown critical role of IL-6 induced STAT3 signal transduction in prostate cancer. As such, DU145 and PC-3 cells might be appropriate tumor models, as they show high STAT3 activity and are of human origin. Usage of DU145 and PC-3 cells in a xenograft model would strengthen the hypothesis developed in this study, due to their improved clinical relevance. In addition, the usage of primary prostate cancer cells derived from human patients might be preferable as well.

To generate a clinical study using the IL-6 receptor fusion protein, it seems necessary to purify the IL-6\textsuperscript{RFP}. Purified IL-6\textsuperscript{RFP} would give the advantage to adjust concentrations and avoid unwanted side-effects potentially induced by conditioned medium of Hek\textsuperscript{IL-6RFP} cells. In this context, it might be preferable to improve the stability of the IL-6\textsuperscript{RFP}. Modifications of the IL-6\textsuperscript{RFP} C-terminus such as the fusion to peptide IG might be an approach to improve protein stability. If inhibitors reveal high stability and pharmacokinetic it might be necessary to evaluate the organ distribution of the IL-6\textsuperscript{RFP} upon continuous treatment to avoid undesired side-effects. Therefore, the occurrence of IL-6\textsuperscript{RFP} in liver and kidney as possible organs for degradation or secretion of the IL-6\textsuperscript{RFP} needs to be determined.

Moreover, it might be possible to expand the application of the IL-6\textsuperscript{RFP} on other tumorigenic diseases. Solid tumors i.e. breast, brain and ovarian cancer have been shown to secrete high levels of IL-6. Multiple myeloma is highly IL-6 dependent and might be therefore a promising cancer model for IL-6\textsuperscript{RFP} therapy. Since many tumors are not strictly IL-6 dependent, STAT3 might get activated by other tyrosine kinases such as the EGFR or Src kinases as well. Considering the complexity of cancer therapy a mono-therapy might not be sufficient to reject tumors. Mono-therapy targeting STAT3 might still result in CSC survival followed
by recurring tumor growth due to other signaling pathways compensating for inhibition of STAT3. Therefore, it might be considerable to investigate a combined therapy (poly-therapy) including the IL-6^{RFP} and i.e. tyrosine kinase inhibitors (TKIs). Dasatinib might be a good candidate, as it predominantly inhibits the Src kinase which is up-regulated in several tumors such as prostate and breast cancer [228]. Furthermore, it might be interesting to evaluate whether the de-differentiation of cancer cells to CSCs as well as the differentiation of CSCs to tumor cell subsets with changed phenotypes is a STAT3 dependent process. In order to further investigate the role of STAT3 in CSC phenotype development, it might be interesting to determine cellular mechanisms involving the crosstalk between constitutive active STAT3 and up-regulated CSC indicating markers. So far, it has been shown, that HA (hyaluronic acid) which is probably secreted by endothelial cells, binds to CD44. Activation of CD44 promotes STAT3-Nanog interaction which leads to nuclear translocation of the interaction partners. In the nucleus, the transcription factors induce gene expression which results in the maintenance of pluripotent CSCs. It has been suggested, that STAT3 does not induce Nanog, but they may have common targets [229]. Moreover, STAT3 has been shown to induce Sox2 to regulate differentiation processes [230]. On the other hand, a STAT3 dependent crosstalk and regulation of other CSC indicating markers such as integrin α2β1 and Musashi-1 remains unclear.
## Abbreviations

### A

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>adenosine</td>
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<tr>
<td>Aa</td>
<td>amino acid</td>
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<tr>
<td>Abl</td>
<td>Abelson leukemia protein</td>
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<tr>
<td>AF</td>
<td>AlexaFluor</td>
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<td>α2M</td>
<td>α2macroglobulin</td>
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<td>AKT</td>
<td>protein kinase B</td>
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<td>ALDH-1</td>
<td>aldehyde dehydrogenase 1</td>
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<tr>
<td>AIPC</td>
<td>androgen independent prostate cancer</td>
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<tr>
<td>APC</td>
<td>allophycocyanin</td>
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<tr>
<td>APRF</td>
<td>acute phase response factor</td>
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<tr>
<td>APS</td>
<td>ammoniumperoxodisulfate</td>
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<tr>
<td>AR</td>
<td>androgen receptor</td>
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<tr>
<td>ARA</td>
<td>androgen receptor activator</td>
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<tr>
<td>ARE</td>
<td>androgen responsive element</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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### B

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<tr>
<th>Abbreviation</th>
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<tr>
<td>Bcl-2</td>
<td>B-cell-lymphoma 2</td>
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<tr>
<td>Bcl-xL</td>
<td>B-cell-lymphoma protein-xL</td>
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<tr>
<td>BCR-Abl</td>
<td>fusion of the breakpoint cluster region (BCR) and Abl</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumine</td>
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<td>C</td>
<td>cytosine</td>
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<tr>
<td>C-terminal</td>
<td>carboxy-terminal</td>
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<tr>
<td>CBP</td>
<td>CREB binding protein</td>
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<tr>
<td>CD31 (PECAM-1)</td>
<td>Platelet Endothelial Cell Adhesion Molecule 1</td>
</tr>
<tr>
<td>CIS</td>
<td>cytokine-inducible SH2-containing protein</td>
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<tr>
<td>CM</td>
<td>conditioned medium</td>
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<tr>
<td>CML</td>
<td>chronic myeloid leukemia</td>
</tr>
<tr>
<td>CMTMR</td>
<td>Orange-fluorescent tetramethylrhodamine</td>
</tr>
<tr>
<td>CNTF</td>
<td>ciliary neurotrophic factor</td>
</tr>
<tr>
<td>CR</td>
<td>cytokine receptor</td>
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<tr>
<td>CRP</td>
<td>c reactive protein</td>
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<td>CSC</td>
<td>cancer stem cell</td>
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<td>CT-1</td>
<td>cardiotrophin 1</td>
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<td>Ctrl</td>
<td>control</td>
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<td>Cy</td>
<td>cyanine-dye</td>
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<tr>
<td>ddH₂O</td>
<td>double distilled water</td>
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<tr>
<td>DHT</td>
<td>5α-dihydrotestosterone</td>
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<tr>
<td>DIC</td>
<td>differential interference contrast</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
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<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>deoxyribonucleoside triphosphate</td>
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<tr>
<td>dsRed</td>
<td>Discosoma spec red fluorescent protein</td>
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<td>DSS</td>
<td>disuccinimidyl suberate</td>
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<tr>
<td>DTSSP</td>
<td>3,3’-dithiobis[sulfosuccinimidylpropionate]</td>
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<td>DTT</td>
<td>dithiotreitol</td>
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<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<td>EPO</td>
<td>erythropoietin</td>
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<td>EtOH</td>
<td>ethanol</td>
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<td>F</td>
<td>phenylalanine</td>
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<tr>
<td>FACS</td>
<td>fluorescent activated cell sorter</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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<td>FRT</td>
<td>flp recombination target sequence</td>
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<td>FSC</td>
<td>forward scatter</td>
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<td>G</td>
<td>guanosine</td>
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<td>G-CSF</td>
<td>granulocyte colony stimulating factor</td>
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<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate-dehydrogenase</td>
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<td>GAS</td>
<td>γ-interferone activated sequence</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GF</td>
<td>growth factor</td>
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<tr>
<td>GFR</td>
<td>growth factor receptor</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage colony stimulation factor</td>
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<tr>
<td>GnRH</td>
<td>gonadotropin releasing hormone</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>H</td>
<td>hour</td>
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<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
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<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
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<td>HIF1α</td>
<td>Hypoxia inducible factor 1α</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>HRPC</td>
<td>Hormone refractory prostate cancer</td>
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<td>HSP</td>
<td>Heat shock protein</td>
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<tr>
<td>HTLV-1</td>
<td>Human T-lymphotrophic virus-1</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin of the subclass G</td>
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<tr>
<td>IF</td>
<td>Immunofluorescence</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IL-R</td>
<td>Interleukin-receptor</td>
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<tr>
<td>IGF</td>
<td>Insulin growth factor</td>
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<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
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<tr>
<td>iPSC</td>
<td>Inducible pluripotent stem cell</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
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<tr>
<td>IVMPM</td>
<td>Intra-vital multiphoton microscopy</td>
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<tr>
<td>ISG</td>
<td>Interferon-stimulated gene</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus Kinase</td>
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<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
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<tr>
<td>KGF</td>
<td>Keratinocyte growth factor</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
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<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
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<tr>
<td>LSM</td>
<td>Laser scanning microscope</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein-kinase</td>
</tr>
<tr>
<td>MEF</td>
<td>Murine embryonic fibroblasts</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MFib</td>
<td>Murine fibrosarcoma cells</td>
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<td>NFκB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B-cells</td>
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<td>poly (ADP-ribose) polymerase</td>
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<td>signal transducer and activator of transcription</td>
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<td>transforming growth factor</td>
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8 References


References


References


162. Veldscholte, J., et al., *The androgen receptor in LNCaP cells contains a mutation in the ligand binding domain which affects steroid binding*


References


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