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Ayush Dagbadorj
Thomas Jefferson University

Robert A. Kirken
University of Texas at El Paso

Benjamin Leiby
Thomas Jefferson University

James Karras
ISIS Pharmaceuticals

Marja T. Nevalainen
Thomas Jefferson University, marja.nevalainen@jefferson.edu

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TRANSCRIPTION FACTOR STAT5 PROMOTES GROWTH OF HUMAN PROSTATE CANCER CELLS *IN VIVO*

Ayush Dagvadorj[1], Robert A. Kirken[2], Benjamin Leiby[3], James Karras[4], Marja T. Nevalainen[1]

1) Dept. of Cancer Biology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA 19107.
2) Dept. of Biological Sciences, University of Texas, El Paso, TX 79968.
3) Dept. of Pharmacology and Experimental Therapeutics, Thomas Jefferson University, Philadelphia, PA 19107.
4) ISIS Pharmaceuticals, Carlsbad, CA 92008.

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Key Words: Stat5a/b, prostate cancer, tumor growth *in vivo*

Abbreviations:
RT - room temperature
Stat5- Signal Transducer and Activator of Transcription 5
DN – dominant negative
WT – wild type
Prl – prolactin

PrlR – prolactin receptor
AS – antisense
MM – mismatch
ODN - oligonucleotides

Address correspondence to:
Marja T. Nevalainen, MD, PhD
Dept. of Cancer Biology,
Kimmel Cancer Center,
Thomas Jefferson University,
233 S. 10th St., BLSB 309
Philadelphia, 19107 PA.

ABSTRACT

Purpose: Stat5a/b is the key mediator of prolactin (Prl) effects in prostate cancer cells via activation of Jak2. Prl is locally produced growth factor in human prostate cancer. Prl protein expression and constitutive activation of Stat5a/b are associated with high histological grade of clinical prostate cancer. Moreover, activation of Stat5a/b in primary prostate cancer predicts early disease recurrence. Here, we inhibited Stat5a/b by several different methodological approaches. Our goal was to establish a proof-of-principle that Stat5a/b is critical for prostate cancer cell viability *in vitro* and for prostate tumor growth *in vivo*.

Experimental Design: We inhibited Stat5a/b protein expression by antisense oligonucleotides or RNA interference and transcriptional activity of Stat5a/b by adenoviral expression of a dominant-negative mutant of Stat5a/b in prostate cancer cells in culture. Moreover, Stat5a/b activity was suppressed in human prostate cancer xenograft tumors in nude mice. Stat5a/b regulation of $BclX_L$ and Cyclin-D1 protein levels was demonstrated by antisense suppression of Stat5a/b protein expression followed by Western blotting.

Results and Conclusions: We show here that inhibition of Stat5a/b by antisense oligonuleotides, RNA interference, or adenoviral expression of DNStat5a/b all effectively kill prostate cancer cells. Moreover, we demonstrate that Stat5a/b is critical for human prostate cancer xenograft growth in nude

mice. Stat5a/b effects on the viability of on prostate cancer cells involve Stat5a/b-regulation of $BclX_L$ and Cyclin-D1 protein levels, but not the expression or activation of Stat3. This work establishes Stat5a/b as a therapeutic target protein for prostate cancer. Pharmacological inhibition of Stat5a/b in prostate cancer can be achieved by small-molecule inhibitors of transactivation, dimerization or DNA-binding of Stat5a/b.

INTRODUCTION

There are currently no effective pharmacological therapies for primary or recurrent prostate cancer. Androgen-deprivation therapy only provides a temporary inhibition of the cancer growth before the hormone-refractory form of prostate cancer develops. Moreover, no effective pharmacological treatments exist for elimination of residual cancer cells after prostate cancer surgery. We propose here transcription factor Stat5a/b as a potential therapeutic target for prostate cancer.

Stat5 is one of the seven members of Stat gene family of transcription factors (1). Two highly homologous isoforms of Stat5, 94-kDa Stat5a and 92-kDa Stat5b, are encoded by separate genes (1). Stat5a and Stat5b (hereafter referred to as Stat5a/b) are latent cytoplasmic proteins that act as both cytoplasmic signaling proteins and nuclear transcription factors. Phosphorylation of a specific tyrosine residue in the carboxy-terminal domain (1) by a tyrosine kinase, typically of the Jak protein family (2, 3), activates Stat5a/b. After phosphorylation, Stat5a and Stat5b homo- or hetero-dimerize and translocate to the nucleus where they bind to specific Stat5a/b response elements of target gene promoters (1).

Stat5 proteins are divided into five structurally and functionally conserved domains. The N-terminal domain is involved in stabilizing interactions between two Stat5 dimers to form tetramers,

which are needed for maximal transcriptional activation of weak promoters (4). Next to the N-terminal domain is the coiled-coil domain which facilitates protein-protein interactions (5, 6) important for transcriptional regulation. The DNA-binding domain mediates direct binding of Stat5a/b to DNA and recognizes members of the GAS family of enhancers (7). The stability of DNA-binding is modified by the adjacent linker domain. The most highly conserved domain of Stat5a/b proteins is the SH2 domain which mediates both receptor-specific recruitment and STAT dimerization (8). Specifically, dimerization requires the binding of a phosphorylated tyrosine residue of one Stat5a/b subunit to the SH2 domain of the other subunit (7). Finally, the carboxy terminus carries a transactivation domain (TAD), which varies considerably in both length and sequence between different Stat family members. TAD binds critical co-activators and is directly involved in facilitating the initiation of transcription (7, 9). Stat5a has 20 amino acids that are unique in its C-terminal sequence, while eight amino acids in the C-terminus are specific to Stat5b. Furthermore, Stat5b has a five residue abbreviation of the Stat5a phosphotyrosyl tail segment between the SH2 and TA domains.

Stat5a/b is constitutively active in human prostate cancer but not in normal prostate epithelium (10, 11). Activation of Stat5a/b in primary prostate cancer predicted early prostate cancer recurrence (12). We have shown in two separate clinical prostate cancer populations that activation of Stat5a/b is associated with high histological grade of prostate cancer (11, 12). Factors that activate Stat5a/b in prostate cancer include prolactin (Prl), a locally produced mitogen in prostate cancer cells (11, 13, 14). Prl expression in prostate cancer, similar to activation of Stat5a/b, is associated with high histological grade of the cancer (11). Transgenic mice overexpressing Prl develop massive prostate enlargement (15-17), and prostates of Prl null mice were smaller than their wild-type counterparts (18). Correspondingly, the prostate acinar epithelium of Stat5a null mice was defective (19).

We have previously shown that adenoviral expression of a dominant-negative (DN) mutant of Stat5a, blocking both Stat5a and Stat5b, induced apoptotic death of human prostate cancer cells in culture (10). This Stat5a mutant lacked the transactivation domain, and thus was able to dimerize and

bind to DNA, but was unable to initiate transcription (10). This finding was later confirmed in TRAMP mouse tumor model using an over-expression model of an analogous truncation mutant of Stat5b that blocked both Stat5a and Stat5b (20). Here, we wanted to establish Stat5a/b as a critical regulator of human prostate tumor growth *in vivo,* and wanted to prove that induction of prostate cancer cell death by inhibition of Stat5a/b is not due to a by-stander effect of over-expression of the

DNStat5a/b protein mutant in prostate cancer cells or to adenoviral gene delivery itself. In order to do this, we set up several different methodological approaches to inhibit Stat5a/b in human prostate cancer cells to demonstrate induction of cell death by suppression of Stat5a/b.

In this work, we show that inhibition of Stat5a/b by antisense oligonuleotides, RNA interference or adenoviral expression of DNStat5a/b all effectively kill prostate cancer cells. Furthermore, we demonstrate that inhibition of Stat5a/b decreases human prostate cancer xenograft tumor growth in nude mice. We show that Stat5a/b regulates $BclX_L$ and Cyclin-D1 protein levels in prostate cancer cells, which likely translates to the Stat5a/b effects on prostate cancer cell viability. In summary, this work establishes that Stat5a/b is highly critical for human prostate cancer cell viability *in vitro* and prostate tumor growth *in vivo*. We propose that Stat5a/b is a potential therapeutic target molecule for prostate cancer.

METHODS

Human prostate cancer cell culture. CWR22Rv and LNCap prostate cancer cells (American Type Culture Collection, Manassas, VA) were grown in RPMI-1640 medium (Biofluids, Rockville, MD). The basal media contained 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 2 mM L-glutamine (Biofluids), 5 mM HEPES (Biofluids), pH 7.3, and penicillin-streptomycin (Biofluids) (50 IU/ml and 50 μg/ml, respectively), at 37°C with 5% CO_2. LNCap cells were cultured in the presence of 0.5 nM dihydrotestosterone (DHT) (5α-androstan-17β-ol-3one; Sigma, St. Louis, MO).

Human prostate cancer xenograft tumors. Castrated male athymic mice were purchased from Taconic (Germantown, NY) and cared for according to the institutional guidelines. Briefly, 20 x 10^6 CWR22Rv cells were mixed with 0.2 ml of Matrigel (BD Bioscience, Palo Alto, CA). One week before the tumor cell inoculation (two sites/mouse), sustained-release testosterone pellets (12.5 mg/pellet, 1 pellet/mouse; Innovative Research of America, Sarasota, FL) were implanted subcutaneously (s.c.). When the tumors reached 15-20 mm in diameter, mice were sacrificed, and the tumor tissues were harvested.

Protein solubilization and immunoblotting. Pellets of prostate cancer cells were solubilized in lysis buffer [10 mM Tris-HCl (pH 7.6), 5 mM EDTA, 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, 1 µg/ml pepstatin A, and 2 µg/ml leupeptin], rotated end-over-end at 4 C for 60 min, and insoluble material pelleted at 12,000 x *g* for 30 min at 4 C. In some of the experiments, protein concentrations of the clarified lysates were determined by a simplified Bradford method (Bio-Rad Laboratories Inc., Hercules, CA) before Western blotting. In Figure 4B, Stat5a and Stat5b were immunoprecipitated 3 h at 4 °C with anti-Stat5a or anti-Stat5b pAb (both 1.2 µg/ml; Advantex Bioreagents, Conroe, TX). Samples were run on a 4–12% SDS-PAGE under reducing conditions. The primary antibodies were used at the following concentrations: anti-Stat5ab mAb (1:250; Transduction Laboratories, Inc., Lexington, KY), anti-BclX_L pAb (1:1000; Cell Signaling, Boston, MA), anti-Cyclin-D1 mAb (1:200; Labvision, Fremont, CA), anti-actin (1:4000; Sigma), anti-phospho-tyrosine-Stat3 pAb (Y705) (1:1000) (Cell Signaling), anti-Stat3 mAb (1:1000) (Santacruz Biotechnologies, Santa Cruz, CA) and detected by horseradish peroxidase-conjugated secondary antibodies in conjunction with enhanced chemiluminescence substrate mixture (Amersham, Piscataway, NJ), and exposed to film.

Stat5a/b antisense transfections. LNCap cells were transfected with Stat5a/b antisense oligodeoxynucleotides (900 pmole, 1050 pmole) with mismatch oligodeoxynucleotides (ODN) as

control using jetPEI™ (QBiogene Inc., Carlsbad, CA) according to manufacturer's instructions. Specifically, Stat5 antisense ODN (5'-GGG CCT GGT CCA TGT ACG TG-3') (a shared sequence within both human Stat5a and Stat5b transcripts) (bp 2153-2173 in open reading frame) were synthesized using a phosphorothioate backbone with 2'-O-methoxyethyl modification of five terminal nucleotides (underlined) to increase their stability (ISIS 130826) as described before (21) (ISIS Pharmaceuticals; Carlsbad, CA). Mismatch ODN for the same chemistry was synthesized as a mixture of all four bases. After 24 h, 48 h, and 72 h the cells were harvested for western blotting, cell viability, and DNA fragmentation assays.

siRNA transfections. The sequence of the Stat5a/b siRNA used was: Sense; r(CCA UAU AUU GUA CAA UGA A) dTdT and antisense; r(UUC AUU GUA CAA UAU AUG G) dGdG targeted to the sequence of Stat5a/b transcript: 5'-CGC CAT ATA TTG TAC AAT GAA -3' (Qiagen Science, Inc., Germantown, MD). The control siRNA (scrambled) target sequence (AAT TCT CCG AAC GTG TCA CGT) (Qiagen Science, Inc.) was screened against the EST Gene bank database. LNCap cells were transfected with Stat5a/b or scrambled control siRNA (70 pmole/well) at 60% confluency using Oligofectamine ™ (Gibco, Grand Island, NY) according to manufacturer's instructions. After 24 h, 48 h, or 72 h, the cells were harvested for western blotting, cell viability and DNA fragmentation assays. In Figure 4B, Stat5a, Stat5b and Stat5a/b were knocked down using commercially available specific Stat5a and Stat5b siRNAs (Dharmacon, Lafayette, CO, USA: catalog numbers: Stat5a; M-005169, Stat5b; M-010539-01-0005).

Generation of adenoviruses for gene delivery of wild-type (WT), dominant-negative (DN) Stat5a/b. pcDNA-CMV-WTStat5b and pcDNA-CMV-DNStat5a/b were cloned into an adenoviral vector using BD Adeno-X™ Expression System 2 (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's protocol. This specific cloning system was chosen because it uses Cre-loxP-mediated recombination, which reduces the likelihood of development of replication-competent adenovirus over time. Briefly, *EcoR*I and *Xba*I digested WTStat5b and DNStat5a/b fragments from

expression vectors of pcDNA-CMV-WTStat5a/b and pcDNA-CMV-DNStat5a/b were subcloned into the *EcoR*I-*Xba*I-digested BD Creator Donor Vector (pDNR-CMV). The expression cassettes were further transferred to BD Adeno-X Acceptor Vector (pLP-Adeno-X-CMV) by Cre-loxP-mediated recombination. The recombinant adenoviruses were purified, linearized by *Pac*I digestion and transfected to QBI-293A cells to produce infectious recombinant adenoviruses. Viral stocks were expanded in large-scale cultures, purified by double cesium chloride gradient centrifugation, and titered side-by-side by a standard plaque assay method in QBI-293A cells as per the manufacturer's instructions.

Adenoviral gene delivery. Adenovirus was delivered to prostate cancer cells in culture by incubation of the cells for 90 min with indicated multiplicity of infection (MOI) of adenovirus expressing wild-type Stat5b (AdWTStat5b), dominant-negative Stat5a/b (AdDNStat5a/b) or beta-galactosidase (AdLacZ) (a gift from Dr. Hallgeir Rui) in serum-free medium at 37°C. Adenovirus was delivered to human prostate xenograft tumors by two different methods. *First*, six hours before subcutaneous inoculation into flanks of castrated nude mice implanted with DHT-pellets (n=5/group, 1 tumor/mouse, 20×10^6 CWR22Rv cells per site), CWR22Rv cells were infected with AdDNStat5a/b, AdWTStat5b or AdLacZ at MOI 5 as described above. Tumor sizes were measured twice a week.

In the second set of experiments, 20×10^6 CWR22Rv cells were injected into right flanks of castrated nude mice implanted with DHT-pellets (n=5/group, 1 tumor/mouse, 20×10^6 CWR22Rv cells per site). When the tumors reached approximately 8 mm in diameter (on day 11), AdDNStat5ab, AdWTStat5b or AdLacZ (2.5×10^6 plaque-forming units/0.2 ml) in plain RPMI 1640 medium was injected every third day (on days 11, 14 and 17) into the tumors. The tumor sizes were measured before each virus injection. The mice were sacrificed and all tumors were harvested on day 20 when the largest tumor in the experiment reached 20 mm in diameter per the IACUC guidelines. Tumor volumes were calculated using the formula (length x width x depth x 0.5236). All tumor growth experiments in nude mice were carried out blind.

Cell viability assay and DNA fragmentation ELISA assay. Cell viability was determined by counting attached cells on a hemacytometer and trypan blue exclusion. Fragmentation of DNA was determined by photometric enzyme immunoassay according to the manufacturers' instructions (cell death detection ELISA^{PLUS}; Roche Molecular Biochemicals, Indianapolis, IN). Briefly, cells were centrifuged at 200 x *g*, and cytoplasmic fractions containing fragmented DNA were transferred to streptavidin-coated microtiter plates that had been incubated with biotinylated monoclonal anti-histone antibody. The amount of fragmented DNA bound to anti-histone antibody was evaluated by peroxidase-conjugated monoclonal anti-DNA antibody using ABTS as a substrate at 405 nm.

Clonogenic survival assay. CWR22Rv were infected with AdDNStat5a/b and AdWTStat5b at MOI 5, with mock-infected cells as an additional control. Infected cells were trypsinized 24 h later, and 100, 200, 400, 800, 1600, and 3200 cells were seeded in triplicate. After 21 days, cells were washed twice with PBS, stained for 30 min in 0.25% crystal violet solution (Sigma), and colonies with more than 30 cells were counted. Plating efficiency (PE) = Colonies counted/cells seeded x (100) and survival fraction (SF) = Colonies counted/cells seeded x (PE/100) per each group was calculated.

Statistical methods. Tumor growth was modeled using mixed effects linear regression analysis. Tumor volumes were log-transformed in order to satisfy the assumption of normality. Separate slopes and intercepts were assumed for each group, and a random intercept term was included to account for correlation among repeated measurements from the same mouse. Stat5a/5b inhibited mice were compared with both control groups with respect to the rate of tumor growth (slopes) and model estimated mean tumor volume at 36 days. All analyses were performed using SAS Version 9.1.3 (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Inhibition of transcription factor Stat5a/b by antisense oligonucleotides or siRNA induces cell death of human prostate cancer cells. Given that the Stat5a/b pathway is highly expressed and active in high grade prostate cancers (11, 12), and that activation of Stat5a/b in primary clinical prostate cancer predicts early disease recurrence (12), we aimed to establish Stat5a/b as a critical survival and potential therapeutic target protein for prostate cancer. We set up Stat5a/b inhibition by several different experimental approaches in prostate cancer cells. First, we tested the efficacy of Stat5a/b antisense oligodeoxynucleotides (ODN) in inducing prostate cancer cell death (Fig. 1A). Transfection of LNCaP cells with antisense ODNs targeted against a homologous region between both Stat5a and Stat5b (21) resulted in a significant decrease in Stat5a and Stat5b protein expression at 48 h demonstrated by Western blotting (Fig. 1A). Stat5a/b inhibition induced significant death of LNCaP cells by 72 hours after transfection as demonstrated by cell morphology (Fig. 1B). Inhibition of Stat5a/b protein expression induced extensive cell rounding, detachment, shrinkage, and blebbing, which are morphological changes consistent with apoptotic cell death. In contrast, there was no evidence of reduced cell viability in response to transfection reagent alone or to mismatch control ODNs. In addition, nucleosomal DNA fragmentation was increased by 4-fold on average in Stat5a/b antisense–treated cells 72 h after the transfection (Fig. 1C). Similar to LNCaP cells, antisense inhibition of Stat5a/b induced cell death in CWR22Rv and DU145 cells (data not shown) which both express active Stat5a/b.

As a second approach, we designed siRNAs to inhibit Stat5a/b protein expression in human prostate cancer cells. Inhibition of Stat5a/b expression in LNCaP cells by Stat5a/b siRNAs, verified by Western blotting at 48 h (Fig. 2A), resulted in induction of morphological characteristics of cell death within 72 h after the transfection (Fig. 2B). Scrambled siRNA was transfected as control. Furthermore, inhibition of Stat5a/b expression by RNA interference induced 3-fold increase in nucleosomal DNA fragmentation at 72 h after transfection indicating cell death caused by Stat5a/b RNA interference

(Fig. 2C). Collectively, these results demonstrate that inhibition of Stat5a/b protein expression by antisense ODNs or RNA interference induces apoptotic death of human prostate cancer cells.

Adenoviral expression of dominant-negative Stat5a/b (DNStat5a/b) inhibits clonogenic survival and growth of CRW22Rv cells. Next, we cloned wild-type-Stat5b (WTStat5b) and dominant-negative-Stat5a/b (DNStat5a/b), which lacks the carboxy-terminal transactivation domain and inhibits both Stat5a and Stat5b, into replication-deficient adenovirus. In this study, we chose an adenoviral vector using Cre-loxP-mediated recombination, reducing the likelihood of replication-competent adenovirus development. Androgen-independent CWR22Rv cells were chosen for this assay because LNCap cells formed colonies poorly in the clonogenic survival assay (data not shown). Infection of CWR22Rv cells with adenovirus expressing beta-Galactosidase (AdLacZ) followed by beta-Galactosidase-staining showed 90-95% transfection efficiency (data not shown). CWR22Rv cells were infected with AdWTStat5b or AdDNStat5a/b, with mock-infected cells as a control, and cells were seeded the next day at different densities for the clonogenic survival assay (Fig. 3A). After 21 days, surviving clones were counted and photographed (Fig. 3A). Inhibition of Stat5a/b resulted in 6-7-fold decrease in the surviving cell fraction compared to mock-infected cells, whereas WTStat5b increased the fraction of surviving cell clones by 30% versus mock-infected cells, and by 20-fold compared to cells in which Stat5a/b was inhibited by AdDNStat5a/b. To test the effect of Stat5a/b inhibition on prostate cancer cell viability in experimental conditions where cells maintain normal cell-cell interactions, CWR22Rv cells were grown at 50% confluence and Stat5a/b was inhibited by adenoviral expression of DNStat5a/b with AdWTStat5a/b and mock-infected cells as controls. The growth curves of the cells were established by determining the number of attached viable cells daily, through manual counting and trypan blue exclusion. On day 6, the number of viable CWR22Rv cells expressing DNStat5a/b reached only 30% of that of mock-infected cells. DNStat5a/b-induced cell death peaked at day 5 (Fig. 3B). In addition, there was approximately 30% more viable cells at each

time point up to the fifth day in the treatment group infected with AdWTStat5b versus mock-infected cells. In conclusion, these results show that adenoviral expression of DNStat5a/b decreases growth and clonogenic survival of human prostate cancer cells in culture. Moreover, the data presented here demonstrate that not only inhibition of Stat5a/b transcriptional activity by DNStat5a/b, but also suppression of Stat5a/b protein expression by Stat5a/b antisense or RNA interference induces rapid apoptotic cell death of human prostate cancer cells. These results are important for establishing the proof-of-principle that Stat5a/b regulates prostate cancer cell viability.

In this study, we inhibited both Stat5 isoforms, Stat5a and Stat5b, collectively in all experimental approaches. Our laboratory is currently in the process of developing new molecular tools for a selective inhibition of Stat5a versus Stat5b, which will allow identification of the individual roles of Stat5a and Stat5b in the promotion of prostate cancer cell growth. This may provide higher specificity for a prostate cancer therapy based on Stat5 inhibition.

All three human prostate cancer cell lines expressing active Stat5a/b, DU145, LNCap and CWR22Rv, responded to Stat5a/b inhibition by cell death (DU145 cell; data not shown), while the viability of PC-3 cells, which are Stat5-negative due to Stat5a/b gene deletion (22) is not affected by Stat5a/b suppression (10). Our findings suggest that Stat5a/b may specifically serve as a survival factor for prostate cancer cells that express Stat5a/b. Therefore, the presence of active Stat5a/b in prostate cancer tissue may serve as a predictive marker for identifying those prostate cancer cases that will respond to a therapy based on inhibition of Stat5a/b. Ongoing studies using immunohistochemical detection of active Stat5 combined with *ex vivo* organ culture testing of clinical primary prostate cancers for Stat5a/b-inhibition will address this question.

Inhibition of Stat5a/b decreases expression of Bcl-X_L and Cyclin-D1 in human prostate cancer cells, but does not affect Stat3 activation or protein levels. To identify mechanisms underlying the critical function of Stat5a/b in the regulation of viability and growth of prostate cancer

cells, we examined whether expression of Bcl-family proteins and Cyclin-D1 are regulated by Stat5a/b. Immunoblotting of LNCaP cell lysates showed a robust decrease in both Bcl-X_L and Cyclin-D1 protein expression at 24 h associated with antisense Stat5a/b inhibition (Fig. 4), while expression of Bcl-2 remained unaffected (data not shown). Re-blotting of the filters with anti-actin antibody shows equal loading of proteins. These results indicate that Stat5a/b promotion of prostate cancer cell viability involves Stat5a/b regulation of Bcl-X_L and Cyclin-D1 expression. Cyclin-D1 and BclX_L represent components of the intracellular growth regulatory mechanisms that drive promotion of growth and viability of prostate cancer cells by Stat5a/b. Future studies using cDNA arrays and bioinformatics are likely to identify additional Stat5a/b-regulated genes in human prostate cancer, which may serve as additional molecular targets for pharmaceutical inhibition of Stat5a/b signaling pathway in prostate cancer. Moreover, this will provide insight to how Stat5a/b regulates prostate cancer cell growth and survival.

To confirm that inhibition of Stat5a/b does not affect the levels or activation of Stat3 in prostate cancer cells, we inhibited Stat5a/b by siRNA in DU145 cells which endogenously express high levels of transcription factor Stat3 in addition to Stat5a/b. As demonstrated in Figure 4B, inhibition of Stat5a, Stat5b or Stat5a/b did not affect the expression or activation of Stat3. These results suggest that the biological effects of Stat5a/b on prostate cancer cell viability are independent of Stat3.

Transcription factor Stat5a/b is critical for human prostate xenograft tumor growth in nude mice. Since inhibition of Stat5a/b induced death of prostate cancer cells in culture, we predicted that Stat5a/b inhibition will decrease prostate cancer xenograft tumor growth *in vivo* in nude mice. To test this hypothesis, we inhibited Stat5a/b by adenoviral expression of DNStat5a/b in CWR22Rv human prostate cancer cells in culture with AdWTStat5b or AdLacZ infected cells as controls. Adenoviral gene delivery was carried out prior (6 h) to inoculation of the cancer cells subcutaneously

into flanks of nude mice. The mice had been castrated and sustained-release 5αDHT (DHT)-pellets were implanted to normalize the circulating androgen levels. Once tumors started to form on day 11, the tumor sizes were measured twice a week until day 36 of the experiment. The mice were sacrificed at that time point because the biggest tumors in the experiment reached 20 mm in diameter. Both incidence and growth of prostate tumors were clearly suppressed in Stat5a/b inhibited group compared to the control groups (Fig. 5A). Specifically, the rate of growth when Stat5 was inhibited was slower than that of both AdLacZ and AdWTStat5b infected cells ($p=0.028$ and 0.0016, respectively). At 36 days, average tumor volume when Stat5 was inhibited was reduced by 65.0% (95% CI: 39.2%-79.8%) when compared with AdLacZ infected cells and 79.6% (95% CI: 64.6%-88.3%) when compared with AdWTStat5b infected cells ($p=0.0011$ and $p<0.0001$).

In the second set of experiments, CWR22Rv tumors were allowed to first grow subcutaneously in castrated nude mice with implanted DHT-pellets. When tumors reached 8 mm in diameter (on day eleven), we injected AdDNStat5a/b into half of the tumors every third day for three consecutive cycles with AdWTStat5b or vehicle (serum-free RPMI1640 medium) as controls. Tumor sizes were measured twice per week. In established human prostate cancer xenograft tumors, inhibition of Stat5a/b attenuated the tumor growth (Fig. 5B). Specifically, the rate of tumor growth for mice injected with AdDNStat5a/b was significantly lower than that of mice injected with AdWtStat5b ($p=0.042$). Moreover, at 20 days, tumors in mice injected with AdDNStat5a/b were 70.1% smaller than tumors in mice injected with AdWtStat5b (95% CI 46.0%-83.4%; $p=0.0003$). In summary, the results presented here show that transcription factor Stat5a/b is critical for for growth of human prostate cancer cells as xenograft tumors *in vivo*.

The findings presented in this work lay the groundwork for development of a therapy for prostate cancer based on inhibition of Stat5. In addition to locally delivered antisense ODNs or

siRNAs in prostate tissue, inhibition of Stat5a/b can be achieved by small-molecule inhibitors for Stat5a/b. The nature of Stat5a/b activity provides multiple levels for rational drug design. First, dimerization of Stat5a/b can be inhibited by targeting the SH2-domain. Second, transactivation of Stat5a/b can be prevented by targeting the C-terminal transactivation domain. Finally, DNA-binding can be blocked by targeting the DNA-binding domain of Stat5a/b (23-28). Since Prl is one of the key factors that activate Stat5a/b in prostate cancer cells (14), Prl-receptor and Jak2 tyrosine kinase may represent additional molecular targets for pharmacological inhibition of a Stat5a/b signaling pathway in prostate cancer. Novel approaches for prostate cancer-specific delivery of pharmacological agents are under development in various laboratories (29-34) which may enable the direct targeting of general Stat5a/b inhibitors to prostate cancer cells. In conclusion, Stat5a/b is critical for prostate cancer cell viability and tumor growth, as established by various experimental approaches.

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FIGURE LEGENDS

Fig. 1. Inhibition of Stat5a/b by antisense oligonucleotides induces rapid apoptotic death of human prostate cancer cells. LNCap cells were transiently transfected with Stat5a/b antisense oligodeoxynucleotides (ODN) or mismatch ODN as control. *A,* At 48 h, whole cell extracts were immunoblotted with a monoclonal anti-Stat5a/b antibody (left panel) and stripped filters were re-blotted with anti-actin antibody to demonstrate equal loading. Densitometric normalization and comparison of Stat5a/b levels is shown in the upper right panel. *B,* Stereomicroscope photographs of Stat5a/b antisense, mismatch ODNs, transfection reagent alone, or non-treated cells 72 h after transfection are shown. The morphology of cell death induced by Stat5a/b antisense ODN in LNCap cells is consistent with apoptosis. *C,* Antisense inhibition of Stat5a/b expression induced DNA fragmentation as demonstrated by nucleosomal ELISA three days after transfection with Stat5a/b antisense or mismatch ODNs. Results are representative of four independent experiments performed in triplicate. Error bars represent mean ±SD.

Fig. 2. Stat5a/b inhibition by RNA interference induces apoptotic death of human prostate cancer cells. LNCap cells were transfected with siRNA targeted to Stat5a/b, with scrambled siRNA, transfection reagent alone, or non-treated cells as controls. *A,* Cells were harvested, lysed and immunoblotted with anti-Stat5a/b monoclonal antibody 48 h after the transfection. Stripped filters were re-blotted with anti-actin antibody to demonstrate equal loading (left panel). Densitometric normalization and comparison of Stat5a/b levels is shown in the left right panel. *B,* Stereomicroscope photographs of LNCap cells 72 h after transfection with Stat5a/b siRNA, scrambled control (control siRNA), media containing transfection reagent, or untreated cells. Inhibition of Stat5a/b by RNA interference induced cell rounding, detachment, shrinkage, and blebbing. *C,* Transfection of LNCap cells with Stat5a/b siRNA, with scrambled siRNA as control, increased DNA fragmentation as quantitated by nucleosomal ELISA. Results representative of four independent experiments performed in triplicate and error bars represent mean ±SD.

Fig. 3. Inhibition of Stat5a/b decreases clonogenic survival of human prostate cancer cells. *A,* CWR22Rv cells were infected with adenovirus expressing dominant-negative Stat5a/b (AdDNStat5) or wild-type Stat5b (AdWTStat5b) at MOI 5, with mock-infected cells as an additional control. For clonogenic survival assay, 100, 200, 400, 800, 1600 and 3200 cells were seeded in triplicate. After 21 days, cells were stained with 0.5% crystal violet and colonies with more than 30 cells were counted. Plating efficiency (PE) = Colonies counted/cells seeded x (100) and survival fraction (SF) = Colonies counted/cells seeded x (PE/100) per each group was calculated (upper panel; bars represent means +/- SD). Photographs of representative wells of mock-, AdWTStat5b- or AdDNStat5a/b-infected CWR22Rv cells seeded at 1600 density (lower panel). *B,* Inhibition of Stat5a/b by adenoviral expression of dominant-negative Stat5a/b (DNStat5a/b) induces death of CWR22Rv human prostate cancer cells. CWR22Rv cells were infected with AdDNStat5a/b, AdWTStat5b or mock-infected at MOI 8 for six days (D1-D6). Attached, viable prostate cancer cells were counted each day. Results are representative of four independent.

Fig. 4. Stat5a/b regulates $BclX_L$ and Cyclin-D1 protein expression in human prostate cancer cells but does not affect Stat3 expression or activation. *A,* Whole cell extracts of LNCap cells, treated either with Stat5a/b antisense olideoxynucleotides (ODN) or with mismatch ODN as controls for 48 h, were immunoblotted with $BcLX_L$ or Cyclin-D1 antibodies. Blots were stripped and re-blotted with anti-actin antibody to demonstrate equal loading. *B,* The expression of Stat5a, Stat5b or both Stat5a and Stat5b were inhibited by siRNA in DU145 cells for 48 h with scrambled siRNA as control and Stat5a and Stat5b were immunoprecipitated and blotted with anti-Stat5ab monoclonal antibody. Whole cell lysates of the same samples were immunoblotted for phospho-Stat3, total Stat3 and actin.

Fig. 5. Inhibition of Stat5 decreases prostate tumor growth in athymic nude mice. *A,* Tumor incidence and growth of CWR22Rv prostate cancer cells infected with adenovirus expressing

dominant-negative Stat5a/b (DNStat5a/b) or wild-type Stat5b (WtStat5b). Stat5a/b was inhibited by adenoviral expression of DNStat5a/b with AdWtStat5b and AdLacZ as controls in CWR22Rv cells in culture at MOI 5. Six hours after infection, the cells were inoculated subcutaneously into flanks of castrated nude mice supplied with sustained-release 5α-dihydrotestosterone (DHT)-pellets (n=5/group, 1 tumor/mouse, 20×10^6 CWR22Rv cells per site). The tumor incidence and growth were measured twice a week for 36 days. Tumor volumes were calculated using the formula (length x width x depth).

B, Growth inhibition of established CWR22Rv tumors in nude mice by injections of adenovirus expressing DNStat5a/b compared to controls. CWR22Rv cells were inoculated subcutaneously into flanks of castrated athymic nude mice (n=5/group, 1 tumor/mouse, 20×10^6 CWR22Rv cells per site) supplied with DHT-pellets (12.5 mg/pellet, 1 pellet/mouse). Once the tumors reached 8 mm in diameter, they were injected with RPMI1640, AdDNStat5a/b or AdWtStat5b at dose of 2.5×10^6 plaque-forming units per tumor every third day for 9 days (days 11, 14 and 17). Tumor volumes were calculated using the formula (length x width x depth x 0.5236).