

## Transcription Factor Signal Transducer and Activator of Transcription 5 Promotes Growth of Human Prostate Cancer Cells *In vivo*

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**Abstract Purpose:** Signal transducer and activator of transcription 5a/b (Stat5a/b) is the key mediator of prolactin effects in prostate cancer cells via activation of Janus-activated kinase 2. Prolactin is a locally produced growth factor in human prostate cancer. Prolactin protein expression and constitutive activation of Stat5a/b are associated with high histologic 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 methodologic 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 Bcl-X<sub>L</sub> and cyclin D1 protein levels was shown 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 oligonucleotides, RNA interference, or adenoviral expression of dominant-negative Stat5a/b effectively kills prostate cancer cells. Moreover, we show that Stat5a/b is critical for human prostate cancer xenograft growth in nude mice. The effects of Stat5a/b on the viability of prostate cancer cells involve Stat5a/b regulation of Bcl-X<sub>L</sub> 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. Pharmacologic inhibition of Stat5a/b in prostate cancer can be achieved by small-molecule inhibitors of transactivation, dimerization, or DNA binding of Stat5a/b.

There are currently no effective pharmacologic 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 pharmacologic treatments

exist for elimination of residual cancer cells after prostate cancer surgery. We propose here transcription factor signal transducer and activator of transcription 5a/b (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 COOH-terminal domain (1) by a tyrosine kinase, typically of the Janus-activated kinase protein family (2, 3), activates Stat5a/b. After phosphorylation, Stat5a and Stat5b homodimerize or heterodimerize 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 NH<sub>2</sub>-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 NH<sub>2</sub>-terminal domain is the coiled-coil domain that 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 IFN- $\gamma$ -activated site

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Received 8/16/07; revised 11/30/07; accepted 12/5/07.

**Grant support:** American Cancer Society grant RSG-04-196-01-MGO, Department of Defense Prostate Cancer grants W81XWH-05-01-0062 and W81XWH-07-1-0411, NIH National Cancer Institute grant 1R01CA113580-01A1, and Pennsylvania Department of Health. Shared Resources of Kimmel Cancer Center are partially supported by NIH grant CA56036-08 (Cancer Center Support Grant, to Kimmel Cancer Center).

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doi:10.1158/1078-0432.CCR-07-2024

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 COOH terminus carries a transactivation domain, which varies considerably in both length and sequence between different Stat family members. The transactivation domain binds critical coactivators and is directly involved in facilitating the initiation of transcription (7, 9). Stat5a has 20 amino acids that are unique in its COOH-terminal sequence, whereas 8 amino acids in the COOH terminus are specific to Stat5b. Furthermore, Stat5b has a five-residue abbreviation of the Stat5a phosphotyrosyl tail segment between the SH2 and transactivation 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 histologic grade of prostate cancer (11, 12). A key factor that activates Stat5a/b in prostate cancer is prolactin, a locally produced mitogen (11, 13, 14). Prolactin expression in prostate cancer, similar to activation of Stat5a/b, is associated with high histologic grade of the cancer (11). Transgenic mice overexpressing prolactin develop massive prostate enlargement (15–17), and prostates of prolactin-null mice were smaller than their wild-type (WT) 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 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 to prove that induction of prostate cancer cell death by inhibition of Stat5a/b is not due to a bystander effect of overexpression of the dominant-negative Stat5a/b (DNStat5a/b) protein mutant in prostate cancer cells or to adenoviral gene delivery itself. To do this, we set up several different methodologic approaches to inhibit Stat5a/b in human prostate cancer cells to show induction of cell death by suppression of Stat5a/b.

In this work, we show that inhibition of Stat5a/b by antisense oligonucleotides, RNA interference, or adenoviral expression of DNStat5a/b effectively kills prostate cancer cells. Furthermore, we show that inhibition of Stat5a/b decreases human prostate cancer xenograft tumor growth in nude mice. We show that Stat5a/b regulates Bcl-X<sub>L</sub> 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.

## Materials and Methods

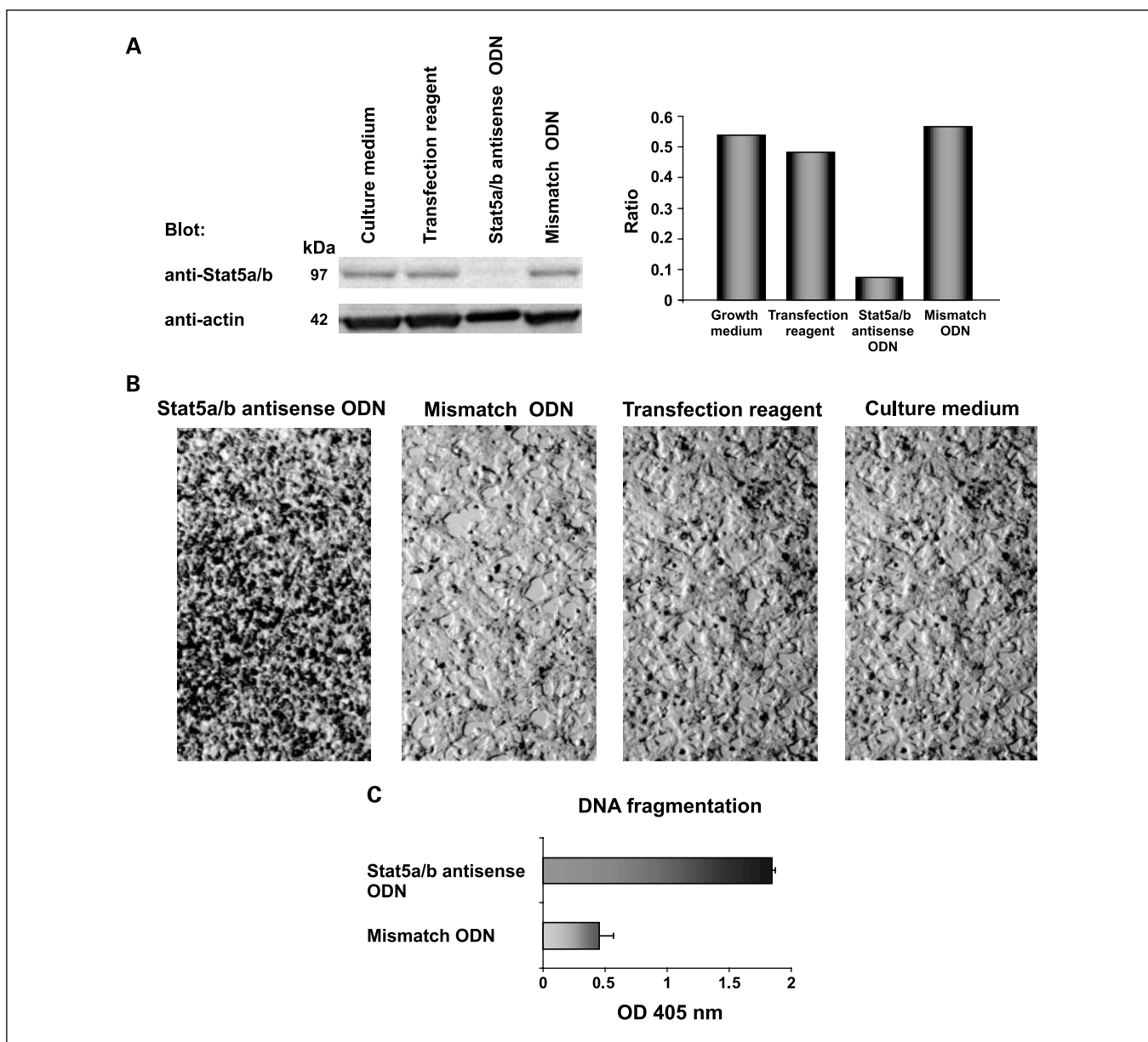
**Human prostate cancer cell culture.** CWR22Rv and LNCaP prostate cancer cells (American Type Culture Collection) were grown in RPMI 1640 (Biofluids). The basal medium contained 10% fetal bovine serum (Atlanta Biologicals), 2 mmol/L L-glutamine (Biofluids), 5 mmol/L HEPES (pH 7.3; Biofluids), and penicillin-streptomycin (50 IU/mL and 50 µg/mL, respectively; Biofluids) at 37°C with 5% CO<sub>2</sub>. LNCaP cells were cultured in the presence of 0.5 nmol/L dihydrotestosterone (DHT; 5α-androstan-17β-ol-3one; Sigma).

**Human prostate cancer xenograft tumors.** Castrated male athymic mice were purchased from Taconic and cared for according to the institutional guidelines. Briefly, 20 × 10<sup>6</sup> CWR22Rv cells were mixed with 0.2 mL Matrigel (BD Biosciences). One week before the tumor cell inoculation (two sites per mouse), sustained-release testosterone pellets (12.5 mg/pellet, 1 pellet per mouse; Innovative Research of America) were implanted s.c. When the tumors reached 15 to 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 mmol/L Tris-HCl (pH 7.6), 5 mmol/L EDTA, 50 mmol/L sodium chloride, 30 mmol/L sodium pyrophosphate, 50 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 5 µg/mL aprotinin, 1 µg/mL pepstatin A, 2 µg/mL leupeptin] and rotated end over end at 4°C for 60 minutes, and insoluble material was pelleted at 12,000 × g for 30 minutes 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.) before Western blotting. In Fig. 4B, Stat5a and Stat5b were immunoprecipitated for 3 hours at 4°C with anti-Stat5a or anti-Stat5b polyclonal antibody (both 1.2 µg/mL; Advantex BioReagents). Samples were run on a 4% to 12% SDS-PAGE under reducing conditions. The primary antibodies were used at the following concentrations [anti-Stat5a/b monoclonal antibody (mAb), 1:250, Transduction Laboratories, Inc.; anti-Bcl-X<sub>L</sub> polyclonal antibody, 1:1,000, Cell Signaling; anti-cyclin D1 mAb, 1:200, Lab Vision; anti-actin, 1:4,000, Sigma; anti-phosphotyrosine Stat3 polyclonal antibody (Y705), 1:1,000, Cell Signaling; and anti-Stat3 mAb, 1:1,000, Santa Cruz Biotechnology], detected by horseradish peroxidase-conjugated secondary antibodies in conjunction with enhanced chemiluminescence substrate mixture (Amersham), and exposed to film.

**Stat5a/b antisense transfections.** LNCaP cells were transfected with Stat5a/b antisense oligodeoxynucleotides (900 and 1,050 pmol) with mismatch oligodeoxynucleotides as control using jetPEI (Qbiogene, Inc.) according to the manufacturer's instructions. Specifically, Stat5 antisense oligodeoxynucleotides (5'-GGGCCTGGTCCATGTACGTG-3'; a shared sequence within both human Stat5a and Stat5b transcripts; 2,153-2,173 bp 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 (ISIS Pharmaceuticals; ref. 21). Mismatch oligodeoxynucleotide for the same chemistry was synthesized as a mixture of all four bases. After 24, 48, and 72 hours, the cells were harvested for Western blotting, cell viability, and DNA fragmentation assays.

**Small interfering RNA transfections.** The sequence of the Stat5a/b small interfering RNA (siRNA) used was r(CCAUUAUUGUACA-AUGAA)dTdT (sense) and r(UUCAUUGUACAUAUUGG)dGdG (antisense) targeted to the sequence of Stat5a/b transcript (5'-CGC-CATATATTGTACAATGAA-3'; Qiagen Science, Inc.). The control siRNA (scrambled) target sequence (AATCTCCGACCGTGTACGGT; Qiagen Science) was screened against the Expressed Sequence Tag Genbank database. LNCaP cells were transfected with Stat5a/b or scrambled control siRNA (70 pmol/well) at 60% confluence using Oligofectamine (Life Technologies) according to the manufacturer's instructions. After 24, 48, or 72 hours, the cells were harvested for Western blotting, cell viability, and DNA fragmentation assays. In Fig. 4B, Stat5a, Stat5b, and



**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 oligodeoxynucleotide as control. *A*, at 48 h, whole-cell extracts were immunoblotted with anti-Stat5a/b mAb (*left*) and stripped filters were reblotted with anti-actin antibody to show equal loading. Right, densitometric normalization and comparison of Stat5a/b levels. *B*, stereomicroscope photographs of Stat5a/b antisense, mismatch oligodeoxynucleotides, transfection reagent alone, or nontreated cells 72 h after transfection. The morphology of cell death induced by Stat5a/b antisense oligodeoxynucleotide in LNCaP cells is consistent with apoptosis. *C*, antisense inhibition of Stat5a/b expression induced DNA fragmentation as shown by nucleosomal ELISA 3 d after transfection with Stat5a/b antisense or mismatch oligodeoxynucleotides. Results are representative of four independent experiments done in triplicate. Columns, mean; bars, SD.

Stat5a/b were knocked down using commercially available specific Stat5a and Stat5b siRNAs (Dharmacon).

**Generation of adenoviruses for gene delivery of WTStat5a/b and DNStat5a/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) 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, *EcoRI*- and *XbaI*-digested WTStat5b and DNStat5a/b fragments from expression vectors of pcDNA-CMV-WTStat5a/b and pcDNA-CMV-DNStat5a/b were subcloned into the *EcoRI/XbaI*-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 *PacI* 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.

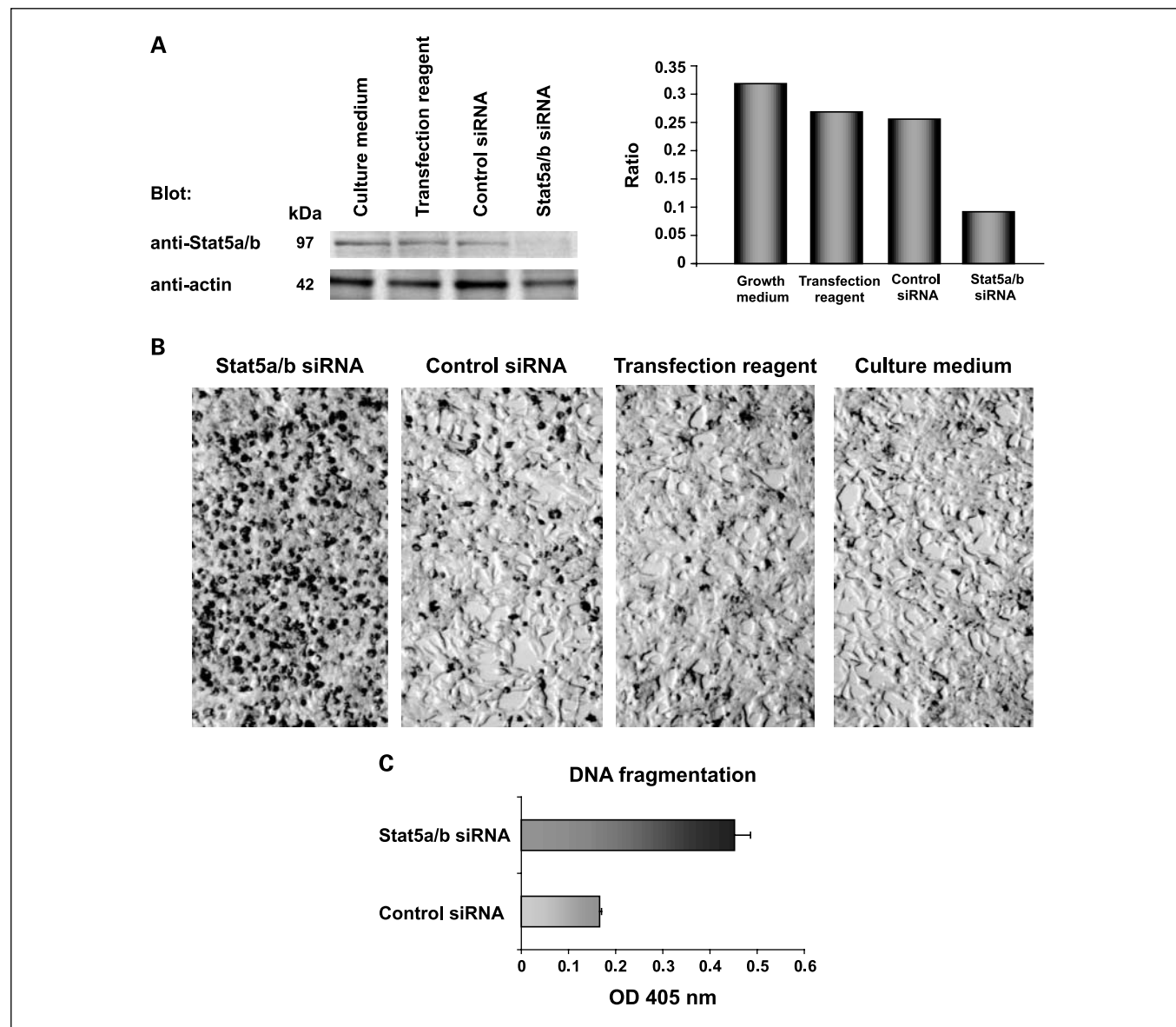
**Adenoviral gene delivery.** Adenovirus was delivered to prostate cancer cells in culture by incubation of the cells for 90 minutes with indicated multiplicity of infection (MOI) of adenovirus expressing WTStat5b (AdWTStat5b), DNStat5a/b (AdDNStat5a/b), or  $\beta$ -galactosidase (AdLacZ;

a gift from Dr. Hallgeir Rui, Department of Cancer Biology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA) in serum-free medium at 37°C. Adenovirus was delivered to human prostate xenograft tumors by two different methods. First, 6 hours before s.c. inoculation into flanks of castrated nude mice implanted with DHT pellets ( $n = 5/\text{group}$ , one tumor per mouse,  $20 \times 10^6$  CWR22Rv cells per site), CWR22Rv cells were infected with AdDN-Stat5a/b, AdWTStat5b, or AdLacZ at MOI 5 as described above. Tumor sizes were measured twice weekly.

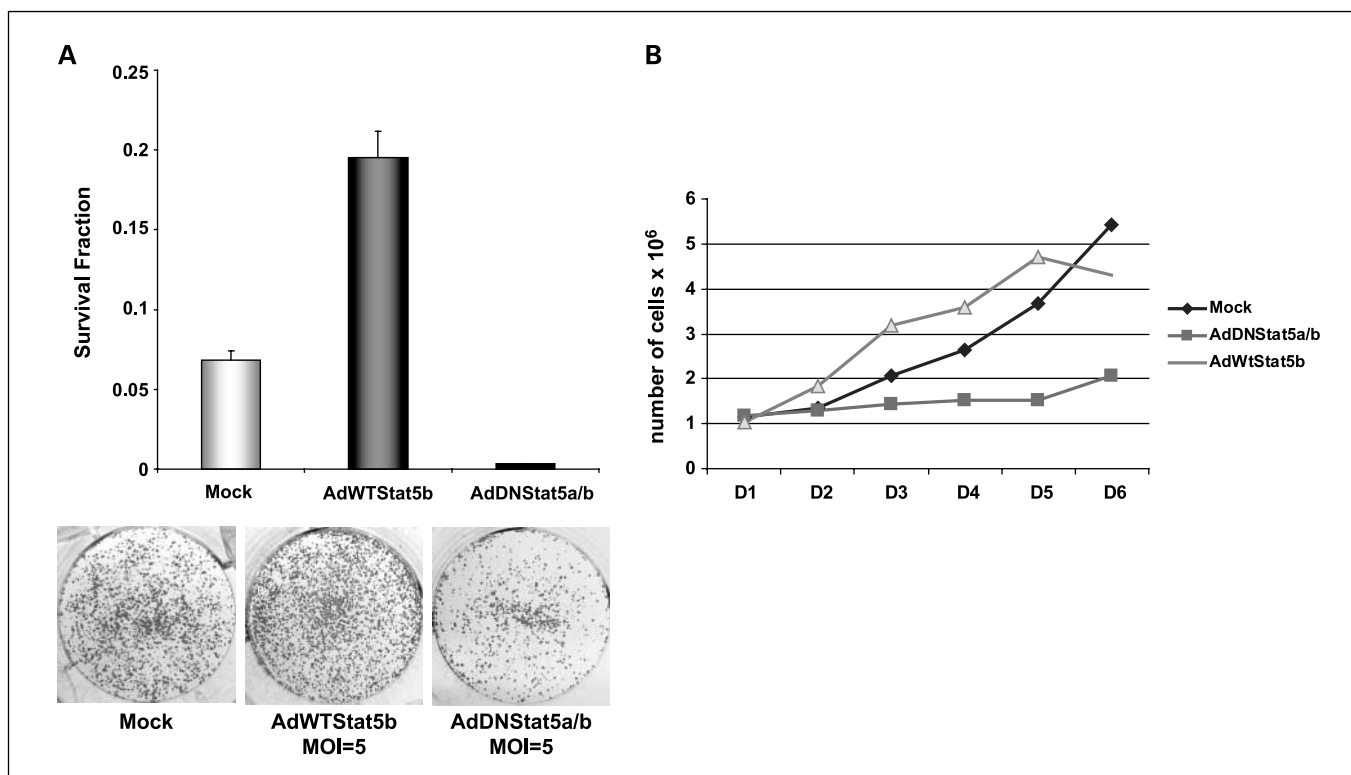
In the second set of experiments,  $20 \times 10^6$  CWR22Rv cells were injected into right flanks of castrated nude mice implanted with DHT pellets ( $n = 5/\text{group}$ , one tumor per mouse,  $20 \times 10^6$  CWR22Rv cells per site). When the tumors reached  $\sim 8$  mm in diameter (on day 11), AdDNStat5a/b, AdWTStat5b, or AdLacZ ( $2.5 \times 10^6$  plaque-forming units/0.2 mL) in plain RPMI 1640 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 Institutional Animal Care and Use Committee guidelines. Tumor volumes were calculated using the following formula: length  $\times$  width  $\times$  depth  $\times$  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<sup>PLUS</sup>, Roche Molecular Biochemicals). Briefly, cells were centrifuged at  $200 \times g$ , and cytoplasmic fractions containing fragmented DNA were transferred to streptavidin-coated microtiter plates that had been incubated with



**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 nontreated cells as controls. **A**, cells were harvested, lysed, and immunoblotted with anti-Stat5a/b mAb 48 h after the transfection. Left, stripped filters were reblotted with anti-actin antibody to show equal loading; right, densitometric normalization and comparison of Stat5a/b levels. **B**, stereomicroscope photographs of LNCaP cells 72 h after transfection with Stat5a/b siRNA, scrambled control (control siRNA), medium 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 are representative of four independent experiments done in triplicate. Columns, mean; bars, SD.



**Fig. 3.** Inhibition of Stat5a/b decreases clonogenic survival of human prostate cancer cells. *A*, CWR22Rv cells were infected with AdDNStat5 or AdWTStat5b at 5 MOI, with mock-infected cells as an additional control. For clonogenic survival assay, 100, 200, 400, 800, 1,600, and 3,200 cells were seeded in triplicate. After 21 d, cells were stained with 0.25% crystal violet and colonies with >30 cells were counted. Plating efficiency (colonies counted / cells seeded  $\times$  100) and survival fraction [colonies counted / cells seeded  $\times$  (plating efficiency / 100)] per each group were calculated. Columns, mean; bars, SD. Bottom, photographs of representative wells of mock-, AdWTStat5b-, or AdDNStat5a/b-infected CWR22Rv cells seeded at 1,600 density. *B*, inhibition of Stat5a/b by adenoviral expression of DNStat5a/b induces death of CWR22Rv human prostate cancer cells. CWR22Rv cells were infected with AdDNStat5a/b and AdWTStat5b or mock infected at MOI 8 for 6 d (D1-D6). Attached viable prostate cancer cells were counted each day. Results are representative of four independent experiments.

biotinylated anti-histone mAb. The amount of fragmented DNA bound to anti-histone antibody was evaluated by peroxidase-conjugated anti-DNA mAb using ABTS as a substrate at 405 nm.

**Clonogenic survival assay.** CWR22Rv cells were infected with AdDNStat5a/b and AdWTStat5b at 5 MOI, with mock-infected cells as an additional control. Infected cells were trypsinized 24 hours later, and 100, 200, 400, 800, 1,600, and 3,200 cells were seeded in triplicate. After 21 days, cells were washed twice with PBS and stained for 30 minutes in 0.25% crystal violet solution (Sigma), and colonies with >30 cells were counted. Plating efficiency (colonies counted / cells seeded  $\times$  100) and survival fraction [colonies counted / cells seeded  $\times$  (plating efficiency / 100)] per each group were calculated.

**Statistical methods.** Tumor growth was modeled using mixed-effects linear regression analysis. Tumor volumes were log transformed 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 done using Statistical Analysis System version 9.1.3 (SAS Institute).

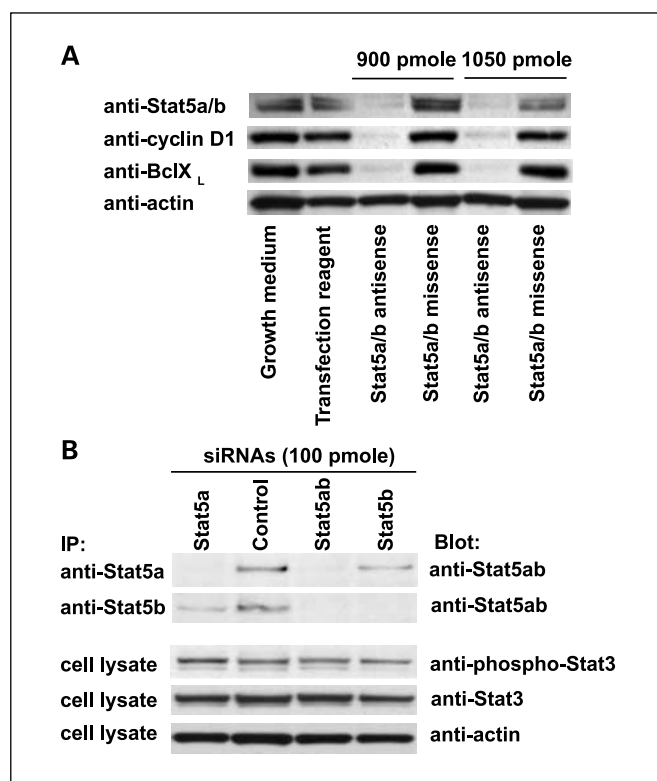
## Results and Discussion

**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 in inducing prostate cancer cell death (Fig. 1A). Transfection of LNCaP cells with antisense oligodeoxynucleotides targeted against a homologous region between both Stat5a and Stat5b (21) resulted in a significant decrease in Stat5a and Stat5b protein expression at 48 hours as shown by Western blotting (Fig. 1A). Stat5a/b inhibition induced significant death of LNCaP cells by 72 hours after transfection as shown by cell morphology (Fig. 1B). Inhibition of Stat5a/b protein expression induced extensive cell rounding, detachment, shrinkage, and blebbing, which are morphologic 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 oligodeoxynucleotides. In addition, nucleosomal DNA fragmentation was increased by 4-fold on average in Stat5a/b antisense-treated cells 72 hours 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 hours (Fig. 2A), resulted in induction of morphologic characteristics of cell death within 72 hours 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 hours after transfection, indicating cell death caused by Stat5a/b RNA interference (Fig. 2C). Collectively, these results show that inhibition of Stat5a/b protein expression by antisense oligodeoxynucleotides or RNA interference induces apoptotic death of human prostate cancer cells.

**Adenoviral expression of DNStat5a/b inhibits clonogenic survival and growth of CWR22Rv cells.** Next, we cloned WTStat5b and DNStat5a/b, which lack the COOH-terminal transactivation domain and inhibit 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).



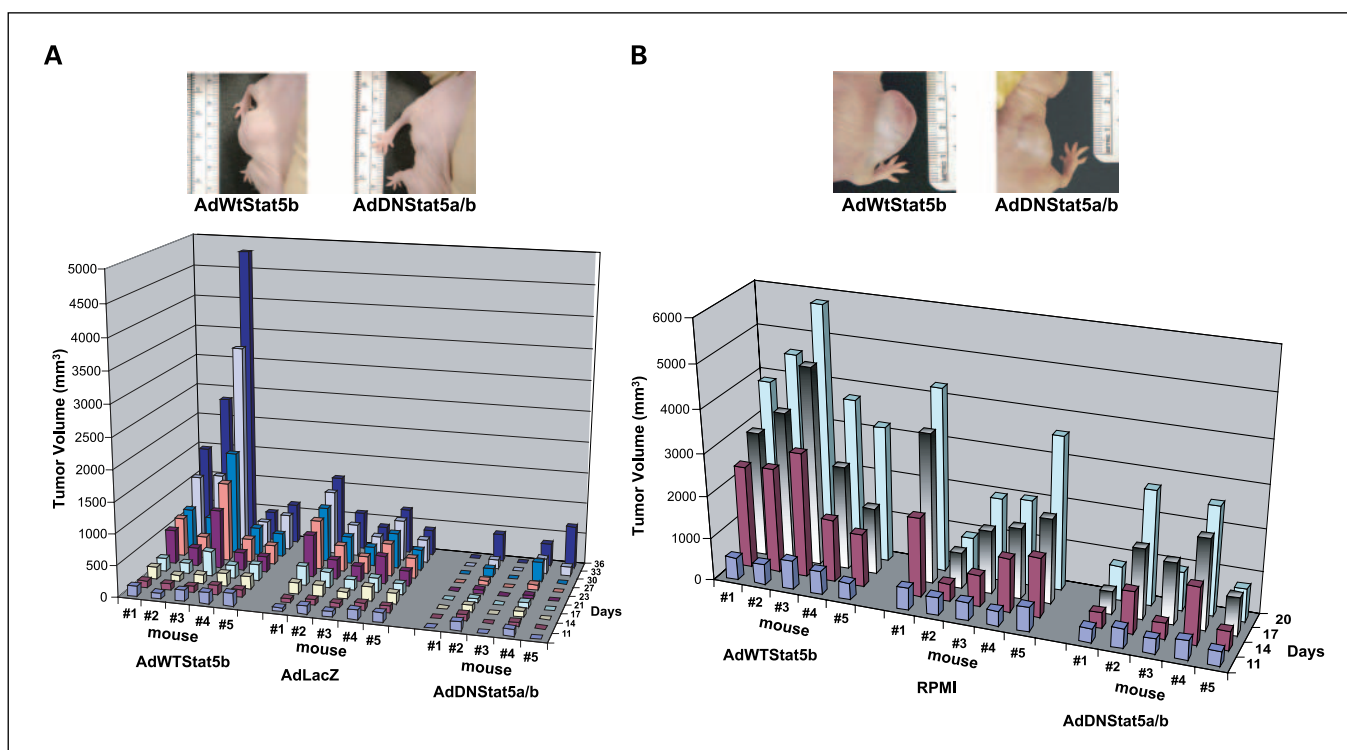
**Fig. 4.** Stat5a/b regulates Bcl-X<sub>L</sub> and cyclin D1 protein expression in human prostate cancer cells but does not affect Stat3 expression or activation. **A**, whole-cell extracts of LNCaP cells, treated either with Stat5a/b antisense oligodeoxynucleotides or with mismatch oligodeoxynucleotide as controls for 48 h, were immunoblotted with Bcl-X<sub>L</sub> or cyclin D1 antibodies. Blots were stripped and reblotted with anti-actin antibody to show equal loading. **B**, the expression of Stat5a, Stat5b, or both Stat5a and Stat5b was inhibited by siRNA in DU145 cells for 48 h, with scrambled siRNA as control, and Stat5a and Stat5b were immunoprecipitated (IP) and blotted with anti-Stat5a/b mAb. Whole-cell lysates of the same samples were immunoblotted for phosphorylated Stat3 (phospho-Stat3), total Stat3, and actin.

Infection of CWR22Rv cells with adenovirus expressing  $\beta$ -galactosidase (AdLacZ) followed by  $\beta$ -galactosidase staining showed 90% to 95% transfection efficiency (data not shown). CWR22Rv cells were infected with AdWTStat5b or AdDN-Stat5a/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- to 7-fold decrease in the surviving cell fraction compared with mock-infected cells, whereas WTStat5b increased the fraction of surviving cell clones by 30% versus mock-infected cells, and by 20-fold compared with 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 ~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 show 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), whereas 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<sub>L</sub> and cyclin D1 in human prostate cancer cells but does not affect Stat3 activation or protein levels.** To identify mechanisms underlying



**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 DNStat5a/b or WTStat5b. Stat5a/b was inhibited by adenoviral expression of DNStat5a/b with AdWTStat5b and AdLacZ as controls in CWR22Rv cells in culture at 5 MOI. Six hours after infection, the cells were inoculated s.c. into flanks of castrated nude mice supplied with sustained-release DHT pellets ( $n = 5$ /group, one tumor per mouse,  $20 \times 10^6$  CWR22Rv cells per site). The tumor incidence and growth were measured twice weekly for 36 d. Tumor volumes were calculated using the following formula: length  $\times$  width  $\times$  depth. *B*, growth inhibition of established CWR22Rv tumors in nude mice by injections of adenovirus expressing DNStat5a/b compared with controls. CWR22Rv cells were inoculated s.c. into flanks of castrated athymic nude mice ( $n = 5$ /group, one tumor per mouse,  $20 \times 10^6$  CWR22Rv cells per site) supplied with DHT pellets (12.5 mg/pellet, one pellet per mouse). Once the tumors reached 8 mm in diameter, they were injected with RPMI 1640, AdDNStat5a/b, or AdWTStat5b at a dose of  $2.5 \times 10^6$  plaque-forming units/tumor every third day for 9 d (days 11, 14, and 17). Tumor volumes were calculated using the following formula: length  $\times$  width  $\times$  depth  $\times$  0.5236.

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 is regulated by Stat5a/b. Immunoblotting of LNCaP cell lysates showed a robust decrease in both Bcl-X<sub>L</sub> and cyclin D1 protein expression at 24 hours associated with antisense Stat5a/b inhibition (Fig. 4), whereas expression of Bcl-2 remained unaffected (data not shown). Reblotting 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<sub>L</sub> and cyclin D1 expression. Cyclin D1 and Bcl-X<sub>L</sub> 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 as 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 that endogenously express high levels of transcription factor Stat3 in addition to Stat5a/b. As shown in Fig. 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.** Because 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 before (6 hours) inoculation of the cancer cells s.c. into flanks of nude mice. The mice had been castrated and sustained-release DHT pellets were implanted to normalize the circulating androgen levels. Once tumors started to form on day 11, the tumor sizes were measured twice weekly 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 with 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% confidence interval, 39.2-79.8%) when compared with AdLacZ-infected cells and 79.6% (95% confidence interval, 64.6-88.3%) when compared with AdWT-Stat5b-infected cells ( $P = 0.0011$  and  $P < 0.0001$ ).

In the second set of experiments, CWR22Rv tumors were allowed to first grow s.c. in castrated nude mice with implanted DHT pellets. When tumors reached 8 mm in diameter (on day 11), we injected AddNStat5a/b into half of the tumors every third day for three consecutive cycles with AdWTStat5b or vehicle (serum-free RPMI 1640) as controls. Tumor sizes were measured twice weekly. 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% smaller than tumors in mice injected with AdWTStat5b (95% confidence interval, 46.0-83.4%;  $P = 0.0003$ ). In summary, the results presented here show that transcription factor Stat5a/b is critical 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 oligodeoxynucleotides 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 COOH-terminal transactivation domain. Finally, DNA binding can be blocked by targeting the DNA-binding domain of Stat5a/b (23–28). Because prolactin is one of the key factors that activate Stat5a/b in prostate cancer cells (14), prolactin receptor and Janus-activated kinase 2 tyrosine kinase may represent additional molecular targets for pharmacologic inhibition of a Stat5a/b signaling pathway in prostate cancer. Novel approaches for prostate cancer-specific delivery of pharmacologic 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.

## Acknowledgments

We thank Dr. Hallgeir Rui for the plasmid encoding DNStat5a/b, Dr. Munkhtsegseg Dorj for help in immunostainings and tumor growth experiments in nude mice, and Jacqueline Lutz for critical reading of the manuscript.

## References

- Ihle JN. The Stat family in cytokine signaling. *Curr Opin Cell Biol* 2001;13:211–7.
- Rui H, Djeu JY, Evans GA, Kelly PA, Farrar WL. Prolactin receptor triggering. Evidence for rapid tyrosine kinase activation. *J Biol Chem* 1992;267:24076–81.
- Rui H, Lebrun JJ, Kirken RA, Kelly PA, Farrar WL. JAK2 activation and cell proliferation induced by antibody-mediated prolactin receptor dimerization. *Endocrinology* 1994;135:1299–306.
- John S, Vinkemeier U, Soldaini E, Darnell JE, Jr., Leonard WJ. The significance of tetramerization in promoter recruitment by Stat5. *Mol Cell Biol* 1999;19:1910–8.
- Chen X, Vinkemeier U, Zhao Y, et al. Crystal structure of a tyrosine phosphorylated STAT-1 dimer bound to DNA. *Cell* 1998;93:827–39.
- Becker S, Groner B, Muller CW. Three-dimensional structure of the Stat3 $\beta$  homodimer bound to DNA. *Nature* 1998;394:145–51.
- Levy DE, Darnell JE, Jr. Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol* 2002;3:651–62.
- Kisseleva T, Bhattacharya S, Braunstein J, Schindler CW. Signaling through the JAK/STAT pathway, recent advances and future challenges. *Gene* 2002;285:1–24.
- Darnell JE, Jr. STATs and gene regulation. *Science* 1997;277:1630–5.
- Ahonen TJ, Xie J, LeBaron MJ, et al. Inhibition of transcription factor Stat5 induces cell death of human prostate cancer cells. *J Biol Chem* 2003;278:27287–92.
- Li H, Ahonen TJ, Alanen K, et al. Activation of signal transducer and activator of transcription 5 in human prostate cancer is associated with high histological grade. *Cancer Res* 2004;64:4774–82.
- Li H, Zhang Y, Glass A, et al. Activation of signal transducer and activator of transcription-5 in prostate cancer predicts early recurrence. *Clin Cancer Res* 2005;11:5863–8.
- Ahonen TJ, Harkonen PL, Rui H, Nevalainen MT. PRL signal transduction in the epithelial compartment of rat prostate maintained as long-term organ cultures *in vitro*. *Endocrinology* 2002;143:228–38.
- Dagvadorj A, Collins S, Jomain JB, et al. Autocrine prolactin promotes prostate cancer cell growth via Janus kinase-2-signal transducer and activator of transcription-5a/b signaling pathway. *Endocrinology* 2007;148:3089–101.
- Wennbo H, Kindblom J, Isaksson OG, Tornell J. Transgenic mice overexpressing the prolactin gene develop dramatic enlargement of the prostate gland. *Endocrinology* 1997;138:4410–5.
- Kindblom J, Dillner K, Sahlin L, et al. Prostate hyperplasia in a transgenic mouse with prostate-specific expression of prolactin. *Endocrinology* 2003;144:2269–78.
- Kindblom J, Dillner K, Ling C, Tornell J, Wennbo H. Progressive prostate hyperplasia in adult prolactin transgenic mice is not dependent on elevated serum androgen levels. *Prostate* 2002;53:24–33.
- Steger RW, Chandrashekar V, Zhao W, Bartke A, Horseman ND. Neuroendocrine and reproductive functions in male mice with targeted disruption of the prolactin gene. *Endocrinology* 1998;139:3691–5.
- Nevalainen MT, Ahonen TJ, Yamashita H, et al. Epithelial defect in prostates of Stat5a-null mice. *Lab Invest* 2000;80:993–1006.
- Kazansky AV, Spencer DM, Greenberg NM. Activation of signal transducer and activator of transcription 5 is required for progression of autochthonous prostate cancer: evidence from the transgenic adenocarcinoma of the mouse prostate system. *Cancer Res* 2003;63:8757–62.
- Behbod F, Nagy ZS, Stepkowski SM, et al. Specific inhibition of Stat5a/b promotes apoptosis of IL-2-responsive primary and tumor-derived lymphoid cells. *J Immunol* 2003;171:3919–27.
- Clark J, Edwards S, Feber A, et al. Genome-wide screening for complete genetic loss in prostate cancer by comparative hybridization onto cDNA microarrays. *Oncogene* 2003;22:1247–52.
- Turkson J, Ryan D, Kim JS, et al. Phosphotyrosyl peptides block Stat3-mediated DNA binding activity, gene regulation, and cell transformation. *J Biol Chem* 2001;276:45443–55.
- Turkson J, Kim JS, Zhang S, et al. Novel peptidomimetic inhibitors of signal transducer and activator of transcription 3 dimerization and biological activity. *Mol Cancer Ther* 2004;3:261–9.
- Jing N, Sha W, Li Y, Xiong W, Tweardy DJ. Rational drug design of G-quartet DNA as anti-cancer agents. *Curr Pharm Des* 2005;11:2841–54.
- Jing N, Zhu Q, Yuan P, et al. Targeting signal transducer and activator of transcription 3 with G-quartet oligonucleotides: a potential novel therapy for head and neck cancer. *Mol Cancer Ther* 2006;5:279–86.
- Jing N, Li Y, Xiong W, et al. G-quartet oligonucleotides: a new class of signal transducer and activator of transcription 3 inhibitors that suppresses growth of prostate and breast tumors through induction of apoptosis. *Cancer Res* 2004;64:6603–9.
- Song H, Wang R, Wang S, Lin J. A low-molecular-weight compound discovered through virtual database screening inhibits Stat3 function in breast cancer cells. *Proc Natl Acad Sci U S A* 2005;102:4700–5.
- Isaacs JT. New strategies for the medical treatment of prostate cancer. *BJU Int* 2005;96 Suppl 2:35–40.
- Denmeade SR, Isaacs JT. The SERCA pump as a therapeutic target: making a "smart bomb" for prostate cancer. *Cancer Biol Ther* 2005;4:14–22.
- Denmeade SR, Jakobsen CM, Janssen S, et al. Prostate-specific antigen-activated thapsigargin prodrug as targeted therapy for prostate cancer. *J Natl Cancer Inst* 2003;95:990–1000.
- Denmeade SR, Litvinov I, Sokoll LJ, Lijja H, Isaacs JT. Prostate-specific antigen (PSA) protein does not affect growth of prostate cancer cells *in vitro* or prostate cancer xenografts *in vivo*. *Prostate* 2003;56:45–53.
- Mhaka A, Denmeade SR, Yao W, Isaacs JT, Khan SR. A 5-fluorodeoxyuridine prodrug as targeted therapy for prostate cancer. *Bioorg Med Chem Lett* 2002;12:2459–61.
- Jakobsen CM, Denmeade SR, Isaacs JT, et al. Design, synthesis, and pharmacological evaluation of thapsigargin analogues for targeting apoptosis to prostatic cancer cells. *J Med Chem* 2001;44:4696–703.