Inhibition of Transcription Factor Stat5 Induces Cell Death of Human Prostate Cancer Cells

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Abbreviated Title: Stat5 inhibition causes apoptosis of prostate cancer cells

Abbreviations:

Stat5 - Signal Transducer and Activator of Transcription-5
DN- dominant-negative
WT - wild-type
Prl - prolactin
MOI - multiplicity of infection
DHT - dihydrotestosterone
RT - room temperature

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ABSTRACT

Identifying regulators of prostate cancer cell survival may lead to new therapeutic strategies for prostate cancer. We now report prevalent activation of transcription factor Stat5 in human prostate cancer and provide novel evidence that blocking activation of Stat5 in human prostate cancer cells leads to extensive cell death. Specifically, Stat5 was activated in 65% of human prostate cancer specimens examined based on nuclear location of tyrosine phosphorylated Stat5. Adenoviral gene delivery of a dominant-negative Stat5 mutant (DNStat5), but not wild-type Stat5, induced cell death of both the androgen-independent human prostate cancer cell line CWR22Rv and the androgen-sensitive LnCap cell line. Endogenous Stat5 was active in both CWR22Rv and LnCap cells. In contrast, only low levels of inactive Stat5 proteins were detected in the PC-3 cell line, which correlated with resistance to DNStat5-induced cell death. In CWR22Rv and LnCap, cells inhibition of Stat5 by expression of DNStat5 induced apoptotic cell death, as judged from morphological changes, DNA fragmentation, and Caspase-3 activation, with evidence of a Caspase-9 dependent mechanism. We propose that blocking Stat5 function may represent a novel therapeutic approach for prostate cancer.
INTRODUCTION

Prostate cancer typically progresses to androgen-independent growth after androgen-ablation therapy. Identification of androgen-independent proteins that control prostate cancer cell survival may lead to more effective therapies. Using long-term organ cultures of human and rat prostate tissues, we have documented direct effects of prolactin (Prl) as a mitogen and survival factor for prostate epithelium (1-4). These observations have been complemented by demonstration of massive hyperplasia of prostates in transgenic mice over-expressing Prl (5,6), as well as reduced prostate sizes in Prl-null mice (7). Importantly, we have also shown local production of Prl (1,8) and expression of Prl-receptors in prostate epithelium (1,9) and, thereby, provided evidence for an autocrine loop of Prl action in prostate. Downstream of Prl-receptor activation, we have demonstrated that Stat5 is a key signaling protein in normal rat prostate epithelium (10), and that deficiency of Stat5 in Stat5a-null mice is associated with defective prostate tissue architecture (11).

Stat5 is one of 7 members of the Stat family of transcription factors in mammals (12), and consists of two distinct, but highly homologous, gene products; the 94-kDa Stat5a and 92-kDa Stat5b (13,14). In response to Prl, Stat5a and Stat5b become activated by phosphorylation on residue Tyr694 and Tyr 699, respectively, in the C-terminal domain predominantly by Janus tyrosine kinase-2 (Jak2), which is preassociated with the cytoplasmic domain of the Prl receptor (15). Tyrosine phosphorylated Stat5 proteins dimerize and translocate to the nucleus, where they bind to specific response elements of target gene promoters to regulate transcription (16).
Based on our previous findings of a survival function of Prl in normal prostate epithelium (4), an autocrine production of Prl by prostate epithelial cells (1,8), and the glandular defect of the prostates of Stat5a knock-out mice (11), we hypothesized that Stat5 may act as a survival protein in human prostate cancer, and that blocking Stat5 function could induce death of prostate cancer cells. We investigated activation of Stat5 in 40 human prostate cancer samples by immunohistochemical analysis using activation-state specific anti-phosphoTyrStat5 antibody. To specifically block Stat5 activity in human prostate cancer cells, we created a dominant-negative mutant of Stat5 (DNStat5) in an adenoviral transfer vector. In this work we demonstrate that transcription factor Stat5 is activated in a significant number of human prostate cancer specimens and that blocking Stat5 activity induces extensive apoptosis of prostate cancer cells.
MATERIALS AND METHODS

Cell Culture. CWR22Rv, LnCap, and PC-3 cells (ATCC, Manassas, VA) were cultured in RPMI-1640 medium (Biofluids, Gaithersburg, MD) and T47D cells (ATCC) in DMEM containing 10% fetal calf serum, 2 mM L-glutamine, and penicillin-streptomycin (50 IU/ml and 50 µg/ml, respectively) at 37 ºC with 5% CO₂. LnCap cells were cultured in the presence of 1 nM dihydrotestosterone (5±-androstan-17²-ol-3-one; Sigma, Chemical Co., MO).

Immunohistochemical Detection of Phosphorylated Stat5 in Human Prostate Cancer. Tissue sections of formalin-fixed prostate cancer samples from 40 patients were immunostained for activated Stat5 as described previously (17). Briefly, tissue sections were de-paraffinized in xylene followed by re-hydration in graded alcohol. To unmask the epitopes, the slides were microwave-treated with antigen retrieval solution AxAR1 (Advantex BioReagents, Conroe, TX) or AxAR2 for use with anti-pTyrStat5 mAb or anti-panStat5 mAb, respectively. Endogenous peroxidase activity was blocked by incubating the slides in 0.3% hydrogen peroxide, and the tissue sections were incubated in normal goat serum (BioGenex Laboratories Inc., San Ramon, CA) for 2 h to block unspecific binding of immunoglobulins (IgGs). The anti-pTyrStat5 mAb was diluted in 1% BSA in PBS at a final concentration of 0.6 ¼g/ml (Advantex BioReagents). The anti-panStat5 mAb was used at a concentration of 2 ¼g/ml (Advantex Bioreagents). Antigen-antibody complexes were detected using anti-mouse biotinylated goat secondary antibody followed by streptavidin-horseradish-peroxidase complex (BioGenex). 3,3’-diaminobenzidine was used as chromogen and hematoxylin as counterstain. For negative controls, parallel slides were immunostained with subtype-specific mouse IgG, and lactating
human mammary gland was used as positive control tissue (17).

**Generation of adenovirus for gene delivery of dominant-negative and wild-type Stat5.**

Expression vector for murine Stat5a (pXM-Stat5a) was kindly provided by Xiwen Liu and Lothar Hennighausen (National Institutes of Health, Bethesda, MD) (14). A dominant-negative (DN) variant of Stat5 (Stat5aΔ713) was derived by truncation after amino acid residue Ala713 of pXM-Stat5a, using a PCR fragment generated using 5-TAATACGACTCACTATAGGG-3 (sense) and 5-GCTCTAGACTAGGCATCTGTGGATGCATTG-3 (antisense) primers, followed by EcoRI and XbaI digestion, and subcloning into the EcoRI-XbaI-digested pXM-Stat5a. The DNA sequence of the resulting construct pXM-Stat5aΔ713 was verified before use. The ability of our DNStat5 (Stat5aΔ713) expression construct to completely suppress both Stat5a and Stat5b-mediated transcriptional activation has been reported (18). Replication-defective human adenovirus (Ad5) carrying wild-type Stat5 (WTStat5) or DNStat5 was generated using the AdEasy Vector system (Qbiogene, Carlsbad, CA). The open reading frame sequences of DNStat5 and WTStat5 were released from respective plasmids by 1) digestion with EcoRI, 2) blunt-ending by Klenow DNA polymerase, and 3) digestion with HindIII, and the resulting fragments were subcloned into the Klenow DNA polymerase blunt-ended BglII site and the unmodified HindIII site of the pShuttle-CMV transfer vector. Homologous recombination of WTStat5 or DNStat5 transfer vectors with the pAdEasy vector was performed in BJ5183 E. coli by electroporation. Recombined clones were screened by Kanamycin-resistant growth, and confirmed by PacI digestion to yield two bands of 30 kb and 4.5 kb. The recombinant viruses were packaged in QBI-293A cells and resulting clones were selected from plaques and
amplified. Expression of WTStat5 and DNStat5 from adenoviral stocks was verified by Western blotting using an anti-panStat5 antibody (Transduction Laboratories, Lexington, KY). Selected recombinant 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.

**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 phenylmethylsulfonylfluoride, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, and 2 μg/ml leupeptin). Cell lysates were rotated end-over-end at 4 °C for 60 min, and insoluble material was pelleted at 12,000 x g for 30 min at 4 °C. For immunoprecipitations, the protein concentrations of clarified cell lysates were determined by simplified Bradford method (Bio-Rad Laboratories, Hercules, CA). The proteins were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting using polyvinylidene difluoride membranes (Millipore, Bedford, MA). Blots were exposed overnight to primary antibodies diluted in blocking buffer at the following concentrations: anti-phosphotyrosine-Stat5a/b (Y694/Y699) mAb (Advantex BioReagents, TX, Conroe; 1 μg/ml), anti-panStat5mAb (Transduction Laboratories, Inc., Lexington, KY; 1 μg/ml) or polyclonal antisera to Stat5a (1:3,000) or Stat5b (1:3,000). The blots were washed in washing buffer [50 mM Tris-HCl (pH 7.6), 200 mM NaCl, 0.25% Tween 20], and incubated with horseradish peroxidase-conjugated goat antibodies to mouse or rabbit IgG (Transduction Laboratories) (5 μg/ml). Enhanced chemiluminescence substrate (Amersham Pharmacia Biotech, Piscataway, NJ) was used according to the manufacturer’s instructions for
antibody detection.

**Cell Viability Assay.** Cell viability was determined by a colorimetric assay in which a tetrazolium compound is bioreduced by cells into a colored formazan product in direct proportion to the number of living cells in culture (Promega, WI). Prostate cancer cells were plated on 96-well plates (10,000 cells/well) and infected the next day with different MOIs (multiplicity of infection) of adenovirus carrying either WTStat5, DNStat5 or LacZ. Cells infected with empty control virus or mock-infected cells served as additional controls. Three days after the infection the assay was performed according to the manufacturer’s instructions and the absorbance was recorded at 490 nm.

**Cell Death Elisa Assay.** Fragmentation of DNA after cell death induced by inhibition of transcription factor Stat5 was determined by photometric enzyme-immunoassay (Cell Death Detection ELISA PLUS; Roche Molecular Biochemicals, Indianapolis, IN). Prostate cancer cells were plated on 6-well plates and infected next morning with adenovirus carrying either DNStat5, WTStat5, LacZ, or with empty control virus. Cells were scraped and 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 of nucleosomes bound to anti-histone antibody was evaluated by peroxidase conjugated monoclonal anti-DNA antibody using ABTS as a substrate at 405 nm.

**Flow Cytometry.** CWR22Rv and LnCap cells (1 x 10^6 cells/sample) were washed once in PBS, trypsinized, pelleted at 1,000 x g, and washed once in 5 ml of cold PBS. After a second centrifugation, cells were resuspended in 0.5 ml of cold PBS and fixed by dropwise addition of
1.5 ml cold 100% ethanol, while slowly vortexing the cell suspension. After having been fixed for 1 h at 4 °C, cells were stained with 100 μg/ml propidium iodide (Roche Molecular Biochemicals) and treated with RNase A (Invitrogen, Carlsbad, CA) for 30 min at 37 °C. The cells were analyzed by flow cytometry using a Coulter EPICS XL cell analyzer (Beckman-Coulter, Brea, CA).

**Caspase-3 and Caspase-9 Activity Assays.** Activation of Caspase-3 (Roche Molecular Biochemicals) and Caspase-9 (Oncogene Research Products, Boston, MA) were determined by fluorometric enzyme assays. Prostate cancer cells were plated on 6-well plates and infected next morning with adenovirus carrying either DNStat5, WTStat5, LacZ or with empty control virus. Cells were scraped and centrifuged at 500 x g, and cytoplasmic fractions containing activated Caspases were transferred to microtiter plates. Caspase-3 substrate (DEVD) or Caspase-9 substrate (LEHD) labeled with the fluorescent molecule 7-amino-4-trifluoromethyl coumarin (AFC) were added to the samples. Free fluorescent AFC was generated proportionally to the amount of activated Caspase in the cell lysate due to proteolytic cleavage of the substrate and determined fluorometrically at 505 nm.

**RESULTS AND DISCUSSION**

**Stat5 is frequently activated in human prostate cancer.** We have recently described a highly sensitive *in situ* detection method for activation of Stat5 in paraffin embedded tissue which is based on immunohistochemical detection of phosphorylated Stat5 that is localized within the cell nucleus (10,17). This method, complemented with immunohistochemical detection of nuclear Stat5 protein, was applied to a material of 40 human prostate cancer specimens, and significant
activation of Stat5 was detected in 65% (26 of 40) of primary human prostate cancer specimens of various Gleason scores. Representative samples illustrating Stat5 activation by either phosphotyrosine detection, or nuclear anti-Stat5 detection, within malignant human prostate epithelia are presented (Fig. 1A, panels a and b, respectively). In contrast, Stat5 phosphotyrosine staining was negative (Fig. 1A, panel c) and nuclear immunostaining for Stat5 was absent (data not shown) in adjacent normal secretory prostate epithelial cells. Lactating human breast epithelium is presented as a positive control for tyrosine phosphorylated, activated human Stat5 (17), and parallel sections of lactating human breast stained with subtype-specific mouse IgG were negative (Fig. 1A, panels d and e, respectively).

Stat5 is activated in CWR22Rv and LnCap cell lines, but not in PC-3 cells. Next, we examined Stat5a and Stat5b protein expression and activation in three human prostate cancer cell lines, including the androgen-independent CWR22Rv and PC-3 cells, and the androgen-sensitive LnCap cell line. For this analysis, immunoprecipitation of either Stat5a or Stat5b was followed by immunoblotting with the anti-phosphoTyrStat5 antibody or with antibodies to either Stat5a or Stat5b. Stat5b was the predominant Stat5 protein expressed in CWR22Rv and LnCap cells both during exponential growth (low density; LD) and in confluent culture conditions (high density; HD; Fig. 1B). In both CWR22Rv and LnCap cells at low and at high cell density, Stat5b was phosphorylated on the critical Y699 residue. In contrast, no activation of Stat5a or Stat5b was detected in PC-3 cells, consistent with low or undetectable levels of Stat5 protein in these cells (Fig. 1B).

Blocking of Stat5 activity in human prostate cancer lines induces cell death. Based on the
prevalent activation of Stat5 in human prostate cancer tissues, we hypothesized that Stat5 may act as a survival protein in human prostate cancer. Therefore, we tested whether blocking Stat5 function in human prostate cancer cells would induce cell death. To block activated endogenous Stat5 proteins in human prostate cancer, we generated an adenovirus for effective delivery of a dominant-negative mutant of Stat5 (DNStat5), and a matching control virus carrying wild type (WT) Stat5. The molecular construction of the adenoviral vector carrying dominant-negative Stat5a (AdDNStat5) and the matching control, AdWTStat5, is described in the Materials and Methods section. Truncation of Stat5a after amino acid 713 removes the entire transcriptional activation domain, and generates a Stat5a mutant that is almost identical to the corresponding Stat5b truncated form. As expected, the Stat5a mutant effectively blocked the function of both WT Stat5a and Stat5b (18). We have further validated both the AdDNStat5 and the AdWTStat5 viral constructs in functional assays, including assays for gene induction, inducible tyrosine phosphorylation, and DNA binding (data not shown).

To determine whether blocking endogenous Stat5 activity would induce death of human prostate cancer cells, we first examined the effect of increasing doses of AdDNStat5 on the viability of the androgen-independent CWR22Rv cell line. For the initial cell viability studies we used an assay determining the metabolic activity of the cells. CWR22Rv cells were cultured to 70% confluence in 96-well plates, and exposed for 90 min to AdDNStat5 or AdWTStat5 at doses up to MOI 10, after which the virus was diluted 6-fold in culture medium as described. After 96 h of infection, cells were analyzed for cell viability. A marked and dose-dependent effect of the expression of DNStat5 on cell viability was observed, with detectable suppression at MOI 2.5,
and suppression of cell viability by more than 90% at MOI 10 (Fig. 1C). In contrast, cells infected with AdWTStat5 or AdLacZ did not show significant loss of viability (Fig. 1C). Viability of CWR22Rv cells infected with an empty control virus (AdCtrl) was not reduced (not shown). The selective effect of AdDNStat5 in CWR22Rv cells was consistently observed in experiments that were repeated and reproduced more than five times, and with four different, independently titered batches of virus.

**Prostate cancer cell death induced by DNStat5 represents apoptosis.** Microscopic assessment of the effect of AdDNStat5 on CWR22Rv cell viability confirmed extensive cell death following expression of DNStat5. CWR22Rv cells exposed to AdDNStat5 at MOI 8 for 72 h displayed extensive cell death as evidenced by cell rounding, detachment, shrinkage, and blebbing (Fig. 1D, panel b), which are morphological changes consistent with apoptotic cell death. In contrast, there was no evidence of reduced cell viability in response to AdWTStat5 (Fig. 1D, panel a). Likewise, AdDNStat5, but not AdWTStat5, induced cell death also in the androgen-sensitive human prostate cancer cell line, LnCap (Fig. 1D, panels c and d). Dose response analyses showed that 2-4 fold higher doses of virus were needed to induce cell death in LnCap cells compared to CWR22Rv cells (not shown), an effect that correlated with less efficient adenoviral gene delivery of Stat5 proteins into LnCap cells. Specifically, dose-response analyses of viral protein delivery revealed that the same amount of virus induced lower levels of DNStat5 or WTStat5 expression in LnCap cells than in CWR22Rv cells shown by immunoblotting of whole cell lysates (Fig. 1E). Figure 1E also indicates that cellular levels of DNStat5 and WTStat5 delivered by the respective adenoviral vectors are comparable, supporting the notion that the
selective cell death observed in response to AdDNStat5 is due to its specific dominant-negative characteristics and not caused by other nonspecific mechanisms. In contrast, there were no signs of cell death in PC-3 cells after 72 h exposure to AdDNStat5 at MOI 32 as judged from cell morphology (data not shown).

To further verify that DNStat5-induced killing of prostate cancer cells was due to apoptotic cell death, prostate cancer cells were analyzed for DNStat5-induced fragmentation of DNA. CWR22Rv cells were exposed to AdDNStat5 and a set of controls at MOI 8 for 48 h. A selective effect of DNStat5 on induction of DNA fragmentation was observed, as analyzed by an enzyme-linked immunosorbent assay (ELISA) of nucleosomal DNA fragments (Fig. 2A, panel a). On average, a seven-fold increase in nucleosomal DNA fragmentation was detected in cells exposed to AdDNStat5 over that detected in WTStat5 expressing cells (6 repeats, each with 3 replicates/treatment group). Furthermore, DNStat5-induced death of LnCap cells was also associated with DNA fragmentation, as evidenced by a consistent and more than six-fold increase in DNA fragmentation over levels in AdWTStat5 treated cells on day 3 at MOI 32 (Fig. 2A, panel b) (6 repeats, each with 3 replicates/treatment group). Therefore, we conclude that suppression of Stat5 induces apoptotic cell death in both CWR22Rv and LnCap cells, as revealed by fragmentation of DNA. In contrast, exposure of PC-3 cells, which do not express active Stat5, to AdDNStat5 did not increase fragmentation of DNA when compared to cells expressing WTStat5 or to cells infected with empty control virus, AdLacZ, or mock-infected cells (Fig. 2A, panel c).
Apoptotic cell death of prostate cancer cells expressing DNStat5 was also verified by cell cycle analysis. In both CWR22Rv and LnCap cells expressing DNStat5, but not in cells expressing AdWTStat5, extensive DNA fragmentation was detected in the form of a large fraction of hypodiploid cell fragments (Fig. 2B). In CWR22Rv cells, this proportion was estimated to be approximately 8% of total cellular DNA at Day 3 of AdDNStat5 infection using MOI of 8, which was 8-fold higher than that observed in parallel AdWTStat5-treated cells (0.9%). In LnCap cells, the fraction of cell fragments with hypodiploid DNA content increased 6-fold from 1.3% in AdWTStat5-treated cells to approximately 8% in DNStat5-treated cells. Mock-infected cells and cells infected with empty control virus or AdLacZ were used as controls, and the fraction of hypodiploid cells consistently remained below 1.3% (data not shown).

**DNStat5-induced apoptosis of CWR22Rv and LnCap cells involves Caspase-3 activation.**

Proteolytic enzymes of the caspase family are critical mediators of programmed cell death. A central role has been ascribed to Caspase-3 as a key executor of a major category of apoptotic cell death (19). To determine whether apoptosis in prostate cancer cells induced by expression of DNStat5 is mediated by activation of Caspase-3, we analyzed Caspase-3 activation by a specific Caspase-3 enzymatic assay. Exposure of CWR22Rv or LnCap cells to AdDNStat5 for 36 h or 48 h, respectively, activated Caspase-3 in both cell lines (Fig. 2C). Specifically, in CWR22Rv cells, activation of Caspase-3 was increased 8-fold in cells expressing DNStat5 compared to AdWTStat5-infected cells. In LnCap cells the corresponding increase was approximately 2-fold (3 repeats each with 3-replicates/treatment group). Therefore, we conclude that DNStat5-induced apoptosis in both CWR22Rv and LnCap human prostate cancer
cells involved activation of Caspase-3.

Caspase-3 mediated apoptosis may be induced through upstream activation of Caspase-9 in a Cytochrome C-dependent manner, or through Caspase-9 independent mechanisms (20). Since suppression of Stat5 activation in CWR22Rv and LnCap cells induced activation of Caspase-3, we wanted to determine whether DNStat5-induced activation of Caspase-3 involved activation of Caspase-9. Caspase-9 activity was monitored in response to DNStat5 in both CWR22Rv and LnCap cells for up to 72 h. A 6-fold induction of Caspase-9 was detected at 36 h in CWR22Rv cells expressing DNStat5 (Fig. 2D). In LnCap cells, the same trend was observed, but the effect was less striking (data not shown). We conclude that DNStat5-induced cell death in CWR22Rv cells is mediated by Caspase-3 through a Caspase-9 dependent mechanism. Caspase-9 activation further indicates a cytochrome C/Apaf-1 dependent apoptotic process (20). Ongoing work aims to define the exact sequence of molecular events that are involved in DNStat5-induced prostate cancer cell apoptosis.

The prevalent detection of activated Stat5 in primary human prostate cancer specimens indicates that Stat5 is a candidate therapeutic target in a high proportion of prostate cancers. A recent study by Gao and colleagues indicated that in human prostate tissue homogenates there was only low level of Stat5 binding to an oligonucleotide probe corresponding to the Stat5 response element of the rat beta-casein promoter in electrophoretic mobility shift assays (21). It is possible that prostate cancer-specific or stromal factors interfere with binding of Stat5 to this promoter when whole tissues are homogenized. In this regard, using our highly sensitive in situ technique, which
detects Stat5 activation at a single cell level, we have recently demonstrated that Stat5 is activated in normal human and mouse mammary epithelial cells outside of pregnancy, an activity that is difficult to detect by EMSA using tissue homogenates of whole mammary glands (17).

The present work suggests that blocking activation of Stat5 in human prostate is a potential new therapeutic approach. Delivery of suicide genes into cancer cells is hampered by lack of effective delivery systems, but gene therapeutic delivery systems are constantly improving and involve both viral and non-viral strategies (22,23). Future prostate cancer gene therapy based on delivery of DNStat5 may therefore be feasible. On the other hand, small molecule inhibitors of the Stat5 tyrosine kinase(s) responsible for Stat5 activation in prostate cancer could represent an alternative and more effective approach to inhibit Stat5 in human prostate cancer with even more attractive pharmacological characteristics. Ongoing work in our laboratory seeks to identify the Stat5 tyrosine kinase(s) responsible for activating Stat5 in prostate cancer. In this respect, the Jak2 tyrosine kinase is a central candidate, since this enzyme is responsible for tyrosine phosphorylation of Stat5 by a series of cytokine receptors, including receptors for prolactin (Prl). Prl-receptor associated signaling pathways are of particular interest, because Prl is produced locally in prostate epithelium indicating an autocrine loop of Prl action in prostate (1,8). In addition to Jak2, other tyrosine kinases, such as Jak1, Jak2, Jak3 or Tyk2, as well as members of the Src tyrosine kinase family, or receptor tyrosine kinases, are also possible Stat5 kinases in prostate cancer (24).

We show in this work using in situ detection of phosphorylated and nuclear Stat5 that Stat5 was
activated in a majority of prostate cancer specimens examined. This suggests that inhibition of Stat5 as a therapeutic approach could have a broad usefulness in the treatment of prostate cancer. The observation that both androgen-refractory CWR22Rv and androgen-sensitive LnCap cells responded to suppression of Stat5 with cell death, implies that apoptosis induced by blocking the activation of Stat5 is independent of responsiveness to androgens. Furthermore, the correlation between endogenous Stat5 activation in prostate cancer cell lines and their sensitivity to DNStat5 suggests that immunohistochemical detection of activated Stat5 in prostate cancer may predict responsiveness to this therapeutic strategy.

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FIGURE LEGENDS

Figure 1.  **A, Stat5 is activated in human prostate cancer.** Detection of activated Stat5 in prostate cancer by immunohistochemical analysis using either anti-phosphoTyrStat5 antibody (panel a) or a pan-Stat5 antibody to detect nuclear localized Stat5 (panel b). Anti-phosphoTyrStat5 staining was negative in normal, secretory human prostate epithelium (panel c). Positive control staining was obtained from normal human breast tissue (panel d) and parallel sections stained with subtype-specific mouse IgG were negative (e). **B, Stat5 is expressed and activated in human prostate cancer cell lines CWR22Rv and LnCap, but not in PC-3.** Protein immunoblotting was performed as indicated with either anti-phosphoTyrStat5 (anti-Stat5pY), anti-Stat5a, or anti-Stat5b on immunoprecipitated (IP) Stat5a or Stat5b from lysates prostate cancer cells that had been harvested during exponential growth (low-density; LD) or at confluency (high density; HD). **C, Adenoviral delivery of dominant-negative Stat5 (AdDNStat5) induced cell death in androgen-independent CWR22Rv human prostate cancer cells.** Representative experiment showing a dose-dependent effect on cell viability of adenoviral gene delivery of a dominant-negative Stat5 mutant (AdDNStat5) introduced into CWR22Rv cells. AdStat5WT and AdLacZ served as controls. The increasing viral doses used were 0, 1, 2.5, 5, and 10 MOI, and cell viability was measured after 96 h by the MTT metabolic activity assay. Error bars indicate SD of triplicate determinations.  **D, Morphology of cell death induced by AdDNStat5 in CWR22Rv and LnCap cells is consistent with apoptosis.** Microphotography of AdWTStat5 or AdDNStat5-treated CWR22Rv (72 h, MOI 8) and LnCap cells (96 h, MOI 16).  **E, Efficiency of adenoviral delivery of Stat5 proteins into CWR22Rv and LnCap cells.** Dose-dependent expression of WTStat5 or DNStat5 in CWR22Rv and LnCap cells as detected by
anti-panStat5 immunoblotting of whole cell lysates after 24 h of adenoviral exposure.

Figure 2.  

A, **DNStat5-induced fragmentation of DNA in human prostate cancer lines CWR22Rv and LnCap, but not PC-3.** DNA fragmentation analyzed by nucleosomal ELISA after exposure to AdDNStat5, AdWTStat5, AdLacZ, AdCtrl, or mock infection in CWR22Rv (48 h, MOI 8), LnCap cells (72 h, MOI 32), or PC-3 cells (72 h, MOI 32). A representative experiment of six independent repeats is shown; error bars represent SD of triplicate determinations.  

B, **DNStat5 induced apoptosis in CWR22Rv and LnCap cells as determined by flow cytometry.** Cellular apoptosis detected as increased hypodiploid fraction after treatment with AdDNStat5 or AdWTStat5, AdLacZ, AdCtrl, or mock infection in CWR22Rv (48 h, MOI 8), LnCap cells (72 h, MOI 32). The respective fractions of hypodiploid cells for CWR22Rv cells treated with AdLacZ, AdCtrl, or Mock, were less than 1.5% for both CWR22Rv and LnCap cells.  

C, **DNStat5-induced apoptosis is associated with Caspase-3 activation.** Caspase-3 activation was determined after exposure to AdDNStat5 and control conditions as indicated in CWR22Rv (36 h, MOI 8) and LnCap cells (48 h, MOI 32). A representative experiment of three independent repeats is shown; error bars represent SD of triplicate determinations.  

D, **DNStat5-induced Caspase-3 activation is associated with activation of Caspase-9.** CWR22Rv cells exposed to DNStat5 showed activation of Caspase-9 at 36 h after initiation of treatment. A representative experiment of three independent repeats is shown; error bars represent SD of triplicate determinations.
Additions and Corrections


Inhibition of transcription factor Stat5 induces cell death of human prostate cancer cells.

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Page 27289, Fig. 1A: Fig. 1A should have been printed in color.

Fig. 1
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