Tumorigenesis and Neoplastic Progression

Transcription Factor Stat3 Stimulates Metastatic Behavior of Human Prostate Cancer Cells *in Vivo*, whereas Stat5b Has a Preferential Role in the Promotion of Prostate Cancer Cell Viability and Tumor Growth

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Identification of the molecular changes that promote viability and metastatic behavior of prostate cancer is critical for the development of improved therapeutic interventions. Stat5a/b and Stat3 are both constitutively active in locally-confined and advanced prostate cancer, and both transcription factors have been reported to be critical for the viability of prostate cancer cells. We recently showed that Stat3 promotes metastatic behavior of human prostate cancer cells not only *in vitro* but also in an *in vivo* experimental metastases model. In this work, we compare side-by-side Stat5a/b versus Stat3 in the promotion of prostate cancer cell viability, tumor growth, and induction of metastatic colonization *in vivo*. Inhibition of Stat5a/b induced massive death of prostate cancer cells in culture and reduced both subcutaneous and orthotopic prostate tumor growth, whereas Stat3 had a predominant role over Stat5a/b in promoting metastases formation of prostate cancer cells *in vivo* in nude mice. The molecular mechanisms underlying the differential biological effects induced by these two transcription factors involve largely different sets of genes regulated by Stat5a/b versus Stat3 in human prostate cancer model systems. Of the two Stat5 homologs, Stat5b was more important for supporting growth of

prostate cancer cells than Stat5a. This work provides the first mechanistic comparison of the biological effects induced by transcription factors Stat5a/b versus Stat3 in prostate cancer. (Am J Pathol 2010, 176:1959–1972; DOI: 10.2353/ajpath.2010.090653)

Presently, there are no effective pharmacological therapies for metastatic and castration-resistant prostate cancer.¹ Prostate cancer develops resistance to androgen-deprivation therapy within two to three years on average.¹ Disseminated prostate cancer, in turn, is the lethal form of the disease. The molecular mechanisms underlying progression of prostate cancer to castration-resistant and metastatic disease are largely unclear, which has inhibited rational-based drug design for advanced prostate cancer.

Transcription factors Stat5 and Stat3 are both constitutively active in clinical prostate cancers^{2–8} and have been reported to promote growth of prostate cancer

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cells.^{2,5,9–12} Stat5 and Stat3 are members of the seven-member Stat gene family of transcription factors.¹³ There are two highly homologous isoforms of Stat5, 94-kDa Stat5a and 92-kDa Stat5b with 93% sequence homology, but are encoded by separate genes.¹³ Stat5a/b and Stat3 share approximately 31% sequence homology and possess a similar structural organization comprising the following domains: N-terminal, coiled-coil, DNA-binding, SH2, and transactivation domain, which are all important for proper functioning.¹⁴ The transactivation domain varies considerably in both length and sequence between different Stat family members. The transactivation domain binds critical co-activators and is therefore directly involved in facilitating the initiation of transcription.^{14,15} Phosphorylation of a C-terminal tyrosine residue activates Stat3 (Y705) and Stat5 (5a, Y694; 5b Y699).¹⁴ Activation of Stat3 is supplemented by phosphorylation of a specific serine residue of Stat3 (S727),¹⁵ whereas the corresponding serine phosphorylation of Stat5 (5a, S725; 5b, S730) might have a negative role in transcriptional activity of Stat5.^{16–18} Once active, Stats dimerize through a phosphotyrosine–SH2 domain interaction^{19,20} and translocate to the nucleus to regulate transcription by direct binding to the target gene promoters.¹⁴ Both Stat5 and Stat3 recognize the same palindromic consensus sequence of TTCN_{2–4}GAA, which is referred to as a GAS element (γ -interferon activation sequence).²¹

Stat5a/b is critical for the viability of human prostate cancer cells in culture, for human prostate xenograft tumor growth *in vivo*^{2,9,10} and for prostate cancer progression in TRAMP mouse model.²² In clinical prostate cancers, active Stat5a/b protein expression is abundant in prostate cancer cells, but not in normal prostate epithelium.² Moreover, expression of active Stat5a/b in prostate cancer is associated with high histological grade of the cancer.^{3,4} We showed recently a co-action between Stat5a/b and androgen receptor (AR) in prostate cancer cells, where Stat5a/b promoted transcriptional activity of AR, and AR, in turn, increased transcriptional activity of Stat5a/b.²³ The transcriptional synergy between Stat5a/b and AR supports the results of our earlier study which showed that active Stat5a/b expression in primary clinical prostate cancer predicted early prostate cancer recurrence.³ Autocrine prolactin in prostate cancer has been demonstrated to activate Stat5a/b^{4,9} via Jak2 tyrosine kinase.⁴ The relative importance of Stat5a versus Stat5b in promotion of prostate cancer cell growth has been unclear.

Besides Stat5a/b, transcription factor Stat3 is implicated in promotion of growth and progression of prostate cancer. Activation of Stat3 has been associated with advanced stage of prostate cancer,^{5–8,24} and Stat3 interacts with AR in prostate cancer cells.^{25–28} Importantly, several reports have demonstrated that Stat3 promotes proliferation and inhibits apoptosis in DU145 and LNCaP prostate cancer cell lines.^{5,11,29,30} Recently, we showed that Stat3 dramatically increases metastases formation of human prostate cancer cells in nude mice in an experimental metastases assay, and Stat3 induced migration of human prostate cancer cells *in vitro*.³¹

In this work, we compared side-by-side the effects of Stat5a versus Stat5b versus Stat3 in the promotion of prostate cancer cell viability *in vitro* and tumor growth *in vivo*. In addition, we compared Stat5a/b to Stat3 in promoting metastatic dissemination of prostate cancer cells *in vivo*. The data indicate that the effects of Stat5a/b-inhibition on reduction of prostate cancer cell viability in culture and subcutaneous and orthotopic prostate tumor growth in nude mice were greater than those of Stat3. Stat3, in turn, was more effective than Stat5a/b in inducing metastases formation of human prostate cancer cells *in vivo*. These findings were supported by the gene expression analysis, which indicated that Stat5a/b and Stat3, despite both recognizing the GAS consensus sequence, regulate to a large extent different sets of genes in human prostate cancer cells. When the two Stat5 homologs were compared, Stat5b was more critical than Stat5a in supporting the viability of prostate cancer cells. Finally, Stat5a/b inhibition did not reduce the viability of normal human prostate epithelial cells or cancer cell lines originating from tissues other than prostate.

Materials and Methods

Cell Culture

DU145, PC-3, LNCaP, and CWR22Rv1 human prostate cancer cells (American Type Culture Collection, Manassas, VA) were grown in RPMI-1640 medium (Mediatech, Inc. Herndon, VA). The growth medium contained 10% fetal bovine serum (FBS; Quality Biological, Inc., Gaithersburg, MD), 2 mmol/L L-glutamine and penicillin-streptomycin (Mediatech, Inc.; 50 IU/ml and 50 μ g/ml, respectively). LNCaP cells were grown in the presence of 0.5 nmol/L dihydrotestosterone (DHT). Normal human prostate epithelial cells RC165N and RC170N³² were grown in keratinocyte-serum-free (keratinocyte-SFM; Gibco, Grand Island, NY) medium supplemented with L-glutamine, epidermal growth factor, and bovine pituitary extract (Gibco). Human breast cancer (T47D and MCF-7), pancreatic cancer (Hs766T and CAPAN), melanoma (A2058), liver cancer (HCT116), and lung cancer (A549) cell lines were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Quality Biological, Inc.), 2 mmol/L L-glutamine, and penicillin-streptomycin (Mediatech, Inc.).

Solubilization of Proteins, Immunoprecipitation, and Immunoblotting

Cells were lysed in lysis buffer (10 mmol/L Tris-HCl [pH 7.5], 5 mmol/L EDTA, 50 mmol/L NaCl, 30 mmol/L sodium pyrophosphate, 50 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 1% Triton X-100, 1 mmol/L phenylmethylsulfonylfluoride, 5 μ g/ml aprotinin, 1 μ g/ml pepstatin A, and 2 μ g/ml leupeptin), and the protein concentrations of the whole cell lysates were determined by the Bradford method (BioRad Laboratories Inc., Hercules, CA). The cell lysates were immunoprecipitated for 2 hours at 4°C with anti-Stat5a or anti-Stat5b pAbs (each

1.2 $\mu\text{g/ml}$; Advantex Bioreagents, Conroe, TX) or anti-Stat3 pAb (1.2 $\mu\text{g/ml}$, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Antibodies were captured by incubation with protein A-Sepharose beads (Pharmacia Biotech, Piscataway, NJ) for 60 minutes. The filters were blotted with anti-phosphotyrosine-Stat5a/b (Y694/Y699) monoclonal antibody (mAb; 1 $\mu\text{g/ml}$, Advantex Bioreagents), anti-Stat5a/b mAb (1:250) (Transduction Laboratories, Inc.), anti-Stat3 pAb (1:1000; Santa Cruz Biotechnology), anti-phospho-tyrosine Stat3 pAb (Y705; 1:1000; Cell Signaling, Danvers, MA) or anti-actin pAb (1:4000; Invitrogen). The immunoreaction was detected by horseradish peroxidase-conjugated secondary antibodies in conjunction with enhanced chemiluminescence substrate mixture (Amersham, Piscataway, NJ), and exposed to film.

Generation of Adenoviruses for Gene Delivery of Wild-Type and Dominant-Negative Forms of Stat5a/b and Stat3

We cloned pcDNA-CMV-WTStat5b (AdWTStat5b) and pcDNA-CMV-DNStat5a/b (AdDNStat5a/b) into adenoviral vector using BD Adeno-X Expression System 2 (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's protocol as described previously.²² Adenoviruses carrying a human wild-type Stat3 (AdWTStat3) and a dominant-negative Stat3 (AdDNStat3; C-terminally truncated at amino acid 715)³³ were kindly provided by Dr. Hallgeir Rui at Thomas Jefferson University. 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 (Qbiogene, Carlsbad, CA).

siRNA Transfection

DU145 and CWR22Rv1 prostate cancer cells were transfected with the scramble siRNA or siRNAs targeted to human Stat3, Stat5a, or Stat5b (Dharmacon, Lafayette, CO; 100 pmol of siRNA) using transfection reagent Lipofectamine 2000 (Invitrogen).

Cell Growth and Viability Assay

The number of living cells was determined by counting the attached cells using a hemacytometer and trypan blue exclusion. In addition, the cell viability was determined by 3-(4,5 Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay (Promega, Madison, WI). In brief, after plating, the cells were infected next day with AdWTStat5b, AdDNStat5a/b, AdWTStat3, AdDNStat3, or AdLacZ at multiplicity of infection (MOI) 5. After 72 hours, the proportion of viable cells in each treatment group in sextuplicates was determined by tetrazolium conversion to its formazon dye (Promega) at 490 nm (POLARstar OPTIMA, BMG Labtech).

Subcutaneous Prostate Cancer Xenograft Tumors

Castrated male athymic mice (Taconic; Germantown, NY) were cared for according to the institutional guidelines. Sustained-release DHT pellets (90-day release, 1 pellet/mouse; Innovative Research of America, Sarasota, FL) were implanted subcutaneously (s.c.) three days before the tumor cell inoculations to normalize the circulating DHT levels. Briefly, DU145 cells were infected 24 hours before the inoculation to the mice with AdWTStat3, AdDNStat3, AdWTStat5b, AdDNStat5a/b, or AdLacZ at MOI 5. DU145 cells (20×10^6) were mixed with one half of the total injection volume (0.2 ml) with Matrigel (BD Bioscience, San Jose, CA), and injected s.c. to the flanks of the nude mice (1 site/mouse). When the tumors reached 15 to 20 mm in diameter, the mice were sacrificed, and the tumor tissues were harvested. The volumes of the tumors were measured twice a week and calculated using the formula $V = (\pi/6) \times d_1 \times (d_2)^2$, with d_1 and d_2 being two perpendicular tumor diameters.

Orthotopic Inoculation of Human Prostate Cancer Cells

Athymic male nude mice (6 to 8 weeks; Taconic, Germantown, NY) were anesthetized with 2% isoflurane, and an incision was made in the lower abdomen through skin and peritoneum to access the dorsolateral prostate. A suspension of DU145 cells (1×10^6) in 20 μl of PBS was injected into the dorsolateral prostate, and the wound was closed by single-stitch technique using a 4-0 suture. Before (24 hours) orthotopic inoculation, DU145 cells had been infected with AdLacZ, AdWTStat3, AdDNStat3, AdWTStat5b, or AdDNStat5a/b at MOI 5. Mice were sacrificed 8 weeks after the tumor cell inoculation, and the tumors were measured and photographed. The volumes of the tumors were calculated using the formula $V = (\pi/6) \times d_1 \times (d_2)^2$, with d_1 and d_2 being two perpendicular tumor diameters.

Tail-Vein Injections of Human Prostate Cancer Cells

Castrated male athymic mice (Taconic) were implanted with DHT pellets (90-day release, 1 pellet/mouse; Innovative Research of America) to normalize the circulating levels of DHT. DU145 cells were infected with AdLacZ, AdWTStat3, AdWTStat5a, or AdWTStat5b at MOI 5. After 24 hours, 1×10^6 cells were suspended in 0.2 ml of PBS and injected into the lateral tail vein using 27-gauge needle. The mice were sacrificed 8 weeks after inoculation, and the lungs were perfused with 1.5 ml of 15% India Ink dye in 3.7% formalin. Lungs were then removed and bleached in Fekete's destaining solution (70% ethanol, 3.7% formaldehyde, 0.75 mol/L glacial acetic acid). Lung surfaces were photographed, and the numbers of metastases nodules were scored.

RNA Preparation and Gene Expression Profiling

Forty-eight hours after the siRNA transfection, total RNA was prepared using the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA). A DNase I digestion step was included to eliminate DNA contamination. Each group (control-siRNA, Stat5a/b-siRNA and Stat3-siRNA) was done in triplicate, and RNA samples from each group were not pooled. RNA was quantified on a Nanodrop ND-100 spectrophotometer, followed by RNA quality assessment by analysis on an Agilent 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA). A total of 2 μ g of RNA from each sample was used for Affymetrix one-cycle target labeling method as recommended by the manufacturer (Affymetrix, Santa Clara, CA). Each Affymetrix Genechip for Human Genome 133 Plus 2.0 was hybridized for 16 hours with biotin-labeled fragmented cRNA (10 μ g) in 200 μ l of hybridization cocktail according to Affymetrix protocol. Arrays were washed and stained using Genechip Fluidic Station 450, and hybridization signals were amplified using antibody amplification with goat IgG (Sigma-Aldrich) and anti-streptavidin biotinylated antibody. Chips were scanned on an Affymetrix GeneChip Scanner 3000 using GeneChip Operating Software version 3.0.

Statistical Analysis

For the analysis of the cell numbers after Stat5a/Stat5b/Stat5ab/Stat3/control siRNA transfections, analysis of variance was used to compare groups with respect to the number of living cells. The residual variance was allowed to vary by group. *P* values were adjusted using Bonferroni method to maintain an overall type I error rate of 5% for this experiment.

The two-sample *t* test was used to compare the number of living cells in the group where Stat5ab was down-regulated by AdDNStat5a/b versus AdLacZ-infected cells, and the group where Stat3 was down-regulated by AdDNStat3 versus AdLacZ-infected cells. *P* values were adjusted using Bonferroni method to maintain an overall type I error rate of 5% for this experiment.

For the analysis of subcutaneous DU145 xenograft tumors, the trajectory of log-transformed tumor volumes over time was modeled in two stages. At the first stage, robust regression was used to estimate the rate of growth (slope) of the log tumor volumes for each tumor. At the second stage, groups were compared with respect to average slopes using linear regression where each mouse's data were weighted by the inverse of the SE of the slope estimate in the first stage. Undetectable volumes were set to four before transformation for purposes of this analysis. The Wilcoxon rank sum test was used to compare groups at day 69.

The distribution of orthotopic tumor volumes by the treatment groups (AdDNStat3, AdWTStat3, AdWTStat5b, AdDNStat5a/b, and AdLacZ) was summarized using the median, interquartile range, and range. Pair-wise group comparisons were performed using the Wilcoxon rank sum test. Tumor volumes from AdDNStat5a/b were compared with AdWTStat5b and AdLacZ, and volumes from

AdDNStat3 were compared with AdWTStat3 and AdLacZ. *P* values for these tests were adjusted using Bonferroni method to maintain an overall type I error rate of 5% for this experiment.

To determine the effects of Stat5a/b inhibition versus Stat3 inhibition on the growth of different cell types, DNStat5a/b expression was compared with WTStat5b, and DNStat3 expression was compared with WTStat3 within each cell type with respect to mean absorption levels using a two-sample *t* test. *P* values for the test were adjusted using the method of Benjamini and Hochberg to control the false discovery rate. *P* < 0.05 was considered significant.

For the statistical analysis of the *in vivo* metastases assay, Wilcoxon rank sum test with exact *P* values was used to compare the groups with respect to the number of lung nodules per mouse.

Microarray Data Analysis

The gene expression profiles of Stat5a/b versus Stat3 were analyzed in parallel in two different human prostate cancer cells lines, DU145 and CWR22Rv1. The chips (*n* = 3 Ctrl siRNA, *n* = 3 Stat3 siRNA, *n* = 3 Stat5a/b) were preprocessed using Robust Multiarray Average.³⁴ The Affymetrix GeneChip for Human Genome 133 Plus 2.0 has 54,675 transcripts, and nonspecific filtering³⁵ was done before analysis resulting in a single transcript for each of 15,992 annotated genes. Specifically, probe sets that did not have an associated Entrez Gene ID and Gene Ontology ID were filtered out. The probe set that exhibited maximal variability was chosen to represent each gene (defined by a unique Entrez Gene ID). Differential expression of each gene between Ctrl siRNA and Stat3 or Stat5a/b siRNA groups was assessed using a linear model with an empirical Bayes correction for the variance, as implemented in the Bioconductor³⁶ package limma.³⁷ This allows a robust analysis of differential expression between Ctrl siRNA and Stat3 or Stat5a/b siRNA groups even in case of small sample size. The *P* values were then corrected for multiple testing using the false discovery rate.³⁸ This analysis establishes whether the fold-change (FC) observed is large enough compared with the variability in the gene expression across the samples to meet our statistical significance criterion. We considered the genes with a false discovery rate-adjusted *P* value of ≤ 0.01 to be statistically significant (see Supplemental Tables S1 and S2 at <http://ajp.amjpathol.org>).

Results

Stat5a/b Has a Preferential Role over Stat3 in Promoting the Viability of Human Prostate Cancer Cells in Vitro and Prostate Xenograft Tumor Growth in Vivo

To determine the biological effects of Stat5a/b versus Stat3 on human prostate cancer cell growth in culture, we

first established the phosphorylation and total protein levels of Stat5a, Stat5b, and Stat3 in CWR22Rv1, LNCaP, and DU145 human prostate cancer cell lines. Immunoprecipitation and immunoblotting of Stat5a/b show persistent activation and expression of Stat5a and Stat5b in all three cell lines (Figure 1A). Immunoprecipitation of Stat3 indicated persistent Stat3 activation in only DU145 cells, whereas Stat3 protein was expressed in DU145, LNCaP, and CWR22Rv1 cells (Figure 1A).

Because persistent activation of both Stat5 and Stat3 was detected in DU145 cells, we decided to use this cell line as our first model system to compare the biological effects caused by inhibition of Stat5a/b versus Stat3. We designed siRNAs to inhibit selectively Stat5a/b or Stat3 in DU145 prostate cancer cells. First, we verified by Western blotting that siRNAs targeted to Stat5a/b did not affect Stat3 protein expression or Stat3 phosphorylation (Figure 1B). Stat3 siRNAs, in turn, did not affect the expression level of Stat5a/b as demonstrated in Figure 1B. DU145 cells were transfected with siRNAs against Stat5a/b or Stat3 for 48 hours, with scrambled siRNA as control, and Stat5a/b was immunoprecipitated and blotted with anti-Stat5a/b mAb. Whole cell lysates of the same samples were immunoblotted for phosphorylated Stat3, total Stat3, and actin (Figure 1B). In the next set of experiments, DU145 cells were transfected with siRNAs against Stat5a/b or Stat3 for 72 hours, after which the cells were photographed (Figure 1, C and D), and the number of attached cells was determined. Inhibition of Stat5a/b by RNA interference resulted in a 70% decrease in attached and viable DU145 cells ($P < 0.0001$), whereas Stat3 inhibition decreased the number of viable cells by 20% compared with cells transfected with control siRNA ($P = 0.15$; Figure 1C). Microscope photographs indicate dead and floating cells in the wells transfected with Stat5a/b siRNA (Figure 1D). Our previous work has demonstrated that prostate cancer cell death induced by Stat5 inhibition is apoptotic involving extensive DNA fragmentation and Caspase-3 and -9 activation.^{2,9,10} In summary, these results demonstrate that Stat5a/b has a more important role than Stat3 in sustaining the viability of DU145 human prostate cancer cells in culture.

To determine the relative importance of the two Stat5 homologs for promotion of the viability of human prostate cancer cells, siRNAs specific to Stat5a or Stat5b were introduced to DU145 cells. The specificity of the siRNAs was verified by immunoprecipitation and Western blotting (Figure 1B). Inhibition of Stat5b by RNA interference resulted in almost similar decrease in the number of attached and viable DU145 cells compared with the group that was transfected with siRNAs targeting simultaneously both Stat5a and Stat5b ($P < 0.0001$; Figure 1, C and D). In contrast, inhibition of Stat5a by Stat5a siRNAs did not affect significantly the cell viability compared with the controls ($P = 1.0$; Figure 1, C and D).

As the second methodological approach, Stat5a/b or Stat3 were inhibited by adenoviral (Ad) gene delivery of dominant-negative (DN) mutants of Stat5a/b¹⁰ and Stat3,³³ respectively, with AdLacZ, AdWTStat5b,¹⁰ AdWTStat3,³³ and mock-infected cells as controls. The transactivation domain was deleted from Stat5a and

Stat3 to create mutants of Stat5a/b and Stat3 that would inhibit transcriptional activity of Stat5a/b and Stat3, respectively.^{2,10,33} All of the adenoviral constructs were propagated and titered side-by-side and tested by Western blotting for transfer of equal gene expression levels for each construct in human prostate cancer cells. DU145 cells were infected with AdWTStat5b, AdDNStat5a/b, AdWTStat3, or AdDNStat3, with AdLacZ (all at MOI 5) or mock-infected cells as controls, and the cells were photographed and the number of attached cells was determined after 72 hours (Figure 1, E and F). Inhibition of Stat5a/b by adenoviral expression of DNStat5a/b decreased the number of viable and attached DU145 cells by 87% compared with AdLacZ-infected cells ($P < 0.0001$), whereas inhibition of Stat3 reduced the cell number by 30% ($P = 0.0014$; Figure 1E). Microscope photographs verify extensive cell death in AdDNStat5a/b-infected wells as evidenced by cell rounding, detachment, and shrinkage (Figure 1F). Collectively, these results suggest Stat5b is the key regulator of DU145 prostate cancer cell viability in culture rather than Stat5a or Stat3.

Given the preferential role of Stat5a/b in promotion of prostate cancer cell viability in cell culture, we hypothesized that Stat5a/b also has a predominant role in the regulation of prostate xenograft tumor growth in nude mice. To test this hypothesis, we inhibited Stat5a/b by AdDNStat5a/b and Stat3 by AdDNStat3 in DU145 prostate cancer cells. Adenoviral gene delivery was performed 24 hours before inoculation of the cancer cells subcutaneously 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 9, the tumor sizes were measured once a week until day 69 of the experiment. The tumor volumes ($P = 0.024$) and the growth rate ($P = 0.016$) of prostate tumors both were significantly suppressed in Stat5a/b-inhibited group compared with the control group (AdWTStat5b), whereas the effects of Stat3 inhibition on the tumor volumes ($P = 0.52$) or the growth rate ($P = 0.90$) of subcutaneously grown prostate xenograft tumors were less consistent (Figure 2A).

In the second set of experiments, we compared the effects of Stat5a/b inhibition versus Stat3 on orthotopic prostate tumor growth in nude mice (Figure 2B). DU145 cells were infected with AdDNStat5a/b, AdWTStat5b, AdDNStat3, AdWTStat3, or AdLacZ 24 hours before inoculation to the dorsolateral prostates of athymic nude mice. After 8 weeks, the mice were sacrificed, the urogenital blocks were photographed, and the prostate tumor sizes were measured. When Stat5a/b was inhibited, the tumors were significantly smaller when compared with the tumors formed from AdWTStat5b-infected cells ($P = 0.0032$). Inhibition of Stat3 by AdDNStat3 moderately decreased the tumor sizes compared with the AdWTStat3-group ($P = 0.112$) or LacZ-group ($P = 1.0$; Figure 2B), but did not reach statistical significance. In conclusion, these results suggest a preferential role for Stat5a/b rather than Stat3 in promotion of subcutaneous and orthotopic xenograft human prostate tumors in nude mice.

Figure 1. Inhibition of Stat5a/b caused a robust decrease in the number of viable DU145 human prostate cancer cells, whereas the effects of Stat3 inhibition were less pronounced. **A:** Transcription factors Stat5 and Stat3 are both constitutively active in DU145 prostate cancer cells. Stat5a or Stat5b were immunoprecipitated (IP) from exponentially growing CWR22Rv1, LNCaP, and DU145 cells using anti-Stat5a or anti-Stat5b pAbs, and blotted (WB) with anti-phospho-Stat5a/b (anti-pYStat5a/b) antibody, as indicated. Whole cell lysates (WCL) were blotted with anti-phospho-Stat3 (anti-pYStat3) antibody. Filters were stripped and re-blotted with anti-Stat5a or anti-Stat3 mAbs, and whole cell lysates before IP were immunoblotted with anti-actin pAb. **B:** To verify the specificity of the siRNAs, DU145 cells were transfected with siRNA targeted to Stat5a, Stat5b, or Stat3, with scrambled siRNAs or nontransfected cells as controls. The cells were harvested 48 hours after the siRNA transfection. Stat5a and Stat5b were immunoprecipitated with anti-Stat5a and anti-Stat5b pAbs, respectively, and blotted (WB) with anti-phospho-Stat5a/b (anti-pYStat5a/b) antibody, as indicated. Whole cell lysates (WCL) of the same samples were blotted with anti-phospho-Stat3 (anti-pYStat3), anti-Stat3, or anti-actin pAbs. **C:** Inhibition of Stat5b by RNA interference decreased the number of viable DU145 cells, whereas the effects of Stat5a or Stat3 inhibition were less evident. The number of live cells after inhibition of Stat5a, Stat5b, Stat5a/b, or Stat3 by specific siRNAs, control group was incubated with transfection reagent only. After trypan blue exclusion, the attached viable DU145 cells were counted 72 hours after the siRNA transfection. The bars indicate mean $\pm$ SD of triplicate wells. **D:** Microscope photographs of the DU145 cells 72 hours after the transfection show the cell morphology. siRNAs targeted to Stat5b or Stat5a/b induced massive detachment and rounding of DU145 cells, whereas Stat5a or Stat3 siRNA caused hardly detectable effects on the cell numbers or the cell morphology. **E:** Inhibition of Stat5a/b by adenoviral (Ad) gene delivery of a dominant-negative (DN) Stat5a/b decreased significantly the number of viable prostate cancer cells, whereas Stat3 inhibition effects were only minor. DU145 cells were infected with AdWTStat5b, AdDNStat5a/b, AdWTStat3, AdDNStat3, or with AdLacZ at MOI 5 using mock-infected cells as an additional control. After trypan blue staining, the cells were photographed and counted after 72 hours. The bars indicate mean $\pm$ SD of triplicate wells. **F:** Microscope photographs demonstrate the cell morphology after 72 hours infection of the cells with AdDNStat5a/b, AdWTStat5b, AdDNStat3, AdWTStat3 versus AdLacZ or mock-infected cells. Inhibition of Stat5a/b induced detachment, rounding, and shrinkage of the cells, which are all characteristics of apoptosis.

Figure 2. Inhibition of Stat5a/b, not Stat3, has a major effect in blocking DU145 prostate cancer subcutaneous and orthotopic xenograft tumor growth in athymic nude mice. **A:** Subcutaneous tumor growth of DU145 prostate cancer cells infected with adenovirus (Ad) expressing DNStat5a/b, WTStat5b, DNStat3 or WTStat3. Twenty-four hours after the infection with AdDNStat5a/b, AdWTStat5b, AdDNStat3, or AdWTStat3 at MOI 5, the cells were inoculated subcutaneously into flanks of castrated athymic nude mice supplied with sustained-release 5 α -dihydrotestosterone (DHT)-pellets ($n = 7$ per group, 1 tumor per mouse, 20×10^6 DU145 cells per site, 1 DHT pellet/mouse). The tumor growth was measured once a week for 69 days. Tumor volumes were calculated using the formula $V = (\pi/6) \times d_1 \times (d_2)^2$, with d_1 and d_2 being two perpendicular tumor diameters. **B:** Orthotopic growth of DU145 prostate cancer cells infected with adenovirus (Ad) expressing DNStat3, WTStat3, DNStat5a/b, WTStat5b, or LacZ. DU145 cells were inoculated orthotopically in the dorsolateral prostates of athymic nude mice (1×10^6 DU145 cells per injection) supplied with DHT-pellets (1 pellet per mouse). Prior (24 hours) to the injection, the DU145 cells had been infected with AdDNStat5a/b, AdWTStat5b, AdDNStat3, or AdLacZ at MOI 5. Mice were sacrificed 8 weeks after tumor cell inoculation, and the tumor volumes were calculated using the formula $V = (\pi/6) \times d_1 \times (d_2)^2$, with d_1 and d_2 being two perpendicular tumor diameters. **C:** Inhibition of Stat5a/b does not affect the viability of normal human prostate epithelial cell lines, but caused a decrease in the number of viable LNCaP and CWR22Rv1 cells. Normal prostate epithelial cell lines, RC165N and RC170N, and human prostate cancer cell lines, LNCaP, CWR22Rv1, and PC-3, were infected with AdWTStat5b, AdDNStat5a/b, AdWTStat3, AdDNStat3, or AdLacZ at MOI 5. The cell viability was determined 72 hours after the adenoviral gene delivery by MTS (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyl-tetrazolium bromide) metabolic activity assay. **D:** Melanoma (A5028), hepatocellular (HepG2), colorectal (HCT116), lung (A549), pancreatic (Hs766T and CAPAN), and breast (MCF-7 and T47D) cancer cell lines were infected with AdWTStat5b, AdDNStat5a/b, AdWTStat3, AdDNStat3, or AdLacZ at MOI 5, and the cell viability was determined 72 hours after the adenoviral gene delivery by MTS assay. The bars indicate mean $\pm$ SD of triplicate determinations. **E:** Stat5a and Stat5b were immunoprecipitated using anti-Stat5a and anti-Stat5b pAbs, respectively, and immunoprecipitates and whole cell lysates (WCL) were blotted with anti-Stat5a/b mAb or anti-Stat3 pAb, as indicated. Anti-actin immunoblotting of whole cell lysates indicates equal level of proteins in the immunoprecipitations.

Table 1. Frequency of Stat5a/b and Stat3 Activation in other Cancer Types than Prostate Cancer

	Number of patients	%		Number of patients	%
Breast cancer			Breast cancer		
Stat5a/b activation status	30	100	Stat3 activation status	34	100
Negative	10	33	Negative	9	9
Positive	20	67	Positive	31	91
Colon cancer			Colon cancer		
Stat5a/b activation status	16	100	Stat3 activation status	19	100
Negative	10	63	Negative	0	0
Positive	6	37	Positive	19	100
Lung cancer (squamous)			Lung cancer (squamous)		
Stat5a/b activation status	16	100	Stat3 activation status	18	100
Negative	2	13	Negative	14	78
Positive	14	87	Positive	4	22
Melanoma			Melanoma		
Stat5a/b activation status	17	100	Stat3 activation status	20	100
Negative	7	41	Negative	10	50
Positive	10	59	Positive	10	50

Inhibition of Stat5a/b or Stat3 Does Not Affect the Viability of Normal Human Prostate Epithelial Cells

We next tested the biological effects of Stat5a/b or Stat3 inhibition on the growth of other human prostate cancer cell lines LNCaP, CWR22Rv1, and PC-3. LNCaP and CWR22Rv1, which are both Stat5a/b and Stat3-positive, responded to Stat5a/b inhibition by significant reduction of viable cells (LNCaP; $P = 0.0163$, CWR22Rv1; $P = 0.0058$), but did not respond (LNCaP; $P = 0.5532$) or responded only moderately to Stat3 inhibition (CWR22Rv1; $P = 0.0213$). Inhibition of Stat5a/b or Stat3 did not affect the viability of PC-3 cells, which do not express Stat5a/b or Stat3 because of gene deletions,³⁹ as expected (Figure 2C).

To determine whether transcription factors Stat5a/b or Stat3 are critical for the viability of normal prostate epithelium, RC165N and RC170N normal human prostate epithelial cell lines³² were infected with adenoviruses expressing DNStat5a/b, DNStat3, WTStat5b, or WTStat3 (Figure 2C). RC165N and RC170N have been established from benign prostate tissue, and both cell lines have been shown to express cytokeratin CK8, PSA, AR, and NKX3.1 characteristic to prostate luminal secretory cells.³² Importantly, neither of the nonmalignant prostate epithelial cell lines responded to inhibition of Stat5a/b or Stat3 by decreased cell viability (Figure 2C). However, the Stat3 protein expression in the nonmalignant prostate epithelial cell lines was below the detection level by Western blotting. Although Stat5b protein expression was detected in nonmalignant prostate epithelial cell lines and Stat5a expression was below the detection level with our antibodies, phospho-Stat5 immunoblotting indicated weak Stat5a phosphorylation in the RC165N and RC170N cells (Figure 2E).

Because transcription factor Stat5a/b may provide a therapeutic target protein for prostate cancer, we next aimed to establish how specific the effects of Stat5a/b inhibition are on the viability of prostate cancer cells compared with other cancers (Figure 2D). We first assessed the frequency of constitutive activation of Stat5a/b and Stat3 by immunohis-

tochemistry in paraffin-embedded specimens of breast, colon, lung cancers, and melanomas (Table 1). Stat5a/b and Stat3 were in the active state in the majority of breast cancers (Stat5a/b 67%; Stat3 91%), and melanomas (Stat5a/b 59%; Stat3 50%), whereas 37% colon cancers expressed activated Stat5a/b and 100% active Stat3. Furthermore, 87% of lung cancers expressed active Stat5a/b and 22% active Stat3 (Table 1). Stat5a/b or Stat3 inhibition by AdDNStat5a/b or AdDNStat3, respectively, did not suppress the viability of breast (MCF-7, T47D), melanoma (A2058), hepatocellular (HepG2), colorectal (HCT116), pancreas (Hs766T, CAPAN), or lung cancer (A549) cells (Figure 2D). Western blotting of immunoprecipitated Stat5a and Stat5b indicated Stat5a/b expression in all of the cell lines except HCT116 and PC-3 cells,² whereas Stat3 was expressed in all cancer cell lines (Figure 2E). Taken together, these results suggest that Stat5b inhibition leads to cell death specifically in prostate cancer cell lines, but not in cell lines established from normal prostate epithelium. Moreover, the data suggest that although Stat5a/b may be constitutively active in cancers originating from other tissue types than prostate, its functional significance in those cancers is different from in prostate cancer.

Stat3 Is the Key Inducer of Experimental Metastases Formation of Human Prostate Cancer Cells, whereas the Effects of Stat5a/b on Induction of Metastases Nodules Were Modest

We recently showed that Stat3 induces a robust increase in lung metastases formation of human prostate cancer after tail-vein injections in athymic nude mice.³¹ Therefore, we next compared the effects of Stat5a/b to Stat3 in promoting the *in vivo* metastasis formation of human prostate cancer cells. We performed an experimental metastases assay by infecting DU145 cells with adenoviruses expressing WTStat5a, WTStat5b, WTStat3, or LacZ at MOI 5. Twenty-four hours after the adenoviral gene delivery, we injected DU145 cells in athymic nude mice through the tail veins. The lungs were harvested after 8

Figure 3. Stat3 has a preferential role in promoting prostate cancer metastases formation in an experimental metastases assay. **A:** Athymic nude mice were injected with DU145 cells infected with adenovirus expressing LacZ, WTStat3, WTStat5a, or WTStat5b at MOI 5 (1×10^6 cells per mouse) through the tail-vein. After 8 weeks, the lungs were harvested and stained with India Ink, bleached with Fekete's solution, and scored for surface lung metastases. **B:** Representative photographs of India Ink-stained lungs derived from athymic nude mice injected with LacZ, WTStat3, WTStat5a, or WTStat5b expressing DU145 cells after 8 weeks.

weeks and stained with India Ink, bleached with Fekete solution, and scored for surface lung metastases. As demonstrated in Figure 3A, the number of metastases in mice injected with DU145 cells infected with AdWTStat3 was increased by approximately 50-fold when compared with mice injected with DU145 cell that had been infected with either AdLacZ. In contrast, WTStat5a increased the number of metastases by only fivefold and WTStat5b by fourfold in comparison with DU145 cells expressing LacZ. Quantitatively, injection of DU145 with AdWTStat3 cells resulted in an average of 137 (SEM = 9.5) metastases per lung, as compared with 2.6 per lung using DU145 cells infected with AdLacZ (SEM = 1.2) or AdWTStat5a (mean, 13, SEM = 6.5) or WTStat5b (mean, 9, SEM = 2.4). Figure 3B demonstrates visually the white metastasis nodules in the lungs of nude mice. In conclusion, the data suggest that Stat3, not Stat5a/b, has a predominant role in promoting metastatic dissemination of human prostate cancer cells in an *in vivo* experimental metastases assay. This is the first side-by-side comparison of Stat5a/b to Stat3 in promotion of metastatic potential of human prostate cancer cells *in vivo*.

Gene Expression Profiles Regulated by Stat5a/b Versus Stat3 in Human Prostate Cancer Cells

To assess gene expression profiles of Stat5a/b and Stat3 in human prostate cancer cells, we inhibited Stat5 gene

expression 48 hours before the gene expression analysis by Stat5a/b siRNA and Stat3 gene expression by Stat3 siRNA using scrambled siRNA as control (Ctrl) in two different human prostate cancer cell lines, DU145 and CWR22Rv1 (Figure 4A). Overall, 1409 genes in DU145 cells and 1344 genes in CWR22Rv1 cells were differentially expressed between Ctrl siRNA versus Stat5a/b and Stat3 siRNA groups using false discovery rate < 0.01 on the full dataset of 15,992 genes (See Supplemental Tables S1 and S2 at <http://ajp.amjpathol.org>).

Importantly, only 15% of the 1409 genes in DU145 cells were regulated by both Stat5a/b and Stat3, whereas 25% were regulated by Stat3 only and 60% were regulated by Stat5 only (Figure 4B). Similarly, only 11% of the 1344 genes in CWR22Rv1 cells were regulated by both transcription factors, whereas 30% were regulated by Stat3 only and 59% by Stat5a/b only (Figure 5A). To define functional groups within the Stat5a/b- and Stat3-regulated genes, we used the descriptions from the Gene Ontology annotations as our tool. The Gene Ontology is constructed in a hierarchical manner with categories corresponding to each gene ontology identifier (GO ID) being potentially subdivided into more precise subcategories, each with its own GO ID. "The metastasis group" of genes was defined as a set of 88 GO IDs and their subcategories (a total of 1975 GO IDs) corresponding to genes encoding proteins related to metastases processes. Correspondingly, "the apoptosis group" of genes was defined as a set of 21 GO IDs and their subcategories (a total of 469 GO IDs) and "the proliferation group" as a set of 47 GO IDs and their subcategories (a total of 943 GO IDs). Somewhat surprisingly, in each of our three comparisons (the genes regulated by Stat5 only, Stat3 only, or by both Stat5a/b and Stat3), the majority of the differentially expressed genes in both DU145 ($n = 1409$) and CWR22Rv1 cells ($n = 1344$) between Ctrl siRNA and Stat5a/b or Stat3 siRNA groups were metastases-related. This was followed by genes related to proliferation, whereas the minority of the genes were apoptosis-related (Figures 4C and 5B).

The heatmaps demonstrate a hierarchical clustering of the expression values of the most differentially expressed genes (as determined by the smallest *P* values) related to metastases, apoptosis or proliferation for the genes regulated by Stat5 only (Figure 4D; DU145, Figure 5C; CWR22Rv1), Stat3 only (Figure 4E; DU145, Figure 5D; CWR22Rv1), or the genes regulated by both Stat5a/b and Stat3 (Figure 4F; DU145, Figure 5E; CWR22Rv1). Red represents higher expression and green represents lower expression in the heatmaps, and the genes (rows) are re-ordered based on hierarchical clustering using the correlation metric. The heatmaps clearly illustrate the unique gene expression patterns of Stat5a/b versus Stat3 in both DU145 and CWR22Rv1 prostate cancer cell lines. Specifically, the majority of the genes that are up-regulated for example by Stat5a/b, and thus coded by red, are either down-regulated or not altered in the groups where Stat3 expression was selectively inhibited (Figures 4 and 5). In addition, the heatmaps (Figures 4 and 5) demonstrate the genes related to metastases, apoptosis, or proliferation, which are similarly regulated by both

Figure 4. Identification of Stat5 and/or Stat3-regulated genes in DU145 human prostate cancer cells. **A:** Inhibition of Stat5a/b or Stat3 protein expression human prostate cancer cells. DU145 and CWR22Rv1 cells were transfected with siRNA targeted to Stat5a/b or Stat3, with scrambled siRNA as control. Cells were harvested 48 hours after the transfection, lysed, and immunoblotted with anti-Stat5a/b mAb or anti-Stat3 pAb, as indicated. The lower parts of the filters were blotted with anti-actin antibody to demonstrate equal loading (**bottom panel**). **B:** Venn-diagrams of Stat5a/b- and/or Stat3-regulated genes in DU145 human prostate cancer cells. **C:** The relative proportions of Stat5a/b versus Stat3 vs. Stat5-Stat3-regulated genes in DU145 prostate cancer cells related to metastases, proliferation, or apoptosis out of total of 1409 genes differentially expressed between control siRNA and Stat5a/b or Stat3 siRNA using false-discovery rate <0.01 on the full dataset of 15,992 genes. The functional groups were identified using the Gene Ontology annotations. **D:** A heatmap showing the top differentially expressed genes by Stat5a/b (determined by the smallest P values) related to metastases, apoptosis, or proliferation. Red represents higher expression, whereas green represent lower expression in the heatmap. For comparison, the levels of the gene expression for the corresponding genes in the groups where Stat3 was inhibited are shown in the middle of the heatmap. **E:** A heatmap showing the top differentially expressed genes by Stat3 (determined by the smallest P values) related to metastases, apoptosis, or proliferation. Red represents higher expression, whereas green represent lower expression in the heatmap. For comparison, the levels of the gene expression for the corresponding genes in the groups where Stat5a/b was inhibited are shown in the left side of the heatmap. **F:** A heatmap showing the top differentially expressed genes by Stat5a/b and Stat3 (determined by the smallest P values) related to metastases, apoptosis, or proliferation. Red represents higher expression, whereas green represent lower expression in the heatmap.

Figure 5. Identification of Stat5 and/or Stat3-regulated genes in CWR22Rv1 human prostate cancer cells. **A:** Venn diagrams of Stat5a/b- and/or Stat3-regulated genes in CWR22Rv1 human prostate cancer cells. **B:** Transcriptional profiles of Stat5a/b versus Stat3-regulated genes in CWR22Rv1 prostate cancer cells related to metastases, proliferation, or apoptosis out of total of 1344 genes differentially expressed between control siRNA and Stat5a/b or Stat3 siRNA using false-discovery rate <0.01 on the full dataset of 15,992 genes. The functional groups were identified using the Gene Ontology annotations. **C:** A heatmap showing the top differentially expressed genes by Stat5a/b (determined by the smallest *P* values) in CWR22Rv1 cells related to metastases, apoptosis, or proliferation. Red represents higher expression, whereas green represent lower expression in the heatmap. For comparison, the levels of the gene expression for the corresponding genes in the groups where Stat3 was inhibited are shown in the middle of the heatmap. **D:** A heatmap showing the top differentially expressed genes by Stat3 (determined by the smallest *P* values) in CWR22Rv1 cells related to metastases, apoptosis, or proliferation. Red represents higher expression, whereas green represent lower expression in the heatmap. For comparison, the levels of the gene expression for the corresponding genes in the groups where Stat5a/b was inhibited are shown on the left side of the heatmap. **E:** A heatmap showing the top differentially expressed genes by Stat5a/b and Stat3 (determined by the smallest *P* values) in CWR22Rv1 cells related to metastases, apoptosis, or proliferation. Red represents higher expression, whereas green represent lower expression in the heatmap.

Stat5a/b and Stat3 (See Supplemental Tables S1 and S2 at <http://ajp.amjpathol.org>). In both cell lines, inhibition of Stat5a/b led to down-regulation of PDCD4, a protein that mediates the inhibition of neoplastic transformation.⁴⁰ In

addition, inhibition of Stat5a/b induced in both cell lines the expression of Kruppel-like factor 4, which is a known repressor of CyclinD1 and mediates growth suppression.^{41,42} VEGF-C and collagen types VI and XII were

Table 2. Primers for Quantitative PCR Analysis

Gene	Direction	Sequence
<i>IL6R</i>	Forward	5'-GGGCTCTGAAGGAAGGCAAGACA-3'
	Reverse	5'-CGGTGGGAGATGAGAGGAACA-3'
<i>PDCD4</i>	Forward	5'-TATGATGTGGAGGAGGTGGATGTGA-3'
	Reverse	5'-CCTTTCATCCAAGGCAAACTACAC-3'
<i>KLF-4</i>	Forward	5'-AGAGGAGCCCAAGCCAAAGAGG-3'
	Reverse	5'-CCACAGCCGTCCAGTCACAGT-3'
<i>MAEA</i>	Forward	5'-GAAGCTCAGCGTCCCTCAAGA-3'
	Reverse	5'-CTGCTATGCTCTTTGAGGTGC-3'
<i>YWHAZ</i>	Forward	5'-CGTTACTTGGCTGAGGTTGC-3'
	Reverse	5'-CAGAGAAAGTTAAGGGCCAGAC-3'
<i>VEGF-A</i>	Forward	5'-CACTGAGGAGTCCAACATCAC-3'
	Reverse	5'-TTCTTGTCTTGTCTCTATCTTTCTTTG-3'
<i>Plexin C1</i>	Forward	5'-GCACCGTGGTAATGAACAGG-3'
	Reverse	5'-GAACGAGTTTGTAGAAAACAGG-3'

identified among the metastases-related genes regulated by Stat5a/b in DU145 cells, whereas inhibition of Stat3 led to down-regulation of Reticulon 4, BCL7B, and Macrophage Erythroblast Attacher⁴³ in both prostate cancer cell lines. We verified by quantitative PCR of a set of six genes that the changes in mRNA levels corresponded with the Affymetrix data (Table 2; see also Supplemental Figure S1 at <http://ajp.amjpathol.org>). In summary, the data presented here demonstrate that the gene expression profiles of transcription factors Stat5a/b and Stat3 differ in human prostate cancer cells.

Discussion

Protein kinase signaling pathways, such as Jak2-Stat5a/b or Jak2-Stat3, are of significant interest in the search for new molecular targets for therapy development for prostate cancer. In this work, we compared side-by-side the biological effects of two transcription factors of the Stat family, Stat5a/b and Stat3, in prostate cancer. We demonstrate that Stat5b has a key role over Stat3 in promoting the viability of prostate cancer cells in culture and growth of subcutaneous and orthotopic prostate tumors in nude mice. Stat3, in turn, is the predominant promoter of metastases formation in an experimental metastases assay *in vivo*. Of the two Stat5 homologs, Stat5b was more important for prostate cancer cell viability than Stat5a. Intriguingly, Stat5a/b regulates largely a different set of genes than Stat3 in prostate cancer cells, which provides the molecular basis for the different biological effects mediated by these two closely related Stat transcription factors in prostate cancer cells.

This is the first side-by-side comparison of the biological effects of Stat5a/b versus Stat3 in human prostate cancer cells *in vitro* and *in vivo*. This is important because it is well established that Stat5a/b and Stat3 are both constitutively active in advanced clinical prostate cancers.^{3-6,23} The relative importance of Stat5a/b versus Stat3 in promoting prostate cancer cell viability and tumor growth has been unclear. Here, our results demonstrate that inhibition of Stat5a/b induced extensive death of prostate cancer cells in culture in three different human prostate cancer cell lines (DU145, CWR22Rv1, and LNCaP), whereas the effects of Stat3 inhibition on prostate cancer cell viability

were modest. Specifically, inhibition of Stat3 had no effect on the number of LNCaP cells, and the effect of Stat3 inhibition on the number of DU145 or CWR22Rv1 cells was clearly less pronounced than that of Stat5 inhibition in the side-by-side comparison. PC-3 cells, which lack Stat5a/b and Stat3 because of gene deletions,³⁹ did not respond to Stat5a/b or Stat3 inhibition. The data presented here were not dependent on the methodology used because similar results were obtained by two different methodological approaches, adenoviral gene delivery of DN mutants of Stat5a/b or Stat3, and Stat5a/b versus Stat3 siRNA inhibition. Moreover, we verified by Western blotting that the siRNAs targeting Stat5a/b or Stat3 did not have cross-reactivity between the three transcription factors. In addition to the effects in cell culture conditions, inhibition of Stat5a/b reduced both subcutaneous and orthotopic prostate tumor growth in nude mice, whereas the effects of Stat3 inhibition were less pronounced. The results of this study also point out that of the two Stat5 homologs, Stat5b may be more important for prostate cancer cell viability and survival than Stat5a. Future work will need to confirm this observation in tumor growth studies *in vivo*.

The second key finding of this work was the demonstration of a predominant role of Stat3, not Stat5a/b, in promoting metastatic dissemination of human prostate cancer cells in an *in vivo* experimental metastases assay. We recently demonstrated that Stat3, through Jak2 activation, induced a migratory phenotype of DU145 and PC-3 cells through regulation of the actin cytoskeleton and the microtubule network.³¹ Moreover, Stat3 promoted metastases formation of human prostate cancer cells *in vivo*. This finding led us to compare Stat3 versus Stat5 in induction of metastatic behavior of human prostate cancer cells. Here we showed that Stat3 induced a 50-fold increase in the number of metastases in the lungs of nude mice after tail-vein injection of DU145 prostate cancer cells, whereas Stat5a/b increased the number of metastases by four- to fivefold. It is important to note that the Stat3 induction of lung metastases formation is unlikely to be attributable to Stat3 promotion of survival of human prostate cancer cells in circulation and in the lung microenvironment, because the effects of Stat3 inhibition on prostate cancer cell viability were only minor. These

observations support the concept that Stat3 induces metastatic behavior of prostate cancer cells by distinct molecular mechanisms that need to be defined in detail.

To understand the molecular background of the differential biological effects of Stat5a/b versus Stat3 in prostate cancer, we examined the gene expression profiles of Stat5a/b versus Stat3 in two different human prostate cancer cell lines. The most important knowledge gained from these studies is that Stat5a/b and Stat3 for the most part regulate different sets of genes in human prostate cancer cells. Both Stat5a/b and Stat3 recognize the GAS consensus sequence²¹ and, therefore, the differential gene expression patterns regulated by Stat5a/b versus Stat3 are likely a result of a number of different factors. These include different expression levels of co-activators, co-repressors, as well as negative regulators of Stat signaling such as phosphatases, PIAS proteins, and cytokine-inducible suppressors of cytokine signaling proteins. Moreover, the spacing between palindromic half-sites within the promoter is important in Stat5a/b versus Stat3 selection by GAS elements. In addition to the sequence, the extent of palindromic sites and also the nucleotides in between and outside the palindrome half-sites can contribute to the strength and selectivity of Stat5/3 binding to GAS elements.²¹ Somewhat surprisingly, the majority of the genes regulated by Stat5a/b as well as Stat3 were metastases-related. This may, however, be affected by the selection of the GO IDs for each functional group of the gene expression analysis.

Finally, Stat5a/b and Stat3 are in the active state in a significant fraction of human melanomas, breast, lung, and colon cancers. At the same time, inhibition of Stat5a/b or Stat3 did not affect the viability of cell lines established from melanomas, liver, colon, pancreas, or lung cancers, suggesting that the biological roles of Stat5a/b and Stat3 in these cancer types are different from in prostate cancer. It is known, however, that presence of active Stat5a/b in breast cancer predicts favorable clinical outcome,⁴⁴ whereas active Stat5a/b expression in prostate cancer predicts early disease recurrence.³ The differential biological effects mediated by Stat5 in various tissues are likely to be attributable to several factors such as epigenetic regulation of chromatin structure and the availability of Stat5-responsive genes for transcription, expression of co-activators and co-repressors in a given tissue, as well as expression levels of negative regulators of Stat5 action.

In conclusion, this work provides the first functional and gene expression profile comparison of transcription factors Stat5a/b and Stat3 in prostate cancer. The results indicate different roles for Stat5a/b and Stat3 in the progression of prostate cancer: Stat5a/b promotes viability and survival of the cells, whereas Stat3 stimulates metastatic behavior of cancer cells. Ongoing work aims to determine whether Jak2 is the common denominator for activation of both Stat5a/b and Stat3 in clinical prostate cancers using organ cultures of clinical prostate cancers as the experimental model system. This is important because the newly developed Jak2 inhibitors⁴⁵ may provide the therapeutic means to target both transcription factors and inhibit two different aspects of prostate cancer progression at the same time.

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