Down-Regulation of Signal Transducer and Activator of Transcription 3 Expression Using Vector-Based Small Interfering RNAs Suppresses Growth of Human Prostate Tumor In vivo

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Abstract

Purpose: Signal transducer and activator of transcription 3 (Stat3) is constitutively activated in a variety of cancers and it is a common feature of prostate cancer. Thus, Stat3 represents a promising molecular target for tumor therapy. We applied a DNA vector–based Stat3-specific RNA interference approach to block Stat3 signaling and to evaluate the biological consequences of Stat3 down-modulation on tumor growth using a mouse model.

Experimental Design: To investigate the therapeutic potential of blocking Stat3 in cancer cells, three small interfering RNAs (siRNA; Stat3-1, Stat3-2, and Stat3-3) specific for different target sites on Stat3 mRNA were designed and used with a DNA vector–based RNA interference approach expressing short hairpin RNAs to knockdown Stat3 expression in human prostate cancer cells in vitro as well as in vivo.

Results: Of the three equivalently expressed siRNAs, only Stat3-3 and Stat3-2, which target the region coding for the SH2 domain and the coiled-coil domain, respectively, strongly suppressed the expression of Stat3 in PC3 and LNCaP cells. The Stat3-1 siRNA, which targeted the DNA-binding domain, exerted no effect on Stat3 expression, indicating that the gene silencing efficiency of siRNA may be dependent on the local structure of Stat3 mRNA. The Stat3 siRNAs down-regulated the expression of Bcl-2 (an antiapoptotic protein), and cyclin D1 and c-Myc (cell growth activators) in prostate cancer cells. Inhibition of Stat3 and its related genes was accompanied by growth suppression and induction of apoptosis in cancer cells in vitro and in tumors implanted in nude mice.

Conclusions: These data indicate that Stat3 signaling is a promising molecular target for prostate cancer therapy and that vector-based Stat3 siRNA may be useful as a therapeutic agent for treatment of prostate cancer.

Prostate cancer is the most common cancer and the second leading cause of cancer-related deaths among men in Western countries (1, 2). More men are currently diagnosed at the early stages of prostate cancer and can be effectively treated by surgery or radiation. However, in one third of the patients, the disease will recur and metastatic prostate cancer remains essentially incurable. Whereas significant progress has been made in defining the molecular mechanisms of prostate cancer development, the specific molecular regulatory pathways involved in prostate cancer progression have not been fully characterized. However, targeting of currently known pathways may lead to effective treatments for prostate cancer.

Signal transducers and activators of transcription (STAT) were identified originally as key components of cytokine signaling pathways that regulate gene expression (3, 4). In mammals, there are seven members of the STAT family. All of them possess a similar modular organization comprised of the following domains: the NH2-terminal, coiled-coil, DNA-binding, SH2, and transactivation domains, which are all important for proper functioning (5). Constitutive activation of one STAT family member, Stat3, has been shown to play a key role in promoting proliferation, differentiation, antiapoptosis, and cell cycle progression (3, 6). Persistently active Stat3 and its overexpression have been detected in human prostate cancers and have been suggested to be associated with prostate cancer progression (7–11). Aberrantly active Stat3 promotes uncontrolled growth and survival through dysregulation of expression of downstream targeted genes, such as cyclin D1, cyclin D2,
c-Myc, and p53 (12–14), and Bcl-xL, Bcl-2, Mcl-1, and Survivin (15–19); these genes influence cell cycle progression or inhibit apoptosis. Stat3 exists in a latent form in the cytoplasm until activated by a wide variety of cell surface receptors via tyrosine phosphorylation, dimerization, and translocation into the nucleus (20), where it binds to STAT-specific DNA response elements in certain promoters. Constitutive Stat3 signaling represents one of the key molecular events in the multistep process leading to carcinogenesis. Thus, Stat3 may represent a new molecular target for therapeutic intervention of prostate cancer.

Several recent reports show that blockade of Stat3 expression in human cancer cells suppresses proliferation in vitro and tumorigenicity in vivo. The approaches include tyrosine kinase inhibitors (21, 22), antisense oligonucleotides (23), decoy oligonucleotides (24), dominant-negative Stat3 protein (8, 25), and RNA interference (RNAi; refs. 26, 27). In the RNAi approach, a sequence-specific posttranscriptional gene silencing is achieved through a small interfering RNA (siRNA), a short double-stranded RNA molecule in which one strand is complementary (i.e., antisense) to the target mRNA of a selected gene (14, 28). In cultured cells, but also for developing highly specific therapeutics. RNAi has been shown to be effective not only in cultured mammalian cells, but also in vivo. Recently, short hairpin RNAs (shRNA) have proven to be effective both in vitro and in vivo (29, 30) at reducing targeted gene expression. These artificial RNAs are apparently transcribed as hairpin RNA precursors from an RNA polymerase III–based vector containing the U6 or H1 promoters in cultured cells, and are processed to their effective mature siRNA forms by Dicer (31, 32). shRNAs are inexpensive to deliver on plasmids and are quite stable relative to antisense RNAs. However, the application of this approach for the treatment of specific diseases has progressed more slowly than initially anticipated. There are only a few studies on the application of siRNA at the organissimal level. However, studies that use RNAi to counteract disease processes in vivo are emerging.

To determine directly the role of Stat3 in prostate cancer, we examined the expression levels of Stat3 and the phosphorylation status of Stat3, as well as expression of its downstream mediators, Bcl-2, cyclin D1, and c-Myc, in prostate tumor-derived cell lines and tissues from prostate cancer patients, comparing them with normal prostate tissue. Furthermore, we applied a DNA vector–based (U6-driven shRNA expression) Stat3-specific RNAi approach to block Stat3 signaling and to evaluate the biological consequences of Stat3 down-modulation on tumor xenografts in a mouse model. We also examined positional effects on the silencing ability of the siRNAs. Results indicate that blockade of Stat3 expression using a specific RNAi approach can significantly reduce prostate tumor growth in nude mice.

### Materials and Methods

**Immunohistochemical detection of Stat3 in prostate tissues.** Primary prostate tumor tissues and adjacent normal tissues were obtained from 23 patients with a prior diagnosis of prostate cancer. Paraffin wax samples of 23 pairs of matched human primary prostate tumors with normal prostate tissue were cut into 5-μm-thick slices. These slices were dewaxed and the endogenous peroxidase activity was quenched after incubation in methanol containing 3% hydrogen peroxide for 10 minutes. The histologic sections were then immunostained using a rabbit anti-human Stat3 polyclonal antibody (Santa Cruz Biotech, Inc., Santa Cruz, CA) to localize Stat3. As a negative control, rabbit immunoglobulins (Vector Laboratories, Burlingame, CA) were used to replace the primary antibody. Goat anti-rabbit IgG conjugated with horseradish peroxidase was used as a secondary antibody. Immunohistochemical staining was done manually at room temperature, using an avidin-biotin-peroxidase complex method (Vectastain Elite ABC kit; Vector Laboratories). The criteria for immunohistochemical assay results are as follows: positive cells contained brown particle staining in the nucleus or cytoplasm. Samples with <5% positive cells were designated as negative (−); samples stained slightly between 5% and 25% positive were designated as (+); samples stained moderately (between 25% and 50% positive) as (++); and stained deeply (>50% strongly positive) as (+++).

**Construction of plasmids that contain DNA templates for the synthesis of small interfering RNAs under the control of the U6 promoter.** The pSilencer 1.0-U6 plasmid (Ambion, Austin, TX) was used for DNA vector–based siRNA synthesis. Three siRNA target sequences were selected from different positions of human Stat3. Stat3-1 siRNA, Stat3-2 siRNA, and Stat3-3 siRNA corresponding to nucleotides 1,571 to 1,590, 438 to 456, and 2,144 to 2,162 (Genbank accession no. NM003150) were located in the DNA-binding, coiled-coil, and SH2 domains of human Stat3, respectively. The sequences of the synthesized oligonucleotides for Stat3-1 are: 5′-GATTTGAGCAGACCCCATACAGTGTTCAGCTGCTGCT-3′ (forward) and 5′-AATTAAAAAGATTGACCTAGAGACCCACTCTCTTGAAGTGGGTCTCT-3′ (reverse); Stat3-2: 5′-GAGTTGAGCAGACCCCATACAGTGTTCAGCTGCTGCTGCGGCC-3′ (reverse); and Stat3-3: 5′-GACGCAGCGCTGCTGCTGCGGCC-3′ (reverse). The three oligonucleotides were annealed and ligated to the U6 promoter of the pSilencer 1.0-U6 plasmid vector to generate the pSilencer1.0-U6-Stat3 siRNA vectors. These vectors produce a short hairpin RNA with the linker sequence (TTCAAGAGA) that forms a looped structure, the linker being processed by Dicer, to generate a Stat3-specific siRNA. A negative control scrambled siRNA (Ambion), which has no significant homology to mouse or human gene sequences, was designed to detect any nonspecific effects.

**Determination of messenger RNA structure.** The possible secondary structures of the targeted mRNA molecule and the minimum free energy (G) values, were predicted using the “Mfold web server,” which provides several closely related softwares for predicting the secondary structure of single-stranded nucleic acids (33). The gateway for the Mfold web server is http://mfold.bioinfo.rpi.edu/applications/mfold.

**Cell culture and transfections.** The human prostate cancer cell lines PC3 and LNCaP were obtained from American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum. For transfection, the cells were plated into either 12-well plates (104 cells per well) or a 100 mm dish (106 cells) and allowed to adhere for 24 hours. LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) was used for...
transfecting cells, and enhanced green fluorescent protein vector (pEGFP; BD Clontech, Inc., Palo Alto, CA) was cotransfected with either pSilencer1.0-U6-Stat3 siRNAs or pSilencer empty vector at ratio of 1:20 to mark the positive transfected cells. Cells were cultured for 5 to 20 hours and then replaced with fresh medium supplemented with 10% fetal bovine serum and lysed for 24 to 72 hours after transfection.

**Messenger RNA quantification.** Quantitation of specific mRNA was done essentially as described previously (34). Briefly, after 72 hours of transfection, cells were collected and total RNA was extracted from cells with Trizol (Invitrogen) following the instructions of the manufacturer. For Northern blot analysis, 20 μg of total RNA were electroeluted on a 1.2% agarose-0.1% Igepal gel, and blotted onto Hybond-N+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Hybridization was done using the express Hyb buffer (BD Clontech) with 32P-labeled cDNA of Stat3 and actin as probes. Blots were exposed to Kodak MS film and then quantitated using a Molecular Dynamics PhosphorImager (Molecular Dynamics, Sunnyvale, CA). For reverse transcription-PCR (RT-PCR) analysis, 3 μg of total RNA were subjected to reverse transcription using a RT-PCR kit (Promega, Madison, WI). For the amplification of Stat3 mRNA, the primer pairs 5'-TGCCTGCCTACAAAGGCTTA-3' and 5'-ATGAGAACCACGAGAGAAAGGAG-3' were used with the following conditions: 94°C for 5 minutes, then 30 cycles of 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 1 minute, followed by 72°C for 5 minutes. For amplification of c-Myc mRNA, the primer pairs 5'-TGGGAGAGGATTCACACGACC-3' and 5'-CTGTTCGCTGCACAACTG-3' were used with the following conditions: 94°C for 5 minutes, then 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, followed by 72°C for 5 minutes. The PCR products were analyzed by standard agarose gel electrophoresis, and the bands were quantified with ImageQuant 5.0 software (Molecular Dynamics).

**Expression of the short hairpin RNA transcripts.** To monitor and determine the expression levels of the Stat3 siRNAs after transfection, three DNA oligonucleotides containing 19 or 20 bp complementary to the antisense of the three Stat3 siRNA were synthesized and used as probes. Their sequences are given as follows: Stat3-1 oligo probe, GAGAGGTTCA-3'; Stat3-2 oligo probe, VTTGCCAGTTGTGGTGATC-3'; and Stat3-3 oligo probe, GCAGCAGCTGAACAACATG. After 72 hours, cells were collected and washed with cold PBS containing 4 mM/L EDTA. After washing, cells were fixed with 70% cold ethanol; collected by centrifugation and washed once with PBS; suspended in PBS containing 20 μL/mL of propidium iodide (Sigma), 0.2% Triton X-100, and 40 μg/mL RNase A; and incubated for at least 30 minutes at 4°C. The cells were then analyzed for cell cycle phase distribution by flow cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ) using CellQuest software (Becton Dickinson). For examination by fluorescence microscopy, cells were stained with 0.1% acridine orange (Sigma), then observed. In proliferation assays, cell numbers were determined at 72 hours posttransfection using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) assays and quantified using a Microplate Reader (Bio-Rad).

**Antitumor effect of Stat3 small interfering RNA in vivo.** PC3 cells (3 × 10⁶/150 μL) were inoculated s.c. into the right flank of nude mice, and establishment of palpable tumors was determined. The tumor volume (m² × m × 0.5236, where m represents the short axis and m2 the longer axis) was measured every 2 to 3 days until day 17. When tumors reached an average volume of ~157.56 ± 28.54 mm³ (on day 17), three experimental groups (five mice per group) were tested as follows: (a) mock transfection (PBS buffer alone), (b) scrambled vector control (20 μg/mouse), and (c) PC3 cells transfected with the U6-vector expressing Stat3-1 siRNA, TCTCTATC; and Stat3-3 oligo probe, GCAGCAGCTGAACAACATG.

**Western blot analysis** of Stat3 expression in PC3, LNCaP, prostate cancer tissue with high density staining for Stat3, 0.5236, where m represents the short axis and m2 the longer axis) was measured every 2 to 3 days until day 17. When tumors reached an average volume of ~157.56 ± 28.54 mm³ (on day 17), three experimental groups (five mice per group) were tested as follows: (a) mock transfection (PBS buffer alone), (b) scrambled vector control (20 μg/mouse), and (c) PC3 cells transfected with the U6-vector expressing Stat3-1 siRNA, TCTCTATC; and Stat3-3 oligo probe, GCAGCAGCTGAACAACATG. For cyclin D1, 50 μg of total protein was resolved on a 10% SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and then probed with anti–cyclin D1 antibody. For Bcl-2, 50 μg of total protein was resolved on a 12% SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and then probed with anti–Bcl-2 antibody. For cyclin D1, 50 μg of total protein was resolved on a 10% SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and then probed with anti–cyclin D1 antibody. For Bcl-2, 50 μg of total protein was resolved on a 12% SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and then probed with anti–Bcl-2 antibody. For cyclin D1, 50 μg of total protein was resolved on a 10% SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and then probed with anti–cyclin D1 antibody. For Bcl-2, 50 μg of total protein was resolved on a 12% SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and then probed with anti–Bcl-2 antibody.

![Image](311x164 to 532x358)

Fig. 1. Stat3 expression in human prostate cancer cells. A, Western blot analysis of Stat3 expression in PC3, LNCaP, primary tumor, and normal prostate tissue with 50 μg of total protein for each sample. B, Stat3 protein levels quantitated from Western blot in PC3, LNCaP cells, prostate tumor, and normal prostate. The absorbance of the bands was measured by densitometric analysis. C, immunohistochemical analysis of Stat3 expression (photographed at ×400) in three experiments; bars, SE (P < 0.05 versus controls). C, immunohistochemical analysis of Stat3 expression (photographed at ×400) in three experiments; bars, SE (P < 0.05 versus controls). C, immunohistochemical analysis of Stat3 expression (photographed at ×400) in three experiments; bars, SE (P < 0.05 versus controls). C, immunohistochemical analysis of Stat3 expression (photographed at ×400) in three experiments; bars, SE (P < 0.05 versus controls).
pSilencer1.0-U6-Stat3-3 siRNA (20 µg/mouse). The samples were diluted in 50 µL of PBS buffer and injected percutaneously into the tumor by using a syringe with a 27-gauge needle. Immediately after injection, tumors were pulsed with an electroporation generator (ECM 830, BTX). Pulses were delivered at a frequency of 1/sec, 150 V/cm, with a length of 50 milliseconds. This process was repeated on day 24. Mice were sacrificed on day 34, and the tumors treated with either scrambled vector control or Stat3-3 siRNA were excised for H&E staining and terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) assay.

Histochemistry and terminal deoxynucleotidyl transferase–mediated nick end labeling assay. Serial sections of tumor tissue excised from animals were fixed in formalin, stained with H&E, and processed for routine histologic examination. TUNEL assay was done by using the In situ Cell Death Detection kit (Roche, Inc., Indianapolis, IN) that relies on fluorescent labeling of DNA strand breaks. Three-micrometer sections from paraffin-embedded specimens were dewaxed and hydrated according to the standard protocol. After incubation with proteinase K (200 µg/mL) for 30 minutes at 21°C, the TUNEL reaction mix containing bromodeoxyuridine triphosphate, terminal deoxynucleotidyl transferase, and reaction buffer was added to the slides and incubated in a humidified chamber for 60 seconds at 37°C, followed by washing and incubation with a FITC-labeled antibr`modeoxyuridine monoclonal antibody for 30 minutes at room temperature. The reaction was visualized by fluorescence microscopy. TUNEL-positive cells exhibit green fluorescence. The apoptotic index was calculated as follows: apoptotic index = (number of apoptotic cells / total cell number counted) × 100%.

Statistical analysis. A χ² analysis was done to evaluate the significance of differences between the experimental groups. For a single comparison of two groups, the Student’s t test was used. Two-way ANOVA using the Student-Newman-Keuls method was used for comparison of tumor size in mice after different treatments. For all analyses, the level of significance was set at P < 0.05. All statistical calculations were done using the SigmaStat statistical software package (SPSS, Chicago, IL). Data are presented as the mean ± SE.

Results

Stat3 is overexpressed in prostate cancer cell lines and prostate cancer tissues. To determine whether Stat3 is overexpressed in prostate cancer tissue, we compared the level of Stat3 expression in normal prostate tissue to prostate cancer tissue and prostate cancer cell lines (PC3, LNCaP) using Western blot and immunohistochemical analyses with an anti-Stat3 antibody. Both approaches revealed that Stat3 was overexpressed in cancer tissues and prostate cancer cell lines (Fig. 1A and C). Stat3 protein levels were measured by densitometric analysis

Table 1. Immunohistochemical analysis of Stat3 expression in normal prostatic tissue and prostatic cancer tissue

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<thead>
<tr>
<th>Tissue Type</th>
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<th>–</th>
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<td>Normal prostatic tissue</td>
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<td>Prostatic cancer tissue</td>
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<td>5</td>
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NOTE: χ² = 21.233, P < 0.001.

Fig. 2. Northern blot and RT-PCR analysis of Stat3 mRNA in human prostate cancer cell lines. A, PC3 cells were transfected with either 2 µg of pSilencer1.0-U6 vector Stat3 siRNAs or negative control vector for 72 hours. Equal amounts of 20 µg of total RNA extract were used for Northern blot analysis. B, the quantified Stat3 mRNA from three separate experiments and normalized to that of β-actin. Columns, mean; bars, SE (*P < 0.05 versus untreated and empty vector). C, 3 µg of total RNA used for RT-PCR analysis of Stat3 mRNA in PC3 cells after transfection with Stat3-2, Stat3-3 siRNA, and pSilencer vectors. Mock = untreated cells.

Fig. 3. Western blot analyses for Stat3, p-Stat3, and Northern blot analysis for shRNA transcripts expression in PC3 cells transfected with Stat3 siRNAs. A, Stat3 protein level in PC3 cells transfected with three Stat3 siRNAs and pSilencer empty vector revealed by Western blot. B, the quantified Stat3 protein level in PC3 cells transfected with Stat3 siRNAs from three separate experiments. Columns, mean; bars, SE (*P < 0.05). C, p-Stat3 protein level in PC3 cells after transfection with Stat3-siRNAs revealed by Western blot analysis. D, the quantified p-Stat3 protein level in PC3 cells transfected with Stat3 siRNAs from three separate experiments. Columns, mean; bars, SE (*P < 0.05). E, PC3 cells transfected with Stat3-3 siRNA for the times indicated and processed for Western blot with anti-Stat3 antibody. F, three shRNA transcripts expression in PC3 cells transfected with U6 vector-Stat3 siRNAs. Mock = untreated cells.
of the Western blots. Quantitative evaluation of the relative expression of Stat3 revealed that this protein is overexpressed by an average of 2.2-fold in the 23 primary prostate tumors, PC3, and LNCaP cells compared with normal prostate tissue (Fig. 1B). As summarized in Table 1, the high Stat3 levels found in both prostate tumor specimens and prostate cancer cell lines were significantly different \((P < 0.001)\) from the lower Stat3 level found in normal cells.

Small interfering RNA constructs specifically reduce Stat3 expression in prostate cancer cell lines. Previous studies provided strong evidence that siRNA specific to the Stat3 gene can significantly suppress Stat3 protein expression \((26, 27, 35)\). To determine if inhibition of Stat3 expression could suppress the prostate tumor via the gene-silencing effect of vector-based RNAi, we synthesized three siRNAs directed at different sites of Stat3 mRNA (Stat3-1, Stat3-2, and Stat3-3).

The DNA oligonucleotides representing the siRNA duplexes were cloned into the pSilencer1.0-U6 vector and transfected into PC3 cells. Specific reduction of Stat3 mRNA level in PC3 cells was first determined by Northern blot analysis 72 hours after transfection. The Stat3-2 and Stat3-3 siRNAs reduced the Stat3 mRNA level to 25% and 20% of the control, respectively (Fig. 2A and B). The Stat3-1 siRNA construct (targeted to DNA-binding domain region) had no significant inhibitory effect on Stat3 mRNA expression. The reduction of Stat3 mRNA was also observed by semiquantitative RT-PCR using the RNA isolated from PC3 cells (Fig. 2C). Furthermore, the effects of siRNAs on Stat3 and p-Stat3, the activated form of Stat3, expression were evaluated by Western blot analysis with antibody against Stat3 or p-Stat3. Cells transfected with the Stat3-1 siRNA vector showed the same level of Stat3 or p-Stat3 expression as the control (Fig. 3A-D). However, Stat3-2 and Stat3-3 siRNA reduced the level of both Stat3 or p-Stat3 in PC3 cells. A comparison of p-Stat3 expression in the cells PC3 cells treated with Stat3-3 siRNA versus control showed that the p-Stat3 almost disappeared in the Stat3-3–treated cells (Fig. 3C and D). The silencing effect of siRNAs was also found to be time dependent, with the maximum effect achieved between 48 and 72 hours posttransfection in PC3 cells (Fig. 3E).

Small hairpin RNA transcript expression in PC3 cells. To determine if the differential effects of these siRNA constructs were due to differential siRNA expression or to differences in their stability, we did Northern blot analyses. In cells transfected with the Stat3-1, Stat3-2, and Stat3-3 plasmid constructs, the shRNAs were expressed at comparable levels at 48 or 72 hours posttransfection (Fig. 3F). However, at 72 hours posttransfection, there was a higher expression of siRNAs than at 48 hours, indicating a sustained, but equivalent, expression of all three siRNAs. Notably, there was no specific decline in the levels of any of the expressed siRNAs.

The silencing efficiency is dependent on the location of the target in Stat3 messenger RNA. The results obtained from above experiments showed that different siRNAs have a different effect on Stat3 expression. This result could be due to the differences in the secondary structure of Stat3 mRNA at the targeted sites. Indeed, using the Mfold program \((33)\), we found a loop-like structure in the mRNA at the region targeted by Stat3-2 and Stat3-3 siRNA. In contrast, a hairpin structure was predicted in the mRNA at the site targeted by Stat3-1 siRNA (Fig. 4). Thus, differences in the local structures of mRNA may explain why the three siRNAs showed different gene-silencing efficiencies. These structural differences are also reflected in the free energy values \((\Delta G)\).

### Table 2. Properties of siRNAs targeted to inactivate the human Stat3 mRNA

<table>
<thead>
<tr>
<th>siRNA</th>
<th>siRNA sequence</th>
<th>Position</th>
<th>RNA structure</th>
<th>(\Delta G) (kcal/mol)</th>
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<tbody>
<tr>
<td>Stat3-1</td>
<td>GAUGACCUAGAGACCCAC</td>
<td>1,571-1,590</td>
<td>Hairpin</td>
<td>3.2</td>
</tr>
<tr>
<td>Stat3-2</td>
<td>GAGUCGAUGUCUCAUAUC</td>
<td>438-456</td>
<td>Loop</td>
<td>–0.8</td>
</tr>
<tr>
<td>Stat3-3</td>
<td>GCAGCAGCUGAACAACUG</td>
<td>2,144-2,162</td>
<td>Loop</td>
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</tr>
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*The RNA structures and the \(\Delta G\) values were determined using the Mfold software.
Stat3-1 siRNA, with high free energy (3.2 kcal/mol; Table 2), showed no reduction in the expression of Stat3 gene. In contrast, Stat3-2 and Stat3-3 had lower free energies and were each equally effective in suppressing prostate tumor cell proliferation.

**Inhibition of Stat3 suppresses Bcl-2, cyclin D1, and c-Myc expression in PC3 cells.** Recent studies indicate that a constitutively active Stat3 induces the expression of several genes, including Bcl-2, which codes for an antiapoptotic protein, and cyclin D1 and c-Myc, which promote cell division. To determine the expression of these gene products, Western blot and RT-PCR analyses were done using the protein and RNA isolated from PC3 cells transfected with the Stat3-3 construct. Western blots showed that the intracellular Bcl-2 and cyclin D1 levels were significantly decreased in Stat3-3 siRNA-transfected PC3 cell (Fig. 5A-D) compared with controls. RT-PCR showed that intracellular c-Myc mRNA was significantly decreased in Stat3-2 and Stat3-3–transfected PC3 cells (Fig. 5E). Thus, Stat3-3 siRNA down-regulates the expression of Bcl-2, cyclin D1, and c-Myc, factors known to ablate apoptosis or stimulate cell cycle progression.

The Stat3-3 small interfering RNA construct inhibits growth and survival of PC3 and LNCaP cells and induces apoptosis and G1 arrest of PC3 cells in vitro. To determine the influence of siRNAs on human prostate cancer cell growth, PC3 cells were transfected with the Stat3-3 siRNA expression vector or empty vector, and analyzed after 72 hours of transfection. After transfection with Stat3-3 siRNA, PC3 cell cultures became less confluent as some cells rounded and detached from the culture plates, compared with a control culture transfected with the empty vector (Fig. 6A). To monitor the growth and cycling status of the siRNA-expressing cells, PC3 cells were stained with acridine orange dye and subjected to flow cytometry. Stat3-3 siRNA induced significant apoptosis in PC3 cells compared with those transfected with the empty vector (Fig. 6B). This siRNA also caused significant growth inhibition of PC3 compared with those transfected with the empty vector.

**Fig. 5.** Western blot analysis for Bcl-2, Cyclin D1, and RT-PCR for c-Myc mRNA in control versus siRNA-treated PC3 cells. A, cells transfected with Stat3-3 siRNA and processed for Western blotting using anti-Bcl-2 antibody. B, quantified Bcl-2 protein levels in cells transfected with Stat3-3 siRNA or controls from three separate experiments. Columns, mean; bars, SE. C, cells transfected with Stat3 siRNAs or control and processed for Western blotting using anti-cyclin D1 antibody. D, quantified cyclin D1 protein level in cells transfected with Stat3 siRNAs from three separate experiments. Columns, mean; bars, SE (* P < 0.05 compared with empty vector). E, RT-PCR analysis for c-Myc mRNA in cells transfected with Stat3 siRNAs or controls. Mock = untreated cells.

**Fig. 6.** Transfection with Stat3-3 siRNA inhibited the growth of PC3 cells and induced apoptosis. A, transfection with Stat3-3 siRNA for 72 hours inhibited the growth of cells (magnification: ×1,000). B, cells were transfected with Stat3-3 siRNA. After 72 hours, cells were fixed in 4% paraformaldehyde and stained with acridine orange to visualize apoptotic cells (orange yellow; ×1,000). C, growth inhibitory ratios for PC3 cells transfected with empty vector or Stat3-3 siRNA, averaged for three separate experiments (* P < 0.05 versus empty vector). Mock = untreated cells.
control (Fig. 6C). To determine whether this growth inhibition was associated with specific changes in cell cycle distribution, cell cycle analysis was done in PC3 cells transfected with Stat3-3 siRNA or scrambled vector control. The data shown in Table 3 indicate that the Stat3-3 siRNA induced ~10-fold more apoptosis in PC3 cells compared with the scrambled control. Cells transfected with siRNA – Stat3-3 accumulated significantly in G1 phase compared with the control. These findings indicate that inhibition of Stat3 suppresses both the growth and survival of prostate cancer cells.

**Antitumor activity of Stat3 small interfering RNA in vivo.** To evaluate the effects of Stat3 siRNA vector on prostate tumor growth in vivo, we used a nude mouse tumor xenograft model. Mice were transplanted s.c. with 3 X 10³ PC3 cells into the right flank. By day 17, palpable tumors had developed at the sites of injection (mean volume, 157.56 ± 28.54 mm³; n = 5). These mice were divided into three groups of five mice each and injected intratumorally with either buffer alone (untreated), Stat3-3 siRNA, or the vector expressing scrambled siRNA. This process was repeated on day 24. Animals were sacrificed on day 34 and tumor sizes were determined. The mean tumor volume of mice treated with buffer control was 1,185.50 ± 147.42 mm³ on day 34 (Fig. 7B). The mean tumor volume in mice treated with scrambled siRNA control was 1,092.09 ± 189.23 mm³ (Fig. 7D) and that of the group treated with Stat3-3 siRNA was 354.25 ± 56.89 mm³ (Fig. 7C). The difference in tumor size between both groups 1 and 2 did not reach statistical significance (P > 0.05; see Fig. 7A-D). The group treated with Stat3-3 siRNA had marked tumor growth suppression compared with the scrambled siRNA control (P < 0.01). To determine the mechanism of tumor growth inhibition in vivo, PC3 tumors treated with buffer alone, Stat3-3 siRNA, or scrambled siRNA control were excised for H&E staining and analyzed with TUNEL assays (Fig. 7E-I). The experiments showed that Stat3-3 siRNA-treated tumors had undergone massive apoptosis with sparsely dispersed chromatin and necrotic tissue (Fig. 7F), and several TUNEL-positive cells or cell clusters (Fig. 7J), compared with the buffer control (Fig. 7E) or scrambled siRNA controls (Fig. 7G), which showed a finely granular cytoplasm with evenly dispersed chromatin and no TUNEL-positive cells, respectively (Fig. 7H and I). These data show that Stat3-3 siRNA, injected into the tumor, exerts strong antitumor effects in this mouse xenograft model.

**Discussion**

Current therapies for advanced androgen-independent prostate cancer are only marginally effective. Thus, it is important to understand the molecular mechanisms involved in the pathogenesis and progression of this disease to identify novel therapeutic targets and develop effective treatment strategies. Elevated STAT activities have been detected in primary tissues and cell lines of prostate tumors. Notably, persistent Stat3 activation is associated with prostate cancer progression (7–11, 35). Stat3 activates several genes whose products promote cell cycle progression, e.g., cyclin D1 or c-Myc (12–14), and prevent apoptosis, e.g., Bcl-2 and Bcl-XL (15–19). Stat3 is also implicated in the expression of genes involved in invasion and metastasis, such as the matrix metalloproteinase-9 (36). In this study, we confirmed that Stat3 plays a key role in promoting prostate tumor proliferation in vitro and in vivo. Immunohistochemical and Western blot analyses showed that Stat3 is overexpressed both in human prostate cancer cell lines and in tissues from human primary prostate cancers. Quantitation revealed an average 2.2-fold increase in the level of Stat3 protein expression in primary prostate tumor and PC3 cells compared with normal prostate tissue. These data are consistent with other studies (7–11, 23) that showed an abnormal activation of Stat3 in a variety of human tumors including prostate cancer.

Using the vector-based RNAi approach, we showed that down-regulation of Stat3 strongly suppresses growth of tumor cells. Western blot analysis with anti-Stat3 or anti–phospho-Stat3 antibodies, Northern blot, and RT-PCR showed that Stat3 siRNAs suppress Stat3 expression in prostate cancer cell lines in vitro. The expression of p-Stat3 in PC3 cells treated with Stat3 siRNAs declined ~90%, indicating a sharp silencing efficiency (Fig. 3C and D). The Stat3-2 and Stat3-3 constructs, but not Stat3-1, strongly reduced Stat3 expression (Figs. 2A, B and 3A, B). The differences in the efficiency of these siRNA constructs in knocking down Stat3 mRNA could not be attributed to differences in their expression or stability intracellularly, because all three siRNAs continued to express at comparable levels at 48 or 72 hours posttransfection (Fig. 3F). At present, it is unclear what factors determine the gene silencing efficiency of a given siRNA. It seems likely that the structure of or access to the target sequence governs the efficiency of a siRNA. Therefore, it is reasonable to presume that sequence-specific interactions between siRNA and its target mRNA may dictate the efficacy. In this connection, the Mfold program (33) may be a useful prognosticator for choosing ideal siRNA targets. Our findings are consistent with recent studies that showed that the local secondary structures of mRNA at regions targeted by siRNAs (Fig. 4) play a critical role in determining the efficiency of gene silencing (37). The targeted sites of mRNA forming a hairpin structure (e.g., Stat3-1 targeted region) and with high free energy values (Table 2), in general, would be less effective in knocking down the

<table>
<thead>
<tr>
<th>Group (n = 3)</th>
<th>Apoptotic cells (%)</th>
<th>G0-G1 (mean ± SD)</th>
<th>S (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mean ± SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scrambled control</td>
<td>1.98 ± 0.24</td>
<td>47.23 ± 2.12</td>
<td>53.25 ± 2.98</td>
</tr>
<tr>
<td>siRNAstat3-3</td>
<td>20.26 ± 2.28*</td>
<td>79.05 ± 4.52</td>
<td>13.97 ± 0.56*</td>
</tr>
</tbody>
</table>

*P < 0.05.
targeted mRNA expression (37–39). Targeted regions that can form a loop and have low free energy values might bind siRNA most efficiently leading to the inactivation of the target mRNA.

Mechanistically, the inactivation of Stat3 mRNA by the siRNAs can cause multiple effects on the growth of a tumor cell. We showed that the expression of Bcl-2, cyclin D1, and c-Myc was greatly diminished in cells transfected with Stat3-3 siRNA (Fig. 5A-E). These results are consistent with other recent reports that showed that ablation of Stat3 signaling was accompanied by growth inhibition and induction of apoptosis (16, 40). Both cyclin D1 and c-Myc respond to mitogenic signals and are required for progression of cells from G1 to S phase of the cell cycle (41, 42). The data shown in Table 3 suggest that inhibition of tumor growth is due to a combination of cell cycle arrest and activation of apoptosis. Ultimately, the Stat3-3 siRNA was found to be effective in suppressing prostate tumor growth in an in vivo xenograft mouse model via both growth inhibition and increased apoptosis.

Chemical synthesis of siRNAs is not cost-effective for large-scale therapeutic applications. RNAi induced by chemically synthesized siRNAs is transient, and reexpression of the target mRNA in mammalian cells usually occurs within a few days (43, 44). In contrast, plasmid-based expression (45–47) produces sustained amounts of siRNA over extended periods utilizing the cellular machinery and is relatively inexpensive to generate (31, 40, 48–50). Two doses were used here to avoid the nonspecific necrotic effects caused by multiple intratumoral injections. Although the data are limited, we have not observed any limitations or toxicities in this treatment model. Most importantly, our data show that inhibition of Stat3 by administration of appropriate vector-based siRNAs into the tumor is an effective and feasible approach to prostate cancer therapy. This is the first study to use DNA injection as a tool for suppressing Stat3 and prostate tumor growth. The feasibility of this approach to treating tumors clinically has not been fully explored, although it seems logical. In an analogous manner, some recent studies have used polymer-conjugated chemotherapeutics (51) for intratumoral administration and obtained significant therapeutic benefits. Perhaps more pertinent, one recent study also showed the intratumoral inhibition of cathepsin B and matrix metalloproteinase-9 expression with plasmid-based siRNA caused a suppression of tumor metastasis (52). Future therapeutic applications of siRNAs, however, are largely dependent on the development of suitable delivery vehicles with appropriate pharmacokinetics and intracellular stability. Electroporation has been shown to facilitate the transfer of plasmid DNA into target cells or tissues. In fact, electroporation has already been applied in vivo to chemotherapy of some malignancies, such as melanoma (53, 54) and colon tumors (55). Much evidence has been accumulated to show the safety of in vivo electroporation for clinical use. Further efforts are clearly necessary for exploring the therapeutic value of injecting naked plasmids into tumors. In summary, we present evidence for a potential use of siRNA for blocking
persistent Stat3 signaling to reverse tumor growth. Plasmid-based siRNA therapy for tumor suppression may offer an effective, inexpensive approach and delivery system for prostate cancer therapy.

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References

Down-Regulation of Signal Transducer and Activator of Transcription 3 Expression Using Vector-Based Small Interfering RNAs Suppresses Growth of Human Prostate Tumor In vivo

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