

RNA Interference Targeting Stat3 Inhibits Growth and Induces Apoptosis of Human Prostate Cancer Cells

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Stat3, a member of the signal transduction and activation of transcription (STAT) family, is a key signal transduction protein that mediates signaling by cytokines, peptide growth factors, and oncoproteins and is constitutively activated in numerous cancers including prostate. Previous studies demonstrated that constitutively activated Stat3 plays an important role in the development and progression of prostate cancer by promoting cell proliferation and protecting against apoptosis. The present study was designed to investigate the potential use of RNA interference to block Stat3 expression and activation and the effect on the growth of human prostate cancer cells. We identified a small interfering RNA (siRNA) specific for Stat3 and demonstrate that blockade of Stat3 activation by the Stat3 siRNA suppresses the growth of human prostate cancer cells and Stat3-mediated gene expression and induces apoptotic cell death. The Stat3 siRNA does not inhibit the proliferation nor induces apoptosis of Stat3-inactive human prostate cancer cells. In addition, the Stat3 siRNA inhibits the levels of androgen-regulated prostate specific antigen (PSA) expression in prostate cancer cells. These results demonstrate that targeting Stat3 signaling using siRNA technique may serve as a novel therapeutic strategy for treatment of prostate cancer expressing constitutively activated Stat3. *Prostate* 60: 303–309, 2004. © 2004 Wiley-Liss, Inc.

KEY WORDS: Stat3; RNA interference; prostate cancer; apoptosis

INTRODUCTION

Stat3, a member of the signal transduction and activation of transcription (STAT) family, is a key signal transduction protein that mediates signaling by numerous cytokines, peptide growth factors, and oncoproteins [1]. Accumulating evidence demonstrates that Stat3 activation plays important roles in cell differentiation, proliferation, development, apoptosis, and inflammation [2]. Elevated activity of Stat3 has been found frequently in a wide variety of human tumors, including hematologic malignancies, head and neck, breast and prostate cancer [2]. Cell lines from multiple myelomas that have become growth factor independent require constitutively active Stat3 to protect against apoptosis [3]. In addition, constitutively activated Stat3 induces cellular transformation in vitro and tumor formation in nude mice [4].

Studies to date provide strong evidence that aberrant Stat3 signaling plays an important role in the development and progression of prostate cancer by

promoting cell proliferation and protecting against apoptosis. Stat3 activity is not only significantly increased in human primary prostate cancer tissues as compared with normal, but it is also increased in androgen independent prostate cancer cells as compared to androgen sensitive cells [5,6]. Stat3 is a major mediator of interleukin-6 (IL-6) induced signaling in prostate cancer cells and that IL-6 induced androgen

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receptor (AR)-mediated gene activation requires the activation of Stat3 in LNCaP prostate cancer cells [7,8]. Constitutive activation of Stat3 promotes androgen independent growth of androgen dependent LNCaP cells in vitro and in vivo [9]. Blockade of Stat3 expression in human prostate cancer cells suppress proliferation in vitro and tumorigenicity in vivo [10]. These studies demonstrate that constitutively activated Stat3 is not only associated with prostate cancer, but also induces prostate cancer cell proliferation. Thus, the Stat3 signaling pathway may represent a new molecular target for novel therapeutic approaches for prostate cancer. Strategies that target Stat3 signaling have been proposed including tyrosine kinase inhibitors such as tyrphostin AG490 and cucurbitacin I [11,12], antisense oligonucleotides and decoy oligonucleotides [6,13], and dominant-negative Stat3 protein [10,14].

Small interfering RNA (siRNAs) are short double-stranded RNA molecules that can target complementary mRNAs for degradation via a cellular process termed RNA interference (RNAi) [15]. RNAi is usually activated by introducing long double-stranded RNA molecules into cells which are cleaved into 21- to 23-nt RNAs referred to as siRNAs by an endonuclease named Dicer in animal cells [15]. The siRNA molecules then serve as a guide for sequence-specific degradation of homologous mRNAs. SiRNA has been used for functional analysis of genes in many species including invertebrates, plants, and mammalian cells [16]. Recently, siRNA has emerged as powerful RNAi reagents for directed posttranscriptional gene silencing and inhibition of viral propagation [17]. The potential of using siRNA for silencing specific genes has been demonstrated in treatment of viral diseases and cancer including HIV, human papillomavirus, and hepatitis C virus [18].

The present study was designed to investigate the potential use of siRNA to block Stat3 expression and the effect on growth of human prostate cancer cells. We identified a siRNA specific for Stat3 and expressed in prostate cancer cells. We demonstrate that blockade of Stat3 expression by this siRNA inhibits the growth of human prostate cancer cells and induces apoptotic cell death. These results demonstrate that targeting Stat3 signaling using siRNA technique may serve as a novel therapeutic strategy for treatment of prostate cancer expressing constitutively activated Stat3.

MATERIALS AND METHODS

Tissue Culture

Human DU145 prostate cancer cells were maintained in RPMI1640 supplemented with 10% of FBS. LN-17 cells were generated from human LNCaP prostate cancer cells stably expressing IL-6 as described

previously [8,19], and cultured in RPMI 1640 supplemented with 10% FBS plus 0.3 mg/ml of G418. Human PC3 prostate cancer cells were maintained in DMEM supplemented with 10% FBS. The cells were cultured in 100 U/ml of penicillin and 100 µg/ml of streptomycin at 37°C in 5% CO₂ incubator.

Plasmids and siRNA Expression

The oligonucleotides containing sequences specific for Stat3 (5'-GATCCCGTGTCTCTATCAGCACAA-TTCAAGAGAATTGTGCTGATAGAGAACATTTT-TTGAAA-3' and 5'-AGCTTTTCCAAAAAATGTTCTCTATCAGCACAAATTCTCTTGAAATTGTGCTGATAGAGAACACGG-3') were synthesized and annealed. Stat3 siRNA expression vector that express hairpin siRNAs under the control of the mouse U6 promoter was constructed by inserting pairs of the annealed DNA oligonucleotides into the pSilencer-neo siRNA expression vector that was digested with BamHI and HindIII (Ambion, Austin, TX). A pSilencer neo vector that expresses a hairpin siRNA with limited homology to any known sequences in the human, mouse, and rat genomes was used as a negative control (Ambion). The negative control siRNAs have been tested in multiple cell lines and they show no toxicity to cells when analyzed by Trypan Blue staining and cell counting 48 hr after transfection. The negative control siRNAs have no effect on the expression of the mRNA levels of "housekeeping" genes, including 28S rRNA, GAPDH, and Cyclophilin (Ambion).

Stat3 siRNA Transfection

DU145, PC3, and LN-17 cells were transiently transfected either with Stat3 siRNA or the negative control plasmid using SuperFect Transfection Reagent (QIAGEN, Inc., Valencia, CA). Briefly, cells were plated into either 12-well plates (10⁵ cells per well) or 100 mm dish (10⁶ cells) and allowed to adhere for 24 hr. Cells were transfected with either Stat3 siRNA expression vector or negative control siRNA plasmids in serum-free medium for 4 hr, incubated with complete medium for 24 hr, and then cells were switched into medium containing G418 (0.3 mg/ml). Two days later, protein extracts and total RNA were prepared for subsequent analysis.

In Vitro Growth Assay

DU145, PC3, and LN-17 cells (1 × 10⁵ per well) were plated in 12-well plates in RPMI containing 10% FBS. Cells were transfected with either Stat3 siRNA expression vector or negative control plasmid as described above. Four days later, cells were counted with a Coulter counter.

Apoptosis ELISA Test

Cell death detection ELISA kit (catalog # 1544675) was obtained from Roche Molecular Biochemicals (Indianapolis, IN) and the protocol was followed according to the manufacturer's instructions. For the preparation of samples, cells were plated and transfected with either 1 μ g of Stat3 siRNA expression vector or 1 μ g of control plasmid as described previously. Three days later, cell lysates were obtained and cell death was analyzed and the absorbance values were normalized by the amount of protein. The protein concentration was determined by Bradford assay (Coomassie Plus, Pierce, Rockford, IL).

Luciferase Assay

DU145 cells were plated (1×10^5 cells per well of 12-well plate) and transfected with 1 μ g of pLucTKS3 reporter plasmid containing specific responsive elements for Stat3 [20], varied amount of Stat3 siRNA expression vector or negative control vector. For control, DU145 cells were cotransfected with 1 μ g of Stat3 unresponsive pLucTK reporter plasmid [20] and Stat3 siRNA expression vector or negative control vector. Total amount of DNA transfected was adjusted using empty vector DNA. After 40 hr of incubation, cell extracts were prepared and luciferase activity was determined according to manufacturer's protocols (Promega, Madison, WI). Luciferase activity was normalized per microgram of protein as determined by Bradford assay (Coomassie Plus, Pierce).

Electromobility Shift Assay

After transfection with either Stat3 siRNA expression vector or negative control plasmid, nuclear extracts were prepared and electromobility shift assay (EMSA) was performed as described previously [10]. For determination of the Stat3 DNA binding activity, nuclear extracts (10 μ g) were incubated in a final volume of 20 μ l (10 mM HEPES, pH 7.9, 80 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 100 μ g/ml poly(dI-dC) with radio labeled double stranded Stat3 consensus binding motif 5'-GATCCTTCTGGGAA-TTCCTAGATC (Santa Cruz Biotechnologies, CA). The protein-DNA complexes were resolved on a 4.5% non-denaturing polyacrylamide gel at room temperature, and the results were autoradiographed using Molecular Imager FX System (Bio Rad, Hercules, CA).

Western Blot Analysis

Cell extracts were prepared and resolved on a 10% SDS-PAGE and blotted onto a membrane. After blocking overnight at 4°C in 5% milk in PBS containing 0.1% Tween 20, membranes were incubated overnight

with either antibodies of cleaved poly (ADP-ribose) polymerase (PARP) (Cell Signaling Technology, Beverly, MA), Bcl-x_L (Santa Cruz, CA), or antibodies against Stat3 or phospho-Stat3 proteins (Cell Signaling Technology). Following secondary antibody incubation, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, England).

Northern Blot Analysis

Twenty micrograms of total RNAs were electrophoresed in 1.2% denaturing agarose gels and transferred to a nylon membrane (MSI, Westborough, MA). PSA cDNA and Stat3 cDNA were labeled with [α -³²P] dCTP (3,000 Ci/mmol, ICN, Costa Mesa, CA) using the Ready-To-Go DNA Labeling Beads (Amersham Pharmacia Biotech). Hybridization was carried out during 3 hr at 65°C in Rapid-hyb buffer (Amersham Pharmacia Biotech). Membranes were washed for 15 min at 65°C in 2 \times SSC, 0.1% SDS (twice), 0.5 \times SSC, 0.1% SDS, and 0.1 \times SSC, 0.1% SDS. Radioactivity in the membranes was analyzed with a Molecular Imager FX System (Bio Rad).

PSA Protein Analysis

PSA secretion was quantitated by ELISA with the use of anti-PSA as primary antibody as described by the manufacturer's protocol (Beckman Coulter, Fullerton, CA). Equal numbers of cells were plated in phenol red-free RPMI containing 10% FBS. Cells were allowed to attach for 24 hr, then the cells were transfected with either 1 μ g of Stat3 siRNA or 1 μ g of control siRNA. After 3 days, 50 μ l of supernatant was assayed for PSA.

Statistical Analysis

Values were expressed as the mean \pm SE. Statistical analyses were performed by one-way ANOVA, followed by the Student-Newman-Keuls test for multiple comparisons, with a $P < 0.05$ significant.

RESULTS

Stat3 siRNA Inhibits Stat3 DNA-Binding Activity

To determine whether transfection of Stat3 hairpin siRNA vector inhibits Stat3 activity, we used several human prostate cancer cell lines expressing high levels of constitutively activated Stat3 including DU145 and LN-17 cells [8,10]. The LN-17 cells were derived from LNCaP cells that were ectopically transfected with IL-6 cDNA and expressed constitutively activated Stat3 [8,19]. Both cell lines were transfected with either the Stat3 hairpin siRNA vector or the negative control siRNA vector, respectively. Antibiotic G418 (300 μ g/ml) was added following 24 hr transfection and the cells

were allowed to grow for another 48 hr. The cells were harvested and the Stat3 activity was evaluated by EMSA. The Stat3 hairpin siRNA vector transfected cells demonstrated a marked decrease in formation of Stat3 DNA–protein complex in the gel shift assay compared to the negative control siRNA vector transfected cells (Fig. 1A).

We also examined the effect of Stat3 siRNA on the levels of endogenous Stat3 mRNA. Figure 1B shows that Stat3 siRNA significantly inhibits the steady-state expression levels of Stat3 mRNA compared to that of negative control siRNA vector.

To determine whether the Stat3 siRNA affect the levels of steady-state Stat3 or phosphorylated Stat3 protein, we performed Western blot analysis using antibodies against either Stat3 or phospho-specific Stat3 protein on the cells transfected with either Stat3 siRNA expression vector or negative control siRNA vector. Figure 1C shows that both the levels of steady-state Stat3 and tyrosine-phosphorylated Stat3 protein were decreased by Stat3 siRNA compared to the negative control siRNA vector. It appears that the reduction in steady state levels of total and phospho-

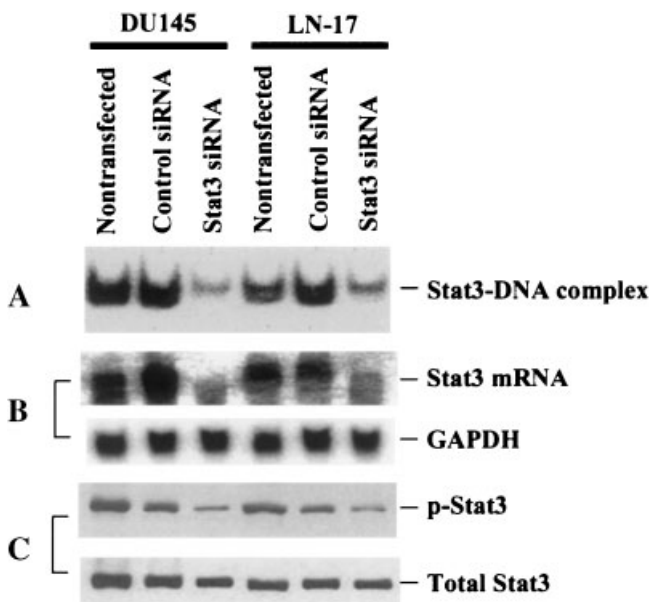


Fig. 1. Effect of Stat3 siRNA on Stat3 expression and activity of human prostate cancer cells. **A:** Stat3–DNA binding activity. The Stat3 activity was analyzed using EMSA as described in Materials and Methods. **B:** Stat3 mRNA levels of human prostate cancer cells transfected with Stat3 siRNA or control siRNA were determined by Northern blot analysis. GAPDH expression was used as an internal control. **C:** Effect of Stat3 siRNA on Stat3 protein expression. Western blots were performed using either antibodies against phospho-specific Stat3 (Tyr-705) or total Stat3 with whole cell extracts isolated from prostate cancer cells transfected with Stat3 siRNA expression vector or control siRNA vector.

lated Stat3 is less than the Stat3-DNA binding activity and Stat3 mRNA levels by siRNA Stat3.

Stat3 siRNA Inhibits Cell Growth and Induces Apoptosis

We have previously demonstrated that constitutively activation of Stat3 promotes human prostate cancer cell growth in vitro and tumor growth in vivo [9]. To determine the effect of the Stat3 siRNA on human prostate cancer cell growth, DU145 and LN-17 cells were transiently transfected with either Stat3 siRNA expression vector or the negative control vector. The cells were counted after 4 days of transfection. Figure 2A shows that Stat3 siRNA expression vector inhibited cellular proliferation by 60% in DU145 cells and 50% in LN-17 cells compared to that of the negative

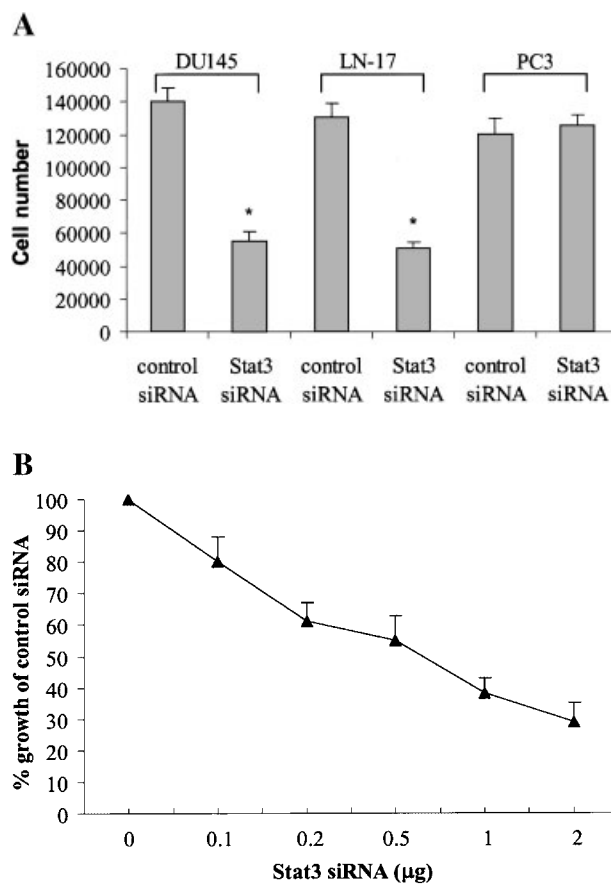


Fig. 2. Effect of Stat3 siRNA on human prostate cancer cell growth. **A:** Human prostate cancer DU145, PC3, and LN-17 cells (1×10^5 per well) were plated in 12-well plates in RPMI 1640 containing 10% FBS. After 24 hr, the cells were transfected with either Stat3 siRNA expression vector or negative control plasmid. Four days later, the cells were counted with a Coulter counter. Columns represent means of data ($n = 4$); bars, \pm SE. *, Significantly different from control siRNA. **B:** Transfected with 0.1–2 μ g Stat3 siRNA expression vector demonstrated a dose-dependent inhibition of proliferation of DU145 cells. The data are expressed as % of the control siRNA.

control vector. To determine whether the growth inhibition by Stat3 siRNA was dose-dependent, prostate cancer cells were transfected with a range of Stat3 siRNA expression vector (0.1–2 $\mu\text{g}/\text{well}$) and the cell number was determined afterwards. As shown in Figure 2B, the growth inhibition by the Stat3 siRNA was dose-dependent.

Activation of Stat3 protects cells from apoptotic cell death, and blockade of Stat3 activation induces apoptosis [3,6]. Therefore, we evaluated the effect of the Stat3 siRNA on apoptosis in human prostate cancer cells. The Stat3 siRNA induced apoptosis was examined using an apoptosis specific ELISA kit. Figure 3A

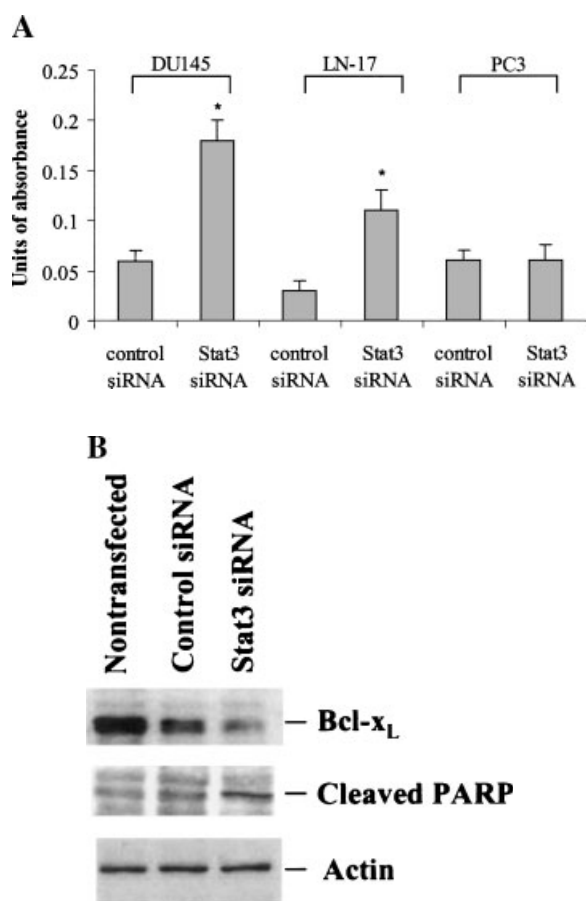


Fig. 3. Effect of Stat3 siRNA on prostate cancer cell apoptosis. **A:** Cell death analysis by a specific ELISA kit. Human prostate cancer DU145, LN-17, and PC3 cells were transfected with Stat3 siRNA expression vector or control siRNA vector. Three days later, cell lysates were obtained and cell death was analyzed and the absorbance values were normalized by the amount of protein. Data are expressed as mean \pm SD of four independent experiments. *, Significantly different from control siRNA. **B:** Western blot analysis of the expression of Bcl-x_L and cleaved PARP proteins in LN-17 prostate cancer cells transfected with Stat3 siRNA expression vector or control siRNA vector. Immunoblots were prepared from 40 μg of whole cell lysate from LN-17 cells transfected with Stat3 siRNA expression vector or control siRNA vector.

shows that the Stat3 siRNA induced apoptosis in DU145 by threefold and in LN-17 by fourfold compared to the negative control vector. Immunoblots using antibodies against Bcl-x_L and cleaved PARP proteins were also performed to determine the effect of the Stat3 siRNA on apoptosis. As shown in Figure 3B, the Stat3 siRNA significantly reduced the expression of Bcl-x_L and enhanced the expression of cleaved PARP compared to the negative control vector, further demonstrating that the Stat3 siRNA induces human prostate cancer cells to undergo apoptotic cell death.

We also evaluated the selectivity of the Stat3 siRNA action. We transfected Stat3 siRNA expression vector into Stat3-negative PC3 human prostate cancer cells. The Stat3 siRNA shows no effect on the proliferation (Fig. 2A) nor apoptosis (Fig. 3A) in PC3 cells compared to the negative control vector, suggesting that the Stat3 siRNA inhibits cell proliferation only in cells that express constitutively activated Stat3.

Stat3 siRNA Inhibits Stat3-Mediated Gene Expression

Stat3 activation contributes to oncogenesis through regulation of its target genes. To examine the effect of the Stat3 siRNA on Stat3-mediated gene expression, DU145 cells were cotransfected with the pLucTKS3 reporter containing the Stat3 responsive elements [20] and with either Stat3 siRNA expression vector or negative control vector, and cytosolic extracts were prepared for luciferase assays. The pLucTK reporter that contains no Stat3 responsive element was used as a control [20]. As shown in Figure 4A, the Stat3 siRNA significantly inhibited the induction of the Stat3-dependent pLucTKS3 luciferase reporter activity without affecting the Stat3-independent pLucTK activity compared to the negative control vector.

We previously demonstrated that activated Stat3 enhances the expression of AR-mediated genes including PSA [9]. To test the effect of the Stat3 siRNA on PSA expression, Northern blot analyses were performed to compare the levels of the expression of PSA mRNA in LN-17 cells that have been transfected with either Stat3 siRNA expression vector or negative control vector. As shown in Figure 4B,C, the Stat3 siRNA significantly reduced the levels of PSA expression compared to the negative vector control.

DISCUSSION

Studies to date provide strong evidence that aberrant Stat3 signaling may play an important role in the development and progression of prostate cancer. We previously demonstrated increase Stat3 activation in prostate cancer and that constitutively activated Stat3 promotes prostate cancer cell tumor growth [5,9].

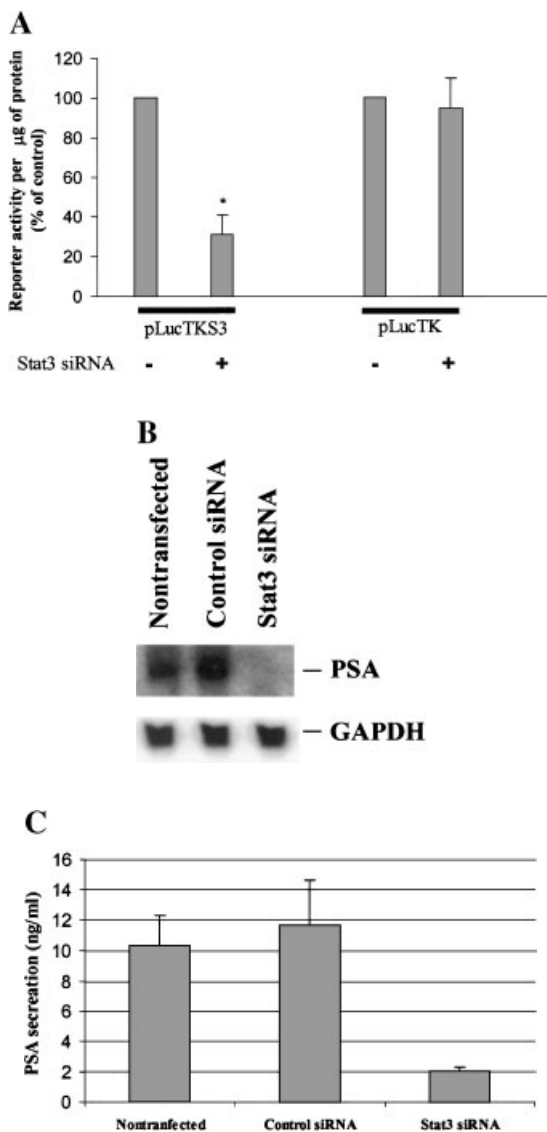


Fig. 4. Effect of Stat3 siRNA on Stat3-mediated gene expression. **A:** DU145 cells expressing constitutively activated Stat3 were cotransfected with the pLucTKS3 reporter (1 μg) containing the Stat3 responsive elements and with either 1 μg of Stat3 siRNA expression vector (+) or 1 μg of negative control vector (-). Cell extracts were prepared for luciferase assays. The pLucTK reporter that contains no Stat3 responsive element was used as a control. Luciferase activity was determined according to manufacturer's protocols and normalized per μg of protein. Data are representative of three independent experiments. *, Significantly different compared to control siRNA. **B:** Stat3 siRNA inhibits PSA mRNA expression in LN-17 cells. Total RNA was isolated from LN-17 cells transfected with either Stat3 siRNA expression vector or control siRNA vector and used for Northern blot analysis as described in Materials and Methods. GAPDH was used as a loading control. **C:** Stat3 siRNA inhibits PSA protein expression in LN-17 cells. The levels of PSA protein expression in the medium was analyzed by PSA ELISA from LN-17 cells transfected with either Stat3 siRNA expression vector or control siRNA vector.

Blockade Stat3 activation by a dominant negative Stat3 mutant resulted in suppression of prostate cancer growth both in vitro and in vivo [10]. Numerous studies also demonstrate that Stat3 activates AR-mediated gene expression and prevents cell from apoptosis [6,7,9]. Collectively, these findings indicate that targeting Stat3 signaling may represent a novel approach to treat prostate cancer.

RNAi represents a promising new technology that could have therapeutic applications for the treatment of diseases including cancer by blocking the action of transcription factors and oncogenes with selective silencing of gene expression with exquisite precision and high efficacy [21]. In this study, we have identified the Stat3 siRNA that specifically inhibits constitutively activated Stat3 and suppresses cell proliferation and induces apoptosis. To compare the relative potency of this siRNA Stat3 with Jak/Stat3 inhibitor AG490, LN-17 cells were transfected with 1 μg of Stat3 siRNA expression plasmid and the cell number was determined, while the same cells were treated with different doses of AG490 (range from 0 to 50 μM). The effect of inhibition of cell growth by expression of 1 μg of Stat3 siRNA (about 60% inhibition) is similar to the effect of 30 μM of AG490.

Previous studies demonstrated that Stat3 is constitutively activated in human prostate cancer compared to normal prostate [5,6], and that constitutively activated Stat3 promotes prostate cancer cell growth both in vitro and in vivo [9]. This study showed that the Stat3 siRNA only inhibits the proliferation and induces apoptosis in cells expressing constitutively activated Stat3, but not in Stat3-inactive PC3 cells, further demonstrating the selectivity of the Stat3 siRNA and potential therapeutic utility of the Stat3 siRNA for prostate cancer expressing active Stat3.

The PSA is synthesized primarily by normal and malignant prostate and the levels of PSA in serum correlate with the clinical stage of the disease. We have previously demonstrated that constitutively activated Stat3 enhances PSA expression in vitro and in vivo and enhances PSA transcription [9]. We showed here that blockade of Stat3 activation by the Stat3 siRNA significantly inhibited PSA mRNA expression in LN-17 cells (Fig. 4B), indicating that targeting Stat3 activation by the Stat3 siRNA could inhibit the AR-mediated gene expression in prostate cancer cells.

In conclusion, we have identified the Stat3 siRNA that specifically inhibits constitutively activated Stat3 and suppresses cell proliferation and induces apoptosis. The Stat3 siRNA resulted in blockade of Stat3 DNA-binding activity and inhibited the levels of Stat3 mRNA. Targeting Stat3 activation with RNAi holds therapeutic promise for prostate cancer with constitutive Stat3 activation. Several strategies have

been reported to block Stat3 activation including using Stat3 decoy oligonucleotides in head and neck cancer cells [13]. Although siRNA method has emerged as powerful RNAi reagents for directed posttranscriptional gene silencing and treatment of viral diseases and cancer, it would be of interest to compare the relative therapeutic potency of using Stat3 decoy oligonucleotides with the Stat3 siRNA identified in this study in prostate cancer cells.

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