

Stat3-siRNA induces Fas-mediated apoptosis *in vitro* and *in vivo* in breast cancer

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Abstract. Stat3, a member of the signal transducer and activator of transcription family, has the potential to mediate cell survival, growth and differentiation. Stat3 is constitutively activated in numerous cancers, including >50% of breast cancers. Previous studies demonstrated that constitutively activated Stat3 plays an important role in breast cancer development and progression by promoting cell proliferation and inhibiting apoptosis. The present study was designed to investigate the potential use of RNA interference (RNAi) to block Stat3 expression and activation, as well as the subsequent effect on human breast cancer cell growth. Our studies show that knockdown of STAT3 expression by siRNA reduced expression of Bcl-xL and survivin in MDA-MB-231 cells, and also led to Fas mediated intrinsic apoptotic pathway by activating caspases -8, -9, -3 and PARP1 cleavage. In nude mice, pRNAi-Stat3 significantly suppressed tumor growth compared with controls. It also suppressed Stat3 expression, and downregulated Bcl-xL and upregulated Fas, Fas-L and cleaved caspase-3 expression within the tumor, which significantly induced apoptosis and led to tumor suppression. Thus, targeting Stat3 signaling using siRNA may serve as a novel therapeutic strategy for the treatment of breast cancers expressing constitutively activated Stat3.

Introduction

The STAT (signal transducer and activator of transcription) family of proteins are transcription factors known for their role as integrators of cytokine and growth factor receptor signaling and are required for cell growth, differentiation, motility and survival (1-3). Among the seven members of the mammalian STAT family, Stat3 has been most strongly implicated in oncogenesis. Stat3 is ubiquitously expressed in mammalian cells and activated by cytokines (e.g., IL-6 family members), growth factors (e.g., EGF, PDGF), and the plasminogen activator system (4,5). Tyrosine phosphorylation is pivotal for STAT activation, which in turn, leads to dimerization and translocation into the nucleus. The translocated activated Stat3 binds to the consensus promoter sequences of target genes and initiates their transcription (6,7). This tyrosine phosphorylation is transient in normal, non-transformed cells. In contrast, it is constitutively activated in the majority of cancer cell lines and a large number of primary tumors (6).

The expression of a constitutively active form of Stat3 (STAT3C) transforms immortalized human breast epithelial cells mediated by active MMP-9 tumorigenesis, thereby demonstrating the sufficiency of this transcription factor in promoting cellular transformation (8). Stat3 is constitutively activated in >50% of primary breast tumors and tumor-derived cell lines. Abundant evidence shows that constitutively activated Stat3 is frequently found in breast cancer samples (9). Thus, the Stat3 signaling pathway may represent a new molecular target for novel therapeutic approaches for breast cancer.

Strategies that target Stat3 signaling have been proposed including tyrosine kinase inhibitors (e.g., tyrphostin AG490 and cucurbitacin I), antisense oligonucleotides, decoy oligonucleotides, and dominant negative Stat3 protein (10-15). A more recent study demonstrated that Stat3 knockdown by RNA interference (RNAi) induced apoptosis in human neuron tumor cells and prostate cancer cells (16,17). Stat3 participates in oncogenesis through the upregulation of genes encoding apoptosis inhibitors (Bcl-xL, Bcl2 and survivin) (18). Inhibition of Stat3 activity reduced the expression of survivin in primary effusion lymphoma-induced cell apoptosis (19). Proteins of the Bcl-2 family are important regulators of the decision between survival or death in response to an apoptotic

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Abbreviations: RNAi, RNA interference; siRNA, short interfering RNA; Stat3, signal and transducer of transcription 3; pRNAi-Stat3, plasmid for small interfering RNA (siRNA) specific for Stat3; pSTAT3C, plasmid for constitutively active form of Stat3; pSV, plasmid for scrambled vector; PBS, phosphate-buffered saline; FITC, fluorescein 5-isothiocyanate; H&E, hematoxylin and eosin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TUNEL terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling

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stimulus (20). Many studies have repeatedly demonstrated that the inhibition of STAT results in growth inhibition and induction of apoptosis in tumor cells harboring constitutive activation of Stat3 (21,22). One possible explanation for the increased sensitivity of transformed cells to apoptosis compared with normal cells is that tumor cells may have become irreversibly dependent on STAT signaling to sustain their survival. The observed dependence of certain tumors, but not normal cells, on constitutive STAT activation for survival offers the potential for preferential tumor cell killing, and therefore, STAT inhibition has broad implications for cancer therapy.

Small interfering RNA (siRNA) are short double-stranded RNA molecules that can target complementary mRNA for degradation via a cellular process termed RNA interference (RNAi). RNAi is usually accomplished by introducing long double-stranded RNA molecules into cells. These RNA molecules are then cleaved into 21-nt to 23-nt RNA (known as siRNA) by an endonuclease (Dicer) in animal cells. The siRNA molecules then serve as a guide for sequence-specific degradation of homologous mRNA (23). The present study was designed to investigate the potential use of siRNA to block Stat3 expression and the subsequent effect on growth of human breast cancer cells. We identified siRNA specific for Stat3 and expressed the siRNA in breast cancer cells. Our results demonstrate that siRNA-mediated downregulation of Stat3 expression inhibits the growth of human breast cancer cells and induces apoptotic cell death. These results demonstrate that targeting Stat3 signaling using the siRNA technique may serve as a novel therapeutic strategy for the treatment of breast cancers expressing constitutively activated Stat3.

Materials and methods

Construction of plasmids that contain DNA templates for the synthesis of siRNAs were constructed under the control of the CMV promoter. pDNR-CMV (Clontech Laboratories, Inc., CA, USA) was used for DNA vector-based siRNA synthesis under the control of CMV promoter *in vivo*. In brief, first, the double-stranded DNA template encoding siRNA oligonucleotides (GeneBank access numbers for the human Stat3: NM003150) that contained a sense strand of 19 nucleotide sequences followed by a short space (TTCAAGAGA), the reverse complement of the sense strand, and five thymidines as an RNA polymerase III transcriptional stop signal were synthesized. The sequences were forward 5'-TCGAGTCAG GTTGCTGGTCAAATTCGAGTCGTCGGAATTTGACCA GCAACCTGACTTTTT-3' and reverse 5'-CTAGAAAAAG TCAGGTTGCTGGTCAAATTCGACGACTCGAATTTG ACCAGCAACCTGAC-3' (located on SH2 domain). The oligonucleotides were annealed in a buffer (potassium acetate 100 and 30 mmol/l HEPES-KOH pH 7.4, and magnesium acetate 2 mmol/l) and the mixture was incubated at 90°C for 3 min and then at 37°C for 1 h. The double stranded oligos were cloned into the *Apal*-*EcoR* I sites of the pDNR-CMV vector (Ambion Inc) where short hairpin RNAs (shRNA) were expressed under the control of the CMV promoter.

Antibodies and reagents. Primary antibodies against Stat3, MMP-2, MMP-9, PARP-1, GAPDH, HRP/FITC/Texas Red

conjugated secondary antibodies were obtained from Biomedica (Foster City, CA); anti-caspases -8, -3 and -9 were from Cell Signaling (Boston, MA); cleaved Bid, Bax, Bcl-xL, Fas-L, FADD and mouse IgG were from Santa Cruz Biotechnology (Santa Cruz, CA); and Fas and cytochrome c were from Calbiochem (San Diego, CA). BCA reagent (Pierce, Rockford, IL), ECL reagent (Amersham Pharmacia, Piscataway, NJ), DAPI nuclear staining mounting solution (Vector Laboratories, Burlingame, CA), DAB peroxidase substrate (Sigma, St. Louis, MO), TUNEL Detection kit (Roche Molecular Biochemicals Indianapolis, IN) and Annexin-V-FITC Apoptosis Detection Kit (BioVision Mountain View, CA) were used for this study. pStat3-C (constructs of constitutively activated Stat3) was purchased from Addgene Inc. (Cambridge, MA) (Addgene plasmid 8722).

Cell culture and treatments. MDA-MB-231 human breast cancer cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% FBS in a humidified CO₂ incubator at 37°C. MDA-MB-231 cells were serum-starved for 12-18 h, transfected with a plasmid siRNA construct against STAT3 using FuGENE HD transfection reagent according to the manufacturer's protocol (Roche Diagnostics Corp., Indianapolis, IN), and incubated in humidified CO₂ atmosphere at 37°C for 6-8 h. The necessary amount of culture medium was then added and cells were returned to the incubator for 36 h for few of the initial experiments and 48h for remaining experiments. For blocking Fas-L on MDA-MB-231 cells, the serum-starved cells were treated with 10 µg/ml of anti-Fas-L antibody or non-specific IgG for 1 h prior to the treatment of pRNAi-Stat3 treatment.

Cytosolic and mitochondrial protein extraction. Cytosolic and mitochondrial fractions were isolated from control, pSV and pRNAi-Stat3-treated cells as described elsewhere (24). Cross-contamination of cytosol and mitochondria was determined on the basis of the amount of activity of marker enzymes for cytosol [lactate dehydrogenase (LDH) and mitochondria (succinate dehydrogenase (SDH)]. Protein levels in the samples were determined with Bio-Rad protein assay kit and analyzed by immunoblot using anti-cytochrome c and anti-smac/DIABLO antibodies.

Western blot analysis. MDA-MB-231 cells were transfected with pSV or pRNAi-Stat3 as described above. After incubation, the cells were washed with ice-cold PBS and lysed in RIPA buffer containing protease inhibitors. Whole cell extracts were subjected to SDS-PAGE and subsequently transferred to a polyvinylidene difluoride membrane (PVDF) (Bio-Rad, Hercules, CA). The membranes were blocked with 7% non-fat dry milk and probed with antibodies (anti-Stat3, pStat3). Appropriate antibody conjugated with horseradish peroxidase was used as the secondary antibody, and membranes were developed according to an enhanced chemiluminescence protocol according to manufacturer's instructions.

Immunocytochemical analysis. MDA-MB-231 cells were transfected with pSV or pRNAi-Stat3 as described above. After incubation, cells were fixed with 4% formaldehyde and

incubated with 3% bovine serum albumin in PBS at room temperature for 1 h for blocking. After the coverslips were washed with PBS, anti-STAT3 mouse IgG (Santa Cruz Biotechnology) was added at a concentration of 1:200. The slides were incubated at 4°C overnight and washed 3 times with PBS to remove excess primary antibody. Cells were then incubated with anti-mouse Texas red conjugate or anti-mouse FITC conjugates IgG (1:500 dilution) for 1 h at room temperature. The slides were then washed 3 times and covered with glass coverslips using Vectashield HardSet mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Cells were viewed under a fluorescent microscope.

Electromobility shift assay (EMSA) and Western blotting. Nuclear extracts were prepared from MDA-MB-231 cells transfected with pSV or pRNAi-Stat3 as described above. Cells were detached with EDTA and resuspended in buffer A [10 mM HEPES (N-2-hydroxyethylenepiperazine-N'-2-ethanesulfonic acid), pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT)] containing protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM iodoacetamide, 0.1 mM quercetin, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 0.3 mM sodium vanadate] and incubated on ice for 15 min. After homogenization using a Wheaton 0.1-ml homogenizer, the nuclei were collected by centrifugation. The pellet was resuspended in buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT) containing protease inhibitors and was incubated on ice for 30 min, followed by centrifugation at 13,000 g (5 min, 4°C). The supernatant was dialyzed against buffer C (20 mM HEPES, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT) containing protease inhibitors for 2 h at 4°C, followed by centrifugation at 13,000 g (5 min at 4°C). The supernatant proteins were used immediately or aliquoted and stored at -80°C.

Binding reaction was performed for 30 min on ice in a volume of 20 µl, which contained 4 µg nuclear protein extracts, 40 ng poly(dI-dC), 4 µl 5X binding buffer (1X binding buffer: 20 mM HEPES, pH 7.9, 50 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 10% glycerol) with or without 20- to 50-fold excess of cold competitor or of unrelated competitor and a [³²P]-labeled probe (3x10⁴ cpm). For the supershift electrophoretic mobility shift assay (EMSA), protein extracts were incubated with 6 µg STAT3 and HIF-1α monoclonal antibody or non-specific IgG as control before the addition of the [³²P]-labeled probe. DNA-protein complexes were separated on 5% polyacrylamide gel in Tris/glycine buffer at 4°C. The following double-stranded oligonucleotides purchased from Santa Cruz Biotechnology were used in this study: Stat3 consensus oligonucleotide: sc-2571, 5'-GAT CC T TCT GGG AAT TCC TAG ATC-3'; 3'-CTA GGA AGA CCC TTA AGG ATC TAG-5'.

Five end-labeled probes were prepared with 40 µCi (1480 MBq) [γ-³²P]-adenosine triphosphate (ATP) using T4 polynucleotide kinase. Probes were gel-purified on NAP-5 Sephadex G-25 DNA-grade columns.

Animal experiments. MDA-MB-231 cells were cultured in complete medium until 70-75% density was reached. Then, cells were trypsinized, washed once with serum-free medium

and counted. Cells were injected bilaterally into the second mammary fat pads of athymic, female, 4-6-week-old nu/nu mice (5x10⁶/100 µl serum-free culture medium). Tumor growth was monitored daily. Once a tumor reached a size of ~6-8 mm, three doses of 150 µg/animal of either pSV or pRNAi-Stat3 were given intraperitoneally on alternate days. One set of animals were sacrificed for tumors after 18 days of treatment while a second set of animals was kept under observation for a period of 6 weeks. Finally, the tumor volume was calculated using the formula: $V = \pi/6 (a \times b \times c)$.

Immunohistochemical analysis. Tumor samples fixed in 10% neutral buffered formalin were embedded in paraffin using automatic embedding equipment. Five µm sections were prepared. Paraffin-embedded breast tumor sections of control mice and mice treated with pSV and pRNAi-Stat3 were analyzed for Stat3, Fas and Fas-L using immunohistochemistry.

Apoptosis assay. Apoptosis was determined through the analysis of externalization of plasma membrane phosphatidylserine (PS) and TUNEL assay.

Annexin V-FITC apoptosis detection assay. An increase in the plasma membrane PS externalization occurs early in apoptosis and can be detected by annexin V staining. MDA-MB-231 cells were treated with pRNAi-Stat3 as described above. After 96 h, cells were isolated and stained with annexin V-FITC (BioVision Research Products, Mountain View, CA). Cells were analyzed using FACS for fluorescence of annexin V-positive cells.

TUNEL assay. TUNEL assay was performed with paraformaldehyde-fixed, MDA-MB-231 cells as per the manufacturer's protocol (Roche Diagnostics Corp., Indianapolis, IN). Briefly, the TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP nick end-labeling) method identifies apoptotic cells *in situ* by using terminal deoxynucleotidyl transferase (TdT) to transfer biotin-dUTP to the free 3'-OH of cleaved DNA. The biotin-labeled cleavage sites are then visualized by reaction with fluorescein-conjugated avidin (avidin-FITC). The cells were visualized using a fluorescent microscope with appropriate filter sets. DNA fragmentation in these treated tumors is indicative of apoptotic cell population. Additionally, TUNEL assay was performed with paraformaldehyde-fixed, paraffin-embedded breast tumor sections.

Statistical analysis. Statistical comparisons were performed using analysis of variance for analysis of significance between different values using GraphPad Prism software (San Diego, CA). Values are expressed as mean ± SE from at least three separate experiments, and differences were considered significant at a p<0.05. Densitometric analysis was performed using ImageJ software (National Institutes of Health) was used to quantify the band intensities.

Results

pRNAi-Stat3 specifically reduces Stat3 expression in MDA-MB-231 cells. Since Stat3 levels are significantly higher in tumor cells (including breast tumor) than in normal cells,

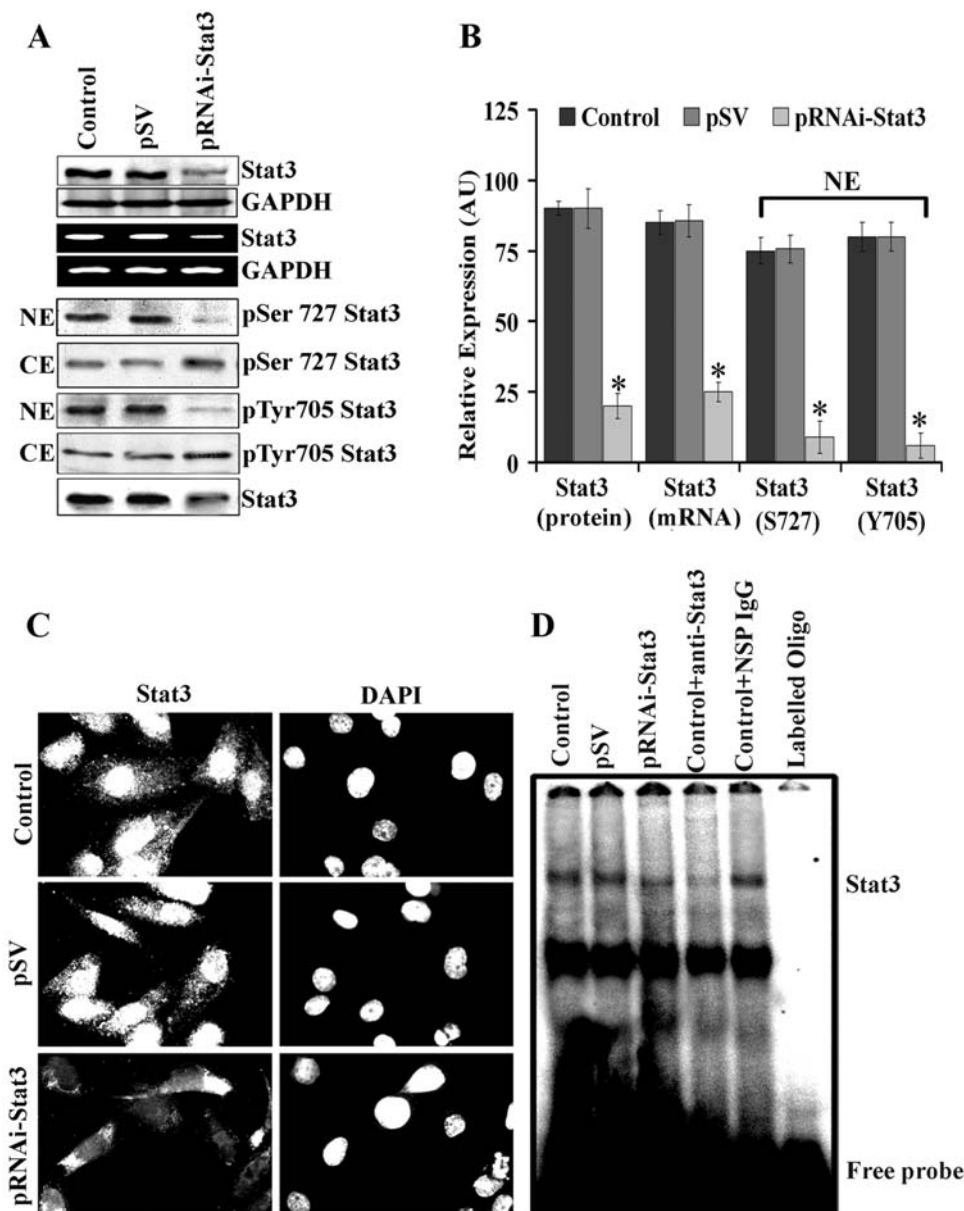


Figure 1. pRNAi-Stat3 specifically reduces Stat3 expression in MDA-MB-231 cells. Downregulation of Stat3 inhibits expression of Stat3 in MDA-MB-231 breast cancer cells. MDA-MB-231 cells were treated with pSV and pRNAi-Stat3. (A) Western blot analysis showing the effect of pRNAi-Stat3 on Stat3. Western blot analysis showing the effect of pRNAi-Stat3 on pSer727 and pTyr705-Stat3 from sub-cellular fractions. Anti-GAPDH antibody is used as a loading control. RT-PCR analysis to show the inhibition of expression of Stat3 at the mRNA level. Expression of GAPDH was verified for the equal loading of cDNA. (B) Densitometric analysis of the Western blot analysis. Bars represents the mean and SE of three experiments (* $P < 0.05$) (C) Immunocytochemical analysis of Stat3 in pRNAi-Stat3 in MDA-MB-231 cells. (D) Nuclear extracts were prepared and subjected to mobility shift assay to assess the DNA binding activity of Stat3 and to check the specificity of the complex formed anti-Stat3 antibody was used in the supershift assay (NE, nuclear extract; CE, cytoplasmic extract).

we attempted to determine whether synthetic Stat3 siRNA could inhibit the expression of Stat3 gene in MDA-MB-231 cells. Treatment of MDA-MB-231 cells with pRNAi-Stat3 (vector expressing siRNA for Stat3) for 36 h only resulted in a significant decrease of Stat3 expression (by $>70\%$) at both protein and mRNA levels compared to the untreated and the vector-treated MDA-MB-231 cells (Fig. 1A and B). Further, we analysed the phosphorylation status of Stat3 in our pRNAi-Stat3-treated MDA-MB-231 cells using sub-cellular fractions. Fig. 1A and B showed that in pRNAi-Stat3-treated MDA-MB-231 cells, Stat3-ser-727 phosphorylation was significantly reduced (by $>75\%$) in the nuclear extract (NE). Similarly, Stat3-Tyr-705 phosphorylation was significantly

reduced (by $>85\%$) in the nuclear extract (NE) in cells treated with pRNAi-Stat3 indicating that pRNAi-Stat3 also inhibited the activity of Stat3. In contrast, pTyr705 and pSer727-Stat3 (activated Stat3) was markedly expressed in the untreated and the vector-treated MDA-MB-231 cells (Fig. 1A and B), thereby supporting previous reports that Stat3 is highly activated in the majority of cancer cell lines and in human cancers. So the Western blot analysis revealed that pRNAi-Stat3 inhibited the translocation of Stat3 into the nucleus in MDA-MB-231 cells. The immunocytochemical (ICC) analysis revealed the localization of the majority of Stat3 in the nucleus of the control and vector-treated cells (Fig. 1C). In contrast, total expression including nuclear localization was reduced

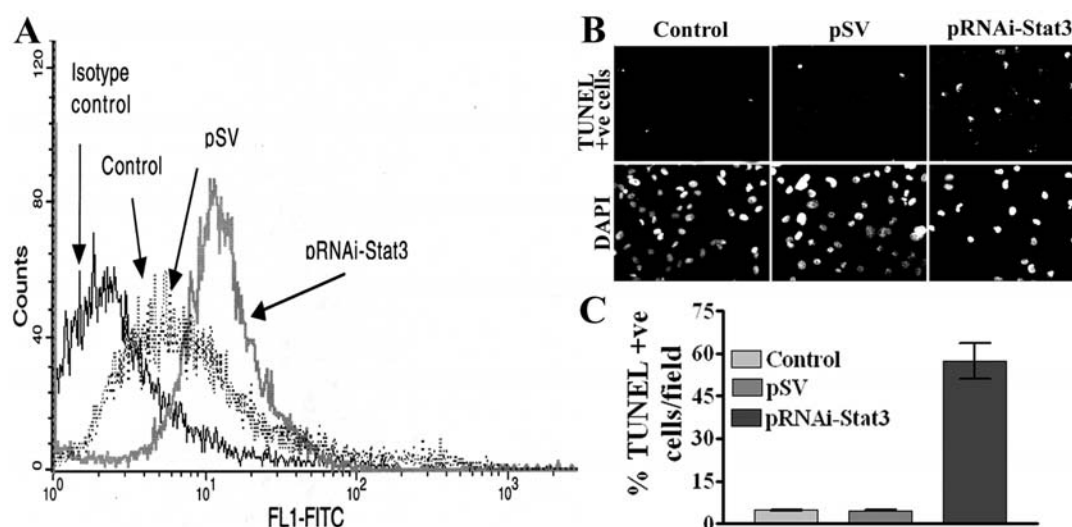


Figure 2. pRNAi-STAT3 induces apoptosis in breast cancer cells. Downregulation of Stat3 induces apoptosis in MDA-MB-231 breast cancer cells. MDA-MB-231 cells were treated with pSV and pRNAi-Stat3. (A) FACS analysis for phosphatidylserine externalization using annexin-V-FITC staining. (B) Cells were stained for apoptosis by TdT-mediated dUTP nick end-labeling assay (TUNEL) staining. Data shown are from a representative experiment. (C) Quantitation of apoptotic cells indicated an increase in number of TUNEL-positive cells in pRNAi-Stat3 treated cells. Bars represent the mean and SE of three experiments (* $P < 0.05$).

in the pRNAi-Stat3-treated cells. Furthermore, as shown by EMSA, treatment of MDA-MB-231 cells with pRNAi-Stat3 resulted in reduced DNA-binding activity of Stat3 as compared to the control and vector-treated cells (Fig. 1D). The specificity of the protein-DNA complex was demonstrated by a decrease in the intensity of the Stat3 signal in the nuclear extracts from the control MDA-MB-231 cells treated with anti-Stat3 antibody (as reported by the manufacturer) in the supershift assay.

pRNAi-STAT3 induces apoptosis in breast cancer cells. In tumors Stat3 is known to regulate the expression of anti-apoptotic genes and to transcriptionally regulate components of the apoptotic pathway (25). We analyzed the effect of treatment of MDA-MB-231 cells with pRNAi-Stat3 on apoptosis. We assessed apoptosis by looking for phosphatidylserine externalization using annexin-V binding and DNA fragmentation (TUNEL) assays. To quantify early and late events in the course of apoptosis, MDA-MB-231 cells were stained with annexin-V-FITC 36 h after pRNAi-Stat3 treatment. As shown by fluorescence-activated cell sorting analysis (Fig. 2A), transfection of MDA-MB-231 cells with pRNAi-Stat3 induced annexin-V expression on the cell surface as compared to control and vector-treated cells. These results confirmed that the cells were undergoing apoptosis. Furthermore, the TUNEL assay results for cells at about 48 h post-transfection indicated a significant increase, nearly 70%, in TUNEL-positive cells in pRNAi-Stat3-treated MDA-MB-231 cells as compared to the controls (Fig. 2B and C). In fact, very few TUNEL-positive cells were present in both the control and vector-treated cells. Taken together, these data indicate that Stat3 plays a pivotal role as an anti-apoptotic molecule in breast cancer and targeting Stat3 activity led to the induction of apoptosis.

Inhibition of STAT3 expression downregulates survival genes and induces the activation of Fas/Fas-L and recruitment of

Fas-associated death domain (FADD) in MDA-MB-231 cells. The activation of Stat3 has been shown to protect cancer cells from apoptotic stimuli emanating from the Fas (CD95/Apo-1) receptor or chemotherapeutic agents (26). This is accomplished as Stat3 activation results in the transcriptional induction of the Bcl-x gene promoter and high levels of the anti-apoptotic protein Bcl-xL. Thus, elevated levels of Stat3 appear to contribute directly to malignancy in multiple cancer cells by the prevention of apoptosis (25,26). To determine whether these anti-apoptotic molecules are involved in the inhibition of apoptosis in MDA-MB-231 cells, we analyzed the expression of Bcl-xL/S. Using Western blotting, we found significant decreased expression of Bcl-xL (~70%) and increased expression of Bcl-xS (~50%), a pro-apoptotic molecule at the protein level (Fig. 3A and B) in MDA-MB-231 cells treated with pRNAi-Stat3. Also, the expression of survivin (~60%) was significantly reduced in MDA-MB-231 cells treated with pRNAi-Stat3 (Fig. 3A and B). This is noteworthy as survivin, a member of the IAP family, has recently been identified as a novel modulator of the cell death/viability balance in cancer (27). Thus, these data show that Stat3 regulates the expression of anti-apoptotic genes in MDA-MB-231 cells.

We sought to further elucidate the molecular signaling involved in the apoptotic pathway. Cell membrane death receptor-induced apoptosis is widely studied in breast cancer, particularly in relation to hormonal disturbances (28). We investigated the involvement of death ligands including the Fas ligand (FasL). We found a significant increase in the expression of Fas (~60%) and Fas-L (~50%) in the pRNAi-Stat3-treated cells as compared to the untreated and vector-treated MDA-MB-231 cells (Fig. 3C and D). We also assessed the immediate downstream molecule in the death receptor-involved apoptotic pathway. An early event in Fas activation of the apoptosis signaling cascade is the interaction or recruitment of FADD, the adaptor protein, following the

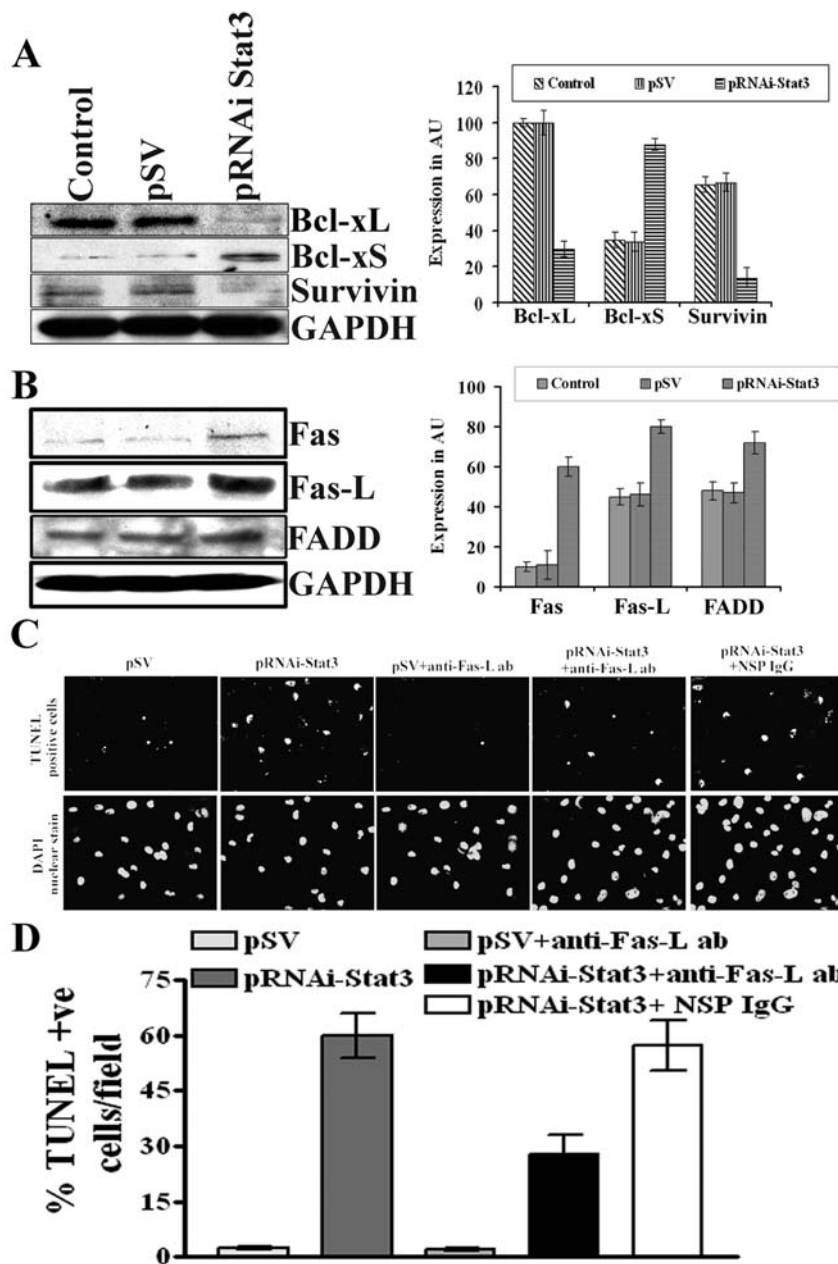


Figure 3. Inhibition of STAT3 expression downregulates survival genes and induces the activation of Fas/Fas-L and recruitment of Fas-associated death domain (FADD) in MDA-MB-231 cells. MDA-MB-231 cells were treated with pSV and pRNAi-Stat3. (A) Cell lysates were used for immunoblot analysis of Bcl-xL, Bcl-xS and Survivin proteins. Densitometric analysis of the Western blot results. The bars indicate SE from the mean of three separate experiments (* $P < 0.05$). (B) Expression of the Fas receptor, Fas-L and FADD was measured by immunoblotting. GAPDH is used as a loading control. Densitometric analysis of the Western blot results. Results are a representative of 3 experiments. Antagonistic Fas-L antibodies block pRNAi-Stat3-induced apoptosis. MDA-MB-231 cells were incubated for 1 h with 10 g/ml of antagonistic Fas-L antibody or nonspecific antibody before transfection with pSV or pRNAi-Stat3. Forty-eight hours later, apoptosis was determined by: (C) TdT-mediated dUTP nick end-labeling assay (TUNEL) staining. Quantitation of TUNEL-positive cells. The bars indicate SE from the mean of three separate experiments (* $P < 0.05$). (D) Immunoblot analysis for cleavage of caspase-8 and PARP. Apoptosis was not antagonized with non-specific antibody. Immunoblots are representative of three experiments.

interaction of Fas and Fas-L (29,30). We found that pRNAi-Stat3-treated MDA-MB-231 cells showed a significant increase in FADD expression (~30%) as compared to the control and vector-treated MDA-MB-231 cells (Fig. 3C and D). To confer the role of Fas/Fas-L in apoptosis in Stat3 down-regulated cells, we treated the MDA-MB-231 cells with Fas-L antibody prior to the pRNAi-Stat3 treatment. Fig. 3D shows pretreatment of MDA-MB-231 cells with Fas-L antibody followed by pRNAi-Stat3 treatment reduced apoptotic cells by >45% as determined by TUNEL staining.

Downregulation of Stat3 alters pro- and anti-apoptotic gene expression and induces cleavage of PARP-1 and caspase-3, -8 and -9. Some members of the Bcl2 family of proteins suppress apoptosis whereas other members promote apoptosis when activated by phosphorylation (unpublished data). An excess of Bax promotes cell death, but expression of Bcl2 can neutralize this effect (unpublished data). Fig. 4A shows that Bax expression increased in pRNAi-Stat3-treated MDA-MB-231 cells as compared to the control and vector-treated cells. On the other hand, Bcl2 expression decreased in pRNAi-

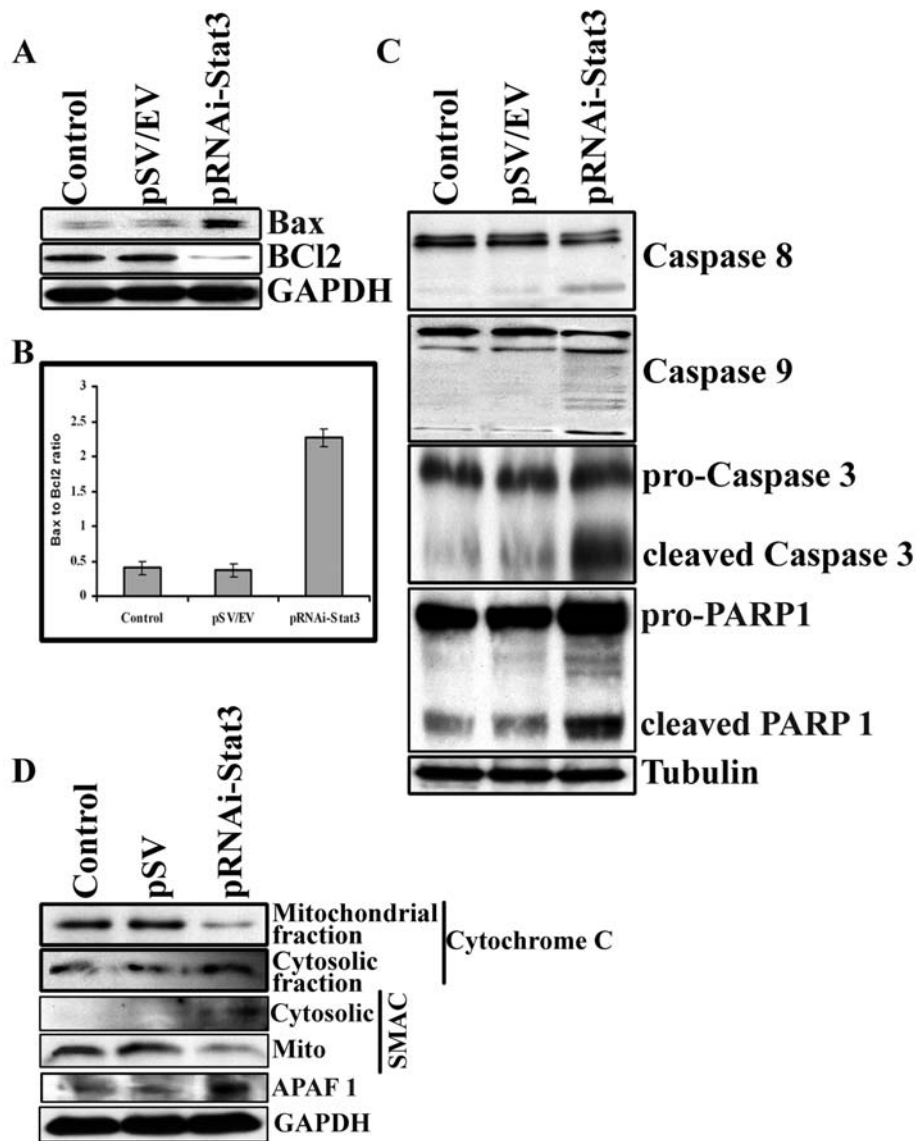


Figure 4. Downregulation of Stat3 alters pro- and anti-apoptotic gene expression and induces cleavage of PARP-1 and caspases -3, -8 and -9. (A) Cell lysates were used for immunoblot analysis of Bax and Bcl-2 proteins. (B) Densitometric analysis showing the Bax/Bcl2 ratio in treatment cells. The bars indicate SE from the mean of three separate experiments. (C) Processing of PARP and caspase-8, -9 and -3 was detected in total cell lysates. (D) Western analysis for SMAC/DIABLO, Cytochrome c release in the cytosol and APAF 1 in the cells treated with pRNAi-Stat3. Anti-GAPDH and anti-tubulin antibodies were used for visualizing equal protein loading. Immunoblots are representative of three experiments (* $P < 0.05$).

Stat3-treated MDA-MB-231 cells while Bcl2 expression was significantly higher in the control and vector-treated cells (Fig. 4A). The functional significance of these expression patterns derives from the potential for heterodimer formation between Bax and Bcl2. A high Bax:Bcl2 ratio enhances the probability for Bax homodimer formation and cell death signaling, whereas a relative abundance of Bcl2 favors formation of Bax/Bcl2 heterodimers and Bcl2 homodimers, which promote cell survival (unpublished data). Hence, we calculated the ratio of Bax expression to Bcl2 expression in the present study. The densitometric analysis showed a high Bax:Bcl2 ratio in the pRNAi-Stat3-treated MDA-MB-231 cells (Fig. 4B).

Since the high Bax: Bcl2 ratio enhances cell death signaling, we next determined whether members of the caspase family were activated. Treatment with pRNAi-Stat3 resulted

in the cleavage of the effector caspase-3 and the initiator caspases -8 and -9 as compared to the control and vector treatments. Further, we found the cleavage of PARP1 in pRNAi-Stat3-treated MDA-MB-231 cells, in particular, the 85 kDa cleaved fragment was significantly higher in these cells as compared to the control and vector-treated cells (Fig. 4C).

The cleavage of caspases is aided by the release of caspase-activating factors, particularly cytochrome c and Smac/Diablo from the mitochondrial membrane into the cytosol (31). In the present study, we found an increased signal for cytochrome c and Smac in the cytosolic fraction of pRNAi-Stat3-treated MDA-MB-231 cells (Fig. 4D). Once released into the cytosol, cytochrome c activates apoptotic protease-activating factor 1 (Apaf-1), which together with pro-caspase 9, forms an active 700-kDa holoenzyme complex known as the

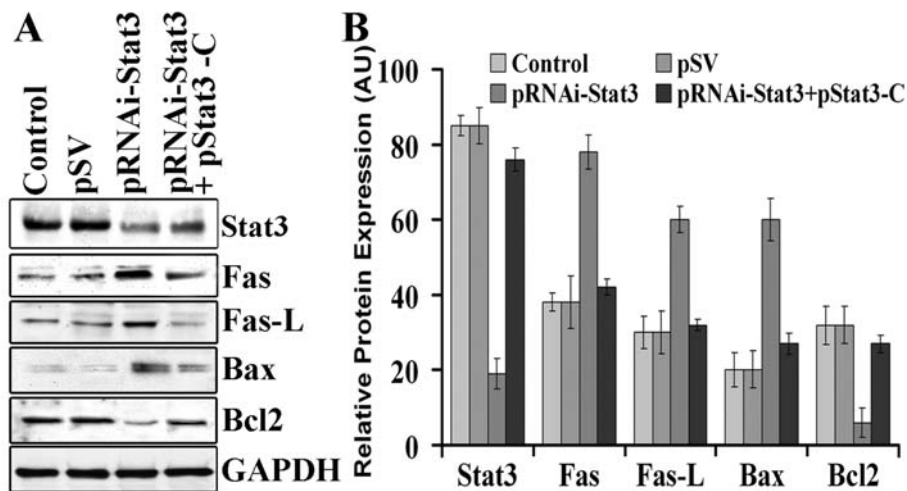


Figure 5. Constitutively activated Stat3 plays a pivotal role in regulating the apoptosis in MDA-MB-231 cells. MDA-MB-231 cells were treated for with pSV, pRNAi-Stat3 and pRNAi-Stat3 + pStat3-C. (A) Cell lysates were used for immunoblot analysis of Fas, Fas-L, Bax and Bcl-2 proteins. Anti-GAPDH antibody was used for visualizing equal protein loading. (B) Densitometric analysis of the Western blot results. Results are a representative of 3 separate experiments (* $P < 0.05$).

apoptosome (31). Fig. 4D shows significantly increased Apaf-1 expression in pRNAi-Stat3-treated MDA-MB-231 cells.

Constitutively activated Stat3 plays a pivotal role in regulating the apoptosis in MDA-MB-231 cells. Groner and Hennighausen (26), have shown that Stat3 protects the cancer cells by regulating the expression of Fas. To show that Stat3 plays an important role in regulating the apoptotic cascade in MDA-MB-231 cells, we used the constructs for constitutively activated Stat3 (pStat3-C). To accomplish this, we co-transfected MDA-MB-231 cells with pRNAi-Stat3 and pStat3-C. Fig. 5 shows the expression of Stat3 is similar to that of control cells in pRNAi-Stat3-treated MDA-MB-231 cells when co-transfected with pStat3-C. Western blot analysis revealed reduced expression of Fas, Fas-L (by >45%), and Bax (by >50%) in the co-transfected cells when compared to the pRNAi-Stat3 transfected cells. Additionally, the expression of Bcl2 drastically increased (reduced only by 10%) in the co-transfected cells almost equaling the expression the pRNAi-Stat3-treated cells. This represents the importance of activated Stat3 in breast cancer cells in protecting the cells from getting apoptosed and aiding in the growth of the tumor.

pRNAi-Stat3 regresses orthotopic breast tumors in nude mice. After 18 days, pRNAi-Stat3 treatment of orthotopic breast tumors reduced tumor size by >60% as compared to the control and vector treatments. Notably, we observed complete tumor regression after 6 weeks of treatment (Fig. 6A). We next determined whether the regression was a result of the induction of apoptosis. To show the efficacy of pRNAi-Stat3 treatment, we used the tumors harvested 18 days after treatment. The tumor tissue was fixed in 10% phosphate-buffered formaldehyde and sectioned. The tumor sections were stained with H&E. Quantification of H&E-stained breast tumor sections by a pathologist (blind review) revealed no difference between the control and empty vector-treated groups, as well as the presence of a high number of mitotic cells and more

aggressive tumor characteristics. However, in the case of pRNAi-Stat3-treated tumor sections nearly a 3-fold increase in apoptotic cells was revealed (Fig. 6B). TUNEL assay of the tissue sections showed that few TUNEL-positive cells were present in the control and vector-treated tumors. In contrast, more than 4-fold more TUNEL-positive cells were present in the pRNAi-Stat3-treated tumors (Fig. 6B). Western blot analysis revealed that pRNAi-Stat3-treated tumors showed decreased Stat3 expression, increased expression of Fas and Fas-L, increased signal for cleaved caspase-3 and decreased expression of Bcl-xL when compared to control and pSV-treated tumors (Fig. 6C). In addition, the immunohistochemical analysis of tumor tissue sections revealed increased expression of Fas and Fas-L and decreased expression of Stat3 in pRNAi-Stat3-treated tumors as compared to the control and vector-treated tumors (Fig. 6D).

Discussion

The evidence that activated Stat3 has a pivotal role in breast tumorigenesis is based on studies of cell lines that express high levels of pStat3 (32). Knocking out Stat3 or blocking the phosphorylation of Stat3 leads to increased apoptosis, chemosensitivity, and decreased angiogenesis in both cell culture and xenograft models (33). Thus, inhibiting Stat3 activity or expression is likely to be an important therapeutic tool for a number of malignancies, including breast cancer.

RNAi represents a promising new technology that could have therapeutic applications for the treatment of diseases by blocking the action of transcription factors and oncogenes with selective silencing of gene expression with exquisite precision and high efficacy (34). In this study, we have identified the Stat3 siRNA that specifically inhibits constitutively activated Stat3 and suppresses cell proliferation and induces apoptosis. One mechanism by which Stat3 participates in tumorigenesis is by inhibiting apoptosis through the induction of anti-apoptotic genes.

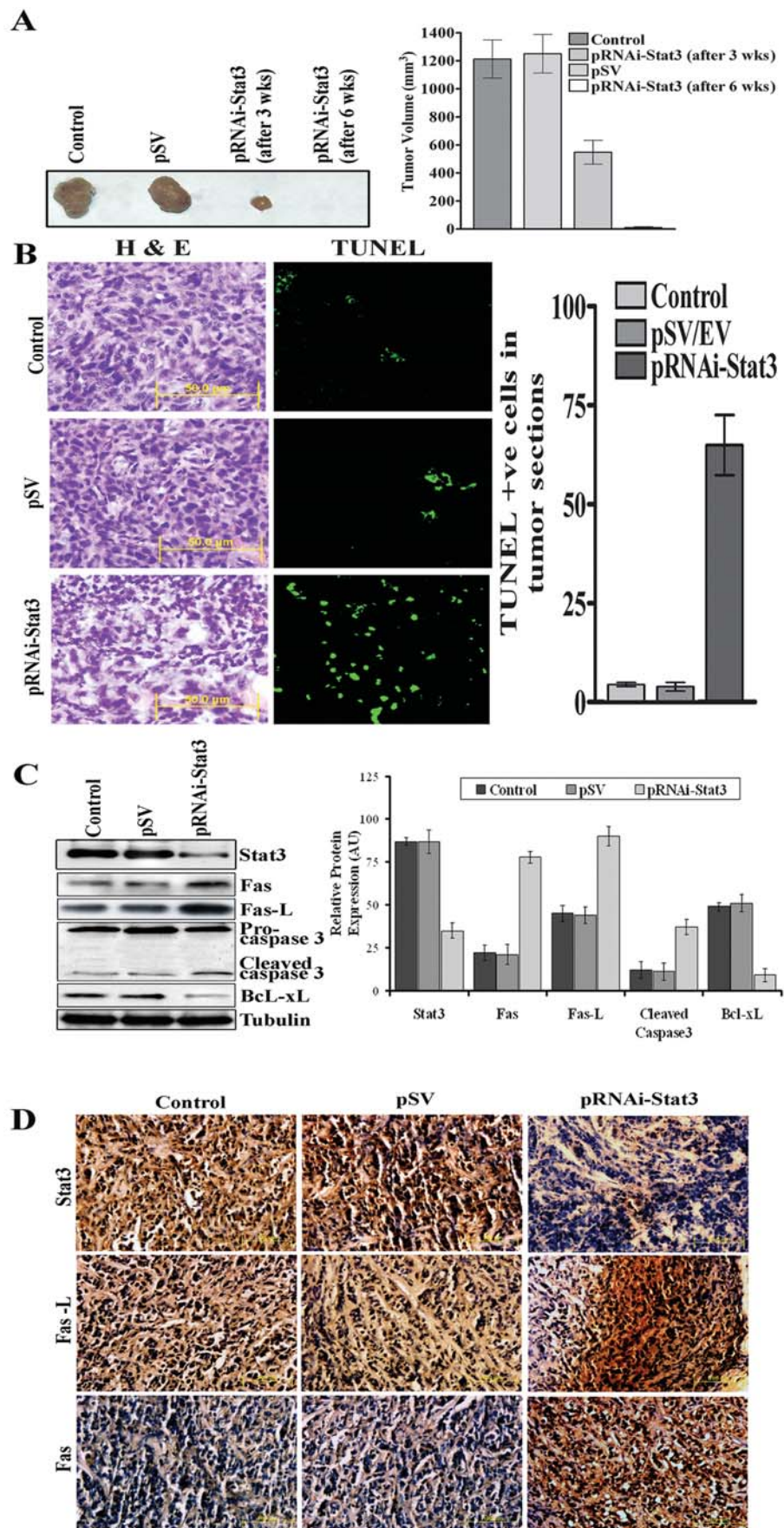


Figure 6. pRNAi-Stat3 regresses orthotopic breast tumors in nude mice. pRNAi-Stat3 treatment induces apoptosis in orthotopic breast tumors. (A) Tumor volume. (B) Tumor sections were subjected to H&E staining and stained for apoptosis by TdT-mediated dUTP nick end-labeling assay (TUNEL) staining. Data shown are representative of 8 fields. Quantification of TUNEL⁺ cells. The bars indicate SE from the mean of 8 microscopic fields. (C) Processing of Stat3, Fas, Fas-L, caspase-3 and Bcl-xL was detected in tumor tissue lysates from the orthotopic breast tumors of mice, which received pSV and pRNAi-Stat3. Densitometric analysis of the Western blot results. Results are a representative of 3 separate experiments. (D) Immunohistochemical analysis Stat3, Fas and Fas-L was performed in paraffin-embedded breast tumor sections of mice treated with pSV and pRNAi-Stat3.

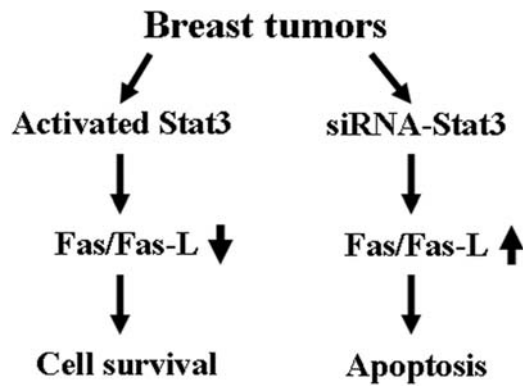


Figure 7. Schematic representation of the effect of pRNAi-Stat3 on apoptotic cascade in breast tumor affecting growth.

The Fas/Fas ligand (Fas L) death pathway is an important mediator of apoptosis. Deregulation of the Fas pathway is reported to be involved in the immune escape of breast cancer and the resistance to anti-cancer drugs. Defects in the Fas/Fas L apoptotic signaling pathway provide a survival advantage to cancer cells and may be implicated in tumorigenesis (35). Significantly, constitutive downregulation of Fas is involved in drug resistance and associated with a poor prognosis in breast cancer. The expression of Fas is downregulated and the expression of Fas-L is upregulated in human breast invasive ductal carcinomas (36).

Numerous investigations have shown apoptosis is mediated by Fas/FasL system in cancer cells treated with anti-cancer agents (37,38). Moreover, anti-cancer drugs are known to enhance FasL expression (39). FasL, which functions as an autocrine/paracrine mediator of apoptosis induced by DNA damaging anti-cancer chemotherapeutic agents, is a member of the TNF superfamily. It induces apoptosis in susceptible cells upon cross-linking to its own receptor, Fas (Apo-1/CD95). Once Fas interacts with Fas-L, it associates with the Fas-associated death domain (FADD) protein through the death domain-death domain interaction. Fas-bound FADD can physically associate with caspase-8. The interaction of caspase-8, an initiator caspase, with FADD results in the activation of caspase-8. This in turn leads to the activation of downstream caspases, including caspase-9 and -3 (40-42), as well as the translocation of Bax to mitochondria and translocation of cytochrome c and Smac/Diablo from the mitochondrial membrane to the cytosol (43-45). Finally, the activated caspases result in the cleavage of PARP-1 (46,47). In the present study, Western blot analysis showed increased expression of Fas and Fas-L in pRNAi-Stat3-treated MDA-MB-231 cells as compared to the control and vector-treated cells. Further, our results showed increases in the expression of Bax in the mitochondrial fraction, Smac/Diablo in the cytosolic fraction, and Apaf-1 in pRNAi-Stat3-treated MDA-MB-231 cells as compared to the control and vector-treated cells.

Moreover, emerging evidence suggests that constitutive activation of Stat3 appears to be ubiquitous in tumors where there is an imbalance in expression levels of anti-apoptotic and apoptotic genes that render tumor cells resistant to

apoptosis (17,48). Recent reports indicate Stat3 siRNA can induce apoptosis in astrocytomas and human prostate cancer (16,49). The induction of apoptosis in all of these studies occurs via inhibition of the anti-apoptotic genes survivin and Bcl-xL (a member of the Bcl-2 family of proteins), which play vital roles in cell survival (16,17). Our results show that expression levels of the anti-apoptotic genes survivin and Bcl-xL were downregulated in pRNAi-Stat3-treated MDA-MB-231 cells as compared to the control and vector-treated cells. Additionally, we found a significant increase in the expression of Bcl-xS in pRNAi-Stat3-treated MDA-MB-231 cells; Bcl-xS is a pro-apoptotic gene induced during apoptosis. The expression of Bcl-xS was negligible in the control and vector-treated cells. Our results strongly support that the constitutively activated Stat3 protects the breast cancer cells from apoptosis.

We suggest a new strategy for treating breast cancer through downregulation of Stat3 using pRNAi-Stat3 (Fig. 7). The decreased Stat3 activity of tumors increased the expression of Fas and Fas-L. Additionally, released the cytochrome c and SMAC into the cytoplasm from the mitochondria activating the intrinsic apoptotic pathway. This in turn led to increased apoptosis, thereby regressing the tumor. Our schematic is based on our *in vitro* and *in vivo* model data showing an increase in apoptosis and tumor reduction.

These results strongly suggest that simultaneous activation of extrinsic and intrinsic pathways is involved in the phenomenon of apoptotic induction. These events are associated with multiple perturbations in apoptotic regulatory proteins including the following: cleavage/degradation of caspase-8, -9, -3, and PARP; cytosolic depletion of Bax; and the release of the mitochondrial proteins Smac/Diablo and Apaf-1. As STAT3 signaling is important for the survival of a number of human tumors, Stat3 siRNA could become an effective therapeutic agent for Stat3-dependent tumors.

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