Persistent Activation of Stat3 Signaling Induces Survivin Gene Expression and Confers Resistance to Apoptosis in Human Breast Cancer Cells

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Abstract

Purpose: Signal transducer and activator of transcription 3 (Stat3) protein is persistently activated in breast cancer and promotes tumor cell survival. To gain a better understanding of the role of constitutive Stat3 signaling in breast cancer progression, we evaluated the expression profile of potential Stat3-regulated genes that may confer resistance to apoptosis.

Experimental Design: Stat3 signaling was blocked with antisense oligonucleotides in human MDA-MB-435s breast cancer cells and Affymetrix GeneChip microarray analysis was done. The candidate Stat3 target gene Survivin was further evaluated in molecular assays using cultured breast cancer cells and immunohistochemistry of breast tumor specimens.

Results: Survivin, a member of the inhibitor of apoptosis protein family, was identified as a potential Stat3-regulated gene by microarray analysis. This was confirmed in Survivin gene promoter studies and chromatin immunoprecipitation assays showing that Stat3 directly binds to and regulates the Survivin promoter. Furthermore, direct inhibition of Stat3 signaling blocked the expression of Survivin protein and induced apoptosis in breast cancer cells. Direct inhibition of Survivin expression also induced apoptosis. Increased Survivin protein expression correlates significantly (P = 0.001) with elevated Stat3 activity in primary breast tumor specimens from high-risk patients who were resistant to chemotherapy treatment.

Conclusions: We identify Survivin as a direct downstream target gene of Stat3 in human breast cancer cells that is critical for their survival in culture. Our findings suggest that activated Stat3 signaling contributes to breast cancer progression and resistance to chemotherapy by, at least in part, inducing expression of the antiapoptotic protein, Survivin.

Signal transducer and activator of transcription 3 (Stat3) protein is a member of a family of latent cytoplasmic transcription factors that convey signals from the cell surface to the nucleus on activation by cytokines and growth factors (reviewed in refs. 1, 2). Engagement of cell surface receptors by polypeptide ligands, such as interleukin-6 (IL-6) or epidermal growth factor, induces tyrosine phosphorylation of Stat3 protein by Janus kinase, growth factor receptor tyrosine kinases, and, in some cases, Src family tyrosine kinases. The phospho-Stat3 protein in the activated dimeric form then translocates to the nucleus and regulates expression of genes harboring Stat3-binding sites in their promoters. Under normal physiologic conditions, the activation of Stat3 protein is rapid and transient and regulates nuclear genes that control fundamental biological processes, including cell proliferation, survival, and development (1, 2).

Numerous studies have detected constitutively active Stat3 in diverse human tumor specimens and established persistent Stat3 activity as essential for malignant transformation of cultured cells by many oncogenic signaling pathways (see refs. 3–7 for reviews). For example, the Src, Janus kinase, and epidermal growth factor receptor family tyrosine kinases are frequently activated in breast cancer cells and induce Stat3 activation (8–11). Blocking of these tyrosine kinase pathways with selective pharmacologic inhibitors results in decreased Stat3 activity, growth inhibition, and apoptosis (11, 12). Persistent activation of Stat3 in tumor cells participates in regulating expression of genes involved in controlling cell cycle progression, apoptosis, and angiogenesis (7). For instance,
an oncogenic mutant of Stat3 induces expression of cyclin D1, Bcl-xl, and c-Myc (13). In human multiple myeloma, head and neck squamous cell carcinoma, and leukemias, disruption of Stat3 signaling induces apoptosis and decreases expression of antiapoptotic genes that encode members of the Bcl-2 family, such as Bcl-xl and Mcl-1 (14–16). In breast cancer, however, the target genes downstream of constitutively active Stat3 and their role in promoting tumor cell survival have not been extensively investigated.

In the present study, we applied microarray gene expression profiling to identify candidate Stat3-regulated genes that may confer resistance to apoptosis in breast cancer cells. Survivin, a member of the inhibitor of apoptosis protein (IAP) family of proteins that is frequently expressed in human tumors (17–19), is identified as a Stat3-regulated gene in breast cancer cells. Direct inhibition of activated Stat3 signaling with antisense oligonucleotides inhibits Survivin expression. Moreover, blockade of constitutively active Stat3 or Survivin expression in breast cancer cells by antisense oligonucleotides induces apoptosis, consistent with the critical role of Stat3-mediated Survivin expression in breast cancer cell survival. We further determined the clinical significance of these findings by investigating Stat3 activation and Survivin expression levels in patients’ tissue samples from 45 high-risk breast cancer patients, as described by Diaz et al., in this issue. Together, these studies reveal that both constitutive Stat3 activation and elevated Survivin expression occur concurrently in high-risk breast tumors that are resistant to neoadjuvant chemotherapy with docetaxel and doxorubicin. Thus, novel therapeutic approaches that down-regulate Stat3 signaling and Survivin expression in breast tumor cells have the potential to induce apoptosis and sensitize tumors to chemotherapy.

Materials and Methods

Tumor specimens. Primary tumors were obtained at H. Lee Moffitt Cancer Center and Research Institute from patients with stage III breast carcinoma before neoadjuvant chemotherapy with sequential docetaxel and doxorubicin in a phase II clinical trial. To preserve the in vivo activated phosphorylation state of Stat3 proteins in tumor cells, specimens were fixed in formalin or snap frozen in liquid nitrogen within 15 to 20 minutes of surgical excision (see Diaz et al., in this issue). Tissue specimens were obtained with written patient consent in this Institutional Review Board–approved clinical trial.

Cell lines and transfections. MDA-MB-435s, MDA-MB-468, MDA-MB-231, MDA-MB-361, HEK-293, and NIH 3T3 cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. MB-231, MDA-MB-361, and Survivin antisense (5'-CCCACTTCTCTAGTTTGGG-3') oligonucleotides were synthesized using phosphorothioate chemistry. To increase stability, oligonucleotides were synthesized with 2'-O-methoxymethyl modification of the five or three underlined terminal nucleotides (21). The final concentration for Stat3 antisense and control mismatch oligonucleotides was 250 nmol/L. For Survivin antisense oligonucleotides, the final concentration was 300 nmol/L. For IL-6 stimulation, cells were serum-starved (DMEM supplemented with 0.1% fetal bovine serum) for 18 hours before IL-6 treatment (20 or 30 ng/mL in DMEM) for 30 minutes [electrophoretic mobility shift assay (EMSA) analysis] or 48 hours (Western blot analysis). Nuclear extracts and cell lysates were prepared for EMSA and Western blot analysis, respectively, as described below.

Western blot analyses. Cells were lysed in a buffer containing 10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L EDTA (pH 8.0), 2 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin, 2 μg/mL leupeptin, and 1 mmol/L Na3VO4. For Western blot analyses, 30 μg of total extracted proteins were applied per lane before SDS-PAGE. Following transfer to nitrocellulose membranes, protein expression levels were detected using polyclonal anti-Stat3 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Survivin (Alpha Diagnostics International, San Antonio, TX), or anti-poly-(ADP-ribose) polymerase (Cell Signaling Technology, Beverly, MA) antibodies. The expression of β-actin (Sigma-Aldrich, St. Louis, MO) was used as a normalization control for protein loading.

Immunohistochemistry and image analysis. Details of the immunohistochemical analysis are described by Diaz et al., in this issue. Survivin monoclonal antibody and polyclonal pY-Stat3 antibody (Cell Signaling Technology) were used for immunohistochemistry of formalin-fixed, paraffin-embedded tissue sections. The Optimas 6.5 software was used to quantify protein expression as described by Diaz et al., in this issue. Results are reported as an index reflecting the intensity of the marker in relationship to the total pixel intensity of the region of interest. The index is an average of the values of three images.

Statistical analysis. Pathologic response was classified as complete pathologic response or partial pathologic response based on the size of residual tumor after treatment (complete pathologic response if 0 cm; partial pathologic response if > 0 cm). Correlations between biomarkers were assessed by Spearman’s rank correlation coefficient for each group and tests of the correlation coefficient equal to zero were carried out. All tests were two sided and declared significant at the 5% level. SAS 8.2 (SAS Institute, Cary, NC) was used for statistical calculations.

Nuclear extract preparation and EMSA. Nuclear extracts were prepared as previously described (9) by high-salt extraction into 30 to 70 μL buffer [20 mmol/L HEPES (pH 7.9), 420 mmol/L NaCl, 1 mmol/L EDTA, 20% glycerol, 20 mmol/L NaF, 1 mmol/L Na3VO4, 1 mmol/L Na2PO4, 1 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 0.1 mmol/L aprotinin, 1 mmol/L leupeptin, and 1 mmol/L antipain]. For EMSA, 5 μg of total nuclear protein were used for each lane. EMSA was done using a 32P-labeled oligonucleotide probe containing a high-affinity six-inducible element (Hsle-6) variant derived from the c-fos gene promoter (sense strand 5'-ACGCTTACCTTCCGGAATTCCTGTCGNTGCCGACGCG-3'; and (−1,105) site #7, 5'-TCTGTCAGAGCGCTTTCGCTTGAAATATGCATTAAATAC-3') that binds activated Stat3 proteins (11). Stat3 protein was supershifted in the EMSA by preincubation with Stat3 antibody (C-20X, Santa Cruz Biotechnology). The oligonucleotides containing the putative Stat3-binding sites in the Survivin promoter used in EMSA are as follows (sense strand): (−1,184) site #1, 5'-TGGAGCTCAGTGGTCTTAAATATCTAC-3'; (−1,143) site #2, 5'-TGGAGCTCAGTGGTCTTAAATCTAC-3'; and (−1,105) site #7, 5'-TCTGTCAGAGCGCTTTCGCTTGAAATATGCATTAAATAC-3' (the bolded and underlined sequences were added to the 5'-end to create overhangs for radiolabeling by Klenow reaction and are not part of the Survivin promoter). Competition analysis was carried out to determine specificity of Stat3 binding sites.
binding to the Survivin promoter was done by preincubating unlabeled hSIE probe with radiolabeled Survivin probe in the EMA. Following incubation of radiolabeled probes with nuclear extracts, protein-DNA complexes were resolved by nonnaturating PAGE and detected by autoradiography.

Cytoplasmic extract preparation and luciferase assays. Cytoplasmic extract preparation and luciferase assays were done as previously described (20). Briefly, cells were lysed in 0.1 mL of low-salt HEPES buffer [10 mmol/L HEPES (pH 7.8), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L DTT, and 20 μL of 10% NP40]. After centrifugation (10,000 × g, 1 minute, 4°C), cytosolic supernatant was used for luciferase assays as described by the vendor (Promega). Experiments were done in triplicate and the average values were determined. To control for transfection efficiency, firefly luciferase values were normalized to the values for β-galactosidase.

In situ terminal deoxynucleoblastosid transferase–mediated dUTP nick end labeling and cellular proliferation assays. MDA-MB-435s and MDA-MB-231 cells were transfected with antisense or control mismatch oligonucleotides. After 48 hours, cells were labeled for apoptotic DNA strand breaks by terminal deoxynucleoblastosid transferase–mediated dUTP nick end labeling (TUNEL) reaction using an in situ cell death detection assay (Roche Applied Science, Indianapolis, IN) according to the instructions of the supplier. TUNEL-positive nuclei were counted and the apoptotic index was expressed as the number of apoptotic cells in one microscopic field. To determine cellular viability, cells were harvested by trypsinization and counted by trypan blue exclusion assay at 24 and 48 hours after transfection. All experiments were done in triplicate.

RNase protection assay. Total RNA was isolated from MDA-MB-435s cells using the RNAeasy mini kit (Qiagen, Valencia, CA). RNase protection assays were carried out with the Riboquant hStress-1 template set containing Bcl-xL and Mcl-1 probes or custom-made multiprobe templates containing Stat3 and Survivin probes (BD Pharmingen, San Diego, CA). Briefly, the multiprobe templates were synthesized by in vitro transcription with incorporation of [32P]dUTP and purified on Quick Spin RNA columns (Roche Applied Science). Labeled probe (1 × 10⁶ cpm) was hybridized with 10 μg of total RNA through a temperature gradient of 90°C to 56°C over a 16-hour period. Unprotected probe was removed by RNase digestion at 30°C for 1 hour followed by separation of protected RNA fragments on a 5% polyacrylamide-urea gel and detection using autoradiography.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation assays were done as previously described (22). Briefly, asynchronously growing HEK-293 cells were incubated with formaldehyde to cross-link protein-DNA complexes. The cross-linked chromatin was then extracted, diluted with lysis buffer, and sheared by sonication. After preclearing with 1:2 mix of protein A/protein G-agarose beads (Life Technologies), the chromatin was divided into equal samples for immunoprecipitation with either anti-Stat3 or anti–immunoglobulin G (negative control) polyclonal antibody (Santa Cruz Biotechnology). The immunoprecipitates were pelleted by centrifugation and incubated at 65°C to reverse the protein-DNA cross-linking. The DNA was extracted from the eluate by the phenol/chloroform method and then precipitated by ethanol. Purified DNA was subjected to PCR with primers specific for a region (−1,231 to −1,009) in the human Survivin promoter spanning three putative Stat3-binding sites. The sequences of the PCR primers used are as follows: Survivin forward primer, 5′-CAGTGAAGACTGATCATCCGC-3′; Survivin reverse primer, 5′-TATAGCCCATCCTACCCAGCG-3′.

Microarray sample processing and data analysis. Five micrograms of total RNA collected from MDA-MB-435s cells treated with antisense or control mismatch oligonucleotides served as the mRNA sources for microarray analysis. The poly(A) mRNA was specifically converted to cDNA and then amplified and labeled with biotin following the procedure initially described by Van Gelder et al. (23). Hybridization with the biotin-labeled DNA, staining, and scanning of the microarray chips followed the prescribed procedure outlined in the Affymetrix technical manual and has been previously described (24).

The oligonucleotide probe arrays were Human Genome U133A chips (Affymetrix, Santa Clara, CA). Scanned output files were visually inspected for hybridization artifacts and then analyzed using Affymetrix Microarray MAS 5.0 software. The MAS 5.0 software identifies the increased and decreased genes between any two samples with a statistical algorithm that assesses the behavior of oligonucleotide probe sets designed to detect the same gene. Probe sets that yielded a change at P < 0.0045 were identified as changed (increased or decreased). In addition, the data were processed using robust multiarray analysis (25). Genes that were significantly changed in their expression were identified. Empirical estimates of the null distribution were determined using permutation analysis, thereby controlling the number of false positives. The significance analysis of microarrays (26) implements this approach to address the multiple testing problem and was also applied to the data analysis. Genes were considered changed if consistent
behavior (increase or decrease) was observed in each of three replicate experiments based on analyses of data by the multiple methods described above.

**Results**

**Direct blocking of Stat3 induces apoptosis in breast cancer cells.** The human breast cancer cell line MDA-MB-435s, harboring activated Stat3, was transfected with Stat3 antisense or mismatch oligonucleotides using Lipofectamine-Plus. Twenty-four and 36 hours after transfection, the mRNA levels and DNA-binding activities of Stat3 were measured by RNase protection and electrophoretic mobility shift (EMSA) assays, respectively. Figure 1 shows that Stat3 antisense diminished Stat3 mRNA expression compared with the mismatch oligonucleotides (Fig. 1A), accompanied by a significant decrease in Stat3 DNA-binding activity (Fig. 1B). Incubation of cells with Stat3 antisense oligonucleotides for up to 48 hours resulted in a marked increase in vacuolated cells and cellular debris (Fig. 2A), indicative of apoptotic cell death. The occurrence of apoptosis was confirmed by *in situ* TUNEL assay (Fig. 2B and C) and by cleavage of poly-(ADP-ribose) polymerase most notably at 48 hours after treatment with antisense oligonucleotides (Fig. 2D). Figure 2E shows that treatment with Stat3 antisense oligonucleotide also induced significant growth inhibition. Both apoptosis and inhibition of cellular proliferation correlated with blockade of Stat3 expression and activation (Fig. 1 and data not shown).

**Inhibition of Stat3 decreases expression of Survivin in breast cancer cells.** We did microarray gene expression profiling analyses to assess the gene expression changes associated with blockade of Stat3 activity. RNA samples derived from MDA-MB-435s cells treated with Stat3 antisense or mismatch oligonucleotides for 24 hours were processed for microarray analysis using Affymetrix Human Genome U133A GeneChips. Experiments were done in triplicates and overlap among the three sets of deregulated genes was determined by using a variety of different microarray data analysis methods (see Materials and Methods). Whereas the biological functions of the genes identified were diverse (see below), we focused on apoptosis-related genes. Previous studies revealed that in some cell types, Stat3 activation prevents apoptosis by regulating expression of the Bcl-2 family of antiapoptotic proteins (26–31). However, microarray analysis did not reveal consistent decreases in the expression of any Bcl-2 family proteins in antisense oligonucleotide–treated breast cancer cells (data not shown). In contrast, expression of Survivin, which is a member of the IAP family of antiapoptotic genes, was found to be diminished by microarray analysis as confirmed below by independent molecular approaches. The microarray analysis revealed no change in other IAP family members including X-linked IAP, cellular IAP-1, and cellular IAP-2 (data not shown).

The microarray data were validated using an RNase protection assay. Results show a decrease in mRNA expression of Survivin, but not of Bcl-xL or Mcl-1, in antisense oligonucleotide–treated breast cancer cells (Fig. 3A). The correlation between Stat3 and Survivin protein expression was further confirmed by Western blot analysis, showing decreased Survivin expression in breast cancer cells that are treated with Stat3 antisense oligonucleotide (Fig. 3B). Because activated Stat3 signaling promotes the survival of breast tumor cells, we determined whether Survivin expression protects breast cancer cells from apoptosis. In MDA-MB-435s and MDA-MB-231 cells transfected with antisense oligonucleotides directed against Survivin, poly-(ADP-ribose) polymerase (PARP) cleavage was detected by Western blot analysis for poly-(ADP-ribose) polymerase (top) and Stat3 (bottom). Blots were normalized for total protein loading.
polymerase cleavage (Fig. 3C) and in situ TUNEL staining (Fig. 3D) were evident following inhibition of Survivin expression, indicative of apoptosis. Thus, expression of the antiapoptotic protein Survivin is associated with constitutive Stat3 activity and survival in breast cancer cells.

**Stat3 directly binds to and regulates the Survivin promoter.**

We examined whether Stat3 regulates Survivin promoter activity. In transient transfection studies with a luciferase reporter gene driven by the human Survivin promoter, we show that cotransfection with a v-Src vector that activates endogenous cellular Stat3 induces expression of the Survivin reporter by 2-fold (Fig. 4A). Moreover, cotransfection with both v-Src and full-length Stat3 vectors further induced the Survivin reporter expression up to nearly 5-fold. By contrast, ectopic expression of the dominant-negative Stat3 variant, Stat3δ, decreased basal levels of Survivin reporter expression by 50% (Fig. 4A).

A search for potential Stat3-binding sites within the Survivin promoter region (18) revealed five candidates with the consensus sequences TT(N₄)AA and TT(N₅)AA (28). To determine whether Stat3 could bind the Survivin promoter under physiologic conditions in intact cells, we did chromatin immunoprecipitation assays using three sets of primers that cover the five candidate Stat3-binding sites. Primers to the region of −1,231 to −1,009 upstream from the ATG translation initiation site yielded Survivin promoter DNA in chromatin immunoprecipitated with an anti-Stat3 antibody (Fig. 4B). This region contains three potential Stat3 binding sites (Fig. 4D). By contrast, primers to the regions of −358/−148 and −938/−759 in the Survivin promoter did not detect promoter DNA in the anti-Stat3 immunoprecipitates (data not shown).

EMSA was done to determine binding of Stat3 to the same Survivin promoter region in vitro. Results show that endogenous activated Stat3 protein, present in nuclear extracts of MDA-MB-435s breast cancer cells, binds to the Survivin promoter fragments −1,174/−1,166 (site #1) and −1,095/−1,087 (site #3) but not to fragments −1,133/−1,126 (site #2), −851/−844 (site #4), and −264/−256 (site #5; Fig. 4C and data not shown). Specificity of Stat3 binding to the Survivin promoter fragments #1 and #3 was shown by competition analysis with unlabeled hSIE probe (Fig. 4C and data not shown). Both site #1 and site #3 are located within the −1,231 to −1,009 region that was detected in the chromatin immunoprecipitation assays above with anti-Stat3 antibody, suggesting it is this region of the Survivin promoter that accounts for Stat3 binding. Taken together, these data provide evidence that Stat3 directly binds the Survivin promoter and induces its expression.

**Stat3 activation correlates with expression of Survivin in breast cancer cell lines.** A unique feature of Survivin is its differential expression in tumor versus normal tissues (17–19) and
overexpression of Survivin has been found in many cancers, including breast cancer (17). We investigated whether Stat3 activation correlates with Survivin up-regulation in a panel of human breast cancer cells harboring constitutively active Stat3. Western blot analysis shows Survivin protein expression in all tested breast cancer cell lines with activated Stat3 (MDA-MB-231, MDA-MB-435s, and MDA-MB-468; Fig. 5A). By contrast, minimal Survivin expression was observed in breast cancer cells (MDA-MB-361) and in normal breast epithelial cells (MCF-10A) lacking detectable Stat3 activation.

We investigated whether cytokine-induced Stat3 activity correlates with increased Survivin expression in breast cancer cells. IL-6 treatment of serum-starved MDA-MB-435s cells increases Stat3 DNA-binding activity within 30 minutes as detected by EMSA (Fig. 5B). In addition, both phospho-Stat3 levels and Survivin protein expression were induced by IL-6 treatment of MDA-MB-435s cells after 48 hours (Fig. 5C). These findings indicate that constitutive and cytokine-induced Stat3 activation correlates with Survivin expression in breast cancer cell lines.

**Stat3 activation and survivin expression in clinical breast tumor specimens.** Based on the above results, we explored the association between constitutive Stat3 activation and Survivin expression in primary breast tumors. Our analysis involved 45 tumor specimens and matched nonneoplastic tissues, which are all from patients with invasive breast carcinoma, obtained before initiation of chemotherapy treatment in a phase II clinical trial with sequential doxorubicin and docetaxel (see Diaz et al., in this issue). Tissue specimens were analyzed by immunohistochemical staining of formalin-fixed, paraffin-embedded sections using phospho-Stat3 or Survivin antibodies. We observed moderate to strong predominantly nuclear staining for phospho-Stat3 and Survivin in a majority of the tumor specimens but not in normal breast epithelial cells (MCF-10A) lacking detectable Stat3 activation.

Importantly, a statistically significant positive correlation ($P = 0.001$) was observed between elevated phospho-Stat3 levels and Survivin expression in $33$ of the $45$ breast cancer patients who displayed a partial pathologic response to this neoadjuvant chemotherapy regimen (see Diaz et al., in this issue). Thus, high levels of phospho-Stat3 and Survivin expression correlate with invasive breast cancer and resistance to chemotherapy.

**Additional candidate Stat3-regulated genes in breast cancer cells.** To increase the likelihood of identifying additional Stat3-regulated genes, we focused on reproducibility of microarray data and intersections between different statistical methods of analysis rather than on fold levels of changes. Microarray samples were obtained from three independent experiments and data were analyzed using standard Affymetrix Microarray MAS 5.0 software, as well as robust multiarray analysis combined with significance analysis of microarrays. The significance analysis of microarrays identifies the most statistically significant, differentially expressed genes between two groups of samples. Potentially interesting genes that were most
consistently changed on inhibition of Stat3 signaling in MDA-MB-435s breast cancer cells (data not shown) include TACC3, SMARCA4, CDC37, and ZNF148 (candidate Stat3 up-regulated genes) and Caspase 4, Histone H2B, RNF6, and GADD45A (candidate Stat3 down-regulated genes).

**Discussion**

We have previously shown that inhibition of activated Stat3 signaling in breast cancer cells by Src and Janus kinase pharmacologic inhibitors induces apoptosis (11). Here, we confirm the critical role of activated Stat3 in breast tumor cell survival by directly blocking its function using antisense Stat3 oligonucleotides. These findings are consistent with earlier studies showing induction of apoptosis following blockade of Stat3 signaling in human head and neck squamous cell carcinoma, multiple myeloma, leukemic large granular lymphocytes, prostate cancer, melanoma, lung cancer, and lymphoma cells (14–16, 29, 32–36). In the present study, we investigated candidate Stat3-regulated genes involved in preventing apoptosis of breast cancer cells. Among the genes of which expression changed on blocking the Stat3 pathway is Survivin, a member of the IAP family of antiapoptotic proteins. Survivin is strongly expressed in embryonic and fetal organs (17–19) but undetectable in most terminally differentiated normal tissues (17, 19, 37). By contrast, dramatic overexpression of Survivin has been shown in many tumors, including breast cancer (19, 38). Our results are consistent with previous findings linking Stat3 activation and Survivin expression in primary effusion lymphoma and gastric cancer cells (36, 39), and provide new evidence that Stat3 directly regulates the Survivin gene promoter.

The present studies establish a correlation between Stat3 activation and Survivin expression in a set of 45 primary tumor specimens from patients with invasive breast carcinoma (see Diaz et al., in this issue). Notably, elevated Stat3 activity is significantly correlated (P = 0.001) with increased Survivin expression in 33 of the 45 breast cancer patients who exhibited a partial response to sequential neoadjuvant therapy with doxorubicin and docetaxel. Previous studies have indicated that Survivin expression correlates with taxol resistance in ovarian cancer (40, 41), providing further support for the potential of Stat3-mediated Survivin expression to modulate response to chemotherapy in breast cancer. Moreover, studies suggest that Survivin directly interacts with the Smac/DIABLO protein in modulating apoptotic responses to taxol (40, 41). Our findings presented here indicate that activated Stat3 signaling in breast cancer cells induces Survivin expression and thereby prevents apoptosis, which potentially contributes to resistance to chemotherapy.

Several lines of evidence suggest that deregulation of Survivin expression occurs in cancer as a result of genetic (amplification of the Survivin locus on 17q25 in neuroblastoma), epigenetic (selective demethylation of Survivin exon 1 in ovarian cancer but not in normal ovaries), transcriptional (transcriptional
repression by wild-type p53), and posttranslational (increased protein stability by phosphorylation on Thr34 of Survivin protein) molecular mechanisms (30, 31, 42–44). Whereas association of Survivin expression with Stat3 signaling has been previously reported (36, 39, 45, 46), we data suggest a new mechanism for overexpression of Survivin that involves direct regulation of the Survivin gene promoter by persistently activated Stat3 protein in tumor cells. This mechanism may be prevalent in other human cancers that exhibit both persistent Stat3 activation and overexpression of Survivin.

Additional candidate Stat3-regulated genes were identified by our microarray analysis.7 Genes of which expression was repeatedly down-regulated by Stat3 antisense treatment (po- 7Our unpublished results.

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