

# Resveratrol inhibits Src and Stat3 signaling and induces the apoptosis of malignant cells containing activated Stat3 protein

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## Abstract

Resveratrol is a naturally occurring phytoalexin with antioxidant and antiinflammatory properties. Recent studies suggest that resveratrol possesses anticancer effects, although its mechanism of action is not well understood. We now show that resveratrol inhibits Src tyrosine kinase activity and thereby blocks constitutive signal transducer and activator of transcription 3 (Stat3) protein activation in malignant cells. Analyses of resveratrol-treated malignant cells harboring constitutively-active Stat3 reveal irreversible cell cycle arrest of v-Src-transformed mouse fibroblasts (NIH3T3/v-Src), human breast (MDA-MB-231), pancreatic (Panc-1), and prostate carcinoma (DU145) cell lines at the G<sub>0</sub>-G<sub>1</sub> phase or at the S phase of human breast cancer (MDA-MB-468) and pancreatic cancer (Colo-357) cells, and loss of viability due to apoptosis. By contrast, cells treated with resveratrol, but lacking aberrant Stat3 activity, show reversible growth arrest and minimal loss of viability. Moreover, in malignant cells harboring constitutively-active Stat3, including human prostate cancer DU145 cells and v-Src-transformed mouse fibroblasts (NIH3T3/v-Src), resveratrol treatment represses Stat3-regulated cyclin D1 as well as Bcl-x<sub>L</sub> and Mcl-1 genes,

suggesting that the antitumor cell activity of resveratrol is in part due to the blockade of Stat3-mediated dysregulation of growth and survival pathways. Our study is among the first to identify Src-Stat3 signaling as a target of resveratrol, further defining the mechanism of antitumor cell activity of resveratrol and raising its potential application in tumors with an activated Stat3 profile. [Mol Cancer Ther 2006;5(3):621–9]

## Introduction

Resveratrol (*trans*-3,5,4'-trihydroxystilbene), a naturally-occurring phytoalexin with antioxidant and antiinflammatory properties, is abundant in red grapes and grape products and is believed to confer cardioprotective effects associated with red wine consumption (1). Initial studies show growth inhibition of human breast cancer (2), oral cancer (3, 4), prostate cancer (5, 6), and leukemia cell lines (7), blockade of angiogenesis, and protection against carcinogenesis (8, 9) by resveratrol, suggesting the potential anticancer properties of this compound. The molecular mechanisms for these resveratrol biological effects are not fully defined, although a few reports indicate that it interferes with the mitogen-activated protein kinase and protein kinase C pathways (7, 9–13), inhibits ribonucleotide reductase and DNA synthesis (14), and disrupts cyclooxygenase activity (15).

The signal transducer and activator of transcription (STAT) family of cytoplasmic proteins is important for promoting the proliferation, survival, and other biological processes triggered by cytokines and growth factors (16–18). STAT activation is initiated upon phosphorylation of a critical tyrosine residue by growth factor receptors, Janus kinases, or the Src family kinases, and involves dimerization between two phosphorylated STAT monomers followed by translocation of the dimers into the nucleus. In the nucleus, STAT dimers bind to specific DNA response elements in the promoters of target genes and regulate their expression. Whereas normal STAT activation is highly regulated and transient, one member of the STAT family, Stat3, is constitutively activated in diverse human tumors largely due to hyperactive tyrosine kinases. Constitutively-active Stat3 induces oncogenic processes, such as dysregulated growth, survival, angiogenesis, and immune modulation, and thereby contributes to malignant transformation and progression (18–22). Given that the Src tyrosine kinase activates Stat3 and the collective roles of both Src and Stat3 in many human tumors (23–25), and the potential for resveratrol to modulate signal transduction pathways, we investigated the effects of resveratrol on Src-Stat3 signaling. We report that resveratrol inhibits

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both Src tyrosine kinase and downstream Stat3 activation in malignant cells and induces cell growth inhibition and apoptosis of malignant cells harboring constitutively-active Stat3.

## Materials and Methods

### Cell Lines, Reagents, and Treatment Conditions

Normal mouse fibroblasts (NIH3T3) and their v-Src-transformed counterparts (NIH3T3/v-Src) have been previously described (23, 26). The human breast cancer MDA-MB-231, MDA-MB-453, MDA-MB-468, pancreatic cancer Panc-1 and Colo-357, and prostate cancer LNCaP and DU145 cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in DMEM or in RPMI 8226 with 10% heat-inactivated fetal bovine serum. *Trans*-resveratrol, RNase, DMSO, propidium iodide, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit were from Sigma-Aldrich (St. Louis, MO), and terminal nucleotidyl transferase-mediated nick end labeling assay kit was from BD Biosciences Pharmingen (San Diego, CA). Each treatment condition is a single dose of resveratrol at the indicated concentration or 0.1% DMSO (vehicle) as control.

### Nuclear Extract Preparation and Gel Shift Assays

Nuclear extracts were prepared from cells and used for electrophoretic mobility shift assay, as previously described (23, 25–28). In some cases, cells were pretreated with resveratrol (30–50  $\mu\text{mol/L}$ , 24 hours) prior to harvesting for nuclear extract preparation. Nuclear extracts (normalized for equal total protein) were incubated with the  $^{32}\text{P}$ -labeled oligonucleotide probe hSIE (high-affinity *sis*-inducible element, m67 variant, 5-AGCTTCATTTCCCGTAAATCCCTA) that binds both Stat1 and Stat3 (28, 29). Supershift assays were done with rabbit polyclonal antibodies specific for COOH-terminal amino acid residues of Stat3 (750–769) or Stat1 (685–710; Santa Cruz Biotechnology, Santa Cruz, CA). Protein-DNA complexes were resolved by nondenaturing gel electrophoresis and detected by autoradiography.

### Western Blot Analysis

Cell lysates were prepared with ice-cold radioimmunoprecipitation assay buffer, clarified by centrifugation, and protein concentrations of supernatants were determined by Bio-Rad protein assay (Hercules, CA). Equivalent amounts of total cellular proteins were separated by SDS-10% PAGE and then transferred onto nitrocellulose membranes. Probing of nitrocellulose membranes with primary antibodies and detection of horseradish peroxidase-conjugated secondary antibodies by enhanced chemiluminescence (Amersham, Piscataway, NJ) were done as previously described (24, 26, 27). The probes used were antibodies against Mcl-1, Bcl-x<sub>L</sub>, cyclin D1, and Stat3 (Santa Cruz Biotechnology), pY-Stat3 and pY-Src (Cell Signaling Technology, Beverly, MA), and Src GD11 monoclonal antibody (Upstate Biotechnologies, Waltham, MA).

### Src Kinase Assay

NIH3T3/v-Src cells were harvested, lysed in radioimmunoprecipitation assay buffer, and protein concentrations

were determined. Equal amounts of total protein (150  $\mu\text{g}$ ) were incubated for 2 hours with 5  $\mu\text{L}$  of Src monoclonal antibody (Upstate Biotechnologies). Protein G-agarose was added and incubation continued for an additional 4 hours. Immunoprecipitated proteins were washed thrice in radioimmunoprecipitation assay buffer and once in kinase buffer (0.1 mol/L HEPES, pH 7.5, 0.02 mol/L  $\text{MgCl}_2$ , 0.01 mol/L  $\text{MnCl}_2$ , and 0.02 mol/L DTT). Equal amounts of immunoprecipitated proteins were incubated in kinase reaction mixture containing kinase buffer, 2  $\mu\text{g}$  denatured enolase (Sigma-Aldrich), 10  $\mu\text{mol/L}$  cold ATP, and 2  $\mu\text{Ci}$  (1 Ci = 37 GBq) [ $\gamma$ - $^{32}\text{P}$ ]ATP, and incubated at 30°C for 30 minutes. The reaction was stopped with gel loading buffer and the proteins were separated by 10% PAGE and subjected to autoradiography.

### Immunohistochemical Detection of Phosphotyrosine Stat3

The indirect peroxidase-antiperoxidase test was done on cytopins prepared from cell lines (control and treated with 30 or 50  $\mu\text{mol/L}$  resveratrol for 24 hours). Immunostaining for pY-Stat3 was done with a rabbit antihuman polyclonal pY-Stat3 antibody (Cell Signaling Technology) with rabbit immunoglobulins as negative controls using avidin-biotin-peroxidase complexes (Vector Laboratories, Burlingame, CA). After incubation and blocking of endogenous peroxidase and nonspecific background staining in 3% hydrogen peroxide and methanol for 20 minutes, slides were washed with PBS, treated with 1.5% normal serum and 3% bovine serum albumin, and incubated with the pY-Stat3 primary antibody overnight at 4°C. Slides were rinsed in PBS and incubated with a biotinylated secondary antibody (Vector Laboratories) for 60 minutes, followed by incubation with avidin-biotin-peroxidase complex for 1 hour at room temperature. Chromagen was developed with Nova-Red. All slides were counterstained with hematoxylin for 30 seconds before dehydration and mounting. PhosphoY-Stat3-positive cells were stained red (due to Nova-Red) and pY-Stat3-negative cells were stained blue (due to hematoxylin).

### Cell Proliferation Assay and Viability Detection by Tetrazolium-Based Calorimetric Assay (MTT)

Proliferating fibroblasts treated (0–50  $\mu\text{mol/L}$  resveratrol) or untreated (0.1% DMSO) for different times were trypsinized and counted by phase-contrast microscopy for viable cells (using trypan blue exclusion). In other studies, resveratrol-containing medium was removed 48 hours after incubation and replaced with fresh resveratrol-free medium for 24 hours. Cell numbers were then counted as above. For MTT assay, the seeding density of the breast, pancreatic, and prostate cancer cell lines ranged from  $8 \times 10^3$  to  $2 \times 10^4$ /well in 96-well plates. After 12 hours of adherence, cells were treated with 0 to 100  $\mu\text{mol/L}$  of resveratrol for different times, then exposed to the MTT dye (5 mg/mL) and incubated at 37°C for 3 hours. The resulting formazan crystals were solubilized with DMSO and the absorbance of each well was measured at 540 nm using a multiscanner autoreader (Dynatech MR 5000, Chantilly, VA).

### Cell Cycle Analysis by Flow Cytometry

Cells (mouse fibroblasts, human breast, pancreatic, and prostate cancer) were treated with 0 to 50  $\mu\text{mol/L}$  of resveratrol or with DMSO for the indicated times. Cells were then fixed with 70% cold ethanol and analyzed after propidium iodide staining. DNA content was determined by a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Cell cycle phase distribution was analyzed using the Cell-Fit program. Data acquisition was gated to exclude cell doublets. For resveratrol recovery experiments, NIH3T3 cells were treated with 20  $\mu\text{mol/L}$  resveratrol or DMSO (control) for 72 hours. Cells were then washed twice with fresh DMEM and cultured in fresh DMEM for 24 hours at 37°C, processed and subjected to cell cycle analysis as described above.

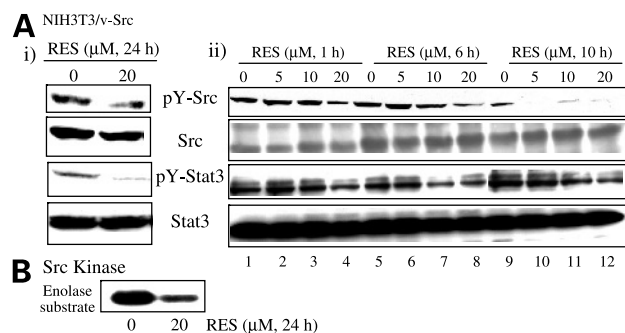
### Apoptosis Detection by Flow Cytometry

Human breast, pancreatic, and prostate cancer cells, were treated with 0 to 50  $\mu\text{mol/L}$  of resveratrol or with DMSO (control) for 72 hours. Cell suspensions were fixed with 1% paraformaldehyde and 70% ethanol, washed with the APO-DIRECT Wash Buffer (BD Biosciences Pharmingen), and incubated with terminal nucleotidyl transferase enzyme and FITC-deoxyuridine 5-triphosphate, and analyzed by flow cytometry. Normal mouse fibroblasts (NIH3T3) and v-Src-transformed counterparts (NIH3T3/v-Src) were treated with or without the caspase inhibitor, zVAD (50  $\mu\text{mol/L}$ ; MP Biomedicals, LLC, Solon, OH), for 2 hours prior to being treated with 20  $\mu\text{mol/L}$  resveratrol for 36 hours. Cells were then harvested, stained with Annexin V and 7-amino-actinomycin D (BD Biosciences Pharmingen) according to the manufacturer's protocol, and analyzed by flow cytometry to quantify the percentage of apoptosis.

## Results

### Resveratrol Inhibits Src Activity and Stat3 Activation in Malignant Cells

Previous reports suggested that resveratrol interferes with Src tyrosine kinase activity (9, 13). We investigated the effects of resveratrol on Src activation by subjecting v-Src-transformed mouse fibroblasts (NIH3T3/v-Src) to resveratrol treatment (5–20  $\mu\text{mol/L}$  resveratrol for 1–24 hours) and preparing cell lysates for SDS-PAGE and immunoblotting with specific antibodies against phosphorylated Src (pY-Src) and Src protein. Compared with levels in control (DMSO-treated) cells, treatment with resveratrol diminished pY-Src level in a time- and dose-dependent manner, whereas total Src protein levels were not affected in treated cells (*top*, Fig. 1A). Reduction of pY-Src levels was evident by 1 hour for 20  $\mu\text{mol/L}$  resveratrol [*lane 4*, Fig. 1(ii)] and persisted for up to 24 hours, suggesting the direct inhibition of Src by resveratrol. In *in vitro* kinase assay with exogenous enolase substrate and Src protein immunoprecipitated from lysates of resveratrol-treated cells, we confirm that resveratrol inhibited intracellular Src activity (Fig. 1B). These results show that resveratrol directly inhibits Src tyrosine kinase activity.

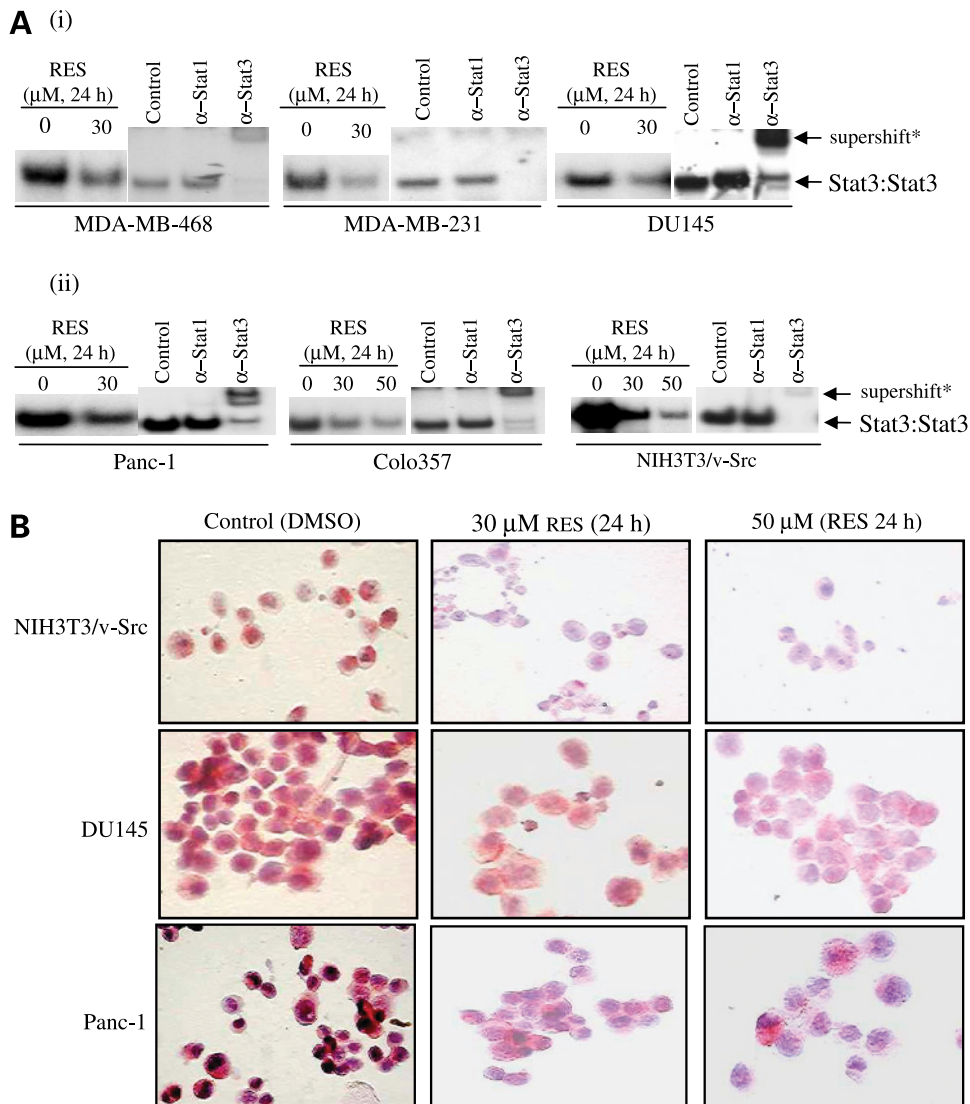


**Figure 1.** Resveratrol inhibits Src activity and Stat3 phosphorylation in v-Src-transformed mouse fibroblasts. NIH3T3/v-Src fibroblasts were treated with or without different concentrations of resveratrol for 1 to 24 h from which lysates were prepared. **A**, Western blot analyses of cell lysates that probed for pY-Src, total Src, pY-Stat3, and total Stat3; **B**, Src immunoprecipitates from NIH3T3/v-Src cells were incubated with acid-denatured enolase in an *in vitro* kinase assay as described in Materials and Methods. Data are representative of two to three independent assays.

We have previously shown that Stat3 is constitutively-activated in v-Src-transformed mouse fibroblasts (NIH3T3/v-Src) and breast carcinoma cell lines in part due to Src kinase activity (23, 25, 26). To investigate if inhibition of Src activity by resveratrol modulates Stat3 phosphorylation, malignant cells were treated with resveratrol and were examined for Stat3 activation. In v-Src-transformed mouse fibroblasts (NIH3T3/v-Src), treatment with resveratrol results in time- and dose-dependent decreases in pY-Stat3 but not total Stat3 levels, as measured by Western blot analysis (*bottom*, Fig. 1A). The decreases in preexisting pTyr-Stat3 levels paralleled reduction in pY-Src levels and occurred as early as 1 hour after resveratrol treatment and persisted for >24 hours (Fig. 1A; data not shown). While this article was being reviewed, a study was published which showed that resveratrol inhibits Stat3 phosphorylation in endothelial cells in response to interleukin-6 (30), which is partly consistent with our findings. Reduction in pTyr Stat3 by resveratrol is further confirmed by immunohistochemical staining in NIH3T3/v-Src and in human prostate and pancreatic cancer cell lines that harbor constitutively-active Stat3 (Fig. 2B). Furthermore, electrophoretic mobility shift assay analysis of nuclear extracts prepared from cells shows a decrease in Stat3 DNA-binding activity in NIH3T3/v-Src, and in human breast, prostate, and pancreatic tumor cell lines harboring constitutively-active Stat3 treated with resveratrol (Fig. 2A). In supershift analysis, Stat3 DNA-binding activity was blocked and supershifted by anti-Stat3 antibody but not by anti-Stat1 antibody (Fig. 2A), confirming that the protein/DNA complex contained Stat3. These results show that resveratrol inhibits Src tyrosine kinase activity and downstream Stat3 signaling in malignant cells.

### Effect of Resveratrol on Proliferation and Viability of Malignant Cells

Given that Src and Stat3 are involved in the growth and viability of malignant cells (25, 26, 31, 32) and previous



**Figure 2.** Resveratrol inhibits Stat3 phosphorylation and DNA-binding activity in malignant cells. NIH3T3/*v*-Src fibroblasts, human breast cancer (MDA-MB-468 and MDA-MB-231), prostate cancer (DU145), or human pancreatic cancer (Panc-1 and Colo-357) cell lines were treated with or without different concentrations of resveratrol for 24 h. Nuclear extracts were prepared from cells or cells were subjected to *in situ* immunohistochemistry. **A**, electrophoretic mobility shift assay analysis of Stat3 DNA-binding activity in nuclear extracts; **B**, immunohistochemical detection of p-Stat3 in NIH3T3/*v*-Src fibroblasts, human prostate cancer (DU145), and human pancreatic cancer (Panc-1) cells. *Intense red staining*, presence of active pY-Stat3; *blue staining*, absence of pY-Stat3; (-) control, DMSO-treated; (+) resveratrol-treated. \*Supershifts (last two lanes) were done with antibodies specifically recognizing either Stat1 ( $\alpha$ -Stat1) or Stat3 ( $\alpha$ -Stat3). \*, position of supershifted complexes. Data are representative of two to three independent assays.

reports that resveratrol inhibits the growth of human tumor cells (2–7), we examined the effects of resveratrol on *v*-Src-transformed fibroblasts (NIH3T3/*v*-Src) as well as human breast cancer MDA-MB-231, MDA-MB-468, prostate cancer DU145, and pancreatic cancer Colo-357 and Panc-1 cell lines that harbor constitutively-active Stat3. Proliferation and viability were measured by trypan blue exclusion with phase-contrast microscopy (Fig. 3A–D) and MTT assay (Fig. 3E–G), respectively. In malignant cells harboring activated Stat3 (NIH3T3/*v*-Src, MDA-MB-231, MDA-MB-468, DU145, Panc-1, and Colo-357), resveratrol induced dose-dependent growth inhibition and loss of viability (Fig. 3B, C, E–G) with extensive fragmentation of cells (data not shown). By contrast, only decreased growth rate with no significant loss of viability is observed with normal mouse fibroblast (NIH3T3, Fig. 3A and C; ref. 26), human breast carcinoma (MDA-MB-453, Fig. 3E; ref. 25) prostate cancer (LNCaP, Fig. 3F; ref. 33) cell lines that do not harbor constitutively-

active Stat3 at 5–50  $\mu$ mol/L of resveratrol treatment. Removal of resveratrol restores the growth rate to normal, as observed for normal NIH3T3 (fifth and eight columns, Fig. 3D; data not shown), suggesting that the decrease in growth rate was reversible. The IC<sub>50</sub> values for loss of viability reveal malignant cells harboring aberrant Stat3 signaling which are two to three times more sensitive to resveratrol than cells that do not (Table 1). Together, these data indicate that resveratrol inhibits Src kinase activity and Stat3 activation, and results in loss of viability of malignant cells that harbor aberrant Stat3 signaling.

#### Effects of Resveratrol on Cell Cycle Phase Distribution

Both Src and Stat3 proteins have key roles in cell cycle progression, whereas inhibition of constitutively-active Stat3 induces cell cycle arrest (25, 31). Flow cytometric analyses were done to further probe the malignant cell growth inhibition induced by resveratrol. In *v*-Src-transformed mouse fibroblasts, treatment with resveratrol

induces an irreversible cell cycle block and accumulation of cells at the G<sub>0</sub>-G<sub>1</sub> phase by 24 hours, compared with nontreated cells (Fig. 4B). This is in contrast with a reversible block at the G<sub>0</sub>-G<sub>1</sub> phase of normal NIH3T3 fibroblasts treated with resveratrol (Fig. 4A), which is reversed after the removal of resveratrol (*bottom*, Fig. 4A). By comparison, the irreversible block at G<sub>0</sub>-G<sub>1</sub> in the resveratrol-treated v-Src-transformed fibroblasts precedes an increased sub-G<sub>1</sub> peak and decreased G<sub>1</sub>, S, and G<sub>2</sub>-M phases at 48 to 72 hours, suggesting DNA fragmentation and apoptosis (Fig. 4B). In human tumor cell lines that harbor constitutively-active Stat3, resveratrol treatment induces cell cycle arrest at the G<sub>0</sub>-G<sub>1</sub> phase with concomitant decrease of cells at the S or G<sub>2</sub>-M phase of human breast cancer (MDA-MB-231), pancreatic cancer (Panc-1), and prostate cancer (DU145) cell lines or an arrest at the S phase with a concomitant decrease of cells in the G<sub>2</sub>-M phase of human breast cancer (MDA-MB-468) and pancreatic cancer (Colo-357) cell lines (Table 2). Although all these cell lines have activated Stat3, we observed differential patterns of change in cell cycle phase distribution in response to resveratrol. At the concentrations used, resveratrol treatment, however, does not significantly affect the cell cycle profile of LNCaP cells which do not harbor Stat3 activity (Table 2), whereas the breast carcinoma MDA-MB-453 cell line is blocked

**Table 1. Inhibition of viability of human tumor cell lines measured by MTT assay following treatment with resveratrol for 72 hours**

Cell line	Stat3 activation status	IC <sub>50</sub> (μmol/L)*
MDA-MB-231	+	40 ± 5
MDA-MB-468	+	60 ± 6
MDA-MB-453	-	90 ± 4
Colo-357	+	45 ± 3
Panc-1	+	70 ± 10
LNCaP	-	100 ± 4
DU145	+	25 ± 11

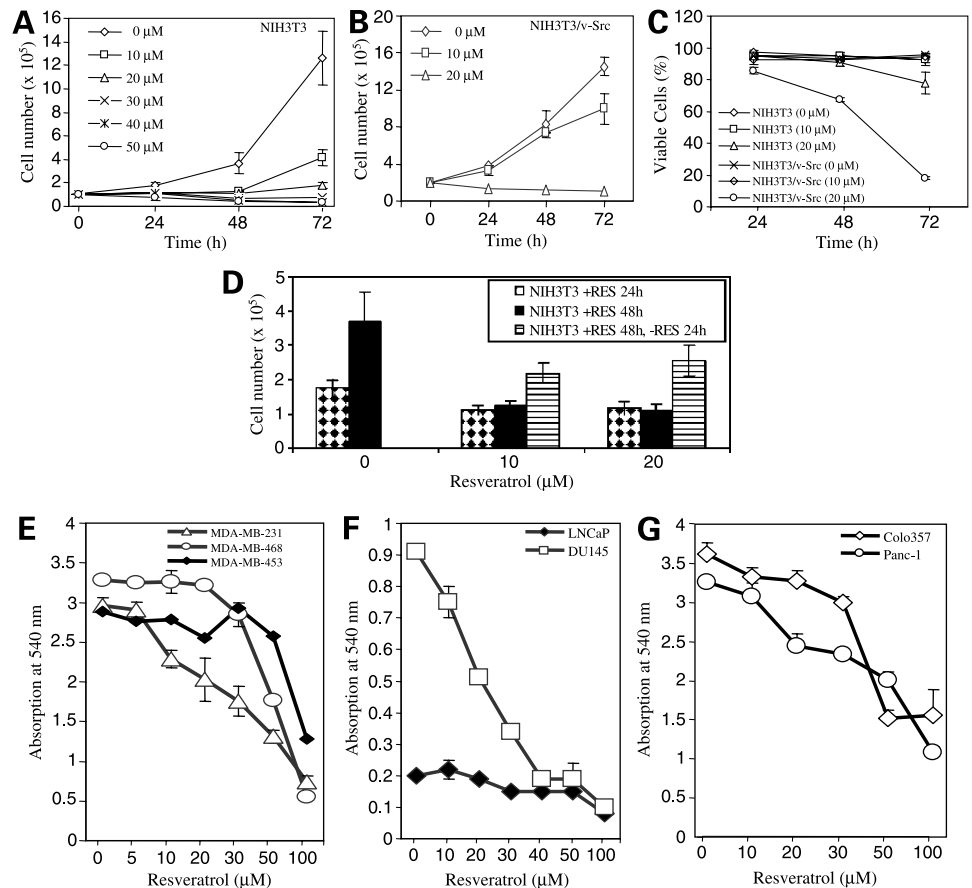
NOTE: (+), cells with constitutively-active Stat3; (-), cells without constitutively-active Stat3. Values are the mean and SD of three independent assays.

\*IC<sub>50</sub>, concentration of resveratrol at which cell viability is inhibited by 50%.

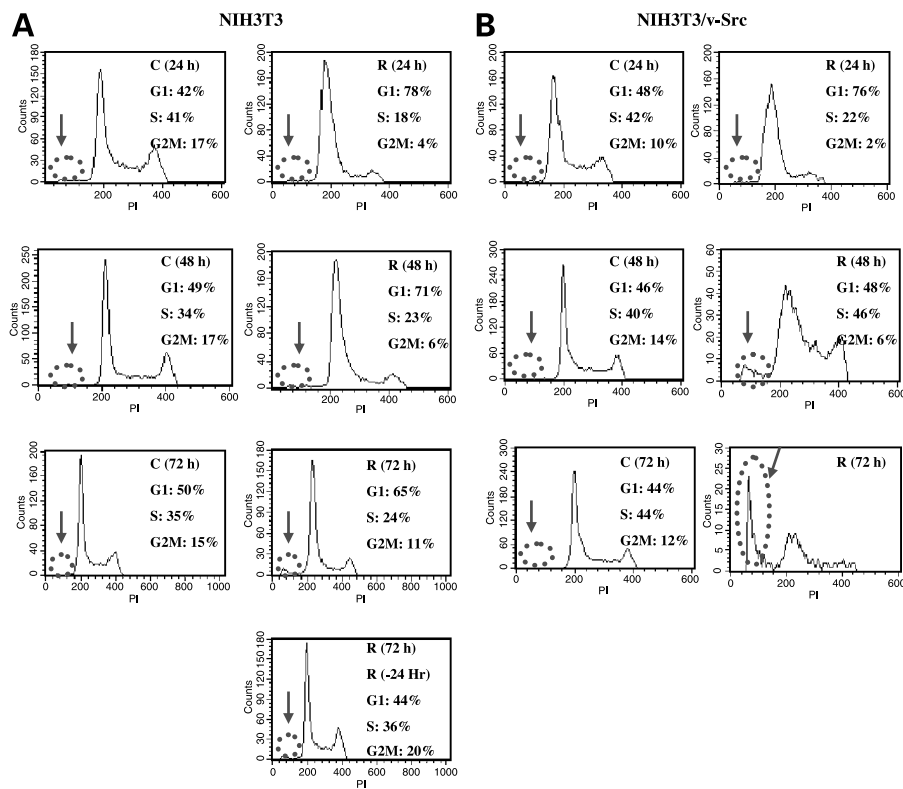
at the S phase (Table 2). These findings suggest that resveratrol-induced inhibition of Src-Stat3 signaling leads to irreversible growth arrest of malignant cells, consistent with the role of aberrant Stat3 signaling in cell cycle and growth control.

### Resveratrol Induces Apoptosis of Human Cancer Cells

With the known importance of Src and Stat3 in survival, we further examined the effects of resveratrol on the



**Figure 3.** Evaluation of the effects of resveratrol on the proliferation and viability of malignant cells. Normal and v-Src-transformed mouse fibroblasts (NIH3T3 or NIH3T3/v-Src), as well as human breast carcinoma (MDA-MB-231, MDA-MB-468 or MDA-MB-453), prostate cancer (LNCaP or DU145), and pancreatic cancer (Colo357 or Panc-1) cell lines were treated with or without different concentrations of resveratrol. **A–C**, fibroblasts were counted by trypan blue exclusion and phase-contrast microscopy on each day of 3 d of treatment; **D**, 48 h after treatment, resveratrol-containing medium was removed and replaced with resveratrol-free medium, and cells were cultured for another 24 h and counted as in **A**; **E–G**, following 72 h of treatment, human tumor cells were subjected to MTT assay. *Points*, means of three independent assays; *bars*, SD.



**Figure 4.** Analysis of cell cycle distribution by flow cytometry. **A**, normal (NIH3T3); and **B**, v-Src-transformed mouse fibroblasts (NIH3T3/v Src) were treated with or without 20  $\mu\text{mol/L}$  resveratrol for 24 to 72 h and processed by propidium iodide (PI) staining for flow cytometric analysis of DNA content. For assessment of reversibility of the effects of resveratrol on NIH3T3, resveratrol-containing medium was removed from cells 72 h after treatment, cells were then cultured in fresh resveratrol-free medium for 24 h and processed for flow cytometry (*bottom, A*). Arrows, region in which fragmented DNA is predicted to be located. Results are representative of three independent assays.

survival of malignant cells. Analyses by terminal nucleotidyl transferase-mediated nick end labeling show that treatment with resveratrol induces apoptosis in the breast (MDA-MB-231 and MDA-MB-468), pancreatic (Colo-357

and Panc-1), and prostate (DU145) cancer cell lines that harbor constitutive Stat3 activity, in contrast with the minimal apoptosis observed in cell lines (MDA-MB-453 and LNCaP) that lack aberrant Stat3 signaling (Fig. 5A-C).

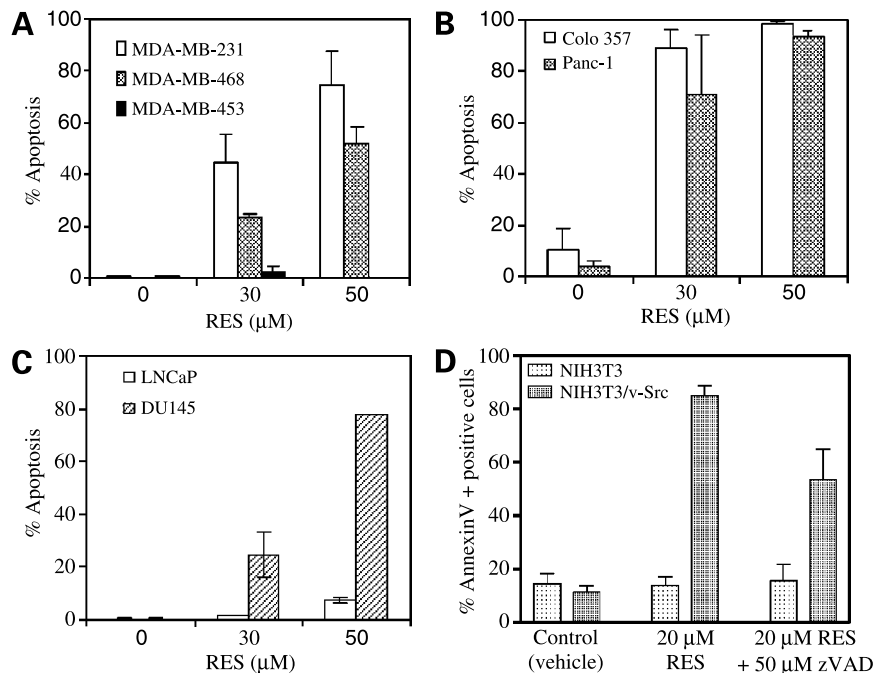
**Table 2.** Effects of resveratrol on cell cycle phase distribution for cell lines treated with 30 or 50  $\mu\text{mol/L}$  RES for 24 hours

Cell line	Resveratrol ( $\mu\text{mol/L}$ )	G <sub>0</sub> -G <sub>1</sub>	S	G <sub>2</sub> -M
<b>A. Human breast cancer cell lines</b>				
MDA-MB-231	0	39.71 $\pm$ 0.69	47.43 $\pm$ 1.56	12.86 $\pm$ 2.52
	50	70.64 $\pm$ 4.82*	29.36 $\pm$ 4.82	0
MDA-MB-468	0	27.76 $\pm$ 1.58	28.57 $\pm$ 1.53	43.67 $\pm$ 0.06
	50	9.27 $\pm$ 0.06	80.06 $\pm$ 2.74*	10.68 $\pm$ 2.67
MDA-MB-453	0	52.29 $\pm$ 2.42	30.58 $\pm$ 3.66	17.14 $\pm$ 4.28
	30	47.88 $\pm$ 7.54	52.12 $\pm$ 7.54	0
<b>B. Pancreatic cancer cell lines</b>				
Colo-357	0	43.77 $\pm$ 6.8	38.97 $\pm$ 4.77	17.26 $\pm$ 2.21
	50	46.61 $\pm$ 5.81	49.43 $\pm$ 7.20	3.96 $\pm$ 1.97
Panc-1	0	39.34 $\pm$ 6.61	45.9 $\pm$ 9.51	14.77 $\pm$ 2.91
	50	51.18 $\pm$ 1.41*	43.59 $\pm$ 3.20	5.23 $\pm$ 1.77
<b>C. Prostate cancer cell lines</b>				
LNCaP	0	64.53 $\pm$ 3.14	18.69 $\pm$ 0.91	16.79 $\pm$ 4.05
	50	65.01 $\pm$ 4.67	21.64 $\pm$ 3.59	13.35 $\pm$ 1.07
DU145	0	49.00 $\pm$ 3.56	29.10 $\pm$ 2.68	21.91 $\pm$ 0.87
	50	69.55 $\pm$ 0.31*	19.93 $\pm$ 0.21	10.52 $\pm$ 0.11

NOTE: Cell cycle phase distribution was analyzed by flow cytometry after propidium iodide staining. Values are the mean and SD of four to six independent assays. Statistical difference at changes in G<sub>0</sub>-G<sub>1</sub> phase for MDA-MB-231, Panc-1, and DU145 and in the S phase for MDA-MB-468.

\* $P < 0.05$ .

**Figure 5.** Analyses of resveratrol-induced apoptosis by terminal nucleotidyl transferase-mediated nick end labeling and Annexin V staining. **A**, terminal nucleotidyl transferase-mediated nick end labeling staining for DNA damage in human breast carcinomas (MDA-MB-231, MDA-MB-468, or MDA-MB-453); **B**, human pancreatic cancer (Colo 357 or Panc-1); **C**, and human prostate cancer (LNCaP or DU145) cell lines; and **D**, Annexin V binding in NIH3T3 and NIH3T3/v-Src fibroblasts treated with or without resveratrol for 36 to 72 h in the presence or absence of zVAD (50  $\mu\text{mol/L}$ , 2 h) and analyzed by flow cytometry. *Columns*, means of three independent assays; *bars*, SD.



Furthermore, Annexin V staining with flow cytometry analysis show that resveratrol selectively induces apoptosis in v-Src-transformed mouse fibroblasts (NIH3T3v-Src) which harbor constitutively-active Stat3 but not in normal mouse fibroblasts (NIH3T3, Fig. 5D). The apoptosis of NIH3T3v-Src fibroblasts is partially blocked by a general caspase inhibitor, zVAD (Fig. 5D), suggesting that resveratrol-induced apoptosis may in part be mediated through the caspase pathway, as previously reported (34).

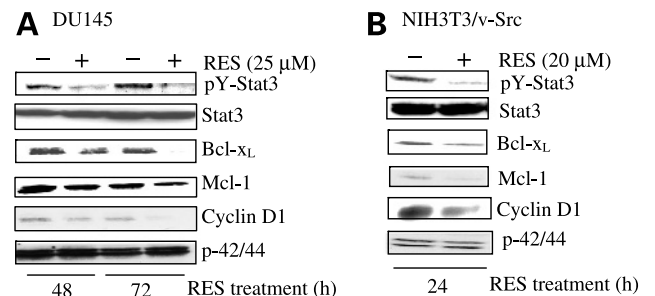
#### Resveratrol Blocks Cyclin D1, Bcl-x<sub>L</sub>, and Mcl-1 Expression in Malignant Cells

To further understand the mechanism by which resveratrol-mediated inhibition of constitutive Stat3 activation leads to cell growth inhibition and apoptosis, we examined the cell cycle regulator, cyclin D1, and the antiapoptotic proteins Bcl-x<sub>L</sub> and Mcl-1, all of which are target genes of Stat3. In the human prostate cancer DU145 cell line and v-Src-transformed mouse fibroblasts (NIH3T3/v-Src) treated with resveratrol, Western blot analysis shows significant reductions in the cyclin D1 as well as Bcl-x<sub>L</sub> and Mcl-1 expression levels (Fig. 6), which correlate with the inhibition of Stat3 phosphorylation and activity by resveratrol (Figs. 1A and 2A and Fig. 6). The activation of mitogen-activated protein kinases, p42/44, is not altered by resveratrol treatment (Fig. 6). These data suggest that resveratrol induces cell growth inhibition and apoptosis of tumor cells at least in part by inhibiting Stat3-dependent induction of cyclin D1, Bcl-x<sub>L</sub>, and Mcl-1.

#### Discussion

Current studies provide new insights into the biological and molecular mechanisms for the antitumor effects of

resveratrol and related stilbene compounds (35). We show that resveratrol inhibits the Src kinase activity, as previously indicated (9, 12) and represses downstream aberrant Stat3 signaling in malignant cells. Inhibition of Src and Stat3 by resveratrol is rapid, occurring in the early hours after treatment. Accordingly, resveratrol induces cell cycle arrest and apoptosis of malignant cells harboring constitutively-active Stat3. Similar biological effects of resveratrol have been reported in the androgen-independent prostate cancer (DU145 or PC-3; refs. 5, 10, 35, 36) and glioma (U251; ref. 37) cell lines, which harbor constitutively-active Stat3 (33, 38). By contrast, minimal apoptosis is observed in cell lines that do not harbor constitutively-active Stat3 at



**Figure 6.** Decrease of levels of phosphotyrosine Stat3, cyclin D1, Bcl-x<sub>L</sub>, and Mcl-1 expression by resveratrol. **A**, human prostate cancer cells (DU145); and **B**, v-Src-transformed mouse fibroblasts (NIH3T3/v-Src) were treated with or without 20 to 25  $\mu\text{mol/L}$  resveratrol for 24 to 72 h. Cell lysates were prepared and subjected to Western blot analysis for pY-Stat3, Stat3, Bcl-x<sub>L</sub>, Mcl-1, and cyclin D1; (-) control, DMSO-treated; (+) resveratrol-treated. Results are representative of two independent assays.

concentrations of resveratrol that otherwise inhibit Stat3 activation. Altogether, our findings suggest that the anticancer properties of resveratrol are in part due to blockade of Src and constitutive Stat3 activities, and are among the first to show cell cycle arrest, growth inhibition, and apoptosis of human cancer cell lines by resveratrol via abrogation of Src-Stat3 signaling.

The potential for resveratrol to modulate gene expression has been previously reported (6, 39). It is conceivable that changes in gene regulation by this compound contribute to its biological effects. Our studies show that resveratrol represses cyclin D1, Bcl-x<sub>L</sub>, and Mcl-1, whereas others have similarly reported that resveratrol inhibits survivin induction (40), all of which are known Stat3-regulated genes (41–44), suggesting that modulation of Stat3-regulated cell cycle control and survival genes constitutes part of its mechanisms of antitumor cell activity. Moreover, given the role of Stat3 in repressing p53 expression and function (45), and in NO production in the tumor microenvironment (46), inhibition of constitutive Stat3 activity by resveratrol could induce antitumor cell effects in part by enhancing p53 function and NO activity, as shown by a previous report (47).

In the LNCaP cell line, which lacks constitutively-active Stat3, reports show that low resveratrol concentrations promoted DNA synthesis and progression into S phase (48), whereas apoptosis occurred at high concentrations (100–1,000 μmol/L; ref. 36) or in parallel with modulation of other factors, such as prostate-specific antigen (5, 6, 49). Previous studies also indicate that resveratrol-mediated apoptosis of breast cancer and endometrial adenocarcinoma cells may be dependent or independent of the estrogen receptor status (2, 50–52). These and our findings together raise the potential for resveratrol to induce differential mechanisms for mediating antitumor cell activity depending on the concentration and the molecular background of the tumor cell (12, 53–56). Consistent with this, previous studies showed that resveratrol inhibits the nuclear factor κB pathway and induces apoptosis of leukemia cell lines via caspases (34). We note in our study that resveratrol-induced apoptosis in v-Src-transformed fibroblasts is partly sensitive to the inhibition of caspases.

Repression of Src-Stat3 by resveratrol parallels similar effects on vascular endothelial growth factor (9), mitogen-activated protein kinase (13, 57, 58), and protein kinase C (10), and together provide evidence that modulation of signal transduction pathways also contribute to resveratrol-induced biological effects (12). Although additional work remains to fully understand resveratrol and its antitumorigenic effects, our studies provide novel potential applications for resveratrol as a small-molecule inhibitor of Stat3 signaling with antitumor cell activity (21, 59–65).

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#### References

- Kopp P. Resveratrol, a phytoestrogen found in red wine. A possible explanation for the conundrum of the 'French paradox'? *Eur J Endocrinol* 1998;138:619–20.
- Mgbonyebi OP, Russo J, Russo IH. Antiproliferative effect of synthetic resveratrol on human breast epithelial cells. *Int J Oncol* 1998;12:865–9.
- ElAttar TM, Virji AS. Modulating effect of resveratrol and quercetin on oral cancer cell growth and proliferation. *Anticancer Drugs* 1999;10:187–93.
- Elattar TM, Virji AS. The effect of red wine and its components on growth and proliferation of human oral squamous carcinoma cells. *Anticancer Res* 1999;19:5407–14.
- Hsieh TC, Wu JM. Differential effects on growth, cell cycle arrest, and induction of apoptosis by resveratrol in human prostate cancer cell lines. *Exp Cell Res* 1999;249:109–15.
- Jones SB, DePrimo SE, Whitfield ML, Brooks JD. Resveratrol-induced gene expression profiles in human prostate cancer cells. *Cancer Epidemiol Biomarkers Prev* 2005;14:596–604.
- Clement MV, Hirpara JL, Chawdhury SH, Pervaiz S. Chemopreventive agent resveratrol, a natural product derived from grapes, triggers CD95 signaling-dependent apoptosis in human tumor cells. *Blood* 1998;92:996–1002.
- Brakenhielm E, Cao R, Cao Y. Suppression of angiogenesis, tumor growth, and wound healing by resveratrol, a natural compound in red wine and grapes. *FASEB J* 2001;15:1798–800.
- Lin MT, Yen ML, Lin CY, Kuo ML. Inhibition of vascular endothelial growth factor-induced angiogenesis by resveratrol through interruption of Src-dependent vascular endothelial cadherin tyrosine phosphorylation. *Mol Pharmacol* 2003;64:1029–36.
- Shih A, Zhang S, Cao HJ, et al. Inhibitory effect of epidermal growth factor on resveratrol-induced apoptosis in prostate cancer cells is mediated by protein kinase C-α. *Mol Cancer Ther* 2004;3:1355–64.
- Stewart JR, Ward NE, Ioannides CG, O'Brian CA. Resveratrol preferentially inhibits protein kinase C-catalyzed phosphorylation of a cofactor-independent, arginine-rich protein substrate by a novel mechanism. *Biochemistry* 1999;38:13244–51.
- Kong AN, Yu R, Hebbar V, et al. Signal transduction events elicited by cancer prevention compounds. *Mutat Res* 2001;480–1:231–41.
- Yu R, Hebbar V, Kim DW, Mandlekar S, Pezzuto JM, Kong AN. Resveratrol inhibits phorbol ester and UV-induced activator protein 1 activation by interfering with mitogen-activated protein kinase pathways. *Mol Pharmacol* 2001;60:217–24.
- Fontecave M, Lepoivre M, Elleingand E, Gerez C, Guittet O. Resveratrol, a remarkable inhibitor of ribonucleotide reductase. *FEBS Lett* 1998;421:277–9.
- Subbaramaiah K, Chung WJ, Michaluart P, et al. Resveratrol inhibits cyclooxygenase2 transcription and activity in phorbol ester-treated human mammary epithelial cells. *J Biol Chem* 1998;273:21875–82.
- Bromberg J, Darnell JE, Jr. The role of STATs in transcriptional control and their impact on cellular function. *Oncogene* 2000;19:2468–73.
- Darnell JE, Jr. Transcription factors as targets for cancer therapy. *Nat Rev Cancer* 2002;2:740–9.
- Yu H, Jove R. The STATs of cancer—new molecular targets come of age. *Nat Rev Cancer* 2004;4:97–105.
- Bowman T, Garcia R, Turkson J, Jove R. STATs in oncogenesis. *Oncogene* 2000;19:2474–88.
- Bromberg J. Signal transducers and activators of transcription as regulators of growth, apoptosis and breast development. *Breast Cancer Res* 2000;2:86–90.
- Turkson J. STAT proteins as novel targets for cancer drug discovery. *Expert Opin Ther Targets* 2004;8:409–22.
- Buettner R, Mora LB, Jove R. Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. *Clin Cancer Res* 2002;8:945–54.
- Yu CL, Meyer DJ, Campbell GS, et al. Enhanced DNA-binding activity of a Stat3-related protein in cells transformed by the Src oncoprotein. *Science* 1995;269:81–3.
- Zhang Y, Turkson J, Carter-Su C, et al. Activation of Stat3 in v-Src transformed fibroblasts requires cooperation of Jak1 kinase activity. *J Biol Chem* 2000;275:24935–44.



25. Garcia R, Bowman TL, Niu G, et al. Constitutive activation of Stat3 by the Src and JAK tyrosine kinases participates in growth regulation of human breast carcinoma cells. *Oncogene* 2001;20:2499–513.
26. Turkson J, Bowman T, Garcia R, Caldenhoven E, De Groot RP, Jove R. Stat3 activation by Src induces specific gene regulation and is required for cell transformation. *Mol Cell Biol* 1998;18:2545–52.
27. Turkson J, Bowman T, Adnane J, et al. Requirement for Ras/Rac1-mediated p38 and *c-Jun* N-terminal kinase signaling in Stat3 transcriptional activity induced by the Src oncoprotein. *Mol Cell Biol* 1999;19:7519–28.
28. Garcia R, Yu CL, Hudnall A, et al. Constitutive activation of Stat3 in fibroblasts transformed by diverse oncoproteins and in breast carcinoma cells. *Cell Growth Differ* 1997;8:1267–76.
29. Wagner BJ, Hayes TE, Hoban CJ, Cochran BH. The SIF binding element confers sis/PDGF inducibility onto the *c-fos* promoter. *EMBO J* 1990;9:4477–84.
30. Wung BS, Hsu MC, Wu CC, Hsieh CW. Resveratrol suppresses IL-6-induced ICAM-1 gene expression in endothelial cells: Effects on the inhibition of STAT3 phosphorylation. *Life Sci* 2005;78:389–97.
31. Niu G, Bowman T, Huang M, et al. Roles of activated Src and Stat3 signaling in melanoma tumor cell growth. *Oncogene* 2002;21:7001–10.
32. Bowman T, Broome M, Sinibaldi N, et al. Stat3-mediated Myc expression is required for Src oncogenesis and PDGF-induced mitogenesis. *Proc Natl Acad Sci U S A* 2000;98:7319–24.
33. Mora LB, Buettner R, Seigne J, et al. Constitutive activation of Stat3 in human prostate tumors and cell lines: direct inhibition of Stat3 signaling induces apoptosis of prostate cancer cells. *Cancer Res* 2002;62:6659–66.
34. Estrov Z, Shishodia S, Faderl S, et al. Resveratrol blocks interleukin-1 $\beta$ -induced activation of the nuclear transcription factor NF- $\kappa$ B, inhibits proliferation, causes S-phase arrest, and induces apoptosis of acute myeloid leukemia cells. *Blood* 2003;102:987–95.
35. Robertson CN, Roberson KM, Padilla GM, et al. Induction of apoptosis by diethylstilbestrol in hormone-insensitive prostate cancer cells. *J Natl Cancer Inst* 1996;88:908–17.
36. Morris GZ, Williams RL, Elliott MS, Beebe SJ. Resveratrol induces apoptosis in LNCaP cells and requires hydroxyl groups to decrease viability in LNCaP and DU 145 cells. *Prostate* 2002;52:319–29.
37. Jiang H, Zhang L, Kuo J, et al. Resveratrol-induced apoptotic death in human U251 glioma cells. *Mol Cancer Ther* 2005;4:554–61.
38. Rahaman SO, Harbor PC, Chernova O, Barnett GH, Vogelbaum MA, Haque SJ. Inhibition of constitutively active Stat3 suppresses proliferation and induces apoptosis in glioblastoma multiforme cells. *Oncogene* 2002;21:8404–13.
39. Narayanan BA, Narayanan NK, Re GG, Nixon DW. Differential expression of genes induced by resveratrol in LNCaP cells: p53-mediated molecular targets. *Int J Cancer* 2003;104:204–12.
40. Aziz MH, Afaq F, Ahmad N. Prevention of ultraviolet-B radiation damage by resveratrol in mouse skin is mediated via modulation in survivin. *Photochem Photobiol* 2005;81:25–31.
41. Epling-Burnette PK, Liu JH, Catlett-Falcone R, et al. Inhibition of STAT3 signaling leads to apoptosis of leukemic large granular lymphocytes and decreased Mcl-1 expression. *J Clin Invest* 2001;107:351–62.
42. Sinibaldi N, Wharton W, Turkson J, Bowman T, Pledger WJ, Jove R. Induction of p21WAF1/CIP1 and cyclin D1 expression by the Src oncoprotein in mouse fibroblasts: role of activated STAT3 signaling. *Oncogene* 2000;19:5419–27.
43. Catlett-Falcone R, Landowski TH, Oshiro MM, et al. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. *Immunity* 1999;10:105–15.
44. Aoki Y, Feldman GM, Tosato G. Inhibition of STAT3 signaling induces apoptosis and decreases survivin expression in primary effusion lymphoma. *Blood* 2003;101:1535–42.
45. Niu G, Wright KL, Ma Y, et al. Role of Stat3 in regulating p53 expression and function. *Mol Cell Biol* 2005;25:7432–40.
46. Burdelya L, Kujawski M, Niu G, et al. Stat3 activity in melanoma cells affects migration of immune effector cells and nitric oxide-mediated antitumor effects. *J Immunol* 2005;174:3925–31.
47. Hsieh TC, Juan G, Darzynkiewicz Z, Wu JM. Resveratrol increases nitric oxide synthase, induces accumulation of p53 and p21(WAF1/CIP1), and suppresses cultured bovine pulmonary artery endothelial cell proliferation by perturbing progression through S and G2. *Cancer Res* 1999;59:2596–601.
48. Kuwajerwala N, Cifuentes E, Gautam S, Menon M, Barrack ER, Reddy GP. Resveratrol induces prostate cancer cell entry into S phase and inhibits DNA synthesis. *Cancer Res* 2002;62:2488–92.
49. Hsieh TC, Wu JM. Grape-derived chemopreventive agent resveratrol decreases prostate-specific antigen (PSA) expression in LNCaP cells by an androgen receptor (AR)-independent mechanism. *Anticancer Res* 2000;20:225–8.
50. Lu R, Serrero G. Resveratrol, a natural product derived from grape, exhibits antiestrogenic activity and inhibits the growth of human breast cancer cells. *J Cell Physiol* 1999;179:297–304.
51. Bhat KP, Lantvit D, Christov K, Mehta RG, Moon RC, Pezzuto JM. Estrogenic and antiestrogenic properties of resveratrol in mammary tumor models. *Cancer Res* 2001;61:7456–63.
52. Bhat KP, Pezzuto JM. Resveratrol exhibits cytostatic and antiestrogenic properties with human endometrial adenocarcinoma (Ishikawa) cells. *Cancer Res* 2001;61:6137–44.
53. Aziz MH, Kumar R, Ahmad N. Cancer chemoprevention by resveratrol: *in vitro* and *in vivo* studies and the underlying mechanisms [review]. *Int J Oncol* 2003;23:17–28.
54. Jeong WS, Kim IW, Hu R, Kong AN. Modulation of AP-1 by natural chemopreventive compounds in human colon HT-29 cancer cell line. *Pharm Res* 2004;21:649–60.
55. Jeong WS, Kim IW, Hu R, Kong AN. Modulatory properties of various natural chemopreventive agents on the activation of NF- $\kappa$ B signaling pathway. *Pharm Res* 2004;21:661–70.
56. Pezzuto JM. Resveratrol: a whiff that induces a biologically specific tsunami. *Cancer Biol Ther* 2004;3:889–90.
57. Reagan-Shaw S, Afaq F, Aziz MH, Ahmad N. Modulations of critical cell cycle regulatory events during chemoprevention of ultraviolet B-mediated responses by resveratrol in SKH-1 hairless mouse skin. *Oncogene* 2004;23:5151–60.
58. Klinge CM, Blankenship KA, Risinger KE, et al. Resveratrol and estradiol rapidly activate MAPK signaling through estrogen receptors  $\alpha$  and  $\beta$  in endothelial cells. *J Biol Chem* 2005;280:7460–8.
59. Turkson J, Jove R. STAT proteins: novel molecular targets for cancer drug discovery. *Oncogene* 2000;19:6613–26.
60. Turkson J, Kim JS, Zhang S, et al. Novel peptidomimetic inhibitors of signal transducer and activator of transcription 3 dimerization and biological activity. *Mol Cancer Ther* 2004;3:261–9.
61. Turkson J, Ryan D, Kim JS, et al. Phosphotyrosyl peptides block Stat3-mediated DNA-binding activity, gene regulation and cell transformation. *J Biol Chem* 2001;276:45443–55.
62. Turkson J, Zhang S, Palmer J, et al. Inhibition of constitutive signal transducer and activator of transcription 3 activation by novel platinum complexes with potent anti-tumor activity. *Mol Cancer Ther* 2004;3:1533–42.
63. Turkson J, Zhang S, Mora LB, Burns A, Sebt S, Jove R. A novel platinum compound inhibits constitutive Stat3 signaling and induces cell cycle arrest and apoptosis of malignant cells. *J Biol Chem* 2005;280:32979–88.
64. Nam S, Buettner R, Turkson J, et al. Indirubin derivatives inhibit Stat3 signaling and induce apoptosis in human cancer cells. *Proc Natl Acad Sci U S A* 2005;102:5998–6003.
65. Darnell JE, Jr. Validating Stat3 in cancer therapy. *Nat Med* 2005;11:595–6.

# Molecular Cancer Therapeutics

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