

Original Article

Effect of Snake Venom on Cancer Growth through Induction of Apoptosis via Down Regulation of NF- κ B and STAT3 in the PA-1, Ovarian Cancer Cells

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국문초록

蛇毒이 난소암세포에 있어서 NF- κ B와 STAT3의 활성억제와 관련된 세포자멸사유도를 통한 암세포 성장에 미치는 영향

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목적 : 최근 NF- κ B와 STAT3의 활성억제와 관련된 항암제 연구가 주목받고 있으며, 본 연구는 사독(蛇毒)이 세포자멸사 관련 단백질의 발현 조절을 통하여 세포자멸사를 유도하고, NF- κ B와 STAT3의 활성억제를 유도하여 난소암 PA-1 세포의 성장을 억제하는지를 확인하고, 해당 기전을 살펴보고자 하였다.

방법 : 사독을 처리한 후 난소암 PA-1 세포의 세포자멸사의 관찰에는 DAPI, TUNEL staining assay를 시행하였고, 세포자멸사 조절단백질 및 NF- κ B, STAT3의 활성 변동 관찰에는 western blot analysis를 시행하였다.

결과 : 1. 사독을 처리한 후 난소암 PA-1 세포에서 세포자멸사가 유도되어 암세포성장이 억제되었다.

2. 사독을 처리한 후 세포자멸사 관련 단백질 중 세포자멸사 촉진 단백질인 cleaved caspase-3, Bax의 발현은 증가되었고, 세포자멸사 억제 단백질인 Bcl-2의 발현은 감소되었다.

3. 사독을 처리한 후 난소암 PA-1 세포의 NF- κ B와 STAT3 발현은 감소되었고, 각각의 길항제인 salicylic

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acid와 static 처리 후 NF- κ B와 STAT3 발현은 더욱 감소되었다.

결론 : 사독은 난소암 세포의 세포자멸사 유발과, NF- κ B와 STAT3의 활성억제를 통해 치료 효율이 높고, 내성이 적은 난소암 치료제의 개발에 도움이 될 것으로 기대된다.

핵심단어 : 사독(蛇毒), 난소암, 세포자멸사, NF- κ B, STAT3

I. Introduction

Ovarian cancer is a major gynecological malignancy and a leading cause of cancer death in women^{1,2)}. Most patients with ovarian cancer are diagnosed at advanced stages, because the disease often does not cause any symptoms until it metastasized³⁻⁵⁾. For the treatment of advanced ovarian cancer, multimodal therapy including surgery and chemotherapy is needed to eradicate the cancer cells^{6,7)}. However, tumor recurrence and metastasis due to the acquisition of chemoresistance is still a major challenge to overcome ovarian cancer⁸⁻¹²⁾. Therefore new treatment strategies that overcome intrinsic and acquired resistance are therefore needed and appropriate chemopreventive compounds reducing or overcoming resistance are also needed to reduce the incidence of ovarian cancer.

A Snake Venom Toxin (SVT) from *Vipera lebetina turanica*, is a group of basic peptides, and important factor V activator composed of 236 amino acids with six disulfide bonds formed by twelve cysteine¹³⁾. Raquel et al¹⁴⁾ elucidated that SVT induce apoptosis related to caspase cascade systems, and a few studies have demonstrated that SVT has apoptosis related anti-cancer effect upon cancers including neuroblastoma¹⁵⁾ and prostate cancer cells¹⁶⁾.

However, experiments demonstrating the molecular mechanisms of the anti-cancer effects of SVT in ovarian cancer cells have not been reported. Moreover, according to the previous reports^{17,18)}, acquired resistance to chemotherapeutics is closely

related to the lower apoptosis rate. Thus, in the present study, I investigated apoptosis related anti-cancer effects of SVT through inhibition of NF- κ B and STAT3 pathway in the human ovarian cancer cells, PA-1.

II. Materials and Methods

A. Materials

SVT from *Vipera lebetina turanica* was purchased from Sigma Chemical Co. (Saint Louis, USA). All of the secondary antibodies such as Bax, Bcl-2, caspase-3, cleaved caspase-3, p50, p65, I κ B, pI κ B and STAT3, used in Western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were purchased from Sigma unless otherwise stated.

B. Cell culture

PA-1 ovarian cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA). PA-1 ovarian cancer cells were cultured in MEM medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 U/ml). Cell cultures were then maintained at 37°C in a humidified atmosphere with 5% CO₂.

C. Cell viability assay

To determine the cell number, PA-1 ovarian cancer cells were plated in 24-well plates (5x10⁴

cells/well), and subconfluent cells were subsequently treated with SVT (2, 4 and 8 $\mu\text{g}/\text{ml}$) for 24 hr. After treatment, cells were trypsinized and pelleted by centrifugation for 5 min at 1,500 rpm, resuspended in 5 ml of phosphate-buffered saline (PBS), and 0.1 ml of 0.2% trypan blue was added to the cancer cell suspension in each of the solutions (0.9 ml each). Subsequently, a drop of suspension was placed into a Neubauer chamber and the living cancer cells were counted. Cells that showed signs of staining were considered to be dead, whereas those that excluded trypan blue were considered viable. Each assay was carried out in triplicate.

D. Apoptosis evaluation

Ovarian cancer cells (2.5×10^5 cells/well) were cultured on 8-chamber slides. After ovarian cancer cells were transfected with siRNA, the cells were treated with SVT (8 $\mu\text{g}/\text{ml}$). The cells were washed twice with PBS and fixed by incubation in 4% paraformaldehyde in PBS for 1 hr at room temperature. Membrane was permeabilized by exposure to 0.1% Triton X-100 in phosphate-buffered saline for 5 min at room temperature. TdT-mediated dUTP nick and labeling (TUNEL) assays were performed by using the in situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. For 4'-6-Diamidino-2-phenyl indole (DAPI) staining, slides were incubated for 15 min at room temperature in the dark with mounting medium for fluorescence containing DAPI (Vector Laboratories, Inc., Burlingame, CA). The cells were then observed through a fluorescence microscope (Leica Microsystems AG, Wetzlar, Germany).

E. Western blot analysis

Western blot analysis was done as described previously. The membrane was incubated for 2 hr at room temperature with specific antibodies: rabbit polyclonal for caspase-3, cleaved caspase-3, Bcl-2 (1:1,000 dilution, Cell Signaling Technology, Inc.,

Beverly, MA), p-STAT3, Bax, I κ B, pI κ B, p50, p65 (1:500 dilution, Santa Cruz Biotechnology, Inc.), and mouse monoclonal STAT3 (1:500 dilution, Santa Cruz Biotechnology, Inc.). The blot was then incubated with the corresponding conjugated anti-rabbit and anti-mouse immunoglobulin G-horseradish peroxidase (1:2,000 dilutions, Santa Cruz Biotechnology, Inc.). Immunoreactive proteins were detected with the ECL Western blotting detection system.

In Synergic Effect, ovarian cancer cells (3×10^4 cells/well) were plated in 24-well plates and transiently treated with salicylic acid, inhibitor of NF- κ B and statins, inhibitor of STAT3, respectively. The NF- κ B and STAT3 inhibitor-treated cells were retreated with 8 $\mu\text{g}/\text{ml}$ SVT for 24 hr.

F. Statistical Analysis

The data were analyzed using the GraphPad Prism 4 ver. 4.03 software (GraphPad Software, La Jolla, CA). Data are presented as mean \pm SD. The differences in all data were assessed by one-way analysis of variance (ANOVA). When the P value in the ANOVA test indicated statistical significance, the differences were assessed by the Dunnett's test. A value of $p < 0.05$ was considered to be statistically significant.

III. Results

A. Cell growth by SVT

To assess the inhibitory effect of SVT on cell growth of PA-1 ovarian cancer cells, we analyzed cell viability. The cells were treated with several concentrations of SVT (2, 4, and 8 $\mu\text{g}/\text{ml}$) for 24 hr. As shown in Figure 1, SVT inhibited cell proliferation of PA-1 ovarian cancer cells in a concentration-dependent manner. 24 hr treatment of SVT inhibited PA-1 ovarian cancer cell growth with IC₅₀ value of 4.5 $\mu\text{g}/\text{ml}$. Morphologic observa-

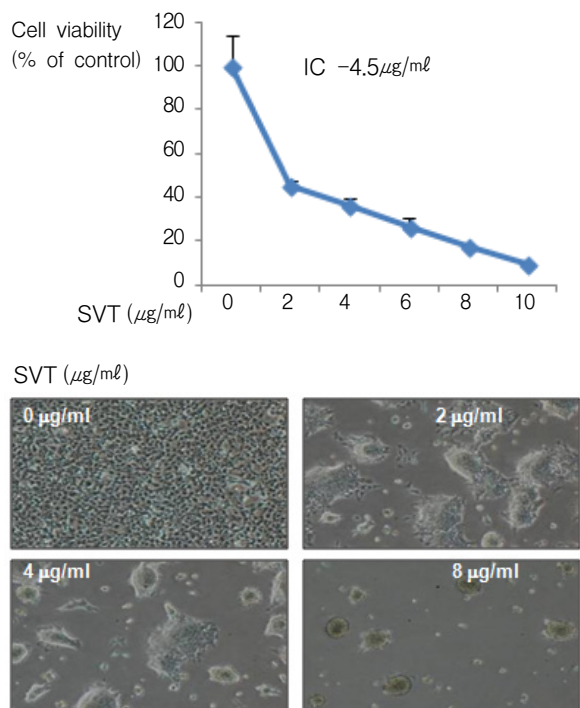


Fig. 1. Effect of SVT on cell viability in ovarian cancer cells

Concentration-dependent effect of SVT on the cell viability assay in PA-1. After treatment of SVT (2, 4 and 8 μg/ml) for 24 hr, the cells were harvested by trypsinization and stained with 0.2% trypan blue. Relative cell survival rate was determined by counting live and dead cells. The results were expressed as a percentage of viable cells. Morphologic observation with the treatment of SVT in PA-1 ovarian cancer cells. Columns, means of three experiments, with triplicates of each experiment; bars, SD. * : $p < 0.05$, significantly different from untreated control cells and SVT.

tion showed that the cells were gradually reduced in size and changed into a small round single cell shape by the treatment of SVT in PA-1 ovarian cancer cells (Fig. 1).

B. Apoptotic cell death by SVT

To determine the inhibition of cell growth by SVT was due to the induction of apoptotic cell death, we evaluated the changes in the chromatin morphology of cells by using DAPI staining followed by TUNEL staining assays, and then the double labeled cells were analyzed by fluorescence microscope. Conversely well with cell growth inhibition, DAPI-stained TUNEL-positive cells were significantly increased in SVT treated cells. The

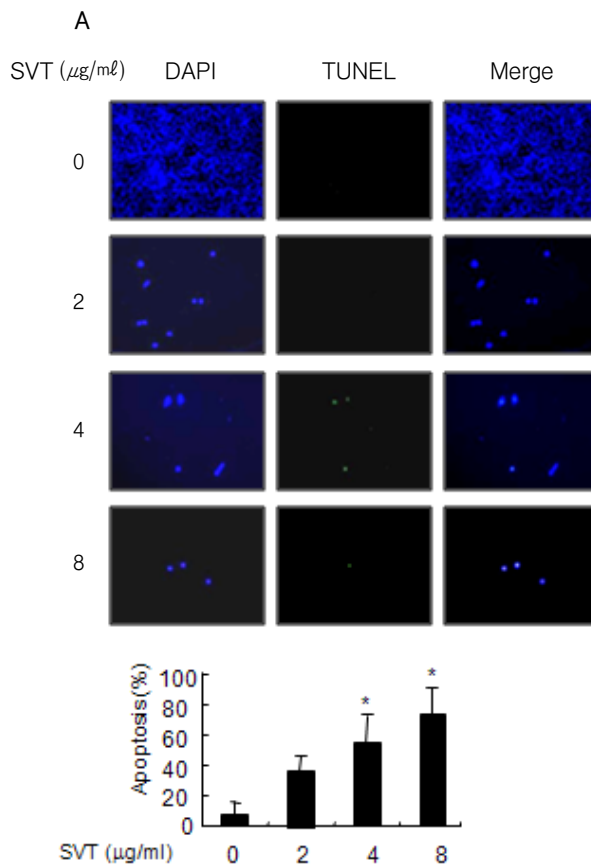


Fig. 2. Effect of SVT on apoptotic cell death

The ovarian cancer cells, PA-1 were treated with SVT (8 μg/ml) for 24 hr, and then labeled with DAPI and TUNEL solution. Total number of cells in a given area was determined by using DAPI nuclear staining (fluorescent microscope). The green color in the fixed cells marks TUNEL-labeled cells. The apoptotic index was determined as the DAPI-stained TUNEL-positive cell number/total DAPI stained cell number (magnification, 200x). Columns, means of three experiments, with triplicates of each experiment; bars, SD.

* : $p < 0.05$, significantly different from SVT-untreated control cells.

treatment of SVT resulted in about 55–65% or 60–80% induction of apoptotic cell death in PA-1 cancer cells (Fig. 2).

C. Expression of apoptotic regulatory proteins by SVT

To figure out the relationship between the induction of apoptotic cell death and the expression of their regulatory proteins by SVT, expression of apoptotic cell death related proteins was investi-

gated by Western blots. The expression of anti-apoptotic protein Bcl-2 was significantly decreased; however, the expression of pro-apoptotic proteins, Bax, cleaved form of caspase-3 were significantly and dose-dependently increased by treatment of SVT in PA-1 ovarian cancer cells (Fig. 3).

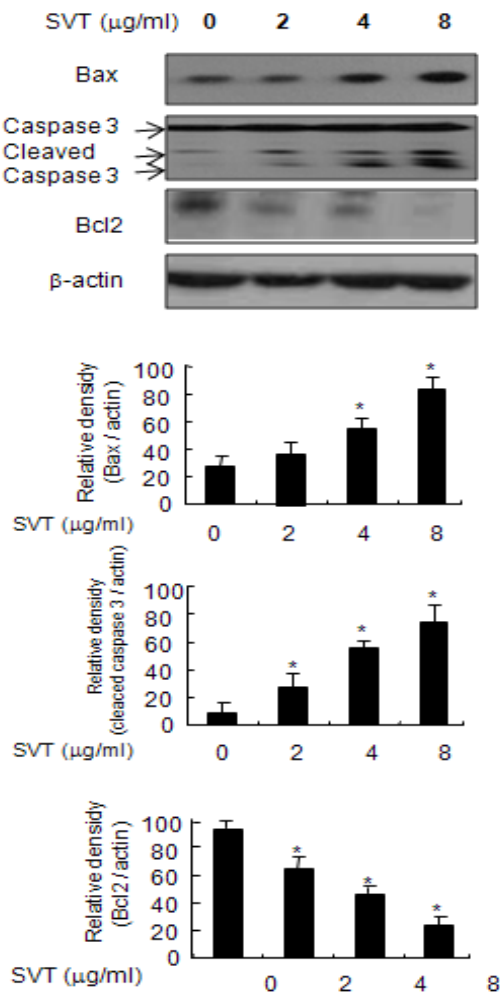


Fig. 3. Effect of SVT on the expression of apoptosis regulatory proteins

Expression of apoptosis regulatory proteins was determined using Western blot analysis. The ovarian cancer cells were treated with different concentrations of SVT (2, 4, and 8 µg/ml) for 24 hr. Equal amounts of total proteins (50 mg/lane) were subjected to 12% or 8% SDS-PAGE. Expression of caspase-3, Bax, Bcl2, and β-actin were detected by Western blotting using specific antibodies. β-actin protein here was used as an internal control. Each band is representative for three experiments.

* : $p < 0.05$, significantly different from untreated control cells and SVT.

D. Effect of SVT on activation of NF-κB signal molecules and STAT3

The phosphorylation of IκB in a NF-κB signal pathway and STAT3 is associated with proliferation and maintenance of tumors. Thus, activation of NF-κB signal molecules and STAT3 pathway was investigated. We found that the expression of NF-κB signal molecules and pSTAT3 were de-

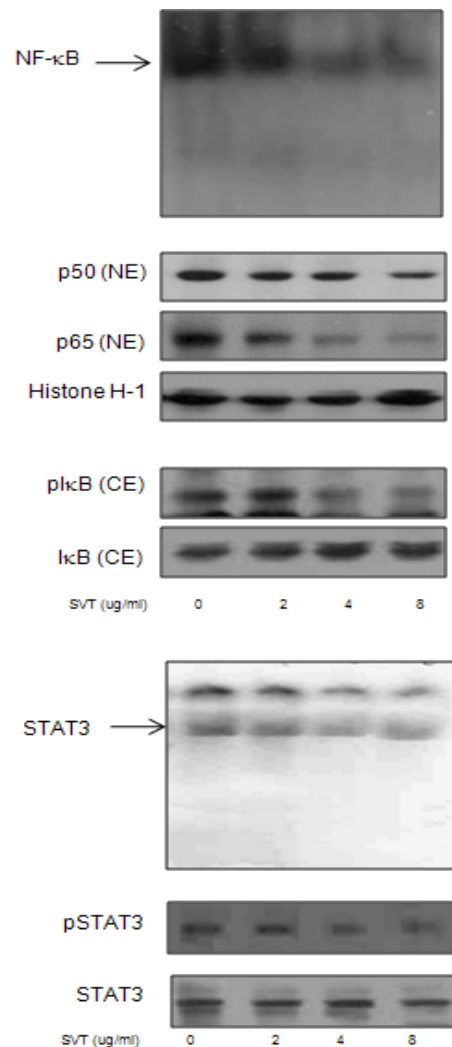


Fig. 4. Effect of SVT on the activation of NF-κB/STAT3 in ovarian cancer cells

The ovarian cancer cells were treated with different concentrations of SVT (2, 4 and 8 µg/ml) for 24 hr. Equal amounts of total proteins (50 mg/lane) were subjected to 8% SDS-PAGE. And β-actin were detected by Western blotting using specific antibodies of p50, p65, pIκB, IκB, STAT3, pSTAT3 and β-actin protein here was used as an internal control. Each band is representative for three experiments.

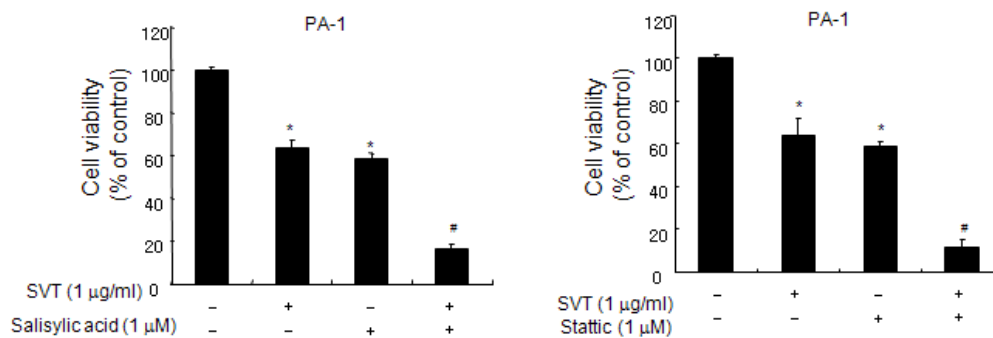


Fig. 5. Effect of salicylic acid and statin, NF-κB and STAT3 inhibitors on the SVT-induced cancer cell growth inhibition in PA-1 ovarian cancer cells

The ovarian cancer cells were treated with salicylic acid or statin (1 µM) for 24 hr, the cells were then and treated with SVT (1 µg/ml) for another 24 hr.

* : $p < 0.05$, significantly different from untreated control cells and SVT.

creased by SVT in ovarian PA-1 ovarian cancer cells dose dependently (Fig. 4).

E. Synergic effect of salicylic acid and statin on SVT-induced cell growth inhibition

To determine the relationship between NF-κB and STAT3 expressions and cell growth inhibitory effect of SVT, we investigated NF-κB and STAT3 expressions in PA-1 ovarian cancer cells using NF-κB and STAT3 inhibitors, such as salicylic acid and statin with SVT concomitantly. As shown in Fig. 5, it was found that knockdown of NF-κB and STAT3 had synergic effect on the cell growth inhibitory effect of SVT in PA-1 ovarian cancer cells. These results demonstrate that decrease of NF-κB and STAT3 expressions is strongly correlated with SVT-induced apoptotic cell death in PA-1 ovarian cancer cells.

IV. Discussion

The central and novel finding in the present study is the identification of anti-cancer efficacy of SVT against PA-1 ovarian cancer cells through induction of apoptosis via inactivation of NF-κB and

STAT3. SVT induces cell death of ovarian cancer cells in a concentration-dependent manner with an IC50 value of 4.5 µg/ml in PA-1 ovarian cancer cells.

Apoptosis plays a crucial role in eliminating the mutated hyperproliferating cells from the system¹⁹⁾. Thus, induction of apoptosis in tumor cells may be considered as a protective mechanism against development and progression of cancer. Apoptosis triggered by various stimuli, is characterized by a series of distinct biochemical and morphological changes²⁰⁾, including increase in ROS level²¹⁾, activation of caspases, cell shrinkage, chromatin condensation and nucleosomal degradation²²⁾. One of the most significant events in apoptosis is mitochondrial dysfunction. Loss of mitochondrial transmembrane potential (MTP) elicits the release of cytochrome c from mitochondria to cytosol²³⁾. After release, it promotes caspase-9 and -3 subsequently activating downstream caspase-3²⁴⁾. Activated caspase-3 cleaves intracellular protein PARP that is an important marker of apoptosis²⁵⁾. It has been evident that the pro-apoptotic protein Bax plays an essential role for the onset of mitochondrial membrane transition potential changes and induces cytochrome c release which is inhibited by the anti-apoptotic protein Bcl-2²²⁾. In this study, SVT treatment resulted in about 55-65% or 60-80% induction of apoptotic cell death in PA-1

ovarian cancer cells, and characteristic apoptotic morphological changes such as cell shrinkage, chromatin condensation and nucleosomal degradation were observed. In addition, SVT increased expression of pro-apoptotic proteins, Bax, cleaved form of caspase-3 and decreased expression of anti-apoptotic protein, Bcl-2.

SVT are greatly dangerous, but many researchers believe natural SVT are useful biological resource, containing several pharmacologically active components that could be of potential therapeutic value²⁶⁻²⁸, and Son et al. demonstrated that SVT from *Vipera lebetina turanica* inhibited NF- κ B activation and growth of cancers such as neuroblastoma and prostate cancer through its interaction with signal molecules (p50 and inhibitory κ B kinases [IKK]) in the NF- κ B signal pathway^{15,16}.

Meanwhile, signal transducers and activators of transcription (STAT) proteins are composed of a family of transcription factors, which is activated by various kinds of cytokines and growth factors via phosphorylation²⁹. Phosphorylated STAT3 forms homodimer or heterodimer, subsequently transfers to nucleus, and it acts as transcription enhancer there³⁰. Phosphorylation of STAT3 is known to be involved in cell growth, proliferation, survival, differentiation, apoptosis, metastasis, and angiogenesis³¹. Several studies have demonstrated that activated STAT3 contributes to development and progression of tumors including breast, prostate, skin, ovary, lung, bone, and blood cancers^{32,33}. Conversely, it was also reported that STAT3 signal pathway is a major target in inhibiting cancer cell growth through apoptosis³⁴, and compounds targeting STAT3 inactivation are available for treating various cancer including ovarian cancer by going apoptosis and hindering cancer growth³⁵⁻³⁷. Consistent with above findings, my data demonstrated that low concentration of SVT inactivated STAT3 as well as signal molecules in NF- κ B signal pathway concomitantly, and it further reconfirmed that inhibition of NF- κ B and STAT3 by SVT was strengthened by employing salicylic

acid and statin the inhibitor of NF- κ B and STAT3 in ovarian cancer PA-1 ovarian cancer cells. Thus, my results indicate that natural toxin SVT inhibit ovarian cancer cell growth through induction of apoptosis via NF- κ B and STAT3 inactivation.

Radiotherapy and chemotherapy are existing useful modalities for a variety of human cancers. However, they also have limitations due to their resistance^{38,39}.

Consequently, these present data provide that SVT could be useful candidate compounds to enhance tumor growth inhibiting ability of chemotherapeutics through overcoming the resistance via inactivation of anti-apoptotic NF- κ B and STAT3.

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