

# Troglitazone induces apoptosis in gastric cancer cells through the NAG-1 pathway

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**Abstract.** The non-steroidal anti-inflammatory drug-activated gene (NAG-1) is a newly identified member of the transforming growth factor (TGF)- $\beta$  superfamily and plays significant roles in regulating proliferation and pro-apoptotic activities. In the present study, we studied the regulation of NAG-1 by troglitazone in the cultured gastric cancer cell line BGC-823. MTT and TUNEL assays demonstrated that troglitazone potentially inhibits the proliferation of the gastric cancer cell line and induces apoptosis *in vitro* in a dose- and time-dependent manner. Troglitazone induced concentration-dependent NAG-1 expression in the BGC-823 cells, as assessed using immunocytochemistry. Furthermore, troglitazone increased Egr-1 protein levels in a concentration-dependent manner. In conclusion, the present study suggests that troglitazone markedly impedes proliferation and pro-apoptotic activities in BGC-823 cells, and the mechanism may partly be through the Egr-1 pathway.

## Introduction

Gastric cancer is one of the most common types of malignancies. It is very difficult to diagnose at earlier stages, and usually no potentially curative therapy is possible at advanced stages. The use of chemotherapy drugs is limited due to serious side effects and intolerance. Therefore, novel therapeutic approaches for the treatment of gastric cancer require immediate investigation.

Troglitazone (TGZ), a thiazolidinedione derivative, is a ligand for PPAR $\gamma$  that binds and activates PPAR $\gamma$  and has wide biological activities (1). Apart from enhancing insulin sensitivity, anti-atherogenesis and anti-inflammation, TGZ has been reported to have a potent anti-tumor effect (2-4). Recent studies have confirmed that thiazolidinediones may inhibit

the growth of cancer cells dependently and independently of the PPAR $\gamma$  pathway *in vitro*. Furthermore, its independence of the PPAR $\gamma$  pathway continues to be an important issue. The up-regulation of NAG-1 by the thiazolidine derivative MCC-555 in a PPAR $\gamma$ -independent manner has been confirmed to promote apoptosis in human colorectal cancer cells (5). However, it is largely unknown whether TGZ induces NAG-1 expression to inhibit growth and induce apoptosis in human gastric cancer cells. In the present study, we examined whether NAG-1 expression is induced in gastric cancer cells by TGZ, and the effect of NAG-1 expression on cancer cell biological behavior.

## Materials and methods

**Cell cultures.** The human gastric cancer cell line BGC-823 (provided by Biotherapy State Key Laboratory, China) was cultured and maintained in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM glutamine, 100 U penicillin/ml, and 100  $\mu$ g of streptomycin/ml, and incubated at 37°C in 5% CO<sub>2</sub>.

**Cell viability assay.** Cell viability was determined by the MTT [3-(4,5-dimethylthiazole)-2,5-diphenyltetrazolium bromide] assay. Briefly, 5x10<sup>3</sup> cells/well were seeded in 96-well plates and allowed to attach overnight. The concentrations of TGZ (Sigma, St. Louis, MO, USA) were 0.5, 1, 5, 15, 25 and 50  $\mu$ mol/l, respectively. Each group contained three wells. After the samples were treated with TGZ for 24 or 48 h, 20  $\mu$ l of MTT (0.5 mg/ml; Sigma) was added to each well, and the cells were incubated at 37°C for 4 h. The reaction was then halted by lysing the cells with 200  $\mu$ l of dimethyl sulfoxide (DMSO) for 15 min. Quantification measurements (optical density) were obtained at a wavelength of 570 nm using spectrophotometric analysis.

**TUNEL assay for detection of apoptosis.** To investigate the apoptosis in gastric carcinoma cells induced by TGZ, the TdT-mediated dUTP nick-end labeling (TUNEL) assay was carried out using an apoptosis detection system (Roche Diagnostics, Indianapolis, IN, USA). Cell culture and treatment were carried out as described above. For quantitative evaluation of apoptotic cells, 10 fields (at least 200 cells) were counted in each preparation. The apoptotic rate was

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calculated as the number of apoptotic cells/number of total cells x 100%.

**Measurement of apoptosis by flow cytometry.** BGC-823 cells were plated at a density of  $4 \times 10^5$  cells/well in 6-well plates, incubated for 24 h, and then treated with different concentrations of TGZ for 48 h. Thereafter, the cells were harvested, washed with phosphate-buffered saline (PBS) and stained using the Annexin V-FITC reagent kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, the cells were stained with 5  $\mu$ l Annexin V-FITC and 5  $\mu$ l propidium iodide (PI) in 500  $\mu$ l binding buffer in the dark at room temperature for 15 min, respectively. They were then examined by FACSscan (BD Biosciences), and data were processed using Cell Quest™ software (BD Biosciences).

**Immunocytochemistry of NAG-1.** BGC-823 cells were plated in 24-well plates containing pre-coated slides at a density of  $5 \times 10^4$  cells/well, incubated for 16 h and then treated with the indicated concentrations of TGZ containing 1% serum for the indicated times. After treatment, the cell samples were washed with PBS, fixed, air dried with 4% paraformaldehyde for 30 min, and then incubated in 0.1% Triton X-100 for 10 min on ice, in 3% H<sub>2</sub>O<sub>2</sub> in the dark for 10 min and in normal goat serum for 15 min. Subsequently, the samples were incubated with anti-NAG-1 antibody (Upstate Biotechnology, NY, USA) at 4°C overnight. After washing with PBS, they were treated with biotinylation secondary antibody and horseradish peroxidase-conjugated streptavidin for 15 min, respectively. The samples were added to the DAB substrate for incubation for 2-5 min, and analysis by light microscopy was carried out after counterstaining. Yellow cytoplasm indicated NAG-1-positive expression. The percentage of positive gastric cancer cells was calculated after each slide was observed in 10 high-power fields.

**Western blot analysis for Egr-1.** BGC-823 subline cells ( $1 \times 10^6$  cells/well) were incubated with 5, 15 and 25  $\mu$ mol/l TGZ and serum-free RPMI-1640 separately for 3 h at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. The concentrations of protein extracted from the BGC-823 cells were determined by Lowry method. Protein (20  $\mu$ g/well) was loaded onto 8% SDS-PAGE gels and separated by electrophoresis. The proteins were then transferred onto PVDF membranes (Roche Diagnostics, Indianapolis, IN, USA), and each blot was immunolabeled with rabbit polyclonal antibody for Egr-1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4°C overnight. Membranes were incubated with horseradish peroxidase-linked immunoglobulin and exposed to enhanced chemiluminescence Western blotting luminal reagent (Santa Cruz Biotechnology, Inc.) followed by autoradiography. Western blotting was performed at least 3 times to ensure reproducibility. To correct for variations in loading, the blots were stripped and incubated with a monoclonal anti-actin antibody (1:3000; Santa Cruz Biotechnology, Inc.). The optical density (OD) of the protein band was analyzed by Band Scan Figure software (Glyko, Novato, CA, USA), and the ratio of the OD for Egr-1 to the OD for actin was calculated.

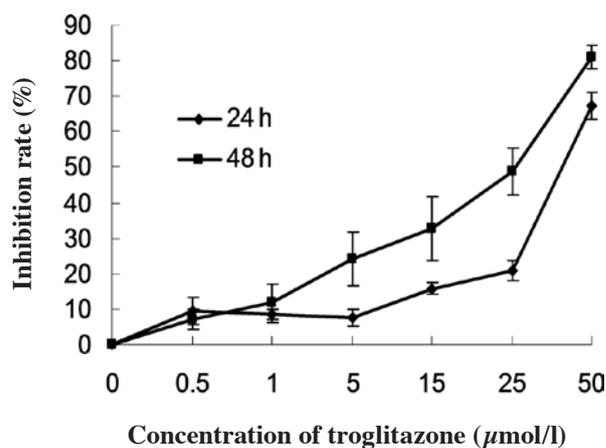


Figure 1. Anti-proliferative effects of troglitazone (TGZ) on BGC-823 cells. Cells were plated at a density of  $5 \times 10^3$  cells per well (6 replicates/group) in 96-well plates and exposed to TGZ treatment for 24 or 48 h. The viable cell number was determined by MTT colorimetric assay and indicated as a percentage of the control. The results represent the average of three independent experiments.

**Statistical analysis.** Statistical significance was determined for P-values <0.05 by one-way ANOVA.

## Results

**Troglitazone inhibits BGC-823 cell growth.** After treatment with TGZ at concentrations ranging from 0.5 to 50  $\mu$ mol/l for 24 and 48 h, the viability of the cells was significantly lower than that of the untreated corresponding control groups ( $P < 0.05$ ). Ratios of cell proliferation inhibition in the BGC-823 cells were linearly positive and dose-dependent (Fig. 1). TGZ inhibited the proliferation of the gastric cancer cell line *in vitro* in a dose- and time-dependent manner.

**Induction of apoptosis by troglitazone.** After 48 h of incubation, TUNEL staining of the BGC-823 cells showed more scattered apoptotic cells with brown nucleoli in the TGZ-treated groups than in the control (Fig. 2). Flow cytometric analyses of the binding of fluorescence-labeled Annexin V to externalized phosphatidylserine was also performed in order to quantify early apoptotic cells. PI uptake was measured to assess cells in the late stages of apoptosis or cells that sustained direct plasma membrane damage. Fig. 3 shows flow cytometric plots obtained with the Annexin V-PI assay after a 48-h exposure to different concentrations (0.5 to 50  $\mu$ mol/l) of TGZ. The number of early and late apoptotic cells or directly damaged cells increased after exposure of the cells to TGZ.

**NAG-1 involvement in troglitazone-induced apoptosis.** Immunocytochemical staining revealed that NAG-1 expression was negative in the BGC-823 cells. After treatment with different concentrations of TGZ (0.5 to 25  $\mu$ mol/l) for 48 h, NAG-1 expression was induced in a concentration-dependent manner. The positive rates of NAG-1 were  $13.3 \pm 4.04$ ,  $30.7 \pm 5.51$ ,  $50.3 \pm 5.86$ ,  $75.3 \pm 5.51$  and  $91.3 \pm 3.06\%$ , separately (Fig. 4).

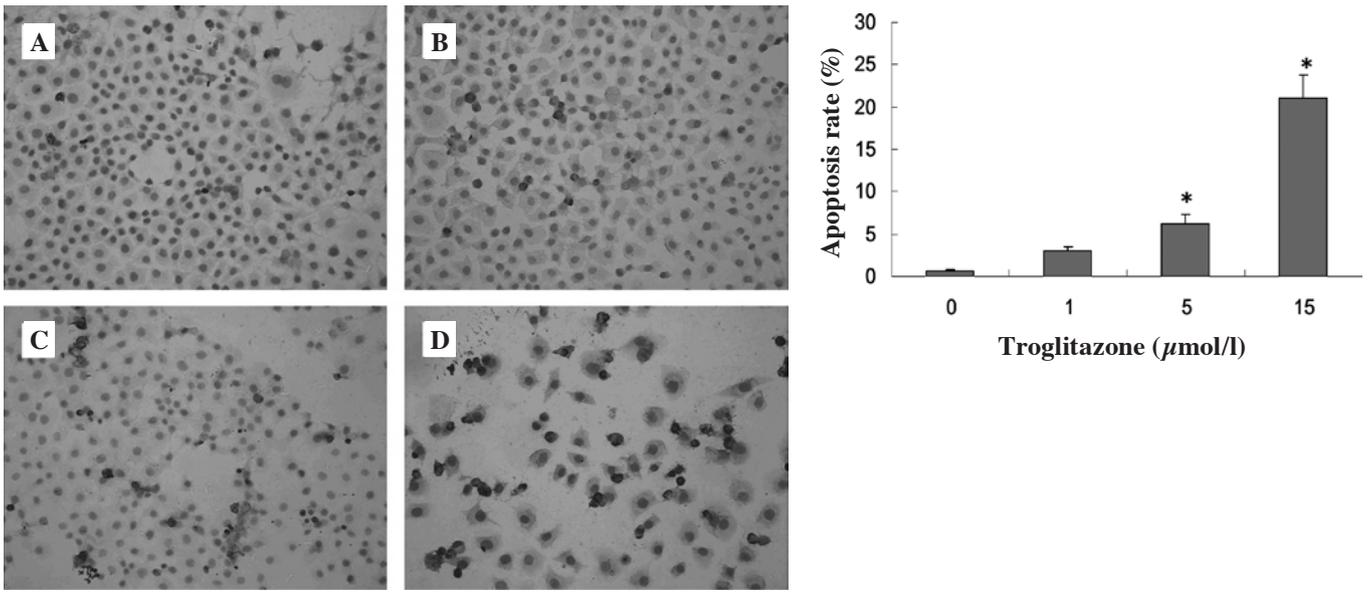


Figure 2. TUNEL staining of apoptotic BGC-823 cells (48 h). Cell apoptosis was assessed by TUNEL assay which showed a markedly greater percentage of TUNEL-positive nuclei in the TGZ groups vs. the control group. (A) Control group. Cells treated with (B) 1, (C) 5 and (D) 15 μmol/l of TGZ, respectively. \*P<0.05, control group compared with the TGZ groups.

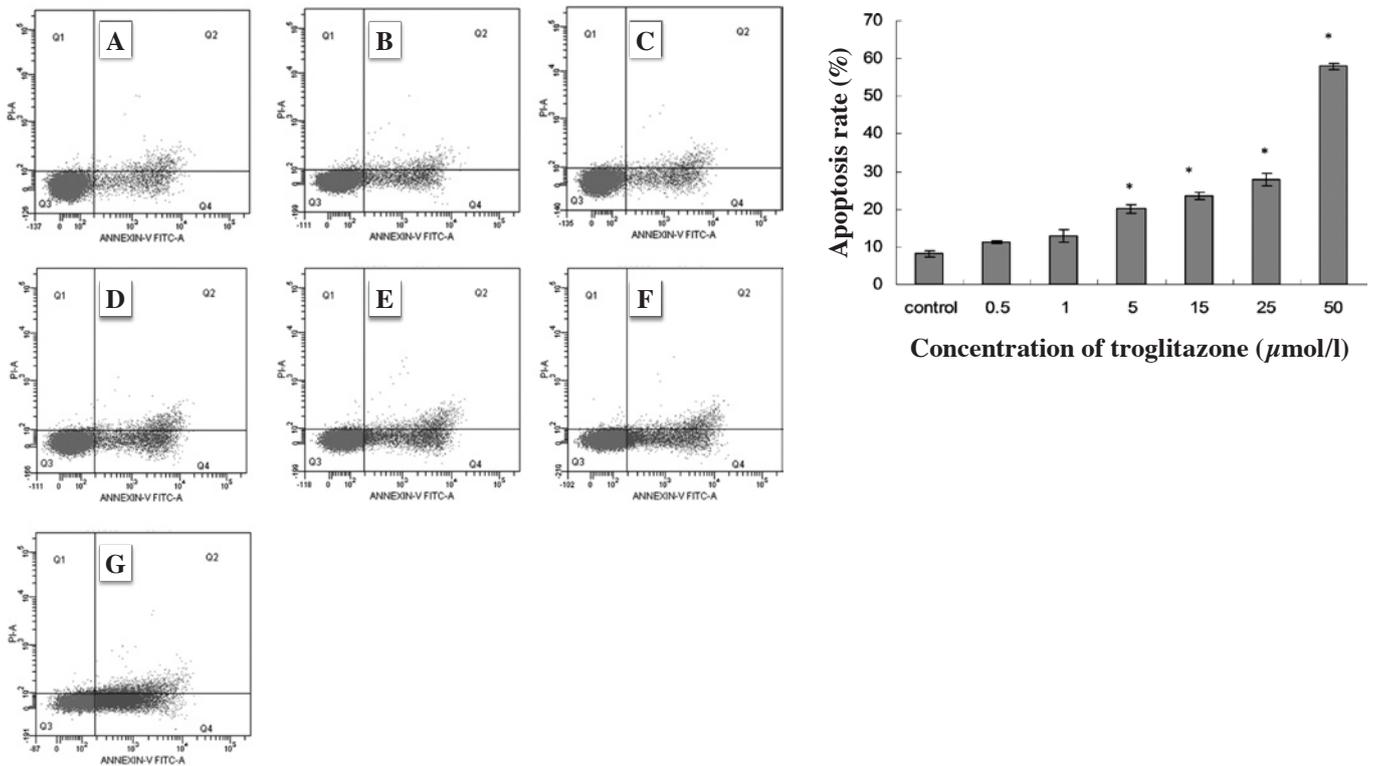


Figure 3. Effect of troglitazone (TGZ) on the apoptosis rate of BGC-823 cells. The rate of apoptosis was detected with FCM using Annexin V-FITC/PI double staining. Stained cell populations were defined as follows: Q2, late apoptotic or necrotic cells (Annexin V<sup>+</sup>, PI<sup>+</sup>); Q3, viable or undamaged cells (Annexin V<sup>-</sup>, PI<sup>-</sup>); and Q4, cells undergoing early apoptosis (Annexin V<sup>+</sup>, PI<sup>-</sup>). BGC-823 cells were treated with various concentrations of TGZ for 48 h. (A) Control group. Cells treated with (B) 0.5, (C) 1, (D) 5, (E) 15, (F) 25 and (G) 50 μmol/l of TGZ, respectively. An increase in apoptosis was noted at 5 μmol/l TGZ and this increase was in a dose-dependent manner. The figures are representative of three separate experiments. \*P<0.05, control group compared to the TGZ groups.

**Induction of Egr-1 by troglitazone.** The protein level of Egr-1 in the BGC-823 cells was measured by Western blotting. As expected, the level of Egr-1 increased in response

to different concentrations of TGZ (Fig. 5). These results suggest that TGZ induces Egr-1 protein expression in BGC-823 cells.

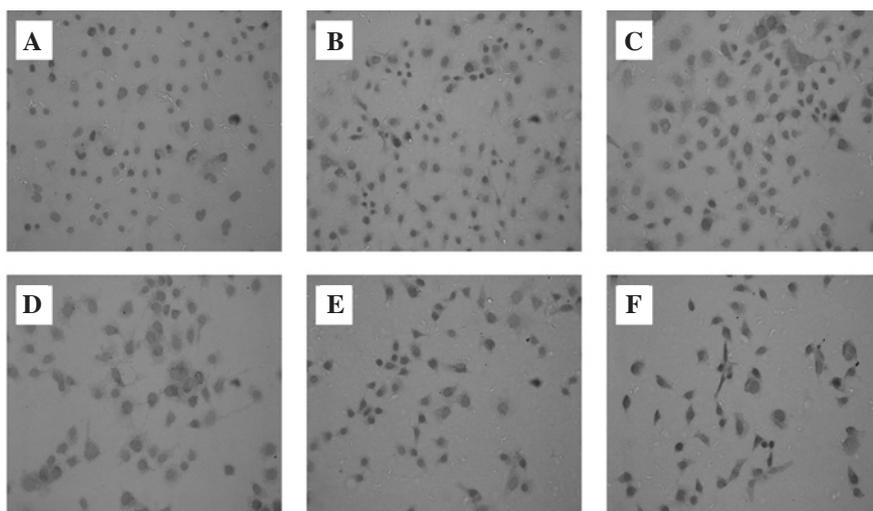


Figure 4. Immunocytochemical staining for NAG-1 in the BGC-823 cells. (A) NAG-1 was negatively expressed in the control groups. Expression of NAG-1 after treatment with (B) 0.5, (C) 1, (D) 5, (E) 15, and (F) 25  $\mu\text{mol/l}$  of TGZ; NAG-1 expression was induced in the cytoplasm in a concentration-dependent manner.

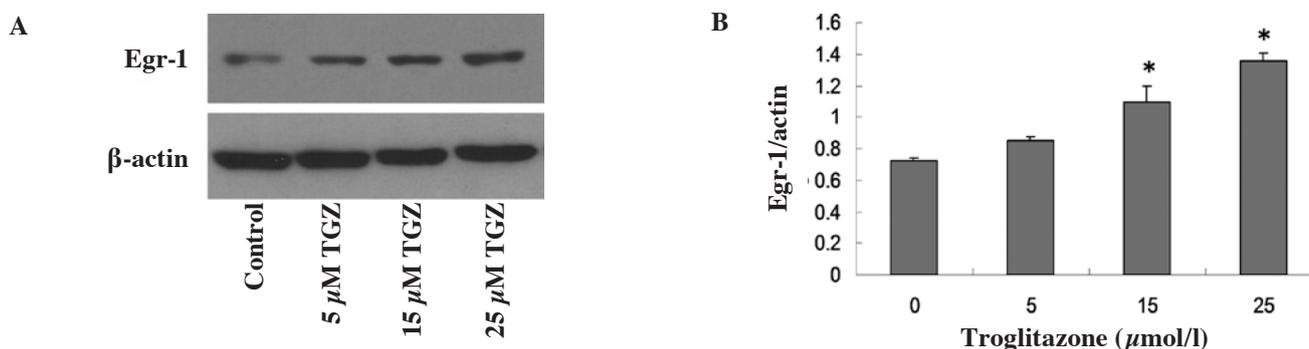


Figure 5. Induction of Egr-1 expression by TGZ in BGC-823 cells. Protein (30  $\mu\text{g}$ ) was loaded on SDS-PAGE gels, and immunoblotting was performed. Rabbit Egr-1 polyclonal antibody and  $\beta$ -actin antibody were used to detect Egr-1 and  $\beta$ -actin, respectively. Expression of Egr-1 protein was calculated by the density of the Egr-1 band over that of the  $\beta$ -actin band. Results represent the average of three independent experiments, and data are shown as the mean  $\pm$  SD. \* $P < 0.05$ , control group compared to the TGZ groups.

## Discussion

Troglitazone is a PPAR $\gamma$  ligand and may be regarded as a novel and promising agent in cancer therapy as it can facilitate differentiation, inhibit growth, induce apoptosis, and inhibit the angiogenesis, migration and invasion of tumor cells (6-9). Although the anti-tumorigenic activities of PPAR $\gamma$  ligands are well-established in human cancer, controversy exists in the literature with regard to the relative contributions of nuclear receptor-dependent and -independent mechanisms (10). A recent study confirmed that a novel peroxisome proliferator-activated receptor gamma ligand, MCC-555, induces apoptosis via post transcriptional regulation of NAG-1 in colorectal cancer cells in a PPAR $\gamma$ -independent manner (5).

NAG-1 (also known as MIC-1, GDF-15, PLAB and placental TGF- $\beta$ ), a member of the TGF- $\beta$  superfamily, is involved in tumor progression and development (11-14). The overexpression of NAG-1 in cancer cells results in growth arrest and an increase in apoptosis, and suggests that NAG-1 has anti-tumorigenic and pro-apoptotic activity (15,16). In our previous study, we found that NSAIDs induced NAG-1 in SW480, LS174T and SW480 colon cancer cells (17). In

the present study, we demonstrated that the BGC-823 human gastric cancer cell line did not express NAG-1, while TGZ induced the expression of NAG-1 protein. NAG-1 protein was located in the cytoplasm and the positive rates of NAG-1 were gradually increased at TGZ concentrations ranging from 0.5 to 25  $\mu\text{mol/l}$  at 48 h. This result illustrates that, apart from NSAIDs, TGZ may also induce the expression of NAG-1 in a dose-dependent manner.

To confirm the relationship between NAG-1 induction and biological activities in BGC-823 cells treated with TGZ, we investigated the inhibition of cell proliferation and the induction of apoptosis by TGZ at concentrations ranging from 0.5 to 50  $\mu\text{mol/l}$ . The percentages of inhibition of cell proliferation were gradually increased at TGZ concentrations ranging from 0.5 to 50  $\mu\text{mol/l}$  at 24 h, and were respectively up-regulated at 48 h. The result is compatible with those obtained in cholangiocarcinoma cells (18). Moreover, apoptosis was induced by TGZ in a dose-dependent manner. In an effort to better understand the mechanisms responsible for these cellular responses, we measured the expression of Egr-1 protein and found that TGZ stimulates the expression of Egr-1 in a concentration-dependent manner. Egr-1 is a member of the immediate early

gene response family and encodes a nuclear phosphoprotein involved in the regulation of cell growth and differentiation in response to signals such as mitogens, growth factors and stress stimuli (19-21). However, evidence suggests that Egr-1 binding sites are located within region -73 to -51 of the NAG-1 promoter, and the overexpression of Egr-1 results in increased NGA-1 expression. We confirmed that TGZ induced expression of NAG-1 in BGC-823 gastric cancer cells and induced apoptosis partly through the Egr-1 pathway.

In conclusion, the PPAR $\gamma$  ligand TGZ may induce NAG-1 expression in gastric cancer cells and is closely related to reduced cell viability and increased apoptosis. The mechanisms of this action may be related to stimulation of the expression of the transcription factor Egr-1, a protein with established tumor suppressor activity.

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