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Implication of Egr-1 in trifluoperazine-induced growth inhibition in human U87MG glioma cells

Soon Young Shin[1], Chang Gun Kim[2], Dong Dae Hong[3], Jung-Hye Kim[3] and Young Han Lee[2,4]

[1]Institute of Natural Science and Technology
[2]Division of Molecular and Life Science
College of Science and Technology
Hanyang University, Ansan
Gyeonggi-do 426-791, South Korea
[3]Department of Biochemistry and Molecular Biology
College of Medicine, Yeungnam University
Daegu 705-717, South Korea
[4]Corresponding author: Tel, 82-31-400-5517;
Fax, 82-31-416-9781; E-mail, younghan@hanyang.ac.kr

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Abbreviations: TFP, trifluoperazine

Abstract

The early growth response gene-1 (Egr-1) is a tumor suppressor which plays an important role in cell growth, differentiation and apoptosis. Egr-1 has been shown to be down-regulated in many types of tumor tissues. Trifluoperazine (TFP), a phenothiazine class of antipsychotics, restored serum-induced Egr-1 expression in several cancer cell lines. We investigated the effect of Egr-1 expression on the TFP-induced inhibition of cell growth. Ectopic expression of Egr-1 enhanced the TFP-induced antiproliferative activity and down-regulated cyclin D1 level in U87MG glioma cells. Our results suggest that antipsychotics TFP exhibits antiproliferative activity through up-regulation of Egr-1.

Keywords: cell cycle; cyclin D1; glioma; trifluoperazine; tumor suppressor proteins

Introduction

Early growth response-1 (Egr-1) gene, which is also known as NGFI-A, zif268, krox24 or Tis8 (Lim *et al*., 1987; Milbrandt, 1987; LeMaire *et al*., 1988; Christy *et al*., 1989), encodes a nuclear protein that has three Cys2-His2 type zinc finger-containing DNA-binding domains in the C-terminal portion of the molecule (Sukhatme *et al*., 1988). Egr-1 preferentially binds to GC-rich regulatory elements, leading to the induction or repression of its target genes (Lim *et al*., 1987; Christy *et al*., 1989). Egr-1 is rapidly induced by a broad spectrum of extracellular signals including nerve growth factor, depolarization, ischemic injury, and differentiation stimuli Tis8 (Gilman *et al*., 1986; Lim *et al*., 1987; Milbrandt, 1987; LeMaire *et al*., 1988; Sukhatme *et al*., 1988; Christy *et al*., 1989), implicating Egr-1 as an important mediator in cell growth, differentiation, and cell survival. Egr-1 expression is absent or reduced in a number of tumor cells (Huang *et al*., 1995; Levin *et al*., 1995; Huang *et al*., 1997) and in PLCγ1-transformed 3Y1 fibroblasts (Shin *et al*., 2002a). Stable expression of Egr-1 inhibited cell proliferation and soft-agar growth in NIH3T3 cells transformed with *v-sis* (Huang *et al*., 1995), suggesting that loss of Egr-1 expression is closely associated with tumor formation.

Trifluoperazine (TFP) is a phenothiazine derivative and a commonly used antipsychotic drug. The clinical potency of phenothiazines may be related to a blockade of the dopamine receptor (Snyder, 1974). In addition to its therapeutic effects, TFP is also widely used as a calmodulin antagonist (Weiss *et al*., 1978; Osborn *et al*., 1980). TFP binds to calmodulin and interferes with Ca^{2+}-calmodulin interactions, and thereby blocks Ca^{2+}-calmodulin dependent cellular events. Calmodulin is a Ca^{2+}-bining protein, which is ubiquitously expressed in almost eukaryotic cells (Cheung *et al*., 1980) and plays a key role in mediating Ca^{2+}-dependent diverse physiological responses including regulation of cell proliferation (Means *et al*., 1980; Lu *et al*., 1993; Schulman, 1993). It has been reported that calmodulin antagonists inhibit cell proliferation in several types of cancer cells (Hidaka *et al*., 1981; Hait *et al*., 1985). Previously, we have reported that calmodulin antagonist TFP induces apoptotic cell death in rat PC12 cells (Kang *et al*., 1999) and TFP stimulates Egr-1 gene expression by modulating Ras-dependent Elk-1 activity in human HT1080 fibrosarcoma cells (Shin *et al*., 2001). However, the functional role of Egr-1 induced by TFP has not been fully elucidated. The aim of this study was to investigate the role of TFP-mediated Egr-1 expression on the tumor cell growth.

Materials and Methods

DNA synthesis assay

Effect of TFP on the DNA synthesis was measured by [^{3}H]-methyl-thymidine incorporation. Cells were cultured in 24-well plates in the presence of increasing concentrations of TFP. After 24 h of TFP treatment, [^{3}H]-methyl-thymidine (247.9 GBq/mmol. NEN) was added at 0.2 μCi/well for 4 h. Cells were then washed with PBS, and precipitated with 15% trichloroacetic acid. The acid-insoluble materials were washed with ether:methanol (2:1), solubilized with 0.4 M NaOH for 2 h at 4°C, and then the radioactivity was counted with a liquid scintillation β-ray counter.

Cell proliferation assay

Cells were seeded at a density of 1×10^5 cells in a 35-mm culture dish and counted in triplicate at 12-h intervals after plating. The viability of cells was estimated by the dye exclusion method after staining with 0.4% trypan blue staining.

FACS analysis

Cells were treated with 20 M TFP for 24 h. The cells were collected and fixed in 70% ethanol. The fixed cells were washed twice with PBS, and stained with 50 μg/ml propidium iodide solution containing 0.1% Triton X-100, 0.1 mM EDTA, and 50 μg/ml RNase A. The propidium iodide stained cells were analyzed at 488 nm using FACScan flow cytometer (Becton Dickson Corp).

Western blot analysis

Cells were lysed in 20 mM HEPES, pH 7.2, 1% Triton X-100, 10% glycerol, 150 mM NaCl, 10 μg/ml leupeptin, and 1 mM PMSF. Protein samples (20 μg of each) were separated by SDS-PAGE (10%) and transferred to nitrocellulose filters (Shin *et al*., 2002b). The blots were incubated with anti-Egr-1 antibodies and developed with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech., Piscataway, NJ). The same blot was reprobed with anti-Erk1/2 antibody for use as an internal control.

Egr-1 promoter assay

U87MG cells were grown in DMEM with 10% heat-inactivated fetal calf serum. One day after seeding cells into 35-mm dishes (6×10^5 cells), the cells were co-transfected with 0.5 μg of 5' deletion constructs of the Egr-1 promoter (Shin *et al*., 2001) and 0.2 μg of pCMV/β-gal plasmid using Lipofectamine 2000 reagents (Life Technologies) according to the manufacturer instructions. At 24 h post transfection, cells were treated with TFP. Cells were harvested after 6 to 12 h of TFP treatment and protein extracts were prepared by three cycles of freezing and thawing. One to five μg of protein was assayed for luciferase activities. Luminiscence was measured using a luminometer model TD 2020 (Berthold, Tubingen, Germany).

Results and Discussion

Restoration of Egr-1 expression by combined treatment with TFP and serum. Previously, we have found that Egr-1 is substantially induced by phorbol myristate acetate (PMA) but not by serum in HT1080 fibrosarcoma cells (Shin *et al*., 2001). This suppression was recovered by combined treatment with TFP and serum (Figure 1, upper left). In this study we investigated whether the effect of combined treatment with serum and TFP on the stimulation of Egr-1 expression is a general phenomenon. U87MG human glioma cells were serum-deprived for 24 h and then treated with 20% serum in the absence or presence of TFP. PMA was used as a positive control. Treatment with serum alone failed to stimulate the induction of Egr-1, however, co-stimulation with serum plus TFP strongly accumulated Egr-1 protein (Figure 1, upper

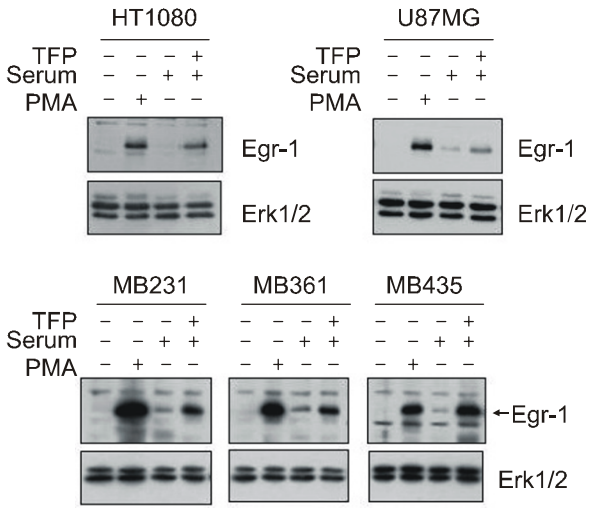

Figure 1. Effect of TFP on the accumulation of Egr-1 protein. Tumor cells starved with 0.5% serum for 24 h were pretreated with TFP (20 μM) for 30 min before addition of 20% serum. PMA (50 nM) was used as a positive control. After 2 h, the amount of Egr-1 protein was detected in whole cell lysates (20 μg) by Western blotting using rabbit anti-Egr-1 antibody (1:1000). The same blot stripped and reprobed with rabbit anti-Erk1/2 antibodies for an internal control of the protein.

right). TFP alone slightly increased the level of Egr-1 protein (data not shown). Similar results were obtained in breast cancer cell lines including MDA-MB 231, -MB 361 and -MB 435 (Figure 1, bottom). The results demonstrate that TFP restores Egr-1 expression, at least, in fibrosarcoma, glioma and breast carcinoma cells.

Effect of TFP on the Egr-1 promoter activity in U87MG glioma cells

To determine whether TFP-mediated induction of Egr-1 occurred at the transcriptional level, U87MG cells were transfected with serial deletion constructs of Egr-1 promoter, p-688egrLuc, p-454egrLuc and p-233egrLuc (Figure 2A), and treated with TFP at a concentration of 30 μM. PMA was used as a positive control. Results were expressed as a fold-increase in luciferase activity normalized for β-galactosidase acti-

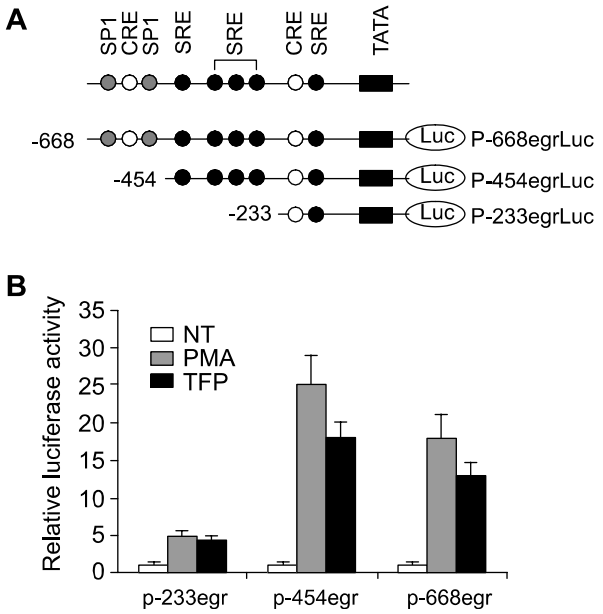

Figure 2. Effect of TFP on human Egr-1 promoter activity. (A) The 5' boundaries of plasmids containing various truncations of the Egr-1 promoter fused to luciferase reporter gene. The numeric designations of each construct refer to the 5' deletion endpoints derived from the transcription start site at +1. The positions of putative regulatory elements (SP1, CRE, and SRE) are indicated by circles. (B) U87MG cells cultured on 35-mm dishes were co-transfected with each construct of the Egr-1 promoter (0.5 mg) and the pCMV/β-gal plasmid (0.2 μg). After 24 h of transfection, cells were treated without (NT) or with TFP (50 μM). PMA (50 nM) was used as a positive control. Cells were harvested after an additional incubation for 12 h and protein extracts were prepared by three cycles of freezing and thawing. One mg of protein was assayed for luciferase and β-galactosidase activities. Results are shown as the amount of induction after correcting for β-galactosidase activity. Error bars represent SD from three independent experiments in duplicate.

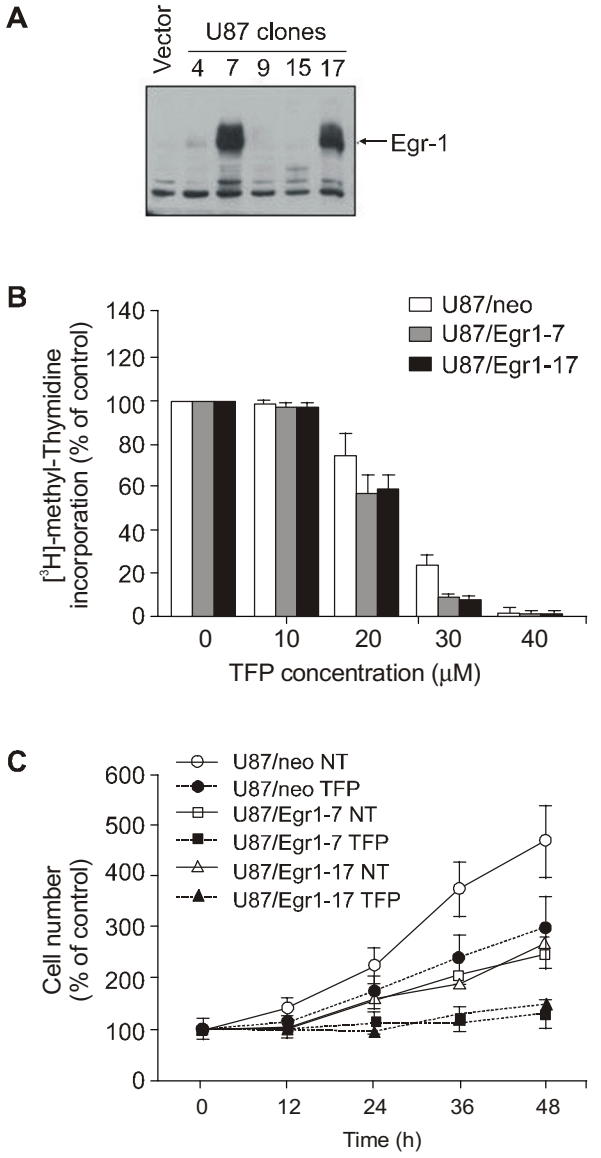

Figure 3. Effect of ectopic expression of Egr-1 on the TFP-induced growth inhibition. (A) U87MG cells maintained in 100-mm culture dish were transfected with an expression vector (30 μg) containing Egr-1 cDNA (pCMVtag2B/Egr-1) or lacking the insert (pCMVtag2B/neo). After selection with 0.7 mg/ml of geneticin (G418), Egr-1 expression was analyzed by Western blotting. (B) U87MG transfectants cultured in 24-well plates in the presence of increasing concentrations of TFP were pulse labeled for 1 h with 0.2 μCi/well of [^{3}H]thymidine. After precipitation with 15% trichloroacetic acid, radioactivity incorporated into the acid-insoluble material was measured with a liquid scintillation β-ray counter. Error bars represents SD from two independent experiments in triplicate. (C) U87MG transfectants were seeded into 35-mm culture dishes and treated with TFP at 20 μM. Every 12 h, the viable cell number was determined by the dye exclusion method after staining with 0.4% trypan blue. Error bars represents SD from two independent experiments in triplicate.

vity. The -668 and -454 constructs exhibited a 28- and 18-fold increase of luciferase activity, respectively, due to the addition of TFP, indicating that TFP stimulates Egr-1 gene expression at the transcription level (Figure 2B).

Effect of ectopic expression of Egr-1 on the cell growth in U87MG glioma cells

To determine the functional role of Egr-1 expression by TFP, U87MG cells were stably transfected with an expression vector containing Egr-1 cDNA (pCMV-tag2B/Egr-1) or lacking the insert (pCMVtag2B/neo). Three weeks after transfection, G418 resistant clones were selected and the elevated level of exogenous Egr-1 was detected by Western blot analysis. From them, we used clone 7 (U87/Egr1-7) and clone 17 (U87/Egr1-17) for the next experiments (Figure 3A). Empty vector-transfected cells (U87/neo) were used as a control. The effect of exogenous expression of Egr-1 on the growth response was assessed by [^{3}H]-methyl-thymidine incorporation into DNA (Figure 3B). Treatment with TFP reduced [^{3}H]methyl-thymidine incorporation in a concentration-dependent fashion. In U87/neo cells, [^{3}H]methyl-thymidine uptake after treatment with TFP at 20 μM or 30 μM was reduced to 75% or 25%, respectively, of that observed in vehicle-treated cells. In contrast, in U87/Egr1 transfectants, [^{3}H]methyl-thymidine uptake after treatment with TFP at 20 μM or 30 μM was reduced to 58% or 25% of that observed in vehicle-treated cells. A complete inhibition resulted when all the U87MG tranfectants were treated with TFP at 40 μM. Cell proliferation as measured by counting of viable cells was also inhibited by treatment with TFP at 20 μM (Figure 3C). U87/neo cells showed about a 470% increase of cell numbers in the absence of TFP, whereas about a 300% increase in the presence of TFP after 48 h of incubation. Compared to U87/neo cells, there was a considerable decrease in cell proliferation in U87/Egr1 transfectants, indicating that forced expression of Egr-1 leads to more sensitive to TFP-inducible inhibition of cell growth. These results suggest that the Egr-1 induction by TFP is required for TFP-inducible antiproliferative activity in U87MG glioma cells.

Effect of TFP and Egr-1 expression on the cell cycle distribution

To determine the possible mechanism involved in the growth inhibition by TFP-induced Egr-1, the progression of cell cycle was determined by a FACS (Figure 4). In U87/Egr1 transfectants, the numbers of cells increased in the Go/G1 phase (63.08% versus 82.4%) but decreased in S (9.25% versus 4.3%) and G2/M (27.76% versus 13.34%) phase of the cell cycle as compared to U87/neo cells (first low panels). Treatment with TFP for 24 h increased G1 populations both in U87/neo (from 63.08% to 74.35%) and in U87/Egr1-17 cells (from 82.4% to 87.04%) but decreased in the cell populations both in the S phase

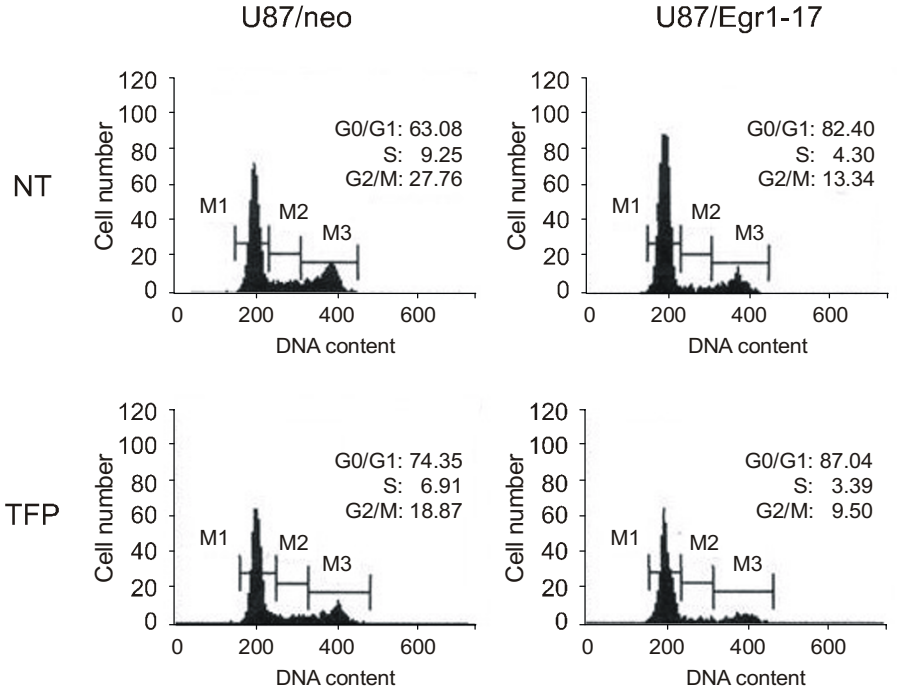

Figure 4. Effect of ectopic expression of Egr-1 on the cell cycle distribution. U87MG transfectants treated with 20 μM TFP for 24 h. DNA content was analyzed by propidium iodide staining at 488 nm using FACScan flow cytometer. Similar results were obtained from two independent experiments.

(from 9.25% to 6.91% in U87/neo, and from 4.3% to 3.39% in U87/ Egr1-17) and G2/M phase (from 27.76% to 18.87% in U87/neo, from 13.34% to 9.5% in U87/Egr1-17) (second row panels). These data suggest that TFP-mediated Egr-1 expression contributes to the inhibition of cell cycle progression.

Effect of ectopic expression of Egr-1 on the cyclin D1 expression

We next analyzed the effect of ectopic expression of Egr-1 on the expression of cell cycle regulators by Western blotting. We found that steady-state level of cyclin D1 protein was decreased in U87/Egr1-17 cells as compared to that in U87/neo cells, whereas cyclin E, cyclin A1 and PCNA were not changed (Figure 5A). Since expression of cyclin D1 is a prerequisite for G1-to-S phase transition, we investigated whether TFP treatment influences the accumulation of cyclin D1 protein (Figure 5B). When serum-starved cells were treated with 20% serum in the absence or presence of 20 μM of TFP, the amount of cyclin D1, but not cyclin E and cyclin A1, was considerably decreased in U87/Egr1-17 cells. Because the transition of cells from G1 to S phase of cell cycle is in part regulated by cyclin D1, it seems likely that Egr-1-induced anti-proliferative activity in U87MG cells is mediated by downregulation of cyclin D1, at least in part. Taken together, our results suggest that TFP controls Egr-1 expression, which in turn leads to a blockade of G1 cell cycle progression and an inhibition of cell proliferation.

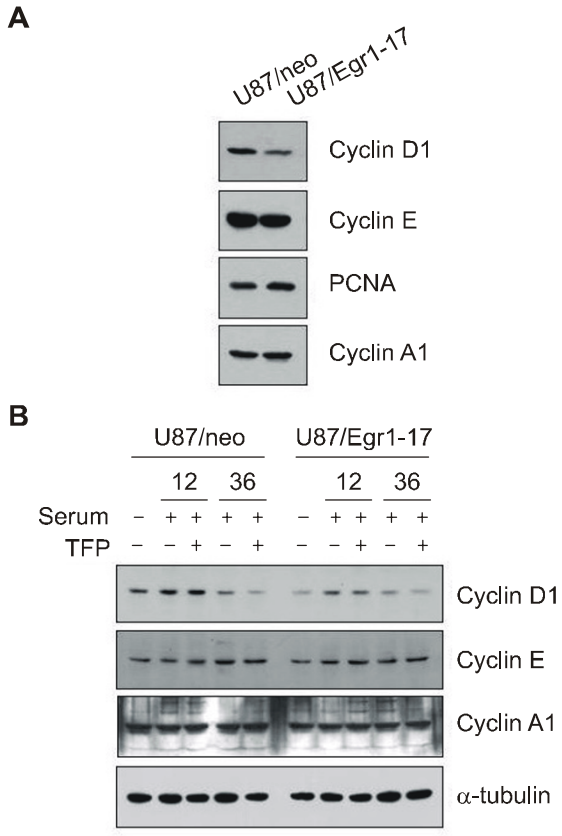

Figure 5. Effect of ectopic expression of Egr-1 on the accumulation of cyclin D1. (A) Exponentially growing U87MG transfectants were harvested and whole lysates (20 μg) were used for Western blot analysis using anti-cyclin D1, -PCNA, and -cyclin A1 antibodies. (B) Serum-starved U87MG transfectants were pretreated or not with 20 μM TFP for 30 min, then stimulated or not with 20% serum for indicated times. Whole lysates (20 mg) were subjected to Western blotting using anti-cyclin D1 or anti-cyclin E antibody. The same blot was reprobed with anti-α-tubulin antibody for internal control. Similar result was obtained from two independent experiments.

It has been reported that introduction of Egr-1 gene into HT1080 fibrosarcoma cells inhibits tumorigenic potential by induction of transforming growth factor-β1 (TGF-β1), fibronectin, and plaminogne activator inhibitor-1 (PAI-1) (Liu *et al*., 1996; 1999). Egr-1 also directly transactivates tumor suppressor gene promoters including PTEN/MMAC1/TEP1 gene (Virolle *et al*., 2001) and p53 gene (Nair *et al*., 1997; Das *et al*., 2001; Krones-Herzig *et al*., 2003), implicating the role of Egr-1 in the regulation of cell death and growth context. Thus, suppression of Egr-1 expression may be the crucial event in the development of malignant transformation. In the present study, we show that Egr-1 expression is suppressed in diverse cancer cell lines, including fibrosarcoma (HT1080), breast carcinoma (MDA MB-231, MDA MB-361, MDA MB-435) and glioma (U87MG) cell lines. This is consistent with a growing body of evidence indicating a role for the Egr-1 gene in the tumor suppressor function. We found that calmodulin antagonist TFP restores Egr-1 expression at the transcriptional level in U87MG glioma cells, and that ectopic expression of Egr-1 in U87 MG cells sensitizes the susceptibility to TFP-induced growth arrest through downregulation of cyclin D1. At present, it is not clear how Egr-1 downregulates cyclin D1 expression. Since inhibition of phosphoinositide 3-kinase (PI3K) inhibits the accumulation of cyclin D1 level (Oh *et al*., 2002), it is plausible to suggest that Egr-1 downstream target genes may deregulate the PI3K signal pathway. The identification of Egr-1 target genes which are associated with the downregulation of cyclin D1 remains to be determined.

In summary, this study demonstrates that TFP-induced elevation of Egr-1 contributes to TFP-mediated inhibition of cancer cell growth. We propose that restoration of Egr-1 function might be of value in treating some tumors in which Egr-1 gene is downregulated, such as breast cancer and glioma.

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