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Chlorpromazine activates p21$^{Waf1/Cip1}$ gene transcription via early growth response-1 (Egr-1) in C6 glioma cells

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Abbreviations: CDKN1A, cyclin-dependent kinase inhibitor 1A; CPZ, chlorpromazine; Egr-1, early growth response protein-1; PPZ, perphenazine; SRF, serum response factor; TCF, ternary complex factor; TFP, trifluoperazine

Abstract

2-Chloro-10-[3(-dimethylamino)propyl]phenothiazinemonohydrochloride (chlorpromazine) is a phenothiazine derivative used clinically to control psychotic disorders. It also exhibits an anticancer activity. Treatment with chlorpromazine (CPZ) results in cell-cycle arrest at the G2/M phase in rat C6 glioma cells. CPZ reduces the expression of cell cycle-related proteins, such as cyclin D1, cyclin A, and cyclin B1, but causes an increase in the p21$^{Waf1/Cip1}$ level. The molecular mechanism by which CPZ regulates p21$^{Waf1/Cip1}$ expression is unknown. Here, we provide evidence that CPZ activates the p21$^{Waf1/Cip1}$ gene promoter via induction of the transcription factor early growth response-1 (Egr-1) independently of p53 in C6 cells. A point mutation in the Egr-1-binding motif within the p21$^{Waf1/Cip1}$ promoter abrogated promoter inducibility due to CPZ. Forced expression of Egr-1 enhanced p21$^{Waf1/Cip1}$ promoter activity. In contrast, knockdown of endogenous Egr-1 by small interference RNA attenuated CPZ-induced p21$^{Waf1/Cip1}$ promoter activity. A chromatin immunoprecipitation assay demonstrated that Egr-1 binds to the p21$^{Waf1/Cip1}$ gene promoter. Further analysis showed that the ERK and JNK MAP kinases are required for induction of Egr-1 by CPZ. Finally, stable silencing of Egr-1 expression lead to attenuated CPZ-inducible p21$^{Waf1/Cip1}$ expression and inhibited G2/M phase cell-cycle arrest. These results demonstrate that a functional link between ERK and JNK MAP kinase pathways and p21$^{Waf1/Cip1}$ induction via Egr-1 contributes to CPZ-induced anticancer activity in C6 glioma cells.

Keywords: chlorpromazine; c-Jun N-terminal kinase; cyclin-dependent kinase inhibitor p21; early growth response-1; extracellular signal-regulated kinase; tumor suppressor protein p53

Introduction

Some cancer patients demonstrate psychological symptoms, such as pain, anxiety, psychosis, and depression with suicidal tendencies (Derogatis *et al*., 1983). Several psychotropic drugs, such as phenothiazine derivatives including chlorpromazine (CPZ), trifluoperazine (TFP), and perphenazine (PPZ), can be useful for controlling psychiatric symptoms or chemotherapy side effects in advanced cancer patients (Noyes, 1981). The pharmacological mechanism of these derivatives to diminish these symptoms is mainly attributed to their ability to block dopamine receptors (Snyder *et al*., 1974). However, these drugs possess a wide range of pharmacological actions that may interfere with calmodulin, channel proteins, DNA topoisomerase, oncogenic K-Ras membrane proteins, and other receptors (Zarnowska and Mozrzymas, 2001; Eisenberg *et al*., 2008). Phenothiazines have been reported to inhibit cell proliferation of various tumor cells (Nordenberg *et al*., 1999), probably through inhibition of the mitotic kinesin KSP/Eg5 resulting in mitotic arrest (Lee *et al*., 2007), or inhibition of PDK1/Akt signaling (Choi *et*

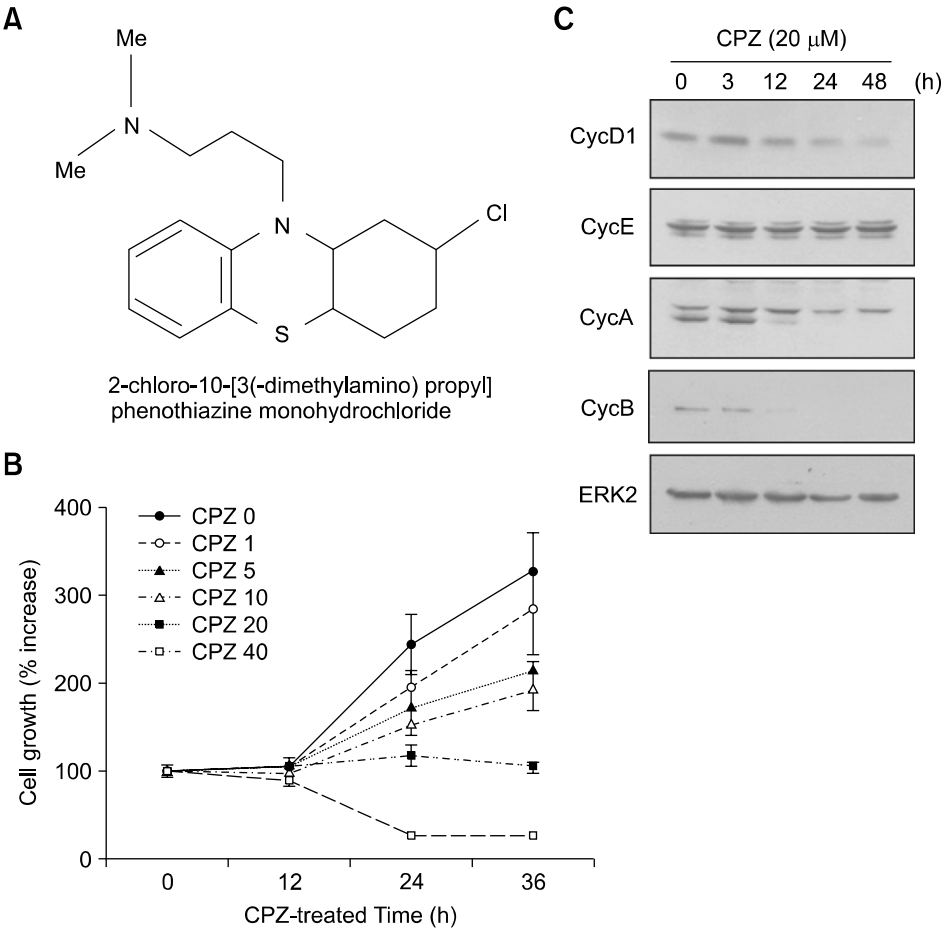

Figure 1. Effects of CPZ on cell proliferation in C6 glioma cells. (A) The chemical structure of CPZ. (B) Cell proliferation assay. C6 cells were seeded into 96-well culture plates and treated with increasing CPZ concentrations for the indicated lengths of time. The cellular proliferation rate was measured using a Cell Counting Kit-8. Data shown represent the mean ± SD of one experiment performed in triplicate. Similar results were obtained from two independent experiments. (C) C6 cells were treated with 20 μM CPZ for the indicated time periods. Cellular extracts were subjected to Western blot analysis using the indicated antibodies. Each blot represents three individual experiments.

al., 2008b).

The cyclin-dependent kinase inhibitor 1A (CDKN1A), also known as p21, Waf1, or Cip1 ($p21^{Waf1/Cip1}$), is a well-characterized cell-cycle regulator that mainly inhibits the activity of cyclin E/cdk2 (Harper *et al*., 1993) or the cyclin B/cdk1 complex (Charrier-Savournin *et al*., 2004) and negatively regulates multiple phases of cell-cycle progression (el-Deiry *et al*., 1994; Waldman *et al*., 1996; Chan *et al*., 2000; Baus *et al*., 2003). $p21^{Waf1/Cip1}$ expression was initially identified as a p53-dependent mechanism; however, multiple signals target $p21^{Waf1/Cip1}$ independently of p53 (Gartel and Tyner, 1999). CPZ exhibits anticancer activities in many types of cancer cells. However, the mechanism of action of CPZ in the regulation of $p21^{Waf1/Cip1}$ expression remains unknown.

The purpose of this study was to characterize the molecular mechanism of action of CPZ in the regulation of $p21^{Waf1/Cip1}$ expression in rat C6 glioma cells. Our results showed that CPZ activates $p21^{Waf1/Cip1}$ promoter activity via Egr-1 independently of p53. In addition, we found that the ERK and JNK MAP kinases are required for CPZ induction of Egr-1 via Ets transcription factor Elk-1. These results provide mechanistic insights into CPZ's antitumor activity and further support for antipsychotic CPZ as a potential therapeutic adjuvant for treatment of glioma.

Results

Effects of CPZ on cell proliferation and cell-cycle progression in C6 glioma cells

To study the mechanism by which CPZ inhibits cell growth, we initially assessed the anti-proliferation efficacy of CPZ using C6 glioma cells. Treatment with CPZ resulted in a concentration- and time-dependent inhibition of cell growth, compared with untreated control cells, reaching a maximum after 24 h with a 20 μM treatment (Figure 1B). At the highest concentration of 40 μM, the level of proliferation was lower than the control levels, probably due to the promotion of cell death. To determine the effect of CPZ on the expression of

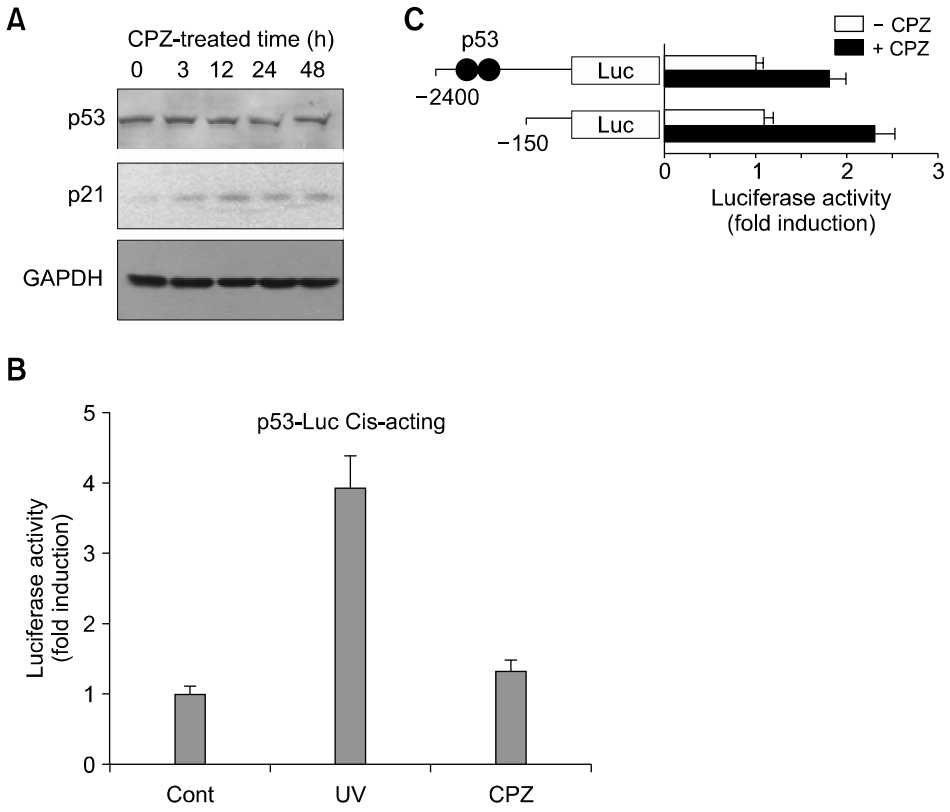

Figure 2. p53-independent activation of p21$^{Waf1/Cip1}$ promoter activity by CPZ. (A) C6 cells were treated with 20 μM CPZ for the indicated time periods. Cellular extracts were subjected to Western blot analysis using the indicated antibodies. Each blot represents three individual experiments. (B) p53-dependent transcriptional activity assay. C6 cells were transfected with 0.5 μg of 5Xp53-Luc *cis*-reporting plasmid containing five repeats of the p53 binding site, along with 50 ng of the pRL-null vector. After 48 h, the cells were irradiated with UV (40 J/m^2) or CPZ (20 μM) for an additional 8 h. (C) C6 cells were transfected with 0.5 μg of p21-Luc (-2400/+1 or -150/+38) plasmid. After 48 h, the cells were either left untreated or were treated with 20 μM CPZ for 8 h, and then analyzed for luciferase activity. The firefly luciferase activity was normalized to the *Renilla* activity. Error bars represent the mean ± SD of three independent experiments performed in triplicate.

cell cycle regulatory proteins, Western blot analysis was performed. Time-response analysis showed that the amounts of cyclin D1, cyclin A1, and cyclin B1 were considerably lower after 12 h of CPZ treatment, compared with control cultures (Figure 1C). In contrast, the cycline E level was unchanged. These results suggest that CPZ inhibits proliferation of C6 rat glioma cells, probably due to inhibition of cell-cycle progression through down-regulation of cell cycle regulatory cyclins.

CPZ activates p21$^{Waf1/Cip1}$ promoter activity independently of p53

p21$^{Waf1/Cip1}$ is an important regulator of cell-cycle progression at the G1 (Harper *et al*., 1993) and G2/M phases (el-Deiry *et al*., 1994; Gartel and Tyner, 1999; Chan *et al*., 2000; Baus *et al*., 2003). Given that accumulation of the p21$^{Waf1/Cip1}$ protein precedes reduction in other cell cycle regulatory protein levels (Figure 2A), we focused on transcriptional regulation of the p21$^{Waf1/Cip1}$ gene by CPZ. p53 is a well-known mediator of p21$^{Waf1/Cip1}$ expression, but a role for p53 in CPZ-induced p21 expression was discounted because (i) the amount of the p53 protein was not changed by CPZ (Figure 2A), (ii) p53-dependent transcriptional activity was not activated by CPZ (Figure 2B), and (iii) CPZ stimulated the promoter activity of the p21-Luc(-150/+38) construct, despite lacking a p53 binding site (Figure 2C). Thus, we suggest that CPZ-induced accumulation of p21$^{Waf1/Cip1}$ is mediated at the transcriptional level independently of p53 and that the proximal region within -150 bp of the p21$^{Waf1/Cip1}$ gene promoter is necessary for CPZ-induced activation of p21$^{Waf1/Cip1}$ expression.

CPZ activates p21$^{Waf1/Cip1}$ promoter activity through an Egr-1 response element

Previous studies have demonstrated that Egr-1 transactivates the p21$^{Waf1/Cip1}$ promoter through a putative Egr-1-response element located between -58 and -51 in the p21$^{Waf1/Cip1}$ promoter (Ragione *et al*., 2003; Kim *et al*., 2007). To evaluate whether an Egr-1-response element between -58 and -51 is involved in mediating CPZ-induced p21$^{Waf1/Cip1}$ promoter activity, we used the Egr-1 mutant construct p21-Luc(-150/+38)mtEgr-1 (Kim *et al*., 2007), which disrupted the core sequence of the Egr-1 binding motif (*gggg to tttt*). Point mutations in the core sequences of the Egr-1 response element resulted in a drastic reduction in CPZ responsiveness (Figure 3A). To further assess the role of Egr-1 in CPZ-induced activation of the p21$^{Waf1/Cip1}$ promoter, the p21-Luc(-150/+38) reporter lacking

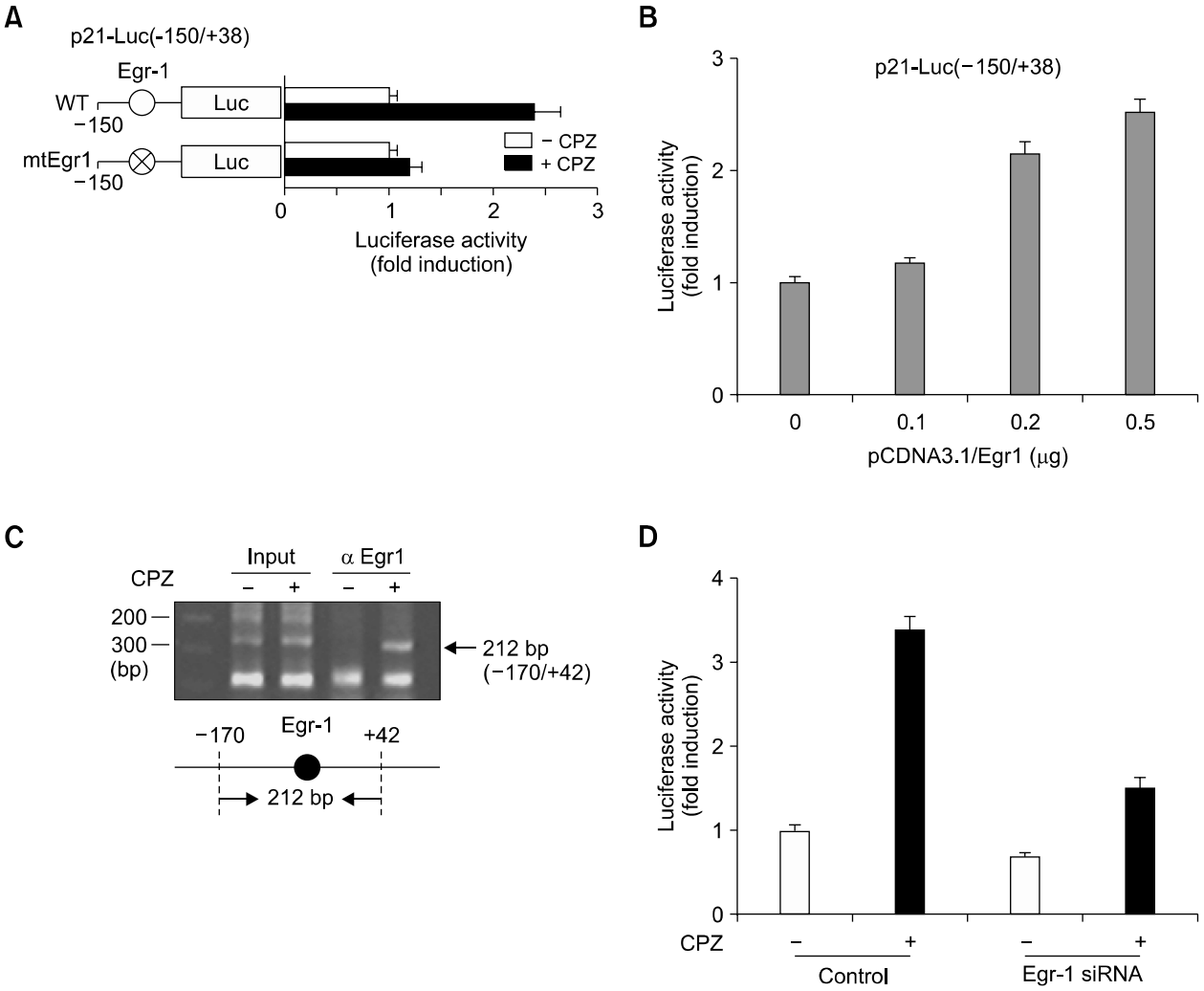

Figure 3. The role of an Egr-1 response element in CPZ-induced activation of $p21^{Waf1/Cip1}$ promoter activity. (A) Promoter reporter assay. C6 cells were transfected with p21-Luc(-150/+38) (wild type; WT) or the Egr-1 mutant construct (mtEgr1). After 48 h of transfection, cells were either left untreated or treated with 20 μM CPZ for 8 h, and then luciferase activities were measured. The firefly luciferase activity was normalized to *Renilla* activity. Error bars represent the mean ± SD of three independent experiments performed in triplicate. (B) C6 cells were co-transfected with 0.5 μg p21-Luc(-150/+38) and the Egr-1 expression plasmid (pcDNA3.1/Egr1) at increasing concentrations. After 48 h, cells were collected and measured for luciferase activity as in (A). (C) ChIP assay. After C6 cells were treated with 20 μM CPZ for 2 h, chromatins were immunoprecipitated using rabbit anti-Egr-1 antibody (αEgr1), and PCR amplified using primers targeted to the promoter elements (-170 to +42) of the $p21^{Waf1/Cip1}$ gene. Total genomic DNA obtained from C6 cells was amplified by using the same primers as a control (Input). (D) C6 cells were transiently transfected with 0.5 μg of p21-Luc(-150/+38) plasmid, along with pSilencer/scrambled (Control) or pSilencer/siEgr1 (Egr-1 siRNA), as indicated. The firefly luciferase activity was normalized to the *Renilla* activity. Error bars represent the mean ± SD of three independent experiments performed in triplicate.

the p53-binding site was transfected into C6 cells together with the expression plasmid for Egr-1. $p21^{Waf1/Cip1}$ promoter activity increased as the amount of transfected Egr-1 increased (Figure 3B), suggesting that Egr-1 mediates $p21^{Waf1/Cip1}$ transcription in response to CPZ treatment.

To determine binding of Egr-1 to the $p21^{Waf1/Cip1}$ promoter, we cross-linked the cellular protein with DNA by using formaldehyde after treatment with CPZ. The cross-linked DNA-protein complexes were subjected to chromatin immunoprecipitation (ChIP) using rabbit anti-Egr-1 antibody, and the immunoprecipitated DNA was amplified using PCR with primers targeted to the promoter elements (from -170 to +42) of the $p21^{Waf1/Cip1}$ gene. The same amount of genomic DNA was used as an input control. After CPZ treatment, we amplified 212-bp fragments of the $p21^{Waf1/Cip1}$ promoter region using ChIP DNA. Comparison with a non-stimulated control (Figure 3C) indicated that Egr-1 forms a complex with the $p21^{Waf1/Cip1}$ gene promoter within the chromatin.

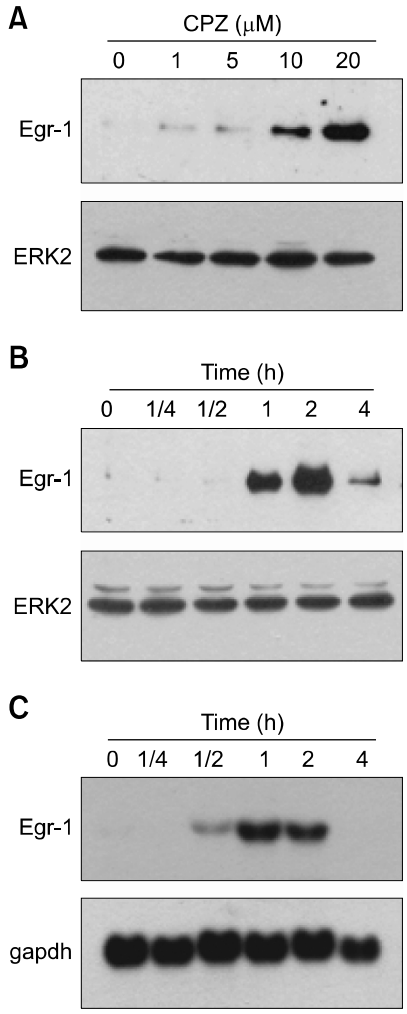

Figure 4. Up-regulation of Egr-1 expression by CPZ. (A, B) Western blot analysis. Serum-starved (0.5% serum for 24 h) C6 cells were treated with different concentrations of CPZ for 1 h (A) or 20 μM CPZ for different times (B). Cellular extracts were analyzed using Western blotting using the anti-Egr-1 antibody. The same blot was re-probed with anti-ERK2 antibody as an internal control. Each blot represents three individual experiments. (C) Northern blot analysis. Serum-starved (0.5% serum for 24 h) C6 cells were treated with 20 μM CPZ for the indicated time periods. Total RNA (10 μg) was isolated and Northern blot analysis was performed with the ^{32}P-labeled Egr-1 cDNA probe. *gapdh* mRNA was used as a control to verify the amount of RNA in each lane. Each blot represents at least three individual experiments.

To verify the functional role of Egr-1 in CPZ-induced activation of p21$^{Waf1/Cip1}$ promoter activity, we used an RNA interference approach. The expression plasmid for Egr-1 shRNA (pSilencer/siEgr-1) and the p21-Luc(-150/+38) promoter reporter were co-transfected into C6 cells. The silencing of endogenous Egr-1 significantly attenuated CPZ-induced p21$^{Waf1/Cip1}$ promoter activity (Figure 3D), suggesting that Egr-1 is required for CPZ activation of p21$^{Waf1/Cip1}$ transcription. These results suggest that the Egr-1 response element in the proximal region of the p21$^{Waf1/Cip1}$ gene is necessary for CPZ inducibility in C6 cells.

CPZ up-regulates Egr-1 expression at the transcriptional level

We investigated whether CPZ induces Egr-1 expression in C6 glioma cells. Serum-starved C6 cells were treated with increasing concentrations of CPZ for 2 h, after which the amount of Egr-1 protein was measured using Western blot analysis. CPZ treatment markedly increased Egr-1 protein amounts in a concentration-dependent manner (Figure 4A). A time-course study showed that CPZ increased the abundance of the Egr-1 protein with a peak at 2 h, and maintained the level for up to 4 h (Figure 4B). Northern blot analysis showed that the abundance of Egr-1 mRNA rapidly increased within 30 min after CPZ treatment with a maximum observed at 1 h, whereas the gapdh mRNA level remained constant (Figure 4C).

CPZ-induced Egr-1 and p21$^{Waf1/Cip1}$ expressions are mediated by ERK and JNK MAP kinases

Elk-1 is a ternary complex factor (TCF) that forms a complex with the serum response factor (SRF) on the SRE of the Egr-1 gene promoter, and plays a crucial role in transcriptional activation of the Egr-1 gene (Watson *et al*., 1997; Schratt *et al*., 2001) Elk-1 is phosphorylated and activated by multiple MAP kinase pathways in response to a variety of extracellular stimuli (Treisman, 1994; Yang *et al*., 1998; Yang and Sharrocks, 2006). To determine whether CPZ activation of Elk-1 is induced by stimulating MAP kinase signaling, serum-starved C6 cells were treated with CPZ for various time intervals, and then the activation status of the MAP kinase pathway was measured using phospho-specific antibodies. The phosphorylated levels of ERK1/2 and JNK1/2 MAP kinases increased in a time-dependent manner in response to CPZ treatment (Figure 5A). In contrast, the total amount of these proteins did not change, demonstrating an actual increase in their phosphorylation status indicative of increased activity. However, the phosphorylation of p38 MAP kinase decreased below basal levels within 15 min of CPZ treatment. These results suggest that CPZ modulates different MAP kinase pathways.

To assess a possible regulatory relationship between MAP kinase activation and Egr-1 expression due to CPZ, we examined the effect of MAP kinase inhibitors on accumulation of the Egr-1

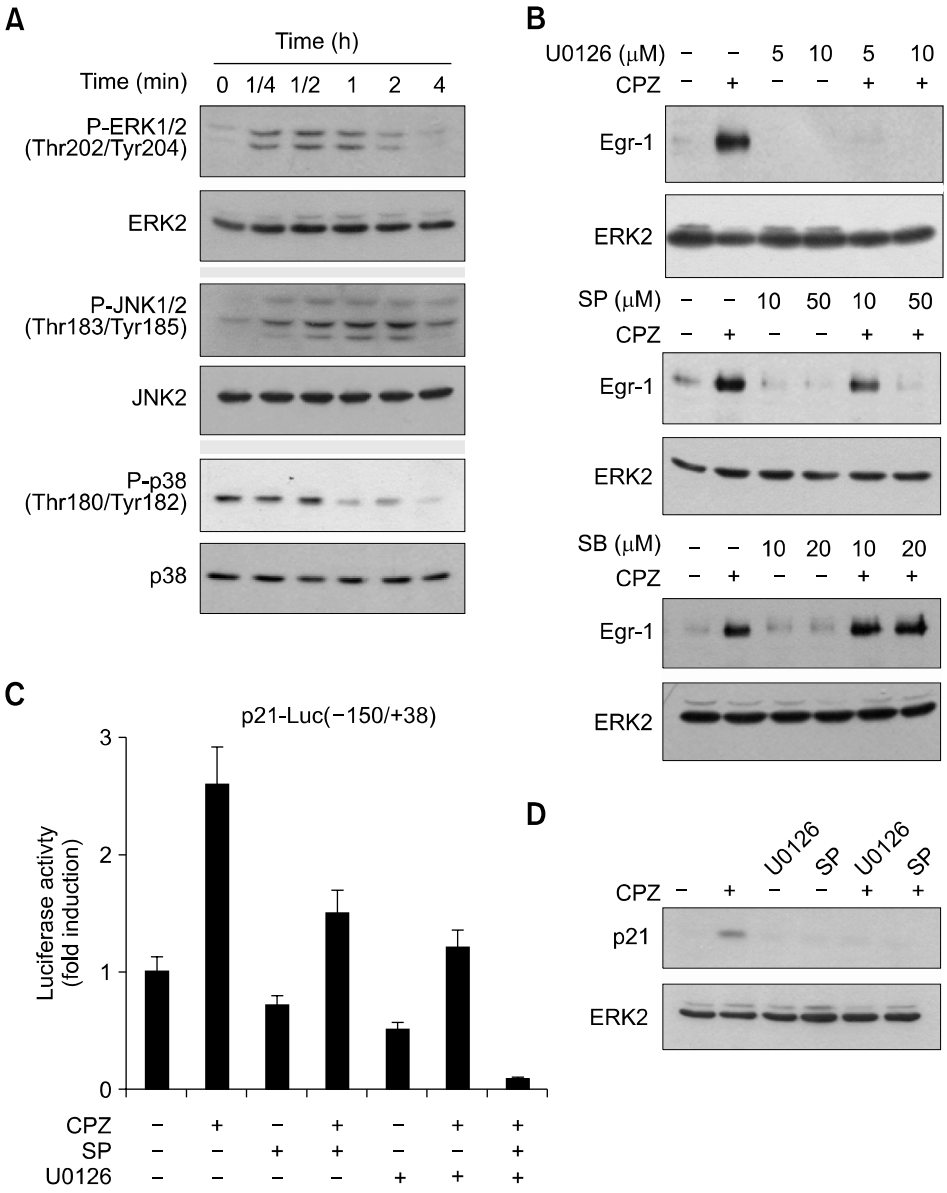

Figure 5. Involvement of the ERK and JNK MAP kinase pathways in CPZ-induced $p21^{Waf1/Cip1}$ expression. (A) C6 cells were serum-starved with 0.5% serum for 24 h, and then treated with 20 μM CPZ for various lengths of time. Western blotting was performed with total protein extracts using antibodies against phospho-ERK1/2 (Thr 202/Tyr 204), phospho-JNK1/2 (Thr183/Tyr185), or phospho-p38 kinase (Thr180/Tyr182), and then probed with an antibody against total proteins as an internal control. Each blot represents at least three separate experiments. (B) Serum-starved C6 cells were left untreated or treated with U0126, SP600125 (*SP*), or SB203580 for 30 min, followed by treatment with 20 μM CPZ for an additional 2 h, as indicated. Total cell lysates were prepared and subjected to Western blotting with anti-Egr-1 antibody. The same blot was re-probed with anti-ERK2 antibody as an internal control. Each blot represents at least three separate experiments. (C) C6 cells were transfected with p21-Luc(-150/+38). After 48 h, cells were left untreated or treated with 20 μM CPZ, SP600125 (*SP*), or U0126, as indicated, for an additional 8 h, and then luciferase activities were measured as in Figure 2C. Error bars represent the mean ± SD of three independent experiments performed in triplicate. (D) Serum-starved C6 cells were left untreated or treated with 10 μM U0126 or 50 μM SP600125 (*SP*) for 30 min, followed by treatment with 20 μM CPZ for an additional 24 h, as indicated. Total cell lysates were prepared and subjected to Western blotting with anti-p21 antibody. The same blot was re-probed with anti-ERK2 antibody as an internal control. Each blot represents at least three individual experiments.

protein. Pretreatment with the MEK inhibitor U0126 or the JNK inhibitor SP600125, but not with the p38 kinase inhibitor SB203580, blocked the CPZ-induced accumulation of the Egr-1 protein (Figure 5B). Furthermore, treatment with U0126 or SP600125 blocked CPZ-induced $p21^{Waf1/Cip1}$ promoter activity (Figure 5C) and accumulation of the $p21^{Waf1/Cip1}$ protein (Figure 5D). These results

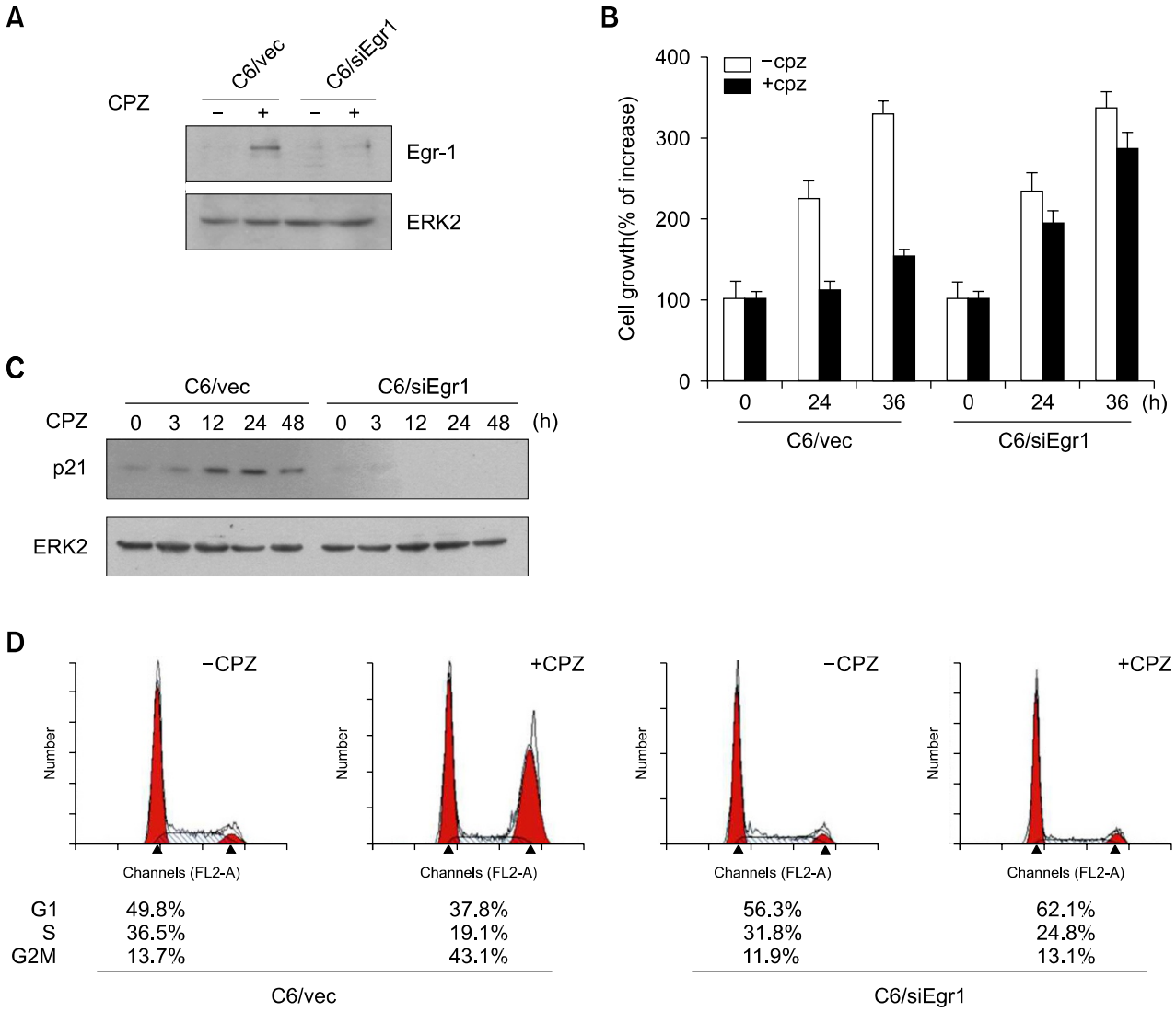

Figure 6. Effects of stable silencing of endogenous Egr-1 by siRNA on CPZ-induced p21$^{Waf1/Cip1}$ expression. (A) Knockdown of endogenous Egr-1 expression was verified in stable transfectants of C6 cells expressing Egr-1 siRNA (C6/siEgr1), as compared with control cells (C6/vec). (B) A cell proliferation assay was performed in C6/vec and C6/siEgr1 cells as in Figure 1B. The data shown represent the mean ± SD for one experiment performed in triplicate. Similar results were obtained from two other independent experiments. (C) C6/vec and C6/siEgr1 cells were left untreated or treated with 20 μM CPZ for the indicated time periods. Cells were collected and subjected to Western blot analysis for p21$^{Waf1/Cip1}$. The same blot was re-probed with anti-ERK2 antibody as an internal control. Each blot represents at least three individual experiments. (D) Exponentially growing C6/vec and C6/siEgr-1 cells were treated with 20 μM CPZ. After 24 h, the cells were harvested and the DNA content was analyzed using FACS analysis. The *x*-axis represents DNA content. The y-axis represents the number of cells. The percentages of the cell population at each phase of the cell cycle are indicated in each histogram. Similar results were obtained from two other independent experiments.

demonstrate that ERK- and JNK-induced Elk-1 transactivation due to CPZ is functionally linked to induction of p21$^{Waf1/Cip1}$ expression via Egr-1.

Stable knockdown of Egr-1 expression attenuates CPZ-induced p21$^{Waf1/Cip1}$ expression

To further corroborate the functional significance of Egr-1 in activation of the p21$^{Waf1/Cip1}$ gene due to CPZ, we evaluated the effect of stable silencing of endogenous Egr-1 expression on CPZ-induced growth inhibition. Stable knockdown of Egr-1 by RNA interference in C6 cells (C6/siEgr1) was verified using Western blot analysis (Figure 6A). Knock-down of endogenous Egr-1 caused resistance to CPZ-induced inhibition of cell growth, compared with control C6/vec cells (Figure 6B), and prevented CPZ induction of p21$^{Waf1/Cip1}$ expression (Figure 6C). Furthermore, silencing of Egr-1 expression abrogated CPZ-induced accumulation

of G2/M arrest (Figure 6D).

Discussion

CPZ is a phenothiazine derivative that is widely used as a neuroleptic drug for control of psychotic disorders. In addition to antipsychotic actions, CPZ also displays effective antiproliferative activity in numerous types of cancer cells. In C6 cells, we found that treatment with CPZ resulted in cell-cycle arrest at the G2/M phase with a concomitant decrease in the G1 cell population. In addition, CPZ reduced expression of the cell cycle-related proteins cyclin D1, cyclin A, and cyclin B1, but promoted an accumulation of the $p21^{Waf1/Cip1}$ protein. $p21^{Waf1/Cip1}$ is an important regulatory element for cell cycle arrest at the G1 or G2/M phase, and its expression is regulated by diverse external stimuli (Harper *et al*., 1993; el-Deiry *et al*., 1994; Waldman *et al*., 1996; Chan *et al*., 2000; Baus *et al*., 2003; Charrier-Savournin *et al*., 2004). It is well established that $p21^{Waf1/Cip1}$ transcription is mediated by the tumor suppressor p53 (El-Deiry *et al*., 1993). However, multiple lines of evidence demonstrate that several transcription factors other than p53, including Egr-1, can bind and activate the $p21^{Waf1/Cip1}$ promoter (Gartel and Tyner, 1999; Ragione *et al*., 2003; Kim *et al*., 2007; Choi *et al*., 2008a). However, the molecular mechanism by which CPZ up-regulates $p21^{Waf1/Cip1}$ expression is unknown. This study focused on the regulatory mechanism of $p21^{Waf1/Cip1}$ induction due to CPZ in C6 cells.

CPZ stimulated $p21^{Waf1/Cip1}$ promoter activity without significantly affecting the expression and *cis*-acting activity of p53 in C6 cells. Thus, we assumed that CPZ-induced $p21^{Waf1/Cip1}$ expression is independent of p53. To test this possibility, we analyzed a response element of the $p21^{Waf1/Cip1}$ promoter. A luciferase reporter assay was used with a deletion mutant of the $p21^{Waf1/Cip1}$ promoter construct that lacked a p53 binding site. Results showed that CPZ still induced comparable promoter reporter activity, compared to the full-length $p21^{Waf1/Cip1}$ promoter construct. This suggested that p53 does not play a central role in CPZ-induced $p21^{Waf1/Cip1}$ promoter activity. Instead, we found that transcription factor Egr-1 mediates CPZ-induced $p21^{Waf1/Cip1}$ expression in C6 cells. The putative consensus Egr-1 binding sequence is located in a region from -58 to -51 within the $p21^{Waf1/Cip1}$ gene (Ragione *et al*., 2003). By using site-directed mutational analysis of the putative Egr-1-binding sequence within the $p21^{Waf1/Cip1}$ promoter, we were able to define an Egr-1-binding site as a cis-acting element responsible for CPZ-inducible promoter activity. A chromatin immunoprecipitation assay revealed that Egr-1 is the transcription factor binding to the $p21^{Waf1/Cip1}$ gene in response to CPZ treatment. The direct role of Egr-1 in regulation of CPZ-induced $p21^{Waf1/Cip1}$ expression was further shown by exogenous expression of Egr-1. Stable knock-down of endogenous Egr-1 using siRNA targeted to Egr-1 mRNA attenuated CPZ-induced $p21^{Waf1/Cip1}$ accumulation and G2/M cell cycle arrest. Thus, Egr-1 directly regulates transcription of the $p21^{Waf1/Cip1}$ gene via an Egr-1 binding cis-acting response element. Our results agree with earlier reports regarding the role of Egr-1 in tamoxifen-induced up-regulation of $p21^{Waf1/Cip1}$ in MDA-MB-361 breast cancer cells (Kim *et al*., 2007), in resveratrol-induced up-regulation of $p21^{Waf1/Cip1}$ in K562 leukemia cells (Ragione *et al*., 2003), and in curcumin-induced $p21^{Waf1/Cip1}$ expression in U-87MG human glioma cells (Choi *et al*., 2008a).

Egr-1 is a Cys2-His2 type zinc finger-containing transcription factor that regulates multiple tumor suppressors, including $p21^{Waf1/Cip1}$, p53, TGFβ1, PTEN, fibronectin, Gadd45, and Sox18 (Virolle *et al*., 2001; Krones-Herzig *et al*., 2003; Thyss *et al*., 2005; Baron *et al*., 2006; Petrovic *et al*., 2010). Thus, Egr-1 appears to be a tumor suppressor. In contrast, Egr-1 plays an essential role in prostate tumor epithelial cells (Baron *et al*., 2003; Virolle *et al*., 2003). Previously, we reported that Trifluoperazine (TFP), a phenothiazine derivative, upregulates Egr-1 expression in cells (Shin *et al*., 2004), suggesting that Egr-1 expresses growth suppressor activity, at least in gliomas. Introduction of functional Egr-1 into human glioma primary cells results in growth arrest and eventual cell death (Calogero *et al*., 2004).

Germline *TP53* mutations are frequently associated with glioma, particularly high-grade glioblastoma multiforme (Li *et al*., 1995), and loss of p53 function affects cellular response to radiation or chemotherapy. Adenovirus-based overexpression of Egr-1 almost completely abolishes glioma cell growth, regardless of the mutational status of the p53 gene (Calogero *et al*., 2004). Our results illustrate a potential use of the old antipsychotic agent CPZ as a chemopreventive and therapeutic supplement for human glioma. Furthermore, CPZ is widely available and can readily pass through the blood-brain barrier, making it an attractive candidate as an anticancer agent in central nervous system tumors.

In summary, our study provides further insight into the molecular mechanism of CPZ anticancer activity. CPZ induction of Egr-1 via the ERK and

JNK MAP kinase pathways plays an important role in CPZ-induced p21$^{Waf1/Cip1}$ expression independently of p53. CPZ appears to be an attractive adjuvant for various anticancer agents in p53-mutated human gliomas.

Methods

Cell culture and reagents

Rat C6 glioma cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT). Chlorpromazine (CPZ; Figure 1A) was purchased from Sigma-RBI (Natick, MA). Antibodies specific to phospho-ERK1/2 MAP kinase (Thr202/Tyr204), phospho-JNK1/2 (Thr183/Tyr185), and phosphor-p38 MAP kinase (Thr180/Tyr182) were obtained from Cell Signaling Technology (Beverly, MA). Antibodies for Egr-1, GAPDH, and ERK2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The Dual-Glo^{TM} Luciferase Assay System for firefly and *Renilla* luciferase activities was purchased from Promega (Madison, WI). pRL-null plasmids encoding *Renilla* luciferase were purchased from Promega (Madison, WI).

Cell proliferation assay

C6 cells seeded into 96-well plates (2×10^3) were treated with CPZ at increasing concentrations for different time periods (0, 12, 24, and 36 h). The proliferation rate was determined by using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD) with water-soluble tetrazolium salt, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-*2H*-tetrazolium, monosodium salt], as a substrate.

Cell cycle analysis

Cellular DNA content was analyzed using flow cytometry as previously described (Choi *et al*., 2008a). Fluorescence was measured and analyzed using a FACSCalibur Flow Cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA).

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 of leupeptin, and 1 mM phenylmethylsulphonyl fluoride. Western blotting was performed as described previously (Choi *et al*., 2008a).

Plasmid DNAs

Generation of the deletion construct of the p21$^{Waf1/Cip1}$ promoter and mutagenesis to disrupt the Egr-1 binding site in the p21$^{Waf1/Cip1}$ promoter are described elsewhere (Kim *et al*., 2007). The full-length and internal deletion constructs of the Egr-1 promoter expressing firefly luciferase were provided by Dr. H. Eibel (Department of Orthopedic Surgery, Research Laboratory University of Tubingen Medical Center, Tubingen, Germany) and are described elsewhere (Aicher *et al*., 1999).

Transient transfection and luciferase reporter assay

For promoter assay, C6 glioma cells were seeded into 12-well plates and transfected with 0.5 μg of Egr-1 or p21$^{Waf1/Cip1}$ promoter constructs using LipofectAMINE 2000 reagents (Invitrogen Life Technologies, San Diego, CA) according to the manufacturer's instructions. To monitor the transfection efficiency, the pRL-null plasmid (50 ng) encoding *Renilla* luciferase (Promega, Madison, WI) was included in all samples. At 48-h post-transfection, cells were treated with CPZ. After 6 to 12 h, the firefly and Renilla luciferase activities were measured sequentially from a single sample using the Dual-Glo^{TM} Luciferase Assay System. Luminescence was measured using a luminometer (Centro LB960; Berthold Tech, Bad Wildad, Germany) as previously described. (Kim *et al*., 2007)

Chromatin immunoprecipitation (ChIP) assay

C6 cells were either untreated or treated with 20 μM CPZ for 12 h. Protein-to-DNA cross-linking was performed by adding formaldehyde directly to the culture medium to obtain a final concentration of 1%. Preparation of crossed-linked chromatin and chromatin immunoprecipitation with anti-Egr-1 antibody was performed as described previously (Shin *et al*., 2009). For PCR amplification of promoter sequences of the p21$^{Waf1/Cip1}$ gene, promoter-specific primers were used (5'-CTGGC-CTGCTGGAACTC-3' as a forward primer, -170/-154, and 5'-CTCCACAAGGAACTGACTT-3' as a reverse primer, +42/+24).

Northern blot analysis

Total RNA (10 μg) for each sample was separated using electrophoresis on formaldehyde/agarose gel, and transferred to a Hybond N$^+$ nylon membrane (Amersham Pharmacia Biotech.). Northern blotting was performed with a [γ-^{32}P]dCTP-labeled Egr-1 cDNA probe, followed by hybridization with a GAPDH cDNA probe, as described previously (Choi *et al*., 2008a).

Expression of Egr-1 siRNA

Generation of the expression plasmid for short hairpin RNA (shRNA)-encoding small interfering RNA (siRNA) targeted to *Egr-1* mRNA (pSilencer/siEgr1) is described elsewhere (Kim *et al*., 2007; Choi *et al*., 2008a). Stable C6 transfectants expressing Egr-1 siRNA are also described elsewhere (Choi *et al*., 2008a).

Statistical analysis

Each experiment was repeated at least three times. Data were plotted as the mean ± SD. Student's *t*-test was used for comparisons, and a value of $P < 0.05$ was considered

significant.

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