

Triptolide downregulates human GD3 synthase (hST8Sia I) gene expression in SK-MEL-2 human melanoma cells

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DOI 10.3858/emm.2010.42.12.088

Accepted 8 November 2010
Available Online 11 November 2010

Abbreviation: TPL, triptolide

Abstract

In this study, we have shown that gene expression of human GD3 synthase (hST8Sia I) is suppressed by triptolide (TPL) in human melanoma SK-MEL-2 cells. To elucidate the mechanism underlying the downregulation of hST8Sia I gene expression in TPL-treated SK-MEL-2 cells, we characterized the TPL-inducible promoter region within the hST8Sia I gene using luciferase constructs carrying 5'-deletions of the hST8Sia I promoter. Functional analysis of the 5'-flanking region of the hST8Sia I gene demonstrated that the -1146 to -646 region, which contains putative binding sites for transcription factors c-Ets-1, CREB, AP-1 and NF- κ B, functions as the TPL-inducible promoter of hST8Sia I in SK-MEL-2 cells. Site-directed mutagenesis and ChIP analysis indicated that the NF- κ B binding site at -731 to -722 is crucial for TPL-induced suppression of hST8Sia I in SK-MEL-2 cells. This suggests that TPL induces down-regulation of hST8Sia I gene expression through NF- κ B activation in human melanoma cells.

Keywords: α -N-acetylneuraminase α -2,8-sialyltransferase; gene expression regulation; melanoma; NF- κ B; triptolide

Introduction

Gangliosides, the sialic acid (NeuAc)-containing glycosphingolipids, are component molecules of the plasma membrane outer leaflet in vertebrate cells (Svennerholm, 1980). They play important roles in a large variety of biological processes, such as cell-cell interaction, adhesion, cell differentiation, growth control, and receptor function (Hakomori and Igarashi, 1993; Varki, 1993). In addition, gangliosides are known to play a pivotal role in tumor formation and progression (Hakomori, 1996) and thus have been studied as tumor markers of neuroectoderm-derived malignant cells (Hakomori, 1981), melanoma cells (Portoukalian *et al.*, 1979; Old, 1981) and neuroblastoma cells (Cheung *et al.*, 1985).

Gangliosides, especially GD3, are highly expressed in human melanoma tissues and melanoma cell lines (Dippold *et al.*, 1980; Pukel *et al.*, 1982). In particular, GD3 and GD2 have been considered important molecules not only as the tumor markers, but also as targets of antibody therapy (Cheung *et al.*, 1985; Fukuda *et al.*, 1998; Zhang *et al.*, 1998). GD3 synthase (ST8Sia I, EC 2.4.99.8) is a key enzyme for the synthesis of whole b-series gangliosides including GD2 as well as GD3 itself (Thampoe *et al.*, 1989). In general GD3 expression in cells is regulated at the transcriptional level of ST8Sia I gene (Haraguchi *et al.*, 1994; Sasaki *et al.*, 1994). Regulatory mechanisms for GD3 expression in human melanoma cells are of particular importance, since GD3 is well known as a human melanoma-specific antigen (Dippold *et al.*, 1980; Yamaguchi *et al.*, 1987). Recently, we elucidated for the first time the transcriptional regulation mechanism of human GD3 synthase (hST8Sia I) expression necessary for GD3 synthesis highly expressed in human melanoma cells (Kang *et al.*, 2007).

Triptolide (TPL), a diterpenoid triepoxide, is the major biologically active component of the Chinese medicine herb *Tripterygium wilfordii* Hook f. that has been used for centuries to treat inflammation and autoimmune diseases (Chen *et al.*, 2001; Qiu and Kao, 2003; Brinker *et al.*, 2007). In recent

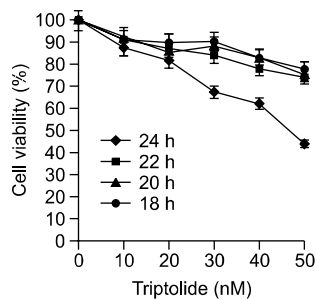


Figure 1. Effect of TPL on cell viability of SK-MEL-2 cells. Cells were treated with the indicated concentrations of TPL for various times. The cell viability was measured by MTT assay. Data were normalized by taking 100% as a viability of non-treated cells. Values were expressed as means \pm SEM of three independent experiments and represented as % of control cell viability.

years, numerous reports demonstrated that TPL could inhibit the proliferation of cancer cells *in vitro* and reduce the growth and metastasis of some tumors *in vivo* (Yang *et al.*, 2003; Carter *et al.*, 2006; Brinker *et al.*, 2007; Phillips *et al.*, 2007; Johnson *et al.*, 2009; Shi *et al.*, 2009; Wang *et al.*, 2009; Zhu *et al.*, 2009b; Chen *et al.*, 2010; Pan, 2010). TPL has shown anti-proliferative and apoptotic effects in a broad spectrum of cancer cells (Pan, 2010). Although anti-cancer effects of TPL by inhibiting the activity of RNA polymerase have been recently reported (Pan, 2010), the exact molecular targets and the precise mechanism of TPL action are still unknown. In addition, the effect of TPL on human melanoma cells showing high levels of GD3 and hST8Sia I expression has not yet been studied.

Recently, we observed a dramatic suppression of hST8Sia I mRNA expression in the presence of TPL in human melanoma SK-MEL-2 cells. In this study, the promoter region to direct down-regulation of hST8Sia I gene transcription by TPL in SK-MEL-2 cells was functionally characterized. The present results clearly indicate that the NF- κ B binding site of the hST8Sia I promoter plays an important role in down-regulation of hST8Sia I gene expression induced by TPL in human melanoma cells.

Results

Effect of TPL on cell proliferation

Prior to the investigation into the regulatory effect of TPL on hST8Sia I expression, we first determined the cytotoxicity of TPL in SK-MEL-2 cells using MTT assay. Relative cell viability was determined by the amount of MTT converted into

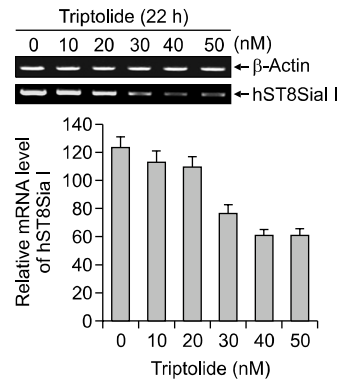


Figure 2. RT-PCR analysis of hST8Sia I mRNA expression levels in TPL-treated SK-MEL-2 cells. Total RNA from SK-MEL-2 cells was isolated after TPL treatment for 22 h at different concentrations (0, 10, 20, 30, 40, 50 nM) and hST8Sia I mRNA was detected by RT-PCR. As an internal control, parallel reactions were performed to measure levels of the housekeeping gene β -actin. The densitometric intensity of hST8Sia I band was shown in the panel below. Data represent the relative values \pm SD of three independent experiments and the mean values from each experiment were compared using ANOVA followed by Duncan's tests. Values not sharing the same letter were significantly different from one another ($P < 0.05$).

formazan salt. SK-MEL-2 cells were treated with TPL in various concentrations for 18 to 24 h. As shown Figure 1, TPL treatment for 22 h at 50 nM had modest cytotoxic effect on cells, whereas 24 h treatment at the same concentration of TPL showed about 45% decrease in cell viability.

Effect of TPL on hST8Sia I mRNA expression in SK-MEL-2 cells

Previous studies have shown that the level of hST8Sia I mRNA expression is distinctively high in human melanoma cell line, such as SK-MEL-2 (Kang *et al.*, 2007) and SK-MEL-28 (Yamashiro *et al.*, 1995; Ruan *et al.*, 1999). To investigate whether TPL can affect hST8Sia I gene expression in SK-MEL-2 cells, cells were treated for 22 h with various concentration of TPL. The hST8Sia I mRNA levels were analyzed by semi-quantitative RT-PCR. Treatment of cells with TPL significantly decreased the levels of hST8Sia I mRNA expression at the concentration of 50 nM (Figure 2). On the other hand, β -actin mRNA expression, as an internal standard, was not affected by treatment of TPL. These results clearly showed that the expression of hST8Sia I was down-regulated by TPL.

Analysis of transcriptional activity of hST8Sia I promoter by TPL in SK-MEL-2 cells

To determine whether the 5'-flanking sequence

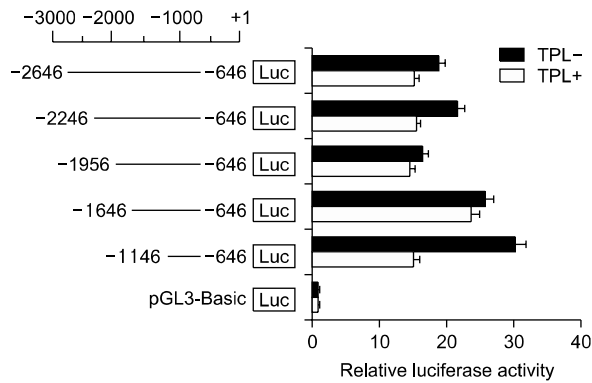


Figure 3. Deletion analysis of hST8Sia I promoter in SK-MEL-2 cells before and after TPL treatment. A schematic representation of DNA constructions containing three equal lengths from different starts of the 5'-flanking region of hST8Sia I gene linked to the luciferase reporter gene is presented. The length sizes are shown and the translation start site was indicated as +1. pGL3-Basic without any promoter and enhancer was used as a negative control. Each construct was co-transfected into SK-MEL-2 cells with pRL-TK as an internal control. The transfected cells were incubated in the presence (open bar) or absence (solid bar) of 50 nM TPL for 22 h. Relative firefly luciferase activity was measured using the Dual-Luciferase Reporter Assay System, and all firefly activity was normalized to the *Renilla* luciferase activity derived from pRL-TK. The values represent the means \pm SD of three independent experiments with triplicate measurements.

of the hST8Sia I gene contained a TPL-responsive promoter, we prepared luciferase constructs carrying serial 5' deletions of the hST8Sia I promoter, transfected them into SK-MEL-2 cells, and then treated the transfected cells with TPL. We monitored the subsequent expression of the luciferase reporter gene using the dual-luciferase reporter assay system, after which we measured luciferase activity with a luminometer. As shown in Figure 3, cells harboring the pGL3-1146/-646 construct showed a remarkable decrease in luciferase activity after TPL treatment, about two-fold higher than untreated transfected cells. In contrast, TPL stimulation did not alter significantly the luciferase activity in cells expressing the pGL3-basic (negative control) or other 5'-deleted hST8Sia I promoter constructs. Based on this result, the effects of different concentrations of TPL on the hST8Sia I promoter activity were investigated using the pGL3-1146/-646 construct in SK-MEL-2 cells. In Figure 4, we demonstrated that TPL suppressed hST8Sia I promoter activity in a dose-dependent manner, with 50% decrease at approximately 30 nM compared to the control (0 nM). These results clearly suggest that the region containing nucleotides -1146 to -646 functioned as the TPL-suppressive promoter of hST8Sia I in SK-MEL-2 cells.

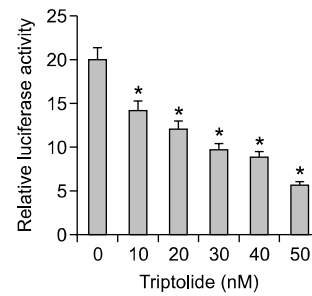


Figure 4. Effects of TPL on transcriptional activity of hST8Sia I in SK-MEL-2 cells. The pGL3-1146/-646 was co-transfected into SK-MEL-2 cells with pRL-TK as the internal control. The transfected cells were incubated in the presence of the indicated concentrations (0, 10, 20, 30, 40, 50 nM) of TPL for 22 h. All firefly activity was normalized to the *Renilla* luciferase activity derived from pRL-TK. The values represent the mean \pm SD for three independent experiments with triplicate measurements. * $P < 0.05$ versus non-treatment.

Identification of TPL-responsive element in nucleotide -1146 to -646 region of hST8Sia I promoter

Previous studies conducted in our lab demonstrated that the region from -1146 to -646 contained putative binding sites such as c-Ets-1, AP-1, CREB and NF- κ B binding sites (Kang *et al.*, 2006, 2007). To determine whether these binding sites contributed to TPL-suppressed expression of hST8Sia I in SK-MEL-2 cells, four mutants (pGL3-1146/-646mtCREB, mtAP-1, mtNF- κ B and mtc-Ets-1) were used, which contained the exact same construct as wild type pGL3-1146/-646 except that combined nucleotides within these binding sites had been changed (Kang *et al.*, 2006). A series of substituted mutations of luciferase constructs were transfected into SK-MEL-2 cells and luciferase assays were carried out. The activity of each construct was compared to that of pGL3-basic and wild type (pGL3-1146/-646) as negative and positive controls, respectively. As shown in Figure 5A, pGL3-1146/-646mtNF- κ B of four constructed mutations markedly reduced transcriptional activity, regardless of TPL treatment, to about 2.5-fold of pGL3-1146/-646wt, whereas the activities of the pGL3-1146/-646mtCREB, mtAP-1 and mtc-Ets-1 constructs were not significantly changed. These results indicate that this NF- κ B site is crucial for the hST8Sia I expression in SK-MEL-2 cells, and that NF- κ B binding to this site is involved in TPL-stimulated SK-MEL-2 cells. We also performed ChIP assay to confirm the binding of NF- κ B to hST8Sia I promoter in SK-MEL-2 cells. An amplification of the hST8Sia I promoter regions was obtained in the presence of antibodies to c-ETS-1, CREB, AP-1, and NF- κ B. As shown Figure 5B, only NF- κ B has the specific amplification and DNA-protein complex observed

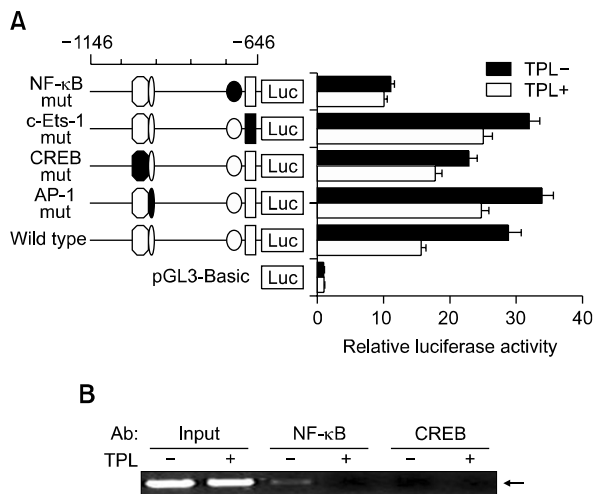


Figure 5. Mutation promoter assay for the transcription factor binding sites in hST8Sia I gene and ChIP analysis of the hST8Sia I promoter. (A) pGL3-Basic without any promoter and enhancer was used as a negative control. Each construct was co-transfected into SK-MEL-2 cells with pRL-TK as an internal control. The transfected cells were incubated in the presence (open) or absence (solid bar) of 50 nM TPL for 22 h. Relative firefly luciferase activity was measured using the Dual-Luciferase Reporter Assay System, and all firefly activity was normalized to the *Renilla* luciferase activity derived from pRL-TK. The values represent the means \pm SD of three independent experiments with triplicate measurements. The mutation mark of promoter construction is indicated by closed form or opened form (wild-type). (B) PCR amplification of the -1146 and -646 region of the hST8Sia I promoter on immunoprecipitated chromatin obtained from SK-MEL-2 cells treated or not treated with TPL. The input (10-fold diluted) represents the positive control.

in SK-MEL-2 cells untreated with TPL to regulate the expression of hST8Sia I gene. There was no detectable binding in a control assay with TPL treatment or CREB antibody. These results indicate that hST8Sia I gene expression was modulated by interaction between the nuclear protein, NF- κ B and NF- κ B elements at nucleotide positions -731 and -722. In addition, we investigated whether TPL has an effect on the transcriptional activity of NF- κ B in SK-MEL-2 cells. TPL significantly inhibited transactivation of NF- κ B in a dose-dependent manner (Figure 6). This result suggests that TPL might suppress hST8Sia I expression through inhibition of NF- κ B transactivation.

Discussion

Previous studies have shown that human melanoma cells have high levels of ganglioside GD3 and hST8Sia I activity, and mRNA levels for the enzyme are coincidentally high (Dippold *et al.*, 1980; Pukel *et al.*, 1982; Ruan *et al.*, 1999). We have also demonstrated that the expression level of hST8Sia I gene is specifically high in human

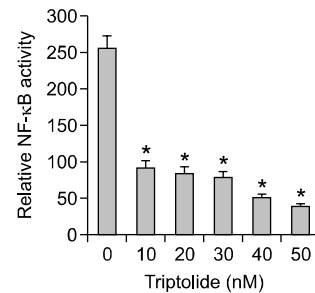


Figure 6. Suppression of hST8Sia I expression by TPL via inhibition of NF- κ B transcriptional activity. The NF- κ B-dependent reporter plasmid pGL2-3 \times NF- κ B was co-transfected into SK-MEL-2 cells with pRL-TK as the internal control. The transfected cells were incubated in the presence of the indicated concentrations (0, 10, 20, 30, 40, 50 nM) of TPL for 22 h. All firefly activity was normalized to the *Renilla* luciferase activity derived from pRL-TK. The values represent the mean \pm SD for three independent experiments with triplicate measurements. *P < 0.05 versus non-treatment.

melanoma cell line SK-MEL-2 (Kang *et al.*, 2007). In this study, we demonstrated for the first time that TPL down-regulated the expression of hST8Sia I mRNA in human melanoma cells. Moreover, this decrease was dose-dependent and the hST8Sia I mRNA signal was significantly decreased after 22 h of 30 nM TPL treatment. We also revealed that the promoter region of the hST8Sia I gene contained a TPL-responsive element. Previous studies have shown that TPL inhibits the proliferation and induces apoptosis in several tumor lines *in vitro* (Yang *et al.*, 2003; Carter *et al.*, 2006; Brinker *et al.*, 2007; Phillips *et al.*, 2007; Johnson *et al.*, 2009; Shi *et al.*, 2009; Wang *et al.*, 2009; Zhu *et al.*, 2009a; Chen *et al.*, 2010; Pan, 2010). In line with these reports, we observed that TPL inhibited the proliferation of SK-MEL-2 cells, and that it induced apoptosis after a 24 h treatment of the cells with 50 nM TPL.

We also unraveled a component of the transcriptional regulation mechanism that underlies hST8Sia I gene repression in response to TPL signaling. In order to investigate TPL-responsive elements involved in the suppression of the hST8Sia I gene expression in SK-MEL-2 cells, we first sought to identify the region within the hST8Sia I promoter that was critical for TPL-induced suppression. We isolated the region between -1146 and -646 as the core promoter; this region was required for transcriptional repression of hST8Sia I in TPL-treated SK-MEL-2 cells. In previous reports, we showed several transcription factor binding sites such as c-ETS-1, AP-1, CREB and NF- κ B binding sites in this region (Kang *et al.*, 2006, 2007; Kwon *et al.*, 2009). We have also demonstrated that only the NF- κ B binding site at position at -731 to -722 in this region contributes to

promoter activity necessary for high expression of hST8Sia I in Fas-induced Jurkat T cells (Kang *et al.*, 2006), human melanoma cells (Kang *et al.*, 2007), and valproic acid-induced human neuroblastoma cells (Kwon *et al.*, 2009). Contrary to these findings, however, our present site-directed mutagenesis and ChIP analysis indicated that binding to this NF- κ B element mediated TPL-dependent down-regulation of hST8Sia I gene expression in SK-MEL-2 cells.

NF- κ B is a crucial transcription factor that controls the expression of various genes involved in immune and inflammatory responses, cell cycle progression, apoptosis, and oncogenesis. Several studies have recently reported that TPL inhibits the transcriptional activation of NF- κ B in various cancer cell lines (Lee *et al.*, 1999; Chang *et al.*, 2007; Jang *et al.*, 2007; Kang *et al.*, 2009; Zhu *et al.*, 2009b; Xu *et al.*, 2010). It is additionally known that TPL down-regulates the various NF- κ B-regulated genes, IL-1, IL-2, TNF, COX-2, bcl-2, cIAP, XIAP (Liu *et al.*, 2000; Choi *et al.*, 2003; Zhou *et al.*, 2003; Yinjun *et al.*, 2005). However, down-regulation of NF- κ B-mediated gene expression by TPL stimulation in human melanoma cells has not been reported. Therefore, it might be important to elucidate which signaling pathways are upstream of this NF- κ B-mediated repression of the hST8Sia I gene expression in human melanoma cells.

Although the precise mechanisms involved in the TPL-mediated activation of NF- κ B leading to a transcriptional down-regulation of the hST8Sia I gene are unknown, we have demonstrated here for the first time that TPL inhibited the expression of hST8Sia I gene in SK-MEL-2 human melanoma cells through the suppression of NF- κ B activation.

Methods

Cell cultures

The SK-MEL-2 human melanoma cell was obtained from American Type Culture Collection (Rockville, MD). This cell line was grown in Dulbecco's modified Eagle's medium (DMEM; WelGENE Co., Daegu, Korea) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C under 5% CO₂.

MTT assay

Cell viability was determined by reduction of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. Briefly, SK-MEL-2 cells were plated in 96-well plates at a density of 1×10^3 cells/well. After 24 h, cells were washed with fresh medium and were treated with various concentrations of TPL (0-50 nM). After incubation

for 18 to 24 h, cells were washed with PBS, and 100 μ l of MTT reagent (5 mg/ml) was added to each well. After incubation for 4 h, DMSO (100 μ l) was added to dissolve the formazan precipitates and the amount of formazan salt was determined by measuring the OD at 570 nm using an ELISA plate reader (Bio-Rad, Hercules, CA). Cell viability was quantified as a percentage compared to the control.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from SK-MEL-2 cells using Trizol reagent (Invitrogen; Carlsbad, CA). Two micrograms of RNA was subjected to reverse transcription with random nonamers utilizing Takara RNA PCR kit (Takara Bio; Shiga, Japan) according to the manufacturer's protocol. The cDNA was amplified by PCR with the following primers: hST8Sia I (460 bp), 5'-TGTGGTCCAGAAAGACATTTG-TGGACA-3' (sense) and 5'-TGGAGTGAGGTATCTTCATCATGGGTCC-3' (antisense); β -actin (247 bp), 5'-CAA-GAGATGGCCACGGCTGCT-3' (sense) and 5'-TCCTTC-TGCATCCTGTCCGCA-3' (antisense). PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized with ethidium bromide. The intensity of the bands obtained from the RT-PCR product was estimated with a Scion Image Instrument (Scion Corp.; Frederick, MD). The values were calculated as a percent of the control and were expressed as means \pm SD.

Transient transfection and reporter assay

The luciferase reporter plasmids used herein, namely pGL3-2646/-646 and its derivatives (pGL3-1146/-646 to pGL3-2246/-646) with base substitutions in the CREB, AP-1, c-Ets-1, NF- κ B binding sites, have been described elsewhere (Kang *et al.*, 2006). To analyze hST8Sia I promoter activity in response to TPL treatment, SK-MEL-2 cells (3.0×10^5 cells/well) were seeded in 24-well tissue culture plates and allowed to grow to 70% confluence, at which point they were transiently co-transfected with 0.5 μ g of the indicated reporter plasmid and 50 ng of the control *Renilla* luciferase vector pRL-TK (Promega; Madison, WI), using 2 μ l VivaMagic reagent (Vivagen, Sungnam, Korea). After a 12 h recovery in normal medium without TPL, the medium was changed to medium containing 50 nM TPL and incubated for an additional 22 h, after which cells were collected and treated with passive lysis buffer (Promega). Firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's instructions, and a GloMaxTM 20/20 luminometer (Promega). Firefly luciferase activity of the reporter plasmid was normalized to *Renilla* luciferase activity and expressed as a fold induction over the empty pGL3-Basic vector, used as a negative control. Independent triplicate experiments were performed for each plasmid. For the NF- κ B trans-activation assay, pGL2-3 \times NF- κ B, which contained three tandem repeats of the NF- κ B binding motif, was used.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using the ChIP kit (Upstate Biotechnology, NY) following the manufacturer's protocol. Briefly, SK-MEL-2 cells (1×10^7 cells for one assay) were cross-linked in 1% formaldehyde at room temperature for 10 min to cross-link proteins and DNAs, followed by sonication to shear the DNAs to an average size of 200-1,000 bp. Immunoprecipitation was carried out using 4 μ g each of CREB and NF- κ B antibodies which were purchased from Santa Cruz Biotechnology (CA). After reversal of cross-linking, the DNA fragments were purified by phenol extraction and ethanol precipitation, followed by PCR analysis using primers flanking CREB and NF- κ B binding sites on the hST8Sia I promoter: CREB, 5'-CGTGTGTGTGTGTGTGTGTGTGTG-3' (forward) and 5'-CCGGTGTGCCAGGCTGT-3' (reverse); NF- κ B, 5'-CTC-CGCCACTCAGGACT-3' (forward) and 5'-ACAAAC-GCCCGGGATTG-3' (reverse).

Acknowledgments

This work was supported by the Korea Science and Engineering Foundation Grant funded by the Korea Government (No. 20100002028).

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