BMB
reports

Transcriptional activation of human GM3 synthase (hST3Gal V) gene by valproic acid in ARPE-19 human retinal pigment epithelial cells

Naree Song[1], *Seok-Jo Kim*[1], *Haw-Young Kwon*[1], *Sung-Wook Son*[1], *Kyoung-Sook Kim*[1], *Hee-Bae Ahn*[2] & *Young-Choon Lee*[1,*]

[1]Department of Biotechnology and Brain Korea 21 Center for Silver-Bio Industrialization, Dong-A University, [2]Department of Ophthalmology, College of Medicine, Dong-A University, Busan 604-714, Korea

The present study demonstrated that valproic acid (VPA) transcriptionally regulates human GM3 synthase (hST3Gal V), which catalyzes ganglioside GM3 biosynthesis in ARPE-19 human retinal pigment epithelial cells. For this, we characterized the promoter region of the hST3Gal V gene. Functional analysis of the 5′-flanking region of the hST3Gal V gene revealed that the -177 to -83 region functions as the VPA-inducible promoter and that the CREB/ATF binding site at -143 is crucial for VPA-induced expression of hST3Gal V in ARPE-19 cells. In addition, the transcriptional activity of hST3Gal V induced by VPA in ARPE-19 cells was inhibited by SP600125, a c-Jun *N*-terminal kinase (JNK) inhibitor. In summary, our results identified the core promoter region in the hST3Gal V promoter and for the first time demonstrated that ATF2 binding to the CREB/ATF binding site at -143 is essential for transcriptional activation of hST3Gal V in VPA-induced ARPE-19 cells. [BMB reports 2011; 44(6): 405-409]

INTRODUCTION

Valproic acid (VPA), a short chain fatty acid, is widely used for the treatment of epilepsy and bipolar disorders due to its anticonvulsant activity (1). VPA possesses anti-cancer activity through several mechanisms, including inhibition of cell growth, induction of differentiation, and apoptosis (2, 3).

Gangliosides, sialic acid-containing glycosphingolipids, are ubiquitous components located in the outer leaflet of the plasma membrane of vertebrate cells and play an important role in a variety of biological processes, including in cell-cell interactions, adhesion, subcellular targeting, growth control, and receptor function (4, 5). In addition, it is well known that the composition and distribution of gangliosides in cells dramatically change during various physiological and pathological processes, such as cell proliferation and differentiation, cell death, development, and oncogenesis (4, 5). These changes reflect the differential regulation of ganglioside biosynthesis catalyzed by a family of glycosyltransferases in the Golgi apparatus. Recently, we demonstrated that VPA modulates the mRNA expression of human GM3 synthase (hST3Gal V) and GD3 synthase (hST8Sia I) in human neuroblastoma cells (6, 7).

In the present study, we investigated whether or not VPA transcriptionally regulates hST3Gal V and hST8Sia I, key regulatory enzymes in the synthesis of nearly all gangliosides, in human retinal pigment epithelial cells. As a result, we have shown for the first time that the mRNA expression of human GM3 synthase (hST3Gal V) is induced by VPA in ARPE-19 human retinal pigment epithelial cells. Furthermore, to investigate the molecular basis of hST3Gal V gene expression induced by VPA in ARPE-19 cells, the promoter region directing the up-regulation of reporter gene transcription in ARPE-19 cells in response to VPA was functionally characterized.

RESULTS AND DISCUSSION

Expression of hST3Gal V gene induced by VPA in ARPE-19 cells

Initial experiments were designed to determine whether or not inducers of cell differentiation or apoptosis modulate the expression of human sialyltransferase genes responsible for ganglioside biosynthesis in ARPE-19 cells. After cells were treated with four different inducers (RA, FenR, TPA, and VPA), we examined the gene expression of human GM3 synthase (hST3Gal V) and human GD3 synthase (hST8Sia I), which are especially key enzymes in the ganglioside biosynthesis pathway. As shown in Fig. 1A, the RT-PCR results show that the mRNA expression of hST3Gal V was 2.4-fold higher in cells treated with 1 mM VPA compared to untreated cells, whereas hST8Sia I expression did not change in response to treatment with inducers. The induction of hST3Gal V mRNA expression re-

*Corresponding author. Tel: 82-51-200-7591; Fax: 82-51-200-6536; E-mail: yclee@dau.ac.kr
DOI 10.5483/BMBRep.2011.44.6.405

Received 8 March 2011, Accepted 15 April 2011

Keywords: ARPE-19, Ganglioside, Human GM3 synthase, Transcriptional regulation, Valproic acid,

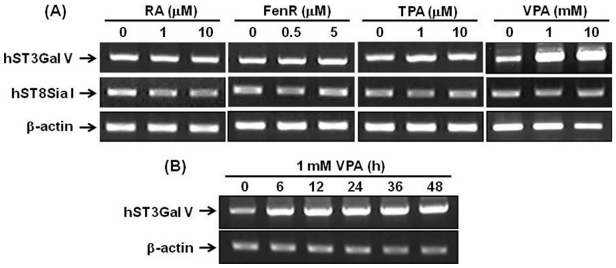

Fig. 1. RT-PCR analysis of hST3Gal V and hST8Sia I mRNA expression levels in ARPE-19 cells after treatment with RA, FenR, TPA, and VPA. Total RNA from ARPE-19 cells was isolated after 24 h of RA, FenR, TPA, and VPA treatment, and hST3Gal V and hST8Sia I mRNAs were detected by RT-PCR (A). Total RNA from ARPE-19 cells was isolated after 0, 6, 12, 24, 36 or 48 h of 1 mM VPA treatment, and hST3Gal V mRNA was detected by RT-PCR (B). The internal control for the RT-PCR reaction was performed by running parallel reaction mixtures with the housekeeping gene β-actin.

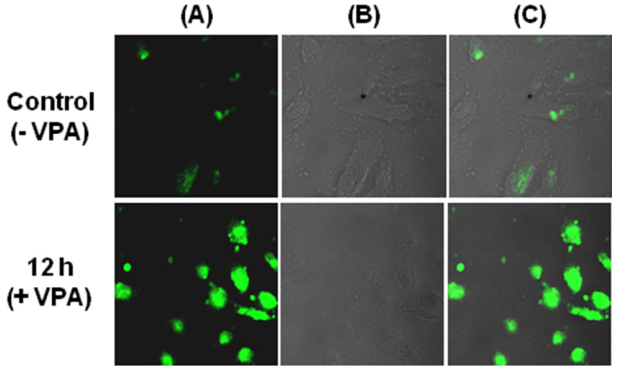

Fig. 2. Confocal analysis of VPA-mediated ganglioside GM3 levels. ARPE-19 cells were grown in standard medium or in medium containing 1 mM VPA for 12 h. A monoclonal antibody (GMR6) was used to analyze the resulting GM3 ganglioside levels. VPA treatment gradually induced strong GM3 up-regulation for 12 h, but the untreated controls exhibited no response. (A), immunofluorescence image; (B), phase-contrast image; (C), merge.

markably increased in response to 1 mM VPA treatment for 6 h and continued for 48 h after VPA treatment (Fig. 1B). These results clearly show that the expression of hST3Gal V is stimulated by VPA.

Expression of ganglioside GM3 in ARPE-19 cells induced by VPA

To investigate whether or not hST3Gal V mRNA induction by VPA increases the cellular expression level of ganglioside GM3, we used immunofluorescence confocal microscopy with mouse anti-GM3 monoclonal antibody and FITC-conjugated goat-anti-mouse IgM as the secondary antibody to visualize GM3 expression in ARPE-19 cells induced by VPA. As shown in Fig. 2, increased expression of ganglioside GM3 was observed in ARPE-19 cells induced by VPA but not in the control without VPA treatment. Previous studies have shown that VPA induces apoptosis and differentiation of neuroblastoma cells both *in vivo* and *in vitro* (8, 9). Based on these observations, the effect of VPA on the morphology of ARPE-19 cells was investigated. The results show that 24 h treatment with 1 mM VPA had no effect on cell morphology (data not shown).

Analysis of transcriptional activity of hST3Gal V promoter induced by VPA in ARPE-19 cells

To understand the transcriptional regulation of the hST3Gal V gene in VPA-induced ARPE-19 cells, luciferase constructs (10-12) carrying a 5′-deleted hST3Gal V promoter were transfected into ARPE-19 cells with or without VPA, and the luciferase activity was measured. As shown in Fig. 3A, transfection of pGL3-177 resulted in a remarkable increase in promoter activity in ARPE-19 cells with VPA treatment, which was higher (about 2-fold) than that in ARPE-19 cells without VPA treatment. However, the promoter activity did not change markedly when pGL3-Basic and the 5′-deleted hST3Gal V promoter without pGL3-177 were transfected into ARPE-19 cells with or without VPA. These results suggest that potential positive regulatory elements exist in pGL3-177 and that the region from -1600 to -177 negatively regulates transcriptional activity. Furthermore, further deletion of nucleotide -83 markedly reduced promoter activity to a level near that of the control vector pGL3-Basic. Therefore, these results clearly suggest that the region containing nucleotides -177 to -83 plays a pivotal role in the expression of the hST3Gal V gene and functions as the VPA-inducible promoter in ARPE-19 cells.

Our previous study (12) showed that that the region from -177 to -83 contains NFY, CREB/ATF, SP1, EGR3, and MZF1 binding sites, although only the CREB/ATF and MZF1 binding sites are present in the correct orientation. Moreover, we also demonstrated that only the consensus CREB/ATF binding site (TGACGTCA) from -143 to -136 is crucial for the TPA-induced expression of hST3Gal V in HL-60 cells (10, 11). To determine whether or not this CREB/ATF binding site contributes to VPA-induced expression of hST3Gal V in ARPE-19 cells, pGL3-1600mtCREB and pGL3-177mtCREB (10, 11) with CREB/ATF binding site mutations in pGL3-1600 and pGL3-177, respectively, were prepared and transfected into ARPE-19 cells with or without VPA. In VPA-treated ARPE-19 cells, these mutations markedly reduced the transcriptional activity of hST3Gal V (Fig. 3B). To confirm whether or not CREB or ATF binds to its site in the region from -177 to -83 in the hST3Gal V promoter in ARPE-19 cells, we also performed ChIP assay. Amplification of the hST3Gal V promoter region was carried out in the presence of antibodies specific to CREB, ATF1, and ATF2. As shown in Fig. 3C, only ATF2 demonstrated specific amplification and formed a DNA-protein complex in ARPE-19 cells treated with VPA for regulation of hST3Gal V gene expression.

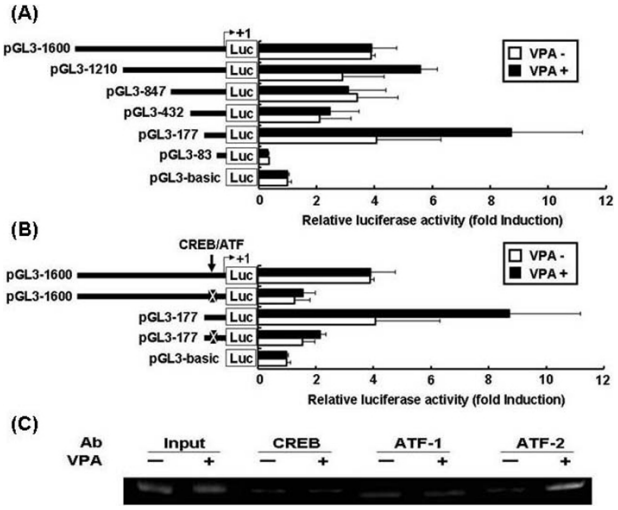

Fig. 3. Analysis of hST3Gal V promoter activity in ARPE-19 cells and ChIP assay. The schematic diagrams represent DNA constructs containing various lengths of the wild-type hST3Gal V promoter (A), or a promoter with a mutant CRE sequence in the 5′-flanking region (B), upstream of a luciferase reporter gene; the transcription start site is designated +1. The pGL3-Basic without any promoter and enhancer was used as a negative control. Each construct was co-transfected into ARPE-19 cells with pRL-TK as the internal control. The transfected cells were incubated in the presence (solid bar) and absence (open bar) of 1 mM VPA for 12 h. Relative luciferase activity was measured using the Dual-Luciferase Reporter Assay System normalized with renilla-luciferase activity derived from pRL-TK. The values represent the mean ± SD of three independent experiments with triplicate measurements. (C) PCR amplification from the -177 and -83 region of the hST3Gal V promoter on immunoprecipitated chromatin obtained from ARPE-19 cells treated or not treated with VPA. The input (10-fold diluted) represents the positive control.

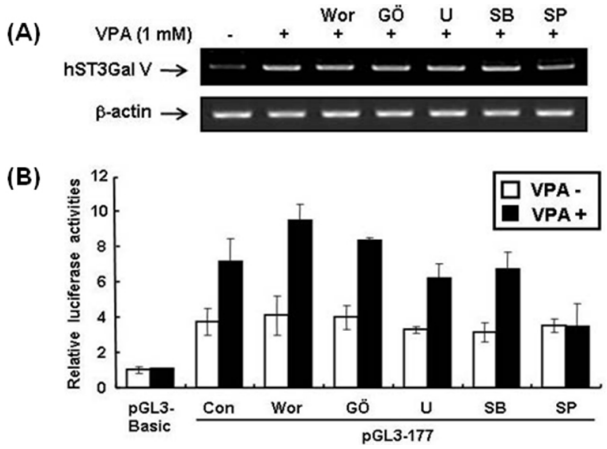

Fig. 4. Transcriptional activation of hST3Gal V through JNK pathway in VPA-treated ARPE-19 cells. (A) ARPE-19 cells were treated with GÖ6976 (5 μM), U0125 (5 μM), Wortmannin (200 nM), SP600125 (10 μM), and SB203580 (20 μM) inhibitors in the absence or presence of VPA (1 mM) for 12 h in serum-free DMEM medium. Total RNA from these cells was isolated, and hST3Gal V mRNA was detected by RT-PCR analysis. Beta-actin was included as an internal control. (B) The pGL3-177 was co-transfected into ARPE-19 cells with pRL-TK as the internal control. The transfected cells were incubated in the presence and absence of 1 mM VPA with each inhibitor for 12 h. All firefly activities were normalized to the *Renilla* luciferase activity derived from pRL-TK. The values represent the mean ± SD of three independent experiments with triplicate measurements.

There was no detectable binding in the ChIP assay using the CREB and ATF1 antibodies. These results show that hST3Gal V gene expression was modulated by the interaction between the nuclear protein ATF2 and the CREB/ATF element from nucleotide positions -143 to -136. Therefore, this CREB/ATF site is crucial for the VPA-induced expression of hST3Gal V in ARPE-19 cells and the binding of ATF2 to this site is involved in the induction of hST3Gal V gene expression by VPA. Our previous studies have demonstrated that only CREB binding to this site contributes to the promoter activity necessary for high expression of hST3Gal V in VPA-stimulated SK-N-BE(2)-C cells (8) and TPA-induced HL-60 cells (10, 11). However, in contrast to these findings, our present site-directed mutagenesis and ChIP analyses found that binding of ATF2 to this site mediated VPA-dependent up-regulation of hST3Gal V gene expression in ARPE-19 cells.

Transcriptional activation of hST3Gal V via JNKs pathway in ARPE-19 cells induced by VPA

It has been reported that ATF2 is a phosphorylation target of the c-Jun N-termainal protein kinases (JNKs) signaling pathway, which is activated in response to external signals (13-15). Therefore, we investigated whether or not the VPA-induced transcriptional activity of a pGL3-177-containing ATF site is stimulated via the JNK signal pathway. As shown in Fig. 4A, RT-PCR revealed that expression of hST3Gal V mRNA was increased in VPA-induced ARPE-19 cells compared to VPA-untreated cells. A specific JNK inhibitor, SP600125, resulted in a decrease in hST3Gal V expression in VPA-stimulated ARPE-19 cells. However, enhanced expression of hST3Gal V in VPA-stimulated ARPE-19 cells was not inhibited by phosphatidylinositol-3 kinase (PI-3K), protein kinase C (PKC), extracellular signal-regulated protein kinase (ERK), and p38 mitogen-activated protein kinase (MAPK) inhibitors (wortmannin, GÖ6976, U0126, and SB203580, respectively) compared to ARPE-19 cells induced by VPA in the absence of chemical inhibitors. The promoter activity of pGL3-177 in ARPE-19 cells stimulated by VPA was not significantly inhibited by PI-3K, PKC, ERK, and MAPK inhibitors compared to ARPE-19 cells induced by VPA in absence of chemical inhibitors, as evidenced by luciferase promoter assay (Fig. 4B). However, only JNK inhibitor (SP600125) resulted in a marked decrease in pGL3-177 activity in ARPE-19 cells induced by VPA. These results indicate that the promoter activity and mRNA transcription of the hST3Gal V gene might be regulated by the JNK signaling path-

way in VPA-stimulated ARPE-19 cells.

In conclusion, in this study, we showed for the first time that the expression of hST3Gal V mRNA is up-regulated in human retinal pigment epithelial ARPE-19 cells induced by VPA. Furthermore, we demonstrated that VPA treatment markedly increases ganglioside GM3 expression in the membrane fraction of ARPE-19 cells. These results suggest that the induction of hST3Gal V gene expression would direct GM3 biosynthesis in response to VPA in ARPE-19 cells, and that VPA-responsive element(s) exists in the promoter region of the hST3Gal V gene. Although the precise mechanisms involved in the VPA-mediated activation of ATF2 leading to the transcriptional up-regulation of hST3Gal V are unknown, we have demonstrated here for the first time that JNK-dependent ATF2 activation regulates the expression of hST3Gal V in VPA-stimulated ARPE-19 cells.

MATERIALS and METHODS

Cell cultures

The human retinal pigment epithelial cell ARPE-19, obtained from American Type Culture Collection (Manassas, VA, USA) was maintained at 37°C in a 5% CO_2 incubator and cultured initially in a 1 : 1 mixture of DMEM and Ham F12-medium (DMEM/F12) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% (v/v) fetal bovine serum (Gibco BRL, Life Technologies; Grand Island, NY, USA). As an initial experiment, valproic acid (VPA), retinoic acid (RA), fenretinide (FenR), and tetradecanoyl phorbol acetate (TPA), all well-known as inducers of cell differentiation or apoptosis, were used to determine whether or not they modulate the expression of human sialyltransferase genes responsible for ganglioside biosynthesis in ARPE-19 cells.

Analysis of ganglioside GM3 expression levels by VPA

Ganglioside GM3 expression levels were analyzed by the same procedure as described previously (6, 7). Mouse anti-GM3 monoclonal antibody (mouse IgM, Kappa-chain, clone: GMR6; Seigakagu; Japan) and FITC-conjugated goat-anti-mouse IgM (Sigma; St. Louis, MO, USA) as the secondary antibody were used to visualize GM3 expression in ARPE-19 cells induced by VPA. The samples were analyzed by confocal laser-scanning microscopy (LSM 510; Zeiss; Jena, Germany).

Reverse transcription-polymerase chain reaction (RT-PCR)

Using Trizol reagent (Invitrogen; Carlsbad, CA, USA), total RNA was isolated from ARPE-19 cells treated with vehicle control or VPA, and 2 μg of RNA was subjected to reverse transcription with random nonamers utilizing a Takara RNA PCR kit (Takara Bio; Shiga, Japan) according to the manufacturer's protocol. The resulting cDNA was amplified by PCR with the following primers: for hST3Gal V (413 bp), 5'-CCCTG CCATTCTGGGTACGAC-3' (sense) and 5'-CACGATCAATGCC TCCACTGAGATC-3' (antisense); hST8Sia I primers (460 bp), 5'-TGTGGTCCAGAAAGACATTTGTGGACA-3' (sense) and 5'-TGGAGTGAGGTATCTTCACATGGGTCC-3' (antisense); and β-actin (247 bp), 5'-CAAGAGATGGCCACGGCTGCT-3' (sense) and 5'-TCCTTCTGCATCCTGTCGGCA-3' (antisense). PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized with ethidium bromide. The intensities of the bands obtained from the RT-PCR product were estimated with a Scion Image Instrument (Scion Corp.; Frederick, MD, USA).

Transfection and luciferase assay

The luciferase reporter plasmids used herein, namely pGL3-1600 and its derivatives (pGL3-83 to pGL3-1210) with base substitutions in the CREB/ATF binding site, have been described elsewhere (10-12). To analyze hST3Gal V promoter activity in response to VPA treatment, ARPE-19 cells (5.0×10^4 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 μg of the indicated reporter plasmid and 50 ng of the control *Renilla* luciferase vector pRL-TK (Promega; Madison, WI, USA) using 1 μl of Lipofectamine 2000 (Invitrogen). After a 12-h recovery period in normal medium without VPA, the medium was changed to medium containing 1 mM VPA and incubated for an additional 12 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 GloMax™ 20/20 luminometer (Promega). Firefly luciferase activity of the reporter plasmid was normalized to *Renilla* luciferase activity and expressed as the fold induction over empty pGL3-Basic vector, which was used as a negative control. Independent triplicate experiments were performed for each plasmid.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using the ChIP kit (Upstate Biotechnology, NY, USA) following the manufacturer's protocol. Briefly, after treatment with 0 mM or 1 mM VPA for 6 h, ARPE-19 cells (1×10^7 cells for one assay) were cross-linked in 1% formaldehyde at room temperature for 10 min to cross-link the proteins and DNAs, followed by sonication to shear the DNAs to an average size of 200-1,000 bp. Immunoprecipitation was carried out using 10 μg of CREB, ATF1, and ATF2 antibodies (Santa Cruz Biotechnology, CA, USA). After reversal of cross-linking, the DNA fragments were purified by phenol extraction and ethanol precipitation, followed by PCR analysis using primers flanking the CREB/ATF binding sites in the hST3Gal V promoter: 5'-GCCCCGGGTGCGTCCCTG-3' (forward) and 5'-AGCGCCGCTCTCGCGCC-3' (reverse).

Acknowledgements

This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and

Technology (MEST) (2009-0070989).

REFERENCES

1. Rogawski, M. A. and Loscher, W. (2004) The neurobiology of antiepileptic drugs. *Nat. Rev. Neurosci.* **5**, 553-564.
2. Blaheta, R. A., Michaelis, M., Driever, P. H. and Cinatl Jr., J. (2005) Evolving anticancer drug valproic acid: insights into the mechanism and clinical studies. *Med. Res. Rev.* **25**, 383-397.
3. Blaheta, R. A. and Cinatl Jr., J. (2002) Anti-tumor mechanisms of valproate: a novel role for an old drug. *Med. Res. Rev.* **22**, 492-511.
4. Hakomori, S. (2003) Structure, organization, and function of glycosphingolipids in membrane. *Curr. Opin. Hematol.* **10**, 16-24.
5. Bektas, M. and Spiegel, S. (2004) Glycosphingolipids and cell death. *Glycoconjugate J.* **20**, 39-47.
6. Kwon, H. Y., Kang, N. Y., Dae, H. M., Kim, K. S., Kim, C. H., Do, S. I. and Lee, Y. C. (2008) Valproic acid-mediated transcriptional regulation of human GM3 synthase (hST3Gal V) in SK-N-BE(2)-C human neuroblastoma cells. *Acta. Pharmacol. Sin.* **29**, 999-1005.
7. Kwon, H. Y., Dae, H. M., Song, N. R., Kim, K. S., Kim, C. H. and Lee, Y. C. (2009) Valproic acid induces transcriptional activation of human GD3 synthase (hST8Sia I) gene in SK-N-BE(2)-C human neuroblastoma cells. *Mol. Cells* **27**, 113-118.
8. Cinatl Jr., J., Cinatl, J., Scholz, M., Driever, P. H., Henrich, D., Kabickova, H., Vogel, J. U., Doerr, H. W. and Kornhuber, B. (1996) Antitumor activity of sodium valproate in cultures of human neuroblastoma cells. *Anticancer Drugs* **7**, 766-773.
9. Cinatl Jr., J., Cinatl, J., Driever, P. H., Kotchetkov, R., Pouckova, P., Kornhuber, B. and Schwabe, D. (1997) Sodium valproate inhibits in vivo growth of human neuroblastoma cells. *Anticancer Drugs* **8**, 958-963.
10. Choi, H. J., Chung, T. W., Kang, N. Y., Kim, K. S., Lee, Y. C. and Kim, C. H. (2003) Transcriptional regulation of the human GM3 synthase (hST3Gal V) gene during monocytic differentiation of HL-60 cells. *FEBS Lett.* **555**, 204-208.
11. Chung, T. W., Choi, H. J., Lee, Y. C. and Kim, C. H. (2005) Molecular mechanism for transcriptional activation of ganglioside GM3 synthase and its function in differentiation of HL-60 cells. *Glycobiology* **15**, 233-244.
12. Kim, S. W., Lee, S. H., Kim, K. S., Kim, C. H., Choo, Y. K. and Lee, Y. C. (2002) Isolation and characterization of the promoter region of the human GM3 synthase gene. *Biochim. Biophys. Acta.* **1578**, 84-89.
13. Gupta, S., Campbell, D., Derijard, B. and Davis, R. J. (1995) Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science* **267**, 389-393.
14. Bhoumik, A. and Ronai, Z. (2008) ATF2: a transcription factor that elicits oncogenic or tumor suppressor activities. *Cell Cycle* **7**, 2341-2345.
15. Morton, S., Davis, R. J. and Cohen, P. (2004) Signaling pathways involved in multisite phosphorylation of the transcription factor ATF-2. *FEBS Lett.* **572**, 177-183.