

The roles of glycosphingolipids in the proliferation and neural differentiation of mouse embryonic stem cells

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DOI 10.3858/emm.2009.41.12.099

Accepted 17 August 2009

Abbreviations: DEAE, diethylaminoethyl; EBs, embryoid bodies; GFAP, glial fibrillary acidic protein; GSLs, glycosphingolipids; HPTLC, high-performance thin-layer chromatography; LIF, leukemia inhibitory factor; MAP-2, microtubule-associated protein 2; MEF, mouse embryonic fibroblast; mESCs, mouse embryonic stem cells; shRNAs, short hairpin RNAs; Ugcg, UDP-glucose:ceramide glucosyltransferase

Abstract

Glycosphingolipids including gangliosides play important regulatory roles in cell proliferation and differentiation. UDP-glucose:ceramide glucosyltransferase (*Ugcg*) catalyze the initial step in glycosphingolipids biosynthesis pathway. In this study, *Ugcg* expression was reduced to approximately 80% by short hairpin RNAs (shRNAs) to evaluate the roles of glycosphingolipids in proliferation and neural differentiation of mouse embryonic stem cells (mESCs). HPTLC/immunofluorescence analyses of shRNA-transfected mESCs revealed that treatment with *Ugcg*-shRNA decreased expression of major ganglio-

sides, GM3 and GD3. Furthermore, MTT and Western blot/immunofluorescence analyses demonstrated that inhibition of the *Ugcg* expression in mESCs resulted in decrease of cell proliferation ($P < 0.05$) and decrease of activation of the ERK1/2 ($P < 0.05$), respectively. To further investigate the role of glycosphingolipids in neural differentiation, the embryoid bodies formed from *Ugcg*-shRNA transfected mESCs were differentiated into neural cells by treatment with retinoic acid. We found that inhibition of *Ugcg* expression did not affect embryoid body (EB) differentiation, as judged by morphological comparison and expression of early neural precursor cell marker, nestin, in differentiated EBs. However, RT-PCR/immunofluorescence analyses showed that expression of microtubule-associated protein 2 (MAP-2) for neurons and glial fibrillary acidic protein (GFAP) for glial cells was decreased in neural cells differentiated from the shRNA-transfected mESCs. These results suggest that glycosphingolipids are involved in the proliferation of mESCs through ERK1/2 activation, and that glycosphingolipids play roles in differentiation of neural precursor cells derived from mESCs.

Keywords: cell differentiation; ceramide glucosyltransferase; extracellular signal-regulated MAP kinases; glycosphingolipids; embryonic stem cells; neurons

Introduction

Glycosphingolipids are primarily found in the outer leaflet of the plasma membrane microdomain on various mammalian cells, and gangliosides are regulated by specific ceramides bound to the Golgi apparatus (Van Meer, 1993). Ceramide, in turn, is modified by the action of glucosylceramide synthase (UDP-glucose:ceramide glucosyltransferase; *Ugcg*) on the cytosolic surface of the Golgi, which results in the production of glucosylceramide (Figure 1A). Glucosylceramide is then converted to lactosylceramide by the addition of a galactose moiety in the luminal leaflet of the Golgi (Allende and Proia, 2002). Glycosphingolipids are converted to gangliosides by carrying one or more sialic acid residues in the carbohydrate moiety. Successive sialylations of lactosylceramide lead to the formation of monosialoganglioside GM3, disia-

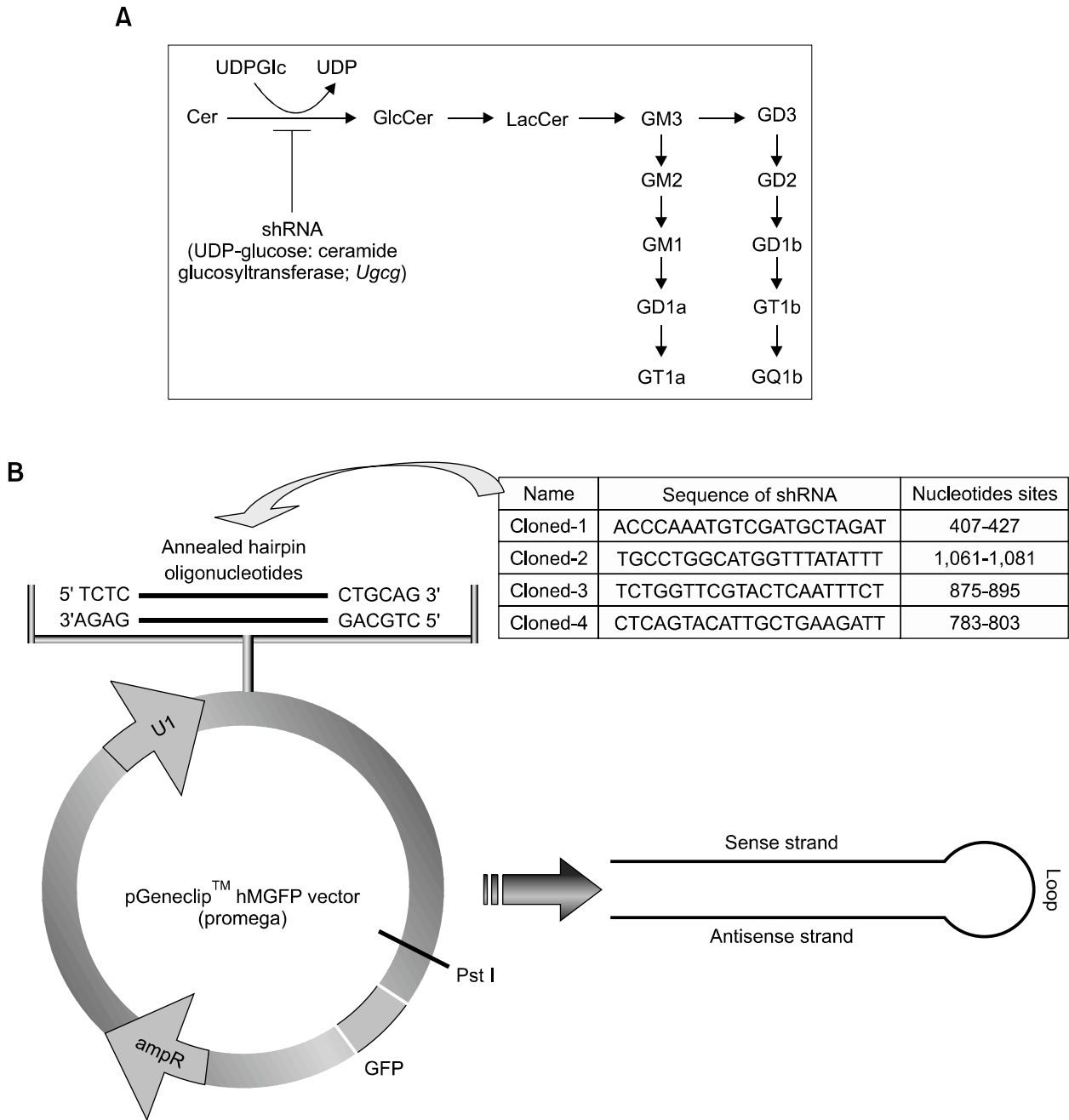


Figure 1. Schematic diagram of the four shRNAs designed to target the *Ugcg* gene in mESCs. (A) Biosynthetic pathway of glucosylceramide-based glycosphingolipids and gangliosides. Glucosylceramide synthase is the enzyme in the biosynthetic first step for the synthesis of most gangliosides and glycosphingolipids. (B) shRNAs vector. Four shRNAs (cloned 1-4) designed to inhibit expression of the mouse *Ugcg* gene (UDP-glucose ceramide glucosyltransferase; GenBank. Accession No. NM_011673, SuperArray Bioscience Corp., Frederick, MD).

loganglioside GD3, and trisialoganglioside GT3 (Van Echten and Sandhoff, 1993).

It is known that the biosynthesis and expression of glycosphingolipids are closely correlated with various cellular processes, such as proliferation, differentiation, and apoptosis. Glycosphingolipids, including gangliosides, are known to regulate

various signal molecules, such as receptor tyrosine kinases of various growth factor receptors and mitogen-activated protein kinase (MAPK), which are key transducers of extracellular signaling during cell growth and differentiation (Fukumoto *et al.*, 2000; Kimura *et al.*, 2001; Maupas-Schwalm *et al.*, 2004; Stoica *et al.*, 2005; Murozuka *et al.*,

2007). The composition of glycosphingolipids in cellular membranes is associated with specific cell types and is drastically changed during embryonic development (Yu, 1994; Draper *et al.*, 2002). Glycosphingolipids also appear to be necessary for the central nervous system to function (Kawai *et al.*, 2001) and to be involved in the formation of myelin during neuritogenesis and in neurite outgrowth (Liour *et al.*, 2000). Functions of glycosphingolipids in stem cells were demonstrated by several groups since the first study of gangliosides expression in mouse embryonic stem cells (mESCs) (Kimber *et al.*, 1993). Many approaches have recently been developed to regulate the expression of glycosphingolipids for evaluating their functions. However, the results are various depending on methods and cell lines used in different studies.

Previously, we reported that ganglioside expression was closely related with neural cell specific gene expression in an *in vitro* model of neural differentiation in mESCs and that neural differentiation was facilitated by increase of ganglioside expression due to daunorubicin treatment (Lee *et al.*, 2007). However, the chemicals that are used to regulate the biosynthesis of glycosphingolipids have been shown to have side effects, such as cytotoxicity and low specificity, particularly alteration of glycosphingolipids biosynthesis may be actively involved in the acquisition of drug-resistance (Kiura *et al.*, 1998; Prinetti *et al.*, 2006). It has been suggested that a genetic approach for regulating biosynthesis of glycosphingolipids would be appropriate in that the role of glycosphingolipids in the ESCs differentiation process can be directly elucidated. (Yamashita *et al.*, 1999; Liu *et al.*, 2004; Diaz-Font *et al.*, 2006). Therefore, in this study, we investigated the roles of glycosphingolipids in the proliferation and neural differentiation of mESCs by direct inhibition of glycosphingolipid biosynthesis using *Ugcg*-shRNA.

Results

Downregulation of the *Ugcg* mRNA level by shRNAs

To induce suppression of the *Ugcg* gene expression, mESCs were transfected with four different *Ugcg*-targeting shRNAs using a GFP vector containing T7 RNA polymerase under control of the U1 promoter (Figure 1B). We evaluated the alteration of *Ugcg* mRNA by transfection with *Ugcg*-shRNAs constructs at 48 h after transfection. The altered *Ugcg* gene expression by transfection had no effect on the morphology of the mESCs (Figure 2A). RT-PCR analysis showed that shRNA clone-1

was the most effective in downregulation of *Ugcg* gene expression in mESCs (Figure 2B). Therefore, shRNA clone-1 was used in the following experiments.

Downregulation of glycosphingolipids expression by shRNA

We expected reduction of glycosphingolipids expression in response to *Ugcg*-shRNA because the biosynthetic pathway of glucosylceramide-based glycosphingolipids begins with glucosylceramide synthase, which is expressed by the *Ugcg* (Proia, 2003). Therefore, the effect of *Ugcg*-shRNA on the expression of glycosphingolipids was investigated by HPTLC and immunofluorescence analysis (Figures 2C-2F). HPTLC analysis demonstrated that the glycosphingolipids expression, particularly ganglioside GM3 and GD3, was reduced by 60% in *Ugcg*-shRNA transfected cells (Figure 2C, lane 5). Conversely, the expression of gangliosides in mESCs transfected with the negative-shRNA was unchanged (Figure 2C, lane 4). The immunofluorescence assay also showed that the expression levels of GM3 and GD3 in the undifferentiated mESCs decreased in shRNA-transfected mESCs in comparison to mock-transfected mESCs and control mESCs (Figure 2D, E and F).

Suppression of the mESC proliferation by inhibition of *Ugcg* gene expression

The effect of suppression of *Ugcg* gene expression on the proliferation of mESCs was investigated by MTT assay. mESCs were transfected with negative-shRNA and shRNA-1 for 24 h, and then cell proliferation was measured at 48 h after transfection. Although the effect was not dramatic, the shRNA-1 significantly reduced mESC proliferation by 86% in comparison to the control cells. Conversely, no significant effect on proliferation was observed in the mock-transfected mESCs when compared to that of the control mESCs (96%).

Suppression of MAPK activation by inhibition of *Ugcg* gene expression

To determine if suppression of *Ugcg* gene expression inhibited cell proliferation via inhibition of the MAPK pathway (ERK1/2), activation of the MAPK pathway was examined by Western blot in the 10 min serum-stimulated mESCs after 6 h serum-starvation. The Western blot using a phospho-ERK1/2 showed that the ERK1/2 was phosphorylated in the cells during maintenance under normal culture conditions (Figure 4A). However, phosphorylation

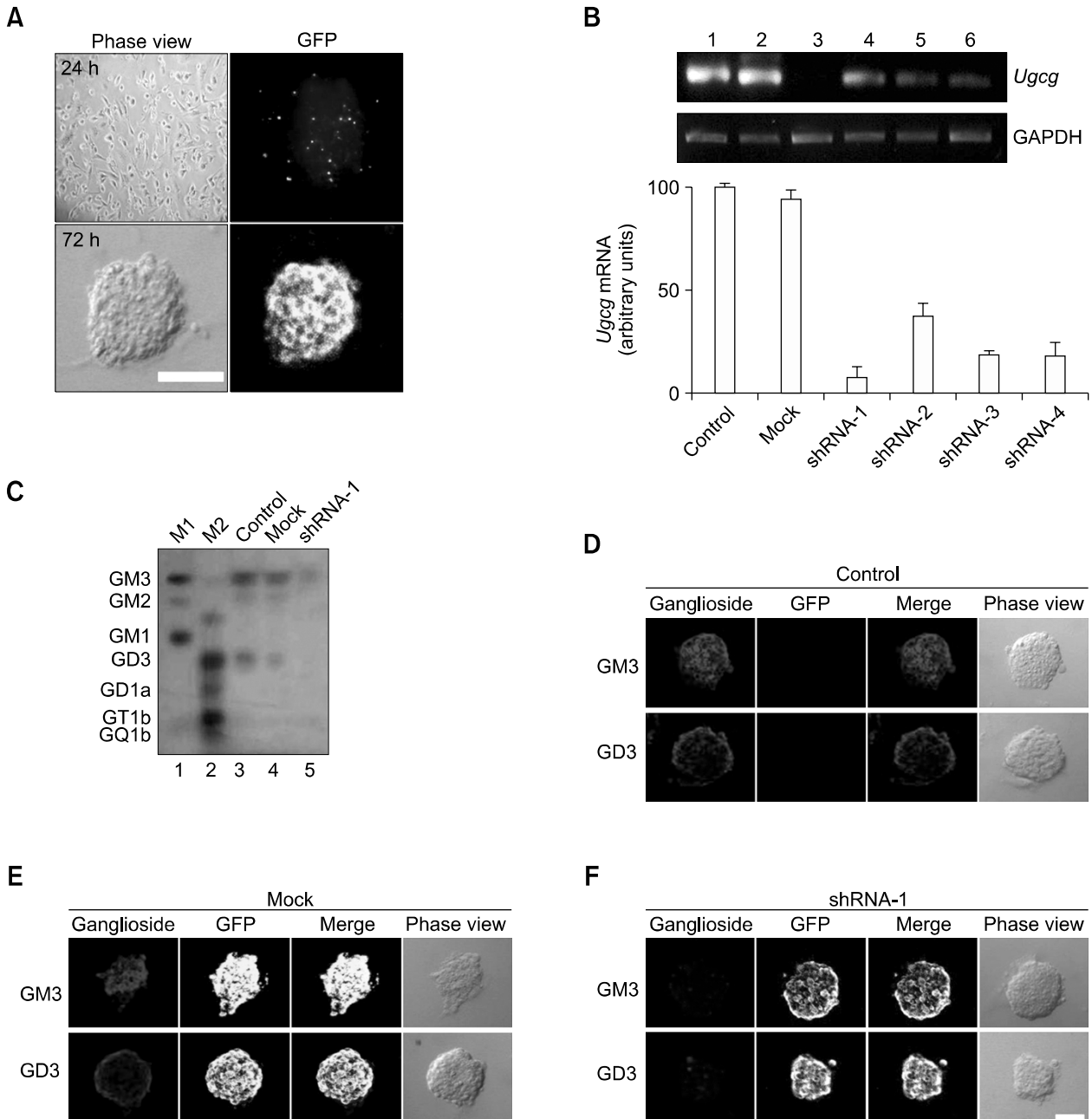


Figure 2. Analysis of *Ugcg* gene and glycosphingolipids expression on *Ugcg* shRNAs-transfected mESCs. (A) Transfected mESCs with a normal morphology and GFP fluorescence. 24 h, 24 h after transfection; 72 h, 72 h after transfection (B) RT-PCR analysis of *Ugcg* gene expression in transfected mESCs. Lane 1, control mESCs; lane 2, mock-transfected mESCs; lane 3, cloned-1 transfected mESCs; lane 4, cloned-2 transfected mESCs; lane 5, cloned-3 transfected mESCs; lane 6, cloned-4 transfected mESCs. RT-PCR was carried out for 35 cycles and expression levels were controlled using GAPDH. (C) Ganglioside extracts were resolved on an HPTLC silica gel plate using chloroform:methanol:0.22% CaCl₂ (55:45:10, v/v/v), and then visualized with resorcinol-HCl reagent. Lane 1 and 2, ganglioside standard marker; Lane 3, control mESCs; Lane 4, mock-transfected mESCs; Lane 5, shRNA-transfected mESCs. (D-F) The mESCs were subjected to immunofluorescence staining with mAb that was specific for the ganglioside GM3 and GD3. The scale bar represents 100 μ m.

of the ERK1/2 was almost undetectable under serum-free conditions (Figure 4B, lanes 1, 3 and 5). Interestingly, the shRNA-1 transfected mESCs showed inhibition of serum-induced ERK1/2 phos-

phorylation ($P < 0.05$) (Figure 4B, lane 6), whereas mock transfection did not affect serum-induced ERK1/2 phosphorylation (Figure 4B, lane 4). In addition, activation of the MAPK was examined

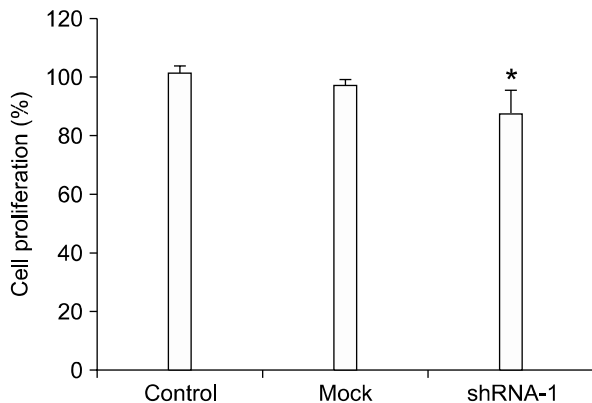


Figure 3. Effect of the suppressed *Ugcg* gene expression on the proliferation of mESCs. Cells were seeded at a density of 4×10^4 cells/well in 24-well plates and then transfected for 24 h. 48 h after transfection, cell proliferation was evaluated by an MTT assay. A low proliferation of mESCs was observed in shRNA-transfected mESCs. The values represent the mean \pm SD of four independent experiments. * $P < 0.05$.

using an immunofluorescence analysis (Figure 4C). This analysis showed that phosphorylated ERK1/2 was also expressed in control mESCs and mock-transfected mESCs. However, the intensity of the phosphorylated ERK1/2 expressed in shRNA-1 transfected mESCs was reduced in comparison to that of mock-transfected mESCs (Figure 4C).

The effects of suppression of *Ugcg* gene expression on neural differentiation of mESCs

To examine the role of the *Ugcg* in neural differentiation, we induced neural differentiation of the *Ugcg*-shRNA transfected mESCs. The EBs derived from shRNA-1 transfected mESCs were adhered on poly-D-lysine/laminin-coated tissue culture dishes for early neural differentiation (Figure 5A). RT-PCR analysis revealed that shRNA-1 transfection resulted in decrease of mRNA expression of the glial marker (GFAP) and the neuronal marker (MAP-2) whereas expression of early neural precursor cell marker gene, nestin, was not affected (Figure 5B).

The effects of suppression of *Ugcg* gene expression on the differentiation of mESCs into neural cells were also evaluated by immunofluorescence staining for nestin, MAP-2, and GFAP. No significant difference was found in nestin expression between the control mESCs and the transfected mESCs (Figure 5C). However, the expressions of GFAP and MAP-2 were significantly reduced in shRNA-1 transfected mESCs in comparison to that of the control mESCs and mock-transfected mESCs (Figures 5D and 5E).

Discussion

Recently, numerous studies have reported that glycosphingolipids play a crucial role during the development of neural cells. Our previous work showed that daunorubicin-induced overexpression of ganglioside enhanced neurite formation in mESCs (Lee *et al.*, 2007). However, the study could not completely exclude the possibility of side effects induced by chemicals, and the exact roles of glycosphingolipids in neural differentiation of mESCs still remained to be clarified. Therefore, in this study we specifically inhibited *Ugcg* gene expression using shRNA transfection to determine if glycosphingolipids are involved in neural differentiation of mESCs. RT-PCR revealed that the shRNA transfection significantly decreased the *Ugcg* gene expression in mESCs. In addition, the expression of the gangliosides, GM3 and GD3, in undifferentiated mESCs was also decreased by *Ugcg*-shRNA transfection. These results confirmed that inhibition of *Ugcg* gene expression decreased the expression of glycosphingolipids in mESCs.

The biological roles of glycosphingolipids in cell culture include modification of cellular growth, proliferation, and differentiation. Although functions of glycosphingolipids might be various depending on the cell type, expression levels of glycosphingolipids, and the cell type-specific MAPK pathway (Kamimura *et al.*, 2005), our current study demonstrated that suppression of *Ugcg* gene expression in mESCs inhibited cellular proliferation possibly by reduction of MAPK activation. These results are consistent with the previous findings that the depletion of glycosphingolipids inhibited proliferation of P19 cells and mESCs (Liour and Yu, 2002; Heo *et al.*, 2006), MAPK pathway in embryonic neural precursor cells (Yanagisawa *et al.*, 2005). However, we can not exclude the possibility that ceramide accumulation caused by suppression of *Ugcg* gene expression results in apoptotic cell death and cell cycle arrest during cell growth and differentiation (Jayadev *et al.*, 1995; Hatanaka *et al.*, 1998). It has been reported that accumulated ceramide concentration might affect the amount of diverse sphingolipid metabolites such as sphingomyelin, sphingosine, and sphingosine-1-phosphate affecting cell growth, apoptosis and the ability of neural cells (Hannun and Linardic, 1993; Pettus *et al.*, 2004; Tani *et al.*, 2007).

To determine the neural differentiation capability of the *Ugcg*-suppressed mESCs, we evaluated the RA-induced neural differentiation of the EBs (Bain *et al.*, 1995; Li *et al.*, 1998). A previous study demonstrated that expression of neural cell markers and glycosphingolipids was closely related to

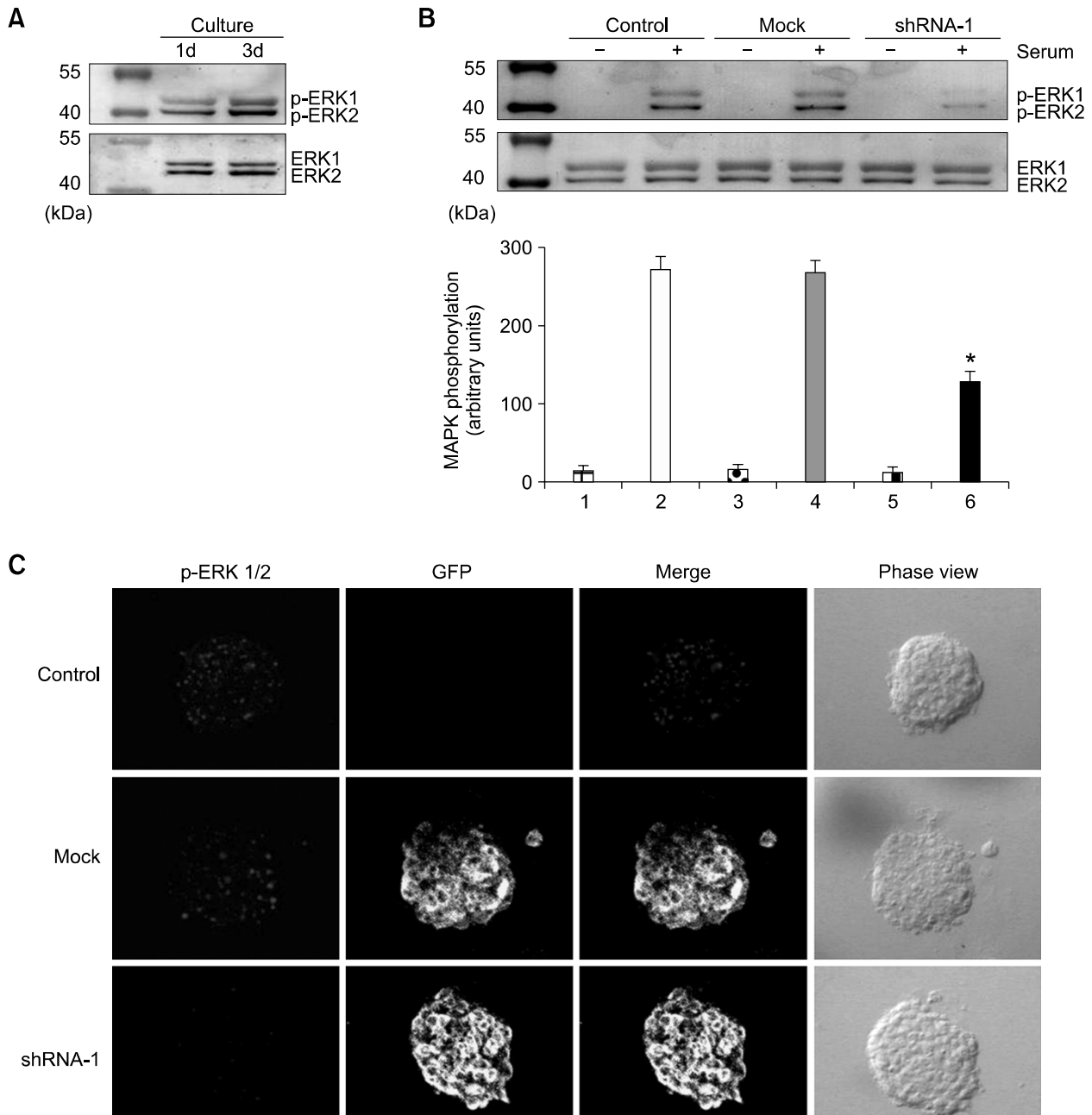


Figure 4. Effect of *Ugcg* shRNA on MAPK activation of mESCs. (A) MAPK activation of control mESCs was examined by Western blot analysis using anti-phospho-ERK1/2. The results were normalized to ERK1/2 expression. The ERK1/2 in the mESCs was phosphorylated during maintenance under normal culture conditions. 1d, 1day in culture; 3d, 3 days in culture. (B) Control mESCs, mock-transfected mESCs and shRNA-transfected mESCs were grown for 6 h in serum-free medium and then stimulated with serum for 10 min. The cells were lysed 10 min after stimulation and then the cell lysates were examined by Western blot analysis. The results represent the mean \pm SD of the level of phosphorylation observed in three experiments. Lane 1, control under serum-free condition; lane 2, control under serum condition; lane 3, mock-transfected under serum-free condition; lane 4, mock-transfected under serum condition; lane 5, shRNA-transfected under serum-free condition; lane 6, shRNA-transfected under serum condition. * $P < 0.05$ (C) phosphorylation of the ERK1/2 was examined by immunofluorescence staining.

the formation of neurites in cultured neuroblastoma cells (Ferreira *et al.*, 1990). Consistently, we found that expression of GFAP and MAP-2 was barely detectable in *Ugcg*-suppressed mESCs

whereas the level of nestin during neural differentiation was not changed in shRNA-transfected mESCs. Our results suggest that although glycosphingolipids may not play critical functional roles in

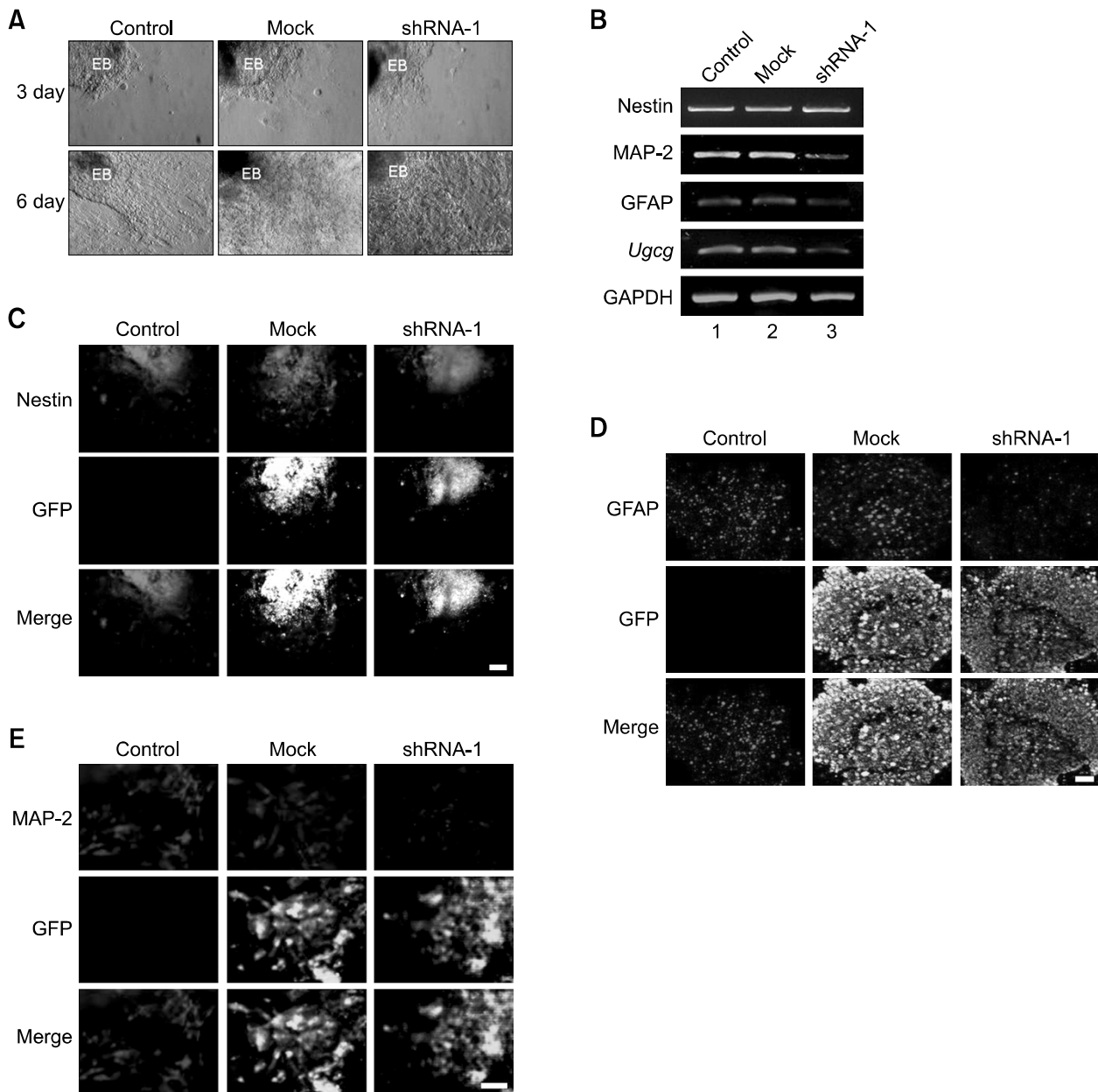


Figure 5. Influence of the suppression of *Ugcg* gene expression on neural differentiation of mESCs. EBs derived from stably transfected mESCs were dissociated and replated onto a gelatin-coated 6 well dish. EBs were incubated for 4 days to induce early neural differentiation. (A) Morphology of neural cells derived from mESCs induced by RA. 3 days, 3 days in culture; 6 days, 6 days in culture. The scale bar represents 250 μ m. (B) RT-PCR analysis of the various genes (nestin, MAP-2, GFAP, *Ugcg*) mRNA level of neural cells derived from control, mock-transfected and shRNA-transfected mESCs. RT-PCR using primer sets for the appropriate genes. (C-E) show an immunofluorescence analysis conducted to confirm the expression of nestin, GFAP and MAP-2, respectively. The cells were analyzed using a confocal fluorescence microscope (Model FV300, Olympus Co., Tokyo, Japan). The scale bar represents 100 μ m.

the maintenance of pluripotency in mESCs, which has also been shown in hESCs (Brimble *et al.*, 2007), they have significant roles in neural differentiation process, especially neural maturation process related to GFAP and MAP-2 expression. The current notion that glycosphingolipids are

involved in later neural maturation process is supported by our previous study that overexpression of glycosphingolipids can facilitate neurite formation, which is one of neural maturation processes (Lee *et al.*, 2007).

In this study, we found that that suppression of

Ugcg gene expression inhibited ESC proliferation, possibly associated with reduction of MAPK activation. It is unknown whether reduction of ESC proliferation is exclusively due to the suppression of the ERK1/2 activity, or possibly also due to alteration in expression of sphingolipid metabolites by inhibition of *Ugcg* gene expression. Therefore, further studies are still need to check whether inhibition of *Ugcg* gene expression alters generation of sphingolipid metabolites, which may affect proliferation of mESCs. Our results demonstrated that inhibition of glycosphingolipid expression by downregulation of *Ugcg* gene expression did not affect differentiation of mESCs to nestin expressing neural precursor cells, suggesting that glycosphingolipids may not have a critical role in early neural commitment of mESCs (Kawai *et al.*, 1998; Lee *et al.*, 2007). Most significant effect of inhibition of glycosphingolipid expression was found in reduction of neural cell marker genes (GFAP and MAP-2). It suggests that the major role of glycosphingolipids is in differentiation of neural precursor cells into neuronal and glial cells. To understand the underlying mechanism by which glycosphingolipids control neural differentiation process in mESCs, future study is required to check whether there is direct regulatory pathway for glycosphingolipids to control expression of later neural cell specific genes.

Methods

mESCs culture and neural differentiation

mESC line J1 was maintained in an undifferentiated status on mouse embryo fibroblast cells in growth medium containing Dulbecco's modified Eagle medium (DMEM) (Gibco, Rockville, MD) supplemented with non-essential amino acids, 2 mM L-glutamine, 0.1 mM β -mercaptoethanol, leukemia inhibitory factor (1,000 unit/ml) (Chemicon, Temecula, CA), penicillin (100 unit/ml), streptomycin (100 μ g/ml), and 15% (v/v) fetal bovine serum (FBS) (Hyclone, Logan, UT), at 37°C in 5% CO₂. The feeder cells were treated with mitomycin solution (10 μ g/ml) for 3 h (Lee *et al.*, 2007). *In vitro* early neural differentiation was induced in mESCs using the 4-/4+ protocol (Bain *et al.*, 1995). The mESCs were grown to form unattached embryoid bodies (EBs) (4-) for 4 days in cell differentiation medium containing DMEM (Gibco, Rockville, MD) supplemented with nonessential amino acids, 2 mM L-glutamine, penicillin (100 unit/ml), streptomycin (100 μ g/ml), and 10% heat-inactivated FBS (Hyclone, Logan, UT). The EBs (4-) were cultured to generate EB (4+) in the presence of all-trans retinoic acid (2 μ M) for 4 days. The EBs (4+) were rinsed off and transferred to poly-D-lysine/laminin-coated tissue culture dishes and then incubated overnight in knockout-DMEM (Gibco) with 10% heat-inactivated FBS to attach the cells to the dish. The EBs (4+) were then rinsed

off and grown to induce early neural differentiation in DMEM/F12 medium (Gibco).

Plasmid construction and cell transfection

A shRNA kit was used to construct the mouse *Ugcg* gene (UDP-glucose ceramide glucosyltransferase; GenBank. Accession No. *NM_011673*) (SuperArray Bioscience Corp., Frederick, MD)-targeted shRNAs expression vectors. *Ugcg*-shRNAs (cloned-1, cloned-2, cloned-3 and cloned-4) were cloned into the linearized pGeneClip™ hMGFP vector (Promega Corp., Madison, WI) (Figure 1B). The most effective shRNA clone was selected based on transcriptional repression of the target gene. For stable transfection with targeting vectors, mESCs (1×10^5 cells/well) were plated in 6-well gelatin-coated plates without mouse embryonic fibroblast feeder cells and then transfected with either circular targeting vectors or linearized targeting vectors using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). After 24 h of incubation at 37°C in 5% CO₂, the medium was changed to the standard medium described above. Individual clones with GFP fluorescence were identified, picked, and expanded.

Cell proliferation assay

Cells were seeded at a density of 4×10^4 cells/well in 24-well plates and then transfected for 24 h at 37°C. Forty eight hours after transfection, cell proliferation was determined by MTT assay. Each well was incubated in MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (Sigma) solution for 4 h, and the absorbance was then determined at 490 nm using a spectrophotometer. The experiments were repeated four times, and the cell proliferation data were analyzed using analysis of variance (ANOVA) followed by Tukey's multiple comparison test to determine if significant differences existed between groups ($P < 0.05$).

Ganglioside extraction

Gangliosides were extracted and purified using previously described methods (Ladisch and Gillard, 1985). Briefly, the cells were homogenized and the total lipids were then extracted in distilled water at 4°C. Next, the extracted total lipid in chloroform/methanol (1:1, v/v) was evaporated under N₂ gas and then dissolved in chloroform/methanol/H₂O (15:30:4, v/v/v). The dissolved total lipid sample was then applied to a diethylaminoethyl sephadex A25 column (Sigma) and washed with chloroform/methanol/H₂O (15:30:4, v/v/v) to remove the neural lipids. The acidic lipids were then eluted by adding chloroform/methanol/sodium acetate (15:30:4, v/v/v) to the column. Next, the eluted samples were dried at 30°C under N₂ for 5 h, dissolved in chloroform/methanol (1:1, v/v), and alkalinized in 12N ammonium hydroxide solution. The acidic lipid samples dissolved in chloroform/methanol (1:1, v/v) were then applied to a Sep-Pak C18 cartridge column, and then washed with H₂O to remove the non-hydrophobic lipids. The gangliosides were then eluted by adding methanol, after which they were dried at 30°C under N₂ for 3 h and

then stored at -80°C .

High-performance thin-layer chromatography (HPTLC)

HPTLC analysis of the gangliosides was performed using 10×10 or 10×20 cm thin-layer chromatography 5651 plates (Merck, Darmstadt, Germany). The purified gangliosides (1 mg protein/lane) were loaded onto thin-layer chromatography 5651 plates (Ladisich and Gillard, 1985), which were then developed in chloroform/methanol/0.25% $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ (50:40:10, v/v/v). The gangliosides were stained with resorcinol-HCl reagent. Bovine brain gangliosides were used as a standard to categorize the individual ganglioside species.

Immunofluorescence analysis

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 mins at room temperature and permeabilized with 0.25% Triton X-100 for 10 mins at 37°C . The cells were then blocked with PBS containing 4% BSA for 20 mins followed by incubation for 16 h at 4°C with primary antibodies specific to neural cell markers [nestin (Chemicon International Inc., Temecula, CA), GFAP (glial fibrillary acidic protein) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and MAP-2 (microtubule-associated protein 2) (Sigma)], stage specific embryonic antigen-1 (SSEA-1) (Chemicon), phospho-extracellular signal-regulated kinase 1/2 (ERK1/2) (Promega) and gangliosides (GM3, GD3) (Seikagaku Corp., Tokyo, Japan) in PBS containing 1% BSA. The cells were washed with PBS containing 1% BSA followed by incubation with secondary antibodies conjugated to fluorescent markers [goat anti-rabbit IgG-Alexa fluor 350, goat anti-rabbit IgG-Alexa fluor 488, goat anti-mouse IgM-Alexa fluor 350 (Molecular Probes, Carlsbad, CA), and goat anti-mouse IgG-TRITC (Sigma)] at 1:500 dilution. To detect alkaline phosphatase (AP), mESC colonies were fixed in 4% formaldehyde for 15 min and stained with Fast red TR/naphthol AS-MX for 15 min (Sigma). After washed with PBS, the cells were analyzed using a fluorescence microscope (Carl Zeiss Inc., Germany) and a confocal fluorescence microscope (Model FV300, Olympus Co., Japan).

Western blot analysis

Cells were homogenized with lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1 mM PMSF, 1 mM Na_3VO_4 , 1 mM NaF, 1% Triton X-100, 0.5 mM EDTA, and 0.5% sodium deoxycholate] and then centrifuged at $15,000g$ for 30 min. The protein concentration was then measured according to the Bradford method. Equal amounts of proteins (40 μg) were separated on a SDS/7%-polyacrylamide gel and then transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The blots were blocked for 2 h with 5% (w/v) non-fat dried milk in Tris-buffered saline (TBS) [10 mM Tris-HCl (pH 8.0) and 150 mM NaCl, 0.05% Tween-20]. The membrane was incubated for 16 h at 4°C with rabbit IgGs specific to ERK1/2, phospho-ERK1/2 (Promega),

respectively. The blot was incubated with anti-rabbit alkaline phosphatase-conjugated secondary antibody (Sigma). After incubation in BCIT/NBT (Zymed, San Francisco, CA), the membranes were scanned with Microtek ScanMaker 5 and quantified by densitometry analysis (Beta 4.0.3 of Scion Image, Frederick, MD).

RNA extraction and reverse transcription-PCR mRNA analysis

Total RNA from the mESCs was isolated using a TRI reagent (Molecular Research Center, Inc., Cincinnati, OH). RT-PCR was conducted to exam expression of the Oct3/4, Sox2, nestin, MAP-2, GFAP and *Ugcg* using the one-step RT-PCR kit (Promega) with a Takara PCR Thermal Cycler DICE Gradient (Takara, Shiga, Japan) under the following conditions: 94°C for 5 mins, followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 7 mins. PCR was conducted using the following primer sets: forward (5'-GGCGTTCTC-TTTGGAAAGGTGTTTC-3') and reverse (5'-CTCGAACCA-CATCCTTCTCT-3') for Oct3/4; forward (5'-GGCAGCTAC-AGCATGATGCAGGAGC-3') and reverse (5'-CTGGTCAT-GGAGTTGTACTGCAGG-3') for Sox2; forward (5'-AGAC-AGTGAGGCAGATGAGT-3') and reverse (5'-ATGAGAG-GTCAGAGTCATGG-3') for nestin; forward (5'-CTGGTGC-TTTTAAACAGGCG-3') and reverse (5'-TTGCAGTTGAT-CCAGGGGTA-3') for MAP-2; forward (5'-GCTCAATGACCGCTTTGCTA-3') and reverse (5'-TGCAAAGTTGTCC-CTCTCCA-3') for GFAP; forward (5'-CCCAAATGTCGAT-GC-3') and reverse (5'-AAGGTGGCAGCAAAG-3') for *Ugcg*; and forward (5'-TTCACCACCATGGAGAAGGCT-3') and reverse (5'-GACAACCTGGTCCTCAGTGA-3') for GAPDH.

Supplemental data

Supplemental Data include two figures and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-41-12-02.pdf.

Acknowledgements

We thank Dr. Naoyuki Taniguchi (Department of Biochemistry, Osaka University Graduate School of Medicine, Osaka, Japan) for numerous suggestions and comments regarding the subjects discussed in this study. This work was supported by Wonkwang University in 2008.

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