Ganglioside GM1 influences the proliferation rate of mouse induced pluripotent stem cells

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Gangliosides play important roles in the control of several biological processes, including proliferation and transmembrane signaling. In this study, we demonstrate the effect of ganglioside GM1 on the proliferation of mouse induced pluripotent stem cells (miPSCs). The proliferation rate of miPSCs was lower than in mouse embryonic stem cells (mESCs). Fluorescence activated cell sorting analysis showed that the percentage of cells in the G2/M phase in miPSCs was lower than that in mESCs. GM1 was expressed in mESCs, but not miPSCs. To confirm the role of GM1 in miPSC proliferation, miPSCs were treated with GM1. GM1-treated miPSCs exhibited increased cell proliferation and a larger number of cells in the G2/M phase. Furthermore, phosphorylation of mitogen-activated protein kinases was increased in GM1-treated miPSCs. [BMB Reports 2012; 45(12): 713-718]

INTRODUCTION

Induced pluripotent stem cells (iPSCs) have been generated from mouse fibroblasts by retroviral introduction of four defined transcription factors: _Oct4, Sox2, Klf4,_ and _c-Myc_ (1). iPSCs are indistinguishable from embryonic stem cells (ESCs) in terms of morphology, self-renewal, and expression of ESC markers (2, 3). In some recent studies, iPSCs were generated from various cell types, including neural stem cells, liver cells, stomach cells, and pancreatic beta cells, and can give rise to chimeric mice that are competent for germline transmission (4, 5). However, other studies found differences between ESCs and iPSCs, including different proliferation rates (2, 6).

Gangliosides are complex glycosphingolipids that contain one or more sialic acids, and which are ubiquitously expressed in the cell plasma membrane (7). The biosynthesis of gangliosides from ceramide involves the stepwise regulation of glycosyltransferases in the endoplasmic reticulum and Golgi apparatus, where diverse forms of gangliosides, including GM3, GD3, and GT3 of the a-, b-, and c-series are assembled (Supplementary Fig. 1) (8, 9). These gangliosides are thought to be involved in the control of several biological processes, including apoptosis, mouse embryonic development, cell proliferation, cell surface interactions, cell differentiation, and transmembrane signaling (10, 11). GM3 is mostly expressed during embryogenesis in mice (12, 13), but its expression is decreased during brain development (14). GD1a is also expressed during late embryogenesis (E9-E16), and increased expression of GD1a is observed during mouse brain development (12, 14). In particular, GM1 has been shown to possess antineurotoxic, neuroprotective, and neurorestorative capabilities, as well as the ability to facilitate neuronal commitment (15, 16). Moreover, GM1 appears to increase neural stem cell, smooth muscle cell, and rat PC-12 cell proliferation (17-19).

Gangliosides regulate mitogen-activated protein (MAP) kinase signaling pathways in fundamental cellular processes (20, 21). In particular, the extracellular signal-regulated kinase (ERK) 1/2 MAP kinase has been reported to regulate cell proliferation. Specifically, GM1 induces vascular smooth muscle cell proliferation via ERK 1/2 (18), and activated ERK 1/2 triggers an increase in DNA synthesis in human glioma cells (22).

In this study, we compared the proliferation rate and ganglioside expression pattern in cultured mouse induced pluripotent stem cells (miPSCs) and mouse embryonic stem cells

(mESCs). We also analyzed the effect of GM1 on the cell proliferation rate and MAP kinase-signaling pathway in miPSCs.

RESULTS

Comparison of miPSC and mESC proliferation and cell cycle

miPSCs and mESCs exhibited the same characteristics, such as expressions of alkaline phosphatase, stage-specific embryonic antigen-1, and pluripotency markers (Supplementary Fig. 2). However, a difference was apparent in the proliferation rate between miPSCs and mESCs (Fig. 1A). The time-dependent cell viability was investigated over 4 days. Although the proliferation rate of miPSCs and mESCs did not differ significantly during the first 2 days, the proliferation rate of miPSCs was lower than that of mESCs on day 3.
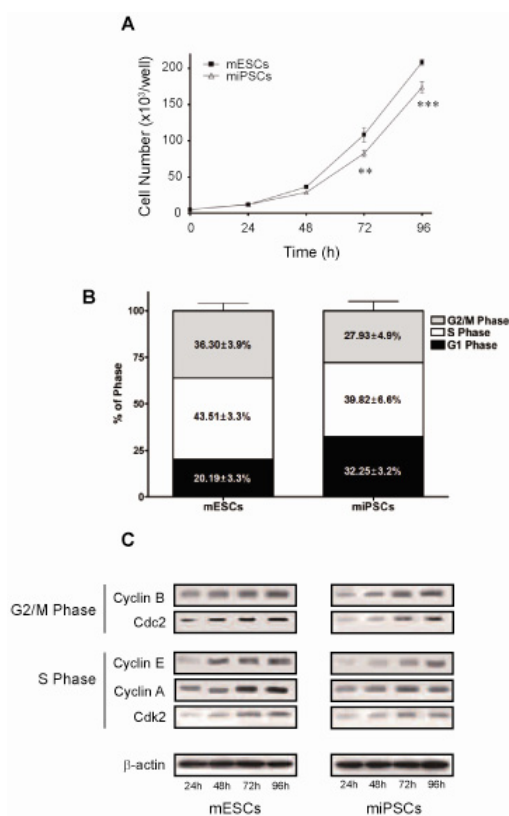
To determine whether cell cycle arrest or apoptosis had been induced, the distribution of cells in each cell cycle phase was analyzed by flow cytometry. As shown in Fig. 1B, the percentage of mESCs and miPSCs in the G1 phase was $20.19 \pm 3.3\%$ and $32.25 \pm 3.2\%$, respectively, at 4 days of culture. The percentage of cells in the S and G2/M phases was also higher in mESCs than in miPSCs. Although the expression level of cell cycle-related proteins increased over 4 days in both cell types, the rate of this increase was higher in mESCs compared with miPSCs. Western blot analysis revealed that the expression of cell cycle progression (S and G2/M phase)-related cyclins (cyclin E, cyclin A, cyclin B, and cdc2) and cyclin-dependent kinase 2 (cdk2) in mESCs was higher than miPSCs (Fig. 1C), whereas the expression of G1 phase-related cyclins and cdks was higher in mESCs (Supplementary Fig. 3).

Differential expression of gangliosides in miPSCs

The expression patterns of gangliosides in miPSCs and mESCs were different, and the GM3 and GD1a expression levels in mESCs were significantly higher than miPSCs (Fig. 2A). Of par-
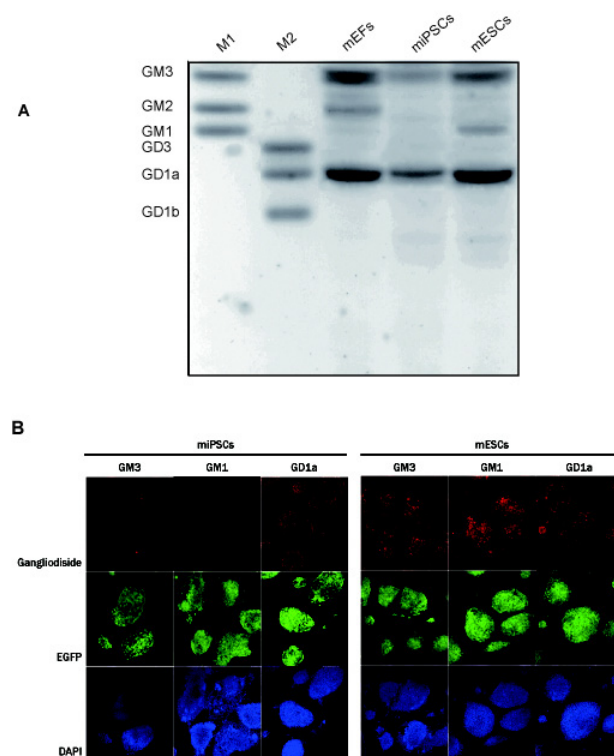

Fig. 1. Comparison of cell proliferation rates and the cell cycle in miPSCs and mESCs. (A) miPSCs and mESCs were seeded at a density of 5×10^3 cells/well in 24-well plates, and the cell proliferation rates were analyzed over 4 days by the MTT assay (**$P < 0.01$, ***$P < 0.001$). (B) Cell cycle distribution profiles determined by fluorescence-activated cell sorting (FACS)/flow cytometry at 4 days (96 h) of culture of miPSCs and mESCs. Data are presented as mean $\pm$ S.D. (C) Expression of G2/M phase- and S phase-related proteins in miPSCs and mESCs. The expression of cyclin B, Cdc2, cyclin E, cyclin A, and Cdk2 proteins was measured by western blotting using specific antibodies.

Fig. 2. Expression of gangliosides in miPSCs and mESCs. (A) Differential expression of gangliosides in miPSCs and mESCs. Lanes 1 and 2, ganglioside standard markers; lane 3, mEFs; lane 4, miPSCs; lane 5, mESCs. (B) Immunofluorescence staining of gangliosides. GM3 and GD1a expression (Cy5, red) and EGFP (green) were detected in miPSCs and mESCs. GM1 expression (Cy5, red) was observed in mESCs but not in miPSCs.

ticular interest, GM1 was expressed in mESCs but not in miPSCs. In addition, immunofluorescence showed the expression of gangliosides in miPSCs and mESCs (Fig. 2B, Supplementary Fig. 4).

Effect of GM1 on the proliferation of miPSCs

To determine whether GM1 increased the proliferation of miPSCs, cells were treated with various concentrations of GM1 over 4 days. The expression of pluripotent markers and the mRNA level were unaltered by the GM1 treatment (Supplementary Fig. 5). Immunofluorescence with antibody against GM1 resulted in the detection of GM1 in GM1-treated miPSCs (Supplementary Fig. 5).

The proliferation rate of miPSCs treated with GM1 was high-

Fig. 3. Effect of GM1 on the proliferation rate and the cell cycle of miPSCs. (A) miPSCs were seeded at a density of 5×10^3 cells/well in 24-well plates with various concentrations of GM1 (0, 1, 2, 5, and 10 μM) for 4 days, and the cell proliferation was then analyzed by the MTT assay (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (B) Cell cycle distribution profiles determined by FACS of a 4-day (96-h) culture of miPSCs and mESCs. Data are presented as mean $\pm$ S.D. (C) Expression of G2/M phase and S phase-related proteins in GM1-treated miPSCs. The expression of cyclin B, Cdc2, cyclin E, cyclin A, and Cdk2 was measured by Western blotting using specific antibodies.

er than that of untreated miPSCs. In particular, the proliferation rate of cells treated with 5 and 10 μM GM1 over 4 days was higher than that of untreated cells (Fig. 3A).

To elucidate the molecular mechanism underlying the proliferation rate of the treated miPSCs, the cell cycle stage was analyzed. As shown in Fig. 3B, the percentage of GM1-treated miPSCs in the G1 phase was lower than untreated miPSCs. However, the percentage of cells in the S and G2/M phases was greater in GM1-treated miPSCs than in untreated miPSCs. In particular, miPSCs treated with 5 and 10 μM GM1 showed a further increased percentage of cells in the S and G2/M phases. In addition, Western blot analysis also revealed the presence of S, G2/M, and G1 phase-related proteins (Fig. 3C, Supplementary Fig. 6).

Increased MAP kinase signaling by GM1

The phosphorylation of ERK1/2 MAP kinase controls cell proliferation and differentiation (23, 24). To determine whether the GM1 treatment increased proliferation rate via the ERK1/2 MAP kinase pathway, activation of this pathway was examined by Western blot analysis of GM1-treated miPSCs over 4 days. Western blotting using antibodies directed against phospho-c-Raf, -MEK1/2, and -ERK1/2 MAP kinases showed that these proteins were phosphorylated in the cells during this time (Fig. 4). GM1-treated miPSCs exhibited increased phosphorylation of c-Raf, MEK1/2, and ERK1/2 MAP kinase.

DISCUSSION

Stem cells are undifferentiated cells endowed with a high potential for proliferation and the capacity for self-renewal. Stem cells are very important from the biological and clinical perspectives. In basic biological studies and clinical use, stem cell surface markers are essential for identification and isolation of stem cells. Gangliosides localized at the cell surface can serve as markers (25). Many gangliosides expressed in pluripotent stem cells, multipotent stem cells, and cancer stem cells have been identified by biochemical and immunological analyses. Some gangliosides are excellent biomarkers of stem
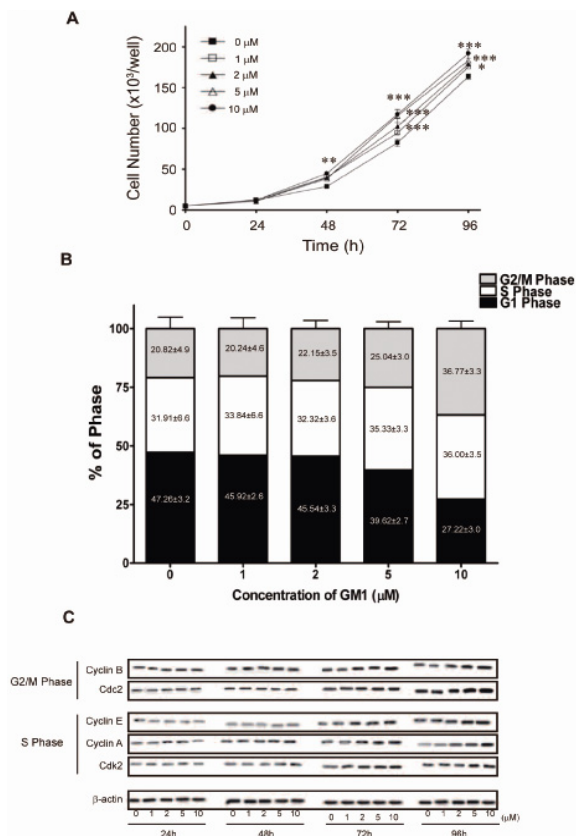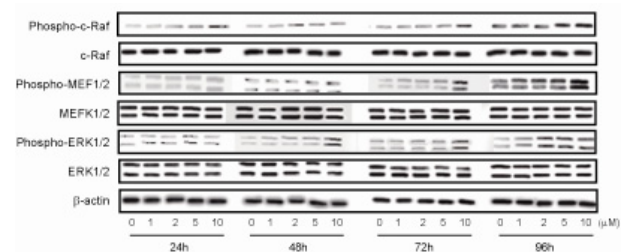

Fig. 4. Effect of GM1 on MAP kinase activation in miPSCs. The activation of MAP kinases, including c-Raf, MEK1/2, and ERK1/2, was analyzed by Western blotting of miPSCs treated with various concentrations of GM1 (0, 1, 2, 5, and 10 μM).

cells (26). miPSCs, which are pluripotent cells artificially generated from somatic cells such as fibroblasts by introducing *Oct4, Sox2, c-Myc,* and *Klf4,* express SSEA-1 (1). Presently, miPSCs expressed stem cell markers including AP, SSEA-1, *Oct4, Sox2,* and *Nanog.* Bhave *et al.* investigated the difference in the proliferation between iPSCs and ESCs (6). Our results revealed a difference in the proliferation rates of these cells. We demonstrated that miPSCs have similar characteristics to mESCs, but different proliferation rates as compared with mESCs.

High performance thin layer chromatography (HPTLC) analyses have revealed the expression of GM3, GM1, and GD1a in E14 mESCs and GM3, GM1, and GD3 in J1 mESCs (27-29). Presently, GM1 was expressed in mESCs, but not in miPSCs. Moreover, our results showed a difference in proliferation rate between miPSCs and mESCs. Therefore, we hypothesize that ganglioside GM1 increases the proliferation rate of miPSCs. GM1 has been implicated in neuronal development and differentiation (30, 31). In addition, GM1 has been shown to activate the high affinity nerve growth factor receptor tyrosine kinase (TrkA) in PC12 cells (32). Exogenous GM1 increases the proliferation of neural stem cells, vascular smooth muscle cells, and brain cells (18, 33-35), and over-expression of GM1 enhances PC12 cell proliferation (19). Our results show that exogenous GM1 enhanced the proliferation rate of miPSCs. Furthermore, on the basis of cell cycle analysis, the number of GM1-treated miPSCs in the G2/M phase was higher than the number of untreated miPSCs in the G2/M phase. These results demonstrate that GM1 induces an increased proliferation rate.

Gangliosides regulate the MAP kinase signaling pathways that are important in fundamental cellular processes (20, 21). In particular, ERK1/2 MAP kinase pathway has been reported to regulate cell proliferation. Treatment with GM1 activates MAP kinase signaling and increases DNA synthesis in human glioma cells (22). GM1 activates growth factor receptor tyrosine kinase, and this activation inhibits apoptosis (36). GM1 treatment of murine neuroblastoma cells increases the ERK1/2 MAP kinase activity (37). Depletion of glycosphingolipids following treatment with a glycosphingolipid synthesis inhibitor, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), represses the activation of the MAP kinase pathway and retards the proliferation of mouse neuroepithelial cells (21). Similarly, in mESCs, knock-down of glucosylceramide synthase using small hairpin RNAs inhibits the Ras-MAP kinase pathway and thereby decreases cell proliferation (38). In addition, dysfunction of the cerebellum and peripheral nerves and alteration of the Ras-MAP kinase pathway have been shown in glycosylceramide synthase-conditional knockout mice (39).

In this study, we showed the correlation between the proliferation rate and ganglioside expression in miPSCs and mESCs. We demonstrated that GM1 induced the miPSCs proliferation rate. Thus, our results indicate that the treatment with GM1 enhances the proliferation rate of miPSCs via activation of the ERK1/2 MAP kinase pathway.

MATERIALS AND METHODS

miPSCs and mESCs culture
miPSCs derived from OG2 (Oct-4GFP) mEFs by retrovirus-mediated delivery and expression Oct-4, Sox-2, Klf-4, and c-Myc, and mESCs derived from OG2 mice were generous gifts from Dr. Jeong Tae Do (CHA Stem Cell Institute and CHA Biotech, CHA University, Seoul, Korea). These cells were maintained on mitomycin C-treated mEF feeder plates in standard mESC medium containing DMEM supplemented with 15% fetal bovine serum (Hyclone, Logan, UT), non-essential amino acids, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, penicillin (100 U/ml), streptomycin (100 μg/ml), and leukemia inhibitory factor (1,000 U/ml; Millipore, Billerica, MA) at 37°C in 5% CO_2.

Viability assay
Cells were seeded at a density of 1×10^4 cells/well in 24-well plates. The cells were then treated with GM1, after which cell proliferation was determined by the established 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based assay. Each well was incubated with MTT solution (Sigma-Aldrich, St. Louis, MO) for 4 h, and the absorbance of each well was measured at 590 nm using a spectrophotometer.

FACS analysis
Cells were trypsinized and harvested in phosphate-buffered saline (PBS) followed by 2 washes with PBS. The cells were then resuspended in 70% ethanol overnight at 4°C. The fixed cells were collected by centrifugation, resuspended in RNaseA solution (0.2 mg/ml RNaseA in PBS), and incubated at 37°C for 30 min. The cells were then pelleted and incubated in propidium iodide (Sigma-Aldrich) staining solution (20 mg/ml propidium iodide in PBS) overnight at 4°C. The cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, Bedford, MA) and Cell Quest software (BD Biosciences) was used for data acquisition and analysis.

Western blot analysis
Cells were homogenized in RIPA buffer (Sigma-Aldrich) and centrifuged at $15,000 \times g$ for 30 min. The protein concentration was measured using the Bradford method. Equal amounts of protein (30 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a Hybond ECL nitrocellulose membrane (Amersham Pharmacia, Piscataway, NJ). Blots were blocked for 2 h at room temperature with 5% (w/v) non-fat dried milk in Tris-buffered saline containing 0.05% Tween 20. Each membrane was then incubated for 16 h at 4°C with specific antibody against cyclin B, Cdc2, cyclin D, Cdk4, Cdk6, cyclin A, cyclin E, Cdk2, c-Raf, phospho-c-Raf, MEK1/2, phospho-MEK1/2, ERK1/2, phospho-ERK1/2, or β-actin (each 1 : 500;

Santa Cruz Biotechnology, Santa Cruz, CA) as specified by the manufacturer. Each blot was then incubated with the corresponding horseradish peroxidase-conjugated anti-mouse, anti-rabbit secondary antibody (Santa Cruz Biotechnology) and proteins were visualized by the enhanced chemiluminescence system (Pierce, Rockford, IL).

HPTLC analysis

HPTLC analysis of the gangliosides was performed using a 10 × 10 cm HPTLC 5651 plate (Merck, Darmstadt, Germany) as previously described (29). Purified gangliosides were applied to the HPTLC plates, which were subsequently developed in chloroform/methanol/0.25% $CaCl_2 \cdot H_2O$ (50 : 40 : 10, v/v/v). The gangliosides were visualized with 0.2% resorcinol. Bovine brain gangliosides were used as markers for individual ganglioside species (Matreya LLC; Pleasant Gap, PA).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and permeabilized in 0.25% Triton X-100 for 10 min at 37^oC. The fixed, permeabilized cells were blocked for 20 min in 5% bovine serum albumin (BSA)/PBS, followed by incubation with primary antibodies against SSEA-1 (Chemicon, Temecula, CA) and gangliosides GM3, GM2, GM1, and GD1a (Seikagaku, Tokyo, Japan) in 5% BSA/PBS overnight at 4^oC. Next, the cells were washed with 1% BSA/PBS. A fluorescent secondary antibody, goat anti-mouse IgM-Cy5 for detecting SSEA-1 and gangliosides (Chemicon), was then applied at a dilution of 1 : 500. Hoechst 33342 reagent (Sigma-Aldrich) was used to stain the nuclei. The stained cells were observed under a model FV300 confocal scanning laser fluorescence microscope (Olympus, Tokyo, Japan).

Statistical analysis

All data are presented as mean ± S.D. Comparisons of multiple groups were performed by one-way analysis of variance (ANOVA), followed by pairwise comparisons with a Bonferroni post hoc test. All data were analyzed using GraphPad Prism version 4.00 software (GraphPad Software, La Jolla, CA).

Acknowledgements

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