

# Dynamic changes of gangliosides expression during the differentiation of embryonic and mesenchymal stem cells into neural cells

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Abbreviations: AP, alkaline phosphatase; EB, embryonic body; GSLs, glycosphingolipids, HPTLC, high performance thin layer chromatography; mESC, mouse embryonic stem cell; MSC, mesenchymal stem cell; RT-PCR, reverse transcriptase-PCR; SSEA-1, stage-specific embryonic antigen

## Abstract

**Stem cells are used for the investigation of developmental processes at both cellular and organism levels and offer tremendous potentials for clinical applications as an unlimited source for transplantation. Gangliosides, sialic acid-conjugated glycosphingolipids, play important regulatory roles in cell proliferation and differentiation. However, their expression patterns in stem cells and during neuronal differentiation are not known. Here, we investigated expression of gangliosides during the growth of mouse embryonic stem cells (mESCs), mesenchymal stem cells (MSCs) and differentiated**

**neuronal cells by using high-performance thin-layer chromatography (HPTLC). Monosialoganglioside 1 (GM1) was expressed in mESCs and MSCs, while GM3 and GD3 were expressed in embryonic bodies. In the 9-day old differentiated neuronal cells from mESCs cells and MSCs, GM1 and GT1b were expressed. Results from immunostaining were consistent with those observed by HPTLC assay. These suggest that gangliosides are specifically expressed according to differentiation of mESCs and MSCs into neuronal cells and expressional difference of gangliosides may be a useful marker to identify differentiation of mESCs and MSCs into neuronal cells.**

**Keywords:** cell differentiation; embryonic development; fluorescent antibody techniques; gangliosides; mesenchymal stem cells; stem cells

## Introduction

Gangliosides are complex glycosphingolipids (GSLs) with N-acetylneuraminic acids (NeuAc), which are major cytoplasmic membrane constituents in mammalian cells, and play important regulatory roles in cell proliferation and differentiation (Svennerholm, 1980; Nojiri *et al.*, 1986; Hakomori, 1990). Cellular gangliosides act as physiological modulators for growth factor mediated cell proliferation (Li *et al.*, 2000). Gangliosides are highly expressed in the vertebrate central nervous system (Ledeen and Yu, 1982), and the antibodies against gangliosides synthases detect the distribution of gangliosides in tissue and cells (Choo *et al.*, 2001). For example, monosialoganglioside 3 (GM3) is expressed in the mouse ovary tissue and rat glomerular mesangial cells (Kwak *et al.*, 2003). Survival of differentiated stem cells depends on the inhibition of the ganglioside biosynthesis (Liour and Yu, 2002). These suggest that gangliosides may regulate the growth and differentiation of stem cells.

Stem cells can be used for the study of developmental processes and offer tremendous potentials for clinical applications as an unlimited source for transplantation and tissue regeneration therapies (Fortier, 2005). Mouse embryonic stem cells (mESCs) are pluripotent cells which are generated from the inner cell mass of blastocysts (Evans and Kaufman,

1981). When mESCs are cultured with mouse embryonic fibroblast, feeder cells, they proliferate indefinitely and retain the potential to differentiate into various lineages of all three primary germ layers (Martin, 1981). Various studies conducted to differentiate mESCs into neuronal cells, cardiomyocytes, muscle cells, and insulin secreting cells (He *et al.*, 2005; Milne *et al.*, 2005; Wei *et al.*, 2005). Mesenchymal stem cells (MSCs) isolated from adult bone marrow are multipotent cells and can be differentiated into cartilage, tendon, fat, or bone marrow stroma (Woodbury *et al.*, 2000). Recent studies demonstrated the possibility that MSCs can be differentiated into other cell types such as cardiac myoblasts, endothelial cells, or neurons (Zhao *et al.*, 2002; Piao *et al.*, 2005). However, expression of gangliosides during the differentiation of mESCs and MSCs into neuronal cells is not known.

In this study, we investigated the spatiotemporal expression of gangliosides during differentiation of mESCs and MSCs into neuronal cells and whether ganglioside expression can be used as a biomarker for identification of neuronal differentiation of mESCs and MSCs in culture.

## Materials and Methods

### Mouse ES cells and mesenchymal stem cell culture

J1 mESCs were maintained in the undifferentiated state with feeder cells and leukemia inhibitor factor and cultured in the mouse ES culture medium at 37°C as described previously (Kawai *et al.*, 1998). MSCs originated from human periodontal bone marrow were primarily cultured as described previously (Woodbury *et al.*, 2000). At passage 6, MSCs were transferred to DMEM media contained 20% FBS, and passages 6-20 were used for experiment.

### Embryonic body formation and neuronal differentiation

Embryonic bodies (EBs) were formed as described previously (Zhang *et al.*, 2005). To differentiate EBs into neuronal cells, retinoic acid (5 µM) was treated for 4 days (Okada *et al.*, 2004). Neuronal differentiation of MSCs was performed as described previously (Kotobuki *et al.*, 2004).

### Stem cell marker staining

Stage-specific embryonic antigen (SSEA-1), mESCs marker, was detected by the antibody against SSEA-1 (Chemicon) and FITC-conjugated IgG (Sigma). 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). CD44 antibody (ImTec Diagnostics, Belgium) was used for a MSCs marker.

### RT-PCR

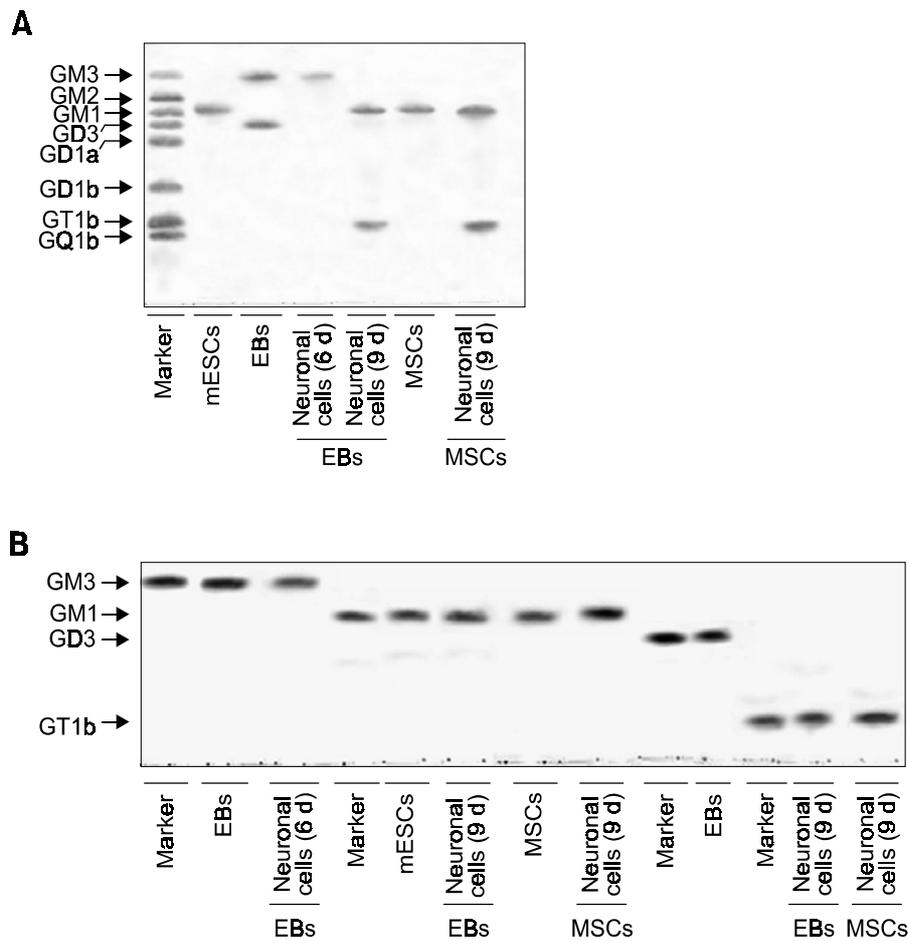
Total RNA from mESCs was isolated with the Trizol Kit (Sangon, China). RT-PCR was carried out using the one-step RT-PCR kit (Promega) with the Eppendorf Mastercycler. PCR conditions were 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 60°C for 1 min, and 68°C for 2 min, and final extension at 68°C for 7 min (Kim *et al.*, 2005). Forward (5'GGCG-TTCTCTTTGGAAAGGTGTTTC3') and reverse (5'CTCGAACCACATCCTTCTCT3') primers were used for the *Oct4* gene (Bortvin *et al.*, 2003), and forward (5'GGCAGCTACAGCATGATGCAGGAGC3') and reverse (5'CTGGTCATGGAGTTGTACTGCAGG3') primers were used for the *Sox2* gene (Zappone *et al.*, 2000).

### Ganglioside purification and high-performance thin-layer chromatography

Gangliosides of mESCs, EBs, MSCs, and differentiated neuronal cells were extracted and fractionated according to the procedure described previously (Svennerholm *et al.*, 1957). Gangliosides were analyzed by high-performance thin-layer chromatography (HPTLC) plates (Merck, Darmstadt, FRG). The solvent system for developing chromatograms was chloroform/methanol/0.22% CaCl<sub>2</sub> in water (55 : 45 : 10, v/v/v). Resorcinol staining was used for detection of gangliosides.

### Immunofluorescence of gangliosides

mESCs, EBs, MSCs, and differentiated neuronal cells were fixed on a gelatin-coated slide chamber. The cells were fixed with 4% formaldehyde at 4°C for 2 h (Graus *et al.*, 1984) and washed twice with PBS at room temperature for 10 min, followed by incubation with 5% BSA in PBS for 15 min at room temperature. After washing with PBS twice, they were incubated with monoclonal antibodies (MAbs) diluted in PBS (1:100) containing 5% BSA at 4°C for overnight. Cells were washed 4 times with cold PBS, and then incubated with FITC-conjugated goat anti-mouse IgM and IgG antibodies (Sigma) diluted in PBS (1:100) for 1 h (Choi *et al.*, 2004), followed by washing and sealing with a cover slip. The fluorescence intensity was measured by confocal laser scanning microscopy (Olympus Co.). MAbs specific for ganglioside GM3, GM2, GM1, GD1a, GD1b, GD3, GQ1b and GT1b were used (Seikagaku Co., Japan). A control was incubated without primary antibodies.



**Figure 1.** Differential expression of gangliosides in mESCs, EBs, MSCs and differentiated neuronal cells. Ganglioside expression (1 µg/lane) was analyzed using HPTLC and resorcinol method (A), and gangliosides on HPTLC plates were reacted with monoclonal antibodies against ganglioside GM3, GM1, GD3 and GT1b (B).

For delipidated controls, the cells were treated, prior to the staining, first with methanol and then with chloroform/methanol (1:1, v/v) for 10 min each.

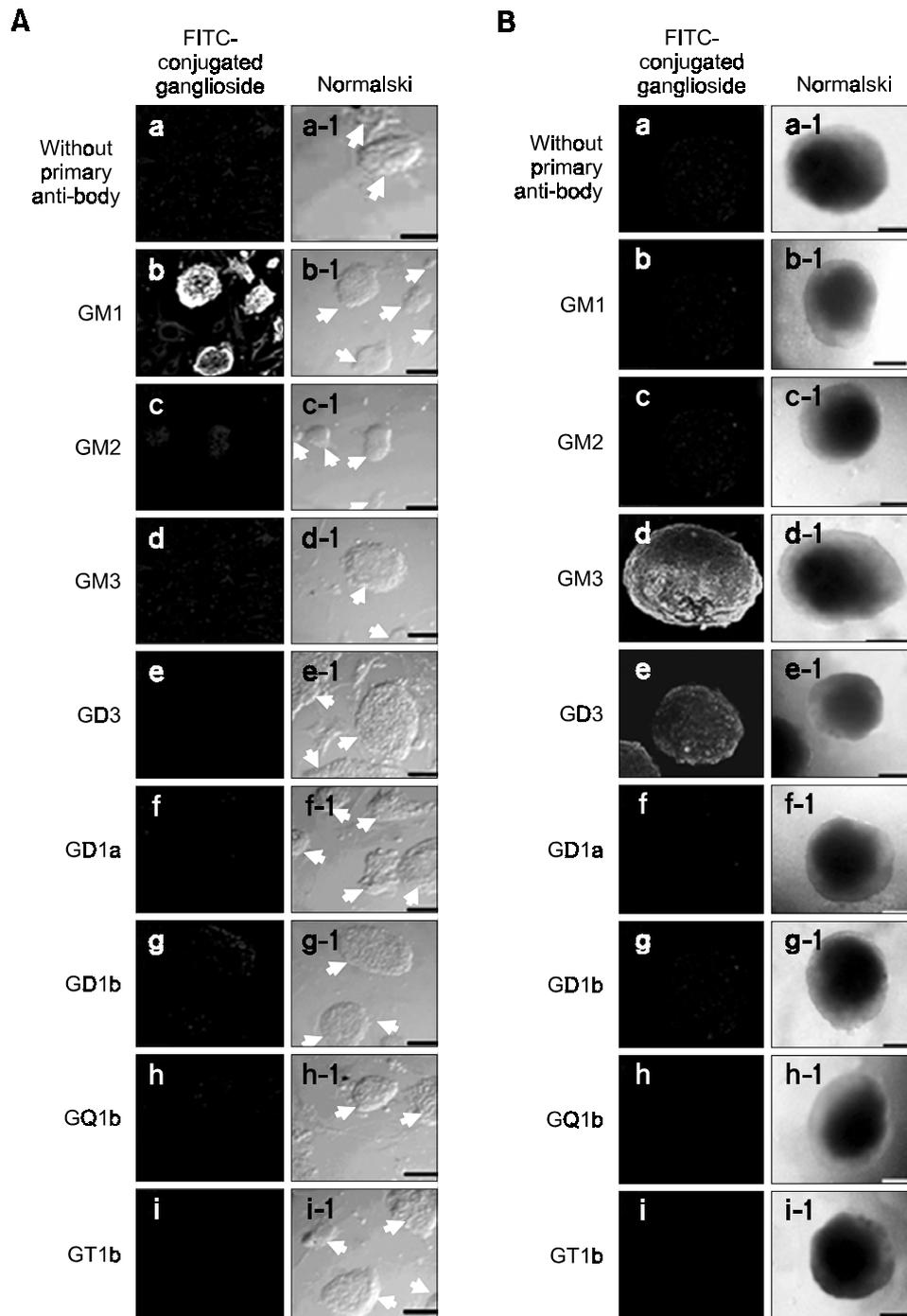
**Immunofluorescence for differentiated neuronal cells**

EBs and differentiated neuronal cells were fixed by 4% paraformaldehyde solution for 2 h and washed with PBS containing 0.1% Triton X-100, followed by blocking with PBS solution containing 5% BSA. Cells were incubated for 1 h with a monoclonal anti-mouse MAP2 antibody. The secondary antibody was goat anti-mouse IgG<sub>1</sub> conjugated with TRICT. The fluorescence intensity was measured by fluorescence microscopy (Olympus Co.). A control was incubated without primary antibodies. For delipidated controls, the cells were treated, prior to the staining, first with methanol and then with chloroform/methanol (1:1, v/v) for 10 min each.

**Results**

**Ganglioside expression**

Gangliosides extracted from mESCs, EBs, MSCs and differentiated neuronal cells were analyzed by HPTLC, and the representative results with 5 times are shown in Figure 1A. Ganglioside GM1 was detected in the mESCs, while gangliosides GM3 and GD3 were detected in the EBs. Ganglioside GM3 was observed in the neuronal cells differentiated from mESCs at 6 days after induction of differentiation, whereas ganglioside GM1 and GT1b were expressed in the neuronal cells differentiated from mESCs at 9 days after induction of differentiation. MSCs expressed ganglioside GM1, and ganglioside GM1 and GT1b were observed in the neuronal cells differentiated from MSCs at 9 days after induction of differentiation. As shown in Figure 1B, these results were also confirmed by the HPTLC immunostaining.

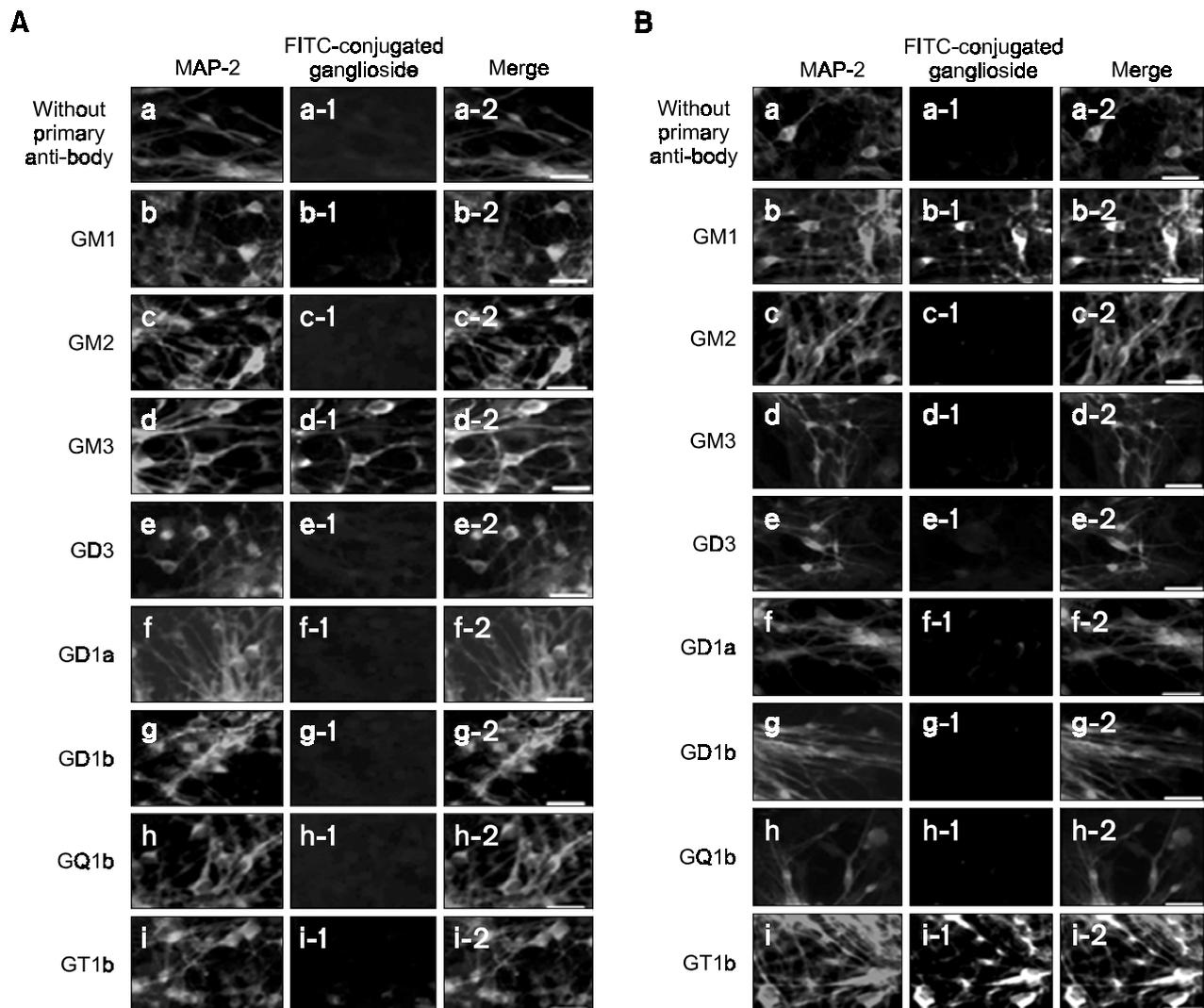


**Figure 2.** Immunostaining of gangliosides in mESCs and EBs. mESCs (A) and EBs (B) were immunostained with monoclonal antibodies specific for gangliosides, and immunoreactivity was observed by laser scanning confocal microscopy. Arrow indicates mES colony. Scale bars are 150  $\mu$ m.

### Immunofluorescence of gangliosides in mESCs and EBs

To confirm the results shown in Figure 1, we investigated the localization of gangliosides in mESCs

and EBs. As shown in Figure 2A, mESCs strongly expressed ganglioside GM1, but other ganglioside GM2, GM3, GD1a, GD1b, GD3 and GQ1b were not detected. In contrast to these, immunostaining



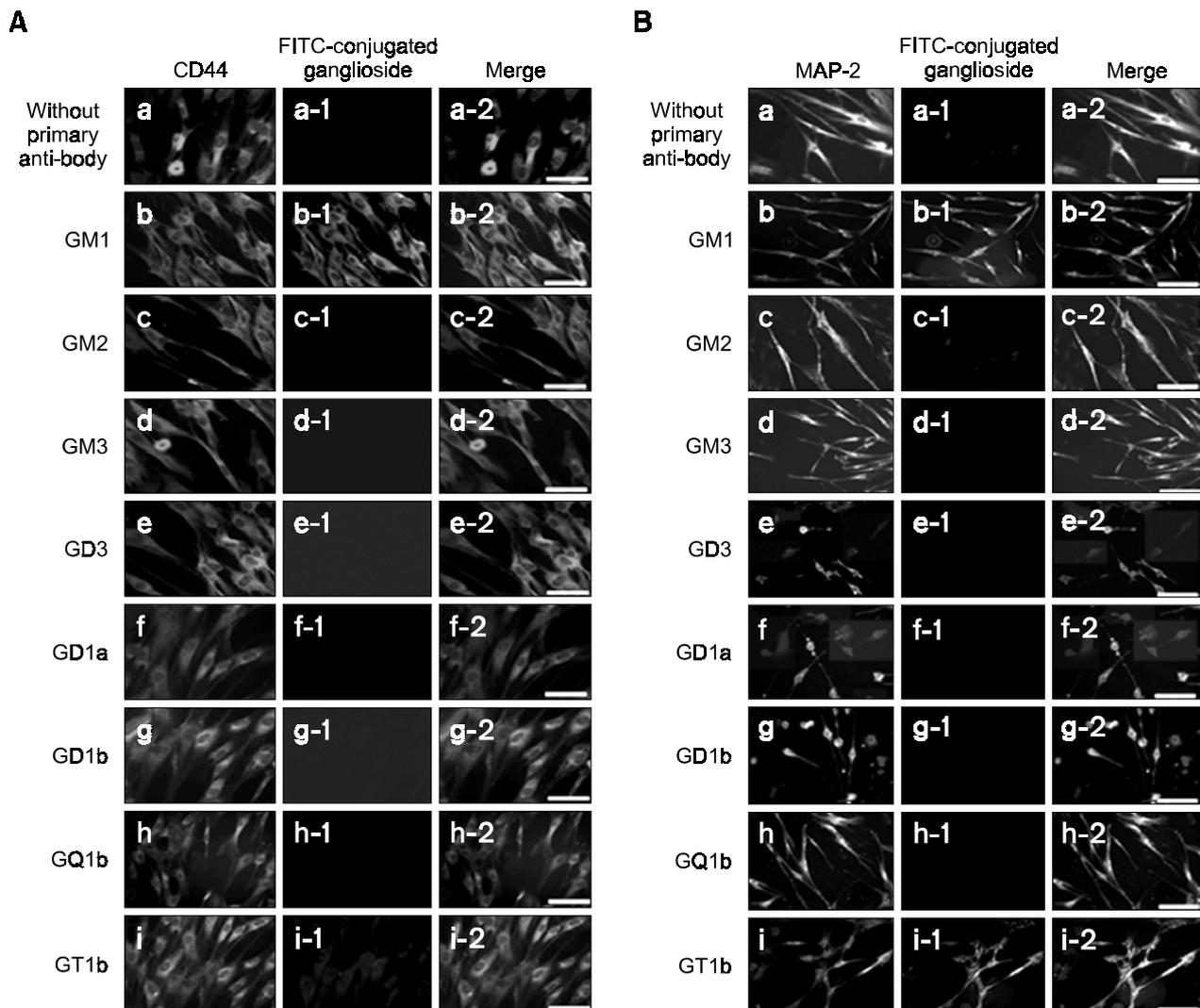
**Figure 3.** Expression of MAP-2 and gangliosides in the neuronal cells differentiated from EBs. At 6 (A) and 9 (B) days after induction of differentiation, neuron-like cells were double-stained with the neuronal marker MAP-2- and ganglioside-specific antibodies. Scale bars are 10  $\mu$ m.

revealed that EBs strongly expressed ganglioside GM3 and GD3, but ganglioside GD1a, GD1b, GM1, and GT1b were not expressed in EB (Figure 2B). These results are consistent with those obtained by HTPLC.

#### Ganglioside expression in the neuronal cells differentiated from EBs

Neuron-like cells appeared at 6 days after induction of differentiation, and these cells strongly expressed MAP-2, a neuronal cell marker (Figure 3A, a-i). Compared with gangliosides expression in EBs, ganglioside GM3 expression was significantly decreased in the neuronal cells at 6 days after differentiation

(Figure 3A, d-1), and other gangliosides in these cells were not detected (Figure 3A, b-1-c-1 and e-1-i-1). The merged image between MAP-2 and ganglioside GM3 (Figure 3A, d-2) showed that GM3 was localized in the membranes of neuronal cell bodies. At 9 days after induction of differentiation, neuronal cells still expressed MAP-2 (Figure 3B, a-i) and ganglioside GM1 (Figure 3B, b-1) and GT1b (Figure 3B, i-1). However, these cells did not express other ganglioside GM2, GM3, GD1a, GD1b, GD3 and GQ1b (Figure 3B, c-1-h-1). The merged images showed that ganglioside GM1 and GT1b were localized mainly in the neuronal cell bodies (Figure 3B, b-2 and i-2).



**Figure 4.** Expression of MAP-2 and gangliosides in the neuronal cells differentiated from MSCs. MSCs (A) and neuronal cells differentiated from MSCs at 9 days after induction of differentiation (B) were double-stained with the neuronal marker MAP-2- and ganglioside-specific antibodies. Scale bars are 10  $\mu\text{m}$ .

#### Ganglioside expression in the neuronal cells differentiated from MSCs

MSCs expressed CD44 (Figure 4A, a-i), a mesenchymal cell marker, and ganglioside GM1 (Figure 4A, b-1). Merged images showed that CD44 and ganglioside GM1 were mainly expressed in the cytoplasm of MSCs (Figure 4A and b-2). However, these cells did not express other gangliosides examined in this study (Figure 4A and c-1-i-1). Neuronal cells differentiated from MSCs at 9 days after induction of differentiation strongly expressed MAP-2 (Figure 4B, a-i) and ganglioside GM1 (Figure 4B and b-1) and GT1b (Figure 4B and i-1), but other gangliosides (Figure 4B and c-1-h-1) were not expressed in these cells. Merged images showed that

ganglioside GM1 and GT1b were mainly expressed in the neuronal cell bodies and axons (Figure 4B, b-2 and i-2).

#### Discussion

Glycosphingolipids are involved in several cellular processes including cell adhesion, cell-cell interaction, embryogenesis and development and differentiation of cells (Bektas and Spiegel, 2004). Because the previous studies suggested that gangliosides are important for neuronal differentiation (Ledeen and Yu, 1982), this study investigated differential expression of gangliosides during neuronal differentiation from mESCs and MSCs. Ganglioside GM1 was expres-

sed in mESCs and MSCs, and EBs mainly expressed ganglioside GM3 and GD3. In contrast to these, neuronal cells differentiated from EBs at 6 days after induction of differentiation expressed only ganglioside GM3, and the neuronal cells differentiated from EBs and MSCs at 9 days after initiating differentiation expressed both ganglioside GM1 and GT1b. These differential expressions suggest that gangliosides may regulate the differentiation of mESCs and MSCs into neuronal cells and that ganglioside expression can be used as a marker for identification of neuronal differentiation from EBs and MSCs.

We firstly examined the cellular biological characteristics of undifferentiated mESCs to be used in this study and observed that mESCs obviously expressed the SSEA-1, a cell surface marker for mESCs and showed a round and compact morphology (data not shown). *Oct4* and *Sox2*, a specific undifferentiated ES cell marker genes (Zappone *et al.*, 2000; Bortvin *et al.*, 2003), in undifferentiated mESCs were detected after RT-PCR with their appropriate sizes, and cells were also stained positively for alkaline phosphate. These results indicate that mESCs used in this study maintains undifferentiated properties of ES cells.

Using HPTLC and immunofluorescence method, this study observed only ganglioside GM1 expression in mESCs and MSCs, and it can be considered that these expressional patterns are relatively simple compared to those observed in the other types of cells such as ovarian and mesangial cells (Choo *et al.*, 2001; Kwak *et al.*, 2003), and we carefully consider that simple pattern of ganglioside expression in mESCs and MSCs may be related with a specific property of cell differentiation. Gangliosides in membranes regulate cell growth through the modulation of protein kinases and growth factor-mediated cell proliferation (Gouni-Berthold *et al.*, 2001). It has been also suggested that ganglioside GM1 is considered to protect cells from apoptosis in neuronal PC 12 cells and heart fibroblasts (Ferrari *et al.*, 1995; Cavallini *et al.*, 1999). In the physiologically relevant study, ganglioside GM1 protects the mouse heart from hypoxic cell death. These may lead us to consider that ganglioside GM1 expressed in mESCs and MSCs may have a protective role against apoptosis, resulted in growth and proliferation of mESCs and MSCs.

Generating differentiated cell types from embryonic stem cells is preformed through the formation of EBs, in which embryonic stem cells spontaneously developed into the multicellular aggregates containing differentiated and undifferentiated embryonic stem cells. They are similar to the embryos of the egg-cylinder stage. EBs recapitulate many aspects of the lineage-specific differentiation and temporal and spatial gene

expression patterns of early embryogenesis (Leahy *et al.*, 1999). In the early stage of embryogenesis, ganglioside GD3 is found in many embryonic tissues. The presence of ganglioside GD3 is generally considered as the high proliferate state of cells (Berra *et al.*, 1985). Ganglioside GM3 is also found in the early stage of embryonic development and plays important roles in cell-cell adhesion, differentiation, and proliferation (Kwak *et al.*, 2003). These suggest that ganglioside GM3 and GD3 expressed in the EBs may regulate the cell differentiation and/or proliferation.

Previous studies suggested that gangliosides are important for neuronal differentiation (Hakomori *et al.*, 1990). For example, ganglioside GM1 is widely distributed throughout the peripheral nervous system and has regulatory roles during neurogenesis and regeneration of injured peripheral nerves (Ogawa-Goto *et al.*, 1992; Stojiljkovic *et al.*, 1996). Ganglioside GT1b is expressed in the synapses of the brain (Kotani *et al.*, 1993). Exposure of the neurons to ganglioside GT1b for 3 days drastically enhances actin-rich dendrite generation (Higashi and Chen, 2004). These reports indicate an important role of ganglioside GM3, GM1 and GT1b in neurogenesis and may support the results obtained in this study. We observed expression of ganglioside GM1 and GT1b in the 9-day old differentiated neuronal cells from EBs and MSCs. These results may suggest that ganglioside GM1 and GT1b may be necessary for differentiation of mESCs and MSCs into neuronal cells. In summary, we provide evidences that ganglioside GM1 and GT1b specifically express during the differentiations of mESCs and MSCs into neuronal cells, and these may offer new information which differential expression of gangliosides can be used as a useful biomarker to identify neuronal differentiation of mESCs and MSCs.

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