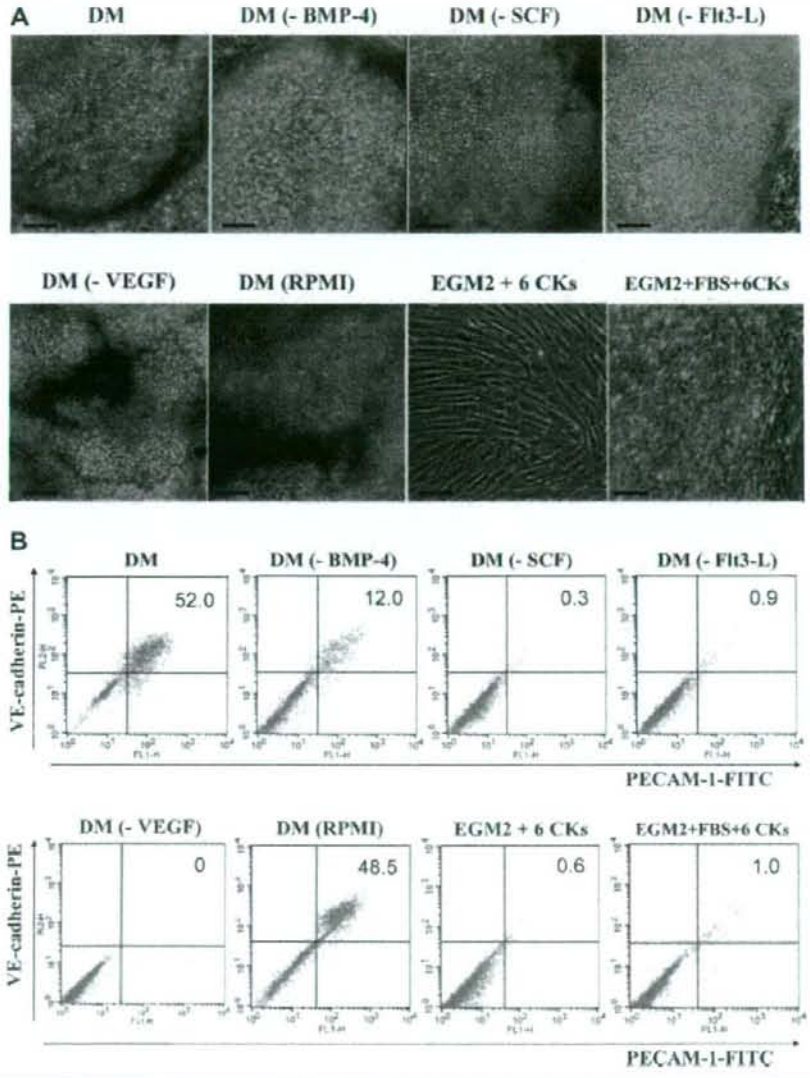

Fig. 9. The roles of medium components for vascular endothelial differentiation. The differentiation procedure was performed by depleting BMP-4 (DM(-BMP-4)), SCF (DM(-SCF)), Flt3-L (DM(-Flt3-L)) or VEGF (DM(-VEGF)) from the differentiation medium (DM), using differentiation medium prepared by using RPMI 1640 in place of IMDM (DM(RPMI)), using EGM®-2 BulletKit medium supplemented with all six cytokines (EGM2 + 6CKs), or using EGM®-2 BulletKit medium supplemented with 10% FBS and all six cytokines (EGM2 + FBS + 6CKs). A: The phase contrast micrographs. Note that BMP-4 depletion resulted in deficient production of inner round cells. Scale bars indicate 50 μm. B,C: Flow cytometric analyses for cell surface VE-cadherin/PECAM-1 expressions. From a number of experiments, we obtained two patterns of results shown in (B) and (C) with 1:2 frequencies (data not shown).

differentiation of ES cells and inhibit the expansion of vascular endothelial cells. Of course, further studies are required for the total understanding of the hematopoietic/endothelial differentiation mechanism from primate ES cells.

In this report, we termed the cells that are cell surface VE-cadherin/PECAM-1-negative but otherwise are equivalent to vascular endothelial cells as "atypical vascular endothelial cells." These cells seem to be frequently produced during primate ES differentiation (Kaufman et al., 2004). Whether the existence of atypical vascular endothelial cells is restricted to in vitro differentiation system or there are in vivo counterparts is a matter of great interest. It is known that fractions of adult human peripheral monocytes can give rise to endothelial-like cells. For example, monocyte-derived immature dendritic cells

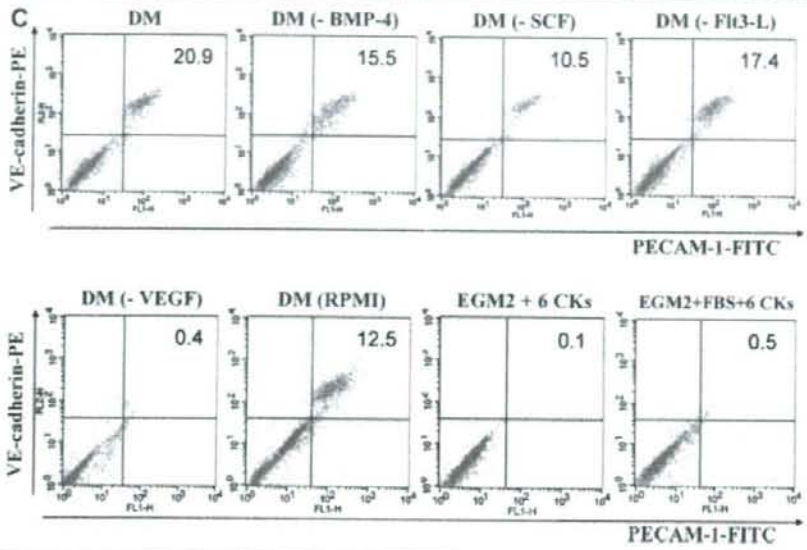

Fig. 9. (*Continued*)

(Fernandez Pujol et al., 2001) and monocyte-derived multi-potential cells (Kuwana et al., 2006) can produce functionally maturated vascular endothelial cells. Our cmES-derived cells are, however, negative for monocyte markers including CD68, CD14 and CD11b (Fig. 6K,L). As for now, it is not yet determined whether they belong to a novel endothelial population or they represent a transient state during which monocytic precursors are differentiating into vascular endothelial cells. Future investigation will be required for the evaluation of atypical vascular endothelial cells in vivo.

The ES-derived vascular endothelial cells generated by our method are subculturable up to eight passages, after which they undergo senescence (unpublished observation). Aging cells show enlarged morphologies and become positive in senescence-associated β-galactosidase activity assays along with p38 activation and p16 induction. The reason for this stress-induced senescence remains elusive. Although the senescence induction in ES-derived vascular endothelial cells may put certain restrictions on their usage, it may exclude the possible tumorigenesis via unlimited proliferation after transplantation. Moreover, our ES-derived vascular endothelial cells can be used for researches on stress-induced senescence, providing tools for investigations on mechanisms of vascular complication development in lifestyle-related stress-associated diseases such as diabetes mellitus (Yokoi et al., 2006) hypertension (Kobayashi et al., 2006) and atherosclerosis (Minamino et al., 2002).

Our differentiation method is applicable to human ES cells: the high efficiency vascular endothelial differentiation (~70%) with proper in vitro and in vivo functions has been achieved by using human ES cells and the same differentiation protocol (M. Nakahara, in preparation). Of course, human ES cells cannot be directly applied to regenerative medicine because of an immunological hurdle. However, when the hurdle will be overcome via a nuclear transfer technique (Byrne et al., 2007) or when iPS cells (Takahashi et al., 2007; Yu et al., 2007) will be improved for safety application to clinical usages, our method will provide the most effective tool for regenerative medicine targeted to vascular endothelial disorders.

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STEM CELLS AND DEVELOPMENT
Volume 18, Number 4, 2009

DOI: 10.1089/scd.2008.0215

Human GPM6A Is Associated With Differentiation and Neuronal Migration of Neurons Derived from Human Embryonic Stem Cells

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Glycoprotein M6A (GPM6A) is known as a transmembrane protein and an abundant cell surface protein on neurons in the central nervous system (CNS). However, the function of GPM6A in the differentiation of neurons derived from human embryonic stem (ES) cells is unknown. To investigate the function of GPM6A in neural differentiation, we generated human ES cell lines with overexpressed (B2h-oeM6A) or suppressed (B2h-shM6A) human GPM6A. Real-time polymerase chain reaction (PCR) showed that overexpression of GPM6A markedly increased the expression of neuroectodermal-associated genes (*OTX1, Lmx1b, En1, Pax2, Sox2*, and *Wnt1*), and the number of neural stem cells (NSCs) derived from B2h-oeM6A cells compared to control vector transfected human ES cells (B2h-Mock1). Our results show an increase in the number of differentiated neuronal cells (cholinergic, catecholaminergic, and GABAergic neurons) from NSCs derived from B2h-oeM6A cells. On the other hand, suppression of human GPM6A expression using a short hairpin RNA (shRNA) in human ES cells led to a decrease in both the expression of neuroectodermal-associated genes and the number of NSCs derived from B2h-shM6A cells. In addition, our results show a decrease in the number of differentiated neuronal cells from NSCs in B2h-shM6A cells compared to control vector transfected human ES cells (B2h-shNSP1). Moreover, overexpression or suppression of human GPM6A in human ES cells led to an increase or decrease, respectively, of neuronal migration. Hence, our findings suggest that expression level of GPM6A is, directly or indirectly, associated with the differentiation and neuronal migration of neurons derived from undifferentiated human ES cells.

Introduction

Embryonic stem (ES) cells are continuously growing pluripotent cells derived from the inner cell mass of preimplantation stage embryos [1,2]. ES cells, which retain the ability to self-renew in vitro, can give rise to various cell types, including neurons, pancreatic cells, and hematopoietic cells [3–7]. The rate of ES cells differentiation is affected by induction or inhibition of certain genes expression. Neurons are generated from ES cells through neural stem cells (NSCs) in embryoid bodies (EB) [5,6]. Nakayama et al. [7] have developed a simple method to efficiently produce NSCs from undifferentiated ES cells under free-floating conditions in an astrocyte-conditioned medium (ACM) without formation of EB.

Glycoprotein M6A (GPM6A) is a tetraspan membrane protein abundant on neurons surface in the central nervous system (CNS) [8], and a member of the proteolipid protein (PLP/DM20) family, which includes GPM6B. GPM6A shares 40% homology with PLP/DM20 and 55% homology with GPM6B. Studies of PLP/DM20 family expression have shown that GPM6A is present in neurons, GPM6B in both neurons and glia, and PLP/DM20 in oligodendrocytes [9]. Besides, it has been reported that chronic psychosocial stress decreases expression level of GPM6A in the hippocampus, and that chronic administration of antidepressants prevents this down-regulation [10,11]. Mukobata et al. have reported that Ca^{2+} influx is increased by treatment with nerve growth factor (NGF) in rat pheochromocytoma PC12 cells with overexpression of GPM6A and that anti-GPM6A antibody suppresses both NGF-triggered Ca^{2+} influx and neuronal differentiation [12]. Overexpression of GPM6A has also been shown to induce filopodium formation in neuronal and

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nonneuronal cell lines, and GPM6A knockdown has been reported to decrease filopodium density and reduce synaptic density in hippocampal neurons [13]. A recent study has shown that GPM6A binds to the μ-opioid receptor and facilitates receptor endocytosis and recycling [14].

To investigate the function of GPM6A in neural differentiation, we generated human ES cell lines with overexpressed or suppressed GPM6A. In the present study, we show that GPM6A is associated with differentiation and migration of neurons derived from both undifferentiated human ES cells to NSCs and NSCs to neuronal cells.

Materials and Methods

Cell culture

Human ES cell line SA002.5 was obtained from Cellartis AB (Göteborg, Sweden) and cultured on mitomycin C (Kyowa Hakko Kirin Co. Ltd., Tokyo, Japan)-treated mouse embryonic fibroblasts in VitroHES (Vitrolife AB, Kungs-Backa, Sweden), supplemented with 4 ng/mL human recombinant basic fibroblast growth factor (bFGF) (R&D Systems, Inc., Minneapolis MN) at 37°C in 5% CO_2 [15–17]. The use of human ES cell lines was performed in conformity with *The Guidelines for Derivation and Utilization of Human Embryonic Stem Cell* (2001) of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Construction of expression plasmids

In order to obtain complete cDNA of human GPM6A, a reverse transcription (RT) polymerase chain reaction (PCR) was performed using human brain mRNA (Takara Bio Inc., Shiga, Japan). RT was carried out using random hexamers at 37°C for 60 min according to the manufacturer's instruction for First-Strand cDNA Synthesis Kit (GE Healthcare UK Ltd., Buckinghamshire, UK). cDNA fragment encoding human GPM6A was amplified using the primer set. PCR was carried out for 30 cycles. The reaction cycle was set at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The PCR-amplified fragment was cloned into pGEM-T Easy (Promega Corp., Madison, WI), giving pGEM-hM6A, and the inserted DNA was sequenced. The fragment of mouse GPM6A was then ligated into pcDNA3.1(+) vector (Invitrogen Corp., Carlsbad, CA), generating phM6A.

To express short hairpin RNA (shRNA) in ES cells, we constructed shRNA expression plasmid under the control of a U6 promoter against GPM6A as previously described [18] and obtained psTN-mG6A1 and psTN-NSP1. All recombinant DNA experiments conformed to National Institute of Health (NIH) guidelines.

Transfection in human ES cells

For stable GPM6A overexpression or suppression by shRNA in SA002.5 cells, 8×10^6 cells were electroporated at 0.25 kV and 500 μF using 20 μg of phM6A, pcDNA3.1(+), psTN-mG6A1, psTN-NSP1, and chicken β-actin (CAG) promoter-derived phrGFP (Promega). After replating, the cells were treated with 200 ug/mL G-418 (Invitrogen) for 2 weeks. GFP-positive and G418-resistant colonies were identified using PCR, and independent colonies were re-seeded in ES growth medium, as described earlier. The established cell lines were stained by antibodies in an ES Cell Characterization Kit (Chemicon International, Inc., Temecula, CA) as described later. Transfected ES cells were differentiated into NSCs and neuronal cells as described later.

Neural differentiation of human ES cells

Undifferentiated ES cells were differentiated into NSCs and neuronal cells according to the method of Nakayama et al. [7]. Briefly, undifferentiated ES colonies were picked up and cultured in ACM (Sumitomo Bakelite Co. Ltd., Tokyo, Japan) supplemented with 20 ng/mL bFGF under free-floating conditions for 2 weeks with human ES cells. Neural stem (NS) spheres, which were induced colonies with undifferentiated ES cells, were plated on poly-L-lysine (PLL) plus laminin (LAM)-coated plates (AGC Techno Glass Co. Ltd., Chiba, Japan) and differentiated with NS medium (NSM: NEUROBASAL medium [Invitrogen] supplemented with B27 supplement [Invitrogen], 2 mM L-glutamine [Sigma Chemical Co. Ltd., St. Louis, MO, USA], 20 ng/mL bFGF, and recombinant human epidermal growth factor [EGF; R&D Systems]) at 37°C in 5% CO_2 for 1 week. NS colonies were then picked up and transferred onto PLL/LAM-coated plates in NSM at 37°C in 5% CO_2 for 1 week, and this process was repeated four additional times. Finally, NSCs were plated on PLL plus LAM-coated plates and differentiated with ACM at 37°C in 5% CO_2 for 2–4 weeks. NSCs were stained with antibody against Nestin (Chemicon International, Inc., Temecula, CA), as described later.

Real-time PCR

Real-time PCR was performed using TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA), TaqMan Gene Expression Assays (Applied Biosystems), and ABI 7500 Fast Real-time PCR system (Applied Biosystems). cDNA synthesis was carried out using the First-Strand cDNA Synthesis Kit, as described earlier, and real-time PCR was performed through 40 cycles. The reaction cycle was set at 95°C for 3 s and 60°C for 30 s. Relative expression levels were calculated using the 2(-Delta Delta C(T)) method with normalization to glyceraldehyde-3-phosphate dehydrogenase transcription [19]. TaqMan probe and primers were purchased from Applied Biosystems.

Immunostaining

Cells were fixed and immunostained with anti-beta III isoform of tubulin (Tubb3; Chemicon International, Inc., Temecula, CA), anti-microtubule-associated protein 2 (MAP2; Chemicon), anti-doublecortin (DCX; Santa Cruz Biotechnology, Santa Cruz, CA), anti-choline acetyltransferase (ChAT; Chemicon), anti-gamma aminobutyric acid (GABA; Chemicon), anti-glutamate decarboxylases (GADs; Chemicon), anti-tyrosine hydroxylase (TH; Chemicon), anti-glial fibrillary acidic protein (GFAP; Chemicon), and anti-O4 (R&D Systems) as previously described [18].

For the neuronal cell proportion study, immunoreactive cells were counted for five randomly selected fields, each of which included 200–1,000 cells of three independent cultures. The number of total cells was counted by the number

of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Dojindo Laboratories, Kumamoto, Japan)-stained nuclei.

Measurement of neuronal migration

NSCs were cultured in NSM under free-floating conditions for 4 days. Reaggregated neurospheres were cultured in ACM under free-floating conditions for 4 days and plated on a PLL/LAM-coated slide glass with ACM at 37°C in 5% CO_2 for 3 days. After completion of the experiment, images were analyzed to measure the distance between the edge of each reaggregated neurosphere and the position of cell body in each DCX-positive neuron.

Statistical analysis

Data are expressed as the mean ± SE. Statistical significance was determined by the unpaired Student's *t*-test. All results were derived from three independent experiments, at least.

Results

Establishment of stable transfected ES cell lines

To investigate the function of GPM6A in neural differentiation, we generated stable-transfected ES cell lines using phM6A, pcDNA3.1(−), psTN-mG6A1, psTN-NSP1, and CAG promoter–derived phrGFP (as a selection marker) plasmids and subjected them to G418 and GFP selection (Fig. 1). In addition, to identify the founder ES cell lines, we performed PCR using a specific primer set for transfected plasmid and genome DNA extracted from each founder ES cell line. PCR results showed that all selected human ES cell lines were inserted with the plasmid into genome DNA (data not shown). In addition, human GPM6A protein was expressed in the established human ES cell lines (data not shown). Hence, both human ES cell lines with overexpressed human GPM6A (B2h-oeM6A1, B2h-oeM6A2, B2h-oeM6A3 [transfected phM6A (B2h-oeM6A)], and B2h-Mock1 cells [transfected pcDNA3.1(+)]), and human ES cell lines expressing shRNA (B2h-shM6A1, B2h-shM6A2, B2h-shM6A3 [transfected psTN-mG6A1 (B2h-shM6A)], and B2h-shNSP1 [transfected psTN-NSP1]) were established. To confirm the undifferentiated state of these cell lines, we performed immunocytochemistry for undifferentiated markers using long-term (passage 30) cultured human ES cell lines. As shown in Figure 2, the established human ES cell lines expressed undifferentiated markers such as SSEA-4, TRA-1-60, and TRA-1-81. To examine these cell lines potential for differentiation, we transplanted all the established ES cell lines into SCID mice to produce teratomas. All established ES cell lines formed teratomas, which following histological examination showed various tissues derived from all three embryonic germ layers, such as squamous, cartilage, and muscle (data not shown). These results indicate that all established ES cell lines retained their pluripotency and that overexpression of human GPM6A and shRNA for human GPM6A transcripts did not influence pluripotency in undifferentiated human ES cells.

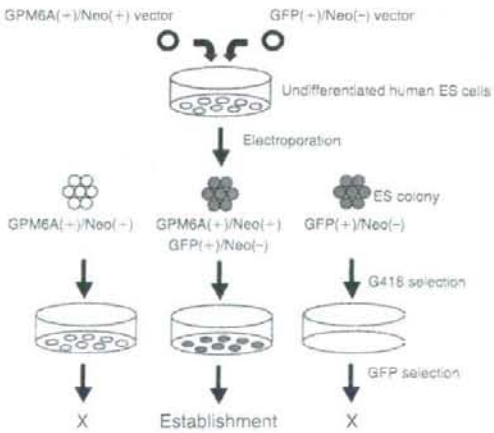

FIG. 1. Clonality of the stable transfected human ES cells. Schematic view of human ES cells clonality.

Effect of GPM6A overexpression or knockdown on generation of NSCs

Undifferentiated ES cell colonies were cultured in ACM under free-floating condition for 2 weeks, and the formed NS spheres were plated on PLL/LAM-coated plates in NSM for 1 week. The differentiated cells (2.5×10^5 cells/mL) containing the NSCs were cultured on PLL/LAM-coated plates in NSM for another 1 week. To investigate the effect of GPM6A overexpression or knockdown by shRNA on neural differentiation, we performed real-time PCR with cDNA from neural-differentiated stable-transfected ES cell lines. After 7 days culture, exogenous human GPM6A transcript was observed in the differentiated cells derived from B2h-oeM6A cells (Fig. 3A). The expression level of endogenous human GPM6A transcripts was increased in neural-differentiated B2h-oeM6A (Fig. 3A) and decreased in B2h-shM6A cells (Fig. 3B). Under these conditions, all examined neuroectodermal markers (OTX1, Lmx1b, En1, Pax2, Sox2, and Wnt1) and Nestin, which is known as NSCs marker [20,21], were increased in neural-differentiated B2h-oeM6A (Fig. 3C) and decreased in B2h-shM6A cells compared with those of B2h-Mock1 and B2h-shNSP1 cells (Fig. 3D). These results indicate that overexpression of human GPM6A transcripts increases neuroectodermal markers and Nestin in neural-differentiated B2h-oeM6A cells and that suppression of these transcripts decreases these markers as well as Nestin in neural-differentiated B2h-shM6A cells. Next, to evaluate the effect of GPM6A overexpression or suppression by shRNA on NSCs generation, we formed neurospheres using NSCs from neural-differentiated stable-transfected ES cell lines. The differentiated cells (1×10^6 cells/mL) containing the NSCs were cultured in NSM under free-floating conditions for another 1 week. Under this culture conditions, it is known that NSCs form neurospheres [22,23]. The neurospheres formed from NSCs derived from undifferentiated B2h-oeM6A cells were clearly large in size and number compared with those formed from undifferentiated

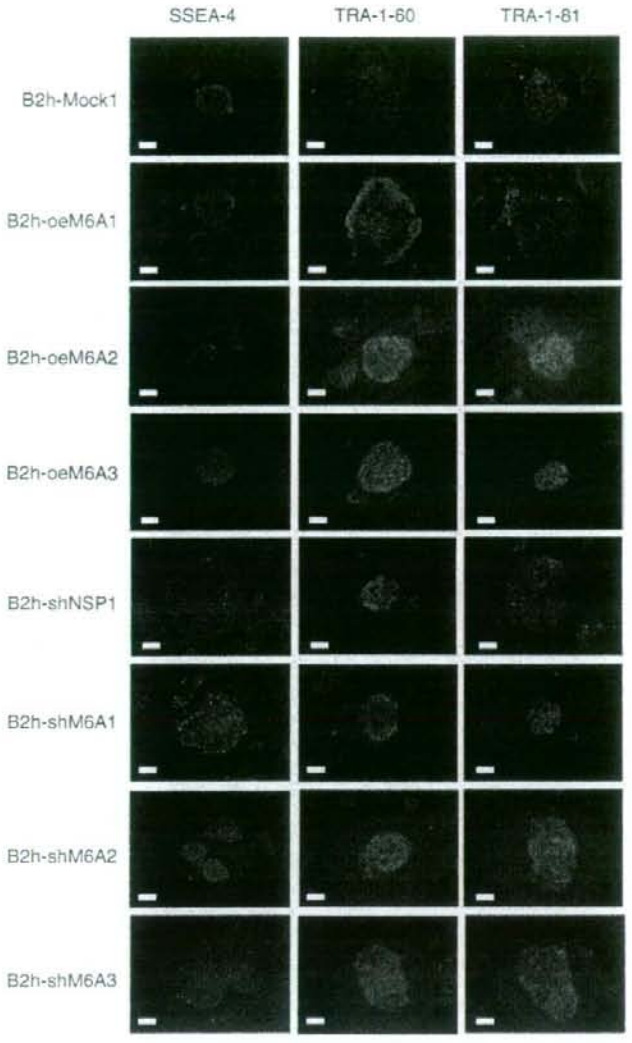

FIG. 2. Establishment of human ES cell lines overexpressing GPM6A or expressing shRNA against human GPM6A transcripts. Detection of undifferentiated markers in B2h-Mock1, B2h-oeM6A, B2h-shNSP1, and B2h-shM6A cells. All established human ES cell lines were positive for SSEA-4, TRA-1-60, and TRA-1-81. Scale bar = 200 μm. All experiments were independently carried out at least three times, and almost the same results were obtained each time.

B2h-Mock1 cells (Fig. 4A and B). On the other hand, the neurospheres formed from NSCs derived from undifferentiated B2h-shM6A cells were clearly small in size and number compared with those formed from undifferentiated B2h-shNSP1 cells (Fig. 4C and D). To confirm whether the formed neurospheres were made of NSCs, we performed immunocytochemical analysis using antibody against Nestin. Immunocytochemical analysis showed that the neurospheres formed from NSCs derived from undifferentiated stable-transfected ES cell lines were stained with antibody against Nestin (Fig. 4E). In addition, no significant difference in Nestin dye pattern was apparent in these neurospheres. Therefore, it is suggested that expression of human GPM6A is associated with generation of NSCs derived from undifferentiated human ES cells.

Proportion of neuronal differentiation in human ES cell lines with overexpression of GPM6A

We indicated earlier that expression of human GPM6A may be associated with generation of NSCs derived from undifferentiated human ES cells. To examine whether the proportion of neuronal differentiation is affected by GPM6A overexpression, we performed neuronal differentiation using NSCs derived from B2h-Mock1 and B2h-oeM6A cells. This neuronal differentiation was carried out using the same

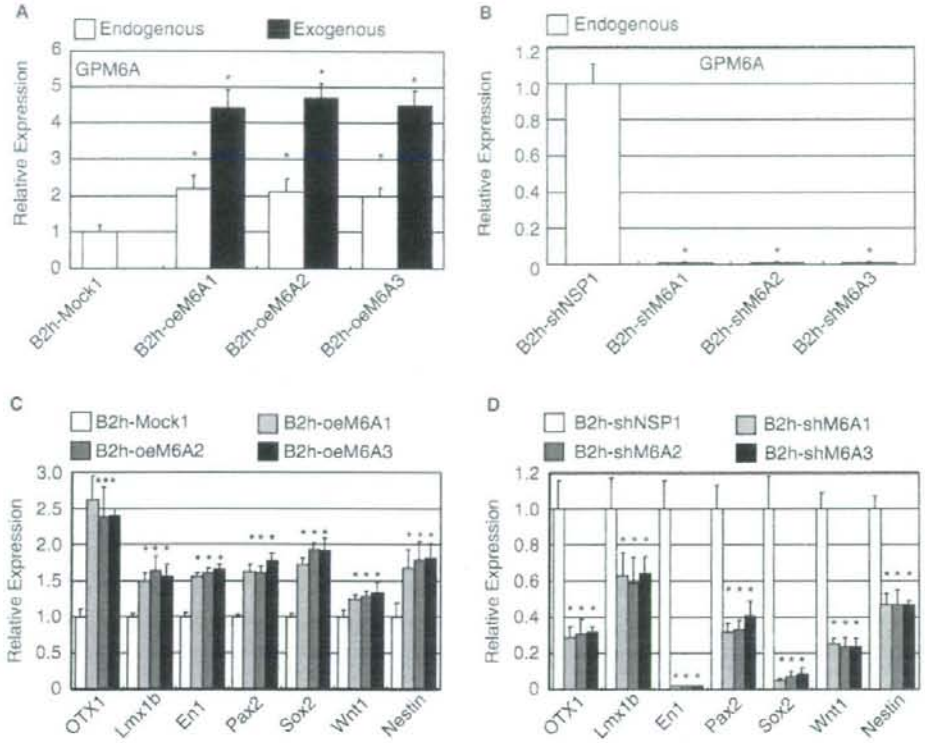

FIG. 3. Effect of overexpression or suppression of human GPM6A on neural differentiation. (**A**) Expression levels of exogenous and endogenous GPM6A in neural-differentiated B2h-Mock1 and B2h-oeM6A cells. (**B**) Suppression of human GPM6A in neural-differentiated B2h-shNSP1 and B2h-shM6A cells. (**C**) Expression levels of neuroectodermal markers in neural-differentiated B2h-Mock1 and B2h-oeM6A cells. (**D**) Expression levels of neuroectodermal markers in neural-differentiated B2h-shNSP1 and B2h-shM6A cells. One week after neural differentiation using NSM, total RNA was extracted from the neuronal-differentiated cells and real-time PCR was performed. Values are expressed as the mean ± SE ($n = 6$). *$p < 0.01$ compared with neural-differentiated control cells. All experiments were independently carried out at least three times, and almost the same results were obtained each time.

number (2.5×10^5 cells/mL) of derived NSCs between B2h-Mock1 and B2h-oeM6A cells. NSCs derived from B2h-Mock1 and B2h-oeM6A cells were cultured on PLL/LAM-coated plates in ACM for 4 weeks. After 4 weeks culture, expression of exogenous and endogenous human GPM6A transcripts was maintained or increased in neuronal-differentiated cells from NSCs derived from B2h-oeM6A cells (Fig. 5A). Expression levels of Tubb3 (an immature neuron marker), MAP2 (a mature neuron marker), and DCX (a migrating neuron marker) were increased in neuronal-differentiated cells from NSCs derived from B2h-oeM6A cells compared with those in B2h-Mock1 cells (Fig. 5B). Further analysis indicated that ChAT, GAD, TH, and dopa decarboxylase (DCC) transcripts, all of which are neurotransmitter-related markers [24–26], were also increased in neuronal-differentiated cells from NSCs derived from B2h-oeM6A cells (Fig. 5B). On the other hand, expression levels of GFAP (an astrocyte marker) and GalC (an oligodendrocyte marker) transcripts [21] were decreased in neuronal-differentiated cells from NSCs derived from B2h-oeM6A cells compared with those in B2h-Mock1 cells (Fig. 5B). To investigate whether protein expression of neuron, astrocyte, and oligodendrocyte markers changed along with overexpression of human GPM6A, we performed immunocytochemical analysis with antibodies against Tubb3, MAP2, DCX, ChAT, GABA, GADs, TH, GFAP, and O4. Immunocytochemical analysis showed that the number of Tubb3, MAP2, DCX, ChAT, GABA, GADs, and TH-positive cells was increased in neuronal-differentiated cells from NSCs derived from B2h-oeM6A cells compared with those in B2h-Mock1 cells (Fig. 5C). Moreover, the number of GFAP and O4-positive cells was decreased in neuronal-differentiated cells from NSCs derived from

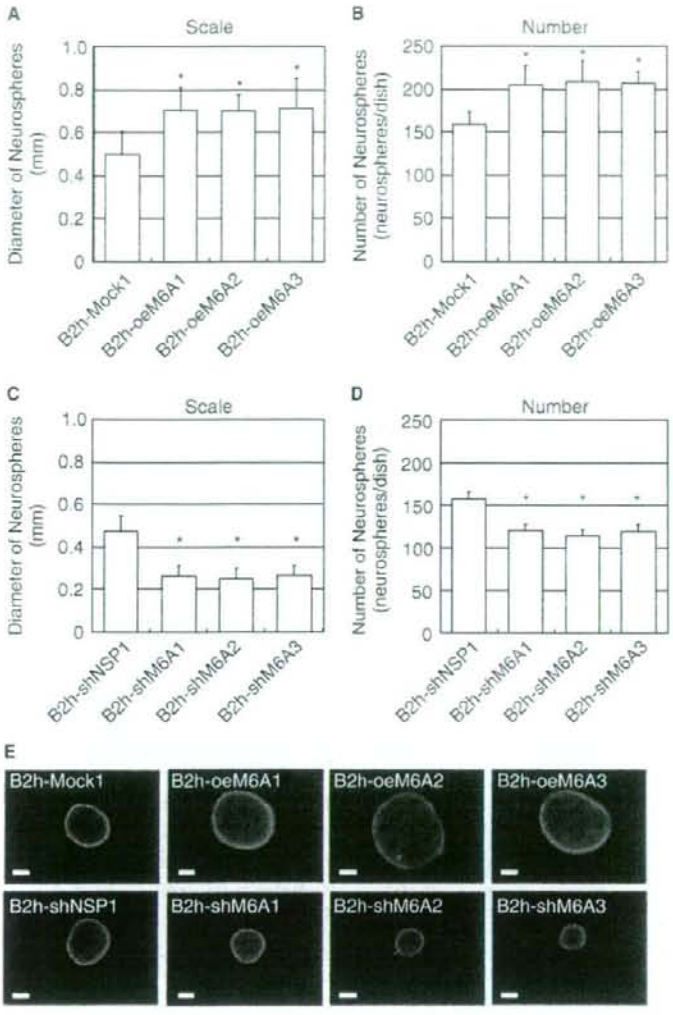

FIG. 4. Effect of overexpression or suppression of human GPM6A on NSCs generation. (**A**,**B**) Scale and number of neurospheres derived from B2h-Mock1 and B2h-oeM6A cells. (**C**,**D**) Scale and number of neurospheres derived from B2h-shNSP1 and B2h-shM6A cells. Differentiated cells (1×10^5 cells/mL) containing NSCs formed neurospheres in NSM after culture for 1 week. Values are expressed as the mean ± SE ($n = 30$). $^*p < 0.01$ compared with neurospheres from control cells. (**E**) Immunocytochemistry of neurospheres derived from B2h-Mock1, B2h-oeM6A, B2h-shNSP1, and B2h-shM6A cells. Neurospheres were fixed and immunostained with Nestin antibody. Scale bar = 200 μm. All experiments were independently carried out at least three times, and almost the same results were obtained each time.

B2h-oeM6A cells (Fig. 5C). These findings indicated that overexpression of human GPM6A increased the proportion of neurons from NSCs to neuronal cells.

Proportion of neuronal differentiation in human GPM6A knockdown ES cell lines

We investigated the effect of GPM6A knockdown on the proportion of neuronal differentiation. Neuronal differentiation was performed using the same method as for GPM6A overexpression in ES cell lines. After 4 weeks culture, the expression of endogenous human GPM6A transcripts was suppressed in neuronal-differentiated cells from NSCs derived from B2h-shM6A cells (Fig. 6A). Expression levels of Tubb3, MAP2, and DCX were decreased in neuronal-differentiated cells from NSCs derived from B2h-shM6A cells compared with those in B2h-shNSP1 cells (Fig. 6B). Further analysis indicated that ChAT, GAD, TH, and DCC transcripts were increased in neuronal-differentiated cells from NSCs derived from B2h-shM6A cells (Fig. 6B). On the other hand, expression levels of GFAP and GalC transcripts were increased in neuronal-differentiated cells from NSCs derived from B2h-shM6A cells compared with those in B2h-shNSP1 cells (Fig. 6B). To investigate whether protein expression of neuron, astrocyte, and oligodendrocyte markers changed along with suppression of human GPM6A, we performed immunocytochemical analysis with antibodies against Tubb3, MAP2, DCX, ChAT, GABA, GADs, TH,

GFAP, and O4. Immunocytochemical analysis showed that the number of Tubb3, MAP2, DCX, ChAT, GABA, GADs, and TH-positive cells was decreased in neuronal-differentiated cells from NSCs derived from B2h-shM6A cells compared with those in B2h-shNSP1 cells (Fig. 6C). Moreover, the number of GFAP and O4-positive cells was increased in neuronal-differentiated cells from NSCs derived from B2h-shM6A cells (Fig. 6C). These findings indicated that suppression of human GPM6A decreases the proportion of neuronal differentiation from NSCs.

Regulation of neuronal migration by expression level of human GPM6A in neurons

In this report, we demonstrated that the expression level of human GPM6A is associated with neural differentiation both from undifferentiated human ES cells to NSCs and from NSCs to neuronal cells. To find more information on the function of GPM6A in neural differentiation, we investigated whether the expression level of human GPM6A affects neuronal migration. Neuronal migration was tested using an in vitro migration assay using neurons derived from each ES cell line. Migration distances in 3 days were binned. Neurons from NSCs derived from B2h-oeM6A cells displayed a rightward shift in bin distribution compared with those in B2h-Mock1 cells (Fig. 7A and B). Mean migration distance increased by 166 μm (80%) in neurons from NSCs derived from B2h-oeM6A cells (Fig. 7C). In contrast, neurons from NSCs derived from B2h-shM6A cells displayed a leftward shift in bin distribution compared with those in B2h-shNSP1 cells (Fig. 8A and B). Mean migration distance decreased by 64 μm (32%) in neurons derived from B2h-shM6A cells (Fig. 8C). These results suggest that expression of human GPM6A positively regulates in neuronal migration.

Discussion

In human ES cells with overexpression or suppression of human GPM6A transcripts, we showed that expression level of NSC marker transcripts Nestin increased or decreased, respectively, compared to that in control human ES cells (Fig. 3C and D). The differentiated cells derived from all stable-transfected ES cell line were able to form neurospheres

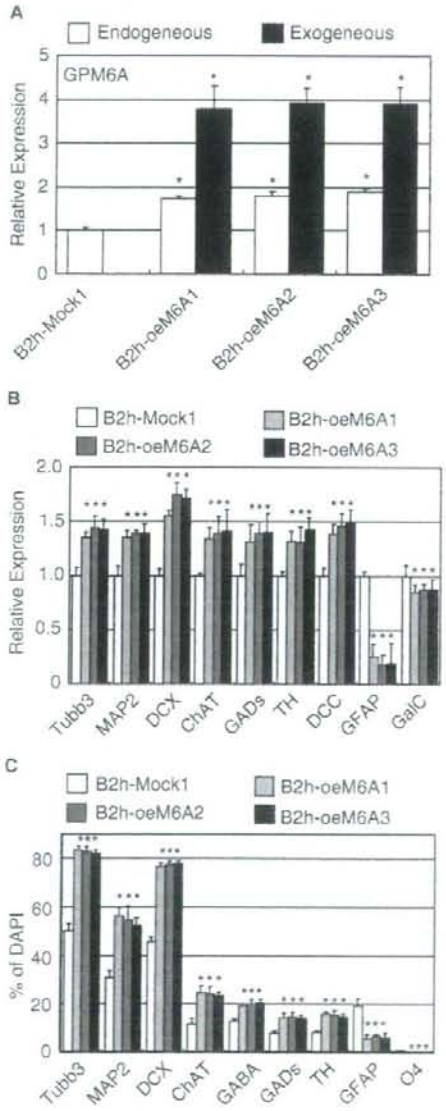

FIG. 5. Alterative expression levels of neuron, astrocyte, and oligodendrocyte markers between neuronal-differentiated B2h-Mock1 and B2h-oeM6A cells. (**A**) Expression levels of exogenous and endogenous GPM6A in neuronal-differentiated B2h-Mock1 and B2h-oeM6A cells. (**B**) Expression levels of neuron, astrocyte, and oligodendrocyte markers in neuronal-differentiated B2h-Mock1 and B2h-oeM6A cells. Four weeks after neuronal differentiation using ACM, total RNA was extracted from the neuronal-differentiated cells and real-time PCR was performed. Values are expressed as the mean ± SE ($n = 6$). $^{*}p < 0.01$ compared with expression levels in B2h-Mock1 cells. (**C**) Proportion of neuronal-differentiated cells from NSCs derived from B2h-Mock1 and B2h-oeM6A cells. Cells were differentiated with ACM for 4 weeks, fixed, and immunostained with Tubb3, MAP2, DCX, ChAT, GABA, GADs, TH, GFAP, and O4 antibodies. The number of total cells was counted by the number of DAPI-staining nuclei. Values are expressed as the mean ± SE ($n = 4$). $^{*}p < 0.01$ compared with neuronal-differentiated cells derived from B2h-Mock1 cells. All experiments were independently carried out at least three times, and almost the same results were obtained each time.

(Fig. 4A–D), and were immunoreactive for anti-Nestin antibody (Fig. 4E). However, neurospheres from B2h-oeM6A cells were clearly large in size and number compared with those in B2h-Mock1 cells (Fig. 4A and B). In addition, neurospheres from B2h-shM6A cells were clearly small in size and number compared with those in B2h-shNSP1 cells (Fig. 4C and D). These results imply that the proportion of NSCs was high in differentiated cells derived from B2h-oeM6A cells and was low in differentiated cells derived from B2h-shM6A cells. There seems to be a correlation between the ratio of expression level of human GPM6A transcripts and the ratio of differentiation of NSCs from undifferentiated human ES cells. We have previously reported the same results using mouse ES cells [18]. Moreover, expression levels of ectodermal marker genes, which are associated with development of the brain [27–30], and Nestin were increased or decreased by overexpression or suppression, respectively, of human GPM6A transcripts (Fig. 3C and D). We also found that expression level of undifferentiated ES marker gene (*OCT3/4*) was barely detected in neuronal-differentiated cells derived from all established human ES cell lines (data not shown), and that the doubling time of all established human ES cell lines was equivalent to that of untransfected human ES cell lines (data not shown). These findings indicate that overexpression or suppression of human GPM6A is irrelevant to maintenance of cells pluripotency or death. It is therefore suggested that human GPM6A is one of the factors that promotes neural differentiation from undifferentiated human ES cells to NSCs.

Mouse GPM6A is known to be expressed in mouse neurons [8,9]. It is also reported that the expression of mouse GPM6A is observed from embryonic day 10 on cells in the marginal zone of the neural tube and is widespread throughout the brain and spinal cord, where it remains throughout adulthood [8,9]. In addition, it has been shown that treatment with anti-GPM6A antibody interferes with neurite extension in cultured neurons [8]. Moreover, a recent study has shown that GPM6A is related to neurite outgrowth, filopodium/spine formation, and synaptic formation in cultured neurons [13]. Furthermore, we have previously reported that suppression of mouse GPM6A transcripts leads to reduction of NSCs generation and the proportion of neuron derived from mouse ES cells [18]. In this study, overexpression or suppression of human GPM6A expression increased or decreased, respectively, generation of NSCs and differentiation into neurons (Figs. 3–6). Our

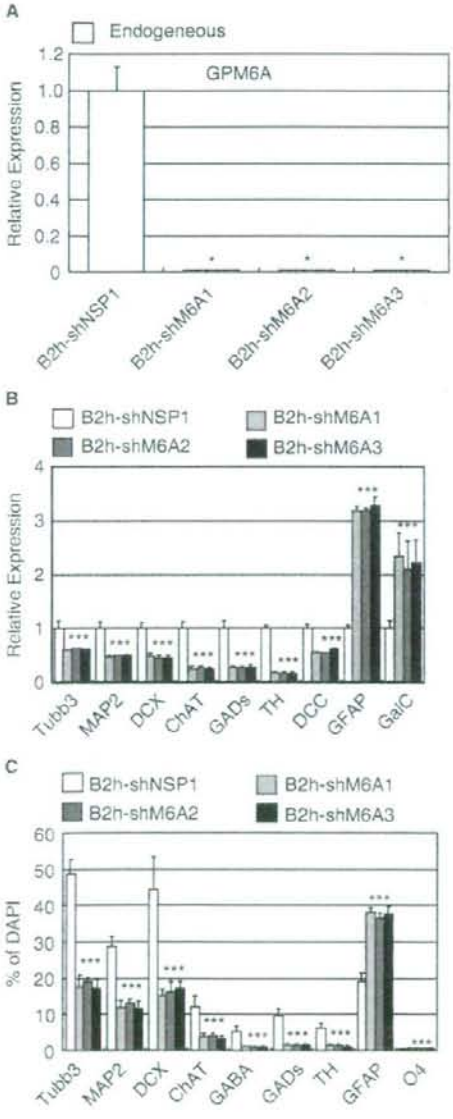

FIG. 6. Alterative expression levels of neuron, astrocyte, and oligodendrocyte markers between neuronal-differentiated B2h-shNSP1 and B2h-shM6A cells. (**A**) Expression level of endogenous GPM6A in neuronal-differentiated B2h-shNSP1 and B2h-shM6A cells. (**B**) Expression levels of neuron, astrocyte, and oligodendrocyte markers in neuronal-differentiated B2h-shNSP1 and B2h-shM6A cells. Four weeks after neuronal differentiation using ACM, total RNA was extracted from the neuronal-differentiated cells and real-time PCR was performed. Values are expressed as the mean ± SE ($n = 6$). $^{*}p < 0.01$ compared with expression levels in B2h-shNSP1 cells. (**C**) Proportion of neuronal-differentiated cells from NSCs derived from B2h-shNSP1 and B2h-shM6A cells. Cells were differentiated with ACM for 4 weeks, fixed, and immunostained with Tubb3, MAP2, DCX, ChAT, GABA, GADs, TH, GFAP, and O4 antibodies. The number of total cells was counted by the number of DAPI-staining nuclei. Values are expressed as the mean ± SE ($n = 4$). $^{*}p < 0.01$ compared with neuronal-differentiated cells derived from B2h-shNSP1 cells. All experiments were independently carried out at least three times, and almost the same results were obtained each time.

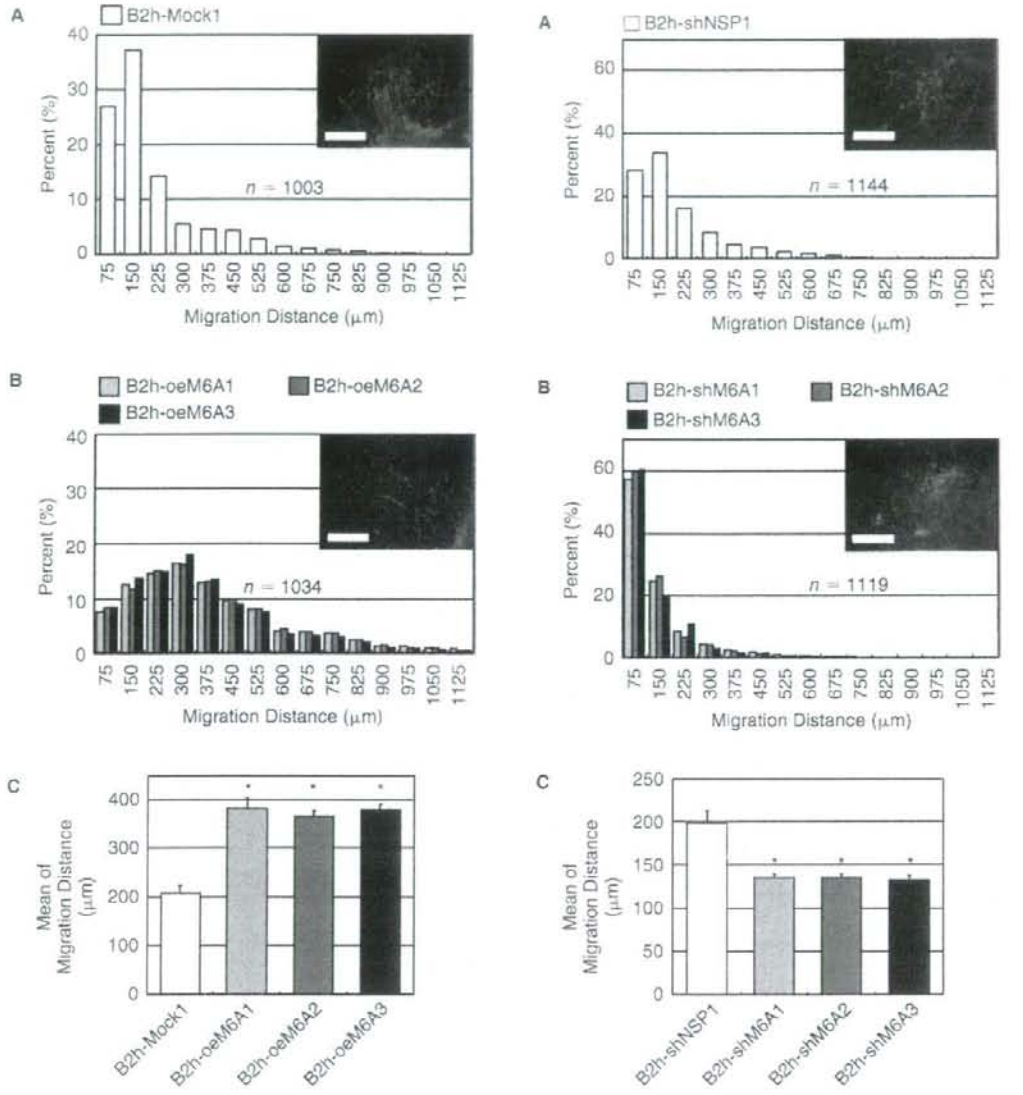

FIG. 7. Effect of human GPM6A overexpression on neuronal migration. (**A**) Distance of neuronal migration in neurons derived from B2h-Mock1 cells. (**B**) Distance of neuronal migration in neurons derived from B2h-oeM6A cells. The *x*-axis represents migration distances. The *y*-axis represents percent of neurons in each bin. The *n* indicates the number of measured neurons. Cells were cultured with ACM for 3 days, fixed, and immunostained with DCX antibody. Scale bar = 200 μm. (**C**) Average migration distance in neurons derived from B2h-Mock1 and B2h-oeM6A cells. Values are expressed as the mean ± SE ($n = 3$). $^*p < 0.01$ compared with neurons derived from B2h-Mock1 cells. All experiments were independently carried out at least three times, and almost the same results were obtained each time.

FIG. 8. Effect of human GPM6A suppression on neuronal migration. (**A**) Distance of neuronal migration in neurons derived from B2h-shNSP1 cells. (**B**) Distance of neuronal migration in neurons derived from B2h-shM6A cells. The *x*-axis represents migration distances. The *y*-axis represents percent of neurons in each bin. The *n* indicates the number of measured neurons. Cells were cultured with ACM for 3 days, fixed, and immunostained with DCX antibody. Scale bar = 200 μm. (**C**) Average migration distance in neurons derived from B2h-shNSP1 and B2h-shM6A cells. Values are expressed as the mean ± SE ($n = 3$). $^*p < 0.01$ compared with neurons derived from B2h-shNSP1 cells. All experiments were independently carried out at least three times, and almost the same results were obtained each time.

results also reveal an associated increase or decrease of differentiation into various neuronal cells, such as cholinergic (ChAT), catecholaminergic (TH), and GABAergic (GABA and GADs) neurons in human ES cells overexpressing human GPM6A or expressing shRNA against human GPM6A (Figs. 5C and 6C). On the other hand, it is known that NSCs can differentiate into neurons, astrocytes, and oligodendrocytes [31,32]. Our results in this study show that the proportion of astrocytes and oligodendrocytes decreased or increased in neuronal differentiated cells from NSCs derived from B2h-oeM6A or B2h-shM6A cells, respectively, (Figs. 5C and 6C). Taken together, these findings suggest that human GPM6A is associated with differentiation into NSC from undifferentiated human ES cells, and is one of the factors that triggers differentiation of NSCs to various neurons but not astrocytes and oligodendrocytes.

Mammalian brain development is characterized by strictly regulated migration of each immature neuron from its birthplace to its final destination, resulting in six distinct layers in the human cerebral cortex [33]. Migration of the earliest-generated neurons from the ventricular zone to the cortical plate during embryogenesis comprises one of the most critical stages in development of mammalian brain [33,34]. Deficiency of this neuronal migration process often results in major brain malformations, including lissencephaly (smooth brain). We have reported that overexpression of human GPM6A enhanced distance of neuronal migration in neurons derived from human ES cells (Fig. 7). In this study, we demonstrated that suppression of human GPM6A expression reduces the distance of neuronal migration in neurons derived from human ES cells (Fig. 8). These results indicate that human GPM6A is associated with neuronal migration in neurons derived from human ES cells. Therefore, it is possible that human *GPM6A* gene may be one of the candidate genes for treatment of lissencephaly and/or other diseases associated with deficiency in neuronal migration.

In the present study, we showed for the first time that human GPM6A expression is associated with NSC generation, neuronal differentiation, and neuronal migration of neurons derived from human ES cells. Further investigation involving GPM6A knockout mice may lead to better understanding of the relationship between brain development and GPM6A function.

Acknowledgments

We thank Yukiko Nishiinkyo for her technical support. We are grateful to Shinji Nito for his useful discussion and continuous interest in this study, and to Gary W. Clendennen and Azedine Barik for their critical reading of the manuscript.

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Received for publication July 31, 2008; accepted after revision December 19, 2008. Prepublished on Liebert Instant Online December 19, 2008.

The Journal of Toxicological Sciences,
Vol.32, No.3, 301-310, 2007

MONITORING OF GENE EXPRESSION IN DIFFERENTIATION OF EMBRYOID BODIES FROM CYNOMOLGUS MONKEY EMBRYONIC STEM CELLS IN THE PRESENCE OF BISPHENOL A

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(Received April 24, 2007; Accepted May 8, 2007)

ABSTRACT — An embryonic stem (ES) cell differentiation model would facilitate analysis of developmental processes at the cellular level and the effects of embryotoxic and teratogenic factors *in vitro*. We explored the use of differentiation of embryoid bodies (EBs) from cynomolgus monkey ES cells for embryotoxicity testing. We determined the mRNA expression of various genes using real-time RT-PCR. Oct-3/4 expression was almost completely suppressed on day 14, suggesting that ES cells reached differentiated status in around 14 days. mRNA expression of E-cadherin, connexin 43, caveolin-1, and argininosuccinate synthetase was reproducibly suppressed during EB differentiation in 7–32% of ES cells in three separate experiments. Although these may not be "general stemness marker genes" such as Oct-3/4, they could play a role in readying stem cells for differentiation in response to deletion of signals from feeder cells. Next, we examined the effects of bisphenol A (BPA) on the mRNA expression of several differentiation marker genes for ES cells. That of PAX-6, an ectoderm marker, with 0, 0.1, and 10 μM BPA in 21-day EBs was 3,500%, 6,668%, and 8,394%, respectively, compared with ES cells. The difference between doses of 0 and 10 μM BPA in 21-day EBs was statistically significant ($p=0.049$). Pax-6 activation in the presence of BPA may interfere with the development of eyes, sensory organs, and certain neural and epidermal tissues usually derived from ectodermal tissues. Differentiation of EBs from cynomolgus monkey ES cells could be a useful model for detecting gene expression changes in response to chemical exposure.

KEY WORDS: Embryonic stem cell, Embryoid body, Differentiation, Monkey, Bisphenol A, Embryotoxicity

INTRODUCTION

Embryonic stem (ES) cells have great potential for cell therapy and regenerative medicine, but also represent a dynamic system suitable for identifying potential molecular targets for the development of novel

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drugs, providing an *in vitro* system to examine safety or potential toxicity in humans (Davila *et al.*, 2004; Wobus and Boheler, 2005). Particularly promising is the ES cell differentiation model, which includes developmental processes from early embryonic stages up to terminally differentiated cell types and which would enable us to analyze developmental processes at the cellular level and to determine the effects of embryotoxic and teratogenic factors *in vitro*. Establishing a reliable *in vitro* embryogenesis model would also contribute to a reduction in the number of animal experiments required for medical and pharmacological testing.

Experimental studies using non-human primate ES cells have advantages such as similar characteristics to human ES cells that are not observed in murine ES cells, and avoidance of the ethical problems caused by the use of human ES cells (Thomson *et al.*, 1995; Suemori and Nakatsuji, 2003; Adachi *et al.*, 2006; Byrne *et al.*, 2006). To date, however, the ES cell test for testing embryotoxicity primarily uses murine ES cells (Spielmann *et al.*, 1997; Scholz *et al.*, 1999; Imai and Nakamura, 2006). As a result, our knowledge on the molecular and cellular aspects of ES cells for embryotoxicity testing is based mainly on murine cells, and information on non-human primate ES cells is limited.

In the course of ES cell differentiation, genes encoding tissue-specific proteins are expressed in a developmentally controlled time pattern that closely resembles what is observed during embryogenesis. ES cells differentiate *in vitro* into embryoid bodies (EBs) comprising endoderm, mesoderm and ectoderm cell layers in the absence of the self-renewal signals provided by feeder layers (Weitzer, 2006). EB formation is considered to mimic embryo development during the stages of pre-gastrulation and early gastrulation. Using a mouse EB model, Wartenberg *et al.* examined antiangiogenic agents in an *in vitro* assay system (Wartenberg *et al.*, 1998). However, to our knowledge, little information is available on the primate EB differentiation model for embryotoxicity screening.

Bisphenol A (BPA) is commonly used in various industries. Its monomer is used for polycarbonate plastic production, and its resin form is used as linings for most food and beverage cans, as dental sealant, and as an additive in other widely used consumer products. BPA is known to elicit weak estrogenic activity in *in vitro* and *in vivo* test systems. Although molecular mechanisms studies have revealed a variety of pathways in which BPA can stimulate cellular responses at very low doses in addition to the effects initiated by its binding to the classical estrogen receptors, there is little information available concerning the effect of BPA on early embryogenesis (vom Saal and Hughes, 2005; Kang *et al.*, 2006).

In the present study, to develop a model system for primate embryotoxicity testing, we used BPA as a model compound and examined changes of gene expression in response to exposure to BPA using cynomolgus monkey EB differentiation. We conducted experiments in three steps. Firstly, we determined the time necessary for EB differentiation in cynomolgus monkey ES cells, examining the expression of Oct-3/4, a POU-class transcription factor. This stemness marker gene was used since loss of pluripotency during spontaneous or induced differentiation has been correlated with progressive loss of Oct-3/4 expression (Niwa, 2001; Mitalipov *et al.*, 2003). In addition to morphological changes, we determined mRNA expression levels using real-time RT-PCR. This quantitative approach is rapid and sensitive and is considered suitable for pharmacological and cytotoxicity screening. Monitoring of ES cell differentiation with quantitative PCR has recently been reported (Noaksson *et al.*, 2005). Secondly, we examined the expression of several genes whose proteins were highly expressed in ES cells compared with EBs. These genes were selected on the basis of preliminary results obtained from a comparison of the protein-expression profiles of various genes in 12–16-day-old CMK-6 ES cells and CMK-6 EBs with Green Fluorescent Protein (Furuya *et al.*, 2003) using Power Blot™, a Western blot array analysis (unpublished result). In the third step, we examined the effects of BPA on mRNA expression of the following differentiation marker genes for ES cells: α-fetoprotein (AFP) and GATA-4 as endoderm markers; BMP-4 and Brachyury as mesoderm markers; and PAX-6 and N-CAM as ectoderm markers.

MATERIALS AND METHODS

Cell culture

The ES cell lines (CMK-6) used in this study were established from blastocysts of the cynomolgus monkey and were kindly provided by Dr Norio Nakatsuji of Kyoto University (Fig. 1A; Suemori *et al.*, 2001).

ES cells were grown on mouse embryonic fibroblast (MEF) feeder cells that were mitotically inactivated by mitomycin C in Dulbecco's Modified Eagle's Medium (DMEM/F12) (SIGMA) supplemented with

20% knock-out serum replacement (Invitrogen), 1% non-essential amino acids (SIGMA) and 2 mM L-glutamine (SIGMA) at 37°C in a humidified 5% CO_2 atmosphere.

For EB differentiation, entire ES cells colonies were loosely detached by 0.1% (w/v) collagenase (Wako Pure Chemical) from the feeder cells and transferred into a feeder-free 25 cm^2 flask (SUMILON) for floating culture. The medium was changed after the first day and half of it was changed every 7 days.

EBs (2×10^5/ml) were incubated for 7, 14 or 21 days with 0.1 μM or 10 μM BPA (SIGMA). In the control group, the same volume of DMSO was added to the media. Stock BPA (100 mM) was dissolved in DMSO and was stored at −20°C. The BPA was further diluted in culture medium just before use, and was sterilized through a filter with a pore size of 0.22 μm. The final DMSO concentration did not exceed 0.1% (vol/vol).

When harvesting ES cells and EBs for mRNA expression analysis, they were washed three times with PBS and then pooled at −80°C until RNA extraction.

Quantitative real-time RT-PCR

Total RNA from ES cells and EBs was isolated using a RNeasy Plus Mini kit (QIAGEN), which includes removal of genomic DNA contamination before cDNA synthesis. Samples were collected from three separate culture experiments.

cDNAs were synthesized from total RNA ranging from 100 ng to 1 μg using QuantiTect Reverse Transcription (QIAGEN) after elimination of genomic DNA contamination.

β-actin and differentially expressed genes were quantitatively detected with a LightCycler Instrument (Roche Diagnostics) using the LightCycler FastStart DNA Master^{PLUS} SYBR Green I (Roche Diagnostics) according to the manufacturer's instructions.

The primers for each gene were designed and synthesized on the basis of respective information in NCBI or ENSEMBL using the software of Premiere Biosoft, so that the targets were 75–200 bp in length (SIGMA GENOSYS, Table 1).

PCR amplification was performed in a total volume of 20 μl containing cDNA and each primer (0.5 μM). The PCR cycling conditions were 95°C for 10 min followed by 45 cycles of 95°C for 10 sec, 60°C for 10 sec, and 72°C for 15 sec. The fluorescent product at the end of the 72°C extension period was detected. All PCR assays were performed in at least duplicate.

The data obtained were analyzed using the LightCycler analysis software. To confirm the amplification specificity, we subjected the PCR products to melting curve analysis. The results are given as the mean ± SE of samples from three separate culture experiments. The statistical analysis was conducted using Kruskal–Wallis test.

RESULTS

As shown in Fig. 1A and B, cynomolgus monkey ES cells spontaneously differentiated into EBs after separation from the MEFs. Fig. 2 shows the time-course of mRNA expression of Oct-3/4 in EBs in relation to that on Day 1 (ES cell). The average mRNA expression levels of Oct-3/4 in EBs were 3%, 0.9%, and 0.4% at 7 days, 14 days and 21 days, respectively, compared with ES cells. No significant effect of BPA on Oct-3/4 expression in EB differentiation was observed.

Next, we examined the mRNA expression of genes selected by the preliminary experiments

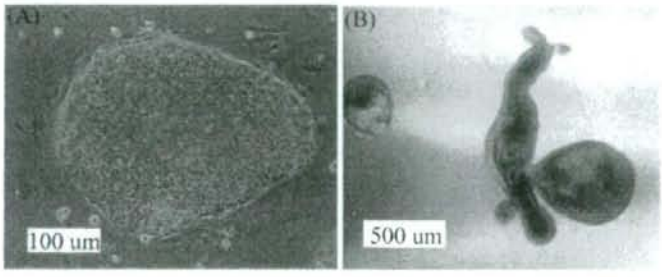

Fig. 1. A: Cynomolgus monkey ES cells, CMK-6.
B: EBs derived from CMK-6 ES cells on Day 14.

described in the Introduction. E-cadherin, connexin 43, caveolin-1, and ASS were consistently suppressed during EB differentiation. In the absence of BPA, the mRNA expression of E-cadherin was suppressed to 12%, 20%, and 21% at 7 days, 14 days and 21 days, respectively, compared with ES cells (Fig. 3A). In the absence of BPA, the mRNA expression of connexin 43 was suppressed to 32%, 32% and 27% at 7 days, 14 days and 21 days, respectively (Fig. 3B). In the absence of BPA, the mRNA expression of caveolin-1 was suppressed to 17%, 26% and 21% at 7 days, 14 days and 21 days, respectively (Fig. 3C). In the absence of BPA, the mRNA expression of ASS was suppressed to 10%, 9% and 7% at 7 days, 14 days and 21 days, respectively (Fig. 3D). There were no detectable differences on microscopy between the BPA treatment and non-treatment groups. In addition, no significant effect of BPA was observed on the expression of these genes in EB differentiation.

Table 1. Primer sequences for real time RT-PCR.

Gene	Primers
β-actin	F: ACCCCGTGCTGCTGACC
	R: CCAGAGGCGTACAGGGATAGC
Oct-3/4	F: GCTCCTGAAGCAGAAGAGGATCACC
	R: GCCCTTCTGGCGCCGGTTACAGAAC
E-cadherin	F: AAGACCAAGTGACCACCTTAGAG
	R: AAACAGCAAGAGCAGCAGAATC
Connexin-43	F: TTCAATGGCTGCTCCTCACC
	R: GCTCACTTGCTTGCTTGTTGTA
Caveolin-1	F: CGGCTCAACTCGCATCTCAAG
	R: GCCAGGAACACCGTCAGGA
ASS	F: TGGCTGAAGGAACAAGGCTATG
	R: GCTGACATCCTCAATGAACACC
AFP	F: AGCTTGGTGGTGGATGAA
	R: CAGCCTCAAGTTGTTCCTCT
PAX-6	F: ACAGACACAGCCCTCACAAAC
	R: ATCATAACTCCGCCCATTCACC

In the third step, we examined the effects of BPA on the mRNA expression of the following differentiation marker genes for ES cells: AFP, GATA-4, BMP-4, Brachyury, PAX-6, and N-CAM. The results for two genes, AFP and PAX-6, which gave reproducible results in three separate cultures, are presented in Fig. 4A and B. The mean AFP mRNA expression in 14-day EBs was 204,132% in the presence of 10 μM BPA and 130,635% in the absence of BPA. The difference was

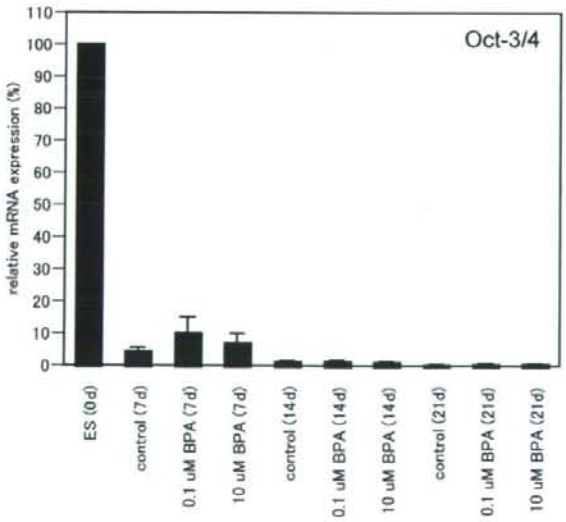

Fig. 2. mRNA expression of Oct-3/4 in EB differentiation at 7, 14 and 21 days. Control: DMSO; 0.1 uM: 0.1 μM BPA; 10 uM: 10 μM BPA. Values are mean ± SE of three independent experiments.

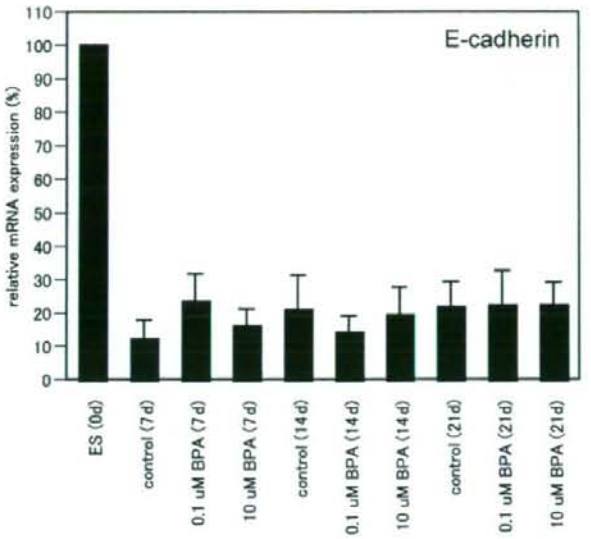

Fig. 3A. mRNA expression of E-cadherin in EB differentiation at 7, 14 and 21 days. Control: DMSO; 0.1 uM: 0.1 μM BPA; 10 uM: 10 μM BPA. Values are mean ± SE of three independent experiments.

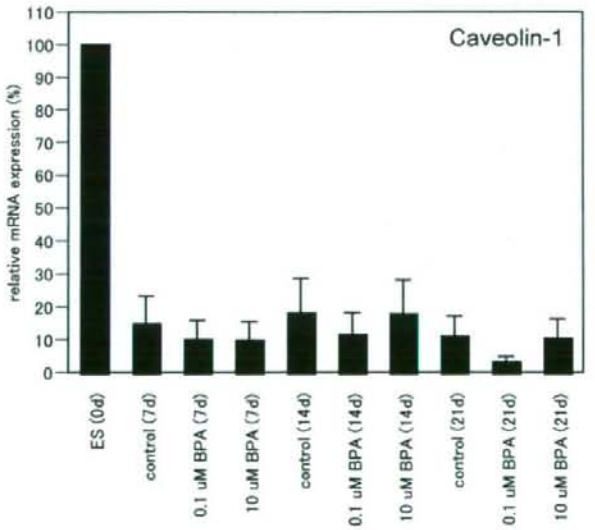

Fig. 3B. mRNA expression of caveolin-1 in EB differentiation at 7, 14 and 21 days. Control: DMSO; 0.1 uM: 0.1 μM BPA; 10 uM: 10 μM BPA. Values are mean ± SE of three independent experiments.

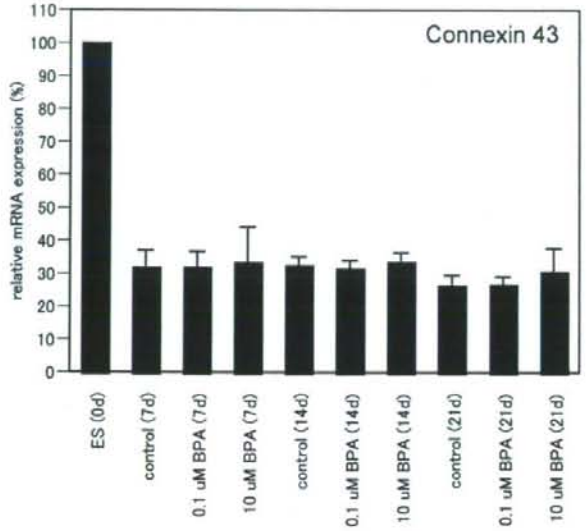

Fig. 3C. mRNA expression of connexin-43 in EB differentiation at 7, 14 and 21 days. Control: DMSO; 0.1 uM: 0.1 μM BPA; 10 uM: 10 μM BPA. Values are mean ± SE of three independent experiments.

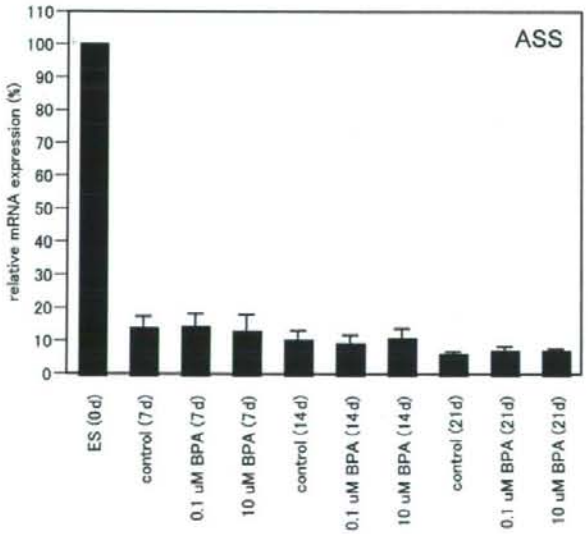

Fig. 3D. mRNA expression of ASS in EB differentiation at 7, 14 and 21 days. Control: DMSO; 0.1 uM: 0.1 μM BPA; 10 uM: 10 μM BPA. Values are mean ± SE of three independent experiments.