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Glycosphingolipid Synthesis Inhibitor Represses Cytokine-Induced Activation of the Ras-MAPK Pathway in Embryonic Neural Precursor Cells

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Neuronal and glial cells in the central nervous system are generated from common neural precursor cells during development. To evaluate the functions of glycosphingolipids (GSLs) in neural precursor cells, neuroepithelial cells (NECs) were prepared from mouse embryos (E14.5), and the effects of an inhibitor of glucosylceramide synthesis, D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), on NECs was investigated. In PDMP-treated NECs, the expression of GD3, a major ganglioside of NECs, disappeared. We found that basic fibroblast growth factor (bFGF)–induced proliferation and extracellular signal–regulated kinase (ERK) activation were repressed in PDMP-treated NECs. Leukemia inhibitory factor (LIF)–induced ERK activation was also abolished in PDMP-treated NECs, suggesting that PDMP specifically represses the Ras-MAPK pathway. bFGF-induced activation of the Ras-MAPK pathway in NECs is dependent on GSL-enriched microdomains, lipid rafts. The organization of lipid rafts and the distribution of Ras and Grb2-SOS in the microdomains were not affected. However, Ras activation was repressed in PDMP-treated NECs. In PDMP-treated NECs, some neuronal genes were up-regulated and glial genes were down-regulated. These results suggest that GSLs might be involved in the proliferation, survival, signal transduction and differentiation of NECs.

Key words: development, glycoconjugates, lipid rafts, proliferation, signal transduction.

Abbreviations: bFGF, basic fibroblast growth factor; bHLH, basic helix-loop-helix; ERK, extracellular signal–regulated kinase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GalCer, galactosylceramide; GalT, UDP-galactose:ceramide galactosyltransferase; GlcCer, glucosylceramide; GSL, glycosphingolipid; JNK, c-Jun N-terminal kinase; LIF, leukemia inhibitory factor; MAP2, microtubule associated protein 2; MBCD, methyl-β-cyclodextrin; NECs, neuroepithelial cells; MAPK, mitogen-activated protein kinase; PDMP, D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; PI3K, phosphatidylinositol-3-kinase; STAT, signal transducers and activators of transcription.

The central nervous system comprises neuronal and glial cells generated from common neural precursor cells during development (*1–7*). The fate of neural precursor cells, including proliferation, differentiation, survival and death, is regulated by multiple intrinsic and extrinsic cellular signals. For instance, basic helix-loop-helix (bHLH) transcription factors have central roles in neural development (*8*). Notch signaling maintains the neural precursor cell pool (*9*) and promotes survival (*10*). Bone morphogenetic proteins and leukemia inhibitory factor (LIF) cooperatively contribute to astrocyte differentiation (*11*). Basic fibroblast growth factor (bFGF) and epidermal growth factor promote the proliferation of neural precursor cells (*2*, *5*, *12*, *13*). Although the importance of these proteinous factors in the regulation of neural precursor cells has been clearly defined, the involvement of non-proteinous molecules remains to be clarified.

Glycosphingolipids (GSLs) are amphipathic molecules consisting of a carbohydrate chain and a ceramide lipid moiety (*14*). GSLs are ubiquitously present in all eukaryotic cells, and mainly localized in the outer leaflet of the plasma membrane (*14*). Recently, GSLs were shown to be clustered with sphingomyelin and cholesterol and form a microdomain, caveolae and lipid raft (also known as GSL-enriched microdomain, detergent-resistant membrane or detergent-insoluble membrane), in the plasma membrane (*15–17*). Microdomains have been suggested to be important for the modulation of signal transduction and cell adhesion (*15–17*). Since GSLs are abundant in neural cells and drastically change during development of the central nervous system (*18*), GSLs are expected to be involved in the regulation of neural precursor cell fate via functions in lipid rafts. However, the functions of GSLs in neural precursor cells remain unclear.

During development, neural precursor cells are present in the neuroepithelium. In this study, to clarify the functions of GSLs in neural precursor cells, we

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treated mouse neuroepithelial cells (NECs) with an inhibitor of glucosylceramide (GlcCer) synthesis, D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) (*19*). Since NECs express no galactosylceramide (GalCer)-based GSL (*20*), PDMP-treatment was considered to induce GSL depletion in NECs. We found that PDMP-treatment retards bFGF-induced proliferation, repress the Ras-mitogen–activated protein kinase (MAPK) pathway and alters gene expression of NECs. This is the first study to evaluate the function of GSLs in primary neural precursor cells.

MATERIALS AND METHODS

NEC Culture—NECs were prepared from telencephalons of ICR mouse embryos (E14.5) as previously described (*21*). Mice were treated according to the guidelines of Kumamoto University Center for Animal Resources and Development. NECs were cultured in N2-DMEM/F12 medium containing 10 ng/ml human recombinant bFGF (R&D Systems or Peprotech) on dishes that had been precoated with poly-L-ornithine (Sigma) and fibronectin (Nitta Gelatin). To inhibit GlcCer synthesis, NECs were cultured in N2-DMEM/F12 medium containing bFGF and 10 μM PDMP (Calbiochem) for 5 d. Immunocytochemical staining using an antibody against GD3 (IgM, Seikagaku Corporation) or nestin (IgG, a rat 401 monoclonal antibody) was performed on cells cultured on precoated Chamber Slides (Nunc). A biotin-conjugated anti-mouse IgM antibody (Jackson ImmunoResearch) and an FITC-conjugated streptavidin (Vector Laboratories) or an Alexa Fluor488–conjugated anti-mouse IgG antibody were used for detection. Hoechst33258 (Nacalai tesque) was used to stain nuclei. Proliferation of NECs was quantitatively measured using a Cell Counting Kit-8 (DOJINDO) (*22*).

Western Blot Analysis—After starvation with serum-free DMEM for 3 h, NECs were stimulated with bFGF or mouse LIF (Chemicon) for 10 min, and then lyzed by sonication (three 30 s bursts) in lysis buffer containing 1% Triton X-100, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 5 μg/ml aprotinin, 2 mM Na_3VO_4, 3 mM APMSF. In some cases, NECs were treated with an inhibitor before stimulation with a cytokine as described below: U0126 (an inhibitor of MEK activation, Promega, 10 μM for 1 h) and methyl-β-cyclodextrin (MBCD, an inhibitor of lipid raft functions, Sigma, 10 mM for 1 h). Incorporation of ceramide was performed by culturing NECs in serum-free DMEM containing *N*-acetyl-D-sphingosine (C_2 ceramide, Sigma) or ceramide (Matreya) for 3 h before stimulation with cytokines. NEC lysates containing equal amounts of protein were subjected to Western blot analysis using antibodies against phospho-extracellular signal-regulated kinase (ERK), phospho-signal transducer and activator of transcription 3 (STAT3), phospho-AKT, phospho-c-Jun N-terminal kinase (JNK) (Cell Signaling), ERK1, ERK2, STAT3, JNK (Santa Cruz Biotechnology) and AKT (Cell Signaling).

Preparation of Lipid Rafts—Lipid rafts were prepared according to the methods of Iwabuchi *et al.* (*23*). NECs were homogenized in 1 ml 0.5 M Na_2CO_3 using a loose fitting homogenizer (20 strokes) and a bath sonicator (three 30 s bursts). An aliquot of NEC homogenate was placed at the bottom of an ultracentrifuge tube and mixed with an equal volume of 90% sucrose (w/v) in 25 mM MES (pH 6.5) and 0.15 M NaCl (MBS). The homogenate was then overlaid with 6 ml 35% sucrose and 4 ml 5% sucrose in MBS containing 0.25 M Na_2CO_3, and centrifuged at 39,000 rpm for 18 h in a Beckman SW41 rotor. After centrifugation, 10 fractions were collected from the top of the gradient. Gradient fractions were directly subjected to Western blot-analysis using antibodies against flotillin-1, Grb2 (BD Biosciences), Ras, and SOS (Santa Cruz Biotechnology).

Ras Activation Assay—Activation of Ras was detected using a Ras activation assay kit (Upstate). Briefly, NECs were stimulated with bFGF (10 ng/ml, 10 min) and then lyzed with Mg^{2+} lysis buffer containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 5 μg/ml aprotinin, 2 mM $NaVO_4$, 3 mM APMSF. GTP-bound Ras (active Ras) in the NEC lysates was pull-downed by Raf-1 RBD agarose and subjected to SDS-PAGE followed by Western blot analysis using an anti-Ras antibody (Upstate).

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)—RT-PCR was performed as previously described (*24*). Primer sets were as follows: 5′-GGC TCA ACT TCA GCG GCT TC-3′, 5′-GTT GGT AAA GTC CAG CAG CTC-3′ (for Mash1); 5′-GGA TCC GGA CCA TGG ACT ACA AGG ACG ACG ATG ACA AGG CGC TGA GCC CGG TGC-3′, 5′-CTC GAG TCA GTG GCA AAA GCT CCT CTT G-3′ (for Id3); 5′-AAA GCC AAG GGC GCT GAT GG-3′, 5′-ATG GAT GTT CCC TAA CGA GCC-3′ (for Tau); 5′-CCT CAG CTG ACA GAG AAA CAG-3′, 5′-CTT GGT TCT GTG CTC TGT TTT C-3′ (for microtubule associated protein 2, MAP2); 5′-ATG AAG AGA GCT CAC CCA GA-3′, 5′-TTA GAA AGC TCC GAT CTC TG-3′ (for Hesr1); 5′-TTG ATA TCA TGG ACT CGG ACG CCA GCC TG-3′, 5′-AAG TCG ACT CAC TTG GCG TCG GAG GTG AG-3′ (for Olig2); 5′-CAC ATG AAG CCA CCC TGG CTC-3′, 5′-GTA GAT CCT GGT ACT CCT GCA G-3′ (for glial fibrillary acidic protein, GFAP); 5′-CTG CAG AGG TGG GTA AGT GG-3′, 5′-GCA GGT CAT TTT GAG GCA GCC-3′ (for UDP-galactose:ceramide galactosyltransferase, GalT); 5′-ACC ACA GTC CAT GCC ATC AC-3′, 5′-TCC ACC ACC CTG TTG CTG TA-3′ (for glyceraldehyde-3-phosphate dehydrogenase, G3PDH).

RESULTS

GSL Depletion by PDMP Represses bFGF-Induced Proliferation of NECs—NECs abundantly contain proliferative immature neural precursor cells capable of differentiating into glial or neuronal cells (*11*, *21*, *25*). To evaluate the functions of GSLs in NECs, these cells were treated with PDMP (10 μM) for 5 d. PDMP is a ceramide analogue that inhibits the synthesis of GlcCer and all GlcCer-based GSLs (*23*). Treatment of NECs with PDMP for 5 d did not severely affect cell viability and adhesion (Fig. 1A), but led to a depletion of GD3, a major ganglioside in NECs (Fig. 1B). NECs express gangliosides (sialic acid–containing GSLs) such as GD3 abundantly (*20*). In contrast, the expression of neutral GSL is weak, and GalCer-based GSLs are not expressed in NECs (*20*). Therefore, these results suggest that PDMP-treatment induces the depletion of whole GSLs in NECs.

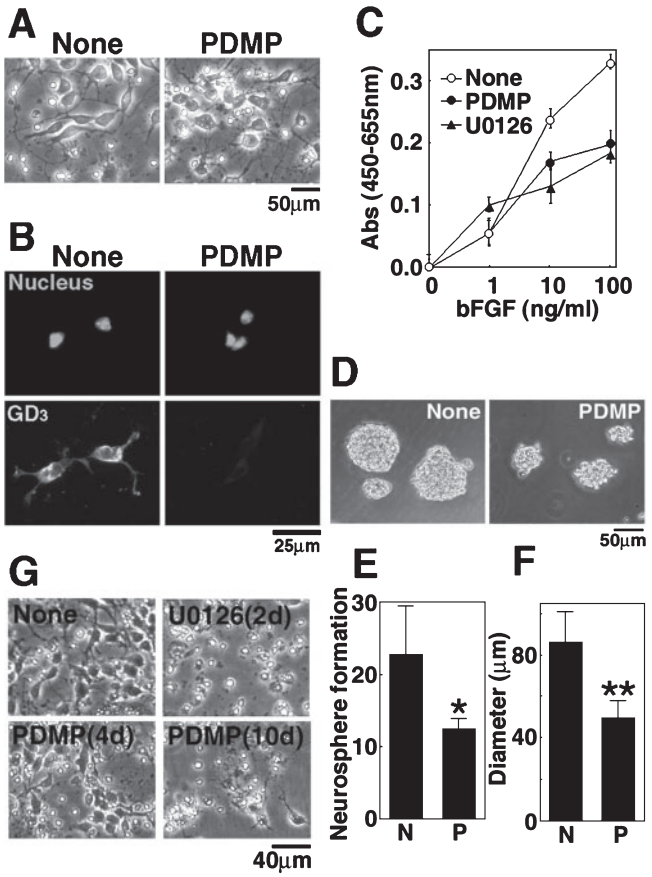

Fig. 1. **Repression of bFGF-induced proliferation of NECs by PDMP-treatment.** (A) NECs were treated with or without PDMP (10 μM) for 5 d, and (B) immunostained for GD3 to confirm GSL depletion. Nuclei were stained with Hoechst 33258. (C) Evaluation of bFGF-induced proliferation of NECs treated with vehicle (open circles), PDMP (closed circles) or U0126 (closed triangles, 10 μM for 1 d). (D) Examination of neurosphere formation by NECs treated with or without PDMP. The number and diameter of neurospheres are indicated in (E) and (F), respectively. $*P < 0.05$; $**P < 0.01$. (G) Most of the NECs died following treatment with U0126 (for 2 d) or PDMP (for 10 d), but not following PDMP treatment (for 4 d).

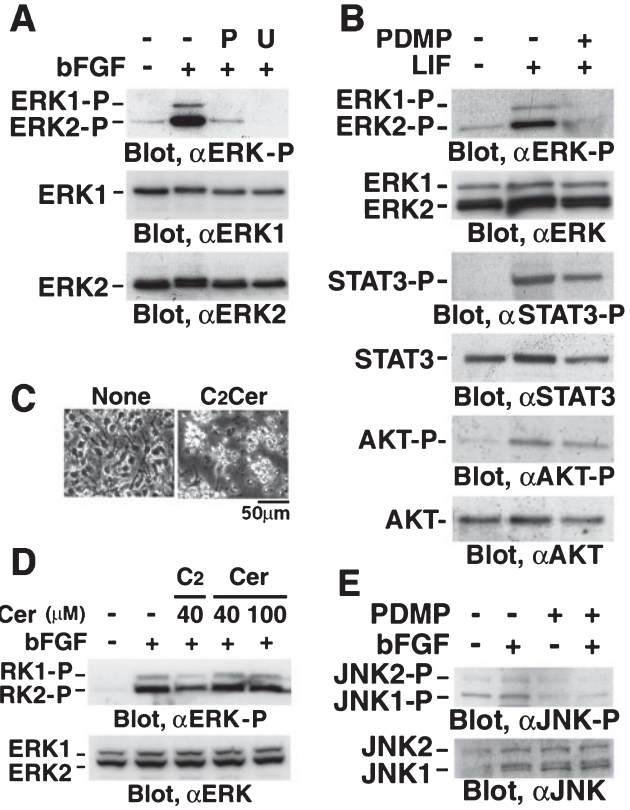

Fig. 2. **Repression of activation of the Ras-MAPK pathway following PDMP-treatment.** Western blot-analysis was performed using cell lysates prepared from (A) NECs treated with PDMP (P. 10 μM for 5 d) or U0120 (U. 10 μM for 1 h) and then stimulated with bFGF (10 ng/ml) for 10 min, or (B) NECs treated with PDMP and then stimulated with LIF (100 ng/ml) for 10 min. PDMP repressed ERK activation induced by bFGF and LIF. (C and D) Ceramides were incorporated by culturing NECs in serum-free DMEM containing ceramides (C_2, C_2 ceramide; Cer, ceramide) for 3 h. Ceramide incorporation was confirmed by culturing NECs for one additional day and detecting ceramide-induced apoptosis (C). (D) Ceramide-incorporation did not affect bFGF-induced ERK activation in NECs. (E) JNK was not activated in PDMP-treated NECs. (A, B, D and E) Cell lysates containing equal amounts of proteins were applied to each lane.

In PDMP-treated NECs, bFGF-induced proliferation was found to be retarded (Fig. 1C, closed circles). Neural precursor cells are known to proliferate and form aggregates, neurospheres, in the presence of a mitogen (*2*, *3*). Following PDMP-treatment, there was a severe reduction in the number and size of neurospheres (Fig. 1, D–F). Proliferation and survival of NECs depends on a Ras-MAPK pathway. The inhibition of bFGF-induced Ras-MAPK pathway activation by the MEK1 inhibitor, U0126, retarded NEC proliferation (Fig. 1C, closed triangles), and then induced cell death (Fig. 1G). In PDMP-treated NECs, the reduction in cell viability was not significant in the 4- or 5-d-culture, but obvious in cells cultured for longer periods (10 d) (Fig. 1G). Thus, these results suggest that PDMP-treatment represses NEC proliferation via inhibition of the bFGF-induced Ras-MAPK pathway.

GSL-Depletion Represses the Activation of the Ras-MAPK Pathway—To confirm directly that GSL-depletion represses bFGF-induced activation of the Ras-MAPK pathway, ERK MAPK activation in PDMP-treated NECs was analyzed. As shown in Fig. 2A, bFGF-induced ERK activation was severely repressed by PDMP treatment. LIF, an interleukin-6 family cytokine, induces the activation of three major signaling pathways: Janus kinases (JAK)-STAT3, phosphatidylinositol 3 kinase (PI3K)-AKT pathway and Ras-MAPK pathways (*26*). In PDMP-treated NECs, while LIF-induced activation of ERKs was repressed, LIF-induced activations of STAT3 and AKT were not affected, (Fig. 2B). These results show that PDMP-treatment specifically represses the Ras-MAPK pathway in NECs.

PDMP, which inhibits a GlcCer synthase, causes an accumulation of the substrate, ceramide. Thus, it was suspected that repression of the Ras-MAPK pathway by PDMP may be due to this side effect. To examine this possibility, ERK activation in ceramide-incorporated NECs was analyzed. The incorporation of exogenous ceramides was performed by culturing NECs in serum-free DMEM

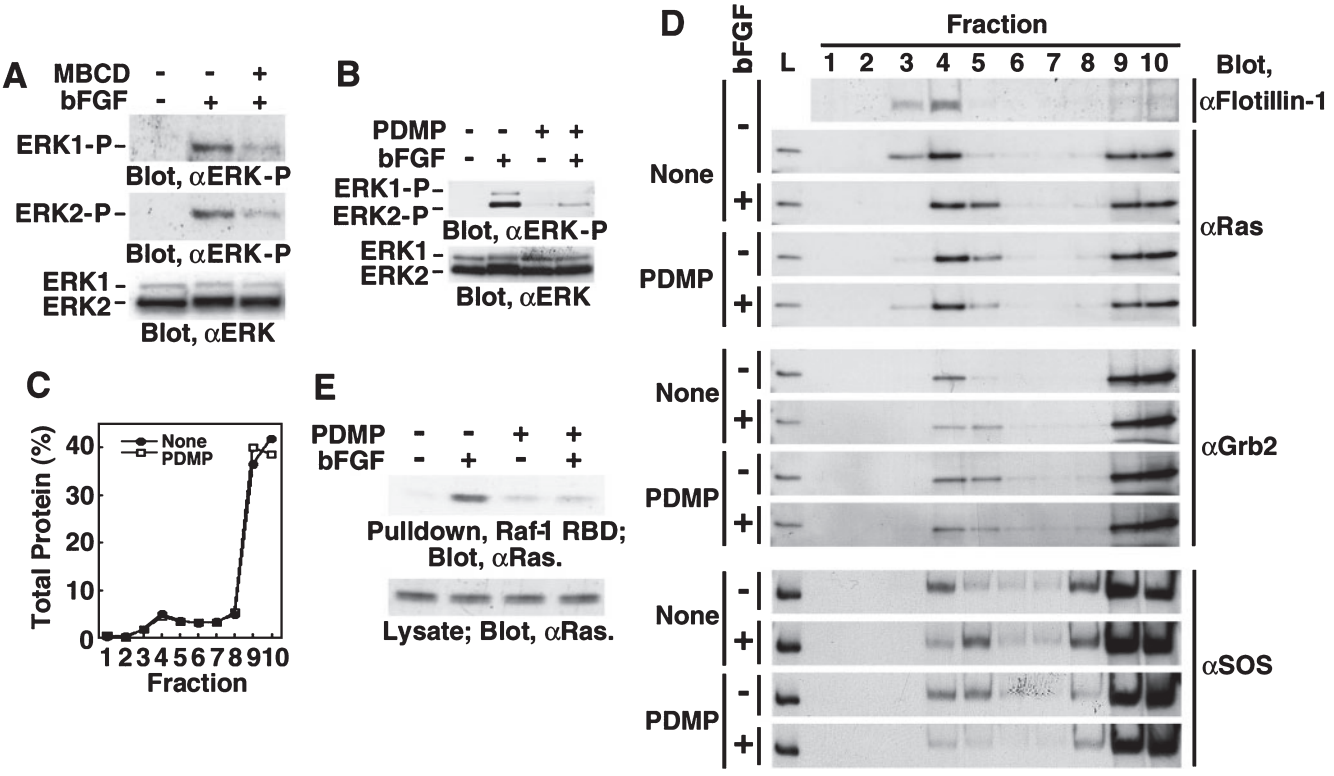

Fig. 3. **Activation of the Ras-MAPK pathway is dependent on lipid rafts in NECs.** (A) Western blot-analysis was performed using cell lysates prepared from NECs treated with MBCD (10 mM, for 1 h) and then stimulated with bFGF (20 ng/ml, for 10 min). MBCD inhibited bFGF-induced ERK activation. (B) NECs treated with PDMP were stimulated with bFGF and lyzed with 0.5 M Na_2CO_3 (pH 11) for lipid raft preparation. NEC lysates in (B) were subjected to sucrose gradient ultracentrifugation and fractionated into 10 fractions. Concentrations of proteins in each fraction prepared from NECs treated with (open squares) or without (closed circles) PDMP are indicated in (C). (D) Each fraction was subjected to Western blot-analysis using antibodies against flotillin-1, Ras, Grb2 and SOS. Lipid rafts were mainly collected in fraction 4. The distributions of Ras, Grb2 and SOS were not affected by PDMP-treatment. L indicates total cell lysates. (E) The GTP-bound form of Ras (active Ras) in NEC lysates was pull-downed by Raf-1 RBD agarose and detected by Western blot-analysis using an anti-Ras antibody. bFGF-induced Ras activation was repressed in PDMP-treated NECs.

containing ceramides for 3 h. The incorporation of ceramide in NECs was confirmed by apoptosis accompanied by DNA fragmentation one day after incorporation (Fig. 2C and data not shown; C_2 ceramide is a permeable analog of ceramide). As shown in Fig. 2D, bFGF-induced ERK activation was not repressed in NECs that incorporated excess C_2 ceramide or ceramide. It has been reported that the activation of JNK blocks ERK activation (*27*). Since ceramide activates the JNK pathway, we examined whether JNK activation was induced by PDMP treatment. As shown in Fig. 2E, PDMP-treatment did not induce JNK activation. Thus, it was considered that the Ras-MAPK pathway repression by PDMP is not caused by ceramide accumulation.

Activation of the Ras-MAPK Pathway Is Dependent on Lipid Rafts in NECs—GSLs are enriched in a plasma membrane microdomain, lipid raft, which is suggested to be important for the modulation of signal transduction and cell adhesion (*15–17*). Molecules triggering activation of the MAPK cascade, such as Ras and a Grb2-SOS complex, are distributed in lipid rafts (*28–30*). Thus, we hypothesized that GSL depletion by PDMP represses the activation of the MAPK cascade via inhibition of Ras activation by disrupting lipid rafts.

First, to confirm the involvement of lipid rafts in the activation of the Ras-MAPK pathway in NECs, the activation of ERKs in MBCD-treated cells was examined. MBCD is a carbohydrate molecule that binds to cholesterol and disrupts lipid rafts (*31*). In MBCD-treated NECs, the bFGF-induced activation of ERKs was repressed (Fig. 3A) as previously reported (*20*). This result indicates that the activation of ERK MAPKs in NECs was dependent on lipid rafts.

Next, we examined the distribution of Ras and a Grb2-SOS complex in lipid rafts of NECs. NECs treated with or without PDMP were stimulated with bFGF and lyzed (Fig. 3B). Then, cell lysates were subjected to sucrose gradient ultracentrifugation (*23*). Following centrifugation, lipid raft-containing, light-scattering bands were observed just above the 5–35% sucrose interface of samples prepared from both PDMP-treated and untreated NECs. Gradient samples were fractionated into 10 fractions from the top. The patterns of the protein concentration were similar between samples prepared from PDMP-treated and untreated NECs (Fig. 3C). As a result of Western blot-analysis with an antibody against flotillin-1, a marker protein of lipid rafts (*32*), lipid rafts were confirmed to be mainly collected in fraction 4 (Fig. 3D). The localization of flotillin-1 in lipid rafts was not affected by PDMP treatment (data not shown). These results suggest that GSLs are not essential for the formation of lipid rafts, as previously reported by Ostermeyer *et al.* (*33*).

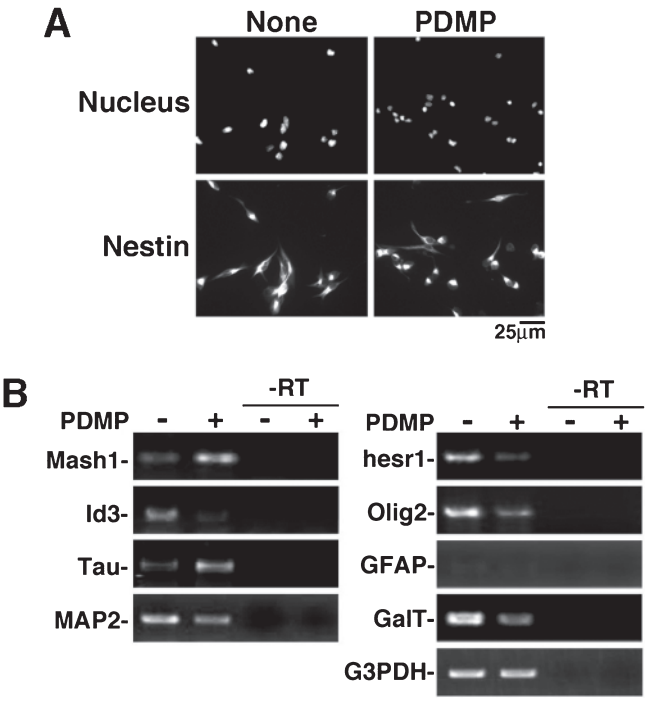

Fig. 4. **Lineage of PDMP-treated NECs.** (A) NECs treated with or without PDMP were stained with Hoechst33258 (for nuclei) and an anti-nestin antibody. (B) Up- or down-regulation of genes expressed in PDMP-treated NECs was analyzed by RT-PCR using primer sets for the appropriate genes. –RT, no reverse transcription (negative control).

Ras, SOS and Grb2 molecules were distributed in lipid raft fractions and soluble fractions (fractions 9 and 10) (Fig. 3D). The distribution of these molecules in lipid rafts was not affected by PDMP treatment and/or bFGF stimulation (Fig. 3D). Thus, it is suggested that the repression of Ras-MAPK pathway activation in PDMP-treated NECs is not due to the dislocation of Ras and a Grb2-SOS complex from lipid rafts. Next, we analyzed the activation of Ras in PDMP-treated NECs. The active form (GTP-bound form) of Ras was pulled down by agarose-conjugated Raf-1 RBD (Ras-binding domain), and detected by Western blot-analysis using an anti-Ras antibody. In PDMP-treated NECs, bFGF-induced Ras activation was repressed (Fig. 3E). bFGF-induced ERK activation was dependent on lipid rafts (Fig. 3A), and signaling molecules triggering the activation of the Ras-MAPK pathway were distributed in lipid rafts (Fig. 3D). Although PDMP-treatment did not affect the distribution of Ras in lipid rafts (Fig. 3D), it might elicit functional defects in lipid rafts.

Disturbance of Differentiation Status of PDMP-Treated NECs—To investigate whether PDMP affects not only proliferation and survival but also differentiation, we examined the lineage of PDMP-treated NECs. Using immunocytochemistry, we found that the number of NECs positive for nestin, a marker of neural precursor cells (*34*), was not significantly affected (Fig. 4A. None, 74.6 ± 9.0%; PDMP-treated, 63.4 ± 9.6%). So, we analyzed gene expression patterns in PDMP-treated NECs by RT-PCR. In PDMP-treated NECs, Mash1 (a bHLH factor important for neuronal differentiation) was up-regulated, and Id3 (an HLH factor inhibiting neuronal differentiation) was down-regulated (Fig. 4B, left panels). In addition, an up-regulation of Tau (a mature neuronal marker protein) was observed, although the expression of MAP2 (an immature and mature neuronal marker protein) was not increased (Fig. 4B, left panels). As for genes involved in glial differentiation of NECs, hesr1 (a bHLH factor that induces astrocyte differentiation and inhibits neuronal differentiation; Manuscript in preparation), Olig2 (a bHLH factor that inhibits astrocyte differentiation) (35) and GalT (a synthase of GalCer, a marker of GSL for oligodendrocytes) were down-regulated in PDMP-treated NECs (Fig. 4B, right panels). The expression of GFAP (a marker protein for astrocytes) was not detected (Fig. 4B, right panels). These results indicate that differentiation status is disturbed in PDMP-treated NECs in which the bFGF-induced activation of the Ras-MAPK pathway is repressed.

DISCUSSION

In this study, we treated NECs with PDMP, an inhibitor of GSL synthesis. In PDMP-treated NECs, (i) depletion of a GD3 ganglioside, (ii) retardation of bFGF-induced proliferation, (iii) repression of bFGF- and LIF-induced activations of the Ras-MAPK pathway, and (iv) disturbance of gene expression patterns were found. These results suggest the functional importance of GSLs in NECs. However, we cannot completely eliminate the possibility that these effects can be attributed to side effects of PDMP, including ceramide accumulation and plasma membrane disruption. Further analysis of NECs treated with other chemical inhibitors of GSL synthesis (*36*) and/or prepared from genetically-engineered mice deficient in GSL synthases (*37*, *38*) will further provide information on the bona fide functions of GSLs in neural precursor cells.

In PDMP-treated NECs, not only bFGF-, but also LIF-induced ERK activation was repressed. LIF activates three distinct signaling pathways: the JAK-STAT pathway, the PI3K-AKT pathway and the Ras-MAPK pathway. Surprisingly, LIF-induced activation of the JAK-STAT pathway was not affected in PDMP-treated NECs. Nishio *et al.* (*39*) reported that the overexpression of a GM1 ganglioside suppresses nerve growth factor–induced activation of a TrkA receptor and ERKs in PC12 cells. Furthermore, it has been reported that the overexpression of a GD3 synthase inhibits cytokine-induced ERK activation in a neural stem cell line (*40*). These facts strongly indicate that a disturbance of GSL expression specifically leads to repression of the Ras-MAPK pathway. Lipid rafts are expected to be responsible for the molecular mechanisms of repression of the Ras-MAPK pathway because (i) in NECs, Ras and a Grb2-SOS complex that trigger activation of the MAPK cascade were found to be distributed in lipid rafts as previously reported in other cells (*28–30*); and (ii) in NECs treated with MBCD, a lipid raft inhibitor, bFGF-induced ERK activation was repressed. In a study by Nishio *et al.* (*39*), the dislocation of TrkA, p75 and Ras from lipid rafts was observed in GM1-overexpressing PC12 cells. However, in NECs, the distribution of Ras and a Grb2-SOS complex was not affected by PDMP-treatment. On the other hand,

bFGF-induced activation of Ras in NECs was repressed in PDMP-treated NECs, suggesting that GSLs are not involved in organizing lipid rafts that harbor these signaling molecules, but are required for the complete function of lipid rafts. Mitsuda *et al.* (*41*) reported that an overexpression of GM1 ganglioside represses platelet-derived growth factor–induced ERK activation by dispersing the receptor molecule in lipid rafts. Also in NECs treated with PDMP, the dispersion of Ras and/or a Grb2-SOS complex might be induced.

Another possibility is an indirect effect of PDMP *via* modification of integrin signaling. Recently, we reported that β1 integrin molecules are localized in lipid rafts of NECs, and integrin-mediated adhesion of NECs to fibronectin substratum is dependent on this microdomain (*20*). ERK activation requires combined stimulation by cytokines and extracellular matrices (*42*). Indeed, in neural precursor cells, β1 integrin has been shown to promote the activation of the cytokine-induced Ras-MAPK pathway (*43*). Therefore, PDMP-treatment may repress the Ras-MAPK pathway by modifying integrin signaling via lipid rafts. Further studies on the molecular mechanism of the Ras-MAPK pathway repression in NECs treated with PDMP or MBCD are required.

In this study, we found that GSLs might be involved in bFGF-induced proliferation and signal transduction. But the activity of bFGF was not solely supported by GSLs. Heparan sulfate proteoglycan (*44*) and a glycosylated form of cystatin C (*45*) has been reported to be important for bFGF signaling in neural precursor cells. Furthermore, GSLs are enriched in lipid rafts, which are important for signal transduction. It is possible that other signal transducers regulating the fate of neural stem cells (for example, Notch, erythropoietin and bone morphogenetic proteins) also mediate signals via lipid rafts. In order to understand neural stem cells and neural development thoroughly, more systematic studies focused on non-proteinous molecules, such as glycoconjugates including GSLs, are necessary.

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