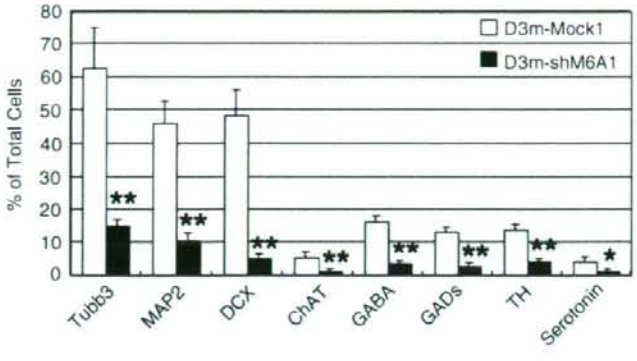

FIG. 7. The proportion of neurons from neural stem cells derived from D3m-Mock1 and D3m-shM6A1 cells. Cells were differentiated with ACM for 2 weeks, fixed, and immunostained with Tubb3, MAP2, DCX, ChAT, GADs, GABA, TH, and serotonin antibodies. The number of total cells was counted by the number of DAPI staining nuclei. Values are expressed as the mean ± SE ($n = 4$). $^{*}p < 0.01$ and $^{**}p < 0.001$ compared with neurons from D3m-Mock1 cells. All experiments were independently carried out at least three times, and almost the same results were obtained each time.

results imply that the proportion of NSC in differentiated cells derived from D3m-shM6A1 cells was smaller than those in D3m-Mock1 cells. Therefore, it was suggested that suppression of GPM6A transcript caused NSC generation from undifferentiated ES cells. There seems to be a correlation between the ratio of suppressed expression of mouse GPM6A transcripts and the ratio of differentiation of NSC from undifferentiated mouse ES cells. Moreover, the expression level of neuroectodermal marker genes, which are associated with the development of the brain [14–24], was reduced by the suppression of mouse GPM6A transcripts using shRNA against mouse GPM6A in neural-differentiated mouse ES cells (Fig. 2B). On the other hand, expression levels of mesodermal marker genes (*Nkx2.5, GATA-4, BMP-4, Flk1* etc. [32,33]) were clearly increased in differentiated cells derived from D3m-shM6A1 cells compared with those in D3m-Mock1 cells (data not shown). In addition, we also obtained results showing that expression level of undifferentiated ES marker gene (*OCT3/4*) was barely detected in differentiated cells derived from both D3m-Mock1 and D3m-shM6A1 cells (data not shown), and the doubling time of undifferentiated D3m-shM6A1 cells was equivalent to that of undifferentiated D3m-Mock1 cells (data not shown). A recent study has shown that transfection of small interference RNA in hippocampal neurons leads to decreased filopodium/spine formation but not to cell death [13]. As a result of these findings, we speculated that reduction of NSC generation by suppression of neural differentiation by GPM6A knockdown might lead to increase of other germ lineages, or ES cells remaining undifferentiated, but not to cell death.

Mouse GPM6A is known to be expressed in mouse neurons [8,9]. It is also reported that 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 extension of neurites in cultured neurons [8]. A recent study has shown that GPM6A is related to neurite outgrowth, filopodium/spine formation, and synaptic formation in cultured neurons [13]. In this study, the inhibition of mouse GPM6A expression decreased the generation of NSC. In addition, expression levels of cholinergic neuron marker (ChAT), catecholaminergic neuron markers (TH and DCC), and GABAergic neuron marker (GADs) [29–31] decreased in neurons derived from D3m-shM6A cells (Fig. 5). Moreover, our results revealed a suppression of differentiation into various neuronal cells, such as cholinergic (ChAT), catecholaminergic (TH and serotonin), and GABAergic (GABA and GADs) neurons, derived from D3m-shM6A1 cells (Figs 6 and 7). Furthermore, there was no difference in differentiation efficiency by neuronal cell type. Taken together, these findings suggest that mouse GPM6A is necessary for differentiation into NSC from mouse ES cells, and is one of the factors that trigger differentiation of NSC to various neuronal cells. However, the effect of shRNAs against mouse GPM6A, in which the level of expression of mouse GPM6A transcripts was maximally suppressed by about 80% in neural-differentiated cells derived from mouse ES cells, was extant (Figs 2A and 5). Therefore, it is uncertain how the remaining mouse GPM6A transcripts influence neural differentiation from NSC.

In the present study, we showed that inhibition of mouse GPM6A transcripts expression reduced NSC generation and neuron proportion derived from mouse ES cells. Further investigation, such as analysis of knockout mice, may lead to a better understanding of the relationship between neural differentiation and GPM6A signal transduction.

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 Azedine Barik and Gary W. Clendennen for a critical reading of the manuscript.

References

1. Evans M, and M Kaufman. (1981). Establishment in culture of pluripotent cells from mouse embryos. Nature 292:154–156.
2. Martin GR. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA 78:7634–7638.

3. Rathjen PD, J Lake, LM Whyatt, MD Bettess and J Rathjen. (1998). Properties and uses of embryonic stem cells: prospects for application to human biology and gene therapy. Reprod Fertil Dev 10:31–47.
4. Nagy A, E Gocza, EM Diaz, VK Prideaux, E Iványi, M Markkula and J Rossant. (1990). Embryonic stem cells alone are to support fetal development in the mouse. Development 10:815–821.
5. Okabe S, K Forsberg-Nilsson, AC Spiro, M Segal and RD McKay. (1996). Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro. Mech Dev 59:89–102.
6. Guan K, H Chang, A Rolletschek and AM Wobus. (2001). Embryonic stem cells-derived neurogenesis. Retinoic acid induction and lineage selection of neuronal cells. Cell Tissue Res 305:171–176.
7. Nakayama T, K Momoki-Soga, K Yamaguchi and N Inoue. (2004). Efficient production of neural stem cells and neurons from embryonic stem cells. Neuroreport 15:487–491.
8. Lagenaur C, V Kunemund, G Fischer, S Fushiki and M Schachner. (1992). Monoclonal M6 antibody interferes with neurite extension of cultured neurons. J Neurobiol 23:71–78.
9. Yan Y, V Narayanan and C Lagenaur. (1996). Expression of members of the proteolipid protein gene family in the developing murine central nervous system. J Comp Neurol 370:465–478.
10. Alfonso J, GD Pollevick, MG Van Der Hart, G Flügge, E Fuchs and AC Frasch. (2004). Identification of genes regulated by chronic psychosocial stress and antidepressant treatment in the hippocampus. Eur J Neurosci 19:659–666.
11. Alfonso J, LR Frick, DM Silberman, ML Palumbo, AM Genaro and AC Frasch. (2006). Regulation of hippocampal gene expression is conserved in two species subjected to different stressors and antidepressant treatment. Biol Psychiatry 59:244–251.
12. Mukobata S, T Hibino, A Sugiyama, Y Urano, A Inatomi, Y Kanai, H Endo and F Tashiro. (2002). M6a acts as a nerve growth factor-gated Ca^{2+} channel in neuronal differentiation. Biochem Bioph Res Co 297:722–728.
13. Alfonso J, ME Fernández, B Cooper, G Flügge and AC Frasch. (2005). The stress-regulation protein M6a is a key modulator for neurite outgrowth and filopodium/spine formation. Proc Natl Acad Sci USA 102:17196–17201.
14. Gammill LS and H Sive. (2001). Otx2 expression in the ectoderm activates anterior neural determination and is required for Xenopus cement gland formation. Dev Biol 240:223–236.
15. Pantò MR, A Zappalà, F Tuorto and F Cicirata. (2004). Role of the Otx1 gene in cell differentiation of mammalian cortex. Eur J Neurosci 19:2893–2902.
16. Arenas E. (2002). Stem cells in the treatment of Parkinson's disease. Brain Res Bull 57:795–808.
17. Guo C, HY Qiu, Y Huang, H Chen, RQ Yang, SD Chen, RL Johnson, ZF Chen and YQ Ding. (2007). Lmx1b is essential for Fgf8 and Wnt1 expression in the isthmic organizer during tectum and cerebellum development in mice. Development 134:317–325.
18. McMahon AP and A Bradley. (1990). The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. Cell 62:1073–1085.
19. Crossley PH and GR Martin. (1995). The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. Development 121:439–451.
20. Joyner AL. (1996). Engrailed, Wnt and Pax genes regulate midbrain-hindbrain development. Trends Genet 12:15–20.
21. Liu A and AL Joyner. (2001). Early anterior/posterior patterning of the midbrain and cerebellum. Annu Rev Neurosci 24:869–896.
22. Sgadò P, L Alberi, D Gherbassi, SL Galasso, GM Ramakers, KN Alavian, MP Smidt, RH Dyck and HH Simon. (2006). Slow progressive degeneration of nigral dopaminergic neurons in postnatal Engrailed mutant mice. Proc Natl Acad Sci USA 103:15242–15247.
23. Kishi M, K Mizuseki, N Sasai, H Yamazaki, K Shiota, S Nakanishi and Y Sasai. (2000). Requirement of Sox2-mediated signaling for differentiation of early Xenopus neuroectoderm. Development 127:791–800.
24. Malas S, M Postlethwaite, A Ekonomou, B Whalley, S Nishiguchi, H Wood, B Meldrum, A Constanti and V Episkopou. (2003). Sox1-deficient mice suffer from epilepsy associated with abnormal ventral forebrain development and olfactory cortex hyperexcitability. Neuroscience 119:421–432.
25. Kaneko Y, S Sakakibara, T Imai, A Suzuki, Y Nakamura, K Sawamoto, Y Ogawa, Y Toyama, T Miyata and H Okano. (2000). Musashi1: an evolutionally conserved marker for CNS progenitor cells including neural stem cells. Dev Neurosci 22:139–153.
26. Strojnik T, GV Røsland, PO Sakariassen, R Kavalar and T Lah. (2007). Neural stem cell markers, nestin and Musashi proteins, in the progression of human glioma: correlation of nestin with prognosis of patient survival. Surg Neurol 68:133–144.
27. Xie Z, Q Zheng, X Guo, C Yi and Y Wu. (2004). Isolation, culture and identification of neural stem cells in new-born rats. J Huazhong Univ Sci Technolog Med Sci 24:75–78.
28. Meng XT, D Chen, ZY Dong and JM Liu. (2007). Enhanced neural differentiation of neural stem cells and neurite growth by amniotic epithelial cell co-culture. Cell Biol Int 31:691–698.
29. Aramant R, M Seiler, B Ehinger, A Bergström, AR Adolph and JE Turner. (1990). Neuronal markers in rat retinal grafts. Brain Res Dev Brain Res 53:47–61.
30. Tashiro Y, JM Drake, S Chakrabortty and T Hattori. (1997). Functional injury of cholinergic, GABAergic and dopaminergic systems in the basal ganglia of adult rat with kaolin-induced hydrocephalus. Brain Res 770:45–52.
31. Maloney KJ, L Mainville and BE Jones. (1999). Differential c-Fos expression in cholinergic, monoaminergic, and GABAergic cell groups of the pontomesencephalic tegmentum after paradoxical sleep deprivation and recovery. J Neurosci 19:3057–3072.
32. Brown CO 3rd, X Chi, E Garcia-Gras, M Shirai, XH Feng and RJ Schwartz. (2004). The cardiac determination factor, Nkx2–5, is activated by mutual cofactors GATA-4 and Smad1/4 via a novel upstream enhancer. J Biol Chem 279:10659–10669.
33. Sakurai Y, K Ohgimoto, Y Kataoka, N Yoshida and M Shibuya. (2005). Essential role of Flk-1 (VEGF receptor 2) tyrosine residue 1173 in vasculogenesis in mice. Proc Natl Acad Sci USA 102:1076–1081.

Address reprint requests to:
Yasushi Kondo, Ph.D.
Advanced Medical Research Laboratory
Mitsubishi Tanabe Pharma Corporation
16-89 Kashima-3-chome
Yodogawa-ku, Osaka 532–8505, Japan

E-mail: kondo.yasushi@mc.mt-pharma.co.jp

Received for publication April 1, 2008; accepted after revision June 3, 2008.