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Wnt proteins promote neuronal differentiation in neural stem cell culture

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Abstract

Wnt signaling is implicated in the control of cell growth and differentiation during CNS development from studies of mouse and chick models, but its action at the cellular level has been poorly understand. In this study, we examine the in vitro function of Wnt signaling in embryonic neural stem cells, dissociated from neurospheres derived from E11.5 mouse telencephalon. Conditioned media containing active Wnt-3a proteins are added to the neural stem cells and its effect on regeneration of neurospheres and differentiation into neuronal and glial cells was examined. Wnt-3a proteins inhibit regeneration of neurospheres, but promote differentiation into MAP2-positive neuronal cells. Wnt-3a proteins also increase the number of GFAP-positive astrocytes but suppress the number of oligodendroglial lineage cells expressing PDGFR or O4. These results indicate that Wnt-3a signaling can inhibit the maintenance of neural stem cells, but rather promote the differentiation of neural stem cells into several cell lineages.

Keywords: Wnt; Neural stem cell; Neurosphere; Neuron; Astrocyte; Oligodendrocyte; Neuronal differentiation

Most of the neuronal and the glial cell types found in the mature vertebrate central nervous system (CNS) originate from neural stem/progenitor cells in the ventricular zone of the fetal brain and spinal cord. Regulated maintenance and expansion of neural stem cells is essential for proper development of the neural tissues. A number of external cues, as well as intrinsic cellular programs, are thought to regulate maintenance and proliferation/differentiation of neural stem/progenitor cells [1]. For instance, bone morphogenic proteins (BMPs) promote neuronal differentiation of cortical ventricular zone precursors [2]. In contrast, BMPs also inhibit the neuronal differentiation of telencephalic neuroepithelial cells due to their promotion of astrocytic differentiation [3–5]. Sonic Hedgehog (Shh) promotes both neuronal and oligodendroglial differentiation of embryonic neural stem cells [6] and regulates proliferation of embryonic and adult hippocampal neural stem cells [6,7]. On the other hand, Notch signaling, as well as FGF2 and EGF, can maintain neural stem cells in an undifferentiated state [8–10].

The Wnt family of secreted cell signaling proteins consists of at least 17 members in vertebrates, some of which are expressed in the developing CNS. Wnt signaling appears to regulate cell growth during CNS development. Overexpression of *Wnt-1* and *Wnt-3a*, as well as their signaling component, β-catenin, causes an increase in the number of cells undergoing mitosis in the ventricular zone of the spinal cord [11,12]. This mitogenic effect of Wnt signaling is partly mediated by transcriptional activation of cyclin D1 and cyclin D2 [12]. Transgenic mice expressing a stabilized form of β-catenin in neuroepithelial precursors show an expansion of the precursor population in the developing brain by enhancing re-entry of the cell cycle [13]. Gene disruption studies also support roles of *Wnt* genes in CNS development. Specifically, the midbrain and the hippocampus are deleted in mice deficient for *Wnt-1* and *Wnt-3a*, respectively [14–17]. Furthermore, *Wnt-1* and *Wnt-3a* double mutants have an additional reduction of

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the rostral hindbrain [18]. On the contrary, Wnt signaling is also implicated in neuronal differentiation. Overexpression of *Wnt-1* in embryonic carcinoma P19 cells promotes neuronal differentiation [19]. Furthermore, Wnt-3a/β-catenin signaling, through the down-regulation of Axin, plays an important role in the neuronal differentiation of P19 cells [20]. Thus, the Wnt signal appears to be involved in mitogenic regulation and differentiation of neural primordia. However, action of Wnt proteins on neural stem cells has not been clarified.

In vitro stem cell culture is a powerful tool for examining the effect of an external signal on neural stem cells. In vitro clonal analysis has shown that, in a serum-free defined medium supplemented with FGF-2, single cells derived from the embryonic or adult brain proliferate and form floating spherical colonies, called neurospheres [8]. Single cells derived from neurospheres self-renew to generate a new neurosphere or differentiate into neurons or glia depending on the culture condition, indicating that they have characteristics of stem cells. In this study, we employed in vitro culture systems to investigate the effect of Wnt proteins on neural stem cells. We show here that Wnt proteins inhibit maintenance of neural stem cells, but rather promote differentiation of neural stem cells into neurons and astrocytes.

Materials and methods

Wnt3a conditioned media. Flag-tagged mouse Wnt3a cDNA was transfected into mouse L cells and expressed under the PGK promoter. Flag-Wnt3a conditioned medium was prepared essentially as described previously [21]. Briefly, transfected cells were cultured in DMEM/F12 with 0.5% fetal bovine serum for 3 days. Cells were switched to serum-free DMEM/F12 and Wnt3a-containing conditioned medium was harvested after 24 h. The concentration of Wnt3a protein in the conditioned medium was estimated at 400 ng/ml. The Wnt3a CM was incubated with Flag-M2 resin (Sigma) overnight at 4 °C to absorb Wnt protein. The amount of Wnt proteins in the absorbed Wnt3a CM was approximately 30% of the original Wnt3a CM as determined by Western blotting using anti-Wnt3a antibodies.

Generation of progenitor cell neurospheres and culture of dissociated cells. Neurosphere culture was performed essentially as described previously [8,9]. The forebrain of E11.5 mouse embryos was dissociated by incubation with 0.025% trypsin for 15 min followed by repeated trituration. Dissociated cells were seeded onto 24-well uncoated multiwell culture plates (Nunc) at a density of 1×10^5/well in serum-free neurosphere medium in the presence of FGF2 (20 ng/ml, Invitrogen). The cells were cultured for 6 days to generate proliferative progenitor neurospheres at the stage of maximal size and viability. The majority of cells in these spheres expressed Nestin, which is expressed in uncommitted neural precursor cells. Cells dissociated from these spheres were differentiated into neurons, astrocytes, and oligodendrocytes in (data not shown). The neurosphere medium consisted of DMEM/F12 (Invitrogen) with nutrient additives (30 nM sodium selenite; 100 μg/ml apo-transferrin; 25 μg insulin; 20 nM progesterone; and 60 μM putrescine). After 6 days in culture, FGF2-generated neurospheres were dissociated again by incubation with 0.025% trypsin for 15 min followed by repeated trituration. Dissociated cells were plated onto uncoated multiwell culture plate or poly-D-lysine-coated (PDL, Sigma, 20 μg/ml for 1 h) coverslips (12 mm) at a density of 5×10^4 cells/well and propagated in neurosphere medium containing FGF2 (20 ng/ml) with Wnt3a CM or control medium. For further examination of Wnt-3a activity in neural differentiation, culture medium was exchanged after 2 days in culture with fresh neurosphere medium containing with Wnt3a CM or control medium, but not supplemented with FGF2.

Immunohistochemistry. Cells were fixed with 4% paraformaldehyde and processed for standard indirect immunofluorescent staining. Fixed coverslips were washed in phosphate-buffered saline (PBS) and incubated with primary antibodies using an appropriate blocking serum (5% normal goat serum) at 4 °C overnight or at room temperature for 2 h. The following primary antibodies were used: MAP2 (mouse IgG, Sigma), Nestin (rabbit IgG, gift from Dr. Tomooka), GFAP (rabbit IgG, Dako), and O4 (mouse IgM, Chemicon). After washing in PBS three times, secondary antibodies conjugated with either Alexa 488 or Alexa 546 were applied (1:1500, Molecular Probes). Following repeated washing in PBS, coverslips were counterstained with Hoechst 33342 to visualize nuclei and to determine total cell numbers. Coverslips were mounted using an antifade reagent (Vector Laboratory). The cell number in each experimental condition was estimated by counting four different fields of view on each coverslip in at least two independent cultures.

Results

To examine effects of Wnt proteins on neural stem cells in vitro, we first generated primary neurospheres derived from the telencephalon of E11.5 mouse embryos. Cells were dissociated from these neurospheres and cultured for 2 days with FGF2 and serum-free conditioned media of cells expressing Wnt-3a (Wnt-3a CM) or not (control CM) (Fig. 1). Treatment of the dissociated cells with Wnt-3a CM decreased the number of newly formed spheres, depending on the concentration of Wnt-3a CM (Figs. 1A, D, and G). In contrast, dissociated cells normally formed new spheres with control CM (Figs. 1C and D). Furthermore, immunodepletion of Wnt-3a CM with anti-Wnt-3a antibody largely recovered the efficiency to form secondary neurospheres (Figs. 1B and E). These results demonstrated that Wnt-3a proteins can suppress neural stem cell maintenance in vitro.

The decrease in the number of neurospheres was highly correlated with an increase in the number of dispersed and adherent cells, many of which showed a neuron-like spindle-shaped morphology (Figs. 1A and E–G). These cells extended neurite-like fibers after 3 days of culturing with Wnt-3a CM (data not shown). An increase in the number of neuron-like cells by Wnt-3a CM was also observed in culture on coverslips coated with poly D- or L-lysine, poly-ethylenimine, collagen, or fibronectin (data not shown). Thus, the effect of Wnt-3a CM is not in promoting adhesion of neural primordial cells to the culture dishes. Rather, it appears that Wnt-3a proteins promote the differentiation of neural stem cells at the expense of neurosphere formation.

To identify the cell types generated by the effect of Wnt-3a CM, cells were stained for immunofluorescence using antibodies against Nestin for uncommitted neural

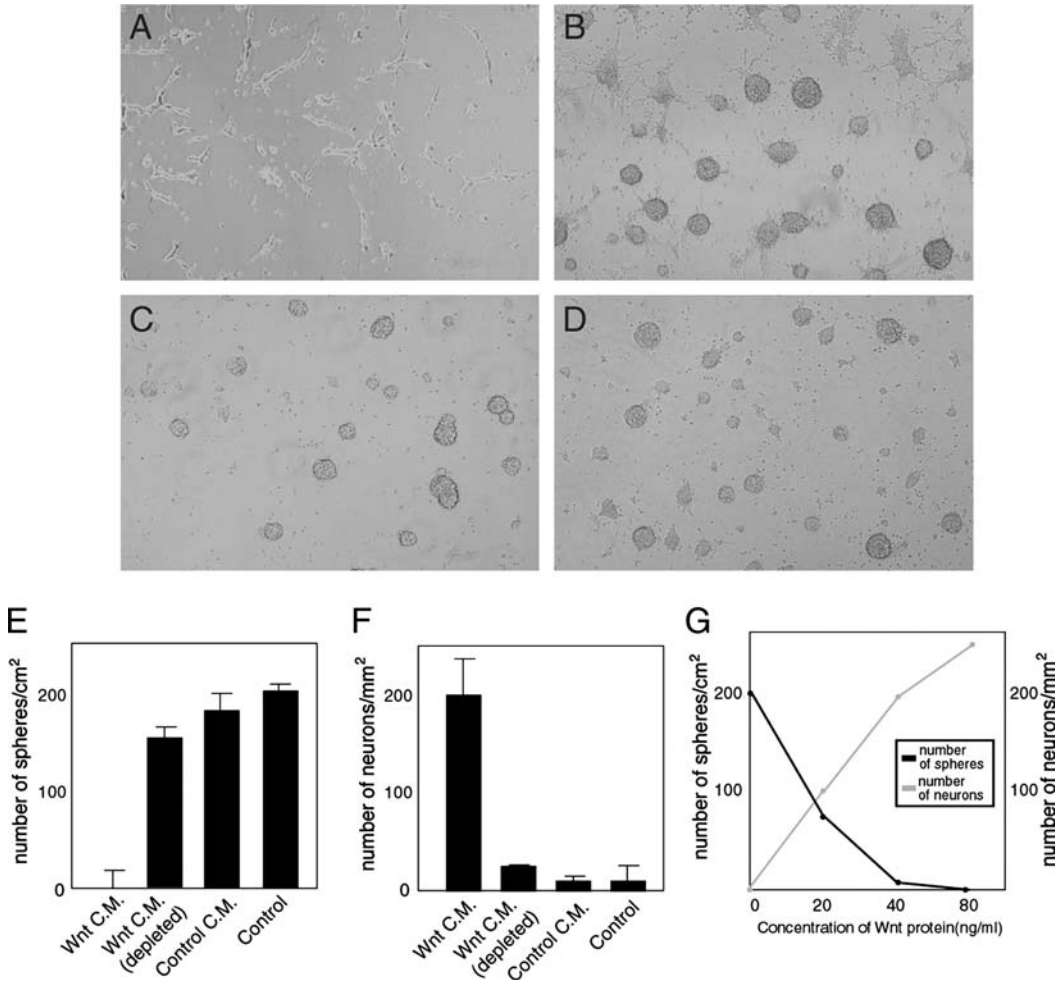

Fig. 1. Inhibition of neurosphere formation and promotion of neural cell differentiation in the presence of Wnt-3a CM. (A–D) Cells dissociated from the E11.5 mouse forebrain were cultured for 2 days in low attachment multiwell plates under various conditions: with Wnt-3a conditioned medium (Wnt-3a CM) (A), with immunoabsorbed Wnt-3a CM (B), with control conditioned medium (control CM) (C), and without conditioned medium (D). (E–G) The numbers of spheres (E) and differentiated cells (F) and the effect of Wnt-3a protein concentration (G) after 2 days in culture are shown. Many large spheres were generated in the culture with control CM (D,E). In contrast, treatment with Wnt-3a CM significantly increased the number of differentiated cells and almost wiped out neurospheres (A,E,F). This effect was dependent on Wnt-3a concentration (G). These effects of Wnt-3a CM were almost eliminated by immunoabsorption, indicating that these effects are due to the Wnt-3a protein (B). Total 10 fields were counted under phase-contrast microscope using 100× magnification.

precursor cells, MAP2 for neurons, GFAP for astrocytes, and PDGFR for immature oligodendrocytes (Fig. 2). Most of the dispersed cells on poly D-lysine coated coverslips expressed MAP2 in the presence of Wnt-3a CM (Fig. 2B), but not in the presence of control CM (Fig. 2E). The number of MAP2-positive cells was increased by the addition of Wnt-3a, whereas the number of Nestin-positive cells decreased (Fig. 2M). Wnt-3a CM did not affect cell proliferation under this culture condition (data not shown). Thus, Wnt-3a enhances the differentiation of neural stem cells into neurons in vitro. However, most of the MAP2-positive cells also expressed Nestin, indicating that neuronal differentiation was not complete in these cells (Figs. 2A and B). On the other hand, the number of GFAP-positive astrocytes and PDGFR-positive oligodendrocyte precursors was not significantly altered by Wnt-3a CM (Figs. 2G–M).

To further examine whether Wnt-3a can promote cells to more differentiated states, FGF2, which usually promotes maintenance of neural stem cells, was removed from the culture in later stages. Primary neurosphere-derived cells were cultured for 2 days, in the presence of FGF2, with Wnt-3a CM or control CM and then further cultured for additional 4 days without FGF2 (Fig. 3). Immunocytochemical analysis indicated that the number of MAP2-positive neuronal cells was remarkably increased in the presence of Wnt-3a CM (4.5-fold increase) while the number of Nestin-positive cells slightly increased (Figs. 3A–F and M). At this stage, many MAP2-positive cells showed attenuated expression of Nestin, indicating that Wnt-3a did promote differentiation of neuronal cells (Figs. 3A–C). Although the number of total cells was increased by 2.5-fold in the presence of Wnt-3a CM (Figs. 3C, F, I and L), the number of

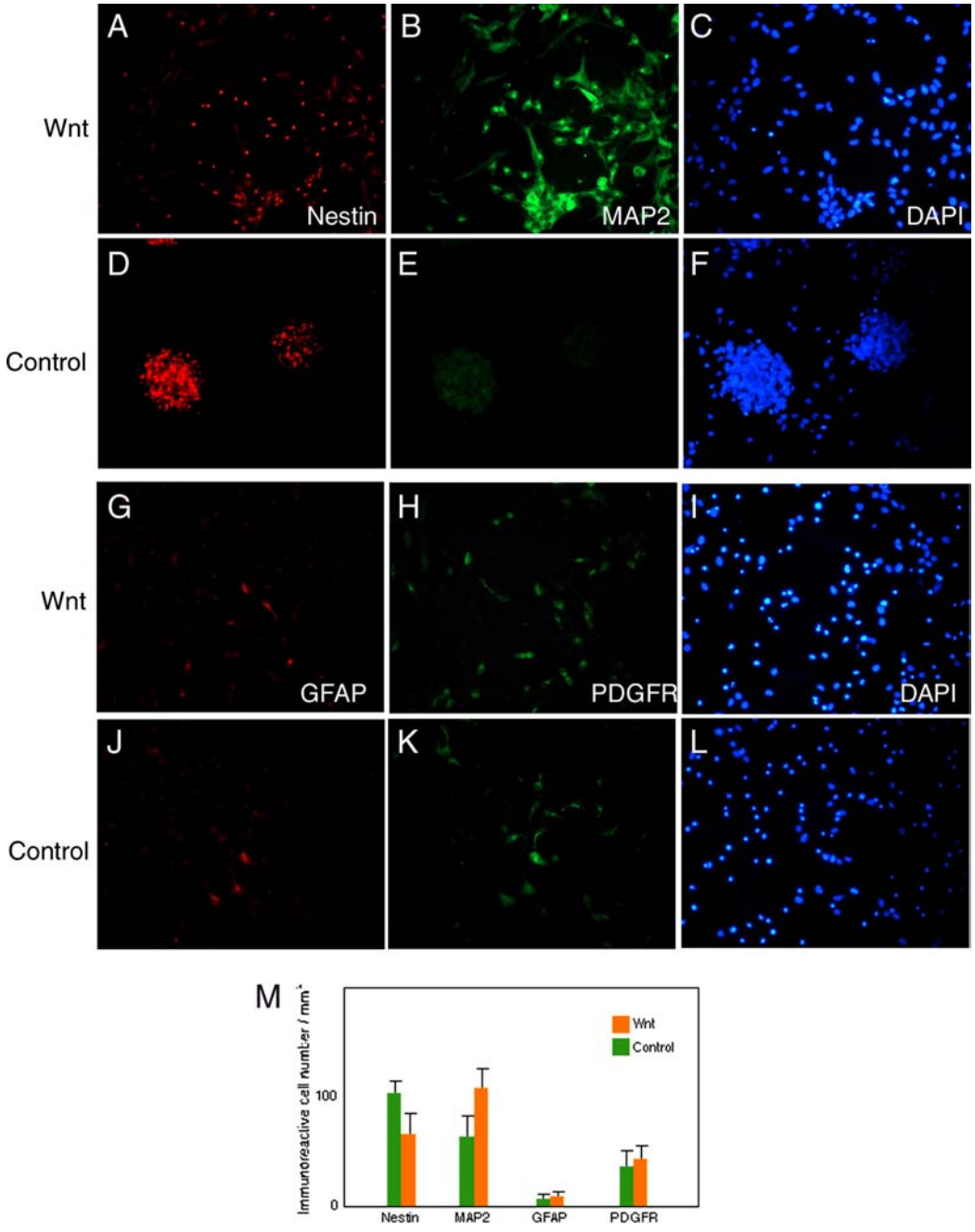

Fig. 2. Wnt-3a CM promotes the differentiation of neural stem cells into neurons. Neurospheres generated from the E11.5 telencephalon were dissociated and plated on poly D-lysine coated coverslips in the presence of Wnt-3a CM (A–C, G–I) or control CM (D–F, J–L). After 2 days in culture, cells were immunostained for Nestin, MAP2, GFAP or PDGFR. (A–F) Double-immunofluorescence microscopy for Nestin (red) (A,D), MAP2 (green) (B,E), and DAPI (blue) (C,F). (G–L) Double-immunofluorescence microscopy for GFAP (red) (G,J), PDGFR (green) (H,K), and DAPI (blue) (I,L). (M) The average numbers of immunoreactive cells that were obtained by counting four different fields of view on each coverslip in at least two independent cultures. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

mitotic cells assessed by BrdU incorporation was almost same between Wnt-3a CM and control CM during the first 1 day after the removal of FGF2 (data not shown). Thus, Wnt-3a CM does not appear to affect cell proliferation primarily under this culture condition, but rather the increase of the total cell number with Wnt-3a CM may be explained as a secondary consequence of differentiation. There was also a 2.7-fold increase in the number of GFAP-positive cells, while O4 positive cells representing differentiated oligodendrocytes decreased (Figs. 3G–M). These results indicated that Wnt-3a selectively enhanced neuronal and astrocyte differentiation.

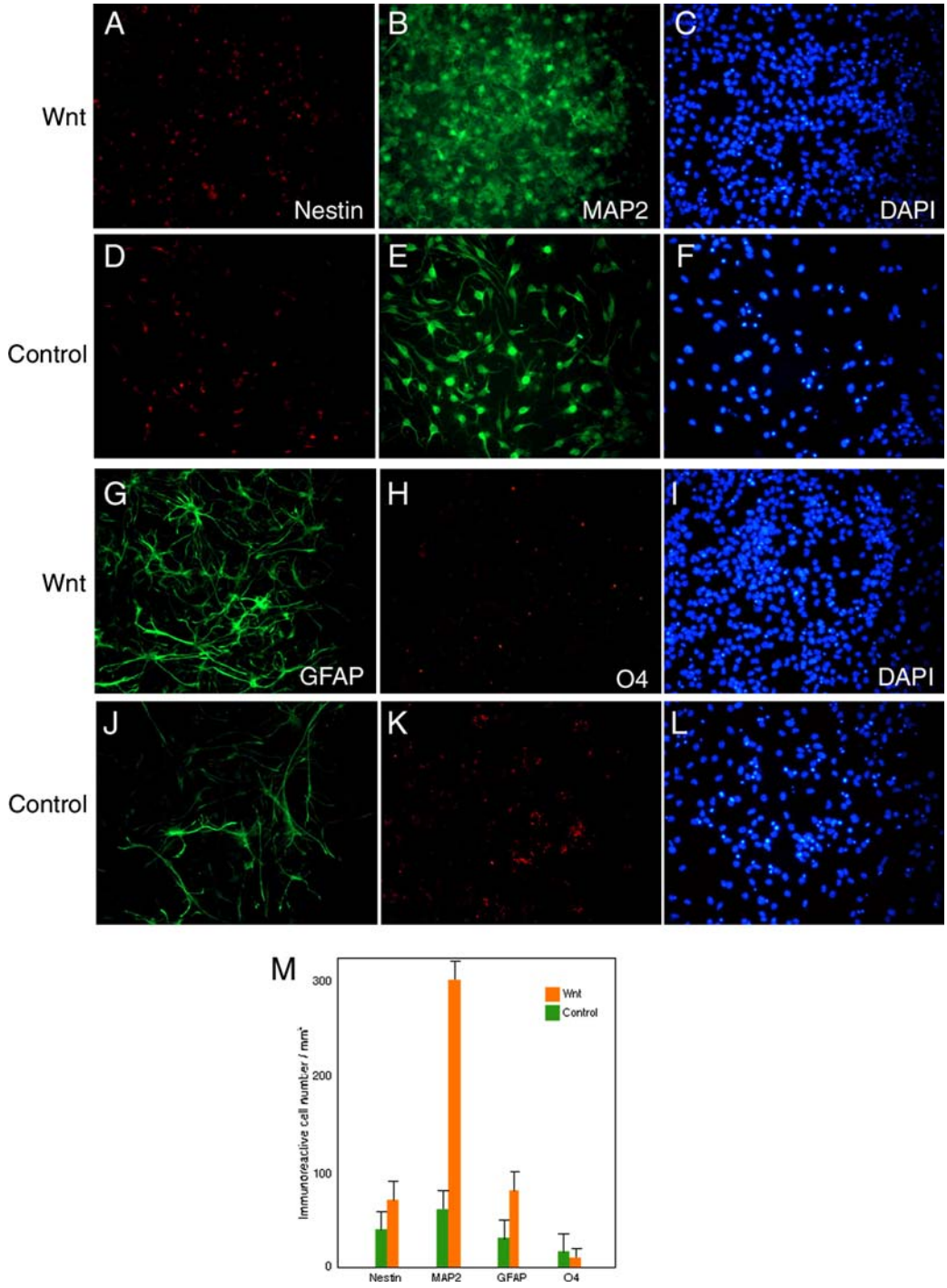

Fig. 3. Wnt-3a CM enhanced the maturation of neuronal and astrocyte lineages after removing FGF2 from cultures. Dissociated cells from the primary neurospheres obtained from the E11.5 telencephalon were cultured for 2 days on poly D-lysine coated coverslips in the presence of Wnt-3a CM and FGF2. Then, these cells were continuously cultured for an additional 4 days with Wnt-3a CM (A–C, G–I) or control CM (D–F, J–L), but without FGF2. After 4 days in culture, cells were immunostained for Nestin, MAP2, GFAP or O4. (A–F) Immunofluorescence microscopy for Nestin (red) (A,D), MAP2 (green) (B,E), and DAPI (blue) (C,F). (G–L) Immunofluorescence microscopy for GFAP (green) (G,J), O4 (red) (H,K), and DAPI (blue) (I,J). (M) The average numbers of immunoreactive cells that were obtained by counting four different fields of view on each coverslip from at least in two independent cultures. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

Discussion

Wnt signaling has been implicated in the expansion of neural precursor cells in the embryo. Ectopically expressed Wnt-1, Wnt-3a, and a constitutive active form of β-catenin, a component of the Wnt signaling pathway, cause expansion of neural precursor cells in the developing brain and spinal cord [11–13]. In addition, gene dis-

ruption studies indicate that Wnt-1 and Wnt-3a are required for the formation of several regions of the CNS, including the midbrain, diencephalon, hippocampus, and dorsal hindbrain [15,17,18]. One possible explanation for the enlargement of precursor population could be an increased number of neural stem cells. Thus, we examined this possibility in in vitro neural stem cell culture, but the results we obtained strongly suggested that expansion of the precursor population by Wnt signaling is not due to an increase in the number of neural stem cells. We demonstrated that Wnt-3a proteins decreased the number of neurosphere-forming neural stem cells derived from E 11.5 telencephalon under in vitro culture condition (Fig. 1). Therefore, the expansion of neural precursor cells in vivo by ectopically activated Wnt signaling seems to be due to another mechanism.

Rather, our results showed that Wnt-3a proteins enhanced differentiation of neural stem cells into neurons and astroglias at the expense of self-renewal of neural stem cells at least in in vitro condition (Figs. 2 and 3). Thus, Wnt signaling may promote differentiation of neural stem cells in vivo at the point when these cells must choose their fates between self-renewing and differentiation. However, so far, no gene disruption study has shown that this function of Wnt signaling is also required for in vivo neural development [15,17,18,22]. One possible explanation for this inconsistency between in vitro and in vivo results is that some other signals may compensate for lack of Wnt signal in differentiation of neural stem cells in mutant embryos lacking a Wnt signal. *Wnt-7b* is overlappingly expressed with *Wnt-7a* in most of E11.5 telencephalon, from which neural stem cells were prepared in our analysis. Although *Wnt-7a* null mutants display no neural defect, *Wnt-7b* may have compensated the loss of *Wnt-7a* by their redundant functions [22]. If so, the in vitro activities of Wnt-3a on neural cells may be related to the functions of these Wnt proteins in vivo.

Several signaling systems are known to play roles in the maintenance and differentiation of neural stem cells. For example, FGF2 and/or EGF are required for neurosphere formation. The components of Notch signaling are also needed for the maintenance of neural stem cells in vitro [9,10]. Thus, Wnt signaling seems to oppose these pathways in neural stem cells, although it is not currently known whether Wnt signaling interacts directly or indirectly with these pathways during neural stem cell development. Extensive examination of the expression of the *Mash* and *HES* gene families, which are activated by Notch signaling during neural development, in neural stem cells treated with Wnt proteins would reveal this question.

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References

[1] T. Edlund, T.M. Jessell, Progression from extrinsic to intrinsic signaling in cell fate specification: a view from the nervous system, Cell 96 (1999) 211–224.
[2] W. Li, C.A. Cogswell, J.J. LoTurco, Neuronal differentiation of precursors in the neocortical ventricular zone is triggered by BMP, J. Neurosci. 18 (1998) 8853–8862.
[3] R.E. Gross, M.F. Mehler, P.C. Mabie, Z. Zang, L. Santschi, J.A. Kessler, Bone morphogenetic proteins promote astroglial lineage commitment by mammalian subventricular zone progenitor cells, Neuron 17 (1996) 595–606.
[4] K. Nakashima, M. Yanagisawa, H. Arakawa, N. Kimura, T. Hisatsune, M. Kawabata, K. Miyazono, T. Taga, Synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300, Science 284 (1999) 479–482.
[5] K. Nakashima, T. Takizawa, W. Ochiai, M. Yanagisawa, T. Hisatsune, M. Nakafuku, K. Miyazono, T. Kishimoto, R. Kageyama, T. Taga, BMP2-mediated alteration in the developmental pathway of fetal mouse brain cells from neurogenesis to astrocytogenesis, Proc. Natl. Acad. Sci. USA 98 (2001) 5868–5873.
[6] G. Zhu, M.F. Mehler, J. Zhao, S. Yu Yung, J.A. Kessler, Sonic hedgehog and BMP2 exert opposing actions on proliferation and differentiation of embryonic neural progenitor cells, Dev. Biol. 215 (1999) 118–129.
[7] K. Lai, B.K. Kaspar, F.H. Gage, D.V. Schaffer, Sonic hedgehog regulates adult neural progenitor proliferation in vitro and in vivo, Nat. Neurosci. 6 (2003) 21–27.
[8] B.A. Reynolds, S. Weiss, Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system, Science 255 (1992) 1707–1710.
[9] T. Ohtsuka, M. Sakamoto, F. Guillemot, R. Kageyama, Roles of the basic helix–loop–helix genes Hes1 and Hes5 in expansion of neural stem cells of the developing brain, J. Biol. Chem. 276 (2001) 30467–30474.
[10] S. Hitoshi, T. Alexson, V. Tropepe, D. Donoviel, A.J. Elia, J.S. Nye, R.A. Conlon, T.W. Mak, A. Bernstein, D. van der Kooy, Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells, Genes Dev. 16 (2002) 846–858.
[11] M.E. Dickinson, R. Krumlauf, A.P. McMahon, Evidence for a mitogenic effect of Wnt-1 in the developing mammalian central nervous system, Development 120 (1994) 1453–1471.
[12] S.G. Megason, A.P. McMahon, A mitogen gradient of dorsal midline Wnts organizes growth in the CNS, Development 129 (2002) 2087–2098.
[13] A. Chenn, C.A. Walsh, Regulation of cerebral cortical size by control of cell cycle exit in neural precursors, Science 19 (2002) 365–369.
[14] A.P. McMahon, A. Bradley, The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain, Cell 62 (1990) 1073–1085.
[15] A.P. McMahon, A.L. Joyner, A. Bradley, J.A. McMahon, The midbrain–hindbrain phenotype of Wnt-1-/Wnt-1-mice results from stepwise deletion of engrailed-expressing cells by 9.5 days postcoitum, Cell 69 (1992) 581–595.

[16] K.R. Thomas, M.R. Capecchi, Targeted disruption of the murine int-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development, Nature 346 (1990) 847–850.
[17] S.M. Lee, S. Tole, E. Grove, A.P. McMahon, A local Wnt-3a signal is required for development of the mammalian hippocampus, Development 127 (2000) 457–467.
[18] M. Ikeya, S.M. Lee, J.E. Johnson, A.P. McMahon, S. Takada, Wnt signalling required for expansion of neural crest and CNS progenitors, Nature 389 (1997) 966–970.
[19] K. Tang, J. Yang, X. Gao, C. Wang, L. Liu, H. Kitani, T. Atsumi, N. Jing, Wnt-1 promotes neuronal differentiation and inhibits gliogenesis in P19 cells, Biochem. Biophys. Res. Commun. 293 (2002) 167–173.
[20] J. Lyu, F. Costantini, E.H. Jho, C.K. Joo, Ectopic expression of Axin blocks neuronal differentiation of embryonic carcinoma P19 cells, J. Biol. Chem. 278 (2003) 13487–13495.
[21] S. Shibamoto, K. Higano, R. Takada, F. Ito, M. Takeichi, S. Takada, Cytoskeletal reorganization by soluble Wnt-3a protein signalling, Genes Cells 3 (1998) 659–670.
[22] B.A. Parr, M.J. Shea, G. Vassileva, A.P. McMahon, Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds, Development 119 (1993) 247–261.