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Current Perspective

Control of Proliferation and Differentiation of Neural Precursor Cells: Focusing on the Developing Cerebellum

Yasuki Ishizaki[1,]*

Department of Molecular and Cellular Neurobiology, Gunma University Graduate School of Medicine, Showamachi 3-39-22, Maebashi 371-8511, Japan

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Abstract. During CNS development, multipotent neural stem cells give rise first to various kinds of specified precursor cells, which proliferate extensively before terminally differentiating into either neurons or glial cells. Control of proliferation of the precursor cells plays a crucial role in determining the number of cells in the CNS. Proliferation is driven by mitogens, but how it is terminated remains a mystery. We examined the role of p27/Kip1 (p27), a cyclin-dependent kinase inhibitor, in the control of proliferation of cerebellar granule cell precursors (GCPs). We found that there is an intracellular mechanism that stops GCP division and causes GCPs to differentiate and that p27 is part of this mechanism. It is still not clear either whether the specified precursor cells are irreversibly determined to differentiate into their particular cell types. We examined the developmental plasticity of GCPs in vitro and found that at least some GCPs are not irreversibly committed to neuronal development but can be induced to differentiate into astroglial cells by appropriate extracellular signals.

Keywords: neural stem cell, neural precursor cell, proliferation, differentiation, plasticity

Introduction

A zygote, or a fertilized egg, divides extensively to form embryonic stem cells, which then become neural stem cells (NSCs) in the nervous system (1). NSCs are then committed to become either neuronal precursor cells or glial precursor cells. Precursor cells then divide extensively before terminally differentiating into neurons or glial cells (Fig. 1). Stem cells have the capacity to divide, but they divide very slowly. By contrast, precursor cells divide rapidly and extensively, and it is generally assumed that it is the proliferation of these precursor cells, together with the programmed cell death of postmitotic neurons that fail to make effective synaptic contacts with their targets, that determines the number of neural cells in the nervous system. Proliferation is driven by mitogens, but how it is terminated remains a mystery. Neither is it known whether such neuronal or glial precursors are irreversibly committed to become neurons or glial cells, respectively, or whether they have the ability to differentiate into other cell types (neuronal precursors to glial cells or glial precursors to neurons) or even revert to NSCs. To help elucidate these problems, we examined the control of proliferation and differentiation of cerebellar granule cell precursors in the developing cerebellum.

There are five types of neurons in the cerebellar cortex: Purkinje cells, granule cells, Golgi cells, stellate cells, and basket cells. Granule cells are most abundant among these neurons. The murine cerebellum contains more than 100 million granule cells, which is more than the number of neurons in the rest of the brain combined. Granule cell precursors (GCPs) arise from the rhombic lip, the dorsal part of the neural tube at the boundary of the mesencephalon and the metencephalon. In rodents, GCPs proliferate extensively in the external germinal layer (EGL) for 2 – 3 weeks after birth, before exiting the cell cycle. Developing GCPs then exit the cell cycle, extend axons that extensively form synapses with the dendrites of Purkinje cells in the molecular layer, and migrate inward to their final destination, the granule layer (GL), where they accomplish their terminal differentiation (2, 3).

*Corresponding author. yisizaki@med.gunma-u.ac.jp
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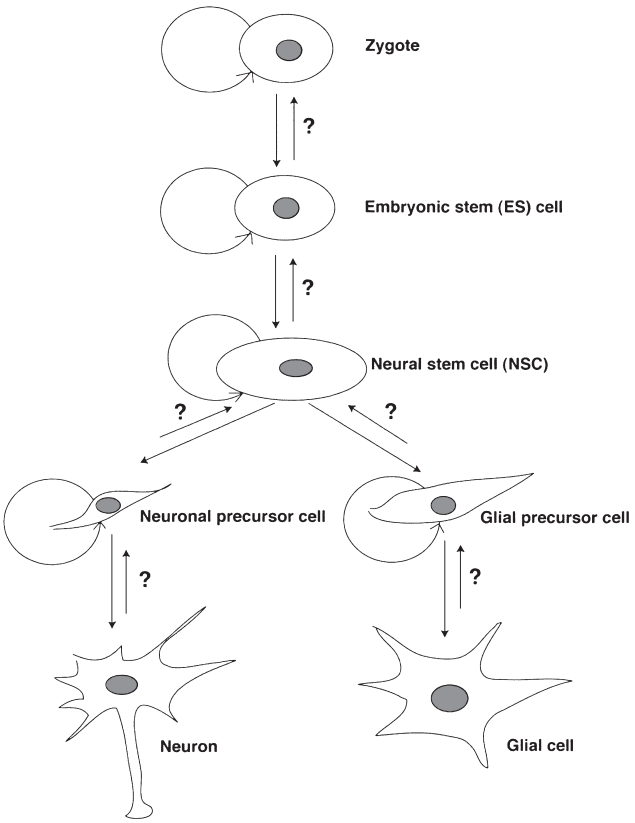

Fig. 1. Development of neural cells. A zygote divides extensively to form embryonic stem (ES) cells, which then become neural stem cells (NSCs) in the nervous system. NSCs are then committed to become either neuronal precursor cells or glial precursor cells. Precursor cells then divide extensively before terminally differentiating into neurons or glial cells. The round arrows denote proliferation of cells. The arrows pointing up suggest the possible dedifferentiation of the more restricted cell. Modified from ref. 1.

Presence of an intracellular mechanism that causes neuronal precursor cells to exit the cell cycle

First, we tried to elucidate the mechanism by which GCPs exit the cell cycle. The eucaryotic cell cycle is controlled by a family of cyclin-dependent kinases (Cdks). Cdk activity is regulated by various proteins, including cyclins that activate Cdks, kinases and phosphatases that activate or inhibit Cdks, and Cdk inhibitors that block the assembly or activity of cyclin-Cdk complexes. Two families of Cdk inihibitors have been identified in mammalian cells, the Cip-Kip family and the Ink4 family. These proteins inhibit the various cyclin-Cdk complexes that control G1 progression and entry into the S phase.

We examined the expression of various Cdk inhibitors, including p18, p21, p27, and p57, in the developing mouse cerebellum (Fig. 2). Only p21 and p27 were significantly expressed in the developing cerebellum.

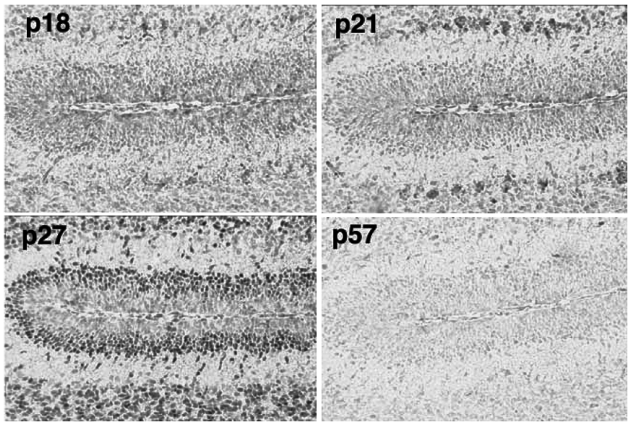

Fig. 2. The expression of Cdk inhibitors in the developing cerebellum. The cerebellum of a P7 mouse was fixed; sectioned sagittally; stained with anti-p18, anti-p21, anti-p27, and anti-p57 antibodies, followed by the incubation with a peroxidase-conjugated secondary antibody; and visualized by 3,3'-diaminobenzidine. Hematoxylin was used as a counterstain.

p21 was expressed predominantly by the Purkinje cells, but not by the cells of granule cell lineage. By contrast, p27 was expressed by the cells of granule cell lineage. It was expressed by the cells in the inner two-thirds of the EGL; those in the superficial areas expressed p27 weakly, whereas those in the deeper areas expressed p27 strongly. Cells in the molecular layer and cells in the GL also expressed p27 strongly. We double-stained the section of the developing mouse cerebellum with an anti-p27 antibody and an anti-PCNA antibody. PCNA is the proliferating cell nuclear antigen, and it is specifically expressed by the cells in the S phase of the cell cycle. PCNA was expressed by the cells in the outer two-thirds of the EGL, indicating that these cells were in the S phase. Cells in the inner third of the EGL, or in the molecular layer, or in the GL did not express PCNA, indicating that these cells were postmitotic. As p27 was expressed by the cells in the inner two-thirds of the EGL, the cells in the middle of the EGL expressed both p27 and PCNA, indicating granule cell precursors begin to accumulate p27 before they exit the cell cycle. In adult mice, granule cells in the GL and Purkinje cells expressed p27 strongly. These results suggest that GCPs begin to accumulate p27 before they exit the cell cycle and that the level of p27 expression remains high in mature granule cells in the adult cerebellum.

We then examined p27 expression by cultured GCPs prepared from cerebella of postnatal day 3 (P3), P7, P11, and P14 mice. Two hours after plating, 60% of the GCPs from P14 mice were p27-positive, whereas only 30% of the cells from P3 mice were p27-positive. The proportion of p27-positive cells increased with the time in culture: 3 days after plating, nearly all of the cells from

P11 or P14 mice, and 80% of the cells from P3 or P7 mice, strongly expressed p27. All of the p27-positive cells had neurites 3 days after plating and showed morphological features that are characteristic of mature granule cells in culture.

We also examined the proliferative capacity of GCPs prepared from developing mouse cerebella. During the first day of culture, the proportion of GCPs that incorporated BrdU was 65%, 53%, 36%, and 25% for cells from P3, P7, P11, and P14 mice, respectively. During the second day of culture, however, only 30% of GCPs from P3 mice incorporated BrdU, and the percentage was smaller than 10 for GCPs from older animals. During the fourth day of culture, only 2% of GCPs from P3 mice, and none of the GCPs from older animals, incorporated BrdU. Thus it was demonstrated that there is an inverse correlation between p27 expression and BrdU incorporation by cultured GCPs. Recently, three groups independently reported that Purkinje cell-derived Sonic hedgehog (Shh) regulates GCP proliferation in the developing cerebellum (4 – 6). Adding Shh significantly increased the proportion of GCPs that incorporated BrdU, confirming these reports. Even in the presence of Shh, however, BrdU incorporation by GCPs from P3 mice stopped on approximately the 11th day of culture. GCPs from P7 mice stopped incorporating BrdU on approximately the seventh day of culture, and GCPs from P10 mice stopped between the fourth and seventh days of culture. GCPs in vivo exit the cell cycle at around P15. Thus the normal timing of exiting of GCPs from the cell cycle was restored in vitro by Shh, suggesting that Shh is the mitogen acting in vivo for GCPs.

We examined the effect of p27 gene inactivation on the developing cerebellum. Cerebella from p27+/– mice appeared to be larger than those from p27+/+ mice and smaller than those from p27–/– mice. This result agrees well with previous reports that the p27–/– mouse has multiorgan hyperplasia (7 – 9). Examination of formalin-fixed, paraffin-embedded sections of the cerebella of P16 mice showed that only a few cells remained in the EGL in p27+/+ mice, whereas many more cells were present in the EGL in p27+/– and p27–/– mice. When we measured the areas of the EGL of the cerebella of P16 mice and the area of the GL of the cerebella of P21 mice, we found that the EGL of the P16 p27–/– mice was significantly larger than that of P16 p27+/+ mice and that the GL of the P21 p27–/– mice was significantly larger than that of P21 p27+/+ mice. These results suggest that p27 is involved in the control of GCP proliferation in vivo. When we isolated and purified GCPs for use in in vitro experiments, the number of GCPs obtained from a p27–/– mouse was always larger than that from a p27+/– or p27+/+ mouse. When cultured at a high density in serum-free medium, the percentage of proliferating (BrdU-positive) cells in the GCP preparations from p27+/– or p27–/– mice was always higher than the percentage in the GCPs from p27+/+ mice. During the period from the 24th to the 72nd h of culture, nearly 20% of the p27–/– GCPs and 10% of the p27+/– GCPs incorporated BrdU, whereas only 2.5% of the p27+/+ GCPs incorporated BrdU. During the 48 – 72-h period of the culture, nearly 10% of p27–/– cells incorporated BrdU, whereas none of the p27+/– or p27+/+ cells incorporated BrdU. During the 72 – 96-h-period of the culture, however, none of the GCPs from p27–/–, p27+/–, or p27+/+ mice incorporated BrdU, and all of the cells showed morphological features characteristic of mature granule cells. Even GCPs from the p27–/– mice ultimately exited the cell cycle and differentiated into mature granule cells, indicating that p27 is not the sole component of the stopping mechanism. In this study, we confirmed the previous finding that cyclin D1 is dismantled in mature granule cells (10, 11), so it is likely that the absence of cyclin D1, together with the presence of p27, prevents mature granule cells from reentering the cell cycle. Huard and colleagues (12) reported that the number of granule cells was reduced in cerebella from mice lacking cyclin D2 and suggested that cyclin D2 is required for GCP proliferation. It remains to be determined, however, whether dismantling of cyclin D1 or D2 is part of the mechanism that results in proliferating GCPs exiting the cell cycle. It is possible that other Cdk inhibitors perform the function of p27 in the p27–/– cells, allowing the cells to stop dividing and to differentiate. We have examined the expression of p18, p21, and p57 in GCPs from p27–/– mice in culture and in frozen sections, but we did not see the upregulation of these Cdk inhibitors.

Taken together, these results suggest that there is an intracellular mechanism that stops the precursor cell division and causes these precursor cells to differentiate and that p27 is part of this mechanism.

Developmental plasticity of neuronal precursor cells

It was previously shown that oligodendrocyte precursor cells (OPCs) can be reprogrammed by extracellular signals to resemble NSCs, which can give rise to both neurons and glial cells (13). To examine whether neuronal precursors have the potential to differentiate into glial cells, we isolated immature GCPs from P7 mice (14). We cultured the cells under various conditions and stained them with anti-GFAP (glial fibrillary acidic protein) antibodies. We focused on two signaling

molecules, Shh and bone morphogenetic proteins (BMPs), as these proteins are known to play crucial roles in the development of the cerebellum. Alder et al. (15) demonstrated that BMPs initiate the program of granule cell specification, and Angley et al. (16) provided evidence that BMPs might participate in regulating postnatal granule cell and astroglial cell differentiation. BMP2, which has been shown to induce OPCs to differentiate into astrocytes (13, 17), failed on its own to induce GCPs to express GFAP. Similarly, Shh alone did not promote differentiation into GFAP-positive cells. When we added Shh and BMP2 simultaneously, however, the number of GFAP-positive cells in the culture increased substantially, although most of the cells still differentiated into β-tubulin III-positive neurons. Although we did not examine the survival rates under the different conditions, the number of total cells within the counted areas was not significantly different between the untreated cultures and the Shh/BMP2-treated cultures. Thus the increase in proportion of GFAP-positive cells resulted from the net increase in the number of GFAP-positive cells. In the presence of a constant concentration of Shh, BMP2 increased GFAP-positive cells in a dose-dependent manner, with an effect at all concentrations greater than10 ng/ml. BMPs 4 and 7 had the same effect as BMP2. Our immature GCP preparation contained less than 1% GFAP-positive cells, when assessed at 2 h after plating, and GFAP-positive cells remained less than 1% for more than 1 week under all conditions examined except when they were cultured with Shh and BMP2. Most of these contaminating GFAP-positive cells had the typical morphological characteristics of astroglial cells and expressed brain lipid-binding protein (BLBP), which is expressed in both astrocytes and Bergmann glia in developing mouse cerebellum (18). Some of these GFAP-positive cells were probably developing Bergmann glia, as they also expressed nestin, a marker for NSCs (19). Nestin has been reported to be expressed in immature Bergmann glia in vivo (20), and we confirmed this by staining frozen sections from P7 mouse cerebellum with an antibody against nestin. In contrast to these GFAP-positive cells, the GFAP-positive cells that developed in the presence of Shh and BMP2 had a distinctive morphology (mostly bipolar with fine processes) and did not express either nestin or BLBP. Ciliary neurotrophic factor (CNTF) has been shown to induce GFAP-negative astrocyte precursor cells to differentiate into GFAP-positive astrocytes (21). In our cultures, however, CNTF did not increase the number of GFAP-positive cells in either the absence or presence of Shh. In addition, although Shh has been reported to induce the expression of BLBP in immature glial cells (6), we did not see an increase of BLBP-positive cells in our GCP cultures treated with either Shh or Shh and BMP2. When we prepared the astrocytes from P7 mouse cerebellum according to the protocol by McCarthy and de Vellis (22) and examined the effect of Shh on BrdU incorporation by these astrocytes, Shh did not stimulate their BrdU incorporation either in the presence or absence of BMP2. Taken together, these results suggest that the GFAP-positive cells induced by the combination of Shh and BMP2 did not develop from either contaminating astrocytes or astrocyte precursors or from contaminating immature Bergmann glia, but instead developed from the immature GCPs themselves. To determine if the GFAP-positive cells could have developed from contaminating NSCs, we searched for nestin-positive cells in our cultures. Except for contaminating immature Bergmann glia, which were nestin-, BLBP-, and GFAP-positive from the start of the culture, as described above, all other cells in our cultures in all conditions were nestin-negative, suggesting that contaminating NSCs were not the source of the GFAP-positive cells that developed in cultures treated with Shh and BMP2.

Simultaneous treatment with BMP2 and Shh significantly decreased the percentage of BrdU-positive cells in our purified GCP cultures compared to treatment with Shh alone. This suggested that BMP2 opposed the mitogenic activity of Shh, thereby helping to induce the terminal differentiation of GCPs into GFAP-positive cells. To test this suggestion, we first cultured GCPs with Shh alone and then added BMP2 at various times after plating. The later we added BMP2 to the Shh-treated cultures, the fewer GFAP-positive cells developed. The decrease in the percentage of GFAP-positive cells paralleled a decrease in the percentage of BrdU-positive cells, suggesting that BMP2 induced GFAP expression only in GCPs that were still proliferating. To confirm this, we used inhibitors of DNA synthesis, Ara-C or aphidicolin. Simultaneous addition of either of these reagents with BMP2 almost completely suppressed the appearance of GFAP-positive cells in GCP cultures that had been treated with Shh alone for 2 days. These reagents had no effect on cells that had already expressed GFAP. As described above, treatment of fresh GCP cultures with BMP2 alone did not increase GFAP-positive cells, even though immature GCPs had a high proliferative rate at the start of culture in the absence of any added signaling molecule (23). Thus, Shh signaling is apparently required for BMP2 to induce immature GCPs to differentiate into GFAP-positive cells.

To confirm that GFAP-expressing cells were derived from immature GCPs, we treated the cells with Shh and BMP2 for 2.5 days and then double-stained them

with antibodies against GFAP and TAG-1, a cell-surface glycoprotein expressed by some CNS neurons (24). In frozen sections, TAG-1 appeared to be transiently expressed by postmitotic GCPs, as it was seen on cells in the deeper zone of EGL but not elsewhere in the EGL or in the molecular layer or in the GL. When purified GCPs in culture were treated with Shh and BMP2, some of the GFAP-positive cells also expressed TAG-1. We further examined whether the GFAP-positive cells expressed other neuronal markers. In frozen sections of P7 mouse cerebellum, β-tubulin III, NeuN, and MAP2 were all strongly expressed by GCPs in the deeper zone of the EGL, in the molecular layer, and in the GL. As was the case with TAG-1, when purified GCPs in culture were treated with Shh and BMP2, some of the induced GFAP-positive cells also expressed these three neuronal markers. In these immunostaining studies, we used mouse monoclonal antibodies against the neuronal markers and rabbit antibodies against GFAP. When we stained the cells with rabbit anti-MAP2 antibodies and a mouse monoclonal anti-GFAP antibody, the results were the same. We searched for the cells that co-expressed neuronal and astroglial markers in our cultures in all conditions and found these cells only in the cultures treated with both Shh and BMP2; even in these cultures, such cells were only seen in a narrow window, between 2 and 7 days. These results strongly suggest that the induced GFAP-positive cells developed from immature GCPs.

To confirm that the induced GFAP-positive cells went on to become bona fide astroglial cells, we continued the cultures for 7.5 days before staining them for other astroglial markers. The induced GFAP-positive cells cultured in Shh and BMP2 for 7.5 days had longer processes and stained more strongly for GFAP than did the induced cells after only 2.5 days of culture. Furthermore, some of them also expressed S100-β, a marker for differentiated astroglial cells. None of GFAP-positive cells at either time point expressed BLBP. By 7.5 days, all of the induced GFAP-positive cells had lost the expression of all of the neuronal markers. Taken together, these results suggest that immature GCPs treated with Shh and BMP2 differentiate first into the GFAP-positive, neuronal marker-positive cells and then finally into GFAP-positive, S100-β-positive, neuronal marker-negative, differentiated astroglial cells (Fig. 3). There is a possibility that the GFAP-positive cells that develop in response to BMPs and Shh are actually NSCs rather than bona fide astroglial cells. There is increasing evidence that at least some NSCs express GFAP (25, 26). Furthermore, Kondo and Raff (13) reported that OPCs can be reprogrammed by extracellular signals to resemble

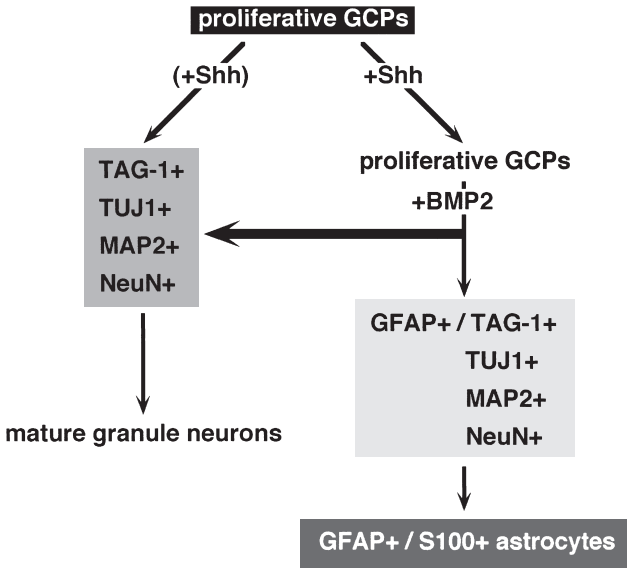

Fig. 3. The developmental plasticity of GCPs. GCPs from the developing cerebellum normally differentiate into neurons [expressing TAG1, TUJ1 (β-tubulin III), MAP2, and NeuN, neuronal markers]. When they are stimulated with Shh and BMP2, however, some of them transiently express both neuron-specific and astrocyte-specific markers, and they finally differentiate into mature astrocytes (expressing S100-β and GFAP, astrocytic markers).

NSCs, and the first signals required for reprogramming include BMPs. We think it unlikely, however, that the GFAP-positive cells induced by BMPs and Shh in our cultures are NSCs for the following reasons. First, when we remove Shh and BMP2 from our cultures and add basic fibroblast growth factor and/or epidermal growth factor to promote NSC proliferation (1), the GFAP-positive cells do not divide or incorporate BrdU. Second, when we remove Shh and BMP2 and add both platelet-derived growth factor and thyroid hormone to promote oligodendrocyte differentiation of NSCs (13, 27), we do not see oligodendrocytes developing in our cultures. Third, some of the GFAP-positive cells induced in our cultures also expressed S100-β, a marker of differentiated astroglial cells.

Together, these results strongly suggest that the GFAP-positive cells are astroglial cells rather than NSCs. To our knowledge, this is the first report to show that neuronal precursor cells can differentiate into glial cells and are therefore not irreversibly committed to differentiate into neurons.

Concluding remarks

Although NSCs have been intensely studied by those interested in either developmental neurobiology or regenerative medicine, the detailed pathways by which NSCs give rise to neurons, astrocytes, or oligodendro-

cytes still remain uncertain. We revealed that neuronal precursor cells have a built-in program that causes their exit from the cell cycle. We also revealed that neuronal precursor cells still have the developmental plasticity to re-differentiate into astrocytes. Recently the list of examples has been expanding where precursor cells, or even differentiated cells, can be diverted from their expected developmental fate by extracellular signals (28). Adult NSCs are rare and divide very slowly, while precursor cells are relatively abundant and divide rapidly. If we can make these precursor cells revert to NSCs, these cells could be exploited as powerful tools of regenerative medicine for CNS injuries and disorders.

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