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CONTROL OF GRANULE CELL PRECURSOR PROLIFERATION IN THE DEVELOPING CEREBELLUM AND IN MEDULLOBLASTOMA

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The cerebellum is essential for the fine motor control of movement and posture. Due to its apparent simplicity and geometrical arrangement, the cerebellum provides an excellent model for studying the mechanisms that control development of the central nervous system (CNS). The cerebellar cortex is formed from two distinct proliferative zones: one ventricular and one superficial called the external granule layer (EGL). Massive clonal expansion of granule cell precursors (GCPs) occurs in the EGL, and ultimately generates by far the most abundant neuronal population in the CNS. In this review, I describe recent advances in understanding the control of GCP proliferation in the developing cerebellum. I also briefly review the uncontrolled GCP proliferation associated with medulloblastoma, the most common brain malignancy in children.
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Key words: brain tumor, cell migration, sonic hedgehog, transcription factor

INTRODUCTION

The cerebellum plays an important role in motor coordination and learning. Moreover, due to its apparent simplicity and geometrical arrangement, the cerebellum provides a useful model system for studies aimed at understanding how the development of the central nervous system (CNS) is controlled. The mature cerebellar cortex consists of three layers: the molecular layer, the Purkinje cell layer, and the granule cell layer (Fig. 1). There are thousands of synaptic connections between granule cell axons and Purkinje dendrites in the molecular layer. The cell bodies of the granule cells lie in the granule cell layer, and the Purkinje cell layer lies between the molecular and granule layers.

Granule cells have a characteristic developmental pattern: in contrast to most other neurons, which are born near ventricles and migrate outward toward the surface of the brain, granule cell precursors (GCPs) are generated outside the cerebellum and migrate inward. GCPs are generated in the rhombic lip and migrate tangentially onto the surface of the cerebellar primordium to form the external granule layer (EGL), from which the postmitotic granule cells later migrate inward to form the inner granule layer (IGL) (1,2). The massive clonal expansion of the GCPs begins postnatally in the EGL, and leads to the generation of by far the most abundant neuronal population of the CNS. The mature cerebellar cortex is established during the first three weeks after birth in mice.

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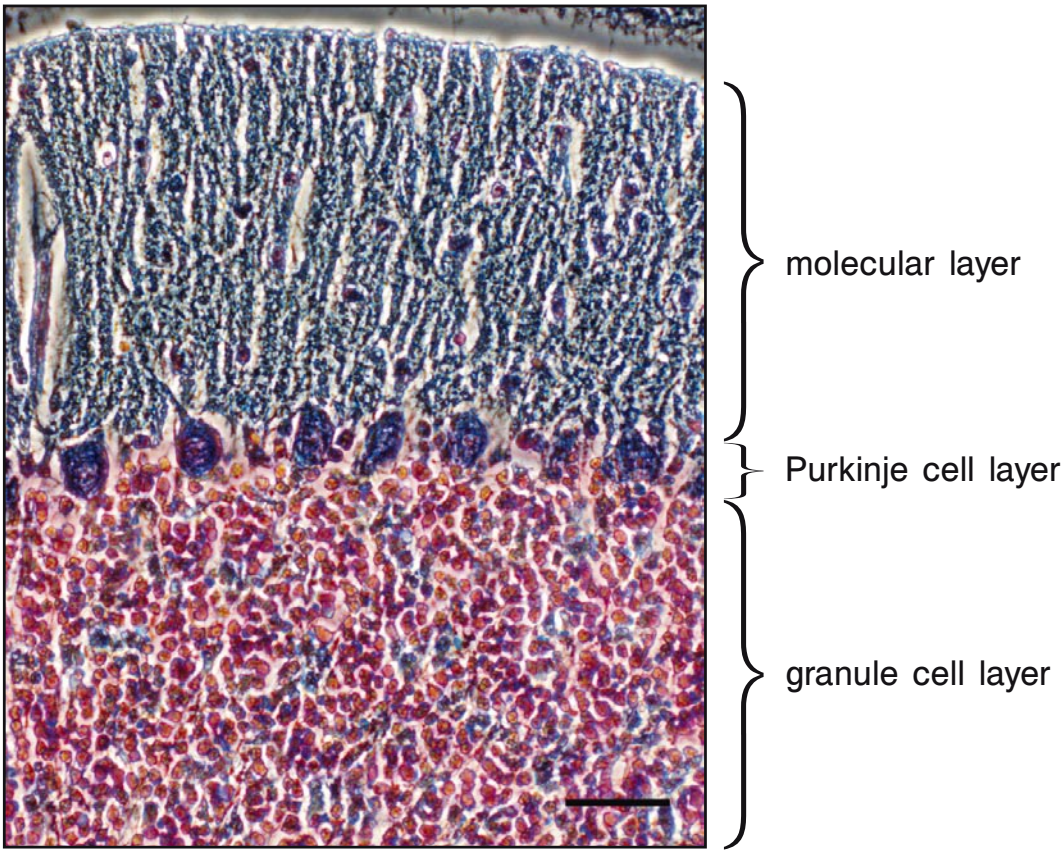

Figure 1. *The three layers of the cerebellar cortex in the adult mouse brain. Scale bar, 50 μm.*

In this review, I focus on the regulation of GCP proliferation in the EGL. Since medulloblastoma (MB), the most common and aggressive brain malignancy in childhood, is believed to arise from GCPs, I will also briefly review recent progress in MB research from the viewpoint of GCP proliferation control.

CONTROL OF GCP PROLIFERATION BY SONIC HEDGEHOG

Studies of mutant mice such as *Lurcher* and *Staggerer* revealed the importance of Purkinje cells in the regulation of GCP proliferation (3,4). Later, cell ablation experiments with transgenic mice in which diphtheria toxin was specifically expressed in Purkinje cells by the L7 promoter, clearly indicated that granule cell proliferation depends on the Purkinje cells (5), as these transgenic mice had less GCP proliferation in the EGL than wild type mice. Subsequently, a role for Sonic hedgehog (Shh), which is secreted from Purkinje cells, in regulating cerebellar growth and development was suggested from studies of Patched (Ptc) antagonizing the Shh signal (6-8). In fact, when GCPs were treated with Shh to block Ptc activity, GCP differentiation was prevented and proliferation stimulated. In contrast, GCP proliferation was dramatically reduced by using anti-Shh antibodies to block Shh function *in vivo* (9-11).

Shh is a secreted glycoprotein that plays a key role in the patterning of various tissues, including the nervous system and the limbs (12,13). Shh binds with high affinity to Ptc, a transmembrane protein at the cell surface (Fig. 2). Vertebrates have two Ptc isoforms, encoded by *Ptc1* and *Ptc2*, and Ptc1 appears to be active in the CNS (7,14-18). Ptc inhibits another transmembrane protein - Smoothened (Smo). The Shh signal is initiated by the binding of Shh to Ptc, which relieves Ptc's inhibition of Smo. The signal relayed by Smo leads to the activation of the Gli zinc-finger transcription factors Gli1, Gli2, and Gli3 (19-24). It is unclear whether the Gli proteins mediate all the aspects of SHH signaling during vertebrate CNS development. However, recently, Bai *et al* (25) demonstrated, using *Gli* knockout and *Gli-lacZ* knock-in mice, that at least

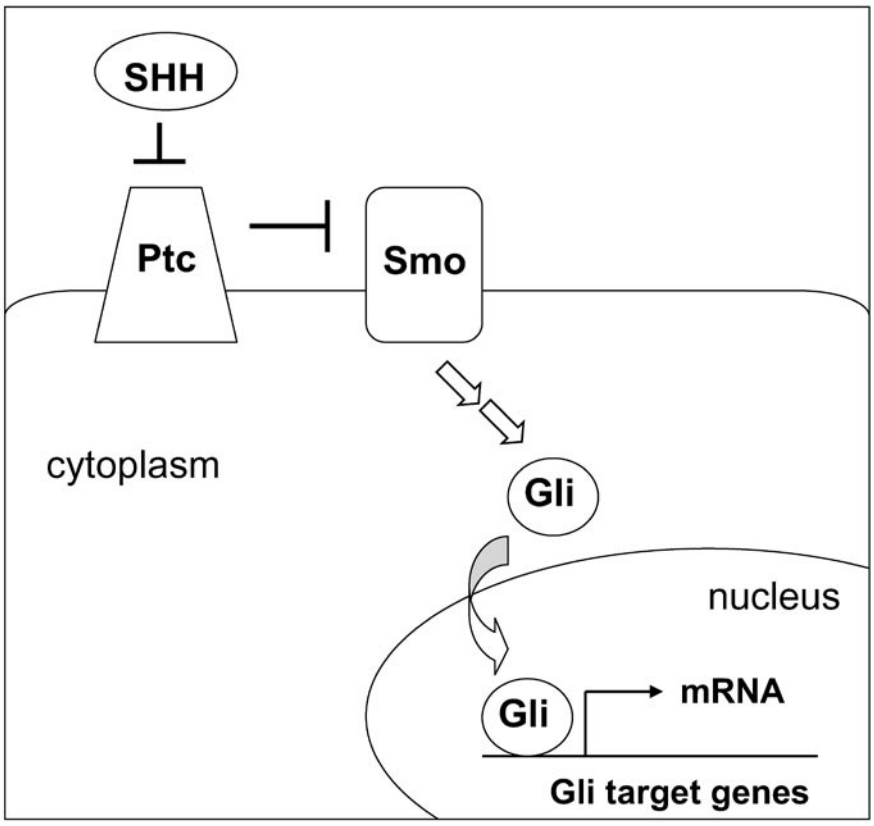

Figure 2. *The Shh signaling pathway. In the absence of Shh, the Ptc transmembrane protein represses the activity of the transmembrane protein Smo. The binding of Shh to Ptc relieves the repression, leading to the activation of the Gli transcription factors, which in turn regulate the transcription of target genes in the nucleus.*

in the spinal cord, all the Shh signaling is dependent on Gli function. Furthermore, the transcription of *Gli1* is dependent on both Gli2 and Gli3 in Shh signaling. Corrales *et al* (26) also analyzed *Gli* knockout and *Gli-lacZ* knock-in mice and showed that positive Shh signaling through Gli2 is required to generate a sufficient number of cerebellar GCPs for normal lobe growth. All the molecules involved in Shh signaling, Ptc, Smo and Gli1-3, appear to be expressed in GCPs (9,11).

OTHER FACTORS INVOLVED IN GCP PROLIFERATION

Although the Shh signaling pathway is crucial for the regulation of GCP proliferation, other factors are also involved in its control. Math1, a basic helix-loop-helix (bHLH) transcription factor, is expressed in the rhombic lip from the beginning of GCP proliferation (27,28). After the tangential movement of GCPs to the EGL, Math1 is selectively expressed in the proliferating GCPs, and not in postmitotic granule cells (27, 29). Strikingly, *Math1*-deficient mice fail to generate granule cells and are born with a cerebellum that is devoid of the EGL (30), indicating the essential role of Math1 in the genesis of granule cells. To date, however, little has been learned about how Math1 expression is regulated or what genes are regulated by Math1. In addition, the Zic zinc-finger transcription factors Zic1 and Zic2 have been shown to mark granule cells in the developing cerebellum (31-33). In *Zic1*-deficient mice, the number of dividing cells in the EGL is significantly reduced (34), implying a regulatory role for Zic1 in GCP proliferation. The same loss-of-function strategy cannot be employed in the case of Zic2, because *Zic2*- knockdown mutant mice die shortly after birth (35). However, both Zic1 and Zic2 appear to have similar functions in the regulation of cerebellar development (33,36).

The chemokine stromal cell-derived factor (SDF-1α) is highly expressed in the pia mater of the developing cerebellum, and its receptor CXCR4 is expressed in the EGL during embryonic development (37-39; also see Lu *et al* on pages 77-81 of this volume). The targeted deletion of murine SDF-1α or CXCR4 results in a premature migration of GCPs out of the

EGL, suggesting a role for SDF-1α-CXCR4 signaling in the retention of GCPs in the EGL (37,40). Furthermore, SDF-1α has been shown to enhance Shh-induced GCP proliferation (41) and to control the axon elongation of cultured cerebellar granule neurons (42). It is well known that increased protein kinase A (PKA) activity antagonizes the mitogenic effect of Shh signaling on GCPs (43-45). However, the relationship between PKA and Shh signaling is unclear. Bone morphogenic proteins, which are members of the transforming growth factor β superfamily, are also reported to inhibit the Shh-mediated proliferation of GCPs, through Smad5 signaling (46).

The Notch signal is a key regulator of cell-fate decisions in species ranging from *Drosophila* to humans (47-49). Murine Notch2 is highly expressed in proliferating GCPs in the EGL, and it is down-regulated in the postmitotic granule neurons in the IGL (50). The expression pattern of Notch2 makes it a marker for dividing GCPs residing in the EGL, in addition to other markers, such as Math1 and Zic. Activation of Notch2 signaling either by adding a soluble form of the Notch ligand Jagged1 or by overexpressing an active form of Notch2 inhibits the differentiation of GCPs by maintaining their proliferative state (50), suggesting an involvement of Notch in the control of GCP proliferation. In addition, the Shh and Notch2 pathways appear to regulate granule cell development through at least some common targets, such as the bHLH transcription factor Hes1 (50).

HOW IS GCP PROLIFERATION TERMINATED?

The EGL is composed of two distinct zones: the outer EGL (oEGL), where GCPs actively proliferate, and the inner EGL (iEGL), which contains the postmitotic and pre-migratory granule cells. To form the internal granule layer (IGL), the proliferating GCPs must undergo several events: cessation of the cell-cycle regulation, movement from the oEGL to the iEGL, differentiation into granule cells, and migration to the final destination (1,2).

Several components of the extracellular matrix (ECM) are instrumental in regulating GCP proliferation and differentiation. Laminin, an ECM glycoprotein, and its receptors (α6 and α7 integrins) are primarily expressed in the oEGL, whereas another ECM glycoprotein vitronectin and its receptors (αv integrins) are highly expressed in the iEGL (51,52). This sharp contrast in localization raised the possibility that laminin and vitronectin regulate distinct aspects of granule cell development. Indeed, laminin has been shown to enhance significantly the Shh-mediated GCP proliferation in primary cultures (51,52). A targeted disruption study provided evidence that α6β1 and α7β1 integrins are crucial for GCP proliferation (52). Conversely, vitronectin can overcome the Shh-induced proliferation. Vitronectin phosphorylates and activates the transcription factor CREB (51), a major target of PKA that antagonizes the mitogenic activity of Shh. Furthermore, the overexpression of CREB was found to be sufficient to induce the neuronal differentiation of granule cells, even in the presence of Shh (51). Thus, when GCPs move to a vitronectin-rich environment such as the iEGL, the vitronectin could function to terminate their proliferation through the PKA-CREB pathway, and to start their differentiation. The cyclin-dependent kinase (Cdk) inhibitor p27/Kip1 has been suggested to possess a similar function along with vitronectin in the regulation of GCP proliferation (53). p27/Kip1 is strongly expressed in the iEGL, and weakly in the oEGL. In addition to this inverse correlation of p27/Kip1 expression with GCP proliferation activity, GCPs in *p27/Kip1*-deficient mice show enhanced proliferation compared with the GCPs from wild-type mice. Therefore, p27/Kip1 is likely to play a role in GCP differentiation by switching off the proliferation program (53).

MEDULLOBLASTOMA

MB is the most common brain malignancy in children. Recent studies have highlighted a deregulation of Shh signaling pathways in MB.

Basal cell nevus syndrome (BCNS), also known as Gorlin's syndrome, is an autosomal dominant disorder characterized by high rates of both basal cell carcinoma and MB. The identification of human *Ptc1* mutations in BCNS patients raised the possibility that MB is associated with uncontrolled Shh-Ptc signaling (54,55). This causal link was confirmed by the development of MB in mice heterozygous for *Ptc1* mutation (56). *Ptc1*-null mice cannot be used as a model system, because they die during embryogenesis (56). Considering the inhibitory activity of Ptc in the Shh signaling pathways, it is easy to imagine that the mutation or deletion of *Ptc* causes the constitutive activation of Shh signaling. In addition, *Ptc1* itself is target of Gli proteins, which are crucial mediators in Shh signaling. Thus, the negative feedback of the Shh pathway is also impaired in animals lacking Ptc function. However, because only 15%-20% of $Ptc1^{+/-}$ mice develop MB and the wild-type *Ptc1* allele is expressed in the majority of tumors, additional genetic alterations are likely to be required for the full development of MB (see below; 57-59).

The most frequently affected genomic region in human MB (30%-50%) is chromosome 17p (60,61). However, none of the Shh signaling genes, such as *Shh*, *Ptc1*, or *Gli1-3*, is located in the 17p region, and *Ptc* mutations account for a

minority of the cases, approximately 10% (57,59). Therefore, the culprit gene(s) in this region is unknown. Recently, Gulino *et al* identified a candidate gene, *REN*, which maps to 17p13.2 (62). *REN* was originally identified as a novel gene involved in neural progenitor cell differentiation (63). The allelic deletion of *REN* occurs in 39% of sporadic human MB cases (62). REN is expressed at higher levels in the non-proliferative cerebellar layers (iEGL and IGL) than in the proliferative oEGL. REN overexpression promotes the growth arrest of GCPs and increases the proportion of GCPs expressing the Cdk inhibitor p27/Kip1 (64). Furthermore, REN antagonizes the Gli-mediated transactivation of Shh target genes (62,64). Collectively, the tumor suppressor REN appears to function to restrain the Shh-sustained proliferation of GCPs from the oEGL to the iEGL, and the loss of REN could release the restraint on Shh pathways, leading to MB.

CONCLUSION

Recently, much progress has been made in understanding the control of GCP proliferation in the developing cerebellum. In particular, Shh signaling undoubtedly plays key roles in the regulation of GCP proliferation. A more precise understanding of the Shh pathways, especially the intracellular signaling that regulates them, will yield important insights into the mechanisms that control development of the mammalian CNS, and will provide more target molecules for therapies to treat MB.

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