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To cite this article: Duancheng Guo, Yanghui Qu, Yijun Yang & Zeng-Jie Yang (2020) Medulloblastoma cells resemble neuronal progenitors in their differentiation, Molecular & Cellular Oncology, 7:6, 1810514, DOI: [10.1080/23723556.2020.1810514](https://doi.org/10.1080/23723556.2020.1810514)

To link to this article: <https://doi.org/10.1080/23723556.2020.1810514>



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Published online: 05 Sep 2020.



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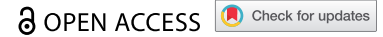


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## Medulloblastoma cells resemble neuronal progenitors in their differentiation

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### ABSTRACT

Medulloblastoma (MB) often originate from cerebellar granule neuron precursors (GNPs). We recently found that medulloblastoma cells undergo differentiation as GNPs. Differentiated MB cells have permanently lost their proliferative capacity and tumorigenicity. The differentiation of MB cells is driven by the transcription factor NeuroD1 (Neurogenic differentiation 1), and NeuroD1 expression in MB cells is repressed by EZH2-mediated H3K27me3.

### ARTICLE HISTORY

Received 10 July 2020  
Revised 10 August 2020  
Accepted 11 August 2020

### KEYWORDS

Medulloblastoma; hedgehog pathway; differentiation; NeuroD1; EZH2

Cerebellar granule neuron precursors (GNPs) represent the most abundant neuronal progenitors in mammalian brains. After birth, GNPs proliferate extensively in the outer part of the external germinal layer in the cerebellum in mice. GNPs then start to migrate inwards and differentiate, expressing *contactin-2* (*Cntn2*), encoding a cell surface glycoprotein called Tag1.<sup>1</sup> Differentiated granule neurons are polarized as they possess with axons and dendrites (Figure 1). The proliferation of cerebellar GNPs is supported by the hedgehog (Hh) signaling, whereas aberrant activation of Hh pathway in GNPs leads to the formation of medulloblastoma, which is the most common malignant brain tumor in children.

Human medulloblastoma (MB) consists of at least four subgroups: Hh, Wnt, group 3, and group 4.<sup>2</sup> Hh-MB accounts for approximately 30% of human MB cases. The most frequently mutated gene in Hh-MB is *Patched1* (*Ptch1*), encoding the antagonizing receptor of Hh pathway. Mutations of *Ptch1* activates Smoothened (*Smo*) that accumulates at the primary cilium. Activated *Smo* initiates the Hh signaling cascade, resulting in the transcription of target genes including *Gli1* and *Gli2*. *Ptch1* deletion in cerebellar GNPs resulted in the formation of Hh-MB in mice with 100% penetrance,<sup>3</sup> indicating that Hh-MB can originate from cerebellar GNPs. Current therapy regimens including surgery, chemotherapy and cranio-spinal radiotherapy, may cure 70–80% of patients with MB. However, MB survivors often suffer long-term side effects from aggressive tumor treatment. Therefore, improved and less toxic approaches are urgently needed to treat this devastating disease.

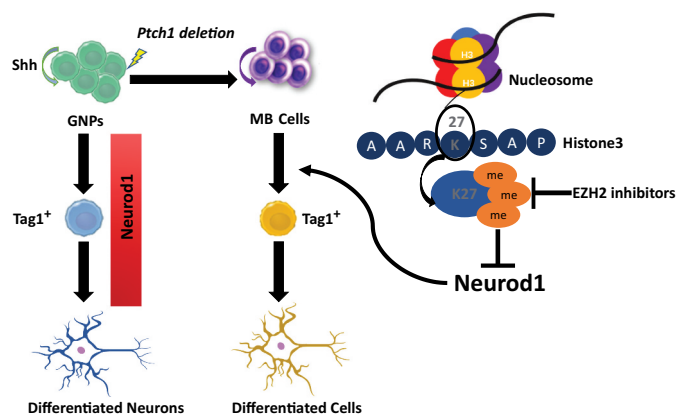
Recently our group revealed that a proportion of tumor cells in human and mouse Hh-MB became differentiated based on single cell RNA sequencing.<sup>4</sup> Those differentiated cells highly expressed genes associated with neuronal maturation and differentiation including *Cntn2*, *Pax6*, *NeuroD1*, *Tubb3*, and *Gap43*. Immunohistochemistry revealed that differentiated MB cells were exclusively negative for Ki67, suggesting that those tumor cells have stopped dividing after becoming differentiated. We purified differentiated MB cells by FACs using an

antibody against Tag1 and found that differentiated MB cells exhibited polarized morphology with long processes (Figure 1). Tag1+ MB cells failed to develop into tumors in *CB17/SCID* mice after intracranial transplantation, suggesting that differentiated tumor cells are not tumorigenic. By RNA sequencing, we demonstrated that differentiated MB cells highly resembled differentiated granule neurons in developing cerebella, in their genetic profiles. These findings demonstrate that MB cells undergo differentiation in a similar manner with cerebellar GNPs. More important, differentiated MB cells have permanently lost their tumorigenicity, implying that MB can be treated by inducing tumor cell differentiation.

Repressed expression of *Gli1* and *Gli2*, was found in differentiated MB cells and granule neurons, suggesting that Hh signaling was compromised following the differentiation. In our studies, MB cells from *Ptch1*-mutant mice can undergo to differentiate despite of *Ptch1* deficiency. Hh pathway can not be activated in differentiated MB cells after forced expression of *Smoothened* (*Smo*) or *Gli1*, downstream of *Ptch1* in Hh pathway. These findings indicate that the differentiation overrides Hh signaling in MB cells.

NeuroD1, a helix-loop-helix transcription factor, is found to be important for the differentiation of cerebellar GNPs. Deletion of *NeuroD1* in cerebellar GNPs impaired their differentiation and enhanced their proliferation *in vivo*.<sup>5</sup> Upregulation of *NeuroD1* transcription was observed in differentiated MB cells, prompting us to further investigate the possible role of NeuroD1 in MB cell differentiation. Forced expression of *NeuroD1* in MB cells repressed tumor cell proliferation and stimulated them to differentiate *in vitro*. More important, no tumors were generated from *NeuroD1*-overexpressed MB cells in *CB17/SCID* mice after the transplantation. In addition, Hh signaling was suppressed in MB cells after *NeuroD1* overexpression. These data suggest that NeuroD1 induces tumor cell differentiation in MB.

Previous studies suggest that NeuroD1 expression in MB cells may be epigenetically regulated by DNA methylation.<sup>6</sup>



**Figure 1.** Medulloblastoma (MB) cells differentiate similarly to cerebellar granule neuron precursors (GNPs). During normal development, the proliferation of cerebellar GNPs is supported by sonic hedgehog (Shh) signaling. After completion of the initial expansion, GNPs express the differentiation marker, Tag1 and start to differentiate. Deletion of *Patched1* (*Ptch1*, the antagonizing receptor of the Shh ligand) in GNPs causes overactivation of Shh pathway, leading to MB formation in the cerebellum. MB cells undergo spontaneous differentiation and express Tag1, thereby losing the capacity to proliferate or form tumors. The transcription factor NeuroD1 drives the differentiation of MB cells as well as cerebellar GNPs. In MB cells, NeuroD1 expression is suppressed by trimethylation of histone 3 lysine-27 (H3K27me3). Inhibition of EZH2 prevents the H3K27me3 in tumor cells, resulting in the upregulation of NeuroD1 expression and enhanced differentiation in MB cells.

However, no difference in the NeuroD1 methylation was observed between differentiated MB cells and bulk tumor cells (Tag1-).<sup>4</sup> By ChIP-PCR experiments, we found that the regulatory region of NeuroD1 was more enriched with histone trimethylation of H3K27 in differentiated MB cells, compared with that in bulk MB cells, suggesting that NeuroD1 expression in bulk tumor cells may be repressed by trimethylation of histone 3 lysine-27 (H3K27me3). EZH2 is a catalytic subunit of PRC2 complex, mediating the histone trimethylation of H3K27.<sup>7</sup> As expected, expression levels of *EZH2* were significantly elevated in bulk MB cells compared with differentiated MB cells. Moreover, EZH2 inhibitors including GSK126, UNC1999, and EPZ6438 stimulated NeuroD1 expression in MB cells *in vitro*. All these data suggest that NeuroD1 expression in MB cells is suppressed by EZH2-mediated H3K27me3.

Finally, we evaluated the efficacies of EZH2 inhibitors including GSK126, UNC1999, and EPZ6438 in repressing MB cell proliferation and *in vivo* growth of MB. Following treatment with EZH2 inhibitors, MB cells underwent extensive differentiation, and the proliferation of tumor cells was substantially repressed. The growth of MB cells was also significantly suppressed by treatment of EZH2 inhibitors. Moreover, EZH2 inhibitors also suppressed the proliferation of MB cells with forced expression of SmoA1, a constitutively activated form of Smo, which mediated the cell resistance to vismodegib, a FDA-approved Smo antagonist.<sup>8</sup> These data suggest that EZH2 inhibition represents a promising avenue to treat MB through inducing tumor cell differentiation.

Our studies demonstrate that MB cells retain their differentiation capacity. In addition, our studies reveal that NeuroD1 drives the differentiation of both MB cells and GNPs, and NeuroD1 expression in Hh-MB cells is regulated by EZH2-mediated H3K27me3. Different with their role in Hh-MB, EZH2 and NeuroD1 were recently found to be required for

the tumorigenesis of group 3 MB, suggesting that functions of EZH2 and NeuroD1 in MB cells appear to be subgroup-dependent.<sup>9,10</sup> Further studies are warranted to investigate the target genes of NeuroD1 and EZH2 in different groups of MB.

## Disclosure of potential conflict of interest

No potential conflicts of interest were disclosed.

## Funding

This work was supported by the American Brain Tumor Association [Discovery Award]; American Cancer Society [RSG1605301NEC]; PA Cure Health Research Fund [CURE 4100068716].

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