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In Search of the Medulloblast: Neural Stem Cells and Embryonal Brain Tumors

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Conceptual links between the malignant cerebellar brain tumor medulloblastoma and neural stem cells extend back to the first report on this neoplasm by Bailey and Cushing in 1925 [1]. In their histogenetic classification scheme, brain tumors were named based on their morphologic resemblance to embryonic or adult neural cell types. These investigators thought that the medulloblast was similar to the ''indeterminate cell of Schapfer,'' which was undifferentiated but had the potential for self-renewal and differentiation into neurons or glia [2]. This correlates nicely with the cellular phenotype of medulloblastoma—a tumor composed of primitive ''embryonal'' cells that retain the ability to produce glia and neurons. Although the ''medulloblast'' as a cell type has not stood the test of time, the name medulloblastoma persists. Today, the scientific community continues to grapple with numerous questions regarding the relation between medulloblastoma and neural stem cells. Do these tumors truly arise from stem cells, or can better differentiated precursor cells (or even fully differentiated cells) also give rise to medulloblastoma? Do the various medulloblastoma subtypes derive from distinct germinal epithelia? To what degree are some or all cells within a medulloblastoma stem-like? If heterogeneity exists within medulloblastomas with respect to a stem-like phenotype, do these features correlate with the ability to propagate lesions in vivo? Similar issues can be raised concerning other primitive-appearing embryonal brain neoplasms, such as the atypical teratoid/rhabdoid tumor (AT/RT), ependymoblastoma, and supratentorial primitive neuroectodermal tumor (sPNET). Although the answers to these questions are not yet clear, the advent of improved molecular markers and novel murine transgenic models has begun to shed light on this complex subject.

Stem and precursor cells in cerebellar development

Before one can determine if medulloblastoma arises from stem cells, it is necessary to understand how stem cells contribute to the development of the brain region from which medulloblastoma forms—the cerebellum. Many excellent reviews of the cellular and molecular basis of cerebellar development have recently been published [3–6]. To briefly summarize, fate mapping experiments in chimeras as well as in studies using retroviral infections to mark specific stem cell populations indicate that most cell types in the cerebellum derive from the ventricular zone (VZ) germinal neuroepithelium. The VZ extends along the entire fetal ventricular system (Fig. 1A, B). The cerebellar anlage at this early stage (gestational week 6) is a cellular oval body bulging into the fourth ventricle just caudal to the midbrain, with VZ cells forming its inner aspect (see Fig. 1B). Cells born in the cerebellar VZ during fetal development migrate outward to form all glia as well as Purkinje, Golgi, basket, and stellate cells.

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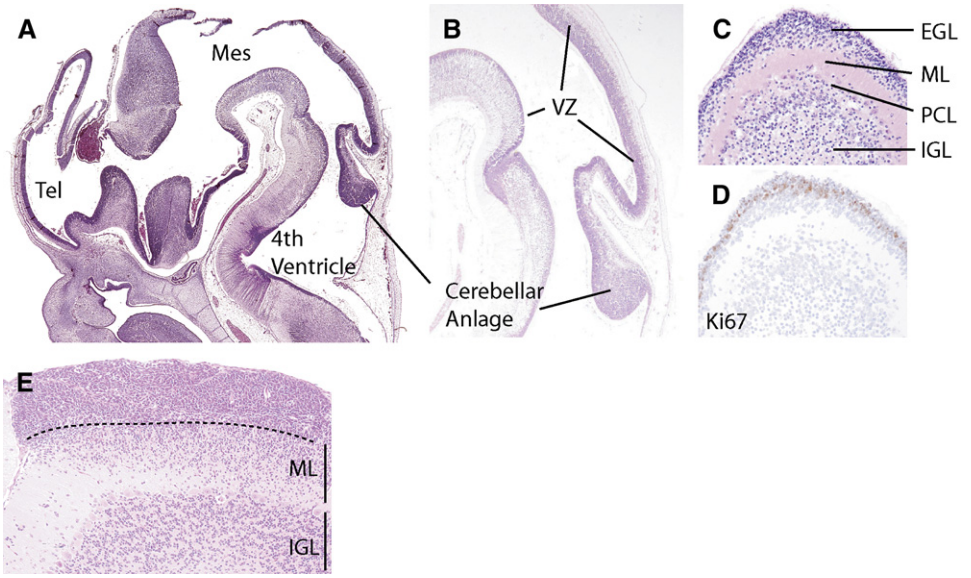

Fig. 1. Stem or progenitor cell populations in the brain. (*A, B*) Sagittal section through the head of a 6-week-old human fetus shows the developing telencephalon (Tel), mesencephalon (Mes), and cerebellar anlage. A dense stripe of proliferating stem or progenitor cells lines the developing ventricular system, forming the VZ germinal epithelium. (*C*) Cerebellar folia from a third-trimester human fetus show the external germinal layer (EGL) below the pial surface membrane and the subjacent molecular layer (ML), Purkinje cell layer (PCL), and internal granule cell layer (IGL). (*D*) Ki67 proliferation marker demonstrates that cycling cells are localized to the outer EGL. (*E*) Medulloblastoma in a PTCH+/− mouse spreads predominantly along the surface of the cerebellum, with the outer limit of the ML highlighted by a dashed line. Tumor cells on the right side of the image have begun to infiltrate inward toward the IGL.

The cerebellum, unlike the rest of the brain, contains a second germinal epithelium on its surface during late fetal and early postnatal life: the external germinal layer (EGL). The EGL is formed by precursors in the rhombic lip that migrate over the surface of the brain below the pial meninges (Fig. 1C). It is believed that the EGL gives rise only to granule neurons in vivo. By definition, stem cells must self-renew and generate multiple cell types as progeny. Because EGL cells are thought to be unipotent, they are generally referred to as granule cell precursors rather than stem cells. In human beings, most of the proliferative activity in the EGL occurs in utero, and although the layer is present for up to 1 year after birth, it is largely quiescent [7]. In contrast, the murine EGL proliferates maximally during the first 2 weeks after birth, making it an ideal system for experimental study. Proliferation occurs in the outer aspects of the EGL, as highlighted by Ki67 immunostaining in Fig. 1D. After several rounds of division, granule cell precursors in the EGL exit the cell cycle and migrate laterally for a short distance before moving inward along radial (Bergmann) glial fibers past the Purkinje cell layer to take up residence in the internal granule cell layer (IGL).

Understanding the cellular and molecular basis of neural stem cell migration may help to control the dissemination of embryonal brain tumors as the avenues by which medulloblastoma spread often recapitulates the migratory patterns of rhombic lip cells and granule neuron precursors. For example, leptomeningeal spread of human medulloblastomas along the surface of the cerebellum is quite common [8]. Mouse models of medulloblastoma also often first spread along the surface of the brain before invading the parenchyma (Fig. 1E and personal observations). Even this inward migration can parallel normal development, because one can sometimes observe movement of medulloblastoma cells from the pial surface down Bergmann glial fibers in human and murine tumors. The same molecular pathways controlling migration of nonneoplastic stem or precursor cells may also play similar roles in medulloblastoma invasion. Some of these migratory signals are likely noncell autonomous, because leptomeningeal cells can secrete factors that promote the migration of granule cell precursors [9].

Interestingly, meningothelial cells also seem to promote survival of human medulloblastoma cells grown in vitro [10].

The molecular and biologic understanding of cerebellar stem and progenitor cells continues to grow, and, in some cases, to change. For example, two groups have recently defined novel stem cell populations arising from the rhombic lip [11,12]. Fink and colleagues [11] used expression of the markers Pax6, Tbr2, and Tbr1 to identify and visualize a population of cells migrating from this region that ultimately give rise to neurons of the deep cerebellar nuclei. Wang and coworkers [12] showed that such cells are also defined by expression of Math1 and do, in fact, depend on this transcription factor. These studies are especially noteworthy, because it was previously believed that the deep cerebellar nuclei were derived not from the rhombic lip but from the VZ. Such distinctions may ultimately prove critical for targeting embryonal brain tumors in a rational fashion, because neoplasms arising from a given neural stem or progenitor cell may still require some of the molecular pathways that control the "normal" developmental program of that specific cell type (Table 1).

The concept that multiple stem cell populations with distinct molecular signatures and functional requirements exist in the cerebellum is perhaps best illustrated in a recent study conducted by the Lee and colleagues [13] of the Wechsler-Reya group. They found that although most of the cells isolated from postnatal cerebellum were CD133-negative granule cell precursors, a much smaller population of CD133-expressing stem-like cells was also present, located predominantly in the white matter. Unlike granule cell precursors, which critically depend on Hedgehog, this CD133-positive stem cell population is Hedgehog insensitive but responsive to basic fibroblast growth factor (bFGF). They also differ from EGL-derived cells in that they are multipotent and capable of extensive self-renewal in culture. These "Wechsler-Reya" cells thus meet the definition of a true neural stem cell and are clearly of great interest as potential cells of origin for medulloblastoma.

Table 1
Potential links between molecular pathways, stem or progenitor cells, and tumor subtypes

Origin	Tumor	Molecular pathway(s)
EGL	Nodular MB	Hh > Notch
Cerebellar VZ	Classic MB	Wnt > Hh, Notch
Extracerebellar VZ	PNET	Wnt, Hh, Notch
Dispersed cerebellar CD133-positive cells	MB	Not Hh

Abbreviations: EGL, external germinal layer; MB, medulloblastoma; PNET, primitive neuroectodermal tumor; VZ, ventricular zone.

Molecular links between neural stem cells and medulloblastoma

At least three canonical signaling pathways playing important roles in neural stem cells are deregulated in medulloblastoma or primitive neuroectodermal tumor (PNET): Hedgehog, Wnt, and Notch. The study of these pathways has provided crucial insights into the relation between stem or progenitor cell biology and medulloblastoma. Two, Hedgehog and Wnt, were initially linked to medulloblastoma because they are activated in inherited syndromes predisposing individuals to tumors [14–16]. The Notch pathway has been implicated more recently in medulloblastoma pathobiology on the basis of gene expression studies and DNA copy number alterations [17,18]. All three pathways represent exciting targets for new medulloblastoma treatments. Given their particular importance in neural stem cells, the Hh, Wnt, and Notch pathways may also be required for the maintenance of stem-like cells within brain tumors [19–24]. Understanding such "developmental" pathways and how they affect neural stem or progenitor cell populations in the brain is therefore potentially critical for the classification and treatment of embryonal brain tumors.

The role of Hedgehog in the cerebellar EGL and in medulloblastoma has been particularly intensely studied. In the developing cerebellum, granule cell precursors proliferate within the EGL in response to Sonic hedgehog (SHH) protein secreted by subjacent Purkinje neurons [25–27]. Prolonged exposure to SHH delays terminal differentiation and causes increased proliferation of cultured granule cell precursors [27]. Although the role of Hh signaling in the cerebral cortex is less well defined than in the cerebellum, several reports strongly support a requirement for the pathway in the viability and proliferation of cerebral periventricular stem cells [28,29], supporting a possible role in sPNET formation as well.

Notch is another critical regulator of neural stem cells [30,31]. Notch1, Notch2, and Notch3 are all expressed in the germinal zones around

ventricles during fetal development, and their expression persists to varying degrees in adult germinal structures, such as the subventricular zone [32]. Precocious neuronal differentiation is observed in mice lacking the Notch1 receptor or two other pathway members, Hes1 and CSL, suggesting that they act to maintain a neuronal precursor population in the embryo [33,34]. Disruption of Notch1 or Hes1 also promotes differentiation of neural stem cells in culture [35,36]. In the cerebellum, only Notch2 is highly expressed in EGL cells, whereas Notch1 is upregulated as granule cell progenitors exit the cell cycle and begin to migrate inward and differentiate [32,37,38]. Introducing activated Notch2 into EGL-derived progenitors promotes their proliferation and inhibits differentiation [37]. Medulloblastoma derived from the EGL seem to recapitulate these developmental differences between Notch1 and Notch2, because it has been shown that the former inhibits proliferation, whereas the latter promotes tumor growth [17].

The Wnt pathway also regulates patterning and growth in the central nervous system, as evidenced by severe abnormalities in the developing midbrain and cerebellum after Wnt1 loss [39,40] and by expanded precursor populations in the VZ after activation of the pathway in transgenic mice [41]. Unlike the Hedgehog and Notch pathways, however, which promote proliferation of progenitor cells in the VZ and EGL, the Wnt pathway is not clearly required in granule cell precursors. Thus, medulloblastoma defined molecularly by Wnt pathway activation may not arise from the EGL but from stem-like cells in the VZ or white matter (see Table 1).

Do medulloblastomas arise from stem or progenitor cells?

Several lines of evidence suggest that medulloblastomas arise from stem or progenitor cells in the brain. First, they are similar to such cells in terms of their microscopic appearance and RNA and protein expression profiles [42,43]. Second, in several murine medulloblastoma models, tumors seem to arise from the cerebellar EGL [44]. Third, multipotent cells with stem-like properties can be isolated from primary medulloblastoma and grown as neurospheres in culture [45,46]. Fourth, neural precursor cells can form medulloblastoma-like lesions when transformed with oncogenes [47]. Although none of these facts prove that medulloblastoma in human beings originates from stem cells, they strongly suggest that this is possible.

It has long been known that medulloblastoma and other embryonal brain tumors bear a strong microscopic resemblance to neural precursor cells. Indeed, the fetal VZ and classic medulloblastoma can appear almost identical (Fig. 2A, B). As further proof of the potential of neural stem cells to recapitulate the appearance of a wide range of embryonal tumors, primitive cellular masses and neuroepithelial structures similar to those seen in medulloepithelioma can be transiently formed by neural stem cells injected into the brain stem of adult rats (Fig. 2C). Given this strong morphologic similarity, it is not surprising that the same RNA species expressed in neural stem and precursor cells are identified in medulloblastoma and other embryonal brain tumors. For example, such genes as nestin, musashi, Bmi1, and CD133, whose expression marks neural stem or progenitor cells, are also actively transcribed in medulloblastoma and PNET [48–50]. The Notch, Wnt, and Hedgehog pathways are also aberrantly activated in embryonal brain tumors [17,18,51–54]. Unbiased high-throughput analyses of gene expression have further linked medulloblastoma to neural stem or precursor cells. Kho and colleagues [42] compared gene expression profiles in human medulloblastoma with those in the developing mouse cerebellum and found that the tumors were most similar to cerebella of postnatal days 1 through 10 (P1–P10), a period during which dividing stem or precursor populations form a large portion of cerebellar cells. A separate gene expression study comparing several murine medulloblastoma models with P5 and adult cerebella also found that tumors were most similar to the P5 stage, containing an actively proliferating EGL [43]. In situ studies by Lee and colleagues [43] confirmed that many of these marker genes mapped to the P5 EGL. Interestingly, the same set of genes that defined the similarity between murine tumors and p5 murine cerebellum was significantly deregulated in only 16 of 50 human tumors examined. One possible interpretation of these data is that only a fraction of medulloblastomas derive from the EGL, and therefore retain gene expression signatures of the germinal epithelia.

Although the EGL has had many champions as a primary source of medulloblastoma, the VZ also seems to be a likely origin for many embryonal brain tumors. This is particularly obvious for PNET arising supratentorially and in other brain

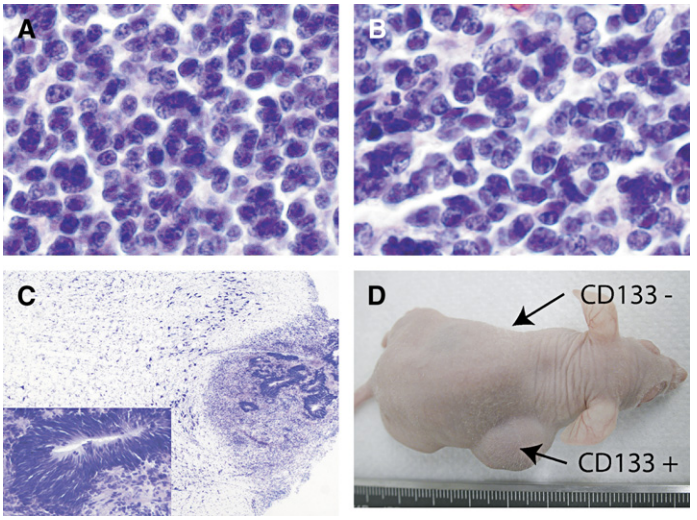

Fig. 2. Neural stem cells and embryonal brain tumors. The tightly packed stem or progenitor cells of the human fetal VZ (*A*) are at high power, essentially identical to classic medulloblastoma (*B*). (*C*) When injected into the rat brain stem, nonneoplastic neural stem cells can transiently form tubular neuroepithelial structures similar to those seen in medulloepithelioma. (*D*) CD133-positive fraction (*arrow*) of DAOY cultures forms large flank xenografts, whereas CD133-negative cells (*arrow*) do not.

regions lacking an EGL, which thus must derive from another cell type. Molecular and immunohistochemical marker studies performed in primary medulloblastoma also suggest that VZ and EGL populations can give rise to tumors. Katsetos and colleagues [55,56] have shown that Calbindin-D, a VZ-associated calcium-binding protein not expressed in the EGL or in granule neurons, was present in 20 of 49 cerebellar medulloblastomas, primarily those of the classic (nonnodular) subtype. Based on this, they suggested that classic medulloblastoma derives from the VZ, whereas nodular tumors come from EGL cells. Buhren and colleagues [57] have advanced a similar "dual-origin" hypothesis based on the elevated percentage of nodular tumors immunoreactive for the neurotrophin receptor p75, which is highly expressed in the cerebellar EGL but not in the VZ. Of the 167 medulloblastomas that they examined, all the nodular lesions expressed p75, whereas only 17% of classic tumors did so, suggesting that p75 expression defines an EGL-derived subset of nodular or desmoplastic tumors.

More recent molecular studies using gene expression arrays also support the concept that medulloblastomas can be clustered into different subtypes, potentially reflecting distinct histogenetic origins. The first such report showed that nodular or desmoplastic tumors were characterized by elevated expression of Hh pathway members [58]. A more recent expression profiling attempt also revealed that RNA signatures characteristic of Hh activity were generally associated with the nodular or desmoplastic subtype [59]. This correlates well with mutational studies of the Hh pathway, in which activating mutations have been observed predominantly in the nodular or desmoplastic subtype in familial and sporadic cases [53,60]. Although the expression profiles of non–EGL-derived tumors are not yet established, at least some may be defined by Wnt pathway activation. Wnt signaling is activated most commonly in nonnodular lesions, and these tumors cluster together in gene expression analyses [51,59]. Tumors in which Wnt is active also seem to show a distinct clinical profile because they tend to be less aggressive than medulloblastomas lacking Wnt activity [61]. Such studies suggest the existence of specific associations between signaling pathways, stem or progenitor cell populations, and embryonal brain tumor subtypes (see Table 1).

If brain tumors arise from neural stem cells, one might predict that they would retain stem-like properties. The neurosphere assay was developed for the analysis of nonneoplastic neural stem cells, and it allows one to assess the clonogenicity and differentiation potential of individual cells. The fact that medulloblastomas can give rise to

multipotent neurospheres supports the concept that they have stem-like characteristics [45,46]. It should be noted, however, that progenitor cells with a more limited capacity for self-renewal can also generate neurospheres, although these are generally smaller in size than those formed by true stem cells [62]. One must therefore be careful in ascribing a stem-cell origin to all spheres generated from tumors. Nevertheless, several groups have shown that freshly resected medulloblastomas contain cells capable of forming multipotent neurospheres that can self-renew and have hypothesized that this ability may be attributable to their origin from neural stem cells [45,46].

Finally, murine medulloblastoma models place these tumors at "the scene of the crime," near to stem or progenitor cell populations. The best-studied murine transgenic medulloblastoma model is based on activation of the Hedgehog signaling pathway in transgenic mice lacking the inhibitory PTCH receptor. It was noted early on that these animals contain small persistent EGL remnants that retain some proliferation potential, and therefore may represent partially transformed precursors of medulloblastoma [44,63]. More sophisticated analyses of preneoplastic and fully transformed cell populations from these mice have largely confirmed this concept [64]. Murine medulloblastoma models driven by genetic alterations affecting DNA repair, apoptosis, the cell cycle, and even interferon-γ production also show early lesions on the surface of the cerebellum [65–69]. Interestingly, when gene expression profiles of such EGL-derived tumors are analyzed, aberrant upregulation of the Hedgehog pathway members and targets important in the proliferation of granule cell precursors stands out as one common theme [43]. Additional proof of the potential of neural stem cells to transform into medulloblastoma comes from the recent studies of Su and colleagues [47]. They have shown that immortalized EGL cells, which normally differentiate when injected into murine cerebellum, generate medulloblastoma when the transcriptional repressor of neuronal differentiation, REST, is forcibly expressed.

Brief overview of cancer stem cells

The cancer stem cell hypothesis postulates that a hierarchy exists within neoplasms. A relatively small population of stem-like cancer cells resides at the apex of this organization and gives rise to a series of more numerous progeny that are better differentiated and lack the ability to self-renew indefinitely [19]. Paradigms explaining the development and repair of normal tissues have been used to generate this model, because only stem cells are thought to have a long-term capacity for self-renewal, and are thus necessary for organogenesis and regeneration. Recent reports indicate that such a hierarchy can persist in established cell lines derived from tumors of the brain or other organs [70,71]. This is potentially critical if effective therapies are to be developed. It has been suggested that many chemotherapeutic agents fail because they remove the bulk of the tumor but do not target stem-like cancer cells, which make up a small percentage of the overall lesion but are absolutely required for long-term tumor growth [19,72–74].

Clonogenic assays provided the initial experimental support for the existence of a discrete cancer cell subpopulation required for tumor propagation, because only a small portion of tumor cells could form colonies in vitro or engraft in vivo [72,75,76]. A stochastic model in which all cells have a low probability of forming new lesions could also explain this functional heterogeneity within tumors, however [74]. Only recently has the prospective isolation of tumor-initiating cells from leukemia been achieved, providing firmer support for the presence of cancer stem cells [77,78]. This has been made possible by the identification of markers by which stem-like cells can be sorted from tumor specimens. Two such markers, CD133 and "side population," prospectively identify a small percentage of cells in brain tumors uniquely able to generate xenografts [70,79–81]. CD133 and side population were initially identified in nonneoplastic stem cells [82–85], highlighting the links between malignant and normal stem cells. These markers are now being used to determine if the stem-like fraction of medulloblastoma or PNET is especially tumorigenic.

Cancer stem cells in medulloblastoma

Singh and colleagues [45] have analyzed newly resected medulloblastomas using flow cytometry and found that CD133 is expressed in only a subset (10%–60%) of cells within these tumors. Only CD133-expressing cells could form multipotent neurospheres in culture capable of being serially propagated, suggesting that they represented

a fraction of the tumor functionally equivalent to a neural stem cell. This same group has subsequently shown that CD133 expression also defines the subset of medulloblastoma cells able to propagate tumors in immunocompromised animals [79]. These experiments need to be repeated by other groups but strongly suggest that although medulloblastomas seem to be largely undifferentiated and "embryonal," many primitive-looking neoplastic cells may be similar to progenitor cells rather than true stem cells in that they cannot self-renew indefinitely and form new tumor masses in vitro or in vivo. In support of this, the authors' have found that CD133-enriched fractions of the established medulloblastoma cell lines DAOY and D425 form large subcutaneous xenografts in immunocompromised mice, whereas CD133-depleted fractions rarely do, suggesting that it may be possible to analyze cancer stem cells in established cells lines (Fig. 2D).

To study cancer stem cells in medulloblastoma effectively, it is necessary to develop a better understanding of how to isolate and manipulate these cells. Although the surface marker CD133 has proven useful, it is expressed in a rather large fraction of medulloblastoma and glioblastoma cells. Such markers as side population identify a much smaller percentage of tumor-propagating cancer stem cells in medulloblastoma and other brain tumors, however, suggesting that CD133 may be somewhat promiscuous [86]. To answer such questions, it is necessary to compare directly how expression-based and functional stem-cell markers, such as CD133, nestin, Bmi1, musashi, Aldefluor, and side population, overlap in primary brain tumors and cell lines derived from such tumors and to determine what markers, alone or in combination, most accurately identify the cells within tumors that can generate new lesions in vivo.

Once a better understanding has been acquired of how to identify and sort stem cells accurately from progenitor and differentiated cells in tumors, it should be possible to ask more detailed questions about the relations between these groups. One critical issue is whether better differentiated cells within the tumor have the capacity to dedifferentiate and repopulate the stem-cell fraction. In normal tissues, it is generally believed that once stem cells give rise to daughters that leave the niche, the developmental current sweeps them inexorably downstream, first through a progenitor phase and, ultimately, into the sea of terminally differentiated cells. Tumor cells, however, may not be bound by the same conventions as their genetically intact cousins. Specifically, they may have the ability to swim upstream in some cases and repopulate the stem cell fraction. This possibility is supported by analyses of nonneoplastic tissues. For example, neural transit-amplifying cells can turn into stem cells when exposed to epidermal growth factor (EGF) [87]. Similarly, differentiating spermatogonia can dedifferentiate into stem cells when the jak-STAT pathway is aberrantly activated [88]. It should be therapeutically critical to determine if similar phenomena occur at any significant frequency in tumors. If better differentiated tumor cells can dedifferentiate, it would indicate a need for combined therapeutic regimens timed to remove multiple cell populations simultaneously.

Can cancer stem cells be targeted in medulloblastoma?

The all too common recurrence of tumors that initially respond to therapy may be attributable to the resistance of cancer stem cells to many standard treatment regimens [89,90]. Cancer stem cells in leukemia, for example, are relatively insensitive to Daunorubicin [89]. One mechanism of chemotherapeutic resistance is enhanced excretion of chemotherapeutic agents by cancer stem cells because of their high expression of ATP-binding cassette (ABC) type drug transporters [91,92]. It has also been suggested that cancer stem cells, like nonneoplastic stem cells, may have an intrinsic resistance to apoptosis that enables them to survive [90]. Clearly, novel therapies must be developed that allow targeting of cancer stem cells if tumor recurrence is to be prevented in lesions perpetuated by this subpopulation. Similarities in the growth characteristics and gene expression patterns of benign neural stem cells and brain tumor stem cells [44,46,93] suggest that the same signaling pathways may be required for survival and growth in both.

Data generated in established medulloblastoma cells lines strongly suggest that Notch pathway blockade may be one means by which stem-like tumor cells can be eliminated, thereby blocking the formation of new tumor lesions [86]. In brief, it has been shown that pharmacologic inhibition of the Notch pathway depletes the stem-like fraction of medulloblastoma cells expressing nestin and CD133. The stem-like side population is also ablated by such therapy. Tumor cultures continue to grow slowly after Notch blockade

but can no longer generate tumor xenografts. Inhibition of the Hedgehog and Wnt pathways may have similar effects on cancer stem cells in medulloblastoma.

Summary

In many ways, medulloblastomas represent the apotheosis of tumor stem cell concepts. They can arise (at least in animal models) from stem or progenitor cells. They resemble stem cells in terms of their primitive appearance and proclivity for divergent differentiation. Perhaps most importantly, they contain a subset of stem-like cells required for tumor propagation. As discussed previously, however, the relatively faithful adherence of medulloblastoma to the developmental program of nonneoplastic neural stem cells may represent an Achilles heel. Therapeutic regimens targeting Notch and other signaling pathways required in nonneoplastic neural stem cells may also be effective in depleting medulloblastoma stem cells. A better understanding of which germinal epithelia or stem cell populations give rise to medulloblastoma and which molecular pathways drive this neoplastic transformation is critical for such therapeutic advances.

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