Stem Cells in Brain Tumour Development and Therapy- Two-Sides of the Same Coin

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ABSTRACT: Primary brain tumours are difficult to manage clinically due to their abilities to invade adjacent tissue and infiltrate distant neuropil. These contribute to challenges in surgical management and also limit the effectiveness of radiotherapy. Despite initial responses to chemotherapy, most tumours become chemo-resistant, leading to relapse. Recent identification and isolation of brain cancer stem cells (BCSCs) have broadened our understanding of the molecular pathogenesis and potential Achilles' heel of brain tumours. BCSCs are thought to drive and propagate the tumour and therefore present an important target for further investigations. This review explores the history of the discovery of BCSCs and the evolving concept of "cancer stem cells" in neuro-oncology. We attempt to present a balanced view on the subject and also to update the readers on the molecular biology of BCSCs. Lastly, we outline the potential strategies to target BCSCs which will translate into specific and effective therapies for brain tumours.

RÉSUMÉ: Rôle des cellules souches dans la formation et le traitement de tumeurs cérébrales, deux faces d'une même médaille. Les tumeurs primitives du cerveau sont difficiles à traiter parce qu'elles envahissent le neuropile tissus adjacent et infiltrent le neuropile à distance, ce qui constitue un défi pour le traitement chirurgical et limite également l'efficacité de la ratiothérapie. Malgré une réponse initiale à la chimiothérapie, la plupart des tumeurs deviennent éventuellement chimiorésistantes ce qui entraîne une récidive. L'identification et l'isolement de cellules souches de cancer du cerveau (CSCC) ont élargi notre compréhension de sa pathogenèse moléculaire et laissent entrevoir la présence d'un talon d'Achille possible de ces tumeurs. On pense que les CSCC gèrent et propagent la tumeur et donc méritent qu'on les étudie davantage. Cette revue explore l'histoire de la découverte des CSCC et la notion de "cellules souches du cancer" en neuro-oncologie. Notre objectif est de présenter une vision équilibrée du sujet et de mettre le lecteur à jour sur la biologie moléculaire des CSCC. Nous donnons enfin un aperçu de différentes stratégies pour cibler les CSCC qui pourront éventuellement mener à des traitements spécifiques et efficaces des tumeurs cérébrales.

Primary brain cancers are tumours that arise from malignant transformation of cells of neuroectodermal lineages within the central nervous system (CNS). These tumours are often aggressive and difficult to treat. Primary brain tumours such as gliomas often show extensive local invasion and distant infiltration, making complete surgical resection difficult to achieve. High-grade gliomas, encompassing both malignant astrocytomas and oligodendrogliomas, often display resistance to therapy in addition to aggressive biological growth[1]. The lower grades gliomas may not always offer a better prognosis because they are frequently surgically incurable and when given enough time, will inevitably progress to high-grade tumours[2,3]. Therefore, challenges in brain cancer treatments can be attributed to a few factors. 1) The infiltrative nature of glioma and intimate involvement of adjacent normal structures precludes effective surgical resection of the tumour and limits the extent and breadth of radiotherapy. 2) Normal brain parenchyma is sensitive to effects from radio- and chemotherapy and has limited capacity to repair itself from damages. Thus far there is still a lack of active therapeutic agents that effectively cross the blood-brain-barrier (BBB) for brain cancer treatment. In addition, drug resistance and disease relapse are common in patients, rendering glioma one of the most lethal and aggressive human malignancies[4].

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Cancer Stem Cell Theory

The concept of "no-neuron dogma", which implicates the absence of brain stem cells in adulthood, was challenged in the 1960's when the generation of new, functional brain cells was described in the adult mammalian CNS[5-7]. In the late 1980's, Nottebohm's group demonstrated the functional relevance of adult neurogenesis in songbirds[8,9]. Subsequently, Reynolds and Weiss reported the isolation of neural stem cells (NSCs) from adult mouse brain[10], followed by a series of *in vitro* experiments which highlighted the presence of human NSCs in adult brain, specifically in the dentate gyrus of the hippocampus[11]. The first tantalizing evidence of the existence of NSCs *in vivo* in adult human brain was described in 2001[12]. The major implication of these studies is the existence of mitotically active stem and progenitor cells within discrete regions of the mature brain. These long-lived cells have high potential in self-renewal and proliferation, with time, hold the greatest opportunity of accumulating genetic defects that transform normal cells [reviewed by Vescovi et al.[13], Al-Hajj et al.[14]].

The "cancer stem cell theory" proposes an hierarchical organization in tumours in which only a specific subset of cells can self-renew, proliferate extensively, establish and maintain a tumour clone while the rest of the variably differentiated cells cannot[15], a concept differs from the traditional view of cancer development. The clonal evolution theory, on the contrary, indicates that all cells in a tumour have similar tumourigenic potential that is activated asynchronously and at low frequency. These cells acquire genetic mutations, each of which confers additional proliferative and survival advantages. The malignant progression towards a disease state proceeds in a manner that resembles Darwinian evolution[15].

Molecular Features of Cancer Stem Cells

The fact that brain tumours tend to recur after surgery may be attributable to the diffuse and infiltrative nature of the tumours and the presence of cancer stem cells (CSCs), which are also known as tumour initiating cells (TICs). We realize that there may be nuances in the definitions of CSCs and TICs; however, these two terms will be used interchangeably in this review for convenience.

The discovery of CSCs was first made by John Dick's group with the isolation of a small population of cells in acute myeloid leukaemia (AML) that were capable of initiating leukaemia after transplantation. These cells self-renewed, bore molecular features of normal hematopoietic stem cells, and were named "leukaemia initiating cells"[16-18]. This exciting result incited the scientific community and a wealth of literature was subsequently published to report the isolation of CSCs in breast[19], skin[20], pancreas[21], colon[22], prostate[23] and brain[24-28]. Cancer stem cells or TICs are defined by the characteristics outlined by Vescovi and colleagues[13]. First, CSCs must be able to self-renew extensively, demonstrated either *ex vivo* by sequential clonogenic and population kinetic analyses[27,29] or *in vivo* by serial, orthotopic transplantation[25,27]. Although transient amplifying progenitors are highly proliferative, they show limited self-renewal and cannot be propagated for an extended period of time. Equally important, stem cells need to be able to generate progenies several orders of magnitude more abundant than the starting population[30]. Furthermore, the term "tumour initiating cells" literally implicates the ability of these cells to instigate tumour formation when implanted in animals. The resulting tumours need to recapitulate the histopathological features of the parental tumours. Cancer stem cells generate tumourigenic as well as non-tumourigenic cells and they are capable of undergoing multi-lineage differentiation, though the latter may not be an absolute requirement for their identification. Unlike normal stem cells, CSCs harbour karyotypic or genetic alterations in addition to aberrant differentiation properties[13].

Despite the phenotypic and functional similarities between normal stem cells and CSCs, it is important to be aware of the fundamental differences between these cells. While normal stem cells are known for the vigilance with which their proliferation is controlled and for the care with which their genomic integrity is maintained, CSCs lack such properties[31]. The perennial nature and high proliferative potential of stem cells has made it tempting to speculate that CSCs may originate from malignant transformation of normal stem cells, yet thus far the cell-of-origin remains elusive. Studies have shown that genetic alterations by way of tumour suppressor gene ablation or oncogene activation increased the frequency of tumour formation in primitive nestin-expressing cells but not in the more differentiated glial fibrillary acidic protein (GFAP)-expressing astrocytes[32,33]. However, conflicting experimental results indicate that differentiated astrocytes and NSCs may be equally permissive to transformation when key genetic alterations are introduced. In particular, mature astrocytes and neural progenitor cells with epidermal growth factor receptor (EGFR) over-expression and p16Ink4a deficiency generate tumours at similar frequencies and intriguingly, the combination of these genetic lesions seem to "re-program" astrocytes and enable them to acquire a "stem-like cell" phenotype[34]. Therefore, experimental evidence has pointed to glioma development from CSCs or from de-differentiation of mature cells within the mature glial population.

Tumour Microenvironment and Brain Cancer Stem Cells (BCSCs)

Brain tumours are formed by a mixed population of cancerous and non-cancerous cells. It is now believed that the non-malignant cells may not be innocent bystanders but accomplices that actively contribute to brain tumour development[35,36]. The tumour microenvironment, endogenous stromal cells and infiltration, support the growth and progression, even acting as part of the "soil" in facilitating the establishment of distant metastases of a tumour.

In the case of CNS malignancies, for instance, tumour cells in glioblastoma multiforme (GBM) can release vascular endothelial growth factor (VEGF), angiopoietin-2 and other factors into the circulation to recruit bone marrow progenitors such as CD45+ myeloid cells to support angiogenesis[37]. Tumour-infiltrating myeloid suppressor cells can mediate immuno-suppression by inactivating dendritic cell maturation and anti-tumour activities of natural killer cells[38]. Endothelial cells have also been shown to promote BCSC proliferation and self-renewal in vascular niche. Increased endothelial cell number in brain tumour xenografts expanded BCSC population and accelerated disease progression[39]. Brain cancer stem cells may

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also create their own tumour-specific niche and engage angiogenesis by secreting VEGF[40]. Bevacizumab, an inhibitor of VEGF-A, suppresses angiogenesis and BCSCs growth *in vivo*[41,42]. Bevacizumab has been part of the treatment regimens for malignant glioma. However, concerns have been raised around its potential to trigger infiltrative tumour growth, resulting in an aggressive disease that is difficult to manage[43,44]. Studies are currently underway to examine the combination of anti-angiogenic therapy with anti-invasion therapy such as cilengitide, an integrin inhibitor, in the hope of delaying disease progression[38]. Thus far, no single molecular-targeted agent has been proven effective and used in clinical setting for brain cancer treatment [reviewed by Van Meir et al.[38]]. Effective management of malignant glioma will likely rely on combinatorial targeting of different pathways to improve cure rate and efficacy.

The Identification and Isolation of BCSCs

Neurosphere Assay

The evidence of the existence of stem-like cells in brain cancer was first reported by Steindler and colleagues who obtained sphere-forming cells from post-operative GBM specimens[45]. Subsequently, Hemmati et al. and Galli et al. grew primary tumour cells isolated from patient specimens in neurosphere assay with conditions permissive for neural stem cell growth.[26,27] The tumour spheres formed expressed NSC markers such as musashi (MSI1), BMI1 and SOX2 and were capable of aberrant multi-lineage differentiation[28]. In addition, the cells dissociated from the spheres were tumorigenic in animals and formed highly infiltrative lesions characteristic of GBM[26,27].

In neurosphere assays, cells dissociated from primary brain tumour specimens were plated in neurobasal medium supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (b-FGF). The variably differentiated cells cannot survive and therefore only neural stem and progenitor cells can proliferate clonally to form free-floating, phase-bright spheres in liquid culture. Tumourspheres can be serially passaged and cells from the spheres can be induced to undergo lineage differentiation upon growth factor withdrawal or addition of serum[10]. It should be noted that there is no one-to-one relationship between neurospheres and NSCs. Estimating stem cell frequency based on sphere forming frequency in the neurosphere assay provides an invalid readout[30]. In other words, sphere-forming frequency approximates progenitor cell activity more closely than stem cell activity. Most of the spheres in this assay are likely derived from progenitor cells but not stem cells, therefore serial passaging is crucial to the enrichment of NSC population[30,46].

Cell Surface Antigen Sorting by CD133 (Prominin 1, PROM1)

In the study of Singh et al., brain tumour initiating cells (BTICs) were enriched by the cell sorting paradigm based on the expression of CD133 (prominin 1), a cell surface glycoprotein. The CD133+ cells exhibited molecular characteristics of NSCs *in vitro* and were much more tumorigenic than the CD133- cells with only 100 CD133+ cells required to form tumours that are phenocopies of the parental tumours in immunocompromised mice. On the contrary, 10^5 CD133- cells engrafted but did not produce tumours[25].

CD133+ cells have subsequently been found to be resistant to chemo- and radiation therapies. CD133+ cell population was enriched in tumours from relapsed patients compared to their initial diagnosis[47]. Transcript levels of drug resistant proteins such as breast cancer resistant proteins (BCRP), O^6-methyl guanine DNA methyl transferase (MGMT) and anti-apoptotic proteins: BCL-2, BCL-xL, MCL-1, and the inhibitor of apoptosis protein (IAP) family are elevated in CD133+ cells which were notably resistant to carboplatin, etoposide, paclitaxel and temozolomide[47]. CD133+ cells survive radiotherapy by preferentially activating DNA repair pathway and radio-resistance could be reversed by treatment with CHK1/2 inhibitors[48].

Recent studies suggest that CD133- cells may also be tumourigenic and that CD133+ and CD133- cells may represent CSCs from distinct cells-of-origin. While CD133+ cells resemble fetal NSCs, display proneural signature genes and grow as neurospheres, CD133- cells resemble adult NSCs, show mesenchymal transcriptional profile and grow semi-adherently[49,50]. Interestingly, CD133low GBM (having ≤ 3% CD133+ cells in the tumour) showed more invasive, proliferative and angiogenic growth than CD133high tumours (having ≥ 3% CD133+ cells in the tumour)[51]. CD133- cells are capable of initiating tumour formation and giving rise to tumours that contained CD133+ cells. Interestingly, *in vivo* passaging of these tumours led to up-regulation of CD133 expression[52].

Despite the uncertainties in the robustness of CD133 as a marker for BCSC identification, experimental evidence has nevertheless lent strong support to the clinical importance of this subset of cells. The presence of >2% CD133+ cells with high Ki67 labelling is an independent prognostic marker of poor survival in GBM patients[53] and the co-expression of nestin and CD133 independently predicts poor clinical outcome of glioma patients[54].

Cell Surface Antigen Sorting by CD15 (Lewis x, Stage-Specific Embryonic Antigen 1, SSEA-1)

In addition to CD133, another cell surface antigen, CD15 (SSEA-1), has recently been studied in the isolation of BCSCs. CD15 is a carbohydrate epitope expressed on normal neutrophils[55], stem and progenitor cells in the adult/embryonic nervous system[56-58] as well as cells from various solid tumours[59,60]. CD15 cell sorting enriched for proliferative and tumourigenic cells *in vitro* and *vivo* in GBM and medulloblastoma[61-63]. However, the fact that CD15 was also expressed in a subset of granular neuronal progenitors (GNPs) may argue against it being a marker exclusive to NSCs. Furthermore, in the study of Read et al., CD15+ cells did not form neurospheres and showed no evidence of multi-lineage differentiation despite being capable of medulloblastoma tumour formation in animals. Therefore, CD15+ cells might be progenitor-like cells with a unique capacity for tumour

propagation[63].

Other Methods Used for the Enrichment of BCSCs

Hoechst 33342 Exclusion- Side Population (SP) Cells

Stem cells and CSCs express elevated levels of multi-functional drug efflux proteins such as the adenosine triphosphate binding-cassette (ABC) transporters on their cell surface. These cells may thus be protected from certain chemotherapeutic agents by "pumping out" drugs from cells[64-68]. Interestingly, Hoechst 33342, a fluorescent DNA binding dye, is also one of the substrates of ABC drug efflux proteins. The unique biological property of stem cells and CSCs in extruding chemotherapeutic agents as well as Hoechst 33342 is exploited for the isolation of this sub-population of cells. In this dye exclusion assay, the cells are stained with Hoechst 33342 and subjected to flow cytometry. Stem cells and CSCs, due to their enhanced capacity in effluxing the dye, will show low Hoechst staining intensity that segregates these cells from the majority of the Hoechst-stained cells (express lower levels of drug efflux proteins) on a flow cytometry dot plot. These Hoechst-effluxing cells are also known as "side-population" (SP) cells[69-75]. This technique has been used to successfully identify CSCs in brain cancers[76]; however it does not provide a pure population of CSCs as normal stem cells are also included and CSCs may not always be in the side population. In addition, the SP cells may contain differentiated, non stem-like cells that are counted as part of the SP merely because of their elevated expression of drug efflux proteins[77].

Aldehyde Dehydrogenase Activity Assay

Aldehyde dehydrogenases are a group of enzymes that catalyze the oxidation of aldehydes. In 1996, Storms et al demonstrated the isolation of hematopoietic progenitor cells on the basis of aldehyde dehydrogenase (ALDH) activity. In the assay they developed, a fluorescent substrate for ALDH, named BODIPY aminoacetaldehyde (BAAA), was examined for its potential for isolating primitive hematopoietic cells. Results indicate that a subset of cells with low orthogonal light scattering and bright fluorescent intensity [$SSC^{lo}ALDH^{br}$] are enriched for hematopoietic progenitor cells[78]. Subsequently, the identification of putative NSCs in the central nervous system was reported by Corti et al. who showed that $SSC^{lo}ALDH^{br}$ cells obtained from murine adult and embryonic neurospheres cells were capable of self-renewal and multi-potent differentiation *in vitro* and *vivo*[79]. The enhanced functional activity of ALDH was not only observed in the progenitor cells in the hematopoietic and central nervous system,[80-82] but also in human malignancies such as cancers of the lung[83,84], breast[85], colon[86], liver[87], brain[88,89], and head and neck squamous cells.[90] It was also associated with disease progression[91], metastasis[92-94] and poor clinical outcome[93,95-98]. Together, these results indicate the clinical importance of ALDH-high cells in the pathogenesis of cancers.

Other novel CSC isolation methods include a marker-independent identification of glioma-initiating cells (GICs). This technique takes advantage of the autofluorescence properties of GICs that emit light at ~520nm upon laser excitation at 488nm. These GICs can therefore be detected by flow cytometry in FL1 channel without the need for cell surface markers[99].

It is likely that a combination of cell surface markers and other techniques will be required to purify CSCs as elegantly exemplified in the hematopoietic system, though challenges arise because of minimal overlap in cell population isolated using different cell surface markers[19,100,101]. Moreover, the fact that brain cancer is a complex disease in which a heterogeneous population of cells contribute to disease progression would refute the idea of targeting solely the CSC population for effective treatments[35]. The study of CSCs is insightful from the scientific standpoint, yet the most effective therapeutic strategy against brain tumours would likely involve targeting most, if not all, cells in a tumour as well as stromal components in the tumour microenvironment.

The "Flipside" of the Story- Debates Around the CSC Theory

CSCs and Aberrant Differentiation

The field of CSC has been an area of intense study. However, as with many other scientific theories, it is not devoid of controversies. A review by Shackleton compares the cancer stem cell theory and the clonal evolution theory[102]. Epigenetic controls are thought to govern the process of differentiation. As such, one could envisage a bias towards a particular epigenetic program that favours an undifferentiated state during the development of CSCs. These epigenetic controls may also be largely reversible such that the majority of the cells in a tumour loses their tumourigenic capacity[102]. Though scientifically sound, thus far there is still a dearth of evidence suggesting that tumourigenic and non-tumourigenic cells are distinguished mainly by epigenetic rather than genetic differences[102]. Furthermore, heterogeneity of cells in tumours is believed to be a result of hierarchical organization established by CSCs, a conclusion drawn from analyses of tumours using only a limited number of cell surface markers that characterize major cell types. It is not known if there is additional genetic heterogeneity that is not captured in these studies[102]. Therefore, tumour heterogeneity may in fact, be partly attributed to genetic diversity of cells in tumours as suggested by the clonal evolution theory.

Controversies of Animal Models

In CSC studies, the use of more immunocompromised mice results in fewer cells being required for tumour formation[17,103-105]. This is exquisitely demonstrated by Quintana et al. that far less cells were required to form detectable melanoma when more highly immunocompromised NOD/SCID mice (IL2γ-null) were used[105]. However, even this experimental system could still under-estimate the number of human tumour cells that are capable of forming tumours when given suitable conditions[102]. Animal models provide valuable information but the differences between mouse and human brain parenchyma more or less limits the extent to which we could interpret the data. The degree of vascularization at sites of tumour cell injections, differences between mouse and human extracellular matrix environment, growth factor requirements for human tumour cells to survive and form tumours in animals, sites of tumour cell implantation (orthotopic or heterotopic) and host immune status are all critical factors that determine the success of engraftment[102,106].

The use of transgenic mouse models may help circumvent the

problem of host-cell compatibility. Transgenic mouse models of medulloblastoma have been developed in which key genetic mutations in the sonic hedgehog pathway are introduced to evoke *in situ* tumour formation. For example, in the study by Weschler-Reya's group, transgenic mouse model with Patched *(Ptch)* mutation was generated to study medulloblastoma in mouse brain and CD15 was identified as a marker that consistently enriches for tumour initiating cells[63]. In addition, *Smo/Smo* transgenic mice were created by Olson's group by expressing a constitutively activated form of the Smoothened gene *(SmoA1)* within cerebellar granule neuron precursors through the regulation of the *neuroD2* (ND2) promoter. The *Smo/Smo* mice exhibit high incidence and early onset of medulloblastoma tumours ideal for preclinical studies[107].

The Functions and Presence of CSCs may be Context-Dependent

The CSC theory proposes that the majority of cells in a tumour lose tumourigenic potential during the process of differentiation. However, the loss of tumourigenicity may not always be due to hierarchical organization but rather a preordained lethal cell fate resulting from the deleterious genetic mutations that tumour cells harbour[102]. Furthermore, the CSC theory addresses the "potential" of cancer cells to contribute to disease but not the actual "fate" of cells in patients. The fact that CSCs have higher potential forming tumours does not mean they actually do so in patients because tumour cells may be held in check temporarily or permanently by constraints from tumour microenvironment and/or host immunity[102]. On the other hand, non-CSCs may become tumourigenic when given the appropriate conditions, as suggested by the "interconversion model"[31] which hypothesizes that cancer cells can interconvert between more or less malignant/proliferative states depending on the contextual signals received. Therefore, a dynamic equilibrium may exist in the conversion of CSCs to non-CSCs and vice versa, in a process controlled by the tissue microenvironment[106].

Notwithstanding the ongoing debates of CSC and clonal evolution theories, cancer is both a proliferation and differentiation disease and these two theories may not be mutually exclusive[108]. Recently, it has become increasingly accepted that the CSC hypothesis may not contradict the clonal evolution view of cancer, but instead suggests a key role for malignant cell hierarchy in tumour evolution and emphasizes the importance of aberrant differentiation programs in tumour-igenesis. Tumour heterogeneity could therefore be contributed by genetic instability of cancer cells, as suggested by the clonal evolution theory, as well as by aberrant differentiation, as proposed by the CSC theory[108].

Aberrant Signaling in CSCs and the Pathogenesis of Brain Cancers

It is now widely hypothesized that CSCs may arise from defects in lineage differentiation. Mutations seem to enable CSCs to "hijack" certain signalling pathways that are endogenous to normal stem cells. Indeed, aberrant activation in the Notch, hedgehog (HH) and WNT pathways, which are instrumental to the homeostasis of normal stem cells, are implicated in the development of brain cancers[109-117]. Cancer stem cells are believed to arise from undifferentiated or poorly differentiated cells that show enhanced self-renewal and thus limited differentiation, and as such, appear to be "trapped" in a perpetual state of cell proliferation. These progenitor cells may further acquire genetic mutations that subsequently facilitate malignant transformation[77].

Notch Pathway

There are four mammalian Notch proteins (Notch 1-4)[118-122] that bind their ligands: Delta-like-1, -3, and -4 (DLL1, DDL3 and DLL4)[123-125] and Jagged 1 and Jagged 2 (JAG1 and JAG2)[126,127]. The signaling is initiated by a ligand-receptor interaction between two adjacent cells, followed by two sequential cleavages of Notch, the receptor. The second cleavage liberates the cytoplasmic domain of the receptor, Notch intracellular domain (NICD), which subsequently translocates into the nucleus and binds to transcription factor CSL, leading to transcriptional activation of downstream target genes [reviewed by Radtke et al[128]].

Notch signaling positively regulates self-renewal of NSCs[129]. Notch pathway activation is not only essential to the maintenance of NSCs but also patient-derived BCSCs[130]. Notch signaling enhances nestin expression and may therefore have a contributing role in the stem-like characteristic of glioma cells[131]. Over-expression of Notch receptors and their ligands Delta-like 1 (DLK1) and Jagged 1 (JAG1) correlates with proliferative capacity of human glioma cells[111]. Inhibition of the pathway by RNA interference or γ-secretase inhibitor provides proof-of-concept of the potential in targeting Notch pathway for brain cancer treatment. A γ-secretase inhibitor, MK-0752 (www.merck.com), is currently evaluated in phase I clinical trial for treatment of T-ALL and advanced/metastatic breast cancers[108]. Pharmacological inactivation of the Notch pathway led to cell cycle exit, apoptosis and differentiation of medulloblastoma cell lines as well as depletion of the CD133+, stem-like cells[113], a result subsequently confirmed in GBM[132,133]. The therapeutic window for γ-secretase inhibitor is narrow due to its broad effects on multiple Notch signaling pathways and normal stem cells[108]. As a result, optimal dosing regimen will be required to avoid undesirable toxicity in normal stem cells. A recent study by Schreck et al suggests that a compensatory signaling via the WNT and hedgehog pathways may play a role in therapeutic resistance against Notch inhibitors. Simultaneous targeting of Notch and hedgehog pathways enhanced apoptosis and growth suppression compared to monotherapy[134].

Sonic Hedgehog (sHH) Pathway and BMI1

The sonic hedgehog pathway (sHH) pathway is initiated by binding of sHH (the ligand) to Patched (PTCH, the receptor). In the absence of pathway activation, PTCH exerts a negative regulatory control on Smoothened (SMO), a transmembrane protein. Upon ligand binding, the repression of SMO by PTCH is relieved and SMO can relay the signaling to downstream components of the sHH pathway. Glioma-associated oncogene (GLI), a downstream target of SMO, is released from the multiprotein complex formed by itself, Fused (FU) and suppressor of fused (SUFU) and enters the nucleus where it

functions as a transcription factor to regulate the expression of growth/angiogenesis-promoting genes such as cyclin D, cyclin E[135,136] and components of the EGFR, platelet-derived growth factor (PDGF) and VEGF pathways [reviewed by Di Magliano et al.[137]].

The secreted protein "sonic hedgehog" (sHH) and its downstream effectors glioma-associated oncogene homologue 1 (GLI1, GLI2 and GLI3) regulate neurogenesis and self-renewal within the external granular layer of the early postnatal cerebellum and control precursor cell proliferation in the adult sub-granular zone[138]. In brain cancer pathogenesis, sHH pathway modulates BCSC proliferation[139] and synergizes with the insulin-like growth factor (IGF) pathway in inducing the formation of medulloblastoma in nestin-expressing progenitor cells in mice[140].

Medulloblastoma, a disease that may be caused by aberrant self-renewal of neuronal progenitor cells in the external granular layer of the cerebellum, often (10-20%) show mutations in *PTCH1, SUFU* or Smoothened (*SMO*) which are components of the sHH pathway[141-144]. Molecular profiling of medulloblastoma classifies the disease into five different subtypes. Class B medulloblastoma is characterized by inactivating mutations of *PTCH1* or *SUFU*, the negative regulators of the sonic hedgehog pathways[145,146]. Results from these molecular profiling studies are highly predictive of patient outcome and may facilitate the development of subclass-specific, patient-tailored therapies.

One of the earliest studies in sHH pathway inhibitors was done by Berman et al. who demonstrated the efficacy of a SMO inhibitor, cyclopamine, in blocking medulloblastoma cell growth *in vitro* and inducing differentiation. Moreover, drug treatment resulted in a concomitant loss of "stem-like" characteristics in the neural progenitor cells and led to tumour regression *in vivo*[116]. Inhibition of the sHH pathway for cancer treatment is an area that has been actively pursued in the pharmaceutical industry. Cyclopamine, the first-in-class sHH pathway inhibitor, has been used for target validation[147] in pre-clinical studies in solid tumours and is also evaluated for its efficacy in basal cell carcinoma in phase I clinical trial[148]. GDC-0449, another SMO antagonist, is currently being assessed in phase II clinical trials for the treatment of basal cell carcinoma and metastatic colorectal cancer (Genentech, www.gene.com). IPI-926, a sHH pathway inhibitor, has been evaluated for the treatment of advanced/metastatic solid tumours in phase I clinical trials (Infinity Pharmaceuticals, www.ipi.com) [reviewed by Zhou et al.[108]].

Recent studies suggest that BMI1 is a key protein required for the hedgehog pathway-driven tumourigenesis[149] and is significantly up-regulated in human medulloblastoma[150]. BMI1, a member of the polycomb group of proteins, enhances cell proliferation and self-renewal of NSCs partly through the repression of tumour suppressor genes *p16INK4a* and *p19ARF*[151,152]. BMI1 and other polycomb group (PcG) proteins form a multi-protein complex, PRC1 (polycomb repressive complex 1) that epigenetically silences gene expression[153-157]. The recruitment of BMI1 to gene promoters is mediated by a number of transcription factors collectively known as "cell fate transcription factors" (CFTFs) for their role in regulating cell fate decision during embryogenesis and differentiation of adult stem cells[158]. Abnormal expression or activity of CFTFs may recruit BMI1 to promoters of genes that encode proteins involved in differentiation. BMI1 and other proteins in PRC1 may cooperatively silence the expression of genes that are required to promote differentiation. As a consequence, cells may be "locked" in a state of relentless self-renewal.

WNT Pathway

Another major self-renewal pathway implicated in brain cancer pathogenesis is the WNT/β-catenin pathway that controls adult neurogenesis[159-162]. This pathway is activated when wingless-type MMTV integration site family (WNT), the secreted ligand, binds to the cell surface receptors of the "Frizzled" (FRZ) family, leading to the activation of Dishevelled (DVL) and subsequent inhibition[163] of the multi-protein complex: axin/adenomatous polyposis (APC)/GSK3β that normally leads to the degradation of β-catenin. When the WNT pathway is "on", the axin/APC/GSK3β complex is inhibited. As a result, β-catenin accumulates and enters the nucleus to function cooperatively with TCF/LEF transcription factors in promoting the expression of specific genes [reviewed by Logan et al.[164]].

Molecular profiling classifies medulloblastoma into distinct subgroups with unique gene signatures. Class A medulloblastoma (WNT subtype) harbours mutations in *CTNNB1*, the gene that encodes β-catenin. Medulloblastoma is believed to arise within the cerebellum, with approximately 25% of the tumours (sHH subtype) originating from the granule neuron precursor cells (GNPCs) due to aberrant sHH signalling[145,146]. However, Gibson et al. recently showed that the WNT subtype of medulloblastoma arose outside of the cerebellum from cells in the dorsal brainstem, suggesting that different subtypes of medulloblastoma may have different cells-of-origin[165]. A clinical study conducted by the U.K. Children's Cancer Study Group Brain Tumour Committee enrolled 109 medulloblastoma patients to examine the relationship between β-catenin expression in tumours and patient survival. Curiously, the results indicated that nuclear accumulation of β-catenin was a marker of favourable outcome in medulloblastoma. Children with tumours that stained positive for nuclear β-catenin (27 of 109, 25%) showed better overall and event-free survival[166], a result subsequently confirmed by Rogers et al.[167].

Deregulation of the WNT pathway is observed in a subset of medulloblastoma[145,146,165,168-171]. Somatic mutations in *APC*, *GSK3b* and *CTNNB1* were identified in primitive neuro-ectodermal tumours (PNETs)[172]. Deletion[173] or mutation of *AXIN1*[168], a negative regulator of the WNT pathway, was reported in medulloblastomas. Overexpression of the components of the pathway, such as Dishevelled and β-catenin was also reported in human astrocytomas[174]. Together these results suggest that activating mutations of the components of the WNT pathway may contribute to the pathogenesis of PNETs and astrocytomas.

Therapeutic strategies targeting the WNT pathway have been investigated for the treatment of brain cancers. For example, small molecule inhibitor OSU03012 which targets PDK1, a protein in the PI3K/AKT pathway, was shown to inhibit WNT signalling by sequestering β-catenin in the cytoplasm. Furthermore, OSU03012 functioned synergistically with chemotherapeutic agents in inducing apoptosis in medulloblastoma cell lines and demonstrated anti-tumour effects

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in vivo[175]. In human glioma cells, the introduction of hDKK-1 (human DKK1), a gene that encodes a negative regulator of the WNT pathway, sensitized brain tumour cells to chemotherapeutic agents that causes DNA alkylation[176].

In addition to the aforementioned developmental signaling pathways, interestingly, concomitant deletion of tumour suppressor proteins *PTEN* and *p53* also promotes an undifferentiated state of cells with high self-renewal potential and elevated MYC expression[177]. Further analyses revealed a key role of MYC up-regulation in sustaining the stem-like state of cancer cells[177], which can be reversed by MYC knock-down[178].

Therapeutic Strategies Targeting BCSCs

Targeting Key Signaling Pathways with Small Molecule Inhibitors

Cancer is a disease of abnormal differentiation and if CSCs is indeed the root of disease recurrence, it would be reasonable to target aberrant self-renewal pathways in the treatment of brain cancers. Cyclopamine, an inhibitor of smoothened (SMO), provides a nice proof-of-principle of the effectiveness of targeting sHH pathway in the treatment of medullo-blastoma[116,179]. GDC-0449 and LDE-225 are examples of hedgehog pathway inhibitors that are currently being evaluated in phase I/II clinical trials in solid tumours and medulloblastoma respectively[180-182]. However, treatment efficacy can be hampered by drug resistance developed as a result of activating mutations in the serpentine receptor Smoothened (*SMO*) or chromosomal amplification of *GLI2* in the pathway[183,184]. Research is underway to examine resistant mechanisms in these hedgehog inhibitor-refractory diseases. In one study, analyses of gene expression signatures indicated a role of the PI3K pathway in mediating drug resistance in medulloblastoma. The combinatorial treatment of PI3K inhibitor NVP-BKM120 or the dual PI3K/mTOR (mammalian target of rapamycin) inhibitor NVP-BEZ235 with SMO antagonist markedly delayed the development of drug resistance[185]. In another study, simultaneous inhibition of the Notch and sHH pathways eliminated GBM-derived cells much more effectively than monotherapy did[134]. Rational drug combinations targeting multiple developmental pathways may thus offer more significant benefit. Resveratrol is an inhibitor of the WNT pathway; a recent study by Yang et al. demonstrated the efficacy of resveratrol in suppressing tumourigenicity and enhanced radiosensitivity of primary GBM TICs by inhibiting the STAT3 signaling axis[186].

Differentiation Therapy

Aggressive brain tumours often include a large proportion of immature, primitive cells that appear to be permanently "locked" in a state of de-differentiation, which causes them to proliferate uncontrollably. An alternative therapeutic strategy is therefore to promote differentiation of these CSCs. By treating GBM CSCs with bone morphogenetic protein 4 (BMP4), Piccirillo et al. were able to decrease proliferation and induce differentiation of CSCs *in vitro*. BMP4 treatment increased tumour latency and prolonged animal survival. *In vivo* serial passaging was unachievable due to the depletion of CSCs by BMP4[187].

Consistent with this, BMP7 released from endogenous neural precursor cells can act as paracrine tumour suppressors to inhibit proliferation, self-renewal and tumour-initiation of stem-like GBM cells[188]. However, if BMP were to be used in clinical setting, it would be important to consider the status of BMP receptor status in patients. Lee et al. noticed that a subset of GBM patients were refractory to BMP4 treatment and curiously showed a paradoxical increase in tumour cell proliferation. These BMP4-resistant cells were subsequently found to express low levels of BMP receptor 1B as a result of epigenetic silencing and could be re-sensitized to BMP4-induced differentiation by ectopic restoration of BMP receptor[189].

Our laboratory has studied the role of a transcription factor named Y-box binding protein 1 (YB-1) in brain cancers. Knock-down of YB-1 by siRNA not only decreased cell proliferation, clonogenicity and invasion *in vitro* but also delayed the onset of tumour formation *in vivo*. Moreover, YB-1 knock-down enhanced the apoptotic effect of temozolomide (TMZ) in adult and pediatric GBM[190]. Further investigation revealed that YB-1 is a critical factor regulating the differentiation of NSCs. YB-1 expression is high in normal NSCs and dramatically decreases when the cells undergo lineage differentiation. In GBM tumours where cells do not undergo normal process of differentiation, the expression of YB-1 is elevated, corresponding to a higher level of cellular proliferation as evidenced by Ki67 staining. Knock-down of YB-1 suppressed the expression of stem cell markers SOX2, musashi and BMI1 and intriguingly, forced GBM brain cancer cells to acquire a more differentiated, astrocytic morphology, accompanied by increased expression of differentiation marker, GFAP[191]. Although thus far there is a lack of small molecule inhibitor targeting YB-1, we have developed in house YB-1 cell permeable peptides that eliminate breast cancer cells. Research is underway to determine if the peptides are equally effective in brain cancer cells.

Eliminating CSCs by Oncolytic Viruses

Additional methods have been designed to target CSCs. With the use of oncolytic herpes simplex virus (oHSV), Wakimoto et al. were able to inhibit self-renewal and killed GBM-derived CSCs *in vitro/vivo*[192]. A recent study on novel virus-gene therapy involved oncolytic virus carrying exogenous endostatin-angiostatin fusion gene (vae) which not only infected and killed glioma stem cells but also inhibited the proliferation of human brain microvascular endothelial cells in the CSC niche[193]. The combination of oHSV and PI3K/AKT inhibitors synergistically induced apoptosis of GBM CSCs but not human astrocytes and prolonged the survival of animals. This therapeutic strategy might also effectively target medulloblastoma CSCs that reside in the perivascular niche and exhibit radio-resistance due to enhanced PI3K/AKT signaling[194]. In another study, Seneca Valley Virus-001 (SVV-001) administered systemically passed the blood-brain-barrier (BBB) and killed the primary xenograft medulloblastoma cells, infected the CD133+ cells and eliminated tumour cells capable of neurosphere formation.

CONCLUSION

Brain cancer stem cells appear to be a Janus-faced entity-looking into the past and future of a perverted path of cell

development and encompassing characteristics of both. Ongoing research into its developmental intricacies and molecular signatures will no doubt help to unravel the biological underpinnings of these cells in brain cancer. Ultimately, it will lead to the development of precise diagnostic tools and more efficacious, targeted therapeutic agents.

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