

# Promises and challenges of exhausting pediatric neural cancer stem cells

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Cancer relapse is one of the major setbacks in pediatric oncology. Cancer stem cells (CSCs) have emerged as a major driving force governing tumor recurrence. CSCs are a small subpopulation of cells capable of regenerating a tumor and are resistant to conventional anticancer therapies. No CSC therapy has been approved by the US Food and Drug Administration. Because CSCs and normal stem cells share many characteristics, CSC-directed therapies have potential detrimental effects on normal stem cells, tissue maintenance, and development. Designing treatments that specifically target neural CSCs while allowing neural tissue stem cells to normally develop the brain is a major challenge in pediatric neuro-oncology. In recent years, better identification and characterization of neural CSCs, together with identifying differences between CSCs and normal neural stem cells, have been key factors in developing tailored therapeutics for these devastating diseases. This review focuses on the promises and challenges of pediatric neural CSC-directed therapies. We delineate the options currently in use to exhaust the ability of neural CSCs to self-renew. Finally, we suggest a comprehensive approach to combine anti-CSC therapies with other therapeutic approaches to prevent tumor recurrence.

Cancer is still the leading cause of nonaccidental death in children (1). Despite significant improvement in survival in some pediatric cancers, which has been achieved by combining chemoradiation approaches (2), a significant percentage of children (20–90% depending on tumor type) will still succumb to their disease. The main cause of death in these children is tumor recurrence. Relapse can occur even when the initial response to therapy results in no evidence of observed disease. The ability of small groups of cells to withstand chemoradiotherapy and subsequently enable tumor regrowth remains a mystery. A major concept that has emerged to explain tumor recurrence is the existence of cancer stem cells (CSCs) (3,4). In this review, we outline the fundamentals of the CSC hypothesis, its role in pediatric neural tumors, and potential approaches to target this cell population. We further discuss the challenges of

CSC inhibition and suggest a more comprehensive approach for the treatment of pediatric neural tumors.

## IDENTIFICATION OF CSCs IN ADULT AND PEDIATRIC TUMORS

Human organs and their corresponding neoplasms are closely related. Both are organized as hierarchical cell populations where stem cells are responsible for self-renewal and differentiation. Thus, stem cells are responsible for the continuous maintenance and growth of both normal tissues and, unfortunately, tumors.

The definition of CSCs (also referred to as tumor-initiating cells, tumor progenitors, or tumor-propagating cells) is controversial and beyond the scope of this review. Here, we will define CSCs as a subpopulation of tumor cells capable of maturing into their lineage and tissue-specific tumor type. CSCs must be able to regenerate a phenotypically heterogeneous neoplasm, mimicking the original tumor, after serial orthotopic transplantations *in vivo*.

The concept of the CSC is an old one and postulates that tumors arise from cells that share hallmark properties with tissue stem cells or their direct progeny through aberrant regulation of self-renewal pathways. Consequently, tumors contain a cellular component that preserve stem cell properties (5). Support for this concept came from early observations that cancers demonstrate morphological heterogeneity (4), that a small subpopulation of cells (down to a single cell) is able to generate tumors in mice (6), and that in some tumors (such as teratocarcinomas) cells can mature into different tissues (7). However, only in recent years has better technology enabled the purification, identification, and propagation of CSCs *in vivo* and *in vitro*. The first breakthrough emerged in the early 1990s when CD34<sup>+</sup>/CD38<sup>-</sup> cells were isolated from human acute myeloid leukemias and shown to initiate similar tumors in NOD/SCID mice (8). Since then, CSCs have been described in multiple tumor types including brain tumors (9), prostate (10), breast (11), colon (12), pancreas (13), liver (14), lung (15), ovary (16), and skin (17).

In contrast to adult tumors, there are relatively few pediatric tumors in which CSCs have been isolated. Pediatric CSCs have thus far been described in leukemias (18), medulloblastomas (19,20), malignant astrocytomas (20), ependymomas (21,22), and neuroblastomas (23). Possible explanations for this discrepancy include a lack of mature markers in pediatric

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cancers, which makes it difficult to sort these cells. Indeed, the majority of pediatric tumors are of embryonic origin, whereas adult cancers are mostly derived from differentiated epithelial tissues, which express more markers of maturation. These differences in tumor biology pose a great challenge in the identification and characterization of pediatric CSCs. Because most pediatric CSCs described to date are of neural origin, we focus our review on these cancers.

**TUMOR RECURRENCE AND THE CSC HYPOTHESIS**

Pediatric tumors generally respond better to treatment than adult tumors. The introduction of multimodal therapy (surgery, radiation, and chemotherapy) has resulted in an extremely high rate of initial tumor response. Specifically, in most leukemias and malignant neural tumors (ependymomas, medulloblastomas, and some malignant gliomas and neuroblastomas), no tumor can be seen at the completion of multimodal therapy in up to 80% of children (24,25). Unfortunately, a significant percentage of these tumors will recur. The conventional thinking regarding this phenomenon was that resistant clones were emerging in unstable cancer cells carrying additional mutations that enabled them to survive the insults of specific therapies (26–28). This concept is supported by the more aggressive behavior of recurrent tumors and their tendency to respond less to therapy. However, the observation that relapsed clones are already present as a small part of the initial tumor (29), which responds to treatment but then recurs, suggests an alternative explanation. The CSC hypothesis, which states that a slow-growing stem cell-like clone is feeding the tumor, fits perfectly with all these findings.

CSCs are insensitive to general metabolic and DNA damage agents such as chemotherapy (30) and radiation (31). Therefore, although the overwhelming majority of tumors cells will die as a result of conventional therapies, CSCs will survive and slowly give rise to recurrent tumors. Support for this concept comes from the correlation between better survival and tumors that contain more mature cells (32). It has also been suggested that tumors containing more CSCs are more aggressive and refractory to therapy (33,34).

Several studies have demonstrated that adult and pediatric neural CSCs are more resistant to cancer therapies than the bulk of tumor cells. In glioblastomas, CSCs contribute to tumor radioresistance because of higher activation of the DNA damage checkpoint proteins ATM and Rad17 in response to radiation. Moreover, CSCs repair DNA damage more efficiently as assessed by the resolution of phosphorylated histone 2AX nuclear foci upon irradiation (31).

In addition, adult glioblastoma CSCs are more resistant to chemotherapeutic agents such as carboplatin, paclitaxel, etoposide, and temozolomide (35,36). The suggested mechanism is overexpression of antiapoptotic factors such as FLIP, BCL-2, and BCL-XL, drug-resistance genes such as BCRP1 and ABCA3, and DNA-repair genes like MGMT, combined with down-regulation of key death effectors to escape the effects of the cytotoxic drugs (35–37). In neuroblastoma cells, treatment with chemotherapies such as cisplatin and doxorubicin results in enrichment of a highly tumorigenic side population of cells

(38) that is capable of forming spheres (39), mimicking CSC behavior. It has therefore been postulated that chemotherapy and radiotherapy increase the percentage of CSCs in a tumor because of their resistance to cancer therapies (36,40).

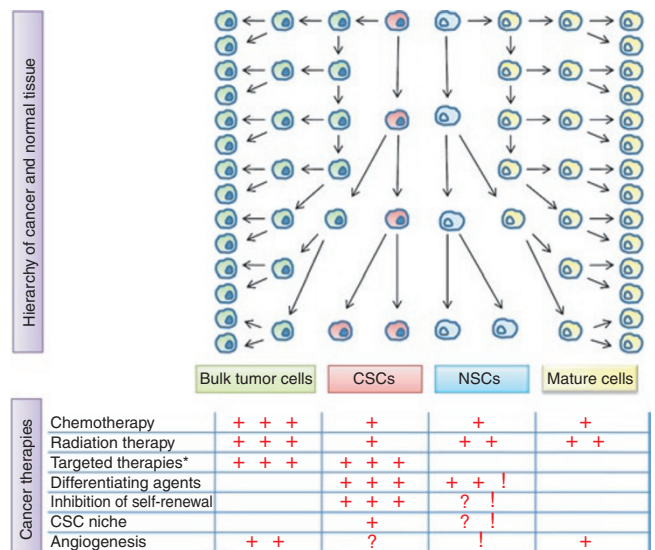
In summary, according to the CSC hypothesis (Figure 1), conventional therapies do not exhaust the CSC subpopulation, and thus additional CSC-specific therapies are required to prevent tumor relapse, even when no evidence of disease exists at the completion of therapy.

**NORMAL STEM CELLS AND CSCs**

Normal tissue stem cells nurture and maintain normal tissues. For children, it is of critical importance to ensure that no harm is done to these cells to ensure normal development and growth.

It is therefore challenging to use anti-CSC therapies that might be nonspecific and have detrimental effects on tissue maintenance and development (Figure 1). This is of particular relevance in pediatric neuro-oncology because therapies toxic to the developing brain cause irreversible and continuous loss of cognition and changes in other aspects of human behavior (41).

Pediatric neural stem cells share with neural CSCs the ability to self-renew and differentiate into neural and glial phenotypes. These two populations of cells share the expression of specific genes including CD133, musashi-1, Sox2, melk, PSP, BMI1, and nestin. This makes distinction and separation of cells difficult both *in vitro* and *ex vivo* (20).



**Figure 1.** Hierarchical scheme of malignant and normal cells and potential therapies. Suggested comparison between different therapeutic options and their efficacy according to the hierarchy of cancer stem cells and normal tissue stem cells. To achieve cure, one must achieve complete tumor remission as well as cancer stem cell exhaustion while sparing normal mature and stem cells. The figure illustrates that only combinational therapies can achieve this complex goal. Green, mature tumor cells; pink, cancer stem cells (CSCs); blue, normal stem cells (NSCs); yellow, mature cells. \*Therapies that target pathways specific for cancer. (+) Represents comparative efficacy of a therapeutic modality against each respective cell population. (?) Unknown effect of the therapeutic agent. (!) The use of the therapeutic agent requires caution because of the potential detrimental effect on normal cells.

Nevertheless, there are several differences between cancer and normal stem cells that can be exploited. Normal neural stem cells are more sensitive to radiation than their malignant counterparts. This may be explained by the inactivation of important checkpoints and the MRN-ATM DNA damage response network in CSCs (42). Moreover, the presence of areas of hypoxia (43) is crucial for the maintenance of glioma CSCs (44) and provides protection against radiotherapy (45). Therefore, certain radioprotective agents may be specific to normal neural stem cells and might enable the use of higher doses of radiation to treat CSCs.

Importantly, pediatric neural CSCs, by virtue of their oncogenic transformation, gain aberrant activation of several pathways that control proliferation, self-renewal, and differentiation. This constitutive activation of oncogenes may make CSCs more susceptible to certain targeted therapies. A perfect example is the Sonic Hedgehog (SHH) oncogenic pathway, which is highly expressed in 35% of medulloblastomas (46). CSCs from these tumors are exquisitely sensitive to SHH inhibitors (47). Moreover, the SHH downstream effector GLI1 was reported to be highly expressed in glioblastoma CSCs (48).

Another illustration that highlights the differences between normal and malignant stem cells is the observation that telomerase activation is specific and critical for neuroblastoma and glioblastoma CSCs but is not required by normal neural stem cells (49). These differences could be used as an Achilles' heel to treat CSCs while preserving the normal brain.

## CSC THERAPIES

Since the turn of the millennium, significant progress in our understanding of the genetic mechanisms governing pediatric neural tumors has been gained (21,22,46,50–54). Unfortunately, this knowledge has not resulted in a change of therapeutic approach and there is a lack of new agents for the treatment of these cancers. Only a few new agents have been approved for general use (55–57), and for some of these, such as temozolomide and bevacizumab, the results in children have been disappointing (58,59). We will outline here some of the CSC-specific novel approaches and preclinical experiments that will hopefully lead to new therapies for children with neural tumors. As outlined above, most of the preclinical studies were done on adult CSCs because of the paucity of pediatric CSC models. We therefore specify studies that used pediatric CSC platforms.

### Inhibition of Specific CSC Signaling

Signaling pathways that control cell differentiation and proliferation, motility, cell survival, and apoptosis during embryonic development have been the subject of intensive studies for developing new therapeutic targets. Aberrant activation of the Notch, Wnt/ $\beta$ -catenin, and SHH pathways have been reported in several pediatric neural tumors including neuroblastomas, ependymomas, medulloblastomas, and primitive neuroectodermal tumors (60). Inhibitors of these pathways are therefore attractive targets for these tumors.

Gamma-secretase inhibitors, which block the Notch pathway, have been described as depleting stem-like cells and

preventing engraftment in pediatric medulloblastomas (61) and glioblastomas (62).

Clinical trials of SHH pathway antagonists are already underway in medulloblastoma (GDC-0449 and LDE225) (63). They have also been shown to have encouraging, although not sustained, results in relapsed cases (47). In malignant gliomas, cyclopamine, which specifically inhibits the membrane protein Smoothed, has been shown to deplete glioma CSCs (48) and, in combination with temozolomide, has shown the ability to suppress CSC proliferation (64). Further work is needed to elucidate whether SHH inhibitors will have a specific and sustained effect on neural CSCs.

CSCs also activate other signaling molecules that could potentially be targeted for CSC exhaustion. Transforming growth factor- $\beta$  has been shown to play multiple roles in gliomagenesis, including increasing CSC self-renewal capacity through induction of the leukemia inhibitory factor (65). Therefore, inhibitors of the transforming growth factor- $\beta$  pathway have been tested for their ability to prevent tumor proliferation, intratumoral angiogenesis, and metastasis. SB-431542 (66) and A-78-03 (67) are compounds that act as inhibitors of the activin receptor-like kinase 5 (transforming growth factor- $\beta$  type I receptor) and induce differentiation of glioma CSCs, thereby diminishing their tumorigenicity (68).

Abnormal regulation of the cytokine interleukin-6 signaling has been associated with poor survival in glioblastoma patients (69), making this molecule an attractive therapeutic target. Using short hairpin RNAs (shRNAs) that recognize the interleukin-6 receptor- $\alpha$  or the interleukin-6 ligand reduced the ability of glioma CSCs to form neurospheres and increased apoptosis while enhancing animal survival in a glioma xenograft model (70).

Hyperactivation of the phosphatidylinositol-3-kinase-AKT pathway is a key alteration in glioma tumorigenesis that promotes cell proliferation, invasion, and angiogenesis. Akt pathway activation has also been described as being responsible for the conversion of anaplastic astrocytomas into glioblastoma multiforme (71). Phosphatidylinositol ether lipid analogs such as the AktIII/SH-6 were shown to be potent Akt inhibitors and able to reduce neurosphere formation and increase apoptosis of glioma CSCs *in vitro*. *In vivo*, Akt inactivation resulted in increase survival of mice bearing human glioma tumors (72).

The antiapoptotic protein A20, or tumor necrosis factor- $\alpha$  inducible protein 3 (TNFAIP3), is a regulator of the nuclear factor- $\kappa$ B pathway that is overexpressed in glioma CSCs and its levels show an inverse correlation with patient survival. Lentiviral-mediated delivery of shRNA targeting A20 resulted in reduced glioma CSC self-renewal capacity and increased animal survival (73).

Bone morphogenetic proteins play a role in the adult brain stem cell niche. Their use as therapeutic agents against glioma CSCs was established when bone morphogenetic protein-4 treatment was shown to reduce the CSC population in glioblastomas and block tumor growth *in vivo* (74).

Interestingly, several pathways that were thought to be specific to one type of cancer may serve as targets for CSC exhaustion in other tumors.

The Myc oncoproteins are highly amplified or constitutively expressed in pediatric lymphomas, neuroblastomas, and medulloblastomas. Interestingly, overexpression of c-Myc has been correlated with a higher histological grade in gliomas (75). c-Myc was described to be highly expressed in glioma CSCs relative to the bulk of tumor cells, thereby suggesting a role in CSC proliferation and survival. Knockdown of c-Myc using shRNAs showed reduced glioma CSC proliferation, increased apoptosis, and cell cycle arrest in the G<sub>0</sub>/G<sub>1</sub> phase. Moreover, downregulation of c-Myc in the CSC population resulted in the inability to form neurospheres or tumors *in vivo* (76).

Polycomb group proteins regulate gene expression through modifications in chromatin structure. The polycomb group gene BMI1 plays a role in proliferation of cerebellar precursor cells and was shown to be overexpressed in pediatric medulloblastoma (77). More recently, BMI1 was found to be highly enriched in glioblastoma CSCs and its downregulation resulted in inhibition of clonogenic ability and tumor formation (78).

Although described in other tumors, it was recently demonstrated that the inhibitor of polo-like kinase 1, BI 2536, inhibited pediatric neuroblastoma CSC tumor growth in a therapeutic xenograft tumor model (79).

The search for cell surface markers that specifically target neural CSCs is a promising avenue for the identification of new molecular targets. These proteins may enable us to block their function or to specifically deliver toxic therapies to CSCs. L1CAM is a neural cell adhesion molecule expressed in the nervous system and described to play a role in adhesion, migration, and invasion of glioma cells (80). Recent studies have shown that the levels of L1CAM are higher in the glioma CSC population than in normal neural progenitors. Also, downregulation of L1CAM through shRNAs resulted in growth inhibition, disrupted neurosphere formation, and induced apoptosis in the CSC population. Moreover, treatment of established tumors with L1CAM shRNAs suppressed tumor growth and increased animal survival (81).

### Induced Differentiation

Retinoic acid modulates cellular proliferation and differentiation and has been successful in treating patients with acute promyelocytic leukemia and pediatric neuroblastoma. In glioblastoma, it was recently shown to sensitize glioma CSCs to therapies while reducing migration, angiogenesis, and tumorigenicity (82).

The dual phosphatidylinositol-3-kinase/mTor inhibitor NVP-BEZ235 was shown to induce differentiation of glioblastoma CSCs and promote a significant decrease in their tumorigenicity (83).

Regulation of gene expression through epigenetic mechanisms also plays an important role in oncogenesis. The Delta/Notch-like epidermal growth factor-related receptor is a gene product that is induced by histone deacetylase inhibition and has been shown to impair the growth of glioblastoma-derived neurospheres, induce their differentiation, and prevent their engraftment *in vivo* (84).

Moreover, treatment of pediatric ependymoma CSCs with the histone deacetylase inhibitor Vorinostat was shown to

induce neuronal differentiation and reduce neurosphere initiation capability (85).

Similar networks of transcription factors are necessary for the regulation of both normal tissue stem cells and CSCs. KLF9 belongs to the Kruppel-like family of transcription factors and was found to be uniquely upregulated in response to differentiation signals. Expression of KLF9 inhibits tumor growth in mice bearing glioblastoma-derived neurospheres (86).

### Inhibition of Self-Renewal

Growing evidence suggests that CSCs have aberrant or constitutively active self-renewal pathways that are controlled by genetic or epigenetic mechanisms and that lead to unrestrained proliferation.

Telomere maintenance is a hallmark of cancer and governs self-renewal in malignant cells (87). Our group and others have demonstrated that both adult and pediatric neural CSCs are addicted to telomerase for their self-renewal (88), whereas normal pediatric neural stem cells lack this dependency. This observation opens avenues for the safe use of telomerase inhibitors in pediatric tumors. In addition, telomerase inhibition by the specific inhibitor imetelstat resulted in proliferation arrest, cell maturation, and DNA damage in pediatric neural CSCs. Strikingly, CSCs exhibited irreversible loss of self-renewal and stem cell capabilities even after cessation of treatment *in vitro* and *in vivo* in a neuroblastoma model (49). This unique observation may indicate that these CSCs have lost the ability to cause tumor recurrence.

CSC exhaustion with telomerase inhibition may be a prototype of a selective CSC therapy that is protective of normal stem cells. We have shown that telomerase is not required for neural, mesenchymal, and neural crest stem cells, but this may not be the case for other organs such as the hematopoietic system. It will be important to demonstrate that such systemic therapies are not detrimental to other normal stem cells.

### The Role of CSC Exhaustion in the Treatment of Pediatric Neural Tumors

Collectively, the data presented above raise hope for better targeting and inhibition of CSCs in pediatric neural tumors. The concepts reviewed also highlight the challenges of such therapies. It should be emphasized that therapies focusing exclusively on CSC exhaustion will not be sufficient to achieve substantial curative results for several reasons. First, without significant tumor reduction, children bearing neural tumors will succumb to tumor burden even before tumors recur. Second, because therapies directed against mature tumor cells seem to be ineffective against CSCs, specific CSC-directed therapies may not be effective on the bulk of tumor cells. Finally, knowledge arising from studies on tumor and stem cell niches and metastases will be fundamental for the design of successful cancer therapies.

Nevertheless, the weight of evidence suggests that to prevent tumor recurrence, CSCs must be exhausted. We suggest that to accomplish this task, it will be necessary to induce cancer remission and to then treat CSCs when minimal residual disease is achieved. Indeed, the optimal timing of CSC-directed

therapies may be as maintenance therapy after the initial phase. This will facilitate CSC exhaustion by allowing longer time and better delivery of therapies directed to the CSC subpopulation.

## CONCLUSION

Over the past two decades, the CSC hypothesis has matured into a major driving force in the field of tumor relapse, perhaps the most devastating event in childhood cancer. New therapies that specifically target CSCs are therefore of great relevance for the field of pediatric oncology. Exploiting the differences between normal and malignant stem cells should result in better protection of normal tissues and thus avoid unacceptable long-term consequences to the developing nervous system and other vital organs. In our opinion, given the heterogeneous nature of pediatric neural tumors, a comprehensive approach should encompass combinations of different modalities (see [Figure 1](#)) to treat the bulk of tumor cells, the CSC subpopulation, and the CSC niche.

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