Targeting cancer cells with stem cell-like properties: the key to preventing recurrence in neuroblastoma?

"Identifying and killing these underlying culprit cells may be crucial to finding more effective treatments."

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The practice of pediatric oncology is at once exhilarating, perplexing and frightening. On the one hand, medical science has advanced to the point where we can actually cure cancer in some cases; nothing could be more exhilarating. Using combinations of chemotherapy, radiation, and surgery, so-called 'conventional' therapy, we are able to achieve long-lasting cures for the vast majority of patients who are diagnosed with cancers such as childhood acute lymphoblastic leukemia, Wilms kidney tumor, Hodgkin's disease, localized sarcomas, and low-risk neuroblastoma. Unfortunately, we have not been so successful with many other childhood cancers such as acute myeloblastic leukemia, metastatic sarcomas, and high-risk neuroblastoma (age >18 months, *MYCN* amplification, unfavorable histology and advanced stage of disease). What is perplexing is that in most cases these diseases do respond to conventional treatment, and most patients achieve remission – their disease is no longer detectable. In these cases the cancer usually returns without warning, any time, even years later. Patients and their families are constantly on edge, living in fear of relapse.

Until recently, medical science has never been able to satisfactorily explain cancer relapse. How is it that we can kill more than 99% of cancer cells but not 100%? It has been assumed that a few cells are able to survive the treatment and re-grow. Did such cells pre-exist in the tumor, or did they develop resistance during the course of treatment? Either way, what is different about those few cells compared with all the others? If a few cancer cells can become resistant to treatment, why don't all cancer cells become resistant? If treatment selects for resistant cells, how is it possible that some patients can be retreated and achieve a second remission, sometimes with the same drugs that were used originally? And finally, why is it that chemotherapy alone is usually not sufficient to cure solid tumors: patients also need good 'local control' using surgery and/or radiation at the site of any bulky disease.

The answers to these questions may lie in the idea of cancer stem cells, which is a new, exciting and potentially far-reaching concept in cancer biology. Normal stem or progenitor cells are known to reside in most if not all normal organs, giving rise to the differentiated 'bulk' cells of the organ. In an analogous way, cancer stem cells are postulated to be the seeds that generate the tumor bulk, most of which is comprised of cells that are partially differentiated and no longer capable of forming tumors themselves. There has been considerable controversy regarding the origin and nature of such cancer stem cells, also known as tumor-initiating cells, and scientists now admit that referring to them as stem cells may be misleading as they do not necessarily exhibit properties of normal stem cells [1]. Regardless of the nomenclature, the fact that most leukemia cells are not tumorigenic and that a subpopulation of self-renewing, tumor-forming cells exists in leukemia is now well established. Mounting evidence suggests that cancer stem cells also lie at the heart of many solid tumors, including neuroblastoma. Identifying and killing these underlying culprit cells may be crucial to finding more effective treatments.

Evidence that neuroblastoma is a cancer stem cell disease

While the heterogeneous nature of neuroblastoma was described long ago, the importance of this phenomenon has not yet been elucidated. Neuroblastoma is thought to arise from cells embryonically fated to contribute to the developing neural crest. Indeed, histopathologic assessment of a large panel of neuroblastoma samples noted that tumors from patients with poor prognoses showed immature cells with enhanced mitotic index and capable of nodule formation [2]. Later it was elegantly shown that neuroblastoma cultures were comprised of morphologically distinct cell populations and that 'I' subtype cells possess increased

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tumor-forming ability in immunodeficient mice [3]. I-type cells expressed stem cell markers (CD133, c-kit), differentiated in the presence of retinoic acid, formed colonies in soft agar and, most importantly, showed increased frequency in tumors from relapse patients by immunohistochemistry [3]. Indeed, the incorporation of retinoic acid therapy has improved survival of patients with high-risk disease [4].

Recent molecular studies from our laboratory have suggested that human neuroblastoma cell lines contain cells capable of multiple stem cell properties including expression of known neural stem cell markers, clonal growth as nonadherent neurospheres, pluripotency, chemoresistance, asymmetric cell division and, most importantly, increased tumorgenicity [5]. Examination of cell surface marker expression in these cell lines revealed variable yet significant expression of the stem cell markers CD133, CD34 and the neural stem cell marker nestin. Upon culturing neuroblastoma cells at clonal densities in serum-free conditions, supplemented with EGF and basic FGF, tumorspheres were readily apparent in a similar fashion to neural stem cells growing in similar conditions that form neurospheres. Tumorsphere formation was dependent upon both notch and EGF receptor signaling in a similar fashion to *bona fide* neural stem cells. After placing neuroblastoma cells from clonally derived tumorspheres into appropriate culture conditions, we observed differentiation of these cells along fibroblastic, gliogenic and neurogenic lineages. Experiments to assess chemoresistance demonstrated that tumorsphere-derived cells were more resistant to doxorubicin than bulk culture-derived cells. The sphere-derived cells also demonstrated increased expression of the drug efflux channel ABCG2. In efflux studies, neuroblastoma cell lines contained a 'side population' (SP) of cells capable of effluxing Hoechst dye, a well-described characteristic of hematopoietic stem cells. These studies also revealed asymmetric cell division in that cultures derived from SP cells could regenerate both SP and non-SP cells; however, non-SP cells could not generate their counterpart SP cells. Finally, neuroblastoma cells that express the stem cell marker CD133 demonstrated increased tumorsphere formation and tumorgenicity in immunodeficient mice. Taken together, these studies support previous findings that neuroblastoma cultures contain cells with stem cell properties.

While our results suggest a stem cell origin for neuroblastoma, other studies suggest more complexity. Primary neuroblastoma samples isolated from patients' bone marrow are highly tumorigenic and grow as tumorspheres, though they do not possess an identifiable SP in efflux studies or express CD133 [6]. It is possible that neuroblastoma cells residing in the bone marrow cavity have been reprogrammed to no longer express CD133 or represent a different subset type of tumor-initiating cells. As others have also noted, the prospective identification of cancer stem cells by marker expression alone may be misleading due to cytoplasmic versus membrane-localized protein and/or plasticity in cell surface marker expression. Functional assays will be required to fully identify neuroblastoma-initiating cells, since cells may differ in marker expression according to their location or clinical setting. Furthermore, incorporation of multiple selection criteria may be required to ensure culture stability. In summary, these results highlight the heterogeneous composition of neuroblastoma and suggest the existence of a subpopulation of pluripotent, tumorigenic cells. Whether or not such cells reside in a geographic or functional niche within the tumor, similar to a normal stem cell niche, is unknown.

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Targeting cancer stem cells: theory & practice

While the true relevance of cancer cells with stem-cell like properties in disease progression and patient survival has yet to be determined, it is likely that these cells are involved in chemoresistance, invasion and metastasis. Therefore, it will be important to target therapy to such cells, without unacceptable side effects for normal stem cells. Cancer cells with stem cell properties may be targeted via multiple nonoverlapping strategies, including enhancing chemosensitization, induction of differentiation, directing therapy toward stem cell surface markers and utilizing inhibitors of signaling pathways required for stem cell maintenance. For instance, strategies to increase chemosensitivity could be achieved by combination of conventional chemotherapy and/or irradiation with efflux-blocking molecules or inhibitors of DNA repair pathway proteins. Targeting current chemotherapy using liposomes that target stem cell receptors and cell surface markers could be employed to increase local concentrations of such agents in the tumor stem cell

niche. Small molecules such as retinoic acid or novel strategies utilizing gene therapy to target and downregulate transcription factors such as Oct-4 or Bmi-1 could be potential strategies to modulate tumor cell differentiation. Inhibiting signaling pathways important for tumor stem cell maintenance, such as notch, wnt and EGF could be achieved using specific small molecule inhibitors. Some have suggested that a niche for cancer stem cells might exist, perhaps in a perivascular location [7], raising the possibility that the niche itself could be a target. Finally, utilization of agents that are cytotoxic for tumor stem cells will be important to destroy these cells instead of merely slowing their division via cytostatic therapies. Examples of cytotoxic agents could include biologics such as cancer cell-killing 'oncolytic' viruses or immunomodulatory strategies to direct the immune system to selectively destroy cancer stem cells.

Combinatorial therapy to simultaneously target multiple tumor-promoting pathways is the future of cancer therapy, so it will be important to also target stem cell properties. Such strategies to combat tumors on various fronts using chemotherapy, irradiation, targeted small molecules, gene therapy, cell therapy, immunotherapy and biologics may be the key to extending progression-free survival for patients with high-risk neuroblastoma.

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Bibliography

1 Jordan CT: Cancer stem cells: controversial or just misunderstood? *Cell Stem Cell* 4(3), 203–205 (2009).

2 Shimada H, Chatten J, Newton WA Jr *et al.*: Histopathologic prognostic factors in neuroblastic tumors: definition of subtypes of ganglioneuroblastoma and an age-linked classification of neuroblastomas. *J. Natl Cancer Inst.* 73(2), 405–416 (1984).

3 Walton JD, Kattan DR, Thomas SK *et al.*: Characteristics of stem cells from human neuroblastoma cell lines and in tumors. *Neoplasia* 6(6), 838–845 (2004).

4 Matthay K, Villablanca J, Seeger R *et al.*: Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group. *N. Engl. J. Med.* 341, 1165–1173 (1999).

5 Mahller YY, Williams JP, Baird WH *et al.*: Neuroblastoma cell lines contain pluripotent tumor initiating cells that are susceptible to a targeted oncolytic virus. *PLoS ONE* 4(1), E4235 (2009).

6 Hansford LM, McKee AE, Zhang L *et al.*: Neuroblastoma cells isolated from bone marrow metastases contain a naturally enriched tumor-initiating cell. *Cancer Res.* 67(23), 11234–11243 (2007).

7 Calabrese C, Poppleton H, Kocak M *et al.*: A perivascular niche for brain tumor stem cells. *Cancer Cell* 11(1), 69–82 (2007).