

Insights into the Cancer Stem Cell Model of Glioma Tumorigenesis

Carol Tang,¹*PhD*, Constance LM Chua,¹*BSc*, Beng-Ti Ang,²*MBBS, FRCSed (SN)*

Abstract

Not all cancer cells are born equal. While the great majority of the cells that make up tumours are destined to differentiate, albeit aberrantly, and eventually stop dividing, a handful of cancer cells appear to possess limitless replicative potential. This review presents compelling evidence to suggest that the bulk of malignant cells of most cancers are generated by a rare fraction of stem cell-like cancer cells. These cells, dubbed cancer stem cells, are phenotypically similar to the normal stem cells of the corresponding tissue of origin, but they exhibit dysfunctional patterns of self-renewal and differentiation. Cancer stem cells that are capable of recapitulating brain tumours as xenografts in mice are characterised by defined stem cell markers. These brain tumour stem cells demonstrate enhanced chemoresistance and radioresistance mechanisms compared to non-stem cells in the heterogeneous tumour, which suggest that they may be the likely candidates for tumour progression and recurrence. Indeed, recent work has shown that such aberrant signalling pathways may be targeted in novel anti-cancer therapeutic strategies. The stem cell concept of tumour progression prompts immediate attention to a new paradigm in cancer research with a focus on this minority subset of cells, and the design of novel therapeutic strategies to target these cells that are insignificant within the population of tumour cells, but that are in fact the relevant cells to be destroyed.

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Introduction

Solid tumours are histologically heterogeneous and include tumour cells, stroma, inflammatory infiltrates and vascular structures. There is now much evidence that in cancers, a minority cell population with stem cell properties (cancer stem cells, or CSCs) is responsible for the maintenance and growth of the tumour.¹ In this view, only CSCs would be able to reproduce the tumour or initiate the tumour if tumour cells were implanted into immunodeficient mice models. From these cells, less malignant or partially differentiated cells contributing to the tumour phenotype could be derived. In recent years, the CSC model of tumorigenesis has received increased attention from work in leukaemia,² tumours of the breast,³ brain,^{4,5} prostate,^{6,7} colon,^{8,9} head and neck,¹⁰ and pancreas.¹¹ The concept of CSCs may have profound implications on our understanding of tumour biology and for the design of novel treatments targeted towards these cells for the complete eradication of tumour growth. In this paper, we will try to integrate evidence from these distinct research fields, in the

framework of the CSC concept, with special attention to CSCs identified and isolated from central nervous system (CNS) tumours.^{4,5,12-16}

CSCs in the Haematopoietic System

Evidence for the existence of CSCs initially arose from studies of acute myelogenous leukaemia (AML), among which a subset of leukaemic cells (leukaemic stem cells, LSCs) was identified that gave rise to AML in immunodeficient mice, and which displayed a similar cell surface immunophenotype to normal hematopoietic stem cells (HSCs).² Since the definition of the HSC by Till and McCulloch,¹⁷ critical change occurred in the late 1980s and early 1990s, when the distinct cell surface marker profile that would allow for the prospective isolation of normal HSCs by fluorescence-activated cell sorting (FACS) became known.¹⁸ These advances led to the isolation of HSCs as well as multipotent and oligopotent progenitors that generate all mature blood cells (Fig. 1¹⁹). Before these discoveries were made, LSCs could only be hypothesised, but with the

¹ Department of Research, National Neuroscience Institute, Singapore

² Department of Neurosurgery, National Neuroscience Institute, Singapore

Address for Correspondence: Dr Beng-Ti Ang, Department of Neurosurgery, National Neuroscience Institute, 11 Jalan Tan Tock Seng, Singapore 308433.

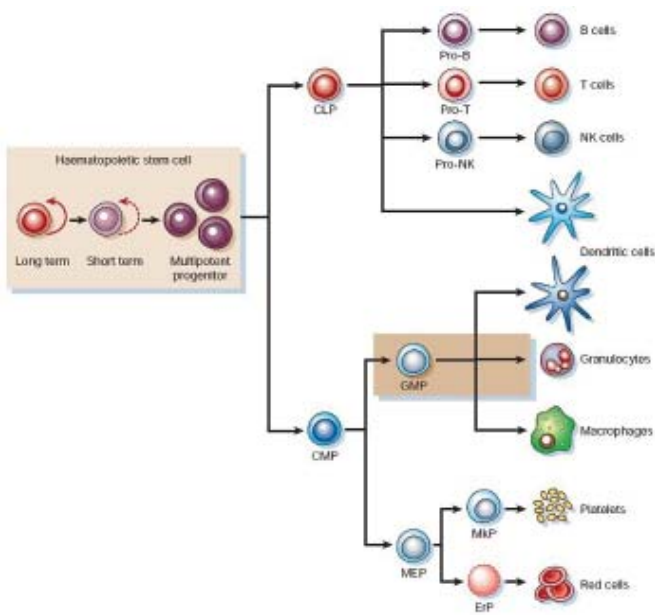


Fig. 1. Comparison between normal hematopoietic stem and progenitor cells (left panel), and leukaemia stem and progenitor cells (right panel). The LSC, similar to the HSC, has the unique ability to self-renew. LSCs give rise to clonogenic leukaemic progenitors that differentiate into leukaemic blasts and more differentiated progeny.

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knowledge of the cell surface phenotypes for HSC and progenitor cells, investigators now had the ability to isolate similar subpopulations from AML. John Dick² and co-workers²⁰ provided the first evidence for LSCs by using FACS to prospectively isolate cells from human AML that were able to initiate leukaemia in transplanted mice. An important aspect of these studies was the use of non-obese diabetic, severe-combined immunodeficient (NOD/SCID) mice, which had been previously shown to support engraftment of normal HSCs.²¹ Evaluating only CD34⁺ AMLs, they showed that the CD34⁺CD38⁻ fraction was highly enriched for leukaemia-initiating activity in transplanted recipients, while both the CD34⁺CD38⁺ and CD34⁻ fractions did not initiate leukaemia. In addition, an engrafted leukaemia could be serially transplanted into secondary recipients, providing functional evidence for self-renewal. This experimental approach – xeno-transplantation followed by serial transplantation – is now widely accepted as an essential criterion in defining CSCs. The ability to recapture tumour pathophysiology is an important defining criterion of CSCs prospectively isolated.

Brain Tumour Stem Cells

Since the work of Al-Hajj et al³ in breast CSCs, the investigation of solid tumour stem cells has gained momentum particularly in the area of brain tumours. Neural

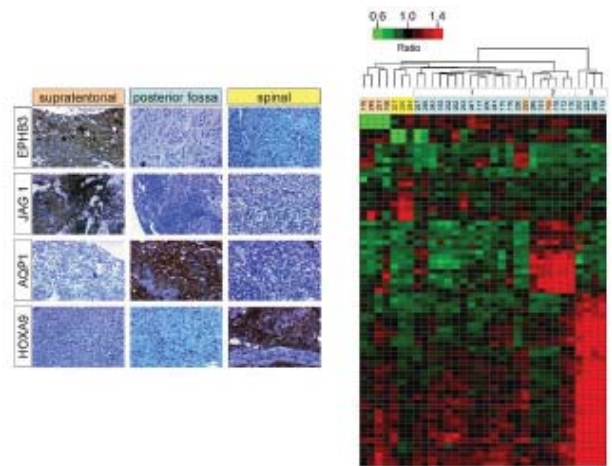


Fig. 2. Identification of ependymoma cancer stem cells and their normal precursors by integrated analysis of the gene expression profiles of human malignant and developing mouse tissues. The left panel illustrates histologically similar but clinically distinct ependymomas; the right panel illustrates the distinct genotypic expression patterns of ependymomas from different regions of the CNS.

Reprinted from: Taylor MD, Poppleton H, Fuller C, Su X, Liu Y, Jensen P, et al. Radial glia cells are candidate stem cells of ependymoma. Cancer Cell 2005;8:323-35, with permission from Elsevier.¹⁵

stem cells have been shown to be enriched in the populations defined by the CD133 marker,²² or by the ability to efflux the Hoechst 33342 dye, the “side population”.²³ Using in vitro serum-free culture conditions in the presence of growth factors, similar to those used for growth of normal neural stem cells as neurospheres,²⁴ 5 groups independently isolated an immature, stem cell-like population from human paediatric, ependymoma and adult glioblastoma multiforme (GBM) brain tumours.^{4,5,12,13,15,16} Hemmati et al¹³ and Singh et al^{4,5} both demonstrated that neurosphere-forming cells expressed CD133, a cell surface marker previously shown to be expressed on normal human neural stem cells.²² Singh et al⁵ later provided more definitive proof for a stem cell population, using antibody-coated magnetic beads to enrich populations of cells that could form tumours in the brains of NOD-SCID mice. Tumorigenic cells were contained entirely within the CD133⁺ fraction, and transplanted CD133⁺ cells gave rise to tumours that contained both CD133⁺ and CD133⁻ cells, recapitulating the original tumour cell heterogeneity. Finally, CD133⁺ cells could be serially transplanted, providing definite proof of self-renewal.

In another work by Taylor et al,¹⁵ the collaboration between developmental and cancer biologists led to the identification of the origin of ependymoma tumour-initiating cells – the radial glia cells. The authors investigated if histologically identical but clinically distinct cancers such as ependymomas might be derived from different progenitor cells in the tissue of origin (Fig. 2). Gene expression profiles of these ependymoma tumour subtypes might give

clues to the identity of their cellular origins. Using gene expression profiling, the authors identified signature genes that most discriminated human spinal, posterior and supratentorial ependymomas. Interestingly, many of the signature genes were known regulators of neural precursor cell proliferation and differentiation in the corresponding region of the CNS. To identify cells in the normal CNS that expressed the signature genes of ependymoma and might therefore represent cells of origin of these tumours, the site of expression of these genes in the embryonic mouse CNS was mapped. The data identified remarkable similarities between the gene expression patterns seen in embryonic radial glia and those observed in ependymomas. Furthermore, the authors showed that CSCs isolated from ependymomas displayed a radial glia-like morphology and immunophenotype (RC2⁺/BLBP⁺/CD133⁺/Nestin⁺) in culture, and recapitulated the primary disease as xenografts in the CNS of immunodeficient mice. Thus, by integrating genomic analyses of malignant and developing nervous tissue, a candidate normal cell of origin of ependymoma and a CSC were identified.

Chemoresistance

Cancer treatment has traditionally been based on the implicit assumption that human cancer populations are homogeneous. At a fundamental level, cancer is resilient to treatment because malignant cells survive chemotherapy and radiation or avoid the immune surveillance of endogenous cytotoxic T cells and natural killer cells. CSCs have the capacity for unlimited self-renewal, as well as the ability to initiate and drive tumour progression in an animal model.⁵ Thus, they would seem to be the most probable candidates responsible for tumour chemoresistance and recurrence.

Work by Hirschmann-Jax et al²⁵ showed that the “side population” (SP) of neuroblastoma cells not only had the characteristics of tumour stem cells (multipotentiality and self-renewal), but were also more resistant to the effects of drugs such as mitoxantrone, and may contribute to the overall drug resistance phenotype of relapsed or resistant cancers. It is certainly clear that neuroblastoma cells cultured in the presence of mitoxantrone showed a progressive increase in the frequency of the SP fraction, indicating that their ability to expel mitoxantrone offered a survival advantage to these putative stem cells. Sorted SP cells were also able to proliferate and establish new colonies in the presence of mitoxantrone, whereas non-SP cells were not, demonstrating stem cell-like properties. Taken together, the data confirm the link between SP and drug resistance, disease persistence and relapse. Relapse following treatment with anti-cancer drugs is already known to be a multi-factorial problem. This study highlights the significance of drug resistant tumour stem cells. Indeed, work by our group

has also shown that SP cells in human malignant glioma cell lines and primary GBM neurosphere cultures increased in the presence of temozolomide treatment, and that this SP fraction correlated with stem cell-like activity as compared to the non-SP fraction (unpublished data).

Much work has also been carried out in brain tumour stem cells identified by another marker, CD133. It is unclear at this time if the SP overlaps with the CD133 population, but both markers have been shown to be highly enriched in neurosphere-forming capacity,^{22,23} one of the defining characteristics of neural stem cells and progenitors. A study by Liu and colleagues²⁶ demonstrated for the first time an increased resistance of CD133⁺ brain tumour stem cells in response to treatment with chemotherapeutic agents such as temozolomide, carboplatin, paclitaxel (Taxol) and etoposide (VP16) compared to autologous CD133⁺ cells. Gene expression studies revealed a higher expression of multi-drug resistance gene BCRP1 and DNA-mismatch repair genes such as MGMT, as well as genes that inhibited apoptosis in the CD133-expressing CSCs. Furthermore, the work showed that CD133 gene expression was significantly higher in recurrent GBM tissue specimens as compared to their respective newly-diagnosed tumours. Clinically, it is observed that tumours respond to chemotherapies only to recur with renewed resilience and aggression. Although chemotherapy kills most of the cells in a tumour, these results suggest that CSCs may be left behind, which then recur due to their enhanced chemoresistance.

A separate study by Fan et al²⁷ implicated the Notch signalling pathway in embryonal brain tumours such as medulloblastoma. The Notch signalling pathway is required in both non-neoplastic neural stem cells and embryonal brain tumours. Using pharmacologic inhibitors of Notch, the authors were able to show depletion of the specific brain tumour stem cell population defined by the CD133 marker or the ability to efflux the Hoechst 33342 dye, the “side population”. Notch was also expressed more highly in the stem cell-like fraction, providing a potential mechanism for their increased sensitivity to inhibition of this pathway. This depletion of stem cell fraction resulted in a loss of tumour-forming capacity. Apoptotic rates following Notch blockade were increased almost 10-fold in primitive nestin-positive stem-like cells compared to nestin-negative cells. Taken together, the data implies that stem-like cells in medulloblastomas seemed to be selectively vulnerable to agents inhibiting the Notch pathway, thereby identifying a novel therapeutic target.

Radioreistance

Radiation biologists were the first to formulate the concept of stem cells. The term “stem cell” was coined in the context of clonogenic cells surviving radiation that were

able to repopulate the spleen.¹⁷ In the gut, cells with a relatively low baseline proliferation rate (which we now know to be “stem cells”) were found to be relatively resistant to radiation and respond to it with cell division, giving rise to cells that repopulate the crypt.^{28,29} Even though gliomas have a dismal prognosis, radiation is the most successful non-surgical treatment. In response to a full course of radiation, gliomas frequently respond but subsequently recur. Medulloblastomas are even more sensitive to radiation than gliomas with cure rates of 70% obtained in children old enough to tolerate the treatment. There has been a long debate over the role of stem or progenitor cells in brain tumours.

In a recent work by Bao et al,³⁰ the authors demonstrated that radiation resistance in highly malignant gliomas (GBM) is most likely mediated by tumour stem cells within. Bao and colleagues³⁰ showed that CD133⁺ CSCs contributed to glioma resistance through preferential activation of DNA damage checkpoint response and an increase in DNA repair capacity compared to CD133⁻ tumour cells. Additionally, the radioresistance of CD133⁺ glioma stem cells could be reversed with a specific inhibitor of Chk1 and Chk2 checkpoint kinases, which are closely associated with cellular resistance to radiation, thereby providing a therapeutic advantage to reduce brain tumour occurrence. As the cell cycle of a normal stem cell is tightly controlled by the checkpoint to maintain genomic stability and integrity, the defective checkpoint responses associated with early cancer development implicate abnormal checkpoint control as a potential contributor to the transformation of normal cells into CSCs.^{31,32}

Radiation resistance conferred by CSCs in the brain has also found support in similar cells isolated from breast cancer.^{33,34} Using conditions that were previously applied for culturing mammospheres from primary breast cancer specimens to culture non-adherent cells that were isolated from 2 adherent breast cancer cell lines,³⁵ the authors showed that cells arising from spheroids were more radioresistant, with an absolute difference in mean survival fraction at 2 Gy of approximately 20%. Importantly, both cell populations were irradiated as single-cell suspensions, thus removing the complicating factor of hypoxia at the centre of spheroids. The authors also examined several molecular assays relevant to radiosensitivity, including levels of reactive oxygen species and phosphorylation of histone H2AX, both of which were decreased in spheroid cultures. Furthermore, fractionated radiation appeared to increase the percentage of non-adherent breast CSCs, suggesting that the relative radioresistance of this subset may lead to their expansion during a course of radiotherapy. The relative resistance of normal mammary stem cells and progenitors to radiation was demonstrated recently in an article by Woodward and colleagues.³⁴ Progenitor cells in

the mammary gland were more resistant to clinically relevant doses of radiation than non-progenitor cells, which constituted the bulk of the mammary gland, and that over-expression of the Wnt/ β -catenin pathway could enhance the radioresistance of progenitor cells. Interestingly, radiation also induced enrichment of “side population” progenitors (a marker of mammary stem cells) in the human breast cancer cell line MCF-7. Taken together, these data indicate that compared with differentiated cells, progenitor cells have different cell survival properties that may facilitate the development of targeted anti-progenitor cell therapies.

Caveats

While candidate CSCs have been identified for several tumour types using xenotransplantation models to demonstrate the presence of a self-renewing population, several concerns remain regarding this experimental strategy. Critics point out that these xenotransplantation assays may introduce a selection bias, as engraftment may depend on other properties of cancer cells, including homing, evasion of host immune response and proliferative capacity. In addition, these xenograft experiments may not adequately model the interaction between tumour cells and the microenvironment that occurs in humans, as highly purified, FACS-isolated tumour cells are used. While such criticisms are not easily ignored, xenotransplantation is currently considered the most physiologically relevant model for human malignancies. Once additional insights into the biology of candidate CSCs become apparent with further characterisation of their gene and protein expression profiles and validation that these molecules play a role in tumour behaviour and clinical responses, it will become clear whether or not engrafting activity is an accurate reflection of stem cell activity. Indeed, a recent study by Calabrese and colleagues³⁶ indicated a potential role for niche microenvironments in the maintenance of brain CSCs, and identified a mechanism by which anti-angiogenic drugs inhibit brain tumour growth. Evidence was presented that brain tumours orchestrate vascular niches that maintain the CSC pool. Disruption of these niche microenvironments ablates the fraction of self-renewing cells in brain tumours and arrests tumour growth.

Additionally, a note of caution in interpreting *in vitro* studies is required. Culture conditions might induce new stem cell properties as reported for normal stem cell induction by growth factors or favour the selection of pre-existing or newly mutated cells.³⁷ However, a recent publication in the area of brain tumour stem cells isolated from human glioblastoma has supported the efficacy of such long-term primary cultures which exhibited the genotype, gene expression profile and biology of their parental primary tumours.³⁸

Clinical Implications of CSCs in the Brain

Despite the recent surge of interest in CSCs, the clinical significance of this population remains unclear. One prediction of the CSC model is that clinical behaviour should be largely dependent on the CSC population, either in quantitative terms such as the relative or absolute number of CSCs, or in the qualitative aspects related to biologic features of CSCs. To date, there is little data addressing this question, although a recent study suggests that in AML, a higher percentage of blasts with the CD34⁺CD38⁻ LSC phenotype is correlated with poorer overall survival.³⁹ Similar studies by Bao et al³⁰ on radioresistance in malignant brain tumours (GBM) showed that the percentage of CD133-expressing cells as analysed by FACS also correlated with the rate of tumour formation when implanted in immunodeficient mice.³⁰

Two recent studies by Bao et al³⁰ and Piccirillo et al⁴⁰ describe the implications of considering the functional hierarchy of the heterogeneous population of tumour cells, and the subsequent identification of potential therapeutic targets for the eradication of malignant brain tumours. The implication of the work by Bao and colleagues³⁰ is that radiation treatment fails in the long run because it cannot kill the subpopulation of CD133⁺ tumour-initiating cells. These cells were rendered less resistant to radiation when 2 pharmacologic inhibitors of Chk1 and Chk2 kinases were added to disrupt the efficient DNA repair mechanisms of CD133⁺ cells. However, further work needs to be carried out on whether these treated cells also lost the ability to subsequently initiate tumours in vivo.

In their research, Piccirillo et al⁴⁰ first showed that human glioblastoma cells expressed bone morphogenetic proteins (BMPs) and their cell-surface receptors; BMPs are the soluble factors that normally induce neural precursor cells to differentiate into mature astrocytes – a subtype of brain cells called glial cells.⁴⁰ The authors showed that BMPs could also prompt the differentiation of CD133⁺ brain tumour stem cells, critically weakening their tumour-forming ability. The results further imply that tumour populations at least partially retain a developmental hierarchy based on stem cells and remain able to respond to the normal signals that induce them to mature. These findings should lead to renewed interest in devising therapies that promote the differentiation of cancer cells.

While the CSC hypothesis has exciting clinical implications, its widespread acceptance and application to the practice of medicine has yet to occur. We anticipate that in the coming years, CSCs will be identified in additional tumour types, and knowledge of the detailed biology and clinical significance of this experimentally defined population will provide further support for the CSC hypothesis. Ultimately, focusing research efforts on the

CSC may drive important advances in our understanding of cancer biology and in developing potential cures for these devastating diseases.

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