

Brain Tumour Stem Cells and Neural Stem Cells: Still Explored by the Same Approach?

W LIU², G SHEN², Z SHI², F SHEN², X ZHENG^{1,2}, L WEN^{1,2} AND X YANG^{1,2}

¹Department of Neurosurgery, First Affiliated Hospital, School of Medicine, and ²Institute of Brain Medicine, Zhejiang University, Hangzhou, China

Brain tumour stem cells (BTSCs) are chiefly responsible for the *in vivo* long-term growth and recurrence of malignant gliomas and may be a potential treatment target. They resemble neural stem cells (NSCs), so their self-renewal and differentiation are currently investigated by the same methods used to study NSCs. There are, however, essential differences between these cell types: in many cases the marker expression pattern of BTSCs does not match the CD133⁺/NSE⁻/FAP⁻ pattern of NSCs; BTSC tumourigenicity is independent of marker expression; and

while attachment, serum-containing medium and withdrawal of mitogens (epidermal growth factor [EGF] and basic fibroblast growth factor [bFGF]) are essential to induce NSCs to differentiate, they do not affect BTSC tumourigenicity. Evidence implies that research on the renewal and differentiation of BTSCs should be orientated towards tumourigenicity and is essentially a pharmaceutical problem. Such an approach may contribute to the development of an accurate definition of BTSCs and to the search for selective differentiation-inducing drugs for BTSCs.

KEY WORDS: BRAIN TUMOUR STEM CELL (BTSC); NEURAL STEM CELL (NSC); MALIGNANT GLIOMA; MARKER EXPRESSION PATTERN; RESEARCH METHODS

Introduction

Cancer stem cell theory suggests that not all cells in malignant tumours share the same ability to initiate and maintain tumour growth; only a small fraction of tumour cells – the cancer stem cells – possess the capacity for infinite proliferation, self-renewal and tumourigenicity. Most ordinary tumour cells lose these abilities and contribute little to *in vivo* long-term growth and recurrence of malignancy.^{1,2}

Cancer stem cells from the glioma –

termed brain tumour stem cells (BTSCs) – have been isolated from low- and high-grade gliomas from children and adults, and normal neural stem cells (NSCs) may be the main source of BTSCs.² In investigating BTSCs, researchers have borrowed many ideas from the field of NSC research. Uchida *et al.*³ directly isolated NSCs from fresh human fetal brain and spinal cord tissue by identifying the cell surface marker, CD133, and it is now a routine approach to enrich CD133⁺ BTSCs using fluorescence-activated

cell sorting or immunomagnetic beads.¹ Kim and Morshead⁴ reported the isolation of NSCs from the neurospheres after exposure to the fluorescent DNA-binding dye, Hoechst 33342, and subsequent analysis via dual wavelength flow cytometry. In the BTSC field, the same strategy was applied extensively in isolating a side population (SP) of cancer stem cells.¹

BTSCs and NSCs resemble one another at cellular and molecular levels, and their similarities in phenotype and signalling suggest that BTSC research could share experimental protocols with NSC research. However, essential differences between both cell types make this impossible. This paper discusses current understanding of the differences in self-renewal and differentiation that exist between BTSCs and NSCs.

BTSCs and NSCs share similarities

Most current studies into BTSC self-renewal and differentiation have drawn upon NSC research methods; they assume that BTSCs and NSCs share specific markers such as nestin, Musashi and CD133. According to prevailing opinion, if the daughter cells of BTSCs maintain the marker expression pattern, the BTSCs self-renew; alternatively, if the daughter cells express the markers for normal neurons or glia (e.g. β -tubulin, non-specific enolase [NSE] and glial fibrillary acidic protein [GFAP]), the BTSCs differentiate.⁵⁻⁷

Another method of studying self-renewal is based on population analysis. Briefly, to determine the self-renewal characteristics of NSCs, primary floating neurospheres are individually dissociated into single cells and replated in identical serum-free media conditions. This passaging assay has been used to assess capacity for self-renewal by the formation of clonally derived secondary

sphere colonies. The longevity of self-renewal ability can be investigated further by passaging single secondary spheres and assessing the formation of tertiary spheres, and so on.⁸ This sphere-passaging assay has also been used to assess BTSC self-renewal.^{7,9} When cultured in serum-containing medium as an attached monolayer, normal NSCs (nestin⁺/CD133⁺) tend to differentiate into neurons (β -tubulin⁺/NSE⁺) and glial cells (GFAP⁺/O4⁺). It is assumed that BTSCs also behave in this way. For example, Singh *et al.*⁵ plated BTSCs onto glass coverslips coated in poly-L-ornithine in medium with 10% fetal bovine serum (FBS) in individual wells of a 24-well culture plate. One week later the cells were processed to detect differentiation using immunocytochemistry for β -tubulin and GFAP.

BTSCs and NSCs – different research goals and clinical implications

It seems evident that many researchers do not discern essential differences between the self-renewal and differentiation capacities of BTSCs and normal NSCs. Research goals and clinical implications should be taken fully into consideration. The ultimate goal of NSC research is to repair damaged or degenerative nervous tissue with endogenous or exogenous NSCs. The NSCs tend to differentiate spontaneously in the natural microenvironment, but it remains very difficult to manipulate the direction of differentiation accurately towards the desired subtype of neural cells.

In the case of BTSCs, however, the situation is completely different. According to cancer stem cell theory, once BTSCs differentiate into ordinary tumour cells they lose the capacity of infinite proliferation and tumourigenicity.^{1,6-8} Thus, it is logical to

deduce that BTSCs seldom differentiate spontaneously under natural conditions, otherwise a malignant tumour could disappear spontaneously. The ultimate goal of BTSC research is to induce BTSCs to differentiate towards ordinary tumour cells or to eradicate BTSCs in the glioma selectively. In the differentiation of BTSCs, the primary interest is in decreasing or eliminating tumourigenic cells and it is clinically meaningless to study the phenotype of the committed progeny cells (ordinary tumour cells). This is the opposite of the situation with NSCs. As for self-renewal, the major concern of BTSC research is the maintenance and inheritance of tumourigenic ability rather than multipotency.^{6,10} Bearing in mind these differences, clearly it is illogical to study BTSC self-renewal and differentiation by the same methods that are used for NSC studies.

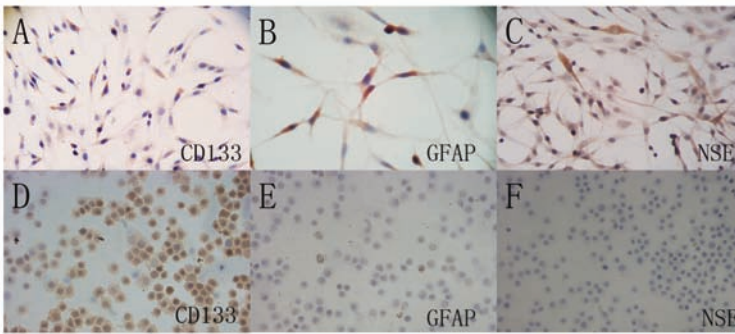
BTSC tumourigenic ability is inconsistent with NSC specific markers

If the tumourigenic ability of BTSCs was exactly consistent with the pattern of expression of NSC specific markers, immunostaining would provide a convenient way of detecting BTSC self-renewal and differentiation. There is, however, considerable evidence that this is not the case.

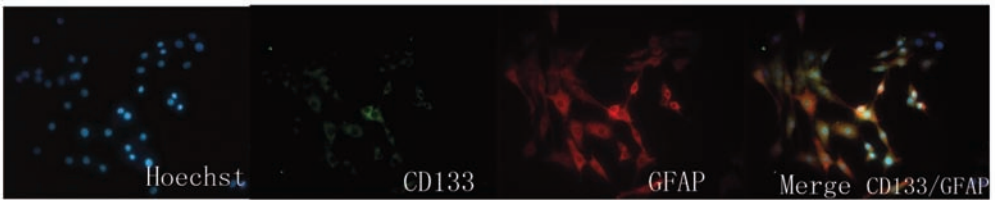
Previously we reported that most C6 cells are cancer stem cells with clonogenic, self-renewal and tumourigenic capacities.¹¹ In FBS-containing medium, however, only about 25.9% were CD133⁺ (Fig. 1A), whereas there were many more GFAP⁺ (Fig. 1B) and a relatively high proportion of NSE⁺ cells (Fig. 1C). When C6 cells were transferred to serum-free medium for 4 weeks, the percentage of CD133⁺ cells increased to

about 80.3% (Fig. 1D), the percentage of GFAP⁺ cells decreased from 45.5% to 2.6% (Fig. 1E), and NSE⁺ cells decreased from 13.8% to 0.5% (Fig. 1F). The clonogenic and tumourigenic capacities, however, remained similar regardless of the different culture media. Zhang *et al.*¹² reported that some human BTSC clones contain cells that are immunopositive separately for GFAP or for neuronal β -tubulin, as well as single cells that co-express both glial and neuronal markers. We have also found that many C6 cells co-express CD133/GFAP (Fig. 1G), or CD133/NSE (Fig. 1H). Beier *et al.*¹³ have isolated both CD133⁺ and CD133⁻ BTSCs from human glioblastoma and, although they showed distinct molecular profiles and growth characteristics *in vitro* and *in vivo*, both populations fulfilled cancer stem cell criteria. Platet *et al.*¹⁴ also demonstrated that SP and non-SP cells have similar tumourigenic capacities. Clearly, many BTSCs do not match the NSC marker expression pattern of CD133⁺/NSE⁻/GFAP⁻ and there are differences in the expression patterns of the markers between BTSCs and NSCs.

The phenotype of stem cells is mainly determined both by the microenvironmental 'niche' and the genotype. For example, a slight change in the microenvironment is sufficient to induce NSCs to decrease nestin expression.¹⁵ Not only is the BTSC genome greatly different from that of a normal NSC, but also the microenvironment (including cytokines, extracellular matrix, intercellular adhesion molecule and pH) in the glioma is very different from the subventricular zone or the dentate gyrus where normal NSCs reside *in vivo*. It should not, therefore, be expected that the expression patterns of the markers of BTSCs and NSCs should resemble each other, even if NSCs may be the origin for BTSCs.



G



H

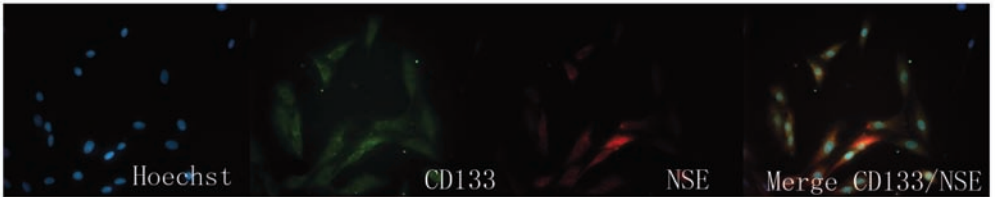


FIGURE 1: Immunocytochemistry for expression of CD133, glial fibrillary acidic protein (GFAP) and neuron-specific enolase (NSE) in C6 cells. For C6 cells cultured in medium containing fetal bovine serum (FBS): (A) approximately 25.9% of cells are CD133⁺; (B) approximately 45.5% cells are GFAP⁺; (C) approximately 13.8% cells are NSE⁺. For C6 cells cultured in serum-free medium: (D) the percentage of CD133⁺ cells increases to 80.3%; (E) the percentage of GFAP⁺ cells decreases to 2.6%; (F) the percentage of NSE⁺ cells decreases to 0.5%. The C6 cells can also co-express (G) CD133 and GFAP or (H) CD133 and NSE in FBS-containing medium

Specific surface markers are needed to distinguish BTSCs from NSCs

In the context of normal development, ‘stem’ refers to multipotentiality, or a cell’s ability to give rise to multiple cell types of a particular lineage. In the context of cancer, it refers to the tumour-forming ability of an undifferentiated cancer cell that might express normal stem cell markers without

necessarily exhibiting other behaviours attributable to normal stem cells.^{2,16} The above evidence taken together, indicates that it is inaccurate and unreliable to investigate BTSCs using NSC research methods. It has been proposed that a single cell capable of giving rise to a tumour of its own origin *in vivo* should be termed a cancer stem cell.^{2,17} We insist that BTSC self-renewal and differentiation should be studied according to this criteria. Single-cell clone and subclone

cultures in a 96-well flask provides a reliable method for detecting the infinite proliferation of BTSCs. Xenograft tumours in immunodeficient mice provide a good tool for *in vivo* study of the tumourigenicity of BTSCs and their progeny. If a tumour stem cell and its progeny lose clonogenic and tumourigenic capabilities in some microenvironments, the BTSC should be regarded as differentiated, otherwise, BTSCs can self-renew spontaneously. This ensures that the clinical goal is reflected and met in the experiment.

As mentioned above, BTSCs have changed marker expression but not tumourigenicity when cultured in serum-containing or serum-free media. Liu *et al.*¹⁸ reported that a large number of CD133⁺ cancer stem cells could be maintained without differentiation in 10% FBS culture medium after 3 – 6 passages. They proposed that some cancer stem cells derived from glioblastoma tumour tissue may be maintained even in FBS-containing medium, which may be a significant characteristic of cancer stem cells as opposed to normal stem cells. Attachment, serum-containing medium and withdrawal of mitogens (epidermal growth factor [EGF] and basic fibroblast growth factor [bFGF]) were shown to be essential to induce NSCs to differentiate.¹⁹ However, although cell lines have been passaged in the form of an attached monolayer in serum-containing medium for several decades, most of these cells were proved to remain undifferentiated.^{7,11,20} Analogously, we have proposed that most factors that induce differentiation (including cytokines such as brain-derived neurotrophic factor, platelet-derived growth factor, glial cell line-derived neurotrophic factor receptors, insulin-like growth factor and nerve growth factor) for normal NSCs can barely promote BTSC

differentiation. We assume that BTSCs that are susceptible to differentiation-inducing factors may have already differentiated and disappeared during tumour initiation and progression and, therefore, the remaining BTSCs in the tumour bulk are resistant to these differentiation-inducing factors.

To deprive the most malignant tumourigenic cells of their tumourigenicity is, of course, very difficult. We imply that BTSC differentiation should be tumourigenicity oriented and is essentially a pharmaceutical problem. In the search for drugs that induce BTSC differentiation we suggest that common cytokines that induce NSC differentiation should be ignored. On the contrary, some exogenic substances that never exist in the BTSC microenvironment may affect BTSC differentiation.

We have mentioned that single-cell clone and subclone culture, and the xenograft tumour in the immunodeficient mouse model, are reliable methods for studying BTSC self-renewal and differentiation. These methods are, however, inconvenient and very time-consuming. If biomarkers that are exactly consistent with the tumourigenicity of BTSCs could be found, this would provide a very convenient way of detecting their self-renewal and differentiation by immunostaining. We suggest using high-throughput methods, such as microarray, two-dimensional electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to identify specific biomarkers for BTSCs.

Acknowledgements

We appreciate the technical expertise of Dr Jiaping Peng and Dr Qi Dong from the Department of Pathology, Second Affiliated Hospital, Zhejiang University School of Medicine. We also deeply appreciate the help of Dr Iain C Bruce at the University of Hong

Kong. This research was funded by the Natural Science Foundation of China (30772243).

Conflicts of interest

The authors had no conflicts of interest to declare in relation to this article.

- Received for publication 26 March 2008 • Accepted subject to revision 28 March 2008
- Revised accepted 9 July 2008

Copyright © 2008 Field House Publishing LLP

References

- 1 Dirks PB: Brain tumor stem cells. *Biol Blood Marrow Transplant* 2005; **11**(suppl 2): 12 – 13.
- 2 Oliver IG, Wechsler-Reya RJ: Getting at the root and stem of brain tumors. *Neuron* 2004; **42**: 885 – 888.
- 3 Uchida N, Buck DW, He D, *et al*: Direct isolation of human central nervous system stem cells. *Proc Natl Acad Sci USA* 2000; **97**: 14720 – 14725.
- 4 Kim M, Morshead CM: Distinct populations of forebrain neural stem and progenitor cells can be isolated using side-population analysis. *J Neurosci* 2003; **23**: 10703 – 10709.
- 5 Singh SK, Clarke ID, Ieraski M, *et al*: Identification of a cancer stem cell in human brain tumors. *Cancer Res* 2003; **63**: 5821 – 5828.
- 6 Yuan XP, Curfin J, Xiong Y, *et al*: Isolation of cancer stem cells from adult glioblastoma multiforme. *Oncogene* 2004; **23**: 9392 – 9400.
- 7 Galli R, Binda E, Orfanelli U, *et al*: Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer Res* 2004; **64**: 7011 – 7021.
- 8 Seaberg RM, van der Kooy D: Adult rodent neurogenic regions: the ventricular subependyma contains neural stem cells, but the dentate gyrus contains restricted progenitors. *J Neurosci* 2002; **22**: 1784 – 1793.
- 9 Ignatova TN, Kukekov VG, Laywell ED, *et al*: Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers *in vitro*. *Glia* 2002; **39**: 193 – 206.
- 10 Lee J, Kotliarova S, Kotliarov Y, *et al*: Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. *Cancer Cell* 2006; **9**: 391 – 403.
- 11 Zheng X, Shen G, Yang X, *et al*: Most C6 cells are cancer stem cells: evidence from clonal and population analyses. *Cancer Res* 2007; **67**: 3691 – 3697.
- 12 Zhang QB, Ji XY, Huang Q, *et al*: A differentiation profile of brain tumor stem cells: a comparative with neural stem cells. *Cell Res* 2006; **16**: 909 – 915.
- 13 Beier D, Hau P, Proescholdt M, *et al*: CD133⁺ and CD133⁻ glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. *Cancer Res* 2007; **67**: 4010 – 4015.
- 14 Platet N, Mayol JF, Berger F, *et al*: Fluctuation of the SP/non-SP phenotype in the C6 glioma cell line. *FEBS Lett* 2007; **581**: 1435 – 1440.
- 15 Chen H, Tung YC, Li B, *et al*: Trophic factors counteract elevated FGF-2-induced inhibition of adult neurogenesis. *Neurobiol Aging* 2007; **28**: 1148 – 1162.
- 16 Fomchenko EI, Holland EC: Stem cells and brain cancer. *Exp Cell Res* 2005; **306**: 323 – 329.
- 17 Al-Hajj M, Becker MW, Wicha M, *et al*: Therapeutic implications of cancer stem cells. *Curr Opin Genet Dev* 2004; **14**: 43 – 47.
- 18 Liu G, Yuan X, Zeng Z, *et al*: Analysis of gene expression and chemoresistance of CD133⁺ cancer stem cells in glioblastoma. *Mol Cancer* 2006; **5**: 67.
- 19 Hsu YC, Lee DC, Chiu IM: Neural stem cells, neural progenitors, and neurotrophic factors. *Cell Transplant* 2007; **16**: 133 – 150.
- 20 Ho MM, Ng AV, Lam S, *et al*: Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. *Cancer Res* 2007; **67**: 4827 – 4833.

Author's address for correspondence

Dr Xiaofeng Yang

Department of Neurosurgery, First Affiliated Hospital, School of Medicine, Zhejiang University, No.79 QingChun Road, Hangzhou 310003, China.

E-mail: alfon.sf@126.com