Response of medulloblastoma cell lines to experimental therapeutics

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Acknowledgements

“First, have a definite, clear practical ideal; a goal, an objective. Second, have the necessary means to achieve your ends; wisdom, money, materials, and methods. Third, adjust all your means to that end.”

*Aristotle (384 BC – 322 BC)*

“Maybe we weren’t meant for paradise. Maybe we were meant to fight our way through – struggle, claw our way up, scratch for every inch of the way. Maybe we can’t stroll to the music of the lute. We must march to the sound of drums.”

*Captain James T. Kirk (2264)*

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Of utmost importance, I dedicate this thesis to my beautiful and wonderful wife, Monisha, and to my precious daughter, Naina, who have been a source of great support throughout every aspect of my life, including my thesis experience. Their constant love and encouragement provided a beacon of hope on days when all my hard work and effort went to naught. Words cannot express the gratitude and love I have for both of them.

Finally, I would like to thank the funding agencies who have supported me financially throughout my doctoral years: Montreal Centre for Experimental Therapeutics in Cancer (MCETC) and the Fonds de la recherche en santé du Québec (FRSQ).
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VK Srivastava is a PhD student and is the primary author of this research article.

J Nalbantoglu is the academic supervisor of VK Srivastava.


VK Srivastava is the primary author of this research article and performed most of the experiments.

Z Yasruel is a research technician within the laboratory of J Nalbantoglu and performed experiments responsible for Figure 6 of the publication.

J Nalbantoglu is the academic supervisor of VK Srivastava.
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<td>4-phenylbutyrate</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
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<tr>
<td>AML</td>
<td>acute myeloid leukaemia</td>
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<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
</tr>
<tr>
<td>AT/RT</td>
<td>atypical teratoid/rhabdoid</td>
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<td>βTrCP</td>
<td>beta-transducin repeat containing protein</td>
</tr>
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<td>BC</td>
<td>blast crisis</td>
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<td>BCA</td>
<td>bicinchoninic acid</td>
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<td>BCRP1</td>
<td>breast cancer-resistant protein 1</td>
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<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<td>BFP</td>
<td>blue fluorescent protein</td>
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<tr>
<td>Bim</td>
<td>Bcl-2–interacting mediator of cell death</td>
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<td>BMP</td>
<td>bone morphogenetic proteins</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>BTSC</td>
<td>brain tumour stem cell</td>
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<td>CBP</td>
<td>CREB-binding protein</td>
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<td>Abbreviation</td>
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<td>CCG</td>
<td>Children’s Cancer Group</td>
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<tr>
<td>CGH</td>
<td>comparative genomic hybridization</td>
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<td>cGMP</td>
<td>cyclic GMP</td>
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<td>Children’s Oncology Group</td>
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<td>Ci</td>
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<td>CKI</td>
<td>casein kinase I</td>
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<td>chronic myelogenous leukaemia</td>
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<td>costal2</td>
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<td>CP</td>
<td>chronic phase</td>
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<td>deep cerebellar nuclei</td>
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<td>DyeCycle Violet</td>
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<td>DLT</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<td>DMSO</td>
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<td>days post-coitum</td>
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<td>Fz</td>
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<td>glioblastoma</td>
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<td>glial fibrillary acidic protein</td>
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<td>glioma associated oncogene homologue</td>
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<td>granulocyte-monocyte progenitor</td>
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<td>histone acetyltransferase</td>
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<td>histone deacetylase inhibitor</td>
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<td>HSC</td>
<td>haematopoietic stem cell</td>
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<td>ICD</td>
<td>intracellular domain</td>
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<td>IGF-1</td>
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<td>IGL</td>
<td>internal granule layer</td>
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<td>IRS</td>
<td>insulin receptor substrate</td>
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<td>JCV</td>
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<td>Jun N-terminal kinase</td>
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<td>LC/A</td>
<td>large cell/anaplastic</td>
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<td>LOH</td>
<td>loss of heterozygosity</td>
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<td>MAP</td>
<td>mitogen-activated protein</td>
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<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<td>MB</td>
<td>medulloblastoma</td>
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<td>MBEN</td>
<td>medulloblastoma with extensive nodularity</td>
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<td>MDR</td>
<td>multidrug-resistant</td>
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<td>MHJ</td>
<td>midbrain-hindbrain junction</td>
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<td>ML</td>
<td>molecular layer</td>
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<td>MOI</td>
<td>multiplicity of infection</td>
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<td>magnetic resonance imaging</td>
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<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<td>NB</td>
<td>neuroblastoma</td>
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<td>NBCC</td>
<td>nevoid basal cell carcinoma</td>
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<td>Ngn</td>
<td>neurogenin</td>
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<td>notch intracellular domain</td>
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<td>NOD/SCID</td>
<td>non-obese diabetic, severe combined immunodeficient</td>
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<td>NSA</td>
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<td>NSCLC</td>
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<td>non side-population</td>
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<td>P-gp</td>
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<td>PACAP</td>
<td>pituitary adenylate cyclase activating polypeptide</td>
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<td>PI3K</td>
<td>phosphatidylinositol-3-kinase</td>
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<td>PKA</td>
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<td>POG</td>
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<td>PR</td>
<td>partial response</td>
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<td>Smo</td>
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<tr>
<td>SSCP</td>
<td>single strand conformational polymorphism</td>
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<td>suppressor of Fused</td>
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<td>subventricular zone</td>
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<td>TA</td>
<td>transiently amplifying</td>
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<td>transcription factor</td>
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<td>vascular endothelial growth factor receptor</td>
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<td>valproic acid</td>
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<td>WHO</td>
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<td>white matter</td>
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<td>2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]2H-tetrazolium-5-carboxyanilide</td>
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Abstract

Medulloblastoma is the most common malignant childhood brain tumour. The discovery of stem-like cells in malignant cancers has provided an alternative hypothesis for tumour initiation and progression. Given the toxicities associated with conventional therapies, developing therapies targeting aberrant molecular pathways or stem-like cells in medulloblastoma may improve overall survival and quality of life.

Numerous reports have indicated that stem-like cells also exist in long-established cultured cell lines. Therefore, we first determined whether the DAOY medulloblastoma cell line contained a stem-like population and how this subset of cells responded to therapeutic modalities. The literature suggests that self-renewal is associated with the CD133 and side population (SP; Hoechst 33342 efflux) phenotypes. However, our results revealed clonogenicity even in the absence of these markers (CD133- and non-SP cells). Furthermore, each compartment demonstrated asymmetric division by regenerating not only itself but the other compartment as well. Expression of the two stem cell markers did not entirely overlap as CD133 expression was present in both SP and non-SP compartments. These results were also supported by decreased cell viability in both the SP and total cell compartments and similar susceptibility of both compartments to chemotherapeutic treatment. Although histone deacetylase (HDAC) inhibitors significantly reduced in vitro tumour cell clonogenicity and increased survival of medulloblastoma-bearing mice, the relationship between HDAC inhibitor treatment and stem-like cell markers was not evident. These
results highlight the fact that care must be used in interpreting preclinical therapeutic studies targeting the stem cell compartment of cancer cell lines.

With a view to developing combinations of targeted therapies, we examined the potential role of transcription factor-based therapies for medulloblastoma. Our results demonstrate that serum starvation induced nuclear translocation of the FOXO1 transcription factor and cell death whereas insulin-like growth factor I (IGF-I) stimulation of serum-starved cells resulted in FOXO1 phosphorylation and an increase in cell viability. Furthermore, expression of constitutively active form of FOXO1 led to a significant reduction in medulloblastoma cell viability.

In conclusion, FOXO1 transcription factor-based therapies or HDAC inhibitors can significantly decrease medulloblastoma cell viability.
Résumé

Le médulloblastome est la tumeur cérébrale maligne la plus fréquente chez les enfants. La découverte de cellules quasi similaires aux cellules souches dans des cancers malins a permis de fournir une autre hypothèse au développement d’une lésion et à sa progression. Compte tenu des toxicités associées aux thérapies conventionnelles, le développement de thérapies qui ciblent des voies moléculaires aberrantes, ou les cellules quasi similaires aux cellules souches du médulloblastome, peut améliorer la survie générale et la qualité de vie.

Dans de nombreux rapports, on a indiqué que les cellules quasi similaires aux cellules souches existent également dans les lignées cellulaires cultivées longuement établies. Par conséquent, nous avons tout d’abord déterminé si la lignée cellulaire des DAOY du médulloblastome contenait une population quasi similaire aux cellules souches et la façon dont ce sous-ensemble de cellules réagissait aux modalités thérapeutiques. La documentation semble suggérer que l’autorenouvellement est associé à des phénotypes CD133 et de population isolée (« side population » ou SP; sortie Hoechst 33342). Toutefois, nos résultats ont permis de révéler une clonogénicité même en l’absence de ces marqueurs (cellules CD133 et non-SP). De plus, chaque compartiment a démontré une division asymétrique en se régénérant non seulement lui-même, mais aussi l’autre compartiment. L’expression de deux marqueurs de cellules souches ne s’est pas entièrement chevauchée, car l’expression du CD133 était présente dans les compartiments SP et non-SP. Ces résultats étaient également appuyés par une diminution de la viabilité cellulaire dans les compartiments SP et de cellule totale,
et dans la susceptibilité similaire des deux compartiments au traitement chimiothérapeutique. Même si les inhibiteurs d’histone désacétylase (HDAC) ont réduit de façon importante la clonogénicité des cellules tumorales in vitro et augmenté la survie des souris atteintes de médulloblastome, la relation entre le traitement aux inhibiteurs HDAC et les marqueurs de cellules quasi similaire aux cellules souches n’était pas évidente. Ces résultats laissent entendre qu’il faut faire attention dans l’interprétation des études thérapeutiques pré-cliniques ciblant le compartiment de cellules souches des lignées cellulaires du cancer.

Dans l’optique de concevoir des combinaisons de thérapies ciblées, nous avons étudié le rôle possible des thérapies fondées sur le facteur de transcription pour le médulloblastome. Nos résultats permettent de démontrer que l’absence de sérum a produit la translocation nucléaire du facteur de transcription FOXO1 et le décès cellulaire, tandis que la stimulation des cellules privées de sérum par le facteur de croissance I analogue à l’insuline (IGF-I) a entraîné la phosphorylation FOXO1 et une augmentation de la viabilité cellulaire. De plus, l’expression de la forme constitutivement active de FOXO1 a mené à une réduction importante de la viabilité des cellules du médulloblastome.

En conclusion, les thérapies fondées sur le facteur de transcription FOXO1 ou les inhibiteurs de HDAC peuvent réduire considérablement la viabilité des cellules du médulloblastome.
Contributions to original knowledge

- Hoechst 33342 efflux and CD133 expression might not be suitable for selectively isolating cancer stem-like cells from cell lines, as shown for the DAOY medulloblastoma cell line, and may constitute two independent compartments; this work was published in the peer-reviewed journal Cytometry Part A. [Srivastava VK and Nalbantoglu J (2008). Flow cytometric characterization of the DAOY medulloblastoma cell line for the cancer stem-like phenotype. *Cytometry Part A* 73A: 940-948].

- CD133 expression mapped to both the side population (SP) and non-SP compartments, with CD133⁺ cells being enriched almost fourfold within the non-SP gate (Srivastava and Nalbantoglu, 2008);

- Slight clonogenic enrichment was observed in only the SP fraction; however, both CD133⁺ and CD133⁻ cells displayed equivalent stem cell-like frequencies (Srivastava and Nalbantoglu, 2008);

- Histone deacetylase (HDAC) inhibitors can effectively suppress DAOY cell survival, clonogenicity and increase survival of mice harbouring intracranial DAOY xenografts;

- No relationship found between HDAC inhibitor treatment and Hoechst 33342 efflux although HDAC inhibitor treatment did slightly upregulate CD133 expression;
The transcription factor FOXO1 may be a critical effector of medulloblastoma cell growth suppression thus highlighting its potential in cancer therapy; this work has been accepted for publication in the peer-reviewed journal entitled International Journal of Oncology. [Srivastava VK, Yasruel Z and Nalbantoglu J (2009). Impaired medulloblastoma cell survival following activation of the FOXO1 transcription factor. *Int J Oncol* 00: 0-00, 0000].

Expression of a constitutively nuclear form of FOXO1 that cannot be phosphorylated by Akt led to a significant reduction in medulloblastoma cell viability, even in the presence of growth factors (Srivastava et al., 2009).
CHAPTER I: INTRODUCTION
I. Rationale and research objectives

Medulloblastoma is the most common malignant brain tumour in children accounting for nearly 25-30% of primary central nervous system (CNS) neoplasms. The peak incidence occurs at about 7 years of age with a higher preponderance in boys; a minor subset of medulloblastomas occurs in adolescents and adults. The classic triad of presenting symptoms usually include headaches, morning vomiting and ataxia with subsequent imaging revealing a space-occupying lesion in the posterior fossa. This tumour generally arises in the midline vermian region of the cerebella and has the propensity for early dissemination throughout the CNS; in adults, the tumour typically arises from one of the cerebellar hemispheres. A 5-year progression-free survival (PFS) rate exceeding 50% is now reported subsequent to the standard regimen of aggressive surgical resection, craniospinal radiotherapy and chemotherapy; however, this comes at a cost. The major trade-off manifesting as endocrine, growth and neurocognitive sequelae has prompted efforts to optimize treatment outcomes by maximizing cure rates while minimizing treatment-related toxicity. This optimization-based approach has lead to the concept of risk stratification that has undergone a further renaissance due to recent advances in histopathology and gene expression profiling. Furthermore, advances in cancer biology have reconceptualized the framework for understanding tumourigenesis as being initiated by a relatively small compartment of cancer stem-like cells. Lastly, the cancer stem cell hypothesis has profound implications in the design of novel therapeutics that could specifically target this minor tumour-initiating
subpopulation and potentially translate into better survival outcomes and reduced toxicity profile.

The research objectives of this thesis were to ascertain the biological characteristics of the stem-like cell compartment in MB cell lines. Gaining an understanding of these characteristics would form the basis for determining the effect of current and experimental therapeutics on the viability of the stem-like compartment.

II. Literature review

This section will present a comprehensive literature review of three major themes pertinent to the formation of this thesis. The first part of the review will focus on reviewing current risk stratification schema for MB classification. The second part of the review will focus on introducing the cancer stem cell hypothesis. The final part of the review will focus on both current and experimental therapeutics for MB.
Medulloblastoma

Risk stratification

The patient’s age is the single most important prognostic factor with children less than three years old, which account for nearly 25-35% of all medulloblastoma cases, facing a dire prognosis (1, 2). For children greater than 3 years of age, other clinical prognostic parameters such as the Chang metastatic stage (Table I) and residual tumour size stratify this age group into two disease-risk categories for relapse: average-risk and high-risk (1). The average-risk category includes children with gross or near-total resection ($\leq 1.5 \text{ cm}^2$).
<table>
<thead>
<tr>
<th>Metastasis stage</th>
<th>Description</th>
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<tbody>
<tr>
<td>M0</td>
<td>No evidence of gross subarachnoid or haematogenous metastasis</td>
</tr>
<tr>
<td>M1</td>
<td>Tumour cells found in cerebrospinal fluid</td>
</tr>
<tr>
<td>M2</td>
<td>Intracranial tumour beyond primary site (e.g., into the aqueduct of Sylvius and/or into the subarachnoid space or in the third or foramen of Luschka or lateral ventricles)</td>
</tr>
<tr>
<td>M3</td>
<td>Gross nodular seeding in spinal subarachnoid space</td>
</tr>
<tr>
<td>M4</td>
<td>Metastasis outside the cerebrospinal axis (esp. bone marrow, bone)</td>
</tr>
</tbody>
</table>

Table I: Chang metastatic staging for medulloblastoma. Adapted from (3)
of residual tumour on post-operative magnetic resonance imaging (MRI)) and no evidence of metastasis whereas the presence of more than 1.5 cm$^2$ of residual tumour or metastasis defines the high-risk category; children younger than 3 years of age are considered high-risk due to higher rates of relapse (1, 2, 4). Despite clinical risk stratification being the mainstay for therapeutic decision-making, significant limitations do exist (5). Firstly, the current clinical paradigm fails to identify that 20-30% of average-risk patients are actually high-risk and thus more aggressive therapy is delayed in a significant number of patients. Conversely, a large number of high-risk patients may be cured with less aggressive forms of therapy and thus do not needlessly be exposed to increased risk of treatment-related toxicity in the form of cognitive decline, intellectual impairment and other unwanted side effects. Finally, a two-level risk categorization, i.e. average-risk and high-risk, could be overly simplistic and may not reflect the underlying heterogeneity of MBs and subsequent prognostic implications. Thus, a further refinement in risk stratification is warranted, which could potentially lead to improved therapeutic efficacy.

There is growing evidence that histopathological grading may increase the predictive power of current stratification schemes thus leading to improved treatment outcomes (6). Presently, there are four histologic subtypes of medulloblastoma identified by the World Health Organization, i.e. medulloblastoma with extensive nodularity (MBEN), desmoplastic/nodular medulloblastoma, classic medulloblastoma and large cell/anaplastic medulloblastoma (7). About 65% of MBs are not categorized as variants by the
WHO classification scheme and hence are referred to as the classic medulloblastomas. This major subtype is characterized by sheets of “small, round, blue cells” that are comprised of both undifferentiated cells and cells differentiating along the neuronal lineage (6). The desmoplastic subtype represents about 25% of MBs and is characterized by reticulin-free nodular islands displaying reduced cellularity and neuronal differentiation surrounded by a reticulin-rich fibre network produced by a population of densely packed, mitotically active cells. These nodules are comprised of cells displaying round, regular nuclei and abundant, fibrillar cytoplasm (6). About 5% of MBs are extreme versions of the desmoplastic subtype, which are characterized by extensive nodularity and neuronal differentiation, and constitute a separate subtype referred to as MB with extensive nodularity (MBEN). Finally, the large cell/anaplastic (LC/A) subtype constitutes about 5% of MBs and consists primarily of undifferentiated cells. These tumours feature large nuclei, prominent nucleoli and display a high mitotic index, frequent necrotic areas, numerous apoptotic figures and cellular wrapping (6). Two other extremely rare variants are MB with melanotic differentiation (noted for intracellular melanin) and MB with myogenic differentiation (displaying focal rhabdomyoblastic elements with immunoreactivity to desmin, myoglobin and fast myosin); however, their rarity precludes their biological behaviour from being assessed as significantly different to that of the classic MB subtype. Linking the histologic snapshot of MBs with their biological behaviour may have significant prognostic value. For example, the pale neurocytic appearance of desmoplastic MBs coupled with low mitotic
activity suggest a relatively benign condition whereas the LC/A subtype histology would suggest otherwise. Indeed, several studies propose that the desmoplastic subtype may have a better prognosis than the classic or LC/A subtypes; MBEN tends to have a better outcome than the other variants because it represents the most differentiated form of nodular desmoplasia (8). Yet, these prognostic values have been initially ascribed to relatively rare subtypes, MBEN and LC/A, which lie on either extremes of the histologic spectrum. Given that the bulk of MBs fall between these two histologic extremes, it has been hypothesized that increased nodularity and anaplasia would be associated with better and worse clinical outcomes, respectively. Interestingly, increasing anaplastic grade and extent of anaplasia was associated with gradually worse prognosis whereas neither increased nodularity nor desmoplasia was associated with better survival; of note, log rank analysis of the anaplastic grade has been reported to be superior to the clinical M stage in stratifying patients with respect to outcome (8). Thus, anaplastic grading may serve an important prognostic role in MB risk assessment and hence, efforts have been directed towards elucidating the cytogenetic and molecular alterations underlying the anaplastic phenotype.

Deletion of the short arm of chromosome 17 (17p) is the most frequent cytogenetic abnormality found in MBs and is accompanied by a duplication of the long arm of chromosome 17 (isochromosome 17q), but this gain need not always accompany the loss of 17p (9). Furthermore, the impact of the deletion of 17p on survival outcome is controversial as some studies suggest that there is a
significant association between loss of 17p and poor prognosis (9) whereas other studies refute this claim (10). Nevertheless, studies have identified several putative genes on 17p, such as p53, HIC1 and KCTD11, that function as tumour suppressors and, when deleted or mutated, may contribute to adoption of an anaplastic phenotype (11-13). For example, KCTD11 inhibits Sonic Hedgehog (Shh) signalling, which is important in controlling granule cell precursor proliferation, through cytoplasmic retention of the Gli1 transcription thus mitigating target gene activity (cyclin D2, Ptc1, IGF-2) associated with cellular proliferation; it has also been put forth that inactivation of this inhibitor may lead to unrestrained mitogenic Shh pathway activity (13, 14). However, additional studies are needed to clarify whether or not KCTD11 haploinsufficiency is sufficient to relieve inhibition of Shh signaling. Furthermore, there is the possibility that additional genes on 17p may act in concert with KCTD11 in promoting tumour suppression. For example, the p53 gene, which plays a central role in tumour suppression, lies centromeric to KCTD11 on chromosome 17p. When activated by cellular stress (DNA damage or oncogenic stress) the transcription factor p53 can elicit cell cycle arrest, senescence or apoptosis (15). Concomitant with 17p loss in MBs is the frequent allelic deletion of p53 with mutational inactivation of the remaining p53 allele occurring in only a minor subset of MBs (16, 17). Thus, p53 heterozygosity may increase at least the susceptibility to MB development; this view is supported by the preferential expression of p53 in Shh-stimulated granule cell progenitors in the external granule layer (EGL) thus implicating its role in maintaining genomic integrity
during rapid clonal expansion (18). Additionally, loss of p53 function has been reported to cooperate with Shh signaling in increasing the frequency of MBs in the Ptc<sup>+/−</sup>p53<sup>−/−</sup> double mutant murine model (19). The major target gene of p53 action implicated in MB development is the tumour suppressor gene Hic1, which is located telomeric to p53 on chromosome 17p (20, 21); Hic-1 tumour suppressor function has been credited to its location in a commonly deleted chromosomal region ie, 17p, and, when transfected, its capacity to inhibit growth in a variety of tumour cell lines (22). However, heterozygous disruption of the murine Hic-1 locus, although predisposing mice to a variety of malignant tumours, does not result in MB formation (23). Promoter hyper-methylation of Hic-1 has been demonstrated in a series of MBs and has also been postulated to inactivate Hic-1 function (12). Interestingly, the presence of aberrant methylation in chromosome 17p11.2, which is a major chromosomal breakpoint cluster associated with loss of heterozygosity (LOH), has fuelled speculation that the hyper-methylation phenotype may be linked to chromosomal instability thus contributing to tumour progression (24, 25).

Aneuploidy is the abnormal number of chromosomes within a cell that is a usual characteristic in most human solid tumours that results from chromosomal instability (26, 27). Aneuploidy as a prognostic marker has met with mixed results with some studies suggesting that aneuploidy is associated with better survival (28, 29) whereas others have demonstrated an association with decreased survival (30). Recently, studies have suggested that chromosomal instability may
generate both chromosomal and transcriptome diversity in tumour cells which can subsequently facilitate clonal expansion and tumour progression (31, 32). Comparative genomic hybridization (CGH) and fluorescent in situ hybridization (FISH) investigations have revealed that about 5-10% of MBs contain amplifications of either c-myc or N-Myc (33). Furthermore, these oncogenic amplifications have been associated with the LC/A MB subtype and the loss of 17p thus adding to their negative prognostic potential (6).

Gene expression profiling has recently emerged as a potentially powerful molecular prognostic marker for disease risk stratification. A landmark study demonstrated that expression profiling could objectively differentiate MBs from other primary brain tumours such as primitive neuroectodermal tumours (PNETs), atypical teratoid/rhabdoid (AT/RTs) and malignant gliomas (34) this is of particular significance because of the histological similarity between anaplastic MBs and AT/RTs (35). Immunohistochemical markers, such as smooth muscle actin and epithelial membrane antigen, could differentiate between the aforementioned tumours for individual cases but in the case of large retrospective studies it is unequivocal that expression profiling would be advantageous. Furthermore, the same study derived a gene expression signature that could differentiate the desmoplastic MB subtype from the other variants; the signature was associated with activation of the Shh signaling pathway such as increased expression of patched, Gli, N-myc and insulin-like growth factor II (34). Investigations have now focused on determining the impact on survival outcome
prediction by combining gene expression profiles with clinical staging criteria (5). Indeed, univariate analysis demonstrated that expression profiling was the only significant prognostic factor in predicting MB outcome whereas clinical criteria (metastatic stage at diagnosis, histologic subtype, age at diagnosis) were not significantly associated with survival; furthermore, multivariate analysis predicted outcome independent of either clinical or histopathological criteria thus implying that clinical criteria could not add prognostic power to expression profiling for risk stratification (36).

C-myc is part of the Mad/Max network that governs cell cycling, differentiation and apoptosis (37). Studies have demonstrated a negative correlation between c-myc mRNA levels and survival outcome; furthermore, these studies have also shown increases in c-myc expression independent of gene dosage (38, 39). Indeed, oncogenic activation of c-myc can occur through either gene amplification, increased message stability or enhanced translation (37). However, N-myc, which acts downstream of the Shh pathway, was not significantly associated with survival outcome; both nodular and anaplastic MBs express N-myc although better survival outcomes are significantly associated with the nodular subtype (6). The Trk receptor family of tyrosine kinases, TrkA, TrkB and TrkC are differentially expressed during CNS development and serve to regulate the proliferation and apoptosis of neuronal progenitor cells; specifically, TrkC receptor ligation can induce apoptosis of MB cells (40). Studies have demonstrated that TrkC over-expression is associated with a good prognosis (41,
The EGFR family of tyrosine kinase receptors, such as EGFR, ErbB2, ErbB3 and ErbB4, have also been examined as a potential molecular prognostic marker for risk stratification. For example, univariate analysis established that tumour expression of the ErbB2 receptor was associated with worse clinical outcomes; interestingly, combining ErbB2 expression data with clinical variables (age, M stage) identified a group of patients (≥3 years, M0, ErbB2-positive) suffering from disease progression within 5 years of diagnosis that were classified as average-risk (5). Furthermore, this study also suggests that c-myc and TrkC do not carry any prognostic weight in determining survival outcome; another study has also refuted a significant interaction between TrkC over-expression and better clinical outcome (6). C-myc was also not prognostic of survival outcome in a recent gene expression profiling study (34). This apparent inter-experimental variability in the predictive power of molecular prognostic markers may arise from differences in technical methods, handling of tissue and small sample sizes (5). Thus, these results will need to be validated by large, multi-institutional, prospective trials that can afford comparisons between the clinical, histopathological and molecular prognostic correlates within a standardized experimental framework.

Although improvements in risk stratification may optimize patient treatment, advancements in therapy can only come from understanding the pathogenesis of medulloblastoma. It is apparent that dysregulation of the Shh pathway plays a common role in MB oncogenesis despite there being a paucity of genetic lesions in components of this pathway. Evidence is now mounting that chromosome 17p
may act either directly or indirectly to modulate Shh signalling. However, reconciling Shh-mediated tumourigenesis with a defective “checkpoint” presumably located in chromosome 17p requires an appreciation of the role these pathway components play within their normal developmental paradigm.

Developmental biology and tumorigenesis

The cerebellum, which harbours medulloblastomas, constitutes about 10% of the entire brain volume and is the region of the brain responsible for integrating sensory information with motor output to fine-tune movement and balance (43). The principal neuronal constituents of the cerebellum are granule neurons (small, glutamatergic, excitatory), Purkinje cells (large, GABAergic, inhibitory) and the GABAergic Golgi and stellate/basket inhibitory interneurons; together, these cell types form the cerebellar cortex (Figure 1) (44). The basic cerebellar circuitry consists of granule neurons, located within the internal granule layer (IGL), receiving mossy fibre afferents from the brainstem precerebellar nuclei.
Figure 1: Organization of the adult cerebellar circuit. Abbreviations: pf, Purkinje fiber; ML, molecular layer; PU, Purkinje cell; GR, granule cell; WM, white matter

Adapted from (44)
The granule cell axons ascend as parallel fibers to terminate within the “cell-poor” molecular layer (ML) and synapse with the Purkinje cell dendritic tree and the various interneurons. The Purkinje cells not only receive granule cell axons but also climbing fibre afferents from the inferior olivary nucleus; these cells are situated at the IGF/ML boundary in a single row. Purkinje cells represent the major output structure of the cerebellar cortex by virtue of their myelinated axons descending to synapse with the deep cerebellar nuclei (DCN), which in turn project to the cerebral cortex. The Golgi interneurons provide feedback inhibition to granule cells whereas the stellate/basket cells provide feed-forward inhibition to the Purkinje cells.

The unique feature of the developing cerebellum is that its neuronal components are derived from two germinal matrices, rather than from a single germinal zone that characterizes cerebral development (45, 46). The ventricular germinal matrix (or ventricular zone) gives rise to cells that migrate laterally and eventually differentiate into the Purkinje cells and the DCN, which form the early cerebellar anlage (43, 46). The formation of the secondary germinal matrix, termed the rhombic lip from which the granule neurons are derived, usually coincides with the ceasing of Purkinje cell/DCN division. These external granule layer (EGL) precursors then migrate over the cerebellar anlage within the same timeframe that the interneurons are generated from the ventricular zone (47). Postnatally, the EGL undergoes clonal expansion to generate post-mitotic neurons within the inner EGL that begin to differentiate and migrate through the ML along
Bergmann glia to form the IGL; the EGL is no longer present after the first year of human life (43, 46). The outer EGL, which contains the proliferating granule cell precursors (GCPs), displays an upregulation in genes that mark the granule neuron lineage such as \textit{math1} (48, 49), \textit{RU49} (50), \textit{zic1}, \textit{zic2} (51, 52) and genes involved in cell proliferation such as cyclin \textit{D2}, \textit{patched}, \textit{smoothened}, \textit{gli1} and \textit{gli2} (53). The early postmitotic granule neurons, which are located in the inner EGL, display increases in gene expression associated with \textit{p27kip1} (54), \textit{Neuro D} (55) and neuron-specific class III \textit{β tubulin} (54), which are markers for cell cycle exit and early neuronal differentiation. Thus, the balance between proliferation and cell cycle exit is crucial to ensure proper cerebellar pattern formation.

The Wnt pathway can regulate early cerebellar pattern formation and has been implicated in medulloblastoma formation. The Wnt genes encode for 24 known vertebrate members of a family of secreted glycoproteins collectively referred to as Wnt proteins (Wnts) (56). In the absence of Wnts, β-catenin is recruited to a cytoplasmic multi-protein complex consisting of the tumour suppressor protein adenomatous polyposis coli (APC), the scaffold protein Axin and the serine-threonine kinase glycogen synthase kinase 3-beta (GSK-3β). β-catenin is then phosphorylated at its N-terminus by GSK-3β and subsequently ubiquitinated and targeted for proteosomal-mediated degradation (57). The Wnt proteins (Wnts) ligate to members of the Frizzled (Fz) family of seven-pass transmembrane receptors and to the low-density lipoprotein receptor related proteins, LRP-5 and LRP-6 resulting in membrane recruitment of Dishevelled (Dsh). Dsh is then able
to recruit Axin to the membrane resulting in its degradation and inhibition of GSK-3β. Non-phosphorylated β-catenin is able to translocate to the nucleus where it can interact with the T-cell factor/lymphocyte enhancer factor (TCF/LEF) family of transcription factors and induce expression of target genes such as \textit{c-myc} and \textit{cyclin D1}. Nuclear β-catenin also regulates the expression of the homeodomain protein Engrailed-1 (En1), which is expressed at the midbrain-hindbrain junction (MHJ) (58, 59). Targeted disruption of Wnt-1 in mice can cause severe midbrain and cerebellar defects (60). Nestin-cre ablation of β-catenin has also revealed severe vermian hypoplasia, failed cerebellar hemispheric fusion and periventricular heterotopias, which suggest disorders of proliferation and migration (61). Wnt signalling involvement in MB formation was first elucidated from studies of the familial Turcot’s syndrome; this syndrome is characterized by multiple colorectal adenomas and increased frequency of MBs (62). Furthermore, germline APC truncating mutations occur in the major inherited syndrome, familial adenomatous polyposis (63). However, about 3-4% of sporadic MBs contain missense APC mutations that imply impaired APC binding to β-catenin or Axin (64). Mutations in other Wnt pathway components also occur in MBs. For example, single strand conformational polymorphism (SSCP) confirmed several point mutations in the β-catenin gene, which translated into loss of serine residues necessary for GSK-3β-mediated phosphorylation resulting in nuclear β-catenin accumulation (65). As stated before, the Axin protein acts as a scaffold to bring β-catenin in close proximity to APC and GSK-3β, which assist in phosphorylation and subsequent degradation. Axin point
mutations in MB occur in about 1-5% of sporadic MBs that can subsequently destabilize the multi-protein complex thus leading to nuclear β-catenin accumulation (66). All together mutations in APC, β-catenin and Axin are generally mutually exclusive occurring in about 15% of sporadic medulloblastomas (67). Given these findings in MB samples, the role that the Wnt pathway plays in MB oncogenesis is still not clear. For example, retroviral-mediated delivery of the β-catenin gene to nestin-positive cells in the postnatal cerebellum did not elicit GCP hyper-proliferation and tumourigenesis compared to transduction of the Shh gene with either the c-myc or the insulin-like growth factor genes (68-70).

The molecular mechanisms regulating granule cell precursor (GCP) proliferation and differentiation have recently begun to be elucidated. Shh, produced by Purkinje neurons, is required for GCP proliferation and also induces the differentiation of Bergmann glia (53, 71). In the absence of Shh, the Patched 1 (Ptc1) receptor indirectly suppresses the activity of the Smoothened (Smo) receptor by possibly acting as a transmembrane gate for a small molecule intermediate that can then directly affect Smo function (72); indeed, synthetic intermediates such as the Hh agonist purmorphamine and the Hh antagonist cyclopamine have been shown to modulate Smo activity (73, 74) Shh binding to Ptc1 relieves Smo suppression presumably by favouring transport of Smo-containing vesicles to the membrane and internalizing Ptc1 from the membrane (75, 76). These membrane dynamics can regulate target gene expression through
regulation of the Cubitus interruptus (Ci)/Glioma associated oncogene homologue (Gli) family of transcription factors. In Drosophila, Ci functions as both a repressor and activator of Shh target genes. Specifically, in the absence of Shh, Ci is tethered to a microtubule-associated multi-protein complex consisting of the kinesin-like protein Costal2 (Cos2), the serine-threonine kinase Fused (Fu) and Suppressor of Fused (SuFu), which is required for Ci cytoplasmic retention (77, 78). Protein kinase A (PKA)-mediated phosphorylation further primes Ci to additional GSK3β- and CKI-mediated phosphorylations. These events then permit binding of Ci to the F-box protein Slimb, which shunts Ci down the ubiquitin-proteosomal pathway to generate cleaved N-terminal repressor fragments; these fragments localize to the nucleus and repress Shh target genes. However, Shh ligation of Ptc1 stabilizes Smo that can recruit Cos2 and Fu to its cytoplasmic tail thus allowing nuclear localization of full-length Ci and subsequent activation of target gene expression (79-82). The vertebrate homologues of Ci such as Gli1, Gli2 and Gli3 cooperate to regulate Shh target gene expression (ptc1, igf-2, Nmyc and cyclin D1) in a remarkably distinct fashion. Gli3, much like the Drosophila Ci, functions as both an activator and a repressor. Gli3 tethering to the cytoplasmic complex under Shh-negative conditions acts as a substrate for phosphorylation by the kinases PKA, GSK3β and CKI, which then promotes binding of βTrCP, the vertebrate homolog of Slimb; subsequent Gli3 processing generates transcriptional repressors (83, 84). Interestingly, nearly all of Gli1 and Gli2 degrade following phosphorylation and ubiquitination; a minor fraction of Gli2 converts to the repressor form. Shh
ligation promotes stabilization and release of full-length Gli1 and Gli2 to activate Shh target genes; full-length Gli3 has a very weak trans-activating potential (83, 85).

The early EGL contains GCPs, which have just completed migration from the rhombic lip; these cells express Ptc, Smo and Gli2 and thus may respond to Shh (86). Indeed, Purkinje cells, which are situated just beneath the EGL, begin secreting Shh that induces a robust expansion of the GCP population for about 2-3 weeks concomitantly with cell cycle exit and differentiation into mature granule neurons; mice lacking Shh or Gli2 show severe deficits in the number of granule neurons (87). The nature of the signals that determine cell cycle exit, whilst the GCPs are still in the presence of Shh, have been forthcoming and include bone morphogenetic proteins (BMPs), fibroblast growth factors, pituitary adenylate cyclase activating polypeptide (PACAP) and extracellular matrix factors such as vitronectin (53, 88-90).

Mutations in the Ptc receptor have been linked to medulloblastoma formation and this connection was first discovered in the rare condition termed nevoid basal cell carcinoma (NBCC) or Gorlin syndrome (91, 92). Patients afflicted with NBCC usually present with skeletal deformities, numerous basal cell carcinomas, rhabdomyosarcomas and a high incidence of sporadic MBs (93); additionally, a subset of sporadic MBs, not associated with NBCC, contain Ptc mutations (94, 95). Thus, Ptc may potentially act as a tumour suppressor in GCPs and, when
mutated, may lead to persistent Smo activation and unrestrained proliferation. Supporting evidence for this view comes from murine transgenic modelling of Ptc mutations where 15-20% of heterozygous Ptc mutants develop sporadic MBs, which share the same molecular and histological features of the human desmoplastic MB variant (96, 97). Mutations in other components of the Shh pathway, aside from Ptc mutations, may also promote MB formation. For example, about 9% of sporadic MBs contain truncated inactivating SuFu mutations, which may lead to constitutive nuclear Gli localization and activation of Shh target genes associated with proliferation (98). No mutations have been observed in other Shh pathway components such as Smo, Gli3 and βTRCP (99-101); however, another study demonstrated activating Smo mutations in about 4% of sporadic MBs (102). Irrespective of the Shh pathway mutation, targets such as Gli1, Gli2, Gli3, N-myc, cyclin D1 and IGF-2 are upregulated in MBs (34, 103-105).

The process of tumourigenesis may result from the stepwise accumulation of genetic and epigenetic defects that convert a normal cell into a malignant one (106). Mutations in proliferative signaling pathways, such as Shh and Wnt, may synergize with pro-survival pathways (apoptosis evasion), such as those mediated by insulin-like growth factor 1-receptor (IGF-IR), to ensure continued survival and propagation; indeed, the Shh pathway activates IGF-II expression, which in turn is an IGF-IR ligand. The IGF-IR is a tetrameric, transmembrane tyrosine kinase receptor that consists of two identical α- and β-subunits (107, 108).
Ligation of the IGFs (IGF-I/II) to the IGF-IR induces a conformational change in the β-subunits resulting in autophosphorylation of tyrosine residues within the cytoplasmic tyrosine kinase domain followed by additional phosphorylations within the juxtamembrane and carboxyl-terminal domains (109). These phosphorylated residues then serve as a docking site for the insulin receptor substrate (IRS) and Shc adaptor proteins. Phosphorylation of IRS and Shc recruit additional factors such as Grb-2/SOS and the p85 regulatory subunit of the phosphatidylinositol-3-kinase (PI3K), which lead to activation of the Ras-Raf-mitogen-activated protein (MAP) kinase and PI3K pathways, respectively (107). The ultimate effectors of the MAPK and PI3K pathways are the Ets and forkhead family of transcription factors, respectively, which control cell proliferation and survival (107).

PI3K activation can activate several serine/threonine kinases, which include both the Akt and the related SGK (serum and glucocorticoid inducible kinase) protein kinases. Akt then phosphorylates a variety of substrates, including the proapoptotic Bcl-2 family member Bad, caspase-9, nuclear factor-κB, mammalian target of rapamycin (mTOR), mdm2 and the Forkhead family of transcription factors, FOXO1, FOXO3a and FOXO4 (110). In established cell lines, the Forkhead transcription factor members augment the expression of the cyclin-dependent kinase inhibitor p27KIP1 causing cell cycle arrest; FOXO-induced transcriptional upregulation of Fas ligand (FasL) and Bim genes can also trigger apoptosis (111). Akt-mediated phosphorylation leads to FOXO nuclear exclusion thereby mitigating apoptosis; furthermore, phosphorylated FOXO is targeted for
ubiquitination and proteosome-mediated degradation (111). Oxidative stress can activate Jun N-terminal kinase (JNK), which then phosphorylates cytoplasmic FOXO, on residues not phosphorylated by Akt, resulting in nuclear import (112). Thereafter, protein acetylation provides an additional layer of regulation whereby nuclear FOXO proteins can bind directly to and are acetylated by the basal transcription machinery (CREB-binding protein (CBP) and p300/CBP-associated factor (PCAF)); CBP-mediated acetylation may interfere with FOXO binding to DNA and prevent transcription of cell cycle arrest and apoptotic genes (113). The constitutively nuclear sirtuin protein, SIRT1, then deacetylates nuclear FOXO resulting in activation of the stress-resistant genes p27KIP1 and GADD45 and preventing transcription of pro-apoptotic genes such as Bim and FasL (Figure 2) (114).
Figure 2: Insulin-like growth factor regulation of FOXO transcriptional activity.

Reproduced from (115)
Cerebellar granule neurons express components of the IGF-IR signalling system with PI3K and Akt activation being essential to granule cell survival (116-119). Furthermore, IGF-IR has also been implicated in Purkinje cell development (120, 121). IGF-IR and IRS-1 upregulation in medulloblastoma biopsies and cell lines relative to control cerebellar tissue, along with activation of Erk-1, Erk-2 and Akt/protein kinase B, suggests the involvement of the IGF-IR axis in MB development (122, 123). Past investigations demonstrated a requirement for functional IGF-IR in the malignant transformation process; IGF-IR-negative cells display resistance to transformation by cellular and viral oncogenes (124, 125). For example, the human neurotropic polyomavirus JC (JCV) encodes for the T antigen, which displays both transforming properties in vitro and a high tumorigenic potential in vivo (126-128). Functional cooperation between the JCV T antigen and the IGF-IR axis results from the requirement of IGF-IR in T antigen-mediated cellular transformation (129). The mechanistic basis for this cooperation apparently lies in the interaction between IRS-1 and JCV T antigen (130). In association with JCV T antigen, IRS-1 may detach from IGF-IR and translocate to the nucleus to initiate cellular transformation (129). Recently, genetic deletion studies revealed the physiological importance of FOXO proteins. For example, FOXO1-null mice display embryonic lethality at 10.5 dpc (days post-coitum) resulting from defects in angiogenesis (131). FOXO1 heterozygote mutants rescue the diabetic phenotype in insulin receptor mutant mice whereas transgenic mice overexpressing a constitutively active FOXO1 mutant result in a severe diabetic phenotype (132). FOXO3-null mice display premature depletion
of ovarian follicles resulting in age-dependent infertility; FOXO4-null mice are viable and do not display an abnormal phenotype (131). As mentioned before, IGF-IR axial engagement leads to cerebellar granule cell survival. The mechanism underlying this neuroprotective role involves Akt-mediated inactivation of FOXO3a resulting in downregulation of Bim, which is a crucial player in the intrinsic death-signalling pathway (133); a similar mechanism operates in hippocampal neurons (134). FOXO factor involvement in tumourigenesis has also come under scrutiny. Chromosomal breakpoints in tumours occur within intron 2 of FOXO1 (childhood alveolar rhabdomyosarcoma), FOXO3 and FOXO4 (acute myeloid leukemia) to generate chimeric proteins consisting of the C-terminus of the FOXO protein fused to the N-terminus of other transcription factors such as PAX3 or PAX7 for FOXO1 (135-137). Although recent studies have revealed inactive FOXO proteins in colon cancer (138), non-small cell lung carcinoma (139), breast cancer (140-142) and prostate cancer (143-145), the status of FOXO proteins in MBs is generally not well appreciated. Interestingly, FOXO proteins have been identified as a partner in a multiprotein complex comprised of SMAD3 and SMAD4, which are regulated in a TGF-β-dependent fashion, to activate p21 expression; FOXG1 binds to this complex thereby inhibiting p21 expression (146). Indeed, a very recent study has demonstrated increased FOXG1 copy gain and immunohistochemical expression in a series of MB cases that is inversely related to p21 expression; FOXG1 appears to be downregulated in normal differentiated cerebellum whereas the converse is true for MB cell lines (147). It has been
suggested that FOXG1 may cooperate with Notch pathway components to negatively regulate neural differentiation (148).

The Notch signalling cascade also plays an important role in cerebellar development and MB tumourigenesis. Both the four vertebrate Notch receptors (Notch1-4) and their ligands, Delta and Jagged, are transmembrane proteins characterized by large extracellular, epidermal growth factor (EGF)-like repeats (149). Signalling commences upon ligand engagement of the Notch receptor to promote two proteolytic cleavage events. The first cleavage, mediated by the ADAM-family of metalloproteases, generates extracellular soluble ectodomain with the second γ-secretase-mediated cleavage generating the Notch intracellular domain (NICD). The NICD translocates to the nucleus and interacts with the transcriptional co-factor CBF1 to effect transcription from the Hes1 and Hes5 genes thus impacting cell fate specification and regulation of differentiation (150). Notch2 marks the proliferating GCPs within the outer EGL of the developing cerebellum; furthermore, Notch2 expression is downregulated in early postmitotic, premigratory granule cells and is absent in migratory granule neurons and in IGL granule neurons (151). Either soluble Jagged1 or overexpression of activated Notch2 (NICD2) can interfere with granule cell differentiation and thus maintain the GCP pool (151). Another study revealed that enforced Notch1 downregulation within the embryonic MHJ neuroepithelium might induce neurogenesis but may not allow progression to complete neuronal differentiation; instead, these Notch1-ablated cells undergo apoptosis leading to a reduction in neuronal number in the adult cerebellum (152). Furthermore, the EGL is devoid
of Notch1 expression (151) whereas its expression is found within the mature IGL (153). Interestingly, Notch1 and Notch2 reveal opposing effects on MB proliferation (154). Specifically, Notch1 activity inhibits MB cell proliferation whereas the converse is true for Notch2 (154). Notch1 and Notch2 expression profiles in primary MB specimens were also consistent with their in vitro tumour growth properties with Notch1 levels being barely detectable and Notch2 displaying robust expression (154).

The ErbB receptor tyrosine kinase receptor system (ErbB-1, ErbB-2, ErbB-3 and ErbB-4) is activated through ligand-mediated homo- or hetero-dimerization. These ligands are neuregulins (NRG1, NRG2 and NRG3), which primarily ligate ErbB-3 and ErbB-4 and EGF/TGF-α, which ligates ErbB-1; there is no known ligand for ErbB-2 (155-158). However, ErbB-2 has high affinity for the other receptors leading to its heterodimerization and increased potency in activating MAP kinase (159). Essentially, ErbB dimers can activate a number of cell signalling cascades, such as the Ras-Raf-MAPK and the PI3K-Akt pathways, important in proliferation, apoptosis, migration and differentiation (160). Studies have demonstrated that upon contact granule neurons can activate Notch1 signaling in cerebellar astrocytes thereby leading to an ErbB-dependent morphological differentiation of these astrocytes into radial glia (161). The functional consequence is the extension of radial processes that permits granule neurons to migrate from the inner EGL to populate the IGL (162). Although ErbB-2 is undetectable in normal human cerebellum, MBs display unusually
intense ErbB-2 levels; in addition, Erb-B4 and NRG-1, which are both expressed in the normal EGL, can also be detected at abnormally high levels in MBs (163). Activation of the ErbB-2-PI3K-Akt pathway can lead to p21WAF1 phosphorylation and cytoplasmic relocalization resulting in unrestrained cellular proliferation (164).

There is ample evidence that the aforementioned signalling pathways (Wnt, Shh, IGF-I/II, Notch, ErbB) may synergize during MB tumourigenesis. As mentioned before, Shh pathway activation leads to upregulation of IGF-II expression, which is a ligand for the IGF-IR axis; indeed, MB formation appears to require IGF-II overexpression (105). Another study demonstrated that the incidence of the MB phenotype in Ptc haploinsufficient mice (15%) significantly increased when co-expressed with IGF-IR axial components such as IGF-II (39%) and Akt (48%); neither IGF-II nor Akt independently formed MBs thus highlighting the synergy between the Shh and IGF-IR signalling pathways in MB pathogenesis (70). One study elegantly demonstrated that IGF-IR activation synergizes with Shh stimulation in promoting N-myc1 stability and subsequent cell cycle progression in GCPs (103). SuFu represses Shh pathway activation by sequestering Gli in the cytoplasm. Furthermore, SuFu can negatively regulate β-catenin activity by complexing with β-catenin thereby preventing TCF/LEF-dependent transcription (165). Indeed, SuFu mutations prevail in a subset of MBs with evidence suggesting that these mutations can lead to activation of both the Shh and Wnt pathways; the SuFu-δex8 mutant, which fails to inhibit Gli-mediated
transcription, can also fail to inhibit β-catenin/TCF/LCF-mediated transcription (98, 166). Finally, both the Notch and Shh signalling pathways synergize in upregulating HES1 expression to maintain the GCP state within the developing cerebellum (151). Constitutive activation of murine Smo leads to GCP hyperproliferation and a high incidence of MBs (48%); significant Notch2 elevation occurs in Smo-induced MBs confirming the synergistic interaction between Shh and Notch signalling pathways (167). Another study has demonstrated using the Ptc MB model that Shh signalling might simultaneously control Notch and Wnt pathway components, such as Notch2, Jagged1, mSfrp1 and mFrz7, resulting in MB formation (168).

Transgenic technology has been particularly useful in modeling MB development. The most widely utilized murine model is the aforementioned Ptc heterozygous model that is consistent with Gorlin’s syndrome (96). Targeted disruptions of genes important in genomic maintenance, such as p53, have yielded further insight into MB development. For example, Ptc heterozygosity synergizes with p53 nullizygosity to increase MB penetrance (19). Other studies have demonstrated that p53 loss can act in conjunction with disruption in DNA repair genes (DNA ligase IV) or cell cycle regulatory genes (Ink4c, Ink4d or Kip1) to result in MB formation (169-171). These studies suggest that genomic instability arising from p53 loss may synergize with the aforementioned disrupted genes in accelerating the onset of MB development. Finally, the observation that concurrent mutations in the previous murine models do not faithfully represent the molecular findings in the vast majority of clinical samples spurred the
development of the aforementioned activated Smo MB model; this model recapitulates Notch and Shh pathway component activation found in MB clinical specimens (167).

Although molecular studies identify the potential series of signalling events or conditions that could transform GCPs into their malignant counterparts, recent evidence has challenged the view that GCPs are the sole cellular targets for transformation.

**Stem cells**

Careful genetic studies and clinical observations of a large variety of cancer types lead to the seminal hypothesis that conversion of a normal somatic cell into a malignant cell requires six essential alterations to its cellular physiology: 1) self-sufficiency in growth signals; 2) insensitivity to anti-growth signals; 3) tissue invasion and metastasis; 4) limitless replicative potential; 5) sustained angiogenesis; 6) evasion of apoptosis (106). The classifications of these molecular events, which are common in most cancers, have still not definitively yielded the target cell for transformation. Despite the observation that these malignant cellular characteristics closely phenocopy the stem-like features of self-renewal and multi-lineage differentiation, the outstanding feature separating the malignant cell from the normal stem cell is the lack of homeostatic balance.
between self-renewal and differentiation. Furthermore, cancer tissue, much like normal tissue, is hierarchically organized according to stage of differentiation and proliferative potential (172). Such close phenotypic parallels imply that the normal stem cell could malignantly transform; however, lineage-restricted or differentiated progeny transformation, through a process of de-differentiation, into malignant cells displaying stem-like features may also be possible (172). Yet, the target stem cell may require fewer alterations to initiate tumourigenesis compared to their downstream progeny (173). These so-called “cancer stem cells” have been isolated from a variety of tumours, including MBs (174).

Neural stem cells (NSCs)

The concept of stem cells existing in the central nervous system (CNS) challenged the dogma that quiescence typified cell turnover rates within the brain (175-177). Rodent studies have demonstrated the existence of NSCs in the early neural tube ectoderm that directly line the ventricular surface and radially orient to contact the pial/basal surface (178, 179); furthermore, symmetric divisions expand the pool of these early NSCs or neuroepithelial cells. These cells may possibly differentiate into embryonic radial glial cells that persist into the early postnatal period, which then undergo asymmetric division, generating radially migrating neuroblasts to form the brain parenchyma; radial glial cells emit basally projecting processes that may aid in neuroblast migration (180-182). Current studies demonstrate that
neurogenesis persists throughout adult life in specialized brain regions, such as the hippocampal dentate gyrus and the subventricular zone (SVZ) of the forebrain lateral ventricles, to subserve activities crucial for optimal brain function (183, 184). The adult SVZ NSCs express the astrocytic marker glial fibrillary acidic protein (GFAP); notably, these astrocyte-like NSCs or Type B cells represent only a minor fraction of the total astrocyte population within the SVZ (185, 186). These SVZ NSCs generate multipotent transiently amplifying (TA) progenitors (or Type C cells), which further generate neuron-restricted precursors that penultimately differentiate into neurons. The hippocampus is likewise hierarchically organized with a subset of subgranular zone (SGZ) astrocytes operating as NSCs, which subsequently give rise to transiently amplifying progenitors; neuron-restricted precursors, derived from these TA progenitors, generate mature neurons that functionally integrate into the hippocampal granule cell layer; glial cells also continue to be generated (184). Morphological and biochemical similarities between SVZ astrocytes and radial glial cells imply that these NSCs may derive from radial glia; in vivo radial glial cell labelling revealed that these cells can generate “neurosphere-forming” SVZ astrocytes (181, 187). If NSCs arise solely from the neuroepithelial → radial glia → astrocyte lineage then the view that neuronal production may occur along the astrogial lineage would challenge the dominant model of separate precursors defining the neuronal and glial lineages. Adult NSCs exposed to growth factors such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) can be expanded in vitro to generate neurospheres thereby making these cells amenable to molecular and
developmental studies; neurosphere formation demonstrates the property of self-renewal (177, 188-190). Interestingly, in vitro studies have revealed cell populations displaying stem-like characteristics (self-renewal and multi-lineage differentiation) within regions defined as non-neurogenic such as the optic nerve and the spinal cord; these findings suggest that local niche-related factors may control progenitor fate (191, 192). Furthermore, committed progenitors, such as the SVZ TA cells and the optic nerve oligodendrocyte precursors, generate tri-potent NSC-like cells when cultured in vitro in the presence of bFGF and EGF (190, 193); parenchymal astrocytes also express FGF receptors (194, 195). Another implication of glial involvement in neuronal production resulted from Cre/loxP fate mapping studies, which utilized the GFAP promoter to drive cre-mediated LacZ expression in astrocytes, which demonstrated that the cerebellar granule neurons may derive from GFAP+ cells (196). The neurosphere formation assay (NSA) has also demonstrated the presence of NSCs within the postnatal cerebellum (197). As stated before, cerebellar neurogenesis arises from either the ventricular zone or the displaced EGL with the majority of the proliferative cells being detected in the EGL; a minor proportion of proliferating cells populate the white matter below the Purkinje cell layer (45). With exception of the granule neurons, most of the other cell types, which include Purkinje cells, interneurons, Bergmann glia and oligodendrocytes, arise from the non-EGL or white matter (WM) proliferative fraction (47). Although it is well established that GCPs generate exclusively granule neurons, ambiguity surrounds the potentiality of the WM progenitors. A recent study identified a subset of cells within the WM
proliferative fraction that express the cell surface glycoprotein prominin-1 (CD133), which is normally found in haematopoietic stem cells and neural precursors, and lack markers for both the neuronal and glial lineages (prominin‘lin’); these cells also form self-renewing neurospheres that display multipotentiality with regards to generation of neurons, astrocytes and oligodendrocytes \textit{in vitro} and after cerebellar transplantation (197). This same study also revealed that EGL-derived GCPs do not form neurospheres and furthermore highlighted bFGF antagonism of Shh-mediated GCP proliferation (197). Thus, the presence of a bona fide stem cell in the cerebellum suggests a cell of origin for MB other than the GCP.

Genes identified as oncogenes or tumour-suppressors also regulate stem cell self-renewal such as Bmi-1, Notch, Shh and Wnt/\(\beta\)-catenin (198-202). \textit{In vitro} \(\beta\)-catenin overexpression expands the epidermal stem cell pool leading to decreased differentiation (203); the \textit{in vivo} correlate results in epithelial cancer (204). Jagged-1 ligand activates the Notch pathway leading to increased primitive progenitor activity and haematopoietic stem cell (HSC) self-renewal and multipotentiality (205). The neural RNA-binding protein Mushashi1, which is highly expressed in NSCs, can activate the Notch pathway leading to NSC self-renewal (206). Although Shh stimulates the proliferation of cerebellar GCPs, which are not bona fide stem cells, only bFGF can stimulate the proliferation of the prominin-positive cerebellar stem cells (197). Bmi-1, belonging to the Polycomb group of genes, enhances cell proliferation and self-renewal through
repressing the tumour suppressors Ink4a and Arf (198). Murine Bmi-1 mutational inactivation revealed the regulatory role of Bmi-1 over normal haematopoietic and leukaemic stem cells (199). Bmi-1 deficient mice, in addition to the observed decrease in haematopoietic cells, develop severe neurological sequelae, such as progressive ataxia and balance disorders; these mice also display significant reduction in cell numbers in the cerebellar IGL and ML (207). Moreover, intense expression in proliferating GCPs within the EGL revealed that committed precursor cells could also be regulated by Bmi-1 (208). The striking resemblance in the cerebella of Bmi-1-deficient mice when compared to N-myc- and Cyclin D2-deficient mice, further illustrates the involvement of Bmi-1 function within the Shh-responsive GCPs (208).

NSC expansion occurs during early development through symmetric divisions to generate daughter progeny, which are identical to the parental cell. Asymmetric division, which characterizes the mode of stem cell self-renewal later in development, generates both a stem cell and a committed progenitor; asymmetric divisions ensure the maintenance of the stem cell pool. Inactivating mutations in the aforementioned signalling pathways can lead to exhaustion of the NSC pool (199, 200, 209). Ultimately, transcription factors (TFs) regulate the cellular fate adopted by NSCs. For example, activation of both gp130/Jak/Stat3 and bone morphogenetic protein (BMP) pathways in NSCs can form a Stat3/CBP/Smad ternary complex, which induces astroglial differentiation (210, 211). On the other hand, neurogenesis appears to be under the control of neuronal basic helix-loop-helix TFs such as Neurogenins 1 and 2 (Ngn1 and 2), Mash1 and Math
Ngn1 can sequester the CBP/Smad complex thereby preventing the activation of STAT TFs and subsequent gliogenesis (212). Despite evidence unequivocally indicating the existence of NSCs in the developing and adult brain, the question still remains whether de-regulated NSCs drive the formation of brain tumours.

Brain tumour stem cells (BTSCs)

Generally, the “cancer stem cell hypothesis” postulates that deregulation of the stem cell self-renewal process initiates tumourigenesis; the corollary is that tumours retain a minor proportion of stem-like cells that drives tumour formation (self-renewal) and contributes to cellular heterogeneity (aberrant differentiation). The roots of the cancer stem cell hypothesis lay in a set of meticulous observations performed by the pathologist Julius Cohnheim in the late 19th century who postulated the following: “The simplest view appears to me undoubtedly to be that in an early stage of embryonic development more cells are produced than are required for building up the part concerned, so that there remains unappropriated a quantity of cells—it may be very few in number—which, owing to their embryonic character, are endowed with a marked capacity for proliferation… The only point on which I lay stress is that the real cause of the subsequent tumour is to be sought in a fault or irregularity of the embryonic rudiment” (213). Subsequently, a seminal investigation directly addressed this
hypothesis by demonstrating, with in vitro clonogenic assays, that only 0.01%-1% of isolated mouse myeloma cells could form colonies (214). Furthermore, in vivo experiments revealed that only 1-4% of transplanted leukaemic cells form spleen colonies (215). Hence, the observation that tumour initiation requires large numbers of cells to be grafted lead to the dilemma of whether there exists only a minor subset of tumour-initiating cells (hierarchical model) or that every cell can initiate a tumour albeit with a low probability (stochastic model). Evidence supporting the hierarchical model stemmed from identification and purification of CD34⁺CD38⁻ cells from patients afflicted with acute myeloid leukaemia (AML) that, when transplanted into NOD/SCID (non-obese diabetic, severe combined immunodeficient) mice, appeared to be the only cellular phenotype capable of initiating tumourigenesis (216). However, proving the existence of cancer stem cells in solid tumours required better characterization and isolation of solid tumour cells.

Seminal observations by pioneer neurosurgeon Harvey Cushing and pathologist Percival Bailey of their series of MB cases lead them to postulate the following: “The belief that the majority of these cerebellar tumours must take their origin from an embryonic rest is inescapable. Pointing in this direction are the facts: that the majority of them give symptoms so early in life; that the cells comprising them are of a germinal and undifferentiated type normally found only in the embryo; that the tumours are median in position and arise from the palaeocerebellum” (217, 218). Recently, studies have demonstrated the existence
of neurosphere-forming cells in GBMs, which, in addition to producing neuronal and astroglial cells in vitro, can also recapitulate the original histological GBM features upon serial transplantation (219, 220). Proving a clonally derived hierarchy of cancer cells required isolation and purification of CSCs. Indeed, as mentioned before, sorting by cell surface marker expression has proven the existence of CSCs in AML. Yet, the best NSC marker appeared to be nestin, which is a cytoplasmic intermediate filament; cell-sorting strategies required that a surface marker be identified on NSCs. CD133, which is a penta-span cell surface protein expressed on haematopoietic stem cells, can also be considered a good NSC marker (221, 222). A recent study has shown that human brain tumours, such as GBM and MB, contain a proportion of cells that express the putative stem cell marker CD133 and that only CD133+ cells exhibit stem cell properties in vitro (223). Furthermore, the capability to initiate tumours in vivo, which could also be serially propagated, solely resides within the CD133+ brain tumour fraction; these serially transplanted tumours recapitulate the phenotype of the original tumour (174). CD133 expression has also been found in tumour-initiating cells of prostate and colon carcinomas (224, 225). Another method to detect potential stem-like cells relies on the capacity of the ATP-binding cassette (ABC) transporters, encoded by the multidrug-resistant (MDR) gene 1, the MDR protein (MRP) and the breast cancer-resistant protein 1 (BCRP1), to efflux drugs out of the cell (226). Labelling bone marrow cells with the Hoechst 33342 dye followed by dual wavelength (red and blue) flow cytometric analysis revealed two populations of cells: 1) a side population (SP) that does not retain a significant
amount of dye and 2) a non-side population (NSP) that displays higher Hoechst fluorescence; verapamil inhibits ABC transporter activity thereby abolishing the SP (227). Furthermore, the SP fraction constitutes about 1% of all bone marrow cells and highly enriches for HSC activity (227, 228). Neurosphere cultures obtained from embryonic and adult murine brains also reveal SP and NSP cells (229-231). An initial study hypothesized that ABCB1/P-glycoprotein (P-gp) expression was responsible for the SP phenotype in murine NSCs (229). Experimental evidence has recently revealed not only the expression of ABCB1 but also ABCG2 expression in human neural stem/progenitor cells that both co-localize with the definitive NSC marker nestin (232, 233). Interestingly, both the embryonic neurosphere-derived SP and NSP express ABCG2 mRNA whereas its expression is only confined to the adult neurosphere-derived NSP (231). However, another study revealed that NSCs do not appear to localize to the SP in freshly harvested developing and adult murine forebrains; the SP phenotype was largely attributed to microglial and endothelial cells expressing ABCB1 and ABCG2 transporters (234). Thus, the molecular basis underlying the SP phenotype still remains uncertain. Nevertheless, SP identification in human primary cancers such as breast, leukaemia, lung and brain, and in a variety of cancer cell lines related to gastrointestinal, CNS, retinoblastoma and ovary has provided further evidence for the CSC hypothesis (235-238). For example, primary neuroblastoma (NB) specimens were capable of \textit{ex vivo} expansion and asymmetric division with only SP cells generating both SP and NSP progeny (239). The widely used C6 glioma cell line also contains a small verapamil-
sensitive SP, which can be both expanded in NSC medium and can also differentiate into neurons and glia; it was shown that only C6 SP cells harbour the potential for malignancy (237). Interestingly, one study demonstrated that the higher tumourigenicity associated with the SP fraction was unrelated to ABCG2 expression; furthermore, both ABCG2\(^+\) and ABCG2\(^-\) cells can reconstitute each other with the ABCG2\(^-\) fraction containing the primitive CSC phenotype and ABCG2\(^+\) expression identifying with fast-cycling TA progenitors (240). The DAOY MB cell line has also been shown to contain a small verapamil-sensitive SP fraction (241).

Despite the abundance of current studies corroborating the hypothesis that the tumour-initiation capability resides within an identifiable tumour stem cell phenotype, much controversy and ambiguity surrounds the cellular origin of this phenotype. Classical hypotheses have centred on genetic mutations dedifferentiating normal somatic cells into potential tumour-initiating cells (242, 243). Furthermore, molecular biological approaches reconceptualized these hypotheses into a set of the aforementioned hallmarks that characterize the phenotypic progression from normalcy to malignancy resulting from acquisition of between four to seven independent mutations (106). However, normal stem cells satisfy a few of these criteria, such as self-renewal and limitless replicative potential, and thus fewer mutations may be needed for progression to a malignant phenotype. Additionally, efficient DNA repair mechanisms ensure that mutations remain rare stochastic events. The immediate downstream progeny, TA
progenitors, may also be targeted by oncogenic mutations. However, transient cell division may not be the ideal environment for the acquisition of multiple mutations; these mutations may be lost through either apoptosis or differentiation (244). Yet, these mutations may also block differentiation, apoptosis or both thereby rendering the cycling TA progenitor immortal. For example, during CML blast crisis, mutations accumulate in the fast cycling progenitor cells making them capable of maintaining the leukaemic state (245). Thus, stem cells and their immediate progeny may be more “transformation-prone” than their differentiated counterparts. Yet, few studies have directly addressed the hypothesis of whether NSCs can transform into BTSCs. However, studies have suggested that adult stem cells may transform into malignant cells after long-term culturing in vitro, resulting in limiting neurosphere-derived NSCs beyond the tenth passage (246, 247). Recently, one study has challenged this dogma by demonstrating stable karyotypic and molecular profiling characteristics from adult NSCs cultured in vitro for over 12 months (> 120 passages); Myc and Ras oncogene transduction did not elicit in vivo tumour formation (248). Indeed, the low incidence of brain tumours may underline the resilience of the NSC compartment to oncogenic insult.

An intriguing study revealed that mature astrocytes treated with transforming growth factor alpha (TGF-α) can dedifferentiate into radial glial cells in an ErbB1-dependent manner and upon prolonged treatment can produce neural stem-like cells, which display neurosphere formation capabilities (249); TGF-α overexpression is detected in the early stages of glioma formation (250). A
related study demonstrated that ErbB1 could cooperate with dually inactivated p16\textsuperscript{INK4a} and p19\textsuperscript{ARF} to dedifferentiate mature astrocytes into multipotent progenitor cells; furthermore, this cooperative system can induce high-grade gliomas \textit{in vivo} (251). A similar study reported that Ink4a-Arf loss could cooperate with KRas in dedifferentiating astrocytes to induce glioma formation (252).

One step towards defining the cell-of-origin may lie in determining the relationship between stem cell markers expressed in both normal and tumourigenic neural tissue. Indeed, one study has provided evidence that CD133, which is defined as the tumour-initiating cell in MB, marks embryonic NSCs, early postnatal radial glial cells and adult ependymal cells; neurogenic SVZ astrocytes lack CD133 expression (253). Furthermore, this same study revealed that the high neurosphere frequencies observed for embryonic and early postnatal CD133\textsuperscript{+} cells (radial glial cells) but not for adult-derived CD133\textsuperscript{+} cells (ependymal cells) suggest that radial glia could be the potential source for CSCs; ependymal cells are postmitotic and thus unlikely to acquire stem-like features relative to mitotically active cells (253). However, \textit{de novo} CD133 production by CSCs derived from CD133\textsuperscript{+} cells (neurogenic SVZ astrocytes) also cannot be ruled out. Despite the controversy surrounding the origin of BTSCs, the therapeutic ramifications of a minor compartment of stem-like cells driving tumourigenesis are considerable.
Medulloblastoma treatment paradigms

Radiotherapy and chemotherapy are commonly instituted post-operatively due to the high propensity for leptomeningeal dissemination (5). This requires irradiation of the entire craniospinal axis and optimal management of both the dosing and the combination of chemotherapeutics. Unfortunately, the debilitating side effects associated with these postoperative regimens include both neuroendocrine and cognitive dysfunction (254). Furthermore, the survival outcomes and recurrence rates still remain unacceptable (255, 256). However, advances in molecular prognostics and stem cell biology have afforded the opportunity to improve patient outcome through better stratification and identification of novel targets for treatment.

Traditional therapeutics

The front-line for MB treatment consists of surgical resection to: 1) establish a definitive diagnosis; 2) relieve obstruction of the cerebrospinal fluid (CSF) pathways; 3) debulk the tumour mass (257). Maximal surgical resection, based on postoperative imaging, correlates with improved prognosis in children greater than 3 years of age, who initially present with nondisseminated (M0) disease (258, 259). Following surgical resection, children less than 3 years of age receive
only chemotherapy since craniospinal irradiation (CSI) can severely damage the infant brain (260); however, localized radiation may improve survival outcome (261). The conventional radiation neuraxis dose of 36 Gy could be reduced to 25 Gy without a significant difference in survival outcome while reducing cognitive dysfunction (262, 263). In addition to CSI, an additional 55 Gy boost to the entire posterior fossa forms part of the post-surgical radiotherapy treatment; recent studies have demonstrated that a localized tumour bed boost proves equally efficacious with a concomitant reduction in toxicity (264, 264-266). Historically, chemotherapy usage started as a front-line treatment for patients with MB relapse (267). Currently, radiation oncologists treat average-risk MB patients with low-dose CSI (25 Gy) in combination with chemotherapy, which can result in survival outcomes of up to 80% (254, 256); incidence of leptomeningeal relapse increases without addition of chemotherapy (262). A landmark study has demonstrated that instituting chemotherapy during and after reduced-dose radiation following surgery may result in better survival outcomes in average-risk patients (268). In high-risk patients, specifically in children younger than 3 years of age, regimens tend to include only chemotherapy to delay or even obviate the use of radiotherapy in the hopes of preserving neuropsychological function (255). One study further stratified the target population with infants having nonmetastatic MB after gross total resection as being most likely to derive benefit from chemotherapy alone; radiotherapy was given to patients not achieving complete remission (269). Interestingly, one trial (POG 9031) revealed no significant difference in the 5-year event-free survival rate in high-risk patients given the
same chemotherapeutic regimen either before or after radiotherapy (270). Yet, some studies have reported induction chemotherapy-related myelosuppression that may result in delaying the initiation of radiotherapy or failure to complete the course of irradiation (271, 272). Another study by the Children’s Cancer Group (CCG) revealed that post-radiation chemotherapy resulted in a progression-free survival rate of 63% compared to both pre- and post-radiation chemotherapy, which resulted in a 45% progression-free survival rate; however, the chemotherapeutic regimens for both treatment arms were not comparable (273). Current trials are focussing on improving survival rates in high-risk patients (> 3 years of age) using high-dose chemotherapy with autologous stem cell rescue following CSI and induction chemotherapy. Furthermore, for infants (< 3 years of age), ongoing trials are also exploring high-dose chemotherapy with autologous stem cell rescue; however, whether or not radiotherapy represents a feasible option is uncertain. Hence, current treatment paradigms will need to reach consensus on a number of issues related to optimizing both CSI dosing and chemotherapeutic combination strategies and their resulting impact on both average- and high-risk patients. As mentioned before, appreciation of the molecular characteristics of MBs may also lead to further risk stratification thereby contributing to a more comprehensive risk-based treatment strategy.
Molecular therapeutics

Given the importance of aberrant signal transduction in MB tumourigenesis, several anti-cancer agents are now being developed to target specific cell-signalling pathways within tumour cells; the overall aim is improvement in survival outcome and reduction in treatment-associated morbidity. For example, cyclopamine, a plant steroidal alkaloid, acts as a negative regulator of Smo activity by competing for vitamin D3 binding; Ptc-mediated gating of vitamin D3 negatively regulates Smo activity but Hh blocks vitamin D3 transport leading to Smo activation (274). Cyclopamine can block in vitro proliferation of murine MB cells and initiate gene expression profiles associated with neuronal differentiation and loss of NSC-like properties; it can also block the growth of in vivo murine tumour allografts and cause loss of tumour cell viability from primary MBs (275-277). However, cyclopamine treatment was unable to cause regression Gli1-transfected tumours thus demonstrating its limitation as a selective upstream inhibitor for MBs that display downstream pathway activation. Indeed, one study has identified, using a cell-based screen for small-molecular inhibitors of Gli1-mediated transcription, a few candidate Gli antagonists, referred to as GANT58 and GANT61, which can inhibit in vitro proliferation of tumour cells with downstream Shh pathway activating mutations; cyclopamine did not significantly reduce the viability of these tumour cell lines (278).
IGF-IR activity may drive MB tumourigenesis and thus strategies directed towards mitigating IGF-IR function may also exhibit antitumoural potential. Recently, a study has demonstrated that NVP-AEW541, a small-molecule IGF-IR inhibitor, can prevent IGF-IR autophosphorylation and also inhibit signalling cascades downstream of IGF-IR (279). Pre-clinical studies have revealed that NVP-AEW541 can significantly attenuate tumour growth in animal models of lymphomas, myelomas and prostate cancer (280). NVP-AEW541 can elicit massive cell death in MB cells growing in suspension that is accompanied by loss of GSK3β phosphorylation; NVP-AEW541 elicits growth arrest in adherent MB cells (281).

Developing cancer therapeutics to reverse the effect of Wnt pathway mutations has also met with some initial success. Deregulated β-catenin activation leading to cyclin D1 and c-myc upregulation may be responsible for initiating tumorigenesis in a subset of MBs; this deregulation occurs through mutations of APC, Axin, GSK3β or β-catenin. The small-molecule inhibitor exisulind and its higher-affinity analogs can directly lower β-catenin levels in a GSK3β-independent manner leading to apoptosis of tumour cells (282, 283). Exisulind, an oxidative metabolite of the non-steroidal anti-inflammatory drug (NSAID) sulindac, along with its higher-affinity analogs CP461 and CP248 can inhibit cyclic GMP phosphodiesterase (cGMP PDE) leading to accumulation of cGMP and subsequent activation of cGMP-dependent protein kinase G (PKG) and β-catenin phosphorylation; these events precede proteosome-mediated apoptosis.
(284). Also, PKG can phosphorylate β-catenin at sites independent of the GSK3β phosphorylation sites; specifically, PKG phosphorylates sites at the C-terminus (285).

Excessive ErbB signalling can arise from mutation(s), autocrine ligand stimulation or receptor overexpression (286). A number of therapeutic strategies are approved or undergoing clinical trials to specifically target oncogenic ErbB signalling. For example, monoclonal antibody (mAb) therapy is directed against the ErbB ecto-domain and functions by recruiting cytotoxic lymphocytes (CTLs) to the target tumour cell, receptor internalization, ligand-binding- or dimerization-inhibition (287). Presently, trastuzumab (Herceptin; Genetech) and cetuximab (Erbitux; Bristol Myers Squibb/ImClone) target ErbB-2 (breast cancer) and ErbB-1 (colorectal cancer), respectively. Pertuzumab is presently in clinical trials used in combination with other therapies for non-small cell lung cancer (NSCLC) and functions by inhibiting ErbB-2 heterodimerization (286). Small-molecule tyrosine kinase inhibitors (TKIs) also target ErbB signalling in tumour cells by crossing the plasma membrane and binding to the ATP- or substrate-binding domains. Two TKIs that target ErbB-1, gefitinib (Iressa; AstraZeneca) and eriotinib (Tarceva; Genentech), have recently been approved for the NSCLC; the latter drug is in ongoing trials for gliomas in combination with other therapies (FDA reference and clinicaltrials.gov). Dual-specific inhibitors, such as lapatinib (GlaxoSmithKline), CI-033 (Pfizer), EKB-569 (Wyeth-Ayerst Research), target both ErbB-1 and ErbB-2 and are in ongoing trials for breast cancer (lapatinib) and
NSCLC (CI-1033, EKB-569). AEE788, which is a dual-specific TKI and also targets the vascular endothelial growth factor receptor (VEGFR), is presently in clinical trials to be used in combination with other therapies for the treatment of glioblastoma (www.clinicaltrials.gov). ErbB therapeutics is often combined with other modalities to overcome or delay the onset of acquired tumour resistance. Combinations may include two different mAbs directed against the same receptor (trastuzumab and pertuzumab) or to different receptors (trastuzumab and cetuximab); mAbs can be combined with TKIs such as trastuzumab and lapatinib (286). Conventional therapeutics such as chemotherapy and radiotherapy can be combined with mAbs to address the widespread genetic heterogeneity that dictate malignancy independent of ErbB signalling (288, 289). Given that ErbB-2 expression may predict aggressive forms of MB and metastasis, a phase I/II trial of the dual TKI lapatinib (GlaxoSmithKline) is ongoing with children afflicted with MB (trial PBTC016) (290, 291).

The Notch signalling pathway is implicated in MB development and thus targeting the signalling steps may demonstrate anti-tumour efficacy. Recently, one experimental approach in the pre-clinical phase utilizes a small-molecule inhibitor GSI-18 to target the γ-secretase-mediated proteolytic step in generating the intracellular Notch ICD (241). Indeed, this study demonstrated that GSI-18 suppresses in vitro MB growth and in vivo MB tumour xenograft growth in nude mice; furthermore, the CD133+ and SP stem-like fractions were depleted (241). The γ-secretase inhibitor, MK0752 (Merck), is currently in phase I clinical trials
for determination of the safety/efficacy profile in patients with metastatic or locally advanced breast cancer or other advanced solid tumours.

Histone deacetylase inhibitors (HDACi) represent a new class of antitumour agents that induce growth arrest and apoptosis in malignant cells. Normally, HDACs impart a positive charge to histone proteins thereby promoting binding to the negatively charged DNA and preventing transcription factor access to DNA; HDACs can also deacetylate other proteins such as transcription factors (292). Three HDAC families exist: Class I (HDAC 1, 2, 3, 8); Class II (HDAC 4, 5, 6, 7, 9, 10); Class III (HDAC NAD⁺ -dependent homologues of the yeast Sir2 protein) (293). There also exist five HDACi classes: short-chain fatty acids (e.g. valproic acid [VPA]), hydroxamic acids (e.g. suberyolanilide hydroxamic acid [SAHA]), electrophilic ketones, aminobenzamides (e.g. MS-275) and natural cyclic peptides (e.g. romidepsin). An in vitro study revealed that HDACi from all classes can inhibit MB cell proliferation albeit at varying efficacies; VPA and trichostatin A (TSA) displayed the lowest and highest cytotoxicities, respectively (294). Preclinical studies have demonstrated efficacy of VPA in inducing significant growth arrest, apoptosis and differentiation of a variety of MB cell lines; VPA also suppresses in vivo tumourigenicity that correlates with histone hyperacetylation (295, 296). A phase I COG trial was initiated to determine the optimal dose and side effects of VPA in treating young patients (2-21 years old) with recurrent or refractory solid tumours or CNS tumours; however, this trial has now been suspended (www.clinicaltrials.gov). SAHA is well tolerated in Phase I
trials and indeed may have contributed to tumour regression and improvements in symptomatology (297, 298). Currently, a phase I trial is recruiting young patients (1-21 years old) to participate in a study for determining optimal dosing of SAHA (Vorinostat) in combination with isoretinoin for treatment of recurrent or refractory solid tumours, lymphoma or leukaemia. A phase I trial is also ongoing for evaluating the optimal dosing and side effect profile of MS-275 in treating patients with advanced solid tumours or lymphoma.

The discovery of a small tumour-initiating compartment within malignant tissue also has profound therapeutic implications. These CSCs may display resistance to conventional chemotherapy and radiotherapy. Indeed, as mentioned before, the presence of a SP, which contains primarily undifferentiated cells or CSCs, is testament to the enhanced drug efflux capacity of this minor subpopulation. Furthermore, the CD133+ stem-like compartment in gliomas is radioresistant and displays enhanced DNA repair capacity; inhibition of DNA checkpoint kinases Chk1 and Chk2 with debromohymenialdisine (DBH) ameliorates the radioresistant phenotype (299). Signalling pathways known to be active in stem cells are the Wnt, Notch and Shh pathways; these pathways are also implicated in the self-renewal phenotype (241, 300). Thus, these pathways may represent attractive targets for the development of novel therapeutics aimed at selectively repressing self-renewal thereby depleting the cancer stem cell fraction.
The side-effect profile from current therapy remains unacceptable and efforts to improve both the mortality and morbidity rates for children afflicted with MB is imperative. Although improvements in risk stratification schemes may lead to improved outcomes in a subset of patients, the most significant advances will come from progress in the understanding of MB biology, which will undoubtedly lead to identification of novel therapeutic targets. In this vein, the contributions of the cancer stem cell hypothesis to MB tumourigenesis may have profound therapeutic implications; therapies that target the tumour-initiating cells may exhaust the proliferative potential of the tumour thereby leading to permanent tumour regression. This thesis aims to investigate the IGF-IR-FOXO1 axial involvement in MB cell survival, to identify, isolate and characterize the stem-like compartment of MB cell lines and finally to determine the effects of histone acetylation on the stem-like potential.
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CHAPTER II
FLOW CYTOMETRIC CHARACTERIZATION OF THE DAOY MEDULLOBLASTOMA CELL LINE FOR THE CANCER STEM-LIKE PHENOTYPE

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ABSTRACT

Background
Side population (SP) analyses and CD133 expression have identified cells with stem-like potential in normal and cancerous tissue. Whether stem-like cells exist in cancer cell lines is hotly debated. We have interrogated the DAOY medulloblastoma cell line with respect to stem-like potential.

Methods
Vital staining for Hoechst 33342 efflux capacity and CD133 immunophenotyping were performed on DAOY cells to assess the presence of the SP and the CD133 stem cell markers, respectively. SP/non-SP and CD133+/CD133- DAOY cells were sorted into separate fractions for limiting dilution analysis (tumour sphere assay) and asymmetric division assessment. SP/non-SP cells were also sorted separately for viability (XTT assay), cell size, cell cycle status and proliferative capacity (carboxyfluorescein succinimidyl ester (CFSE)) evaluation.

Results
A minor proportion of cells displayed either the SP or the CD133+ phenotypes. CD133 expression mapped to both the SP and non-SP compartments, with CD133+ cells being enriched almost 4-fold within the non-SP gate. The SP, non-SP, CD133+ and CD133- fractions were all capable of reconstituting the original parental DAOY population. Slight clonogenic enrichment was observed in only the SP fraction; however, both CD133+ and CD133- cells displayed equivalent stem cell-like frequencies. SP cells were resistant to Hoechst 33342-mediated
toxicity relative to the parental population and differed from the non-SP cells with respect to increased cell size, decreased S-phase and slightly decreased proliferative capacity.

**Conclusions**

The multiparametric strategy described in this study revealed that the SP and CD133\(^+\) subset may be two independent compartments. Our results highlight the need for new reliable specific cancer stem cell marker(s) as Hoechst 33342 efflux and CD133 expression might not be suitable for selectively isolating cancer stem-like cells from cell lines, as shown for the DAOY cells. As such, care must be used in interpreting therapeutic studies targeting the stem cell compartment of cancer cell lines.

**Keywords:** flow cytometry; side population; CD133; cancer stem cell; medulloblastoma; tumour sphere
INTRODUCTION

Despite the observation that malignant cellular characteristics closely phenocopy the stem cell-like features of self-renewal and multi-lineage differentiation, the outstanding feature separating the malignant cell from the normal stem cell is the lack of homeostatic balance between self-renewal and differentiation (1). Furthermore, cancer tissue, much like normal tissue, is hierarchically organized according to stage of differentiation and proliferative potential (2). Such close phenotypic parallels imply that the normal stem cell could transform malignantly; however, it is also possible that lineage-restricted or differentiated progeny transform, through a process of de-differentiation, into malignant cells displaying stem-like features (2). Yet, the target stem cell may require fewer alterations to initiate tumourigenesis compared to its downstream progeny (3). This so-called “cancer stem cell” has been isolated and characterized from a variety of haematological malignancies and solid tumours including medulloblastomas (4).

However, it must be understood that the term ‘cancer stem cell’ is an operational term for a tumour cell that displays the ability to self-renew and also divide to generate another stem cell and a progenitor cell, which generates the multitude of cell types comprising the bulk of the tumour (5). Definitive proof for the existence of cancer stem cells was first elucidated in acute myeloid leukaemia (AML) whereby xenotransplantation of human AML cells into immunodeficient mice revealed the frequency of leukaemic stem cells to be about 0.1 to 1% of all the tumour cells; specifically, only the CD34+CD38- cells were able to reconstitute the leukaemic phenotype whereas the CD34+CD38+ and CD34- cell
were unable to do so (6). More recently, using the neural stem cell marker CD133, a putative brain tumour stem cell has been isolated from medulloblastoma that is capable of self-renewal and multilineage differentiation (4). Although immunophenotyping has demonstrated that many malignancies may be organized hierarchically, functional assays using vital fluorescent dyes have also revealed that the cancer stem cell phenotype may be defined by high expression of various types of ATP-binding cassette (ABC) transporters. Indeed, seminal experiments using the Hoechst 33342 dye, which can be effluxed by ABCG2 transporters, have identified an unlabeled side population (SP) that is enriched for stem cells in the bone marrow (7). These observations have been extended by an increasing number of studies that have identified the SP phenotype in a variety of primary tumour tissues and cancer cell lines.

Remarkably, the presence of stem-like cells has also been reported in long-established cell lines (8-10). Thus, the major objective of this study was to characterize the established DAOY medulloblastoma cell line for cancer stem-like cells using both immunophenotypic (CD133) and physiological substrate (Hoechst 33342) markers and to determine the cellular properties of the isolated putative stem-like fraction with respect to proliferative capacity, cell cycle status and reconstitution of the original phenotype. Our results demonstrate a weak relationship between the expression of these stem cell markers and clonogenicity, indicating that these markers may not target the stem cell compartment in established cancer cell lines.
MATERIALS AND METHODS

Cell culture
The DAOY human medulloblastoma cell line was obtained from the American Type Culture Collection (Manassas, VA). DAOY cells were routinely propagated in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) foetal bovine serum (FBS; Sigma Corp., Oakville, ON) and penicillin-streptomycin (1X final concentration; Invitrogen, Carlsbad, CA). Cultures were grown at 37°C in a humidified 5% CO₂ incubator and passaged when 70-80% confluent.

Hoechst 33342 staining
DAOY cells were detached with 0.5 mM EDTA/PBS to better preserve the integrity of cell surface molecules, and resuspended at 0.5-1.0 x 10⁶ cells/mL in 0.1% BSA/PBS. Hoechst 33342 was added at a final concentration of 0.5, 1.5, 2.0, 3.0, 5.0, 6.0, 7.5 and 10.0 µg/mL. In selected samples, verapamil was additionally added to a final concentration of 100 µM. Cells were then incubated for 90 min at 37°C/5% CO₂ with intermittent agitation. After incubation, cells were washed twice with cold PBS and resuspended at 2 x 10⁶ cells/mL supplemented with propidium iodide (PI) (2 µg/mL). The cell suspensions were then analyzed with a flow cytometer equipped with an ultraviolet (UV) laser (described below).
**Immunophenotyping**

Cells, grown in 10-cm tissue culture dish (Sarstedt Inc., Montreal, QC), were detached with 0.5 mM EDTA/PBS for 15 min at 37°C/5% CO₂ prior to washing and reconstituting to a final concentration of 0.5-1.0 x 10⁶ cells/mL in 0.1% BSA/PBS. After additional washing in PBS, cells were reconstituted in 0.1 mL of 0.1% BSA/PBS and labelled with 10 µL of anti-CD133-PE (Miltenyi Biotec, Auburn, CA) for 30 min at 4°C; the isotype IgG1-PE was added at a similar concentration. Cells were then washed twice with PBS and resuspended in 0.3 mL of 0.1% BSA/PBS and analyzed by flow cytometry.

**Cell sorting strategy**

Cells were sorted on a MoFlo™ High Performance Cell Sorter (Dako, Fort Collins, CO) either into tubes or into 96 well microwell plates using the Cyclone system. The machine was equipped with three excitation lines which included a water cooled Coherent Innova 90C Argon 488 nm (blue) laser (150 mW), a Spectra-Physics Helium-Neon 635 nm (red) laser (35 mW) and a 351 nm (UV) water-cooled Innova 90 Krypton laser. Five fluorescent channels (FL1 to FL5) can pick up the 488 nm laser, two channels (FL6, FL7) the 351 nm laser and one the 635 nm laser (FL8). Hoechst fluorescence was measured at both 424/44 nm and above 670 nm (split by a 510 nm long-pass dichroic mirror) resulting from UV excitation. PI fluorescence, measured at 610 nm in the FL3 channel, excluded dead cells during sorting. For cell cycle distribution, 5 x 10⁴ cells from the SP and non-SP (NSP) fractions were sorted, using Summit software version.
4.3 (Dako), into Modified Krishan’s buffer (0.1% sodium citrate, 0.3% NP-40, 0.05 mg/mL PI, 0.02 mg/mL RNase) and incubated on ice for at least 15 min prior to flow cytometric analysis using the BD FACScan (Becton Dickinson, San Jose, CA), which is equipped with a single argon 488 laser and can read from 3 different fluorescent channels (FL1, FL2, FL3) and 2 scatter gates (FSC – forward scatter; SSC – side scatter) simultaneously.

**CFSE labelling**

Cell division tracking was carried out in the presence of the vital dye carboxyfluorescein succinimidyl ester (CFSE, Invitrogen, Carlsbad, CA), which is converted intracellularly into a membrane impermeable fluorescent dye allowing the monitoring of cell division through a decrease in its intensity in subsequently cell populations. DAOY cells were detached with 0.5 mM EDTA/PBS and resuspended in 0.1% BSA/PBS (10⁶ cells/mL). A stock solution of 10 mM CFSE was adjusted to a final concentration of 10 µM in the cell suspension and incubated for 10 min at 37°C/5% CO₂. Subsequently, the staining was quenched by addition of 5 volumes of ice-cold DMEM/10% FBS and incubated for 5 min on ice in the dark. The cells were washed thrice in PBS and cultured in 10-cm dishes for about 36 hr at 37°C/5% CO₂. Cells were then harvested by EDTA dissociation, resuspended to about 10⁶ cells/mL and stained with Hoechst 33342, as described above. Data from over 20 000 cells were collected for each fraction (SP and non-SP) with aggregates and cell doublets excluded from the analysis using an FL2-Area versus FL2-width histogram plot. SP and non-SP dye dilution
profiles were analyzed using ModFit LT™ 3.2 software packages (Verity Software House Inc., Topsham, ME, USA).

**XTT viability assay**

The XTT assay measures the viability of living cells by quantitatively assessing the production of soluble formazan product resulting from the mitochondrial dehydrogenase-mediated cleavage of the tetrazolium sodium salt XTT. Briefly, after the indicated experimental treatments, buffer exchange into OPTIMEM was performed. Sterile XTT was prepared at 1 mg/mL in pre-warmed (37°C) OPTIMEM whereupon 50 µL of the XTT solution was added to each 200 µL of culture. It must be noted that PMS was prepared at 5 mM (1.53 mg/mL) in PBS and was added to the XTT solution at a final concentration of 25 µM (5 µL of 5 mM PMS added to 1 mL of XTT (1 mg/mL) prior to addition to the culture media; PMS acts as an electron coupling agent thereby potentiating XTT reduction. After incubating at 37°C for 1.5 h, the optical density (OD) of each well was measured at both 450 nm (reference 620 nm) for quantification of formazan production using an EAR 400AT 96-well plate reader (SLT Labinstruments, Gröding/Salzburg, Austria). XTT assays were always performed in triplicate with the one-way analysis-of-variance (ANOVA) and the Bonferroni post-test determining statistical significance.
Limiting dilution analysis – neurosphere assay

Cellular suspensions were labelled with propidium iodide (PI) with PI-negative (viable) cells being sorted by the MoFlo™ flow cytometer (Dako, Fort Collins, CO) into 96-well microwell plates (Sarstedt Inc., Montreal, QC) containing neural stem cell (NSC) media (epidermal growth factor (EGF; 20 ng/mL), basic fibroblast growth factor (bFGF; 20 ng/mL), heparin (2 µg/mL), 2 mM L-glutamine, B-27 (1x), penstrep (1x) and DMEM/F12); final cell numbers ranged from 10 cells per well to 1 cell per well in 0.2 mL volume. Cultures were incubated for 7 days prior to counting the fraction of wells without spheres as plotted against the number of cells plated per well. The proportion of stem-like cells (or the number of cells required to form one sphere) in the DAOY cell line was determined from the Poisson distribution of stem-like cells where \( F_0 = e^{-x} \) (\( F_0 \) represents the fraction of wells without spheres and \( x \) is the average number of stem-like cells per well); \( F_0 = 0.37 \) corresponds to the number of cells required to be plated in order for one stem-like cell to be present (11).

RESULTS

Stem cell marker expression in the DAOY medulloblastoma cell line

Immunophenotypic stem cell markers or physiological markers of stem cell function can address the existence of putative stem-like cells in the DAOY cell line. To this end, the side population protocol was first established on the MoFlo™ flow cytometer using DAOY medulloblastoma cells titrated with
varying concentrations (0.5 - 10.0 µg/mL) of the Hoechst 33342 dye (data not shown). Incubation at a Hoechst concentration of 5.0 µg/mL for 90 min at 37°C/5% CO₂ was utilized to ensure consistency amongst the various experiments. Control incubations were performed with the ABCB1 inhibitor verapamil to verify the specificity of the SP gate. As shown in Figure 1a, the flow cytometric profile of cells labelled with Hoechst 33342 demonstrates that a minor proportion of DAOY cells (mean: 21%; range: 12.4% - 39.1%; n = 13 independent experiments can exclude the Hoechst 33342 dye; the SP was also sensitive to the ABCB1 inhibitor verapamil (Figure 1a). Expression of the primitive stem and progenitor cell marker, CD133, was also evaluated in the DAOY cell line. As illustrated in Figure 1b, the DAOY cell line contains a minor proportion of CD133⁺ cells (mean: 0.5%; range: 0.36% - 0.58%; n = 3 independent experiments); control IgG1 antibody staining did not reveal any significant staining. Furthermore, co-localization of the two stem cell markers, CD133 and SP, was also investigated in the DAOY cell line. After Hoechst 33342 staining, DAOY cells were labelled with anti-CD133 and processed for flow cytometry. Interestingly, CD133⁺ cells are present in both the SP and non-SP fractions and the proportion of CD133⁺ cells is almost 4-fold greater in the non-SP relative to the SP (Figure 1c, d).
Cell cycle parameters of DAOY SP and NSP

Given the relatively large proportion of SP cells present within the DAOY cell line, characteristics such as cell size, cell cycle status and proliferative capacity were evaluated within both the SP and non-SP fractions. Cell size data were captured during cell acquisition and the forward scatter (FSC) profiles of both the SP and non-SP cells were compared and analyzed (Figure 2). These studies revealed that SP cells are larger than the non-SP cells as evidenced by the significant increase of SP cells in the larger FSC region relative to non-SP cells (Figure 2a, b). Subsequently, the cell cycle status of the SP and non-SP fractions was analyzed through isolation and staining of the individual fractions with Krishan's modified cell cycle buffer for about 15 minutes prior to cell cycle analysis. Figure 2c demonstrates that the only significant difference in the cell cycle parameters between the two fractions lay in the increased number of non-SP cells residing in the S phase; one-way ANOVA failed to reveal any significant differences in the G0/G1 and G2/M phases. Finally, the proliferative potential of each fraction was assessed by first labelling the cells with 10 μM CFSE and culturing the CFSE-labelled cells for 72 hours prior to Hoechst 33342-staining to evaluate CFSE dilution in SP and non-SP cells. Both Figure 3 and Table I illustrate that the CFSE dye appears to be slightly more diluted in non-SP cells, thereby potentially reflecting increased cell divisions within this fraction relative to SP cells. These analyses revealed that in addition to the expression of ABC transporters, the SP cells also had different cell cycle characteristics than non-SP cells.
Since Hoechst-labeled cells (primarily non-SP) may undergo apoptosis when cultured for extended periods of time by virtue of binding to DNA, a bias in favour of improved SP cell viability relative to non-SP cells could potentially exist. Prior to undertaking further studies, we tested this hypothesis by determining the effects of the Hoechst 33342 dye on cellular viability (Figure 4). Standard gating facilitated sorting of SP and non-SP cells (Figure 4a) into regular culture media containing 10% FBS; parental cells (+/- Hoechst 33342 labelling) were also sorted into separate wells. Furthermore, additional gating divided both SP and non-SP fractions into lower and upper regions (Figure 4b). As seen in Figure 4c Hoechst labelling significantly decreased the viability of parental DAOY cells; non-SP cells also displayed a significant reduction in viability compared to SP cells. No significant differences in viability were observed between the lower SP and upper SP cells; interestingly, the converse was true for lower and upper non-SP cells (Figure 4d). Therefore, during subsequent studies, if long-term viability were a concern, SP cells were compared to non-SP cells as well as parental cells that were isolated through flow cytometry in the absence of Hoechst 33342.

**Asymmetric division capacity and tumour sphere formation of DAOY cell fractions**

In the classical view, through cell division a stem cell may generate a daughter stem cell as well as a progenitor cell that can then proceed through lineage
restriction to generate a mature differentiated cell. Therefore, we compared the ability of SP/non-SP and CD133+/CD133− cells to regenerate the entire cellular population. DAOY cells were cultured in DMEM/10% FBS, stained with the fluorescent Hoechst 33342 dye and sorted into SP and non-SP fractions by flow cytometry and then further expanded in the same medium for an additional two weeks. Subsequently, upon restaining with Hoechst 33342 and reanalysis by flow cytometry, it was revealed that both fractions have the capability of regenerating each other (Figure 5). A similar finding was observed for DAOY cells stained with anti-CD133 whereby upon isolation, culture and restaining, both the CD133+ and CD133− fractions were able to regenerate each other (Figure 5).

To determine the stem cell-like capacity of each fraction in terms of tumour sphere expansion, the limiting dilution analysis method was used (12). DAOY cells were stained with Hoechst 33342 and gated by flow cytometry into separate SP and parental cell fractions (no Hoechst 33342 treatment) that were sorted into separate 96 well microplates containing neural stem cell media at different cell densities. Similarly, DAOY cells were stained with anti-CD133 and sorted by flow cytometry into CD133+ and CD133− populations. Both the CD133+ and CD133− fractions demonstrated nearly equivalent stem cell-like frequencies (Figure 6) whereas stem-like cells appeared to be slightly enriched in the SP fraction as compared to the parental cell population (Figure 6). These results indicate that the capacity for tumor sphere formation is not restricted to CD133+ cells alone.
DISCUSSION

The technique for stem cell labelling and sorting based on cells displaying low red and blue fluorescence subsequent to incubation with the Hoechst 33342 dye has been appreciated for quite some time (7). With resurgence of the cancer stem cell hypothesis, the Hoechst-based technique has now been recently applied to assessing the stem cell frequency of cancer cell lines and primary tumours. However, a recent study has demonstrated that not all cancer cell lines display an SP phenotype (10). An explanation for this discrepancy may lie in re-visiting the basic premise or assumptions underlying the association between the SP phenotype and stem cell activity. In addition to displaying the classical features of self-renewal and quiescence, stem cells also express high levels of certain members of the ATP-binding cassette (ABC) transporter family, which includes ABCB1 (P-glycoprotein), ABCC1 (multidrug-associated protein 1 or MRP1) and ABCG2 (breast cancer-related protein or BCRP) (13). Interestingly, integrity of the stem cell compartment is not affected in mice with disruptions in Abcb1, Abcc1, Abcg2; however, there is increased sensitivity to chemotherapeutics such as mitoxantrone, vinblastine and topotecan suggesting that drug transporters may not play an essential role in stem cell development but only subserve xenobiotic resistance (14-16). Clinically, the SP phenotype may reflect selection of drug transporter expression leading to evolution of treatment-resistant cells in a variety of cancers (13). Previous studies have marked putative stem cells with fluorescent dyes (e.g. Hoechst 33342, rhodamine 123) and cytotoxic compounds (e.g. mitoxantrone, methotrexate) and characterized self-renewal properties
(7,15,17). However, our results have demonstrated Hoechst 33342-mediated toxicity of DAOY cells and that only SP cells display resistance to this toxicity (Figure 4), presumably attributed to Hoechst 33342 efflux. Furthermore, DNA-binding affinity of Hoechst 33342 may interfere with cellular replication and differentiation thus confounding the ability to detect relevant biological differences between the SP and non-SP fractions. Indeed, an early study revealed that Hoechst 33342 can induce the F9 embryonal carcinoma cell line to differentiate along the endodermal pathway (18). Likewise, a recent study reported that nuclear Hoechst 33342 staining can have a dramatic impact on C2C12 myogenic differentiation and PC12 neuritic differentiation (19).

Regarding our results, it is possible that Hoechst 33342 may interfere with differentiation and thus affect non-SP parameters; interestingly, this may explain the slight stem-like cell enrichment in the SP fraction whereas both CD133$^+$ and CD133$^-$ fractions have equivalent stem-like cell frequencies. The tumor sphere limiting dilution assay revealed that SP cells were more clonogenic when compared to the parental unlabelled tumour cell population (Figure 6). Although not presented here, preliminary experiments did reveal that non-SP cells were also able to generate tumour spheres. As well, the cancer stem cell hypothesis would predict that the SP fraction should be able to regenerate both the SP and non-SP fractions whereas the non-SP fraction should only be able to regenerate itself. Interestingly, the results presented here demonstrate that both the SP and the non-SP fraction have the capacity to completely regenerate both fractions (Figure 5). A recent paper has also observed this same finding in the C6 glioma cell line
where either fraction was capable of reconstituting the parental cellular population (20).

Immunophenotyping is an alternative method for assessing the existence of stem-like cells. Indeed, this method may bypass the limitations of current physiological-based dye efflux assays by marking stem-like cells extracellularly thereby potentially avoiding toxicity associated with DNA-dye intercalation. The CD133 extracellular receptor is an established primitive stem cell marker that has now found application for cancer stem cell analysis in a variety of cancer cell lines and primary tumours. A recent report has also shown CD133 expression in all tested medulloblastoma cell lines (8). In the present study, CD133+ cells displayed tumour sphere-like growth (Figure 6) thereby initially confirming previous findings that CD133+ cells display stem-like activity (4,21,22). However, CD133- DAOY cells were able to form tumour spheres with a calculated stem-like frequency comparable to that of CD133+ cells (Figure 6). Also, similar to the SP and non-SP fractions, either the CD133+ or the CD133- fraction was capable of reconstituting both fractions (Figure 6). A similar study has also reported that in the C6 glioma cell line CD133- cells displayed self-renewal and tumorigenic features (23). Limiting dilution analysis of the tumour sphere assay revealed that not all cells were capable of clonogenic expansion. Thus, rather than the entire culture being made up of stem cells, it appears that a relatively large minority of DAOY cells display clonogenicity. The present study also suggests that plasticity or stem-like potential of tumour cells, as defined by Hoechst 33342 efflux or CD133 expression, is variable and dependent on
environmental factors; indeed, the Hoechst 33342 technique appeared to be highly variable with the percentage of SP cells varying significantly with tumour cell density.

Another novel aspect of this study was the finding that CD133 expression mapped to both the SP and non-SP gates (Figure 1). Initially, the anticipation was that CD133 expression would be localized primarily to the SP gate; however, CD133 expression appears to be enriched almost 4-fold within the non-SP gate. Moreover, given both the very low abundance of CD133 expression in both gates and that non-SP cells constitute the vast majority of the parental cell population, this may explain the similar tumour sphere formation efficiencies that were observed between both the CD133⁺ and the CD133⁻ fractions (Figure 6).

In conclusion, this study highlights the novel application of flow cytometric methods in assessing the extension of the cancer stem cell hypothesis into cancer cell lines. Using the established DAOY medulloblastoma cell line, our results do not demonstrate a strong relationship between stem cell marker expression and clonogenicity; both non-SP and CD133⁻ cells also display stem-like characteristics. The DAOY cell line contains a relatively large minority of cells that display clonogenicity independent of stem cell marker expression. Thus, Hoechst 33342 and CD133 expression may not be suitable in selectively isolating stem-like cells in the DAOY cell line. This may be of concern when studies begin to utilize these markers to demonstrate efficacy of targeting the cancer stem cell compartment in cancer cell lines such as DAOY (9). This study provides evidence that cancer cell lines may not recapitulate the hierarchical model of
stemness observed \textit{in vivo}. This may be of fundamental clinical importance as many non-clinical testing strategies utilize established cancer cell lines in modelling tumour biology and in screening anti-cancer therapies.

\section*{Acknowledgements}

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REFERENCES


FIGURES
a

b

- verapamil

+ 100 μM verapamil

Total CD133

c

d

CD133/SP

CD133/non-SP
Figure 1. Stem cell marker expression and co-localization in the DAOY cell line. DAOY cells were first labeled with Hoechst 33342 for 1.5 hours at 37°C/5% CO₂ followed by labeling with anti-CD133-PE for 30 minutes at 4°C. The cells were analyzed by a flow cytometer and the following regions were gated on R1 (parental cell population excluding cellular debris): (a). R3 (SP) and R6 (non-SP); (b). R5 (total CD133⁺ cells); (c). R7 (proportion of CD133⁺ cells that are SP) and R8 (proportion of CD133⁻ cells that are non-SP); (d). R2 (proportion of SP cells that are CD133⁺) and R4 (proportion of non-SP cells that are CD133⁺).
Figure 2. Phenotypic and cell cycle analysis of SP and non-SP cells isolated from the DAOY cell line. (a) DAOY cells were labeled with Hoechst 33342 and then propidium iodide staining was used to define the gate R3 for live cells. The R1 and R2 gates were used to define the SP and non-SP cells. Forward scatter and side scatter density plots of all live cells (R3) and either SP cells (R1) or non-SP cells (R2) were used to define R6, R7, R8 and R9 (small SP, large SP, small non-SP and large non-SP, respectively). (b) Graphical representation of the proportion of SP and non-SP cells in either the small (R6, R8) or large cell (R7, R9) size gates. (c) SP and non-SP cells were sorted separately into modified Krishan’s buffer for analysis of cell cycle phase distribution. * p < 0.05 as determined by Student’s t-test.; NSP: non-SP.
Figure 3. Comparison of CFSE profiles of SP and non-SP cells. CFSE-labeled DAOY cells were incubated at 37°C/5% CO₂ for 36 hours and subsequently labeled with Hoechst 33342 for 1.5 hours under the same conditions prior to analysis by flow cytometry. Over 20 000 events were acquired for both SP and non-SP fractions. The reduced chi-square was 2.19 and 1.78 for SP cells and non-SP cells respectively.
<table>
<thead>
<tr>
<th>Generation</th>
<th>SP (%)</th>
<th>Non-SP (%)</th>
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<tbody>
<tr>
<td>Parent</td>
<td>0.00</td>
<td>0.52</td>
</tr>
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Table I. Tabulated summary of the generational history distribution of CFSE-labeled SP and non-SP cells
**Figure 4. Cell viability of defined Hoechst 33342 fractions.** DAOY cells were first labeled with Hoechst 33342 and then propidium iodide was used to define live cells from which further gates were defined. Cells from each gate, including parental cells (non-gated), were sorted into separate 96 microwell plates and cultured at 37°C/5% CO₂ for 4 days at which point viability was measured by the XXT assay as described in the Methods section.  
(a) SP (R1) and non-SP (R2).  
(b) Lower SP (LSP; R2), upper SP (USP; R7), lower non-SP (LNSP; R1) and upper non-SP (UNSP; R6) gates.  
(c) XTT viability of SP, non-SP and total (parental) cells +/- Hoechst 33342, p < 0.0001 from one-way ANOVA.  
(d) XTT viability of LSP, USP, LNSP and UNSP cells, p < 0.01 from one-way ANOVA.  
P = parental cells.
a

Hoechst blue
Hoechst red
Hoechst blue
Hoechst red

SP re-label
NSP re-label

b

FL3 Log
FL2 Log

CD133− re-label
CD133+ re-label
Figure 5. *Asymmetric divisional capacity of (a) SP/non-SP and (b) CD133⁺/CD133⁻ fractions.* DAOY cells were labeled with Hoechst 33342 or immunolabeled with anti-CD133-PE (with propidium iodide defining the gate for live cells). The R1 and R3 gates defined the SP and non-SP cells, respectively and R2 defined the CD133⁺ cells. 1000 events from each gate were sorted separately into separate wells of a 24-well culture plate; parental cells gated from the forward scatter and side scatter plot were also sorted separately. The sorted fractions were incubated at 37°C/5% CO₂ and repeatedly passaged for about 2 weeks. The separate fractions were then relabeled with Hoechst 33342 and anti-CD133-PE and subjected to flow cytometric analysis.
a

\[ \text{log}_e \text{ fraction of wells without spheres} \]

\[ \# \text{ of cells/well} \]

\[ \bullet \text{ SP} \]
\[ \bullet \text{ P} \]

Stem-like frequency in:

\[ \text{SP} \quad 24.7\% \]
\[ \text{P} \quad 6.72\% \]

b

\[ \text{log}_e \text{ fraction of wells without spheres} \]

\[ \# \text{ of cells/well} \]

\[ \bullet \text{ CD133}^+ \]
\[ \bullet \text{ CD133}^- \]

Stem-like frequency in:

\[ \text{CD133}^+ \quad 23.2\% \]
\[ \text{CD133}^- \quad 24.4\% \]
Figure 6. Tumour sphere assay of (a) SP/parental cell fractions and (b) CD133+/CD133- fractions. DAOY cells were labeled with Hoechst 33342 or anti-CD133 mAb and subjected to flow cytometric analysis. SP/total cell or CD133+/CD133- fractions were identified and sorted into 96 well culture plates containing neural stem cell media (20 ng/mL EGF and 20 ng/mL bFGF); cells were incubated at 37°C/5% CO₂ for 7 days to produce spheres as depicted in (c). The fraction of wells without spheres was plotted against the number of cells plated per well. The proportion of stem-like cells (or the number of cells required to form one sphere) in the DAOY cell line was determined as described in the Methods section.
APPENDIX

Effect of chemotherapeutics on DAOY SP viability

Introduction

Stem cells can be identified by the ability of drug transporters to efflux the Hoechst 33342 dye giving rise to the characteristic “side-population” (SP) tail observed in flow cytograms (Goodell et al., 1996). Given that numerous studies have documented the presence of stem-like cells in solid tumours and the sina que non stem cell characteristics of quiescence, DNA damage repair and overexpression of drug efflux transporters, it has been hypothesized that cancer stem cells (CSCs) may remain relatively unaffected, in contrast to their rapidly cycling progeny, when exposed to conventional chemotherapeutics and radiation treatment (1-3). However, we have shown that the SP fraction is not comprised primarily of quiescent cells and displays cell cycle properties that are indistinguishable from the total cell population (4). Thus, the objective of this study was to test whether SP cells as defined in vitro are selectively spared during treatment with traditional chemotherapeutics.

Materials and Methods – Chemotherapeutic drug treatment

Side population (SP) cells or total cells were sorted into 96-well plates (Sarstedt Inc., Montreal, QC) at a density of $2\times10^3$ cells per well in complete serum (DMEM supplemented with 10% FBS) in triplicate. Cells were allowed to adhere
to the well for about 4-6 h prior to exchanging into drug-containing media for about 72 h at 37°C/5% CO₂. The drugs used were thiotepa (0, 0.5, 5.3, 13.2, 26.4 and 52.9 µM dissolved in PBS), carboplatin (0, 25, 50, 75, 100 and 125 µM dissolved in PBS), etoposide (0, 0.5, 1.0, 2.5, 5.0 µM dissolved in DMSO), and vincristine (0, 1, 5, 10, 20 nM dissolved in PBS).

**Results**

The effects on cell viability of traditional chemotherapeutics such as vincristine, thiotepa, carboplatin and etoposide were compared between SP cells and total cell (TC) population using the XTT assay. Thiotepa, carboplatin and etoposide had a significant dose-dependent impact on the viability of SP cells (Figure 7A-C). The response of the SP cells did not differ from that of the TC population (Figure 7A-C). In contrast, SP cells appear to be slightly resistant to the effects of vincristine and thus display a slight survival advantage relative to the total cell population at concentrations below 10nM (Figure 7D).

**Significance**

In this study we have explored whether the SP fraction is resistant to traditional chemotherapeutic agents utilized in the treatment of medulloblastoma. Indeed, our study reveals that SP cells are not resistant to these agents, which is in stark contrast to the majority of recent studies using other cancer cell lines (5-8).
Furthermore, the significance of this finding is bolstered by the multiple chemotherapeutic agents with different mechanisms of action utilized in this study as opposed to the majority of studies in the literature that have utilized only one chemotherapeutic agent. Given our previous finding that the DAOY SP fraction essentially does not differ from the total cell population with regards to cell cycle properties, then it can be reasonably hypothesized that chemotherapeutic agents may not spare the SP fraction in the DAOY medulloblastoma cell line, which we have now shown in this study. Thus, this study taken together with other studies suggests that Hoechst 33342 efflux capacity may be necessary but not sufficient for chemotherapeutic resistance.

REFERENCES


Comment [J6]: The figures have to be standardized.
Figure 7: Exposure of DAOY side population (SP) and total cells (TC) to conventional chemotherapeutics results in significant reduction in XTT viability. A-D: Both SP and TC fractions were sorted into 96-well plates at a density of 2x10^3 cells per well in complete serum followed by buffer exchange into drug-containing media for about 72 hours. Cells were assayed for viability utilizing the XTT protocol. ** p<0.01, ***p<0.001 (one-way ANOVA).

Comment [7]: What are the stats – ANOVA?, what’s the post-hoc for multiple comparisons, what’s the p-value?
LINKER

Reliably predicting the association between a treatment and a true endpoint through the use of surrogate markers demands that: firstly, the surrogate marker is associated with the endpoint; secondly, the treatment is associated with the surrogate marker; and thirdly, that the endpoint and treatment become unrelated once surrogate marker status is factored in (1). This thesis has already demonstrated that the CD133 and SP markers are not exclusively associated with clonogenicity in the DAOY cell line. Thus, the next logical step was to further interrogate the validity of these putative cancer stem-like cell markers by ascertaining their relationship with experimental intervention.

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CHAPTER III
ABSTRACT

Side population (SP) analyses and CD133 expression have identified cells with stem-like potential in normal and cancerous tissue and in long-established cultured cancer cell lines. We have previously shown for the DAOY medulloblastoma cell line that these specific markers do not enrich for stem-like potential (1). In this study, we further examined the surrogacy of these markers by assessing their relationship to experimental intervention. Here we show that histone deacetylase (HDAC) inhibitors (suberylanilide hydroxamic acid [SAHA], 4-phenylbutyrate (4-PB), valproic acid (VPA)) can effectively suppress DAOY cell survival, clonogenicity and increase survival of mice harbouring intracranial DAOY xenografts; however, this is not accompanied by a selective decrease in SP and CD133 marker expression. These data suggest cautious interpretation of studies intending to utilize in vitro cancer stem cell markers as surrogate measures for therapeutic efficacy.
INTRODUCTION

Mounting experimental evidence suggest that cancer stem cell markers, such as CD133 expression and the side population (SP) compartment, may selectively tag the tumour-initiating compartment (T-IC) of many cancers (2-5). However, a few studies have recently demonstrated, at least in cancer cell lines, that clonogenicity may be independent of stem cell marker expression (1, 6, 7). Nevertheless, therapeutics targeting the T-IC may exhaust the cancer’s self-renewal potential leading to tumour regression and possibly improved survival. By extension, therapeutics decreasing the T-IC (even though the T-IC has not been selectively targeted) should also theoretically decrease the tumour’s self-renewal capacity along with decreases in cancer stem cell marker expression. Numerous studies have documented chemoresistant or radioresistant phenotypes associated with the putative stem-like markers in different cancer cell lines exposed to conventional treatments such as chemotherapy or radiotherapy (8-12). However, there have been few studies examining the effects of promising new investigational therapies with regards to marker expression and self-renewal. Notch pathway blockade using an experimental γ-secretase inhibitor was able to cause growth arrest, differentiation and apoptosis in medulloblastoma cell lines associated with significant reductions in the CD133 and SP compartments (13). Similarly, hedgehog pathway blockade achieved utilizing cyclopamine was able to deplete the stem-like fraction in glioblastoma cell lines accompanied by the absence of neurosphere formation and lack of tumour development in athymic mice injected intracranially with glioblastoma cells (14). We chose to concentrate on one group
of novel therapeutics, histone deacetylase (HDAC) inhibitors given their impressive antitumour activity in a variety of preclinical animal models coupled with a low toxicity profile (15-17). The objective of this study was to investigate the effects of HDAC inhibitors on CD133 and SP expression in the DAOY medulloblastoma cell line.

MATERIALS AND METHODS

Cell lines and cell culture

The DAOY (American Type Culture Collection; Manassas, VA) cell line was cultured in 10-cm plates containing Dulbecco's Modified Eagle Medium (DMEM) (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Sigma Corp., Oakville, ON) at 37°C in 5% CO₂ atmosphere. Cells were grown at 37°C in a humidified 5% CO₂ incubator and routinely passaged when 90–95% confluent.

Hoechst 33342 staining

DAOY cells were treated with 0.5 mM EDTA/PBS and resuspended at 0.5-1.0 x 10⁶ cells/mL in 0.1% BSA/PBS. Hoechst 33342 was added at a final concentration of 5.0 µg/mL. Cells were then incubated for 90 min at 37°C/5% CO₂ with intermittent agitation. After incubation, cells were washed twice with cold PBS and resuspended at 2 x 10⁶ cells/mL supplemented with PI (2 µg/mL).
The cell suspensions were then taken to a flow cytometer equipped with an ultraviolet (UV) laser.

**Cell sorting strategy**

Cells were sorted on a MoFlo™ High Performance Cell Sorter (Dako, Fort Collins, CO) either into tubes or into 96 well microwell plates using the Cyclone system. The machine was equipped with three excitation lines which included a solid state Coherent Enterprise II 488 nm (blue) laser (200 mW OPSL), a solid state 635 nm (red) laser (25 mW diode) and a 351 nm (UV) water-cooled laser (5 W Innova 90C). Three fluorescent channels (FL1, FL2, and FL3) can pick up the 488 nm laser, two channels (FL4, FL5) from the 351 nm laser and one from the 633 nm laser (FL6). Hoechst fluorescence was measured at both 424/44 nm and above 670 nm (split by a 610 nm short-pass dichroic mirror) resulting from UV excitation. PI fluorescence, measured at 617 nm at the FL3 channel, excluded dead cells during sorting.

**Immunophenotyping**

Cells, grow in 10-cm tissue culture dish (Sarstedt, Montreal, QC), were detached with 0.5 mM EDTA/PBS for 15 min at 37°C/5% CO₂ prior to washing and reconstituting to a final concentration of 0.5-1.0x10⁶ cells/mL in 0.1% BSA/PBS. After additional washing in PBS, cells were reconstituted in 0.1 mL of 0.1% BSA/PBS and labelled with 10 µL of anti-CD133-PE (Miltenyi Biotec, Auburn, CA) for 30 min at 4°C; the isotype IgG1-PE was added at a similar concentration.
Cells were then washed twice with PBS and resuspended in 0.3 mL of 0.1% BSA/PBS and analyzed by flow cytometry.

**HDAC inhibitor treatment**

Side population (SP) cells or total cells were sorted into 96-well plates (Sarstedt Inc., Montreal, QC) at a density of 2x10³ cells per well in complete serum (DMEM supplemented with 10% FBS) in triplicate. Cells were allowed to adhere to the well for about 4-6 h prior to exchanging into drug-containing media for about 72 h. The drugs used were suberoylanilide hydroxamic acid (SAHA; CedarLane Laboratories, Burlington, ON) (0, 0.2, 1.0, 5.0, 10.0 µM dissolved in DMSO) and 4-phenylbutyric acid (4-PB; Sigma Corp., Oakville, ON) (0, 0.5, 1.0, 2.0, 4.0 mM dissolved in DMSO).

**XTT viability assay**

The XTT assay measures the viability of living cells by quantitatively assessing the production of soluble formazan product resulting from the mitochondrial dehydrogenase-mediated cleavage of the tetrazolium sodium salt XTT. Briefly, after the indicated experimental treatments, buffer exchange into OPTIMEM was performed. Sterile XTT was prepared at 1 mg/mL in pre-warmed (37°C) OPTIMEM whereupon 50 µL of the XTT solution was added to each 200 µL of culture. It must be noted that PMS was prepared at 5 mM (1.53 mg/mL) in PBS and was added to the XTT solution at a final concentration of 25 µM (5 µL of 5 mM PMS added to 1 mL of XTT (1 mg/mL) prior to addition to the culture.
media; PMS acts as an electron coupling agent thereby potentiating XTT reduction. After incubating at 37°C for 1.5 h, the optical density (OD) of each well was measured at both 450 nm (reference 620 nm) for quantification of formazan production using an EAR 400AT 96-well plate reader (SLT Labinstruments, Gröding/Salzburg, Austria). XTT assays were always performed in triplicate with the one-way analysis-of-variance (ANOVA) and the Bonferroni post-test determining statistical significance.

**Western blotting**

SP cells from different experimental conditions (SAHA or DMSO-vehicle) were rinsed in ice-cold phosphate-buffered saline (PBS) and lysed in sample buffer (30 mM Tris (pH 8.0), 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1% Triton X-100, 0.5% sodium deoxycholate, complete EDTA-free protease inhibitor tablet(s)). The bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL) was used to quantify protein levels in the cell lysates with equal amounts of protein (20 µg) being resolved by denaturing 10% polyacrylamide gel electrophoresis and electrotransferred to Hybond-ECL nitrocellulose membranes. Membranes were then incubated in 0.1% Ponceau red to determine protein loadings followed by incubation in 5% nonfat milk in TBST (20 mM Tris-Cl (pH 8.0), 150 mM NaCl and 0.2% Tween-20) for 1 hr. at room temperature. Incubation with the appropriate dilution of the primary antibody (rabbit-anti-histone H3, 1:1000; rabbit-anti-acetyl histone H3 (Lys 9), 1:1000; mouse-anti-p21 Waf1/Cip1, 1:1000) was performed at 4°C. The membranes were then rinsed thrice in TBST and probed with the corresponding secondary
antibodies (anti-rabbit (1:1000; Cell Signaling Tech., Danvers, MA)/anti-mouse (1:1000; Dako, Glostrup, Denmark)) conjugated with horseradish peroxidase at room temperature for 1 hr. Membranes were finally washed thrice in TBST to remove unbound secondary antibodies prior to revelation using the SuperSignal West Femto substrate kit (Pierce Biotechnology Inc., Rockford IL) and chemiluminescent imaging using the GeneGnome system (Syngene, Frederick, MD).

**Limiting dilution analysis – neurosphere assay**

Cellular suspensions were labelled with propidium iodide (PI) with PI-negative (viable) cells being sorted by the MoFlo™ flow cytometer (Dako, Fort Collins, CO) into 96-well microwell plates (Sarstedt Inc., Montreal, QC) containing neural stem cell (NSC) media (epidermal growth factor (EGF; 20 ng/mL), basic fibroblast growth factor (bFGF; 20 ng/mL), heparin (2 µg/mL), 2 mM L-glutamine, B-27 (1x), penstrep (1x) and DMEM/F12); final cell numbers ranged from 10 cells per well to 1 cell per well in 0.2 mL volume. Cultures were incubated for 7 days prior to counting the fraction of wells without spheres as plotted against the number of cells plated per well. The proportion of stem-like cells (or the number of cells required to form one sphere) in the DAOY MB cell line was determined from the Poisson distribution of stem-like cells where $F_0 = e^{-x}$ ($F_0$ represents the fraction of wells without spheres and $x$ is the average number of stem-like cells per well); $F_0 = 0.37$ corresponds to the number of cells required to be plated in order for one stem-like cell to be present.
Animal experimentation

DAOY cells were harvested at 60-70% confluency with 0.5 mM EDTA/PBS and resuspended in Hank’s Buffered Saline Solution (HBSS). Stereotactic injections were based on the work of Segal’s laboratory (18). Briefly, 6-week old CD1 \textit{nu/nu} mice were anaesthetized with ketamine/xylazine i.p. and placed in a stereotactic apparatus (Kopf). A burr hole was drilled 2 mm lateral and posterior to the bregma and 2x10^5 cells in 4 \( \mu \)L HBSS were injected 3.1 mm below the dura with a 26-gauge Hamilton needle over 10 min. Tumours were allowed to grow for 2 weeks prior to treatment with 500 mg/kg daily i.p. valproic acid (2-propyl-pentanoic acid; Sigma Corp., Oakville, Ontario) until animals displayed signs of neurological deficit.

RESULTS

Effects of HDAC inhibitors on DAOY SP viability

The relative abundance of SP cells in the DAOY medulloblastoma cell line made this cellular population particularly amenable to experimental investigation. The effect of HDAC inhibitors, such as SAHA and 4-phenylbutyric acid (4-PB), on SP viability were assessed. Indeed, SAHA and 4-PB can both significantly reduce the viability of SP cells and furthermore reduce the viability of the total cell population to the same extent (Figure 1). It must be noted that the range of 4-PB concentrations utilized in these experiments encompassed the serum therapeutic range reported in the Buphenyl Product Monograph. There appeared to be a
concentration-dependent effect of SAHA on XTT reduction with lower concentrations (1 µM) eliciting a reduction in proliferation rate and the higher concentrations (10 µM) resulting in massive cell death. FACS analysis revealed that at low SAHA concentrations there was a slight increase in propidium iodide-positive cells indicating cell death; however, at high SAHA concentrations FACS analysis could not be performed due to insufficient cell numbers (data not shown).

SAHA induces H3 acetylation and p21 expression in DAOY SP cells

To determine whether histone acetylation levels were changed upon incubation with an HDAC inhibitor, we chose to analyze the effect of SAHA because of its robust impact on the viability of MB cells. DAOY cells were first labelled with Hoechst 33342 with SP cells being flow cytometrically sorted into a 24 well plate containing 1 µM SAHA. SP cells were treated for 8 hours prior to Western blot analysis which revealed that 1 µM SAHA can significantly increase the acetylation of histone H3 in the DAOY SP cell fraction Figure 2; DMSO vehicle treatment did not elicit histone H3 acetylation. Furthermore, there was a concomitant increase in expression of the cell cycle growth arrest regulator, p21, with SAHA treatment of SP cellsFigure 2B. These observations are consistent with the mechanism of action of HDAC inhibitors (19).
Effect of HDAC inhibitors (SAHA and 4-PB) on DAOY clonogenicity, CD133 expression, and Hoechst 33342 dye exclusion

Next we assessed whether the tumour sphere potential of DAOY cells would be altered in the presence of HDAC inhibitors. Limiting dilution analysis experiments revealed that treatment of DAOY cells with HDAC inhibitors (1 µM SAHA or 2 mM 4-PB) for 7 days could result in a significant reduction in tumour sphere formation efficiency compared to DMSO vehicle treatment (Figure 3). In addition, we used an HDAC inhibitor, valproic acid that has had a long history of clinical usage to examine in vivo effects in a xenograft model. DAOY cells were implanted intracerebrally into athymic mice, allowed to proliferate for 10 days and then the animals were administered i.p. injections of either 500 mg/kg VPA or control PBS. Log-rank analysis revealed an extension of median survival from 22 days to 28 days in mice bearing intracranial DAOY xenografts (p<0.05; Figure 4). Finally, we determined whether treatment of the total cell population would have an impact on the level of the stem-like markers CD133 and Hoechst 33342 dye exclusion. As shown in Table I, the various HDAC inhibitors had different effects on the SP fraction while both 2 mM 4-PB and 1 µM SAHA slightly increased the proportion of CD133+ cells in the DAOY cell line after 72 hours of treatment. These results indicate that although these HDAC inhibitors have profound effects on cell survival, tumour sphere formation and in vivo tumour growth, non-concordant results are obtained when marker phenotype is used for assessing potential efficacy.
DISCUSSION

In this study, we have demonstrated that HDAC inhibitor treatment is associated with a slight increase in CD133 expression; these changes were not mirrored by increases in Hoechst 33342 efflux. We have also shown that HDAC inhibitors can significantly attenuate clonogenicity and increase survival of medulloblastoma-bearing mice.

A recent study demonstrated reactivation of CD133 in U251MG glioma cells upon either 5-azacytidine or VPA treatment suggesting epigenetic regulation of CD133 expression; it was speculated that epigenetic therapies may be effective for differentiated cancer cells but not for cancer stem cells (20). However, another study using neural stem cell-like cells (NSCLCs) revealed that VPA treatment significantly inhibited Bmi1-induced astrocytic dedifferentiation and self-renewal as assessed by reductions in stem cell marker expression and absence of neurosphere formation; CD133 expression was upregulated upon Bmi1 transduction of astrocytes, but CD133 levels were not reported after VPA treatment (21). Indeed, short hairpin RNA-mediated knockdown of Bmi1 in the DAOY cell line can result in inhibition of proliferation, loss of clonogenic survival and suppression of subcutaneous DAOY xenograft growth in nude mice (22). Interestingly, VPA has been shown to increase the number of neurons derived from rat stem cells with a concomitant decrease in astrocytic differentiation thus apparently influencing stem cell fate (23). Our results revealed HDAC inhibitors could significantly attenuate tumour sphere formation that was not accompanied by decreases in the CD133+ fraction.
A recent study has shown that, at least for pancreatic carcinoma cell lines, 4-PB was able to inhibit cellular export as evidenced by intracellular calcein-AM dye retention (24). However, the 4-PB-mediated decreases of both the SP compartment and clonogenicity do not constitute definitive proof that the SP is solely responsible, if at all, for clonogenicity given that upon SAHA treatment, the Hoechst 33342 efflux profile remained unchanged whereas tumour sphere formation decreased. Furthermore, we have shown previously that there is no association between Hoechst 33342 efflux and clonogenicity in the DAOY cell line (1). Yet, a recent study suggested that Notch pathway inhibition may deplete the stem-like cell population in the DAOY cell line, as evidenced by decreases in the side population; however, the side population was not interrogated for stem-like potential (13). VPA has been shown to upregulate p21 expression that is accompanied by growth arrest, differentiation, apoptosis in medulloblastoma cell lines and extended survival of medulloblastoma-bearing mice (17, 25). Our results revealed that Hoechst 33342 efflux remained unchanged at a therapeutically relevant concentration, although survival time was significantly prolonged upon VPA treatment. Supratherapeutic VPA treatment appeared to increase Hoechst 33342 efflux and the overall SP compartment; a recent study demonstrated that therapeutic VPA concentrations were able to upregulate ABCB1 expression (26).

The observed reduction in viability of DAOY SP cells when treated with SAHA was accompanied by an increase in both H3 acetylation and p21 expression. The p21 promoter contains the SAHA responsive elements (REs), Sp1-3 and Sp1-4
sites, which are also REs for other HDAC inhibitors (butyrate and trichostatin A) and bind Sp transcription factors thereby promoting p21 activation (27-29). Previous studies have demonstrated that SAHA can induce growth arrest and apoptosis in breast cancer cells accompanied by pronounced p21 activation (30). SAHA also has been shown to initiate apoptosis in medulloblastoma cells (31, 32). Thus, it appears that SAHA may at least lead to p21-mediated cell cycle arrest, which is reflected in the reduction of both the SP and TC DAOY cell proliferation and it is possible that p21 expression may mitigate self-renewal leading to decreased clonogenicity.

Taken together, these results suggest that changes in marker expression occur independently of the changes in clonogenicity in response to HDAC inhibitor treatment. This study has provided further evidence that illustrates the divergence between cancer stem cell marker expression and clonogenicity in vitro and warrants caution for using well-established cancer stem cell markers in evaluating the efficacy of experimental therapeutics on tumour-initiating cells in vitro.
REFERENCES


FIGURES
Figure 1: SAHA (A) and 4-PB (B) treatment of the DAOY SP and TC for 72 h. DAOY side population (SP) and total cells (TC) were sorted into 96-well plates at a density of 2000 cells per well in complete serum followed exchange into drug-containing media at the indicated concentrations for about 72 h prior to XTT viability analysis; Suberoylanilide hydroxamic acid (SAHA – 0, 0.2, 1.0, 5.0, 10.0 µM) and 4-phenylbutyric acid (4-PB – 0, 0.5, 1.0, 2.0, 4.0 mM). * p<0.05, ** p<0.01, *** p<0.001 (one-way ANOVA compared to the mock condition for each fraction).
SAHA

1 μM DMSO

AcH3

panH3

p21
Figure 2: SAHA-mediated upregulation of p21 levels in DAOY SP cells. DAOY cells were first labeled with Hoechst 33342 for 1.5 h at 37°C/5% CO₂ and subjected to flow cytometric analysis. SP cells were identified and sorted into a 24 well plate containing 1 µM SAHA or DMSO vehicle and incubated at 37°C/5% CO₂ for 8 h. Lysates were collected and western blotting was carried out using anti-acetyl-histone H3, anti-p21 and anti-histone H3.
A

![Graph A](attachment:image.png)

- untreated
- DMSO
- 1 uM SAHA

B

![Graph B](attachment:image.png)

- DMSO
- 2 mM 4-PB
Figure 3: Effects of (A) SAHA and (B) 4-PB on DAOY tumour sphere formation. DAOY cells were sorted into 96 well culture plates containing neural stem cell media (20 ng/mL EGF and 20 ng/mL bFGF); cells were incubated at 37°C/5% CO₂ for 7 days to produce spheres. The fraction of wells without spheres was plotted against the number of cells plated per well. The proportion of stem-like cells (or the number of cells required to form one sphere) in the DAOY cell line was determined as described in the Materials and Methods section. The tumour sphere efficiencies for both treatments were as follows: A) SAHA - untreated (38.9%), DMSO (42.6%), 1 µM SAHA (3.81%); B) 4-PB - DMSO (18.2%), 2 mM 4-PB (0.46%).
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Figure 4: Valproic acid (VPA) significantly enhances the survival of mice implanted with DAOY cells. $2 \times 10^5$ DAOY cells were injected intracranially into 6-week old anaesthetized nude mice and allowed to grow for 2 weeks. 500 mg/kg VPA was administered intraperitoneally until signs of neurological deficit was observed. Inset photomicrograph displaying nuclear staining of medulloblastoma specimen at the injection site area.
<table>
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Table I: Effect of HDAC inhibitor on SP and CD133 marker expression in the DAOY cell line. DAOY cells were separately treated for 72 h at 37°C/5% CO₂ with the following HDAC inhibitors: 2 mM 4-PB, 1 µM SAHA, 1 mM VPA, 5 mM VPA. Subsequently, cells were harvested and labeled with either Hoechst 33342 (1.5 h at 37°C/5% CO₂) or anti-CD133-PE (30 min at 4°C) and subjected to flow cytometric analysis.
Extensive preclinical studies, including this thesis, have demonstrated that HDAC inhibitors may represent a promising avenue in drug development for cancer treatment. Synergistic combinations with other molecular targeted therapies may serve to increase the efficiency of tumour cell kill. Given that transcription factors serve as a central node for multiple upstream signalling pathways ultimately controlling cell behaviour and that they are frequently deregulated in many cancers, there has been a recent surge of interest in developing transcription factor-based therapies for cancer treatment. Forkhead box O (FOXO) transcription factors regulate a wide variety of fundamental cellular processes including cell cycle arrest, DNA repair, apoptosis, cell differentiation and metabolism. The last chapter of this thesis will explore the potential of delivering Forkhead box O (FOXO) transcription factors using adenoviruses to suppress medulloblastoma cell viability.
IMPAIRED MEDULLOBLASTOMA CELL SURVIVAL FOLLOWING ACTIVATION OF THE FOXO1 TRANSCRIPTION FACTOR

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Running Title: FOXO1 and medulloblastoma survival

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Keywords: medulloblastoma; forkhead; FOXO; IGF-I, adenovirus
ABSTRACT

Medulloblastoma is the most frequent type of childhood brain tumor. The insulin-like growth factor I receptor (IGF-IR) plays a significant neuroprotective role in medulloblastoma survival through regulation of the downstream effectors of the phosphoinositide-3-kinase-protein kinase-B (PI3K-PKB/c-Akt) pathway. One such target is Forkhead box O1 (FOXO1; FKHR), which is part of the FOXO family of Forkhead transcription factors. Phosphorylation by Akt results in cytoplasmic sequestration of FOXO1 thus inhibiting the expression of genes controlling cell death, cell proliferation, differentiation, cellular metabolism and oxidative stress. Here we show that serum starvation of medulloblastoma cells is accompanied by nuclear translocation of FOXO1. IGF-I stimulation of serum-starved cells resulted in rapid phosphorylation of Akt and FOXO1, and was associated with a significant increase in cell viability. In contrast, expression of a constitutively active form of FOXO1 that cannot be phosphorylated led to a significant reduction in medulloblastoma cell viability, even in the presence of growth factors provided by fetal bovine serum (FBS). These data suggest that the transcription factor FOXO1 may be a critical effector of medulloblastoma growth suppression.
INTRODUCTION

Medulloblastoma is a highly malignant tumour of the cerebellum that represents about 20-25% of all paediatric intracranial neoplasms with the peak incidence occurring between 5 and 9 years of age.\(^1,2\) Despite aggressive therapy, which includes surgical debulking, craniospinal irradiation and chemotherapy, the 5-year survival rate hovers around 50-70% with children under 3 years of age faring much worse.\(^3,4\) Furthermore, there are considerable neuropsychological sequelae amongst survivors secondary to both the disease and the treatment.\(^5\)

The exact origin of medulloblastoma remains unknown although evidence suggests derivation from the granule cell precursors found in the external germinal layer of the developing cerebellum.\(^6,7\) A few potential underlying mechanisms of medulloblastoma initiation and progression have been revealed, which include chromosomal alterations and dysregulation of cell signaling systems.\(^8,9\) The PI3K/Akt pathway has been implicated in medulloblastoma cell survival and medulloblastoma tumor formation in humans and in an animal model of the tumour.\(^10,11\) The majority of tumour specimens examined to date have shown immunohistochemical staining for activated Akt (phosphorylation of Ser473).\(^12,13\) Furthermore, overexpression of constitutively active Akt in medulloblastoma cell lines reverses the proliferative arrest elicited by the PI3K inhibitor, LY294002.\(^12\) As well, in the ptc\(^{+/-}\) mice, which spontaneously develop medulloblastoma at a somewhat low frequency (15-20%), activation of the Akt pathway increases tumour incidence.\(^10,14\)
Upon phosphorylation by PI3K, activated Akt can phosphorylate a variety of substrates, including the proapoptotic Bcl-2 family member Bad; caspase-9; nuclear factor-κB; mammalian target of rapamycin; mdm2; and the Forkhead family of transcription factors, FOXO1 (FKHR), FOXO3a (FKHRL1) and FOXO4 (AFX) suggesting a multifactorial mechanism in mediating cell survival through blockade of apoptosis. (15,16) In established cell lines, the Forkhead family of transcription factors augments the expression of the cyclin-dependent kinase inhibitor p27kip1 causing cell cycle arrest; further FOXO-induced transcriptional upregulation of FasL and Bim genes can trigger apoptosis. (17) Phosphorylation by Akt leads to nuclear export and cytoplasmic retention of FOXO members, preventing activation of gene targets related to growth arrest and apoptosis thereby allowing the target cells to proliferate and/or survive. (15,16)

There is currently a dearth of information on the effects of the Forkhead family of transcription factors on the survival of medulloblastoma cells. Consequently, the major aim of this study was to assess the effects of the FOXO transcription factors on medulloblastoma cell survival. We have found that upon serum starvation of the DAOY and UW228-3 human medulloblastoma cell lines, FOXO1 (FKHR) translocates to the nucleus. Furthermore, a constitutively active form of FOXO1 (FOXO1-AAA) that cannot be phosphorylated by Akt was sufficient in significantly reducing medulloblastoma cell viability.
MATERIALS AND METHODS

Cell lines

The DAOY (American Type Culture Collection; Rockville, Md.) and UW228-3 (18) (a gift of Dr. Rolando Del Maestro; McGill University, Montreal, Canada) medulloblastoma human cell lines were cultured in 10-cm plates containing Dulbecco’s Modified Eagle Medium (DMEM) (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum at 37°C in a 5% CO2 atmosphere.

Treatments

Cells were first grown in 10-cm plates until 70-80% confluency followed by cellular detachment with 5mM EDTA and plating into 6-well plates at a density of 5x10^4 cells per well in complete serum (DMEM supplemented with 10% FBS). In order to make cells quiescent, after 24 h, the culture medium was replaced with serum-free medium solution (DMEM supplemented with 0.1% bovine serum albumin) for 48 h. Cell viability response to IGF-I (Intergen Co., Purchase, NY) by stimulation of quiescent serum starved DAOY cells with 10 and 100 ng/mL IGF-I, was assessed by a colorimetric assay with the dye (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]2H-tetrazolium-5-carboxanilide) sodium salt (XTT; Sigma, St. Louis, MO). To investigate the short-term kinetics of phosphorylation of Akt (Cell Signaling Tech., Beverly, MA) and p44/42 MAPK (Cell Signaling Tech., Beverly, MA), cells were exposed to IGF-I for 2, 10, 60 and 120 min; cell lysates were subsequently collected for western blot analysis. To study the effect of IGF-I on the phosphorylation of Akt, p44/42 MAPK and FOXO1 (Cell Signaling
Tech., Beverly, MA), cells were treated with either LY294002 (25 µM; Calbiochem, San Diego, CA) or PD98059 (50 µM; Calbiochem) for 2 min, 10 min, 1 h and 24 h following stimulation with IGF-I (100 ng/mL) for 24 h. Subsequently, lysates were collected for western blot analysis.

**Western Blotting**

Briefly, cells from different experimental treatment conditions were rinsed in ice-cold PBS and lysed in sample buffer (30 mM Tris (pH 8.0), 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1% Triton X-100, 0.5% sodium deoxycholate, complete EDTA-free protease inhibitor tablet(s)). Samples with equal amounts of protein were then resolved by denaturing 10% polyacrylamide gel electrophoresis and electrotransferred to Hybond-ECL nitrocellulose membranes. Membranes were then incubated in 0.1% Ponceau red to determine protein loadings followed by incubation in 5% nonfat milk in TBST (20 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.2% Tween 20) for 1h at room temperature and then incubation with the appropriate primary antibody (anti-phospho-Akt[Ser 473], 1:1000; anti-phospho-FOXO1[Ser 256], 1:1000; anti-Akt, 1:1000; anti-FOXO1, 1:1000; anti-caspase-9, 1:500, Cell Signaling Tech., Beverly, MA); anti-p27, 1:1000 (Santa Cruz Biotechnology, Santa Cruz,CA); anti-cyclin D1, 1:1000 (NeoMarker, Fremont, CA) and anti-β-actin, 1:1000 (abcam, Cambridge, UK) at 4°C overnight. The membranes were then rinsed thrice in TBST and probed with the corresponding secondary antibodies (anti-rabbit (1:1000; Cell Signaling Tech., Beverly, MA)/anti-mouse (1:1000; Dako, Glostrup, Denmark)) conjugated with
horseradish peroxidase at room temperature for 1h. Membranes were finally washed thrice in TBST to remove unbound secondary antibodies prior to revelation using the BLAZE kit (Pierce Biotechnology Inc., Rockford, IL).

**Immunofluorescence studies**

DAOY cells were plated into wells within the Lab-Tek chamber slide system (Nalge Nunc International, Naperville, IL) at a density of $10^4$ cells per well in complete serum. After 24 h, serum starvation was carried out in which the medium was replaced with DMEM (containing 0.1% BSA) for varying times (1, 3, 12 and 24 h). Subsequent after each time point cells were rinsed twice in ice-cold PBS and fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with ice-cold acetone: methanol (1:1) for 10 min. After blocking with 5% BSA in PBS containing 0.1% Triton-X-100 for 1 h at room temperature, primary antibody incubation was performed for 16 h at 4°C using anti-FOXO1 (1:100) diluted in 5% BSA in PBS. After two washes in PBS, incubation in the appropriate secondary antibody (Alexa Fluor 555; Molecular Probes, Oregon) diluted in 5% BSA (1:1000) was done for 1h at room temperature. During the last 10 min of the incubation, Hoechst 33258 (1:1000) was added. Slides were visualized on a Leica DMIRE2 (Richmond Hill, ON, Canada) wide-field fluorescence microscope equipped with a CCD camera. The same exposure time was used for all samples. The images were processed using Openlab (Lexington, MA) software. A similar procedure was also utilized for observing FOXO1 translocation during serum-starvation of UW228-3 medulloblastoma cells.
Adenovirus preparation and infection

Ad-FOXO1-AAA (Ad-FKHR-AAA) was a kind gift of Dr. W.R. Sellers (Cambridge, MA). It was generated with the pAD-Easy system described previously. (19) Ad-FOXO1-AAA, and Ad-LacZ were amplified in 293A cells and purified by freeze-thaw extraction followed by CsCl gradient purification. Viruses were titered by monitoring cytopathic effect on 293A cells. DAOY cells were grown in 10-cm dishes until 80-90% confluency. These cells were then serum starved for 2 days prior to plating the cells in 6-well plates containing serum-free medium. Selected wells were supplemented with IGF-I (100 ng/mL) and allowed to grow for 24 h at 37°C/5% CO₂. Subsequently, Ad-FOXO1-AAA was added at MOI 100 and the control Ad-LacZ was added at the same MOI to ascertain the possibility of non-specific viral toxicity. DAOY cells were exposed to the viral treatment for 24, 48 and 72 h at 37°C/5% CO₂. For each time point, cell viability was assessed with the XTT assay (Sigma). For adenoviral transduction into 10% FBS containing media, DAOY or UW228-3 cells were first counted and allowed to adhere for about 6 h prior to transduction with Ad-FOXO1-AAA, Ad-LacZ or Ad-BFP at their respective MOIs. Both medulloblastoma cell lines were exposed to viral treatment for 24, 48 and 72 h at 37°C/5% CO₂ prior to XTT viability assessment.
RESULTS

FOXO1 undergoes nuclear translocation during serum starvation

Medulloblastoma cell lines in culture are sensitive to serum deprivation. In both the DAOY and UW228-3 cell lines, upon withdrawal of serum, metabolic activity diminishes and cell numbers decrease (Fig. 1).

As the PI3K/Akt pathway has been implicated in medulloblastoma survival, we examined a distal target of the PI3K/Akt pathway, FOXO1 during serum starvation of DAOY and UW228-3 cell lines. FOXO1 was chosen as a representative of the FOXO family of Forkhead transcription factors, which include FOXO1, FOXO3a and FOXO4. To examine the localization of FOXO1 transcription factor upon growth factor withdrawal, DAOY cells were placed into serum-free media for varying periods of time. Endogenous FOXO1 was primarily confined to the cytoplasm of DAOY cells proliferating in 10% fetal bovine serum (Fig. 2). Transfer of these cells to serum-free medium [containing 0.1% bovine serum albumin (BSA)] resulted in FOXO1 re-localization to the nucleus after 24 h with progressively stronger FOXO1-positive immunoreactivity being observed within Hoechst-positive nuclei (Fig. 2A). Analysis of 6 high-power fields (hpf) per time point revealed that most of the FOXO1-positive nuclei are observed after 24 h (Fig. 2A). Nuclear FOXO1 immunoreactivity was also observed in serum-starved UW228-3 medulloblastoma cells, which displayed reduced viability (Figs. 1 and 2C). These results show that serum starvation of the medulloblastoma cells is accompanied by nuclear translocation of FOXO1.
Expression of IGF-IR and IGF-I-dependent survival signaling in human medulloblastoma cells

The insulin-like growth factor I (IGF-I) is a potent survival factor which signals through the PI3K/Akt pathway. (20,21) The human medulloblastoma cell line, DAOY, expresses IGF-IR suggesting the potential for these cells to respond to IGF-I (Fig. 3A). The DAOY cells express almost equivalent levels to that of the rat glioma cell line, C6LacZ, which was used as a positive control for IGF-IR (22); IGF-IR expression was also observed in the UW228-3 human medulloblastoma cell line (data not shown). The IGF-IR in these cells is functional as IGF-I can significantly increase the viability of serum-starved DAOY in response to IGF-I stimulation (Fig. 3B). When the DAOY cell line was cultured in serum-free medium for 48 h prior to stimulation with IGF-I (10 ng/mL and 100 ng/mL) for 24, 48 and 72 h, IGF-I rescued DAOY cells in a dose-dependent manner. These results indicate that IGF-I can lead to survival of medulloblastoma cells under low-serum conditions.

Treatment with IGF-I was associated with a rapid phosphorylation of Akt within 2 minutes of stimulation with IGF-I (100 ng/ml) and which was sustained for at least 24 h post-IGF-I stimulation (Fig. 3C). Of interest, IGF-I induces rapid FOXO1 phosphorylation (Fig. 3D), which is sensitive to treatment with the PI3K/Akt inhibitor LY294002, but insensitive to the Ras-MAPK inhibitor, PD98059, as unphosphorylated FOXO1 appears within 10 min of LY294002 inhibitor administration while it remains phosphorylated in the presence of
PD98059 (Fig. 3D). Thus, IGF-I rescues DAOY cells from serum starvation, possibly through the downstream phosphorylation of FOXO1.

**Activated FOXO1-AAA significantly suppresses medulloblastoma viability**

A mutant form of FOXO1 (FOXO1-AAA), containing three modified Akt phosphorylation sites (Ser, Thr to Ala), cannot be negatively regulated by Akt. Thus, adenoviral expression of FOXO1-AAA was utilized to restore FOXO1 activity to DAOY cells in an attempt to ascertain the effects of constitutively active FOXO1 on medulloblastoma viability. DAOY cells were first serum starved for 48 h prior to treatment with IGF-I for 24 h. These cells were then exposed to either Ad-FOXO1-AAA (Ad-A3) or Ad-LacZ at MOIs (multiplicity of infection) of 100 and assessed for viability after 24, 48 and 72 h. [All DAOY cells are transduced at this MOI (Fig. 4A)]. Cells infected with Ad-LacZ showed similar viability to that of mock-infected cells (Fig. 4B). However, the viability of Ad-FOXO1-AAA infected DAOY was significantly reduced compared to that of both Ad-LacZ treated and mock-infected cells (Fig. 4B). Interestingly, Ad-FOXO1-AAA infected DAOY which were exposed to complete serum displayed a similar trend. Cells that were infected with Ad-LacZ at MOIs of 100 and 500 showed similar viability to mock-infected cells (Fig. 5A). In contrast, the viability of Ad-FOXO1-AAA infected DAOY grown in complete serum was significantly reduced at both MOIs as compared to cells that were infected with Ad-LacZ (Fig. 5A). Furthermore, Ad-FOXO1-AAA infections carried out at MOI 500 were able to reduce DAOY viability constantly over a three-day period.
Similarly, viability of UW228-3 medulloblastoma cells growing in complete serum significantly decreased after Ad-FOXO1-AAA transduction at MOIs of 50 and 100 (Fig. 5B). In cells transduced with Ad-FOXO1-AAA, Western blot analysis of selected FOXO1 targets showed an increase in the cyclin-dependent kinase inhibitor p27 and a concomitant decrease in cyclin D1 (and cyclin D2, data not shown) as compared to cells transduced with Ad-LacZ or mock treated (Fig. 6). Importantly, only Ad-FOXO1-AAA-transduced cells had activated caspase 9 (Figure 6). Thus, a FOXO1 construct that cannot be negatively regulated by Akt is a critical direct regulator of medulloblastoma cell survival in the absence or presence of growth factors.
DISCUSSION

Data from studies in nematode and mammalian cells suggest that regulation of the Forkhead family of transcription factors is key to many cellular responses which include apoptosis, cell cycle function, stress and metabolic responses. (17,23) The major targets of the serine-threonine kinase Akt are the Forkhead factors; however there is a dearth of data regarding the involvement of the IGF-I-FOXO1 axis in the survival of medulloblastoma. Here we report that retention of FOXO1 in the cytoplasm is key to medulloblastoma survival and that enforced expression of a constitutively active FOXO1 can dramatically reduce medulloblastoma cell viability. It has been shown that IGF-I can stimulate FOXO3a/FKHRL1 phosphorylation in neuronal cells via the PI3K-Akt pathway thus inhibiting this pro-apoptotic transcription factor. (24) The common mechanism for inhibition has been attributed to phosphorylation of the FOXO factors via insulin or other growth factor signaling cascades leading to their retention in the cytoplasm and thus repression of pro-apoptotic genetic programs. (17) For example, in cerebellar granule cells, the ultimate phenotypic response for FOXO regulation by the IGF-I signaling cascade is the blockade of FOXO3a/FKHRL1-dependent transcription of Bim, which is a principal player in initiating the intrinsic (mitochondrial) apoptotic pathway. (25) Furthermore, nuclear exclusion of FOXO1 induced via phosphorylation by insulin serves to target FOXO1 for ubiquitin-mediated degradation. (26) We have shown for the first time in medulloblastoma cells that FOXO1 is phosphorylated upon sole IGF-I stimulation subsequent to serum starvation (Figure 3D). Furthermore, IGF-I-induced FOXO1 phosphorylation
appears to be PI3K-dependent rather than dependent on the MAPK pathway. Thus, in the medulloblastoma cells, integrity of the PI3K-Akt pathway is required for FOXO1 phosphorylation, in accordance with previous findings in other cell types. (17,23,24,26,27)

Upon serum starvation of the medulloblastoma cells, the subcellular localization of FOXO1 was primarily nuclear. This observation is in agreement with the commonly held hypothesis that in the absence of IGF-I signaling FOXO1 is no longer phosphorylated and thus can be imported into the nucleus to effect target gene expression. Serum starvation led to a drastic reduction in medulloblastoma cell viability (Figure 1). The findings of nuclear FOXO1 localization and low medulloblastoma viability upon serum starvation prompted us to investigate the link between the regulation of FOXO1 status and medulloblastoma survival. Since phosphorylated FOXO1 has been hypothesized to lead to cell survival, a dominant-negative form of FOXO1 (FOXO1-AAA), in which the three Akt phosphorylation sites are altered, was used to test the null hypothesis that medulloblastoma cell death requires FOXO1 to be constitutively non-phosphorylated in the presence of growth factor signaling. We report here that adenovirus-mediated delivery of FOXO1-AAA into medulloblastoma cells can reduce cell viability (Figures 4, 5 and 6), which is in keeping with other published reports demonstrating a regulatory role for FOXO1 in apoptosis. (28-31) Indeed, a recent study demonstrated that pharmacological inhibition of the p85 subunit of PI3K in human breast carcinoma cells leads to activation of FOXO transcription factors and subsequent transcriptional induction of cell cycle arrest and apoptotic
genes. As well, dramatic apoptosis of LAPC4 prostate carcinoma cells occurred upon adenovirus-mediated overexpression of FOXO1 and FOXO3a. Interestingly, in our study, Ad-FOXO1-AAA treatment significantly abrogated medulloblastoma viability in the presence of complete serum (Figures 5 and 6), which suggests that FOXO1 may represent an important convergence point for multiple signaling pathways.

Taken together, the data presented here support a novel role for FOXO1 in medulloblastoma viability and furthermore, demonstrate that adenoviral expression of a mutant form of FOXO1 (FOXO1-AAA), which cannot be phosphorylated, can induce efficient growth suppression of medulloblastoma cell lines. Thus, means of inhibiting FOXO1 nuclear export might provide important therapeutic applications, allowing the activity of a single downstream transcription factor, such as FOXO1, to overcome defects in tyrosine kinase receptor signalling through the PI3K-Akt pathway.

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FIGURES
Fig. 1. Serum starvation decreases both (A) DAOY and (B) UW228-3 medulloblastoma cell viability. The cells were plated at $5 \times 10^4$ cells/well in the presence of 10% FBS in a 6-well plate. The cells were then buffer exchanged into serum-free (SF) medium and cell viability was assessed at 24, 48, 72 and 96 h using the XTT assay. The results are representative of three experiments and are shown with standard deviation.
Fig. 2. FOXO1 nuclear localization upon serum-starvation of DAOY and UW228-3 medulloblastoma cells. (A) DAOY cells were serum starved for varying times (1, 3 and 24 h) followed by fixation and permeabilization (a)-(d). Subsequently, cells were stained with anti-FOXO1 (Alexa Fluor 555; red) and Hoechst 333248 (nuclear stain; blue) and images were captured with a fluorescent microscope as described in Materials and Methods. (B) Graphical representation of FOXO1 nuclear localization in DAOY medulloblastoma cells; bars represent mean +/- SEM from at least six independent high-power field. P-value was determined by using one-way ANOVA with Bonferroni’s multiple comparison post-test. (C) Endogenous FOXO1 nuclear localization in UW228-3 cells after 24 h serum starvation as visualized by 63x oil immersion.
Fig. 3. Effect of IGF-I on medulloblastoma cell survival. (A) Expression of IGF-IRβ in the DAOY medulloblastoma cell line; C6LacZ was used as a positive control for IGF-IRβ expression. (B) IGF-I promotes survival of DAOY medulloblastoma cells under serum-free conditions. Complete serum (10% FBS) was replaced with serum free medium (SF) and cells were cultured for an additional 48 h under SF conditions. Cells were subsequently stimulated with either 10 ng/mL or 100 ng/mL of IGF-I; control cells did not receive IGF-I stimulation. Cell viability was determined at 24, 48 and 72 h post-stimulation using the XTT assay. The results are representative of three experiments and are shown with standard deviation. (C) Phosphorylation of Akt upon IGF-I stimulation (100 ng/mL) of serum starved DAOY cells for 2 min, 10 min, 1 h and 24 h. Lysates were collected and electrophoresed on a 10% reducing polyacrylamide gel. Proteins were transferred onto nitrocellulose and the blots were probed with anti-phospho-Akt (Ser473) and total Akt. (D) DAOY cells were treated with IGF-I (100 ng/mL) for 24 h followed by exposure to LY294002 (25 μM) or PD98059 (50 μM) for 2 min, 10 min, 1 h and 24 h. Lysates were collected and western blotting was carried out using anti-phospho-FKHR/FOXO1 (Ser256).
Fig. 4. Transduction of medulloblastoma cells with Ad-FOXO1-AAA abrogates the survival effect of IGF-I. (A) Bright-field/GFP overlay of DAOY medulloblastoma cells transduced with Ad-FOXO1-AAA at multiplicity of infection (MOI) 100 for 24 hours. (B) Graphical representation of Ad-FOXO1-AAA transduction of IGF-I treated DAOY cells; bars represent mean +/- SEM from at least three experiments. DAOY cells were plated at $2.5 \times 10^4$ cells per well and buffer exchanged into SF medium for 48 h prior to IGF-I treatment for 24 h. Adenovirus infection was then carried out with either FOXO1-AAA or LacZ expression cassettes at MOIs of 100 and assessed for viability after 24, 48 and 72 h.
Fig. 5. Effect of Ad-FOXO1-AAA transduction on survival of medulloblastoma cells cultured in serum. Graphical representation of Ad-FOXO1-AAA treatment of (A) DAOY and (B) UW228-3 medulloblastoma cells grown in complete serum; bars represent mean +/- SEM from at least three experiments. Cells were plated at $2.5 \times 10^4$ cells per well and adenoviral infection was then carried out with FOXO1-AAA, LacZ or BFP cassettes at the indicated MOIs and assessed for viability after 24, 48 and 72 h.
Fig. 6. Effect of Ad-FOXO1-AAA transduction on selected FOXO1 targets. UW228-3 cells were infected at MOI 100 for 24 h prior to cell lysis and western blot analysis of FOXO1, cyclin D1, p27 and caspase 9; actin was used as a loading control. Abbreviations: M, mock (PBS); L, Ad-LacZ; F, Ad-FOXO1-AAA.
APPENDIX

Trypan blue assessment of DAOY cell survival following serum starvation

**Rationale**

To demonstrate that nuclear localization of endogenous, physiological levels of FOXO1 and a consequent reduction in XTT viability following serum starvation of DAOY cells is also accompanied by a significant increase in cell death.

**Materials and Methods – Trypan blue protocol**

DAOY cells were grown in 10-cm plates until 70-80% confluency in DMEM/10% FBS after which the cells were serum starved by replacing the medium with DMEM/0.1% BSA and incubating for 24, 48 and 72 hours. Cells were then detached with 0.5 mM EDTA and resuspended and diluted in PBS at each time point. 1 part of 0.4% trypan blue and 1 part diluted cell suspension were mixed and incubated for about 3 minutes prior to viability counts with a haemocytometer.

**Results**

When the DAOY cell line was cultured in serum-free medium for 48 hours, IGF-I rescued DAOY cells in a dose-dependent manner (10 ng/mL and 100 ng/mL); the
majority of DAOY cells remained adherent during IGF-I incubation (data not shown). Serum starvation resulted in a time-dependent progressive loss of viable DAOY cells whereas treatment with complete serum resulted in an expected increase in the number of viable cells.

**Significance**

These results demonstrate that serum starvation can lead to bona fide cell death in the DAOY cell line that is accompanied by nuclear localization of endogenous FOXO1 and reduction in XTT metabolism.

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**Comment [J8]:** You didn’t do trypan blue on the IGF-I treated cells, so these results do NOT directly indicate that IGF-I prolongs survival.
Figure 7: Serum starvation induced decrease in DAOY cell viability. DAOY cells initially grown in complete serum were serum starved (DMEM/0.1% BSA) for 24, 48 and 72 hours, detached at each time point, resuspended in PBS, and incubated in trypan blue solution for about 3 minutes prior to viability counts with a haemocytometer.

Comment [J9]: Were the cells starved for 48hrs, and then viability assessed for a further 75 hrs? or is time 0 at the beginning of serum starvation? The figure legend doesn’t make sense.
CHAPTER V
INTEGRATED DISCUSSION

The cancer stem cell hypothesis may provide an alternative framework in understanding the mechanisms of tumourigenesis and the evolution of drug resistance. This hypothesis can be substantiated only by judicious application of the tools utilized to investigate the stem cell compartment of cancer cell lines and primary tumours. Although numerous studies have utilized the Hoechst 33342 dye in stem-like cell enrichment, its mechanism of action must warrant caution in the interpretation of results especially from studies comparing stem-like potential of both the SP and NSP compartments. There are currently less cytotoxic vital dyes such as calcein-AM and rhodamine 123 that can also identify stem-like cells thereby potentially circumventing toxicity associated with long-term culture of Hoechst 33342-labeled cells. Reliance on solely one method of labelling stem cells may not be sufficient in comprehensively assessing the stem-like fraction in solid tumours. For example, numerous cell surface markers in addition to functional markers such as the side population define both normal haematopoietic and leukaemic stem cells. However, unlike haematological malignancies, there is a marked absence of a well-defined hierarchy of lineage marker expression for both neural stem cells and CNS malignancies. This has been largely attributed to technical difficulties in extracting stem cells from solid tissue compared to the relative ease of stem cell extraction from the peripheral blood compartment and bone marrow. Furthermore, CD133 has also come under intense scrutiny as a putative stem cell marker given that a few recent studies have demonstrated that CD133- cells also display stem-like characteristics (1-3). Further experimental
investigations into the nature of the target cell of transformation may provide an explanation for this phenomenon. Recent studies have now provided experimental proof that committed progenitors can acquire self-renewal characteristics. For example, granulocyte-monocyte progenitors in blast crisis chronic myelogenous leukaemia patients display self-renewal \textit{in vitro} whereas in chronic phase (CP) chronic myelogenous leukaemia, they do not exhibit this stem-like feature (4). It is quite conceivable that for some solid tumours, the target cell receiving the initial transforming insult may be a lineage-restricted precursor that has acquired the stem-like features of self-renewal and multilineage differentiation without acquiring CD133 expression; CD133+ and CD133- cells may arise from different cells of origin. However, the field of neural stem cell biology is in a current state of flux with evidence accumulating that astrocyte-like cells in the subventricular zone are bona fide neural stem cells thus blurring the distinction between the dichotomous neuronal and astrocytic lineages (5, 6). It can be surmised that tumours with either a CD133+ or a CD133- stem-like cell compartment may present with different clinical pictures in terms of biological aggressiveness (1).

While the serum-free neurosphere culture methodology has been extensively utilized as an \textit{in vitro} assay for self-renewal, its limitations highlight our incomplete understanding of the rich interactions between tumour cells and the stromal microenvironment (7, 8). Particularly, further work is needed to elucidate the self-renewal strategies (e.g. symmetric versus asymmetric divisions) adopted both at the individual cell and population levels in response to environmental
signals. Indeed, it seems plausible that a structured signalling environment \textit{in vivo} may lead to a hierarchical organization with respect to self-renewal and differentiation. However, the relative homogeneity of an \textit{in vitro} cell culture system makes it difficult to reconcile with the preservation of a tumour cell hierarchy \textit{in vitro}; this view implies that the original hierarchical organization may be largely driven by intrinsic mechanisms. A cancer stem cell is defined as “a tumour cell that displays the ability to self-renew and also divide to generate another stem cell and a progenitor cell, which generates the multitude of cell types comprising the bulk of the tumour” (9); strictly speaking, this definition implies asymmetric division of the cancer stem cell that is counterintuitive within an \textit{in vitro} context. It may be posited that progenitor cells may divide symmetrically thereby increasing cell number within the culture, but then repeated passaging would rapidly dilute out the “cancer stem cell”. Cultures solely consisting of symmetrically dividing cancer stem cells also seem unlikely given the existence of differentiating elements within the culture. Thus, symmetric and asymmetric divisions may be regulated by extrinsic factors such as cell density and growth factor availability. This obviously implies that the hierarchy observed \textit{in vitro} may not recapitulate the original hierarchy. Another limitation of this assay relates to the capacity of progenitor/non-stem cells to also produce spheres through limited self-renewal (10). Dissociation and replating of DAOY spheres at clonal and single-cell densities (greater than 5 times) resulted in repeated sphere production illustrating the ability to self-renew (data not shown); however, sphere propagation was not tested for the isolated fractions based on CD133 expression.
or dye exclusion. There is a dearth of data regarding the molecular mechanisms regulating self-renewal. It has been shown that enforced expression of β-catenin in normal granulocyte-monocyte progenitors can lead to induction of self-renewal whereas viral-mediated Axin expression can inhibit nuclear β-catenin in blast crisis granulocyte-monocyte progenitors leading to subsequent loss of self-renewal (11). Furthermore, β-catenin is localized solely within the HSC compartment in chronic phase chronic myelogenous leukaemia whereas it is also localized to the granulocyte-monocyte progenitors in blast crisis chronic myelogenous leukaemia patients. Self-renewal initiation in committed progenitors undoubtedly would involve oncogenic-mediated reprogramming. In the case of chronic myelogenous leukaemia, it has been suggested that the BCR-ABL oncogene may interact with Wnt signalling in promoting granulocyte-monocyte progenitor self-renewal (4). Thus, elucidating the transcription factor signalling network underlying the self-renewal phenotype may provide further molecular insight into the cancer stem cell hypothesis, which may in turn offer new molecular targets for experimental therapy.

Identification of a minor subpopulation of stem-like cells that display drug resistance will undoubtedly provide additional targets for cancer therapeutics; in this case, the objective is to “nip it in the bud”. However, this view may be overly simplistic considering that firstly, drug resistance is an innate property of normal stem cells as well and secondly, therapeutics designed to target cancer stem cells may also inadvertently target normal stem cells potentially resulting in dose-limiting toxicities; traditional chemotherapeutics elicit dose-limiting toxicities at
concentrations that damage normal tissue stem cells. Presently, clinical trials have provided preliminary evidence that HDAC inhibitors may offer an acceptable safety profile; however, their overall risk-benefit profile remains to be assessed. However, there is ample historical precedent for the development of resistance to any novel forms of therapy. For example, treatment with the HDAC inhibitor depsipeptide can result in ABCB1 upregulation with consequent drug efflux resulting in acquired resistance to depsipeptide (12). There was no cross-resistance to other HDAC inhibitors, such as trichostatin A implying that the large size of depsipeptide may serve as an effective ABCB1 substrate; the small molecule HDAC inhibitors may differ in their resistance mechanisms. HDAC inhibitors regulate cellular gene expression globally and thus it is a reasonable assumption that resistance can also be independent of drug transporter expression. For example, in chronic myelogenous leukaemia, resistance to the tyrosine kinase inhibitor, imatinib, in addition to acting as an ABCG2 substrate, is further conferred by BCR-ABL mutations thus producing resistant tumour clones; however, the mechanism underlying genomic instability that generates these resistant clones is unclear (13). Interestingly, within the cancer stem cell paradigm, imatinib appears to be more toxic towards the differentiated progeny relative to the stem cells in chronic myelogenous leukaemia. Furthermore, the BCR-ABL target is required for chronic myelogenous leukaemia progenitor expansion whereas its expression in stem cells is negligent. Thus, imatinib may in fact selectively target the differentiated cells while sparing the stem cells, which may explain the objective clinical response to therapy but no significant
difference in survival outcome (14). It is possible that mutations in the HDAC enzymes may be selected upon continuous exposure to HDAC inhibitors thereby rendering this particular class of drugs ineffective. The search for additional mechanisms of drug resistance to HDAC inhibitors will undoubtedly be connected to basic research aimed at providing a more comprehensive explanation of the mechanism of action behind HDAC inhibitors. The present accepted mode of action of HDAC inhibitors to globally increase histone acetylation cannot explain the tissue- or tumour-specific gene expression changes associated with HDAC inhibition. Interestingly, a number of studies have revealed that less than 10% of active genes in a malignancy are affected by HDAC inhibitors with concomitant transforming oncogene suppression and upregulation of epigenetically-silenced tumour suppressors (15); a growing list of nonhistone proteins are also discovered to be acetylated subsequent to HDAC inhibition (16). It is now being recognized that preclinical strategies of combining HDAC inhibitors with other therapeutic agents (e.g. chemotherapy, radiotherapy, demethylating agents, small-molecule kinase inhibitors) may further subvert the development of drug resistance. As alluded to earlier, transcription factors play a significant role in malignant transformation and thus may serve as potential targets for therapeutic intervention.

The role that FOXO transcription factors play in tumorigenesis was a direct consequence of their discovery in chromosomal translocations associated with cancers such as leukaemia and alveolar rhabdomyosarcoma. In several types of
cancer, FOXO factors can be inactivated by a plethora of post-translational modifications that serve to tip the balance towards increased cellular proliferation and survival. A few recent studies have begun to highlight the importance of FOXOs in maintaining the integrity of the embryonic and adult stem cell compartments (17, 18). This is consistent with regulation of dauer formation and life span extension by the FOXO ortholog, DAF-16 (19). For example, FOXO factors may regulate the quiescent stem cell phenotype through protection from physiological oxidative stress and inhibiting cell cycle progression through upregulation of “detoxification” genes such as MnSOD and Gadd45 (18). Given the known widespread tissue distribution of FOXO expression, it is somewhat surprising that FOXO-deficient (conditional FOXO1, FOXO3 and FOXO4 knockout) mice display tumours at selected tissue sites that further underscore the context-dependency of FOXO action (20). Given that the mammalian Sir2 ortholog, SIRT1, represses the pro-apoptotic activity of FOXO factors under conditions of oxidative stress, it may be of interest to investigate the role of SIRT1 in the cancer stem cell compartment with regards to regulation of FOXO factors in stress resistance and self-renewal (21, 22). Interestingly, mammalian sir2α null embryonic stem (ES) cells, unlike yeast sir2 mutants, do not require the SIR2α protein in maintaining gene silencing and were able to differentiate normally in culture (23). Also, no differences were observed in HSC or progenitor frequencies in young Sirt1+/− mice, although Sirt1-deficient HSCs displayed higher in vitro proliferative capacity and reduced dependency on growth factors (24).
Although the results presented in this thesis are novel within the medulloblastoma context, these findings could be extended to a greater number of medulloblastoma cell lines and documenting FOXO1 expression and activation in primary human medulloblastoma specimens. Given that the objective to document response of medulloblastoma cells to adenovirally administered FOXO1 was demonstrated, the next logical step is to better understand the physiological role that FOXO1 plays in medulloblastoma cell viability. FOXO1 expression levels should be quantified before and after serum starvation and/or IGF-I treatment. Furthermore, nucleo-cytoplasmic shuttling should also be demonstrated upon IGF-I treatment of serum-starved cells. The majority of studies, including this one, have focused on nucleo-cytoplasmic FOXO1 shuttling consequent to growth factor signalling and there is a relative paucity of studies investigating FOXO1 transcription regulation and expression. A previous study demonstrated that generally most CNS-PNET (Primitive Neuroectodermal Tumour) cell lines (including the DAOY cell line) displayed minimal FOXO1 expression (25). Thus, it is plausible to hypothesize that serum starvation may not only alter FOXO1 nucleo-cytoplasmic shuttling dynamics but may also affect FOXO1 expression. Given the approximately 48 hour lag between the start of serum starvation and observable cell death, the results from this study indirectly suggest upregulation of FOXO1 expression. Future experiments should directly assess and quantify FOXO1 message and protein expression in relation to serum starvation and FOXO1 infection of medulloblastoma cells. An extension of this study would be to investigate whether the mechanism of cell death differs between serum starvation and
overexpressing a constitutively active FOXO mutant with regards to gene and protein expression patterns and levels of FOXO1 expression. RNAi-mediated FOXO1 knockdown in serum-starved medulloblastoma cells may help to better elucidate the contribution of endogenous FOXO1 in serum starvation-mediated cell death. Given the potential of other FOXO factor members such as FOXO3a and FOXO4 to contribute likewise to the initiation of cell death programs, it may also be necessary to document their expression and contribution to medulloblastoma cell viability. Inhibiting endogenous FOXO1 export through the use of psammaplysene A in medulloblastoma cells growing in complete serum may also afford a complementary perspective in assessing the contribution of FOXO1 in medulloblastoma cell viability (26). Thus, means of inhibiting FOXO1 nuclear export might provide important therapeutic applications, allowing the activity of a single downstream transcription factor, such as FOXO1, to overcome defects in tyrosine kinase receptor signalling through the PI3K-Akt pathway. Penultimately, it would be of interest to investigate the physiological role of FOXO factors in the stem-like compartment of in vivo medulloblastomas and other brain tumours with respect to quiescence, cell cycling and target gene expression.

It has become increasingly apparent that both the molecular and phenotypic characteristics exhibited in serial passaged cancer cell lines vary substantially from the parental tumour (27-29). Given the importance of Shh signalling in MB oncogenesis, small-molecule inhibitors of Smoothened (Smo) have been designed and tested in pre-clinical MB animal models to suppress the growth of these Shh-
dependent tumours; it must be noted that mouse transgenic models and primary
tumours were utilized in these studies (30-32). Yet, in many cultured MB cell
lines, the Shh pathway is downregulated rendering them ineffective to potentially
promising therapeutics (31). In this light, the clinco-biological relevance of in
vitro and in vivo cancer line-based preclinical models may need to be re-
evaluated. However, the access to freshly resected primary tumours is a major
obstacle to both fundamental research into the molecular mechanisms governing
tumourigenesis and the preclinical development of cancer therapeutics. The
cancer stem cell hypothesis predicts that the minor stem-like cell population is
responsible for tumour initiation and propagation. Most of the cultured cell lines
are propagated in differentiation medium (complete serum) that may in fact
differentiate the tumour stem cells. Indeed, an elegant study has demonstrated
that cancer cells isolated from primary GBM tumours, when grown under
standard serum-containing conditions, are depleted of the original stem-like cells
and are constituted by cells that significantly differ from the parental tumour in
terms of their genetic and phenotypic profile (27). However, cancer cells
propagated in NSC media (Neurobasal media supplemented with bFGF and EGF)
closely phenocopy the original tumour with no evidence of genomic alterations
and gene expression changes with serial passaging (>70 passages) surpassing the
one-year mark. Therefore, establishment of cancer stem cell-lines may provide a
biologically and clinically relevant in vitro and in vivo model system for
understanding the molecular basis of tumourigenesis and furthering the preclinical
development of therapeutic strategies.
In conclusion, the cancer stem cell hypothesis offers an intriguing perspective on the hierarchical organization of cancers that may lead to a better understanding of cancer initiation, maintenance and penultimately, strategies to decrease the tumour burden. This thesis has broadly examined the implications of the cancer stem cell hypothesis with specific attention paid to the experimental tools utilized to define the cancer stem cell population in vitro, the role that FOXO factors may play in the transcriptional network governing medulloblastoma cell survival and evaluation of the impact of HDAC inhibitors on cancer stem cell marker expression and clonogenicity. Further work will evaluate the role of FOXO factors and the influence of HDAC inhibition in modulating FOXO factor activity in brain tumour stem cell maintenance and expansion.
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