REVIEW OF LITERATURE
Central Nervous System [CNS]

The Human Brain

The human brain is a complex organ. Much of our understanding of human brain function has come through the use of in vivo and in vitro non-human models. These models have been used to unravel intricate signaling processes, as well as being used to screen therapeutic agents. However, a multitude of candidate drugs that have shown promise in non-human models have not been successful when taken through to human clinical trials – a failure of translation (1). This failure to translate laboratory-based results to the clinic may be due to many factors including species differences, brain complexity, age, patient variability and disease-specific phenotypes that cannot easily be modelled in a chosen non-human system. Therefore it is unlikely that animal studies alone will unravel the complexities of the human brain and alternative approaches are required. Traditionally, gliomas were thought to derive from the transformation and dedifferentiation of glial cells residing within the brain parenchyma (2, 3) and classified according to the cell type that they resembled (2). Currently, cancer is increasingly being viewed as an aberrant organ (4), containing a stem-like cell population that drives tumor growth (5); divides asymmetrically, giving rise to differentiated progeny with varied morphological features, cell-specific antigens, and functional properties that are characteristic of the tumor (6); and may be responsible for tumor recurrence (7, 8). This latter view is supported by analogies between normal stem cells and cancer stem cells (9). Both cell types self-renew, proliferate, and differentiate, with normal cells behaving in a highly regulated manner and tumor stem cells in an aberrant fashion.

In vitro models have been used extensively to study normal brain cell behavior and disease pathogenesis. These systems can comprise 2-dimensional individual or mixed cultures of primary cells isolated from mammalian brains at different developmental stages, or immortalized cell lines. Alternatively 3-dimensional (3D) cultures can be established using whole organs, slice cultures or cultures of a single or multiple cell types grown to high density in a 3D matrix to re-create a component of the organ or tissue under study (Freshney, 2001). Studies using primary cultures and cell lines of neurons, microglia, astrocytes and oligodendrocytes are numerous and have been used to investigate many processes such as neurotoxicity, inflammation and neuroprotection as well as being used to screen for novel drugs to treat neurodegenerative disorders (10-14). There are many advantages to using in vitro cell culture models although as with in vivo models, their limitations must be recognized. To overcome these limitations much more work should be undertaken using primary adult
human brain cells to study neurodegenerative disorders.

Figure 1: The Human Central Nervous System and Neurodegenerative Disorders

Culturing of adult human brain cells

In vitro culture systems of adult human brain cells have been in use for many decades. The majority of the early work in the 1940s and 1950s used cells derived from explant cultures prepared using either a hanging drop method or roller tube method to bathe the tissue in culture media (15-17). In the 60s and 70s, scientists began to complement the
research performed on cells originating from the tissue explants with monolayers of cells derived from mechanically or enzymatically dissociated tissue (18, 19). Many of these early studies successfully isolated, cultured and characterised cells from both biopsy and short post-mortem tissue, but were unsuccessful in culturing cells from tissues with a long post-mortem delay (18). Following on from the advent of immunocytochemical techniques, the different cellular components of the explants and dissociated cultures could readily be identified, although not without controversy (20). Immunocytochemical markers used to complement morphological characterization included glial fibrillary acid protein (GFAP) and glutamine synthetase to detect astrocytes, galactocerebroside (GC) for oligodendrocytes and fibronectin to detect the presence of fibroblasts in the culture (21) (22). In addition to isolating, maintaining and expanding adult human brain cells from fresh tissue, researchers have been able to build up frozen cell stocks (19), isolate cells from specially cryopreserved human tissue (23), and generate immortalized cell lines for further studies (24). Research groups throughout the world use adult human cell culture systems to study aspects of the normal and diseased human brain and in doing so build on these culture techniques developed over the past 60 years.

![Figure 2: Schematic representation of types of Brain cells.](Courtesy: stanmed.stanford.edu)

**Astrocytes**

Astrocytes play varied roles in the healthy and diseased human brain. Evidence has emerged to suggest that astrocytes have multiple housekeeping functions which include modulation of synaptic function, intracellular communication via gliotransmission and gap junctions,
regulation of cerebral blood flow and maintenance of the extracellular environment. Astrocytes can undergo morphological and functional changes (reactive gliosis) in response to disease-specific stimuli and have been implicated in AD (Alzheimer Disease), PD (Parkinson's disease), HD (Huntington disease) and amyotrophic lateral sclerosis (ALS) (25-27, 27, 28).

Since the 1940s researchers have determined the presence of astrocytes in cell cultures derived from the adult human brain. Many studies have successfully used mixed cultures to characterize or investigate the functions of astrocytes without the use of any enrichment or purification steps (29). Where the isolation of astrocytes was preferred researchers utilized adherence methods to allow them to selectively remove other cell populations (30-32). Although the yield of astrocytes isolated from human brains differed for individual donors (29, 33), astrocytes of varying morphologies could be detected. Although its know that the human brain comprises at least four populations of astrocytes (34), morphologically astrocytes are generally described as either having a protoplasmic or fibrous (also described as stellate) morphology (16), the latter being further characterized in vitro by the number and complexity of their processes (35).

Astrocytes can be characterized by examining their morphological, immunocytochemical and functional properties, although when reviewing the literature relating to their characterization there are some discrepancies in the identity of the cells, the morphology of the putative astrocytes and their ability to proliferate in culture. Immunochemically, adult human astrocytes in culture can be labeled with antibodies against GFAP, a well characterized marker used extensively for this cell type (36), alone or in combination with other astrocytic markers such as glutamine synthetase or vimentin (22, 30, 37, 38). Some researchers demonstrated that they could obtain highly enriched astrocyte cultures that stably expressed GFAP through successive sub-culturing (30, 31).

Astrocytes can also be neuroprotective and this has been demonstrated by a study showing that activation of Toll-like receptor-3 in adult human astrocytes induced the expression of a range of neuroprotective mediators that could enhance neuronal survival (39), thus highlighting the diverse roles for astrocytes in the brain. Collectively these studies using adult human astrocyte cultures highlight the diverse roles of astrocytes in the CNS and the complexity of their responses that can confer both protective and toxic phenotypes as well as showing how they can be used for investigating cellular processes and for targeted therapies.

**Oligodendrocytes**

Oligodendrocytes are the myelin forming cells of the CNS, insulating axons and enabling fast and accurate conduction of neuronal electrical action potentials.
Oligodendrocyte death or dysfunction has been associated with inflammatory demyelinating diseases of the CNS such as multiple sclerosis (MS) (40-42). Oligodendrocytes can be cultured and can account for approximately 30% of the total cells in mixed glial cultures (22). Cultures derived from white matter only often contain oligodendrocytes and astrocytes and very few microglia. These cultures can be further enriched for oligodendrocytes by utilising their lack of adherence to cell culture plastic ware (43, 44). Morphologically, freshly isolated oligodendrocytes are small round cells that begin to extend processes after a few days in culture, and with increased time in culture, form an interlacing multipolar morphology (43). The classical immunophenotypic marker for mature oligodendrocytes is the expression of GC (also known as O1) and is used alone or alongside other oligodendrocytes markers such as 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) and myelin basic protein in the characterisation of cultured adult human oligodendrocytes (45) (43) (31, 44). In addition to mature oligodendrocytes in culture, researchers detected the presence of pre-oligodendrocytes reminiscent of rodent early progenitor cells called oligodendrocyte-type 2 astrocyte progenitors that had the capacity to preferentially mature into oligodendrocytes with time in culture (45), as well as transitional cells that co-expressed the oligodendrocytic marker GC along with astrocytic GFAP (43). These studies suggest the potential for a mixed population of oligodendrocytes in cultures derived from the adult human brain. The few studies that use dissociated adult human oligodendrocytes cultures as their model system tend to focus on inflammatory processes in MS, with a specific emphasis on investigating the mechanisms of oligodendrocyte cell death. Inflammatory cytokines have been implicated in MS pathogenesis (46). An in vitro study showed that adult human oligodendrocytes treated with tumour necrosis factor (TNF)-α and activated T-cell factors resulted in nuclear damage thus highlighting their vulnerability to inflammatory mediators (47).

Neurons

Neurons, or nerve cells, are highly specialized cells in the CNS that process and transmit information. The loss of neurons and their connectivity is the central cause of brain disorders such as AD, PD, HD and ALS (48-51). The disorder depends on the location and function of the susceptible neurons where, for example, the loss of DA containing striatal projection neurons in the SNpc (Substantia Nigra Pars Compacta) causes PD, while the loss of upper and lower motor neurons is responsible for ALS. Understanding the mechanisms of neuronal toxicity and researching ways to protect against neuronal cell death is a central theme in neuroscience research. Protocols for isolating neurons from the adult human brain have been published (52-56), although there are few when compared to studies using glial cell cultures. This may be indicative of the complexity of their isolation and maintenance in culture, their post-mitotic phenotype and their susceptibility to changes in the environment.
Early neuronal isolation methods from fresh or frozen biopsy and/or post-mortem tissue utilized mechanical dissociation and filtration through descending pore sizes followed by density centrifugation to obtain cellular fractions containing neurons (54) (56). Although the purity and yield of neurons seemed to differ between cases, these early studies demonstrated the ability to isolate and culture adult human neurons. More recently researchers have demonstrated the ability to culture and maintain adult human neurons from surgical tissue using protocols previously developed for adult rat neurons (52). Using media optimized for adult rodent neurons that was supplemented with human recombinant basic fibroblast growth factor and the steroid hormone dehydroepiandrosterone 3-sulfate the researchers found that in about 40% of cases an average of 20% of the viable cells were immunopositive for the neuronal markers neurofilament, microtubule associated protein (MAP)-2 and Tau and could be maintained in culture for many weeks (52). The percentage of neurons that retained their phenotype and function in culture could be increased to approximately 80% by enrichment using an immunomagnetic sorting DNA-linker technique utilizing the binding of tetanus toxin C (TTC) to neuronal specific cell surface gangliosides and the subsequent immunological labelling with an anti-TTC antibody and separation using magnetic Dynabeads (55). This small number of studies suggests that it is possible to obtain dissociated cultures of adult human neurons that can be used as a model system to study cellular and molecular processes in neurons derived from the normal and diseased human brain.

**Adult neural stem cells**

Neurogenesis is currently one of the most studied topics in Neurosciences. In particular, since the initial observation that the adult human brain contains regions of neurogenesis (confirming results in other species) (57, 58) there has been a surge in research in this area. Furthermore, studies showing that neurogenesis is altered in human neurodegenerative disorders such as HD (57) indicate the clinical importance of this work. There have been a number of papers published over the past few years that have provided good evidence that the culture of adult human neural precursor cells from neurogenic regions of the adult human brain is feasible (59-61). Although these in vitro studies of adult human brain neurogenesis are at an early stage they have shown the great potential that this area holds for studying basic mechanisms of neurogenesis and gliogenesis in the adult brain and for studying disease-specific abnormalities in these processes. Future studies may reveal disease-specific deficits in neurogenesis and/or gliogenesis that may play causative roles in brain disorders.
Tumors of the CNS

Primary tumors of the CNS

Primary tumors of the CNS are common, and form the second most common group of tumors occurring during childhood (after lymphoproliferative tumors). The annual incidence of primary tumors of the CNS is approximately 12.5 per 1,00,000 in the U.S. Primary tumors of the CNS rarely metastasize. A benign tumor located in a critical area may be associated with a very poor prognosis (malignant by position). General features of malignancy include rapid growth, and invasion /dissemination.

Common types of CNS tumors occurring during adult include glioblastoma, metastases, malignant astrocytoma, meningioma, pituitary adenoma and schwannoma. In children, the most common CNS tumors include medulloblastoma, astrocytoma,
ependymoma, craniopharyngioma and glioblastoma. Gliomas comprise the largest group of primary intracranial tumors and consist of astrocytoma, oligodendroglioma, ependymoma and mixed glioma. Astrocytomas may be broadly sub-classified into two categories: diffuse and circumscribed. An example of a “circumscribed” astrocytoma is the pilocytic astrocytoma which occurs at a young age, is low grade, and is usually located in the cerebellum, inferior third ventricle, or optic nerve. The prognosis is usually very good. “Diffuse” astrocytomas are infiltrative, usually occur in an older age group, and are of all histologic grades. The prognosis is generally unfavorable. Astrocytomas may be of low grade, anaplastic or high grade, such as glioblastoma multiforme. Factors important for grading astrocytomas include: increased nuclear pleomorphism, mitotic activity, vascular neogenesis, and the presence or absence of necrosis. Meningiomas are more common in women. 90% are intracranial, 9% are spinal and 1% occur in unusual locations such as the nasal pharynx or peri-orbital region. Meningiomas may be classified as being benign, atypical or malignant. Most meningiomas are benign.

PNET: The term Primitive Neuroectodermal Tumor is applied to tumors showing similar histologic characteristics (“small blue cell tumor”), occurring in different locations. For instance, in the cerebellum: medulloblastoma, with nesopharynx or periobital regions. Neuroblastomas are described within cerebral hemispheres, pineoblastoma in pineal gland, and retinoblastoma within retina. PNET’s are located in the following parts of the CNS in descending order of frequency: cerebellum, cerebral hemispheres, pineal gland, spinal cord, and brain stem. Schwannomas may be peripheral or intracranial. The most common intracranial locations are 8th followed by 5th cranial nerves.

Haemangiopericytomas were previously confused with meningiomas, but they are distinct from meningiomas and make up 1 – 5% of tumors occurring within the meninges. They are aggressive highly recurrent tumors, which frequently metastasize. They may occur at any age, with a peak in the 4 – 5th decades. They are more common in men than women. Primary CNS Lymphoma was previously rare, but has become much more common within the last 20 years, to the point that they make up approximately 15% of primary CNS tumors. Primary CNS lymphomas may be divided into non-Hodgken’s Lymphoma, of which the vast majority are B cell type, and Hodgken’s disease.

Germ Cell Tumors of the CNS: These include: germinoma, embryonal carcinoma, endodermal sinus tumor, choriocarcinoma, and teratoma. Other important but less common tumors of the CNS include: Choroid Plexus Papilloma, Colloid cysts, Craniopharyngioma, Epidermoid and Dermoid, and Chordoma.
**Metastatic tumors to the CNS**

Metastatic tumors to the CNS are common. The origin of metastases to the CNS includes the following in descending order: respiratory system, breast, gastrointestinal, and genitourinary systems. 80 – 90% of metastases occur to the brain, 10 – 20% to the spine.

![Diagram: Genetic pathways to primary (de novo) and secondary glioblastomas at the population level. LOH 10q is frequent in both primary and secondary glioblastomas. TP53 mutations are early and frequent genetic alterations in the pathway leading to secondary glioblastomas. *Genetic alterations that are significantly different in frequency between primary and secondary glioblastomas.*](Image)

**Glioma**

Glioma, the most common type of primary brain tumor, is classified by the World Health Organization into 4 distinct grades based on histological features of cellularity, nuclear morphology, mitotic activity, necrosis, and vascular proliferation (62). A higher histologic grade corresponds to a less differentiated phenotype and to increasing malignancy. The most common form, a grade IV glioma, called glioblastoma multiforme (GBM) has a median survival of 14-15 months in spite of aggressive multimodality treatment by surgery, external beam radiation therapy, and chemotherapy (63). As asserted nearly seventy years ago by
GBM can either develop by dedifferentiation from a lower grade tumor ("secondary GBM") or can arise "de novo" ("primary GBM"). The differences in clinical and molecular features of the two types of GBM hint at a distinct pathogenesis.

Although gliomas are a relatively rare form of cancer, they account for disproportionately high morbidity and mortality because their location in the brain prevents adequate surgery and other therapies are largely ineffective. Gliomas rarely metastasize outside of the brain, but instead, infiltrate extensively into surrounding normal brain. Thus, surgery is not curative but can establish the diagnosis and relieve symptoms by decompressing the brain located in a poorly compliant intracranial cavity. Radiation therapy and chemotherapy increase survival; however, disease recurrence is virtually inevitable. Both the invasive nature of the tumor and its heterogeneity probably contribute to the poor response to currently available treatment regimens. Heterogeneity is traditionally believed to result from regional variations in the tumor microenvironment and the diversity of cancer cell subpopulations that results from progressive stochastic genetic alterations. The recent reports describing the presence of cancer-initiating stem-like cells or cancer stem cells (CSC) may help to explain cellular heterogeneity (stem cells have an indefinite lifetime and reproduce over long periods of time making them likely to accumulate mutations that could lead to genetic instability) and explain resistance to therapy (4). The observations that cancer cells and stem cells share the common defining features of incompletely differentiated state and unlimited self-renewal capacity, have led to the cancer stem cell hypothesis.

**Introduction to the cancer stem cell hypothesis**

Recent evidence suggests that a subset of cancer cells might underlie the growth of different types of cancer and be responsible for their resistance to therapy (65). The terms cancer stem cells (CSCs), cancer stem-like cells, or tumor initiating cells are variably used to describe tumor cells with stem-like properties (66) (Figure 6). The new concept that a subset of cells within tumors might possess significant expansion capacity and the power to generate new tumors has been dubbed the cancer stem cell hypothesis (4). This postulate also implies that the bulk of cancer cells within a solid tumor are progeny of CSCs, which cannot form new tumors and might represent a mix of partially differentiate cancer progenitor-like cells with limited proliferative capacity and terminally differentiated cancer cells (Figure 5). CSCs have been isolated from different types of tumors, including primary brain tumors such as GBM, MB and ependymoma (67-70) (Figure 1). These cells constitute a variable fraction of the total cell population within brain tumors, yet may be the drivers of their growth. They share characteristics similar to those of normal NSCs, including self-renewal and the proliferative ability for the generation of many progeny. Multipotency (the capability to differentiate into multiple cell types) is not a requirement for CSCs because some tumor types
might have a single differentiated state (Queen model in Figure 5). The gold standard assay for the functional evaluation of both self-renewal and tumor propagation of CSCs is the ability to propagate serially in an undifferentiated state and form tumors in animals upon transplantation (71). CSCs are isolated from dissociated tumors, propagated as neurospheres in specific neurobasal medium, and a subset express NSC surface markers such as Nestin and CD133 (7).

Definition and source of neural stem cells and progenitor cells in the brain

Current hypotheses postulate that brain cancer propagating cells (BCPCs) either originate from transformed NSC or neural progenitor cell populations in the brain or that they dedifferentiate from mature brain cells and reacquire phenotypic and functional similarities of NSCs (72) (Figure 2). Individual adult NSCs were initially isolated from the adult striatum and shown to possess the ability to proliferate and generate clones of cells that showed multipotency, and grew as spheroids in defined medium supplemented with growth factors epidermal growth factor (EGF) and fibroblast growth factor (FGF)-2, termed neurospheres (73). In this neurobasal medium, NSCs can be propagated and expanded indefinitely, whereas most differentiating or differentiated cells rapidly die (74, 75). Upon growth factor removal or addition of serum, NSCs differentiate into neurons, astrocytes and oligodendrocytes. NSCs are found in different areas of the brain, including around the ventricular system (subventricular zone (SVZ), subependymal zone, lining of the lateral ventricles and cerebellar ventricular zone), dentate gyrus, hippocampus and subcortical white matter (76) (77). During human development, the one important source of NSCs is the SVZ, a region localized between the lateral ventricle and parenchyma of the striatum. This region is widely viewed as the source of cells that can initiate GBM and ependymomas (78, 79). The SVZ contains astrocyte-like stem cells (also known as type B cells in mice) identified through their expression of the astroglial marker glial fibrillary acidic protein (GFAP). In contrast to mature astrocytes of the brain parenchyma, which also express GFAP, these cells can function as mature NSCs (77). The murine postnatal cerebellum has been shown to contain multipotent NSCs that lack markers of neuronal and glial lineages and can differentiate into glial cells (astrocytes and oligodendrocytes) and neurons in vitro and in vivo (80). NSCs generate transit amplifying cells (TACs; also known as type C cells in mice) from which PGCs are derived.

Prior evidence suggested that two types of Primordial germ cells (PGCs) are formed: those producing progeny along either the neuronal or glial lineages (Figure 6). New evidence suggests more plasticity, for example oligodendrocyte precursor cells, which are dispersed throughout the CNS, and initially thought to be lineage-restricted precursors that terminally
differentiate to postmitotic oligodendrocytes seem to have the potential to form neuronal cells and might qualify as NSCs (81, 82). Although PGCs do replicate, their self-renewal capacity is finite in contrast to de facto stem cells. In principle, any of these cell types might be subject to neoplastic transformation and engender brain tumors, although the rapidly expanding TACs are the most likely source (83). In rodents, embryonic radial glial cells are believed to engender adult NSCs that share the functional and molecular characteristics of astroglial cells present in the SVZ of the lateral ventricles (84). (85) (86).
Figure 5: Different models of CNS tumor formation.

Courtesy: Hadjipanayis et al 2009
Figure 6: Normal CNS differentiation and transformation

Cancer Stem Cells (CSC)

Cancers generally retain histological and gene expression programmatic resemblance to the tissue of origin. Usually, surgical pathologist can identify the organ for which a neoplasm arose. When anatomic review alone is insufficient, molecular studies such as genomic profiling and gene expression analysis are often successful in determining the tissue of origin. Close examination of cancer histopathology often demonstrates an attempt to recapitulate the organ-specific functional morphology such as formation of follicles in thyroid cancer. Such tissue specific growth patterns exhibited by cancers suggest a pathologic regenerative process. The observation that stem cells and some cancers cells share the common defining features of incompletely differentiated state and self-renewal capacity, in conjunction with technical developments for comparative studies, have led to the cancer stem cell hypothesis.
supported by numerous compelling studies. The recent attention to cancer stem cells as the source of various malignancies represents a rebirth of an old idea. The German physician considered by many to be the father of pathology, Rudolf Virchow, suggested as early as 1858 that cancers arise from embryonic-like tissue (3). Virchow's assertion, driven by observations of histologic similarities between developing normal tissues and poorly differentiated cancers, was further extended by subsequent investigators who proposed that cancer results from "embryonal rests" due to disrupted developmental programs in which tissues fail to appropriately differentiate to instructive specification (87). The histopathologic terms "poorly differentiated," and "de-differentiated" used to describe some cancers invoke early developmental processes. The link between cancer and primordial cells is also suggested by analysis of germ cell tumors where multipotent teratomas exhibit differentiation into tissues of all three germ layers. This finding strongly suggests that certain cancers likely of monoclonal origin retain pluripotency. Thus, the belief that cancer may be initiated and maintained by uncommitted self-renewing cells was not common, even though definitive proof was lacking.

Evidence for the above concept came from technical advances in in vitro cell propagation and from improved understanding of normal developmental processes. Refinements of in vitro cell culture methods, and identification and cloning of key growth factors permitted isolated studies of cancer cells. Fidler and Kripke observed striking heterogeneity of dissociated tumor cells with respect to the clonogenic ability to form metastasis (88, 89). Clonogenicity may in some cases, serves as a surrogate marker of self-renewal capacity. Recognition of clonogenic cells in cancers combined with the enhanced understanding of lineage development of normal cells allowed subsequent investigators to examine cancers with the cancer stem cell hypothesis in mind. Perhaps in part due to easy access to normal stem cells, the hematopoietic developmental patterns were first characterized (90). Relying on established markers of normal hematopoietic development, John Dick identified a leukemic cancer stem cell capable of recapitulating the disease in an immunodeficient mouse (91). This was a seminal paper in the development of the cancer stem cell field. Subsequently, the existence of "cancer stem cells" or "cancer initiating cells" have been identified in a variety of solid tumors including gliomas, medulloblastomas, breast cancer, lung cancer, prostate cancer, and colon cancer (69, 92-97).

CSC in gliomas

Aggressive brain tumors are well known to contain poorly differentiated cells, reflected in the use of the term “blastoma” in glioblastoma, pineoblastoma, neuroblastoma, and medulloblastoma. Percival Bailey and Harvey Cushing suggested an embryonic origin for medulloblastoma as early as 1926. They hypothesized that medulloblastomas may originate
within the embryonal rests in the roof and the ependymal lining of the fourth ventricle. Ronald McKay's group eventually provided experimental evidence (98). Expression of nestin, a marker of neural stem-progenitor cells was subsequently demonstrated in a variety of neuroepithelial brain tumors (99, 100). The renewed interest in applying developmental biology to cancers, perhaps fueled by John Dick's work on leukemia, led to the identification and in vitro propagation of malignant cells with stem cell-like properties of undifferentiated state, self-renewal capacity, and multipotency in clinical neuroepithelial tumor specimens (70, 97, 101, 102). Singh et al. demonstrated that the expression of a putative neural stem cell marker, CD133, in malignant tumor cells was both sufficient and necessary to initiate and recapitulate the tumor upon transplantation into immunodeficient mice (72, 103). Since these initial observations, numerous laboratories have joined the effort to further investigate and clarify the field of CSC in brain tumors.

However, the suggestion that only CD133 positive cells are capable of recapitulating the parental tumor in immunodeficient animals has been disputed (104-107). A clear separation of the CD133 positive and negative populations is technically difficult (108). Therefore, in working with impure populations of cells, a definitive conclusion concerning respective subpopulations is murky at best. In addition, a host of surrogate markers such as BMI-1, Nestin, Sox2, Musashi, SSEA-1 (CD15), and activated Notch pathway have also been suggested to identify the glioma CSC. Some of these findings have created confusion and disagreements rather than adding clarity, possibly because of a lack of uniform definition of exactly what constitutes a glioma cancer stem cell.

**Significance of glioma cancer stem cells**

The presence of glioma cells of variable differentiation status that correlates with self-renewal capacity has been demonstrated by numerous laboratories. Detailed studies with glioma cells enriched for the CSC subpopulation show increased resistance to irradiation, a major therapeutic modality for the treatment of malignant gliomas, because they activate the DNA damage response pathway, rapidly repairing the DNA damage induced by the radiation (7). These cells also seem to play a critical role in recruitment of blood vessels, a necessary task to promote tumor growth (7). Consonant with these observations, the presence of embryonic stem cell-like gene expression signatures in human cancers (GBM, breast, and bladder) is associated with aggressive histopathology, confirming clinical-prognostic significance for the stem-like phenotype of cancers (109).

CSC subpopulation must be targeted to achieve complete and durable response, and considerable research efforts have been and continue to be devoted to this cause (110-112). According to a hierarchical model, only an identifiable CSC subpopulation is endowed with
self-renewal capacity sufficient to repopulate the tumor. A competing stochastic idea suggests that self-renewal capacity is linked to the presence of key genomic alterations that may occur in a variety of cells. Therefore, non-CSC may form or develop into CSC. The argument is not simply a conceptual exercise because adoption of each model dictates obviously distinct therapeutic strategies. The available data, including clinical evidence for progression of well differentiated low grade gliomas to poorly differentiated gliomas suggest that both hierarchical and stochastic mechanisms may be involved (113). The CSC hypothesis represents an opportunity to emphasize developmental process in the study of tumorigenesis. Such effort involves examination of the stem cell niche and early developmental signaling pathways. Similar to normal neural stem cells, glioma-derived cancer stem cells seem to reside within a perivascular niche (114-116). This may suggest that therapeutic targeting of the tumor-associated vasculature may at least indirectly interfere with glioma CSC growth. The recent demonstration of clinical effectiveness of antiangiogenic strategy with bevacizumab, a monoclonal antibody directed against vascular endothelial growth factor (VEGF), may in part be mediated by effect on the CSC (117). The significant therapeutic response in those patients receiving bevacizumab led to approval by the Food and Drug Administration of the United States of America for use of this agent in recurrent glioblastoma. There are ongoing studies of other anti-angiogenic agents in malignant gliomas that may provide pharmacodynamic insight into dependence of CSC on the perivascular niche for survival, growth, and proliferation.

These studies may also provide an opportunity for comparative analysis and identity of cancer cells that adopt an enhanced migratory-invasive phenotype after exposure to antiangiogenic agents. In addition to addressing the niche, the CSC may be directly targeted. Signaling cascades that are emphasized by normal stem cells, such as notch, hedgehog, Wnt, and the PI3K-Akt axis, have been a focus of increasing interest in cancer therapy because manipulation of these pathways may preferentially deplete the CSC (118-123). Because cells at different phases of development may rely on adoption of serial signaling pathways, it is possible that activation of particular pathways may aid in the identification and classification of CSC subtypes. For instance, glioma cancer cells that express similar markers indicative of the undifferentiated state may be further divided on the basis of activated signal transduction pathways. Cancer cells bearing identical surface markers may turn-on different pathways for self-renewal versus quiescence. In combination with surface markers, identification of activated signal transduction pathways may be used to provide highly discriminating targets and biomarkers of therapeutic response.
Signaling pathways leading to the genesis of glioblastoma

There has been a longstanding debate as to whether the cell of origin for the formation of GBM results from the transformation of NSCs or through the dedifferentiation of a mature glial cell in the brain (124, 125). Genetic studies have demonstrated that a number of signaling pathways are commonly altered in human GBM, namely the p53/mdm2/ARF, pRb/p16/INK4a, PTEN/PI3kinase and HIF/IDH1/2 pathways. Concurrently, oncogenic signals are activated such as those deriving from EGF, c-Met and platelet-derived growth factor (PDGF) tyrosine kinase receptors. The alteration of the same pathways in various NSC and PGC populations in the brain using genetically engineered mice with multiple gene combinations EGF receptor/ARF loss, PDGF overexpression, Akt/Ras activation, NF1/p53 losses and PTEN/p53 losses has demonstrated that they can lead to transformation in rodents (126-128). These models confirm that NSCs and PGCs initiate the tumorigenic process. Accordingly, the gene expression profiles of GBM resemble those of NSCs and PGCs of the developing forebrain (129). OLIG2, a transcription factor that can promote the
proliferation of neural progenitors by repressing the p21 tumor suppressor, has similar effects on GBM stem cells (127). MELK, a cell cycle modulator, regulates NSC self-renewal and has been shown to regulate BCPC proliferation (69, 130). The commonality of signaling pathways activated in NSCs and GBM stem cells (131) has important implications for potential differentiation therapy. For example, blockage of the Sonic hedgehog (SHH) signaling pathway depletes BCPC cell populations in GBM (119). The evidence for the formation of gliomas from mature differentiated glial cells is not as extensive, perhaps because it is a rare event. Mature, differentiated astrocytes or oligodendrocytes might be less prone to transformation. They could be intrinsically more resistant to carcinogenesis and thereby necessitate more stochastic transformation steps. The activation of two oncogenic pathways, such as ras and Akt in conjunction with p53 and Rb inactivation and expression of hTERT, can lead to the transformation of human astrocytes in vitro. The inactivation of the pRb and p53 pathways through the combined genetic loss of p16INK4a and p19ARF in mouse astrocytes leads to dedifferentiation in response to EGF receptor activation; and these cells can induce high-grade gliomas (132). The dedifferentiation of astrocytes into glial progenitors or stem cells can produce gliomas when infected by PDGF-encoding or EGF-encoding retroviruses (133).

**Ras and the MAP kinases**

Growth factors and hormones exert their effects through the receptor-mediated activation of signal transduction pathways such as Grb2, SOS and Ras, which then signal to Raf, MEK and the MAPKs Erk 1 and Erk2. The Ras p21 proto-oncogenes, including K-ras, H-ras and N-ras, encode a family of proteins that are central to the signal transduction pathways that mediate cell growth (134). The Ras proteins are anchored to the cytoplasmic side of the cell membrane through a covalently attached lipid group. Ras proteins function as GTPases and function by transducing signals from the cell surface by the modulation of two activation states. Mutations in Ras proteins have been shown to constitutively activate growth signals, resulting in tumor development in many organ systems (135). Tumors without Ras mutations can have elevated levels of Ras activity through growth-factor-receptor gene mutation or amplification (136). The oncogenic potential of Ras activation clearly relates to the activity of transcription factors such as Jun and Fos. However, Ras pathway activation can also increase gene expression by increasing the translational efficiency of existing RNAs. In this regard, translation initiation, elongation and ribosome biogenesis all increase to improve the translational capacity of the cell (137). The predominant mechanism by which Ras achieves changes in global protein synthesis is through Erk1/2 activation of Mnk 1 and 2, which in turn mediate elf4E phosphorylation (138). Cap-dependent translation rates of existing mRNAs subsequently increase.
Cooperation between Ras and Akt: glioblastoma as a model

There is ample evidence that the regulation of oncogene expression can occur at the level of translation. Relating these observations to aberrant signaling pathways requires the identification of differential effects on the steady-state pool of RNA transcripts. The global analysis of mRNAs regulated at the translational level in glia as a function of combined Ras and Akt signaling underscores the specificity and magnitude of differential global translational regulation (139).

Much of the understanding regarding the pathophysiology of many tumor types has come from genetic studies identifying specific defects in tumor cells. This is specifically the case for glioblastoma multiforme (GBM), the most aggressive of the primary brain tumors. Two examples of important mutations in GBM include mutant or amplified versions of the epidermal growth factor receptor and the deletion or mutation of PTEN (140). Each of these genetic defects has significant consequences, affecting other proteins in the cell and ultimately leading to tumor formation. EGFR signaling results in Ras activation whereas PTEN negatively regulates Akt. The activation of Ras and Akt together has recapitulated glioma formation in human cell cultures and in transgenic mice (126).

An important goal in glioma biology has been to identify the mechanism by which Ras and Akt cooperate so effectively. The impact of Ras and Akt signaling pathways in gliomas has recently been implicated in the regulation of B7-H1, a potentially immunosuppressive protein (141-143). B7-H1 transcript was detected at low levels in both normal and genetically altered human astrocytes. However, B7-H1 protein was significantly detected on the cell surface only after the introduction of a constitutively active Akt construct.

When Ras and Akt pathways are activated simultaneously they can increase protein expression by optimizing cap-dependent translation initiation through a variety of overlapping pathways. The eukaryotic mRNA cap-binding translation initiation factor (eIF4E) is induced and phosphorylated by ERK after Ras activation. The mammalian target of rapamycin (mTOR) can diminish 4E-BP-mediated inhibition of eIF4E after Akt pathway activation. Akt also works through mTOR to activate S6 ribosomal protein (S6RP). Translational regulation by Ras and Akt might have profound implications for oncogenesis in many solid tumors and, in particular, GBM. Akt activation is sufficient to convert anaplastic astrocytoma to GBM, and this conversion has been associated with gene products that have proliferative, invasive and angiogenic properties (126).
Figure 8: Ras and Akt cooperation in translational regulation: glioblastoma as a model.
Figure 9: p53 Signaling pathway.

**P53**

The TP53 gene encodes a protein that plays a role in several cellular processes, including the cell cycle, response of cells to DNA damage, cell death, and cell differentiation (144). Following DNA damage, TP53 is activated and induces transcription of genes such as p21Waf1/Cip1 (145, 146). MDM2 is induced by wild-type TP53, (147) which binds to mutant and wild-type TP53, thereby inhibiting the ability of wild-type TP53 to activate transcription (148, 149). Approximately 20% of oligodendrogliomas showed p14ARF promoter methylation (150), and p14ARF homozygous deletion or promoter methylation were observed in 40% of anaplastic
oligodendrogliomas (150). The overall frequencies of alterations in the TP53/MDM2/p14ARF pathway were 21% in oligodendrogliomas and 50% in anaplastic oligodendrogliomas (150, 151). The TP53 pathway plays a crucial role in the development of secondary glioblastomas. TP53 mutations are the first detectable genetic alteration in two-thirds of precursor low-grade diffuse astrocytomas; this frequency is similar to that in anaplastic astrocytomas and secondary glioblastomas derived thereof. TP53 mutations also occur in primary glioblastomas, but at a lower frequency (<30% of cases). The less specific pattern of TP53 mutations in primary glioblastomas may constitute, at least in part, secondary events due to increasing genomic instability during tumor development. Germline TP53 mutations are associated with glioma and in particular the high-grade glioblastoma multiforme (GBM) (151). These brain tumors are associated particularly with TP53 mutations in the DNA binding loop that contacts the minor groove \((P=0.01)\) and with null mutations. The latter are associated with earlier onset brain tumors \((P=0.004)\) (152). GBMs are the most prevalent primary brain neoplasm in adults and their incidence is inexplicably on the increase. They have a disproportionate socio-economic ranking compared to their incidence, being the fourth greatest impacting cancer. Treatments are not curative and mortality rates are high, with very little improvement in years. However, length of survival varies greatly, with the median being 9 months and a small but significant percentage of patients living for 2–3 years post diagnosis. Somatic mutations in p53 are found in approximately 50% of all human cancers. The frequency varies according to tumor type and ranges from about 10% in leukemia, 20% in breast cancer, 30% in gastric cancers and up to 60% in ovarian and colorectal cancers. Wide variations have been reported between different studies of the same tumor type, probably reflecting methodological, geographic and case mix differences. One of the major issues has been the use of immunohistochemical (IHC) technique as a surrogate marker for p53 abnormality. Concordance between the over-expression of p53 protein detected by IHC and the presence of gene mutation detected by molecular methods is approximately 70% (153). Considerable optimism initially surrounded the possibility that p53 mutation status could provide prognostic information for the outcome of cancer patients that was independent of conventional histopathological parameters. A study of breast cancers found that p53 mutation frequency was not related to nodal involvement or tumor size (154), although another study found a marginally increased frequency in recurrent tumors (155). The many
studies to investigate prognostic significance of p53 mutation in specific tumor types have often yielded inconsistent results. Reasons for this are likely to include the methodology used to detect mutations, small sample size, short length of patient follow-up, use of adjuvant therapies and variable case mix. Meta-analyses combine the results from individual studies in order to produce more reliable estimates of the prognostic significance of p53 mutation.

**p21 deregulation in cancer**

Much of the understanding about the role of p21 in cancer has come from knockout mouse studies combined with biochemical and functional analysis of cells in culture. Groundbreaking work came from the initial discovery of p21 as a potential mediator of the tumour suppressor activity of p53. Subsequent work showed that, although deletion of Cdkn1a (cyclin-dependent kinase inhibitor). The encoded protein binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes, and thus functions as a regulator of cell cycle progression at G1.) In mice abrogated DNA damage-induced and p53-dependent growth arrest, it had no effect on p53-dependent apoptosis. p21 could not, therefore, account for all the tumour suppressor activities of p53. Nevertheless, p21 is a major determinant of tumour protection by p53 (156), as Cdkn1a deletion drastically accelerated tumour formation in mice expressing a mutant form of p53 (Trp53R172H) that is incapable of inducing apoptosis but retains partial growth arrest activity.

The first genetic evidence supporting a tumour suppressor activity for p21 came from the discovery that Cdkn1a−/− mice developed spontaneous tumours. The late onset of these tumours (average age of 16 months) compared with those arising in mice deficient in other tumour suppressor genes such as Trp53, p16 or Arf suggests that the loss of Cdkn1a by itself is insufficient to promote malignancy. Although many human cancers such as colorectal, cervical, head and neck, and small-cell lung cancers are associated with reduced p21 expression the extreme rarity of loss-of-function mutations in CDKN1A in human cancer argues that p21 may not be a classical tumour suppressor. Instead, p21 synergizes with tumour suppressors and antagonizes oncogenes to protect against cancer. Furthermore, Cdkn1a deficiency accelerates the development of chemically induced tumours in mice (157). Additional in vivo evidence for tumour suppressor activity for p21 comes from studies using the transplantation of Cdkn1a−/− cells in mice with defined genetic
alterations. For example, although the leukaemogenic fusion protein AML1–ETO (AML1 is also known as RUNX1) does not promote leukaemia without secondary mutations, fetal liver haematopoietic cells isolated from Cdkn1a−/− mice and transduced with AML1–ETO promoted leukaemogenesis when transplanted into mice (158). Cdkn1a deficiency also cooperates with the co-expression of HRAS and MYC, the expression of BCR–ABL1 (BCR is breakpoint cluster region) or with Ink4 deletion to promote transformation and proliferation of cells in culture. Together, these data are consistent with the multi-step tumorigenesis theory and a role for p21 in this process.

A significant insight into the role of p21 in tumour suppression came from a study by Shen et al (159), demonstrating a prominent tumour suppressor role for p21 in a genomically unstable background. Cdkn1a deficiency cooperated with the loss of the DNA damage checkpoint protein ATM (ataxia–telangiectasia mutated) in promoting aneuploidy that preceded tumour development (159). Furthermore, although malignancies developing in the aforementioned Trp53R172P+/+ mice retain stable genomes, lymphomas and sarcomas arising in Trp53R172P+/+;Cdkn1a−/− mice had an earlier onset and exhibited chromosomal aberrations and marked aneuploidy(137). The finding that p21 downregulation inversely correlates with microsatellite instability in colorectal cancer, irrespective of the p53 status (160), adds support to the conclusion that the loss of protection against genomic instability by p21 contributes to human malignancy.

p21 also promotes genomic stability in stem cells, both maintaining the self-renewal capacity of stem cells, and possibly contributing to its oncogenic potential. For example, although haematopoietic stem cells (HSCs) derived from mice that are engineered to express PML–RAR (retinoic acid receptor) the initiating oncogene of human acute promyelocytic leukaemia (APL) exhibit relatively moderate DNA damage foci, those derived from PML–RAR;Cdkn1a−/− mice exhibit a significantly higher rate of DNA damage foci, with more than 95% of cells exhibiting multiple foci per cell. Thus, at least in the context of overexpression of this oncogene, p21 seems to limit DNA damage and protect against genomic instability in HSCs. Although there is currently no evidence to suggest that the increase in genomic instability in the absence of p21 in HSCs results in increased tumorigenesis, it is conceivable that the acquisition of additional genetic alterations, under these circumstances, may uncover a protective role for p21.
**p21 in cancer**

Figure 10: The molecular basis of p21 function in cancer.

**PTEN Controlled Signaling Pathways.**

The PTEN tumor suppressor gene encodes a lipid and protein phosphatase and is one of the most frequently mutated genes in human cancers (161). As a lipid phosphatase PTEN dephosphorylates phosphatidylinositol 3,4,5-triphosphate (PIP3), a product of phosphatidylinositol-3-kinase (PI3Kinase). PTEN loss results in an accumulation of phosphatidylinositol-3,4,5-triphosphate (PIP3) which activates a cascade of signaling molecules including the phosphatidylinositol-dependent kinases (PDKs), the serine/threonine kinases AKT/Protein kinase B, S6 kinase, and mTOR, as well as small GTPases Rac1 and Cdc42. PTEN exerts a wide range of effects on cell growth, cell migration, cell death, and cell differentiation (162). Activation of AKT, one of most studied PTEN downstream effectors,
leads to inhibition of proapoptotic factors such as BAD and caspase 9, and stimulates cell cycle progression through downregulation of G1 cell cycle inhibitor p27. In addition, recent work has demonstrated the role of PTEN in regulating p53 protein level and activity (163), the expression of NKX3.1 in prostate cancer development (164), in controlling genomic stability (165) and senescence (166). Taken together, it is clear that PTEN is responsible for regulating a variety of cellular processes (Figure 11). Emerging evidence shows that PTEN and its controlled pathway also control stem cell homeostasis and PTEN’s role in the development of CSCs in various malignancies is presented herein (167).

**PTEN and Neural Stem Cells.**

Previous research has shown that only a small percentage of brain tumor cells possess the ability to self-renew and propagate tumor growth (97), suggesting that brain tumors contain CSCs. PTEN is frequently mutated in glioblastomas. Pten deletion in embryonic neural stem cells results in brain enlargement, increased cell proliferation, decreased cell death, and enlarged cell size (168). Using an in vitro neurosphere culture system to study stem cell proliferation, it was observed that there were more stem/progenitor cells in the Pten deficient brains (169). Microarray analysis revealed prominent dysregulation of cell cycle-related genes in Pten deficient neurospheres. In addition, flow cytometric analysis indicated that Pten deficiency mediates enhanced neural stem/progenitor cell self-renewal by promoting G0-G1 cell cycle transition. Taken together, these data suggest that the loss of PTEN confers an increased self-renewal capacity to neural stem/progenitor cells, a potentially important mechanism for brain tumorigenesis. Brain cells positive for nestin, a neuronal stem and progenitor cell marker (102), and the stem cell marker CD133 occupy a region adjacent to the blood vessels called the perivascular niche (PVN). In a mouse model of medulloblastoma with Pten loss (170), cells in the PVN are highly proliferative and escape cell cycle arrest that would normally have occurred in the presence of functional PTEN. It was observed that while the majority of the cells in the tumor bulk underwent apoptosis upon irradiation treatment, the cells in the PVN survived and showed neither cell cycle arrest, nor elevated p53 levels that are normally associated with both radiation-induced cell cycle arrest and apoptosis (170), strongly suggesting that the radiation induced cell cycle arrest via p53 is PTEN-dependent. Moreover, it was shown that AKT inhibition could potentially be an effective adjunct treatment that may increase the efficacy of radiation treatment, highlighting the importance of the PI3K/AKT pathway in CSC radiation resistance.
Figure 11: Simplified representation of the PI3K pathway.
WNT PATHWAY

The WNT pathway, so named in humans because of homologs to the Drosophila wingless (wg) and mouse int-1, is involved in many key developmental processes such as proliferation, stem cell maintenance, pattern formation, and differentiation (171). Canonical, or WNT/β-catenin, signaling is the most thoroughly studied WNT pathway, but the molecule has also been shown to function through two other pathways: the WNT/Ca²⁺ and planar cell polarity (PCP); (171-173). In the canonical pathway, WNT acts as a secreted molecule and begins signaling by binding to a seven-transmembrane receptor called Frizzled (FZD) and a low-density lipoprotein receptor-related protein (LRP). Activation of this complex by WNT leads to phosphorylation of Dishevelled (Dsh). In the cytoplasm of the cell, β-catenin usually exists as part of a destruction complex including Axin, adenoma polyposis coli (APC) and glycogen synthase kinase-3β (GSK3 β). Without WNT signaling, β-catenin is phosphorylated by GSK3 β, slating it for destruction by means of the ubiquitin-proteasome pathway. In the
presence of WNT signaling, Dsh inhibits the activity of GSK3β leading to accumulation of β-catenin and eventually its transport into the nucleus in a concentration-dependent manner. In the nucleus, β-catenin associates with T-cell factor/lymphoid-enhancer factor (TCF/LEF) transcription factors leading to transcription of downstream targets (171, 174), including proliferation genes such as cyclin D1 and MYC (175) (176). Inhibitors of WNT signaling exist at multiple levels of the pathway. LRP-binding factors (Dickkopf, DKK) and secreted FZD-related proteins (sFRP) prevent formation of the WNT/receptor complex to block signaling at the receptor level (171, 177). At the nuclear level, complex formation between TCF/LEF and β-catenin can be inhibited by "inhibitor of β-catenin and TCF-4" (ICAT). DNA-binding affinity of the β-catenin/TCF complex is modulated by Nemo-like kinase (NLK) and can indirectly regulate nuclear WNT signaling. The transcriptional repressor Pitx2 also works in the nucleus to modulate WNT signaling. WNT signaling has been shown to be involved in multiple cancer types (174). Aberrant WNT signaling was first implicated in brain tumors through study of patients with Turcot’s syndrome, which involves an APC mutation linked to a high incidence of colorectal cancers and brain tumors, particularly medulloblastoma (178, 179). Mutations in Axin, β-catenin, and APC were also found in sporadic medulloblastomas (180-183), nuclear localization of β-catenin suggesting constitutive WNT signaling has also been found (184). Mutations of the WNT signaling pathway in medulloblastomas may represent a distinct molecular subgroup with its own set of genomic aberrations (185, 186). The WNT antagonist DKK-1 has been implicated as a medulloblastoma suppressor that is epigenetically silenced during tumorigenesis, and its re-activation decreased tumor cell growth by 60% while increasing apoptosis fourfold (187). Some evidence also exists for WNT signaling in astrocytomas. The WNT receptor FZD9 was immunohistochemically detected and up-regulated in human astrocytomas, with staining intensity correlated to histological grade (188). The production of sFRPs by human malignant astrocytoma cell lines has been demonstrated and shown to modulate growth and inhibit motility (189). Ectopic expression of DKK-1 in the U87MG astrocytoma cell line sensitized these cells to DNA damage and induced much higher levels of apoptosis after chemotherapy (190). It was reported that blockade of Wnt signaling with antagonists WIF1 and sFRP1 could inhibit tumor angiogenesis and induce antitumor activity in Hepatocellular Carcinoma, (191).

sFRP-1

The secreted frizzled related proteins (sFRP) are soluble proteins thought to interfere with the Wnt signaling. Our group previously demonstrated that one of these members, sFRP-1/FrzA, is strongly expressed during early phases of the vascularization process in embryonic vasculature and in the endothelium of arteries and capillaries in adults and modulated vascular cell proliferation (192). Wnt signaling is involved simultaneously in the
regulation of migration and proliferation of malignant glioma cells. Recently identified group of soluble proteins, the soluble Frizzled-related proteins (sFRPs; secreted apoptosis-related proteins, SARPs) (193-195). These proteins interfere with Wnt signaling presumably by binding to Wnt proteins, or, alternatively, directly by binding to Frizzled receptors (196). Importantly, sFRP-1 has been shown to act as a biphasic modulator of Wnt signaling, promoting Wnt-induced effects at low concentrations and counteracting them at higher concentrations (197). The interaction between Wnt proteins and Frizzled receptors triggers a multistep cascade of hitherto unknown events that lead to the inhibition of the serine-threonine kinase glycogen synthase kinase 3β (GSK-3β), resulting in decreased phosphorylation of adenomatous polyposis coli protein (APC) and β-catenin. Due to dephosphorylation or other as yet ill-defined events, β-catenin becomes less prone to degradation by the proteasome pathway. Instead, free β-catenin translocates to the nucleus where it serves as a transactivator for the transcription factor T-cell factor (TCF), leading to the transcription of several target genes. On the other hand, a change in the rheostat of complex-bound, cytoplasmic, and free, uncomplexed β-catenin also affects its role as a structural protein in the cell adhesion complexes (198). Frizzled. sFRPs are soluble proteins that bind to Wnt and modulate Wnt signaling (195-197). The biological role of Wnt signaling in human malignant gliomas has not been examined in detail. It is reported that the majority of long-term as well as freshly prepared ex vivo glioma cells produce sFRPs. The extent of sFRP expression correlates with cell density in vitro in that exponentially proliferating cells produce less sFRPs than confluent cell cultures. Activation of the Wnt pathway may result in accumulation of the free pool of cytoplasmic β-catenin, translocation into the nucleus and, consecutively, increased transactivation of the transcription factor TCF by virtue of the transactivator function of β-catenin. On the other hand, β-catenin belongs to structural proteins like the other catenins and the cadherins. By constituting the cell-adhesion complex, these proteins are responsible for cell-cell adhesion and more complex processes such as cellular motility (198). Therefore, investigations were done whether interfering with the Wnt pathway by ectopic expression of the soluble sFRPs would lead to a modulation of cellular motility. In such studies, it for the first time presented evidence that interfering with Wnt signaling results in a modulation of growth and motility of malignant glioma cells. Data support a role for the Wnt family and its natural modulators, sFRPs, in the pathophysiology of malignant brain tumors. Taken together, sFRPs inhibit migration and promote proliferation under non-supportive conditions. The underlying mechanisms may involve sFRP-induced actions to maintain β-catenin in a dephosphorylated state, thereby promoting its signaling functions.
NOTCH PATHWAY

The Notch signaling pathway plays several significant roles in both the development and maintenance of organisms. In development, Notch is responsible for lateral inhibition of surrounding cells, a significant mechanism of cell fate determination and proliferation (199). Notch expression may be used to locate neural precursors within embryonic tissues (200) and has been used to track the mechanism of division in neural precursors (201). Notch signaling is vital to organogenesis during embryonic development (202), this pathway is involved in determining cell fate by amplifying variation in nearly homogenous cell populations. Notch also participates in cell fate determination during creation and repair of differential tissue layers, as seen in epidermal and gut epithelia (199, 203, 204). The Notch pathway is expressed both in vitro and in vivo in adult stem cells (205, 206). It is required for the maintenance of general stem cell populations, which undergo terminal differentiation without Notch expression (205, 205, 205, 207-209). Notch also has a role during the renewal of existing cell populations through proliferation of progenitor cells, particularly during epidermal wound repair or in neuronal cell replacement (203, 205, 205, 205, 210, 211). The Notch signaling pathway resembles other developmental signaling pathways, with some unique differences. The Notch protein family consists of four transmembrane receptor proteins that share several key features, including a repeating EGF domain in the extracellular component (199, 212, 213). The Notch protein is targeted by the ligands Delta (DLL) and Jagged (JAG), which are transmembrane proteins integrated into the membrane of neighboring cells (214). When one of these ligands binds to Notch, two proteolytic cleavage events are promoted. The first of these cleavage events is linked to the sheddase tumor necrosis factor alpha converting enzyme (TACE), a member of the ADAM metalloprotein family (215). TACE allows the cell to shed the extracellular domain of Notch, which is taken up by the ligand-expressing cell through endocytosis. Following TACE action, the γ-secretase complex, an integral membrane protein associated with a wide range of cellular actions, liberates the Notch intracellular domain (NICD) from the cell membrane through a second cleavage event (216). Once NICD has been released into the cytoplasm, it is transported to the nucleus where it forms a coactivator complex with Mastermind-like (MAML) and activates DNA binding protein CSL complex CSL; (212, 213). This process causes expression of several of the target genes of Notch, including HES1 and HES5, and continues until NICD is phosphorylated by cyclin-dependent kinase 8 (CDK8). Phosphorylated NICD is then ubiquitinated and undergoes proteolysis, allowing repressor complexes to halt further CSL function. Aside from the primary pathway, several other factors activate or suppress Notch function within the cell, including several major signaling pathways (206, 212, 217, 217). In addition to participating in stem cell proliferation, the Notch pathway has also been associated with tumor development, potentially as an oncogene (218, 219). However, the
exact nature of oncogenic Notch signaling is unclear, as it has also been shown to act as a tumor suppressor. Notch and its family members have different effects on cell fate during development (220), and Notch's involvement in tumorigenesis may also be dependent on the cellular and tissue context (221). The correlation between brain tumor growth and notch expression is well documented for several tumor types. In medulloblastomas grown in transgenic mice, high levels of NOTCH1 signaling were detected in the tumor mass compared with unaffected tissue, whereas NOTCH2 was down-regulated (222). NOTCH1 and NOTCH2, as well as the notch ligand JAG1, have been found up-regulated in malignant meningiomas cultured from fresh tumor specimens (223). Primary GBM tissue has been shown by reverse transcriptase-polymerase chain reaction to express high levels of NOTCH3 and increased HES1 transcription compared with typical neural tissue (224). Expression of NOTCH1 and several of its ligands was shown to be required for continued survival and proliferation of astrocytoma cells (224, 225). By silencing the expression of any of these proteins, cell growth was inhibited both in culture and in xenograft. In the established U373 astrocytoma line, a subpopulation of cells has been isolated that exhibit stem-like properties, increased rates of growth and high levels of Notch expression (226). Evidence is growing in support of notch signaling leading to brain tumor development driven by BTSC. In GBM tissue samples, high nestin levels have been associated with high levels of Notch expression, suggesting correlation between BTSC and Notch expression (227). After global gene microarray analysis, it was found that Notch expression in brain tumors correlated with good versus poor prognosis (129). Pharmacologically blocking the γ-secretase complex in medulloblastomas decreased the quantity of CD133+ BTSCs in culture and inhibited proliferation (228). When implanted into nude mice, cells treated with a γ-secretase inhibitor demonstrated reduced ability to form tumors.
Evidence shows that Notch signaling in various tumor cells is able to activate endothelial cells and trigger angiogenesis in vitro and in xenograft mouse tumor models, which might be one of the mechanisms for promoting tumor growth (229, 230). Recent findings in Drosophila melanogaster indicated that constitutive activation of the Notch pathway or ectopic ligand Delta expression cooperating with the deregulation of Psq and Lola convert eye tissue overgrowth to tumors. The Notch pathway initiates the repressor of target gene rbf, just like its human counterpart RB1 gene, by epigenetic silencing of the promoter and transcription start region of rbf. This finding links the Notch pathway to the epigenetic silencing pathway in promoting tumorigenesis and may provide a new focus for studying Notch signaling (231). Because Notch activation plays a role in the initiation and progression of many human malignancies, it is suggested that Notch may be a novel cancer therapeutic target. It has been shown that c-secretase inhibitors block Notch pathway induced apoptosis and growth inhibition in Kaposi's sarcoma tumor cells with Notch activation [miele 2006], and it has also been reported that curcumin inhibits pancreatic cell growth and induces apoptosis by down-regulation of Notch1 signaling (232).
Cell cycle

Figure 13: A schematic representation of the mammalian cell cycle. In each cell division cycle, chromosomes are replicated once (DNA synthesis or S-phase) and segregated to create two genetically identical daughter cells (mitosis or M-phase). These events are spaced by intervals of growth and reorganization (gap phases G1 and G2). Cells can stop cycling after division, entering a state of quiescence (G0). Commitment to traverse an entire cycle is made in late G1. Progress through the cycle is accomplished in part by the regulated activity of numerous CDK-cyclin complexes, indicated here and described in the text.

The process of replicating DNA and dividing a cell can be described as a series of coordinated events that compose a "cell division cycle," illustrated for mammalian cells in Figure 13. At least two types of cell cycle control mechanisms are recognized: a cascade of protein phosphorylations that relay a cell from one stage to the next and a set of checkpoints that monitor completion of critical events and delay progression to the next stage if necessary. The first type of control involves a highly regulated kinase family (233). Kinase activation generally requires association with a second subunit that is transiently expressed at the appropriate period of the cell cycle; the periodic "cyclin" subunit associates with its partner "cyclin-dependent kinase" (CDK) to create an active complex with unique substrate specificity. Regulatory phosphorylation and dephosphorylation fine-tune the activity of CDK-cyclin complexes, ensuring well-delineated transitions between cell cycle stages. A second type of cell cycle regulation, checkpoint control, is more supervisory. It is not an essential part of the cycle progression machinery. Cell cycle checkpoints sense flaws in critical events such as DNA replication and chromosome segregation (234). When checkpoints are activated, for example by under replicated or damaged DNA, signals are relayed to the cell cycle-progression machinery. These signals cause a delay in cycle progression, until the danger of mutation has been averted. Because checkpoint function is not required in every cell cycle,
the extent of checkpoint function is not as obvious as that of components integral to the process, such as CDKs.

Superficially, the connection between the cell cycle and cancer is obvious: cell cycle machinery controls cell proliferation, and cancer is a disease of inappropriate cell proliferation. Fundamentally, all cancers permit the existence of too many cells. However, this cell number excess is linked in a vicious cycle with a reduction in sensitivity to signals that normally tell a cell to adhere, differentiate, or die. This combination of altered properties increases the difficulty of deciphering which changes are primarily responsible for causing cancer.

DNA REPAIR

DNA repair is essential as DNA is highly susceptible to spontaneous damage (thousands of lesions occur in a normal cell per day as a result of oxidative radical generation, spontaneous chemical modifications and replication errors). Cellular DNA is also highly susceptible to carcinogens, and the target of a broad range of anticancer agents. It is therefore not surprising that a number of cancer susceptibility genes encode for DNA repair and DNA damage response (DDR) factors. Oncogenic defects in such genes enable the generation of cells with a mutator phenotype, which gives rise to transformed cells that escape the normal homeostatic processes. A large number of hereditary cancers are rooted in genetic defects of DNA repair factors. Germ line mutations in the XP nucleotide excision repair factors lead to Xeroderma Pigmentosum with high incidence of skin cancer and visceral tumors; defects in mismatch repair to HNPCC (human nonpolyposis colorectal cancer); defects in crosslink repair to Fanconi anemia with increased risk of acute leukemia and squamous cell carcinoma; and defects in DSB repair (BRCA2, BRCA1) to breast and ovarian cancers. Defect in replication and repair RecQ helicases (BLM and WRN) lead to Bloom and Werner syndrome with an early incidence of broad range of cancers. DNA repair is coupled with DNA damage responses that are commonly referred to as checkpoint response. Those checkpoints enable cell cycle arrest, which provides time for repair and avoids further damage until the DNA damaging agent is cleared from the cell. Hereditary defects in DDR are exemplified by ataxia telangiectasia. Inactivation of the ATM gene confers high risk of tumors, in particular lymphomas. Genetic inactivation of p53 is the cause of Li-Fraumeni syndrome. Defects in Chk2, the downstream effector kinase from ATM leads to Li-Fraumeni syndrome with normal p53, and defects in Mre11 and Nbs1 (both ATM cofactors) in ATLD (Ataxia-like-disorder) and Nijmegen breakage syndrome, which both predispose to cancers. Somatic mutations of the cancer predisposing genes listed above, especially in patient heterozygote for those genes, is likely to contribute to oncogenesis. For instance, defects in NER genes has a high incidence in ovarian and colorectal cancers.
(1) and defects Mre11 and mismatch repair has a high incidence in colorectal cancers
(2) p53 is mutated/inactivated in approximately 50% of sporadic tumors. Thus, it seems that characterization of tumors should include genetic status of the DNA repair and DDR genes in order to stratify tumors and rationalize therapy.
Figure 14: Mammalian Cell Cycle Checkpoint Pathways. In response to DNA damage, ATM and/or ATR trigger the activation of a checkpoint that leads to cell cycle arrest or delay. Checkpoint pathways are characterized by cascades of protein phosphorylation events (indicated with a "P") that alter the activity, stability, or localization of the modified proteins. A general overview of the G1, S, and G2 cell cycle checkpoint pathways is indicated (left, center, and right panels, respectively). See main text for additional details.

CHECKPOINT DEFECTS AND GENOMIC INSTABILITY IN CANCER

DNA repair and DDR are tightly coupled. Indeed, DNA repair requires cell cycle checkpoints to arrest cell cycle progression and enable DNA repair to take place without interference from replication of the damaged DNA template. For instance, p53 (and its downstream target p21CIP1/WAF1) is a key factor for cell cycle arrest in G1, while ATM, BRCA1, Mre11 and Nbs1 arrest S-phase progression. Inactivation of ATM, BRCA1, Mre11 and Nbs1 result in radioresistant DNA synthesis (RDS) (235), and leads to oncogenic and mutagenic DNA lesions. In addition, DDR can act as death effector and induce apoptosis in case of failure to repair DNA accurately. This is a well-known function of p53 in addition to its cell cycle arrest function. Thus, DNA repair and DDR are functionally linked and combination of agents that modulate DNA repair and DDR is likely to yield potent antiproliferative regimens.
DNA repair modulation

Genomic instability

Genetic instability is a paramount feature of cancer, which leads to accumulation of genetic alterations that varies from subtle changes in DNA sequence to chromosomal abnormalities (236). Microsatellite instability (MSI) is a particular type of genetic instability affecting short sequences of DNA repeats (microsatellites) found throughout the genome. MSI was first described in hereditary nonpolyposis colorectal cancer (HNPCC) and is present in the majority of these patients; currently, MSI analysis of this malignancy is standardized by the Bethesda guidelines (237). In colorectal cancer (CRC), the MSI phenotype appears to be related to particular clinical and histopathological features, including location in the proximal colon, tumors poorly differentiated with mucinous and signet ring cells, high tumor lymphocyte infiltration, low frequency of distant metastasis, and a comparably good prognosis (238). The MSI phenotype is a consequence of deficient DNA mismatch repair (MMR), which fails to recognize errors introduced in microsatellite regions during DNA replication. The loss of function of MMR family genes (MLH1, MLH3, MSH2, MSH3, MSH6, PMS1, and PMS2) is caused by germline mutations in hereditary malignancies, whereas in sporadic cancers, MLH1 (mutL homolog 1, colon cancer, nonpolyposis type 2) promoter methylation has been shown to be the main cause of gene silencing (239). As naturally occurring replication errors are not efficiently repaired, tumors with MMR deficiencies have a higher number of nucleotide insertions/deletions in genes harboring microsatellites (236, 239). The accumulation of activating or inactivating frameshift mutations in genes that regulate cell functioning, such as TGFB2 (transforming growth factor b type II receptor) and BAX (BCL2-associated X protein), is thought to be responsible for the tumorigenic process of MSI in MMR-deficient cells. Particularly important to oncological research is the evidence that many of these mutated genes, already identified in different tumors, also appear to have a role in the therapeutic response of different anticancer drugs (240-242).
Figure 15: Schematic representation of DNA double stranded break repair and checkpoint.
Reasons for modeling gliomas

One way of determining which genetic alterations identified in human gliomas are the actual etiological events, is to model the formation of gliomas by mimicking those genetic alterations in mice. Moreover, accurate animal models are powerful tools to investigate important aspects of glioma biology that can not be studied in cell culture systems, such as angiogenesis, invasion, and metastasis. Animal models that accurately duplicate human gliomas will hopefully provide excellent opportunities for identifying the most promising therapeutic targets for this disease and for testing any potential therapeutic strategies in vivo.

Strategies for modeling gliomas

For decades people have been modeling gliomas using various techniques and animal species. To date, four major strategies have been successfully employed to reach the goal: chemical mutagen-induced models, xeno- or allograft transplantation-induced models, germline genetic modification-induced models, and somatic genetic modification-induced models. Each of these strategies has characteristic features that are advantages or disadvantages depending on the application for which they are being used (Table 1).

Table 1

Summary of strategies used to model gliomas

<table>
<thead>
<tr>
<th>Mutagens/Transplantation</th>
<th>Principles</th>
<th>Primary genetic modifications</th>
<th>Cell-of-origin</th>
<th>Secondary mutations</th>
</tr>
</thead>
<tbody>
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<td>Unknown</td>
<td>Likely</td>
</tr>
<tr>
<td>Xeno-or allografts,</td>
<td>Unknown</td>
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<td>Unknown</td>
<td>Less likely</td>
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Applications of animal models

There are several reasons why modeling human cancer in animals is important. First, genetically accurate animal modeling allows us to demonstrate the casual relationship between mutations found in human tumors and the formation of those tumors. Second, many of the biological pathways involved in cancer biology have been studied extensively in cell culture. However, not all of the complex biology of cancer is experimentally approachable with cell culture systems. For example, identification of signaling pathways that affect the biologic behavior of tumors and their interactions with the host cell environment requires experiments to be carried out in *in vivo* systems. Third, identification of targets for cancer therapy that are likely to be effective if attacked by therapeutic strategies requires testing in animal systems that validate these targets as essential for the disease process. Finally, once these targets are identified and animal models for the disease are generated by activation of these targets, such preclinical animal models will be excellent test animals for therapeutics aimed at those targets.

In the murine system, the criteria defining the hematopoietic stem cell are clear. A single cell must be able to rescue and establish long-term reconstitution of the lymphohematopoietic system in the recipient (243). Such a simple and precise assay does not exist for brain tumor models. Instead, the model relies on recapitulation of the original tumor upon transplantation into immunodeficient mice. The xenograft model is an imperfect assay in determining the identity of "a CSC" because formation of brain tumor from single cell injection is not yet feasible. At best, one can demonstrate that a pool of tumor cells as few as 100, if tumorigenic, is enriched with glioma cancer stem cells (103). Also, because a stem cell is capable of generating the heterogeneous cellular constituents of a tissue or an organ, the CSC must demonstrate recapitulation of the parental tumor in addition to mere tumorigenicity. Additional technical refinements are likely to provide methodological tools to define the identity of glioma cancer stem cells with greater resolution. For instance, Sean Morrison's group has recently shown that single cell injections of melanoma cells can be tumorigenic at an unexpectedly low frequency of 1 in 4 (244). They used a highly immunocompromised mouse strain consisting of combination of sub acute combined immunodeficiency disease and interleukin-2 receptor gamma-null features. Because the authors did not observe a relationship between tumor cell surface marker expression and tumorigenicity, the result has
been used to call in question, the hierarchical interpretation of the CSC hypothesis. There are two important considerations concerning this study. First, the highly permissive in vivo condition used reflects an in vitro environment that lacks micro-environmental feedbacks that are believed to be critical for both normal and cancer stem cells (114). As such, this highly artificial animal model may not represent the true in vivo niche. Second, the CSC hypothesis can function in concert with both hierarchical and stochastic models (4, 113). Depending on the particular context, both hierarchical and stochastic processes are in play.

Experimental assays are helpful in improving understanding of concepts, define glioma CSC remains unclear. Reliance on expression of intracellular and/or surface markers alone is insufficient in defining glioma CSC because unlike normal stem cells, the genetic dysregulation that occurs in cancer may lead to ectopic protein expression. Particular caution is warranted in using criteria that simply combine a “stem cell marker” and in vitro proliferative rate because enhanced division may instead identify a “transit amplifying fraction” capable of proliferation but not extensive self-renewal. The in vitro environment assumes a reductionistic view that ignores the critical role of niche interactions with other cells (this may also apply to use of highly immune-compromised animals). The various putative markers of CSC may only identify subpopulations with enhanced in vitro propagation capability. Enhanced in vitro growth may also represent an artifact resulting from physical detachment of the cell from its native microenvironment. One potential means to overcome this difficulty is by performing live cell imaging to determine lineage mapping over generations of divisions to exclude transit amplifying cells, which may retain multilineage differentiation capacity, but only for brief periods (245, 246). At this time it is suggested heuristically that the CSC is characterized by persistent self-renewal capacity and tumor initiation in orthotopic animal models (71). It is difficult to know how best to study putative CSC. There is widespread agreement that long term in vitro exposure leads to irreversible changes in the identity of the cultured cells. For this reason, in vivo passage may be preferable. However, as discussed, even the in vivo conditions of rodent brains do not faithfully represent the native human brain microenvironment. This may be particularly true for artificially immune-deficient animals. Examination of human glioma specimens often demonstrates presence of CD45 positive (common leukocyte antigen) cells, implying interaction of cancer cells with the immune system. The in vivo animal model is incapable of providing this potentially critical input. In addition, we have observed that the molecular profile changes after treatment in unpredictable ways, suggesting evolution of over time (247). For example, some tumors acquire amplification of EGFR whereas others lose it. That the molecular profile of gliomas changes over time is hardly surprising considering that lower grade gliomas can transform to higher grades. Histopathologic grades represent an arbitrary classification applied to a process that is not punctuated, but rather existing on a spectrum, and the microenvironment
likely plays a key role in the constant evolution of gliomas, an effect that cannot be reproduced in immune-deficient animals. The role of the microenvironment (cell non-autonomous influence) in tumorigenesis and maintenance is critical because cancer is more complex than a mere cell autonomous entity strictly dictated by intrinsic genetic and epigenetic programs. While recognizing the limits of both in vitro and in vivo systems individually, we submit that the combination of both methods is effective in advancing the field.

**Therapeutic implications**

Despite advances in standard therapy, including surgical resection followed by radiation and chemotherapy, the prognosis for patients with glioblastoma multiforme (GBM) remains poor. Unfortunately, most patients die within 2 years of diagnosis of their disease. Molecular abnormalities vary among individual patients and also within each tumor. Indeed, one of the distinguishing features of GBM is its marked genetic heterogeneity. Nonetheless, recent developments in the field of tumor biology have elucidated signaling pathways and genes involved in the development of GBM, and several novel agents that target these signaling pathways are being developed. As new details on the genetic characteristics of this disease become available, innovative treatment regimens, including a variety of traditional treatment modalities such as surgery, radiation, and cytotoxic chemotherapy, will have to be combined with newer targeted therapies (248).

Another challenge for the successful treatment of GBM is the diversity of cell types and mutations in the tumor. These tumors are composed of highly heterogeneous cell populations that are often characterized by high chemoresistance (249). Furthermore, because a variety of genes may be mutated or overexpressed in different areas of the tumors, no one treatment is likely to destroy the tumor. Although significant progress has been made, further elucidation of signaling pathways responsible for the malignant phenotype of GBM will represent a significant advance in the field. Once tumors can be more accurately classified by mutations, treatment regimens can be tailored to individual tumors. It is likely that the most effective treatments will combine traditional interventions such as surgery, irradiation, and chemotherapy with the newer targeted therapies.
Development of DNA- and RNA-based therapies represents a future direction for GBM therapy. Although the use of gene therapy is still in the experimental stages, it is a promising new area of research. Theoretically, gene therapy strategies can be designed on the basis of unique cytogenic and molecular characteristics of the tumor and can improve the selectivity and safety of treatment [lawler 2005]. Similarly, antisense therapy is a promising new treatment strategy that is currently under investigation. Several genes, including TGF-β, bFGF, EGFR-1, VEGF, telomerase, topoisomerase II β subunit, PKC-β, and microtubule-associated protein 1A have been targeted by antisense technology in glioma cells (250).

Therapeutic vaccination of patients with cancer also represents an encouraging experimental approach to treating malignant gliomas (250). Antigen-presenting dendritic cells are designed to potently stimulate antitumor T-cell responses that in turn destroy the tumor (251). A recent phase I study demonstrated the ability of a tumor lysate-pulsed dendritic cell vaccine to generate antigen-specific cytotoxicity in patients with GBM and anaplastic astrocytoma (251).

In summary, recent therapeutic approaches are based on a greater understanding of the molecular and cellular biology of GBM. Some of the new cytostatic and noncytotoxic targeted agents currently under investigation may eventually add to the armamentarium of agents that can be used in combination with surgery, RT, and conventional cytotoxic agents for improved treatment of patients with GBM.