The Role of the Bmi1-GSK3β pathway in Glioblastoma

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophish-Naturwissenschaftlichen

Fakultät der Universität Basel

Von

Serdar Korur

von Edirne, Türkei

Basel, 2010

Genehmigt von der Philosophisch-Naturwisseschaftlichen Fakultät
auf Antrag von Prof. Heinrich Reichert, Prof. Adrian Merlo, Prof. Ruth Chiquet-Ehrismann und Dr. Brian Hemmings.

Basel, den 13. Oktober 2009

Prof. Dr. Eberhard Parlow
Dekan

Table of contents

Summary

Malignant gliomas remain one of the deadliest of all cancers despite maximal therapy. They present unique challenges to therapy with a median survival of 12 months.

Simultaneous activation of several growth promoting and anti-apoptotic pathways represents the basis for the failure of monotherapies against this disease. In order to efficiently block growth of glioblastoma (GBM) cells, we have applied several combinatorial approaches. We have found that combination of histone deactylase inhibitors along with the glycolytic inhibitor 2-deoxyglucose (2DG) efficiently induced apoptosis in GBM cells. Furthermore, combination of the microtubule inhibitor patupilone and AEE788 –an inhibitor of EGFR, which is frequently activated in gliomas, induced apoptosis in GBM cells at doses that as single drugs were not effective. In GBM and other cancers, subpopulations of tumor cells with stem cell properties that are believed to constitute a tumor cell reservoir, have been identified. GBM cells frequently express the progenitor cell markers Nestin and Sox2 and low levels of the differentiation markers CNPase, GFAP and β-tubulin III. Bmi1 and Glycogen synthase kinase 3 (GSK3) has been implicated in stem cell maintenance, but how Bmi1 regulates differentiation is still unknown. We have identified a link between Bmi1 and GSK3 and showed that blocking GSK3 may be instrumental to reduce the GBM cancer stem cell pool. We found that the GSK3 inhibitors SB216763 as well as Lithium chloride depleted the cancer stem cell population in GBM cells and induced tumor cell differentiation, irrespective of the CD133 status. Cell proliferation and colony formation were markedly reduced in a dose-dependent manner.

Future work giving a deeper insight into the regulatory mechanisms of the receptor tyrosine kinases and downstream effectors will help us to identify more specific targets. Understanding the mechanisms why some targeted therapies work and others fail will finally bring us to the level that efficient long-term treatment strategies can be envisaged.

1. Introduction

1.1. Cancer

Cancer is the main cause of death after circulatory diseases in western societies, estimated to be the cause of death in one quarter of the population in the EU (Niederlaender, 2006). In spite of the advances in the understanding of the molecular biology of cancer and of the development of novel therapeutics, cancer remains one of the deadliest of all diseases (Maher et al., 2001). Critical factors are to be identified prior to the successful introduction of therapeutic interventions.

Cancer can be viewed as the backside of evolution, which maximizes the probability of an organism to survive in a hostile environment (Maynard Smith and Szathmáry, 1995). Life originated in an environment with dramatically changing conditions, caused in part by exposure to toxic chemical compounds and by continuous ultraviolet and gamma-radiation (Maynard Smith and Szathmáry, 1995; Ridley, 1993). In order to maintain survival and stability, cells had to repair damage induced by external forces, endogenous metabolic toxins and reactive oxygen species, formed during normal metabolism. On the other hand, precise cellular repair systems would not allow genetic variation of the gene pool and thus, will lead to lack of adaptability (Maynard Smith, 1989). Perfect organisms with a constant gene pool over their lifetime might extinct when exposed to a different environmental parameter. Optimal organisms are formed by a trade-off between genetic variability and stability, which includes the risk of acquiring mutations that can give rise to cancer (Ridley, 1993). Those mutations may result in formation of cells that ultimately break the most basic rules of the organism and exploit every possibility of cellular regulatory pathways in order to proliferate indefinitely. The huge research effort to understand and combat cancer has tremendously increased the general knowledge in cell biology, as most of the cancer genes discovered play an important role in pathways regulating DNA repair, cell signaling, cell cycle, programmed

cell death and tissue architecture (Alberts, 2002). Normal cells have to receive and interpret an elaborate set of signals for the good of the organism, and damaged cells must be sacrificed in order to maintain stability of the organism. Only a few cells that can evade those protective mechanisms may constitute the candidate cancer initiating cells. Those cells may further develop through a microevolutionary process governed by several mutations, each conferring a growth advantage progressively leading to a selection of ever more aggressive clones (Nowell, 1976). As a result, cancer cells gain the ability to reproduce without restraint and colonize foreign tissues leading to death of the organism by eventually causing malfunctioning of a vital organ (Knudson, 2001; Knudson, 1971; Friend et al., 1986).

A fundamental feature of most cancer cells is that they are genetically unstable and have high mutation rate caused by impaired DNA repair systems and increased replication errors paving the way to the microevolutionary selection process. The fact that cancer is a multistep process is reflected by the requirements needed by a cell to be capable of cancerous growth (Alberts, 2002; Hanahan and Weinberg, 2000):

1. *Insensitivity to extrinsic and intrinsic signals regulating cell proliferation*
2. *Evasion of apoptosis*
3. *Ability to overcome replicative senescense and avoid differentiation inducing signals*
4. *Genetic instability*
5. *Invasion*
6. *Survival in foreign sites*.

Genetic alterations needed to push normal cells to a cancerous state can be induced in different ways: *i)* direct environmental factors (e.g. radiation) *ii)* genetic susceptibility to certain environmental factors (e.g. haploinsufficency of a gene involved in DNA repair) *iii)* induction by genetic factors (e.g. presence of an oncogenic mutation in the germline). Environmental factors might directly induce genetic alterations that target genes involved in the regulation of the cell cycle, survival and genome integrity (e.g. induction DNA adducts by cigarette smoking). Main environmental factors leading to cancers are cigarette smoking (Witschi et al., 1995), UV-light (Fisher and Kripke,

2002) nuclear accidents, nuclear bombs (Little, 2000), and certain chemicals that industrial workers are exposed to (e.g. asbestos, benzene, benzidine, vinyl chloride etc.) (Jameson, 2000). On the other hand, alterations in genes involved in cancer might already be present in germline causing inherited cancer syndromes. Genetic studies of families with inherited cancer syndromes have led to the identification of many important genes. Among those familial diseases, Li-Fraumeni syndrome (*p53*), Retinoblastoma (*Rb*), neurofibromatosis (*NF1* and *NF2*), Breast cancer (*BRCA1*), Colorectal cancer (*APC*), Von-Hippel Lindau syndrome (*VHL*), Wilms tumor (WT1-4), Xeroderma pigmentosum (*XP genes*), Ataxia-telengiectasia (*ATM*) and Bloom syndrome (*BLM*) (Fearon, 1997) represent typical examples. Mutations can also occur in somatic cells, causing sporadic forms of cancers, which constitutes the majority. Rb (Friend et al., 1986; He et al., 1995), p16/p14 (Merlo et al., 1995; Labuhn et al., 2001) p27 (Alleyne et al., 1999) and HDM2 (Vogelstein and Kinzler, 2004) are examples of those genes that, when mutated, are able to equip the cells with a growth advantage and induce the cancerous process.

1.2. Cancer stem cells

What are the normal cells of origin of cancer and why is this question so important? The cancer-initiating cell could be a normal stem cell, a progenitor cell, or a differentiated cell. This question was highly debated in recent years after the discovery of cancer stem cells in leukemia (Lapidot et al., 1994) that was followed by the identification of cancer stem cells in numerous solid tumors including glioblastoma (Ignatova et al., 2002; Lochhead et al., 2001; Singh et al., 2003; Singh et al., 2004; Al-Hajj et al., 2003; Gibbs et al., 2005; O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Xin et al., 2005; Burger et al., 2005). The failure to eradicate cancer may be as fundamental as a misidentification of the target. Identification of a defined cell that could function as a therapeutic target would facilitate development of successful treatment strategies (Figure 1). Conventional non-specific cancer treatments such as chemotherapy and radiotherapy, which act on all dividing cells, usually fail, and the disease recurs.

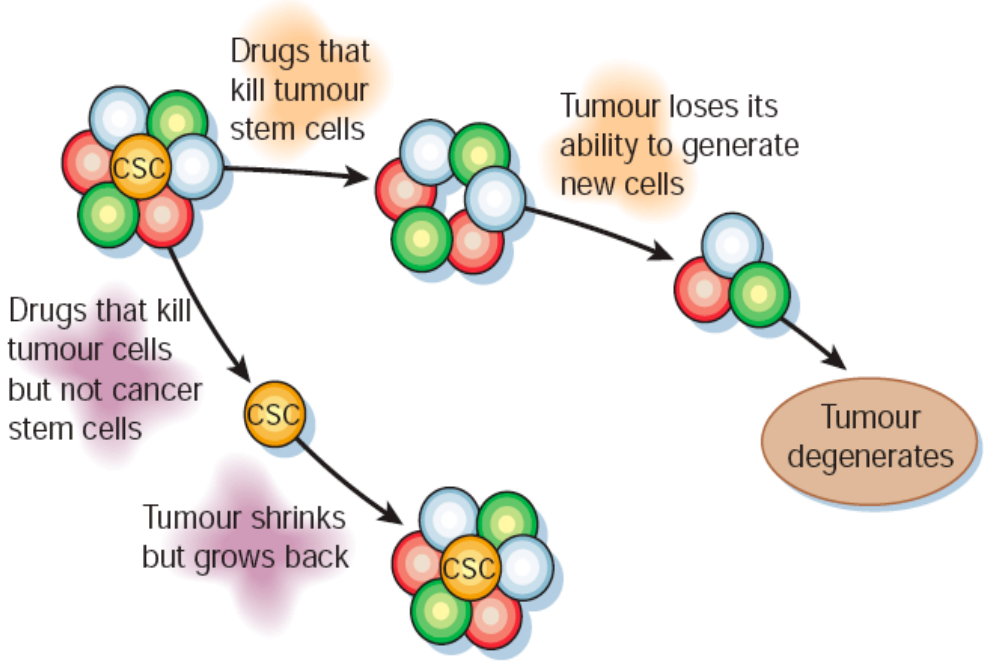


Figure 1: Tumors are maintained and driven by a rare population of cancer cells termed – cancer stem cells. Conventional therapies may kill tumor cells with limited proliferative potential but if the cancer stem cells remain viable they will reform the tumor. On the other hand, cancer stem cell specific therapies may lead to cures by extinguishing renewal potential of the tumor (Reya et al., 2001).

One of the most typical and interesting features of stem cells is the self-renewal characteristic that is also found in the cancer cells. Tumors might arise from the transformation of normal stem cells into cancer cells since they share many genetic and phenotypic features (Austin and Kimble, 1987; Bhardwaj et al., 2001; Chan et al., 1999; Ellisen et al., 1991; Gailani and Bale, 1999; Henrique et al., 1997; Korinek et al., 1998; Polakis, 2000; Varnum-Finney et al., 2000; Wechsler-Reya and Scott, 2001; Wechsler-Reya and Scott, 1999; Zhang and Kalderon, 2001; Zhu and Watt, 1999; Figure 2). Those cancer initiating cells are the driving force behind tumor propagation as well as the critical mediators of both drug- and radiation resistance (Visvader and Lindeman, 2008) and the reason behind the failure of conventional therapies.

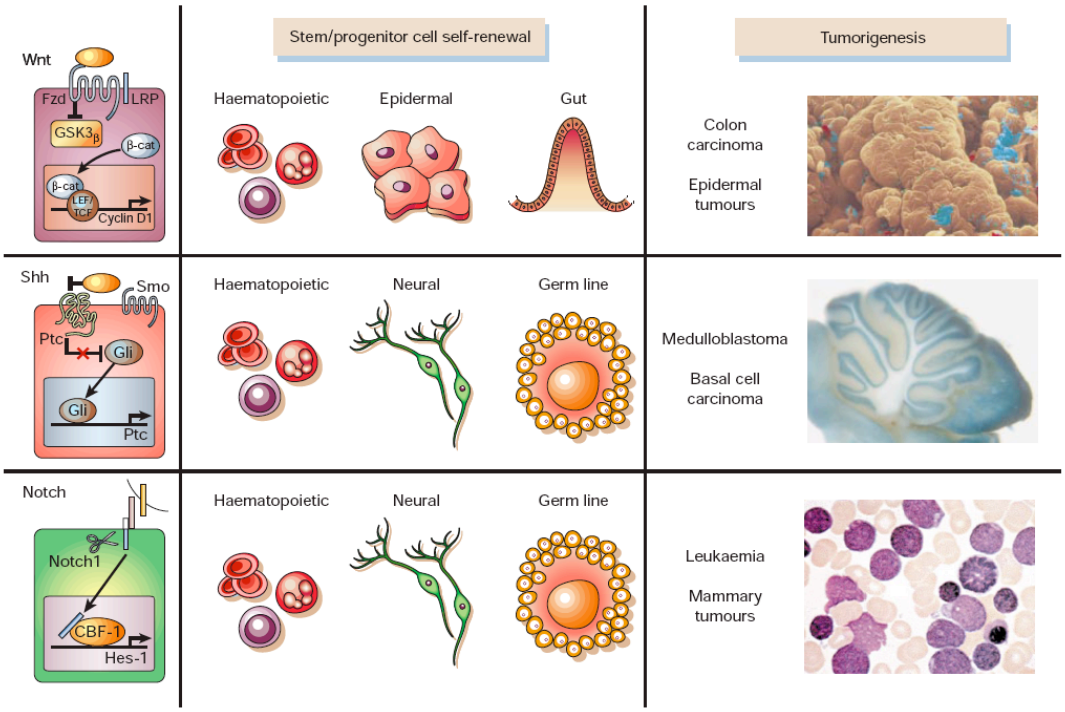


Figure 2: Several signaling pathways regulating normal stem cells found to be deregulated in cancer (Reya et al., 2001)

Singh and colleagues were able to identify CD133 (also known as Prominin 1) as a surface marker of cancer stem cells in brain tumors. As few as 100 of these CD133-positive cells found to be able to induce tumors in transplantation experiments and yielded phenocopies of the initial neoplasia (Singh et al., 2003; Singh et al., 2004). The expression of multi drug resistance proteins (Dean et al., 2005) and efficient DNA repair mechanisms (Bao et al., 2006) render CD133-positive cells highly resistant to chemo- and radiotherapeutic regimens. However, CD133 may not be a reliable stem cell marker for brain tumors as recent studies showed CD133-negative cells that are able to form tumors in immunocompromised mice. The other hypothesis consists of the concept that an adult astrocyte can dedifferentiate becoming a cancer cell as shown in an animal model (Bachoo et al., 2002). If cancer originates from cancer stem cells then any successful therapy will have to also eradicate this tumor promoting cell population to prevent recurrence.

1.3. General features of brain tumors

Any brain tumor having the histological, immunohistochemical and ultrastructural proof of glial cell differentiation is defined as "glioma". Gliomas are classified into different groups according to their degree of malignancy. The most widely accepted classification sytem is based on World Health Organization (WHO), which classifies glial tumors into four basic grades (I-IV astrocytoma) according to the degree of malignancy defined by histopathological criteria. Grade I gliomas are usually benign, well circumscribed and seldom progress into more advanced stages, whereas grades II to IV are malignant and readily infiltrative into the brain parenychma. Survival ranges from 3 to 10 years in low-grade astrocytoma (grade II) from 2 to 5 years in Grade III anaplastic astrocytomas, and about 1 year in grade IV tumors also known as glioblastomas (GBM) (Maher et al., 2001). In Switzerland the incidence rate per 100,000 population/year, was estimated as 3.32 in males and 2.24 in females (Ohgaki et al., 2004).

The blood-brain barrier (BBB) is an important cellular structure that prevents toxic substances from entering the brain and allows passage of nutrients and small compounds. On the other hand, it constitutes a major obstacle to the delivery of pharmacological agents into the tumor tissue, an important problem in the treatment of brain tumors (Sathornsumetee et al., 2007). The blood-brain barrier is formed by the tight junctions made by endothelial cells, other vascular cells and astrocytic foot processes and involves several active efflux transport systems including the prototype member P-glycoprotein (P-gp) (Pardridge, 2003). Drugs that could have been invaluable for the treatment of brain tumors either fail to pass the BBB or fail to pass blood-tumor barrier, which is limited by the fact that tumors have a high interstitial pressure (Boucher et al., 1997). 100% of large molecular and 98% of small molecular drugs do not cross the BBB. Another approach to overcome BBB is a bur hole-based drug delivery via intracerebral catheters (Merlo et al., 1999). Although those methods might efficiently supply drugs into the tumor bed, they may not readily target metastatic cells as the diffusion of the drugs to other areas of the brain than the tumor could be limited. A further development is direct intra-tumoral injection of small peptides that are distributed in the tumor mass

prior to resection (Kneifel et al., 2006). Efficient BBB drug targeting strategies can be built with the knowledge of the endogenous transporters within the brain capillary endothelium. The development of novel carrier molecules such as conjugating a tumor-targeting domain to a protein that can bind to molecules expressed on the BBB, and mediating their entry into tissue are strongly needed.

1.4. Glioblastoma

Glioblastoma (GBM) is the most frequent and most aggressive type of primary brain tumor in humans, accounting approximately for 50% of all tumors of glial origin and 20% of all intracranial tumors (Louis et al., 2007). Any disease with prevalence of less then 50 in 100.000 is classified as an orphan disease. Those diseases have not often been adopted by the pharmaceutical industry, as the number of patients affected is too low to make the drug-development cost-effective. Glioblastoma belongs to this class of diseases with an occurence of about 5-10 per 100,000 persons (Rich et al., 2004).

Malignant gliomas present unique challenges to therapy and remain one of the deadliest of all cancers with a median survival of 12 months. Even in the most favorable cases patients die within two years (Deorah et al., 2006). The duration of survival associated with malignant gliomas has improved only minimally despite tremendous efforts of therapy and improvement in the understanding of the molecular biology of cancer and in molecular medicine in the last decades (Rich and Bigner, 2004). Unique challenges in combating GBM are associated with; i) high vulnerability of the tissue where the tumor mass resides ii) diffuse invasiveness of tumor cells into the adjacent brain parenchyma iii) recurrence of the disease by rapid growth of the infiltrating cells (Merlo, 2003), resulting in very poor prognosis. GBM can manifest as *de novo* lesion (primary GBM, >90%) or progress from less undifferentiated low-grade astrocytoma (secondary GBM) (Ohgaki and Kleihues, 2007). Primary GBM usually develops in older patients as a highly aggressive and invasive de novo lesion, without any clinical or histological evidence of a less malignant precursor lesion. Secondary GBM manifest in

younger patients and develop through progression from low-grade to high-grade astrocytoma in a time range of 5 to 10 years. Although there are common pathways employed, primary and secondary GBMs develop through distinct molecular pathways (Collins, 1998; Rasheed et al., 1999). Primary GBM manifests with loss of heterozygosity at 10q (70% of cases), EGFR amplification (36%), p16INK4a deletion (31%) and PTEN mutations (25%). On the other hand, in secondary GBM etiology TP53 mutations are the most frequent and earliest detectable genetic alterations, already present in 60% of precursor low-grade astrocytomas. Additionally, primary and secondary glioblastomas manifests significant differences in their pattern of promoter methylation and in expression profiles at RNA and protein levels. (Ohgaki and Kleihues, 2007; Maher et al., 2001; Wechsler-Reya and Scott, 2001; Zhu and Parada, 2002; Figure 3). Recently, in a cancer genome-sequencing project, the IDH1 gene was identified as a gene, which is somatically mutated predominantly in secondary glioblastomas (Parsons et al., 2008). It was later found that IDH1 mutations are a strong predictor of better prognosis and a highly selective molecular marker to distinguish primary glioblastomas from secondary glioblastomas that complements clinical findings (Nobusawa et al., 2009). The IDH1 gene which encodes isocitrate dehydrogenase (IDH) 1 catalyzes the oxidative carboxylation of isocitrate to α-ketoglutarate, resulting in the production of NADPH in the Krebs cycle (Devlin, 2006). IDH1 mutations dominantly inhibit the function of the enzyme through the production of catalytically inactive heterodimers (Zhao et al., 2009). Further studies will provide molecular explanations for the role of IDH1 mutations in GBM.

It is predictable that the mutations occurring in the precursor cancer cell in primary GBM creates a much more unstable genetic background that facilitates further mutations which accelerates tumor growth by selection of more malignant clones (Nowell, 1976). In secondary GBM, specific founder mutations might cause milder instability and may require longer time lapse in order to gain further mutations to progress to a GBM (Ohgaki and Kleihues, 2007).

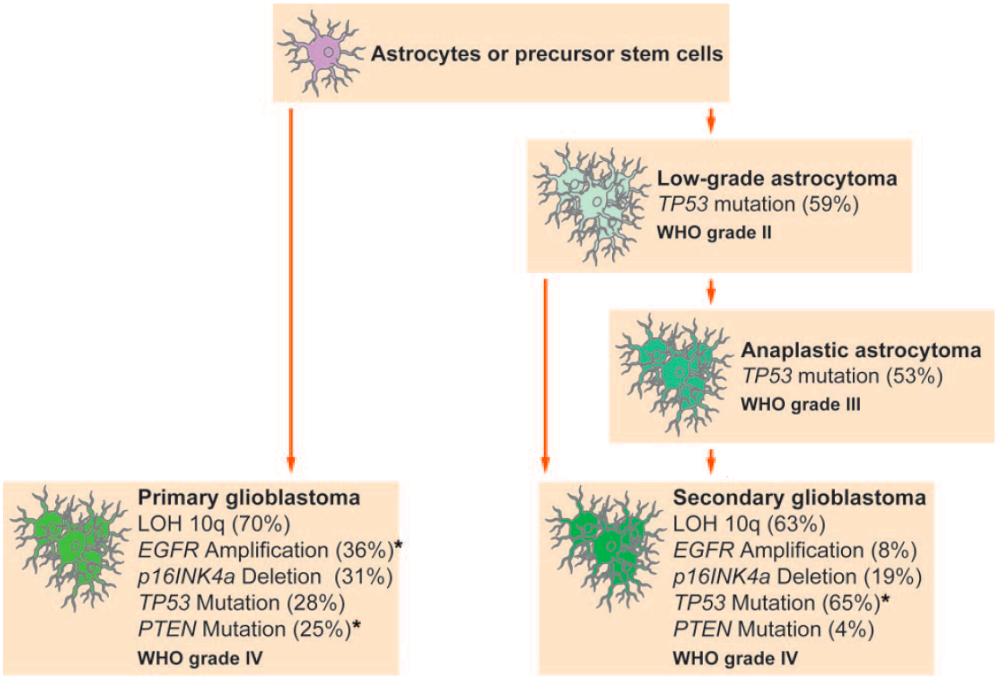


Figure 3: Genetic pathways to primary and secondary glioblastomas (Ohgaki and Kleihues, 2007).

Standard therapy, including complete surgical resection is not successful because of the infiltrative behavior of the tumor cells, which already invade multiple parts of the brain during the progression of the disease even long before the time of the diagnosis. Surgical intervention is usually followed by aggressive chemo- and radio-therapeutic regimens, which has proven limited efficacy because of *i)* the expression of multi-drug resistance proteins (Dean et al., 2005), *ii)* efficient DNA repair mechanisms of glioblastoma cells (Bao et al., 2006), *iii)* serious side effects induced from the therapy. Identifying novel molecular targets, and therapeutical strategies with improved efficacy and reduced toxicity, are strongly demanded.

1.5. Main pathways affected

Key cellular pathways, controlling apoptosis, cell cycle arrest, proliferation, survival and DNA repair are the most frequently disrupted pathways in GBM due to alterations in *TP53*, *p16*/*p14*, *RB*, *PTEN*, *EGFR* and *PDGFR* genes (Figure 4).

EGFR/*PTEN*/*PKB pathway*

Epidermal growth receptor (EGFR) is a key protein involved in the development of primary GBM (Kita et al., 2007), its overexpression occurs in about 60% of primary GBMs but rarely in secondary GBM (ca. 10%) (Dropcho and Soong, 1996). The most frequent mutant form is the constitutively active variant 3 (EGFRvIII) with the deletions of exons 2 to 7 (Huang et al., 1997). Activation of the EGF receptor in turn promotes cell proliferation in part through the suppression of the p27 gene via the PI3K/PKB pathway and partly due to the activation of the Ras/MAPK pathway (Narita et al., 2002). PKB activation due to the constitutive active EGFR results in increased cell proliferation and cell survival. LOH at chromosome 10 is the most frequent genetic alteration in GBM. PTEN is located at chromosome 10 and negatively regulates PI3K by dephosphosphorylating phosphatidyl inositol triphosphate (PIP_3). In PTEN mutant cells, PKB is hyperphosphorylated by PI3K (Maehama and Dixon, 2000) and this leads to increased proliferation and inhibition of apoptosis.

TP53/*HDM2*/*p14ARF Pathway*

The most frequent alteration found in diffuse astrocytoma is on the TP53 gene (60%). TP53 mutations are also found in primary GBM but at a lower frequency (ca. 30%) and with a different distribution pattern through the gene. In the cases where p53 is not mutated Hdm2 mutations have been detected (Maher et al., 2001) (less than 10% of GBM). In addition p14/arf is frequently deleted (76%) in GBM. Disruption of the p53 pathway leads to evasion of apoptosis and allows proliferation of damaged cells.

p16INK4a/RB1 Pathway

A hallmark of astrocytomas is the high mitotic activity, a characteristic shared also by primary and secondary GBM. Homozygous deletions of p16 is also frequently detected, it is deleted in 31% in primary GBM and 19% secondary GBM. Promoter methylation of RB1 gene occur 43% of secondary and in 14% of primary GBM (Ohgaki and Kleihues, 2007; Labuhn et al., 2001). RB1 and p16 tumor suppressor proteins control the progression through G1 to S phase of the cell cycle. Therefore inactivation of this pathway allows G1/S phase progression leading to high mitotic activity.

Despite many efforts, median survival of GBM patients has not improved more than a few months (Rich and Bigner, 2004). Thus, the development of specific bioactive molecules that selectively target and inhibit tumor initiation and propagation capacity of brain tumor stem cells might allow reduction or elimination of tumor establishment, growth and recurrence (Reya et al., 2001). The pursuit of novel agents that fulfill these criteria will allow a big leap towards successful treatment of brain tumor patients.

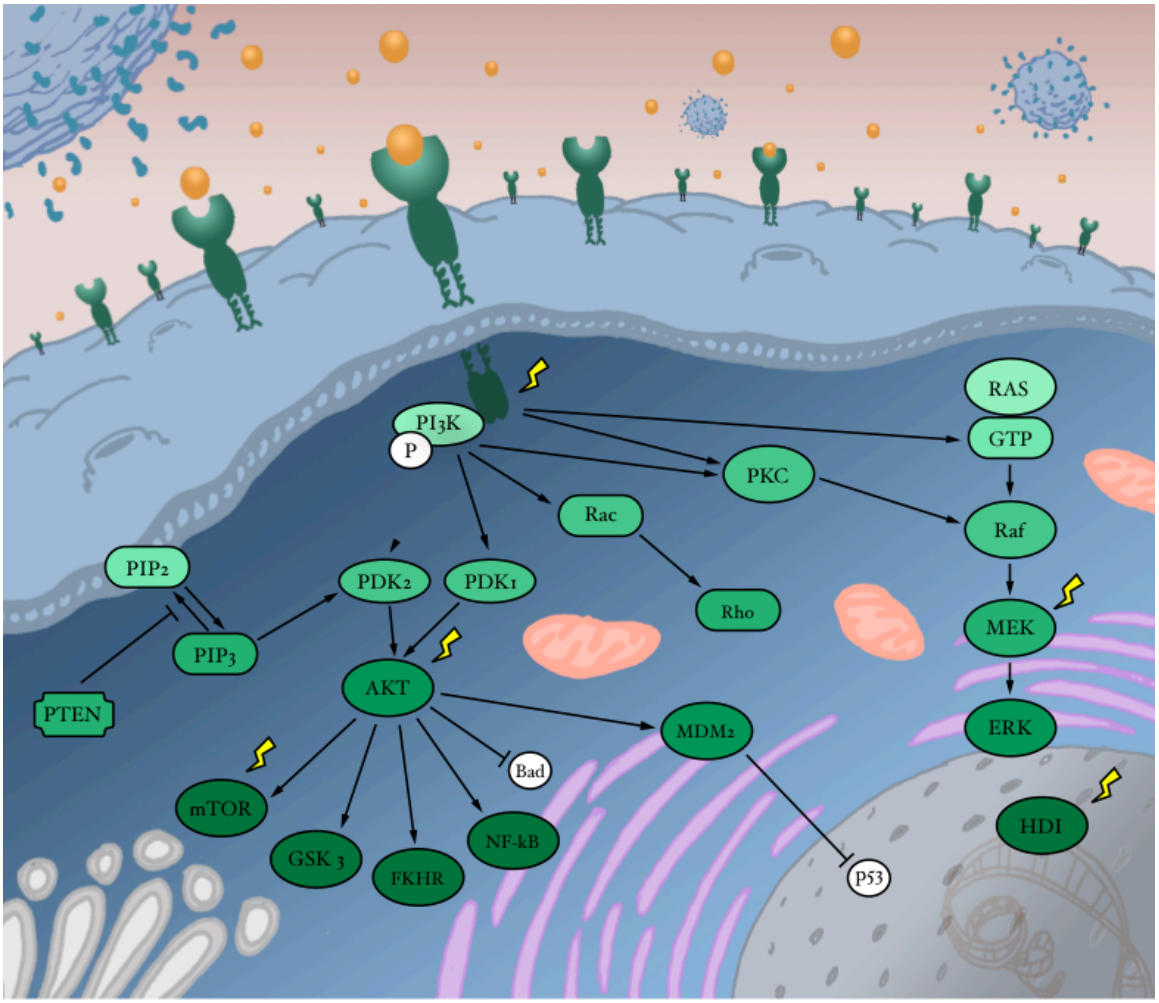


Figure 4: Main signaling pathways activated by growth factors. PI3K pathway and the MAP kinase pathway are indicated. Designed by Emmanuel Traunecker (mac_manus@hotmail.com)

1.6. Glycogen synthase kinase 3 pathway

Glycogen synthase kinase 3 serine/threonine kinase was first identified as an enzyme phosphorylating and inactivating glycogen synthase (Doble and Woodgett, 2003). Far behind its role in glycogen metabolism, further studies showed that GSK3 is a key protein in the regulation of numerous signaling pathways. It was shown to be inhibited in response to insulin signaling from PKB (Cross et al., 1995). It integrates several signaling pathways and regulates many aspects of cell behavior such as cell cycle, proliferation, differentiation and apoptosis (Cohen and Goedert, 2004; Doble and Woodgett, 2003). Two mammalian GSK3 isoforms are known: GSK3α and GSK3β.

Knocking out the GSK3β isoform in mice is embryonically lethal due to massive liver degeneration. The presence of the normal α isoform in the GSK3β KO animals is not able to rescue the phenotype (Hoeflich et al., 2000) indicating that at least some of the functions of the two isoforms are not redundant. The two isoforms share 97% sequence similarity within their kinase domains, but differ significantly outside this region, with GSK3α containing an extended N-terminal glycine-rich tail (Frame and Cohen, 2001). Controversial findings have been reported regarding the influence of GSK3 on the induction of apoptosis. GSK3 has been shown to act as a pro-survival factor in pancreatic cancer (Ougolkov et al., 2005) and as a proapoptotic factor in colorectal cancer (Tan et al., 2005). These opposite findings indicate that the biological function of GSK3 depends upon cellular context and microenvironment. Consequent to its key functions GSK3 is involved in the etiology of several diseases such as Alzheimer's disease (Ryder et al., 2003), diabetes (Cline et al., 2002), bipolar disorder (Gould and Manji, 2002), and recently cancer (Wang et al., 2008b).

Pathways regulating normal stem cell behavior are also utilized by cancer cells. GSK3 is involved in the regulation of Wnt (Miller and Moon, 1996; Yost et al., 1996); (Polakis, 2000), Shh (Jia et al., 2002) and Notch pathways (Foltz et al., 2002) which are important for embryonic cell fate determination and normal stem cell maintenance. Therefore we decided to investigate its role in brain tumor cell identity and maintenance of the cancer stem cell pool.

1.7. Bmi1 pathway

In the recent years it became evident that cancer is not only a disease due to genetic mutations but also epigenetic changes play a crucial role influencing malignant transition (Jones and Baylin, 2002). Maintenance of chromatin structure is essential for appropriate gene expression and every perturbation of the epigenetic regulations can lead to inappropriate gene expression and genomic instability, driving normal cells into a cancerous state. Polycomb group proteins are epigenetic gene silencers implicated in

neoplastic transformation. Bmi1, a member of the polycomb group (PcG) proteins is involved in brain development (Leung et al., 2004). PcG proteins maintain embryonic and adult stem cells by forming multiprotein complexes that function as transcriptional repressors (Park et al., 2003; Zencak et al., 2005; Liu et al., 2006). Bmi1 was first indentified as an oncogene due to its cooperation with Myc in lymphoma formation (Jacobs et al., 1999b). It has been shown to block senescence in immortalized mouse embryonic fibroblast through the repression of INK4A/Arf (Jacobs et al., 1999a) and it is amplified and/or overexpressed in non small cell lung cancer (Vonlanthen et al., 2001), colorectal carcinoma (Kim et al., 2006), medulloblastoma (Leung et al., 2004), lymphoma (Haupt et al., 1993), multiple myeloma (Matsui et al., 2004) and primary neuroblastoma (Nowak et al., 2006). Bmi1 regulates the Ink4a/Arf-locus that is a frequent target for homozygous deletions in glioblastoma. Whether Bmi1 is expressed in GBM is being debated (Leung et al., 2004) and its role in GBM is not well delineated. In a mouse glioma model, Bmi1 had been implicated in brain tumorigenesis in an Ink4a/Arf-independent manner (Bruggeman et al., 2007). In addition, it was recently shown that inhibition of Bmi1 by micro RNA-128 attenuates glioma cell proliferation and self renewal (Godlewski et al., 2008).

1.8. Combinatorial therapies to overcome therapeutic resistance

The glucose analog 2-deoxy glucose (2-DG) is a competitive inhibitor of glucose uptake and metabolism. Once entering the cells 2-DG is metabolized by the hexokinase to 2-deoxy glucose-6-phosphate (2-DG-6-P) which is not a substrate for glucose-6-phosphate dehydrogenase or phosphohexoisomerase (Wick et al., 1957), therefore cannot be further metabolized and accumulate in the cell until dephosphorylation by phosphatases. GBM cells are highly proliferative they rely on high sources of energy. A cardinal feature of glioblastoma cells is increased glucose uptake aided by high levels of hexokinase and glucose transporters. Most glioblastoma cells maintains a huge part of their energy supply from the glycolytic pathway as this pathways leads to faster production of ATP when compared to oxidative phosphorylation and in order to maintain their high growth and proliferation rates. Cancer cells can overcome drug effects by

turning on and off different genes to adapt changes in the environment, which requires ATP. Thus, blocking cells' energy production machinery in combination with another cytotoxic drug might sensitize the effects of the therapeutics by not giving opportunity to turn on and off redundant pathways. Tertiary structure of chromatin is a crucial factor in determining whether a particular gene is expressed or not. The accessibility of DNA wrapped around nucleosomes determines how efficiently a gene can be transcribed. This is regulated in part by a series of chromatin modifying enzymes such as histone acetlytransferases (HATs) and histone deactylases (HDAs). The alterations in chromatin structure by mutations or aberrant transcription of genes involved in the control of histone modifications are key events in cancer initiation. Changes in chromatin structure might lead to silencing of tumor suppressor genes and activation of oncogenes leading to carcinogenic progression. Various structurally diverse compounds (such as TSA, SAHA, trapoxin A, Laq842, sodium butyrate) are available which can bind to histone deactylases (HDACs) and induce histone acetylation and consequent reactivation of 2-10% of all genes (Mariadason et al., 2000; Egler et al., 2008).

EGFR, activation is one of the most frequent alterations in primary GBM, resulting in simultaneous activation of PKB and RAS pathways (Barker et al., 2001). The failure to induce efficient cell death in GBM suggested additional crosstalk between downstream pathways. Bioactive compounds such as PKI-166 or AEE788 with EGFR protein kinase inhibitory (PKI) activity have been designed and found to have a cytostatic effect in vitro on tumor cells that overexpress EGFR (Lane et al., 2001; Traxler et al., 2004). In addition, the EGFR PKI imatinib (gefitinib) allowed tumor growth control in 10% of patients with non–small cell lung cancer who carried specific mutations in the tyrosine kinase domain (Lynch et al., 2004). Several small molecular weight compounds, of tyrosine kinase inhibitors such as Gleevec or erlotinib/gefinitib, when applied as monotherapies, only resulted in limited efficacy in the treatment of GBM (Wen et al., 2006). Highly mutator phenotype of GBM cells enabled them to gain alterations in several growth promoting pathways and there is an obvious cross talk between several signaling pathways. In conclusion, these findings supported the hypothesis that in order to efficiently induce cell death in GBM cells, combination of two or more drugs is required (Failly et al., 2007).

2.Results

Part 1: GSK3β regulates differentiation and growth arrest in glioblastoma

Serdar Korur, Roland M. Huber, Balasubramanian Sivasankaran, Michael Petrich, Pier Jr. Morin, Brian A. Hemmings, Adrian Merlo and Maria Maddalena Lino.

PLoS One. 2009 Oct 13;4(10):e7443

GSK3β Regulates Differentiation and Growth Arrest in Glioblastoma

Serdar Korur[1], Roland M. Huber[1], Balasubramanian Sivasankaran[1], Michael Petrich[1], Pier Morin Jr[2], Brian A. Hemmings[2], Adrian Merlo[1]* and Maria Maddalena Lino[1]

1: Laboratory of Molecular Neuro-Oncology, University Hospital Basel, Hebelstrasse 20, CH-4031 Basel, Switzerland

2: Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, CH-4058 Basel, Switzerland

* Proofs should be addressed to Adrian Merlo

Tel.: +41 61 265 93 11; Fax: +41 61 265 90 60; E-mail: adrian.merlo@gmx.ch

Running title: GSK3β regulates differentiation and growth arrest in glioblastoma

Key words: Glioblastoma, cancer stem cells, glycogen synthase kinase 3β, Bmi1, differentiation therapy

Abstract

Cancers are driven by a population of cells with the stem cell properties of self-renewal and unlimited growth. As a subpopulation within the tumor mass, these cells are believed to constitute a tumor cell reservoir. Pathways controlling the renewal of normal stem cells are deregulated in cancer. The polycomb group gene Bmi1, which is required for neural stem cell self-renewal and also controls anti-oxidant defense in neurons, is upregulated in several cancers, including medulloblastoma. We have found that Bmi1 is consistently and highly expressed in GBM. Downregulation of Bmi1 by shRNAs induced a differentiation phenotype and reduced expression of the stem cell markers Sox2 and Nestin. Interestingly, expression of glycogen synthase kinase 3 beta (GSK3β), which was found to be consistently expressed in primary GBM, also declined. This suggests a functional link between Bmi1 and GSK3β. Interference with GSK3β activity by siRNA, the specific inhibitor SB216763, or lithium chloride (LiCl) induced tumor cell differentiation. In addition, tumor cell apoptosis was enhanced, the formation of neurospheres was impaired, and clonogenicity reduced in a dose-dependent manner. GBM cell lines consist mainly of CD133-negative (CD133-) cells. Interestingly, *ex vivo* cells from primary tumor biopsies allowed the identification of a CD133- subpopulation of cells that express stem cell markers and are depleted by inactivation of GSK3β. Drugs that inhibit GSK3, including the psychiatric drug LiCl, may deplete the GBM stem cell reservoir independently of CD133 status.

Introduction

Recent studies suggest that cancer stem cells are the driving force behind tumorigenesis [1]. CD133 (also known as Prominin 1) was identified as a surface marker of cancer stem cells in brain tumors [2]. As few as 100 CD133-positive (CD133+) cells were shown to induce tumors in transplantation experiments giving rise to a phenocopy of the initial neoplasia [2,3]. CD133+ cells, which express multi-drug resistance and DNA repair proteins [4], are highly resistant to chemo- and radiation therapy. However, stemness is not restricted to the expression of the CD133 marker, since CD133-negative (CD133-) cell populations were also found to be tumorigenic [5]. Cancer stem cells have also been detected in glioblastoma (GBM), the most malignant human brain tumor, with an annual incidence of 36 per million and a mean survival of less than 1 year [6-8]. GBM, a highly invasive and proliferative tumor, manifests itself as a *de novo* lesion or progresses from less undifferentiated low-grade astrocytoma.

Bmi1 is a member of the polycomb group of proteins involved in brain development [9]. Polycomb group proteins maintain embryonic and adult stem cells by forming multi-protein complexes that function as transcription repressors [10-17]. Bmi1 is also involved in cancer by cooperation with Myc in lymphoma formation [18] and blocking of senescence in immortalized mouse embryonic fibroblasts through repression of the Ink4a/Arf-locus [19]. It is also amplified and/or overexpressed in non-small-cell lung cancer, colorectal carcinoma, nasopharyngeal carcinoma, medulloblastoma, lymphoma, multiple myeloma and primary neuroblastoma [9,13,19-22]. Whether Bmi1 is expressed

in GBM is controversial [9]. In a mouse glioma model, Bmi1 was implicated in tumorigenesis in an Ink4a/Arf-independent manner [23]. Furthermore, it was shown recently that microRNA-128 inhibits proliferation and self-renewal in glioma at least partially by downregulating Bmi1 [24].

Glycogen synthase kinase 3 (GSK3), a serine/threonine kinase, regulates numerous signaling pathways involved in cell cycle control, proliferation, differentiation and apoptosis [25,26]. The mammalian isoforms GSK3α and GSK3β are functionally independent as GSK3α cannot rescue the embryonically lethal phenotype of GSK3β (-/-) mice [27]. GSK3 has been described as a pro-survival factor in pancreatic cancer [28] and as a pro-apoptotic factor in colorectal cancer [29] and is interconnected with several pathways and implicated in Alzheimer's disease [30], diabetes [31], bipolar disorder [32], and more recently cancer [33].

We have analyzed the role of GSK3 in malignant gliomas and its links to critical signaling proteins. Downregulation of Bmi1 reduced GSK3β levels and induced the differentiation of malignant glial cells. Direct inhibition of GSK3β by lithium chloride (LiCl), SB216763 and siRNA decreased Nestin and Sox2 levels and induced the cell differentiation markers CNPase, glial fibrillary acidic protein (GFAP) and β-tubulin III. In addition, LiCl and SB216763 depleted cancer stem cells grown as human GBM *ex vivo* cell cultures, induced differentiation and inhibited neurosphere formation. Thus, GSK3 may represent a novel therapeutic target for malignant gliomas.

Materials and Methods

Patients

Tumor samples obtained from patients during a neurosurgical procedure were immediately frozen and kept at -80°C. All patients gave their written consent for the neurosurgical procedure and for anonymous scientific analysis of diseased tissue according to the guidelines of the Ethics Committee of Basel, Switzerland (EKBB).

Cell culture and reagents

LN319, LN18, LN215, U373, LN229, LN401, U343, U87, BS125 and Hs683 glioma cell lines with defined genetic status of *TP53, p16/p14* and *PTEN* [34], DAOY medulloblastoma and B104 neuroblastoma cell lines were cultured in Eagle medium supplemented with 25 mM glucose, glutamine, standard antibiotics, and 10% FCS. BS287 cells were cultured in Neurobasal medium (Invitrogen) supplemented with basic fibroblast growth factor (20 ng/ml, Invitrogen), epidermal growth factor (20 ng/ml, R&D Systems), B27 (1x) and N2 supplement (0.5x) (Invitrogen). All cells were maintained at 37°C in 5% CO_2. The cell lines were seeded in 6-cm plates at 5'000-10'000 cells/cm^2 and grown for 24 h prior to treatment. For cell counting, cells were treated for 72 h as described in the figure legends and counted by hemocytometer. Lithium chloride (LiCl) was obtained from MERCK and SB216763 from Tocris. Drug concentrations used are indicated in the figure legends. LiCl was dissolved in PBS and SB216763 in DMSO and stored at -20°C. The EGFR inhibitor AEE788 was provided by Novartis Pharma. The

γ–secretase inhibitor DAPT was obtained from Roche.

Colony formation assay

For each cell line, 500 cells were plated in triplicate into 94-mm Petri dishes containing 10 ml of culture medium with 10% FCS. Cells were grown for 14 days at 37°C and 5% CO_2, during which period the medium was not changed. Cells were then fixed with 4% formaldehyde in 1x PBS and stained with crystal violet.

BS287 "*ex vivo*" cell line formation and neurobasal medium

Following informed consent, a tumor sample classified as GBM based on the WHO criteria was obtained from a patient undergoing surgical treatment at the University Hospital, Basel, Switzerland. Within 1-3 h after surgical removal, the sample was treated with the Neural Tissue Dissociation Kit (Miltenyi Biotec GmbH) according to the manufacturer's protocol. Tumor cells were cultured in NBE media. Uncoated plastic dishes were used for neurosphere culture of NBE cells.

Plasmids, lentiviruses and transfection

The lentiviral vectors pLKO.1-puro-scrambled-shRNA (Addgene) and pLKO.1-puro-shRNA (Sigma, sh1061: *CCGGCCTAATACTTTCCAGATTGATCTCGAGATCAATCTGG AAAGTATTAGGTTTTT*, sh693: *CCGGCCAGACCACTACTGAATATAACTCGAGTTATA TTCAGTAGTGGTCTGGTTTT*) targeting Bmi1 were transfected into HEK293 cells

together with plasmids encoding the packaging (pCMV_dr8_91) and envelope proteins (pMD2-VSV-G) using $CaCl_2$ precipitation. The concentration of infectious particles in the supernatant was titrated using HeLa cells. Glioma cells were transduced with infectious viral particles. Stably transfected clones were selected with 2 μg/ml puromycin. Bmi1 overexpression was obtained with pBABE-puro and pBABE puro-Bmi1 using $CaCl_2$ precipitation for 8 h. Stably transfected clones were selected with 2 μg/ml puromycin. siRNA transfection for GSK3 was performed using the GSK-3α/β siRNA SignalSilence Kit (Cell Signaling Technology) according to the manufacturer's instructions. Cells were transfected using the Amaxa Nucleofector device (Lonza). Cells transfected with non-specific siRNA were used as a control.

Transwell migration assays

Transwell migration assays were performed using modified Boyden chamber units with polycarbonate filters of 8-μm porosity (Costar). The lower side of the filter was coated with 10 mg/ml fibronectin for 2 h at 37°C. The bottom chamber was filled with DMEM containing 10% FCS. Cells (10^4 per well in serum-free DMEM) were plated in the upper chamber and incubated for 24 h with or without GSK3β inhibitors. After removal of the remaining cells from the upper surface of the filter, migrated cells at the bottom of the filter were fixed with 3.7% formaldehyde in PBS and stained with 0.1% crystal violet. For each treatment, cells in 10 fields of view were counted in three independent experiments.

Western blot analysis and antibodies

Cells were washed with 1x PBS, lysed in buffer containing 2% sodium dodecyl sulfate (SDS), 50 mM Tris pH 6.8, 0.1 M dithiothreitol (DTT), boiled and used either immediately or frozen at -20°C. Protein lysates were resolved on denaturing 8-12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (iBlot Gel transfer stacks, Invitrogen). The following primary antibodies were used: anti-Bmi1 (Upstate), anti-β-catenin and anti-Nestin (Santa Cruz Biotechnology); anti-β-tubulin III and anti-GFAP (Sigma); anti-CNPase (Chemicon); anti-GSK3β (Cell Signaling); anti-Notch2 (Developmental Studies Hybridoma Bank); anti-Sox2 (R&D systems); anti-CD133 (Miltenyi Biotec); anti-Akt and phospho-Akt (Ser-473) (Millipore, Billerica MA, USA), anti-p16/p14, anti-Bcl2, anti-Erk and anti-phospho-Erk (Santa Cruz Biotechnology, Santa Cruz CA, USA), anti-Actin (Sigma-Aldrich, St. Louis, USA). Decorated proteins were revealed using horseradish peroxidase-conjugated anti-mouse, anti-rabbit, anti-rat (New England Biolabs) or anti-goat (Pierce) secondary antibodies and visualized by the chemoluminescence detection system SuperSignal West Pico (Thermo Scientific). Protein bands were quantified with ImageJ software (http://rsb.info.nih.gov/ij/). Results were normalized to actin levels.

Cell sorting and flow cytometry

Cell DNA content and apoptosis were analyzed by flow cytometry (CyAn ADP Analyzer, Beckman Coulter) and the results statistically evaluated with Summit v4.3 software. Cells were trypsinized, fixed in ice-cold 70% ethanol for 1 h and stained with 50 μg/ml propidium iodide for FACS analysis. Percent dead cells was determined from

the proportion of cells in sub-G1 phase. Results are given as mean values from three independent experiments. For CD133 analysis, isolated cells were labeled with anti-CD133 antibody (1:10) for 10 min at 4°C, washed with PBS and sorted (INFLUX Cell Sorter by BD Biosciences).

For BrdU analysis, cells were pulsed with 10 μM bromodeoxyuridine for 2 h and processed with the APC-BrdU kit according to the manufacturer's instructions (BD Pharmingen). For fluorescent labeling with GFAP, cells were fixed and permeabilized with the Cytofix/Cytoperm kit (BD Pharmingen). Permeabilized cells were incubated with anti-GFAP (1:200) or matching isotype control antibody for 30 min on ice, washed twice and incubated with the corresponding secondary antibody for 30 min on ice and analyzed by flow cytometry (CyAn ADP Analyzer, Beckman Coulter).

Immunocytochemistry

Cells were grown to 80% confluency as a monolayer as described above. Cells were then fixed in 4% PFA in PBS for 15 min at room temperature, washed with PBS and incubated with the primary antibody overnight at 4°C in PBS + 1% BSA + 0.1% Triton X100. After thorough washing with PBS, the secondary antibody was added for 3 h at room temperature. Cells were imaged by confocal microscopy. All tumor samples analyzed were stained with hematoxylin-eosin.

Microarray analysis of glioma

BS series are primary tumor tissues obtained from patients diagnosed with primary CNS

tumors classified according to the WHO grading system. Normal brain tissue used as a template for microarray was obtained from samples of brain surgery for non-neoplastic disease. Total RNA from two normal brains, 12 GBM and eight astrocytoma samples was amplified and labeled using the Affymetrix 2-cycle amplification protocol according to the manufacturer's instructions (Affymetrix). Samples were hybridized to Affymetrix U133v2.0 GeneChips and scanned using an Affymetrix Gene Chip scanner following the manufacturer's instructions. Expression values were estimated using the GC-RMA implementation in the Genedata Refiner 4.1 (Genedata, Basel, Switzerland) package. Data-mining and visualization was performed using the Genedata Analyst 4.1 package. All samples were quantile normalized and median scaled to correct for minor variation in expression distribution. All microarray data reported in the manuscript are in accordance with the MIAME guidelines.

Results

Bmi1 is overexpressed in GBM, oligodendroglioma and astrocytoma

Bmi1 overexpression has been reported in several different tumor types including medulloblastoma and neuroblastoma. In an analysis of Bmi1 mRNA and protein expression in GBM cell lines and primary brain tumors, all GBM cell lines expressed high Bmi1 levels, with the LN319 line having the highest expression comparable to the reference line DAOY [9] (Figure 1A and data not shown). In primary brain tumor samples, Bmi1 expression was marked in 16/19 (84%) of GBM, 5/7 (71%) of oligodendroglioma and 3/7 (42%) of astrocytoma. In contrast, fully differentiated normal brain tissue had no Bmi1 protein (Figure 1B, C).

shRNAs against Bmi1 downregulates GSK3β

To study the role of Bmi1 in GBM, Bmi1 expression was knocked down using lentiviral-mediated delivery of shRNAs. Bmi1 was efficiently downregulated in the Hs683, U373, U87, and U343 GBM cell lines (Figures 2A and S1A and data not shown) using different shRNA sequences (Figure S1B). Since the Polycomb group gene Bmi1 is involved in the regulation of development and tumorigenesis, the effect of Bmi1 downregulation in GBM cells was screened by analysis of proteins involved in key cellular pathways of the cell cycle, development, metabolism, apoptosis and growth, including Erk, Akt, GSK3β, p16 and p14, Bcl-2, c-Myc, Nestin and Sox2. In contrast to non-neoplastic cells in Bmi1 knockout mice [19], Bmi1 downregulation in GBM cells did not affect Ink4a/Arf protein levels (Figure 2A). Bmi1 downregulation induced cell differentiation associated with morphological changes and decreased expression of the stem cell-related proteins Nestin

and Sox2, accompanying induction of an astrocytic fate in U373 glioma cell line, determined by increased levels of the astrocyte-specific marker GFAP, and decreased levels of oligodendrocyte-specific marker CNPase (Figures 2A-D and S2B). In contrast, Bmi1 overexpression accompanied dedifferentiation as shown by increased Nestin expression (Figure S1C). Interestingly, GSK3β levels were markedly reduced (Figures 2A and S1A-B). This raised the question of whether GSK3β mediates the effects observed on cell differentiation. To this end, we used the small molecules LiCl and SB216763 as well as siRNA to interfere with GSK3β activity in GBM cells.

GSK3β is expressed in GBM

Thirty-two primary tumor tissues obtained from patients diagnosed with GBM were studied using microarray and western blot analysis to measure GSK3β mRNA and protein levels. GSK3β mRNA were higher in GBM and astrocytoma patients compared with the control (Figures 2E, F and S2) and protein was found to be expressed in the majority of the tumors analyzed. The high GSK3β expression in astrocytoma was most probably due to greater necrosis in the GBM, with increased protein degradation (Figure S2B).

siRNA- and drug-induced inhibition of GSK3 increases differentiation markers

The effect of the inhibition of GSK3 on protein levels of progenitor (Nestin and Sox2) and differentiation (CNPase, GFAP, and β-tubulin III) markers in GBM cell lines U373, LN319, BS125 and LN18 was analyzed using the drugs LiCl and SB216763 or siRNAs. β-catenin is targeted for degradation upon phosphorylation by GSK3β [25]. Blocking

GSK3β therefore leads to accumulation of β-catenin, which was used as a read-out for the effects of LiCl and SB216763 on GSK3 activity (Figure 3B). After 72 h treatment with LiCl and SB216773, Nestin protein level decreased in the Nestin-expressing U373, LN319 and LN18 cell lines (Figures 3A, B), which was confirmed by immunocytochemistry (Figure 4B). Nestin and Sox2 were not expressed in the "*ex vivo*" BS125 GBM cell line. Sox2 levels were reduced in LN18, U373 and LN319 upon GSK3 inhibition (Figure 3A, B). The oligodendrocyte specific marker 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) and the neuronal marker β-tubulin III increased in BS125, LN18 and LN319, (Figure 3A, B). GSK3 inhibition also increased the protein levels of the astrocytic lineage-specific marker GFAP in LN319 and BS125 (Figure 4A). Downregulation of GSK3 activity in LN18 by siRNA reduced Nestin and Sox2 protein levels (Figure 4C), confirming the specificity of the inhibitory drug SB216763 in blocking GSK3β. The effect of the specific GSK3 inhibitor SB216763 was more pronounced and consistent than the effect of LiCl, which is known to target other signaling molecules [26]. Thus GSK3 inhibition specifically decreased the expression of progenitor markers (Nestin and Sox2) and induced the expression of differentiation markers (neuronal marker β-tubulin III, oligodendrocyte-specific marker CNPase and the astrocytic marker GFAP) in a cell line-dependent manner.

Inhibition of GSK3 depletes GBM cells with a stem cell signature

The phenotypic switch towards differentiation in GBM cells following inhibition of GSK3 raises the question of whether GSK3 activity regulates cancer stem cell

populations. CD133+ and CD133- cancer stem cells have been described in GBM [2,3,35-37]. In an analysis of eight different tumorigenic GBM cell lines (LN18, LN215, LN319, U373, LN229, U343, BS125 and Hs683) [34] for the presence of CD133+ cells, only LN319 contained CD133+ cells, at approximately 12%. To test whether the CD133+ population possessed a cancer stem cell-like character, the expression levels of stem cell markers were analyzed in the CD133-enriched population. Nestin, Notch2 and Bmi1 were highly expressed relative to the CD133- fraction (Figure 5C). GSK3β and β-catenin protein levels were also higher in the CD133+ population than in the control (Figure 5C). Inhibition of GSK3 in cell line LN319 with either LiCl or SB216763 showed a selective effect on the cancer stem cell-like population. LiCl at 10 mM and SB216763 at 20 μM induced a 50-60% depletion of CD133+ cells (Figure 5A). The effects of GSK3 inhibitory drugs were found to be specific in that the epidermal growth factor receptor inhibitor (AEE788) and the γ-secretase inhibitor (DAPT) did not significantly alter the CD133+ population (data not shown). To further consolidate this observation, we enriched the LN319 CD133+ population by FACS sorting, obtaining a CD133+ cell population of approximately 80%. This was maintained for several passages and then subjected to inhibition of GSK3 by LiCl or SB216763, which depleted the CD133+ fraction (Figure 5B).

It has been argued that GBM cells grown for many passages in standard medium do not mirror the stem cell compartment within the original tumor [38]. To examine this, the "*ex vivo*" cell line BS287 was analyzed which had been isolated from a fresh tumor biopsy and directly grown as neurospheres in neurobasal medium supplemented with bFGF and EGF, thus favoring expansion of cancer stem cells. Interestingly, the population with a

stem cell-like signature in this cell line was represented by the CD133- and not by the CD133+ population (Figure 6A-C). Only the CD133- population expressed elevated levels of Nestin, Sox2 and Bmi1 and formed neurospheres (Figure 6B, C). Inhibition of GSK3 decreased the stem cell like (CD133-) population (Figure 6D) and also altered protein levels of stem cell and differentiation markers, mainly decreased Sox2 levels (Figure S3). Induction of differentiation impairs the ability of precursor cells to form neurospheres. Inhibition of GSK3 significantly reduced the number and volume of neurospheres in BS287 cells (Figure 6E, F). The results show that inhibition of GSK3 reduces the cancer stem cell pool and that CD133 may not be a reliable cancer stem cell marker.

GSK3 inhibition reduces colony formation and induces apoptosis in GBM cells

The effects of GSK3 inhibition on cell proliferation and apoptosis in the GBM cell lines LN18, U373, LN215 and LN319 were analyzed using colony formation, relative cell number and cell death as readout. In a colony formation assay, both the number and size of colonies formed after 14 days of drug treatment were measured. GSK3 inhibitors significantly reduced colony formation in all GBM cell lines tested compared with the untreated control (Figure 7A). The GSK3 inhibitor concentrations used were in the non-toxic range; cell proliferation and survival of the human adipose tissue-derived progenitor cells (A111) were not negatively affected (Figure 7A). GSK3 inhibition by LiCl or SB216763 induced a slight increase in cell death for GBM cell lines LN319, LN18, U373 and BS125 after 72 h (Figure 7B). Induction of cell death was significantly elevated

when GSK3 inhibitor LiCl was combined with the standard GBM therapeutic temozolomide in the LN18 cell line (Figure 7D). Cell death was dose-dependent and varied from cell line to cell line. Direct cell counting after exposure of cells to LiCl or SB216763 for 72 h showed inhibition of the proliferation of LN18, LN319, U373 and LN215 cells (Figure 7C). G2-M accumulation was recorded in LN319, LN18, U373 and G2-M arrest in LN215 (Figure S4). In a migration assay, LN319 showed a significant reduction in cell migration following GSK3 inhibition (Figure 7E). These results show that GSK3 strongly reduces colony formation and induces cell death in GBM cell-lines.

Discussion

Bmi1, a member of the polycomb group proteins, is required for self-renewal of neural stem cells and is upregulated in several cancers. It is also known to repress Ink4a/Arf locus inhibiting progenitor cell proliferation during neural differentiation [39]. In differentiated cells, Bmi1 levels decrease while Ink4a/Arf protein levels increase [40]. As the Ink4a/Arf locus is frequently deleted in brain tumors [41], the role of Bmi1 overexpression in GBM cells appears to be distinct from its repression of the Ink4a/Arf locus. For example, downregulation of Bmi1 did not influence Ink4a/Arf protein levels in tumor cells that retained the Ink4a/Arf locus. Thus, in GBM cells, Bmi1 targets a different pathway. Screening of several key proteins controlling cell cycle, development, metabolism, apoptosis and growth, including Erk, Akt, GSK3β , p16 and p14, Bcl-2, c-Myc, Nestin and Sox2, showed that downregulation of Bmi1 reduced GSK3β protein levels and induced differentiation in cancer cells. In addition, tumor cell proliferation,

survival, migration and clonogenicity were markedly reduced.

Discovered some 25 years ago [42], GSK3 has been considered only recently as a therapeutic target for cancer [33]. It has been shown that this enzyme negatively regulates the Wnt, Hedgehog and Notch pathways, which are aberrantly activated in several cancers [33,43]. This suggests that GSK3 inhibitors could exert a therapeutically negative, pro-survival effect on tumor cells. However, the long-term medical use of the GSK3 inhibitor LiCl for the treatment of psychiatric disorders did not lead to an increase in cancer incidence [44], arguing against an oncogenic effect of GSK3 inhibitors. On the contrary, Cohen *et al*. demonstrated that cancer prevalence in psychiatric patients on long-term LiCl medication was lower than in the general population [44], suggesting even a protective effect of LiCl. The results presented here offer a molecular explanation of this epidemiological observation: administration of LiCl induces differentiation and inhibits proliferation and, thereby, might effectively inhibit tumor formation and progression. Furthermore, the plethora of clinical data on LiCl offer solid information about potential side-effects and it appears safe to assume that normal adult stem cells are not negatively affected, even by long-term use of the drug. The very similar phenotypic and functional alterations induced by either inhibiting GSK3 or by downregulating Bmi1 in the present study points to a functional link between Bmi1 and GSK3. However, further studies are needed to analyze whether there is a direct interaction between Bmi1 and GSK3. Downregulating GSK3 specifically decreased the subpopulation of cancer cells that contained a cancer stem cell-like signature by driving them into differentiation.

Sox2 protein is widely expressed in the early neural plate and early neural tube of several species [45]. In the developing central nervous system, Sox2 expression becomes

restricted to the neuroepithelial cells of the ventricular layer, which continue to divide and exhibit an immature phenotype. Cells that leave the ventricular layer lose Sox2 expression [45]. Interestingly, Sox2 has also been implicated in GBM [46,47] as downregulation of Sox2 reduced cell proliferation and tumorigenicity in GBM cells. Therefore, Sox2 was proposed as a new GBM therapeutic target [46]. At present, inhibitors of Sox2 are not available but the data presented here show that inhibition of GSK3 strongly downregulates Sox2 in GBM cells. This raises the possibility that LiCl or more specific GSK3-inhibitory drugs could be used to decrease the Sox2-dependent tumorigenic potential of GBM cells.

Two main strategies are currently being exploited to eradicate the cancer stem cell (CSC) pool: *i)* chemotherapeutic regimens that specifically drive CSC into apoptosis and thereby deplete the CSC reservoir of the tumor, and *ii)* strategies aiming to drive CSC into differentiation and thereby increase their susceptibility to pro-apoptotic treatments [1]. Given the high degree of drug resistance and the shared cellular and gene expression profiles of adult and cancer stem cells [48], targeting CSC has proven to be difficult. However, induction of differentiation remains a therapeutic strategy for CSC as Piccirillo *et al*. showed that bone morphogenetic proteins can induce differentiation of CD133+ GBM cells, thereby reducing their tumorigenic potential [49]. However, the use of morphogens bears the risk of interfering with the tightly regulated adult stem cell niches. Any strategy to induce differentiation in cancer stem cells must be carefully assessed for any adverse effects on the adult stem cell population.

Our results show GSK3 inhibition to be an attractive strategy for specifically targeting a subpopulation of cancer cells with stem cell-like characteristics. Expression of stem cell

and differentiation markers more accurately defined the subpopulation of cells within GBM cell lines and *ex vivo* tumor cells than expression of the CD133 marker. Inactivation of both Bmi1 and GSK3 depleted precursor cells required for tumor maintenance and progression. These data add another facet to the many effects of GSK3 as a regulator of cancer cell identity. Here, GSK3 activation is identified as a key element in maintaining stem cell-like characteristics in a subset of cancer cells, providing these cells with a higher self-renewal capacity. Recently, downregulation of GSK3 was shown to induce apoptosis in glioma cells and to have an anti-migratory effect in glioma sphreoids [50,51]. The role of GSK3 inhibiton on differentiation was not analyzed. Optimal therapies for cancer aim to spare normal cells with minimal or no general toxicity while depleting malignant cells. The Wnt pathway is involved in regulating cell processes as proliferation, apoptosis, differentiation, mobility and stem cell self-renewal and has been described also as a major regulator of adult neurogenesis in the hippocampus [52]. In the Wnt/β-catenin pathway GSK3β mediates β-catenin degradation. Use of GSK3 inhibitors leads to the accumulation of β-catenin, which then drives cells into proliferation but this effect was not observed in the present study. This may be explained by the constitutive activation of several growth-promoting pathways, such as EGFR and PI3K, commonly found in GBM. This could lead to maximal Wnt signaling target activation masking additional activation. Conversely, differentiation- and apoptosis-inducing programs, which are low in cancer cells, could be influenced by GSK3 inhibition and are, therefore, directly detectable. On the opposite in normal system as in the A111 cells GSK3 inhibition lead to an increased cell proliferation in accordance with the previously described results in neural progenitor cells [53].

shRNA against Bmi1 downregulated not only GSK3 but also Bcl2, Nestin, Sox2 and not p16 and p14 (Figure 2A). The microarray data showed higher levels of GSK3 expression in brain tumors than in normal brain tissue, and protein was found to be expressed in the majority of the tumors analyzed indicating a role for GSK3 in GBM (Figure 2). GSK3 can thus be regarded as an important regulator of tumor cell identity in GBM.

In conclusion, we propose GSK3 inhibitory drugs, e.g. LiCl, as possible first- and/or second-line treatments complementing standard cancer therapy.

The additive effect of combining the GSK3 inhibitor LiCl and the standard GBM therapeutic temozolomide suggests possible sensitization due to the induction of differentiation by interference with GSK3 activity. In addition, the vast clinical experience of this drug with psychiatric patients indicates safe application and the lower cancer prevalence in LiCl-treated patients than in the general population suggests a protective effect of the drug [44]. Clinically, LiCl could be tested in patients receiving standard treatments in an additional therapeutic arm, the clinical hypothesis being that long-term LiCl therapy in stabilized GBM patients may delay tumor recurrence from the residual cancer stem cell pool by driving cancer stem cells into differentiation and apoptosis.

References

1. Visvader JE, Lindeman GJ (2008) Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer 8: 755-768.
2. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, et al. (2004) Identification of human brain tumour initiating cells. Nature 432: 396-401.
3. Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, et al. (2003) Identification of a cancer stem cell in human brain tumors. Cancer Res 63: 5821-5828.
4. Dean M, Fojo T, Bates S (2005) Tumour stem cells and drug resistance. Nat Rev Cancer 5: 275-284.
5. Joo KM, Kim SY, Jin X, Song SY, Kong DS, et al. (2008) Clinical and biological implications of CD133-positive and CD133-negative cells in glioblastomas. Lab Invest 88: 808-815.
6. Merlo A (2003) Genes and pathways driving glioblastomas in humans and murine disease models. Neurosurg Rev 26: 145-158.
7. Newton HB (1994) Primary brain tumors: review of etiology, diagnosis and treatment. Am Fam Physician 49: 787-797.
8. Newton HB (2004) Molecular neuro-oncology and the development of targeted therapeutic strategies for brain tumors. Part 3: brain tumor invasiveness. Expert Rev Anticancer Ther 4: 803-821.
9. Leung C, Lingbeek M, Shakhova O, Liu J, Tanger E, et al. (2004) Bmi1 is essential for cerebellar development and is overexpressed in human medulloblastomas. Nature 428: 337-341.
10. Orlando V (2003) Polycomb, epigenomes, and control of cell identity. Cell 112: 599-606.
11. Nowak K, Kerl K, Fehr D, Kramps C, Gessner C, et al. (2006) BMI1 is a target gene of E2F-1 and is strongly expressed in primary neuroblastomas. Nucleic Acids Res 34: 1745-1754.
12. Valk-Lingbeek ME, Bruggeman SW, van Lohuizen M (2004) Stem cells and cancer; the polycomb connection. Cell 118: 409-418.

13. Park IK, Morrison SJ, Clarke MF (2004) Bmi1, stem cells, and senescence regulation. J Clin Invest 113: 175-179.
14. Molofsky AV, Pardal R, Iwashita T, Park IK, Clarke MF, et al. (2003) Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. Nature 425: 962-967.
15. Zencak D, Lingbeek M, Kostic C, Tekaya M, Tanger E, et al. (2005) Bmi1 loss produces an increase in astroglial cells and a decrease in neural stem cell population and proliferation. J Neurosci 25: 5774-5783.
16. Park IK, Qian D, Kiel M, Becker MW, Pihalja M, et al. (2003) Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. Nature 423: 302-305.
17. Liu S, Dontu G, Mantle ID, Patel S, Ahn NS, et al. (2006) Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. Cancer Res 66: 6063-6071.
18. Jacobs JJ, Scheijen B, Voncken JW, Kieboom K, Berns A, et al. (1999) Bmi-1 collaborates with c-Myc in tumorigenesis by inhibiting c-Myc-induced apoptosis via INK4a/ARF. Genes Dev 13: 2678-2690.
19. Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M (1999) The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. Nature 397: 164-168.
20. Vonlanthen S, Heighway J, Altermatt HJ, Gugger M, Kappeler A, et al. (2001) The bmi-1 oncoprotein is differentially expressed in non-small cell lung cancer and correlates with INK4A-ARF locus expression. Br J Cancer 84: 1372-1376.
21. Shakhova O, Leung C, Marino S (2005) Bmi1 in development and tumorigenesis of the central nervous system. J Mol Med 83: 596-600.
22. Haupt Y, Bath ML, Harris AW, Adams JM (1993) bmi-1 transgene induces lymphomas and collaborates with myc in tumorigenesis. Oncogene 8: 3161-3164.
23. Bruggeman SW, Hulsman D, Tanger E, Buckle T, Blom M, et al. (2007) Bmi1 controls tumor development in an Ink4a/Arf-independent manner in a mouse model for glioma. Cancer Cell 12: 328-341.

24. Godlewski J, Nowicki MO, Bronisz A, Williams S, Otsuki A, et al. (2008) Targeting of the Bmi-1 oncogene/stem cell renewal factor by microRNA-128 inhibits glioma proliferation and self-renewal. Cancer Res 68: 9125-9130.
25. Doble BW, Woodgett JR (2003) GSK-3: tricks of the trade for a multi-tasking kinase. J Cell Sci 116: 1175-1186.
26. Cohen P, Goedert M (2004) GSK3 inhibitors: development and therapeutic potential. Nat Rev Drug Discov 3: 479-487.
27. Hoeflich KP, Luo J, Rubie EA, Tsao MS, Jin O, et al. (2000) Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. Nature 406: 86-90.
28. Ougolkov AV, Fernandez-Zapico ME, Savoy DN, Urrutia RA, Billadeau DD (2005) Glycogen synthase kinase-3beta participates in nuclear factor kappaB-mediated gene transcription and cell survival in pancreatic cancer cells. Cancer Res 65: 2076-2081.
29. Tan J, Zhuang L, Leong HS, Iyer NG, Liu ET, et al. (2005) Pharmacologic modulation of glycogen synthase kinase-3beta promotes p53-dependent apoptosis through a direct Bax-mediated mitochondrial pathway in colorectal cancer cells. Cancer Res 65: 9012-9020.
30. Ryder J, Su Y, Liu F, Li B, Zhou Y, et al. (2003) Divergent roles of GSK3 and CDK5 in APP processing. Biochem Biophys Res Commun 312: 922-929.
31. Cline GW, Johnson K, Regittnig W, Perret P, Tozzo E, et al. (2002) Effects of a novel glycogen synthase kinase-3 inhibitor on insulin-stimulated glucose metabolism in Zucker diabetic fatty (fa/fa) rats. Diabetes 51: 2903-2910.
32. Gould TD, Manji HK (2002) The Wnt signaling pathway in bipolar disorder. Neuroscientist 8: 497-511.
33. Wang Z, Smith KS, Murphy M, Piloto O, Somervaille TC, et al. (2008) Glycogen synthase kinase 3 in MLL leukaemia maintenance and targeted therapy. Nature.
34. Ishii N, Maier D, Merlo A, Tada M, Sawamura Y, et al. (1999) Frequent co-alterations of TP53, p16/CDKN2A, p14ARF, PTEN tumor suppressor genes in human glioma cell lines. Brain Pathol 9: 469-479.

35. Beier D, Hau P, Proescholdt M, Lohmeier A, Wischhusen J, et al. (2007) CD133(+) and CD133(-) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. Cancer Res 67: 4010-4015.
36. Pfenninger CV, Roschupkina T, Hertwig F, Kottwitz D, Englund E, et al. (2007) CD133 is not present on neurogenic astrocytes in the adult subventricular zone, but on embryonic neural stem cells, ependymal cells, and glioblastoma cells. Cancer Res 67: 5727-5736.
37. Galli R, Binda E, Orfanelli U, Cipelletti B, Gritti A, et al. (2004) Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. Cancer Res 64: 7011-7021.
38. Lee J, Kotliarova S, Kotliarov Y, Li A, Su Q, et al. (2006) Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell 9: 391-403.
39. Molofsky AV, He S, Bydon M, Morrison SJ, Pardal R (2005) Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16Ink4a and p19Arf senescence pathways. Genes Dev 19: 1432-1437.
40. Molofsky AV, Slutsky SG, Joseph NM, He S, Pardal R, et al. (2006) Increasing p16(INK4a) expression decreases forebrain progenitors and neurogenesis during ageing. Nature.
41. Labuhn M, Jones G, Speel EJ, Maier D, Zweifel C, et al. (2001) Quantitative real-time PCR does not show selective targeting of p14(ARF) but concomitant inactivation of both p16(INK4A) and p14(ARF) in 105 human primary gliomas. Oncogene 20: 1103-1109.
42. Hemmings BA, Yellowlees D, Kernohan JC, Cohen P (1981) Purification of glycogen synthase kinase 3 from rabbit skeletal muscle. Copurification with the activating factor (FA) of the (Mg-ATP) dependent protein phosphatase. Eur J Biochem 119: 443-451.
43. Foltz DR, Santiago MC, Berechid BE, Nye JS (2002) Glycogen synthase kinase-3beta modulates notch signaling and stability. Curr Biol 12: 1006-1011.

44. Cohen Y, Chetrit A, Sirota P, Modan B (1998) Cancer morbidity in psychiatric patients: influence of lithium carbonate treatment. Med Oncol 15: 32-36.
45. Wegner M (1999) From head to toes: the multiple facets of Sox proteins. Nucleic Acids Res 27: 1409-1420.
46. Gangemi RM, Griffero F, Marubbi D, Perera M, Capra MC, et al. (2008) SOX2 Silencing in Glioblastoma Tumor Initiating Cells Causes Stop of Proliferation and Loss of Tumorigenicity. Stem Cells.
47. Fong H, Hohenstein KA, Donovan PJ (2008) Regulation of self-renewal and pluripotency by Sox2 in human embryonic stem cells. Stem Cells 26: 1931-1938.
48. Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, et al. (2008) An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. Nat Genet 40: 499-507.
49. Piccirillo SG, Vescovi AL (2006) Bone morphogenetic proteins regulate tumorigenicity in human glioblastoma stem cells. Ernst Schering Found Symp Proc: 59-81.
50. Nowicki MO, Dmitrieva N, Stein AM, Cutter JL, Godlewski J, et al. (2008) Lithium inhibits invasion of glioma cells; possible involvement of glycogen synthase kinase-3. Neuro Oncol 10: 690-699.
51. Kotliarova S, Pastorino S, Kovell LC, Kotliarov Y, Song H, et al. (2008) Glycogen synthase kinase-3 inhibition induces glioma cell death through c-MYC, nuclear factor-kappaB, and glucose regulation. Cancer Res 68: 6643-6651.
52. Byfield MP, Murray JT, Backer JM (2005) hVps34 is a nutrient-regulated lipid kinase required for activation of p70 S6 kinase. J Biol Chem 280: 33076-33082.
53. Nusse R, Fuerer C, Ching W, Harnish K, Logan C, et al. (2008) Wnt signaling and stem cell control. Cold Spring Harb Symp Quant Biol 73: 59-66.

Figure Legends

Figure 1. Bmi1 was highly expressed in GBM. Bmi1 expression in (A) GBM cell lines and (B) primary brain tumors. (C) Ratio of Bmi1-positive primary tumors. NB: normal brain. EPEN: Ependymoma

Figure 2. GSK3β was downregulated by reduction of Bmi1 expression. (A) GBM cell lines U373 and Hs683 transduced with shRNA against Bmi1 (sh1061) or with scrambled shRNA (scr). Bmi1 downregulation induced downregulation of the following proteins: p-AKT, Nestin (in U373), Bcl2, and GSK3β. No effects are evident on the p16/p14^{ARF}, or p-ERK protein levels. (B) Scrambled control or U373 Bmi1-downregulated cells at subconfluency (sub-con) or confluency (con). Bmi1-downregulated cells show differentiated morphology (arrow: long, branched processes). (C) The glial fibrillary acidic protein (GFAP) levels in U373scr and U373sh1061 cells measured by flow cytometry. (D) Relative CNPase protein levels in U373scr and U373sh1061 measured by western blotting, quantified using ImageJ software and normalized to β-actin levels. (E) GSK3β mRNA values from a series of primary brain tumors (GBM and astrocytoma) and normal brain. (F) GSK3β protein is expressed in primary GBM. *P<0.05; one-way ANOVA (Newman-Keuls Multiple Comparison Test).

Figure 3. GSK3 inhibitors specifically induced differentiation in GBM cell lines. Western blot analysis of Nestin, Sox2, CNPase, β -tubulin III and β -actin (loading control) protein levels after treatment of GBM cell lines with GSK3 inhibitors (10 mM

LiCl; 20 μM SB216763). Each cell line showed a pro-differentiation response to GSK3β inhibitor application. (A) Protein bands quantified with ImageJ software. Protein levels were normalized to β-actin for each cell line. (B) Corresponding western blots.

Figure 4. GSK3β downregulation by siRNA treatment induced differentiation. (A) The intensity of GFAP, an astrocyte-specific marker, was measured by FACS analysis of GBM cell lines treated with 10 mM LiCl for 72 h. (B) Nestin immunostaining of cell lines U373 and LN18 after GSK3β inhibition by LiCl (Li) or SB216763 (SB) for 72 h. (C) GSK3β siRNA reduced Sox2 and Nestin expression in the LN18 cell line. **P*<0.05; one-way ANOVA (Newman-Keuls Multiple Comparison Test). Scale bar in B is 30 μm.

Figure 5. GSK3β inhibition reduced the CD133+ cell population of a GBM cell line. (A) LN319 GBM cell line was treated for 72 h with 10 or 20 mM LiCl or with SB216763 at 10 or 20 μM. GSK3β inhibition reduced the CD133-positive (CD133+) population. (B) LN319 CD133+ cells were enriched to 80% purity by cell sorting. The enriched population was treated for 72 h with 10 or 20 mM LiCl or with SB216763 at 10 or 20 μM. (C) Western blot analysis of Notch2, Nestin, Bmi1, β-catenin, GSK3β and β-actin in CD133+ and CD133-negative (CD133-) enriched populations compared with the control. ***P*<0.01; ****P*<0.001; one-way Anova (Newman-Keuls Multiple Comparison Test).

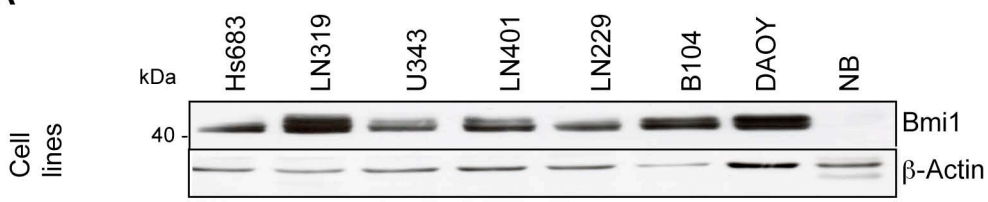
Figure 6. GSK3β inhibition reduced stem cell characteristics in an "*ex vivo*" GBM cell line. (A) CD133 sorting of the BS287 "*ex vivo*" GBM cell line. (B) Growth characteristics of the CD133+ and CD133- populations in BS287. (C) Western blot analysis of Nestin, Sox2, Bmi1 and β-actin in BS287. The CD133- population showed stem cancer-like characteristics. (D) Cells in the CD133+ population with a more

differentiated geno/phenotype increased after GSK3β inhibition. (E, F) Inhibition of GSK3β led to a reduction in the number and volume of neurospheres in the BS287 "*ex vivo"* cell line.

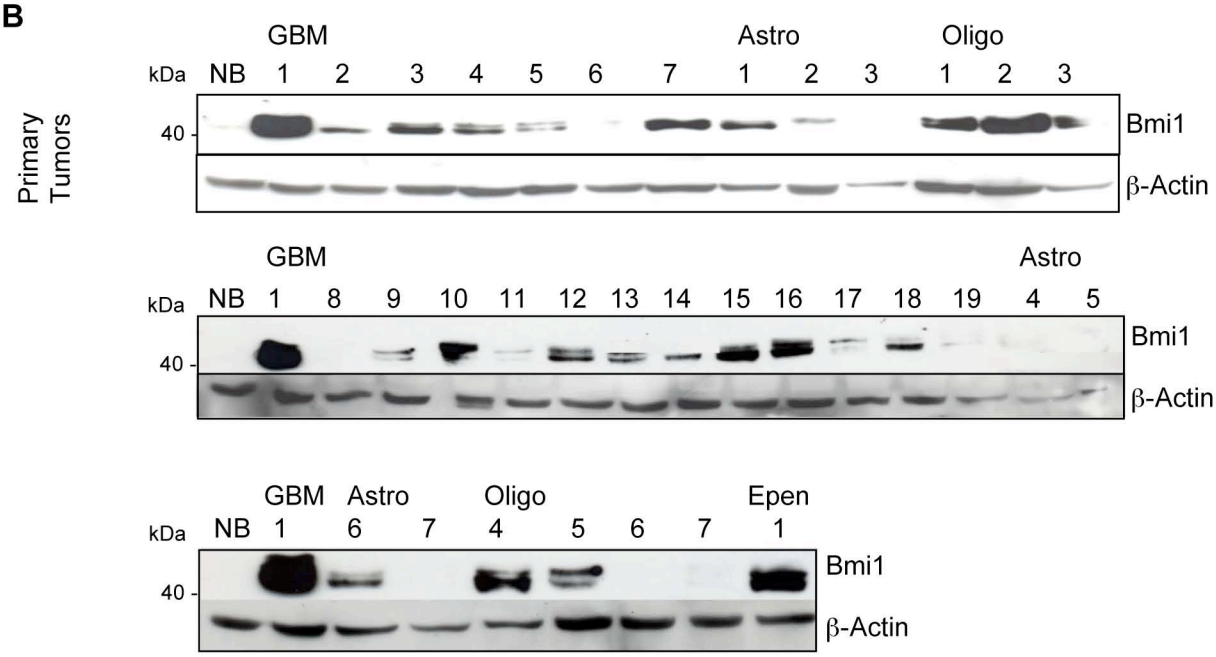
Figure 7. GSK3β inhibition reduced colony formation of GBM cells. (A) A colony formation assay was performed on GBM cell lines treated with 10 mM LiCl or 20 μM SB216763 for 14 days. (B, C) GBM cell lines treated with LiCl or SB216763 for 72 h. (B) Percent cell death and (C) relative cell number relative to the initial seeding. (D) Cell death determined by PI staining of the LN18 cell line after treatment with 10 mM LiCl with or without 50 μg/ml temozolomide (TMZ) for 72 h. **P*<0.05, for the combination of LiCl and TMZ compared with each drug alone. (E) GSK3β inhibition significantly reduced migration of the LN319 cell line. (B, C and E) Treated samples were compared to the corresponding control: **P*<0.05; ***P*<0.01; ****P*<0.001; one-way ANOVA (Newman-Keuls Multiple Comparison Test).

Acknowledgements: This work was supported by Oncosuisse (OCS - 01613-12-2004) and the Regional Cancer League of Basel Stadt and Basel Land. The funding agencies played no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We thank Beatrice Dolder and Dr. Elisabeth Taylor for valuable technical assistance, Emmanuel Traunecker and Verena Jäggin for cell sorting, Dr. Ivan Martin (University of Basel) for adipose tissue derived progenitor cells, Dr. Goberdhan P. Dimri (Northwestern University, Evanston, USA) for the pBabe-puro-Bmi1 vector, and Michal Grzmil (FMI, Basel) for bioinformatics. We thank Dr Patrick King (FMI) for editing the manuscript.

A



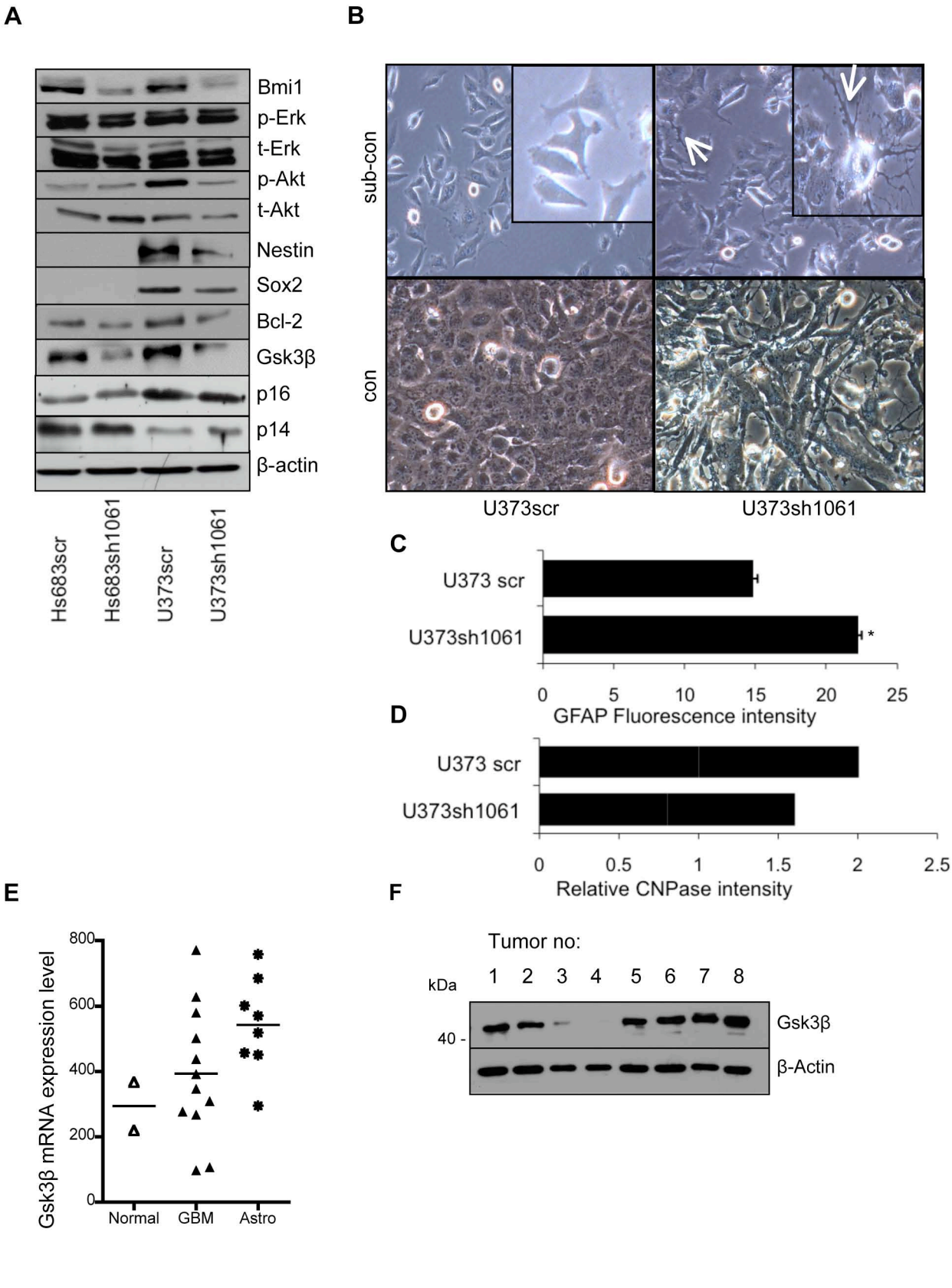
B

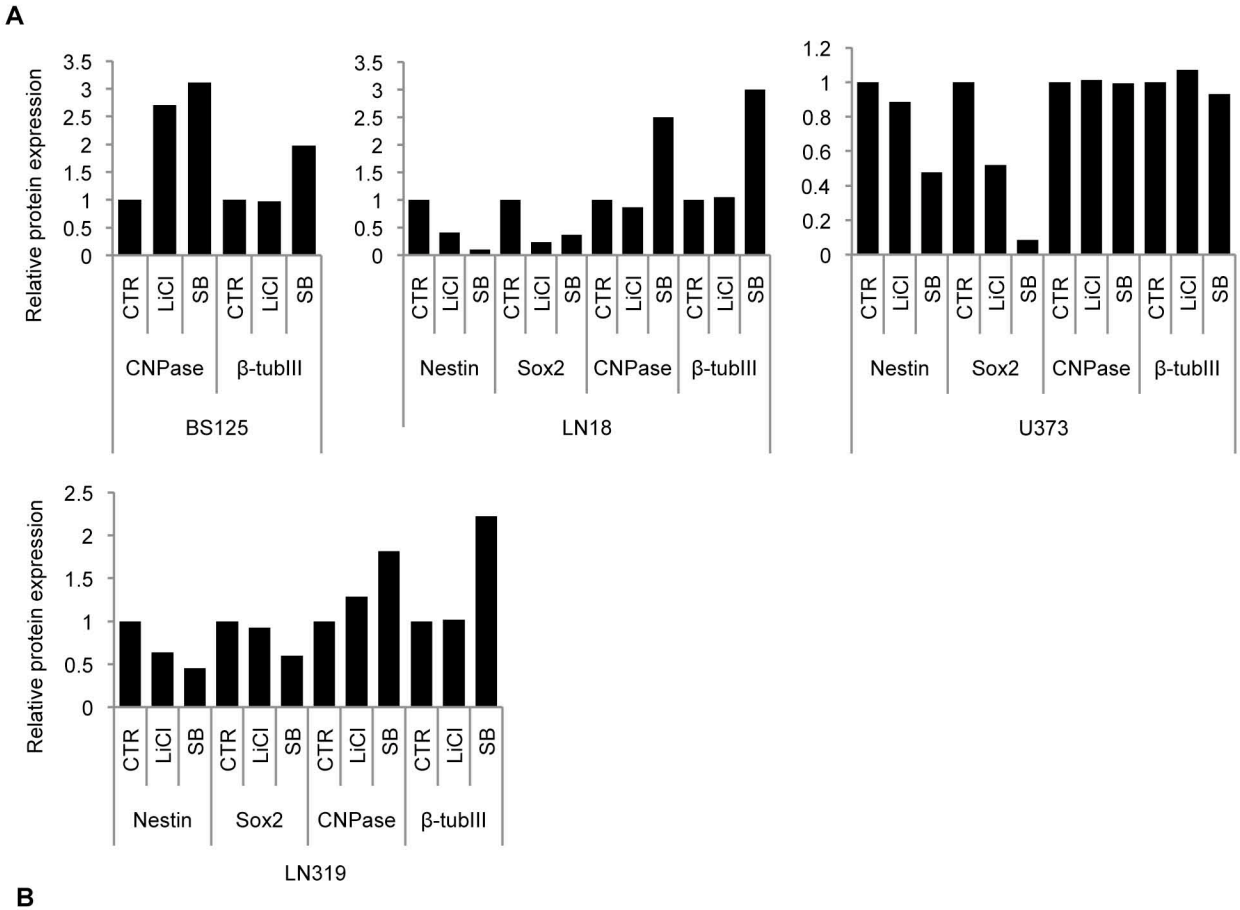


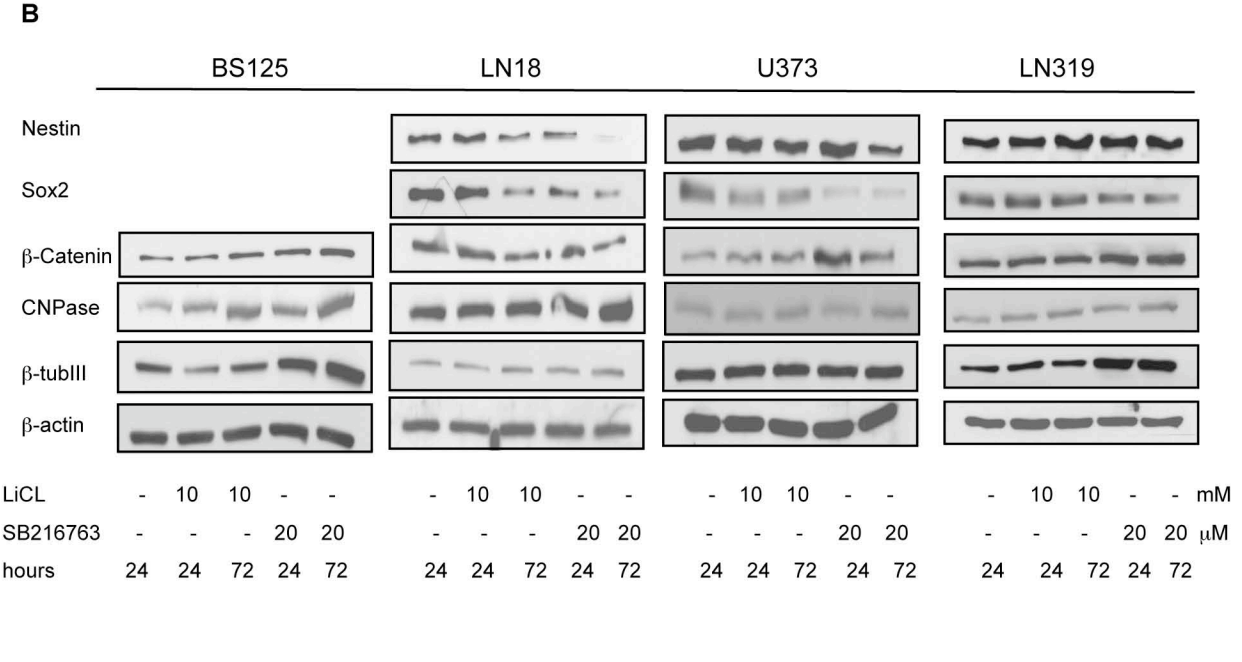
C

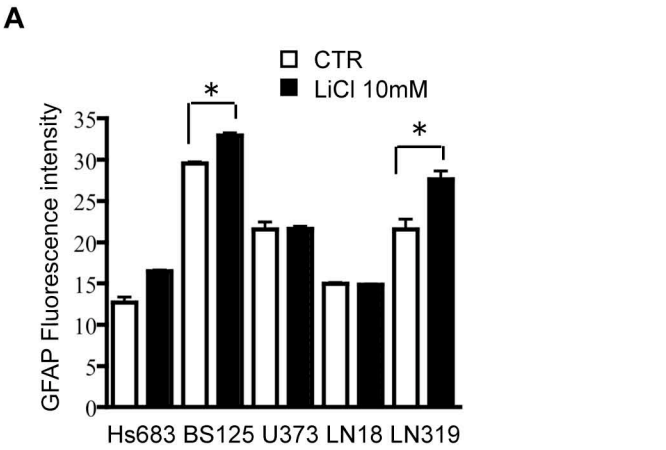
Primary tumors

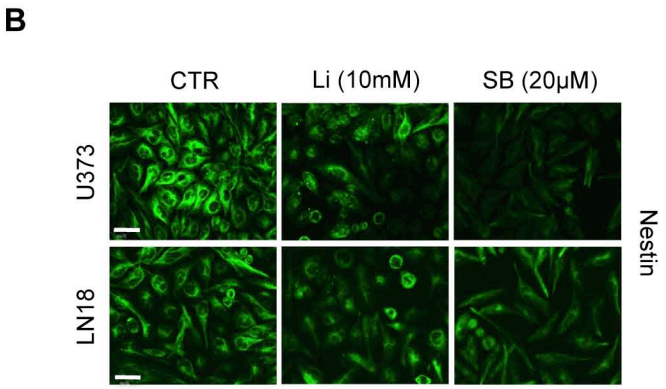
GBM	16/19
Astro	3/7
Oligo	5/7

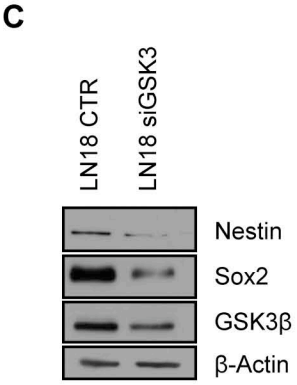

A
Bmi1
p-Erk
t-Erk
p-Akt
t-Akt
Nestin
Sox2
Bcl-2
Gsk3β
p16
p14
β-actin
Hs683scr
Hs683sh1061
U373scr
U373sh1061
B
sub-con
con
U373scr
U373sh1061
C
U373 scr
U373sh1061
*
0
5
10
15
20
25
GFAP Fluorescence intensity
D
U373 scr
U373sh1061
0
0.5
1
1.5
2
2.5
Relative CNPase intensity
E
Gsk3β mRNA expression level
800
600
400
200
0
Normal
GBM
Astro
F
Tumor no:
kDa
1 2 3 4 5 6 7 8
40 -
Gsk3β
β-Actin


A
Relative protein expression
CTR
LiCl
SB
CNPase
β-tubIII
BS125
Nestin
Sox2
LN18
U373
LN319

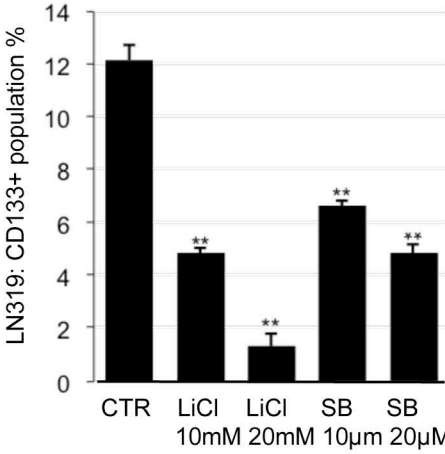

B
BS125
LN18
U373
LN319
Nestin
Sox2
β-Catenin
CNPase
β-tubIII
β-actin
LiCL
SB216763
hours
mM
μM


A
CTR
LiCl 10mM
*
*
GFAP Fluorescence intensity
35
30
25
20
15
10
5
0
Hs683 BS125 U373 LN18 LN319

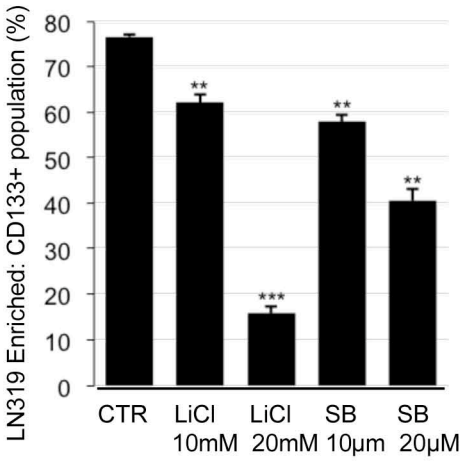

B
CTR
Li (10mM)
SB (20μM)
U373
LN18
Nestin


C
LN18 CTR
LN18 siGSK3
Nestin
Sox2
GSK3β
β-Actin

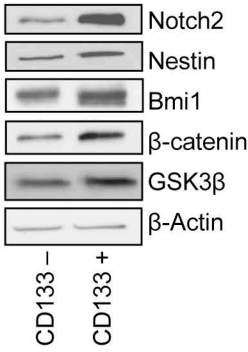
A

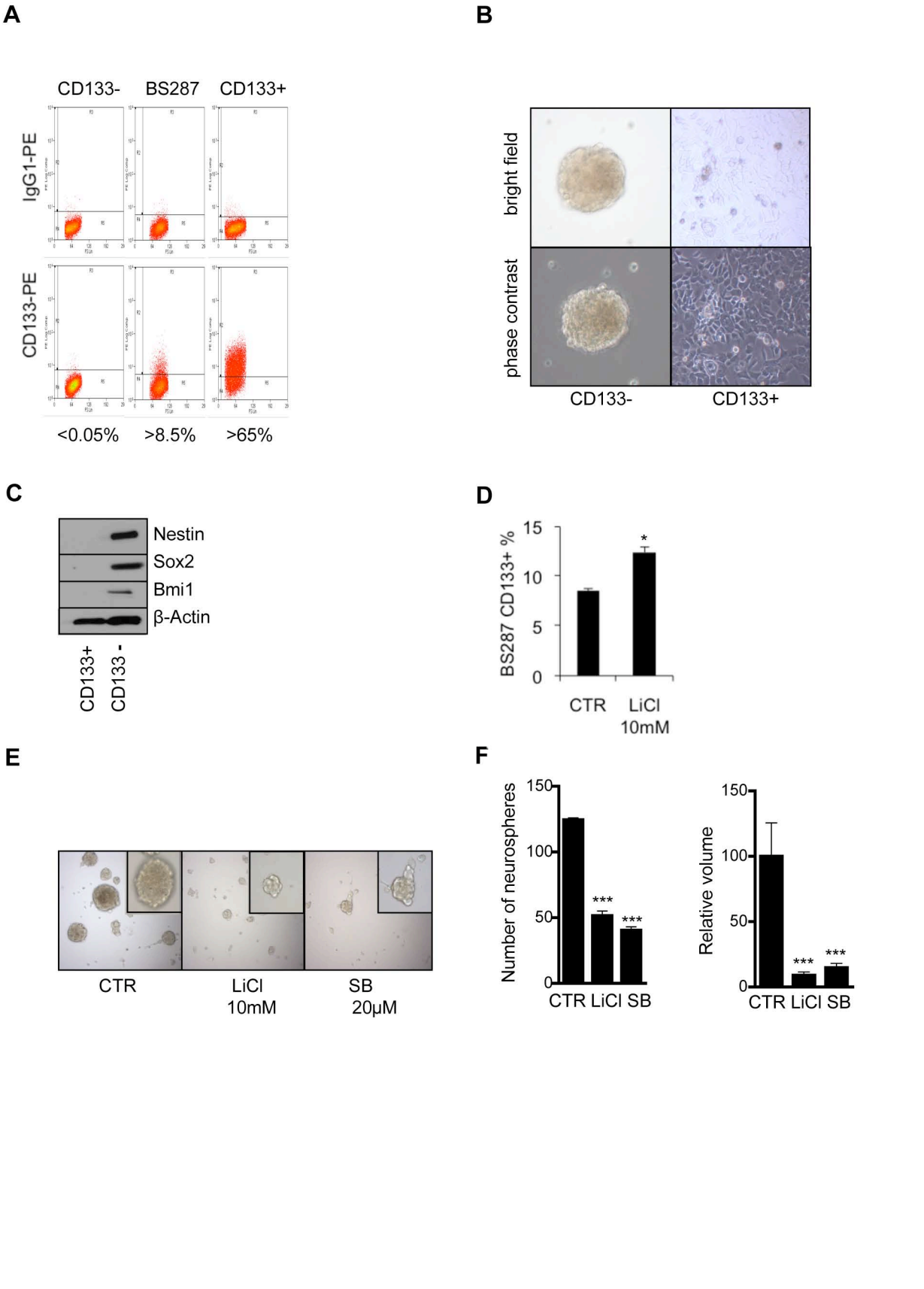

LN319: CD133+ population %
14
12
10
8
6
4
2
0
**
**
**
**
CTR
LiCl 10mM
LiCl 20mM
SB 10µm
SB 20µM

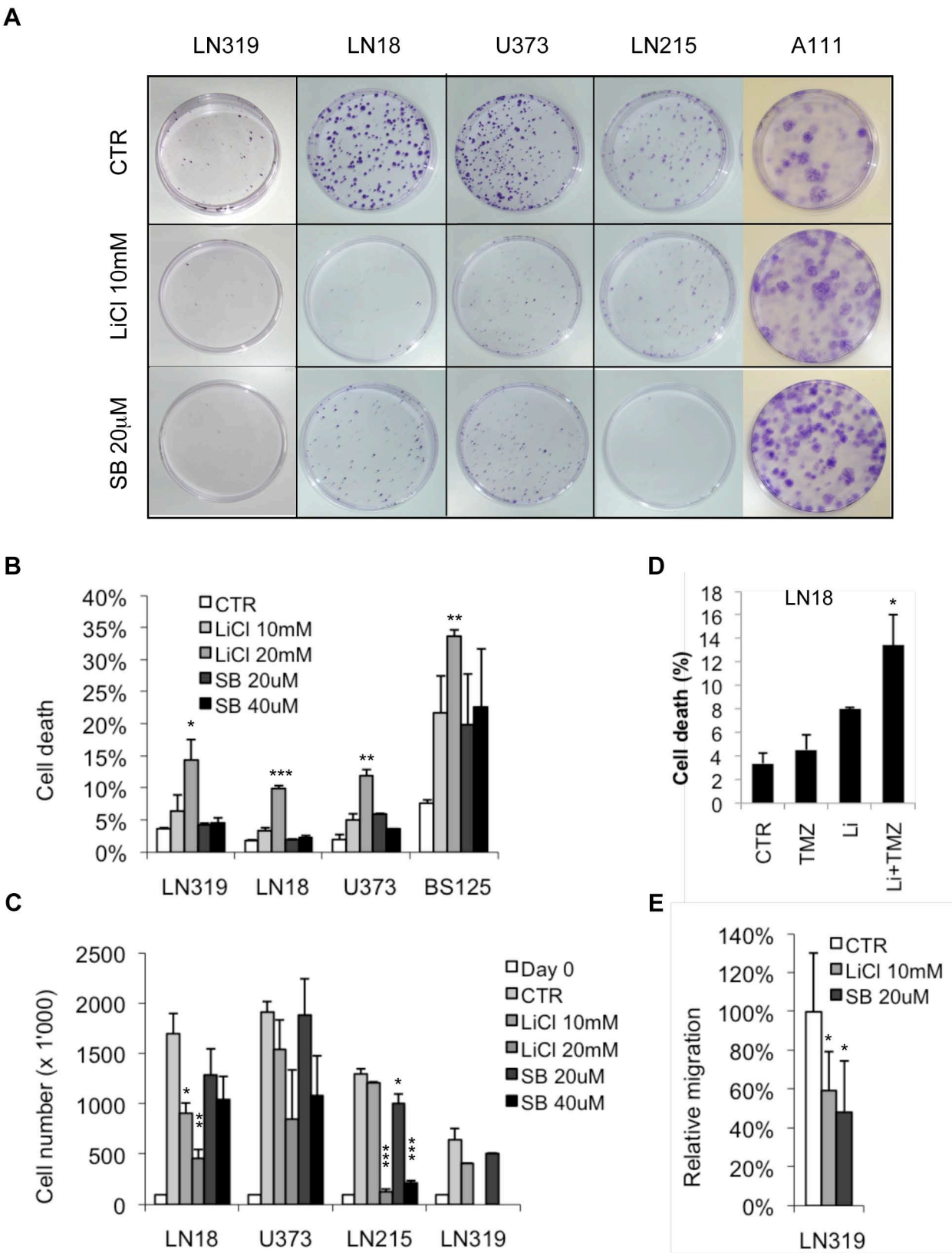
B


LN319 Enriched: CD133+ population (%)
80
70
60
50
40
30
20
10
0
**
**
***
**
CTR
LiCl 10mM
LiCl 20mM
SB 10µm
SB 20µM

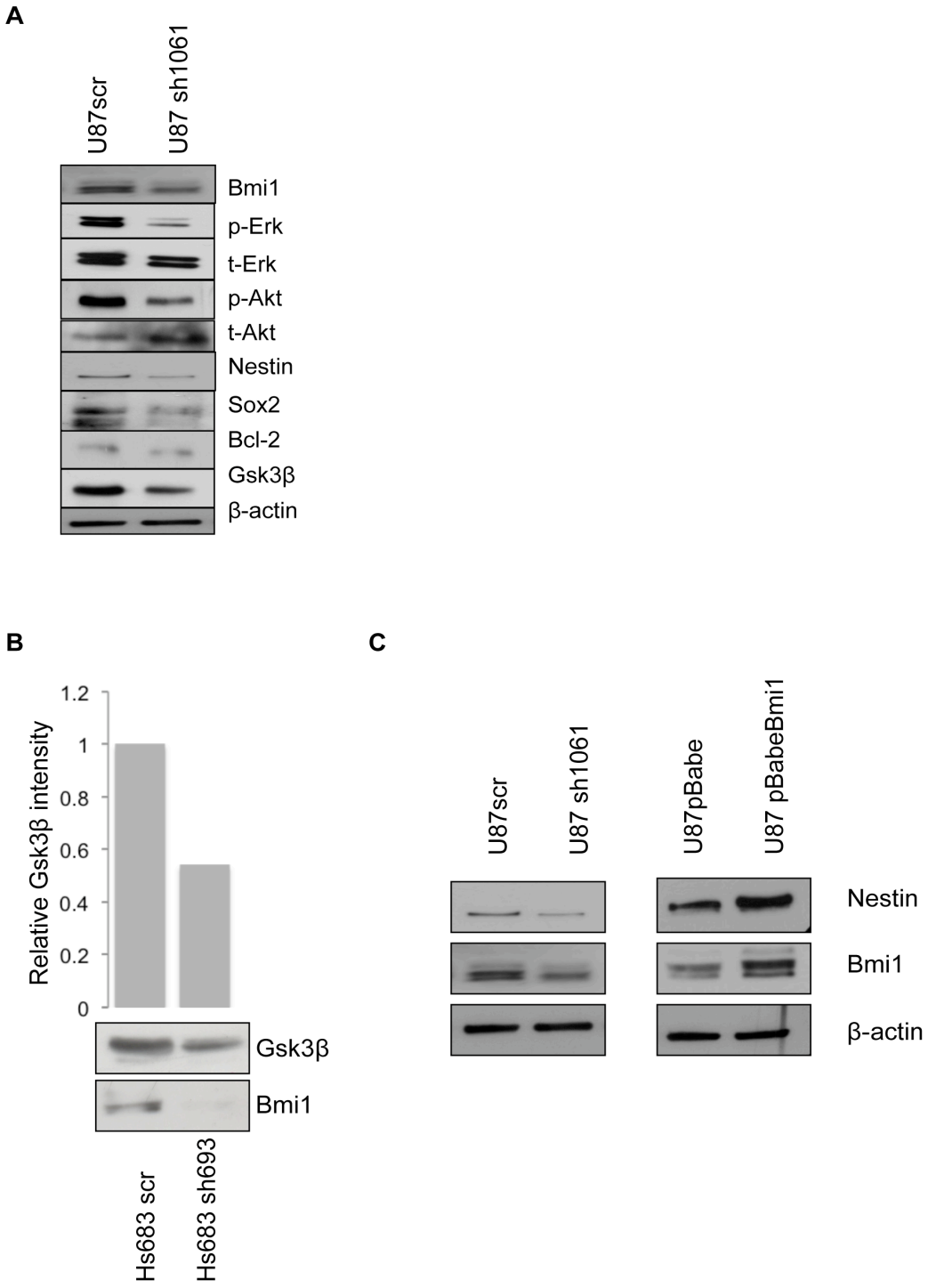
C


Notch2
Nestin
Bmi1
β-catenin
GSK3β
β-Actin
CD133 –
CD133 +

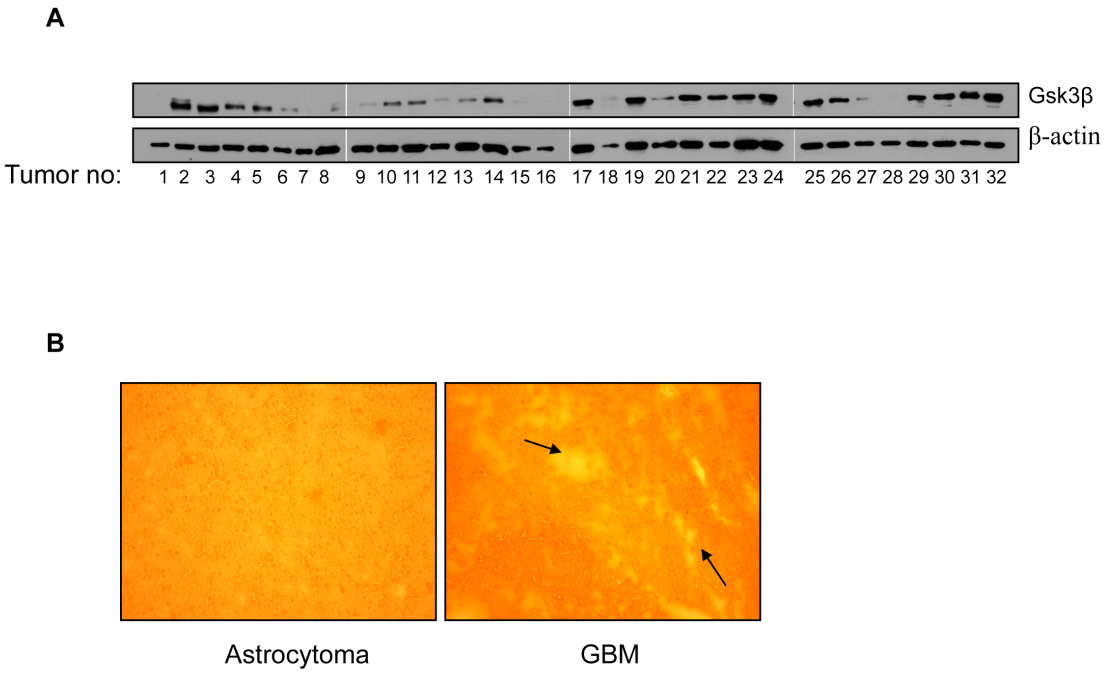

A
CD133-
BS287
CD133+
IgG1-PE
CD133-PE
<0.05%
>8.5%
>65%
B
bright field
phase contrast
CD133-
CD133+
C
Nestin
Sox2
Bmi1
β-Actin
CD133+
CD133 -
D
BS287 CD133+ %
15
10
5
0
*
CTR
LiCl
10mM
E
CTR
LiCl
10mM
SB
20μM
F
Number of neurospheres
150
100
50
0
***
***
CTR LiCl SB
Relative volume
150
100
50
0
***
***
CTR LiCl SB


A
LN319
LN18
U373
LN215
A111
CTR
LiCl 10mM
SB 20µM
B
Cell death
40%
35%
30%
25%
20%
15%
10%
5%
0%
CTR
LiCl 10mM
LiCl 20mM
SB 20uM
SB 40uM
*
***
**
**
LN319
LN18
U373
BS125
D
LN18
Cell death (%)
18
16
14
12
10
8
6
4
2
0
*
CTR
TMZ
Li
Li+TMZ
C
Cell number (x 1'000)
2500
2000
1500
1000
500
0
Day 0
CTR
LiCl 10mM
LiCl 20mM
SB 20uM
SB 40uM
*
**
*
***
***
LN18
U373
LN215
LN319
E
Relative migration
140%
120%
100%
80%
60%
40%
20%
0%
CTR
LiCl 10mM
SB 20uM
*
*
LN319

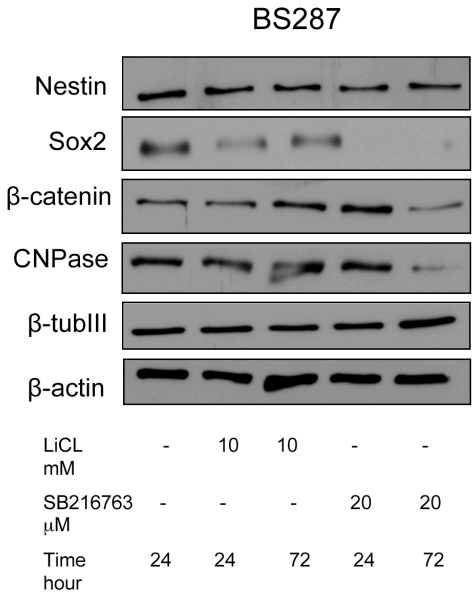
Supporting information Figure 1



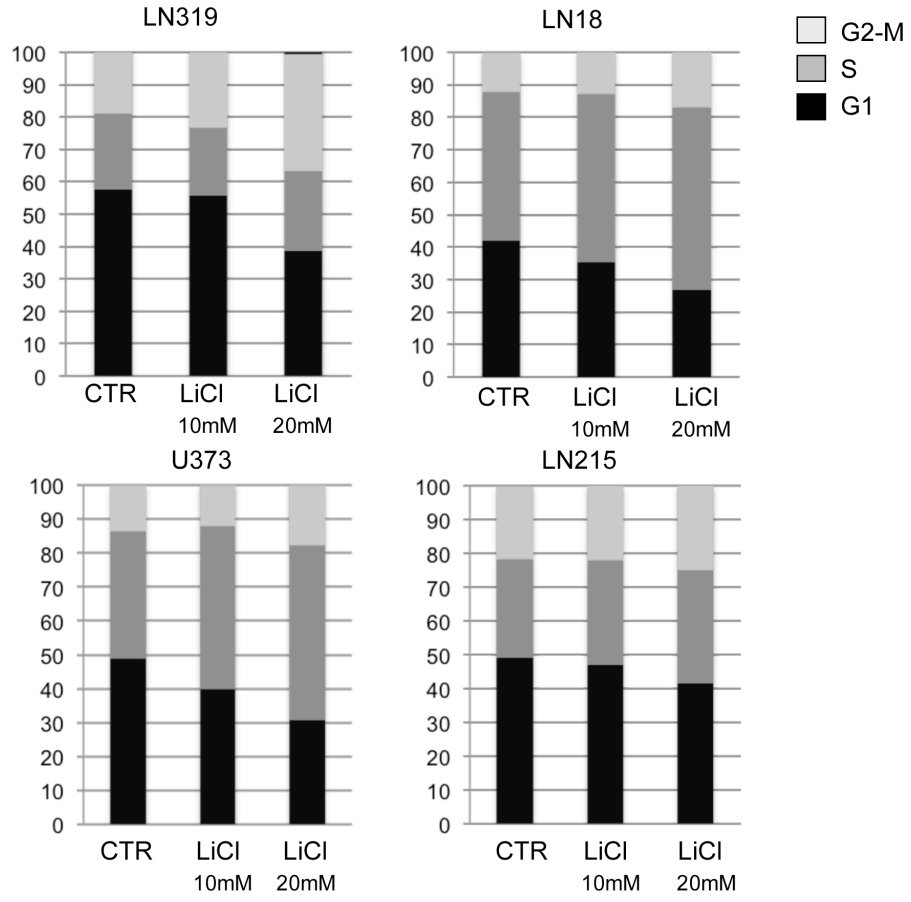
Supporting information Figure 2



Supporting information Figure 3



Supporting information Figure 4



Part 2: Combination of sublethal concentrations of epidermal growth factor receptor inhibitor and microtubule stabilizer induces apoptosis of glioblastoma cells

Mike Failly, Serdar Korur*, Viviane Egler, Jean-Louis Boulay, Maria Maddalena Lino, Roland Imber, and Adrian Merlo

Clin Cancer Res. 2008 May 15;14(10):3132-40

* Author contributed to: experimental design, performing experiments, data analysis and writing the paper

Combination of sublethal concentrations of epidermal growth factor receptor inhibitor and microtubule stabilizer induces apoptosis of glioblastoma cells

Mike Failly, Serdar Korur, Viviane Egler, Jean-Louis Boulay, Maria Maddalena Lino, Roland Imber, and Adrian Merlo

Laboratory of Molecular Neurooncology, Departments of Research and Surgery, University Hospitals, Basel, Switzerland

Abstract

The oncogenic epidermal growth factor receptor (EGFR) pathway triggers downstream phosphatidylinositol 3-kinase (PI3K)/RAS-mediated signaling cascades. In transgenic mice, glioblastoma cannot develop on single but only on simultaneous activation of the EGFR signaling mediators RAS and AKT. However, complete blockade of EGFR activation does not result in apoptosis in human glioblastoma cells, suggesting additional cross-talk between downstream pathways. Based on these observations, we investigated combination therapies using protein kinase inhibitors against EGFR, platelet-derived growth factor receptor, and mammalian target of rapamycin, assessing glioblastoma cell survival. Clinically relevant doses of AEE788, Gleevec (imatinib), and RAD001 (everolimus), alone or in combinations, did not induce glioblastoma cell apoptosis. In contrast, simultaneous inactivation of the EGFR downstream targets mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase and PI3K by U0126 and wortmannin triggered rapid tumor cell death. Blocking EGFR with AEE788 in combination with sublethal concentrations of the microtubule stabilizer patupilone also induced apoptosis and reduced cell proliferation in glioblastoma cells, accompanied by reduced AKT and ERK activity. These data underline the critical role of the PI3K/AKT and the RAS/RAF/mitogen-activated protein/ERK kinase/ERK signaling cascades in the cell-intrinsic survival program of sensitive glioblastoma cell lines. We conclude that drug combinations, which down-regulate both ERK and protein kinase B/AKT activity, may prove effective in overcoming cell resistance in a subgroup of glioblastoma. [Mol Cancer Ther 2007; 6(2):773–81]

Introduction

Glioblastoma multiforme is the most frequent malignant neoplasm of the human central nervous system. Surgery can only control the highly proliferative component of the disease, whereas widespread tumor cell infiltration into normal brain areas resists radiotherapy and chemotherapy (1, 2). Amplification and overexpression of the gene encoding the epidermal growth factor (EGF) receptor (EGFR) are detected in ~50% of glioblastomas and are mainly associated with disease progression (3, 4). EGFR is a member of the ErbB family of receptor tyrosine kinases (5). Small molecular weight compounds with EGFR protein kinase inhibitory (PKI) activity, such as PKI-166 or AEE788, have a cytostatic effect *in vitro* on tumor cells that overexpress EGFR (6, 7). In addition, treatment of non–small cell lung cancer with the EGFR PKI imatinib (gefitinib) resulted in tumor growth control in 10% of patients (8). In fact, tumors that responded to gefitinib specifically carried specific mutations in the EGFR tyrosine kinase domain (8–10). Although responses to gefitinib have also been observed in a limited number of glioblastoma cases (11), a specific molecular profile is associated with response that differs from the lung signature (12–15). To date, several small molecular weight inhibitors, such as Gleevec or erlotinib/gefinitib, applied as monotherapies for the treatment of gliomas, only resulted in limited effectiveness (16). This has supported the hypothesis that combination of drugs would be a more appropriate treatment for glioma.

EGF mediates signaling via phosphatidylinositol 3-kinase (PI3K)/AKT and RAS/mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK pathways (17–19), which are redundantly stimulated by other activated growth factor receptors (e.g., the insulin-like growth factor receptor-I; ref. 20). In at least 50% of glioblastoma, PI3K is activated either by loss of function of the tumor suppressor PTEN (21) or by gain-of-function mutations in the *PIK3CA* gene, which encodes the p110 catalytic subunit of PI3K (22). Interestingly, activated protein kinase B (PKB)/AKT cooperated with RAS in the induction of malignant gliomas in a murine brain tumor model (23). Furthermore, activation of PKB/AKT has been detected in several types of human cancers and found to be associated with poor clinical outcome (24–26) and resistance to chemotherapy and radiotherapy (24, 27, 28).

Received 9/13/06; revised 11/22/06; accepted 12/21/06.

Grant support: Swiss National Foundation grant 31-67123.01, Oncosuisse grant 01338, Regional Cancer League of Basel, and Novartis Oncology (Basel, Switzerland).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Adrian Merlo, Laboratory of Molecular Neurooncology, Departments of Research and Surgery, University Hospital Basel, Spitalstrasse 21, 4031 Basel, Switzerland. Phone: 41-61-265-71-82; Fax: 41-61-265-71-38. E-mail: amerlo@uhbs.ch

doi:10.1158/1535-7163.MCT-06-0566

Although no RAS mutations have been detected in glioblastoma, high levels of RAS-GTP have been documented in high-grade astrocytomas (29, 30). It has been suggested that ERK plays a critical role in cellular transformation and resistance to apoptosis (31). Evidence for a cooperation between AKT and RAS has been proven by a transgenic mouse model in which both oncogenes are ectopically expressed in normal astrocytes, giving rise to glioblastoma, whereas neither AKT nor RAS alone was sufficient for tumor induction (23).

Several examples of combined therapies have recently undergone advanced clinical trials in cancer treatment. For example, lapatinib with tamoxifen has been tested together in breast cancer (32), and in xenografted tumors (32), PKI AEE788 has been associated with the rapamycin-derivative RAD001 for the treatment of glioblastoma (33–35). Successful drug combinations allow the use of lower doses, possibly reducing toxicity and limiting the degree of acquired drug resistance. Given the limited efficacy of PKI to induce cell death in rapidly proliferating tumor cells, these inhibitory drugs may depend on the coadministration of a cytotoxic drug for the induction of cell death rather than on the coadministration of a second cytostatic compound. The principle of cytotoxicity of patupilone (epothilone B, EPO906) relies on the inhibition of microtubule depolymerization, which is lethal for all dividing cells. The antitumor activity of patupilone has been proven *in vitro* and *in vivo* in lung, breast, colon, and prostate cancers (36). Patupilone also shows clinical activity in a range of solid tumors (37) and is now in phase III clinical development.

In the present study, we examined the induction of cell death on glioblastoma lines *in vitro* by targeting EGFR/ErbB2 by the receptor tyrosine kinase inhibitor AEE788 alone or in combination with the cytotoxic compound patupilone. We further evaluated the activation status of the EGFR downstream signaling mediators PKB/AKT and ERK following combined treatment with patupilone and AEE788. Our study points to a critical role of both PI3K and ERK in glioblastoma cell survival.

Materials and Methods

Cell Culture

All cell lines were grown at 37°C in a 5% CO_2 humidified atmosphere. DMEM culture medium was supplemented with 10% FCS and standard antibiotics. Cells were detached with 1 mL trypsin-EDTA (1×) for 5 min at 37°C. The genetic status of all cell lines at *p53, p16, p14ARF*, and *PTEN* tumor suppressor genes has been previously reported (38, 39).

Pharmacologic Inhibitors

AEE788, RAD001, and patupilone (provided by Novartis Pharma AG, Basel, Switzerland) and U0126 (Promega, Madison, WI) were dissolved in DMSO as 10 mmol/L stocks and stored as aliquots at −20°C. Wortmannin (Sigma, St. Louis, MO) was prepared as a 1 mmol/L stock solution in DMSO and stored at 4°C, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA; LC Laboratories, Woburn, MA) was prepared as a 2 mmol/L stock solution in DMSO, aliquoted, and stored at −20°C. Treatment of the cells with TPA, U0126, or wortmannin consisted of daily additions without replacing the medium.

Measurement of Cell Proliferation and Cell Cycle Profile

In experiments shown in Fig. 1B, cells were grown for 24 h in DMEM supplemented with 10% FCS and for an additional 24 h in the presence of the drug. Bromodeoxyuridine (BrdUrd) was added 1 h before cell harvesting to a 10 μmol/L final concentration. Fluorescence-activated cell sorting analysis was done according to the manufacturer's instructions (Becton Dickinson, Franklin Lakes, NJ). The cell proliferation assays presented in Figs. 2C and 3B were done with the Biotrak ELISA System version 2 (Amersham Biosciences Corp., Piscataway, NJ) according to the manufacturer's instructions. Cells (5×10^3) were seeded in 96-well plates and grown for 24 h, and drugs were applied for an additional 24 h. BrdUrd incorporation was allowed during the last 2 h of treatment. In experiments shown in Fig. 1D, cells were grown for 24 h in DMEM supplemented with 10% FCS and for an additional 24 h in the presence of the drug. Cell DNA content and apoptosis were analyzed on a FACSCalibur. Cells were trypsinized and fixed in 70% ice-cold ethanol for 1 h and stained with 50 μg/mL propidium iodide for fluorescence-activated cell sorting analysis. The percentages of cells in the cell cycle phases are reported (results from three independent experiments).

Measurement of Apoptosis

Expanding cells were subjected to drug treatment for 24 h, briefly washed with PBS, and trypsinized. Cell suspension was pelleted at 800 rpm for 5 min, fixed in ice-cold 70% ethanol, and kept at 4°C for 30 min. Cells were resuspended in a 1× PBS solution containing 50 ng/mL propidium iodide and 50 μg/mL RNase. Percentage of cells in sub-G_1 for apoptosis was determined by flow cytometry.

Protein Extraction and Western Blot Analysis

Cells were washed with PBS, resuspended in 1× SDS sample buffer (62.5 mmol/L Tris-HCl, 2% SDS, 10% glycerol, 50 mmol/L DTT), and boiled at 95°C during 5 min; aliquots were stored at −20°C. Proteins (30 μg) were separated by size on SDS-PAGE gels (10%) and transferred to nitrocellulose membranes (Hybond ECL, Amersham Biosciences). Western blot analysis was done using antibodies against the following proteins: phosphorylated AKT (Upstate Biotechnology, Lake Placid, NY), ERK1/2, phosphorylated ERK, phosphorylated EGFR (Tyr^{1173}), and EGFR (Santa Cruz Biotechnology, Santa Cruz, CA) and actin (Sigma). The anti-AKT antibody was a gift from Dr. Brian Hemmings (Friedrich Miescher Institute, Basel, Switzerland). Western blots were developed with enhanced chemiluminescence reagents (Pierce, Rockford, IL).

Results

Specific Targeting of the EGF Signaling Pathway by AEE788

Nearly 50% of primary glioblastomas exhibit amplification of the *EGFR* gene (3, 4), which is also associated with

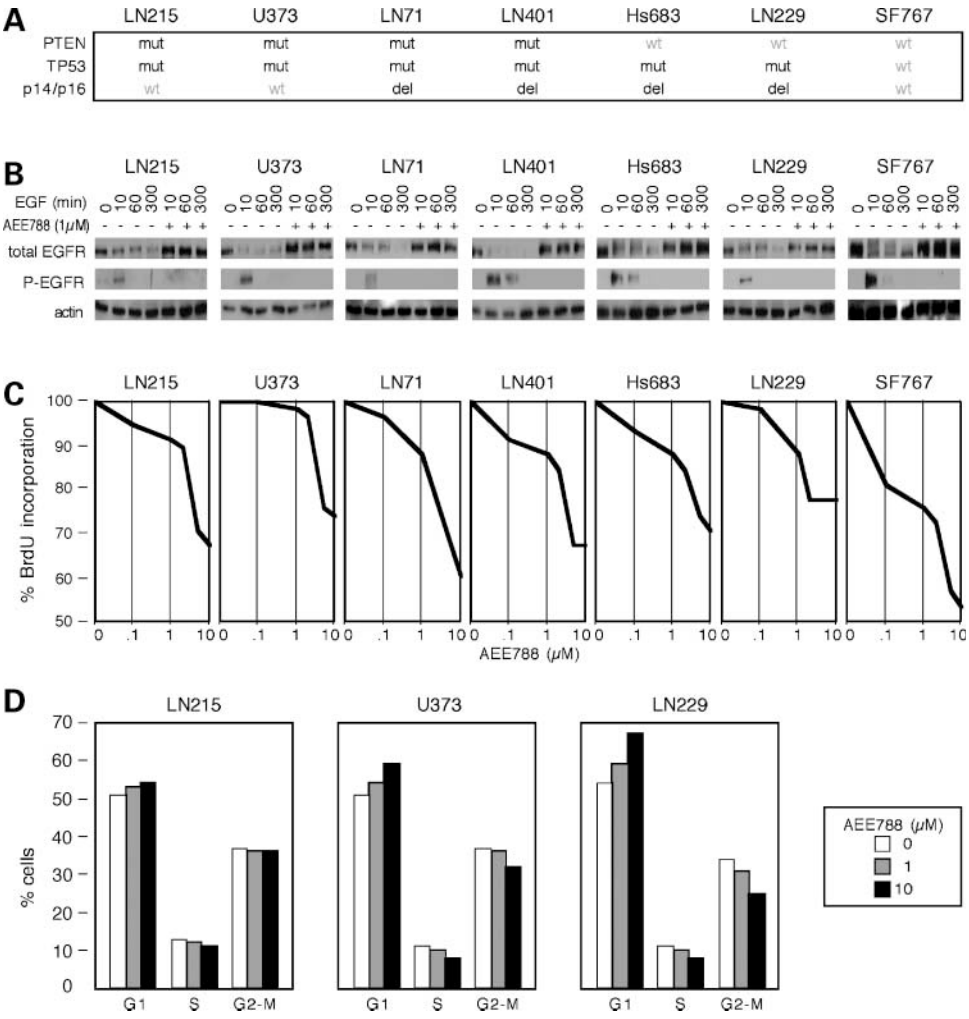


Figure 1. Treatment of glioblastoma cell lines with EGFR inhibitor. **A,** genotyping of used glioma cell lines by Ishii et al. (39). **B,** cells were preincubated in the presence or absence of 1 μmol/L of the EGFR inhibitor AEE788 during 30 min and stimulated with 100 ng/mL EGF for 10, 60, and 300 min. EGFR, phosphorylated EGFR (Tyr^{1173}), and actin were detected by Western blot analysis of cell lysates. **C,** cells were incubated during 4 d with increasing concentrations of AEE788 (0, 1, 2, 5, and 10 μmol/L). Proliferation was measured by BrdUrd and flow cytometry. Experiments were done in triplicate. *Points,* average of three independent determinations; *bars,* SD. **D,** cell cycle phase analysis on glioblastoma cell lines on 1 and 10 μmol/L of AEE788 treatment.

poor prognosis. For this reason, the inhibitor of EGFR and of ErbB receptor protein kinase activities AEE788 has been considered to be a potent drug to induce cell death in glioblastoma cell lines. AEE788 was tested for its ability to induce cell growth arrest and apoptosis in various cell lines derived from glioblastoma, for which the genetic status of established cancer genes had been previously defined (Fig. 1A; refs. 38, 39). For a preliminary determination of the minimal drug concentration needed to fully prevent EGFR activation by EGF, increasing concentrations of AEE788 were applied to the LN229 glioblastoma cell line, and EGFR/ErbB receptor activation status was monitored by the presence of EGFR phosphorylated at Tyr^{1173}. To completely block receptor phosphorylation, 1 μmol/L was needed (data not shown). Further time course experiment done with 1 μmol/L AEE788 showed a strong inhibition of the transient phosphorylation of Tyr^{1173} as well as of the degradation of the receptor protein in all seven glioblastoma cell lines tested (Fig. 1B).

Increasing concentrations of AEE788 were applied to the glioblastoma cell lines, and proliferation was assayed by integration of BrdUrd after 1 day. At a 1 μmol/L concentration, AEE788 gives up to 20% of proliferation reduction compared with the control cell population (Fig. 1C). Cell cycle analysis after 24 h of 1 and 10 μmol/L of AEE788 treatment shows an increase of cell population in the G_1 phase (Fig. 1D).

Cell viability was assayed by flow cytometry after 24, 48, and 96 h. Very low or no apoptosis was induced at drug concentrations <2 μmol/L as late as 96 h (Fig. 2A). High levels of apoptosis were observed at unphysiologically high concentration (10 μmol/L) in five of the seven cell lines (SF767, LN215, U373, LN229, and LN71). Under the same condition, LN401 and Hs683 did not show significant levels of apoptosis. Because AEE788 was not capable of inducing strong apoptosis in glioblastoma cells when used as a single drug, we tested whether a specific double or a triple combination of compounds would significantly increase the induction rate of apoptosis. For this purpose, we combined suboptimal doses of AEE788 (0.5 μmol/L) with two additional biological drugs: Gleevec (imatinib, 0.5 μmol/L) and RAD001 (everolimus, 20 nmol/L). In gliomas, Gleevec is being explored as a PKI of platelet-derived growth factor receptor (34), which is activated in

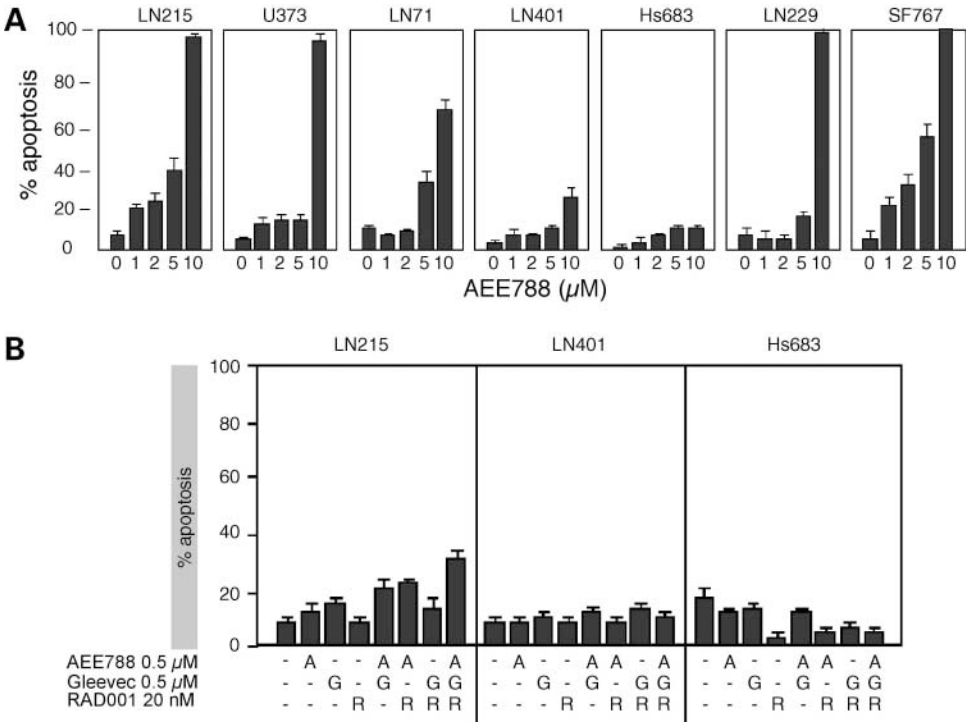


Figure 2. Survival of glioblastoma cell lines on treatment with EGFR inhibitor and in combination with Gleevec and RAD001. **A,** cells were incubated during 4 d with increasing concentrations of AEE788 (0, 1, 2, 5, and 10 μmol/L). Apoptosis was measured by flow cytometry. Experiments were done in triplicate. *Columns,* average of three independent determinations; *bars,* SD. **B,** combinatorial study of biological drugs AEE788 (A), Gleevec (G), and RAD001 (R) on glioma cell survival.

primary tumors (40, 41). Platelet-derived growth factor receptor phosphorylation levels correlated with sensitivity to Gleevec in glioma primary cultures (42). RAD001, a derivative of rapamycin, inhibits mammalian target of rapamycin, leading to inactivation of ribosomal S6K1 and inhibition of cap-dependent translation (43).

The application of the combined drugs to 10 glioblastoma cell lines for 4 days revealed that there was a combination-specific and a cell line–specific response with regard to induction of cell death (Fig. 2B). The cell lines could be divided into three categories. One group (SF767, LN18, and LN215; Fig. 2B, *left*) showed a modest additive induction of apoptosis by all of the three drugs, reaching a maximum of 35% of cell death with the triple combination. A second group of glioblastoma cell lines (LN401, LN71, LN229, and LN319; Fig. 2B, *middle*) was basically insensitive to all drug combinations, showing the same rate of apoptosis as untreated cells. The third group (Hs683, U373, and U343; Fig. 2B, *right*) exhibited a relatively high level of basal apoptosis (15–20%), which was, however, decreased by the three drugs applied together. Especially, RAD001 exhibited an antiapoptotic effect, alone or in combination. No differences in apoptosis rate were evidenced between PTEN-mutant (LN215, LN401, LN71, and U373) and wild-type (Hs683, SF767, and LN229) cell lines on RAD001 application. Although, in theory, RAD001 was expected to overcome the constitutive AKT activation resulting from loss of PTEN activity.

Taken together, the results indicated that neither single nor combined application of the three biological drugs, at physiologic concentration, was able to strongly induce cell death in glioblastoma cell lines.

Induction of Apoptosis in Glioblastoma Cell Lines by Patupilone

Because neither the single nor the combined application of the three biological drugs led to a consistent induction of apoptosis, we tested whether the combination of AEE788 with a cytotoxic drug could result in a more cooperative induction of apoptosis. Patupilone is a member of the group of epothilones, which represent a new class of low molecular weight compounds that target microtubules by inhibiting their depolymerization and therefore impairs cell division (44). Patupilone prevents chromosome alignment at metaphase and drives cells to undergo apoptosis (45). It has been shown to exhibit antitumor activity *in vitro* and *in vivo* (36, 37). As a result of inhibition of microtubule depolymerization, after addition of patupilone, dramatic changes in cell morphology occurred within 18 h (Fig. 3A, *right*). To investigate its ability to induce apoptosis, glioblastoma cells were treated with increasing concentrations of patupilone in the picomolar and nanomolar concentration range for 4 days (Fig. 3A, *left*). Although 100% apoptosis was induced in all cell lines when the highest drug concentration (35 nmol/L) was applied, levels of apoptosis induced by 0.7 nmol/L patupilone strongly varied between the cell lines, from Hs683 and LN401, the most resistant, to LN229, the most sensitive one.

Combined Treatment with AEE788 and Patupilone Strongly Induced Apoptosis and Reduced Proliferation of Glioblastoma Cells

AEE788 (1 μmol/L) was applied together with increasing concentrations of patupilone on cell lines LN71, LN229, Hs683, and SF767, and cell survival was determined after 4 days (Fig. 3B). The combined drugs induced apoptosis in

a cooperative manner in LN71 and SF767 but not in Hs683 and LN229 cell lines. In LN71 and SF767 lines, the patupilone concentration required to induce apoptosis in 50% of cells (AC_{50}) revealed that AEE788 acted as a sensitizer, reducing the patupilone concentration from a nanomolar to a picomolar concentration range. Thus, addition of AEE788 shifted down the amount of patupilone required to induce apoptosis in 50% of cells, up to 2 orders of magnitude in SF767 cells. It is noteworthy that the synergistic effect of patupilone on those glioma cell lines seemed independent of their respective sensitivities to AEE788. Consistent with the absence of synergy of both compounds on LN229 cells, also proliferation was not affected either by AEE788 or patupilone or by the combination of both drugs. In contrast, SF767 cells, on which the AEE788/patupilone synergized the best, had a significant reduction of proliferation up to 80% ($P < 0.001$) after each individual or combined drug application (Fig. 3C).

Simultaneous Inhibition of Both ERK1/2 and AKT Kinase Activities Parallels the Induction of Apoptosis

Both signaling pathways, PI3K/AKT and RAS/MEK/ERK, are under the control of EGF-directed activation of ErbB receptors. To investigate the activation of these two pathways, phosphorylation status of the kinases AKT and ERK was chosen as readouts. AEE788 (1 μmol/L) and patupilone (0.7 nmol/L), alone and in combination, were applied for 24 and 72 h to the four glioblastoma cell lines that differed remarkably in their sensitivities toward those drugs. In contrast to LN229 and Hs683, LN71 and SF767

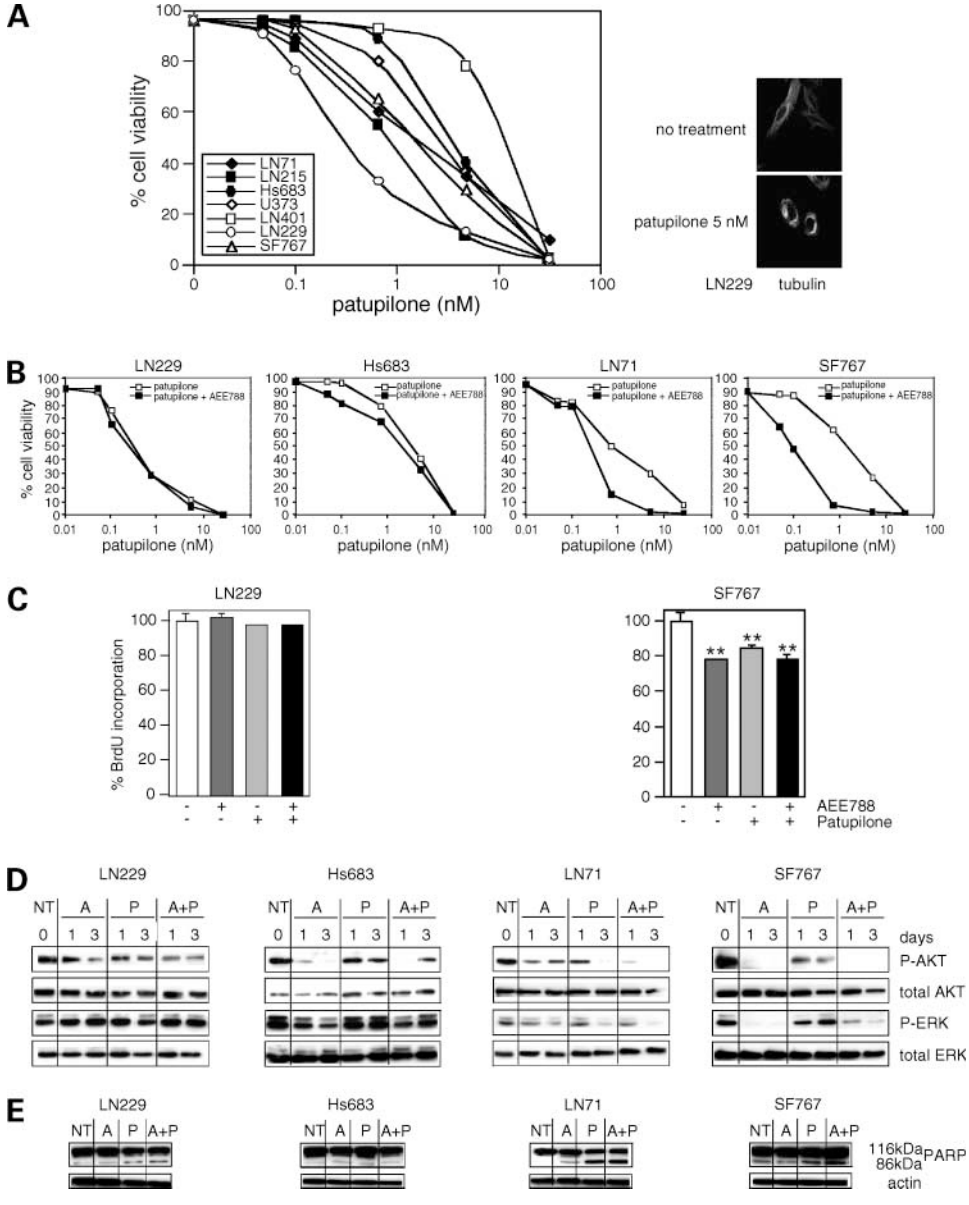


Figure 3. Combined treatment of glioblastoma cells stabilizing microtubules and blocking EGFR. **A,** increasing concentrations of patupilone (0.05, 0.1, 0.7, 5, and 35 nmol/L) were applied to seven cell lines for 4 d and the percentage of viable cells was determined using flow cytometry. *Points,* average of three independent determinations; *bars,* SD. Confocal microscopy of LN229 cells after 18 h of patupilone treatment. **B,** cells were treated for 4 d either with increasing concentrations of patupilone alone or in combination with 1 μmol/L AEE788. Cell survival was measured by flow cytometry. *Points,* average of three independent experiments; *bars,* SD. **C,** cells were treated with 1 μmol/L AEE788 and/or 0.7 nmol/L patupilone and assayed for proliferation with BrdUrd by ELISA. **D,** *top,* cells were incubated during 24 h and 3 d with patupilone (0.7 nmol/L), AEE788 (1 μmol/L), or both. Western blot analysis was done on cell lysates. *NT,* no treatment; *A,* AEE788; *P,* patupilone. *Bottom,* poly(ADP-ribose) polymerase (*PARP*) cleavage after 24 h.

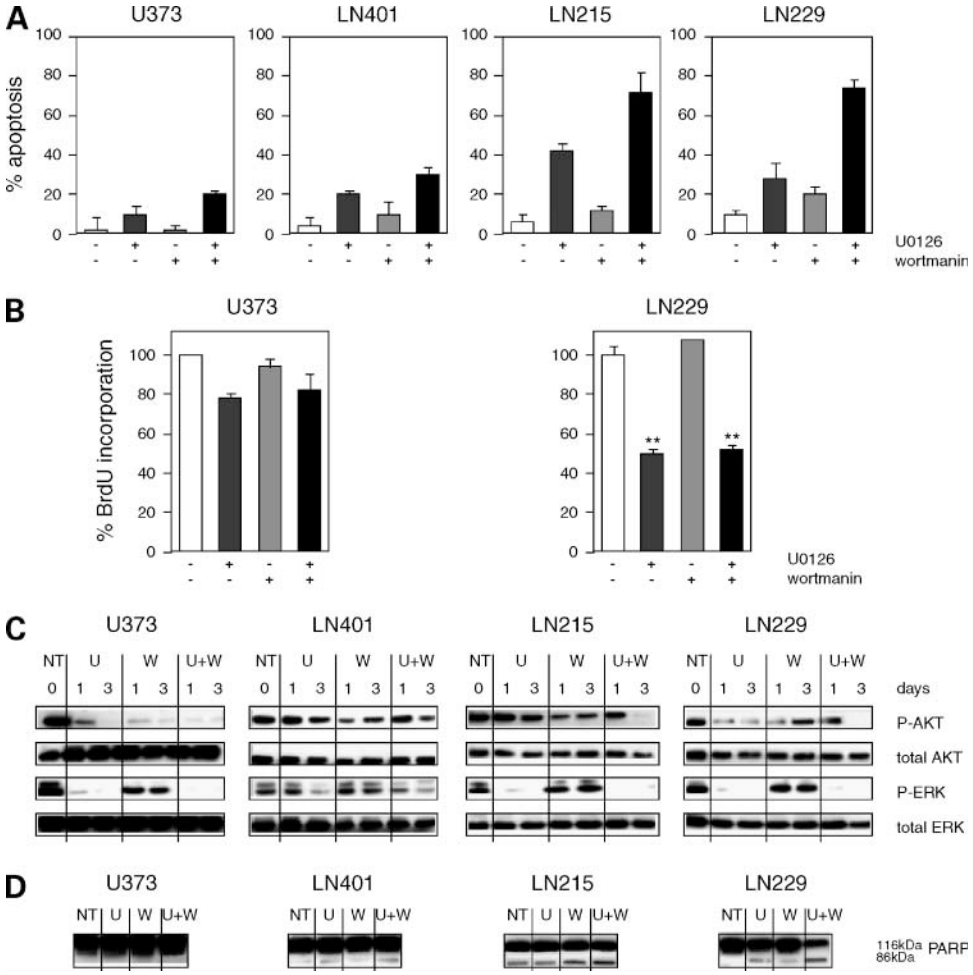


Figure 4. Combined treatment of glioblastoma cells blocking PI3K and MEK. **A,** cell lines were treated with the MEK inhibitor U0126 (20 μmol/L) and/or the PI3K inhibitor wortmannin (1 μmol/L) for 3 d, whereby the addition of both drugs was repeated every day. Percentage of apoptotic cells was measured using flow cytometry. *Columns,* mean of three independent experiments; *bars,* SD. **B,** cells were treated with 20 μmol/L U0126 and/or 1 μmol/L wortmannin and assayed for proliferation with BrdUrd by ELISA. **C,** Western blot analysis was done to determine the relative phosphorylation levels of ERK1/2 and AKT after 3 d of treatment. *U,* U0126; *W,* wortmannin. **D,** poly(ADP-ribose) polymerase (PARP) cleavage after 24 h.

showed full down-regulation of phosphorylation of both protein kinases after 3 days of combined treatment (Fig. 3D), accompanied by widespread apoptosis between 85% and 95% of cells. In contrast, LN229 and Hs683 still retained strong phosphorylation of the two protein kinases and showed much lower levels of apoptosis. These results suggested a link between the simultaneous inhibition of the two EGF-dependent signaling pathways and the induction of apoptosis triggered by the combination of patupilone and AEE788. To support this hypothesis, we analyzed the cleavage status of poly(ADP-ribose) polymerase, which triggers caspase-dependent apoptosis (46, 47). The cleaved form of poly(ADP-ribose) polymerase was indeed detected in glioblastoma cells undergoing apoptosis (Fig. 3E).

Simultaneous Inhibition of AKT and MEK Cooperatively Induces Apoptosis in a Subgroup of Glioblastoma Cell Lines

To test whether sensitivity of LN71 and SF767 cells to the AAE788-patupilone combination is due to low phosphorylation levels of AKT and/or ERK, inhibitors of PI3K (wortmannin) and MEK (U0126) were used to abrogate activation of AKT and ERK pathways. The potential to induce cell death was tested by applying single or combined inhibitors to four glioblastoma cell lines and measuring the proportion of apoptotic cells (Fig. 4A). When used as a single compound, U0126 was able to induce a significant level of apoptosis in LN215 and LN229 cells but to a much lesser extent in U373 and LN401 cells, whereas wortmannin alone had no or very little effect. However, strong induction of apoptosis was caused by the combination of the two inhibitors. On U0126 application, proliferation was significantly decreased up to 50% in LN229 cell line ($P < 0.001$), whereas U373 cells showed a 20% reduction. Again, wortmannin alone had no effect on reduction of proliferation on both LN229 and U373 cells, and no synergistic effect was present after drug combination (Fig. 4B).

U373 and LN401 generally exhibited much less sensitivity toward the two inhibitors. To examine the long-term effect of the inhibitors on phosphorylation of AKT and ERK, the two most sensitive and the two much less sensitive cell lines were treated for 24 h as well as for 3 days by applying fresh doses of the compounds daily. The activation status of AKT and ERK was determined as a readout for drug efficiency (Fig. 4C). In response to the combination of U0126 and wortmannin, a decrease of

phosphorylated ERK and phosphorylated AKT occurring in all glioblastoma lines confirmed the activity of the two drugs used. Unexpectedly, on treatment with the MEK inhibitor, U0126, U373, and LN229 cell lines show a decrease of phosphorylated AKT levels, suggesting a cross-talk between the RAS/MEK/ERK and PI3K/AKT pathways.

In the sensitive cell lines LN215 and LN229, simultaneous inactivation of protein kinases AKT and MEK was associated with poly(ADP-ribose) polymerase cleavage and apoptosis (Fig. 4D). However, in the resistant cell lines U373 and LN401, AKT and MEK inactivation was not sufficient to trigger apoptosis. There was no correlation between glioblastoma cell line sensitivity or resistance to apoptosis and a given genotype (Fig. 1A).

Phorbol Ester TPA Restores Activation of MEK and Inhibits Induction of Apoptosis

Phorbol ester TPA activates protein kinase C and ERK1/2, thereby modifying mitogenic signaling pathways (44, 48, 49). When exposed to 20 or 100 nmol/L of TPA, LN215 and LN229 cells showed a strong increase of phosphorylated ERK levels (Fig. 5A). Treatment of the cell lines with combined U0126 and wortmannin for 4 days in the presence of TPA (20 or 100 nmol/L) led to a substantial increase of activated ERK1/2, whereas phosphorylation was completely down-regulated without TPA (Fig. 5A). In parallel, the extent of the induction of apoptosis was determined after 4 days under the same conditions, revealing that the addition of TPA to both glioblastoma cell lines substantially decreased the induction of apoptosis caused by the two inhibitors (Fig. 5B). Taken together, the results further supported the model of a correlation between down-regulation of the two signaling pathways and induction of apoptosis.

Discussion

We report that the combination of the inhibitor of EGFR kinase AEE788 and of the microtubule depolymerization inhibitor patupilone synergistically induced death of glioblastoma cells. Of high interest is that this synergy occurred at drug concentrations that were not effective to induce cell death when each drug was applied alone. AEE788 alone at 1 μmol/L only induced low levels of apoptosis in glioblastoma cells *in vitro*. Its main antitumor activity consists of inhibition of cell growth and motility (50). Consistently, survival of animals bearing intracranial tumors had been extended by giving AEE788 at concentration below the maximally tolerated dose (34) and confirmed a role of AEE788 as an antitumor agent. However, lasting responses cannot be obtained with a cytostatic effect but require induction of tumor cell death, which may only be achieved by drug combinations. Therefore, we designed a strategy to trigger glioblastoma cell apoptosis by combining the PKI AEE788 with patupilone, a compound with strong cytotoxic potential against various cancer types and also against multidrug resistance cancer cell lines (51). Glioblastoma cell death could be triggered in all tumor cell lines *in vitro* by 35 nmol/L patupilone, a dose that can already be toxic *in vivo*. Interestingly, synergistic induction of apoptosis was observed in glioblastoma cells when using 1 μmol/L AEE788 in combination with only 0.1 nmol/L patupilone. Of interest, the genetic backgrounds of the cell lines that responded the best to the combination therapy with AEE788 and patupilone were different with regard to the three main glioma pathways: TP53, PTEN, and p14/p16 (Fig. 1A). Whereas SF767 cells are wild-type at all three loci, LN71 cells are either mutated or null (39), suggesting that such a combination could be applied to a wide spectrum of gliomas. When analyzing the activation status of EGFR, PKB/AKT, and ERK as readouts for critical pathways in the glioblastoma signaling network, we found that synergistic induction of apoptosis by combining patupilone and AEE788 was paralleled by inactivation of PKB/AKT and ERK. Conversely, the two critical cell signaling mediators remained active in glioblastoma cells that did not show this drug synergism. Although AEE788, patupilone, and U0126, applied individually, reduced glioblastoma cell growth, AEE788/patupilone and U0126/wortmannin combinations

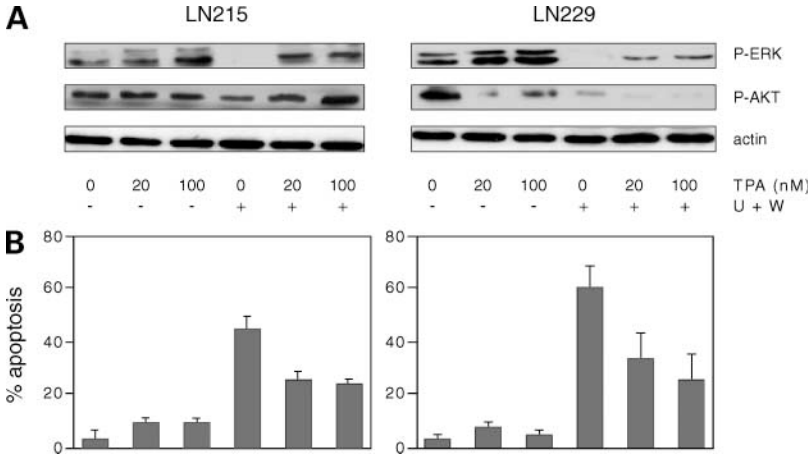


Figure 5. Phorbol ester TPA antagonizes induction of apoptosis and maintains AKT and ERK1/2 activities. **A,** cells were treated with 20 and 100 nmol/L of TPA in the presence or absence of wortmannin (W) and U0126 (U) for 3 d, whereby the additions were renewed every day. Cell extracts were prepared by the addition of SDS sample buffer, and Western blot analysis was done. U0126, 20 μmol/L; wortmannin, 1 μmol/L; TPA, 20 or 100 nmol/L. **B,** cells were treated in the same way for 4 d and the percentage of dead cells was determined by flow cytometry.

did not affect cell growth in a synergistic manner, suggesting that these processes are driven by distinct pathways. The importance of the two signaling pathways PI3K/PKB and RAS/RAF/MEK/ERK for glioblastoma signaling is further supported by the finding that PI3K is activated in the majority of human glioblastoma either by loss of PTEN function (38, 39) or by activating mutations of PI3KCA (22), whereas growth factor stimulation causes activation of RAS in glioblastoma (52). Further evidence stems from a murine transgenic glioblastoma model, in which simultaneous activation of RAS and PKB gives rise to malignant gliomas (53, 54). Glioma animal models have further shown that the combination of ectopic expression of activated RAS and AKT in the glial lineage (23) led to spontaneous induction of murine gliomas, whereas an activated allele of either RAS or AKT alone failed to induce tumor formation. In analogy to this animal model and the effects on signaling induced by the described synergism, we hypothesized that direct inhibition of signaling mediators downstream of EGFR within the PI3K/PKB and RAS/RAF/MEK/ERK pathways will overcome glioblastoma cell resistance. Therefore, we specifically blocked activity of both MEK and PI3K, which induced strong apoptosis in a subgroup of glioblastoma cell lines, indicating a critical role of these two pathways in glioblastoma signaling. Combining the MEK inhibitor U0126 and the PI3K inhibitor wortmannin led to synergistic induction of apoptosis in LN215 and LN229 cell lines. Interestingly, although phosphorylation of ERK1/2 and PKB/AKT was decreased in all cell lines, it was not associated with apoptosis in resistant lines, suggesting additional defects in the proapoptotic machinery. Activation of protein kinase C and ERK1/2 by TPA counteracted U0126- and wortmannin-dependent apoptosis, confirming the specificity of drug effect.

An unresolved question is at which cellular level the intervention has to take place. Our data argue that combined blocking of signaling mediators downstream of growth factor receptors, interfering with the signaling cross-talk, may be more effective than inhibition of a single cell surface receptor (e.g., EGFR; ref. 13). This view is supported by other findings in glioblastoma cell lines, where inhibition of PKB/AKT could be counteracted by a stimulation of insulin-like growth factor receptor-I, resulting in sustained activation of PI3K (19, 20).

In conclusion, our results indicate that the induction of apoptosis in glioblastoma cell lines requires drug combination, which down-regulate distinct pathways. Blocking EGFR activation alone did not induce apoptosis unless complemented with the microtubule stabilizer patupilone. If PKIs were targeted without the help of cytotoxic drugs, combined inhibition of MEK and PI3K was found to be the most efficient combination to induce glioblastoma cell death. Drug effects were independent of mutation statuses at the major glioblastoma pathways (Fig. 1A). Additional studies are needed to develop other target for treatment of resistant glioblastoma cells to directly down-regulate key members of the critical pathways.

Acknowledgments

We thank Elisabeth Taylor for critical reading of the manuscript.

References

1. Shapiro WR, Green SB, Burger PC, et al. Randomized trial of three chemotherapy regimens and two radiotherapy regimens and two radiotherapy regimens in postoperative treatment of malignant glioma. Brain Tumor Cooperative Group Trial 8001. J Neurosurg 1989;71:1 – 9.

2. Burger PC, Heinz ER, Shibata T, Kleihues P. Topographic anatomy and CT correlations in the untreated glioblastoma multiforme. J Neurosurg 1988;68:698 – 704.

3. Libermann TA, Nusbaum HR, Razon N, et al. Amplification, enhanced expression, and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. Nature 1985;313:144 – 7.

4. Wong AJ, Ruppert JM, Bigner SH, et al. Structural alterations of the epidermal growth factor receptor gene in human gliomas. Proc Natl Acad Sci U S A 1992;89:2965 – 9.

5. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer 2005;5:341 – 54.

6. Lane HA, Motoyama AB, Beuvink I, Hynes NE. Modulation of p27/Cdk2 complex formation through 4D5-mediated inhibition of HER2 receptor signaling. Ann Oncol 2001;12 Suppl 1:S21 – 2.

7. Traxler P, Allegrini PR, Brandt R, et al. AEE788: a dual family epidermal growth factor receptor/ErbB2 and vascular endothelial growth factor receptor tyrosine kinase inhibitor with antitumor and antiangiogenic activity. Cancer Res 2004;64:4931 – 41.

8. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129 – 39.

9. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304: 1497 – 500.

10. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from ''never smokers'' and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci U S A 2004;101:13306 – 11.

11. Rich JN, Reardon DA, Peery T, et al. Phase II trial of gefitinib in recurrent glioblastoma. J Clin Oncol 2004;22:133 – 42.

12. Barber TD, Vogelstein B, Kinzler KW, Velculescu VE. Somatic mutations of EGFR in colorectal cancers and glioblastomas. N Engl J Med 2004;351:2883.

13. Lassman AB, Rossi MR, Raizer JJ, et al. Molecular study of malignant gliomas treated with epidermal growth factor receptor inhibitors: tissue analysis from North American Brain Tumor Consortium Trials 01-03 and 00-01. Clin Cancer Res 2005;11:7841 – 50.

14. Marie Y, Carpentier AF, Omuro AM, Sanson M, Thillet J, Hoang-Xuan K. EGFR tyrosine kinase domain mutations in human gliomas. Neurology 2005;64:1444 – 5.

15. Mellinghoff IK, Wang MY, Vivanco I, et al. Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. N Engl J Med 2005;353:2012 – 24.

16. Wen PY, Yung WK, Lamborn KR, et al. Phase I/II study of imatinib mesylate for recurrent malignant gliomas: North American Brain Tumor Consortium Study 99-08. Clin Cancer Res 2006;12:4899 – 907.

17. Carpenter G. The EGF receptor: a nexus for trafficking and signaling. Bioessays 2000;22:697 – 707.

18. Graves LM, Guy HI, Kozlowski P, et al. Regulation of carbamoyl phosphate synthetase by MAP kinase. Nature 2000;403:328 – 32.

19. Sibilia M, Fleischmann A, Behrens A, et al. The EGF receptor provides an essential survival signal for SOS-dependent skin tumor development. Cell 2000;102:211 – 20.

20. Chakravarti A, Loeffler JS, Dyson NJ. Insulin-like growth factor receptor I mediates resistance to anti-epidermal growth factor receptor therapy in primary human glioblastoma cells through continued activation of phosphoinositide 3-kinase signaling. Cancer Res 2002;62:200 – 7.

21. Choe G, Horvath S, Cloughesy TF, et al. Analysis of the phosphatidylinositol 3′-kinase signaling pathway in glioblastoma patients *in vivo*. Cancer Res 2003;63:2742 – 6.

22. Samuels Y, Wang Z, Bardelli A, et al. High frequency of mutations of the PIK3CA gene in human cancers. Science 2004;304:554.

23. Holland EC, Celestino J, Dai C, Schaefer L, Sawaya RE, Fuller GN. Combined activation of Ras and Akt in neural progenitors induces glioblastoma formation in mice. Nat Genet 2000;25:55 – 7.

24. Brognard J, Clark AS, Ni Y, Dennis PA. Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation. Cancer Res 2001;61:3986 – 97.

25. Lee JI, Soria JC, Hassan KA, et al. Loss of PTEN expression as a prognostic marker for tongue cancer. Arch Otolaryngol Head Neck Surg 2001;127:1441 – 5.

26. Perez-Tenorio G, Stal O. Activation of AKT/PKB in breast cancer predicts a worse outcome among endocrine treated patients. Br J Cancer 2002;86:540 – 5.

27. Ermoian RP, Furniss CS, Lamborn KR, et al. Dysregulation of PTEN and protein kinase B is associated with glioma histology and patient survival. Clin Cancer Res 2002;8:1100 – 6.

28. Clark AS, West K, Streicher S, Dennis PA. Constitutive and inducible Akt activity promotes resistance to chemotherapy, trastuzumab, or tamoxifen in breast cancer cells. Mol Cancer Ther 2002;1:707 – 17.

29. Guha A, Dashner K, Black PM, Wagner JA, Stiles CD. Expression of PDGF and PDGF receptors in human astrocytoma operation specimens supports the existence of an autocrine loop. Int J Cancer 1995; 60:168 – 73.

30. Rajasekhar VK, Viale A, Socci ND, Wiedmann M, Hu X, Holland EC. Oncogenic Ras and Akt signaling contribute to glioblastoma formation by differential recruitment of existing mRNAs to polysomes. Mol Cell 2003; 12:889 – 901.

31. Wu CJ, Qian X, O'Rourke DM. Sustained mitogen-activated protein kinase activation is induced by transforming erbB receptor complexes. DNA Cell Biol 1999;18:731 – 41.

32. Chu I, Blackwell K, Chen S, Slingerland J. The dual ErbB1/ErbB2 inhibitor, lapatinib (GW572016), cooperates with tamoxifen to inhibit both cell proliferation- and estrogen-dependent gene expression in antiestrogen-resistant breast cancer. Cancer Res 2005;65:18 – 25.

33. Doherty L, Gigas DC, Kesari S, et al. Pilot study of the combination of EGFR and mTOR inhibitors in recurrent malignant gliomas. Neurology 2006;67:156 – 8.

34. Goudar RK, Shi Q, Hjelmeland MD, et al. Combination therapy of inhibitors of epidermal growth factor receptor/vascular endothelial growth factor receptor 2 (AEE788) and the mammalian target of rapamycin (RAD001) offers improved glioblastoma tumor growth inhibition. Mol Cancer Ther 2005;4:101 – 12.

35. Nguyen TD, Lassmann AB, Lis E, et al. A pilot study to assess the tolerability and efficacy of RAD-001 (everolimus) with gefitinib in patients with recurrent glioblastoma multiforme (GBM). Neuro-oncol 2006;8:447.

36. Altmann KH, Wartmann M, O'Reilly T. Epothilones and related structures—a new class of microtubule inhibitors with potent *in vivo* antitumor activity. Biochim Biophys Acta 2000;1470:M79 – 91.

37. Goodin S, Kane MP, Rubin EH. Epothilones: mechanism of action and biologic activity. J Clin Oncol 2004;22:2015 – 25.

38. Maier D, Zhang Z, Taylor E, et al. Somatic deletion mapping on chromosome 10 and sequence analysis of PTEN/MMAC1 point to the 10q25-26 region as the primary target in low-grade and high-grade gliomas. Oncogene 1998;16:3331 – 5.

39. Ishii N, Maier D, Merlo A, et al. Frequent co-alterations of TP53, p16/CDKN2A, p14ARF, PTEN tumor suppressor genes in human glioma cell lines. Brain Pathol 1999;9:469 – 79.

40. Hermanson M, Funa K, Hartman M, et al. Platelet-derived growth factor and its receptors in human glioma tissue: expression of messenger RNA and protein suggests the presence of autocrine and paracrine loops. Cancer Res 1992;52:3213 – 9.

41. Westermark B, Heldin CH, Nister M. Platelet-derived growth factor in human glioma. Glia 1995;15:257 – 63.

42. Hagerstrand D, Hesselager G, Achterberg S, et al. Characterization of an imatinib-sensitive subset of high-grade human glioma cultures. Oncogene 2006;25:4913 – 22.

43. Huang S, Houghton PJ. Targeting mTOR signaling for cancer therapy. Curr Opin Pharmacol 2003;3:371 – 7.

44. Bollag DM, McQueney PA, Zhu J, et al. Epothilones, a new class of microtubule-stabilizing agents with a taxol-like mechanism of action. Cancer Res 1995;55:2325 – 33.

45. Kamath K, Jordan MA. Suppression of microtubule dynamics by epothilone B is associated with mitotic arrest. Cancer Res 2003;63: 6026 – 31.

46. Alano CC, Ying W, Swanson RA. Poly(ADP-ribose) polymerase-1-mediated cell death in astrocytes requires NAD^+ depletion and mitochondrial permeability transition. J Biol Chem 2004;279:18895 – 902.

47. Cregan SP, Fortin A, MacLaurin JG, et al. Apoptosis-inducing factor is involved in the regulation of caspase-independent neuronal cell death. J Cell Biol 2002;158:507 – 17.

48. Crews CM, Erikson RL. Purification of a murine protein-tyrosine/threonine kinase that phosphorylates and activates the Erk-1 gene product: relationship to the fission yeast byr1 gene product. Proc Natl Acad Sci U S A 1992;89:8205 – 9.

49. Lang FF, Miller DC, Koslow M, Newcomb EW. Pathways leading to glioblastoma multiforme: a molecular analysis of genetic alterations in 65 astrocytic tumors. J Neurosurg 1994;81:427 – 36.

50. Maher EA, Furnari FB, Bachoo RM, et al. Malignant glioma: genetics and biology of a grave matter. Genes Dev 2001;15:1311 – 33.

51. Chou TC, Zhang XG, Balog A, et al. Desoxyepothilone B: an efficacious microtubule-targeted antitumor agent with a promising *in vivo* profile relative to epothilone B. Proc Natl Acad Sci U S A 1998;95: 9642 – 7.

52. Guha A, Feldkamp MM, Lau N, Boss G, Pawson A. Proliferation of human malignant astrocytomas is dependent on Ras activation. Oncogene 1997;15:2755 – 65.

53. Ding H, Roncari L, Shannon P, et al. Astrocyte-specific expression of activated p21-ras results in malignant astrocytoma formation in a transgenic mouse model of human gliomas. Cancer Res 2001;61: 3826 – 36.

54. Lassmann AB, Holland EC. Molecular biology and genetic models of gliomas and medulloblastomas. In: MCLendon RE, Bigner DD, Rosenblum MR, Bruner JM, editors. Russel and Rubinstein's pathology of tumors of the nervous system. London: Arnold Health Sciences; 2006. p. 1039 – 91.

Part 3: Histone deacetylase inhibition and blockade of the glycolytic pathway synergistically induce glioblastoma cell death

Viviane Egler, Serdar Korur*, Mike Failly, Jean-Louis Boulay, Roland Imber, Maria Maddelena Lino, and Adrian Merlo

Mol Cancer Ther. 2007 Feb;6(2):773-8

* Author contributed to: experimental design, performing experiments, data analysis and writing the paper.

Histone Deacetylase Inhibition and Blockade of the Glycolytic Pathway Synergistically Induce Glioblastoma Cell Death

Vivian Egler, Serdar Korur, Mike Failly, Jean-Louis Boulay, Roland Imber, Maria M. Lino, and Adrian Merlo

Abstract

Purpose: High-grade gliomas are difficult to treat due to their location behind the blood-brain barrier and to inherent radioresistance and chemoresistance.

Experimental Design: Because tumorigenesis is considered a multistep process of accumulating mutations affecting distinct signaling pathways, combinations of compounds, which inhibit nonoverlapping pathways, are being explored to improve treatment of gliomas. Histone deacetylase inhibitors (HDI) have proven antitumor activity by blocking cell proliferation, promoting differentiation, and inducing tumor cell apoptosis.

Results: In this report, we show that the HDIs trichostatin A, sodium butyrate, and low nanomolar doses of LAQ824 combined with the glycolysis inhibitor 2-deoxy-D-glucose induce strong apoptosis in cancer cell lines of brain, breast, and cervix in a p53-independent manner. HDIs up-regulate p21, which is blocked by concomitant administration of 2-deoxy-D-glucose.

Conclusions: We propose simultaneous blockade of histone deacetylation and glycolysis as a novel therapeutic strategy for several major cancers.

Glioblastoma multiforme (GBM) are the most frequent human brain tumors. These aggressive, highly invasive, and neurologically destructive tumors are among the deadliest of human cancers, with a median survival ranging from 9 to 12 months (1). Despite treatment efforts including new technological advances in neurosurgery, radiation therapy, and clinical trials with novel therapeutic agents (2), median survival has not changed significantly over the past two decades. Meanwhile, cancer drug development has been moving from conventional cytotoxic chemotherapeutics to more sophisticated drugs exploiting biological mechanism of tumorigenesis (3).

In high-grade gliomas, several genes and pathways are altered due to a severe mutator phenotype that leads to the accumulation of mutations in critical regulatory genes. Mutations of *PTEN*, *RB*, *p16/p14*, *p53* (4), and receptor tyrosine kinase (5–7) result in up-regulation of pathways that promote tumor growth, invasion, and resistance to apoptotic stimuli (8).

Besides classic mutations, epigenetic silencing of tumor suppressor genes frequently leads to dysregulation of signaling pathways promoting tumorigenesis. Histone acetyltransferase and histone deacetylase (HDAC) catalyze the acetylation and deacetylation of lysine residues in the tails of histone proteins (e.g., lysine residue in histones 3 and 4), regulating the affinity of the protein transcriptional complexes to the DNA (9). Thus, the recruitment of histone acetyltransferase and HDAC is considered as a key element in the dynamic regulation of genes involved in cellular proliferation and differentiation during normal development and carcinogenesis (10). HDAC inhibitors (HDI) induce re-expression of silenced tumor suppressor genes (11, 12). HDIs induce differentiation and promote apoptosis in transformed and cancerous but not in normal cells (11, 13). HDIs have been classified in different classes: short-chain fatty acid (as sodium butirrate, valproic acid, etc.), Epoxides (as depudecin and trapoxin), cyclic peptides, Benzamides, and Hydroxamic acids [as trichostatin A (TSA), SAHA, and LAQ824]. Besides the chemical TSA, sodium butyrate (NaB), and SAHA (Vorinostat; Food and Drug Administration approved in 2006), a new potent cinnamic hydroxamic acid derivate, LAQ824 (13), is currently used in clinical trials for the treatment of leukemia (14–16). Because this drug passes the blood-brain barrier, it may potentially be useful for the treatment of malignant gliomas. *In vitro* treatment of tumor cell lines with the LAQ824 caused hyperacetylation of histones, restoring the expression of <2% of human genes, including the cell cycle kinase inhibitor $p21^{WAF1/Cip1}$ (17). High concentrations of LAQ824 induce accumulation in G_2 phase of the cell cycle and are selectively toxic for transformed and cancer cells, whereas lower concentrations induce cell cycle arrest in G_1, increase p21 expression, and only weakly induce apoptosis (18). The presence or the absence of p21 seems to play a critical role for the commitment of the targeted cell to either undergo growth arrest or apoptosis in response to LAQ824.

Maintenance of growth and proliferation of tumor cells requires high cellular energy levels. Tumor cells have abnormal mitochondrial functions and essentially rely on glycolysis to provide ATP for their metabolic requirements even under

Authors' Affiliations: Laboratory of Molecular Neuro-oncology, Departments of Research and Surgery, University Hospitals, Basel, Switzerland

Received 9/12/07; revised 12/10/07; accepted 1/3/08.

Grant support: Swiss National Foundation (Grant 31-67123.01), by Oncosuisse (01338; OCS-01613-12-2004), Regional Cancer League of Basel-Stadt and Baselland (No. 7-2004), and by Novartis Oncology, Basel, Switzerland.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Adrian Merlo, Departments of Research and Surgery, University Hospital, Spitastrasse 21, CH-4031 Basel, Switzerland. Phone: 41-61-265-74-56; Fax: 41-61-265-71-38; E-mail: amerlo@uhbs.ch.

doi:10.1158/1078-0432.CCR-07-4182

aerobic condition (Warburg effect). As a consequence, tumor cells have relatively low oxygen needs and can survive in a hypoxic environment that is not suitable for normal cells. Human malignant gliomas were shown by 18 F-fluoro-2-deoxyglucose positron emission tomography studies to be much more avid of glucose than the normal cortex (19). This high energy demand of cancer cells is the rational basis to block glycolysis in cancer cells (20). Glucose metabolism and ATP production are inhibited by the glucose analogue 2-deoxy-D-glucose (2-DG). Upon phosphorylation by hexokinase, 2-DG selectively accumulates in cancer cells by increased glucose transporter expression, high hexokinase activity, and low phosphorylase activity (21, 22). 2-DG strongly reduced growth of tumor cells, when used as single agent (23). Moreover, 2-DG impairs repair of radiation-induced DNA damage in tumor cells and promotes tumor cell apoptosis by lowering intracellular energy levels (24). In the treatment of human GBM, 2-DG is used as a radiosensitizer in combination with radiotherapy (24, 25). Moreover, at least two clinical trials using 2-DG for solid tumor and intracranial metastases are ongoing.

Because HDIs induce re-expression of tumor suppressor genes in several distinct pathways and cancer cells critically depend upon continuous energy supply, we explored the therapeutic potential of a combination of two compounds that simultaneously target the epigenetic status and the energy demand of cancer cells. Synergistic effects may even allow to reduce the drug

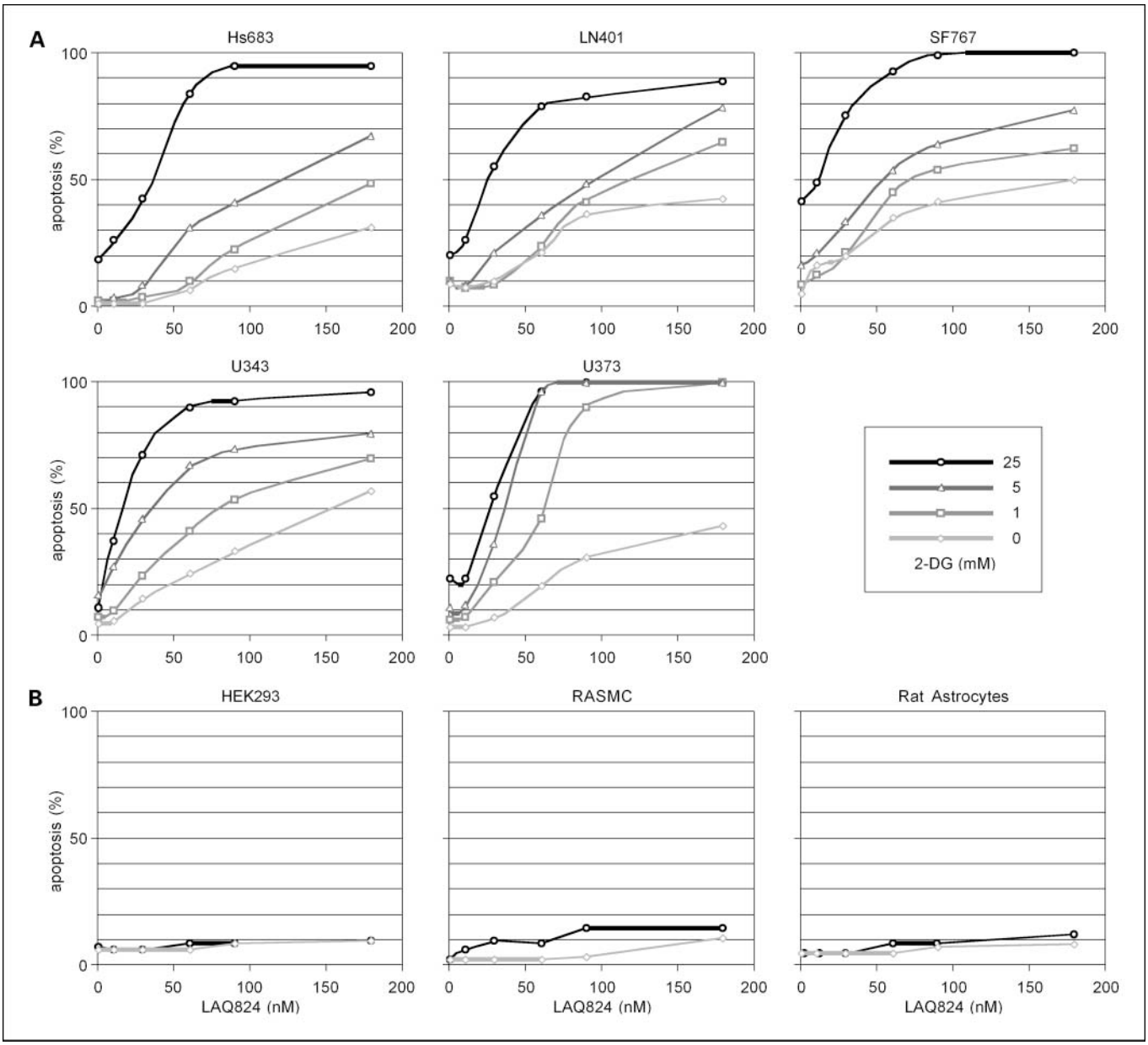


Fig. 1. Synergistic induction of apoptosis by 2-DG and HDAC inhibitor LAQ824 in glioma cell lines. Glioma cell lines Hs683, LN401, SF767, U343 and U373 (*A*), nontumor lines HEK and rat aorta smooth muscle cell, and normal rat astrocytes (*B*) were precultured for 48 h in standard medium and were exposed to various concentrations of LAQ824 in the presence of 2-DG for 72 h. Apoptosis was determined by FACS analysis. The CI calculated for the combination of 25 mmol/L 2-DG and 60 nmol/L LAQ824 were 0.085 (Hs683), 0.066 (LN401), 0.018 (SF767), 0.053 (U343), and 0.005 (U373), respectively. CIs lower than 1 indicate synergy in all cases (see the Materials and Methods section for cutoffs).

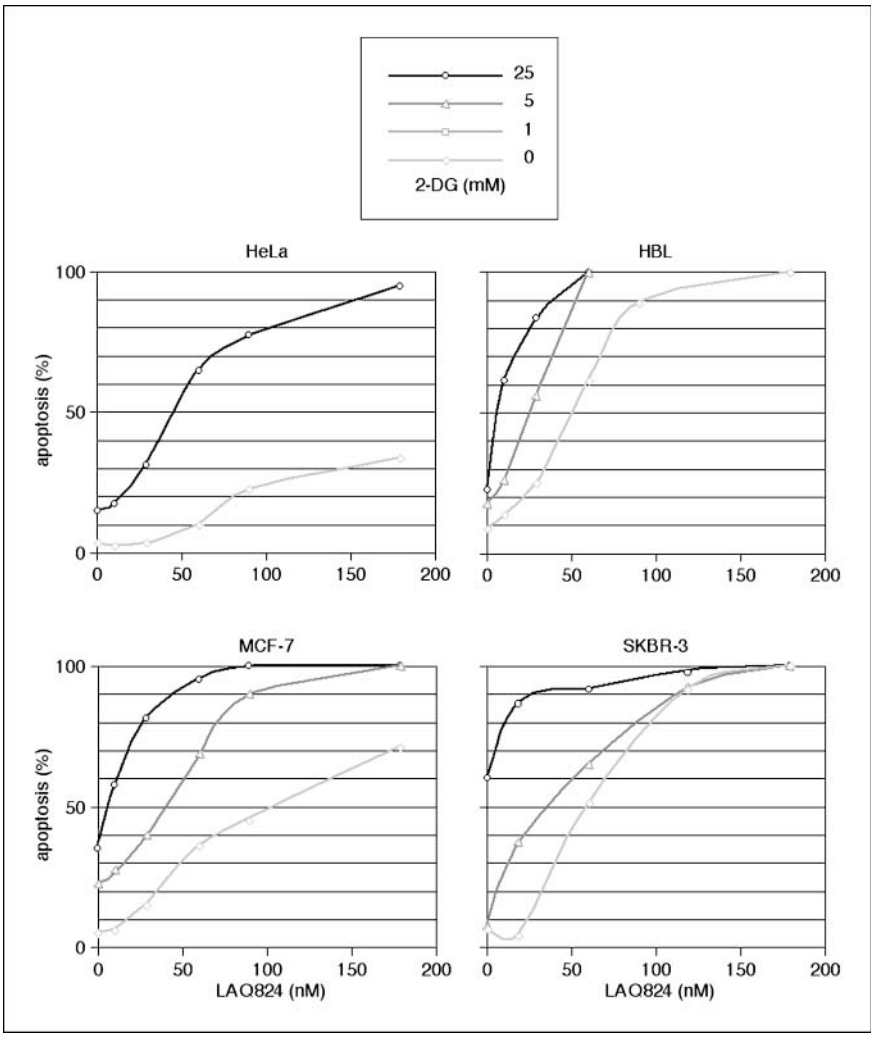


Fig. 2. Synergistic induction of apoptosis of 2-DG and HDAC inhibitor LAQ824 in nonglioma cell lines. HeLa (cervix) and HBL, MCF-7, and SKBR-3 (breast) cancer cell lines were precultured for 48 h in standard medium and were exposed to drugs at various concentrations for 72 h. Apoptosis was determined by FACS analysis. The CI calculated for the combination of 25 mmol/L 2-DG and 60 nmol/L LAQ824 were 0.05 (HeLa), 0.4 (HBL), 0.1 (MCF-7), and 0.6 (SKBR-3), respectively, indicating synergy in all cases.

dosage of potentially toxic HDIs (26). We found that the combination of 2-DG and HDIs lead to a strong synergistic effect resulting in widespread apoptosis of tumor cells of the brain, breast, and cervix, while sparing normal rat astrocytes.

Materials and Methods

Cell lines and reagents. Hs683, LN401, SF767, U373, U343 glioma cell lines, for which the genetic status of established cancer genes *TP53*, *p16/p14*, and *PTEN* has been defined (4), were cultured in Eagle medium supplemented with 25 mmol/L glucose, glutamine, standard antibiotics, and 10% FCS. HCT116, HeLa, MCF-7, HBL, and SKBR-3 cells were cultured in RPMI containing 25 mmol/L glucose, glutamine, standard antibiotics, and 5% FCS. Parental $p21^{+/+}$ and engineered $p21^{-/-}$ HCT116 (27) were a generous gift from Prof. Bert Vogelstein (Johns Hopkins University, Baltimore MD). Fresh rat astrocytes were cultured as described (28). All cells were maintained at 37°C in 5% CO_2. All cell lines were seeded in 3-cm plates at 35% density and grown for 48 h before treatment with the indicated drugs. LAQ824, RAD001, and Imatinib (Gleevec) were kindly provided by Novartis-Pharma AG. 2-DG, TSA, and NaB were purchased from Sigma (Saint-Louis). Drug concentrations described are indicated in the figures.

Western analysis and antibodies. Cells were washed with 1× PBS; lysed in a buffer containing 2% SDS, 50 mmol/L Tris (pH 6.8), and 0.1 mol/L DTT; boiled; and either used immediately or frozen at -20°C. Protein lysates were resolved on denaturing SDS-polyacrylamide gels ranging from 8% to 13% and transferred to nitrocellulose membranes (Hybond; enhanced chemiluminescence; Amersham Biosciences). Membranes were probed with the following primary antibodies: anti-Akt (kindly provided by Dr. Brian Hemmings, Friedrich Miescher Institute Basel, CH); anti-S6 (kindly provided by Dr. George Thomas, Friedrich Miescher Institute Basel, CH); antibodies against phosphorylated S6 protein (Ser-240/244), S6K and phospho-S6K (Ser-389), phospho-Akt (Ser-473), and poly(ADP)ribose polymerase (PARP) were purchased from Cell Signaling; and antibodies against p21, Cyclin A, extracellular signal-regulated kinase (p42 and p44), and Phospho–extracellular signal-regulated kinase (Tyr-204 of p42 and p44) were purchased from Santa Cruz Biotechnology. Decorated proteins were revealed using horseradish peroxidase–conjugated anti-mouse or anti-rabbit immunoglobulins (New England Biolabs) and visualized by enhanced chemoluminescence (Amersham Biosciences).

Cell viability assay. Cell DNA content and apoptosis were analyzed with a Flow cytometer (FACS Calibur; BD Biosciences), and statistics were determined with Cell Quest software. Cells were trypsinized and fixed in ice-cold 70% ethanol for 1 h, stained with 50 μg/mL propidium iodide for fluorescence-activated cell sorting (FACS) analysis. The percentage of dead cells was determined by the proportion of cells in pro-G_1 phase. Percentages reported result from three independent experiments.

Combinatorial index calculation. Apoptosis resulted from the combination of two drugs was analyzed for each cell line using

CalcuSyn Software (Biosoft) to generate a combination index (CI). A CI greater than one indicates antagonism. A CI of one indicates an additive effect. A CI less than one indicates synergism. Specifically, a value between 0.85 and 0.90 indicate slight synergism, between 0.70 and 0.85 indicates a moderate synergism, between 0.30 and 0.70 indicates synergism, between 0.10 and 0.30 indicates strong synergism, and <0.1 indicates a very strong synergism.

Cell proliferation assay. Cells (5×10^3) were seeded in 96-well dishes and grown for 8 h before treatment. Cells were then exposed to 60 nmol/L LAQ824 and 25 mmol/L 2-DG for 24 h. Bromodeoxyuridine incorporation was allowed for the last 2 h. Cell proliferation Biotrak Elisa system (Amersham Biosciences) was done according to manufacturer's instructions. Experiments were done in triplicate. Data were analyzed with GraphPad prism4 software (GraphPad Software Corporation).

ATP measurement. Cells cultured in 6-well dishes were treated with 25 mmol/L 2-DG and 60 nmol/L LAQ824, lysed, and incubated at room temperature for 5 min. ATP levels were measured using a luciferase-based assay kit (ATP Bioluminescence Assay kit CLS II; Roche). For each well, ATP levels were normalized by comparison with the amount of total protein by the Bio-Rad protein assay reagent. All ATP level measurements and protein assays were done in triplicate.

Results

Blocking glycolysis strongly enhances apoptosis induced by HDAC inhibition. In malignant glioma cells, we targeted the histone acetylation pathway with the HDI LAQ824, a known inducer of cell death in tumor and immortalized cell lines, and blocked the energy pathway with the glucose analogue 2-DG. Increasing amounts of LAQ824 and 25 mmol/L 2-DG were applied to 5 glioma cell lines during 72 hours of treatment, and apoptosis was monitored by FACS analysis (Fig. 1A). The use of

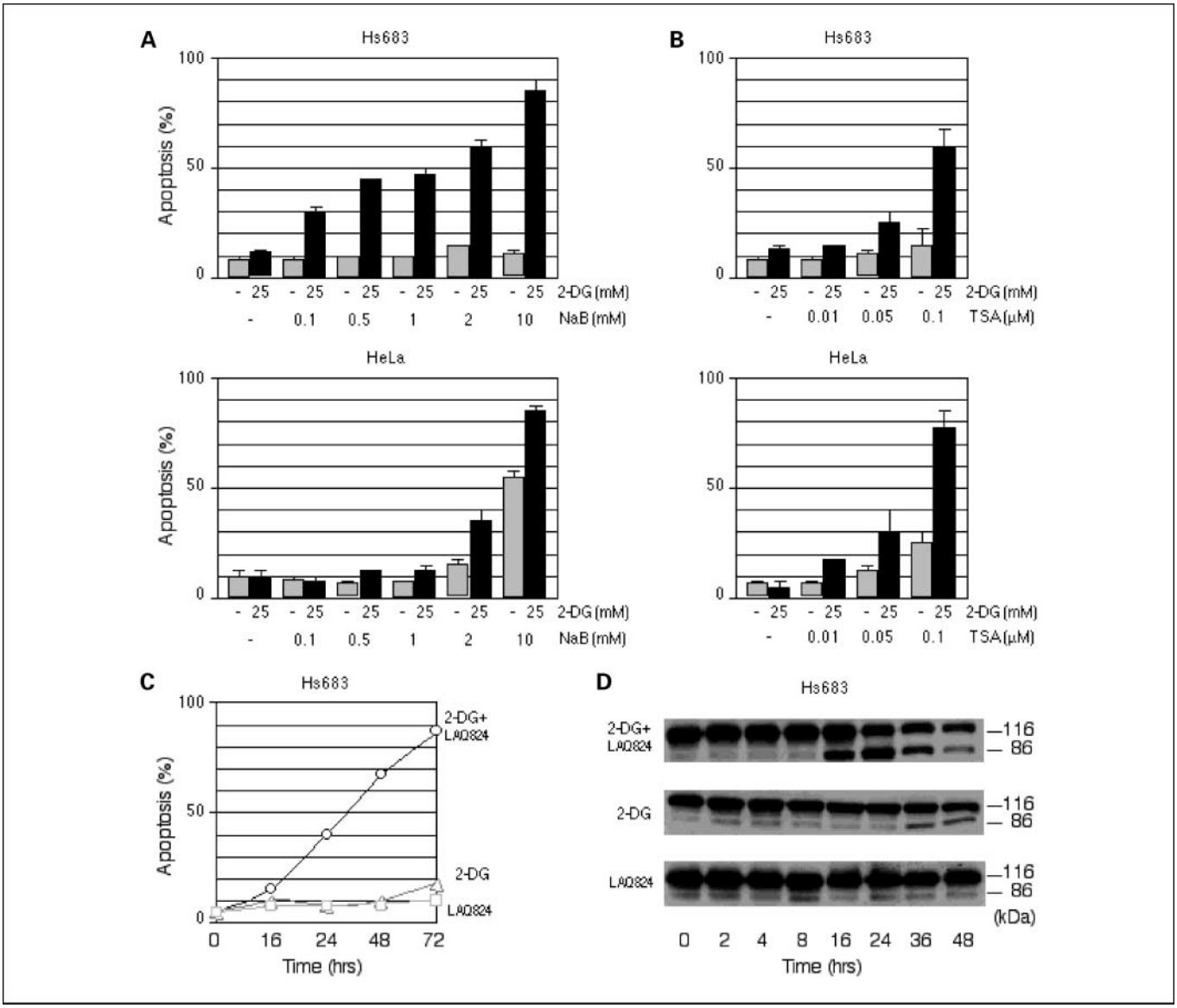


Fig. 3. HDI TSA and NaB combined with 2-DG synergistically induce apoptosis. *A* and *B,* combination of 25 mmol/L 2-DG with 0.1 to 10 mmol/L NaB (*A*) or 10 to 100 nmol/L TSA (*B*) on Hs683 and HeLa cells. *C,* time course of apoptosis. Hs683 cells precultured for 48 h in standard medium were treated with 25 mmol/L 2-DG and 60 nmol/L LAQ824 for various times. Apoptosis was determined by FACS analysis. *D,* time course of PARP cleavage. Hs683 were treated as described in 2*C*. PARP cleavage was assessed by immunoblotting with an anti-PARP antibody that recognizes the native (116 kDa) and the cleaved forms (86 kDa).

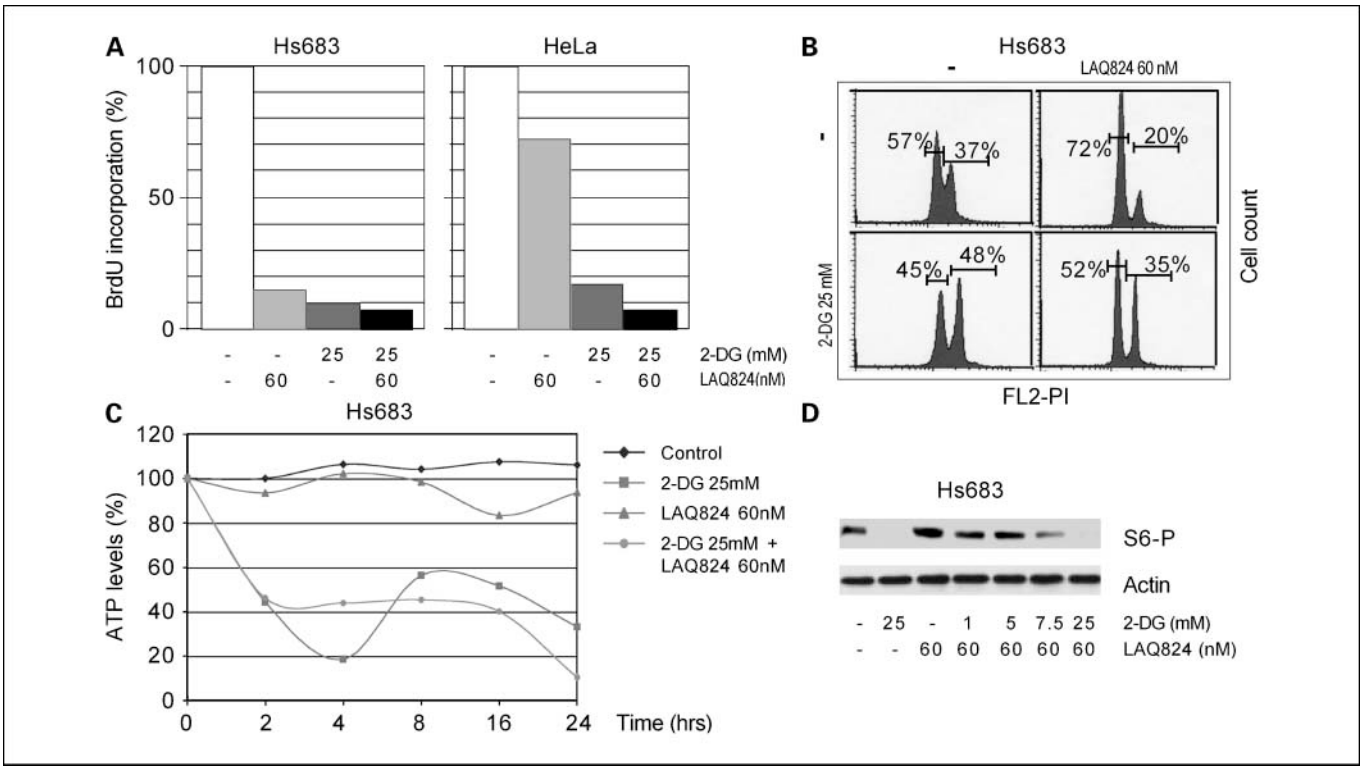


Fig. 4. Combination of 2-DG and LAQ824 impairs tumor cell proliferation. Cellular responses to 2-DG/LAQ824 combined treatment for 24 h. *A,* Hs683 and HeLa cell proliferation. *B,* Hs683 cell cycle. *C,* ATP levels in Hs683 cells. ATP level of untreated cells at time t = 0 was set to 100%. *D,* S6 phosphorylation in Hs683 cells.

LAQ824 alone induced relative low levels of apoptosis in a concentration-dependent manner in all glioma cell lines tested, whereas 2-DG alone triggered cell death just above baseline. However, the combination of LAQ824 and 2-DG significantly enhanced cell death in all lines tested. The extent of synergism was dependent on the concentration of both drugs but predominantly on the concentration of 2-DG. The observed synergism was found to be independent of the p53 status because p53–wild-type SF767 and U343 and p53-mutant Hs683, LN401, U373 tumor cells (4) responded in a similar way (Fig. 1A). The noncancer cell lines HEK293, rat aorta smooth muscle cells, and rat astrocytes do not show an enhanced cell death upon combining 2-DG with LAQ824 (Fig. 1B).

To test whether this synergism is also observed in other tumor types, we tested the 2-DG/LAQ824 combination in breast and cervix cancer cells MCF-7, HBL, SKBR-3, and HeLa. We found that these epithelial cancer cells exposed to 2-DG together with increasing doses of LAQ824 exhibited the same synergistic induction of cell death as we had consistently observed in a panel of glioma cells (Fig. 2).

To establish whether the synergistic induction of apoptosis triggered by the association of LAQ824 and 2-DG represents a general mechanisms not limited to the drug LAQ824, we used other HDIs such as TSA, used as a antifungal antibiotic, and the chemical compound NaB, currently used in clinical trials, in combination with 2-DG. Consistent with the results obtained with LAQ824, the association of 2-DG with TSA or NaB strongly induced apoptosis in glioma and cervical cancer lines (Fig. 3A and B).

Titration curves established that concentrations of 25 mmol/L 2-DG and 60 nmol/L LAQ824 on Hs683 cells were optimal for synergy (Fig. 1A). Using these conditions, apoptosis started as early as 16 hours after treatment and constantly increased until 72 hours, although application of each drug alone induced little or no cell death at these concentrations (Fig. 3C). The appearance of the cleaved 86 kDa form of PARP at 16 hours indicated the onset of apoptosis induction resulting from the combined treatment. Only low levels of cleaved PARP appeared after 36 hours of 25 mmol/L 2-DG treatment alone, and no evidence for cleavage was present upon LAQ824 application alone (Fig. 3D).

2-DG and LAQ824 affect cell cycle regulation. Next we studied the effect of LAQ824 on Hs683 and HeLa cell cycle as single agent or combined with 2-DG. After 24 hours of treatment, both 2-DG and LAQ824 reduced Hs683 and HeLa proliferation rate (Fig. 4A). We also analyzed the effect of 2-DG and LAQ824 on G_1 and G_2 phases of cell cycle. 2-DG and LAQ824 as single agents induced G_1 and G_2 accumulation, respectively, whereas the combination had minimal effect of G_1 and G_2 distribution but strongly inhibited the S phase of the cell cycle (Fig. 4B).

ATP levels and S6 phosphorylation are affected by 2-DG. Decreased ATP levels lead to the activation of the energy sensoring kinase AMP-protein kinase. Its activation blocks the high energy consuming mechanisms by reducing the activation of S6K and phosphorylation of the ribosomal protein S6 at serine residues 240 and 244, thereby blocking global protein translation. To establish whether synergistic induction of apoptosis by 2-DG and/or LAQ824 involves the energy

regulatory pathway, ATP levels and the S6 protein phosphorylation status were studied. Upon application of 25 mmol/L 2-DG, a dramatic decrease in ATP levels was measured while the presence of LAQ824 did not alter ATP levels (Fig. 4C). A comparable decrease was observed by combining both drugs (Fig. 4C). We observed a decrease in intracellular phosphorylated S6 protein in cells treated with 2-DG, whereas the application of LAQ824 did not alter the phosphorylation of S6 protein (Fig. 4D). Reduction of phosphorylated S6 protein levels were also observed in HeLa cells line after 2-DG treatment (data not shown). These results show that 2-DG dramatically inhibits the energy regulatory pathway and may considerably block translation by reducing the amounts of phosphorylated S6 protein.

2-DG abrogates p21 expression induced by LAQ824. We then investigated the role of p21 in the synergistic induction of apoptosis by following levels of p21 protein in the glioma cell line Hs683 after addition of the HDI LAQ824. Starting 4 hours after LAQ824 treatment, p21 levels showed a transient prominent increase dropping again to undetectable protein levels at 48 hours (Fig. 5A). When 2-DG and LAQ824 were applied together, the p21 increase disappeared (Fig. 5B). To test whether lowering 2-DG concentrations would reduce p21 protein levels, intermediate doses of 2-DG were combined with 60 nmol/L of LAQ824. After 8 hours of treatment, p21 levels were significantly augmented in presence of LAQ824 and reduced with increasing doses of 2-DG (Fig. 5B). Consistent increase of p21 levels upon addition of LAQ824 alone and its disappearance after 2-DG treatment was observed in HeLa cells (Fig. 5B) and all other glioma cell lines tested (data not shown). Moreover, as with LAQ824, the HDIs TSA and NaB caused an increase in p21 protein levels that was strongly reduced in the presence of 2-DG both on the glioma line Hs683 and the cervix cancer line HeLa (Fig. 5C).

Lack of p21 protein sensitizes cancer cells to apoptosis. To test whether the 2-DG effect on p21 protein is a critical factor in the synergistic induction of apoptosis, we tested the effect of the HDI LAQ824 on the parental colon cancer cell line HCT116, which is wild-type for p21 ($p21^{+/+}$), and its $p21^{-/-}$ derivate (27). HCT116 $p21^{+/+}$ cells were very sensitive to LAQ824, undergoing rapid apoptosis. Nevertheless, HCT116 $p21^{-/-}$ cell line showed a 15% to 20% increase of apoptosis compared with the wild-type constellation HCT116 $p21^{+/+}$, both after 24 hours and after 72 hours of application of the HDI LAQ824 (Fig. 6A). Combination of LAQ824 and 2-DG on HCT116 $p21^{+/+}$ and $p21^{-/-}$ cells did not change the different apoptosis rates observed (Fig. 6B). These data show that the reduction of p21 protein levels may increase apoptosis rate upon treatment with the HDI LAQ824.

Synergistic induction of apoptosis in fresh* ex vivo *GBM cell cultures after the combined treatment of 2-DG with LAQ824. Because commonly used cell lines undergo many cycle divisions with a high probability to increase their genetic mutator phenotype, we tested LAQ824 and 2-DG in combination on an *ex vivo* GBM-derived cell line BS153. BS153 cell line showed a synergistic response upon LAQ824/2-DG combination when compared with the single drug application (Fig. 6C). Thus, targeting the energy and the epigenetic pathways may be an efficient treatment also in *ex vivo* GBM cell lines.

LAQ824 specifically synergizes with 2-DG but not with the rapamycin derivative RAD001 or Imatinib. We tested the specificity of the synergy between LAQ824 and 2-DG by measuring apoptosis of glioma cells Hs683, U87, and U373 in response to LAQ824 in combination with other compounds that target alternative nonoverlapping glioma pathways such as the mammalian target of rapamycin inhibitor RAD001 or the platelet-derived growth factor inhibitor Imatinib. Neither combination increased the apoptosis rate in GBM cell lines (Fig. 6D), showing the specificity of synergy between combined

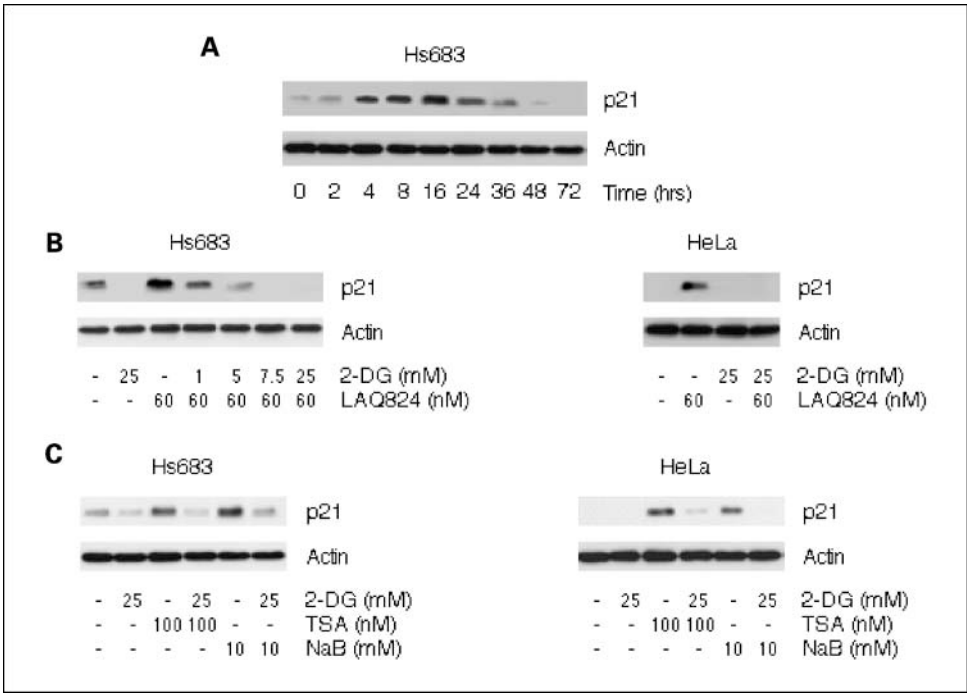


Fig. 5. p21 levels are increased in the course of the synergistic induction of apoptosis. *A,* time course of Hs683 p21 levels in response to 60 nmol/L LAQ824 (*A*). *B* and *C,* p21 status of Hs683 and HeLa cells after 8 h of 2-DG/LAQ824 (*B*) and 2-DG/TSA or 2-DG/NaB (*C*) combined treatment.

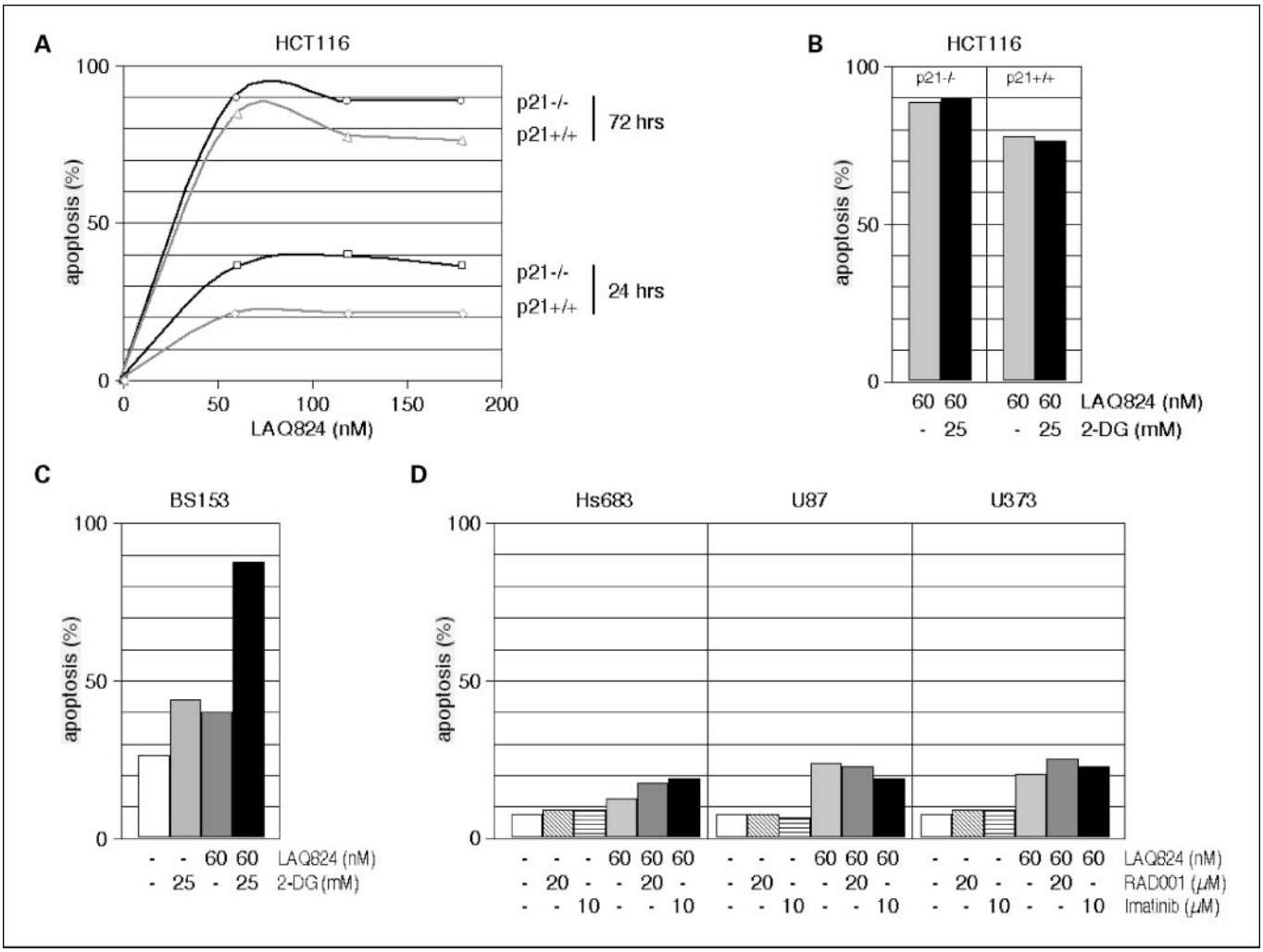


Fig. 6. HCT116 $p21^{-/-}$ cells show increased apoptosis rate upon LAQ824 treatment. *A,* apoptotic responses of $p21^{-/-}$ and $p21^{+/+}$ HCT116 colon cancer cells treated with 60 nmol/L LAQ824 for 24 or 72 h. *B,* apoptotic responses of $p21^{-/-}$ and $p21^{+/+}$ HCT116 cells treated with 60 nmol/L LAQ824 alone or in combination with 25 mmol/L 2-DG for 72 h. Apoptosis was determined by FACS analysis. *C,* apoptotic responses of fresh *ex vivo* derived glioblastoma cell line BS153 to 60 nmol/L LAQ824 and/or 25 mmol/L 2-DG treatment for 72 h. *D,* RAD001 or Imatinib do not synergize with LAQ824 to induce apoptosis in glioma cell lines. Apoptotic responses of glioma lines Hs683, U87, and U373 in the presence of 60 nmol/L LAQ824 combined with either 20 μmol/L RAD001 or 10 mmol/L Imatinib.

inhibition of histone deacetylation and of the glycolytic pathway in glioma cells.

Discussion

Combination of drugs that inhibit nonoverlapping cancer pathways is a rational strategy to control tumor growth. A synergistic effect after the use of two drugs can lower the single dosage and increase the patient tolerance, reducing collateral effects. We have shown that 2-DG, an inhibitor of the glycolytic pathway, sensitizes glioma cells treated with HDI to undergo apoptosis. Epigenetic silencing of tumor suppressor genes leads to dysregulation of cellular processes promoting tumorigenesis. It is well-established that 2-DG and HDIs such as Vorinostat (butyrates) are able to pass the blood-brain barrier, a prerequisite for glioma treatment, and are also well-tolerated by patients. We found that HDIs such as TSA or NaB strongly synergize with 2-DG in the induction of tumor cell death. We then tested LAQ824, an HDI that is currently in clinical trial against leukemia and displays good blood-brain barrier penetration, for its potential as a cancer drug against malignant glioma. In response to higher nanomolar doses of LAQ824, glioma cell lines underwent apoptosis, whereas lower nanomolar doses only had a cytostatic effect with markedly reduced cell death. However, a combination of 2-DG and low nanomolar concentrations of LAQ824 showed a marked synergism in the induction of tumor cell apoptosis, not only in GBM cells, but also in breast and cervix cancer cells, regardless of their p53 status. The observed synergism seems to be specific because combination with other drugs that target glioma pathways such as RAD001 and Imatinib did not lead to any increased apoptosis rate when combined with LAQ824.

The glycolysis demand of many tumors represents the rationale to block glucose metabolism in addition to histone deacetylation inhibition. We first tested the effect of 2-DG per se on human GBM cell line survival, which induced apoptosis in GBM cells only at high concentrations (25 mmol/L) but

efficiently inhibits proliferation of all glioma cell lines. Glycosylation of essential proteins such as transcription factors can be profoundly affected by the absence of glucose (29) or by blocking glycolysis with 2-DG (30). Under conditions of energy starvation, the decreased ATP to AMP ratio activates the first cellular energy sensor, the tumor suppressor LKB1 (31), and the Tuberous Sclerosis Complex TSC1/2 (32) activate 5′AMP-protein kinase. Activation of AMP-protein kinase leads to a reduction of global translation and cell size by inhibiting phosphorylation of the downstream effectors such as the mammalian target of rapamycin (33), the S6 40S ribosomal kinase protein kinase, and the eukaryotic initiation factor 4E binding protein. 2-DG attenuates the transcription factor activity of Sp1 by influencing its *O*-GlcNAcylation levels (30). Because many transcription factors are modified by *O*-GlcNAc, it is likely that *O*-GlcNAcylation of other transcription factors could regulate gene expression in response to glucose use.

HDIs are able to restore the expression of 2% of the total number of human genes (17), e.g., the cyclin-dependent kinase inhibitor p21 whose up-regulation is considered to be responsible for the antiproliferative effect of the drug (17, 34). A number of studies have shown that p21 expression causes G_1 cell cycle arrest and protects cells against chemotherapeutic agents (18, 35, 36). However, among cells that have HDI-induced p21 expression, only cells in late G_1 and S phase proceed through an aberrant mitosis and rapid apoptosis, whereas cells in G_1 phase accumulate p21 and undergo cell cycle arrest. Our results show that LAQ824, TSA, and NaB applied alone induced a transient increase of p21 protein levels that is completely abolished upon addition of 2-DG, which then drives the cells into apoptosis.

Other drug combinations (37), e.g., cisplatin and the rapamycin derivative RAD001 (38), the HSP90 antagonist 17-AAG, and NaB (39, 40), can induce similar synergism regarding the implication of p21 proteins in the regulation of cell survival. We therefore conclude that the p21 response to some drug combinations including HDIs is not the causative factor of induction of tumor cell apoptosis because marked decrease of p21 levels also accompanied induction of widespread tumor cell death in the combination of 2-DG with HDI. Nonetheless, p21 background slightly influences cell death response upon drug application, as found when exposing p21 – wild-type and p21-knock out cancer cells.

In conclusion, we propose to develop a clinical protocol that explores the therapeutic potential of a combination of 2-DG and LAQ824 for glioblastoma that can be extended to other malignancies such as breast and cervix cancer.

Disclosure of Potential Conflicts of Interest

Novartis covered 25% of all expenses and 75% of noncommercial expenses of the research.

Acknowledgments

We thank Prof. Bert Vogelstein for the HCT 116 $p21^{+/+}$ and $p21^{-/-}$ cell lines, Dr Brian Hemmings for the anti-Akt antibodies, Dr George Thomas for the anti-S6 antibodies, Novartis Pharma AG, Switzerland for providing the LAQ824 compound, and Dr. Kenneth Takeda for supervising the thesis work of Viviane Egler.

References

1. Wechsler-Reya R, Scott MP. The developmental biology of brain tumors. Annu Rev Neurosci 2001;24:385 – 428.
2. Zhu Y, Parada LF. The molecular and genetic basis of neurological tumours. Nat Rev Cancer 2002;2:616 – 26.
3. Kondo Y, Hollingsworth EF, Kondo S. Molecular targeting for malignant gliomas (Review). Int J Oncol 2004;24:1101 – 9.
4. Ishii N, Maier D, Merlo A, et al. Frequent co-alterations of TP53, p16/CDKN2A, p14ARF, PTEN tumor suppressor genes in human glioma cell lines. Brain Pathol 1999;9:469 – 79.
5. Mellinghoff IK, Cloughesy TF, Mischel PS. PTEN-mediated resistance to epidermal growth factor receptor kinase inhibitors. Clin Cancer Res 2007;13:378 – 81.
6. Nicholas MK, Lukas RV, Jafri NF, Faoro L, Salgia R. Epidermal growth factor receptor - mediated signal transduction in the development and therapy of gliomas. Clin Cancer Res 2006;12:7261 – 70.
7. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer 2005;5:341 – 54.
8. Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. Nature 2001;411:342 – 8.
9. de Ruijter AJ, van Gennip AH, Caron HN, Kemp S, van Kuilenburg AB. Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem J 2003;370:737 – 49.
10. Cress WD, Seto E. Histone deacetylases, transcriptional control, and cancer. J Cell Physiol 2000;184:1 – 16.
11. Johnstone RW. Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. Nat Rev Drug Discov 2002;1:287 – 99.
12. MacLachlan TK, Sang N, Giordano A. Cyclins, cyclin-dependent kinases and cdk inhibitors: implications in cell cycle control and cancer. Crit Rev Eukaryot Gene Expr 1995;5:127 – 56.
13. Atadja P, Gao L, Kwon P, et al. Selective growth inhibition of tumor cells by a novel histone deacetylase inhibitor, NVP-LAQ824. Cancer Res 2004;64:689 – 95.
14. Weisberg E, Catley L, Kujawa J, et al. Histone deacetylase inhibitor NVP-LAQ824 has significant activity against myeloid leukemia cells *in vitro* and *in vivo*. Leukemia 2004;18:1951 – 63.
15. Nimmanapalli R, Fuino L, Bali P, et al. Histone deacetylase inhibitor LAQ824 both lowers expression and promotes proteasomal degradation of Bcr-Abl and induces apoptosis of imatinib mesylate-sensitive or -refractory chronic myelogenous leukemia-blast crisis cells. Cancer Res 2003;63:5126 – 35.
16. Nimmanapalli R, Fuino L, Stobaugh C, Richon V, Bhalla K. Cotreatment with the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) enhances imatinib-induced apoptosis of Bcr-Abl-positive human acute leukemia cells. Blood 2003;101:3236 – 9.
17. Richon VM, Sandhoff TW, Rifkind RA, Marks PA. Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. Proc Natl Acad Sci U S A 2000;97:10014 – 9.
18. Burgess AJ, Pavey S, Warrener R, et al. Up-regulation of p21 (WAF1/CIP1) by histone deacetylase inhibitors reduces their cytotoxicity. Mol Pharmacol 2001;60:828 – 37.
19. Novak L, Molnar P, Lengyel Z, Tron L. Does increased 18FDG uptake reflect malignant transformation of a low-grade glioma? A diagnostic dilemma. Neurol India 2005;53:112 – 4.
20. Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis? Nat Rev Cancer 2004;4:891 – 9.
21. Kaplan O, Navon G, Lyon RC, Faustino PJ, Straka EJ, Cohen JS. Effects of 2-deoxyglucose on drug-sensitive and drug-resistant human breast cancer cells: toxicity and magnetic resonance spectroscopy studies of metabolism. Cancer Res 1990;50:544 – 51.
22. Gallagher BM, Fowler JS, Gutterson NI, MacGregor RR, Wan CN, Wolf AP. Metabolic trapping as a principle of oradiopharmaceutical design: some factors responsible for the biodistribution of [18F] 2-deoxy-2-fluoro-D-glucose. J Nucl Med 1978;19:1154 – 61.
23. Cay O, Radnell M, Jeppsson B, Ahren B, Bengmark S. Inhibitory effect of 2-deoxy-D-glucose on liver tumor growth in rats. Cancer Res 1992;52:5794 – 6.
24. Dwarkanath BS, Zolzer F, Chandana S, et al. Heterogeneity in 2-deoxy-D-glucose-induced modifications in energetics and radiation responses of human tumor cell lines. Int J Radiat Oncol Biol Phys 2001;50:1051 – 61.
25. Mohanti BK, Rath GK, Anantha N, et al. Improving cancer radiotherapy with 2-deoxy-D-glucose: phase I/II clinical trials on human cerebral gliomas. Int J Radiat Oncol Biol Phys 1996;35:103 – 11.
26. Failly M, Korur S, Egler V, et al. Combination of sublethal concentrations of epidermal growth factor receptor inhibitor and microtubule stabilizer induces apoptosis of glioblastoma cells. Mol Cancer Ther 2007;6:773 – 81.
27. Waldman T, Kinzler KW, Vogelstein B. p21 is necessary for the p53-mediated G_1 arrest in human cancer cells. Cancer Res 1995;55:5187 – 90.

28. Fischer M, Kaech S, Knutti D, Matus A. Rapid actin-based plasticity in dendritic spines. Neuron 1998;20:847–54.
29. Han I, Kudlow JE. Reduced O glycosylation of Sp1 is associated with increased proteasome susceptibility. Mol Cell Biol 1997;17:2550–8.
30. Kang HT, Ju JW, Cho JW, Hwang ES. Down-regulation of Sp1 activity through modulation of O-glycosylation by treatment with a low glucose mimetic, 2-deoxyglucose. J Biol Chem 2003;278:51223–31.
31. Shaw RJ, Bardeesy N, Manning BD, et al. The LKB1 tumor suppressor negatively regulates mTOR signaling. Cancer Cell 2004;6:91–9.
32. Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. Cell 2003;115:577–90.
33. Dennis PB, Jaeschke A, Saitoh M, Fowler B, Kozma SC, Thomas G. Mammalian TOR: a homeostatic ATP sensor. Science 2001;294:1102–5.
34. Blagosklonny MV, Robey R, Sackett DL, et al. Histone deacetylase inhibitors all induce p21 but differentially cause tubulin acetylation, mitotic arrest, and cytotoxicity. Mol Cancer Ther 2002;1:937–41.
35. Fueyo J, Gomez-Manzano C, Puduvalli VK, et al. Adenovirus-mediated p16 transfer to glioma cells induces G_1 arrest and protects from paclitaxel and topotecan: implications for therapy. Int J Oncol 1998;12:665–9.
36. Schmidt M, Fan Z. Protection against chemotherapy-induced cytotoxicity by cyclin-dependent kinase inhibitors (CKI) in CKI-responsive cells compared with CKI-unresponsive cells. Oncogene 2001;20:6164–71.
37. Kim MS, Blake M, Baek JH, Kohlhagen G, Pommier Y, Carrier F. Inhibition of histone deacetylase increases cytotoxicity to anticancer drugs targeting DNA. Cancer Res 2003;63:7291–300.
38. Beuvink I, Boulay A, Fumagalli S, et al. The mTOR inhibitor RAD001 sensitizes tumor cells to DNA-damaged induced apoptosis through inhibition of p21 translation. Cell 2005;120:747–59.
39. Rahmani M, Yu C, Dai Y, et al. Coadministration of the heat shock protein 90 antagonist 17-allylamino-17-demethoxygeldanamycin with suberoylanilide hydroxamic acid or sodium butyrate synergistically induces apoptosis in human leukemia cells. Cancer Res 2003;63:8420–7.
40. Burgess A, Ruefli A, Beamish H, et al. Histone deacetylase inhibitors specifically kill nonproliferating tumour cells. Oncogene 2004;23:6693–701.

3. Future Perspectives

Malignant gliomas remain one of the deadliest of all cancers despite most aggressive therapy. Traditional treatments are based on surgical resection and on nonspecific, cytotoxic regimens mediating DNA damage. The combat against brain tumors experiences particular challenges such as the highly infiltrative, chemo- and radio-resistant nature of the cancer cells in addition to the vulnerability of normal brain tissue to any intervention (Merlo, 2003). The probability of 2-year survival for a GBM patient is not more than 10% (Stupp et al., 2005) and recurrence occurs in 80% of patients within 2 cm from the margins of the surgical resection reflecting the highly infiltrative behavior of glioma cells (Wallner et al., 1989). Advances in our understanding of the molecular biology and characterization of the genetic lesions of brain tumors led the opportunity for specific targeting of those tumors with novel agents. However, monotherapies against GBM did not show promising effects (Sathornsumetee et al., 2007; Wallner et al., 1989). No subtype of GBM has been found to depend on a single tumor suppressor or oncogene pathway. The frequent alterations in several non-redundant signaling pathways in GBM pathogenesis gave those cells their unique malignant characteristics. It seems unlikely that therapeutics towards a single target will help to achieve tumor control in GBM patients. The clinical evaluation of several single agents showed only modest effects in a minority of the GBM patients (Sathornsumetee et al., 2007). In order to efficiently induce cell death combinatorial therapies targeting non-overlapping pathways will be a prequisite. In addition, cancer cells may compensate for a single specific molecular inhibition by activating upstream feedback loops from the primary target and thus, dual targeting in a pathway upstream and downstream might be more successful.

Another attractive strategy would be targeting the same signaling pathway at two different levels to maximize the level of inhibition. Novel preclinical studies showed the success of that strategy, for example one study showed that combination of inhibitors of translation initiation; MNK (CGP57380) and mTOR (Rapamycin) both acting on the same pathway but at different levels had an additive effect on growth inhibition in

glioblastoma cell lines. MNK inhibitor reduced the light ribosomal fractions (40/60/80S) and the latter inhibitor reduced the global ribosome abundance explaining the mode of additive effect (Grzmil et al., 2009). In another report two drugs targeting EGFR; cetuximab (blocking ligand binding) and gefinitib (inhibitor of tyrosine kinase activity) offered combinatorial benefit in head and neck cancers by showing more pronounced down regulation of downstream pathways (Huang et al., 2004). Two different monoclonal antibodies against ERBB2: trastuzumab and pertuzumab showed synergistic effects on breast cancer cells (Nahta et al., 2004) and the reason behind this phenomenon was shown that ERBB receptors can utilize two different activation modes for relaying downstream signals and combination of those antibodies inhibit both modes by binding to different critical domains on ERBB2. Trastuzumab inhibits ligand independent dimerization of ERBB2 with ERBB3, whereas pertuzumab inhibits ligand dependent ERBB2 dimerization with other growth factor receptors including ERBB3. Thus, inhibiting different modes of activation of a single receptor may enhance the success of the therapy by limiting compensating mechanisms. On the other hand, it was found that ERBB2 overexpressing cells with PI3K mutations were insensitive to trastuzumab because PI3K resides downstream of ERBB2 and trastuzumab could not prevent activation of PKB (Junttila et al., 2009). Thus, it is required to inhibit PI3K to block growth of those kinds of cancer cells. Future work giving a deeper insight into the regulatory mechanisms used by receptor tyrosine kinases and downstream effectors, and analysis of efficiency of targeted therapies and identification of the mechanisms behind the failure and/or resistance will help us to develop more efficient treatments.

Using combinatorial approaches in our lab, we discovered a specific strong synergistic induction of cell death upon targeting energy metabolism together with disruption of epigenetic regulation with histone deactylase inhibitors. We speculate that re-expression of the silenced genes by HDIs lowered the apoptotic threshold of glioma cells which becomes even more pronounced under energy deprivation leading to a synergistic induction of cell death. The other synergistic effect we found by combining a cytotoxic drug with a targeted drug (AEE788 and patupilone) proves the potential of those kinds of combinations as well. A strategy is needed to determine the most promising combinations because the possible combinations are unlimited. The successful

therapeutic agent for GBM must be highly selective for cancer cells relative to the normal cells. In addition, efficient delivery across blood brain barrier into the brain parenchyma is required. Furthermore, the synergistic effects shown by combining two drugs might allow lowering the doses of the single agents and increase the patient tolerance.

Growing evidence indicates that many types of tumors are maintained by a rare population of stem cell like cancer cells which are thought to be the reason behind the chemo- and radio- resistant behavior of the tumor (Bao et al., 2006; Dean et al., 2005; Visvader and Lindeman, 2008). Many pathways involved in the control of normal stem cells are often found to be deregulated in cancer. Tumor development involves properties such as self-renewal and unlimited growth potential. Elucidating regulatory mechanisms shared between normal and cancer stem cells might facilitate the identification of novel therapeutic targets. To this end, it is necessary to further identify better markers for cancer initiating cells.

Recently, it was shown that cells derived from glioblastoma tumor samples grown in the neurobasal medium with bFGF and EGF more closely mirror primary tumors than serum cultured cell lines. This fact is represented by the phenotypic and genotypic similarities of those cells with the original tumor, unlike the highly discrepant genomic profile with the corresponding serum grown cell lines (Lee et al., 2006). Thus, it is crucial to expand findings with serum-cultured cells to neurobasal medium-grown *ex vivo* cell lines. Growing evidence challenged the initial judgment about CD133 positive cells as the universal cancer stem cell marker in brain tumors. Our finding that the cancer stem cells in a primary glioblastoma sample are contained in the CD133-negative population in BS287 *ex vivo* cell line, also contradicted the initial reports. Indeed, CD133-negative cells were also found to have stem cell characteristics, which differ, from the CD133+ cells. Beier et al proved that the CD133+ CSCs represent only a subset of primary GBM. CD133- tumor cells also possess stem cell like characteristics but with distinct genomic profiles and growth characteristics (Beier et al., 2007). Interestingly, Wang et al showed that CD133- cells are tumorigenic and CD133+ cells can be obtained from the tumors formed by the CD133- cells. CD133+ cells start to appear coinciding with angiogenesis and shorter survival suggesting that they are not required to initiate the tumor, but are

involved during brain tumor progression (Wang et al., 2008a). Griguer et al showed that CD133 expression is elevated under hypoxic conditions and with mitochondrial dysfunction, suggesting that CD133 is a marker of bio-energetic stress in GBM (Griguer et al., 2008). Additionally, the fact that xenotransplantation assays are used in order to identify the tumor initiating cells may lead to incorrect conclusions as these assays may select for cells that are able to survive in a foreign host but not represent cells with superior abilities (Read et al., 2009). Read et al also have shown that in a mouse model of medulloblastoma CD133- cells were the cells capable of initiating tumors. For all of the above-mentioned reasons the concept that CD133 is a CSC marker needs to be revised.

Cancer cells usually upregulate several pathways in order to increase their apoptotic threshold, and the efficacy of cell death inducing drugs by causing cell damage has proven limited efficacy (Hanahan and Weinberg, 2000). Another attractive approach for the treatment of GBM is differentiation therapy that is based on the concept that neoplastic cells are normal cells that have been arrested in an immature or less differentiated state. Differentiation therapy aims to force neoplastic cells to resume their maturation process (Pierce, 1983). This approach results in tumor reprogramming and a concomitant loss in proliferative capacity and induction of terminal differentiation or cell death (Leszczyniecka et al., 2001). Although differentiation therapy does not necessarily induce cell death, it restrains cell growth and allows the application of the standard therapies under more favorable conditions. The first such agent found to be effective was all-trans-retinoic acid (ATRA) which is used in the treatment of acute promyelocytic leukemia (APL), causing premyelocytes to mature and stop proliferating (Huang et al., 1988). An agent that showed promising effects in GBM was BMP4. It has an instructive role in the development of normal brain. However, it was also found to induce differentiation of brain tumor initiating cells and prolonged survival in the mice transplanted with GBM (Piccirillo and Vescovi, 2006). It is well plausible that the future work utilizing differentiation inducing therapeutic approaches will aid us to make bigger steps in the race for more efficient treatment options.

Bmi1, is a member of the polycomb group proteins, which are involved in the regulation of chromatin structure to fine-tune the expression of specific sets of genes

required for a certain developmental stage (Orlando, 2003). One major function of Bmi1 is to repress Ink4a/Arf locus, which is frequently deleted in brain tumors (Labuhn et al., 2001). These data commended that high Bmi1 protein levels could influence brain tumor development by repressing the Ink4a/Arf locus. Nonetheless, Bmi1 down-regulation did not affect the Ink4a/Arf expression in the tumor cell lines analyzed. Hence, in GBM, we proposed that Bmi1 is possibly influencing distinct pathways. We have shown that down-regulation of Bmi1 reduced GSK3β protein, and in turn, induced differentiation in cancer cells, and markedly reduced tumor cell proliferation, survival, migration and clonogenicity without affecting Ink4a/Arf expression. Clonogenic potential of cancer cells is reflected on their self-renewal capacity. One important role of Bmi1 is to regulate stem cell self-renewal, which is also a cardinal feature of cancer cells. Thus, our results identified a critical function of Bmi1-GSK3 route in GBM cancer cell identity. Inhibition of GSK3 induced tumor cell reprogramming with a concomitant loss of stem cell related markers and a stronger expression of differentiation markers. This led to a decrease in the stem cell like population and a reduction in the colony-forming abilities. The efficacy of differentiation therapy has not yet been broadly analyzed in cancers. Our findings show that inhibiting pathways controlling normal stem cell self-renewal, also affects growth and renewal of cancer cells and might lead to novel therapeutic approaches against GBM.

Although discovered three decades ago (Hemmings et al., 1981), glycogen synthase kinase 3 has only recently been considered as a therapeutic target for cancer therapies (Ougolkov et al., 2005; Wang et al., 2008b). GSK3 inhibits Wnt, Hedgehog and Notch signaling, which are activated in several cancers (Foltz et al., 2002; Polakis, 2000) This suggests a therapeutically negative pro-survival effect of GSK3 inhibitors in cancer cells. This, however, may not be true since long-term medical use of the GSK3 inhibitor lithium chloride in psychiatric patients did not result in increased cancer incidence (Cohen et al., 1998) and also normal cells might well be equipped with preventive mechanisms to balance activation of oncogenic pathways by GSK3 inhibitors. Likewise, Cohen et al. demonstrated that psychiatric patients on long-term Lithium chloride medication have a lower cancer prevalence compared to the general population (Cohen et al., 1998), Surprisingly, this would suggest a protective effect of Lithium chloride. Our results represent a molecular explanation for this epidemiological observation:

administration of Lithium chloride drives differentiation, inhibits proliferation and thereby might effectively inhibit tumor formation and progression. Additionally, excessive clinical data on Lithium chloride offer solid information on potential side effects, and it appears safe to assume that normal adult stem cells are not negatively affected even by long-term use of Lithium chloride. The complementary phenotypic and functional alterations induced by either inhibiting GSK3 or by down-regulating Bmi1 in this study points to a functional link between Bmi1 and GSK3. Nonetheless, further studies are needed to clarify the nature of the bond between Bmi1 and GSK3. In essence, as inhibitors of Bmi1 activity do not exist, GSK3 down-regulation can be viewed as a novel approach to efficiently block the subpopulation of cancer cells that contain a cancer stem cell like signature, inducing differentiation.

Sox2 protein is important for self-renewal of embryonic stem cells and has also been implicated to play a role in GBM (Fong et al., 2008; Gangemi et al., 2008). Down-regulation of Sox2 reduced cell proliferation and tumorigenicity in GBM. Sox2 was therefore proposed as a new therapeutic target for GBM (Gangemi et al., 2008). At present, inhibitors of Sox2 are not available. Our data show, however, that inhibition of GSK3 strongly down-regulates Sox2 in GBM. Thus, by applying Lithium chloride or SB216763 we can interfere with Sox2-dependent tumorigenic potential in GBM tumors.

Two main approaches are currently being investigated to eradicate the cancer stem cell (CSC) pool. One method is based on chemotherapeutic regimens, which specifically drive CSCs into apoptosis and thereby deplete the CSC reservoir of the tumor. The other method, aims to drive CSC into differentiation along with lowering their apoptotic threshold, thereby increasing their susceptibility to pro-apoptotic stimuli (Visvader and Lindeman, 2008). Two important facts -high degree of drug resistance and the shared cellular and gene expression profile between adult and cancer stem cells (Ben-Porath et al., 2008)- made specific targeting of CSCs difficult. Induction of differentiation represents a therapeutic strategy for CSC as Piccirillo et al. showed that bone morphogenetic proteins (BMP) can induce differentiation of CD133-positive GBM cells, thereby reducing their tumorigenic potential (Piccirillo and Vescovi, 2006). One concern with the use of morphogens is the risk of interfering with the tightly regulated

adult stem cell niches. Therefore, a strategy to induce differentiation in cancer stem cells has to be carefully assessed for any adverse effect on the adult stem cell population.

Our findings proposes GSK3 inhibition as an attractive strategy to specifically target a subpopulation of cancer cells with stem cell like characteristics. Expression of stem cell and differentiation markers define more accurately the subpopulation of cells within GBM cell lines and the *ex vivo* tumor cells than expression of the CD133 marker. Inactivation of either Bmi1 or GSK3 depleted precursor cells required for tumor maintenance and progression. Our data add another facet to the diverse effects of GSK3 as a regulator of cancer cell identity. Here, we identified GSK3 activation as a key element in maintaining stem cell like characteristics in a subset of cancer cells with higher self-renewal capacity. Recent reports corroborated our findings on the importance of GSK3 in GBM, as down-regulation of GSK3 was shown to induce apoptosis and to have an anti-migratory effect in glioma spheroids (Nowicki et al., 2008; Kotliarova et al., 2008), however, its effect on cell identity had not been analyzed.

GSK3 inhibitors consistently lead to accumulation of β-catenin protein, which then might drive cell proliferation. However, we did not observe increased proliferation. This may be explained by the constitutive activation of several growth promoting pathways such as EGFR and PI3K commonly activated in GBM, which could lead to saturation of Wnt signaling and masking additional activation. Conversely, pro-differentiation and pro-apoptotic programs, which have to be kept low in cancer cells, could be influenced by shRNAs against Bmi1, downregulating not only GSK3, but also Bcl2, Nestin, Sox2. Our microarray and protein data showed increased levels of GSK3β expression in brain tumors compared to normal brain tissue indicating a role of GSK3β in GBM. Down-regulation of GSK3 depleted the cancer stem cell population. GSK3 can therefore be regarded as an important regulator of tumor cell identity in GBM. Therefore, we propose GSK3 inhibition (e.g. Lithium chloride) as a complementary approach to standard therapy. Following control of the nodular tumor component, second line therapy with GSK3 inhibition may prevent tumor recurrence by the residual cancer stem cells that readily resist standard therapy.

Although analysis of glioma cells *in vitro* and *ex vivo* represents a fast and reliable model to assess therapeutic agents, future studies using an orthotopic rodent GBM model are required. An animal model would confirm the effects of our differentiation therapy with GSK3 inhibition and combinatorial approaches with histone deacetylase inhibitors and 2-DG. These animal studies will also enable us to better evaluate potential side effects. In the combat against GBM, a better identification of the tumor initiating cell along with an in depth study of the pathways regulating brain tumor initiating cells, would open new windows for development of novel drugs. In addition, recent studies showed that cancer stem cells as normal stem cells require a niche, specific extracellular signals in order to maintain their growth and renewal potential (Sneddon and Werb, 2007). Inhibition of niche-specific signals and of mechanisms regulating migration of normal stem cells might be applicable to limit invasive behavior of brain tumor cells.

The cancer stem cell field and our knowledge on cancer biology is expanding rapidly which will ultimately enable us to interfere and destroy the devastating potential of tumor cells in the near future.

References

Al-Hajj, M., Wicha, M.S., Benito-Hernandez, A., Morrison, S.J., and Clarke, M.F. (2003). Prospective identification of tumorigenic breast cancer cells. Proceedings of the National Academy of Sciences of the United States of America *100*, 3983-3988.

Alberts, B. (2002). Cancer. In Molecular Biology of The Cell, B. Alberts, ed. (New York, Garland Science), pp. xxxiv, 1464 p.

Alleyne, C.H., Jr., He, J., Yang, J., Hunter, S.B., Cotsonis, G., James, C.D., and Olson, J.J. (1999). Analysis of cyclin dependent kinase inhibitors in malignant astrocytomas. International journal of oncology *14*, 1111-1116.

Austin, J., and Kimble, J. (1987). glp-1 is required in the germ line for regulation of the decision between mitosis and meiosis in C. elegans. Cell *51*, 589-599.

Bachoo, R.M., Maher, E.A., Ligon, K.L., Sharpless, N.E., Chan, S.S., You, M.J., Tang, Y., DeFrances, J., Stover, E., Weissleder, R.*, et al.* (2002). Epidermal growth factor receptor and Ink4a/Arf: convergent mechanisms governing terminal differentiation and transformation along the neural stem cell to astrocyte axis. Cancer cell *1*, 269-277.

Bao, S., Wu, Q., McLendon, R.E., Hao, Y., Shi, Q., Hjelmeland, A.B., Dewhirst, M.W., Bigner, D.D., and Rich, J.N. (2006). Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature *444*, 756-760.

Barker, F.G., 2nd, Simmons, M.L., Chang, S.M., Prados, M.D., Larson, D.A., Sneed, P.K., Wara, W.M., Berger, M.S., Chen, P., Israel, M.A.*, et al.* (2001). EGFR overexpression and radiation response in glioblastoma multiforme. International journal of radiation oncology, biology, physics *51*, 410-418.

Beier, D., Hau, P., Proescholdt, M., Lohmeier, A., Wischhusen, J., Oefner, P.J., Aigner, L., Brawanski, A., Bogdahn, U., and Beier, C.P. (2007). CD133(+) and CD133(-) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. Cancer Res *67*, 4010-4015.

Ben-Porath, I., Thomson, M.W., Carey, V.J., Ge, R., Bell, G.W., Regev, A., and Weinberg, R.A. (2008). An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. Nat Genet *40*, 499-507.

Bhardwaj, G., Murdoch, B., Wu, D., Baker, D.P., Williams, K.P., Chadwick, K., Ling, L.E., Karanu, F.N., and Bhatia, M. (2001). Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. Nature immunology *2*, 172-180.

Boucher, Y., Salehi, H., Witwer, B., Harsh, G.R.t., and Jain, R.K. (1997). Interstitial fluid pressure in intracranial tumours in patients and in rodents. British journal of cancer *75*, 829-836.

Bruggeman, S.W., Hulsman, D., Tanger, E., Buckle, T., Blom, M., Zevenhoven, J., van Tellingen, O., and van Lohuizen, M. (2007). Bmi1 controls tumor development in an Ink4a/Arf-independent manner in a mouse model for glioma. Cancer Cell *12*, 328-341.

Burger, P.E., Xiong, X., Coetzee, S., Salm, S.N., Moscatelli, D., Goto, K., and Wilson, E.L. (2005). Sca-1 expression identifies stem cells in the proximal region of prostatic ducts with high capacity to reconstitute prostatic tissue. Proceedings of the National Academy of Sciences of the United States of America *102*, 7180-7185.

Chan, E.F., Gat, U., McNiff, J.M., and Fuchs, E. (1999). A common human skin tumour is caused by activating mutations in beta-catenin. Nature genetics *21*, 410-413.

Cline, G.W., Johnson, K., Regittnig, W., Perret, P., Tozzo, E., Xiao, L., Damico, C., and Shulman, G.I. (2002). Effects of a novel glycogen synthase kinase-3 inhibitor on insulin-

stimulated glucose metabolism in Zucker diabetic fatty (fa/fa) rats. Diabetes *51*, 2903-2910.

Cohen, P., and Goedert, M. (2004). GSK3 inhibitors: development and therapeutic potential. Nat Rev Drug Discov *3*, 479-487.

Cohen, Y., Chetrit, A., Sirota, P., and Modan, B. (1998). Cancer morbidity in psychiatric patients: influence of lithium carbonate treatment. Med Oncol *15*, 32-36.

Collins, V.P. (1998). Gliomas. Cancer surveys *32*, 37-51.

Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., and Hemmings, B.A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature *378*, 785-789.

Dean, M., Fojo, T., and Bates, S. (2005). Tumour stem cells and drug resistance. Nat Rev Cancer *5*, 275-284.

Deorah, S., Lynch, C.F., Sibenaller, Z.A., and Ryken, T.C. (2006). Trends in brain cancer incidence and survival in the United States: Surveillance, Epidemiology, and End Results Program, 1973 to 2001. Neurosurgical focus *20*, E1.

Devlin, T.M. (2006). Textbook of biochemistry : with clinical correlations, 6th edn (Hoboken, N.J., Wiley-Liss).

Doble, B.W., and Woodgett, J.R. (2003). GSK-3: tricks of the trade for a multi-tasking kinase. J Cell Sci *116*, 1175-1186.

Dropcho, E.J., and Soong, S.J. (1996). The prognostic impact of prior low grade histology in patients with anaplastic gliomas: a case-control study. Neurology *47*, 684-690.

Egler, V., Korur, S., Failly, M., Boulay, J.L., Imber, R., Lino, M.M., and Merlo, A. (2008). Histone deacetylase inhibition and blockade of the glycolytic pathway synergistically induce glioblastoma cell death. Clin Cancer Res *14*, 3132-3140.

Ellisen, L.W., Bird, J., West, D.C., Soreng, A.L., Reynolds, T.C., Smith, S.D., and Sklar, J. (1991). TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. Cell *66*, 649-661.

Failly, M., Korur, S., Egler, V., Boulay, J.L., Lino, M.M., Imber, R., and Merlo, A. (2007). Combination of sublethal concentrations of epidermal growth factor receptor inhibitor and microtubule stabilizer induces apoptosis of glioblastoma cells. Mol Cancer Ther *6*, 773-781.

Fearon, E.R. (1997). Human cancer syndromes: clues to the origin and nature of cancer. Science (New York, NY *278*, 1043-1050.

Fisher, M.S., and Kripke, M.L. (2002). Systemic alteration induced in mice by ultraviolet light irradiation and its relationship to ultraviolet carcinogenesis. 1977. Bulletin of the World Health Organization *80*, 908-912.

Foltz, D.R., Santiago, M.C., Berechid, B.E., and Nye, J.S. (2002). Glycogen synthase kinase-3beta modulates notch signaling and stability. Curr Biol *12*, 1006-1011.

Fong, H., Hohenstein, K.A., and Donovan, P.J. (2008). Regulation of self-renewal and pluripotency by Sox2 in human embryonic stem cells. Stem Cells *26*, 1931-1938.

Frame, S., and Cohen, P. (2001). GSK3 takes centre stage more than 20 years after its discovery. Biochem J *359*, 1-16.

Friend, S.H., Bernards, R., Rogelj, S., Weinberg, R.A., Rapaport, J.M., Albert, D.M., and Dryja, T.P. (1986). A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature *323*, 643-646.

Gailani, M.R., and Bale, A.E. (1999). Acquired and inherited basal cell carcinomas and the patched gene. Advances in dermatology *14*, 261-283; discussion 284.

Gangemi, R.M., Griffero, F., Marubbi, D., Perera, M., Capra, M.C., Malatesta, P., Ravetti, G.L., Zona, G.L., Daga, A., and Corte, G. (2008). SOX2 Silencing in Glioblastoma Tumor Initiating Cells Causes Stop of Proliferation and Loss of Tumorigenicity. Stem Cells.

Gibbs, C.P., Kukekov, V.G., Reith, J.D., Tchigrinova, O., Suslov, O.N., Scott, E.W., Ghivizzani, S.C., Ignatova, T.N., and Steindler, D.A. (2005). Stem-like cells in bone sarcomas: implications for tumorigenesis. Neoplasia (New York, NY *7*, 967-976.

Godlewski, J., Nowicki, M.O., Bronisz, A., Williams, S., Otsuki, A., Nuovo, G., Raychaudhury, A., Newton, H.B., Chiocca, E.A., and Lawler, S. (2008). Targeting of the Bmi-1 oncogene/stem cell renewal factor by microRNA-128 inhibits glioma proliferation and self-renewal. Cancer research *68*, 9125-9130.

Gould, T.D., and Manji, H.K. (2002). The Wnt signaling pathway in bipolar disorder. Neuroscientist *8*, 497-511.

Griguer, C.E., Oliva, C.R., Gobin, E., Marcorelles, P., Benos, D.J., Lancaster, J.R., Jr., and Gillespie, G.Y. (2008). CD133 is a marker of bioenergetic stress in human glioma. PloS one *3*, e3655.

Grzmil, M., P., M.J., Lino, M.M., Wang, Y., Merlo, A., and Hemmings, B.A. (2009). Targeting overexpressed MNK1 sensitizes glioblastoma cells to rapamycin.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Haupt, Y., Bath, M.L., Harris, A.W., and Adams, J.M. (1993). bmi-1 transgene induces lymphomas and collaborates with myc in tumorigenesis. Oncogene *8*, 3161-3164.

He, J., Olson, J.J., and James, C.D. (1995). Lack of p16INK4 or retinoblastoma protein (pRb), or amplification-associated overexpression of cdk4 is observed in distinct subsets of malignant glial tumors and cell lines. Cancer research *55*, 4833-4836.

Hemmings, B.A., Yellowlees, D., Kernohan, J.C., and Cohen, P. (1981). Purification of glycogen synthase kinase 3 from rabbit skeletal muscle. Copurification with the activating factor (FA) of the (Mg-ATP) dependent protein phosphatase. Eur J Biochem *119*, 443-451.

Henrique, D., Hirsinger, E., Adam, J., Le Roux, I., Pourquie, O., Ish-Horowicz, D., and Lewis, J. (1997). Maintenance of neuroepithelial progenitor cells by Delta-Notch signalling in the embryonic chick retina. Curr Biol *7*, 661-670.

Hoeflich, K.P., Luo, J., Rubie, E.A., Tsao, M.S., Jin, O., and Woodgett, J.R. (2000). Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. Nature *406*, 86-90.

Huang, H.S., Nagane, M., Klingbeil, C.K., Lin, H., Nishikawa, R., Ji, X.D., Huang, C.M., Gill, G.N., Wiley, H.S., and Cavenee, W.K. (1997). The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling. The Journal of biological chemistry *272*, 2927-2935.

Huang, M.E., Ye, Y.C., Chen, S.R., Chai, J.R., Lu, J.X., Zhoa, L., Gu, L.J., and Wang, Z.Y. (1988). Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. Blood *72*, 567-572.

Huang, S., Armstrong, E.A., Benavente, S., Chinnaiyan, P., and Harari, P.M. (2004). Dual-agent molecular targeting of the epidermal growth factor receptor (EGFR): combining anti-EGFR antibody with tyrosine kinase inhibitor. Cancer research *64*, 5355-5362.

Ignatova, T.N., Kukekov, V.G., Laywell, E.D., Suslov, O.N., Vrionis, F.D., and Steindler, D.A. (2002). Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. Glia *39*, 193-206.

Jacobs, J.J., Kieboom, K., Marino, S., DePinho, R.A., and van Lohuizen, M. (1999a). The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. Nature *397*, 164-168.

Jacobs, J.J., Scheijen, B., Voncken, J.W., Kieboom, K., Berns, A., and van Lohuizen, M. (1999b). Bmi-1 collaborates with c-Myc in tumorigenesis by inhibiting c-Myc-induced apoptosis via INK4a/ARF. Genes Dev *13*, 2678-2690.

Jameson, C.W. (2000). Environmental Carcinogens. In Environmental health secrets, R.L.L. Luanne Kemp Williams, ed. (Elsevier Health Sciences).

Jia, J., Amanai, K., Wang, G., Tang, J., Wang, B., and Jiang, J. (2002). Shaggy/GSK3 antagonizes Hedgehog signalling by regulating Cubitus interruptus. Nature *416*, 548-552.

Jones, P.A., and Baylin, S.B. (2002). The fundamental role of epigenetic events in cancer. Nat Rev Genet *3*, 415-428.

Junttila, T.T., Akita, R.W., Parsons, K., Fields, C., Lewis Phillips, G.D., Friedman, L.S., Sampath, D., and Sliwkowski, M.X. (2009). Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941. Cancer cell *15*, 429-440.

Kim, T.Y., Zhong, S., Fields, C.R., Kim, J.H., and Robertson, K.D. (2006). Epigenomic profiling reveals novel and frequent targets of aberrant DNA methylation-mediated silencing in malignant glioma. Cancer Res *66*, 7490-7501.

Kita, D., Yonekawa, Y., Weller, M., and Ohgaki, H. (2007). PIK3CA alterations in primary (de novo) and secondary glioblastomas. Acta neuropathologica *113*, 295-302.

Kneifel, S., Cordier, D., Good, S., Ionescu, M.C., Ghaffari, A., Hofer, S., Kretzschmar, M., Tolnay, M., Apostolidis, C., Waser, B.*, et al.* (2006). Local targeting of malignant gliomas by the diffusible peptidic vector 1,4,7,10-tetraazacyclododecane-1-glutaric acid-4,7,10-triacetic acid-substance p. Clin Cancer Res *12*, 3843-3850.

Knudson, A.G. (2001). Two genetic hits (more or less) to cancer. Nat Rev Cancer *1*, 157-162.

Knudson, A.G., Jr. (1971). Mutation and cancer: statistical study of retinoblastoma. Proceedings of the National Academy of Sciences of the United States of America *68*, 820-823.

Korinek, V., Barker, N., Moerer, P., van Donselaar, E., Huls, G., Peters, P.J., and Clevers, H. (1998). Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. Nature genetics *19*, 379-383.

Kotliarova, S., Pastorino, S., Kovell, L.C., Kotliarov, Y., Song, H., Zhang, W., Bailey, R., Maric, D., Zenklusen, J.C., Lee, J.*, et al.* (2008). Glycogen synthase kinase-3 inhibition induces glioma cell death through c-MYC, nuclear factor-kappaB, and glucose regulation. Cancer Res *68*, 6643-6651.

Labuhn, M., Jones, G., Speel, E.J., Maier, D., Zweifel, C., Gratzl, O., Van Meir, E.G., Hegi, M.E., and Merlo, A. (2001). Quantitative real-time PCR does not show selective targeting of p14(ARF) but concomitant inactivation of both p16(INK4A) and p14(ARF) in 105 human primary gliomas. Oncogene *20*, 1103-1109.

Lane, H.A., Motoyama, A.B., Beuvink, I., and Hynes, N.E. (2001). Modulation of p27/Cdk2 complex formation through 4D5-mediated inhibition of HER2 receptor signaling. Ann Oncol *12 Suppl 1*, S21-22.

Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., Minden, M., Paterson, B., Caligiuri, M.A., and Dick, J.E. (1994). A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature *367*, 645-648.

Lee, J., Kotliarova, S., Kotliarov, Y., Li, A., Su, Q., Donin, N.M., Pastorino, S., Purow, B.W., Christopher, N., Zhang, W.*, et al.* (2006). Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer cell *9*, 391-403.

Leszczyniecka, M., Roberts, T., Dent, P., Grant, S., and Fisher, P.B. (2001). Differentiation therapy of human cancer: basic science and clinical applications. Pharmacology & therapeutics *90*, 105-156.

Leung, C., Lingbeek, M., Shakhova, O., Liu, J., Tanger, E., Saremaslani, P., Van Lohuizen, M., and Marino, S. (2004). Bmi1 is essential for cerebellar development and is overexpressed in human medulloblastomas. Nature *428*, 337-341.

Little, J.B. (2000). Radiation carcinogenesis. Carcinogenesis *21*, 397-404.

Liu, S., Dontu, G., Mantle, I.D., Patel, S., Ahn, N.S., Jackson, K.W., Suri, P., and Wicha, M.S. (2006). Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. Cancer Res *66*, 6063-6071.

Lochhead, P.A., Coghlan, M., Rice, S.Q., and Sutherland, C. (2001). Inhibition of GSK-3 selectively reduces glucose-6-phosphatase and phosphatase and phosphoenolypyruvate carboxykinase gene expression. Diabetes *50*, 937-946.

Louis, D.N., Ohgaki, H., Wiestler, O.D., Cavenee, W.K., Burger, P.C., Jouvet, A., Scheithauer, B.W., and Kleihues, P. (2007). The 2007 WHO classification of tumours of the central nervous system. Acta neuropathologica *114*, 97-109.

Lynch, T.J., Bell, D.W., Sordella, R., Gurubhagavatula, S., Okimoto, R.A., Brannigan, B.W., Harris, P.L., Haserlat, S.M., Supko, J.G., Haluska, F.G., *et al.* (2004). Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med *350*, 2129-2139.

Maehama, T., and Dixon, J.E. (2000). [PTEN tumor suppressor: functions as a lipid phosphatase]. Tanpakushitsu kakusan koso *45*, 2405-2414.

Maher, E.A., Furnari, F.B., Bachoo, R.M., Rowitch, D.H., Louis, D.N., Cavenee, W.K., and DePinho, R.A. (2001). Malignant glioma: genetics and biology of a grave matter. Genes Dev *15*, 1311-1333.

Mariadason, J.M., Corner, G.A., and Augenlicht, L.H. (2000). Genetic reprogramming in pathways of colonic cell maturation induced by short chain fatty acids: comparison with trichostatin A, sulindac, and curcumin and implications for chemoprevention of colon cancer. Cancer research *60*, 4561-4572.

Matsui, W., Huff, C.A., Wang, Q., Malehorn, M.T., Barber, J., Tanhehco, Y., Smith, B.D., Civin, C.I., and Jones, R.J. (2004). Characterization of clonogenic multiple myeloma cells. Blood *103*, 2332-2336.

Maynard Smith, J. (1989). Evolutionary genetics (Oxford [Oxfordshire] ; New York, Oxford University Press).

Maynard Smith, J., and Szathmáry, E. (1995). The major transitions in evolution (Oxford ; New York, W.H. Freeman Spektrum).

Merlo, A. (2003). Genes and pathways driving glioblastomas in humans and murine disease models. Neurosurg Rev *26*, 145-158.

Merlo, A., Hausmann, O., Wasner, M., Steiner, P., Otte, A., Jermann, E., Freitag, P., Reubi, J.C., Muller-Brand, J., Gratzl, O., *et al.* (1999). Locoregional regulatory peptide

receptor targeting with the diffusible somatostatin analogue 90Y-labeled DOTA0-D-Phe1-Tyr3-octreotide (DOTATOC): a pilot study in human gliomas. Clin Cancer Res *5*, 1025-1033.

Merlo, A., Herman, J.G., Mao, L., Lee, D.J., Gabrielson, E., Burger, P.C., Baylin, S.B., and Sidransky, D. (1995). 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nature medicine *1*, 686-692.

Miller, J.R., and Moon, R.T. (1996). Signal transduction through beta-catenin and specification of cell fate during embryogenesis. Genes & development *10*, 2527-2539.

Nahta, R., Hung, M.C., and Esteva, F.J. (2004). The HER-2-targeting antibodies trastuzumab and pertuzumab synergistically inhibit the survival of breast cancer cells. Cancer research *64*, 2343-2346.

Narita, Y., Nagane, M., Mishima, K., Huang, H.J., Furnari, F.B., and Cavenee, W.K. (2002). Mutant epidermal growth factor receptor signaling down-regulates p27 through activation of the phosphatidylinositol 3-kinase/Akt pathway in glioblastomas. Cancer research *62*, 6764-6769.

Niederlaender, E. (2006). Atlas on mortality in the EU. In European Commission Eurostat

Nobusawa, S., Watanabe, T., Kleihues, P., and Ohgaki, H. (2009). IDH1 Mutations as Molecular Signature and Predictive Factor of Secondary Glioblastomas. Clin Cancer Res.

Nowak, K., Kerl, K., Fehr, D., Kramps, C., Gessner, C., Killmer, K., Samans, B., Berwanger, B., Christiansen, H., and Lutz, W. (2006). BMI1 is a target gene of E2F-1 and is strongly expressed in primary neuroblastomas. Nucleic Acids Res *34*, 1745-1754.

Nowell, P.C. (1976). The clonal evolution of tumor cell populations. Science (New York, NY *194*, 23-28.

Nowicki, M.O., Dmitrieva, N., Stein, A.M., Cutter, J.L., Godlewski, J., Saeki, Y., Nita, M., Berens, M.E., Sander, L.M., Newton, H.B.*, et al.* (2008). Lithium inhibits invasion of glioma cells; possible involvement of glycogen synthase kinase-3. Neuro Oncol *10*, 690-699.

O'Brien, C.A., Pollett, A., Gallinger, S., and Dick, J.E. (2007). A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature *445*, 106-110.

Ohgaki, H., and Kleihues, P. (2007). Genetic pathways to primary and secondary glioblastoma. The American journal of pathology *170*, 1445-1453.

Orlando, V. (2003). Polycomb, epigenomes, and control of cell identity. Cell *112*, 599-606.

Ougolkov, A.V., Fernandez-Zapico, M.E., Savoy, D.N., Urrutia, R.A., and Billadeau, D.D. (2005). Glycogen synthase kinase-3beta participates in nuclear factor kappaB-mediated gene transcription and cell survival in pancreatic cancer cells. Cancer Res *65*, 2076-2081.

Pardridge, W.M. (2003). Molecular biology of the blood-brain barrier. Methods in molecular medicine *89*, 385-399.

Park, I.K., Qian, D., Kiel, M., Becker, M.W., Pihalja, M., Weissman, I.L., Morrison, S.J., and Clarke, M.F. (2003). Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. Nature *423*, 302-305.

Parsons, D.W., Jones, S., Zhang, X., Lin, J.C., Leary, R.J., Angenendt, P., Mankoo, P., Carter, H., Siu, I.M., Gallia, G.L.*, et al.* (2008). An integrated genomic analysis of human glioblastoma multiforme. Science *321*, 1807-1812.

Piccirillo, S.G., and Vescovi, A.L. (2006). Bone morphogenetic proteins regulate tumorigenicity in human glioblastoma stem cells. Ernst Schering Found Symp Proc, 59-81.

Pierce, G.B. (1983). The cancer cell and its control by the embryo. Rous-Whipple Award lecture. The American journal of pathology *113*, 117-124.

Polakis, P. (2000). Wnt signaling and cancer. Genes Dev *14*, 1837-1851.

Rasheed, B.K., Wiltshire, R.N., Bigner, S.H., and Bigner, D.D. (1999). Molecular pathogenesis of malignant gliomas. Current opinion in oncology *11*, 162-167.

Read, T.A., Fogarty, M.P., Markant, S.L., McLendon, R.E., Wei, Z., Ellison, D.W., Febbo, P.G., and Wechsler-Reya, R.J. (2009). Identification of CD15 as a marker for tumor-propagating cells in a mouse model of medulloblastoma. Cancer cell *15*, 135-147.

Reya, T., Morrison, S.J., Clarke, M.F., and Weissman, I.L. (2001). Stem cells, cancer, and cancer stem cells. Nature *414*, 105-111.

Ricci-Vitiani, L., Lombardi, D.G., Pilozzi, E., Biffoni, M., Todaro, M., Peschle, C., and De Maria, R. (2007). Identification and expansion of human colon-cancer-initiating cells. Nature *445*, 111-115.

Rich, J.N., and Bigner, D.D. (2004). Development of novel targeted therapies in the treatment of malignant glioma. Nature reviews *3*, 430-446.

Rich, J.N., Reardon, D.A., Peery, T., Dowell, J.M., Quinn, J.A., Penne, K.L., Wikstrand, C.J., Van Duyn, L.B., Dancey, J.E., McLendon, R.E*., et al.* (2004). Phase II trial of gefitinib in recurrent glioblastoma. J Clin Oncol *22*, 133-142.

Ridley, M. (1993). Evolution (Boston, Blackwell Scientific).

Ryder, J., Su, Y., Liu, F., Li, B., Zhou, Y., and Ni, B. (2003). Divergent roles of GSK3 and CDK5 in APP processing. Biochem Biophys Res Commun *312*, 922-929.

Sathornsumetee, S., Reardon, D.A., Desjardins, A., Quinn, J.A., Vredenburgh, J.J., and Rich, J.N. (2007). Molecularly targeted therapy for malignant glioma. Cancer *110*, 13-24.

Singh, S.K., Clarke, I.D., Terasaki, M., Bonn, V.E., Hawkins, C., Squire, J., and Dirks, P.B. (2003). Identification of a cancer stem cell in human brain tumors. Cancer Res *63*, 5821-5828.

Singh, S.K., Hawkins, C., Clarke, I.D., Squire, J.A., Bayani, J., Hide, T., Henkelman, R.M., Cusimano, M.D., and Dirks, P.B. (2004). Identification of human brain tumour initiating cells. Nature *432*, 396-401.

Sneddon, J.B., and Werb, Z. (2007). Location, location, location: the cancer stem cell niche. Cell stem cell *1*, 607-611.

Stupp, R., Mason, W.P., van den Bent, M.J., Weller, M., Fisher, B., Taphoorn, M.J., Belanger, K., Brandes, A.A., Marosi, C., Bogdahn, U*., et al.* (2005). Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. The New England journal of medicine *352*, 987-996.

Tan, J., Zhuang, L., Leong, H.S., Iyer, N.G., Liu, E.T., and Yu, Q. (2005). Pharmacologic modulation of glycogen synthase kinase-3beta promotes p53-dependent apoptosis through a direct Bax-mediated mitochondrial pathway in colorectal cancer cells. Cancer Res *65*, 9012-9020.

Traxler, P., Allegrini, P.R., Brandt, R., Brueggen, J., Cozens, R., Fabbro, D., Grosios, K., Lane, H.A., McSheehy, P., Mestan, J*., et al.* (2004). AEE788: a dual family epidermal growth factor receptor/ErbB2 and vascular endothelial growth factor receptor tyrosine kinase inhibitor with antitumor and antiangiogenic activity. Cancer research *64*, 4931-4941.

Varnum-Finney, B., Xu, L., Brashem-Stein, C., Nourigat, C., Flowers, D., Bakkour, S., Pear, W.S., and Bernstein, I.D. (2000). Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. Nature medicine *6*, 1278-1281.

Visvader, J.E., and Lindeman, G.J. (2008). Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer *8*, 755-768.

Vogelstein, B., and Kinzler, K.W. (2004). Cancer genes and the pathways they control. Nature medicine *10*, 789-799.

Vonlanthen, S., Heighway, J., Altermatt, H.J., Gugger, M., Kappeler, A., Borner, M.M., van Lohuizen, M., and Betticher, D.C. (2001). The bmi-1 oncoprotein is differentially expressed in non-small cell lung cancer and correlates with INK4A-ARF locus expression. Br J Cancer *84*, 1372-1376.

Wallner, K.E., Galicich, J.H., Krol, G., Arbit, E., and Malkin, M.G. (1989). Patterns of failure following treatment for glioblastoma multiforme and anaplastic astrocytoma. International journal of radiation oncology, biology, physics *16*, 1405-1409.

Wang, J., Sakariassen, P.O., Tsinkalovsky, O., Immervoll, H., Boe, S.O., Svendsen, A., Prestegarden, L., Rosland, G., Thorsen, F., Stuhr, L.*, et al.* (2008a). CD133 negative glioma cells form tumors in nude rats and give rise to CD133 positive cells. International journal of cancer *122*, 761-768.

Wang, Z., Smith, K.S., Murphy, M., Piloto, O., Somervaille, T.C., and Cleary, M.L. (2008b). Glycogen synthase kinase 3 in MLL leukaemia maintenance and targeted therapy. Nature.

Wechsler-Reya, R., and Scott, M.P. (2001). The developmental biology of brain tumors. Annual review of neuroscience *24*, 385-428.

Wechsler-Reya, R.J., and Scott, M.P. (1999). Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog. Neuron *22*, 103-114.

Wen, P.Y., Yung, W.K., Lamborn, K.R., Dahia, P.L., Wang, Y., Peng, B., Abrey, L.E., Raizer, J., Cloughesy, T.F., Fink, K.*, et al.* (2006). Phase I/II study of imatinib mesylate for recurrent malignant gliomas: North American Brain Tumor Consortium Study 99-08. Clin Cancer Res *12*, 4899-4907.

Wick, A.N., Drury, D.R., Nakada, H.I., and Wolfe, J.B. (1957). Localization of the primary metabolic block produced by 2-deoxyglucose. The Journal of biological chemistry *224*, 963-969.

Witschi, H., Pinkerton, K.E., Coggins, C.R., Penn, A., and Gori, G.B. (1995). Environmental tobacco smoke: experimental facts and societal issues. Fundam Appl Toxicol *24*, 3-12.

Xin, L., Lawson, D.A., and Witte, O.N. (2005). The Sca-1 cell surface marker enriches for a prostate-regenerating cell subpopulation that can initiate prostate tumorigenesis. Proceedings of the National Academy of Sciences of the United States of America *102*, 6942-6947.

Yost, C., Torres, M., Miller, J.R., Huang, E., Kimelman, D., and Moon, R.T. (1996). The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in Xenopus embryos by glycogen synthase kinase 3. Genes & development *10*, 1443-1454.

Zencak, D., Lingbeek, M., Kostic, C., Tekaya, M., Tanger, E., Hornfeld, D., Jaquet, M., Munier, F.L., Schorderet, D.F., van Lohuizen, M.*, et al.* (2005). Bmi1 loss produces an increase in astroglial cells and a decrease in neural stem cell population and proliferation. J Neurosci *25*, 5774-5783.

Zhang, Y., and Kalderon, D. (2001). Hedgehog acts as a somatic stem cell factor in the Drosophila ovary. Nature *410*, 599-604.

Zhao, S., Lin, Y., Xu, W., Jiang, W., Zha, Z., Wang, P., Yu, W., Li, Z., Gong, L., Peng, Y.*, et al.* (2009). Glioma-derived mutations in IDH1 dominantly inhibit IDH1 catalytic activity and induce HIF-1alpha. Science (New York, NY *324*, 261-265.

Zhu, A.J., and Watt, F.M. (1999). beta-catenin signalling modulates proliferative potential of human epidermal keratinocytes independently of intercellular adhesion. Development (Cambridge, England) *126*, 2285-2298.

Zhu, Y., and Parada, L.F. (2002). The molecular and genetic basis of neurological tumours. Nat Rev Cancer *2*, 616-626.

Abbreviations

2DG: 2-deoxyglucose

ATP: adenosine triphosphate

BBB: Blood-brain barrier

CSC: Cancer stem cell

DNA: Deoxyribonucleic acid

EGFR: Epidermal growth factor receptor

GBM: Glioblastoma

GSK3: Glycogen synthase kinase 3

HDM2: Human double minute 2

IDH: Isocitrate dehydrogenase

KO: Knockout

LOH: Loss of heterozygosity

MAPK: Mitogen activated protein kinase

NF1-2: Neurofibromatosis 1-2

PcG: Polycomb group genes

PKB: Protein kinase B

PKI: Protein kinase inhibitory

Rb: Retinoblastoma

UV: ultra-violet

WHO: World health organization

Acknowledgements

I would like to express my gratitude to my thesis supervisor, Prof. Adrian Merlo for his continuous guidance and helpful advices. This study could never have been achieved without his supreme experience that he never refrained from sharing with me. His valuable criticisms helped me in every step of my study.

I would like to gratefully thank Dr. Maria Maddalena Lino for her advices in my experimental work and subsequent manuscripts, as well as her supervision during my study.

I owe grateful thanks to the other members of the thesis evaluation committee, Prof. Heinrich Reichert, Prof. Ruth Chiquet-Ehrismann, and Dr. Brian A. Hemmings, who gave their valuable time to read, examine and evaluate this thesis.

I would like to express my thanks to Beatrice Dolder and Elisabeth Taylor sharing their knowledge and experience all throughout my studies. They were always giving a hand at all the steps of this study.

I would like to express my thanks to Emmanuel Traunecker and Mustafa Sirin. They were also great friends who were always with me during the hard times of my laboratory work.

Last but not the least, I express my sincere thanks to all my colleagues, Family and friends for their constant support and encouragement during my Ph.D career.

SERDAR KORUR

E-mail: serdar.korur@unibas.ch
Address: Klybeckstrasse 40, 4057 Basel, SWITZERLAND
Phone: +41 76 262 89 62

PERSONAL INFORMATION

Date and Place of Birth: November 08, 1979 – Merkez, Edirne, TURKEY

Nationality: Turkish

Marital Status: Single

EDUCATION

2004 – 2009

- PhD. in Department of Molecular Neuro-oncology, Basel University, Basel, SWITZERLAND

2002 – 2004

- M.S. in Department of Molecular Biology & Genetics, Bogaziçi University, Istanbul, TURKEY

1999 – 2002

- B.S. in Department of Molecular Biology & Genetics, Bogaziçi University, Istanbul, TURKEY

1997 – 1999

- Department of Chemistry, Bogaziçi University, Istanbul, TURKEY

LABORATOY EXPERIENCE

August, 2004 – Present **PhD. Student**
Prof. Adrian Merlo (Basel, Switzerland)

- The role of Bmi1-GSK3β in brain tumors

December, 2002 – 2004 **Masters project**
Prof.Aslı Tolun, Supervisor

- Analysis of Seckel Syndrome: Linkage Analysis, Gene localization, Mutation screening.
- Mutation screening of a new candidate gene leading to Azoospermia

August, 2002 – December, 2002 **Research Scientist**
Biotechnology company

- Biological kit design (Iontek, TURKEY)

Summer 2002, **Visiting Laboratory student**
Prof. Adrian Merlo (Basel, Switzerland)

- Role of PTP epsilon in glioblastoma

Summer 2001, **Visiting Laboratory student**
Prof. Adrian Merlo (Basel, Switzerland)

- Role of focal adhesion kinase in brain tumors

Fall 2000 – Spring 2001 **Project student**
Prof. A.Nazlı Basak (Istanbul, Turkey)

- Mutation analysis in Frontotemporal Dementia

PUBLICATIONS

1. Korur S, Huber R, Sivasankaran B*, et al.* GSK3β Regulates Differentiation and Growth Arrest in Glioblastoma. PLoS One. 2009 Oct 13;4(10):e7443.

2. Egler V, Korur S, Failly M, *et al.* Histone deacetylase inhibition and blockade of the glycolytic pathway synergistically induce glioblastoma cell death. Clin Cancer Res 2008;14(10):3132-40.

3. Failly M, Korur S, Egler V*, et al.* Combination of sublethal concentrations of epidermal growth factor receptor inhibitor and microtubule stabilizer induces apoptosis of glioblastoma cells. Mol Cancer Ther 2007;6(2):773-81.